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Emerging and Traditional Technologies for Safe, Healthy and Quality Food



Springer

Food Engineering Series

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Emerging and Traditional Technologies for Safe, Healthy and Quality Food

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Preface

This book is based on selected papers presented at the 6th Central European Congress on Food (CEFood), held in 2012 in Novi Sad, Serbia. CEFood is a biennial event, the first one being at Ljubljana, Slovenia, in 2002, gathering scientists from universities, research institutes, food industry, as well as food producers and distributors. The 6th CEFood was among the most successful ones with close to 600 participants from 36 countries from all around the world. This 6th Congress emphasized novel and traditional technologies to enhance food safety and competitiveness in European and global markets. It is worth mentioning all the authors upgraded and updated their respective contributions which later on were peer-reviewed by highly qualified colleagues.

This reference book will be very useful to food scientists/engineers from academia, research institutions, and the food industry and, at the same time, to practitioners from the food sector. The aim of this book is to present fundamentals and recent developments in food science and technology that will help advance research, development, innovation, and education. It is divided into four well-intertwined parts as follows: Safe and Healthy Foods, Food Quality, Food Biotechnology, and Food Engineering. Topics addressed in this book include, among others, novel technologies to process foods, food safety and quality, food ingredients, trends in nutrition and health, functional foods, bioactive compounds, and regional and global food markets.

The first part is a thorough and vast updating on how to have healthy foods while being very safe, in other words, how to find a sound balance to advance food science and nutrition at the same time. Chapter 1 presents the bird's-eye view of the link between safe food and healthy diets, elaborating on challenges in food safety and food security, food safety and quality control, safe food vs. healthy nutrition, and rights and responsibilities of the consumer. Chapter 2 elaborates on the advantages of supplying foods via networks rather than chains. Traditional foods and their safety are extensively addressed in Chap. 3 including legislation, the hazards coming from raw materials, as well as hazards from processing. Chapter 4 is dedicated to analyze the role of selected chemical contaminants promoting the formation of carcinogenic compounds, i.e., polycyclic aromatic hydrocarbons (PAHs) in smoked meat products, liquid smoke flavors and vegetable oils, as well as their elimination.

The mechanism of acrylamide formation and factors affecting its concentration in thermally processed foods are discussed in detail in Chap. 5, as well as methods for its mitigation by means of recipe and process modifications. Chapter 6 presents contemporary methods for the analysis of bioactive compounds, i.e., polyphenols, tocopherols, and carotenoids in food products. The beneficial aspects of beer as an integral part of healthy diets are discussed in Chap. 7 followed by the significance of beer vs. other alcoholic beverages, potential harmful components, and the development of new beer types with new sensory and functional properties. Chapter 8 deals with the screening of antioxidant peptides in protein hydrolysates using the structural descriptors of antioxidant peptides following a knowledge-based strategy.

The second part of the book, “Food Quality,” starts with Chap. 9 which presents current findings about heat-induced casein-whey protein interactions in caprine milk, as well as means to better control the quality of caprine dairy by identifying similarities and differences to bovine milk. Chapter 10 also deals with whey proteins but in this case with an update on their use on edible films. Chapter 11 analyzes the impact of process parameters and material characteristics on structural, textural, and sensory attributes of rice extrudates, whereas Chap. 12 addresses the standardization of traditional dry fermented sausages in terms of safety and quality.

The part “Food Biotechnology” includes four chapters where Chap. 13 explores the possibility to utilize autochthonous strains of lactic acid bacteria, isolated from traditional Serbian cheeses and prepared by traditional and emerging technologies, in cheese production. Chapter 14 reviews the effect of cell immobilization on the properties of presumptive probiotics with emphasis on tolerance to simulated GI tract conditions, adhesion attributes, and modulation of microbial intestinal flora. The following two chapters are focused on foodborne pathogens, the first one, Chap. 15, on the resistance of *Campylobacter jejuni* and *Campylobacter coli* from food, animal, human, and environmental water sources to some biocides and antibiotics. Chapter 16 discusses recent developments, specificity, and application of microbial polysaccharides as promising and versatile materials for future use in food systems.

Chapter 17 initiates the “Food Engineering” part covering several aspects dealing with the cold chain including shelf life monitoring, the use of time-temperature integrators, and other advanced strategies to properly manage the handling and storage of frozen foods. Chapter 18 reviews benefits of microencapsulated ingredients in the food industry, materials used for encapsulation, and encapsulation technologies which are scalable and acceptable by the food industry. Chapter 19 presents the main types of barrier packaging materials for food application, special functions of new packaging materials, and innovative designs including eco-design aspects. Chapter 20 explores extraction by supercritical fluids from solids or liquids of specific ingredients and their incorporation into the formulation of certain products with desired properties. Chapter 21, the last one, evaluates the capability of a wet germ processing method to increase the purity of dry-milled corn germ to make it suitable for food applications.

The editors of this book are very grateful to all authors for the high quality of their contributions, as well as to all reviewers for their time and thorough criticism of the chapters. We consider that by selecting outstanding authors and reviewers, in addition to our own work, we managed to develop a quality book.

We hope the body of knowledge of all disciplines covered in this book will be expanded in a meaningful way. We also hope readers will find this book interesting, challenging, informative, and appealing, as well as encouraging to closely follow future Central European Congresses on Food.

Belgrade, Serbia
Izola, Slovenia
Novi Sad, Serbia
Novi Sad, Serbia
Pullman, WA, USA

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Part I

Safe and Healthy Food

Chapter 1

Safe Food and Healthy Diets

Elke Anklam

1.1 Introduction

Consumers in all parts of the world request enough safe and nutritious food, which ideally should be available wherever they travel and for an affordable price. Furthermore, consumers expect that the foods (and other products) they buy are genuine, i.e. are not subject to fraud and are of as high quality as possible. This holds especially true for high priced products such as wine, honey and olive oil. For such products, not only the composition and taste is important, but also the geographical and botanical origin, the kind of production process used, e.g. for extra virgin olive oil or the age of a product, e.g. wines, for which consumers are willing to pay. Moreover, there is a growing demand for organic food as more and more consumers have a growing interest to buy ‘healthy’ food. However, it must be stressed that there is—despite considerable research—no substantial evidence that organic food is safer than conventionally produced food. Moreover, can we speak about healthy or unhealthy food in general terms? Is the consumption of an apple per se more healthy than a piece of chocolate?

There is a growing emphasis on health risks from food in the public debate. This is due to a number of recent food scandals that have been extensively discussed and debated in the media such as the Enterohaemorrhagic *Escherichia coli* (EHEC) crisis, dioxins in eggs and horsemeat in food preparation. The latter case did not represent a health risk even though this was the perception of many consumers.

Consumers receive a lot of information on food nowadays from a variety of information sources. This is not only through food labelling on the products, but also through ‘apps’ on mobile phones and scanners placed in supermarkets. Due to the fact that food is globally marketed, food labels contain information in a variety

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of languages. As this information can be regarded as factual requiring only the understanding of consumers of, e.g. ingredient names and impact of calories, there is much more—and sometimes contradictory—advice given on nutritional values and diets. However, products with health claims do not necessarily lead to a healthy diet. Safe food is the prerequisite of a healthy nutrition. Food safety and quality requires the appropriate control to ensure that the food bought by consumers complies with legislation set in the interest of consumers.

This chapter will elaborate on the challenges of safe and authentic food products to deliver the appropriate (healthy) diets for consumers.

1.2 Challenges in Food Safety and Food Security

Due to globalisation and the availability of new technologies, the food sector has grown increasingly complex. This holds especially true when it comes to adulteration of food. In this respect, food control must look out for the unknowns. Today, laboratories around the world become increasingly well equipped to master the manifold analytical challenges. However, most food products are highly complex as they consist in many cases of hundreds of chemical compounds. This complex composition, e.g. of the same plant species is dependent on a huge number of factors such as geographical origin, climate condition and storage conditions, age of the product, year of the product and time of harvesting. This makes authenticity testing and detection of frauds a huge challenge.

It should be noted in this respect that food frauds occur since food is marketed and high value products such as wine, spirits, olive oil, meat and dairy products are still today at high risk of being adulterated. Therefore, the challenge of official food control is to keep pace with scientific and technological developments to understand and foresee potential fraudulent practices and to inform regulatory bodies as quickly as possible in order to take countermeasures.

The other big challenge has been, and is still today, the provision of enough safe food for the world's increasing population, especially as food is increasingly competing with plant products for biofuel production. Consumers worldwide change their diet patterns by, e.g. increasing their meat consumption subsequently requiring increasing amounts of feeding stuffs. Consumers need to have the right information and education to understand these changes in diets not only impacting their health status but also the environment and society.

1.3 Food Safety and Quality Control

To ensure and regain consumers' trust in the food they buy, it is important to control products on the market throughout the whole production chain, i.e. to apply the farm-to-fork principle. Food policy and resulting legislation must consider consumers' interests, expectations and rights to buy fresh, wholesome and safe products.

In this respect, it is important to note that European legislation is one of the toughest around the world. The European Union has established an appropriate framework to deal with issues, setting maximum limits, for example, contaminants (man-made and natural) and residues as well as establishing procedures for authorisation of certain products that arrive into the food chain. The implementation of this strict legislation is performed by the EU Member States for which the legislative framework has foreseen requirements such as accreditation of official food control laboratories and the provision of quality assurance tools through the establishment of National and European Reference Laboratories (NRLs and EURLs). Official food control measures are laid down in Regulation (EC) Nr. 882/2004. Control laboratories need to follow harmonised procedures and the results obtained need to be trustable, reproducible and of high quality. Laboratories need to follow internationally harmonised and recognised standard methods for analysis. As already mentioned above, laboratories need to comply with quality criteria, e.g. accreditation according to ISO 17025. Whenever possible, methods used should be internationally validated and standardised. European Reference Laboratories support the National Reference Laboratories of the European Union to obtain high quality and harmonised results by the provision of reference methods, reference materials, proficiency testing schemes and training to laboratory staff.

This coordinated networking supports the harmonisation of analytical methods performed in field laboratories throughout the European Union, as standardised methods lead to robust and reliable analytical results. The impact of the work performed by the EURLs and NRLs is a better implementation of EU legislation, e.g. by controlling legislative limits by likewise reducing the number of analyses such as harmonisation of methods and results lowers the number of repetitions and increases the mutual recognition. This finally results in safe food and consumer products on the market.

Due to increasing European and worldwide standardisation of analytical methods and the provision of quality assurance tools such as test materials and certified reference materials, validated methods and proficiency testing schemes, the quality of analytical data obtained in the laboratories is becoming more and more comparable. Consequently, this leads to the improvement of the quality of data in, e.g. monitoring databases being of utmost importance for exposure and risk assessment. Food business operators are responsible for EU food safety. This starts with controls carried out by farmers and the industry of raw materials, moving into quality control of the food during processing and before leaving the production hall, controls carried out by the industry, then by retailers for trade and competent authorities to ensure the safety of products for purchase by consumers.

Systems established within the EU for rapid alerting of other Member States and countries on problems arising from food control ensure a quick response and consequently ensure the availability of safe and high-quality food products on the market.

1.4 Safe Food vs. Healthy Nutrition

Safe food products are the prerequisite for a healthy diet. It is of utmost importance that consumers have access to products that are not harmful to their health. As discussed above, food safety is ensured by appropriate production and control.

It seems, however, that there is ambiguity in understanding what food safety really encompasses. One often sees and hears the term ‘healthy food’. It is important to achieve some harmonised agreement on what should be included into the term food safety and what not. Of course one can argue that products containing huge amounts of, e.g. fat, sugar or salt are not safe for consumers and that other products such as vegetables or fruits would be *per se* healthy.

However, there is a common understanding that, e.g. olive oil has a great value for human nutrition due to its nutrient balance despite the fact that it is composed of almost 100 % fat. Its positive scientifically recognised attributes are, e.g. enhancement of cognitive function and therefore moderation of the ageing process, reduction of risk of cancers, anti-inflammatory and anti-clotting properties. Together with tomatoes it assists gut absorption and especially makes vegetables taste better, thereby attracting consumers to make vegetables a high part of their diet.

Using an apple vs. a piece of chocolate in behavioural studies by assuming that the results of the study will show that the apple is the healthy choice is not fully correct. An apple *per se* is not healthy as the human diet requests many other nutrients not being present in the apple to be a standalone dietary component. An apple may also contain pesticides or due to some imperfection may contain patulin deriving from mould. On the other hand, chocolate *per se* is not unhealthy as it contains a number of beneficial substances such as antioxidants. Therefore, it is not fully correct to assume that there is healthy food or unhealthy food. If consumed in moderation and being part of a balanced diet, food containing high-fat content such as chocolate, oils and fats or high sugar content such as breakfast cereals and confectionary can be regarded as safe for the consumer—of course, provided that there are not toxic constituents such as residues or contaminants (man-made or natural). This includes fast food that is regarded as ‘unsafe’ or ‘unhealthy’ for consumers.

I postulate that it may not be correct to talk about ‘healthy food products’ nor to state that food products are unsafe due to their nutritional composition. The term food safety should be exclusively used in the context of absence of harmful substances or when those are above legal limits. The term food quality encompasses freshness, authenticity, wholesomeness and absence of frauds.

It is the overall diet that can be regarded as healthy and unhealthy. As already discussed above, there is no risk for healthy consumers to consume products containing, e.g. high amounts of sugar and fat and even sometimes salt, when done in proportion and moderation. The knowledge of the right amounts in the overall diet is the responsibility of the consumers themselves. Even by consuming only safe food products, the nutrition can be unbalanced and this results in an unhealthy diet. It is the amount of products in the diet that make the difference. It is therefore important for consumers to understand the amount of calories and nutrients in their

meals. The portion size is the major problem on energy intake. Portion sizes are prefixed by ready-to-eat food products that can be purchased. Consumers may not divide, e.g. smaller size bottles or packages of yogurt into several portions and may therefore take those sizes as a standard.

1.5 Rights and Responsibilities of the Consumer

Although appropriate infrastructure is in place to ensure that the food consumers buy starts out safe, it is not granted that the food finally consumed is still safe nor the overall diet is healthy. It is the responsibility of the individual consumer to ensure that the quality and safety of food finally ending up in his/her body is still as high as possible. Even though consumers may spend less time in preparation of dishes by buying convenience food requiring only little processing in the home kitchen, food must be properly stored after purchase and carefully handled and cooked before consumption. This holds especially true when handling fresh products such as meat, chicken, eggs and dairy products, as it is quite easy to spread potential microbial contamination throughout the whole kitchen. Increasing consumer education on appropriate handling of food, especially with regard to impact on health from microbial contamination, is necessary to ensure a high quality not only from the farm to the supermarket but also from the market to the consumer's plate.

Furthermore, consumers need to understand the potential risk of interactions of medical products with food. One example would be the interaction of antibiotics and iron or of blood thinners that can have an interaction with vitamin K present in green vegetables which in turn can have inhibiting properties for blood thinners leading to clotting. Dietary supplements such as ginkgo and ginseng have blood thinning promoting properties and thereby could lead to bleeding.

Scientists and teachers have the responsibility to ensure that accurate and consistent information about scientifically sound food safety aspects, including nutritional matters, is not only communicated to consumers but also to the media. It is of utmost importance that contradictory results of scientific investigations, e.g. on risks and health claims are evaluated with care and not arbitrarily communicated to consumers. The impact of scientific results with regard to food safety and nutrition has to be studied from a holistic point of view.

In addition, consumers need to have the right understanding about the amounts of calories in their daily diets and have an idea about the nutrient intake to ensure a healthy diet. A harmonised approach for nutritional surveillance worldwide is important as food is marketed globally and moreover there is the right for safe and enough food for every human being worldwide.

Communication to the consumer has to be done appropriately to avoid misunderstanding and frustration. Consumers need to get the best recommendations and subsequently need to make the right implementation to achieve a nutritious and healthy diet.

1.6 Conclusion

Thanks to appropriate legislation and increasingly improved food control, it can be concluded that food in general—especially the food marketed within the European Union—can be regarded as safe for consumers. Criminal tampering with food—whether leading to unsafe products or just not delivering what is promised on the labels—needs to be detected and prevented. This is the responsibility of the food producer and food control authorities. However, the individual consumer is still responsible for his or her own safe food products and healthy diet. Food safety continues to be an issue in the consumer's home. It is therefore important that consumers have the knowledge and education on appropriate handling of food, especially with regard to impact on health from microbial contamination. However, safe food products alone do not guarantee a healthy nutrition. Appropriate nutrition, i.e. a healthy diet, is the consumer's responsibility. It is the portion size and frequency of certain foods in the diet that have a significant impact on a healthy nutrition. Moreover, an appropriate and consistent diet is important, especially when consumers are under medication. A healthy diet is composed of safe food products tailored to the individual needs of the consumer. Food products need to be affordable but not cheap, and above all safe. Cheap food may not give incentives for appropriate portion sizes and responsible food handling especially with regard to food waste. Consumers need to be empowered by transparent information to make the right decision for healthy choices in their diets.

Disclaimer The views in this article are of the author and do not necessarily reflect those of the European Commission.

Chapter 2

Food Supply Chains vs. Food Supply Nets

Peter Raspor and Mojca Jevšnik

2.1 Introduction

Today's food industry and its sophisticated processing and distribution technology produce a variety of foodstuffs available to the consumer at rapidly growing commercial centres. Development of food and related sciences and technologies provides a more in-depth knowledge of health risks; however, the ongoing interventions in technology and the distribution of food innovations are causing new risks.

Federal and international agencies are acting to encourage better public health protection. One of the principal actions has been the development of HACCP (Hazard Analysis and Critical Control Point) based regulations or recommendations by federal agencies and the United Nations Codex Alimentarius Commission (Sperber 1998). To control and comprehend safety in European Union (EU), «White Paper on Food Safety» is an important document that was published in January 2000 (EC 2000). After that regulation 178/2002/EC and decision 97/579/EC were published, which exactly define «European Food Safety Authority». The use of HACCP principles at all levels of the food chain is however compulsory under EU Directive 93/43/EEC and Regulation 852/2004/EC (EU 1993; EC 2004). There will be soon new EU legislation on food control. The new food safety legislation package provides a modernized and simplified, more risk-based approach to the protection of health and more efficient control tools to ensure the effective application of the rules guiding the operation of the food chain. It is a responsibility of all

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included parties in the food chain to ensure food traceability and food safety by internal control in all production phases.

Since April 2004, when the European Parliament adopted Regulation (EU) No 853/2004 on the hygiene of foodstuffs, through its adoption on 1 January 2006 by all food operators, there has been a strong focus on the system of food safety management. The main change to the law relates to food safety management systems, i.e. risk-based methodologies to ensure the safety of food. Successful implementations of the procedures based on HACCP principles require the full cooperation and commitment of food business employees. To this end, employees should undergo training (EC 2004; Jevšnik et al. 2008c; Raspor 2008).

‘Food safety’ is a broad term, which means an assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use. Providing the consumer with safe and healthy food is, in the age of globalization, linked with different styles of food habits and responsibilities and represents an ongoing endeavour in developed and developing countries. Currently, food systems represent a historical collection of knowledge and skills, which are necessary to handle food ‘from stable to table’, ‘from farm to fork’ and ‘from spring to drink’ (Raspor 2004a, b, 2006) what also reflects in professional and communication language and courses substantial problems in communication in food safety area (Ambrožič et al. 2010).

Food safety is of crucial importance to the consumer, the food industry and the economy of each country. Despite significant investment, the incidence of Food-Borne Diseases (FBD) continues to increase. FBD caused by microbiological hazards are a public health problem in Europe and throughout the world.

The inability to effectively improve the situation is a matter of major concern despite the significant resources allocated to the problem of FBD. A closer look at the field of food, from the technical sciences to the social sciences, yields a broad spectrum of possibilities on how to completely maintain food safety. Food safety represents a cross section of four important fields: food regulation, food technology, analytics, and finally, public food safety knowledge and awareness. The purpose of these four fields is to protect human health. Today, we master food safety with different good practices, which are the products of human culture, history and lifestyle. If we analyse good practices in the broad spectrum of the food, we could arrange them in three categories. The first category is directly connected with food technology (i.e. Good Manufacturing Practice (GMP)). The second category is indirectly connected with food issues (i.e. Good Research Practice (GRP), Good Educational Practice (GEP), Good Training Practice (GTrP)). The third category deals with all the activities regarding consumers’ handling of food (Good Housekeeping Practice (GHKP)).

Tradition, practice and a wide variety technical and scientific knowledge have helped shape principles and techniques of how to achieve acceptable food safety in a given environment. Heterogeneous environmental conditions, a wealth of different materials, a diversity of cultures and ways of practical work have helped shape the principles, some of which were later included in legislation. Today, we manage food safety through the good practices at different levels of food production, cater-

ing, distribution and consumption. The current maintenance of food safety in food supply chain can easily break down because of the different kinds of barriers or simple misunderstandings amongst the people involved in food supply chain, including consumers (Raspor and Jevšnik 2008; Jevšnik et al. 2008a, b). The HACCP system, supported with good practices, represents the clearest example of this development (Raspor 2004b). The previous quality control system was based on the finished product. A new food safety philosophy is based on the appropriateness of the technological process in the chain through which food passes, which significantly reduces the risk of inadequate health final product (Sperber 2005a, b; Raspor and Jevšnik 2008). Food safety, synonymous with food hygiene, embraces anything in the processing, preparation or handling of food to ensure it is safe to eat (Griffith 2006), therefore the emphasis of this review paper on food hygiene.

Finally, food safety has not been mastered according to the ‘from farm to fork’ concept, because consumers are not properly connected to the food supply chain (Raspor and Jevšnik 2008; Raspor 2008).

This chapter clusters the main issues and consequently outlines new platform within food safety area based on networking structure and not any longer on linear food supply chain approach. Such systemic approach is underpinned with comprehensive and critical review of relevant publications in the last decade enriched with author’s own findings in research and practice.

2.2 Food-Borne Diseases Arising from Food Supply Chain

FBD are associated with microbial pathogens, biotoxins and chemical contaminants in food. According to the WHO definitions, a ‘food-borne disease’ is any disease of an infectious or toxic nature caused by the consumption of food, whilst a ‘food-borne disease outbreak’ is classified as the occurrence of two or more cases of a similar food-borne disease resulting from the ingestion of the same food. A ‘food-borne outbreak’ is also defined by the European Union Directive 2003/99/EC as an incidence, observed under given circumstances, of two or more human cases of the same disease and/or infection, or a situation in which the observed number of human cases exceeds the expected number and where the cases are linked, or are probably linked, to the same food source. Whereas, ‘food’ is defined in Regulation (EC) No 178/2002 as any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be, ingested by humans; this definition also includes drinking water and covers single food items as well as meals consisting of various types of food (Ambrožič et al. 2010).

Consumer concern about the threats associated with food is growing. Due to recent food crises in Europe, food quality and food safety have become a hot topic in mass media. Food safety is of crucial importance to the consumer, food industry and economy. It is commonly known that the levels of FBD are increasing in both developed and developing countries. The calculation of annual cases of salmonellosis and campylobacteriosis shows that the yearly number of cases in Europe is

likely to exceed five million, demonstrating that the economic losses and human distress resulting from food-borne diseases can no longer be neglected (Raspor 2004a). Food contamination creates an enormous social and economic burden on communities and their health systems. The incidence of food-borne diseases is rising in developing countries, as well as in the developed world (Redmond and Griffith 2003). The cause can be found in changing lifestyles, increasing consumption of ready-to-eat foods, consumers neglecting the principles of GHKPs, improved laboratory diagnostics and an increasing number of infections involving new or more virulent types (Tauxe 2002; Smole Možina and Hočevar Grom 2004; Jevšnik et al. 2008b, 2011). According to epidemiologists, the recent emergence of infectious diseases can be considered a third epidemiological transition, characterized by a globalization of human disease ecology and the evolution of considerable technological and social-economic changes. The changing epidemiology of food-borne diseases and the increase in knowledge concerning emerging food-borne pathogens require a re-examination of food safety educational messages to ensure that the guidance given to consumers is appropriate for controlling pathogens that are prevalent in the food supply chain (Hillers et al. 2003).

Correct handling of food during all stages of its preparation and storage is vital in reducing the incidence of food-borne diseases. To achieve satisfactory level of food safety at home, consumers should be well informed regarding basic principles of food safety practice (Raspor and Jevšnik 2008). Despite significant advances in public health, in 2010, the European Food Safety Authority (EFSA 2012) registered in total 5262 (1.1/100,000) reported food-borne outbreaks, in which 43,473 people were affected, amongst which 4695 were hospitalized, and 26 died. Apart from households, the most common settings of outbreak were restaurants/cafes and similar premises. Nevertheless, these numbers probably do not reflect the real epidemiological picture, because only reported outbreaks are recorded in the official reports. Therefore, the importance of unreported cases should not be ignored, whilst people with mild medical symptoms often do not seek medical assistance and are therefore not registered in official statistics.

In the current organization of everyday life, there is an increased prevalence of eating away from home and the use of partly or fully cooked food (Haapala and Probart 2004; Byrd-Bredbenner et al. 2007), which is more a reaction to daily time constraints than a result of any increasing popularity of such foodstuffs (Tivadar 2003).

Consumers need knowledge and skills for effective food handling, but also they have to be motivated to act upon that knowledge as a precondition to behaviour change (Hillers et al. 2003; Redmond and Griffith 2003). It is obvious that consumers are not provided with sufficient and easy-to-understand information (Banati and Lakner 2006).

The field of food science and technology is a part of the natural sciences and is thus mainly researched with quantitative methodology (Jevšnik et al. 2006). It is understandable that complex behavioural barriers require detailed diagnostic tools and matching interventions to effectively overcome them, especially in the field of food safety. Behavioural research offers an innovative, yet logical approach to the

problems existing within the field of food safety management, and one that has thus far been mostly untouched (Gilling 2001; Gilling et al. 2001). People do not react to external signals automatically but individually interpret their meaning. Consequently, it is important to learn in detail about various ways of signal interpretation, which can be done with qualitative research techniques. Quantitative and qualitative methodologies have their advantages and disadvantages; neither of the two methodological techniques can assure completely valid and reliable data, but if combined, they can provide important insights into the dynamics of a society. In general, quantitative data offer more static insights but enable the research of basic patterns and structures. Qualitative data, in contrast, are less appropriate for determining patterns and structures in general but enable a more thorough and in-depth understanding of the process of changes in social life (Haralambos and Holborn 1999). Therefore, further multidisciplinary food safety research should be encouraged to comprehend the importance of individual people in units of the food chain. Formal and informal organizational structures and relationships should be taken into strong consideration. Due to a significant increase in the volume of information that scientists from different fields are facing today, a systematic approach to the analysis of published discoveries has become essential. A multidisciplinary approach, including experts for food safety, food technology, psychology, sociology and public health, is thus of great importance (Jevšnik et al. 2006).

2.3 Food Supply Chains vs. Food Supply Nets

Globalization and increased urbanization, especially in developing countries, influence the organization of food supply chains and networks with increasingly complex relationships. Globalization is a historical process that began as early as the first movement of people out of Africa into other parts of the world. Migrants and merchants, who travelled short and gradually longer distances, have always taken their ideas, customs and products into new lands. The global food supply system has undergone dramatic changes in recent decades. The increasing integration of both cross-border and local food supply chains can be considered both a threat and a challenge for food safety (Ambrožič et al. 2010).

Porter (1990) and Selvan (2008) described the meaning of supply chains. Supply chains are understood as transformation processes from inputs through primary production, processing and marketing to the final consumption (Porter 1990). A food supply chain is a network of food-related business involved in the creation and consumption of food products, through which food products move from farm to table (Selvan 2008). Supply chain management is the integrated planning, coordination and control of all business and activities in the supply chain to deliver superior consumer value at the lowest cost to the supply chain as a whole whilst satisfying the variable requirements of other stakeholders in the supply chain, such as governments and NGOs (van der Vorst 2006). In this definition, the supply chain is a series of physical and decision-making activities connected by material and information

flows and associated flows of money and property rights that cross organizational boundaries. The supply chain includes all parties involved in any operation within food circle from production to consumption. Only one insufficient or truncated piece of information or just simply miscommunication in the supply chain can result in unsafe and dangerous food. For this reason, transparency and traceability along food supply chain is one of the most important elements in the food supply chain in order to ensure product and process integrity, improve consumer trust and maintain quality and safety standards (Ambrožič et al. 2010).

Assessing all interactions within food supply chains, we see that many contact points do not receive the attention that they deserve. This complexity raises the question of how we discuss food safety management in food chains. Specifically, we comprehend current food systems running on a linear basis. We know from daily practice that this is not the case. Therefore, we shall start to redesign our approach in thinking, and we shall start to think about food supply networks. It is very common to speak about networking when discussing people, organizations, companies, various subjects in different areas of expertise. With regard to food, nutrition and health, however, there seems to be a desire for a one-dimensional or linear system that would be very practical to handle. Unfortunately (or fortunately), this is not the case. We should implement at least a two-dimensional principle. This shows that we can connect activities in food supply area via activities at contact points, which represent the fusion of some activities on facing sides. This implies that we have active node that integrates the activities of all relevant sides and consequently to more dimensions of activities with different professional stakeholders.

This calls for a network. The network approach is so much more relevant to interconnecting all nodes existing in current food supply systems. Three groups of good practices are controlling food of plant and animal origin within production, processing, storage and distribution, trade and catering. Food supply network is controlled by regulated elements (environment, food premises, conveyances and containers, working utensils and equipment, water supply, pests, food waste and food handlers) and is of crucial importance for stabilizing particular food path in food supply continuum (Fig. 2.1).

When will we include this thinking in practice? Moreover, when will we adopt this practice in flexible thinking?

2.4 Personnel as Main Food Safety Actor

The acceptance of food safety systems has put employee training under the spotlight (Collis and Winnips 2002). Under the personnel programme of HACCP, employees must be trained in areas such as food safety, manufacturing controls and personnel hygiene. Once HACCP plans have been established, employees must be trained to manage any critical control points (CCPs). Though numerous companies have developed, documented and implemented training programmes, few understand why employee training is important, what their training requirements are or how to

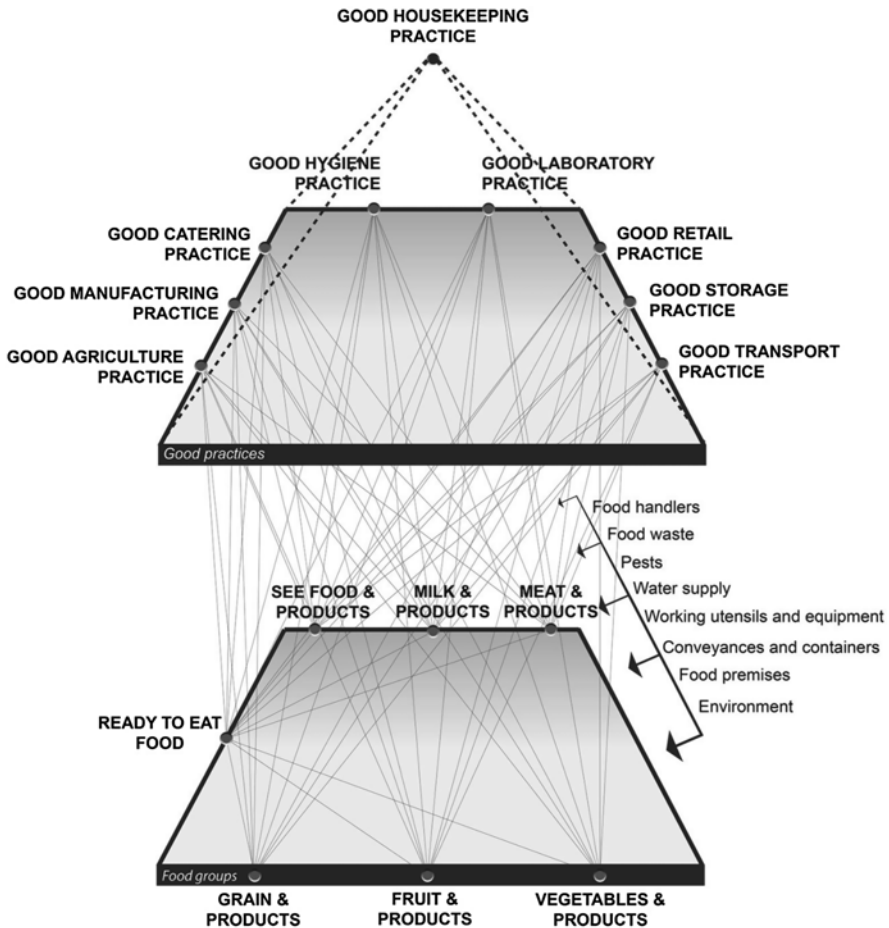


Fig. 2.1 Systems approach in food safety management asks for integration of food chains into food supply networks. Activities are transparently connected via nodes to complex structure of traceable food supply network

assess the effectiveness of in-house training programmes. Thus far, most publications about HACCP training have described what should be done, but little has been written about the effectiveness of such training or how to motivate employees to follow all food safety requirements. Food business operators have to engage with these issues in their own way, as every company has its own specific ways of ensuring safety. HACCP has been described as a philosophy in theory and a tool in practice (Gilling et al. 2001). Bryan (1981) pointed out: 'It should therefore come as no surprise that there can be different opinions on how it should be applied.'

HACCP problems are a complex mix of managerial, technical and behavioural issues requiring specific remedies (Gilling 2001). By taking a psychological approach and utilizing practical experience and theoretical knowledge of HACCP,

Gilling et al. (2001) identified 11 key barriers and organized them around knowledge, attitude and behaviour frameworks. The proposed Behavioural Adherence Model, therefore, acts as a diagnostic tool, identifying progressive stages to successful HACCP guideline adherence. The authors emphasized that the model should be of significant help to those offering advice and guidance to food operators undertaking HACCP implementation.

A problem that has considerable influence on the acceptance of the introduced 'new' food safety system, especially at the beginning, was the way of presenting HACCP and the qualification of trainers. Mortimore and Smith (1998) mentioned that many trainers had been willing to provide HACCP training without considering the scope (what had to be taught and what need not have been) and the depth of coverage. They also described that a wide disparity in content and quality between courses. Moreover, several authors suggested that most managers in the food industry have limited understanding of the global food safety strategy (Ehiri et al. 1995; Mortimore and Smith 1998; Khandke and Mayes 1998; Williams et al. 2003). MacAuslan (2003) who wrote that the majority of food businesses do not have satisfactory training policies for all their staff. He emphasized that too much reliance is placed upon attaining a certificate rather than attention paid to achieving competency in food hygiene practice. He suggested that greater emphasis and more resources be diverted towards assisting managers to become highly motivated food hygiene managers who develop and maintain a food safety culture within their business. A small business owner may be tempted to place the burden of training responsibility on an external employer, and not shoulder any responsibility themselves. According to MacAuslan (2003), the problem can have two sides: firstly, the employer lacks key management skills in leadership, motivation, training and evaluation; secondly, going for a training course just to obtain a certificate. The responsibility for food safety has been put on food business operators, who do not have sufficient knowledge and skills for human resource management.

Factors that have a significant impact on employers' behaviour are correlated with the organizational climate in the company, the level of job satisfaction and labour conditions, and with relations between employees. Marolt and Gomišček (2005) described a new management approach to employees, one which stimulates employees to take initiative, to learn, to be devoted to the company, to be self-confident, to achieve higher efficiency and better teamwork, which all contribute to the greater success and effectiveness of the organization. They emphasized the function of leadership, which plays a key role in realization of the new principles into practical work and can, therefore, significantly contribute to better usage of existent resources. A leader should persuade employees to fulfil their needs and desires by working effectively and should enable them to reach their potential, and by doing so to contribute to achieving the goals of the team and organization. It would be ideal if people were motivated to such level that they would not work just because they have to but would work with eagerness and with trust. For efficient food safety management, Jevšnik et al. (2007) suggested that food business operators follow the model of 'four elements analysis' for efficient hygiene-technical situation management in food processing plants. The model includes equally important

elements, in which each requires the involvement of a competent and trained person. The model's benefit is the importance of the human factor in food safety assurance. The first element includes an estimation of the current hygiene-technical situation in the food processing plant. Hygiene-technical deficiencies and/or irregularities have to be analysed, and a plan of improvements has to be made. The second element includes the establishing of hygiene basics, the so-called prerequisite programmes, which are the basis for establishing the HACCP system, i.e. a tool for food safety management. The third element includes the planning and execution of periodical training and education, adapted to specific work tasks, for employees at all food hygiene levels. The fourth element notifies employees' knowledge about food handling of an individual worker at a specific work task. This requires a professionally trained, competent person who possesses adequate technical and pedagogical knowledge, practical experiences and knowledge of human resource management. Various techniques and methods of training involvement and control of the work process performance are also required. With the fourth element, the human factor as a risk for food safety assurance is indicated. In the future, a discussion of the human risk factor as being equal to the other risk factors in production processes (biological, chemical and physical) is suggested.

Based on the results of the Jevšnik et al. (2007) research, it is determined that hygiene education and individual awareness are the most important tools for food safety assurance; therefore, every food handler requires a complex and individual management. The human factor must be discussed equally amongst all the other risk factors, e.g. hygiene, technical and technological factors. For food safety, it is essential that every person in the food supply chain understands and fulfils his responsibilities and relies upon the previous and the next step in the chain.

2.5 Human Resources (Personnel Management and Education)

We are facing both insufficient knowledge and awareness of food safety issues amongst food workers and with insufficiently informed consumers about food safety principles at home. It is truly astonishing that so much activity has been invested in this area from childhood onward, but the effect somehow remains minor (Ovca et al. 2014).

In daily practice, most of the critical points depend on a particular person at a particular place. If we do not perform adequate training and appropriate education within human resources, we cannot expect to have professionals with highly developed skills or high knowledge; this makes the control and documentation of food handlers by human resource management relevant (Jevšnik et al. 2006, 2008c).

Human resource management and education of food safety managers on food premises has not captured any significant attention of researchers until recently (Jevšnik et al. 2008c). The strict performance of working procedures in accordance with HACCP system principles and food hygiene is essential for the prevention of

food-related diseases and the efficient assurance of safe food. To achieve this purpose, two basic conditions must be assured: (1) a suitable working environment from the hygienic-technical perspective, and (2) motivated, satisfied and qualified personnel as indicated by Latham and Ernst (2006). It is interesting that many understand the HACCP system as a novelty 15 years ago, when in fact it is a much more complete approach to food safety assurance, as stated by Ehiri et al. (1995). The HACCP system assures more structured surveillance over determined hazards than was the case with the typical type of surveillance. Hazards and corrective actions are not something new. What is new is how separate activities and procedures are logically arranged. The approach is multidisciplinary. It requires personal responsibility, monitoring of documents and records, and rapid action when non-conformities are discovered. It also enables traceability. Its greatest ability lies in responding to changes and in enabling continuous checking and efficiency confirmation. It brings changes to thinking, organizing, managing, education and training at all levels, from employers to employees (Likar et al. 2001; Likar and Jevšnik 2004). The system becomes efficient when it is understandable to employees and when the responsible parties perform their duties. Then the requirements of the system are not considered to be irrational, unnecessary or burdensome, but as a desire for the continuous improvement of one's own work. Consequently, training, from top management to all employees, is crucial for food safety what was already indicated in 1988 by Bryan.

Legislative changes in 2004 required that all food premises provide food hygiene training appropriate for the work activities of their staff (EC 2004, EC (2014)). Jevšnik et al. (2008c) showed that training carried out by company experts and by supervisors directly in working place is the most efficient. Mortlock et al. (2000) suggested that it is also important to recognize that whilst formal training might ensure greater consistency and quality (Manning 1994), improper training could present a greater risk to food safety than no training at all. In a study by Cohen et al. (2001), the impact of an in-house food sanitation training programme on the performance of a catering company was analysed. It was concluded that for a fully effective sanitation programme, the different environments and circumstances in which the departments operate must be taken into consideration. It is very important that those performing any training have suitable food safety knowledge as well as skills in pedagogical/andragogical field. Such people have to be competent experts in their field, so that adequate knowledge and skills can be passed on to the employees.

A problem is found in small and medium-sized enterprises whose owners are usually the responsible persons for food safety programmes, including training. Because of a lack of time or poor knowledge, such trainings are not carried out as required by the law. The results of the Jevšnik et al. (2008c) study show poor knowledge about microbiological hazards and their control amongst employees in retail, catering and food production units. MacAuslan (2003) emphasized the importance of helping managers to understand what is expected of them, and of giving them support in managing effective food hygiene. He pointed out that too much reliance has been placed upon certificates and not enough on competence. In his opinion,

this is defined as the ability of an individual to demonstrate the activities within their workplace, or to function to the standards expected in a food business.

The purpose of internal surveillance is to identify specific hazards in a particular company and then to establish a strategy of efficient control or successive elimination of the hazards, as stated by Jevšnik et al. (2008c).

Strict performance of working procedures in accordance with HACCP system principles and food hygiene is essential for the prevention of food-related diseases and the assurance of efficient safe food. A novel food safety concept for safe food separate activities and procedures is logically arranged. The approach is multidisciplinary, and it requires personal responsibility, monitoring of documentation and records, and rapid action when non-conformities are discovered. It also enables traceability. Its greatest ability lies in responding to changes as well as in enabling continuous checking and efficiency confirmation. It brings changes in thinking, organizing, managing, education and training at all levels, from employers to employees (Likar et al. 2001; Likar and Jevšnik 2004; Jevšnik et al. 2008c).

2.6 Current Limitations in Food Safety Management

The occurrence of intense globalization and urbanization is having a major impact on food systems worldwide. Food systems are changing and consequently resulting in consistent quality, enhanced safety, greater availability and diversity of broad assortments of products throughout the year. Consumers have become increasingly concerned and demanding about the quality and safety of food they are eating. The increased demand for safer food has resulted in the development and introduction of quality management systems, which are used to control the quality and safety of products, such as standards and good practices (Raspor and Ambrožič 2012). Food safety requirements with changes in food supply chains, social, health and demographic situations, lifestyle and environmental conditions have led to significant efforts in the development of quality management system in agribusiness and food industry worldwide. Because quality systems differ in several aspects, they are combined or integrated to assure more aspects of food quality. Quality is divided into aspects of product safety, product quality and total quality, which embrace products' safety and quality (Raspor and Jevšnik 2008; Vefflen Olsen and Motarjemi 2014).

At present, quality assurance systems, such as GMP, HACCP, International Organization for Standardization (ISO), British Retail Consortium (BRC) and International Food Standard (IFS), are applied for assuring food safety (van der Spiegel et al. 2003; Raspor and Ambrožič 2012). Each quality assurance system is focused on a particular one. For example, GMP and HACCP were specifically developed to assure food safety (Hoogland et al. 1998; Raspor 2004b). Like HACCP, BRC deals with food safety and product quality but also evaluates management aspects (like ISO does) and facility condition (like GMP does). Additionally, ISO and Total quality management (TQM) focus more on management aspects, whereas GMP and HACCP focus on technological aspects (Barendsz (1998), Hoogland et al.

1998; ISO 9000:2005 (2005), Moy and Motarjemi 2014). Food manufacturers have to decide which quality assurance system is most suitable to their situation and how this system should be implemented. In recent years, a large number of companies have implemented quality assurance systems and TQM systems in order to introduce effective quality systems and consequently produce and distribute high-quality products (Raspor 2008). The vast numbers of laws, regulations, standards, good practices and codes can be quite confusing, even for those who are working in the field on a regular basis and are forced to keep up with the developments (Ambrožič et al. 2010). The challenge for the food supply chain is to satisfy and meet consumers' needs, wants and even their desires. The food supply chain embraces a wide range of disciplines. The creation, operation and evaluation of food supply chains are key dimensions in food safety management (Motarjemi 2014).

In most Small Enterprises (SEs), there are specific limitations (e.g. insufficient training, inadequate or insufficient control of a catering process, inadequate cleaning of working utensils and equipment), and they are not constructive-technically suitable for performing food-related activities (Baş et al. 2006, Jevšnik et al. 2007). In small plants, technical and hygiene conditions for handwashing were estimated as being inadequate and of concern. A non-negligible share (14 %) of small plants did not meet even minimal hygiene-technical requirements for food handling (e.g. wash-hand basin is missing or is not installed properly, thereby enabling cross-contamination between high- and low-risk areas; unsuitable and worn-out materials do not enable efficient sanitation and maintenance). Aarnisalo et al. (2006) summarize the results of many studies that have shown that food processing equipment could be a source of contamination, e.g. *Listeria monocytogenes*. Hygiene problems in equipment are caused when microorganisms become attached to surfaces and survive on them and later become detached from them, thereby contaminating the product (Aarnisalo et al. 2006). In some medium enterprises (MEs) as well as in some SEs, the basins for handwashing do not prevent cross-contamination between high- and low-risk areas. Hygienic equipment of basins is inadequate mainly in SEs, since in more than a third of (39 %) plants necessary hygienic equipment by the basins was missing (e.g. liquid soap, paper towels). In regulation (EC) No 852/2004, it is stated that an adequate number of basins is to be available, suitably located and designated for cleaning hands. Washbasins for cleaning hands are to be provided with hot and cold running water and materials for cleaning hands and for hygienic drying. Where necessary, the facilities for washing food are to be separated from the handwashing facility (EC 2004).

In observing employees during their work, the fact that most of workers in both groups do not wash their hands after performing any dirty work (e.g. when changing between high- and low-risk phases of work, after handling packaging) or do not wash hands properly (e.g. they do not use liquid soap, negligent handwashing technique) was determined. It was concluded that employees do not understand the meaning of proper handwashing and are not aware of microbiological hazards that can occur due to dirty hands. The causes for the latter can be found amongst insufficient hygiene training, negligent, insufficient employees' knowledge and/or inefficient control by supervisors (Jevšnik et al. 2007; Jianu and Goleţ 2014; Pichler et al. 2014).

Ambrožič et al. (2010) summarized research results regarding hand hygiene and pointed out that microorganisms are always present on hands because they are a part of the normal microflora of the human body; nevertheless, in food production and trade, the presence of some bacteria is not allowed. In the research, blood agar plates were used for bacteriological analyses of hands, which enabled the quick estimation of hygiene condition in the selected plants. In further analyses, a selective growth medium would be used only for bacteria considered dangerous; this would show the hygienic status of food processing plants. It was determined that on the right hands of employees there were fewer microorganisms than on the left hands. When studying an individual person, in most of the cases it was observed that they have either low or high bacteria count on both hands. Therefore, it may be wise to take swabs from workers' hands more frequently and to communicate the results, which could be a motivation for better hand hygiene at work. However, as shown in previous studies of food handlers' beliefs and self-reported practices (Clayton et al. 2002), food handlers were aware of the food safety behaviours they should be carrying out, but 63 % of respondents admitted that they did not always carry out these behaviours. Food handlers also reported carrying out food safety practices, particularly handwashing, much more frequently than they actually implemented them (Manning and Snider 1993; Walker et al. 2003; Jianu and Goleţ 2014; Pichler et al. 2014). This suggests that food handlers could be carrying out food safety practices less frequently than the self-reported data implies (Clayton et al. 2002). Shojaei et al. (2006) cited the fact that many authors emphasized that the hands of food handlers are an important vehicle of food cross-contamination and that improved personal hygiene and scrupulous handwashing would lead to the basic control of faces-to-hand-to-mouth spread of potentially pathogenic transient microorganisms. Lues and Van Tonder (2007) summarized the results of several studies in which it was established that various bacteria, amongst others *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp., survive on hands and surfaces for hours or even days after initial contact with the microorganisms.

Every person working in a food-handling area is to maintain a high degree of personal cleanliness and is to wear suitable, clean and (where necessary) protective clothing (EC 2004). It was determined that personal hygiene is significantly poorer in SEs than in MEs. More than a third (36 %) of workers in SEs did not wear clean and suitable overalls, and more than half (52 %) performed work with no head-covering. The cause of the problem contributing to the stated results in SEs is lack of control by trained and responsible persons. Workers are to a large extent left on their own; moreover, the owners do not provide necessary means for the safe food handling. In MEs, the situation regarding personal hygiene is better (Jevšnik et al. 2007). In most of the MEs, there is a responsible person authorized by management, who is responsible for hygiene and has required professional education. A periodical training for workers is performed in accordance with a plan, and work performance is checked daily. The main problem identified amongst food handlers in SEs is related to the fact that they receive no specific or insufficient knowledge about food hygiene (Jevšnik et al. 2007).

Knowledge and training for working according to the HACCP system were estimated using questions that had been designed prior to the research. By asking the question: 'How do you record temperatures in cooling appliances and during heat treatment?', it was determined that in 12 % of SEs and in 20 % of MEs temperatures in cooling appliances were registered in advance (e.g. as it seems to be the next day) or for the past (e.g. the person responsible for monitoring the temperature value forgot to write the temperature of cooling appliances) (Jevšnik et al. 2007). From the results, it is concluded that the majority of workers follow instructions, but are not familiar with or do not understand why they are necessary and are not aware of hazards in case of hygiene violations and non-fulfilment of the requirements. This finding was consistent with the findings of Panisello and Quantick (2001), Vela and Fernández (2003), Yapp and Fairman (2006) in which they established that smaller companies may lack knowledge and expertise in HACCP and appropriate resources to obtain knowledge, both resulting in insufficient understanding of functions of HACCP principles. It was established that education and training is not efficient mainly in SEs, since it is carried out by incompetent persons without suitable professional and pedagogical knowledge. Yapp and Fairman (2006) pointed out that in some cases SEs do not realize that they are breaking the law and often do not understand what is required of them. It is particularly evident when recording parameters according to an HACCP plan. It was determined that documentation regarding prerequisite programmes in both types of food enterprises is incomplete, but in SEs the situation is worse. Mitchell (1998) stated that the HACCP plan is sometimes a 'paper exercise' that overburdens the needs of small and medium-sized enterprises and it is not implemented in practice.

With Regulation (EC) No 852/2004, the responsibilities for food safety lay entirely on food business operators, which mean that operators are also responsible for education and training of their employees (EC 2004).

Which training type will prove to be more effective in the future remains a question. Irrespective of that, the most important fact according to Seaman and Eves (2007) is that the training will only lead to an improvement in food safety if the knowledge imparted leads to desired changes in behaviour in the workplace. For conscientious hygiene, it is not important in which enterprise people work, but it does depend upon hygiene awareness and education of an individual person.

2.7 Consumers: A Neglected Link but Essential Node in Food Supply Chains vs. Food Nets

Ensuring safe food for the consumer is, in the era of globalization, the responsibility of every link in food supply chain and constant task in developed and developing countries. Definitions of food safety are generally written, thereby allowing the possibility of many interpretations (Raspor and Jevšnik 2008). A variety of dictionary items and interpretations from different perspectives could be cited, but the point is

that we do not treat food safety as a food safety cycle ‘from the farm to the table’, because we often focus on it partially (only individual segments of the food chain), and we neglect consumers. Each of us is a consumer, regardless of which stage of the food chain we enter the safety cycle (Jevšnik et al. 2011).

The principal objective of the general and specific hygiene rules is to ensure a high level of consumer protection with regard to food safety (EU 2004). Foodstuffs can become a risk factor for consumers if they are not handled and treated along the food supply chain in accordance with the principles of good practices and the HACCP system. The food supply chain does not exclude consumers, but the question is whether consumers are sufficiently informed to assure food safety at the end of the food supply chain. Redmond and Griffith (2003) demonstrated that multiple food safety responsibilities are held by consumers, because consumers not only purchase and receive products but also process and provide foods for themselves and for others. They also emphasized that the implementation of proper food-handling practices can prevent cases of food-borne disease, and the way in which consumers handle food in the kitchen affects the risk of pathogen multiplication, cross-contamination to other products and the destruction of pathogens via thorough cooking procedures (Redmond and Griffith 2003; Griffith and Redmond 2014).

What do consumers know about food safety principles and what do they do to protect themselves from food-borne diseases? The meaning of the term ‘Food Safety’ is well known and defined in expert circles, but, when analysing how it is interpreted by consumers, new dimensions are opening, which can be used as a guide in preparation of educational material for consumers. Jevšnik et al. (2008a, b) analyse statements made by consumers when answering the question, ‘How do you interpret the term food safety?’ The findings show considerable terminological diversity amongst statements made by respondents regarding a description of the term ‘safe food’. The results show a connection between 38.4 % of consumers’ statements in Category A, (harmless for health), and a definition of food safety that mentions the term ‘without hazards’. The results of food safety consumer studies concerning knowledge and practices have shown that consumers are aware of and are thinking about food safety, although there are also many gaps in food safety knowledge and practices that may result in food-borne diseases (Jevšnik et al. 2008a, b; Badrie et al. 2006; Medeiros et al. 2004; Patil et al. 2004; Marklinder et al. 2004; Redmond and Griffith 2003).

Epidemiologic surveillance summaries of food-borne diseases clearly indicate that consumer behaviours, such as the ingestion of raw/undercooked foods, and poor hygienic practices are important contributors to outbreaks of food-borne diseases (Patil et al. 2004). Unusan (2007) reported that people of all ages seem to think they know how to handle food safely, but their self-reported food-handling behaviours do not support this confidence. A review of the consumer food safety literature indicates many gaps that have an impact on food-borne diseases at home (Unusan 2007; Kenedy et al. 2005; Garayoa et al. 2005; Kendall et al. 2004, 2013; Marklinder et al. 2004; Redmond and Griffith 2003; Hillers et al. 2003; Li-Cohen and Bruhn 2002; Yang et al. 2000; Jay et al. 1999a, b; Ergönül 2013). Wilcock et al. (2004) demonstrated that, overall, consumer attitudes towards food safety in general differ accord-

ing to demographic and socio-economic factors, such as gender, age, educational level and economic status. Consumers need to know which behaviours are most likely to result in illness in order to make decisions about food handling and consumption behaviours (Hillers et al. 2003), and then need to be motivated to act on that knowledge as a precondition for behavioural change (Medeiros et al. 2004).

It is very important to investigate consumers' knowledge, behaviour and attitudes towards food safety. Redmond and Griffith (2003) noted that targeted social marketing of food safety strategies is required, because they found differences in perceived responsibility between males and females and consumers from different age groups. They also emphasized that consumers need to perceive interventions as personally relevant for there to be effective food safety education (Griffith and Redmond 2014).

One important perspective is to educate the public about safe food handling and the preparation of foods through different kinds of educational models (Griffith and Redmond 2014), which emphasize hazardous food handling techniques and the microbiological causes of food-borne disease.

Teaching food hygiene on a primary level is crucial, because such behaviour is more easily changed at that stage and also more resistant to alterations later on. Learning about food hygiene and food safety in schools makes it possible to influence children's behaviour with systemic measures, whilst school-based education (on a primary level) as a rule reaches all social classes in developed countries. Children educated in an effective way can also act as facilitators at home through the messages conveyed to family members (Egan et al. 2008) and will hopefully develop to adults who continue to implement proper behaviour at home as caregivers for family members or as employees in the food business. School is, therefore, recognized as an important institution for influencing this kind of behaviour (Moon et al. 1999); it must be noted that the key elements are qualified teachers and quality curriculum. Additionally, the food hygiene content has been restricted in some national curriculums or moved from compulsory to elective subjects and is therefore no longer mandatory for all (Griffith and Redmond 2001; Byrd-Bredbenner et al. 2007). A combination of problems regarding the organization of everyday life in the families and restrictions or even withdrawal of food hygiene content in schools could lead to extreme situations in which children will not be included at all or not in the correct way in food preparation, neither at home nor at school, and will, therefore, not value these topics in their future life (Ovca et al. 2014).

2.8 Good Housekeeping Practice: A key Node in Health Maintenance

To achieve global food safety, consumers should be well informed regarding basic principles of food safety practice at homes (food housekeeping practice), because food safety begins and ends with consumers' daily practices (Raspor and Jevšnik 2008).

To achieve adequate food safety, a coordinated plan is needed for all parties involved in the food chain, including primary and secondary production, distributors, and consumers (Garayoa et al. 2005), which requires a more comprehensive systemic approach. This can be delivered by a food network platform that includes nodes as active points and links as passive points in the food safety management structure. Jones (1998) emphasized that it is extremely important to pay attention to hygienic measures and that they can decrease numerous potential risk factors, which underlines the importance of acknowledging HACCP principles at home (Griffith and Worsfold 1994; Beumer 2003). In the previous 20 years, most of the work has been centred on hazard control in the production sector, but the government has not dedicated the same effort to improving food safety education of consumers. Effective risk communication to inform consumers of the possible health risks of food-borne illnesses and to encourage safer food handling practices in the home is probably the best way to ensure food safety at the consumer end of the food chain (Patil et al. 2005; Griffith and Redmond 2014).

In the classic food chain strategy, all relevant activities are taken for the benefit of human beings but the consumer is located outside the system. The consumer should be an integral part of food safety systems, because he/she is a vital link between retail and home. We expected that a well-informed consumer would start to follow 'Good Housekeeping Practice' (GHKP), which is a selection of the principles and techniques of food storage and preparation at home performed directly by consumer. Given the considerable number of food-borne diseases occurring in domestic food preparation (Ergönül 2013; Kendall et al. 2013), it is obvious that we do not have GHKP, and we neglect the fact that the consumer is crucial link in food supply chain. Consumer behaviour and attitudes towards food safety shows that the levels of understanding, motivation and trust need to be further cultivated. It has been shown that the present maintenance of food safety in the food chain can easily break down because of different kind of barriers or simple misunderstanding. Therefore, a new approach called 'Good Nutritional Practice' (GNP) should be adopted to enhance food safety (Raspor and Jevšnik 2008; Raspor 2008). In all of mentioned practices are HACCP elements that compose HACCP system as main system in food practice today. All practices are partial and are not connected in comprehensive system. For solving the existing barriers in implementing and maintaining food safety system in all steps in food chain, it is necessary to linkup all relevant good practices to the one, named GNP, which could solve many issues in it (Raspor and Jevšnik 2008; Raspor 2008).

Jones (1998) warned against focusing on particular (sensitive) groups and proposed applying HACCP to identify hygiene risks in the home. She suggested drawing up hygiene codes of practices and thus forming the basis of educational material aimed at different target groups. International studies indicated that a significant proportion of food-borne diseases arise from practices in home kitchens (Scott et al. 1982; Bryan 1988; Scott 1996; Wilcock et al. 2004; Patil et al. 2004; Unusan 2007; Jevšnik et al. 2008a, b, c; Nesbitt et al. 2014). Domestic food preparation can negate much of the efforts of primary and secondary food producers to provide safe food

(Oosterom 1998; Jay et al. 1999a, b). The fact is that household food safety education is needed to minimize the risk of exposure to food-borne pathogens.

2.9 Food Safety Management in the Future

As Raspor stated in 2008, food safety is a result of several factors: legislation should establish minimum hygiene requirements; official controls should be in place to check food business operators' compliance; food business operators should establish and operate food safety programmes and procedures. In theory, it seems that we manage food safety completely but practical experiences show some deviations. For that reason, we have to proceed to new solutions that are based on a synthesis of all relevant key factors included in food supply chain. One possibility is to link all relevant good practices in GNP (Raspor 2008; Raspor and Jevšnik 2008).

Currently, we master food safety with different good practices, which are the consequence of human culture, history and lifestyle. If we analyse good practices in the broad range of the food area, we could arrange them in three categories. The first category of good practices is directly connected with food technology (i.e. GMP). The second category is indirectly connected with food issues (i.e. GRP, GEP, GTrP). The third category deals with all the activities regarding consumers' food handling (GHKP). Consumers are currently not connected to food supply chain according to chain principles.

However, it has been shown that present maintenance of food safety in food supply chain can be easily broken down because of different kind of barriers or simple misunderstanding. Therefore, GNP was developed to manage food safety (Raspor 2008; Raspor and Jevšnik 2008). It is important to reconstruct the existent food safety system with GNP, which includes consumers, and that it be based on a model that covers subsystems from other good practices.

New techniques for reducing pathogen contamination in different kinds of food-stuffs are developed every day. It is difficult to cope with all the novelties and innovations since is not always totally clear what is actually new and what is merely an improvement of existing techniques or protocols. The compilations of different authors or authorities around the world are attempting to solve this issue. However, such information can provide a reference for processors worldwide searching for better ways to improve food safety in their plants. The new technologies have to bring significant improvements to the safety of food. Increased public and industry awareness of the new technologies being used could further promote their use, by small and very small plants in particular, towards improving the safety of food products. The new technologies listed should be viewed as information of the current state of the art (Raspor and Jevšnik 2009).

Global food safety will be achieved only when every single link in the food chain systems will master his/her particular area and will trust in the activity of both the previous and following links in the food safety circle 'from farm to table', not ignoring consumer as the one who should be aware of potential risks, proper handling and preparation of food for safe and balanced everyday meal (Raspor and Jevšnik

2008). For this advancement, we need education, training and regular practicing of all the basic principles of food safety.

2.10 Conclusion

Assessing all interactions within food supply chains, we see that many contact points do not have the attention they would deserve. This complexity opens questions: shall we really discuss the future of food safety management in food chain? This implies that we accept linearity as a key principle in current food systems. We know from daily practice that this is not the case. Thus, we shall start to redesign our approach and thinking, and we shall start to think about food supply networks. It is very common that we speak about networking when we speak about people, organizations, companies and various subjects in different areas of expertise.

It seems that the chain approach is slowly fading into history since it focuses primarily on food, food ingredients and food products as passive elements in the food system. These became more evident with the industrial revolution and even more with information revolution. The active player, i.e. the person, who has most important part in the traditional food chain, was pushed aside. People, with many different professions and educations, sometimes far from food, nutrition or health deep professional knowledge, monitor and decide on all actions and reactions in food supply chains. To mitigate this stage of development, it is essential to begin to see both sides passively and actively and simultaneously synchronized to the greatest degree as possible.

With the industrial approach, the primary contact was taken from man by machine. This will not change although the decision is drafted by people, realized by machines and even inspected by machines. This is why a food network active node system must be applied in all practices of current food and nutrition space. In particular is this the issue when we go to international or even global food trade. The challenge question is: When will we include systemic thinking into the practice? Or even more relevant: When we will adopt this practice in flexible thinking?

Finally, it is also important to be aware that people are active twice: once in producing and second in eating food. Do we always have this as primary challenge?

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Chapter 3

Food Safety Aspects Concerning Traditional Foods

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3.1 Introduction

European consumers, in their multicultural diversity, are more and more interested about traditional foods that make food to be a way of communication and socialization, but also a means of having jobs, business, and profit.

Traditional foods are linked to local/regional specificities and cultural and gastronomic heritage (Banterle et al. 2008) and are produced locally through an authentic, traditional process transmitted from generation to generation, using locally raw materials and ingredients. Traditional producers are mainly SMEs with an important role within EU food sector.

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The EU definition of traditional foods shows that traditional means proven usage in the community market for a time period showing transmission between generations. The time period should be the one generally ascribed as one human generation, at least 25 years.¹

Traditional foods are also linked to a positive general image with distinct characteristics and a high sensorial, nutritional as well as safety quality (Almli et al. 2011). They are related to a place, a good taste, freshness, a specific recipe, uniqueness, something traditional, healthy and pleasure.

All published definitions of traditional foods include temporal, territorial, and cultural dimensions through idea of a transmission from generation to generation and elaborative statements about traditional ingredients, traditional composition, and traditional production and/or processing.

In this sense, a definition was formulated within TRUEFOOD project in which: “A traditional food product is a product frequently consumed or associated with specific celebrations and/or seasons, normally transmitted from one generation to another, made accurately in a specific way according to the gastronomic heritage, with little or no processing/manipulation, distinguished and known because of its sensory properties and associated with a certain local area, region or country.”²

Projects were funded at European level on this topic, as the following: TYPIC, DOLPHINS, TRUEFOOD, TRACE, EuroFIR, Tol4FOOD.

Because of interest of consumers in traditional foods and because of specificities of these products, three European schemes for traditional foods protection were developed:

- Protected Designation of Origin (PDO) used to describe an agricultural product or a foodstuff originating in that region, specific place, or country. The quality or characteristics are essentially or exclusively due to a particular geographical environment with its inherent natural and human factors, and the production, processing, and preparation are taken place in the defined geographical area.³
- Protected Geographic Indication (PGI) used to describe an agricultural product or a foodstuff originating in that region, specific place, or country, and which possesses a specific quality, reputation, or other characteristics attributable to that geographical origin, and the production and/or processing and/or preparation are taken place in the defined geographical area (EC Regulation No. 510/2006).
- Traditional Specialties Guaranteed (TSG) means a traditional agricultural product or foodstuff recognized by the European Community for its specific character.⁴

¹Council Regulation (EC) No. 509/2006 on agricultural products and foodstuffs as traditional specialties guaranteed.

²TRUEFOOD—“Traditional United Europe Food”—an Integrated Project financed by the European Commission under the sixth Framework Program.

³Regulation (EC) No. 510/2006.

⁴Council Regulation (EC) No. 509/2006.

3.2 Food Safety Concerns in Traditional Processing

Safety of food means safety of raw materials, of methods of processing, transport, distribution, and retail. Safety of food includes food hygiene that is consisting in five key principles according to World Health Organization (WHO): preventing contaminating food with pathogens and cross-contamination by separation raw and cooked foods, using the appropriate time and temperature for cooking food as well as storing them and using safe water and cooked materials.

ISO 22000 is a standard developed by the International Organization for Standardization dealing with food safety, which specifies the requirements for a food safety management system that involves interactive communication, system management, prerequisite programs, and HACCP principles. In 2003, WHO and International Food and Agriculture Organisation under United Nations (FAO) published the *Codex Alimentarius* which serves as a guideline to food safety through Codex standards.

Traditional foods have an assumed “history of safe use” in the country in which they are used. Although, no food can be considered to be absolutely safe under all circumstances, individuals may tolerate the same food differently. Traditional foods are considered safe within the context of its traditional use by the consuming population group and prevailing dietary, preparation and processing regimes and cultural practices.

Foods prepared and used in traditional ways have therefore been considered to be safe for the consuming population on the basis of long-term human experience. However, foods, in general, may contain natural toxicants, anti-nutrients, or allergens that would cause concern if they are present above accepted levels or consumed by sensitive individuals.

Traditional foods are associated with high quality and healthy food. By increasing the interest of more and more consumers in traditional food, food safety can be an important issue in this sector. SMEs, the most important player on the traditional foods market, often lack the facilities or capital to establish the best practices for microbiological or toxicological safety assurance systems and cannot afford to meet, in this sense, the needs of scale-up production and processing systems in traditional foods.

3.2.1 EU Food Safety Legislation

EU Hygiene rules are established for all kind of food even are conventional, organic, traditional, or novel with some specific requirements for each of them, especially for the way of producing and labelling.

The General Principles of Food Law (Articles 5–10) entered into force the 21th of February 2002 with the general framework established by Regulation EC/178/2002.

General Objectives. The food law aims at ensuring a high level of protection of human life and health taking into account the protection of animal health and welfare, plant health, and quality of the environment. This integrated “farm to fork” approach is now considered a general principle for EU food safety policy. Food law, both at national and EU level, establishes the rights of consumers to safe food and to accurate and honest information. The EU food law aims to harmonize existing national requirements in order to ensure the free movement of food and feed in the EU.

The food law recognizes the EU’s commitment to its international obligations and will be developed and adapted taking international standards into consideration, except where this might undermine the high level of consumer protection pursued by the EU.

Risk Analysis. The Regulation establishes the principles of risk analysis in relation to food and establishes the structures and mechanisms for the scientific and technical evaluations which are undertaken by the European Food Safety Authority (EFSA).

Depending on the nature of the measure, food law, and in particular measures relating to food safety must be underpinned by strong science. The EU has been at the forefront of the development of the risk analysis principles and their subsequent international acceptance. Regulation EC 178/2002 establishes in EU law that the three interrelated components of risk analysis (risk assessment, risk management, and risk communication) provide the basis for food law as appropriate to the measure under consideration. Clearly not all food law has a scientific basis, e.g., food law relating to consumer information or the prevention of misleading practices does not need a scientific foundation.

Scientific assessment of risk must be undertaken in an independent, objective, and transparent manner based on the best available science.

Risk management is the process of weighing policy alternatives in the light of results of a risk assessment and, if required, selecting the appropriate actions necessary to prevent, reduce, or eliminate the risk to ensure the high level of health protection determined as appropriate in the EU.

In the risk management phase, the decision makers need to consider a range of information in addition to the scientific risk assessment. These include, for example, the feasibility of controlling a risk, the most effective risk reduction actions depending on the part of the food supply chain where the problem occurs, the practical arrangements needed, the socioeconomic effects and the environmental impact. Regulation EC/178/2002 establishes the principle that risk management actions are not just based on a scientific assessment of risk but also take into consideration a wide range of other factors legitimate to the matter under consideration.

Transparency. Food safety and the protection of consumer interests are of increasing concern to the general public, nongovernmental organizations, professional associations, international trading partners, and trade organizations. Therefore, the Regulation establishes a framework for the greater involvement of stakeholders at all stages in the development of food law and establishes the mechanisms necessary to increase consumer confidence in food law.

This consumer confidence is an essential outcome of a successful food policy and is therefore a primary goal of EU action related to food. Transparency of legislation and effective public consultation are essential elements of building this greater confidence. Better communication about food safety and the evaluation and explanation of potential risks, including full transparency of scientific opinions, are of key importance.

Food Additives. Food additives are:

- Sweeteners to sweeten foods or in tabletop sweeteners;
- Colors adding or restoring color in a food;
- Preservatives prolonging shelf life of foods by protecting them against deterioration by microorganisms;
- Antioxidants prolonging shelf life of foods by protecting them against oxidation, e.g., fat rancidity and color changes;
- Stabilizers to maintain the physicochemical state of a foodstuff;
- Emulsifiers to maintain the mixture of oil and water in a foodstuff;
- Added to food for technological purposes in its manufacture, processing, preparation, treatment, packaging, transport or storage, food additives become a component of the food. EU legislation describes 26 different technological functions.

All additives in the EU must be authorized and listed with conditions of use in the EU's "positive" list based on:

- A safety assessment;
- The technological need;
- Ensuring that use of the additive will not mislead consumers.

Regulation EC 1333/2008 sets the rules on food additives: definitions, conditions of use, labelling, and procedures.

It contains:

- Technological functions of food additives: Annex I;
- Union list of food additives approved for use in food additives and conditions of use: Annex II;
- Union list of food additives approved for use in food additives, food enzymes and food flavorings, and their conditions of use: Annex III;
- Traditional foods for which certain Member States may continue to prohibit the use of certain categories of food additives: Annex IV;
- Additives labelling information for certain food colors: Annex V.

Food labelling. Legislation is applicable until 12 December 2013 and covers:

- General rules on food labelling;
- Rules for specific foods, e.g., beef or chocolate.

Directive 2000/13/EC on labelling, presentation, and advertising of foods is the main EU legislation on the subject.

EU rules after 13 December 2013: The new EU Regulation 1169/2011 on the provision of food information to consumers considerably changes existing legislation on food labelling including:

- Nutrition information on processed foods;
- Origin labelling of fresh meat from pigs, sheep, goats, and poultry;
- Highlighting allergens, e.g., peanuts or milk in the list of ingredients;
- Better legibility, i.e., minimum size of text;
- Requirements on information on allergens also cover non-prepacked foods including those sold in restaurants and cafés.

Health and Nutrition Claims. In December 2006, the Regulation (EC) No 1924/2006 on nutrition and health claims made on foods was adopted by the Council and Parliament. For the first time, this Regulation lays down harmonized rules across the European Union for the use of nutrition claims such as “low fat,” “high fiber,” or health claims such as “reducing blood cholesterol.”

This Regulation foresees implementing measures to ensure that any claim made on foods’ labelling, presentation, or marketing in the European Union is clear, accurate, and based on evidence accepted by the whole scientific community. Consequently, foods bearing claims that could mislead consumers will be eliminated from the market. In addition, in order to bear claims, foods will have to have appropriate nutrient profiles which will be set. This will enhance the consumers’ ability to make informed and meaningful choices.

Further, this Regulation respects fair competition and protects innovation in the area of foods. It also facilitates the free circulation of foods bearing claims as any food company will be able to use the same claims on its products everywhere in Europe.

Many traditional foods have resisted on the market because of a high nutritive value or for providing well-being evidence in people. Some European projects in bioactive compounds were developed to find the benefits of some local/regional traditional foods (e.g., bioactive compounds in food from the Black Sea area, subject included in FP6).

Nutrition Labelling is governed by Council Directive 90/496/EEC, as amended by Commission Directives 2003/120/EC and 2008/100/EC.

In January 2008, the Commission adopted a proposal for a Regulation of the European Parliament and of the Council on the provision of food information to consumers to update and revise the Community legislation on general food labelling and nutrition labelling. The proposals for the amendment of the nutrition labelling aspects of the Community rules took into account consultations in 2003 and 2006, and impact assessments prepared in 2004 and 2007.

Food Contaminants. The basic principles of EU legislation on contaminants in food are in Council Regulation 315/93/EEC of 8 February 1993:

- Food containing a contaminant to an amount unacceptable from the public health viewpoint, and in particular at a toxicological level, shall not be placed on the market;

- Contaminant levels shall be kept as low as can reasonably be achieved following recommended good working practices;
- Maximum levels must be set for certain contaminants in order to protect public health.

Maximum levels for certain contaminants in food are set in Commission Regulation (EC) No 1881/2006. This Regulation entered into force on 1 March 2007. Maximum levels in certain foods are set for the following contaminants: nitrate, mycotoxins (aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins), metals (lead, cadmium, mercury, and inorganic tin), 3-MCPD, dioxins and dioxin-like polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (benzo[*a*]pyrene).

Pesticide Residues. Directive 91/414/EEC laid down the evaluation, authorization, and approval of active substances at EU level and national authorizations of products—shows in its Annex 1, the list of Approved substances.

Food contact materials are materials and articles intended to come into contact with foods such as:

- Packaging materials;
- Cutlery and dishes;
- Processing machines;
- Containers;
- Materials and articles in contact with water for human consumption.

The term does not cover fixed public or private water supply equipment.

New legislation:

- Regulation EU 1183/2012 for plastic materials and articles intended for contact with food amending Regulation (EU) No 10/2011;
- Corrigendum to Regulation EU 1183/2012 for plastic materials and articles intended for contact with food amending Regulation (EU) No 10/2011;
- Regulation EU 1282/2011 for plastic materials and articles intended for contact with food amending Regulation (EU) No 10/2011;
- Regulation EU 321/2011 for restricting Bisphenol A use in plastic infant feeding bottles;
- Regulation EU 284/2011 for import procedures for polyamide and melamine plastic kitchenware from China and Hong Kong.

Food Hygiene. In the White Paper on Food Safety, the Commission outlined a radical revision of the Community's food safety hygiene rules, under which food operator's right through the food chain will bear primary responsibility for food safety. The new regulations merge, harmonize, and simplify detailed and complex hygiene requirements previously contained in a number of Council Directives covering the hygiene of foodstuffs and the production and placing on the market of products of animal origin. They innovate in making a single, transparent hygiene policy applicable to all food and all food operators right through the food chain "from the farm to the fork," together with effective instruments to manage food safety and any future food crises throughout the food chain.

Community legislation covers all stages of the production, processing, distribution, and placing on the market of food intended for human consumption. “Placing on the market” means the holding of food for the purpose of sale, including offering for sale, or any other form of transfer, whether free of charge or not, and the sale, distribution, and other forms of transfer themselves.

The new hygiene rules were adopted in April 2004 by the European Parliament and the Council. They became applicable on 1 January 2006. They are provided for in the following key acts:

- Regulation (EC) 852/2004 on the hygiene of foodstuffs, 29 April 2004
- Regulation (EC) 853/2004 laying down specific hygiene rules for food of animal origin, 29 April 2004
- Regulation (EC) 854/2004 laying down specific rules for the organization of official controls on products of animal origin intended for human consumption, 29 April 2004
- Directive 2004/41/EC repealing certain Directives concerning food hygiene and health conditions for the production and placing on the market of certain products of animal origin intended for human consumption and amending Council Directives 89/662/EEC and 92/118/EEC and Council Decision 95/408/EC, 21 April 2004

Inspection fees—Articles from 26 to 29 of Regulation EC no 882/2004.

Article 65 of Regulation (EC) No 882/2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health, and animal welfare rules requires the Commission to submit a report to the European Parliament and the Council to review the experience gained from the application of the Regulation itself.

The report is to consider, among other things, the issue of inspection fees or charges (Articles from 26 to 29 of the Regulation).

Inspection fees or charges imposed on feed and food business operators are among the tools that Member States can use to make adequate financial resources available for organizing official controls.

Geographical indications and traditional specialties. Three EU schemes known as PDO (protected designation of origin), PGI (protected geographical indication), and TSG (traditional speciality guaranteed) promote and protect names of quality agricultural products and foodstuffs.

More information can be found in the following link: http://ec.europa.eu/agriculture/quality/schemes/index_en.htm.

Traditional specialties guaranteed of food and agro food products are governed by the following laws:

- Regulation (EU) No 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs
- Commission Regulation (EC) No 1216/2007 of 18 October 2007 laying down detailed rules for the implementation of Council Regulation (EC) No 509/2006 on agricultural products and foodstuffs as traditional specialties guaranteed

New framework for Quality schemes in agriculture: Regulation (EU) No 1151/2012 of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs.

Guaranteeing **quality to consumers** and a **fair price for farmers** are the twin aims of the new Quality Regulation on quality schemes for agricultural products and foodstuffs that entered into force in the beginning of 2013.

It encourages the diversification of agricultural production, protects product names from misuse and imitation, and helps consumers providing information on product characteristics and farming attributes.

The new Regulation on quality schemes for agricultural products and foodstuffs achieves a **simplified regime for several quality schemes** by putting them under one single legal instrument. Furthermore, it creates a more **robust framework for the protection and promotion of quality agricultural products**.

The key elements of the new Regulation include:

- More coherence and clarity to the EU quality schemes
- A reinforcement of the existing scheme for protected designations of origin and geographical indications (PDOs and PGIs)
- Overhauling the TSG scheme
- Laying down a new framework for the development of optional quality terms to provide consumers with further information, it creates and protects the optional quality term “mountain product.”

This Regulation establishes for PDOs and PGIs (excluding wines, aromatized wines and spirits which remain covered by separate legislation) the following:

- Faster registration procedures as in particular the opposition period is halved from 6 to 3 months
- The rules on controls are clarified
- The use of the PDO and PGI logos will become compulsory for products of EU origin from 4 January 2016 onwards
- A legal basis for inserting third country GI protected through bilateral agreements into the EU register is created
- A legal basis for financing the defense of the EU logos is established
- The role of producer groups is recognized

3.2.2 The Main Hazards in Traditional Processing

The main hazards for traditional foods are the same for other foods (conventional, organic, and so on) and are coming from outside processing area or from processing technological steps. Hazards that are coming from outside processing area usually are coming through raw materials and ingredients, water, and food contact materials. During the technological processing, other hazards can occur by using inappropriate technological parameters (time, temperature, and pressure) or by different unexpected causes (as different accidental contamination with copper or other metal chips from the equipments, lubricants, cleaning and sanitizing agents).

3.2.2.1 Hazards Coming from Outside Traditional Foods Processing (Raw Materials, Ingredients, Others)

The hazards coming from outside processing area are both chemical and microbiological hazards and are influenced by how much traditional foods producers are informed and trained in food safety and the quality of supplier services.

These hazards have chemical or microbiological origin, and it very much depends on the type of raw material, ingredients, and food contact materials.

By ISO 22000 standard implementation and HACCP quality system, most of these hazards are minimizing or even removed.

Some high incidence hazards are summarized below.

Chemical Hazards

Mycotoxins are mostly found in cereals and cereal derivatives, milk and dairy products, dried fruits, spices, and so on. Mycotoxins are secondary metabolites produced by molds either in crops during unfortunate weather conditions or during storage of crops or foods under humid conditions. There were found about 400 mycotoxins but only several are very toxic for human body. Some of these are:

Fumonisins are mycotoxins produced by genera *Fusarium*. Fumonisin B1 is the most prevalent and toxic of the fumonisins; it can appear especially in maize and maize-based products, and it is produced by *Fusarium verticillioides*. Maize can be contaminated during growth, storage, and processing and fumonisins are heat stable, light stable, water soluble, poorly absorbed, poorly metabolized, and rapidly excreted by animals.

Another mycotoxins are the four major **aflatoxins** called B₁, B₂, G₁, and G₂ based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. Aflatoxin B₁ is the most potent natural carcinogen known and is usually the major aflatoxin produced by toxicogenic strains. Aflatoxins are difuranocoumarin derivatives produced by many strains of *Aspergillus flavus*, a common contaminant in agriculture, and *Aspergillus parasiticus*; Natural contamination of cereals, oilseeds, nuts, dried fruits, spices etc. is a common occurrence. Sometimes crops become contaminated with aflatoxin in the field before harvest, where it is usually associated with drought stress or during storage under specific conditions that favor mold growth. Dairy products can be indirectly contaminated by aflatoxin M₁, less carcinogenic hydroxylated form of B₁, which is metabolically biotransformed in cow's body. The International Agency for Research on Cancer has classified aflatoxin B₁ as a group I carcinogen.⁵

Ochratoxin A is a metabolite of *Aspergillus ochraceus*. It has been found in barley, oats, rye, wheat, etc. There is also concern that ochratoxin may be present

⁵International Agency for Research on Cancer. 1982. The evaluation of the carcinogenic risk of chemicals to humans. IARC Monograph Supplement 4. International Agency for Research on Cancer, Lyon, France.

in certain wines, especially those from grapes contaminated with *Aspergillus carbonarius*. The International Agency for Research on Cancer has rated ochratoxin as a possible human carcinogen (category 2B) (Beardall and Miller 1994). Ochratoxin can be transmitted to meat through improper quality of fodder.

Citrinin was identified in *Penicillium* species and several *Aspergillus* species (e.g., *Aspergillus terreus* and *Aspergillus niveus*). Citrinin can act synergistically with ochratoxin A and was found in wheat, oats, rye, corn, barley, rice but also in naturally fermented sausages. There is not known very well its significance for human health.

The **ergot alkaloids** found in the sclerotia of *Claviceps* species, which are common pathogens of various grass species and rye.

Patulin, 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one, is produced by several genera: *Penicillium* (e.g., *Penicillium patulum*/*Penicillium griseofulvum*), *Aspergillus*, and *Byssosclamyces*. *Penicillium expansum*, the blue mold that causes soft rot of apples, pears, cherries, and other fruits, is recognized as one of the most common mold in patulin contamination. Patulin is regularly found in unfermented apple juice, and it does not survive the fermentation into cider product. Joint Food and Agriculture Organization-World Health Organization Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for patulin of 0.4 mg/kg of body weight per day (Trucksess and Tang 2001).

The **trichothecenes**, a family of more than 60 sesquiterpenoid metabolites produced by a number of fungal genera, including *Fusarium*, *Trichoderma*, *Trichothecium*, and others, are commonly found as food and feed contaminants. Diacetoxyscirpenol, deoxynivalenol, and T-2 are the best studied of the trichothecenes produced by *Fusarium* species. Deoxynivalenol is one of the most common mycotoxins found in grains: barley, corn, rye, safflower seeds, wheat, and mixed feeds.

Zearalenone, ZEN, (6-[10-hydroxy-6-oxo-*trans*-1-undecenyl]-B-resorcylic acid lactone) is a secondary metabolite from *Fusarium graminearum*. The zearalenones are also biosynthesized by *Fusarium culmorum*, *Fusarium equiseti*, and *Fusarium crookwellense*. All these species are regular contaminants of cereal crops worldwide. The recommended safe human intake of ZEN is estimated to be 0.05 µg/kg of body weight per day, but in foodstuffs the level of ZEN is not yet regulated anywhere.

Dioxins are mainly found in fatty tissues of animals. Dioxins, a group of chemically related compounds, are found throughout the world in the environment, being persistent environmental pollutants. Over 90 % of human exposure to dioxins is through food, mainly through animal origin food.

The name “dioxins” is often used for the family of structurally and chemically related polychlorinated dibenzo para dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Certain dioxin-like PCBs with similar toxic properties are also included under the term “dioxins.” Four hundred nineteen types of dioxin-related compounds have been identified but only about 30 of these are considered to have significant toxicity, with TCDD (2,3,7,8-tetrachlorodibenzo para dioxin) being the most toxic.

Dioxins are coming from different industrial processes (e.g., paper or pesticides manufactures and so on) through water or soil contaminated but can also result from natural processes (e.g., forest fires) or by burning dried herbs or leaves when countryside people clean the yards (or backyard burning of trash). In this way, the soil is contaminated and domestic animals can cumulate dioxins in their fatty tissues. Exposure of humans to high levels of dioxins may result in skin lesions, altered liver function, impairment of the immune system, or even several types of cancer.

Concerning animal origin raw materials, it is important to know the hazard of some diseases that animals can have and also the way of treatments of these diseases. In this sense, other chemical hazards in food are **veterinary pharmaceuticals or drug-resistant pathogens** (including antibiotics as oxacillin, methicillin, oxazolidinones, fluoroquinolone, and so on) which are used in animal treatments and could be transmitted to food if the animal is slaughtered after inappropriate period of time (less few weeks) from a specific treatment.

Depending on the level of quantity of traditional foods processed, vegetal origin raw materials from small or large farms produced in conventional or organic systems agriculture can be used and they can introduce on the food chain different type of contaminants.

Agrochemicals as insecticides, herbicides, fungicides, fertilizers, rodenticides and plant growth regulators, and nitrates can also be chemical hazards and contaminate the raw materials which will be further used for producing food. If they are not used according with the best agricultural practices, it is possible to be found residues of these chemicals in agro foodstuff.

Heavy metals are sometimes polluting soil and air, very much depending on the industrial level of the area. The main hazard from heavy metals is associated with lead, cadmium, mercury, and arsenic contamination. Food is the most important source of cadmium, mercury, lead, and arsenic exposure. People are primarily exposed to mercury via fish, especially shark, swordfish and tuna or pike, walleye and bass.

Packaging materials can come with several chemical hazards as the following substances: antimony, tin, lead, perfluorooctanoic acid (PFOA), semicarbazide, benzophenone, isopropyl thioxanthone (ITX), Bisphenol A. Sometimes, traditional food are coming with traditional ways of packaging but, the testing of any food contact material is necessary in order to assure food safety and food integrity.

Microbiological Hazards

The microbes are everywhere: in soil, water, air. These are very useful for the environment, but some of it are pathogen or toxicogen as was described already for introducing mycotoxins. Yeast and molds that are included in fungi category and bacteria, viruses and protozoa, even prions,⁶ with a higher pathogenity and harmful effect in human health are the main category of microorganisms that can be hazards

⁶EFSA (2013) The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011. EFSA Journal 11(4):3129, <http://www.efsa.europa.eu/en/efsajournal/doc/3129.pdf>.

for food area. These microbes are hazards because they can survive, multiply, and spread through cross-contamination.

Depending on agrifood raw materials (animal or vegetal origin), some microbiological hazards can be: *Salmonella*, *Yersinia enterocolitica*, *Campylobacter*, *Escherichia coli*, *Shigella*, *Vibrio parahaemolyticus*, *Aeromonas hydrophila*, *Clostridium perfringens* and *Bacillus cereus* which are multiplying, invading the host body or *Staphylococcus aureus* and *Clostridium botulinum* which are producing very harmful toxins.

3.2.2.2 Hazards Coming from Traditional Foods Way of Processing

Diethyl carbamate is formed during fermentation process and it can be found in wines, beer, and other fermented alcoholic beverages, often providing the most significant part as well as in bread and other fermented grain products, orange juice, and commonly consumed foods. Ethyl carbamate is not acutely toxic to humans, as shown by its use as a medicine. Heating (e.g., cooking) the beverage increases the ethyl carbamate content. Due to the omnipresence of diethyl carbamate during fermentation process, all people have background exposure, which is not expected to affect human health.

Heterocyclic aromatic amines (HCAs) and Polycyclic aromatic hydrocarbons (PAH) are chemicals formed when muscle meat, including beef, pork, fish, and poultry, is cooked using high-temperature methods, such as pan frying or grilling directly over an open flame (Cross and Sinha 2004). HCAs are named “cooked food mutagens.” HCAs is a group of 20 chemical compounds, some of it is labelled to be carcinogenic to humans. The most potent carcinogenic of the HCAs, MeIQ, (2-Amino-3,4-dimethylimidazo[4,5-f]quinoline), is almost 24 times more carcinogenic than aflatoxin, a mycotoxin produced by mold (Sugimura 1997).

PAH are formed in appreciable amount during food processing, roasting, frying, baking, smoking, and barbecuing of food.⁷ There are 33 non-heterocyclic PAH, with the most known compound benzo[a]pyrene.

The reduction of heterocyclic amines and the concomitant mutagenic activity is possible by eliminating the known precursors of heterocyclic amine formation. J.S. Felton et al. (1994) showed that increasing of the temperature processing of beef from 200 to 250 °C increases the mutagenic activity about threefold.

Acrylamide is formed in appreciable amount during heating treatments of carbohydrate-rich food (potatoes, cereals, coffee). It was found acrylamide in certain foods that were heated to a temperature above 120 °C, but not in foods prepared below this temperature (Stadler et al. 2002). Potato chips and French fries were found to contain higher levels of acrylamide compared with other foods (FAO 2008). The World Health Organization and the Food and Agriculture Organization of the United Nations stated that the levels of acrylamide in foods pose a “major concern”

⁷ Biology and Biotechnology Research Program, L-452, Lawrence Livermore National Laboratory, P.O. Box 808, Livermore, CA 94551-9900, Running title: Microwave pretreatment of beef.

and that more research is needed to determine the risk of dietary acrylamide exposure.

Asparagine is an amino acid that is found in many vegetables, with higher concentrations in some varieties of potatoes. When heated to high temperatures in the presence of certain sugars, asparagine can form acrylamide. High-temperature processing by frying, baking, or broiling leads to produce acrylamide (Mottram et al. 2002), while boiling appears less likely to do so. Longer cooking times can also increase acrylamide production when the cooking temperature is above 120 °C.

Acrylamide has been found in products such as potato crisps, French fries, bread, biscuits, and coffee. It was first detected in foods in April 2002.⁸

Nitrosamine is produced from nitrites and amines, which often occur in the form of proteins. Under acidic conditions (i.e., human stomach) or at high temperatures, as in frying, nitrosamines can occur. Nitrosamines can occur in many foodstuffs, especially beer, fish, and fish by-products and also in meat and cheese products preserved with nitrite pickling salt. Cured meats can contain nitrosamines because meats contain amines, and sodium nitrite which is added to cured meats as a preservative. The very high cooking temperatures used to fry bacon lead to nitrosamine formation. Removal of sodium nitrite would prevent nitrosamine formation, but it might also increase the risk of botulism poisoning. Sodium nitrite and sodium chloride together are particularly effective against *Clostridium botulinum*. The solution to the dilemma was to limit the addition of sodium nitrite to 120 parts per million (ppm), the lowest level found to be effective in controlling growth and toxin production by *Clostridium botulinum* (Moore 2004).

Benzene is ubiquitous in the atmosphere. It has been identified in air samples of both rural and urban environments and in indoor air. Although a large volume of benzene is released to the environment, environmental levels are low because of efficient removal and degradation processes. The U.S. Food and Drug Administration funded for 5 years period a study to determine the amount of volatile organics in food from 1996 to 2000. Benzene was found in over 40 types of foods. Foods with the highest level of benzene were ground beef (maximum 190 ppb), raw bananas (maximum 132 ppb), carbonated cola (maximum 138 ppb), and coleslaw with dressing (maximum 102 ppb).⁹

During food processing, food can be accidentally contaminated by **copper or other metal chips** and **lubricants** coming from the equipments and its maintenance and **cleaning, and sanitizing agents** or another **chemical substances used against pests and rodents**.

Trans fatty acids (TFA). Unsaturated fatty acids are fatty acid molecules containing at least one double bond. They can be classified as *cis* or **trans** according to the structure of the double bonds within the molecule. Most unsaturated fats in the

⁸Acrylamide in food—EFSA to publicly consult on draft opinion in 2014, News Story, 15 July 2013.

⁹Greenberg, A, Weisel, CP, Benzene, POTENTIAL FOR HUMAN EXPOSURE, 2006, USDA, U.S. Department of Agriculture.

diet exist in the cis form while a small proportion can be found in the trans form. TFA originate in foods from three main sources:

- Bacterial transformation of unsaturated fatty acids in the rumen of ruminant animals such as cows and sheep (passing to the fat, meat, and ruminant's milk)
- Industrial hydrogenation of oils for margarine producing
- Heating and frying of oils at high temperatures

Thus, TFA are present in ruminant fat and products derived from their meat and milk, in some fat spreads and bakery products, such as crackers, pies, cakes, and biscuits, and fried foods.

Microbiological hazards in processing are the same as for raw materials including also *Listeria monocytogenes* and *Clostridium botulinum* which are the most harmful bacteria that can contaminate food during processing and storage.

More, into the processing area, biofilms occur when bacteria form a slime layer upon a surface and provide an environment for pathogens to proliferate. The adhesion of pathogenic bacteria to a biofilm is a food safety hazard because the biofilm can detach and become a significant source of food contamination.

3.3 Some Technological Solutions for Making Traditional Foods Safer in Longer Time

Traditional products have an extremely high intrinsic value, both culturally and economically, not only due to their highly appreciated organoleptic properties, but also because of their manufacturing technologies that are the result of hundreds of years of evolution of ancestral practices through empirical experience of several generations. These products have gained economic importance, to the point of being considered a catalyst for the development and a factor of economic changes in some regions. They are important in the dissemination of local tourism and in an effort to reverse the trend of depopulation that had been accentuated in recent decades in various regions, valuing family labor by creating local employment.

However, the high organoleptic and nutritional quality of these foods is not sufficient to ensure their safety. Food safety is a fundamental right of consumers and, as such, it should be the main concern of the food industry. Traditional products are generally considered safe by the consumers, as, with a few exceptions, there are not many known cases of foodborne diseases attributed to the consumption of these products. Empirical knowledge of producers and a set of technologies that, while not having as the main objective, the quality of products, have nevertheless, contributed to this safety. It is however worth noting that foodborne outbreaks associated with the consumption of these products have occurred, e.g., attributed to *Escherichia coli* and *Listeria monocytogenes*, respectively, in fermented sausages and in soft cheeses (Bille et al. 2006). Cases of botulism have also been attributed to the consumption of traditional meat products. Moreover, several foodborne pathogens have been isolated from these products worldwide.

Beyond the obvious consequences inherent to foodborne diseases in terms of mortality and morbidity and financial costs directly associated with any outbreak, there are other consequences associated with diseases caused by the consumption of these types of products. Thus, an accident caused by a traditional product from a particular region will have an impact not only on the acceptance of the specific brand but also on products from that region and also on the product category. At a time when the concept of “traditional” is establishing itself as an option for the modern consumer, this kind of problem can be disastrous.

Even if it is compulsory to follow the authentic recipe and technology, some small innovation would be necessary, for example in packaging, for better preservation of the traditional foods in order to meet consumer’s demands in having larger quantities of traditional foods on the market.

Consumer’s demands are directed to traditional foods generally produced in small farms, but the market trends are the opposite: market globalization. This is a challenge for small and medium traditional food producers because a traditional food from the south of the country may be requested in the north of the country or even abroad. This means that food safety must be assured for a longer time. This implies that technological innovations must be implemented in the companies always assuring the traditional methodologies, raw materials, etc.

Knowing that traditional foods producers cannot make big investments in their companies, these are some of the technologies that may play an important role in assuring food safety and increasing shelf life of traditional foods. All these technologies are not effective if the raw material is contaminated or if the elaboration process itself produces food contaminants:

3.3.1 Vacuum Packaging

The first protective atmosphere packaging method ever commercially used was the vacuum packaging (VP). It is a really simple system, consisting in evacuating the air inside the container. If the process is made properly, the residual amount of oxygen is lower than 1 %. The packaging material, due to the low inner pressure, fixes to the food product. This packaging material must have a very low gases and steam permeability. Initially, VP was limited to red meats, cured meats, hard cheeses, and ground coffee. However, at present it is applied to a wide variety of food products.

The most important innovation in VP is the SKIN technology. It is one of the fastest growing areas of food packaging. Skin Packing is where a highly transparent film is sealed to the top of a pre-made tray, but before the film is sealed to the tray a vacuum is drawn and the film is sucked down to follow the contours of the tray and its contents. This technology is used for both fresh and frozen products and can greatly extend the shelf life of many food types.

3.3.2 *Modified Atmosphere Packaging*

Modified atmosphere packaging (MAP) consists in the evacuation of the air inside the container and the injection of one gas or a mixture of gases adequate to the food product requirements.

If food with an important metabolic activity (like fruits or vegetables) is packaged MA, it is essential to use materials with selective permeability. Otherwise the shelf life of the food products is very short. The structure of these polymeric layers allows gas exchange between the container's headspace and the outside atmosphere. In other types of food, changes in the inner atmosphere are due to enzymatic reactions and high barrier films are selected because gases emissions are really low.

In ready-to-eat meals, it is advised to work with atmospheres with 20–40 % CO₂ and the rest N₂.

3.3.3 *Active Packaging*

Active packaging (AP) of foods is one of the most innovative ideas that has been introduced the last years. It is defined as a kind of package that changes packaging conditions during the product shelf life, improving its safety, or sensory characteristics and maintaining its quality.

Active component of the container could be part of the packaging material or some other material added to it. Consequently, we can mention several possibilities. The “classic” method is the usage of sachets or bags that contain the active product (e.g., oxygen absorber or drying material) and that are generated within the containers. These sachets must be made of a permeable material in order to allow active compound activity and to prevent it from being in direct contact with food. They must also be very resistant to avoid breaks and must be labelled correctly to prevent their consumption by accident.

In other cases, active compounds are incorporated into the container layers either synthetic or edible. This technique allows the active component to be in contact with all the surface of the product and not only with a restricted area as it is the case of sachets. Sometimes direct contact between active compound of the container and the product is necessary. Otherwise (in the case of gas disposal) it is enough to have it on a multilayer film, leaving a permeable material in contact with the product. This is usually the case of synthetic films. Edible coating and films have been used traditionally to improve the appearance and preservation of foods such as confectionery (chocolate), fruit (wax), and cold meat (natural guts). Due to active packaging development, these films have become more popular as nutrient conductors, antimicrobial agents, oxygen absorbers, etc. since with them it is easier to control component spreading than with synthetic ones.

Furthermore, there are cases where active materials are not added to the package but appear as labels, inks, and polishes on its surface. One example is the time/temperature indicator.

Antimicrobial and antioxidant packaging (AM/AO) is one of the several packaging application of active packaging on foods. It is a packaging system that can kill or inhibit microorganism and pathogens that contaminate food and prevents darkening and smells. This is done by adding antimicrobial/antioxidant agents to the packaging system and/or using antimicrobial polymers that comply with traditional packaging.

When the packaging system has antimicrobial activity, it inhibits or prevents microbe growth due to extending delay period, reducing the growth rate and decreasing the number of living microorganisms.

Material used in AM/OM food packaging changes the food product condition in order to increase or extend its shelf life and/or improve its microbiological safety and/or improve its sensory characteristics.

The aims of conventional food packaging are to extend shelf life, keep quality, and ensure health control. Antimicrobial packaging is specially designed to control microorganisms that usually affect the above-mentioned objectives. As a result, some food products which are not affected by microbe decaying or contamination may not need antimicrobial packaging. But most foods are perishable. As a consequence, the main objectives of an antimicrobial packaging system are: to guarantee food safety, to keep quality of products, and to extend shelf life, which is the reversed order of what conventional packaging aimed at. Nowadays, food safety is a highly important issue and antimicrobial packaging could have an important role in this field.

AM/AO packaging technology could be classified as barrier technology, where the combination of various effects (storage temperature, moisture control, etc.), controls, and keeps the product within certain quality measures and food safety requirements.

3.3.4 Freezing

Food freezing is a preservation method based on the solidification of the water contained in food in order to stop or slow down bacterial and enzymatic processes that spoil food.

There are different freezing technologies:

- Mechanical refrigeration systems: They use like freezing medium cold air, a liquid, or cold surfaces.
- Cryogenic Freezers: They use carbon dioxide, liquid nitrogen, or Freon in direct contact with food.

For the selection of the appropriated freezing technology, the following points have to be taken into account:

- Freezing velocity required by the food product
- Food size, shape, and packaging system
- Continuous or discontinuous

If the freezing technology is appropriated for the product, its sensorial properties are maintained and shelf life improved. Now, it is a technology widely used for high added value products like bakery, desserts, frozen dishes, fruits, and vegetables.

An inappropriate application of the freezing technology may produce some undesirable effects like: changes in texture, pH, viscosity and color, losses of vitamins.

3.3.5 Use of Bacteriocins to Increase Microbiological Safety of Traditional Products

Despite the implementation of several control measures in the food industry, such as effective sanitation processes, or good manufacturing practices (GMPs) or HACCP program, to reduce the risk of food contamination, foodborne outbreaks have occurred. Thus, the development of food preservation techniques is of great importance to control foodborne pathogens, such as *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella* spp., *L. monocytogenes*, or *E. coli*. Chemical preservatives are often added to inhibit the growth of bacteria; however, consumers' awareness of chemical hazards and an increasing demand for more "natural" and less processed products have instigated the development of novel bio-preservation techniques.

Some bacterial species, such as the Lactic Acid Bacteria (LAB), have an important role in the preservation and fermentation processes of several traditional products, as they are naturally present in the raw materials and processing environment. Some species of LAB (e.g., *Lactobacillus sakei*, *Lactococcus lactis*, *Pediococcus*) are frequently used as starter cultures to enhance the organoleptic characteristics of the product; furthermore, some of these bacteria present a bio-preservative activity (Albano et al. 2009). Specific properties of LAB are their GRAS (generally regarded as safe) status and their ability to produce inhibitory compounds effective against foodborne pathogens, such as organic acids, hydrogen peroxide, diacetyl, enzymes, and bacteriocins. Bacteriocins are defined as biologically active peptides or proteins with a bactericidal mode of action, produced by different groups of bacteria. They differ in size, diversity, and mode of action. Bacteriocins represent a potentially valuable biological tool for use in the food industry as natural preservatives, since they exhibit several advantages, including recognized as safe, not active against eukaryotic cells, stable to different pH and temperature conditions, are inactivated by the digestive proteases, do not affect the intestinal microflora, and do not present resistance problems like antibiotics.

Bacteriocins can be incorporated in the food product to improve its safety by (1) using a bacteriocin-producer starter culture, (2) as an ingredient by means of a purified preparation during product manufacture, or (3) incorporating an ingredient previously fermented with a bacteriocin-producing strain. For example, *L. sakei* strains producing bacteriocins have been used as starter cultures by the meat industry, as it demonstrates inhibitory activity against Gram-positive bacteria, such as *L. monocytogenes*. Bacteriocins added in the form of an ingredient are mandatory to be labelled as an additive, which can be unattractive to the food industry.

The GMPs have the highest importance in food processing with the aim to prevent any contamination of food through technological steps, from the raw materials, ingredients, and food contact materials reception till the consumers.

GMPs is a guide which is elaborated by each company for its specific conditions including the type of foods that are processed. It should comprise all technological specifications and general and operational procedures necessary to prevent food against contamination.

The general procedures are referring to reception of raw materials, ingredients, food contact materials and avoiding cross-contamination, how the suppliers are selected, hygiene practices, records, and so on.

For avoiding both microbiological and chemical contamination, the reception of raw materials, ingredients, and food contact materials has to be made by following a special procedure which includes the criteria of supplier selection and the quality requirements for all the materials (i.e., laboratory tests).

For avoiding cross-contamination (concerning allergenic and/or microbiological aspects) on the production lines, it is necessary to have some specific procedures concerning very strict separation between different flows: flow of personnel directly involved in production, flow of raw materials, flow of food contact materials and flow of end-products.

Microbiological safety hazards include pathogenic bacteria, viruses, and parasites. For better prevention against microbiological hazards periodically training programs and effective hygienic practices for employees are necessary. The hygienic practices have to include both the hygiene of employee and labor working environment hygiene (cleaning and sanitation). Hygienic design is very important, especially to prevent contamination into the sites where may be impossible to reach and clean with normal cleaning and sanitizing procedures. Examples include hollow rollers on conveyors, cracked tubular support rods, the space between close-fitting metal-to-metal or metal-to-plastic parts, worn or cracked rubber seals around doors, and on-off valves and switches. Good hygienic design of equipment requests that the materials used for food processing equipments to be easily cleanable for better sanitation but also the equipments has to be built by appropriate, non-corrosive materials to avoid metal chips contamination which means a chemical contamination.

Moreover, it is necessary to be developed a preventive maintenance program procedure in order to keep an appropriate hygiene in the processing area and surroundings.

Microbiological contamination can occur also because of insects or rodents; in this sense, a procedure for keeping the processing area free of these sources of contamination is necessary. Another important source of microbiological contamination, which is very important to be taken into account, can be production line staff through protection clothes or some injuries or diseases if there are not declared before entering in the processing area.

Chemical safety hazards during the food processing include intentionally added chemicals (e.g., allergens), unintentionally added chemicals (e.g., cleaners and solvents), and natural toxins (e.g., mycotoxins). Chemicals can also contaminate food through corrosion of metal processing equipment/utensils and residues of cleaning chemicals left on processing equipment. Further, adding too much of an approved ingredient, such as a vitamin in vitamin-fortified products, or additives may compromise the safety of foods.

Another chemical hazard can be the substances used against pests. The way of using the facilities for controlling pests is needed to be procedure.

Chemical hazards are occurring in food also when the technological parameters are not well optimized in order to prevent high-temperature chemical compounds formation, as HCAs and PAH, acrylamide or nitrosamine, and so on. In this respect, the technological specifications have to be developed in order to be followed, very strictly, the optimized technological parameters.

Another kind of contamination is **physical contamination** with materials that do not belong to food, like glass, wood, rocks, or metal and which cause physical safety hazards.

Production line staff can be a major source of contamination. Jewelry can fall off or break, fingernails can break, and pens can fall into food. Jewelry removal is required under GMPs. If pens are metallic, a metal detector can detect them. Production workers' fingernails should be cut short and gloves should be worn under certain processing conditions.

Additional to GMP, implementation of **HACCP system** makes possible that prevention against contamination to be more efficient. Standard ISO 22000 is a tool to be used for implementing a very effective food safety management system.

3.4 Conclusions

The more and more increasing of consumer's demands in Traditional Food Products makes producers and policy makers to be more aware about food safety issues in this sector. The hazards that can occur in traditional processing area the same as those that can occur in any other production systems, as conventional or organic food. In this respect, GMP implementation in traditional food companies would be necessary in order to prevent food contamination. Additional, improving the policies in the area while its harmonization at the European level would be supporting for the producers. Also, training programs and periodically information of traditional food producers with novelties and scientific evidences about new improvements in food safety are needed.

It cannot be changed the technology or recipe in order Traditional Food to be more safer but small changes or even small innovations in packaging could be implemented.

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Chapter 4

Factors Affecting Elimination of Carcinogenic Compounds from Food Products

Peter Šimko

4.1 Introduction

Meat smoking belongs to the oldest food technologies that have been used by mankind at minimum for 10,000 years. So far, techniques of smoking have been gradually improved and various procedures have been developed in different regions for treating meat and fish (Arvanitoyannis and Kotsanopoulos 2012). However, during the smoking, there are also conditions suitable for formation of food contaminants such as PAHs. These ones are being formed during the thermal decomposition of wood, especially at limited access of oxygen and then depositing on food surface during smoking (Purcaro et al. 2013). Apart from smoked foods, PAHs are frequently occurred also in other food matrix, especially in vegetable oils (Purcaro et al. 2007). Since benzo[*a*]pyrene (BaP) has the most carcinogenic potential of all PAHs compounds, it had been set as the reference compound for overall risk assessment of food contamination by PAHs (Šimko 2002). Also, the Scientific Committee on Food (European Commission 2002) confirmed it as suitable marker for the occurrence of PAHs in foods on the European market. Later, the content of BaP in smoked meat products was limited to 5 µg kg⁻¹ according to the European Commission's Regulation No. 1881/2006 (2006). On the basis of a comprehensive survey of PAH presence in foods, the European Food Safety Authority (2008) proposed—apart from BaP—also including other reference compounds, such as benzo[*a*]anthracene (BaA), chrysene (CHR), and benzo[*b*]fluoranthene (BbF). Consequently, both maximum content of BaP and sum of all four compounds (PAH4) were established by the European Commission's Regulation No. 835/2011 (2011). For smoked foods, the maximum BaP content remained at 5 µg kg⁻¹ and

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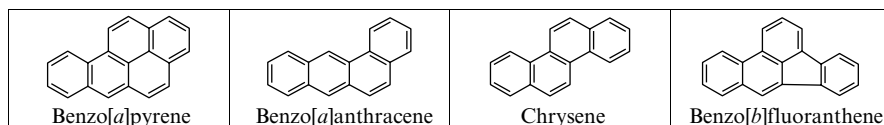


Fig. 4.1 Structural formulas of PAHs limited by European Commission's Regulation No. 835/2011

PAH4 content was set to $30 \mu\text{g kg}^{-1}$ until 31 August 2014. From 1st September 2014, maximum limit of BaP content has been lowered to $2 \mu\text{g kg}^{-1}$, while the allowable content of PAH4 has been limited to $12 \mu\text{g kg}^{-1}$. For oils and fats intended for direct human consumption or use as an ingredient in foods, maximum limit of BaP content has been limited to $2 \mu\text{g kg}^{-1}$, while the allowable content of PAH4 has been limited to $10 \mu\text{g kg}^{-1}$ (European Commission 2011). Structural formulas of these compounds are shown in Fig. 4.1.

4.2 Behavior of PAHs in an Organism

According to current knowledge, some PAHs are able to interact in organisms with enzymes (such as aryl hydrocarbon hydroxylases) to form PAHs dihydrodiol derivatives. These reactive products (the so-called bay region dihydrodiol epoxides) are believed as ultimate carcinogens that are able to form covalently bonded adducts with proteins and nucleic acids. In general, DNA adducts are thought to initiate cell mutation which is resulting in a malignancy (Šimko 2005).

4.3 Stability of PAHs Content in Smoked Meat Products

In general, the PAHs content in foods had been commonly considered as a constant value, not affected by environmental factors and additional operations, e.g., cooking, or even packaging. However, latest studies show that photodegradation of PAHs by UV light is possible (Sklářšová et al. 2010) and the formation of oxidative products has already been proven (Bednářiková et al. 2011). Due to tendency to migrate into nonpolar parts of food matrix, PAHs content can be also decreased by removal of fat during cooking (Šimko et al. 1993). PAHs content also depends on physical parameters (such as volume, diameter, and packaging material) of food matrix (Gomes et al. 2013).

4.4 Changes in BaP Content in Fish Smoked with Hot Smoke

To obtain data regarding possible effects of environmental factors on BaP content in hot smoked meat products, an Atlantic herring (*Clupea harengus*) was treated with hot smoke at the temperature of 82°C in a plant smoke house for 50 min

(Šimko 1991). After finishing the smoking, BaP content was determined immediately. Next, the samples were hung in the laboratory at unlimited access of oxygen and daylight at 18 °C, and the BaP content was determined after 1, 2, 3, 4, 6, and 7 days of exposition. During this time, the BaP content was lowered from 0.58 to 0.12 $\mu\text{g kg}^{-1}$. Simultaneously, BaP methanolic solution was transferred into far UV silica cells and, after evaporation of the solvent, BaP at amount of 1 μg was exposed to the same conditions as used for the smoked fish. The contents of the cells were analyzed for amount of BaP at the same time intervals. As found, BaP content was affected by environmental factors, especially at initial time of exposition, when all BaP was localized on the fish surface. But, the process of BaP light decomposition was not the only physicochemical process to be taken place in the fish. Analyzing surface and internal layers at the beginning and at the end of experiment, it was found that part of BaP diffused into the fish bulk. So, while BaP content in fish skin decreased from 10.6 to 1.3 $\mu\text{g kg}^{-1}$, BaP in the bulk increased from the zero value to 0.1 $\mu\text{g kg}^{-1}$. It was found that BaP degradation in silica cells could be described, in general, by reaction of zero order what is typical for photolytic degradations; it means that the amount of decomposed BaP was proportionally equal to the time of light exposition. On the other hand, the diffusion inside of fish bulk brought about the protection of BaP against environmental (decomposition) factors and the stabilization of its residual content in the fish. This course is typical for reaction of first order, and it is clear that two different physicochemical processes took place there—light decomposition and diffusion—independently to each other. It is clear that extent of BaP content decrease can be affected by intensity of light and, in final, also by antioxidant activity of phenol derivates and other antioxidants to be present in the food matrix.

4.5 Changes in BaP Content in Fermented Salami Smoked with Cold Smoke

Fermented meat products belong to the group of special meat products that are not treated by heat during technological production. Required ripeness is achieved by controlled fermentation of microbial cultures added into minced raw meat mixtures. To do not inactivate microbial cultures by heat, a flavor of fermented products is reached by aromatization using cold smoke procedure. Cold smoke, in general, consists of 0.5 h of smoking regime twice daily for several days. In the experiment, the salami was then put into an air-conditioned plant for drying and ripening. After 35 days, the final product was either in the laboratory with exposure to oxygen and light, or wrapped in aluminum foil and kept in a refrigerator at 3 °C for 31 days, respectively. For the first sight, BaP content did not change very much because it was compensated by the water content, successively being lowered during the ripening. In principle, these losses in weight should bring about the increase in BaP content. But, the BaP content was decreased due to photodegradation of BaP, as

already previously mentioned. The final result of these processes was, more or less, constant BaP content in the salami. However, a far more objective view of changes in BaP contents in the salami can be obtained after recalculation of the BaP content on a dry weight basis. This recalculation eliminates the effect of variable water content on changes in BaP content during production. So, while in the first case the average BaP content in the final product was lowered only by 17 % compared with the sample analyzed immediately after smoking, after recalculation on dry basis the decrease in BaP content corresponded to the value of 40 %, when BaP content itself changed only in the period of ripening, while during the whole interval of storage BaP content was already constant, although the salami was stored either in laboratory exposed to light or in dark at lowered temperature. This proved the reality that BaP already migrated into the salami bulk, and its content was stabilized due to protective effects of the salami matrix against the light (Šimko et al. 1991).

4.6 Effect of Cooking on BaP Content in Sausages Smoked with Hot Smoke

Sausages belong to a group of typical meat products to be consumed in considerable quantities in the region of central Europe. They are commonly made from pork—boneless hams, shoulders, and slab bacon. The proper amounts of each are cut and ground through a 7-mm plate of a meat grinder. The mixture is then combined with seasoning ingredients (NaCl, black and red pepper, garlic, etc.), and stuffed into natural casings. The linked sausages are smoked using hot smoke for 7 h. The sausages are consumed directly, or after cooking in boiling water. To follow the effect of cooking on BaP in sausages, a sausage was analyzed for BaP content, fat content and dry weight prior the cooking (Šimko et al. 1993). The BaP content was determined not only on the whole sausages, but also in a casing and peel off sausage separately, and also in cooked-out fat to be remained in water after cooking. The sausage was cooked in water at the boiling point, taken off at set time intervals, and analyzed. As followed from experiment, cooking of sausage could be an effective tool for lowering of BaP content, because the BaP content was lowered from 4.8 to final value of 1.9 $\mu\text{g kg}^{-1}$. The maximum drop in BaP content was observed during the first 20 min of cooking, after which it already remained at a constant level. The decrease of BaP content during cooking was directly proportional to the amount of cooked-out fat, released from sausages during cooking. Although the BaP content found in the sausage casing corresponded to a level of 86 $\mu\text{g kg}^{-1}$, in real terms this represented only 21 % of the total BaP content in the sausage; this means that 78 % of the total amount of BaP had diffused into the sausage bulk before the cooking. Evidence about diffusion of BaP and its high affinity to nonpolar parts, i.e., its nonhomogeneous distribution in the sausage was also proven by determination of BaP content in cooked-out fat when BaP content at the level of 7.7 $\mu\text{g kg}^{-1}$ was determined.

4.7 Effect of Cooking on BaP Content in Frankfurters Smoked with Hot Smoke

With regard to the finding that BaP content can be influenced by cooking, frankfurters were also treated by cooking, and BaP and fat content were monitored. In comparison to the sausage, it was found that BaP and fat content in frankfurters were not affected by cooking in boiling water. Comparing data and production technologies, it was found that although sausages and frankfurters are visually similar, they are very different due to the way of their production. While production of sausages is finished immediately after smoking, the frankfurters are still cooked in water steam. For this reason, the BaP content did not change in the frankfurters during additional cooking because the “redundant” fat had already been removed from them during their technological production. Moreover, while the sausages consist of ground pork meat and bacon, frankfurters represent the typical fine cut homogeneous emulsified system, where the “residual” fat (“locked” in denaturated proteins) is in equilibrium with other matrix components. Because the fat content had been stabilized in the frankfurters during production, it was not already changed considerably during cooking. Then, with regard to the high affinity of BaP to nonpolar parts, its content was also not influenced by additional cooking and it remained at a constant level (Šimko and Knežo 1992).

4.8 Effect of Package on BaP Content in Roasted Meats

Freshly roasted duck skin was analyzed for the presence of BaA, BbF, and BaP, which were determined at level of 143, 3.7, and 3.5 $\mu\text{g kg}^{-1}$, respectively. The skin was then packed into LDPE pouches and the PAHs content determined again after 24-h storage. During this time, the contents were lowered to 130, 1.7, and 0.9 $\mu\text{g kg}^{-1}$ due to migration of PAHs into LDPE, where the compounds were then determined (Chen and Chen 2005). It seems that the package of smoked meat products into appropriate packaging material could reduce significantly PAHs content in these products with regard to high affinity of PAHs to LDPE. However, it is obviously, that the extend of PAHs removal would depend on beginning time of interaction with package material with regard to PAHs diffusion into meat bulk, taking place not only after finishing the smoking, but during the smoking as well. So, to reach the most effective extend of PAHs removal from smoked meat products, it would be necessary to carry out package operations of smoked meat products as quickly as possible, or to use packaging material with higher affinity to PAHs in comparison to meat matrix, respectively.

4.9 Changes in BaP Concentration of Liquid Smoke Flavorings (LSF)

LSF are alternative to traditional procedures and their application accelerates the process of smoking and decreases considerably the level of total contamination in comparison to traditional smoking procedures (Šimko et al. 1992). Similarly to smoked meat products, PAHs concentrations in LSF had also been understood as a constant value, independent of physicochemical factors and effects of package material properties. However, the changes of PAHs concentrations could take place even more intensively due to higher values of diffusion coefficients of PAHs in liquid media in comparison to the values in solid matrix of smoked meat products.

4.10 Effect of LDPE Packing on PAHs Concentration in LSF

During experiments with LSF, it was found that PAHs concentration is variable when LSF were packed into LDPE receptacles (Šimko and Bruncková 1993). To find out possible effect of the packaging on PAHs concentration, the LSF were spiked with pyrene (Py), BaA, dibenzo[*a,c*]anthracene (DBaA), benzo[*e*]pyrene (BeP), BaP, and dibenzo[*a,h*]anthracene (DBaH) at a level of $45.6 \mu\text{g kg}^{-1}$ and filled into LDPE receptacles. However, after 14 days the PAHs concentration in LSF was lowered by two orders when PAHs migrated into LDPE. The ability of LDPE was also tested in three various liquid systems with different polarity and viscosity for BaA, BbF, BaP, indeno[*c,d*]pyrene (IcdPy), and DBaH (Chen and Chen 2005). It was found that, the most intensive removal processes of PAHs from liquid media took place within 24 h when an important role played by the polarity as well as the viscosity of liquid media. During this period, the PAHs concentration was lowered in all studied systems by more than 50 % in comparison to initial concentration of $50 \mu\text{g kg}^{-1}$ for each compound.

4.11 Characterization of Physicochemical Processes in LSF Packed in LDPE

To characterize physicochemical processes taking place in liquid media, the experiment was carried out at PAHs concentrations of $91.1 \mu\text{g kg}^{-1}$, and the changes were followed for 164 h (Šimko et al. 1994). During this time, the PAHs concentration in LSF dropped to the zero value. As the spiked samples were not stirred during the experiment, it was reasonable to assume that the factor limiting the rate of PAHs concentration decrease is the diffusion in liquid media. Because the LDPE receptacles were of cylindrical shape, it was therefore used for quantitative description of the relationship derived for the diffusion in a cylinder:

$$\frac{n_t}{n_\infty} = 1 - \sum_{n=1}^{\infty} \frac{4}{a^2 \alpha_n^2} \exp[-D \alpha_n^2 t] \quad (4.1)$$

where n_t is the amount of diffused PAHs, which has left the sample as a consequence of the diffusion into PET at time t , and n_∞ is the amount of PAHs corresponding to infinite time, D is the diffusion coefficient, a is the radius of the cylinder, and α_n are the roots of the equation:

$$J_0(a \alpha_n) = 0 \quad (4.2)$$

where J_0 is the zero-order first-class Bessel function. In the experiments, not the amount of PAHs adsorbed on PET was measured, but their residual concentration in the liquid media. After recalculation of the amount of PAHs to their concentration, Eq. (4.1) could be modified into the form

$$c_t = c_0 \cdot \sum_{n=1}^{\infty} \frac{4}{a^2 \alpha_n^2} \exp[-D \alpha_n^2 t] \quad (4.3)$$

where c_0 and c_t are the initial concentration and concentration at time t , respectively. Diffusion coefficients of the PAHs were calculated by the nonlinear least-squares method by minimizing the sum of squares of differences between the PAHs concentrations measured experimentally and those calculated by Eq. (4.3). Comparing measured and calculated data, it was found a very high agreement between them, what approved either diffusion process in liquid media or the suitability of this equation for predictive purposes. By numerical solution of Eq. (4.3) for known values of the diffusion coefficient and given initial concentration c_0 , it is possible to calculate the time of interaction with PE at which PAHs concentration decreases to a required value c_t , eventually the time interval just necessary to reach the zero concentration.

4.12 Characterization of Physicochemical Processes in LDPE Package

To define the physicochemical processes taking place after leaving liquid phase, water was spiked with fluorene (Fl), Py, and BaP and filled into a diffusion cell made of stainless steel. One of the walls was replaced by LDPE sheet composed of 5 layers of LDPE foil, made as follows: LDPE sheet (thickness 1 mm), composed of 5 layers (thickness 200 μm), was prepared from LDPE granules using a hydraulic press under the following conditions: preheating period 5 min, pressing temperature 200 $^{\circ}\text{C}$, period of pressing 4 min, and period of cooling 10 min. Cooled LDPE (thickness roughly 2 mm) was then pressed again to obtain LDPE foil with a thickness 200 μm under these conditions: preheating period 3 min, period of pressing 3

min, and temperature of pressing 200 °C. Finally, after cooling, five pieces of this foil were pressed together using a preheating period 1.25 min, period of pressing 0.75 min, and temperature of pressing 102 °C. This way prepared PE sheets were free of air bubbles, compact, but it was easily possible to peel off individual foils from sheets after finishing the experiment. A depth of PAHs migration into this sheet was followed for 143 h. The solution of the second Fick law for the diffusion of PAHs in polymer in the diffusion cell gives the equation

$$c = S \cdot \operatorname{erfc}\left(\frac{x}{2\sqrt{Dt}}\right) \quad (4.4)$$

where c is the concentration of PAHs in the polymer at time t and at the distance x , D is the diffusion coefficient, and S is the solubility of PAHs in the polymer. The amount of diffusant in the region $x - h$ to x is given by formula (4.5)

$$Q = A \int_x^{x-h} c(x,t) dx \quad (4.5)$$

where A is the area and h is the thickness of the polymer layer. It can be derived from Eqs. (4.4) and (4.5) that the amount of PAHs in the n th polymer layer expressed as a weight of PAHs per a weight of polymer, w

$$w = S \int_n^{n-1} \operatorname{erfc}\left(\frac{hn}{2\sqrt{Dt}}\right) dn \quad (4.6)$$

where n is the number of the layer.

The experimental data have been treated using Eq. (4.6), and the solubilities of individual PAHs in PE and the related diffusion coefficients have been obtained from the comparison of the theoretical values given by Eq. (4.6) and the experimental values using the nonlinear least-squares method. Integration of Eq. (4.6) has been performed using the Simpson formula. As followed from the values of standard deviations, the agreement between experimental and calculated values was fairly good. As the results revealed, PAHs after leaving the water were primarily adsorbed on the LDPE surface, with subsequent migration into LDPE bulk. After 143 h of the experiment, the PAHs already diffused through all sheet and PAHs were presented also in the last layer of the sheet in the concentration profile and diffusions for individual compounds were different and depended on values of diffusion coefficients, which decreased in the order Fl > Py > BaP, what was due to increasing molar masses of the compounds (Šimko et al. 1999). The evidence about migration of PAHs into bulk of LDPE was also proven indirectly by decomposition of PAHs by UV light, when only partial decomposition has been observed in LDPE (Chen and Chen 2005) because of the fact that UV light has a low energy radiation in comparison to, for example, γ radiation and it is not able to penetrate into LDPE bulk. Moreover, LDPE polymer can be stabilized against light decomposition effects by

addition of UV filter components to polymer matrix. From these reasons, UV decomposition is an effective tool only for PAHs to be deposited just on the LDPE surface but not in the bulk.

4.13 Characterization of Physicochemical Processes in PET Package

Apart from LDPE, PET is also a material widely being used for packaging purposes, especially for vegetable oils. To evaluate interactions of PAHs with PET, rapeseed oil and water were spiked with PAHs at the total level of 955.1 and 711.4 $\mu\text{g kg}^{-1}$, respectively, and filled into PET cylindrical shape receptacles, and the PAHs concentrations in both liquid media were followed for 90 h. During this time, the PAHs concentrations decreased by 315.1 in oil and 212.7 $\mu\text{g kg}^{-1}$ in water due to an interaction of PAHs with PET. Using derived kinetic equation (4.7),

$$c_t = c_\infty + (c_0 - c_\infty) \sum_{n=1}^{\infty} \frac{4}{a^2 \alpha_n^2} \exp[-D \alpha_n^2 t] \quad (4.7)$$

diffusion coefficients for PAHs in both liquid media were determined. Values of the diffusion coefficients obtained indicate that the polarity of medium did not affect the rate of PAHs removal. Calculation of the area occupied by PAHs molecules on PET surface suggests that either multilayer adsorption or limited diffusion into PET bulk came into account as the decisive factors causing the decrease of PAHs concentrations in both media (Šimko et al. 2004). To more precise these interactions, commercially available rapeseed oil, the same oil additionally refined physically, and paraffin oil for comparison purpose were spiked with BaP at the level of 29.4, 34.3, and 50.4 $\mu\text{g kg}^{-1}$, respectively, and filled into PET cylindrical shape receptacles, and the BaP concentrations were followed within 73 h. During this time, the BaP concentrations decreased to 22.9, 25.4, and 23.5 $\mu\text{g kg}^{-1}$. Using the modified kinetic equation (4.7), diffusion coefficients of BaP in all oils were calculated. The values of diffusion coefficients and distribution coefficients indicated that other compounds present in oils (e.g., vitamins, sterols) competed with BaP, and affected a rate of BaP diffusion in the oils and extend of adsorption onto PET. For these reasons, the final state of PAHs–PET interactions could be classified such as the surface adsorption, what limits considerably the PET capacity in removal processes of PAHs from liquid media in comparison with LDPE (Šimko et al. 2005).

4.14 Light Decomposition of BaP

Decomposition of BaP was studied at two different light wavelengths, 254 and 365 nm, in a nonpolar medium at concentrations 50, 100, and 150 $\mu\text{g L}^{-1}$. At chosen time intervals, BaP concentration was measured by HPLC using fluorescence detection.

Comparing rate constants k and half-lives $\tau_{1/2}$, it was found that decomposition at 365 nm was 15.3 times faster in comparison with the decomposition at 254 nm. The decompositions obeyed the first-order kinetics. Considerable effect had addition of food antioxidants, 2,6-di-*tert*-butyl-4-methylphenol (BHT) and *o*-methoxyphenol (guaiacol), which both accelerated the rate of BaP decomposition—BHT by 1.17 times and guaiacol even 1.45 times. This means that both antioxidants exhibited considerable pro-oxidant effects on BaP. These findings may represent a basis for a new approach to decrease PAHs content in foods, where their presence is inevitable due to the applied production technology (Skláršová et al. 2010). To derive kinetic equation and eliminate errors associated with non-isothermal heating procedures, BaP dissolved in glyceryl trioctanoate was heated in a glass reaction vessel within temperature range 297.95–361.85 K with heating rate of 1 K min⁻¹ and simultaneously exposed to UV light at wavelength $\lambda = 365$ nm at radiation power 20 mW cm⁻². On the base of experimental data, the adjustable parameters making possible description the non isothermal kinetics of BaP photooxidation by kinetic Eq. (4.8) were calculated

$$c = c_0 \exp\left[-\frac{aT_r \vartheta^{m+1}}{\beta(m+1)}\right] \quad (4.8)$$

where T_r is a reference temperature, c_0 and c are the concentrations of BaP in vessel at the temperatures T_0 and T , ϑ is reduced temperature, a and m are adjustable parameters.

The parameters were tested at two various isothermal conditions (290.16 and 323.26 K) to verify a suitability of derived parameters. Comparing calculated and measured data of half-lives of BaP decrease at mentioned temperatures, it was found that calculated half-lives are in a good accordance with those experimentally obtained values when relative standard deviations at 290.16 K were 17.0 % and 5.4 % at 323.26 K, respectively. The kinetic parameters enable the calculation of the rate constant for any temperature in isothermal regime and make possible to model, in general, the kinetics of such processes without a deeper insight into their mechanisms (Skláršová et al. 2012).

4.15 Conclusion

According to recent findings and summarized data about PAHs in foods and LSF discussed above, it is possible to generalize following conclusions:

1. Meat products are contaminated during smoking by PAHs which are being formed in the meantime by wood combustion.
2. In general, a content of PAHs in smoked meat products is not constant and depends on effects of environmental factors, e.g., light and oxygen.

3. The environmental factors cause decomposition PAHs and a formation of their oxidized products that may have even more hazardous effects on living organisms.
4. With regard to the ability of PAHs to accumulate in lipophilic tissues, it is possible to remove partially PAHs contained in fat by removing the fat during additional cooking operations.
5. It seems that an effective tool for PAHs removal from foods may be PAHs interaction with an appropriate plastic package in which foods would be wrapped.
6. Concentrations of PAHs in LSF can be lowered by sorption into LDPE.
7. High efficiency of PAHs removal into LDPE is possible due to the combination of such physicochemical processes as a surface adsorption and subsequent diffusion into the polymer bulk.
8. Concentration of PAHs in vegetable oils can be lowered by PET. However, this is less effective material, because only surface adsorption comes into account. Moreover, other compounds presented in liquid media are able to affect extend of the removal due to their reciprocal competition for adsorption center on the PET surface.
9. Effective way of PAHs removal is also photodegradation, which can be accelerated by addition/presence of antioxidants, as these behave at given conditions as pro-oxidants of PAHs, while oxidation of other food compounds (e.g., unsaturated fat acids) at these conditions is negligible.
10. Derived kinetic equations make possible to predict/model changes in PAHs concentrations knowing initial concentrations, or set the time interval needed for lowering of PAHs to acceptable level.
11. It seems that these new procedures could be applicable in food processing as effective tools of food contaminants' removal and increase in such a way food safety of some food products.

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Chapter 5

Acrylamide Formation in Foods: Role of Composition and Processing

Vural Gökmen

5.1 Introduction

Thermal processing is indispensable to develop sensorial characteristics, in particular color, taste and flavour in food products. In addition, it may also induce the formation of health-promoting antioxidative compounds (Lingnert and Wailer 1983). However, the formation of potentially harmful compounds is one of the significant consequences of thermal processing from the viewpoint of food safety. In 2002, the discovery of acrylamide in cooked foods by Swedish researchers raised an alarm over the safety of such foods (Tareke et al. 2002). The presence of acrylamide in common heated foods has been considered an important food-related crisis by international authorities due to following reasons: (1) the foods that contain acrylamide are extensively consumed; (2) acrylamide is a probable human carcinogen and (3) the levels of acrylamide found in food are higher than a large number of other known food-borne carcinogens (Friedman 2003). Assessments by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) confirmed that a risk couldn't be excluded for dietary intake of acrylamide, because it is classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC 1994).

5.2 Occurrence of Acrylamide in Heated Foods

Detection of acrylamide levels in processed foods has become an intensive area of research shortly after its discovery in heated foods. A lot of studies confirmed the presence of acrylamide in nearly all fried, baked and roasted foods sold in Germany

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Table 5.1 Distribution of acrylamide levels in certain heated foods in 2010 (EFSA 2012)

Foods	n	Median (µg/kg)	Mean (µg/kg)	P90 ^a (µg/kg)	Maximum (µg/kg)
French fries	256	240	338	725	2174
Potato crisps	242	450	675	1538	4533
Soft breads	150	18	30	63	425
Breakfast cereals	174	91	138	293	1290
Biscuits, crackers	462	129	333	833	5849
Roasted coffee	103	200	256	462	1932
Instant coffee	15	520	1123	2629	8044
Coffee substitutes	24	870	1350	3300	4200
Baby foods (cereal)	128	24	51	144	578

^a90th percentile

(Gutsche et al. 2002), the UK (Ahn et al. 2002), Sweden (Svensson et al. 2003), the Netherlands (Konings et al. 2003), Hong Kong (Leung et al. 2003), Japan (Ono et al. 2003), Austria (Murkovic 2004), Australia (Croft et al. 2004), Turkey (Şenyuva and Gökmen 2005), Spain (Rufian-Henares et al. 2007), Finland (Eerola et al. 2007), Italy (Tateo et al. 2007), Brazil (Arisseto et al. 2007), China (Zhang et al. 2007) and Korea (Koh 2007). Acrylamide exposure varies depending on the population's eating habits, and the way the foods are processed and prepared. In general, fried potato products, bakery products and roasted coffee products are important dietary sources of acrylamide. Table 5.1 summarizes the descriptive statistics of acrylamide levels in different food groups for the data collected in 2010 (EFSA 2012).

5.3 Mechanism of Acrylamide Formation

Initial results on acrylamide indicated that carbohydrate-rich foods generate relatively more acrylamide (Tareke et al. 2002). Researchers have established that the main pathway of acrylamide formation in foods is linked to the Maillard reaction (Mottram et al. 2002; Stadler et al. 2002). The amino acid asparagine has a crucial role in this reaction (Yaylayan et al. 2003; Zyzak et al. 2003). The first step is the amino-carbonyl reaction between asparagine and a carbonyl compound at temperatures higher than 100 °C. This leads to the formation of Schiff base (Fig. 5.1). Both the *N*-glycosyl conjugation and the Schiff base are relatively stable under low moisture conditions (Robert et al. 2004). However, in aqueous systems the Schiff base may hydrolyse to the precursors or rearrange to the Amadori compound, which is not an effective precursor in acrylamide formation (Yaylayan et al. 2003; Stadler et al. 2004). Most of the Schiff base formed rearranges to Amadori compound, which contributes to colour and flavour formation. Rate limiting step of acrylamide formation is decarboxylation of Schiff base (Blank 2005). Decarboxylation of Schiff base can occur directly through Schiff betaine or Schiff betaine rearranges to

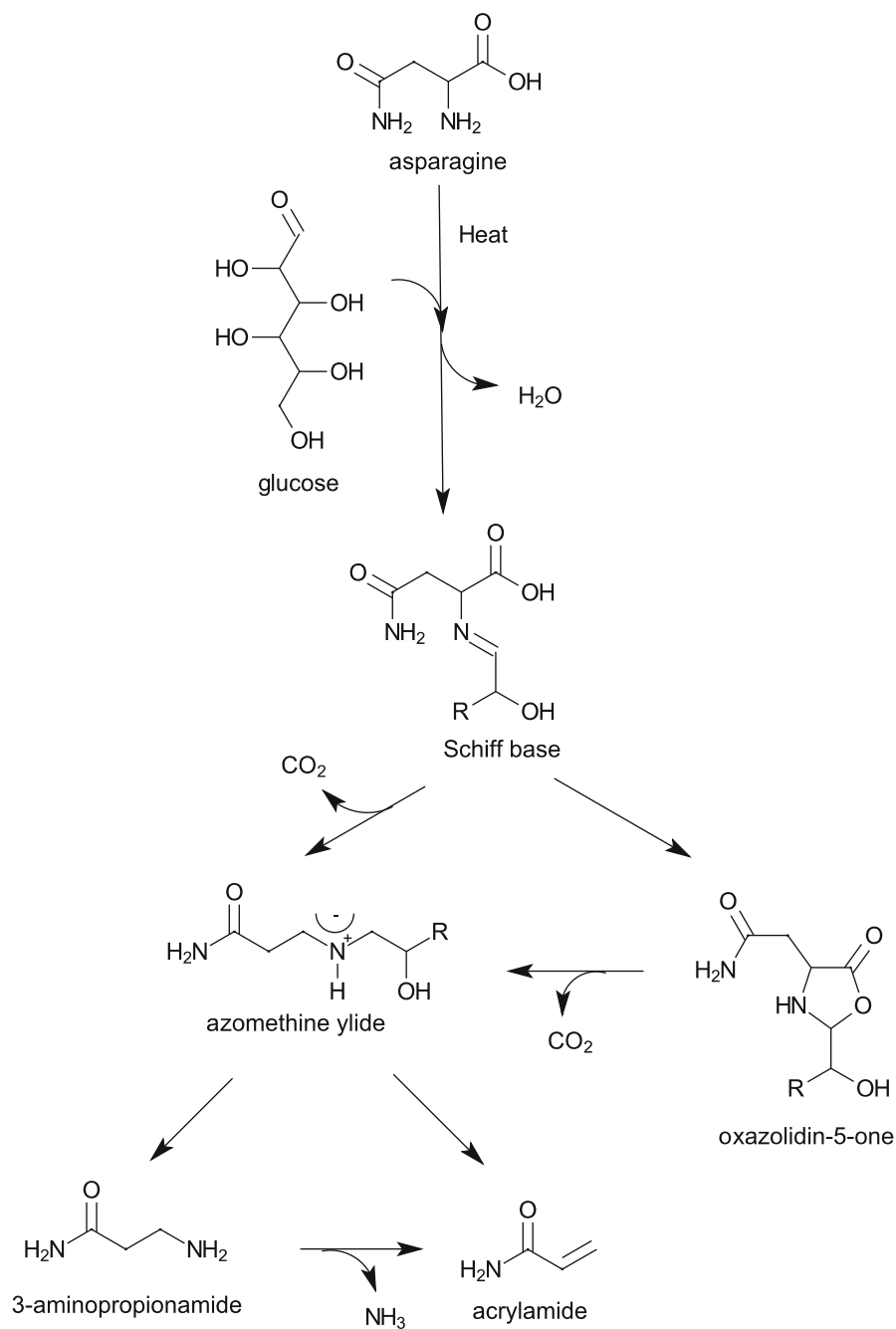


Fig. 5.1 Mechanisms of acrylamide formation via the Maillard reaction (adapted from Zyzak et al. 2003; Yaylayan et al. 2003; Stadler et al. 2004)

oxazolidine-5-one intermediate, which is known to decarboxylate easily to form azomethine ylide (decarboxylated Schiff base) (Yaylayan et al. 2003; Zyzak et al. 2003). Azomethine ylide rearranges by tautomerization to form decarboxylated Amadori compound, which then forms acrylamide via β -elimination (Stadler et al. 2004). This rearrangement occurs if there is hydroxyl functionality on the β position to the nitrogen atom. This case happens with α -hydroxycarbonyls like glucose. On the other hand, azomethine ylide might decompose directly to acrylamide or 3-aminopropionamide (Zyzak et al. 2003). 3-APA easily deaminates to form acrylamide (Granvogl and Schieberle 2006). This reaction has been shown to proceed favourably under aqueous conditions in the absence of sugars (Granvogl et al. 2004).

5.4 Factors Affecting Acrylamide Formation in Foods during Heating

Several factors related to food composition and processing conditions have been shown to influence the formation levels of acrylamide, but also other quality characteristics such as browning in thermally processed foods (Friedman 2003).

5.4.1 Agronomical and Recipe Factors

Agronomical and recipe factors, such as the initial concentrations of the reactants (asparagine and reducing sugars) and their ratio, pH and water activity of the medium, type of leavening agents used, the presence of amino acids other than asparagine, mono-, di- and polyvalent cations, oxidizing fatty acids and antioxidant compounds, have been shown to influence the amounts of acrylamide formed in foods during thermal processing (Brathen et al. 2005; Kim et al. 2005; Gökmen and Şenyuva 2006; Fink et al. 2006; Low et al. 2006; Gökmen et al. 2007; Gökmen and Palazoğlu 2008; Mestdagh et al. 2008; Zamora and Hidalgo 2008; Capuano et al. 2009, 2010; Hidalgo et al. 2009, 2010; Koutsidis et al. 2009; Zamora et al. 2010).

The link of acrylamide to asparagine, which directly provides the backbone of the acrylamide molecule, has been established by labelling experiments (Stadler et al. 2002; Zyzak et al. 2003). Studies to date clearly show that the amino acid asparagine is mainly responsible for acrylamide formation in heated foods after condensation with reducing sugars or other carbonyl sources. Moreover, the sugar–asparagine adduct, *N*-glycosylasparagine, generates high amounts of acrylamide, suggesting the early Maillard reaction as a major source of acrylamide (Stadler et al. 2002). In addition, decarboxylated asparagine can generate acrylamide in the absence of reducing sugars (Zyzak et al. 2003).

Carbonyl compounds become limiting in the formation of acrylamide in foods during heating when asparagine is present in excess amounts. α -Hydroxycarbonyl compounds such as fructose and glucose are usually found in high amounts

compared to other carbonyls, and contribute to acrylamide formation significantly (Yaylayan and Stadler 2005). Glyoxal, methylglyoxal, 5-hydroxymethyl-2-furfural and other sugar degradation products containing carbonyl groups promote acrylamide formation (Amrein et al. 2004, 2006a, 2006b). Additionally, carbonyls originating from lipid oxidation or bioactive compounds also participate in Maillard reaction yielding to acrylamide formation (Hamzaloğlu and Gökmen 2012; Hamzaloğlu et al. 2013; Hidalgo et al. 2010; Kocadağlı et al. 2012a, b).

Stadler et al. (2002) investigated different sugars for their efficiencies in acrylamide formation. Lactose, galactose, fructose and sucrose form acrylamide with similar yields. Although many researchers have found that acrylamide is formed when a specific amino acid reacts with a reducing sugar in the presence of heat, the reaction of sucrose and an amino acid in the presence of heat resulted in acrylamide formation comparable to the levels formed by fructose and glucose (Stadler et al. 2002). This could be due to the hydrolysis of sucrose at high temperatures into glucose and fructose, both of which are reducing sugars. In theory, one sucrose molecule could give rise to two reducing hexoses resulting in a molar ratio of 2:1 sugar to amino acid (Taeymans et al. 2004). Tripling the concentration of sucrose from 10 to 30 % in a sweet cookie formulation resulted only 60 % increase in acrylamide formation during baking. However, significantly higher amounts of acrylamide formed in cookies formulated with glucose instead of sucrose (Gökmen et al. 2007).

Concerning reducing sugars as carbonyl source, fructose is more efficient than glucose in forming acrylamide. The melting points of fructose and glucose are 126 and 157 °C, respectively (Robert et al. 2004). This explains why fructose is more reactive than glucose during heating under low moisture conditions. Biedermann et al. (2002) has also reported that fructose is more efficient than glucose in a potato model. Frying, baking and roasting are simply characterized as open processes in which heat and mass transfers occur simultaneously. As the moisture reduces due to evaporation, sugars initially dissolved in water form a saturated solution and then crystallize. After crystallization, melting is required to change their state to liquid, so to make them chemically reactive. In this respect, reducing sugar having a lower melting point is expected to form acrylamide earlier during heating.

It is well known that the rate of Maillard reaction is strongly dependent on pH of the reaction environment. It has been reported that lowering pH by means of the addition of organic acids decreased the amount of acrylamide formed in foods during heating (Rydberg et al. 2003; Jung et al. 2003; Surdyk et al. 2004; Kita et al. 2004; Low et al. 2006). The protonation of the α -amino group of asparagine hinders the formation of the N-substituted glycosylamine, which may explain the reduced acrylamide content of acid treated foods. However, the addition of citric acid into dough comprising sucrose has been shown to increase the susceptibility of acrylamide formation in cookies due to the excessive hydrolysis of sucrose, which increased the concentration of reactive sugars (Gökmen et al. 2007).

It has been shown that treatment of potato slices with non-transition state cations such as Ca^{2+} reduces acrylamide formation in foods during heating (Lindsay and Jang 2005). In the presence of cations in a binary reaction mixture composed of glucose and asparagine, acrylamide formation is remarkably reduced depending on

the type and concentration of cations, but the reaction proceeds mainly toward the dehydration of glucose leading to hydroxymethylfurfural. Cations prevent the formation of Schiff base, which is a key intermediate leading to acrylamide during the Maillard reaction (Gökmen and Şenyuva 2007).

One potential strategy to mitigate acrylamide formation in heated foods is to reduce the levels of precursors in the raw materials prior to thermal processing. Asparaginase, an enzyme that hydrolyses asparagine to aspartic acid, is a very effective mean for reducing acrylamide formation (Zyzak et al. 2003; Ciesarová et al. 2006, 2009, 2010; Pedreschi et al. 2008; Capuano et al. 2009). The use of asparaginase is very advantageous because it has no significant impact on sensorial characteristics of the final product. Furthermore, fermentation by using certain yeasts and bacteria may also reduce the concentrations of reducing sugars and asparagine (Huang et al. 2008; Sadd et al. 2008; Mustafa et al. 2009).

Minimization of acrylamide formed during thermal processing of foods is of great importance from the viewpoint of food safety. To date, several approaches have been found to lower the levels of acrylamide formed in foods. Table 5.2 summarizes the most important compositional and conditional factors affecting the formation of acrylamide in foods with potential chemical interventions and process modifications for mitigation.

5.4.2 Processing Factors

Acrylamide formation depends on the time–temperature history of the processed foods. Its formation generally increases with increase in thermal load as in bakery and fried potato products. Temperature has an important role in the formation and elimination of acrylamide. It is well known that acrylamide forms in foods that are cooked at temperatures exceeding 120 °C (Mottram et al. 2002; Tareke et al. 2002; Becalski et al. 2003; Biedermann and Grob 2003; Rydberg et al. 2003). The fact that acrylamide is not formed during boiling indicates that higher temperatures and/or low moisture conditions are needed for its formation. During heating under atmospheric conditions, higher temperatures can be reached only if simultaneous drying takes place. The loss of water as the food dries during heating extracts a large amount of the incoming energy, and hence the bulk of the product is at a temperature very much lower than that of the heating medium. In this respect, temperature, time and moisture are key drivers of acrylamide formation in foods during heating. The moisture content determines the physical state and mobility of chemical constituents in the food matrix. In addition, water alone affects the chemical route and the mechanistic pathway for acrylamide formation.

Fried potatoes are in the food category with probably the highest concentrations of acrylamide recorded so far (Friedman 2003). During frying, all the heat transferred from the hot oil is utilized to increase the internal energy of potato strip until the surface reaches slightly above the boiling point of water. After this point, moisture evaporation starts extracting a large amount of the incoming energy.

Table 5.2 Effects of compositional and conditional factors on acrylamide formation with their mode of action and practical meaning

Factor	Mode of action and practical meaning	References
Asparagine	• It provides backbone of acrylamide	Mottram et al. (2002), Stadler et al. (2002),
	• More asparagine produces more acrylamide upon heating	Yaylayan et al. (2003), Zyzak et al. (2003),
	• It is in excess amounts in most potato varieties, but limited in cereals	Becalski et al. (2003), Surdyk et al. (2004)
	• Whole grain flours contain more asparagine than refined flours	
Sugars	• Reducing sugars strongly accelerate acrylamide formation from asparagine during heating	Biedermann et al. (2002), Biedermann and Grob (2003), Surdyk et al. (2004), Graf et al. (2006),
	• Sucrose is least effective, but becomes reactive after its hydrolysis into glucose and fructose	Vass et al. (2004), Gökmen et al. (2007)
	• Fructose is more effective than glucose due to its lower melting point	
	• More reducing sugars produce more acrylamide upon heating	
Raising agents	• NH_4HCO_3 has strong influence on acrylamide formation in bakery products	Amrein et al. (2006a, b), Gökmen et al. (2007), Kukurova et al. (2013)
	• Elimination of NH_4HCO_3 from the recipe or replacing it with other raising agents is practically useful mitigation measure for bakery products	
	• Other amino acids compete with asparagine for carbonyl compounds during the Maillard reaction	
Amino acids	• The presence of other amino acids such as glycine and cysteine may be an effective way to mitigate acrylamide formation	Rydberg et al. (2003), Kita et al. (2004), Brathen et al. (2005), Kim et al. (2005), Fink et al. (2006),
	• Higher glycine concentrations cause darker colour that is disadvantageous for consumer perception	Low et al. (2006), Mestdagh et al. (2008),
	• Cysteine forms Michael adduct with acrylamide at high temperatures. This may be the reason for the reduction of acrylamide concentration in certain products like roasted coffee during prolonged heating or storage periods	Capuano et al. (2009), Koutsidis et al. (2009), Zamora et al. (2010)

(continued)

Table 5.2 (continued)

Factor	Mode of action and practical meaning	References
Cations	• Cations such as Ca^{2+} form complex with asparagine and prevent the formation of Schiff base, which is an intermediate leading to acrylamide • Certain ingredients may form chelates with cations. Chelate formation reduces the effectiveness of cations on acrylamide mitigation	Gökmen and Şenyuva (2007), Lindsay and Jang (2005)
Lipid oxidation	• Polyunsaturated fatty acids readily oxidize to reaction carbonyls compounds such as decadenals during heating • Lipid-derived carbonyl compounds increase the risk of acrylamide formation • The presence of antioxidants may prevent lipid oxidation, so the formation of acrylamide	Zamora and Hidalgo (2008), Hidalgo et al. (2009, 2010), Capuano et al. (2010)
Antioxidants	• The presence of antioxidants may prevent lipid oxidation, so the formation of acrylamide • Some phenolic antioxidants have carbonyl functions, and may react with asparagine increasing the risk of acrylamide formation	Capuano et al. (2010), Hamzaloğlu and, Hamzaloğlu et al. (2013)
Organic acids	• Low pH may limit the rate of the Maillard reaction, so the formation of acrylamide • Reducing the pH by adding organic acid accelerates sucrose hydrolysis, increasing the risk of acrylamide formation	Jung et al. (2003), Low et al. (2006), Gökmen et al. (2007)
Asparaginase	• Asparaginase enzyme converts asparagine to aspartic acid and prevents acrylamide formation	Zyzak et al. (2003), Ciesarová et al. (2006), Pedreschi et al. (2008), Ciesarová et al. (2010), Capuano et al. (2009)
Fermentation	• Fermentation by yeasts or lactic acid bacteria reduces significantly the reducing sugars, and limits acrylamide formation • The yeasts consume acrylamide present in instant coffee	Huang et al. (2008), Mustafa et al. (2009), Akilloğlu and Gökmen (2014)

Temperature and thermal load	- Thermal load can be correlated with the resulting acrylamide concentration of heated foods - When asparagine is in excess amounts, increasing the thermal load also increases the amount of acrylamide formed in foods - When asparagine is in limited amounts, increasing the thermal load may lower the concentration of acrylamide in the final product	Taubert et al. (2004), Amrein et al. (2006a, b), Gökmen et al. (2006)
	Acrylamide formation is accelerated at higher temperatures	
	Microwave heating of potato strips to gelatinize starch may reduce significantly the frying time of French fries, so the formation of acrylamide	
Microwave heating		Erdoğan et al. (2007)
Programmed frying	Using temperature-programmed frying starting with high temperature (185–190 °C) and short time (1–2 min), and finishing with lower temperature (150–160 °C) and longer time (2–3 min) is beneficial for crust formation and starch gelatinization, respectively	Palazoglu et al. (2012), Kocadağlı et al. (2012a, b)
	Doing so, crust layer of potato strips is prevented to face to high temperatures, and acrylamide formation is limited	
Vacuum post-processing	Vacuum post-processing may remove acrylamide from roasted coffee and bakery products	Anese et al. (2010, 2014)
	This treatment may also cause the loss of volatile aroma compounds, which is disadvantageous for consumer perception of the final product	
Radio frequency post-baking	Partial baking under conventional conditions followed by radio frequency post-baking reduces the thermal load during processing, so the acrylamide formation in bakery products	Anese et al. (2008), Palazoglu et al. (2012), Kocadağlı et al. (2012a, b)

The energy input to potato strip is limited during frying at lower temperatures ($<150^{\circ}\text{C}$). When the oil temperature is increased to high enough ($\geq 170^{\circ}\text{C}$), the energy input is sufficient for both moisture evaporation and temperature increase to take place in the same duration, which favours the formation of acrylamide (Gökmen et al. 2006).

The formation of acrylamide takes place mainly at the surface and in the near-surface regions, because the conditions in this part of the potato strip become favourable for acrylamide formation as a result of simultaneous drying. As a consequence, any treatment like washing of the cut-surface of potato strips may decrease concentrations of precursors on the surface. In deep-frying, conduction within the food material is the rate-controlling heat transfer mechanism, which implies that the frying time can be reduced if the potato strips are cooked before going into the fryer. This eliminates the need to rely on conduction of heat during frying to render the interior cooked, and frying is performed just to bring about the desirable surface characteristics of the already-cooked strips. Microwave pre-cooking is effective in reducing acrylamide levels in the surface region of French fries (Erdoğan et al. 2007). Potato strip is rendered cooked volumetrically in a very short time. The reduction of acrylamide is a consequence of the combined effect of reduced frying time and surface temperature.

The thermal processing of foods is rather complex due to a wealthy composition of the reaction pool. Therefore, the results of a single treatment would be diverse in terms of safety concerns as well as organoleptic properties. Hence, any approach putting forward to prevent adverse effect like acrylamide formation during thermal processing should also consider other possible changes to be made on the resulting food product.

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Chapter 6

Detection of Bioactive Compounds in Plants and Food Products

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6.1 Introduction

Creating a scientifically valid distinction between food and medicine has never been easy. Centuries ago, Hippocrates advised, “Let food be thy medicine and medicine be thy food.” (IFT Expert Report 2005). Today, food has emerged beyond its basic function—nutrition. Increased health awareness and consumer confidence coupled with an aging population have driven demand for products that improve the quality of life, have specific health benefits, and can be used by consumers to self-medicate (Kosseva 2013). Functional foods are defined as being similar in appearance to conventional foods, are consumed as part of a usual diet, and are known to improve health status and render physiological effects beyond basic nutritional function expected of conventional foods (Shahidi 2007).

The European Commission’s Concerted Action on Functional Food Science in Europe (FuFoSE), coordinated by the International Life Science Institute (ILSI) Europe, defined functional food as follows: “A food product can only be considered functional, if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. Functional foods must remain foods and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet: they are not pills or capsules, but part of a normal food pattern. Therefore, it could not be in the form of pill or capsule just as normal food form” (Diplock et al. 1999). European legislation, however, does not consider functional foods as specific food categories but rather as a concept (Coppens et al. 2006).

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Functional food consumption is increasing in almost all industrialized countries. The functional food industry, consisting of food, beverage, and supplement sectors, is one of the several areas of the food industry that is experiencing fast growth in recent years. It was estimated by BCC Research that the global market of functional food industry will reach 176.7 billion in 2013 with a compound annual growth rate (CAGR) of 7.4 % (Japan Ministry of Health, Labour and Welfare 2014). In this large and burgeoning market place, the food industry is demanding economical, high-quality, novel, and substantiated ingredients (Smithers 2008).

In ancient times, people had no idea about bioactive molecules but the use of these compounds was sufficiently diverse in different prospect. Thus a simple definition of bioactive compounds in plants is: secondary plant metabolites eliciting pharmacological or toxicological effects in human and animals (Bernhoft 2010). Most of the beneficial properties of fruits, vegetables, and whole grains have been attributed to bioactive non-nutritional chemical compounds commonly named phytochemicals. Whole foods have been estimated to have between 5000 and 25,000 individual phytochemicals (Acosta-Estrada et al. 2014). Many phytochemicals found in plants are reported to have antioxidant activity. The addition of antioxidants to food is an effective way to prevent the development of various off-flavors and undesirable compounds that result from lipid peroxidation. There is considerable interest in preventive medicine and in the food industry in the development of natural antioxidants obtained from botanical sources (Čanadanović-Brunet et al. 2006).

The trend to produce functional foods by adding bioactive compounds has entailed numerous investigations on the extraction of secondary plant metabolites (Kosseva 2013). Therefore, accurate identification of bioactive compounds is essential to identify relationships between different food components and their health benefit, and also in establishing appropriate dietary intake and safety guidelines and understanding their role in human health and nutrition (Luthria 2006).

According to the European regulation on nutrition and health claims made on foods (ECNo. 1924/2006), a list of authorized claims has to be published for all member states, and nutrient profiles also have to be established for foods containing health claims (Siro et al. 2008). New, validated, standardized, and harmonized protocols for determining bioactives in foods have to be advised for quality control in the growing functional food industry. Moreover, fast, accurate, and nondestructive detection approaches need to be developed as methods of quality control in the production of functional ingredients or applied to raw materials and finished products for functional food industries (Azmir et al. 2013). Further, education and knowledge transfer in the field of functional foods are essential in order to inform consumers to make adequate choices and learn about safety and health impacts of food they eat.

The Institute of Food Technologists (IFT) reported the scheme for designing, developing, and marketing of functional foods. The expert panelists describe that this process consists of seven key-steps:

- Identification of the relationship between food components and health benefits
- Demonstration of the efficacy and determination of intake level necessary to achieve desired effect

- Demonstration of safety at efficacious levels
- Development of suitable vehicle for bioactive components
- Demonstration of scientific sufficiency of evidence for efficacy
- Communication of benefits to consumers
- Conducting in-market confirmation of efficacy and safety

Every one of these steps is highly dependent on the same factor: development of rapid, cost effective, accurate, and rugged analytical procedures (Luthria 2006).

Numerous investigations have proved that medicinal plants contain compounds such as phenolics, tocopherols, carotenoids, terpenoids, etc., which show beneficial biological effects. Due to revival of plant extracts, many questions arise, such as:

- What kind of plant extracts can be used for the food industry?
- What technical data or physical prerequisites does a certain plant extract need for being applicable in different types of food?
- Which chemical and physicochemical measurements can be applied to evaluate their effect in food products? (Djilas et al. 2003)

According to Mitra and Brukh (2003), the common steps for any analytical method are suggested to be: sampling, sample preservation, sample preparation, and analysis (separation and detection). However, working with bioactive compounds, from sampling and sample preparation and to the final detection, requires special caution because of their high sensitivity, ensuring that experimental conditions are sufficiently mild to avoid any changes in the analyte structure. Poole et al. (1990) emphasized that the development of modern chromatographic and spectrometric techniques make bioactive compound analysis easier than before but the success still depends on the extraction methods, input parameters, and exact nature of plant parts.

6.2 Sampling and Sample Preparation

Considering that the composition of foods is affected by many factors and compounds are not uniformly distributed within and between samples of a given food, statistically valid sampling and sample preparation to obtain representative and homogeneous samples for analysis are of utmost importance (Rodriguez-Amaya 1999). The major causes of data inaccuracy at the present time are that a single sample lot is analyzed per food and errors are not observed in intralaboratory and interlaboratory evaluations in which the same homogenized samples are analyzed.

Sample selection, number of samples, sample handling, and sample preparation prior to extraction are important factors affecting data quality. Sample preparation may consist of multiple steps such as sample drying, homogenization, sieving, extraction, pre-concentration, derivatization, and hydrolysis (Luthria 2006).

The type of sample will determine the sampling protocol and how the samples are handled. The main considerations are the degree of homogeneity of the material and the possible variation in the compounds content, not only between different

materials, but also between different samples of the same material. Also the variety, origin, season of year, growing conditions, etc., will affect the bioactives content. It is essential that the sampling protocol and the number of samples collected reflect the composition of the whole sample. The time between sample collection and analysis should be as short as possible (Mitra and Brukh 2003). The protocol should minimize any effects that may cause undesirable losses prior to analysis.

The qualitative and quantitative studies of bioactive compounds mostly rely on the selection of proper extraction method (Smith 2003; Sasidharan et al. 2011). Extraction is the first step in the study of bioactive compounds and crucial for the final result and outcome. Therefore extraction should be carefully optimized and performed. Polarities of different bioactive compounds, which vary significantly due to their conjugation status and their association with the sample matrix, will determine the solvents for extraction to be used. Specific pretreatments are needed to preserve the raw materials, such as storage under modified atmosphere, microbial or chemical acidification, and pasteurization (Laufenberg et al. 2003). Milling, crushing, steam explosion, and/or use of depolymerizing enzymes are essential to favor the extraction (Benoit et al. 2006). Also, additional procedures (shaking, rotary shaking, stirring, sonication, or reflux) can be used to improve the extraction. Selection of solvent for bioactive compound extraction should also consider molecular affinity between solvent and solute, mass transfer, use of co-solvent, environmental safety, human toxicity, and financial feasibility. Hernández et al. (2009) reported that the most common factors affecting extraction processes from plants are matrix properties of the plant part, solvent, temperature, pressure, and time. Optimization of extraction parameters not only increases extraction efficiency of the analyte of interest but also reduces the solvent consumed and the waste generated during the extraction process making it more environmental friendly.

The main objectives of extraction are (a) to extract targeted bioactive compounds from complex sample, (b) to increase selectivity of analytical methods, (c) to increase sensitivity of bioassay by increasing the concentration of targeted compounds, (d) to convert the bioactive compounds into a more suitable form for detection and separation, and (e) to provide a strong and reproducible method that is independent of variations in the sample matrix (Smith 2003).

Conventional extraction techniques are based on the extracting power of different solvents and the application of heat and/or mixing. Classical conventional techniques for extraction of bioactives are: (1) Soxhlet extraction, (2) Maceration, and (3) Hydrodistillation. Besides being simple, conventional extraction requires longer extraction time, costly and high purity solvents, evaporation of large volumes of solvent, low extraction selectivity, and thermal decomposition of thermo labile compounds (Luque de Castro and Garcia-Ayuso 1998).

Some of the most promising *nonconventional extraction techniques* are:

- Ultrasound-assisted extraction involves the diffusion across the cell wall and rinsing the contents of cell after breaking the walls (Mason et al. 1996). Ultrasound energy for extraction also facilitates more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, selective

extraction, reduced equipment size, faster response to process extraction control, quick start-up, increased production, and eliminates process steps (Chemat et al. 2008).

- Enzyme-assisted extraction includes the addition of specific enzymes (i.e., cellulase, α -amylase, and pectinase) during extraction enhances recovery by breaking the cell wall and hydrolyzing the structural polysaccharides and lipid bodies (Rosenthal et al. 1996; Singh et al. 1999).
- Microwave-assisted extraction involves three sequential steps (a) separation of solutes from active sites of sample matrix under increased temperature and pressure; (b) diffusion of solvent across sample matrix; (c) release of solutes from sample matrix to solvent (Alupului 2012). This extraction technique enables quicker heating, reduced thermal gradients and equipment size, and increased extract yield (Cravotto et al. 2008).
- Pulsed electric field-assisted extraction is based on the cell membrane structure destruction, thus increasing extraction efficiency (Bryant and Wolfe 1987).
- Supercritical fluid extraction is usually performed with carbon dioxide. It is suitable for extracting nonpolar substances, however this limitation has been overcome by a chemical modifier (Lang and Wai 2001; Ghafoor et al. 2010). Advantages are: higher rate of penetration to sample matrix and favorable mass transfer, decreased extraction, complete extraction due to the repeated reflux of supercritical fluid to the sample, optimized selectivity by modifying temperature and/or pressure, complete and fast separation of solute from solvent, convenient for thermo labile compounds (operated at room temperature), the amount of sample can range from milligrams to tons, environment friendly, possibility for on-line coupling with chromatographic process, recycling and reuse of supercritical fluid.
- Pressurized liquid extraction include the extraction techniques using high pressure: pressurized fluid extraction, accelerated fluid extraction, enhanced solvent extraction, and high pressure solvent extraction. High pressure keeps the solvent liquid beyond its normal boiling point and therefore facilitates the extraction. These techniques require small amounts of solvents and provide faster extraction (Ibañez et al. 2012).

6.3 Identification and Characterization of Bioactive Compounds

For identification and characterization of an isolated substance usually the critical point is the selection of the right measuring method. Spectroscopic methods usually permit a crude identification of the bioactives present in the food, but in most cases the specific composition can remain obscure. Therefore, detailed information about the composition requires additional methods like chromatography and mass spectrometry to fully characterize the structures of the molecules in sample.

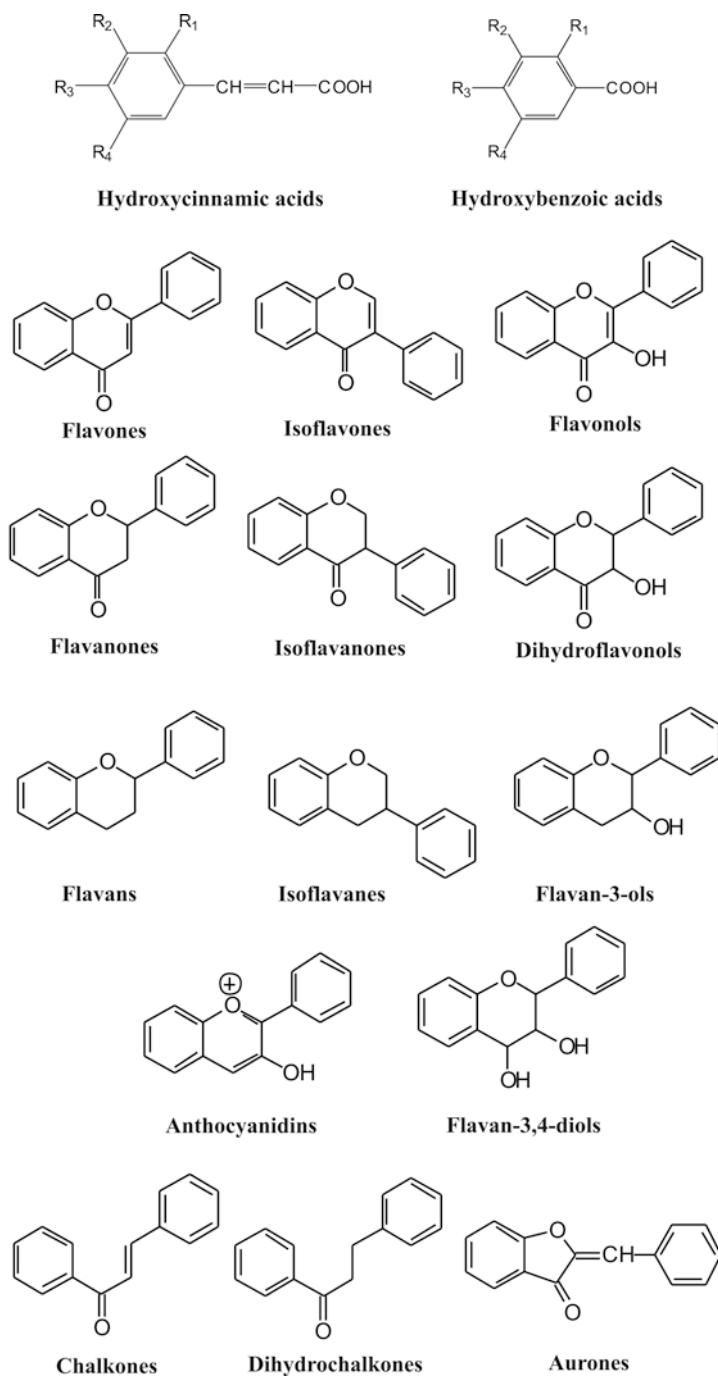
High performance liquid chromatography (HPLC) is the most popular analytical method in separation science and is regarded as the gold standard in the authentication of pharmaceuticals and herbal medicines due to its precision, sensitivity, and reproducibility. However, some of the pitfalls of HPLC include high instrumental cost, relatively long analytical time, and large organic solvents consumption (Sherma 2012). Thin-layer chromatography (TLC) is often used as a first, effective, and relatively inexpensive analytical method. As a consequence of the relatively “weak” resolution power of TLC, these methodologies have been progressively supplemented by more efficient separation techniques, such as liquid chromatography. TLC method is regarded as one of the fundamental authentication methods in the Chinese and European Pharmacopoeias, and the development of high performance thin-layer chromatography (HPTLC) has made it an attractive alternative for the quality control of bioactive compounds. Advantages of HPTLC are reliability, simplicity, flexibility, fast analytical time, low running cost, low organic solvent consumption, and the possibility of analyzing numerous samples simultaneously (Marston 2011). However, HPTLC is based on the visualization of the plate and comparison to a reference sample, which makes it imprecise. Furthermore, it is not suitable to be adopted for a daily routine procedure in industry. Recently, the procedures involved in the digitalization and transformation of HPTLC plate images into chromatographic fingerprint matrices) using Matlab pre-processing algorithms are developed (Wonga et al. 2014).

When analyzing samples with complex matrices and low analyte contents, direct measurement is rarely possible and a separation (chromatography, solid phase extraction, etc.) followed by a sensitive detection is usually required.

6.4 Analysis of Bioactive Compounds

6.4.1 Polyphenols

Polyphenolic compounds are secondary metabolites and are derived from phenylalanine. Phenolics found in vegetables and fruits have been proposed as one of the major bioactive compounds providing health benefits (Vulić et al. 2014). Plant polyphenols are a very diversified group of phytochemicals, which includes phenolic acids, flavones, isoflavones, flavonols, flavanones, isoflavanones, dihydroflavonols, flavans, isoflavanes, flavan-3-ols, anthocyanidins, flavan-3,4-diols, chalcones, dihydrochalcones, and aurones (Fig. 6.1). Identification and quantification of phenolic compounds can give vital information relating to antioxidant function, food quality, and potential health benefits. Many separation techniques such as thin-layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have been proposed to separate and determine phenolic compounds in various plant materials (Četković et al. 2007). Figure 6.1 presents basic and the most abundant classes of phenolic compounds.

**Fig. 6.1** Basic classes of phenolic compounds

Polyphenolic compounds have been reported to have multiple biological effects, such as antibacterial, anti-allergic, antiviral, antioxidant, anti-inflammatory, and even anti-aging activity (Četojević-Simin et al. 2010). Phenolic compounds are suggested as substitutes for preservatives in food due to their antioxidant and antimicrobial properties (Fazary and Ju 2007; Oliveira et al. 2012; Ou and Kwok 2004), but also in cosmetics (Sancho et al. 2001), nutraceuticals and pharmaceuticals. For example, FDA classifies ferulic acid as an antioxidant in the list of food additives (Fazary and Ju 2007), and it is also used as precursor of the flavoring agent vanillic acid. Based on these facts, there is a need to develop new extraction methodologies for phenolic compounds so that they can effectively be used as functional ingredients.

Phenolic compounds possess one or more aromatic rings bearing a hydroxyl substituent, including their functional derivatives such as esters, methoxy compounds, and glycosides. Based on their structures, most of them could be extracted using solvents of different polarities (Čanadanović-Brunet et al. 2008). Freeze-drying is usually employed prior to extraction in order to prevent oxidation in material.

Hydrolysis has been used to simplify the chromatographic analysis of phenolic compounds. Bound phenolics comprise an average of 24 % of the total phenolics present in the food matrices (Adom and Liu 2002; Sun et al. 2002). Alkaline and acidic hydrolyses are the most common means of releasing bound phenolic compounds (Stalikas 2007). Acid hydrolysis cleaves glycosidic bonds between the phenolic compound and the sugar molecule attached to it and alkaline hydrolysis has been employed in order to hydrolyze ester bonds releasing phenolics bound to cell wall (Kylli 2011). However, the hydrolysis loses the valuable information of the naturally occurring glycosylates, as well as of other conjugates and their bioactivities. Prevention of degradation during the hydrolysis can be achieved by adding antioxidants (*tert*-butylhydroquinone, ascorbic acid alone or with EDTA). Some food processes, such as fermentation, malting and extrusion cooking, enhance the liberation of bound phenolics. The release of bound phenolics can also effectively be achieved by carbohydrate-hydrolyzing enzymes such as pectinases, cellulases, amylases, hemicellulases, and glucanases. Microwave-assisted extraction and ultrasound-assisted hydrolysis are effective in extracting bound phenolics as well (Acosta-Estrada et al. 2014).

Polyphenols are extracted with polar solvents such as aqueous acetone (hydroxycinnamic acids and anthocyanins), methanol or ethanol (flavan-3-ols and proanthocyanidins) and ethyl acetate (phenolic acids) (Heinonen et al. 1998; Kähkönen et al. 2001). Kylli (2011) has found that in most cases ethanol was the best solvent, ethyl acetate was moderate, and water was the least effective. Ethanol extracted more polar compounds such as anthocyanins, while ethyl acetate extracted more nonpolar flavonols. Määtä-Riihinen et al. (2004) extracted flavan-3-ols and low-molecular-weight proanthocyanidins with ethyl acetate. Proanthocyanidins from the grape seeds are extracted with methanol, ethanol, acetone and ethyl acetate, or with their mixtures, for analytical and preparative purposes (Escribano-Bailon and Santos-Buelga 2003). Proanthocyanidins are well soluble in ethyl acetate and this solvent possesses significant selectivity towards the extraction of the flavan-3-ols of

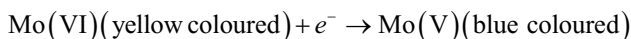
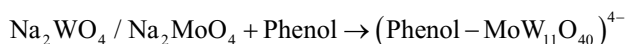
lower molecular masses (Pekić et al. 1998). Mandić et al. (2008) extracted grape seeds with 90 % ethanol and 90 % ethyl acetate. The yields of ethyl acetate extracts were lower than those of ethanol extracts because of the higher selectivity of ethyl acetate. By using vanillin assay, it was further validated that ethyl acetate is a better solvent for isolation of flavan-3-ols. HPLC analysis of grape seed extracts confirmed that the content of total soluble polyphenols in ethyl acetate extracts was higher than in ethanolic extracts. Extremely polar phenolic acids, such as benzoic and cinnamic acids, cannot be effectively extracted with pure organic solvents so mixtures of alcohol–water or acetone–water are recommended (Stalikas 2007). Aqueous acetone has been shown to be more efficient extraction solvent than aqueous methanol for hydroxycinnamic acids and anthocyanins (Heinonen et al. 1998; Kähkönen et al. 2001). On the other hand, aqueous methanol may be better for flavan-3-ols and proanthocyanidins (Kähkönen et al. 2001). Stanojević et al. (2009) have extracted higher amounts of chlorogenic acid, umbelliferone, and apigenin-7-O-glucoside using 50 % ethanol in a Soxhlet apparatus at a boiling temperature compared to the same extraction procedure with 80 % methanol and pure water. In addition, acidified or chilled solvents are frequently used (Rochín-Medina et al. 2012; Singh-Gujral et al. 2012). It was found that a small addition of organic acids (formic acid, acetic acid) may stabilize anthocyanins and enhance their extractability (Gao and Mazza 1994; Kalt et al. 2008). High-molecular-weight proanthocyanidins are extracted more efficiently with a combination of acetone, methanol, and water (2:2:1) containing 0.01 % formic acid (Hellström and Mattila 2008; Kalt et al. 2008). The portion of organic acids in extraction solvents varies from 0.01 to 10 %. Extractions are almost always repeated 2–3 times with extracts, and then combined. The phenolic profiles are different when different solvents are used. Fractionation of phenolics can be achieved by successive extraction with solvents of different polarities. Čanadanović-Brunet et al. (2009) extracted horsetail (*Equisetum arvense* L.) with 70 % methanol, and after removing methanol successively treated the extract using solvents with increasing polarities: petroleum ether, chloroform, ethyl acetate, and *n*-butanol. Total phenol content measured by Folin–Ciocalteu assay showed that the content of phenolic compounds was increased with increasing polarity of solvents. HPLC analysis revealed different phenolic compounds only in ethyl acetate, *n*-butanol and remaining water extract, where *n*-butanol extracted highest amount of phenolic compounds.

Column chromatography can be used for purification of phenolics from extracts as well as for fractionation and isolation of specific group of phenolic compounds. Further purification of extracted high-molecular-weight proanthocyanidins can be achieved on Sephadex LH-20 columns. For example, ellagitannins have been extracted with 70 % aqueous acetone followed by fractionation on Sephadex LH-20 column and by subsequent elution with water, aqueous methanol, or ethanol and aqueous acetone (Hager et al. 2008; McDougall et al. 2008; Gasperotti et al. 2010; Karonen et al. 2010). Ellagitannins can be also extracted using ethanol:water:formic acid (80:20:1) as a solvent and separation of ellagitannins from anthocyanins could be done by the ion exchange column by elution with the extraction solvent and anthocyanins ethanol:water:HCl (50:50:1) (Kool et al. 2010).

The raw extracts also contain non-phenolic substances such as sugars, organic acids, proteins, and pigments, which can interfere during analysis of polyphenolics

(Macheix et al. 1990). Solid phase extraction (SPE) is useful for removing sugars, and to some extent, organic acids from phenolic extracts. Tumbas et al. (2012a) performed a clean-up of the rosehip extract using C-18 solid phase extraction to remove organic acids, residual sugars, amino acids, proteins, and other hydrophilic compounds. C18 column was further used for fractionation of phenolic compounds to acidic and neutral phenolics. For separation of neutral phenolics from the extract adjusted to pH 7, the column was preconditioned with methanol and water, followed by washing with water and elution with methanol. Acidic phenolics were isolated from the effluent portion adjusted to pH 2, loaded on the column preconditioned with methanol and 0.01 M HCl, followed by washing with 0.01 M HCl and elution with methanol. Recoveries of the phenolic extracts after SPE are reported to be almost 100 % (Głowniak et al. 1996; Benassi and Cecchi 1998; Pinelo et al. 2006).

The identification and quantification of polyphenols in food and biological samples is difficult due to the wide variety of polyphenol structures found in nature and the lack of commercially available standards. Several methods have been used, including spectrophotometric methods, capillary electrophoresis, nuclear magnetic resonance spectroscopy, near-infrared spectroscopy, and chromatographic techniques such as high- and ultra-high-performance liquid chromatography, high-speed counter-current chromatography, supercritical fluid chromatography, and gas chromatography (Lamuela-Raventós et al. 2014). The most common assay for measurement of the total amount of polyphenolic compounds in a sample is Folin–Ciocalteu method. It is based on the reduction of phosphomolybdic-phosphotungstic acid reagent (Folin reagent) in alkaline solution by phenolic compounds:



Absorbance is measured at 755–765 nm and the results are most commonly expressed as gallic or chlorogenic acid equivalents. Measurement of total phenolic content by the Folin–Ciocalteu method may be interfered by other chemical components present in the extract, such as sugars, aromatic amines, sulfur dioxide, ascorbic acid, organic acids, and Fe(II), as well as non-phenolic organic substances that react with Folin–Ciocalteu reagent, giving the false overestimated result. In order to remove these interferences, as well as to remove the polar substances, purification of extract can be conducted by SPE (Tumbas et al. 2012b).

Anthocyanins can be identified and quantified spectrophotometrically due to their property that in acidic aqueous media (pH 1.0) they exist mainly as red flavylium cation, and when pH is increased (pH 4.5), the colorless carbinol form prevails. pH-Differential method uses two wavelengths for measurement: 510–550 nm (absorbance maximum for anthocyanins) and 700 nm (for haze correction), and at two different pH levels: 1.0 and 4.5. The absorbance is calculated as follows: $A = (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH } 1.0} - (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH } 4.5}$. The concentration of anthocyanins is then calculated by the Lambert–Beer equation (Lee 2005).

Vanillin assay has been commonly used for determination of proanthocyanidins. The aldehyde vanillin (0.5–2 % in acidic methanol) reacts with *meta*-oriented

hydroxyl groups on the flavanol A-ring. After 15 min incubation at 30 °C, the absorbance is measured at 500 nm (with HCl or H₂SO₄) or 510 nm (with glacial acetic acid) (Sun et al. 1998).

Puškaš et al. (2010) have determined total tannins in red wine enriched with solid parts, stems, and seeds, using spectrophotometrical method measuring the absorption at 280 nm (total tannins = $A_{280} \times 100$).

TLC is a simple and fast method that can be used for preliminary identification of phenolic compounds in extracts, but the main disadvantage is low resolution. Čanadanović-Brunet et al. (2005) have evaluated qualitative composition of wormwood (*Artemisia absinthium* L.) extracts using TLC in one and two dimensions. TLC plates were precoated with microcrystalline cellulose. One-dimension TLC was performed with ethyl acetate:formic acid:acetic acid:water (100:11:11:26, v/v) as mobile phase, while for two-dimension analysis the mobile phases were: (1) *tert-butanol*:acetic acid:water (3:1:1, v/v) and (2) 15 % acetic acid. Spots were observed under UV light at 366 nm and sprayed with DPPH reagent and compared with reference substances. The one-dimensional TLC method was used in the study of Četković et al. (2004) for identification of flavonoids and phenolic acids in water and methanolic extracts of cultivated marigold (*Calendula officinalis* L.) and wild growing marigold (*Calendula arvensis* L.). After separation, TLC plates were additionally sprayed with DPPH (2,2-diphenylpicrylhydrazyl radical) for antioxidant activity screening. The reduction of the color could be observed visually as a yellowish spot on a purple background. Vladimir-Knežević et al. (2011) have performed thin-layer chromatographic (TLC) analysis of different polyphenol subclasses in *Micromeria* plant species on precoated silica gel 60 F254 TLC plates with the mobile phases: ethyl acetate–formic acid–acetic acid–water (100:11:11:27, v/v/v/v), diisopropyl ether–acetone–water–formic acid (50:30:10:10, v/v/v/v), and ethyl formate–formic acid–water (80:10:10, v/v/v) for analysis of flavonoids, phenolic acids, and tannins, respectively. Flavonoids and phenolic acids were detected under UV light at 365 nm after spraying them with natural products–polyethylene glycol reagent. For visualization of tannins in visible light, plates were sprayed with 10 % ethanolic iron(III) chloride.

Nowadays, the best analytical tool to quantify and characterize phenolic compounds is considered to be HPLC combined with ultraviolet-photodiode array detection or mass spectrometry (Lamuela-Raventós et al. 2014). Reversed-phase nonpolar columns C-18 or C-8 are used, with gradient of polar, mostly water based, solvents as eluent and acetonitrile or methanol as organic modifier. Usually, eluents contain 0.1–5 % aqueous acetic, formic, or trifluoroacetic acid to enhance the retention to nonpolar column (Kylli 2011). Due to aromatic ring in their molecular structure detection with ultraviolet–visible (UV–Vis) is carried out with absorbance maximum at the 280–520 nm wavelength range (Table 6.1).

Fluorescence detection is more suitable for proanthocyanidins, where excitation wavelength is set to 275–280 nm and emission wavelength to 315–325 nm. The main advantage of fluorescence detectors is sensitivity which is much higher than that of UV–Vis.

For full detection of the polyphenolic structures in the sample mass spectrometry has to be used. Electrospray ionization (ESI) and atmospheric chemical ionization

Table 6.1 Characteristic UV–Vis absorbances of phenolic compounds

Phenolic compounds	$\lambda_{\max}$ (nm)
Hydroxybenzoic acids	280
Hydroxycinnamic acids	320
Flavonols and ellagic acid	365
Anthocyanidins	520
Proanthocyanidins	280

(APCI) are the main ionization techniques for HPLC. ESI is applicable for polar compounds, while APCI is better for neutral and nonpolar compounds. Limitations of this technique are:

- Detection of anthocyanins is difficult because of high molecular weight.
- Inability to differentiate between various diastereoisomeric forms of sugars; therefore this method cannot provide information on the exact glycosidic substitution(s) other than the number of carbon atoms and the presence of methyl side group(s) bound to sugar moiety (Kylli 2011).

Using MS-MS technology these limitations can be avoided. Tumbas Šaponjac et al. (2014b, 2015) used HPLC-DAD-ESI/MS n technique for identification of anthocyanins in caneberry pomaces. The full-scan mass covered the range from m/z 100 up to m/z 1200. Collision-induced fragmentation experiments were performed in the ion trap and MS data were acquired in the positive ionization mode. LC-MS with ESI and time-of-flight (TOF) mass detector was used for identification of phenolic compounds in *Boletus* mushrooms, being variegatic acid characteristic phenolic compound (Vidović et al. 2010).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) with quadrupole and ion trap mass analyzers are appropriate for detection of polymeric, nonvolatile, and polar phenolic compounds such as proanthocyanidins and ellagitannins. MALDI-TOF or HPLC coupled to time-of-flight mass spectrometry can detect up to 300 kDa. Conventional LC-MS coupled to quadrupole or ion trap mass analyzers are not suitable for analyzing higher molecular weight compounds (limit of quadrupole and ion trap mass analyzers is around 2000–4000 Da) (Ketola et al. 2010).

Nuclear magnetic resonance (NMR) spectroscopy is well established as a tool for the rapid compositional analysis of foods and beverages. However, a limiting factor in understanding the information from NMR spectra of foods and beverages is their complexity. Because of the large number of components within the mixture, besides low peak intensity, signal overlap and crowding arises in the low-field spectral region of the ^1H NMR spectra for many of the aromatic compounds. ^1H NMR spectroscopy in principle would provide a rapid, nondestructive, and straightforward method for profiling of polyphenols (Humpfer et al. 2008; Biais et al. 2009; del Campo et al. 2006; Gil et al. 2000). Savage et al. (2011) described the development of an extraction method for polyphenol profiling and identification in grape juices prior to analysis using ^1H NMR spectroscopy. Liquid/liquid partitioning and solid phase extraction (SPE) were used for selective removal of heavily dominating com-

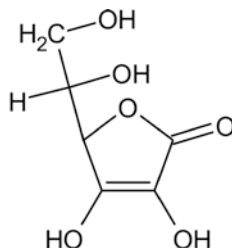
pounds such as carbohydrates. However, limitations in detection of some classes of phenolic compounds (e.g., anthocyanins) using NMR are relatively high amount of samples (mg range) and long acquisition which is undesirable because of their critical stability (Kylli 2011).

Gas chromatography can also be used for polyphenol profiling. Generally, gas chromatography systems are more stable, repeatable, and selective. Analysis of phenolic compounds in the gas phase requires a chemical modification step—derivatization. The most common procedure for derivatization is silylation. Compared with their parent compounds, trimethylsilyl derivatives are more volatile, less polar, and more thermotolerant. Pejin et al. (2009) have investigated the changes in phenolic acids content during malt production using GC-MS method. In this study, barley, steeped barley, green malt, and malt samples were subjected to hydrolysis by acid (to release simple esterified phenolic acids), α -amylase (to release bound phenolic acids from starch and similar polysaccharides), and cellulase (to degrade barley and malt cell walls and release insoluble-bound phenolic acids). After hydrolysis, phenolic acids were extracted with ethyl acetate. Trimethylsilyl derivatives of phenolic acids were analyzed by quadrupole mass analyzer. Nevertheless, GC-MS methods are suitable only for relatively small molecules (molecular weight below 600 Da) due to the need for volatility.

6.4.2 Vitamin C

Vitamin C—ascorbic acid (2-(1,2-dihydroxyethyl)-4,5-dihydroxyfuran-3-one) (Fig. 6.2) is one of the most important water-soluble vitamins, naturally present in fruits and vegetables, and it is widely used as a food additive and antioxidant (Versini et al. 2006). Vitamin C plays crucial roles in electron transport, hydroxylation reactions, and oxidative catabolism of aromatic compounds in animal metabolism. In cells, the other role of vitamin C is to reduce hydrogen peroxide (H_2O_2), which preserves cells against ROS, and also recycling vitamin E. The only way humans can uptake vitamin C is via food, since it is not synthesized in the human body. Vitamin C can be mostly found in fruits and vegetables. The main sources of vitamin C are citrus fruits, hips, strawberries, peppers, tomatoes, cabbage, spinach, and others, but also liver and kidney tissues (Davey et al. 2000).

Fig. 6.2 Structure of ascorbic acid



Vitamin C has two forms that are biologically active, ascorbic acid, and dehydroascorbic acid (Gokmen et al. 2000). Ascorbic acid plays a crucial role in several biochemical processes in the human body and is reversibly converted into dehydroascorbic acid, under oxidative stress (Wilson 2002).

Instability of vitamin C is the main problem of ascorbic acid or dehydroascorbic acid assays. Vitamin C analysis in foods requires care that can vary depending on the matrix under study. Cell structure disruption during the extraction processes allows enzymes responsible for vitamin C degradation to come into contact with the substrate. Therefore it may be necessary to prevent the enzymatic action by reducing the pH, what favors vitamin C stability. Campos et al. (2009) tested different solvents (water, 4.5 % metaphosphoric acid and the solution consisting of 3 % metaphosphoric acid, 8 % acetic acid, 1 mM EDTA, and 0.15 M H₂SO₄) to extract vitamin C from vegetables. They concluded that adding metaphosphoric acid to the extracting solution contributes to vitamin C preservation during the extraction process. However, to improve the repeatability of the method, it was necessary to reduce the proportion of metaphosphoric acid and add other acids and EDTA to stabilize vitamin C. In this study, the amount of vitamin C in water extract of collards (*Brassica oleracea*) was about 30 times less than the quantity detected when the samples were extracted with a 4.5 % metaphosphoric acid solution (pH 2.0). Franke et al. (2004) assumed that during extraction with water the ascorbate oxidase enzyme was not completely inactivated. The optimum pH range of reaction of the enzyme is between 5.0 and 6.5 (Saari et al. 1999). Metals such as iron and copper increase vitamin C oxidation and a metal chelating agent is usually recommended (Ball 1994; Hernández et al. 2006).

HPLC is the most widely used for vitamin C analysis because of the high sensitivity and selectivity of this technique. Due to absorbance of ascorbic acid at 265 nm, HPLC methods with ultraviolet detection have been developed. However, there are many interfering substances absorbing in the same region and a sample clean-up can resolve interferences. Tumbas Šaponjac et al. (2014a, b, 2015) separated polar compounds, including vitamin C, from polyphenols in dried bilberry extract, using SPE (Fig. 6.3).

The classical method for vitamin C analysis in foods is the titulometric that does not quantify dehydroascorbic acid but only ascorbic acid. Spectrophotometry, fluorimetry, amperometry, electrophoresis, chromatography, and enzymatic methods have also been used, but some of these methods have limitations regarding specificity (Kall and Andersen 1999; O'Connell et al. 2001; Furusawa 2001). As dehydroascorbic acid detection is hindered by its weak molar absorptivity, most researchers choose to reduce it to ascorbic acid before chromatographic separation, making indirect quantification by difference. Dithiothreitol (DTT) has been widely used as reducing agent (Furusawa 2001). Also, standard procedure for ascorbic acid determination according to method of AOAC Method 967.21, 45.1.14 is based on oxidation of L-ascorbic acid to L-dehydroascorbic acid by 2,6-dichloroindophenol (AOAC 1995) and the method can be conducted as titrimetric or colorimetric where L-dehydroascorbic acid is further converted into oxazone with 2,4-dinitrophenylhydrazine, recolored with sulfuric acid and measured at 520 nm. Microfluorimetric method of AOAC 967.22, 45.1.15 can be used for measurement both ascorbic acid and dehydroascorbic acid by

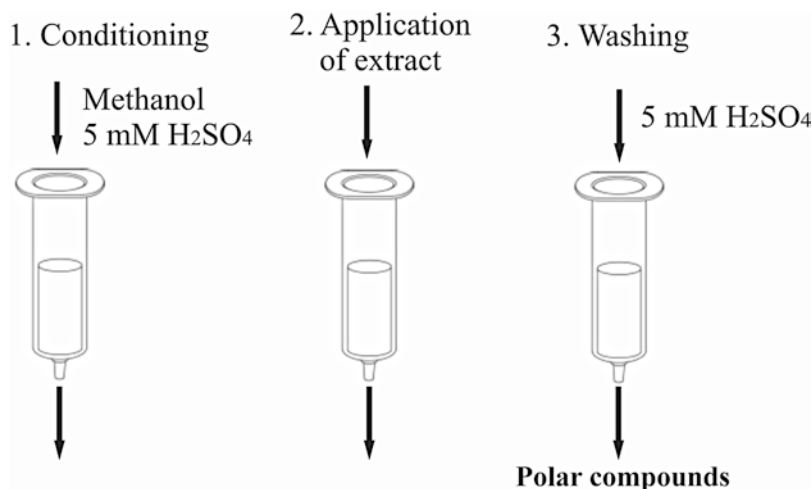


Fig. 6.3 Separation of polar compounds from polyphenols using SPE

previous oxidation of ascorbic acid to dehydroascorbic acid. A fluorescent quinoxaline compound is formed upon reaction of ascorbic acid with *o*-phenylenediamine and measured at $E_x = 356$ nm, $E_m = 440$ nm (AOAC 1995). These assays lack sensitivity and specificity because of interfering compounds such as sugars, amino acids, and glucuronic acid (Fernández-Muñoz et al. 2008).

Giannakourou and Taoukis (2003) proposed the chromatographic conditions for vitamin C analysis consisting of isocratic elution, detection in the UV range, using a mobile phase of water with pH adjusted to 2.2 with metaphosphoric acid. The mobile phase proposed by Franke et al. (2004) included the buffering agent 1 mM monobasic sodium phosphate with 1 mM EDTA, pH adjusted to 3.0 with phosphoric acid, isocratic elution and detection at 245 nm.

Versini et al. (2006) proposed methods for fruit juice clean-up procedure for detection of artificially added vitamin C. Also, a new approach for the stabilization of L-ascorbic acid against its easy oxidation by adding metabisulfite to the juice before any processing step was suggested. Juice was treated with pectolytic enzyme, sodium metabisulfite, and bentonite prior to isolation of ascorbic acid (along with malic and citric acid) by preparative HPLC and purification by ion exchange. Another method for purification consists of loading the sample (pH 7.0) on the cartridge, pre-washed with methanol, and conditioned with 0.6 N HCl and water, end eluting ascorbic acid with 0.05 N Na₂SO₄.

Electrochemical detection can be an alternative method for detection of vitamin C due to its electroactivity. There are many advantages of electrochemical detectors such as simplicity, ease of miniaturization, high sensitivity, and relatively low cost. Gazdik et al. (2008) optimized a method for determination of vitamin C using HPLC coupled with electrochemical detection. Then the concentration of ascorbic

acid was determined in various samples including food (orange and apples) under the optimized experimental conditions: detector potential 100 mV, temperature 25 °C, mobile phase 0.09 % TFA:ACN, 3:97 (v/v). They concluded that coulometric detectors are more sensitive to presence of vitamin C than amperometric detectors.

6.4.3 Carotenoids

Carotenoids are certainly among the most widespread and important pigments in living organisms. Carotenoids are fat-soluble food components that are categorized as either xanthophylls (oxygen-containing carotenoids) or carotenes (hydrocarbon carotenoids) according to their chemical composition (Zeb and Murkovic 2010). They are yellow or red photosynthetic pigments that serve as light absorbers and protect plants and microorganisms against excessive irradiation. They strongly interact with ROS and thus act as antioxidants. Some of them are vitamin A precursors (Feltl et al. 2005). Aside from the provitamin A activity, these compounds have health-promoting effects: immunoenhancement and reduction of the risk of developing degenerative diseases such as cancer, cardiovascular diseases, cataract, and macular degeneration (Krinsky and Johnson 2005).

Carotenoids, vitamin A precursors, and vitamin A are poorly soluble in water and relatively unstable during food processing and storage due to its chemical structure, which contains many double bonds susceptible to degradation. The stability of vitamin A is also affected by acidity (at pH below 5.0 this compound is more easily destroyed), trace metals, and ultraviolet light. To minimize the degradation of vitamin A, industry employs more stable esters and antioxidants (BHA, BHT, vitamin E, etc.) (Andrés et al. 2014).

Databases on carotenoid composition (Holden et al. 1999; O'Neill et al. 2001) focus on α -carotene, β -carotene, β -cryptoxanthin, lycopene, lutein, and zeaxanthin, the major carotenoids in foods and the most studied in terms of human health. Figure 6.4 presents structures of the most abundant carotenoids.

Carotenoids exhibit pronounced photo- and thermal sensitivity. Irradiation and an increased temperature lead mainly to *trans*–*cis* isomerization which causes bending of the originally rod-like molecule and may also cause cleavage of the molecule, especially in the presence of air and catalysts (Feltl et al. 2005). Thus, special attention during sampling and measures during the pretreatment are needed, including (Oliver and Palou 2000; Britton et al. 1991):

- Addition of antioxidants.
- Laboratory operations should be carried out in dimmed, yellow or red light.
- Evaporations should be carried out under a protective nitrogen or argon atmosphere at temperatures below ca. 40 °C.
- The samples should be stored in darkness, under nitrogen or argon, at temperatures around –20 °C.

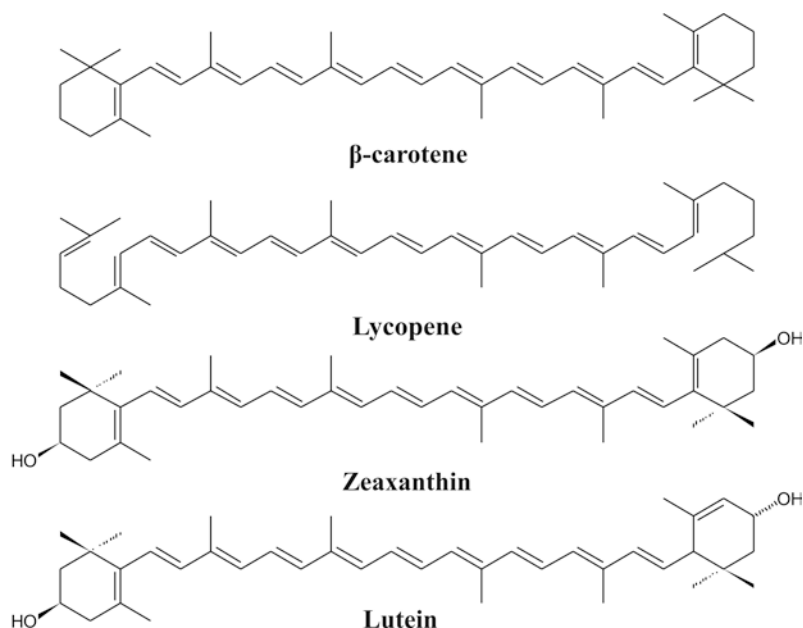


Fig. 6.4 Structures of the most abundant carotenoids

Their insolubility in water and poor solubility in many organic liquids place considerable demands on proper selection of extraction and pre-concentration agents and impose severe limitations on the composition of HPLC mobile phases (Feltl et al. 2005). Various organic solvents, for example acetone, tetrahydrofuran (THF), n-hexane, pentane, petroleum ether, methanol, and ethanol, and mixtures of these solvents can be used for the extraction of carotenoids, as described in detail by Rodriguez-Bernaldo de Quiros and Costa (2006). Different solvent systems are used for different samples. The same solvent system in the same or a different ratio can be used for a variety of foods. The main concern about these extraction solvents is their noxiousness; therefore optimization of the extraction process is necessary. To isolate carotenes, the sample should be extracted first with a polar solvent (e.g., methanol), which removes water and more hydrophilic xanthophylls, followed by extraction with a suitable solvent of low polarity (Feltl et al. 2005).

Different extraction procedures are used to isolate carotenoids, including simple solvent extraction (Soxhlet), lipid phase distribution, solid-phase extraction, accelerated solvent extraction, and supercritical fluid extraction (Minguez-Mosquera and Garrido-Fernandez 1989; Gutierrez et al. 1989; Breithaupt 2004; Gomez-Prieto et al. 2004). Tumbas Šaponjac et al. (2014a, b, 2015) reported that hexane shows higher selectivity for paprika pigments—carotenoids, than supercritical carbon dioxide. The color intensity in oleoresins obtained by supercritical fluid extraction was increased three times as a result of increase in extraction pressure from 20 to 40 MPa and mimics the increase in the solubility of pigments in supercritical CO₂.

with pressure. In accordance with this, it was reported by Tepić et al. (2009) that supercritical CO₂ extracted the least carotenoids under the lowest extraction pressure (20 MPa), whereas the extraction of carotenoids was increased with the increase in pressure. Other investigators also confirmed the improvement of carotenoid pigment extraction from paprika with increasing process pressure. A threefold increase was found by Jaren-Galan et al. (1999) with increase in pressure from 137.8 to 413.4 bar. Gnayfeed et al. (2001) showed a significant increase in carotenoid extraction in the experimental range from 100 to 400 bar at 40 °C, and fivefold increase was found by Daood et al. (2002) for the same pressure range, at the temperatures from 35 to 55 °C. The main advantage of supercritical extracts is that they are solvent free, CO₂ is nontoxic and is considered a GRAS solvent, which means that it is acceptable for use in food. On the contrary, extracts obtained by conventional extraction using organic solvents can contain some residues of solvents. Additionally, supercritical extraction is compatible with supercritical fluid chromatography, because they can share the mobile phase and some devices. Supercritical fluid extraction is rapid, readily automated, and selective where the extraction selectivity can easily be varied by varying the carbon dioxide density (i.e., by varying the temperature and pressure) or addition of co-solvent (Gnayfeed et al. 2001; Modey et al. 1996). The most important sources for carotenoids are plants; however, a variety of microorganisms and small animals also synthesize carotenoids. Carotenoids from animal and microbial sources are usually extracted with acetone, or a mixture of hexane with petroleum ether and ethanol (Jaime et al. 2005). After washing the petroleum ether extract with water, the pigments are stored at low temperature (below −20 °C).

Chromatography is the technique of choice for the analysis of carotenoids (Rivera and Canela-Garayoa 2010), although due to their chromophores carotenoids can easily be detected by TLC. There are some advantages of HPTLC over HPLC in qualitative and quantitative analysis, such as lower solvent consumption, minimal sample preparation, and concurrent analysis of high throughput with minimal costs (Sherma 2000). TLC analysis of carotenoids is demanding due to their instability. Sorbent itself can play a crucial role as its active surface can accelerate the degradation of carotenoids (Rivera and Canela-Garayoa 2010). On C18 reversed-phase silica gel plates, where carotenoids are more stable than on ordinary silica gel, methanol, acetonitrile, acetone, and petrol ether or n-hexane are used in different combinations and ratios as developing solvents for their separation (Rodić et al. 2012).

The use of TLC chromatography in carotenoid analysis of plant and animal samples has been reviewed by Zeb and Murkovic (2010). Carotenoids extracted from red pepper could easily be separated on silica gel plates by use of petroleum ether–hexane–acetone 2:1:1 (v/v). The separation of color pigments of paprika has been performed on many stationary phases, while petroleum ether with acetone was used as major organic mobile phase and hexane as the second major mobile phase used in TLC of carotenoids from plant sources.

The existence of an extensive system of conjugated double bonds is responsible for strong absorption of carotenoids in the visible region, between 400 and 500 nm (three absorption bands or two bands plus a shoulder, Table 6.2) (Scott 2005).

Table 6.2 Spectral characteristics of selection of carotenoids

	MW	$A_1\%$	$\epsilon^1 \text{ mM}$	λ (nm)	Solvent	Absorption peaks		
α -Carotene	537	2710	145	445	Hexane	422	445	473
β -Carotene	537	2592	139	450	Hexane	425	450	478
β -Cryptoxanthin	553	2460	136	450	Hexane	428	450	478
Lutein	569	2550	145	445	Ethanol	421	445	474
Lycopene	537	3450	185	470	Hexane	444	470	502
Zeaxanthin	569	2480	141	450	Hexane	425	450	478
<i>Natural carotenoids as food colors</i>								
Bixin (Bixa orellana)	395	4200	166	456	Petroleum ether	432	456	490
Capsanthin (paprika)	585	2072	121	483	Benzene	450	475	505
Capsorubin (paprika)	601	2200	132	489	Benzene	445	479	510
<i>Synthetic food colors</i>								
β -apo-8'-carotenal	417	2640	110	457	Petroleum ether	405	430	460
Canthaxanthin	564	2200	124	466	Petroleum ether		466	

This property has long been used for the routine quantification of carotenoids in solution, according to the law of Lambert-Beer. The amount of carotenoids X (mg) of a carotenoid present in a volume V (mL) of solution can be calculated from the following equation:

$$X = \frac{A \times V \times 1000}{A_{1 \text{ cm}}^{1\%} \times 100}$$

In practice, the main problem is to know the exact value of the specific absorption coefficient ($A_{1 \text{ cm}}^{1\%}$) for a given carotenoid. When $A_{1 \text{ cm}}^{1\%}$ of the specific carotenoid is not known, or when the sample is a mixture of pigments, an average value of 2500 is used for calculation.

Chlorophylls, usually present along with carotenoids, have absorption bands in the same region as the carotenoids. Saponification of the sample will remove the chlorophylls. The other way to avoid interferences is to use an alternate wavelength for the carotenoids: most of the major carotenoids of interest in foods have an absorption peak around 480 nm, where any absorption of chlorophylls causes less interference (Scott 2005).

Chromatographic techniques are used for detection of the carotenoid structures and chromatographic analysis of carotenoids have been reviewed by Su et al. (2002). C18 RP columns are commonly used; however, C30-RP column have shown to be particularly efficient (Rouseff et al. 1996). In reversed-phase systems, nonaqueous mobile phases are recommended, e.g., mixtures of solvents, mostly of methanol, acetonitrile, and THF, except when polar molecules such as glycosyl esters of carotenoids are present in the mixture. Some of the carotenoids present in foods are esters and they can be determined by comparing the chromatograms before and after saponification (10 % KOH+antioxidant BHA in MeOH for 3 h). Peaks of esterified carotenoids are replaced with peaks of free carotenoids with shorter

retention times, after saponification. Saponification can degrade carotenoids to some extent (Oliver and Palou 2000). One of the great problems of carotenoid analyses lies in the unavailability of standard compounds caused by natural instability of carotenoids. The conjugated double-bond system of carotenoids causes their spectral activity, but also their electroactivity enabling their electrochemical detection (Jeevarajan et al. 1994). Mass spectrometry enables analyte quantification and elucidation of its structure, on the basis of the molecular mass and of fragmentation. Most of the methods applied to carotenoids use the API mode, due to the absence of protonation sites in carotenoids. Combinations of HPLC-MS and HPLC-NMR for identification and determination of carotenoid stereoisomers are the most reliable (Feltl et al. 2005).

IR spectroscopy is not very applicable for the identification of carotenoids, but it is especially selective in determining the presence of particular functional groups such as acetylenic, allenic, hydroxyl, and carbonile (Mínguez-Mosquera et al. 2008). Raman scatterers and Raman spectroscopy is usually the method of first choice for analyzing carotenoids. Resonance Raman spectroscopy produces strong lines even at concentration as low as 10–5 mol/L. The strongest lines are observed within the range of C=C and C–C stretchings of the conjugated backbone (1550–1500 and 1200–900 cm^{-1}) when excited with a wavelength corresponding to the main electronic transition ($1\text{Bu}(S_2) \leftarrow 1\text{Ag}(S_0)$ ($p-p^*$)) but an intensity enhancement can also be attained outside this resonance condition (Abramczyk et al. 1999).

6.4.4 Tocopherols

Tocopherols are fat-soluble compounds having vitamin E activity, and they are necessary for normal cell differentiation and function. The term vitamin E refers to a group of eight fat-soluble compounds with different biological activities, which are divided into two groups: tocopherols and tocotrienols. All feature a chromane ring, with a hydroxyl group and phytyl side chain. Tocotrienols differ from the analogous tocopherols by the presence of three double bonds in the phytyl side chain. Both the tocopherols and tocotrienols occur in α , β , γ , and δ forms, determined by the number and position of methyl groups (Me) on the chromanol ring. Tocotrienols occur at very low levels in nature. α -Tocopherol presents the highest biological potential (Luzia and Rondo 2014). Figure 6.5 presents structures of four tocopherol forms.

One of the main biological functions of vitamin E is as an antioxidant which protects the polyunsaturated fatty acids of cell membranes from free-radical damage. Product fortification with DL- α -tocopheryl acetate or other forms of vitamin E or other antioxidants can be a required practice in manufacturing of functional foods. The tocopheryl esters are usually applied for the purpose of fortification since they are more stable to oxidative degradation (Andrés et al. 2014). Tocopherols are found predominantly in corn, soybean, and olive oils (Roy et al. 2002). Olive oil adulteration can be traced based on its tocopherol and tocotrienol contents (Aparicio and Aparicio-Ruiz 2000). The importance of vegetable oils minor constituents such

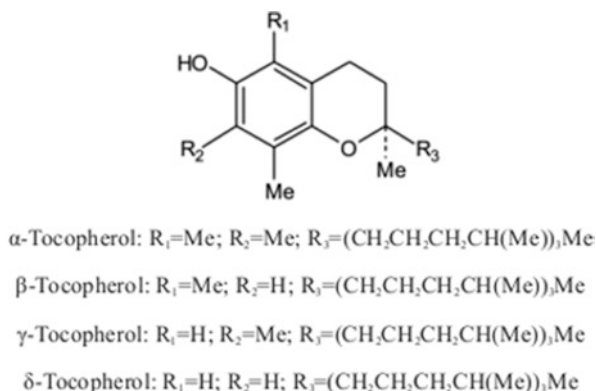


Fig. 6.5 Structures of tocopherol isomers

as tocopherols and tocotrienols to their oxidative stability and possible health benefits have prompted the need for rapid and reliable analytical methods for their detection (Psomiadou and Tsimidou 1998; Guthrie et al. 1997).

The official method of measurement of tocopherols in foods and oils involves alkaline saponification performed before extraction as a separate step and is designed to overcome interactions between lipids, lipid-soluble vitamins, and the matrix. Saponification is often regarded as a lengthy and tedious procedure with the potential to cause degradation of tocopherols. As a precaution against damage to tocopherols, it is performed under an inert gas and always in the presence of antioxidants such as pyrogallol, BHT, and ascorbic acid (Korchazhkina et al. 2006). Souza et al. (2014) investigated the influence of two factors, agitation time, and percentage of KOH, in the saponification procedure for the determination of tocopherols in foods. Peanut samples were saponified using 60 or 80 % KOH in aqueous solution of ethanol with added ascorbic acid for 2 or 4 h. Tocopherols were analyzed in hexane extract of saponified peanut samples by RP-HPLC on C18 column and UV-detection at 295 nm, with mobile phase methanol/dichloromethane in a 85:15 (v/v) ratio. The response surfaces analysis showed that the most efficient saponification procedure was obtained using a 60 % (w/v) solution of KOH and with an agitation time of 2 h. In the case of lipid environment, such as milk, tocopherols are extracted with hexane, after disruption of milk fat globules with ethanol or methanol without saponification. Korchazhkina et al. (2006) compared the recovery of tocopherols extraction from milk with or without saponification and concluded that the method including saponification gave higher and more consistent recoveries, and therefore more reliable data. After extraction, tocopherols were identified and quantified using RP-HPLC method including integration at 295 nm.

Generally, tocopherols can be detected by light scattering, spectrophotometry, fluorimetry, and electrochemistry. Tocopherol acetate (commonly used in food, dietetic and pharmaceutical preparations) is not fluorescent and may be detected by UV photometry. Wavelength for detection of free tocopherols is 292 nm and 284 nm for tocopherol acetate. With fluorescence detection excitation wavelength is set at

292 nm, and emission at 327 nm. Using the evaporative light-scattering detector, linear responses are obtained only in the range 0.1–0.5 μg (Edison 2009).

Andrés et al. (2014) developed a method for simultaneous detection of tocopherols and tocopheryl acetate, retinol and retinyl palmitate, and β -carotene, often present in fortified milk- and soy-juice-based beverages. Analytes were extracted with hexane:acetone 1:1 (v/v). Organic phase was analyzed by RP-HPLC method, in isocratic mode with methanol: THF: water 67:27:6 (v/v/v) as mobile phase. The eluted compounds were monitored by a DAD at 290 nm (tocopherols and tocopheryl acetate), 325 nm (retinol and retinyl palmitate), and 440 nm (β -carotene). This method was shown to be simple with good linearity, precision, accuracy, and sensitivity. The sample preparation is simple and eliminates the saponification step, minimizing manipulation and, thereby, reducing sample loss and allowing for optimum analyte recoveries. Direct dilution method was used also in the study of Tumbas Šaponjac et al. (2014a, b, 2015) for determination of tocopherols in paprika oleoresins. Prior to analysis, oleoresin samples were diluted with methanol. α -Tocopherol separation was performed on a reversed-phase column with an isocratic elution using methanol. The chromatograms were acquired in the range 294 nm by DAD detector. López-Ortiz et al. (2008) developed a method for identification of tocopherol isomers in almonds. Almond oil was extracted with petroleum ether as solvent. To simplify the tocopherol extraction method, two different methods were compared: alkaline digestion of the almond oil prior to extraction of the unsaponifiable compounds with hexane and direct dilution of almond oil by 1-propanol prior to injection into the chromatographic system. Recovery percentages obtained using the saponification method were lower than the ones obtained when direct dilution of the almond oil was applied. Detection of tocopherol homologues was carried out by the DAD detector set at 295 nm. Simultaneous fluorescence detection for tocopherol homologues was performed at an excitation of 290 nm and an emission wavelength of 325 nm, due to its higher sensitivity. A mixture of acetonitrile and methanol (1:1) was used as mobile phase. Sedej et al. (2010) developed a method for determination of tocopherols in flours. In this study, free tocopherols in buckwheat and wheat flours were extracted after alkaline hydrolysis with *n*-hexane. Extract was redissolved in methanol and analyzed by RP-HPLC, on an C18 column with methanol as mobile phase. Tocopherols were detected at 295 nm.

Because of their low oxidative potential, the various forms of vitamin E can be analyzed by electrochemical detection. Puspitasari-Nienaber et al. (2002) have developed a method for simultaneous determination of tocopherols, tocotrienols, carotenoids, and chlorophylls in vegetable oils by C30 RP-HPLC with coulometric electrochemical array detection (a PDA equivalent of an electrochemical detector containing a serial array of coulometric electrodes with improved resolution). Oil samples were dissolved in the mobile phase and directly injected without extraction and saponification preventing adverse effects on and possible loss in these compounds. The use of a coulometric electrochemical array detector enabled construction of the characteristic current–voltage curves of each tocopherol form. α -Tocopherol exhibited a dominant response oxidation potential at around 260 mV and γ - and δ -tocopherol at around 320 mV and 380 mV, respectively.

Psomiadou and Tsimidou (1998) have detected simultaneously tocopherols, carotenoids, and chlorophylls in virgin olive oil by a C18 HPLC method coupled with a photodiode array detector. Their method, however, was not able to separate the *cis*-isomers of carotenoids.

6.5 Conclusion

Reliable quantitative data on bioactive compounds in food are essential in agriculture in choosing cultivars/varieties and optimizing production and post-harvest handling conditions; in food technology in selecting raw materials and in monitoring and controlling degradation during processing and storage; in nutrition and public health in assessing adequacy of dietary intake and formulating dietary recommendations/guidelines; in the medical sciences in establishing associations between dietary intake and disease prevention. Health claims resulting from the data obtained in the studies on bioactive compounds composition in food have large influence on public opinion, industrial sector and market as well. This fact calls for great responsibility of food professionals in using analytical methods for quality control in the growing functional food industry. Therefore, fast, accurate and nondestructive detection approaches are required as methods of quality control in the production of functional ingredients or applied to raw materials and finished products for the pharmaceutical, cosmetic or functional food industries. Accurate estimation of bioactive compounds will enable researchers to correctly evaluate the role of bioactive compounds in health, provide precise dietary and safety guidelines on consumption of these compounds, allow manufacturers, consumers, and marketing professionals to differentiate quality value-added products from the conventional ones.

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Chapter 7

Beer as an Integral Part of Healthy Diets: Current Knowledge and Perspective

Ida J. Leskošek-Čukalović

7.1 Introduction

People have been drinking beer for more than 8000 years, from the time when it was first made probably unintentionally. For centuries, it has played an important part in many cultures, serving as a safe drink in an age when the purity of water was uncertain and drinks like coffee and tea were still unknown. Ever since, it has been accepted not only as a warming and refreshing drink, but also as liquid bread, a source of energy, and a drink that can promote well-being (Bamforth 2000; Nelson 2005). For the last decade, it has been the focus of significant medical research. Numerous studies have given the scientific confirmation that beer is far more than a thirst-quenching low-alcohol beverage. Published data have indicated that beer contains a wide range of nutrients with bioactive properties, and if consumed moderately and in a responsible manner, it can be a useful part of a healthy diet, it can improve well-being, and reduce risk of various types of diseases. However, beer like any other alcohol beverage may contain some substances with potential harmful effects as well. In the case of beer, they are biogenic amines, nitrosamines, purine, gluten, and sulfur dioxide. They may cause the problems, especially for individuals with gout and intolerance to gluten. So talking about beer as a part of the diet, several facts deserve to be discussed: the latest information considering the beer's beneficial action, the meaning of moderation in each particular case, the significance of beer versus other alcohol beverages, potential harmful components, and finally, the perspective in terms of the new beer types with new sensory and functional properties.

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7.2 Bioactive Components in Beer

Beer is an extremely complex beverage. Apart from water, which normally represents more than 90 % and about 5 %v/v of ethanol, beer contains around 800 organic compounds, and many of them are biologically active. Potentially beneficial effects on the human body are the consequents of two facts, the small amount of alcohol and the presence of other compounds, such as vitamins, minerals, trace elements, phytoestrogens, and antioxidants.

The most important vitamins in beer are B vitamins. Numerous studies (Van der Gaag et al. 2000; Mennen et al. 2003) confirmed that beer consumption in moderation could provide considerable increase of vitamin B in the human body (Table 7.1) (Bamforth 2002).

From the nutritional point of view and the position of beer as part of the diet, the most important minerals are potassium, magnesium, sodium, and phosphorus (Buiatti 2009; Leskošek-Čukalović 2009). Beer is rich in potassium and magnesium, low in sodium and calcium, and may be a significant dietary source of phosphorus, and even selenium, and silicon (Table 7.2) (Buiatti 2009).

Beer contains phenolic acids and flavonoids derived principally from barley and hops (*Humulus lupulus* L.). There are many references in the literature determining their content in beer. Identified are 78 different phenolic compounds including simple phenolics, aromatic carboxylic and phenol carboxylic acids, such as anthocyanin, chalcone, flavonol, flavan-3-ol, procyanidin, and isoflavone classes (Shahidi and Nacz 2004; Gerhäuser and Becker 2009; Mayer 2009; Gorjanović et al. 2010; Piazzon et al. 2010). The antioxidant capacity of beer depends on the type of beer (Lugasi and Hóvári 2003; Saura-Calixto and Goñi 2006; Saura-Calixto et al. 2009). Dark beers seem to be considerably inferior to coffee, red wine and tea, but can be compared to rose wine (Table 7.3) (Saura-Calixto et al. 2009).

Prenylflavonoids (PF) in beer that deserve special attention are xanthohumol (XH), isoxanthohumol (IX), and 8-prenyl-naringenin (8-PN). They are present

Table 7.1 The composition of beer relative to recommended dietary intakes of vitamins

Parameter	Daily adult (age 25–50) requirement		Range in beer (per L)
	Male	Female	
Energy (kcal)	2550	1940	150–1100
Protein (g)	63	50	3–5
Thiamine (mg)	1.5	1.1	0.003–0.08
Riboflavine (mg)	1.7	1.3	0.02–0.8
Niacin (mg)	19	15	3–8
Vitamin B6 (mg)	2	1.6	0.07–1.7
Folate (μg)	200	180	40–600
Vitamin B12 (μg)	2	2	3–30
Biotin (μg)	30–100	20–100	2–15

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Table 7.2 Mineral content of beer

Mineral	Beer (mg/L)	Mineral	Beer (mg/L)
Potassium	200–600	Silica	40–120
Sodium	10–100	Phosphate	260–995
Magnesium	60–250	Sulfate	60–300
Calcium	20–160	Chloride	150–400
Iron	0.01–0.3	Selenium	<0.0004–0.0072
Copper	0.02–0.4	Lead	<0.01–0.1
Zink	0.02–4.5	Fluoride	0.09–0.2
Manganese	0.03–0.2	Cobalt	0.01–0.11

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Table 7.3 Antioxidant capacity of beverages (FRAP and ABTS assays)

Beverage	FRAP ($\mu\text{mol Trolox}/100 \text{ mL}$)	ABTS ($\mu\text{mol Trolox}/100 \text{ mL}$)
Coffee	2267 $\pm$ 18.9	1328 $\pm$ 5.1
Tea	601 $\pm$ 5.5	631 $\pm$ 8.0
Red wine	1214 $\pm$ 24.5	1093 $\pm$ 54.2
Rose wine	286 $\pm$ 39.2	261 $\pm$ 23.7
White wine	154 $\pm$ 36.8	181 $\pm$ 22.2
Orange juice	515 $\pm$ 41.5	249 $\pm$ 3.4
Cola	20.7 $\pm$ 0.7	≤ 10
Lager beer	139.6–149.5	220.0–305.6
Dark beer	278.8	259.0–536.5
Alcohol-free beer	75.6–91.2	155.8–175.3

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almost exclusively in hops and beer is practically the only foodstuff in which they can be found. Their final content in beer strongly depends on the production process, the type of used hops product (cones, pellets, or extracts), and the way hops are introduced in the brewing kettle. Although XN and lower amounts of desmethylxanthohumol (DMX) are the main PF in fresh and properly preserved hops (more than 95 % of the PF fraction, present at a concentration of 0.01–0.5 %), their abundance in beer is much lower in favor of the corresponding prenylflavanones. During the wort boiling much of the XH and all of the DMX are converted by thermal isomerization into IX and a mixture of 6-PN and 8-PN (Possemiers et al. 2009; Česlova et al. 2009). Therefore, IX and lower quantities of 8-PN are the main prenylflavonoids in beers (Table 7.4) (Česlova et al. 2009).

Beer, like any other beverage, contains some substances with potential harmful effects: biogenic amines, nitrosamines, purine, gluten, and sulfur dioxide.

Biogenic amines (BAs) form a group of undesirable natural components widespread in foods and beverages, e.g., scombroid fish (histamine fish poisoning), meat and meat products, cheeses, vegetable products, wine, and beer. They are essential

Table 7.4 Prenylflavonoids) content in beer and dietary supplements

Beer (µg/L)	Xanthohumol	Isoxanthohumol	8-Prenyl-naringenin	Total
Pils	9–34	400–1060	13–21	460–1100
Lager	0.2–28	20–1910	2–175	24–2342
Porter	690	1330	240	2900
Stout	340	2100	69	2680
Wheat beer	5	300	8	330
Strong ale	240	3440	110	4000

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Table 7.5 Biogenic amines in 195 European beers from 1996 and 114 beers from Czech Republic in 2011

Biogenic amines	Kalač and Křižek (2003)	Buňka et al. (2012)
	Range (mg/L)	Range (mg/L)
Histamine	nd–21.6	nd
Tyramine	0.6–67.5	nd–100
Tryptamine	nd–5.4	2.7–58.7
Phenylethylamine	nd–8.3	>6
Cadaverine	nd–39.9	nd–100
Putrescine	1.5–15.2	nd–62.2
Spermine	nd–3.9	nd–100
Spermidine	nd–6.8	nd–100

(C J. Inst. Brew. 2003, 2012), reprinted with permission

Note: Values are expressed as mean ± SD

for the human organism, but their excessive intake can cause many health problems, such as headaches, fluctuation of blood pressure, breathing problems and vomiting. Histamine (HI) and tyramine (TY) are especially known for their psychoactive and vasoactive effects. The nine determined amines in beer can be divided into two groups. Group one includes putrescine (PUT), spermidine (SPD), spermine (SPM), and agmatine (AGM) and can be considered as natural beer constituents primarily originating from the malt, whilst Group two, mainly HI, TY, and cadaverine (CAD), usually indicate the activities of contaminating lactic acid bacteria during brewing. The bottom brewing yeasts do not appear to produce TY or HI and even, yeast recycling for several fermentations did not influence amine levels. Also wild yeasts were not found to produce TY, while a significant positive relationship was observed between amine formation and lactic acid bacteria. The major biogenic amines found in different types of beer as the results from surveys in several European countries are given in Table 7.5 (Kalač and Křižek 2003; Buňka et al. 2012).

Nitrosamines are the most mentioned carcinogens in beer. The major nitrosamine in beer is N-nitrosodimethylamine (NDMA). Under the International Agency for Research on Cancer (IARC), which is part of the World Health Organization (WHO), NDMA is classified as Group 2A substances (probably carcinogenic to

humans). The European Union categorizes NDMA as category 1B (presumed to have carcinogenic potential for humans; largely based on animal evidence) (World Health Organization/International Agency for Research on Cancer 2010; Selin 2011). The NDMA is formed from phenolic alkaloids in green malt (hordenine and gramine formed in malt roots during germination) and nitrogen oxide during kilning by direct firing, which was the predominant production method in the past. For a long time, beer was regarded as one of the major source of NDMA in human nutrition. It was estimated that the 65 % of the daily intake of NDMA (1.1 µg/day), by men in West Germany resulted from the consumption of beer (only 10 % from cured meat products) (Tricker et al. 1991). However, since 1980 malsters have altered the process of kilning to indirect techniques (air heating by steam or hot water) and considerable reduction in volatile nitrosamine content in beer has been achieved.

The established limiting concentrations of NDMA in beer are regulated by local legislations. It is 5 µg/kg in the United States, 3 µg/kg in Estonia, 2–15 µg/kg in Russia, but only 0.5 µg/kg in Italy, Switzerland, Germany, and most other countries. The situation now is quite different, than it was before. A range of 138 beers from 42 countries analyzed in 2007 for the presence of NDMA, showed that the overwhelming majority of samples (79 %) did not contain detectable NDMA (less than 0.1 µg/L), that only three samples exceeded 0.5 µg/L (less than 3 %), and only one sample exceeded 1.0 µg/L. The determined range was from <0.1–1.9 µg/L (Baxter et al. 2007). The similar results were obtained in 2005 for 264 analyzed beers from nine different countries. The determined range was from <0.15–1.31 µg/L with an average of 0.21 µg/L (Yurchenko and Moölder 2005). No association was found between NDMA content and beer strength, type or geographical origin. However, it was noted that water could be a potential source of NDMA in beverages (Baxter et al. 2007).

For moderate beer drinkers, determined levels of nitrosamines in beers available on the market are unlikely to constitute a hazard. The contribution of beer in daily intake of NDMA is now much lower than different products undergone drying, kilning, salting, smoking, or curing. Especially taking into account exposure via smoking and drinking water (Table 7.6) (U.S. Department of Health and Human Services 2011).

Other beer constituents that deserve attention are purine. They are converted to uric acid in the body and increase plasma concentration of uric acid. Elevated serum uric acid (hyperuricaemia) is a metabolic disorder that is associated with gouty arthritis urolithiasis, kidney, and cardiovascular disease including coronary heart disease and stroke (Nakamura et al. 2012; Cortacero-Ramirez et al. 2004; Yamamoto and Moriwaki 2009). Beer is the alcoholic beverage acknowledged to contain considerable amounts of purine hypoxanthine, xanthine, guanine, and particularly guanosine (dominant beer purine more readily absorbed than other nucleosides, nucleotides, or bases) (Table 7.7) (Yamamoto and Moriwaki 2009).

Two potential allergens must be considered in relation to beer as well. One is gluten and the other is sulfur dioxide.

Gluten intolerance (Celiac disease) is an autoimmune disease induced in genetically susceptible individuals by exposure to dietary gluten. Gluten is the generic

Table 7.6 Intake of nitrosodiethylamine from exposure via diet, smoking, and water

Alcohol	Alcoholic beverages	0.1 µg/kg
Diet	Cheese	0.5–30 µg/kg
	Soybeans	0.2 µg/kg
	Soybean oil	4 µg/kg
	Various fish	<147 µg/kg
	Salt-dried fish	1.2–21 mg/kg
	Cured meats	<40 µg/kg
Tobacco per cigarette	Tobaccos health and disease prevention smoke condensate	1.0–28 ng
	Mainstream smoke	<8.3 ng
	Side stream smoke	8–73 ng
Water	High-nitrate well water for drinking	0.010 µg/L
	Deionized water	0.33–0.83 µg/L

(Data from U.S. Department of Health and Human Services 2011), fair use

Table 7.7 Purine content in a selection of beer

Beer	Concentration (mg/L)		
	Guanosine	Xanthine	Adenine
Belgian beer	72.8 ± 1.2	94.0 ± 5.1	nd
British beer	10.0–17.3	2.6–3.8	0.4–3.0
Japanese beer	174 ± 16.1	58.5 ± 3.6	17.2 ± 2.3
Lager beer	6.9–38.1	3.5–34.7	4.3–4.8
Wheat beer	5.1 ± 7.5	35.3 ± 5.9	nd
Guinness	10.6	5.5	nd

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Note: nd not detected; Values for Belgian, Japanese, and wheat beer are expressed as mean ± SD

term for several hundred homologous seed storage proteins in wheat, rye, oats, and barley. It includes the two groups of proteins known as glutelins and prolamins. The most troublesome component of gluten is the prolamin. Prolamins from barley are known as hordeins, from wheat gliadins, from rye secalin, from oats avenins. Gluten is the dominant barley protein (about 75 %), with 50 % prolamins and 25 % glutelins. As a result, barley and wheat were considered the foods and ingredients, to be excluded in the case of gluten intolerance. The same opinion was established for beer.

Different analytical methods have been developed for measuring the prolamin content in beer. Most of them were antibody-based like radioimmunoassay (RIA), enzyme immunoassay (EIA), and enzyme-linked immunosorbent assay (ELISA). The Sandwich ELISA-Ridascreen Gliadin for gluten analysis test is effective for intact prolamins. Nineteen commercial beers, which represent all principle brands of beers available on the Czech market, were analyzed for gluten content using immunochemically the gluten content method. Gluten concentrations were found to

Table 7.8 Gliadin levels in the different types of beer

Beer	Gliadin mg/L (ppm)
Gluten-free beers	<3
Alcohol-free	<5
Light lager	3.9±0.1 to 12.2±0.18
Lager	7.1±0.39 to 12.3±0.68
Pale Ale	7.17±0.32 to 15.3±0.53
Pale lager	8.26±0.39 to 14.9±0.64
Stout	18.9 to 21.3±0.15
Wheat beer	98.2±0.1 to 145.8±0.69

(C Food Chem. 2011), reprinted with permission

increase from alcohol-free beer (<3.0 mg/L), lager beers (<3.0–8.7 mg/L), stouts (9.0–15.2 mg/L), to wheat beers (10.6–41.2 mg/L). It was concluded that only 84 % of 19 beer samples was safe for consumption of coeliacs from the point of view of the legislation limit (20 mg/L) (Dostálek et al. 2006). The number of safe beer samples dramatically decreased to 30 %, if the samples were analyzed by competitive ELISA test. Sandwich ELISA-RIDASCREEN gliadin for gluten analysis is effective for intact prolamins, but not for hydrolyzed ones.

Competitive ELISA-RIDASCREEN Gliadin for celiac-toxic peptide analysis uses the R5 monoclonal antibody capable of recognizing immunostimulatory epitopes that are rich in proline and glutamine. It enables the evaluation of hydrolyzed products from wheat gliadin and related proteins in both rye and barley. The analysis of gliadin content of 28 commercially available beers using this method, showed that 34 % of the beers contained less gluten than the guidelines established by the Codex Alimentarius Standard (Table 7.8) (Guerdrum and Bamforth 2011). However, this study did not assess the hordein proteins, only proteins containing the motifs specific for the R5 antibody (Colgrave et al. 2012).

Similar results were obtained in a study of more than 40 Belgian brewed commercial beers with the Sandwich ELISA-Ridascreen Gliadin test (gluten-free labeled beers and malt beers: pils/lager, abbey, trappist, strong blond, amber, old brown, kriel, and gueuze) and some foreign commercial beers. The gluten content of the gluten-free labeled beers and the malt beers were in the range of 5–8 mg/L and beneath the quantitative detection limit (5 mg/L) to 101 mg/L, respectively; 45 of the 58 examined beers were gluten-free (<20 ppm gluten). The results of the Competitive ELISA-Ridascreen Gliadin test suggest that not all of these “gluten-free” beers would still be gluten-free in case the beers were analyzed by Competitive ELISA (Van Landschoot 2011).

The other allergenic reaction, which may be associated with beer, is a reaction to sulfur dioxide. This is an approved additive, which has been used as a preservative in a wide range of foodstuffs. In alcoholic beverages, natural sulphite concentrations emerge during fermentation by the metabolism of yeasts (beer yeasts produce

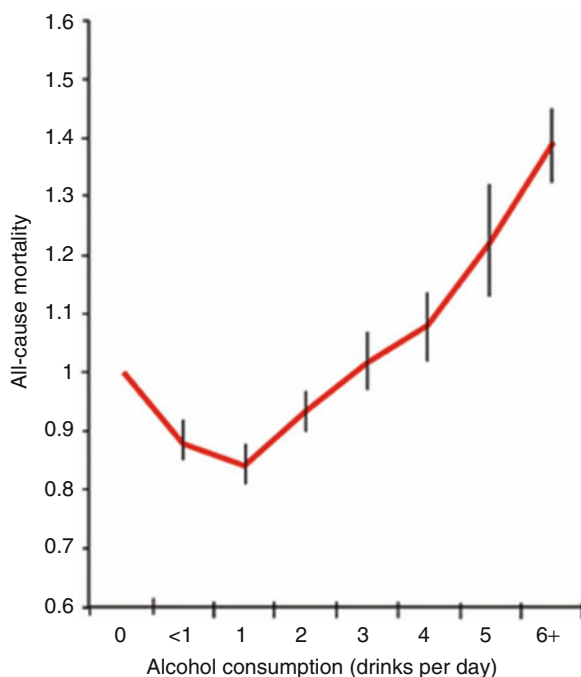
approximately 2–6 mg/L SO₂). In beer sulphite is a desired substance because of its antioxidative effect as scavenger and the binding of stale flavor causing carbonyl compounds. At the levels that are usually present, pose no health hazards. However, a small number of individuals are hypersensitive to sulphite and for these people it may be fatal, even at low levels of exposure. For alcohol beverages, sulfur dioxide and sulfites are the most relevant group of allergens and have been associated with the triggering of asthmatic responses in certain individuals. In beer, the levels of sulphite are controlled by EU law with the limit for packaged beers being 20 mg/kg (50 mg/kg in cask-conditioned beers). Those levels are relatively low in comparison with some other foods. Despite the worldwide consumption of beer, allergic reactions following beer ingestion and uncommon are very rare (particularly compared with allergic reactions following wine consumption) (Lachenmeier and Nerlich 2006; Diel et al. 2009).

7.3 Beer in Relation to Health

Since ancient times, people have attributed a variety of health benefits to moderate consumption of different fermented beverages, mostly beer and wine. For millennia, they were used in religious ceremonies, social events, and for healing purposes as well. Nowadays, we know that the correlation between alcohol consumption and health outcomes is complex and multidimensional. There is a basic disparity in alcohol–health relations between effects of heavier and lighter drinking. Alcohol and heavy uncontrolled drinking carried major medical and social risks. Approximately 4.5 % of the global burden of disease and injury is attributable to alcohol and it is a causal factor in more than 60 major types of diseases and injuries. It is the third highest risk for disease and disability (World Health Organization 2011). However, the effect it has in any particular case is attributed to a dose-related combination of beneficial and harmful effects.

The health effects of ethanol depend on the amount of alcohol consumed and the pattern of intake. The relationship between alcohol consumption and all-cause mortality has often referred as a J- or U-shaped curve, in which the morbidity or mortality is lower in people who consume some alcohol than in abstainers, but increases when an inflexion point is passed (Fig. 7.1) (Grønbaek 2009). This concept is developed from evidence with respect to lifestyle-related diseases, caused by unsuitable dietary habits, the lack of exercise, or stresses. They include coronary heart disease, stroke, and other cardiovascular disorders (CVDs), osteoporosis, many types of cancer, etc. It has also been suggested that favorable action of alcohol beverages might not be due to ethanol per se but to other confounding factors (Van der Gaag et al. 2000; Marmot and Brunner 1991; Jackson et al. 1991; Doll et al. 1994; Pitsavos et al. 2004; Ghiselli et al. 2000; Rehm et al. 2007; Kondo 2004; Tousoulis et al. 2008; Arranz et al. 2012a).

Fig. 7.1 The relationship between alcohol consumption and all-cause mortality. (C JIM 2009), reprinted with permission



7.3.1 Beer and Cardiovascular Diseases

The possible CV benefits appear to be the most important health effects of light to moderate drinking. It has been evident for a long time that heavy uncontrolled drinking is one of the leading causes of preventable deaths by cardiovascular diseases CVDs and other athero-thrombotic diseases, strongly in conjunction with obesity, inappropriate diet, hypertension, diabetes, and sedentary living. However, there is also substantial evidence that the intake of light to moderate amounts of alcohol is associated with reduced morbidity and mortality from CVDs and stroke. The interpretation of these beneficial effect has been extensively analyzed and discussed. It has been suggested that it might be related with a vasodilatation action, and known risk factors, such as blood lipoproteins, increase in HDL and decrease in LDL, their susceptibility to oxidation, coagulation and fibrinolytic factors, insulin sensitivity, endothelin, etc. Unfavorable ones could be mediated through increased blood pressure or by changes in the number, size, or atherogenicity of lipoprotein particles (Mukamal et al. 2001; Klatsky 2007; Brien et al. 2011; Whitfield et al. 2013).

The main beer constituents responsible for the beneficial action on the CVDs are alcohol, polyphenols, a well-balanced array of minerals, and vitamins. Beer is an alcoholic beverage with an alcohol content that much lower comparing with wine and spirit. The alcohol content of beers depends of beer type, but in beers, dominantly present on the market is not higher than 5 %v/v. The effect of beer cannot be

explained only by the vasodilatation action of alcohol, because changes also occur in the hormone, water and electrolyte balance, due to the presence of other beer constituents.

Numerous studies have associated polyphenol consumption from beer with reduced risk for CVDs. Anti-atherogenic, and anti-thrombotic effects and regulation of endothelial function were mainly ascribed to polyphenolic and phenolic constituents (Piazzon et al. 2010; Martinez et al. 2011; Van Duynhoven et al. 2011). The kinetics and extent of polyphenols absorption were the focus of many studies. The flavan 3-ol monomers, (+)-catechin and (–)-epicatechin, and the oligomers, such as procyanidin dimer, trimer, and tetramer that are linked by C4–C8 bonds, are particular bioactive. It seems that they may improve hypertension, dyslipidemia, insulin resistance, and obesity induced by inappropriate daily habits (Schewe and Sies 2009; Osakabe 2013). Measured were plasma concentrations and/or urinary excretion among adults after the ingestion of a single dose of polyphenols, provided as a pure compound or whole beverage. It was found that subjects who showed a higher absorption of polyphenols showed a lower serum concentrations of inflammatory biomarkers than their counterparts (Williamson and Manach 2005). The results clearly show wide variability in the bioavailability of the different polyphenols and complexity of their action. The polyphenols present in beer are well absorbed (most well absorbed in humans are isoflavones and gallic acid, followed by catechins, flavanones and quercetin glucosides). The least well-absorbed polyphenols are proanthocyanidins, the galloylated catechins and the anthocyanins. Moreover, the metabolism by the gut microbiota probably plays a major role in the biological activity of many polyphenols as in the case of prenylflavonoids of beer. The different profiles of their biological activity obtained in vitro and in vivo are the result of their possible synergistic action (Arranz et al. 2012b).

Isohumulones, which impart the bitter flavor of beers, are generated from humulones (also known as α -acids) in the hops during wort boiling. Based on current findings, it is likely that they function to normalize lipid metabolism. Supplementation with isohumulones decreases hepatic triglyceride and cholesterol contents and increases HDL-cholesterol in blood (Miura et al. 2005; Yoshida 2009).

Phosphoric acid and magnesium are known to be important for proper blood vessels functioning, while the high potassium and low sodium content is the right balance for a healthy, low blood pressure (Winkler et al. 2006).

The most important beer vitamins are folate, B12 and B6. Folate, vitamin B12 and B6 are associated with homocysteine (tHcy) as an independent risk factor of CVDs involved in mechanisms of thrombosis (Winkler et al. 2006; Cravo 2005). Whereas heavy alcohol abuse undoubtedly resulted in markedly elevated tHcy, the effect of moderate ethanol consumption did not show a consistent pattern. Several studies have examined the relation between alcohol intake and plasma tHcy concentrations, but the results were rather contradictory. The intriguing question is whether the inverse relation can be ascribed to ethanol intake or to the type of alcoholic beverages consumed, as some trials suggest. Several studies are consistent with the observation that tHcy concentrations are significantly lower in beer drinkers than in wine or spirits consumers (Mennen et al. 2003; Cravo et al. 1996; Ganji and Kafai

2003). The beneficial effect of beer drinking could be due to its folate, vitamin B12, and vitamin B6 content, all important for the enzymatic homocysteine conversion. The consumption of beer in small amounts, despite its being an alcoholic beverage, could have a null or even a lowering effect on tHcy. The inverse relation with beer was independent of these nutrients, which might indicate a dose effect of ethanol (Van der Gaag et al. 2000). However, novel results indicate that intervention with B-vitamin supplementation successfully restore normal tHcy concentrations, but without concomitant reductions in disease risk (Williams and Schalinske 2010; Saposnik 2011). Thus, the mechanistic relation between tHcy balance and disease states, the value of tHcy management, as well as the effect of vitamins B supplementation should be an area of further intense investigation.

It is obvious that moderate beer consumption might have beneficial effects on diseases of vascular system. Brien et al. identified 4690 articles that examined the effect of alcohol consumption and their analysis showed that moderate consumption of alcohol could act favorable on a variety of biomarkers linked to the risk of coronary heart disease. The beneficial effects are recognized up to one drink or 15 g alcohol a day for women and up to two drinks or 30 g alcohol a day for men (Brien et al. 2011). However, possible cardioprotective effects of alcohol beverages that are seen in observational studies continue to be hotly debated in the medical literature and popular media. The protective effects of moderate alcohol intake on CV diseases still puzzle researchers. A cardioprotective relationship between alcohol use and vascular disease cannot be assumed for all drinkers, even at low levels of intake. The relationship between alcohol and CV diseases has to be perceived based not only on average volume of consumption, but on patterns of drinking as well. Although some form of a cardioprotective association was confirmed in all strata, substantial heterogeneity across studies remained unexplained and confidence intervals were relatively wide, in particular for average consumption of 1–2 drinks/day. It seems that the drinking pattern has great influence on obtained effects and in great dill determines possible beneficial action. Particularly in relation to food consumption and irregular heavy drinking occasions.

A study designed to investigate the association of current alcohol consumption and aspects of the drinking pattern with hypertension risk indicates that drinking without food may counteract any benefit associated with moderate alcohol consumption (<2 drinks/day) on the CV system. Several plausible physiological mechanisms have been put forward to explain the observed association the between drinking pattern in relation to food consumption and CV risk. Drinking with meals has been shown to exert beneficial effects on fibrinolysis and lipids, on absorption and intragastric metabolism of ethanol leading to a slower increase and lower peak of blood alcohol and to increase alcohol elimination rates (Stranges et al. 2004).

Other studies designed to investigate the association of irregular heavy drinking occasions and the risk of CV disease, showed that the cardioprotective effect of moderate alcohol consumption disappeared when light to moderate drinking is mixed with irregular heavy drinking occasions. A heavy drinking occasion, or binge drinking, is defined as drinking at least 60 g of pure alcohol or five standard drinks in one sitting. The results indicate that even one heavy drinking occasion per month

can markedly contribute to the associated burden of disease and injury (Gmel et al. 2011; Roerecke and Rehm 2010).

7.3.2 *Beer and Cancer Diseases*

Concerning malignancy there is convincing proof that high alcohol intake is related to carcinogenesis. The available evidence indicates that the alcoholic beverage consumption considered to be causally related to cancers of the upper aerodigestive tract (oral cancer and cancers of the oropharynx, hypopharynx, larynx, and esophagus), liver, and probable to colorectal cancer in men, and breast cancer in women (Gerhäuser 2005). The available knowledge on the relationship between the consumption of alcoholic beverages and a variety of human cancers is based primarily on epidemiological evidence. The results obtained for moderate consumption (2–3 drinks/day for men and 1–2 drinks/day for women) indicate that risks never increase above twofold and are mostly less than 25 % above baseline. The evidence from the available literature suggests that 25 g/day of alcohol is associated with a relative risk of 1.9 for cancers of the oral cavity and pharynx, 1.4 for cancers of the esophagus and larynx, about 10 % for colorectal cancer (mechanisms unclear), and 20 % for liver cancer (mechanisms well described). The association between alcohol and breast cancer is not strong and not necessarily causative, at least for moderate consumption (World Health Organization/International Agency for Research on Cancer 2010).

Beer drinking is almost invariably related to alcohol consumption. However, in 1996 beer was first reported to possess antimutagenic components when prenylated flavonoids (PF) from hops were first described to modulate carcinogen metabolism in vitro and to possess antioxidant, anti-proliferative, and cytotoxic activity (Arimoto-Kobayashi et al. 1999, 2006; Miranda et al. 1999; Henderson et al. 2000). Scientific evidence accumulated so far points to the cancer preventing potential of XH and hop bitter acids. The latest studies, have proven that XH, show cancer chemopreventive activities, antimutagenic and anticarcinogenic properties with an exceptional broad spectrum of inhibitory mechanisms at all three stages of the carcinogenesis, initiation, promotion, and progression, appearing as a broad-spectrum chemopreventive agent (Gerhäuser 2005; Ferk et al. 2010). Anticancer activities of XH have been investigated in various cell lines. For example, XH inhibits human breast, colon, ovarian, prostate cancer, B-chronic lymphocytic leukemia cell proliferation, and malignant glioblastoma cells (Lust et al. 2005; Vanhoecke et al. 2005; Zanolli and Zavatti 2008; Strathmann and Gerhäuser 2012; Benelli et al. 2012; Festa et al. 2013). 8-PN is of special interest as one of the most potent known plant-derived estrogens (phytoestrogen) found in nature which potency can be ranked with coumestrol and genistein (Chadwick et al. 2004). There is a large body of evidence from both animal experiments and epidemiological studies that phytoestrogens and especially 8-PN exhibit several biological activities including various

forms of antioxidant activity in the micromolar range, and are weakly cytotoxic to certain cancer cell lines (Gerhäuser 2005).

For a long time, hop prenylflavonoids were not considered relevant phytoestrogens in the human diet because 8-PN concentrations in beer were considered too low to affect human health. The final concentrations in beers are generally below 100 µg/L, and total estrogenicity of beers is 500- to 1000-fold lower than the concentration needed for harmful *in vivo* activity (~100 mg/L) in rat experiments (Milligan et al. 2002). Moreover, many beers are now made with CO₂ hop extracts instead of whole hops, giving lower 8-PN concentrations or even no 8-PN. However, the latest studies give more light to this approach. Data show that moderate beer consumption can lead to 8-PN exposure values of 1–2 mg/day that might fall within the range of human biological activity (Arimoto-Kobayashi et al. 2006; Possemiers et al. 2005, 2006). Scientific evidences accumulated over the past decade point to the microbial O-demethylation of IX in the human intestine. According to the obtained results, this could readily increase intestinal 8-PN concentrations tenfold, leading to the uptake of active estrogen doses after moderate beer consumption that might fall within the range of biological activities (Possemiers et al. 2005, 2006). Therefore, moderate beer intake could provide a daily intake of 8-PN in the same range as that provided by some “breast enhancement” products. As an estrogenic chemical, 8-PN has the potential to interact with the estrogen-signaling systems within the body. Therefore, it can modulate responses in the reproductive tract, breast, bone, or any other tissue. However, there is no evidence that exposure to 8-PN via beer is of any adverse significance (for instance, feminization effects of excessive drinking in male alcoholics). It should be noted that there are no reports of clinical trials demonstrating either the effectiveness or oestrogenic activity of such herbal preparations in humans (Possemiers et al. 2009). It is generally agreed that with the current knowledge, no detrimental health effects are to be expected through moderate beer consumption, but some cautions have to be taken considering breast cancer in females (Milligan et al. 2002; Stevens and Page 2004; Overk et al. 2008).

7.3.3 *Beer and Osteoporosis*

Osteoporosis is a disease characterized by reduced bone mass and altered microarchitecture, which results in diminished bone strength and increased fracture risk. With CVDs, it becomes the major disease that causes marked morbidity, mortality, disability, and a large socioeconomic burden worldwide. It is a progressive bone disease with aging in both men and women, but particular in post-menopausal women. There are many factors that mutually contribute to the risk of its development including genetics, autoimmune, endocrine (hyperparathyroidism, hyperthyroidism) and gastrointestinal disorders, chronic diseases (such as hepatic and renal disease), hormones (mainly estrogen and calcitonin), as well as lifestyle factors such as diet, physical activity, smoking, corticosteroid medication use, and

nutritional deficiency (such as Ca and vitamins) (Cashman 2007; Dempster 2011; McLernon et al. 2012; Nguyen and Eisman 2013).

Alcohol consumption and human skeletal health also exhibit a J-shaped curve. The evidence in the literature suggests that chronic, heavy alcohol consumption has serious negative skeletal consequences and increased fracture risk in both men and women. Different mechanisms may be responsible. The decrease in bone mass and strength following alcohol consumption is mainly due to a bone remodeling imbalance, with a predominant decrease in bone formation by violating the proper balance between the activity of bone-forming cells (i.e., osteoblasts) and bone-resorbing cells (i.e., osteoclasts). Alcohol may directly impair proliferation and function of osteoblasts, modulate specific signaling pathway due to increased oxidative stress. The change in cell differentiation is associated with an increase of fat accumulation in the bone marrow, causing malabsorption, increased renal excretion, and disruption of Ca-regulating hormones such as parathyroid hormone, calcitonin, and vitamin D metabolites (Turner 2000).

The effects of alcohol consumption on bone are linked to the dose ingested and the duration of consumption (Maurel et al. 2012). Epidemiological studies have shown that low-to-moderate drinking has no detrimental effects and may even be beneficial in certain populations. In post-menopausal women and elderly men, moderate alcohol consumption (1 drink/day) is associated with higher bone mass and with smaller decreases in bone mineral density (BMD) and bone mineral content (BMC) compared to nondrinkers (Mukamal et al. 2007; Berg et al. 2008; Marrone et al. 2012; Sommer et al. 2012). It seems that alcohol at appropriate doses is a powerful stimulant of calcitonin secretion. It has been shown that calcitonin both inhibits the resorption and stimulates the formation of bone (Pedrera-Zamorano et al. 2009). However, when the number of drinks is increased to 2 or 3 drinks/day, the effects can be positive or negative, depending on the sex, age, hormonal status of the patient, and the type of beverage consumed (Maurel et al. 2012). In younger and middle-aged men, the effects of moderate alcohol consumption on BMD are less clear, with some studies showing a positive relationship (Wosje and Kalkwarf 2007; Tucker et al. 2009; Venkat et al. 2009) but others showing no effect (Malik et al. 2009). In the case of the growing skeleton, the evidences strongly support the conclusion that alcohol has detrimental effect.

In the case of beer, there are other nutrients other than alcohol with contributing effects on bone health: phytoestrogens, B12 vitamin, and silicon. Estrogen plays an important role in maintaining bone mass in women, stimulating calcitonin secretion, slowing bone remodeling, and maintaining the proper balance between the activity of osteoblasts and osteoclasts. Moderate beer consumption may be beneficial in part by low-alcohol intake, which counteracts the effects of estrogen deficiency on bone turnover rate and in part by direct estrogenic properties of flavonoids, IX, and 8-PN (Pedrera-Zamorano et al. 2009; Turner and Sibonga 2001). Vitamin B12 seems to have direct effect on bone metabolism via osteoblast and osteoclasts and indirect effect via hyperhomocysteinemia. Accumulating evidence suggest that its poor dietary intake and low blood concentration may associated with decreased

Table 7.9 Common dietary sources of silicon

Dietary source	Portion size	mg/portion
Beer	350 mL	8.25
Red wine	118 mL	1.70
Raisins	100 g	8.25
Green beans	250 g	6.10
Whole grain bread	200 g	4.50
Mineral water	0.5 L	0–40
Brown rice with husk	100 g	2.07
Bananas	100 g	5.44

(C Int J Endocrinol 2013), open access

BMD, greater bone loss, and higher risk of osteoporotic fracture (Naharci et al. 2012; Alharbi et al. 2012).

Silicon is present in all body tissues, but the tissues with the highest concentrations of silicon are bone and other connective tissue including skin, hair, arteries, and nails. It is an essential mineral for bone formation. Published data suggest that silicon increases type I collagen synthesis, promotes the differentiation of osteoblast-like cells, improves calcium incorporation in bone, and even protects the human body from the toxic effects of aluminum (Jugdaohsingh et al. 2004; Price et al. 2013; Gonzalez-Munoz et al. 2008). Silicon has also been found at the mineralization front of growing bone suggesting also an involvement in early calcification/mineralization of the bone matrix (Jugdaohsingh 2007). Major sources of dietary Si are cereals and their products (breakfast cereals, bread, beer), some fruits and vegetables (bananas, raisins, beans, lentils), and unfiltered drinking water (depending on the source and method of processing) (Table 7.9) (Price et al. 2013).

The absorption of silicon, however, is strongly influenced by the form of silica ingested and the rate of production of soluble and absorbable species of silica in the gastrointestinal tract. Bananas are rich in silicon, having 5.44 mg of silicon per 100 g portion, but it is highly polymerized, poorly absorbed, and only about 5 % is bioavailable. The most available source of silicon is orthosilicic acid $[\text{Si}(\text{OH})_4]$, the major silica species present in beer, some beverages, and some drinking water (from volcanic areas). It is readily absorbed and more than 50 % is bioavailable (Jugdaohsingh 2007; Jugdaohsingh et al. 2002). Beer, due to its high level of silicon in this form, has been claimed to be the most important sources of silicon in the Western diet (Jugdaohsingh et al. 2002). Daily silicon intakes are usually 20–33 % higher in men (approximately 40 mg/day) than in women (18.9 mg/day). The primary reason for this difference supposedly is the higher beer consumption by men, which accounts for approximately 45 % of their silicon intake (Jugdaohsingh et al. 2002; Casey and Bamforth 2010).

Commercial beers ranged from 6.4 to 56.5 mg/L in silicon (Table 7.10) (Casey and Bamforth 2010). There is yet no recommended daily intake level for silicon. However, one finding suggests that diets containing more than 40 mg/day of silicon have been positively associated with increased femoral BMD compared to dietary

Table 7.10 Silicon content of commercial beers

Category	Si, average (ppm)	Range (ppm)
Ales	32.8	11.1–55.5
Lagers	23.7	10.1–56.4
Light lagers	17.2	14.1–23.4
Non-alcoholic	16.3	6.4–25.7
Wheat	18.9	14.3–23.4

(C J Sci Food Agric 2010), reprint with permission

intake of less than 14 mg/day (Jugdaohsingh et al. 2004). Based on the average daily intake of 20–50 mg, it can be inferred from Table 7.5 that on average, 2 L of beer will satisfy that requirements, while 1 L of some beers will provide an even higher level of intake.

7.3.4 Beer and Stone Diseases

Urinary stone disease is common and a very painful medical condition that poses a significant health-care burden in a working age population. They are typically classified by their location (kidney, ureter, gallbladder, or bladder) or by their chemical composition (calcium-containing, struvite, uric acid, or other compounds). In humans, calcium oxalate is a major constituent of most urinary stones. Average global prevalence (frequency in population) has risen from 3.2 to 5.6 % in just over one decade from the mid-1980s to the mid-1990s, and continues to grow until now (Romero et al. 2010). Several factors may have contributed to these observed elevations in prevalence, such as lifestyle and environmental factors. Epidemiologic studies have demonstrated that increased consumption of fast foods and refined carbohydrates, increased sodium, oxalate and animal proteins, as well as diminished fluid and calcium consumption are risk factors. Global climate change is another environmental factor that affects stone disease rates. Hot climate or just working in hot environments with high fluid loss and sun exposure (higher vitamin D production increase intestinal calcium absorption) can promote stone formation. Some non-lifestyle factors can increase the risk of stone formation and recurrence as well. Conditions in which there is insulin resistance, such as obesity, the metabolic syndrome, and type 2 diabetes mellitus are now known to be associated with increased stone risk (Romero et al. 2010; Taylor et al. 2005; Moe 2006; Scales et al. 2012). Dietary recommendation includes fluid intake to 2.5–3 L daily, reduces sodium intake to 2300 mg and protein intake to 0.8–1 g/kg of body weight/day (Finkelstein and Goldfarb 2006; Taylor and Curhan 2006; Worcester and Coe 2010; Nouvenne et al. 2013).

There is concern that the diuretic effect of alcohol may cause dehydration and be a risk for stone formation. However, several epidemiological studies have indicated

a reduced risk of stone formation in subjects with moderate alcohol consumption, particularly decreased risk for gallstones (Leitzmann et al. 2003; Cuevas et al. 2004). It has been suggested that this beneficial effect might be related with known risk factors, such as decrease in cholesterol saturation, but also the fact that alcohol inhibits secretion of vasopressin (antidiuretic hormone, which regulates the body's retention of water; released when the body is dehydrated causing the kidneys to conserve water), leading to increased urine flow and decreased urinary concentration (Curhan et al. 1998; Ferraro et al. 2013).

The study involved 194,095 participants, follow-up of more than 8 years analyzed the association between different types of beverages and the incidence of kidney stones in individuals without a history of nephrolithiasis. The study confirms that some beverages are inversely associated with kidney stone formation, whereas others are associated with a higher risk. Several beverages showed significant trends for decreased risk of developing kidney stones with increasing consumption. Participants consuming one or more servings of coffee (8 oz $\approx$ 2.5 dL) per day had a 26 % lower risk compared with those participants consuming less than one serving per week. Significant trends were also found for tea (8 oz $\approx$ 2.5 dL) (11 % risk reduction for the highest category) and wine. Participants consuming one or more servings of red wine (5 oz $\approx$ 1.5 dL) per day had a 31 % risk reduction compared with those participants consuming less than one serving per week, while those who preferred white wine had a 33 % risk reduction. The highest decrease for risk of developing kidney stones was found among beer consumers. Participants consuming one or more servings of beer (12 oz, 3.5 dL) per day had a 41 % lower risk compared with those participants consuming less than one serving per week. Higher risk for kidney stones formation was found for beverages like sugar-sweetened soda and punch. Participants consuming one or more sugar-sweetened servings (12 oz, 3.5 dL) of cola, sugar-sweetened noncola (carbonated beverages like clear soda), and punch (1.5 oz $\approx$ 0.4 dL) had higher risk compared with those participants consuming less than one serving per week. Consumption of artificially sweetened sodas (cola and noncola) was marginally associated with kidney stones. There were no significant interactions between consumption of beverages and the risk of developing kidney stones with age, body mass index, and diabetes (Ferraro et al. 2013).

The protective effect of beer could be mediated through water and alcohol content, mineral composition (high potassium and magnesium and low sodium content), and diuretic properties. The role of potassium and magnesium is probably connected with calcium and oxalate action. Potassium supplementation decreases calcium excretion and increases urinary citrate due to their alkali content, all of which decrease the risk of stone formation. Magnesium like calcium complexes with oxalate, potentially reduces oxalate absorption in the gastrointestinal tract (the amount of oxalate available for absorption into the bloodstream) and decreases calcium oxalate supersaturation in the urine (in the urine, oxalate is a very strong promoter of calcium oxalate precipitation, about 15 times stronger than calcium). On the other hand, the low sodium and high potassium content (the potassium: sodium ratio is typically 4:1) promote strong dehydrating and diuretic effects (Curhan et al. 1997; Taylor et al. 2004; Curhan 2007).

7.3.5 *Beer and Cognitive Functioning*

Cognition is a term used for complex and dynamic mental processes associated with the storage and processing of information. It usually refers to mental functions such as reasoning, planning, judgment, organizing, concept formation, and problem solving. Dementia as a serious loss of global cognitive ability is not a single disease, but rather a set of signs and symptoms in which affected areas of cognition may be memory, attention, language, and problem solving. It has been associated with increased prevalence of disability and need for institutional care, as well as increased morbidity and mortality. Alzheimer's dementia (AD) is the most common pathology, accounting for 50–75 % of cases. The prevalence of dementia in developed countries rises with age, from about 1 % in those aged 60–64 years to over 45 % in those aged 95 years and over. Together with other dementias, it is projected to show a 66 % increase from 2005 to 2030 (World Health Organization and World Federation of Neurology 2004; World Health Organization 2006).

Alcohol consumption may impair cognition in many ways, including performance decrements on cognitive and psychomotor tasks such as divided attention, digit-symbol substitution, mental rotation, strategic decision making, simple and choice reaction time, compensatory tracking and fine motor movement, but also depression, permanent brain damage leading to problems with memory, learning capacity, and verbal skills (Eckardt and Martin 1986; Gallimberti et al. 2011). The consequences of alcohol abuse and binge drinking can be very serious, leading to alcohol-induced disorders comprise delusions and delirium, memory disorder, and sleep disorders appearing during intoxication or withdrawal and, in addition, anxiety, mood, and psychotic disorders. These disorders also include the typical microzooptic hallucinations, delirium tremens and Korsakoff's syndrome, which may occur in the alcohol withdrawal syndrome (Pompili et al. 2010). However, several studies have investigated the association between alcohol consumption and cognitive functioning leading to the conclusion that alcohol can have opposite effects as well. It has been concluded that alcohol consumption is associated with either decreased or increased risk of cognitive impairment, depending on the alcohol dosage and the way of drinking (Zuccalà et al. 2001).

Although the adverse effects of excessive alcohol intake are well known, results of many studies suggest that in older people, small to moderate amounts of alcohol consumption are associated with reduced incidence of dementia and AD (Stampfer et al. 2005). Several mechanisms have been proposed to explain this phenomenon. The well established properties that contribute to the protective effect of alcohol against CVD, stroke, and diabetes certainly contribute to the protective effects against dementia as well. Atherosclerosis has been associated with both Alzheimer's disease and vascular dementia, so that any beneficial effect of alcohol on atherosclerosis would be expected to affect the risk (Zuccalà et al. 2001). On the other hand, AD has been termed "type 3 diabetes" (because of the weakening effect of amyloid beta oligomers on neuronal insulin receptors in the brain) and control with insulin and antidiabetic medications results in less AD neuropathology (Zhao

et al. 2008). The results indicate that the benefits of moderate drinking apply to all forms of dementia and to cognitive impairment, but there is no significant benefit against cognitive decline. Both light and moderate drinking provided a similar benefit, but heavy drinking was associated with higher cognitive risk for dementia and cognitive impairment (Neafsey and Collins 2011).

The connection between alcohol consumption and intelligence quotient (IQ) deserves attention as well. Existing evidence suggests that the development of IQ is associated with exposures affecting brain growth during the fetal period, but also factors operating during an individual's life course. It is known that excessive alcohol use in pregnancy can cause a variety of poor developmental outcomes and cognitive difficulties. A study conducted on 4332 participants with complete data on maternal use of alcohol and followed prospective association of alcohol consumption (measured repeatedly in a longitudinal study with domain-specific cognitive abilities assessed up to 17 years after the first measure of alcohol intake), has indicated that frequency of drinking four or more units on a single occasion by mothers was associated with lower scores on verbal and total IQ scales in children (Alati et al. 2008). However, the study aimed to explore the relationship between IQ and alcohol consumption in a large sample of young males confirmed the positive link between IQ and moderate drinking. It was a psychiatric-epidemiological survey using a cross-sectional design IQ-tests were administered to approximately 50,000 conscripts at age of about 20 years. Self-reported information about the frequency of alcohol consumption was split into five categories: "non-drinking," "rare drinking (1–5 times/year)," "occasional drinking (1–5 times/month)," "moderate drinking (1–5 times/week)," and "daily drinking." Results have shown that distributions of full- and subscale IQ follow a reverse j-shaped relationship with alcohol consumption with lowest scores in daily drinkers and highest in occasional and moderate drinkers. Splitting up nondrinkers revealed similar scores for former drinkers and daily drinkers, whereas lifetime abstainers were similar to rare drinkers (Fig. 7.2) (Müller et al. 2013). Studies like this should be taken with a grain. There are lot of limitations, which deserve attention, and factors that have influence on the obtained results. Nevertheless, they indicate the difference between abstainers, former drinkers, and individuals with different drinking habits.

In the case of beer, a possibly protective effect for preventing dementia and AD were found due to its alcohol, polyphenols, and bioavailable silicon content. The low content of polyphenols makes it unlikely that they act purely through their antioxidant activity, but they play a part in increasing levels of endothelial nitric oxide and in reducing endothelin-1 synthesis, which influences atherosclerotic processes in dementia (Doré 2005). Bioavailable Si in the form of silicic or ortho-silicic acid, found in beer, may decrease aluminum bioavailability. Aluminum is a highly neurotoxic element that may be involved in neuronal degeneration in human brains. By blocking its uptake through the gastrointestinal tract and by impeding reabsorption Si may reduce one of the risk factor for Alzheimer's disease (González-Muñoz et al. 2008).

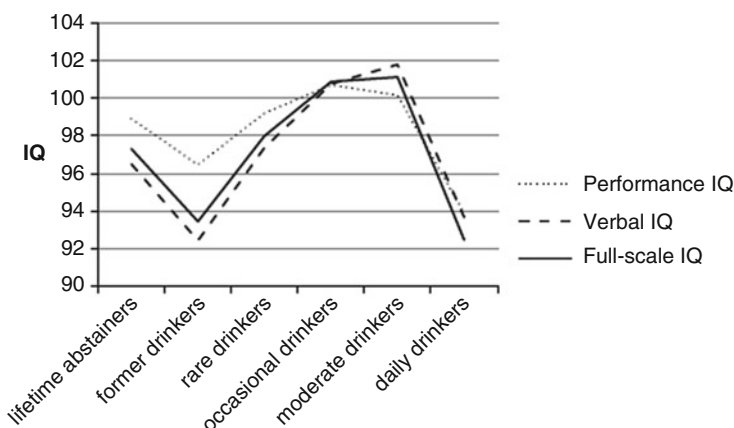


Fig. 7.2 IQ by drinking categories with two nondrinkers subgroups: lifetime abstainers and former drinkers. (C SPPE 2013), reprinted with permission

7.3.6 Beer and Gout

Gout is a common form of inflammatory arthritis, often causing recurrent episodes of pain and swelling of certain joints. It affects 1–2 % of adults in developed countries and are increasing in prevalence, likely as a result of a changing pattern of risk factors (longevity, current dietary and lifestyle choices, obesity, metabolic syndrome genetic disorder, and genetic predisposition) (Fam 2005). The prevalence of self-reported, physician-diagnosed gout survey was found to be greater than 2 % in men older than 30 years of age and in women older than 50 years of age. The prevalence increased with increasing age and reached 9 % in men and 6 % in women older than 80 years of age (Choi et al. 2005).

Hyperuricemia is considered the precursor of gout, causing deposition of monosodium urate crystals within joints and connective tissue (Weaver 2008). It is a disorder of the purine metabolism or renal excretion of uric acid (humans do not express the enzyme uricase, which degrades uric acid). Uric acid is the end-product of purine degradation in humans and its plasma concentration is regulated by both the production and excretion of uric acid (Fam 2005; Neogi 2011). Alcohol intake is one of the main causes of hyperuricemia. Alcohol consumption increases the level of serum uric acid and even at light-to-moderate consumption triggers recurrent gout attacks (especially in the case of genetic predisposition). Results from the Health Professionals Follow-up Study showed that the risk of incident gout attack increased as the amount of alcohol consumed. Compared with abstinence, daily alcohol consumption of 10–14.9 g increased the risk for gout by 32 %; daily consumption of 15–29.9 g, 30–49.9 g, and 50 g or greater increased the risk by 49 %, 96 %, and 153 %, respectively. Several mechanisms of ethanol-induced increase in plasma uric acid concentration have been reported. Ethanol ingestion stimulates an increase of lactic acid in blood with inhibitory effect on the urinary excretion of



Fig. 7.3 Multivariate difference in serum uric acid per daily serving of alcoholic beverages. (C Art. Care Res. 2004), reprinted with permission

uric acid and induces and enhances adenosine triphosphate (ATP) consumption resulting in purine degradation, accelerated uric acid production, increase of plasma concentration of uridine, increase of plasma concentration of oxypurines, hypoxanthine and xanthine, and slightly inhibition of xanthine dehydrogenase activity (Yamamoto et al. 2002).

Available data suggest that risk of gout attack varied according to type of alcoholic beverage as well. Beer conferred a larger risk of gout than other alcoholic beverages (Choi et al. 2005). Beer contains not only alcohol, but also considerable amounts of various purines that might augment the hyperuricemic effect of alcohol. Several previous cross-sectional observations and experimental studies suggested that beer intake had a greater effect on inducing hyperuricaemia than liquor, whereas moderate wine drinking did not increase risk (Fig. 7.3) (Choi and Curhan 2004; Choi et al. 2004). Therefore, patients with gout should be cautious regarding drinking large amounts of beer or should even avoid it to lower their risk of recurrent gout attacks (Zhang et al. 2006). Beer intake may increase serum uric acid even more than the intake of common purine-rich foods such as meat and seafood (Nakamura et al. 2012; Yamamoto et al. 2002; Choi and Curhan 2004).

7.3.7 Potentially Hazardous Effects of Beer Biogenic Amines

Biogenic amines play important roles in human body where they have important metabolic and physiological roles, such as the regulation of growth (PUT, SPD, SPM), control of blood pressure (indoleamines and histamine), and neural transmission (catcholamines and serotonin). They are involved in brain activity, the regulation of body temperature and stomach pH, gastric acid secretion, the immune response, cell growth, and differentiation, etc. (Karořičova and Kohajdova 2005). BAs are normally metabolized in the body to keep their steady-state concentrations

low. After food consumption, small quantities are commonly metabolized in the human gut to physiologically less active forms through the action of amine oxidases. However, an excessive intake of BAs (particularly TY and HI) can cause several deleterious effects and a wide range of health problems.

The intake of foods with high BA loads, or inadequate detoxification, either for genetic reasons or because of the inhibitory effects of some medicines can lead to BA entering the systemic circulation and causing the release of adrenaline and noradrenaline. Psychoactive amines can affect the neural transmitters in the central nervous system, while vasoactive amines can act directly or indirectly on the vascular system as vasoconstrictors (namely TY) or vasodilators (HI). They can induce and provoke gastric acid secretion, increased cardiac output, migraine, tachycardia, increased blood sugar levels, and higher blood pressure, particularly when accompanied by alcohol and acetaldehyde (alcohol and acetaldehyde enhance the toxic effects by increasing the permeability of the intestinal wall) (Kalač and Križek 2003; Jayarajah et al. 2007; Spano et al. 2010; Ladero et al. 2010).

Foods likely to contain high levels of BA are especially risky for the individuals with lack of monoamine oxidase (MAO) activity or expression and individuals under treatment with MAO inhibitors (MAOI) (antidepressant). As new MAOI were developed for treating depression, TY rich foods were observed to cause a serious hypertensive crisis. Consequently, it is important for individuals who suffer from a dietary TY intolerance syndrome or those on MAOI to pay attention to their dietary intake of TY. BA levels are also higher in patients with Parkinson's disease, schizophrenia, and depression (Spano et al. 2010; Ladero et al. 2010; Caston et al. 2002; Russo et al. 2010).

Foods accepted to contain higher levels of BA include fish, fish products, and fermented foodstuffs, such as meat, dairy products and vegetables, and beverages: wine, cider, and beer (Table 7.11) (European Food Safety Authority (EFSA) 2011). Based on mean content in foods and consumer exposure data, fermented food categories were ranked in respect to HI and TY, but presently available information was insufficient to conduct quantitative risk assessment of BA, individually and in combinations. Based on limited published information, no adverse health effects were observed in healthy individuals not taking MAOI drugs after exposure to following BA levels in food (per person per meal): 50 mg HI and 600 mg TY. For individuals taking classical MAOI drugs, no adverse health effects have been observed after exposure to a level of 6 mg of TY per person per meal. Whereas from 50 up to 150 mg of TY would be well tolerated by patients under new generation MAOI treatment, so-called reversible inhibitors of MAO (European Food Safety Authority (EFSA) 2011).

Beer has been reported as a possible health risk for some consumers due to BAs intake. However, these high BAs intake levels are not usually caused by a high amine content in individual beers, but rather by a very high beer consumption during a very short time interval. Alcohol and probably some other BAs present in beer can potentiate TY effects. However, no risk was reported for healthy consumers. Beer has been reported as a trigger for headaches with patients susceptible to migraines. Histamine in alcoholic drinks was capable of triggering of allergic and

Table 7.11 Summary table of 95-percentile of biogenic amines expressed in mg/kg

Food class	Subcategory	Occurrence of biogenic amines (95-percentile) mg/kg			
		HI	TY	PUT	CAD
Alcoholic beverages	Beer	4.8	24.7	8.3	5.3
	Fortified and liquor wines	2.8	21.3	3.6	0.3
	Red wine	12.3–12.4	7.8–8.5	9.5–11.5	0.6–1.6
	White wine	2.6	26.4	15	0.2
Sauce	Fish sauce	597	421	167	502
	Other savor sauces	<13.3	18.6	24.2	<20
Fish and fish products	Dried anchovies	1440	–	–	–
	Fermented fish meat	34.9	251	75.1	34.5
Meat products	Fermented sausages	149	397	334	154
	Other ripened products	35	149	136	84.1
Dairy products	Cheese	130	440	143	470
	Yoghurt	1	5.2	1.1	10.3
Vegetables and vegetable products	Fermented vegetables	92	91	549	94
	Other vegetables	<0.5	25.4	310	85

(Data from EFSA 2011), fair use

allergic-like adverse responses; however, beer has been a less common source than wine. Sensitivities to wine appear to be mainly due to pharmacological intolerances to specific components, such as biogenic amines and the sulphite additives (<http://www.efsa.europa.eu/en/search/doc/2393.pdf>2013).

7.3.8 Beer and Celiac Disease

Celiac disease (CD) is a complex disease of the small intestine caused by a permanent intolerance to gluten. Once considered a gastrointestinal disorder that mainly affects caucasian children, celiac disease is now known to affect persons of different ages, races, and ethnic groups. It is currently one of the most common chronic inflammatory conditions of the digestive system, although it may be manifested without any gastrointestinal symptoms. Epidemiological studies in Europe and the United States indicate that CD prevalence is approximately 1 % in the general population and increases significantly over the last three decades (Vally and Thompson 2003; Dube et al. 2005). It is characterized by an autoimmune response in genetically susceptible individuals resulting in small intestinal mucosal injury. As a consequence, malabsorption develops which results in malnutrition-related problems including anaemia, vitamin deficiencies, osteoporosis, and neurological disorders.

Long delays between onset of symptoms and diagnosis often occur, and the condition remains underdiagnosed. The only currently available treatment is lifelong adherence to a glutamin-free diet and exclusion of grains containing gluten (Vilppula et al. 2009; Niewinski 2008).

There is still no full agreement on the amount of dietary gluten that CD subjects may ingest without damaging the mucosa of the small intestine. The clinical sensitivity toward gluten differs considerably amongst patients. It has been reported that the prolonged ingestion of 50 mg gluten daily may cause significant damage to the architecture of the small intestine in patients with CD, while a high consumption of 1–5 g gluten per day, may cause relapse of the disease. However, there is a study, which suggests that even 10 mg should be considered as the maximum tolerable daily intake in treated coeliacs (Tack et al. 2010). Codex Alimentarius Standard and EU Regulation concerning the composition and labeling of foodstuffs suitable for people intolerant to gluten define “gluten-free” food as foods containing less than 20 ppm gluten, made only from one or more ingredients that do not contain wheat, rye, barley, oats, or their crossbred varieties, as well as foods that contain one or more of the aforementioned ingredients that have been specially processed to remove gluten (Catassi et al. 2007; Codex Alimentarius Commission: ALINORM 08/31/26 2008).

As most beers are brewed from barley, it has been inferred that beer is not suitable for people suffering from CD. However, this conclusion has been questioned, taking in consideration in mind some important facts. Considerable protein degradation modification and removal occur during the malting and brewing process. Many of the large barley storage proteins (in particular hordeins—which are hydrophobic and scarcely soluble in a lowalcoholic solution such as beer, ca. 4–7 % ethanol) are precipitated and removed during the mashing and the boiling stages (EU Regulation (EC) 2009). In beer, the content of proteins recognized by anti-gliadin antibodies has been estimated to be approximately three orders of magnitude lower than in raw malt (Bamforth 2009). On the other hand, the substantial quantities of adjuncts that are not derived from barley, wheat, oats, or rye as malt substitutes are present in variety of beers on the market, which means lower gluten content. However, although hordeins appear in beer at low levels, the levels are still too high for gluten intolerants and coeliacs to safely consume. Proteins maybe hydrolysed. However, it is not yet clear if the hydrolysed peptides remain immunogenic to coeliacs, or not. At least some of peptide fragments may contain potentially coeliac-toxic epitopes (part of an antigens that are recognized by the immune system). Determination of hordein by ELISA-based methods report hordein concentrations in beer in trace amounts, in the ppm range (Dostálek et al. 2006; Van Landschoot 2011; Picariello et al. 2011). However, it is suspected that these levels are underestimated (Guerdrum and Bamforth 2011). The question of presence, amount, and toxicity of gluten in beers is still open. In order to answer this question, it is necessary to develop highly sensitive and selective analytical procedure.

Over the last few years, a number of studies have examined the beer proteome for the presence of potential allergens, especially the proteinaceous gluten components, using an antibody-based screening. While ELISA technology has been successfully applied to the quantification of wheat gluten in underivatised products, a number of

issues limit the usefulness of ELISA in the analysis of hydrolysed and/or modified gluten present in food and beverages. Mass spectrometry is the only non-immunological method presently available to detect with high specificity glutenins, gliadins, and related prolamins in food samples. Quantitative proteomics may overcome some of the issues with ELISA; however, there are still many significant challenges to overcome, namely the unambiguous identification of proteins and the definitive characterisation of their toxicity (Guerdrum and Bamforth 2011; Colgrave et al. 2012; Tanner et al. 2013; Haraszi et al. 2011; Picariello et al. 2012a, b).

7.4 New Beer Types with Functional Properties

According to market statistics, consumers are increasingly interested in the health benefits of foods. This interest combined with a more widespread understanding of how diet affects disease, rising health-care costs, and an aging population are the reason behind a growing market for products with functional properties. During the past decades, public interest in natural therapies, namely herbal medicine, has increased dramatically. This fact became the challenge for brewing industry to make a breakthrough for the growing functional food market. Beer as a natural drink, which already possesses functional properties can be an excellent base for developing new products with additional benefits and functionality. Several products like this have been already present on the market.

In 2004, two new beverages were introduced in the German market. Weihenstephan brewery launched, XAN Hefeweissbeer (XAN wheat beer) with elevated levels of XN up to 1.4 mg/L, and XAN Wellness (beermix with 40 % of alcohol-free wheat beer and 60 % natural multi-fruit juice—apple, acerola, lemon—and, advertised as the first ever soft drink to contain XN) with 4 mg/L of XN. Unfortunately, these beers are retired; no longer brewed. Soon after Weihenstephan brewery, Žatecký brewery in Czech Republic promoted ZATEC Xantho, dark beer (5.7 %vol alcohol) with 0.3 mg/L of XN and 3 mg/L of IXN. There is also ZATEC Dark, dark lager beer with nearly doubling content of XN than standard dark lagers. The fate of these beers was different and they are still present in the market.

German brewer Carlsberg (not to be confused with Danish Carlsberg) has launched functional beer for women Karla—the mixed drink in two varieties. Both were low in alcohol content (1 %v/v) and a blend of beer and fruit juices. *Karla Balance* claimed to provide “peace and balance” by mixing hops with lemon balm, an herb well known for its sedative properties. *Karla Well-Be* was enriched with ingredients such as soy-derived lecithin (which may positively affect cholesterol levels), folic acid (recommended for women considering pregnancy), and other vitamins. Emphasis on health, Carlsberg supplied its beer through an atypical distribution channel: pharmacists. However, although singled out by Polish business magazine *Handel* as Poland’s best new FMCG product of 2006, Karla cannot be found any more (Halbmayer-Jech et al. 2013).

There are also several beers with added functional additives or modified content to satisfy defined target group of costumers. One example is *Spirulina beer*. Spirulina is blue-green algae that contain numerous bioactive components and can be consumed as a dietary supplement, as a functional food additive, as well as a whole food. It is supposed to promote health in many ways: protects from oxidant stress and supports the immune system and a healthy inflammatory response. It is cultivated worldwide and is available in tablet, flake, and powder form, now as a beer as well. Spirulina beer is claimed to keep the nutritional components and flavor of traditional beer and increases its nutrient content derived from Spirulina (www.karlsberg.de). Gluten-free beer is another example. These beers are produced with less immunogenic grains such as oats and gluten-free grains such as buckwheat, sorghum, and millet as the substitutes for barley malt or by specially modified technological procedure to minimize gluten content (EU Regulation (EC) 2009).

A variety of plants, grapes, medicinal herbs, and mushrooms give a lot of possibilities to create a new beer line with defined sensorial, functional, and therapeutic properties. Possibilities are enormously high (Chen et al. 2004; Despotović et al. 2007; Leskosek-Cukalovic et al. 2010; Leskošek-Čukalović et al. 2010).

7.5 Conclusions

Studies focusing on the associations between different health conditions and alcohol consumption confirm the hazards of excess drinking but also indicate the existence of potential windows of alcohol intake that may confer a net beneficial effect of drinking, at least in terms of survival, both in men and in women. However, available epidemiological studies in many cases have provided contradictory results, showing positive, or negative, or nonsignificant associations between alcohol consumption and prevalence for given diseases, differences between type of alcohol beverage, active constituents, and mechanisms of their beneficial action. In many cases, they have yielded no conclusive evidence that alcohol either promotes or prevents diseases. Therefore, the question, whether to drink or not, is still open. Moderate drinking seems to be beneficial in prevalence of some diseases but to recommend anyone to drink alcohol because of that would be very dangerous. All studies found a negative association between alcohol consumption and health, especially in heavy drinkers and binge drinkers, regardless the type of alcohol.

Beer is a drink with numerous constituents important for healthy body functioning, and regarding alcohol content, it is less hazardous than other alcohol beverages. Therefore, moderate beer consumption could be included in the dietary habits of the population as a possible protective factor, an aspect that supports the recent inclusion of beer in the food guide pyramid. However, awareness of limitations and the potential health problems associated with its consumption is certainly necessary.

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Chapter 8

The Strategy for Screening of Antioxidant Constituents in Protein Hydrolysates

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8.1 Introduction

Oxidative stress is one of the typical responses of living organisms on negative environmental factors. Oxidative stress is believed to play important role in the pathogenesis of inflammatory, neurodegenerative, cardiovascular, oncological diseases, premature aging, and is associated with increased production of reactive oxygen species (ROS) (Arts and Hollman 2005; Skulachev 2007; Knasmüller et al. 2008). Exogenous and endogenous antioxidants are involved in the protection of biomolecules from ROS-mediated modifications (Frankel and Finley 2008). Antioxidants are the substances capable of preventing the oxidation of target molecules (e.g., lipids, proteins, nucleic acids) efficiently in a concentration range comparable to that of target molecules oxidized (Huang et al. 2005; Frankel and Finley 2008; Moon and Shibamoto 2009). In humans, ROS-mediated

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oxidation of biomolecules is prevented by both enzymes of the antioxidant defense system (e.g., catalase, glutathione peroxidase, superoxide dismutase) and nonenzymatic exogenous and endogenous antioxidants. The latter include amino acids, peptides, and proteins; those provide substantial input to the antioxidant capacity (AOC) of the biological liquids (van Overveld et al. 2000; Bartosz 2003). Therefore, peptides are important for maintenance of antioxidant state in the living organisms.

In spite of structural diversity of peptides, the major mechanisms of ROS scavenging include hydrogen atom transfer (HAT) and electron transfer (ET). Moreover, the ET mechanisms can be divided into: sequential electron transfer with proton loss or sequential proton loss with electron transfer. Besides the direct ROS scavenging, the antioxidant effect of peptides in heterogeneous systems containing water and lipid phases can be also attributed to their surface metal-chelating activities (Mendis et al. 2005; Xiong 2010). Chelation of transitory metal ions (e.g., $\text{Cu}^+/\text{Cu}^{2+}$, $\text{Fe}^{2+}/\text{Fe}^{3+}$) inhibits the Fenton-like reactions during the lipids peroxidation. N and O atoms of peptide bonds, as well as the side chains of H, D, E residues, are believed to be responsible for chelation of transitory metal ions by peptides (Sarmadi and Ismail 2010; Samaranayaka and Li-Chan 2011). Despite of huge massive of literature data, the action mechanisms and structure–activity relationships in antioxidant peptides still remain challenging problems.

Antioxidant peptides are usually produced by fermentation or enzymatic conversion of proteins. Obvious advantages of the latter approach are preservation of labile redox-active amino acids (Y, W, C) due to low hydrolysis temperatures; fine tuning of hydrolysis degree by application of certain enzyme preparation; and obtaining of protein hydrolysates with tailored peptide profiles by variation of hydrolysis conditions (Klompong et al. 2007; Xiong 2010). Increasing of AOC during the enzymatic hydrolysis can be attributed to release of functional groups by peptide bonds cleavage. Bacterial enzyme preparations (e.g., Corolase PP, Pronase, Alcalase, Neutrase) as well as proteolytic enzymes of plant (papain) and animal (pepsin, trypsin, chymotrypsin) origin are most widely used for production of protein hydrolysates with enhanced antioxidant properties due to formation of small peptide fragments (Kim et al. 2001; Li et al. 2007). Commonly, protein hydrolysates are further subjected to micro- and ultrafiltration for obtaining the fractions enriched in antioxidant peptides, removal of enzymes used for hydrolysis, and potentially allergenic high molecular weight constituents (Kim et al. 2001, 2007; Hernandez-Ledesma et al. 2005).

More than 100 antioxidant peptides have been identified in protein hydrolysates (Dziuba and Darewicz 2007; Sarmadi and Ismail 2010). The majority of them contain from 2 to 20 a.a.r., and their average chain length is around 10 a.a.r. However, structure–activity relationships for antioxidant peptides have not been established yet in particular the preferential positions of redox amino acid residues (a.a.r.) in peptides and their superposition.

Current study was aimed to develop a knowledge-based strategy for screening of antioxidant peptides in protein hydrolysates using structural descriptors of antioxidant peptides followed by analysis of the hydrolysates peptide profiles and their antioxidant effects *in vitro* and *in vivo*.

8.2 Materials and Methods

8.2.1 Reagents

Proteinogenic L-amino acids and L-norleucine were purchased from Fluka (Germany), standard mixture of L-amino acids—from Sigma (USA). The derivatives of amino acids: L-cysteic acid, L-methionine sulfone, N-acetyl-L-TRYPTOPHANAMIDE were supplied by Sigma (USA), N-acetyl-L-tryptophan, N-acetyl-L-tyrosine, L-tyrosinamide, L-tryptophanamide, N-acetyl-L-TYROSINAMIDE—from Bachem (Switzerland). The dipeptides: AY, YA, HY, VY (Sigma, USA), YG, GY, YK, KY, YE, EY, YV, YW, WY, YH, MY, YY were supplied by Bachem (Switzerland). Other reagents used were at least of analytical grade and purchased from Sigma (USA).

8.2.2 Preparation of Peptides Compositions

Poultry protein hydrolysate (PH1) was obtained from poultry meat and bone trimmings using multienzyme cocktail containing four commercially available enzyme preparations—Alcalase 2.4 L, Neutrase, Protamex, Flavourzyme 500 L (Novozymes, Bagsvaerd, Denmark). The hydrolysis was performed under mild conditions: pH (7.0) and temperature (55 °C) as has been previously described by Nikolaev et al. (2008). The poultry protein hydrolysate PH2 was produced from PH1 by means of micro- and ultrafiltration (final cutoff 10 kDa) (Nikolaev et al. 2008).

8.2.3 Characterization of Protein Hydrolysates

Proximate composition of PH1 and PH2 (protein, moisture, ash, and fat content) was determined according to Association of Official Chemists (2000), methods 976.05 (protein), 934.01 (moisture), 942.05 (ash), and 920.39 (fat). Total nitrogen content was determined by Kjeldahl method, and protein content was calculated using conversion factor 6.25.

Free amino acid composition of PHs was determined by AccQ Tag method (Waters, USA) according to Cohen and De Antonis (1994) using L-norleucine as internal standard and the standard amino acids mixture for quantification. Total amino acid composition was determined by the same procedure after hydrolysis with 6 M hydrochloric acid at 120 °C for 23 h. The sulfur-containing amino acids (C, M) were quantified as described above with performic acid pretreatment before the hydrolysis. Total tryptophan content was measured by derivative spectrophotometry according to Fletouris et al. (1993) with N-acetyl-L-tryptophanamide as a standard. The content of free and total amino acids was expressed in mg of amino acid per g of PH.

8.2.4 Fractionation of Poultry Protein Hydrolysates

Prior to fractionation, PHs were defatted by 2-step extraction of lipids with hexane–chloroform mixture 1/1 (vol./vol.). 4 g of PH were suspended in 40 mL of hexane–chloroform mixture and shaken for 1 h on a Rotamix (Elmi, Latvia) at a speed of 90 rpm. The solid residue was separated by filtration and dried at 45 °C. Defatted PH1 and PH2 were dissolved in 25 mM potassium phosphate buffer, pH 6.8 with final concentration 50 mg/mL. Solutions (2 mL of each PH) were filtered through 0.45 µm PVDF syringe filter (Carl Roth, Germany) and fractionated by SE-LC on HiLoad 26/60 Superdex 75 column (GE Healthcare, USA) with 25 mM phosphate buffer, pH 6.8 as the mobile phase, and flow rate of 1.5 mL/min. The fractions with the highest AOC values were subjected to peptides identification.

8.2.5 Peptides Identification by HPLC-ESI-FT-ICR-MS/MS

The fractions containing the peptides with high AOC values were separated using an Agilent 1100 system autosampler/nanoHPLC (Agilent Technologies, Palo Alto, CA, USA). A sample volume of 1 µL was loaded by autosampler onto a homemade capillary column (75 µm id × 12 cm, Reprosil-Pur Basic C18, 3 µm; Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany), and separation was performed at a flow rate of 0.3 mL/min using 0.1 % formic acid (v/v, solvent A) and acetonitrile/0.1 % formic acid (v/v, solvent B). The column was pre-equilibrated with 3 % (v/v) solvent B, and linear gradient from 3 to 50 % (v/v) of solvent B in 30 min followed by isocratic elution (95 %, v/v, of solvent B for 10 min) was used.

Mass spectrometric analysis was performed on a 7-Tesla Finnigan LTQ-FT Ultra (Thermo Electron, Bremen, Germany) mass spectrometer equipped with a homemade nanoelectrospray ion source. The following conditions were used for electrospray: positive ion mode; needle voltage 1.9 kV; no sheath and auxiliary gas flow; tube lens voltage 45 V; heated capillary temperature 250 °C. Data were acquired in data-

dependent mode using Xcalibur (Thermo Finnigan, San Jose, CA, USA) software. The precursor ion scan MS spectra (m/z 100–1600) were acquired with resolution of $R = 50,000$ at m/z 400 (number of accumulated ions 5×10^5).

Data evaluation was performed by using Mascot Daemon 2.2.2 (Matrix Science, London, UK) software for automated database searching from LS-MS/MS data. Mass tolerance for protein identification was 5 ppm for MS and 0.5 Da for MS/MS. Methionine oxidation was used as variable modification. A specific chicken (*Gallus gallus*) protein database was created (2144 sequences; 1,025,668 residues) using entries from UniProtKB_SwissProt sprot_v.57.9 (510,076 sequences; 179,409,349 residues). Significance thresholds were less than 0.05. Peptides with Mascot probability-based score >24 were accepted to be identified.

8.2.6 Protocol in Animals

A set of 40 male Wistar rats (A.N. Bach Institute of Biochemistry, Research Center of Biotechnology of the Russian Academy of Sciences, Moscow, Russian Federation) with initial weight 145–150 g were used. The animals were housed at a temperature 20 °C with 12/12 h light/dark cycles. Prior to experiments, the rats consumed a standard diet (PK-120 formula, Laboratorkorm LLC, Russia) for 2 weeks. The animals had free access to meal and drinking water. During the experiment, the animals consumed the semisynthetic diets containing (g/100 g of feed): protein source—25.0; maize starch—58.0; non-refined sunflower oil—5.0; lard—5.0; minerals mixture—4.0; water soluble vitamins mixture—1.0; fat soluble vitamins mixture—0.1; microcrystalline cellulose—2.0. The animals were randomly divided into four experimental groups. The first group was fed with the diet, containing bovine casein as a protein source (25.0 g/100 g of feed). The second and the third groups of animals were fed with the diets containing PH1 and PH2 as a single protein source (25.0 g/100 g of feed), respectively. Animals from the fourth group were maintained on protein-free diet (protein source was replaced with corn starch). After 4 weeks period, the rats were euthanized in a CO₂ chamber.

Blood samples were collected by heart puncture. After clotting, serum samples were separated by centrifugation on CM-6 M centrifuge (Elmi, Latvia) at 2500 rpm for 10 min. 100 μ L of each serum sample was immediately frozen in the liquid nitrogen and stored at -80 °C for subsequent analysis of AOC. For the analysis of thiobarbituric acid reactive species concentration (TBARS assay), 1 mL of serum was supplemented with 10 μ L of 54 mM 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) solution in methanol, frozen in the liquid nitrogen, and stored at -80 °C.

Liver and brain were removed from the animal immediately after obtaining of blood samples. The organs were washed with ice-cold 0.9 % sodium chloride solution, weighted, frozen in the liquid nitrogen, and stored at -80 °C.

All the experiments were performed as authorized for scientific research (European Directive 86/609/CEE).

8.2.6.1 Determination of Antioxidant Capacity by TEAC Method

The AOC was determined in accordance with the method of Re et al. (1999), modified for 96-well microplates using 50 mM phosphate buffer saline pH 7.40 (PBS) as a solvent. The reaction was recorded at 25 °C for 40.5 min with 60 s measurement interval in a Synergy 2 microplate reader (BioTek, USA). All measurements were carried out in four replicates.

Trolox was used as a standard, and the calibration curve for AOC determination was constructed for the concentration range 1–10 mM. The AOC of compounds studied was expressed in μmol Trolox equivalents (TE) per μmol of antioxidant, and AOC of PHs in μmol TE per g of PH.

Prior to AOC determination, serum samples were thawed on ice and diluted with 50 mM PBS buffer, pH 7.4. Working serum dilution range comprises 180–250 times. AOC was expressed as TE concentration (mM).

For AOC determination, 200 mg of organ (liver or brain) was homogenized with 8 mL 1.15 % potassium chloride solution at +4 °C for 300 s using Silent Crusher S homogenizer (Heidolph, Germany), operating at a speed of 75,000 rpm. The homogenates were centrifuged at 30,000 g and +4 °C for 20 min. The supernatant was separated and diluted in 50–60 folds with 50 mM PBS buffer, pH 7.4. The AOC was expressed in μmol TE per g of tissues.

8.2.6.2 Determination of TBARS in Serum and Tissue Homogenates

TBARS in serum were determined according to Sattler et al. (1998). 1,1,3,3-Tetraethoxy-propane (TEP) was used as a calibration standard. In order to produce malondialdehyde (MDA) TEP was hydrolyzed according to Scmedes and Holmer (1989) and the resultant MDA stock solution was further used for preparation of working dilutions (0.125–15,000 μM). Condensation of TBARS with thiobarbituric acid was carried out in 1.5 mL PP-tubes with screw caps and rubber O-ring. The reaction mixture contained 200 μL of serum or standard solution, 30 μL of 54 mM methanolic BHT solution, 200 μL 200 mM phosphoric acid, and 200 μL of thiobarbituric reagent (0.11 M solution of 2-thiobarbituric acid in 0.1 M sodium hydroxide). The reaction mixtures were incubated on a water bath (90 °C) for 45 min with subsequent cooling up to ambient temperature. 500 μL of 1-butanol and 50 μL of saturated sodium chloride solution were added for extraction of pink-colored chromogen. The mixtures were shaken vigorously and centrifuged at 7000 $\times$ g for 10 min. 200 μL of the upper layer was transferred into a 96-well plate with subsequent reading of the absorbance at 535 and 572 nm on Synergy 2 plate reader (BioTek, USA). TBARS concentration was calculated using the linear regression equation between difference of absorbance at 535 and 572 nm and concentration of MDA. TBARS content in serum was expressed in μM of MDA equivalents.

For TBARS assay, 100 mg of organ (liver or brain) was weighted in a 1.5 mL Eppendorf tube with subsequent addition of 900 μL of ice-cold homogenization buffer (1.15 % potassium chloride, 2.0 mM EDTA and 2.5 mM EGTA, pH 7.40).

EDTA (ethylenediaminetetraacetic acid) and EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) were incorporated into homogenization buffer as chelating agents preventing oxidation of lipids during homogenization step. The tissues were homogenized at +4 °C for 120 s using Silent Crusher S homogenizer (Heidolph, Germany), operating at a speed of 75,000 rpm. The reaction mixture contained 80 μ L of homogenate or standard solution (0.125–15,000 μ M MDA), 20 μ L of 54 mM methanolic BHT solution, 90 μ L of deionized water, 40 μ L of 8.1 % SDS (sodium dodecyl sulfate) solution, 200 μ L of 200 mM phosphoric acid, and 200 μ L of thiobarbituric reagent. Further analytical procedures were completely the same as described above. TBARS content in homogenates of different organs was expressed in nmol of MDA equivalents per g of tissue.

8.2.7 Statistical Analysis

The results were expressed as mean values $\pm$ SD for ten rats. Significant differences were evaluated by one-way analysis of variance (ANOVA), followed by Student *t*-test. $p < 0.05$ was used as the threshold for statistically significant differences. The SPSS Statistics 17.0 software was used for data processing.

8.3 Results and Discussion

The AOC of amino acids, their derivatives, and Y-containing dipeptides was determined by TEAC assay utilizing ABTS radical cation (Fig. 8.1). Under the physiological concentration range (up to 1 mM) among 20 proteinaceous amino acids only 3 (Y, W, C) scavenged ABTS radical cation (Fig. 8.1). AOC values of amino acids

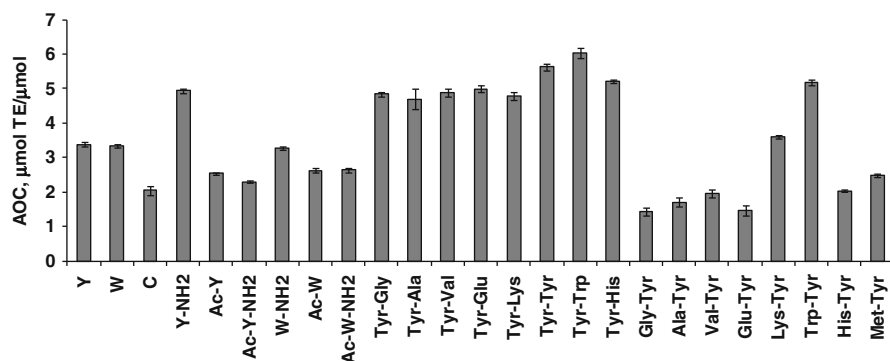


Fig. 8.1 Antioxidant capacity (AOC) of amino acids, their derivatives, and tyrosine-containing peptides

decreased in the following order: $Y \approx W > C$, that is in line with the data of Clausen et al. (2009). Other commonly recognized redox-active amino acids (M, H) were lacking of ABTS-scavenging ability that met well the data previously reported by Walker and Everette (2009), and Güngör et al. (2011).

Antioxidant activity of thiol-based peptidic antioxidants has been intensively studied recently by Güngör et al. (2011). Conversely, antioxidant properties of tyrosine- and tryptophan-containing peptides are less known. Therefore, the further experiments were carried with tyrosine and tryptophan acetyl- and amide-derivatives. Tryptophan and tyrosine acetylation resulted into 1.3-fold decrease of their AOC values comparably to non-modified amino acids (Fig. 8.1). Amidation had no effect on AOC value in case of tryptophan, but caused 1.5-fold increase in AOC value in case of tyrosine with respect to non-modified tyrosine (Fig. 8.1). Tryptophan and tyrosine with modified both α -amine-groups and carboxyls retained only 79 % and 68 % of initial AOC, respectively. Thus, it can be proposed that the peptides with N-terminal Y or W positions would exhibit higher AOC values comparably to peptides with C-terminal or internal positions of those amino acids. Those findings are in contradiction with the data on higher importance of C-terminal comparably to N-one a.a.r. for antioxidant peptides scavenging ABTS radical action as reported recently by Li and Li (2013) based on the data of QSAR modeling. It should be underlined that the authors included only five peptides with N-terminal position of Y or W residues in the database of 126 peptides considered for QSAR modeling experiments.

Dipeptides of monoamino-monocarboxylic acids with N-terminal tyrosine residues exhibited on average 1.4 times higher AOC values ($4.81 \pm 0.10 \mu\text{mol TE}/\mu\text{mol}$) comparably to tyrosine, while dipeptides with C-terminal tyrosine residues possessed nearly twice lower AOC values ($1.70 \pm 0.27 \mu\text{mol TE}/\mu\text{mol}$). The finding on preferred N-terminal tyrosine position in antioxidant peptides reactive with ABTS radical cation meets well the data of Liu et al. (1997) on susceptibility of tyrosine, GY, and YG to photo-induced oxidation under alkaline conditions (pH 10). The rate constant (k) of YG photo-induced oxidation was 1.1 times higher with respect to these of tyrosine, while dipeptide GY exhibited 1.7 times lower k value. Therefore, it can be proposed that presence of free α -amine-group is essential for antioxidant properties of tyrosine residues in peptides.

Analysis of AOC of dipeptides incorporating the residues of tyrosine and amino acids with ionizable groups in the side chains (E, K) revealed positive effect of K residue on AOC value of dipeptide KY. The AOC value of dipeptide KY was equal to that of tyrosine and was from 1.8 to 2.5 folds higher with respect to those of dipeptides with C-terminal Y position (Fig. 8.1). Probably, interaction of tyrosine phenol group with ϵ -amino group of lysine can affect the AOC of this peptide. The AOC of dipeptides YK, YE, and EY has been determined only by Y positions (Fig. 8.1).

Theoretical AOC values for dipeptides consisting of tyrosine and other redox-active amino acids (W, M, H) were calculated as a sum of AOC for corresponding Y residue and certain amino acid. Comparison of the theoretically calculated and

Table 8.1 Proximate composition and antioxidant capacity (AOC) of poultry protein hydrolysates

Parameter	PH1	PH2
Moisture (%)	4.0±0.5 ^a	5.0±0.6 ^a
Fat (%)	0.50±0.05 ^a	0.12±0.02 ^b
Protein (N×6.25) (%)	86.5±0.5 ^a	85.5±0.7 ^a
Ash (%)	6.19±0.08 ^a	1.80±0.07 ^b
AOC (μmol TE/g)	638±13 ^a	484±19 ^b

^aMean±standard deviation (*n*=4)Values in the same rows followed by different letters are significantly different at *p*<0.05 by *t*-test

experimental data (Fig. 8.1) revealed additivity of antioxidant effects of Y and W residues in dipeptide WY (theoretical and experimental values comprised 4.97 and 5.17±0.09 μmol TE/μmol, respectively). Infraadditivity (experimental AOC values lower than theoretical ones) was observed for dipeptides YY (theoretical and experimental values comprised 6.48 and 5.62±0.11 μmol TE/μmol, respectively), and YW (7.41 and 6.04±0.15 μmol TE/μmol, respectively). Infraadditive effects can be attributed to possible interactions of redox-active a.a.r. and their primary oxidation products with each other leading to reduction of AOC values of the corresponding dipeptides. Supraadditive (experimental AOC values higher than theoretical ones) effect was observed for dipeptides MY (theoretical and experimental values comprised 1.64 and 2.48±0.05 μmol TE/μmol, respectively), and HY (theoretical and experimental values comprised 1.64 and 2.03±0.05 μmol TE/μmol, respectively) because of intermolecular tunnel electron transition from M and H residues on Y phenoxyl radical. Thus the data obtained revealed the following structural descriptors of tyrosine-containing antioxidant peptides: preferred N-terminal Y position, presence of sequences (YH, MY) with intermolecular synergistic effects between neighboring redox-active a.a.r., presence of KY sequence with positive effect of neighboring K residue on antioxidant properties of tyrosine residue.

The molecular descriptors established above were further used for identification of novel antioxidant peptides in poultry protein hydrolysates (PH1 and PH2). The data on proximate composition of PH's (Table 8.1) clearly indicate that sequential micro- and ultrafiltration of PH1 resulted into partial separation of fat and mineral compounds. PH1 and PH2 were characterized by nearly the same contents of free R, Q, T, and Y, while free G and C contents in PH2 were correspondingly 1.8- and 3-fold higher (Table 8.2). Content of other free amino acids in PH2 was shown to be 1.6–3.4 times lower with respect to PH1 (Table 8.2). Comparison of total amino acids compositions of PHs revealed nearly equal total contents of K, P, T and sum of AA. Total content of A, F, V, I, L, S, and W in PH1 was in 1.3–1.8-fold higher; however, the total content of G, H, M, and Y in PH1 was in 1.5–2.7 times lower (Table 8.2). The analysis of PHs amino acid compositions revealed that in PH1 redox-active amino acids, Y, M, and H are predominantly (>50 % of total content)

Table 8.2 Amino acid composition (mg/g) of poultry protein hydrolysates PH1 and PH2

Amino acid	PH1		PH2	
	Free	Total	Free	Total
R	16.27±0.41 ^a	49.55±2.75 ^b	18.66±1.16 ^a	71.47±2.72 ^c
D	14.56±0.77 ^a	121.57±0.88 ^b	4.25±0.49 ^c	67.56±3.01 ^d
N	13.22±0.53 ^a		6.04±2.30 ^c	
A	20.36±1.27 ^a	75.38±1.60 ^b	5.99±0.27 ^c	54.97±5.30 ^d
C	0.53±0.01 ^a	4.41±0.33 ^b	1.58±0.01 ^c	6.17±0.48 ^d
Cystine	0.80±0.04 ^a		0.54±0.36 ^a	
G	4.80±0.13 ^a	49.55±2.75 ^b	8.85±1.89 ^c	13.79±3.32 ^d
E	23.60±0.86 ^a	151.82±1.45 ^b	14.20±0.66 ^c	141.72±1.65 ^d
Q	15.60±0.70 ^c		16.02±4.24 ^c	
H	5.12±0.26 ^a	22.17±1.18 ^b	2.72±0.10 ^c	37.36±2.58 ^d
I	20.66±1.34 ^a	43.22±0.42 ^b	8.96±0.01 ^c	25.35±0.93 ^d
L	34.26±2.01 ^a	65.24±0.65 ^b	15.40±0.30 ^c	41.36±1.42 ^d
K	27.21±1.48 ^a	60.47±7.88 ^b	16.59±1.58 ^c	62.68±3.13 ^b
M	8.96±0.58 ^a	9.79±0.97 ^b	5.65±0.01 ^c	14.98±2.97 ^d
F	14.61±1.09 ^a	30.35±1.98 ^b	8.22±0.15 ^c	22.09±1.27 ^d
P	7.35±0.26 ^a	47.40±0.28 ^b	4.74±0.22 ^c	54.08±2.29 ^b
S	16.01±0.64 ^a	44.40±1.43 ^b	6.05±0.32 ^c	26.40±0.93 ^d
T	8.38±0.39 ^a	33.26±1.58 ^b	8.11±0.61 ^a	34.06±2.17 ^b
Y	11.23±0.56 ^a	21.01±1.86 ^b	9.95±1.13 ^a	37.57±2.39 ^c
V	13.89±0.63 ^a	37.38±0.50 ^b	8.23±0.14 ^c	29.00±0.99 ^d
W	3.76±0.41 ^a	8.52±0.31 ^b	2.34±0.21 ^a	6.45±0.28 ^b
Sum	281.2±13.9 ^a	873.8±37.4 ^b	173.1±19.5 ^c	864.1±33.7 ^b

^aMean±standard deviation ($n=4$)

Values in the same rows followed by different letters are significantly different at $p<0.05$ by t -test

in a free form, while in PH2 those amino acids are mostly present in peptides. Taking into account AOC values of tyrosine and tyrosine-containing peptides (Fig. 8.1), one can expect higher AOC values in PH1. Really, AOC of PH1 was 1.3 times higher in comparison with PH2 (Table 8.1). Relative contributions of free Y, W, and C to the AOC of PHs were calculated based on their molar AOC values (Fig. 8.1) and the data of free amino acids analysis (Table 8.2). Free Y contributed nearly one-third (29.1–32.9 %) to the AOC of PH's under the study. Free W contributed 9.6 % and 6.0 %, whereas free C contributed 1.4 % and 4.2 % in AOC of PH1 and PH2, respectively. The overall input of redox-active free amino acids to the AOC of PHs resulted into 39–44 %, and the rest is contributed by antioxidant peptides. It should be noted that AOC values of the PHs (Table 8.1) laid within the range common for protein hydrolysates of animal origin (260–1000 $\mu\text{M TE/g}$) as reported by Hernandez-Ledesma et al. (2007), Dryakova et al. (2010), and Samaranayaka et al. (2010). However, correct comparison of protein hydrolysates AOC values reported by different authors is hampered by differences in TEAC

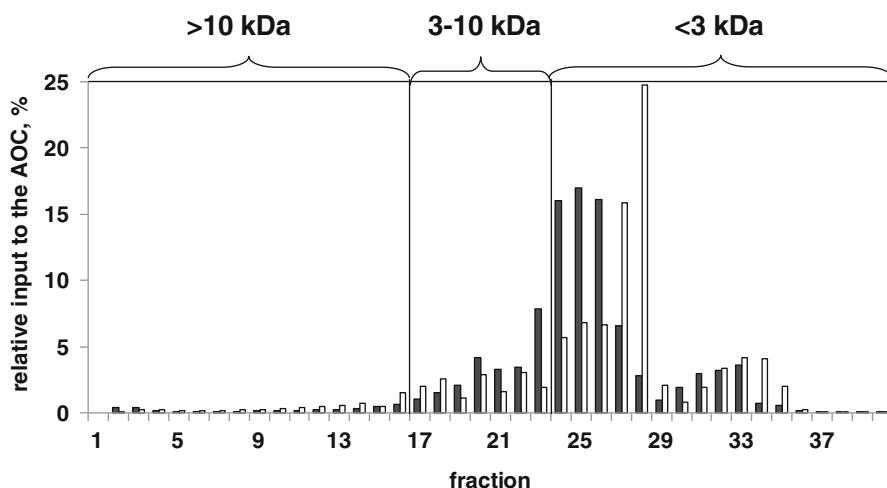


Fig. 8.2 Relative contribution of fractions obtained by SE-LC to the antioxidant capacity (AOC) of poultry protein hydrolysates PH1 (black bars) and PH2 (white bars)

assay procedures, such as duration of the assay and working concentrations of the hydrolysates in the reaction mixtures.

PHs were subjected to fractionation by semipreparative SE-LC with subsequent analysis of the AOC in the fractions obtained (Fig. 8.2). High molecular weight fractions (N 1–16, M_w > 10 kDa, Fig. 8.2) of PHs exhibited low AOC values and contributed only 3.5 % and 6.1 % to the AOC of PH1 and PH2, respectively. Relative input of medium molecular weight fractions (N 17–23, M_w 3–10 kDa, Fig. 8.2) to the AOC of PHs comprised 23.3 % and 15.1 % for PH1 and PH2, respectively. Low molecular weight fractions (N 24–40, M_w < 3 kDa, Fig. 8.2) containing free amino acids and oligopeptides contributed >70 % to the AOC of PHs (Fig. 8.2). The finding on predominant input of low molecular weight constituents to the AOC of PHs is in line with the literature data on antioxidant properties of low molecular weight fractions of protein hydrolysates obtained from milk, fish, and soy proteins (Pena-Ramos et al. 2004; Je et al. 2005; Beerman et al. 2009). For instance, low molecular weight constituents (0.35–1.50 kDa) made up the major (44–55 %) input to the AOC of α -lactalbumin and β -lactoglobulin hydrolysates determined in the Fe^{2+} -induced liposome oxidation system (Pena-Ramos et al. 2004). Je et al. (2005) also reported the major contribution of low molecular weight constituents (1–3 kDa) to the AOC of hoki frame protein hydrolysate determined by DPPH assay, hydroxyl radical scavenging assay, and in the linoleic acid auto-oxidation system Je et al. (2005). The major contribution of low molecular weight (<1 kDa) fraction to the AOC of tilapia muscle proteins hydrolysate had been recently demonstrated by Foh et al. (2010) in the linoleic acid auto-oxidation system, TEAC, and DPPH assays.

PH1 was characterized by bimodal distribution of the relative input of the fractions with different molecular weight constituents in AOC with maximum contributions of fractions 23–28 and 30–33 (Fig. 8.2). The AOC distribution of PH2 was slightly different: maximum contributions of fractions 24–28 and 31–34; however, the fractions 27 and 28 contributed more than 40 % of PH2 AOC (Fig. 8.2). PH's fractions with the highest AOC mentioned above were subjected to peptides identification by HPLC-ESI-FT-ICR-MS.

The fractions 30–34 of PHs were composed mainly of free tryptophan, while fractions 23–28 contained free amino acids (M, L/I, H, Y, F, R, K), meat-originating peptides of non-proteolytic origin (anserine, carnosine) and some phenylalanine-containing dipeptides (SF, GF, AF), and oligopeptides. The HPLC-ESI-FT-ICR-MS analysis enabled identification of 108 and 123 oligopeptides (5–24 a.a.r.) in PH1 and PH2, respectively. Sarcoplasmic (β -enolase, creatine kinase M-type, glyceraldehydes-3-phosphate dehydrogenase, pyruvate kinase), myofibrillar (α -actin, myosin heavy and light regulatory chains, C and T troponins, α -1 tropomyosin chain), and stromal (ovotransferrin, α -A- and β -hemoglobin subunits, serum albumin, α -2(I) and α -1(I) collagen chains) proteins of *Gallus gallus* were the major precursors for the peptides identified in PHs.

As indicated above, reactivity of protein hydrolysates with ABTS radical cation can be primarily attributed to the tyrosine-, tryptophan-, and cysteine-containing peptides. Therefore, corresponding peptides identified in PH1 and PH2 fractions 23–28 were selected for further consideration. Among the peptides identified both in PH1 and PH2, 44 contained Y and W residues. Besides, 15 tyrosine- and tryptophan-containing peptides were found only in PH2. That can be attributed to low concentration of these peptides in PH1.

Based on the structural descriptors of tyrosine-containing antioxidant peptides established above, the screening of perspective antioxidant peptides in PHs was carried out. The sequences of 16 novel antioxidant peptides selected are listed in Table 8.3. α -actin, myosin heavy chain, α -A-hemoglobin subunit, and serum albumin are the major parent proteins for these antioxidant peptides. Among the peptides selected, oligopeptides NVPAMY, NVPAMYVAIQ, and TFNVPAMY contain the sequence MY. According to the data of Erdman et al. (2006), this dipeptide (MY) possessed antioxidant activity in human endothelial cells by attenuation of NADPH-mediated free radicals formation via upregulation of hemeoxygenase 1 and ferritin genes expression. Oligopeptides TNPYDYHY and TTPYDYHYVSQ have sequences YHY and YDY (Table 8.3). Antioxidant properties of those tripeptides in TEAC assay and linoleic acid oxidation system were shown by Saito et al. (2003). Dodecapeptide YPPTKTYFPHFD has tripeptide motif YFP (Table 8.3), and this sequence was found in peptide YFPVGGDRPESF possessing antioxidant activity in a linoleic acid auto-oxidation system (Adebiyi et al. 2008). The tripeptide motif YVP was found in oligopeptide YVPPFPNPFDMFSF (Table 8.3). Antioxidative hexapeptide NPYVPR identified recently by Tsopmo et al. (2011) in the human milk subjected to simulated digestion contained the same tripeptide motif. Based on the data obtained, we can hypothesize that the tripeptide motifs YFP, YVP, YHY, and YDY are the

Table 8.3 Antioxidant peptides identified in poultry protein hydrolysates PH1 and PH2

Parent protein of <i>Gallus gallus</i>	Peptide sequence	Mascot score	Observed mass (Da)
Peptides identified in fractions 23–28 of both PH1 and PH2			
α -Actin	NVPAMY	25.60	693.3
	NVPAMYVAIQ	63.91	1104.6
	TFNVPAMY	37.06	941.4
	YELPDGQ	32.35	820.3
	YVGDEAQSQRG	81.40	1208.6
β -Enolase	YPVVS	29.48	563.3
Creatine-kinase M-type	GDDLDPKY	55.09	921.4
Glyceraldehydes-3-Phosphate dehydrogenase	YDSTHGHFK	45.65	1090.5
α -A-hemoglobin subunit	YGAETLERMF	35.41	1215.6
	YPPTKTYFPFHD	58.23	1511.7
Myosin heavy chain	TNPYDYHY	44.99	1071.4
	TTNPYDYHYVSQ	59.29	1486.6
Peptides identified only in fractions 23–28 of PH2			
α -Actin	YDEAGPS	32.62	737.3
	DLDPKY	24.21	749.3
Serum albumin	YQDNRVSF	26.50	1027.5
	YVPPPFNPDMFSF	63.33	1556.7

smallest information-bearing units in antioxidant peptides. However, further *in vivo* experiments should be conducted for the analysis of their bioaccessibility, bioavailability, and efficiency.

Antioxidant properties of PHs were verified *in vivo* on male Wistar rats. Serum, liver, and brain were examined for AOC and TBARS content (Table 8.4). AOC and TBARS content in the biological samples collected in animals of the one group decreased in the following order: liver>brain>serum. The same tendency was observed in all other groups studied (Table 8.4). No significant differences in serum and liver AOC values and TBARS content were observed among all four experimental groups of animals (Table 8.4). It can be connected with duration of experiments. The significant increase of serum AOC along with decrease of liver TBARS content was demonstrated by Manso et al. (2008) on spontaneously hypertensive rats after 17 weeks of egg protein hydrolysate passive ingestion with drinking water.

AOC values and TBARS content in brain homogenates were notably different in the experimental groups of animals under the study. The two and the three groups of animals exhibited significantly ($p<0.05$) lower TBARS content in brain homogenates comparably to the other groups (Table 8.4). Moreover, the third group of animals was characterized by significantly ($p<0.06$) higher AOC of brain extracts with respect to those for the one and the four groups (Table 8.4). Overall those findings indicated that *in vivo* antioxidant effects of PHs are more evident in nervous system. Probably, the early effects were observed in central nervous system, while systemic antioxidant state would be affected only after prolonged consumption of PHs.

Table 8.4 Antioxidant capacity and TBARS content in serum, liver, and brain of experimental animals

Parameter	Group (M±SD)			
	1 (n=10), casein	2 (n=10), PH1	3 (n=10), PH2	4 (n=10), protein free diet
Serum AOC (mM)	10.29±0.70 ^a	9.89±0.34 ^a	10.08±0.43 ^a	9.68±0.57 ^a
Serum TBARS (μM MDA equivalents)	1.22±0.20 ^a	1.45±0.28 ^a	1.14±0.22 ^a	1.23±0.26 ^a
Liver AOC (μmol TE/g)	79.2±13.6 ^a	69.6±4.2 ^a	64.6±2.8 ^a	64.4±6.0 ^a
Liver TBARS (μM MDA equivalents)	59.0±8.7 ^a	55.2±12.7 ^a	57.9±4.1 ^a	54.4±10.2 ^a
Brain AOC (μmol TE/g)	38.1±4.8 ^a	43.5±3.1 ^a	49.9±3.2 ^b	37.2±5.6 ^a
Brain TBARS (μM MDA equivalents)	44.8±4.4 ^a	32.9±4.2 ^b	36.3±4.1 ^{ab}	41.2±7.7 ^a

^aMean± standard deviation (n=10)

Values in the same rows followed by different letters are significantly different at $p<0.05$ by *t*-test

8.4 Conclusions

The structural descriptors were established for tyrosine-containing antioxidant peptides: preferred N-terminal Y position, presence of sequences (YH, MY) with intermolecular synergistic effects between neighboring redox-active a.a.r., presence of KY sequence with positive effect of neighboring K residue on antioxidant properties of tyrosine residue.

PHs obtained from poultry meat and bone trimmings were characterized in terms of their AOC, amino acid and peptide compositions. Low molecular weight ($M_w<3$ kDa) constituents were shown to contribute >70 % to the AOC of PHs, while the contribution of redox-active free amino acids (Y, W, C) reached 39–44 %. Among >100 peptides identified in the low molecular weight fractions of PHs, 16 novel antioxidant peptides were selected based on the structural descriptors established. The tripeptide motifs YFP, YVP, YHY, and YDY were hypothesized as the smallest information-bearing units in antioxidant peptides. These motifs can be considered as valuable starting points for design of small molecule biological modulators with antioxidant activity.

The data on *in vivo* antioxidant effects of PHs highlighted the significant role of antioxidant peptides for maintenance of antioxidant state in central nervous system. Therefore, further *in vivo* experiments should be conducted in order to elucidate the mechanism of PHs effects in central nervous system. The authors acknowledge the Center of Collective Use at IBCP RAS for LC-MS/MS analysis.

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Part II

Food Quality

Chapter 9

Heat-Induced Casein–Whey Protein Interactions in Caprine Milk: Whether Are Similar to Bovine Milk?

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9.1 Introduction

Milk is one of the most abundant food in human nutrition either as a fluid/dried milk or in the form of wide array of dairy products. The term milk is commonly related to bovine milk and for many years the focus of dairy scientists was on this type of milk. But, an increasing interest for caprine milk was present in last decade owing to its beneficial properties compared to bovine milk. Every year, more and more scientific papers referring to the caprine milk and dairy products are published and many of them are well reviewed (Barac et al. 2013; Tamime et al. 2011; Raynal-Ljutovac et al. 2007, 2008; Park et al. 2007; Park 2007). The significance of caprine milk in human nutrition is evidenced by the fact that the bovine milk adulteration in caprine milk occurs in dairy industry. As a consequence, different methods have been developed and are used for species identification in milk and dairy products (Chen et al. 2004; López-Calleja et al. 2007; Mayer 2005; Müller et al. 2008; Pešić et al. 2011a; Commission Regulation 1996).

Compared to bovine milk, caprine milk shows lower allergenicity (Haenlein 2004; Park 1994) and better lipid and protein digestibility (Tomotake et al. 2006; Almaas et al. 2006; Jenness 1980; López-Aliaga et al. 2010). Also, caprine milk and dairy products were recognized as a valuable source of bioactive peptides which exert ACE inhibitor, antihypertensive, antimicrobial, antithrombotic, immunomodulant

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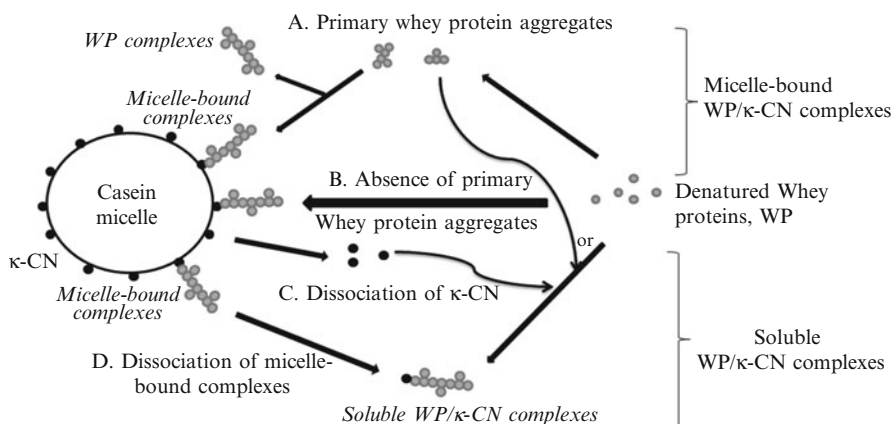


Fig. 9.1 A schematic representation of the formation of micelle-bound and soluble whey protein/κ-CN (WP/κ-CN) complexes (adopted from Donato and Guyomarc'h 2009)

and opioid activities (Park et al. 2007; Hernández-Ledesma et al. 2002, 2008, 2011; Slačanac et al. 2010). Furthermore, according to recent data, the caprine milk casein hydrolysates show similar to or greater antioxidant activity than some chemical antioxidants. These results indicate that the caprine milk casein hydrolysates can be used as functional food ingredients, food additives and pharmaceutical agents in the future (Li et al. 2013).

In many dairy processes, heat treatment of milk and dairy products is important operation designed to eliminate potential pathogenic and spoilage microorganisms and/or to enhance desirable properties of the final products, such as texture and taste. During heat treatment, the formation of protein complexes among denatured whey proteins, mainly β-lactoglobulin (β-LG) and α-lactalbumin (α-LA), and κ-casein (κ-CN) was occurred. The several possible mechanisms that lead to their formation were proposed and were reviewed by Donato and Guyomarc'h (2009) (see Fig. 9.1). In bovine milk, depending on pH of milk, the heat-induced whey protein/κ-CN (WP/κ-CN) complexes could be located at the surfaces of casein micelles as micelle-bound complexes and in serum phase of milk as soluble complexes (Donato and Dalgleish 2006; Guyomarc'h et al. 2003a, 2010; Vasbinder and de Kruif 2003). Different technological factors such as pH (Anema 2007, 2008; Anema and Li 2003b; Donato and Dalgleish 2006; Vasbinder and de Kruif 2003; Anema et al. 2004), temperature (Corredig and Dalgleish 1996; Patel et al. 2006; Vasbinder et al. 2003), time (Anema and Li 2003a; Oldfield et al. 2000) and composition of milk (Anema et al. 2006; Dalgleish et al. 1997; Donato et al. 2007; Guyomarc'h et al. 2003b) strongly affect the amount of formed complexes, their composition and distribution between the micellar and the serum phases of milk.

In contrast to the vast literature on heat-induced protein interactions in bovine milk, there is paucity of publications concerning the same interactions in caprine milk. The current knowledge about heat-induced casein–whey protein interactions in caprine milk, similarities and differences compared to bovine milk are discussed in this chapter.

9.2 Significance of WP/ κ -CN Complexes in Heated Bovine and Caprine Milk

The importance of WP/ κ -CN complexes in heated bovine milk is well known. They affect, positively or negatively, many dairy processes.

1. *Cheese production.* Denaturation of whey proteins and formation of complexes cause higher yield, longer rennet coagulation time and weaker curd structure (Ménard et al. 2005; Singh and Waungana 2001).
2. *Yoghurt production.* The earlier onset of gelation, higher firmness, increasing viscosity and lower syneresis of acid gels has been linked to the occurrence of complexes in heated milk (Guyomarc'h et al. 2003b, 2007; Mottar et al. 1989).
3. *UHT milk.* The gelation of UHT milk during storage provoked by heat-induced protein interactions in milk is a major factor which limits its shelf life (Chavan et al. 2011).
4. *Skim milk powder.* The technological-functional properties of skim milk powders and their suitability for different applications are strongly influenced by degree of whey protein denaturation and formation of complexes (Patel et al. 2007).

The available literature about the heat-induced changes in caprine milk mainly is referred to the influence of heat treatment on heat stability of caprine milk, its rennet clotting and gelation properties as well as to solutions for improving these properties (Raynal-Ljutovac et al. 2007; Li and Guo 2006; Herrero and Requena 2006; Montilla et al. 1995; Park et al. 2007; Ham et al. 2007; Park 2006, 2007; Domagała et al. 2013; Domagała 2009; Raynal and Remeuf 1998). The available reports indicated that:

1. *Cheese production.* The rennet coagulation time is not affected by heating regime (Montilla et al. 1995; Calvo and Balcones 1998), but curd structure is weaker than in heated bovine milk (Park et al. 2007; Park 2007). The differences in the ratio of caseins in the bovine and caprine milk are marked as a possible explanation.
2. *Yoghurt production.* The weakness and lack of consistency in curd tension or viscosity upon agitation are recognized as the main problems compared to bovine yoghurt, mainly due to differences in protein composition between two types of milk (Park 2006, 2007; Domagała 2009).
3. *Heat stability.* The heat stability of caprine milk at its natural pH is lower compared to bovine milk. Some of the reasons, which are proposed by several authors, could be the differences in micellar structures, partition of salts between colloidal and aqueous phases and protein interactions (Anema and Stanley 1998; Bouhallab et al. 2002; Morgan et al. 2001; Raynal-Ljutovac et al. 2007).

According to the current literature data, the essential differences between caprine and bovine milk are in the proportions of the individual proteins present in milk which could lead to differences in their technological-functional properties. β -casein (β -CN) is the primary casein in caprine milk (Park 2006), whereas α_{s1} -casein

Table 9.1 Reported values of the percentages of major proteins in caprine and bovine milk

	Casein fraction (% of total caseins)				References
	α_s -CN	β -CN	κ -CN	β -CN/ α_s -CN	
Bovine milk	56	33	11	0.58	Slačanac et al. (2010)
	33.8	41.9	n.a.	0.81	Ham et al. (2010)
	50	36	14	0.72	Park (2006)
	50.3	33.0	16.7	0.66	Pesic et al. (2012)
	48–49	33–39	11–13	n.a.	Tamime et al. (2011)
Caprine milk	26	64	10	0.41	Slačanac et al. (2010)
	13.3	54.7	n.a.	0.24	Ham et al. (2010)
	24.31	54.8	20.4	0.44	Park (2006)
	23.8	56.1	20.1	0.42	Pesic et al. (2012)
	26–30	50–64	10–20	n.a.	Tamime et al. (2011)
	28.8–38.7	51.0–58.4	9.9	0.49–0.76	Moatsou et al. (2004)
Whey protein (% of total whey proteins)					
Bovine milk	β -LG	α -LA	β -LG/ α -LA		
	50	33	1.51		Pintado and Malcata (1996)
	60.6	28.6	2.12		Pešić et al. (2011b)
	59.51	30.77	1.93		Lin et al. (2010)
Caprine milk	45	42	1.07		Pintado and Malcata (1996)
	45.9	33.5	1.37		Pešić et al. (2011b)
	43.64–65.77	8.97–22.03	2.02–6.65		Moatsou et al. (2005)

n.a. not available

α_s -CN α_s -casein, β -CN β -casein, κ -CN κ -casein, β -LG β -lactoglobulins, α -LA α -lactalbumin

(α_{s1} -CN) is most abundant casein in bovine milk (Farrell et al. 2004). Depending on goat breeds, the content of α_{s1} -CN in caprine milk could vary from zero to high levels (Caroli et al. 2006; Marletta et al. 2007). On the other hand, the significant amount of α_{s2} -casein could be present in caprine milk (Park 2006). Also, this type of milk could have higher content of α -LA and lower ratio of β -LG to α -LA than bovine milk, which could reflect to CN/WP ratio (Pešić et al. 2011b). Furthermore, the differences exist not only quantitatively than also in the primary structures of the same proteins in caprine and bovine milk which induce structural and electrophoretic differences (Jenness 1980; Pešić et al. 2010; Pesic et al. 2011b; Mayer 2005). The composition of major proteins in caprine and bovine milk is shown in Table 9.1.

Regarding bovine milk, it is known that formation of WP/ κ -CN complexes during heating dramatically affects its technological-functional properties (Donato and Guyomarc'h 2009), but how these complexes influence technological properties of caprine milk is not enough investigated. Obviously, the results obtained from the researches on bovine milk cannot be directly applied to caprine milk.

9.3 Formation of WP/ κ -CN Complexes in Heated Caprine Milk

Unlike detailed studies on the formation and technological consequences of heat-induced WP/ κ -CN complexes in bovine milk, there have been a few investigations concerning the occurrence of these complexes in caprine milk and their possible impact to its properties.

Anema and Stanley (1998), investigating the reasons of different heat stability of caprine milk compared to bovine milk, postulated that the interactions between β -LG and κ -CN occurred during heating of caprine milk and were pH dependent. After heating of milk at 120 °C for 10 min at pH below 6.8, these interactions occurred to a lesser extent than in bovine milk because the high level of dissociated κ -CN (40 %) and low level of α -LA and β -LG in ultracentrifugal supernatants of heated milk were registered (ultracentrifugation was performed at 65,000 g for 1 h at 20 °C). Differences in interaction behaviour of major caprine and bovine milk proteins were attributed to variations in the primary structures of the respective proteins of these species. They also reported that the reduced interactions between denatured whey proteins and κ -CN in heated caprine milk at pH below 6.8 may explain why heat treatment has little effect on rennet coagulation time of this milk. When the pH of milk was increased above pH 6.8, the level of dissociated κ -CN further increased up to 75 % of the total κ -CN at pH 7.6, whereas the levels of β -LG and α -LA in ultracentrifugal supernatants also increased up to 60 % of the total β -LG and 50 % of the total α -LA, respectively. Whether the WP/ κ -CN complexes were formed and in which extent at pH above 6.8, the authors did not report.

Morgan et al. (2001) also investigated the role of the heat-induced interactions between whey proteins and casein micelles in the heat stability of caprine milk. According to obtained results, it was suggested that the heat-induced interactions of β -LG with κ -CN were of minor importance at natural pH of milk, but were promoted at more elevated pH.

On the other hand, using multiple approach based on ultracentrifugation of heated protein suspension, chromatographic fractionation, sequential enzyme digestion of disulfide-linked oligomers and identification of disulfide-linked peptides by online liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS) and tandem MS, Henry et al. (2002) first identified the presence of covalent complexes in heated caprine milk suspension. After heating of the suspension of β -LG and purified casein micelles in the milk ultrafiltrate at 80 °C for 10 min and at 115 °C for 20s at natural pH of milk (6.7), three main covalent links were evidenced:

1. Intermolecular bridges between β -LG molecules
2. Disulfide bond involving two κ -CN molecules
3. A disulfide bond between β -LG (Cys¹⁶⁰) and κ -CN (Cys⁸⁸)

These results were the first direct evidence of the heat-induced interactions between β -LG and casein micelles derived from goat milk.

At the same year, the same group of authors examined how modification of the environment of the casein micelles affected the heat stability of caprine milk by combined membrane processes (Bouhallab et al. 2002). It was shown that increase of the CN/WP ratio increases the heat stability of goat milk. One of possible explanation was that the reduction of the whey protein content reduces the amount of heat-sensitive denatured whey proteins- κ -casein complexes.

In the review concerning the heat stability and enzymatic modification of goat and sheep milk, Raynal-Ljutovac et al. (2007) reported that the effect of the concentration of protein on heat-induced changes on surface hydrophobicity of casein micelles and interactions of milk proteins are of critical importance for heat stability. The heat stability of milk increases with decreasing concentration of protein (mainly β -LG).

Although it is known that heat treatment of bovine milk due to interactions between whey proteins and κ -CN increases the size of casein micelles, no information on the effect of heat treatment on the mean size of caprine milk casein micelles was reported, until 2011 year. Devold et al. (2011) firstly estimated the size of caprine casein micelles after heat treatment of caprine milk at 90 °C for 30 min. They concluded that the size of casein micelles increased and is not related to the amount of α_{s1} -CN present in milk. The effect of complex interactions between serum whey proteins and casein micelles was not discussed.

A study on casein-whey protein interactions in heat-treated caprine and bovine milk has been recently carried out (Pesic et al. 2012; Pesic et al. 2011a). In this study, the occurrence of high amount of whey protein-casein complexes in caprine milk was detected after heat treatment at 90 °C for 10 min at natural pH of milk (6.7). The composition of formed complexes and the distribution of major whey proteins between serum and micellar phases of milk were determined using three electrophoretic techniques: native PAGE, SDS-PAGE under reducing and non-reducing conditions and fractionation technique based on renneting. It was established that the micelle-bound complexes in caprine milk, apart from denatured whey proteins, included all three dominant caseins, κ -CN, β -CN and α_{s2} -CN, which were different compared to complexes in heated bovine milk that contained denatured whey proteins and κ -CN (Fig. 9.2). The differences also found in the distribution of denatured whey proteins between soluble and micellar phases of heated milk. About 30 % of total bovine whey proteins were involved in soluble complexes, whereas about 65 % of β -LGs and about 40 % of α -LA were found in micelle-bound complexes (Fig. 9.3). Conversely, denatured whey proteins were not detected as a part of soluble complexes in caprine milk. Only native whey proteins were detected in serum phase of heated caprine milk at very low percentage. All denatured whey proteins, more than 95 % of the total major whey proteins present in caprine milk, were located on the surface of caprine casein micelles.

Based on these results, the models of distribution of whey proteins and κ -CN in both heated milk species have been proposed (Fig. 9.4). It was hypothesized that the main reason for such significantly different composition and the distribution of whey proteins and κ -CN in two analysed milk was the differences in the casein micelle structure of these two types of milk caused by different casein composition.

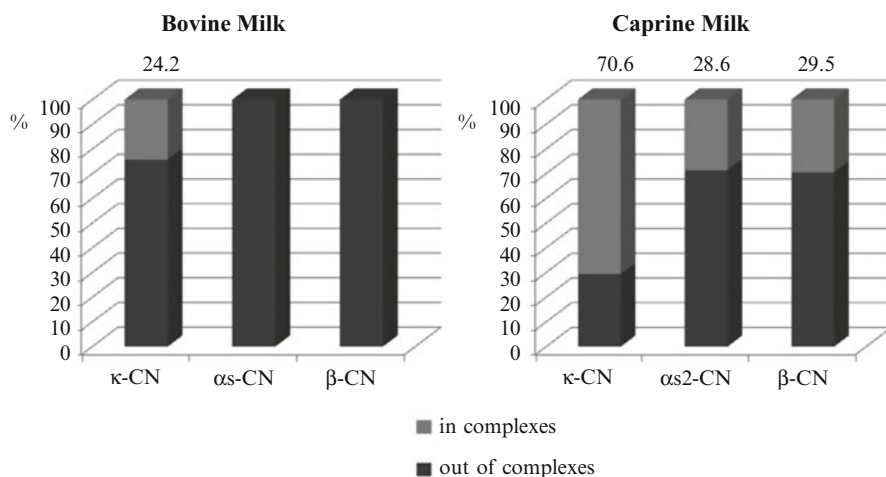


Fig. 9.2 The participation of caseins in heat-induced complexes in caprine and bovine milk (adopted from Pesic et al. 2012)

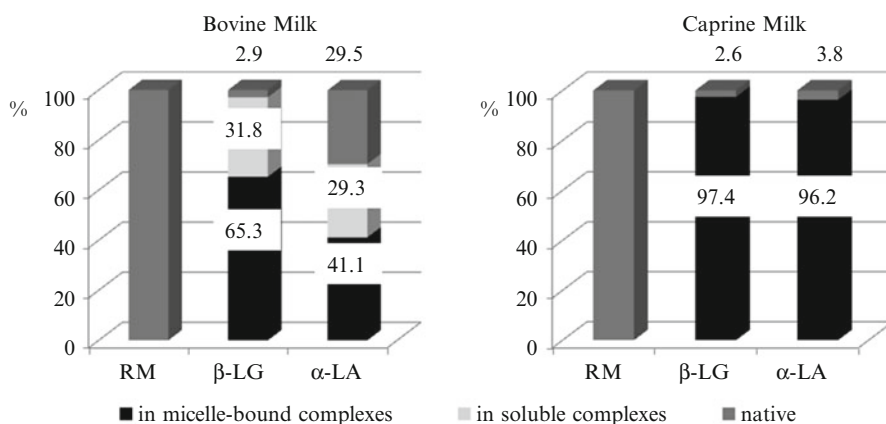


Fig. 9.3 Distributions of β-lactoglobulins (β-LG), α-lactalbumins (α-LA) and κ-caseins (κ-CN) among the native form, soluble complexes and micelle-bound complexes after heat treatment of caprine and bovine milk at 90 °C for 10 min at pH 6.71 (adopted from Pesic et al. 2012). RM row milk, β-LG β-lactoglobulin, α-LA α-lactalbumin

According to the obtained results, β-CN represented the main component of caprine casein micelle, whereas that in analysed bovine milk was αs-CN. Concerning that these caseins have completely different structure, with different number of phosphoserine regions which enabling cross-linked network in the interior of casein micelles (according to the Holt model of network formation, Fig. (Horne 2006)), β-CN possibly cannot provide the same cross-linked network in the interior of casein micelles as can the αs-CN. This is probably the main reason why many researchers found that the native caprine casein micelle is larger than the native

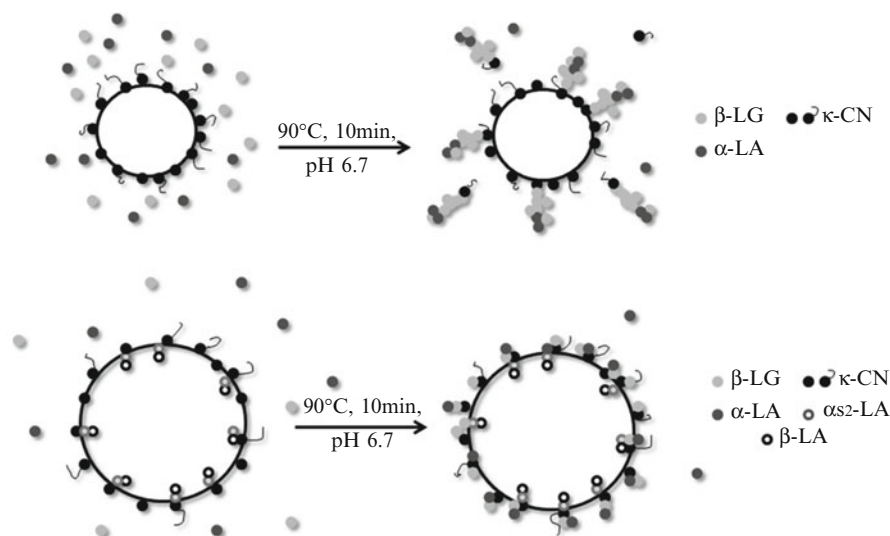


Fig. 9.4 A schematic representation of the interactions between casein micelles and denatured whey proteins occurred in bovine (a) and caprine (b) milk after heat treatment at 90 °C for 10 min at pH 6.71 (©Small Ruminant Research 2012), reprinted with permission

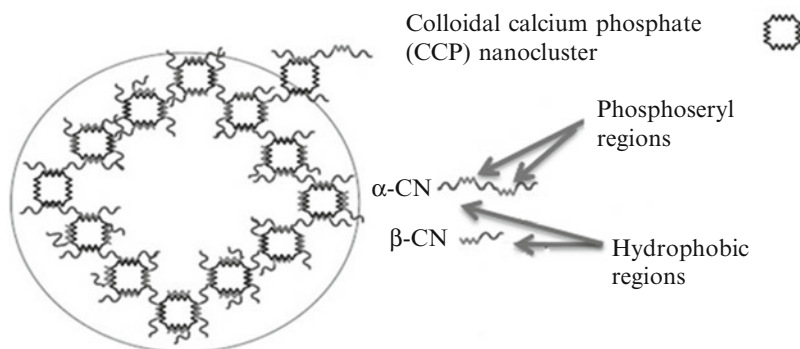


Fig. 9.5 Illustration of network formation in the Holt model of casein micelle (adopted from Horne 2006)

bovine micelle and strong influence of the content of α_{s1} -CN in caprine milk to the casein micelle size (with higher amount, the size is lower) (Dalglish et al. 1997; Pierre et al. 1998, 1995, 1999; Tziboula and Horne 1999). The reported average size of the native caprine and bovine casein micelles was listed in Table 9.2.

As a consequence of different casein micelle structures, it was assumed that:

1. Apart from κ -CN, the α_{s2} -CN and partially β -CN were present on the surface of caprine casein micelles.

Table 9.2 Reported average size of caprine and bovine native casein micelles

Bovine (nm)	Caprine (nm)	References
183.9	220.4	Inglingstad et al. (2010)
180	260	Park (2006)
n.a.	195–216	Devold et al. (2010)
n.a.	211–263	Pirisi et al. (1994)
n.a.	225–294	Tziboula and Horne (1999)
178–204	n.a.	Glantz et al. (2010)

n.a. not available

2. All three caseins were present in micelle-bound heat-induced complexes.
3. Micelle-bound complexes were stronger attached to casein micelles than in heated bovine milk which resulted in absence of soluble complexes.
4. Denatured whey proteins were probably uniformly distributed on the surface of caprine casein micelles implicated to the formation of small, well-distributed micelle-bound complexes.

So, differences between caprine and bovine heat-induced complexes were in composition, distribution on the surface of casein micelles and distribution between micellar and serum phases of milk.

9.4 Concluding Remarks

This chapter demonstrates that heat treatment of caprine milk had different effect on the casein–whey protein interactions compared to those interactions in heat-treated bovine milk. It seems that in the basis of the different protein interactions in caprine and bovine milk are differences in the structure of casein micelles. Different structures result to different properties of casein micelle surfaces which further affect the distribution of denatured whey proteins on them. Concerning that the aggregation properties of the casein micelles are of primary importance in many dairy processes as in cheese and yoghurt productions, the structure and properties of micelle interior and surface should be well understood. Hence, further research will be needed to reveal the structure of caprine casein micelles, native and thermally treated, and how it influences the technological-functional properties of caprine milk. The answers to these questions could be of crucial importance for understanding and control of the dairy processes.

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Chapter 10

Whey Protein Edible Coatings: Recent Developments and Applications

Marta Henriques, David Gomes, and Carlos Pereira

10.1 Functions of Edible Films and Coatings

The main purpose of edible films and coatings is to control the mass transfer of gases (e.g. O₂, CO₂), aromas, water and oil from or into food, thus preserving its quality and increasing its shelf life and safety. Edible coatings involve the formation of a film directly on the surface of the object they are intended to protect or enhance in some manner. For this reason, coatings are typically thinner than films and remain on the product throughout its use and consumption, whereas films should be removed before use. Edible coatings can enhance the nutritional value of foods, not only by increasing their composition but also through the incorporation of nutritional supplements. Additionally, they can improve the appearance and quality of a product and make it more appealing to consumers by adding gloss or colour or preventing microbiological development.

Protein-based films and coatings can be *edible* and/or *biodegradable* depending on their formulation (Li and Chen 2000), the formation method or modification treatments they undergo. If the compounds and additives involved in the formulation are food-grade and the film formation is only achieved by heating, pH modification, enzymatic treatment, UV irradiation and water/solvent removal, the film or

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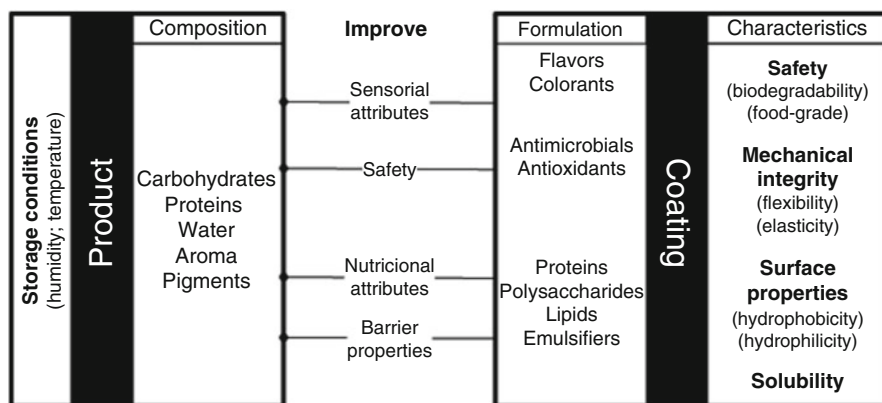


Fig. 10.1 Functions and attributes of edible coatings applied to food products

coating produced is edible, and of course biodegradable. However, if the proteins react with other chemicals before or during the formation of the film or coating (e.g. chemical cross-linking), or when non-edible compounds are added to the formulation, they are no longer edible (Krochta 2002). The challenge for biodegradable food packaging development is to ensure that biodegradation only begins after the film or coating has safely and effectively served its purpose within the prescribed time for product consumption.

In order to meet the desired requirements, films or coatings must be carefully selected (formulation) on the basis of the chemical composition of the food product matrix, the storage conditions and the characteristics to be maintained or improved (Fig. 10.1). It is possible to identify four specific functions for films and coatings used in food systems: (1) appearance and sensorial improvement, (2) safety, (3) nutritional enhancement and (4) reduction or prevention of mass transfer. The main features to be considered when selecting coatings for particular applications are: mechanical integrity (flexibility and elasticity), good surface properties, coating solubility and, the most important one, safety.

10.2 Composition of Films and Coatings

Proteins, polysaccharides and lipids are the main materials available for producing edible films and coatings. Although the first two are the most widely used and considered the basis of formulations, it is common to find certain plasticizers used to reduce brittleness, surface-active agents to aid film or coating adhesion as well as flavours and colourings to improve sensorial attributes. In some cases, especially for active food packaging, other compounds are also included, such as antioxidants, antimicrobials, fatty acids and preservatives.

10.2.1 Proteins

The proteins used to produce films and coatings may be derived from animal or vegetal sources. The former include collagen, gelatin, myofibrillar proteins, keratin, egg white protein, casein and whey proteins. Corn zein, wheat gluten, soy protein, peanut and cotton protein are examples of vegetal materials. Although collagen and gelatin have been widely employed in meat products (such as casings and wraps), the use of proteins as the basis for edible films and coatings has not been studied as comprehensively as it occurred with polysaccharides. More recently, whey proteins have attracted increasing interest, not only because they can be obtained from renewable resources (dairy industry by-products) but also due to their recognized oxygen barrier properties (Baldwin et al. 1995).

The main factor responsible for the characteristics of edible whey protein-based films and coatings is the difference in chemical composition of whey protein products (whey protein concentrates—WPC and isolates WPI), mainly with regard to the protein amounts and types. According to the nature and sequence of membrane processing technology (e.g. ultrafiltration, diafiltration), the proportions of whey proteins are not preserved either in WPC or WPI, which are generally richer in β -lactoglobulin and α -lactalbumin but proportionally poorer in immunoglobulins, lactoferrin and other minor proteins (Foegeding and Luck 2003). However, the presence of fat, lactose, salts (NaCl, KCl and CaCl_2), non-nitrogen compounds and vitamins can also influence their performance as edible coating materials (Banerjee and Chen 1995). One extra advantage cited for whey proteins is related to their intrinsic bioactive properties, due to the presence of enzymes such as lactoperoxidase and lactoferrin.

10.2.2 Polysaccharides

The most common polysaccharide film-forming materials studied include starch and its derivatives, cellulose derivatives, gums (e.g. arabic gum, guar gum and xanthan gum), agar, alginate, carrageenan, chitosan/chitin, pectin, gellan and pullulan (Liu 2005; Lacroix and Le Tien 2005, Cerqueira et al. 2008). Proteins can be combined with polysaccharides to modify and enhance the mechanical and barrier properties of films and coatings (Le Tien et al. 2000; Krochta 2002; Erdohan and Turhan 2005; Gounga et al. 2007). The use of polysaccharides in protein film-forming solutions increases their viscosity, a desired feature in coating formation in order to improve adherence between the food and the coating.

10.2.3 Lipids

Lipids are not polymers and they do not form cohesive stand-alone films. Their poor film-forming ability and weak mechanical properties restrict their use as coatings to a few applications, such as fruit and meat products (Ramos and Malcata 2011).

However, they can be included in the film matrix to provide some desirable characteristics such as gloss or nutritional value or to increase moisture barrier by reducing water vapour permeability, as a result of their hydrophobicity (Morillon et al. 2002; Fernández et al. 2007). The most popular lipids included in edible coatings are waxes (e.g. beeswax, carnauba wax, candelilla wax), triglycerides (e.g. milk fat fractions), acetylated monoglycerides, oils (vegetable and mineral oil), fatty acids and surfactants.

10.2.4 Plasticizers

When proteins are used as the base material for manufacturing films and coatings, plasticizers are usually required because the final film structures are normally stiff and brittle (Ramos 2011). The result of adding plasticizer to films or coatings is a decrease in protein chain-to-chain interaction, thus increasing free volume and chain movements (Daniels 1989). As a result, the protein glass transition temperature (T_g) is lower, film elongation (stretchiness) and flexibility increase (lowering of the film elastic modulus), and film strength decreases. The most negative impact associated with the use of plasticizers is the reduced capacity of the film to act as a barrier to moisture, aroma, oxygen, antioxidants, bacteriocins, oils and other solutes (McHugh et al. 1994; Bodnar et al. 2007). The most common plasticizers used in whey protein-based edible films are monosaccharides or disaccharides, polyols, lipids and, of course, water. However, water can easily be lost by dehydration at low relative humidity. The film moisture content is also affected by the relative humidity (RH) of the surroundings which significantly affects film properties. The use of hydrophilic plasticizers attracts additional moisture to the film, in particular increasing water activity, which can reduce the shelf life of food products (Krochta 2002).

The plasticizers used in edible whey protein films and coatings referred to in the literature are: propylene glycol (PG), glycerol, xylitol, sorbitol, sucrose, polyethylene glycol 200 (PEG 200) and polyethylene glycol 400 (PEG 400). Their various chemical structures, molecular weight and number of hydroxyl groups (involved in hydrogen bonds with biopolymers) are responsible for the different solubility, mechanical and barrier properties of the films. It is also possible to incorporate lipids into protein-based films and this may provide a plasticizer effect (Shellhammer and Krochta 1997; Anker et al. 2002; Reinoso et al. 2007). The most commonly used fatty acids are: myristic, palmitic, stearic, arachidic, behenic and lauric acids. They have been studied as plasticizers in WPI films (Sherwin et al. 1998) and WPI-lipid emulsions films (McHugh and Krochta 1994a). Normally, larger chain length fatty acids reduce moisture permeation through the film.

Combining a variety of plasticizers is another possible way of obtaining average film properties. For example, it is possible to increase flexibility and reduce water vapour permeability by using a polyol and a fatty acid simultaneously. Edible whey protein films and coatings (produced mainly from WPI) usually require a plasticizer content ranging from 10 to 60 % (w/w). However, these levels depend on the desired

properties of the films and the choice of plasticizer (McHugh and Krochta 1994b; McHugh et al. 1994; Sothornvit and Krochta 2005). When WPCs are used as protein source, lower amounts of plasticizers are required (Banerjee and Chen 1995). In this case, the non-protein compounds (e.g. lactose and fat) present in the formulation act as plasticizers.

10.2.5 Other Additives

Some proteins are sufficiently surface-active to form well-dispersed composite films or provide good surface wetting and adhesion. However, when the molecules do not have this property, an emulsifier is necessary. Surfactants such as sodium dodecyl sulphate (SDS) may also be used to enhance mechanical properties (Fairley et al. 1996). Edible films and coatings have the potential to enhance food safety, nutrition and quality by incorporating antimicrobial compounds (Seydim and Sarikus 2006; Min et al. 2008; Lee et al. 2008; Zinoviadou et al. 2009), antioxidants (Lee et al. 2003; Pérez-Gago et al. 2006; Min and Krochta 2007), nutraceuticals such as essential fatty acids, flavours (Lanciotti et al. 2004) and colourants.

10.3 Film and Coating Formation

Film formation and properties depend on two types of interaction: cohesion (attraction forces between polymer molecules) and adhesion (attraction forces between the film and substrate). The first phenomenon, in which polymer properties such as molecular weight, polarity and chain structure are relevant (Sothornvit and Krochta 2005), is the most extensively investigated.

Edible protein films are produced by two different mechanisms, namely wet and dry processing. The wet process, also called the solvent casting method, is the traditional and most widely used method. This process is preferred for applying coatings in liquid form directly onto food products by dipping, brushing or spraying. In the dry process, which is now attracting attention, films are produced in lower water contents by extrusion and compression-moulded, using the thermoplastic properties of polymers.

10.3.1 Solvent Casting

When films and coatings are produced by solvent casting, proteins are firstly dissolved into the solvent that is normally water. Plasticizers, lipids, polysaccharides or emulsifiers are added during this phase and homogenization takes place. This is followed by a pH adjustment, if necessary, or the induction of protein cross-linking

specially by heating in order to enhance film formation. Finally, the protein coating or film is formed by applying the prepared formulation to the desired product surface or casting, respectively, and allowing the solvent to evaporate. The formation of food coatings is achieved by solvent drying, after dipping, spraying or enrobing the food in the film-forming solution.

In terms of equipment, the solvent casting of whey protein films and coatings can be performed on various scales. From a research point of view, a very simple methodology is used as it is effective and cost-efficient. Usually, the film-forming solutions are manually spread into levelled teflon plates or Petri dishes and left to dry at specific conditions. On a larger scale, whey protein films are mechanically produced in batch or continuous coaters at fixed thicknesses (Dangaran and Krochta 2008).

10.3.2 Extrusion and Compression-Moulding

Extrusion and compression-moulding are common industrial techniques used to form films and containers. Their adaptation to the production of whey protein-based films allows for the mass manufacture of products such as water-soluble pouches and cups for individual servings of various dry ingredients and foods (Balagtas et al. 2003).

Thermoplastic extrusion would be an attractive way to produce protein casings and films, avoiding the need to add and then removing the solvent. Although various researchers suggest that some proteins have thermoplastic behaviour, this property has not been explored much in edible film production (Hernandez et al. 2005, 2006). Solvent evaporation, in the solvent casting method, is time consuming (especially for aqueous based film-forming solutions involving lower drying rates than organic solvents) and energy expensive, due to the cost and maintenance of the drying oven. On the other hand, extrusion is a faster and energy cheaper method that may reduce biopolymer production costs to levels competitive with synthetic polymers. Hernandez (2007) successfully produced extruded, homogeneous, transparent and flexible whey protein sheets using glycerol as a plasticizer (46–52 % on a dry basis). The extruder configuration and operation conditions allowed for heating denaturation and cross-linking of whey protein sheets produced with similar or enhanced mechanical properties compared to solvent casting heat-denatured films. The elongation of the extruded films was not affected by the amount of plasticizer (Hernandez et al. 2006) and had higher values than solvent casting films. The tensile strength was also higher. It was also found that whey protein extruded sheets displayed thermoplastic behaviour that enabled them to be compression-moulded to form thinner films or to be heat-sealed. Depending on the operation temperatures and type of plasticizer, Sothornvit et al. (2003) reported that compression-moulded whey protein films plasticized with water were more brittle and insoluble than those plasticized with glycerol. In the latter, elongation could be increased (from 85 to 94 %) by increasing the glycerol contents from 40 to 50 %, whilst tensile strength decreased (from 8 to 4 MPa). Despite these promising results, the properties of extruded and compression-moulded whey protein films need to be better understood (Dangaran and Krochta 2008; Hernandez and Krochta 2008).

Extensive work has been published on solvent casting and drying polymerization as well as on the influence of processing conditions (such as pressure, temperature and time), due to their direct effects on the denaturation of proteins via the unfolding of their globular structure which promotes interaction and entanglement between protein chains (Ghanbarzadeh and Oromiehi 2009; Denavi et al. 2009). However, in order to enhance film formation, other cross-linking processes, such as chemical, enzymatic or even irradiation processes, have also been documented. Very little information exists on the application of UV irradiation in the production of WPC-based films (Hettiarachchy and Eswaranandam 2005). For that reason, it is our intention to describe in greater detail the cross-linking mechanism induced by UV irradiation (Sect. 10.3.3) and its application to WPC films through a case study (Sect. 10.5).

10.3.3 UV Polymerization

UV irradiation technology uses the energy of photons from radiation sources in the short wavelength region of the electromagnetic spectrum (200–400 nm) to form reactive species which trigger a fast chain growth reaction. This is based on a fast, room temperature process with low energy consumption, requiring little equipment space (Schwalm 2006). The more expensive and high energy e-beam and X-ray photons are sufficient to cleave C–C or C–H bonds and therefore do not need photoinitiator species to form the radicals required as initiators for polymerization. In the case of UV exposure (300–400 nm), the cleavage of C–C bonds is possible although photoinitiators are commonly used, since the direct cleavage processes are not efficient enough.

The chemistry involved in radical UV-induced cross-linking starts with the absorption of a photon by the photoinitiator molecule which results in the excitation of an electron into higher singlet states, following the intersystem crossing an electron spin inversion by producing the excited triplet state (Oadian 2004; Schwalm 2006). From the triplet state, two main reactions can lead to initiating species (free radicals) which can start radical polymerization, the intramolecular scission of an α -bond or the intermolecular abstraction of a hydrogen atom, depending on the photoinitiator type. Intramolecular scission is the most effective process in the formation of radicals, since hydrogen abstraction is a bimolecular-type reaction in which diffusion is controlled and may be accompanied by several deactivation reactions. Thus, only the initiation step is different from thermal-initiated radical polymerization, whereas the polymerization reactions pursued follow almost exactly the same rules.

Photoinitiators are molecules that absorb photons on irradiation with light and form reactive species from their excited state, which then initiate consecutive reactions. For this reason, it is essential that they are selected to match with the output spectrum of the UV light source. They may be classified as cations, anions or radical photoinitiators, the latter representing more than 90 % of the commercially used

initiators. Almost all radical photoinitiators contain the benzoyl (phenyl-CO-) structure element. The two most important classes are the α -cleavable (type I) and the non-cleavable (hydrogen abstraction—type II) photoinitiators. The type I photoinitiators are very versatile, exhibiting higher efficiency in comparison to the type II photoinitiators due to the unimolecular cleavage reaction and consequently the most widely used.

The efficiency of UV irradiation in terms of the properties of films and coatings depends on several factors: the amino acid composition and molecular protein structure; the pH of the film-forming solution and the time when irradiation is applied to the film solution (e.g. before dissolving the raw protein, before heating, before casting, before or after drying). Hettiarachchy and Eswaranandam (2005) studied the use of UV irradiation applied to soy protein films. They found that if the UV treatment was performed before heating, no significant differences were found in comparison to the control—without UV treatment. However, if it was applied after heating, the drastic conformational changes in protein molecules during the thermal treatment affected the cross-links formed during UV irradiation and significantly increased film tensile strength. It was also pointed out that the high levels of tyrosine and phenylalanine in soy protein involved in the cross-linking reactions induced by UV irradiation are responsible for this behaviour. The lack of tyrosine residues in whey proteins may be the main reason why there has been no significant research into the implementation of this clean technology in the production of whey protein-based films and coatings. However, this drawback can be overcome with the use of photoinitiators, as previously mentioned, which create radicals under UV irradiation that start the polymerization reaction.

10.4 Applications and Opportunities

Taking into account the desired functions of whey protein coatings in terms of food or packaging materials, it is possible to categorize six different groups of applications:

- Moisture barrier coatings on foods
- Intend to prevent the movement of moisture from one component to another, avoiding sogginess, unwanted chemical reactions and microbial growth. Whey protein coatings were tested in fresh cut fruit, eggs or breakfast cereals. In general, the hydrophilicity of whey proteins has a negative impact regarding this application. Improvements on this property are therefore needed, especially in the case of foods kept on high moisture environments.
- Oxygen barrier coatings on foods
- Coating foods that are prone to oxidation (rancidity) will prolong their shelf life. Lee and Krochta (2002) and Lee et al. (2002a) have applied whey protein coatings on snack peanuts and nuts for confectionery bars, with very good results. Since only non-toxic edible films and coatings can be ingested with the food, the use of whey coatings seems very promising as an alternative to synthetic plastics.

- Oxygen barrier coatings on plastics
- Most plastics that are good moisture barriers are poor oxygen barriers. Thus, they may be coated with whey proteins to provide a good oxygen barrier (Hong and Krochta 2003) and to allow for contact between the packaging and the food.
- Grease barrier coatings on paper and paperboard
- Whey protein coatings are used in packaging for products such as fast food and pet food (Chan and Krochta 2001).
- Gloss coatings on foods
- Confectionery manufacturers are looking for an alternative glaze that will not be subject to the restrictions that accompany the use of shellac (resin). The specific application that has been most thoroughly investigated is whey protein gloss coatings for chocolate-panned confectionery (Lee et al. 2002b). Gloss coatings on other non-panned confectionery have not yet been investigated but can potentially be viable. Increasing the gloss on fruit, especially minimal processed fruit, is also of great interest.
- Active films and coatings
- Flavour, appearance, chemical protection and microbial safety are probably the most attractive properties to enhance in foods and, for this reason, practically all applications of whey protein films and coatings aim to make use of their carrier properties. Flavours and colourants, antioxidants, functional ingredients and antimicrobial compounds are the active agents usually applied.

In terms of the first five functions described above, whey protein films and coatings can be classified as passive barriers that compete directly with traditional packaging materials, whose main purpose is to offer mechanical and barrier protection. However, this does not indicate any economic potential for biopolymers, due to their high production costs in comparison to synthetic materials, at least in the short term (Weber et al. 2002). Indeed, the added value of bio-based edible films and coatings, particularly whey protein-based, as opposed to traditional packaging is, firstly their edible nature and inherent biodegradability and secondly their capacity to incorporate functional compounds that offer further protection for systems, creating the so-called active packaging (Brody et al. 2001), both allied to their intrinsic bio-active properties. Therefore, the active packaging category is the most promising application for whey protein-based edible films and coatings. By definition, active packaging interacts directly with the food or headspace of the product in a positive way to extend shelf life or create certain characteristics that cannot be obtained otherwise without raising concerns about toxicity.

10.4.1 Antimicrobial Agents

Food safety is an important issue worldwide and many technologies based on temperature changes, reduction of water activity, pH control and irradiation have been developed. However, their combination with antimicrobial agents incorporated into films and coatings allows for the use of less severe treatments and leads to more

specific growth control of the pathogenic microorganisms in each particular application. Several factors should be considered in developing antimicrobial films or coatings, such as the effect of the antimicrobial agent on the mechanical and physical properties of the coatings, the spectrum of antagonistic microorganisms, the antimicrobial mechanism, migration into the food and toxicological issues, as well as their effect on food product composition (Regalado et al. 2006).

Antimicrobial agents may be grouped as bacteriocins, fungicides, enzymes, organic acids, salts, oil extracts and polysaccharides. Evidence of the antimicrobial properties and concentrations used to control various target food-borne microorganisms is widely available in the literature (Ramos et al. 2012; Henriques 2012). Organic acids, for example, are protective compounds when sprayed onto food surfaces, but can quickly diffuse into the food interior leaving the surface susceptible to bacterial contamination. Little information is available on the antimicrobial activity of bioactive agents when incorporated into whey protein films, and it has been mainly evaluated *in vitro* using the film disc agar diffusion assay (Lee et al. 2003; Min et al. 2005; Cagri et al. 2001; Seydim and Sarikus 2006; Ko et al. 2001). There is even less antimicrobial evaluation of active whey protein coatings in real food applications. Only a few studies have been carried out into cheese, meat or fish products (Alcantara 1996; Stuchell and Krochta 1995; Samelis et al. 2001; Cagri et al. 2002; Franssen 2002; Min et al. 2006, 2008; Zinoviadou et al. 2009; Ramos 2011). In these food products (solids and semisolids), microbiological growth normally starts on the surface, due to post-processing steps and handling. The use of appropriate coatings that can retain antimicrobial compounds on the surface can reduce the amount of antimicrobial compounds used, as well as eliminate the need to compensate for the amount moving into the products. The inhibition of *L. monocytogenes* in hot dogs was more effective using WPI casings with *p*-aminobenzoic acid than with sorbic acid. However, the opposite was observed in Bologna and summer sausages in the case of *L. monocytogenes*, *E. coli* and *Salmonella typhimurium*, which could have been influenced by the natural pH of each product (Cagri et al. 2001, 2002). These findings indicated that organic acids in their undissociated form (at pH 5.2) are more effective and may be very attractive as antimicrobial agents for use in coated food products such as cheeses or fermented meat with lower pH values.

The use of lysozyme and lactoperoxidase in WPI films extended the shelf life of smoked salmon in terms of aerobic microorganisms (yeasts and moulds) and *L. monocytogenes* through their growth reduction or even inhibition (Min et al. 2005, 2008; Neetoo et al. 2008). Lysozyme hydrolyses linkages in peptidoglycan cell walls causing cell lysis and lactoperoxidase systems oxidize thiocyanate to hypothiocyanate, which then oxidizes sulphhydryl groups in microbial enzymes (Dangaran and Krochta 2008). Nisin is a natural bacteriocin that has also been investigated in WPI films. A reduction in *L. monocytogenes* counts at pH 3 and 6000 IU/g in WPI-nisin films was observed. Concerning the effect of the composition of whey protein films and coatings on the diffusion of bioactive compounds in food, Franssen et al. (2004), Ozdemir and Floros (2003) and Min et al. (2006) determined the diffusion coefficient of potassium sorbate, natamycin (antimycotic used by the cheese industry),

lysozyme and lactoperoxidase, respectively. The type of plasticizer used (glycerol or sorbitol) and the molecular weight of the bioactive compound are responsible for the different diffusion coefficients.

Finally, it is not possible to disregard the nutritional value provided by whey proteins as source of edible films and coatings, coupled with their intrinsic biological activities such as anticarcinogenic and immunomodulatory properties (Morris and FitzGerald 2008), bioactive properties (due to the presence of lysozyme, lactoferrin and lactoperoxidase) as well as antioxidant properties due to the presence of cysteine (Lacroix and Crooksey 2005).

10.5 Edible Whey Protein Coatings with Antimicrobial Activity Applied to Ripened Cheese: A Case Study

This case study refers to the efficacy of edible whey protein coatings with antimicrobial properties produced by heat denaturation and UV irradiation applied to ripened cheese as alternatives to commercial cheese coatings (Henriques et al. 2013). Edible whey protein coatings were produced from whey protein concentrate with the composition referred by Henriques et al. (2011) (WPC—10 % w protein/w coating solution), glycerol as a plasticizer (50 % w/w, protein basis), guar gum (0.7 % w/w), tween 20 (0.2 % w/w), sunflower oil (10 % w/w), lactic acid (6 gL⁻¹) and natamycin (0.125 gL⁻¹) as antimicrobials. Two methods of coating production were used separately: the heat denaturation method (HD) and the UV modification method (UV) using the commercial radical photoinitiator Irgacure® 2959 (2-Hydroxy-1-[4-(2-hydroxyethoxy) phenyl]-2-methyl-1-propanone) and in combination (HD+UV). Their effectiveness was evaluated by measuring the physicochemical, microbiological and sensorial properties of coated cheeses during 45 days of storage and comparing these properties with those of uncoated cheeses and cheeses with commercial coatings (whose commercial composition is polyvinyl alcohol (PVA) and natamycin (2.5 gL⁻¹) as the antimicrobial agent).

The use of two antimicrobial components in the tested formulation (lactic acid and natamycin) allowed for the significative reduction of the natamycin levels when compared to the commercial formulation. The choice for the concentration of natamycin in the formulation tested was based on previous results of Ramos (2011).

10.5.1 Cheese Physicochemical Profile Influenced by the Coating Type

Our analyses provided information on how cheese weight loss over 45 days was affected by the presence of a particular type of coating, and the influence of the coating production method used. Weight loss increased in ripened cheeses

throughout storage. It was more pronounced ($p < 0.05$) during the first 30 days, exception made to the commercial coating in which, weight loss tends to stabilize after 15 days. At the end of the ripening period, no differences were observed among the coated cheeses, but the WPC coating produced by the HD+UV method and the commercial coating showed the best performance in terms of moisture retention (Henriques et al. 2013).

The moisture loss profile was different between cheeses during the ripening period, displaying losses of approx. 33.4 to 40.0 % for the coated ones. Cheeses with WPC coatings produced by the UV and HD+UV method had the highest levels of humidity, followed by the commercially coated cheese. Fat, protein and salt contents increased during the ripening period in all the cheese samples, whether coated or not. However, this evolution was coating type dependent and varied in inverse proportion to the cheese moisture content.

Figure 10.2 presents the hardness values for cheese samples throughout storage, which increased significantly ($p < 0.05$) in all cases. It was also observed that the presence of a coating, its nature and the coating production method considerably influenced hardness. Surprisingly, the most dehydrated cheeses (uncoated and HD)

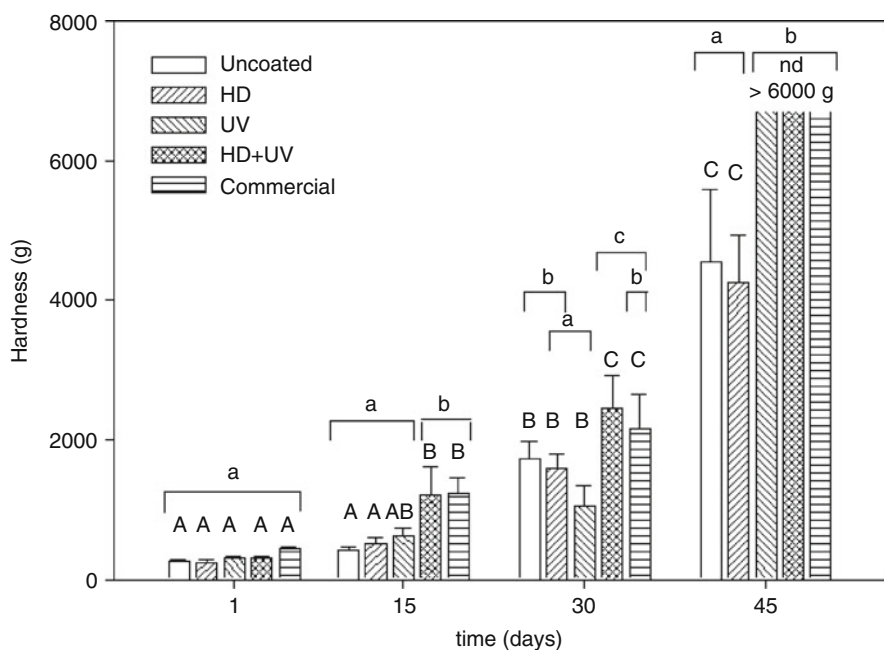


Fig. 10.2 Hardness (g) of cheese samples coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV methods compared with uncoated cheese and cheese with commercial coating, during 45 days of ripening at 11 °C and 85 % RH. Means with different capital letters differ significantly ($p < 0.05$) during ripening time for the same coating type, means with different small letters differ significantly ($p < 0.05$) between coating types at the same ripening day (adapted from Henriques et al. 2013)

had the lowest hardness values, whereas cheeses that had significantly higher humidity (UV, HD+UV and commercial) presented higher hardness values (Henriques et al. 2013). This finding can be explained by the influence of the coating on this parameter. In fact, cheese hardness does not depend exclusively on cheese bulk consistency but is also influenced by rind consistency, in which the polymeric material used in coating formulation and the type of chemical interactions occurring during the formation of the coating play an important role.

The similarity in hardness profiles obtained for cheeses coated with commercial coatings and with the WPC coating produced by the HD+UV method (Fig. 10.2) may indicate that the nature of the molecular and chemical interactions is similar, despite the different polymeric base materials (PVA for the commercial coating). The faster drying and coating formation during the first days of ripening was evident in both cheeses, allowing a harder crust to form which prevented further dehydration in the cheese bulk.

Coatings produced only by heat denaturation, did not significantly improve coating efficiency, whereas UV modification in combination with thermal treatment (HD+UV) enhanced cheese attributes. With regard to physicochemical evaluation, after 45 days of storage no significant differences were found ($p > 0.05$) between the cheese samples with commercial coatings and those with edible coatings (produced exclusively by UV modification or in combination with heat denaturation) not only concerning weight loss and hardness, but also regarding fat, protein and salt contents, as well as a_w and pH, which indicates that the antimicrobial edible coatings applied could be used as an alternative to their commercial counterpart(s) (Henriques et al. 2013).

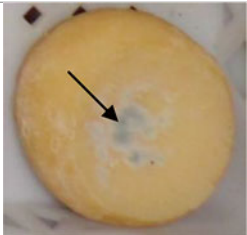
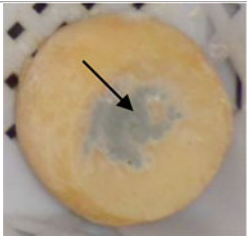
10.5.2 Cheese Appearance and Sensorial Evaluation

The appearance of the cheeses coated with antimicrobial WPC-based coatings produced by the HD+UV coating method was compared with uncoated and commercially coated cheeses during the 45 days of ripening (Table 10.1).

The most relevant visual changes in cheese throughout ripening occurred during the first 15 days when the initial white colour of the cheese and its wet appearance changed to a dry appearance and a light yellow colour. It was not easy to detect colour differences between uncoated and coated cheeses by visual inspection, but with regard to microbial growth, the uncoated cheese presented a significant amount of moulds on its surface after 30 days, which increased over the 45 days. Visually, the presence of moulds was not detected in the commercial coated cheese and cheese with the antimicrobial WPC coating produced by the HD+UV method during the 45 days (Henriques et al. 2013).

Sensory assessment was performed for the external cheese attributes (whole cheese evaluation) and also for the internal attributes (sliced cheese). With regard to external cheese evaluation, no differences were observed between cheeses in terms of shape and rind colour. Sensorial differences were only found for colour homoge-

Table 10.1 Appearance of cheeses coated with antimicrobial whey protein edible coatings produced by HD+UV method compared with uncoated cheese and cheese with commercial coating, during 45 days of storage at 11 °C and 85 % RH

Ripening time (days)	Coating		
	Uncoated	HD+UV	Commercial
1			
15			
30			
45			

Arrows presence of moulds (adapted from Henriques et al. 2013)

neity and hardness. It was observed that the commercial coated cheese had the lowest score for colour homogeneity and the cheese with the HD coating was classified as the most uniform. These results were consistent with the values obtained for colour measurement, which indicated that the commercial coating was the lightest and the HD coating the darkest. In some applications, darker coatings benefit cheese surface homogeneity, since smaller defects may be masked, making them more attractive to consumers.

Cheeses were classified as hard by the panellists. These results corroborate the behaviour of the cheeses according to the hardness measurements, which produced values of over 3000 g (Fig. 10.2). In terms of the internal cheese evaluation, no differences were found in colour difference between paste and rind, odour, consistency and flavour. With regard to the general acceptance, the cheese with the commercial coating was evaluated as the best by panellists. However, there were no significant differences in cheeses with antimicrobial edible coatings and commercial coatings.

10.5.3 Cheese Coatings Antimicrobial Performance

The microbiological results show that Gram-positive bacteria (*Staphylococcus* spp.) were found in lower levels ($<6.5 \log \text{ CFU/g}$) than Gram-negative bacteria (*Pseudomonas* spp. and *Enterobacteriaceae*) or even yeasts and moulds.

Cheese coated with the antimicrobial WPC-based edible coating produced by the HD+UV method exhibited the best results for microbial growth control or inhibition, regardless of the microorganism evaluated. The microbial inhibition of *Staphylococcus* spp. and growth control of *Enterobacteriaceae* were very clear in this coating type (Fig. 10.3), in contrast to the performance of the remaining coatings in terms of Gram-positive and Gram-negative bacteria. The results obtained for yeasts and moulds revealed a similar performance for HD+UV and commercial coatings. In both cases, there was no evidence of the growth of yeasts and moulds (Table 10.1). The application of the HD+UV coating with a significative lower amount of natamycin (0.125 gL^{-1}) compared to 2.5 gL^{-1} for the case of the commercial coating, which is a well-established success concentration in preventing growth of yeast and moulds on cheese surfaces (Amefia et al. 2006), turns it extremely attractive as an efficient substitute.

Microbiological analysis proved that the edible antimicrobial coatings prevented the growth of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae*, yeasts and moulds, thus demonstrating their ability to ensure safety of cheese. In fact, the coatings produced by HD+UV inhibited or reduced microbial growth, probably as a result of the synergistic effect of antimicrobial and UV light. The commercial coating proved to have a good performance against yeasts and moulds, due to the higher amounts of natamycin (Henriques et al. 2013).

The best coating performance was obtained with the antimicrobial whey protein coating produced by the HD+UV method, which prevented water loss more efficiently, led to a physicochemical cheese composition similar to the commercial coating, inhibited microbial growth and offered a good visual appearance. These results indicate that the antimicrobial whey protein-based edible film produced by the HD+UV method is an effective alternative to commercial coatings.

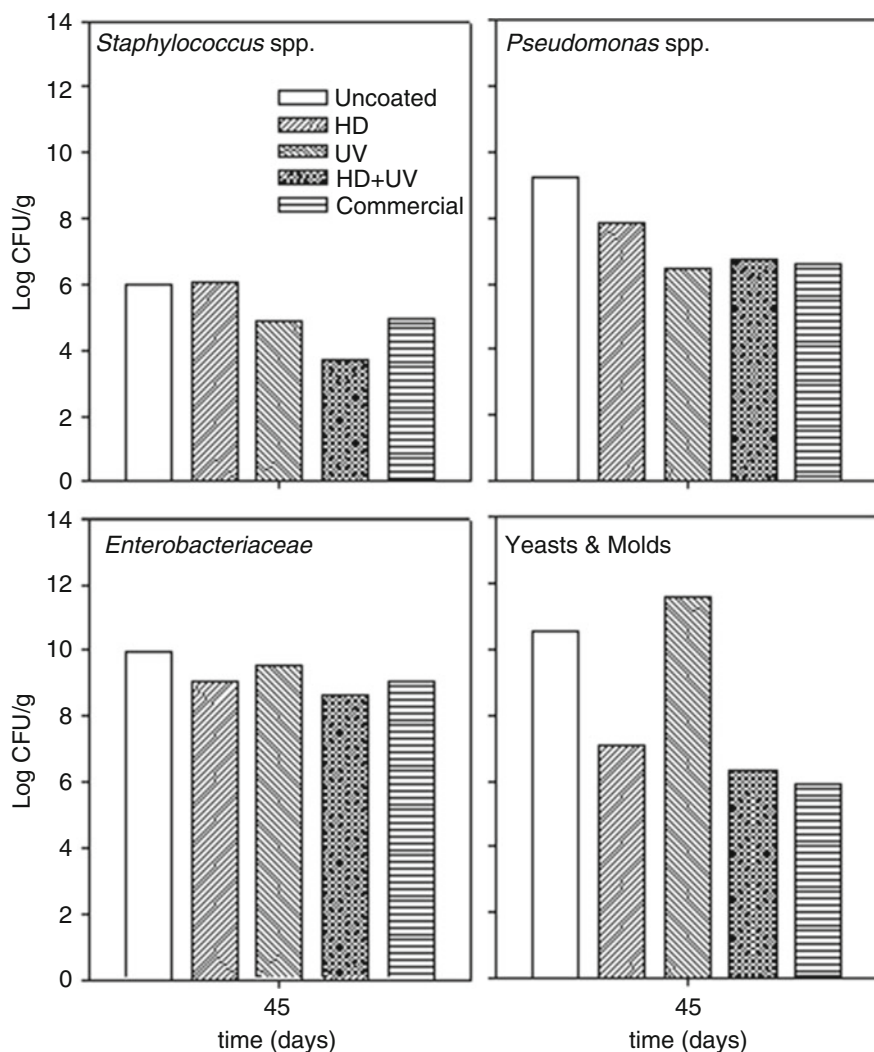


Fig. 10.3 Viable cell counts (log CFU/g) of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and yeasts and moulds of cheese samples coated with antimicrobial whey protein edible coatings produced by HD, UV and HD+UV methods compared with uncoated cheese and cheese with commercial coating, after 45 days of storage at 11 °C and 85 % RH (adapted from Henriques et al. 2013)

10.6 Conclusions

Whey protein films and coatings are attractive alternatives to petroleum-based packaging materials. They simultaneously solve two environmental problems, whey disposal and the treatment of packaging waste, due to the fact that they are biodegradable

and edible. They are excellent oxygen and oil barrier films with very attractive visual properties. Their hydrophilic nature is responsible for their poor moisture barrier properties, but the use of some specific compounds may improve this, as well as their mechanical properties.

Antimicrobial edible coating solutions based on WPC proved to be a suitable alternative to edible coatings based on WPI, as well as commercial coatings, since the cheese samples with either coating displayed similar values in terms of physico-chemical, microbiological and sensorial properties, in particular the edible whey protein coating produced simultaneously by heat denaturation and UV modification (HD + UV). UV treatment of WPC-based coatings with microbial activity may offer improved functionality and provide opportunities for extending the use of this technology in the food industry, and therefore warrants research attention. However, the great challenge involved in applying this technology is the development of reliable modification and polymerization methods that could be implemented on an industrial basis.

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Chapter 11

Physical and Sensory Properties of High Added Value Rice Extrudates

Vasiliki Oikonomopoulou, Asterios Bakolas, and Magdalini Krokida

11.1 Introduction

The current way of life has imposed the consumption of food products that need no or require limited preparation time. Food industries are trying to cover consumers' needs by producing ready-to-eat products that are characterized by superior quality and advanced characteristics. Extrusion minimizes processing times and economically produces a diverse range of high quality products. Various raw materials with low moisture are mixed in powder form and treated under high temperatures and short times and under very strong compressive stresses. Several unit operations and physicochemical changes (mixing, kneading, heating, starch gelatinization, protein denaturation, sterilization, plasticization) take place during extrusion cooking (Ding et al. 2006; Anton et al. 2009). The raw materials are converted into a dough which is extruded through a die. The rapid fall in pressure on the exit vaporizes the superheated water, leading to bubble growth, expansion, and creation of porous structure (Hutchinson et al. 1987). The extruder type, feed moisture, feed composition, temperature profile, residence time, screw speed, and feed rate affect the quality and properties of final products (Thymi et al. 2005).

Extrusion cooking can be used for the production of a wide variety of foods, such as snacks, pastas, ready-to-eat cereals, baby foods, and bakery products (Riaz 2000). Extrusion is usually used for the processing of starch-based raw materials, such as corn flour, rice flour, and wheat flour. Among them, rice flour is attractive for the formulation of various extruded snacks. It contains high amount of carbohydrates, has a unique, attractive white color and soft flavor, is hypoallergenic and is easily digested (Kadan et al. 2003). The increased consumer demands for the consumption of food products with high quality and nutritional characteristics have forced industries to the addition

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of several ingredients, such as beans, peas, apple pomace, cactus pear, grape seed, and grape pomace (Anton et al. 2009; Sarkar et al. 2011; Karkle et al. 2012). Considering the nutritional characteristics of carrots, the incorporation of dehydrated carrot powder into ready-to-eat snacks is very promising. Carrots contain considerable amounts of bioactive compounds (carotenoids, dietary fibers, and natural antioxidants) and have significant anticancer and health-promoting properties (Sharma et al. 2012). The addition of carrots in the extrusion mixture can be succeeded after dehydration. Freeze drying is considered as the gentlest method for food dehydration, leading to products with advanced characteristics similar to those of raw materials (Krokida et al. 1998).

The addition of proteins and fibers in the extruded mixture has a strong impact on final products' properties. Structural and textural properties are of high importance for the extrudates' characterization. Density and porosity characterize the texture and quality of expanded products (Hagenimana et al. 2006). Expansion is also desirable and is associated with other important properties of produced snacks, such as crispness and water absorption (Chen and Yeh 2001; Jyothi et al. 2009). Textural properties of foods are related to their sensorial characteristics and finally to their quality and acceptance by consumers. Texture can be determined using a texture analyzer or a universal testing machine, etc. (Hutchinson et al. 1987; Vincent 1998; Mazumder et al. 2007; Chakraborty et al. 2009). Shear compression measurement is indicative for textural analysis, since compression and shear are the main processes that occur during mastication. The correlation between structure and texture of extrudates is of high importance (Agbisit et al. 2007). Understanding the effect of process parameters on final products' properties, and the correlation among these properties, is beneficial for process optimization and development of products with high quality.

The acceptance of extruded snacks is also related to sensory attributes. Structural and textural properties are also being evaluated by sensory panels through attributes that characterize the food products (Cheng et al. 2007). Extrudates with low apparent density and high porosity gain global consumer preference. Crispness and crunchiness are also sensory attributes that are preferable for the acceptance of extruded products.

As a result, the objective of the present work is the determination of the impact of process parameters and material characteristics on expansion and microstructure formation of rice extrudates. Compression analysis was used for the examination of textural properties. Sensory analysis was also performed for the characterization of rice extrudates. Process optimization was conducted using simple mathematical powder models, containing parameters with physical meaning. The selected models were used for the prediction of the examined properties correlated with process conditions and material characteristics.

11.2 Materials and Methods

11.2.1 Mixtures Preparation

Rice flour was provided from Ev.Ge. Pistiolas (Agrinio) S.A. (Agrinio, Greece) and fresh carrots were purchased from the local market. Carrots were washed, peeled, cut in pieces, and stored at -30°C for 72 h, in a biomedical freezer (SANYO,

MDF-236, Osaka, Japan). The samples were dehydrated for 48 h using a laboratory freeze-dryer (Leybold-Heraeus GT 2A, Koln, Germany) operated at 6 Pa absolute pressure. Dehydrated products were ground to powder, sealed in opaque polyethylene bags, and stored at -18°C until further use.

Carrot powder and rice flour were brought to room temperature for mixtures preparation. Their moisture content was determined using the oven method at 70°C under vacuum for 24 h. The moisture content of rice flour was 11 % (wet basis (w.b.)), whereas carrot powder contained 3 % moisture (w.b.). Carrot powder and rice flour were mixed in different ratios (5, 7.5, and 10 % carrot) and simultaneously, sufficient amount of distilled water was added to the mixture to raise the moisture content of the samples at three different levels: 14, 17, and 20 %. The mixtures were mixed with a laboratory mixer in order to ensure uniform moisture distribution. Then, they were stored in opaque plastic bags at low temperatures (5°C) for 24 h in order to equilibrate and their actual moisture content was determined again. If moisture was far from the desired levels, correction was succeeded by adding calculated amounts of water or materials.

11.2.2 Extrusion

A co-rotating twin screw extruder (Prism Eurolab, model KX-16HC, Staffordshire, UK) was used for the production of extrudates. The extruder consisted of six independent temperature control zones, electrically heated and cooled by water. The two screws had 40 cm length, 16 mm external diameter, 11 mm internal diameter and their maximum rotation speed was 500 rpm. The products were extruded through a die with 3 mm diameter. Extrusion was conducted in three different levels of temperature and screw speed, varied from 140 to 180°C and from 150 to 250 rpm, respectively. The produced extrudates were cooled to room temperature and placed in laminated bags until required for analysis.

11.2.3 Experimental Design

A central composite design (CCD) (Table 11.1) with 26 runs was used for the determination of the effect of process conditions and materials characteristics on extrudates' final properties. Process variables (extrusion temperature, screw speed, moisture content, and carrot concentration) varied over three levels. Samples were extruded in two replicates.

11.2.4 Structural Analysis

11.2.4.1 Apparent Density

Extruded snacks were cut in cylinders of approximately 4 cm length and apparent volume was obtained by measuring their actual geometric characteristics (diameter, length), using a digital Vernier caliper. Apparent volume was determined using the equation:

Table 11.1 Process conditions and materials characteristics (CCD)

Moisture content X (% w.b.)	Carrot concentration C (%)	Extrusion temperature T (°C)	Screw speed N (rpm)
20	10	140	150
20	10	140	250
20	5	140	250
20	5	140	150
17	7.5	140	200
14	10	140	150
14	10	140	250
14	5	140	250
14	5	140	150
20	7.5	160	200
17	10	160	200
17	7.5	160	200
17	7.5	160	200
17	7.5	160	250
17	7.5	160	150
17	5	160	200
14	7.5	160	200
20	10	180	250
20	10	180	150
20	5	180	150
20	5	180	250
17	7.5	180	200
14	10	180	250
14	10	180	150
14	5	180	150
14	5	180	250

$$V_{\text{ap}} = \frac{\pi \cdot d^2 \cdot h}{4} \quad (11.1)$$

where V_{ap} (kg/m³) is the apparent volume, d (m) is the diameter, and h (m) is the length of each extrudate.

The mass of the extrudates was measured using an electronic balance with an accuracy of 10⁻⁴ g. Apparent density was calculated using the equation:

$$\rho_{\text{ap}} = \frac{m_{\text{s}}}{V_{\text{ap}}} \quad (11.2)$$

where ρ_{ap} (kg/m³) is the apparent density and m_{s} (kg) is the mass of the extrudates. Seven replications were made for each sample.

11.2.4.2 Porosity

The samples that were used for the calculation of apparent density were then ground to powder in order to eliminate their internal pores, and their true volume was estimated using a stereopycnometer (Quantachrome multipycnometer MVP-1) utilizing helium gas, with an accuracy of 0.001 cm³. True density was expressed by the equation:

$$\rho_{ts} = \frac{m_s}{V_s} \quad (11.3)$$

where ρ_{ts} (kg/m³) is the true density, m_s (kg) is the mass of powdered extrudates, and V_s (m³) is the volume of powdered extrudates. Three replications were made for each sample.

Extrudates' porosity ε was estimated using the equation:

$$\varepsilon = 1 - \frac{\rho_{ap}}{\rho_{ts}} \quad (11.4)$$

11.2.4.3 Expansion Ratio

Expansion ratio was determined as the ratio of the extrudate's diameter to the diameter of the die exit, according to the equation:

$$\text{Exp} = \frac{d}{d_o} \quad (11.5)$$

where d (m) is the diameter of the extrudates and d_o (m) the diameter of the die. Seven replications were made for each sample.

11.2.4.4 Mercury Porosimetry

Mercury intrusion porosimetry (MIP, Fisons, Porosimeter 2000, Milano, Italy) was used for the evaluation of porosity and pore size distribution of representative samples (Karathanos and Saravacos 1993; Aggelakopoulou et al. 2011). The unit is comprised from the macropores unit 120 and the micropores unit Porosimeter 2000. The pressure range of the porosimeter was 0.01–200 MPa.

11.2.4.5 Microscopy

The extrudates' microstructure was visualized using scanning electron microscopy (SEM). Samples were cut with a blade at a thickness of 1–2 mm. The samples were sputtered with gold particles creating a layer with 15 nm

thickness, using a SC7620 Mini Sputter Coater (Quorum Technologies, West Sussex, UK), in order to make their surface reflect the electron beam. The specimens were then scanned at $\times 25$ magnification, using a scanning electron microscope (Quanta 200 FEI (2004), OR, USA) operated at 25 kV, using a large field detector (LFD).

11.2.5 Textural Analysis

Textural analysis was performed using a universal testing machine (Zwick model Z2.5/TN1S, Ulm, Germany). The uniaxial compression tests were performed at room temperature (25 °C). The instrument was fitted with parallel plates for uniaxial compression, using a 100 N load cell. Extrudates were cut in cylinders of approximately 5 cm height and their dimensions were measured prior to each experiment, using a digital Vernier caliper. Extrudates were compressed at a constant loading rate of 5 mm/min. A stress–strain curve was constructed from the force and deformation data and sample dimensions, according to the equations:

$$\sigma = \frac{F}{A} \quad (11.6)$$

$$\varepsilon_n = \frac{\Delta L}{L_o} \quad (11.7)$$

where σ (Pa) is the stress, ε_n (mm/mm) is the strain, A (mm²) is the cross section area, L_o (mm) is the initial diameter of the samples, and F (N) and ΔL (mm) are the force and deformation, respectively. Five replications were made for each sample.

Elasticity modulus was calculated as the slope of the initial linear elastic region before first rupture.

11.2.6 Sensory Evaluation

A ten-member trained panel, both male and female, was chosen to evaluate the appearance and texture of rice extrudates using a nine-point scale ranging from 1 (low) to 9 (high) for each sensorial characteristic. Each sample was coded using a three-digit random number, placed into white styrofoam plates, and was served to the panelists. Evaluation was conducted in individually partitioned booths at room temperature. The panelists rinsed their mouth with water after tasting each sample. Definitions for each sensorial characteristic are shown in Table 11.2 (Liu et al. 2000).

Table 11.2 Sensory characteristics, definitions, and anchors

	Characteristic	Definition	Anchor
Appearance	Porosity	Number of pores per unit area	Low to high
	Expansion	Diameter of the sample	Low to high
	Uniformity	Uniformity of the samples	Low to high
Texture	Crunchiness	Degree of grinding noise perceived when chewing with molar teeth	Low to high
	Hardness	Force required to bite through the sample	Low to high
	Adhesiveness	Degree to which sample sticks to mouth surface or teeth	Low to high
	Cohesiveness	Degree to which particles of a sample stick together	Low to high
	Hydration	Degree to which sample mixes with saliva	Low to high

Table 11.3 Reference process conditions and materials characteristics

Moisture content X (% w.b.)	Carrot concentration C (%)		Extrusion temperature T (°C)	Screw speed N (rpm)
17	7.5		160	200
ρ_o^a (kg/m ³)	Exp_o^a	σ_o^a (kPa)	ϵ_o^a (mm/mm)	E_o^a (kPa)
274.67	2.26	206.00	0.26	762.01

^aMean values of experimental structural and textural properties

11.2.7 Mathematical Modeling

Simple power models, containing parameters with physical meaning, have been selected in order to correlate structural and textural properties of extrudates with process conditions and materials characteristics. The models are expressed with the equation:

$$Y = Y_o \left(\frac{X}{X_o} \right)^{Y_X} \left(\frac{100 - C}{100 - C_o} \right)^{Y_C} \left(\frac{T}{T_o} \right)^{Y_T} \left(\frac{N}{N_o} \right)^{Y_N} \quad (11.8)$$

where Y is the measured property; X (% w.b.) is the feed moisture content; C (%) is the carrot concentration; T (°C) is the extrusion temperature; N (rpm) is the screw speed; Y_o is a constant; X_o , C_o , T_o , and N_o are the corresponding values of experimental parameters at reference conditions (Table 11.3); Y_X , Y_C , Y_T , and Y_N are the exponents of feed moisture, material concentration, extrusion temperature, and screw speed and are constants dependent on the properties and the materials. The reference conditions are estimated as the central value of each parameter according to the experimental design.

In addition, based on the analysis of Ashby and Medalist (1983), textural properties are correlated with extrudates' structure. A simple power model was selected for the correlation of structural with textural properties:

$$I = I_o \left(\frac{\text{Exp}}{\text{Exp}_o} \right)^a \quad (11.9)$$

where I is the textural property, Exp is the expansion ratio, Exp_o is the average expansion ratio, and I_o , a are constants.

11.2.8 Statistical Analysis

Nonlinear regression analysis was used in order to estimate the models' parameters, according to Maroulis et al. (1988). Data from structural and textural analysis were analyzed using Statistica software (Statistica Release 7, Statsoft Inc, Tulsa, OK, USA) in order to determine whether the process conditions and material characteristics significantly influence the final properties. Analysis of variance (ANOVA) was conducted to assess the effects of independent process variables on sensory characteristics. Significant differences were considered when $p < 0.05$.

11.3 Results and Discussion

11.3.1 Structural Properties

The obtained experimental data were statistically analyzed in order to reveal the effect of process conditions and material characteristics on structural properties. Regression analysis showed that all parameters (feed moisture content, material concentration, extrusion temperature, and screw speed) significantly affected extrudates' apparent density, porosity, and expansion ratio as shown in Figs. 11.1, 11.2, and 11.5, respectively. These figures present the central points of the experimental data. The solid lines represent the predicted values obtained from the model fitting to the experimental data.

Extrudates' apparent density ranged from 143 to 600 kg/m³. The experimental data were modeled using a simple power model containing parameters with physical meaning. As it can be seen in Fig. 11.1, the model describes well the data, giving the opportunity of predicting apparent density's values from process parameters. The results of parameter estimation are presented in Table 11.4. The value of calculated constant ρ_o is close to the average value of experimental apparent density, showing its physical meaning and indicating the importance of the selected model. As can be observed in Fig. 11.1, apparent density showed an increasing trend with the increment of the initial mixture's moisture content and the concentration of

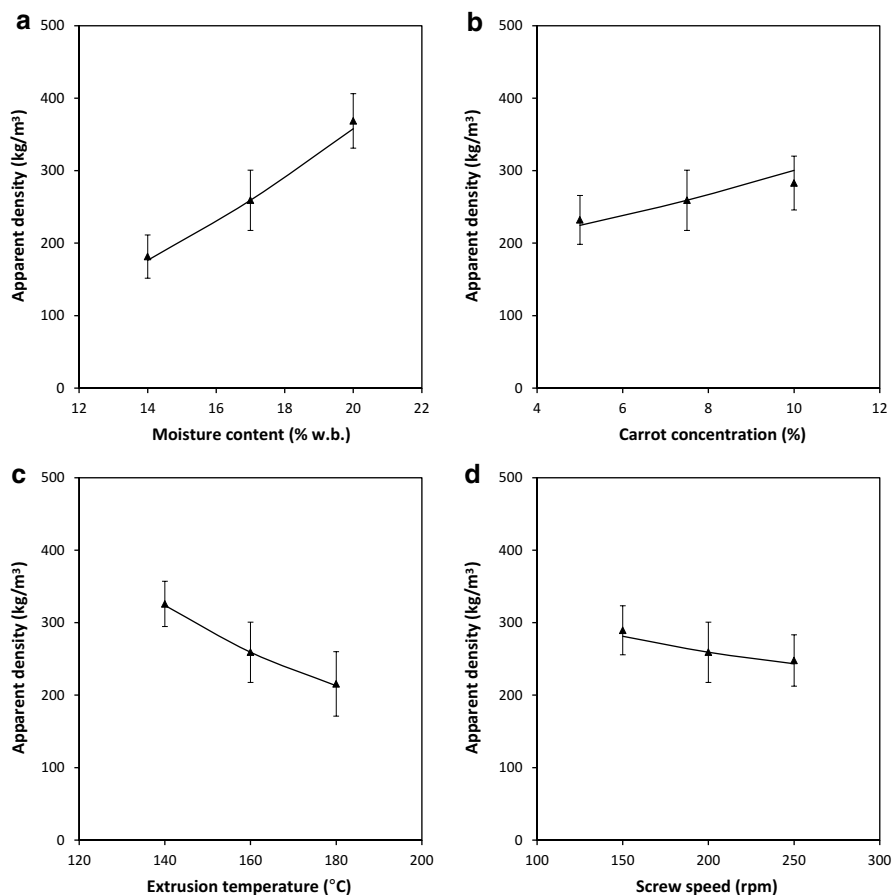


Fig. 11.1 Effect of (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) carrot concentration ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (c) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (d) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) on apparent density of extrudates

carrot. The results are in accordance with previous studies (Hutchinson et al. 1987; Chiu et al. 2012; Karkle et al. 2012; Robin et al. 2012). The added dehydrated carrot powder contains proteins and high concentration of fibers. The addition of fibers and proteins in starch-rich products ruptures the cell walls and prevents air bubbles to expand, causing an increase in their apparent density. Several works have shown similar results (Sacchetti et al. 2004; Anton et al. 2009; Yu et al. 2013). Apparent density increase, with the increment of feed moisture content, is attributed to the alteration of the mixture's rheological characteristics. Moisture reduces the viscosity of the dough and the friction between the dough and the barrel, having a negative impact on starch gelatinization and leading to more compact final products (Liu et al. 2000). In addition, high water content enables the material to undergo a glass transition temperature during extrusion, facilitating the matrix deformation and creating high dense structures.

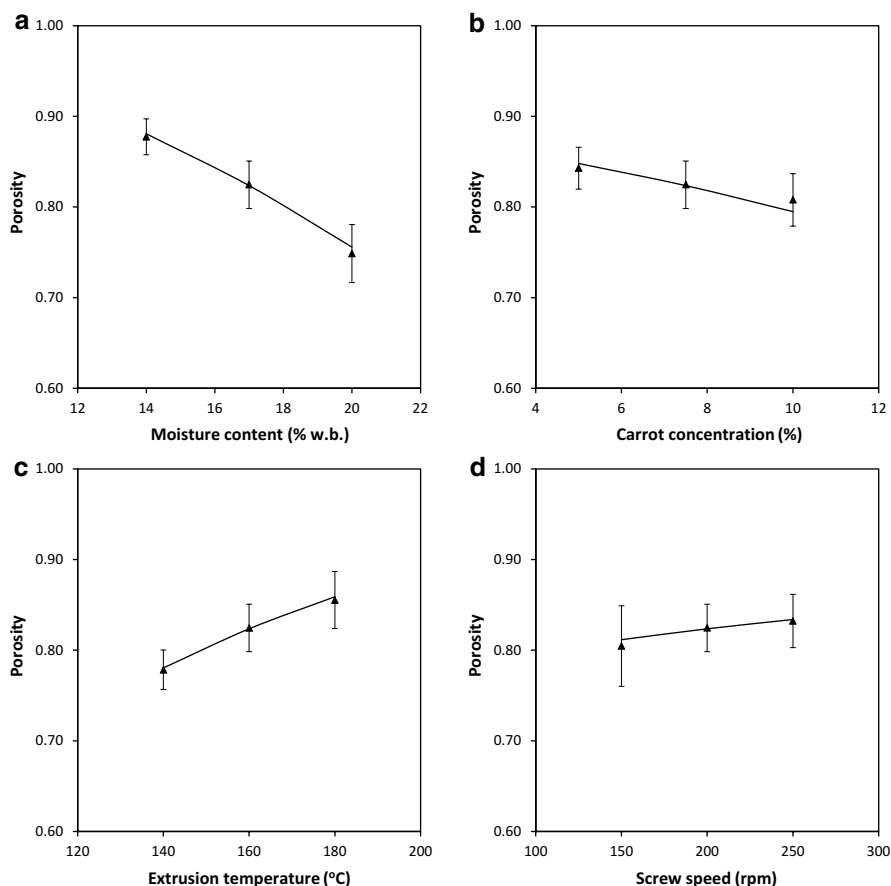


Fig. 11.2 Effect of (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) carrot concentration ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (c) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (d) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) on porosity of extrudates

On the other hand, the process parameters (extrusion temperature and screw speed) led to apparent density's decrement. The increment of extrusion temperature decreases the melt viscosity of the mixture and helps bubble growth during extrusion. As a result, products with higher void space and lower density are produced (Ding et al. 2005). Moreover, increased temperature causes higher vapor pressure which enhances the puffing of the dough and decreases products' density (Ding et al. 2005; Jyothi et al. 2009). The reduction of extruded products' density due to screw speed increment can be attributed to the reduction of viscosity, the increment of dough elasticity, and the starch gelatinization (Sacchetti et al. 2004; Hagenimana et al. 2006). Kumar et al. (2010) studied the properties of rice extrudates enriched with air dried carrot that were produced at lower temperatures and higher screw speeds than those examined in the present study. The lower temperatures resulted in increased density; however, further increment reduced the density and increased the void space.

Table 11.4 Parameter estimation for apparent density's and expansion ratio's models

<i>Mathematical models</i>						
Apparent density						
$\rho_{ap} = \rho_o \left(\frac{X}{X_o} \right)^{\rho_x} \left(\frac{100-C}{100-C_o} \right)^{\rho_c} \left(\frac{T}{T_o} \right)^{\rho_T} \left(\frac{N}{N_o} \right)^{\rho_N}$						
Expansion ratio						
$Exp = Exp_o \left(\frac{X}{X_o} \right)^{Exp_x} \left(\frac{100-C}{100-C_o} \right)^{Exp_c} \left(\frac{T}{T_o} \right)^{Exp_T} \left(\frac{N}{N_o} \right)^{Exp_N}$						
<i>Parameter estimation</i>						
Apparent Density						
	ρ_o	ρ_x	ρ_c	ρ_T	ρ_N	R^2
	259.18	1.98	-5.38	-1.67	-0.28	0.821
<i>p</i> -Value	0.000	0.000	0.000	0.000	0.000	
Expansion ratio						
	Exp_o	Exp_x	Exp_c	Exp_T	Exp_N	R^2
	2.24	-0.43	2.53	-0.80	0.21	0.824
<i>p</i> -Value	0.000	0.000	0.000	0.000	0.000	

In addition, porosity of rice/carrot extrudates presented particularly high values, as illustrated in Fig. 11.2. The alteration of porosity with process conditions and material characteristics showed the opposite trend with apparent density as expected, since porosity is inversely related to apparent density. Samples with low initial moisture content and low carrot concentration presented higher porosity. On the other hand, higher extrusion temperatures and higher screw speeds led to the formation of products with higher porosity. The porosity distribution of produced extrudates was evaluated with mercury porosimetry and is illustrated in Fig. 11.3. Due to the fact that mercury porosimetry measures pores smaller than 0.15 mm (mercury is easily penetrated to larger pores before any pressure is applied), the comparison among the samples will be on that basis (Jamroz et al. 1999). Figure 11.3a depicts that samples which contained 14 % w.b. initial moisture content and were characterized by high porosity, presented pores with larger radius. The addition of carrot powder created pores with reduced diameters. Moreover, lower extrusion temperatures and screw speeds resulted in the formation of smaller pores.

SEM pictures (Fig. 11.4) provided a window into the cellular architecture of the extrudates. The images enhance the previously described results. Indicatively, moisture content and carrot concentration rise led to the development of more compact structures. The addition of carrot powder caused some discontinuities and reduced the dough elasticity, having as a result the decrement of porosity (Karkle et al. 2012). Increased operating conditions (extrusion temperature and screw speed) led to products with higher porosity and more open air cells.

Expansion ratio of extruded products is shown in Fig. 11.5. The selected mathematical model contains parameters with physical meaning, since the calculated constant Exp_o is close to the average value of the experimental expansion ratio.

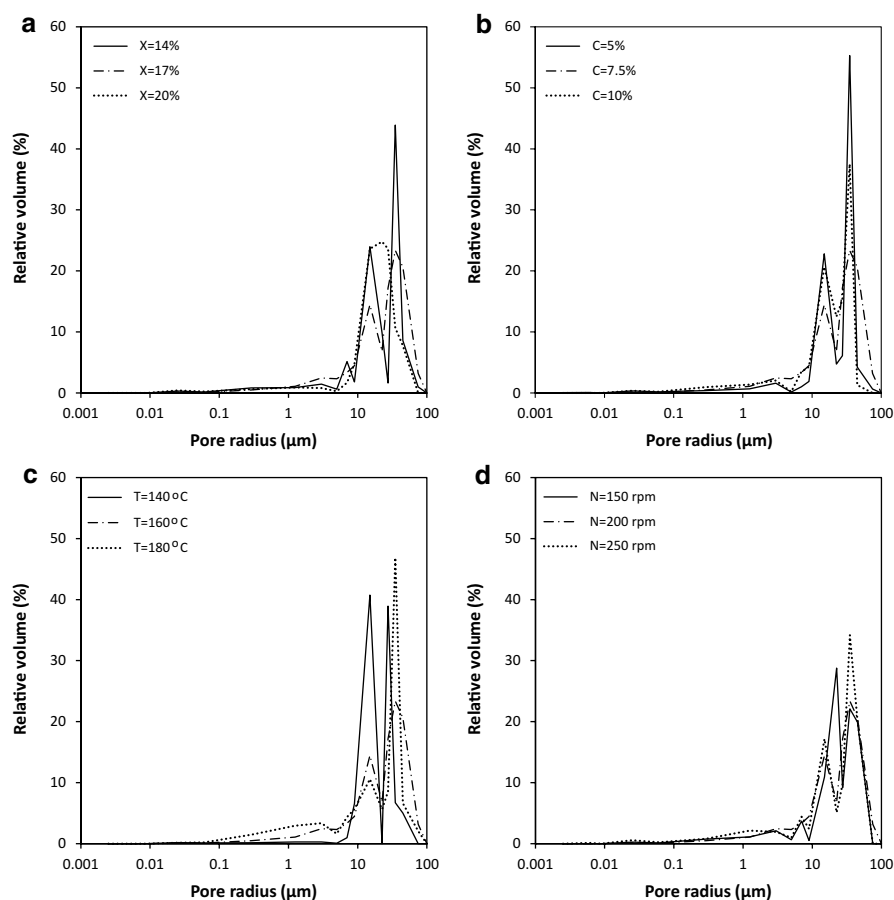
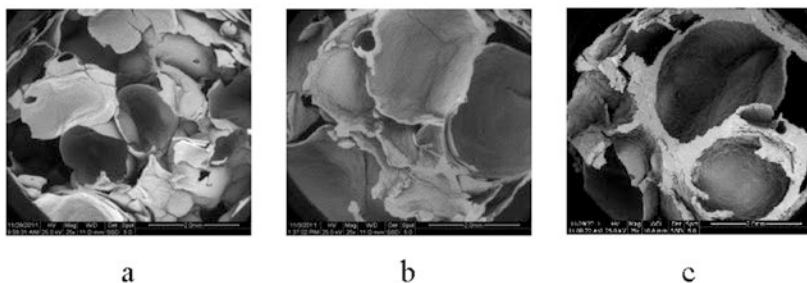


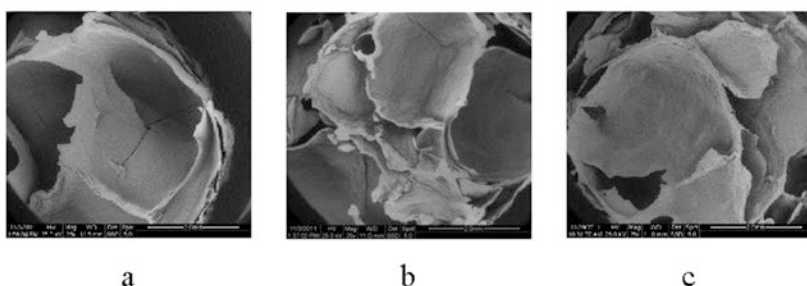
Fig. 11.3 Pore size distribution of extrudates correlating with (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) carrot concentration ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (c) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (d) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$)

Statistical analysis showed that carrot concentration and extrusion temperature had the most significant effect on expansion. Extrudates' expansion decreased with moisture content, carrot concentration, and temperature and increased with screw speed. The values of expansion ranged from 1.68 to 3.00 and are in accordance with previous studies (Hagenimana et al. 2006). In general, temperature increment causes the increase of extrudates' expansion, because the overheating of water at higher temperatures favors the bubble growth. Kumar et al. (2010) studied the effect of lower temperatures on the expansion ratio of rice/air dried carrot extrudates and reported the increase of expansion with temperature. The decrease of expansion with increasing temperature, which was observed in the current study, is possibly attributed to greater

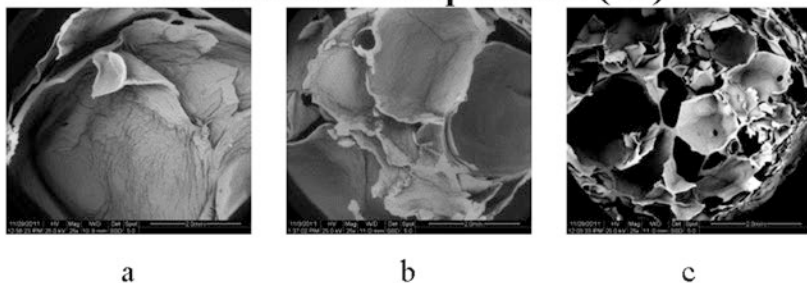
A. Moisture Content (% w.b.)



B. Carrot Concentration (%)



C. Extrusion Temperature (°C)



D. Screw Speed (rpm)



Fig. 11.4 Effect of process conditions and material characteristics on the microstructure of extrudates: **(a)** ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$) (a) $X=14\%$, (b) $X=17\%$, (c) $X=20\%$; **(b)** ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$) (a) $C=5\%$, (b) $C=7.5\%$, (c) $C=10\%$; **(c)** ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$) (a) $T=140\text{ }^{\circ}\text{C}$, (b) $T=160\text{ }^{\circ}\text{C}$, (c) $T=180\text{ }^{\circ}\text{C}$; **(d)** ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) (a) $N=150\text{ rpm}$, (b) $N=200\text{ rpm}$, (c) $N=250\text{ rpm}$

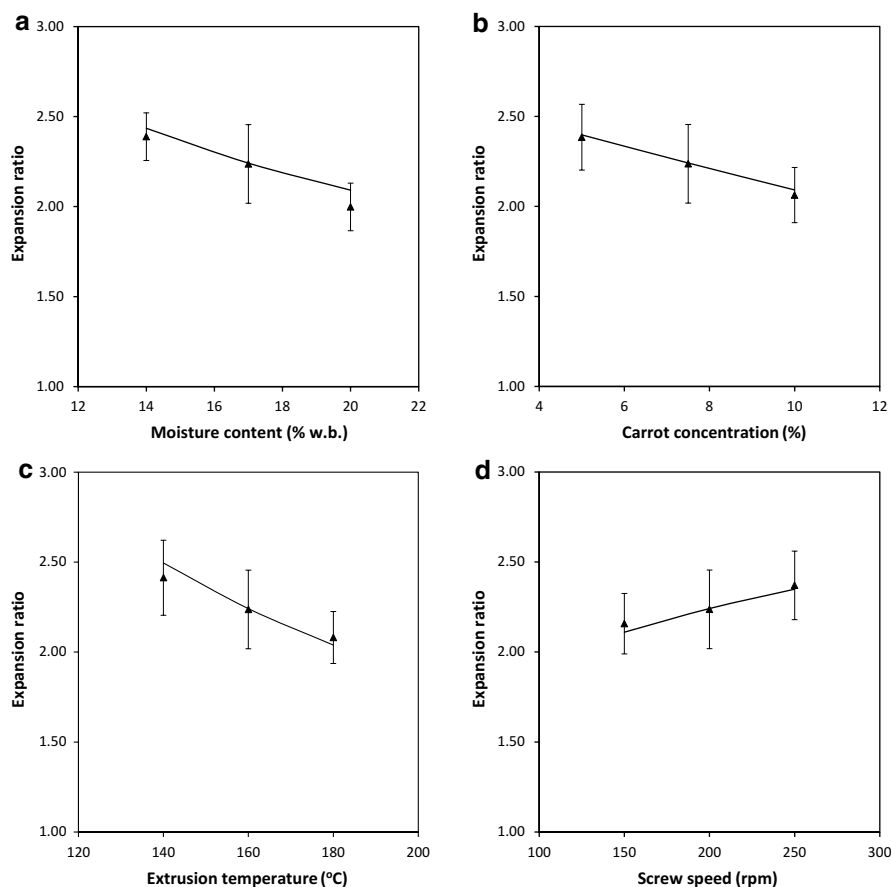


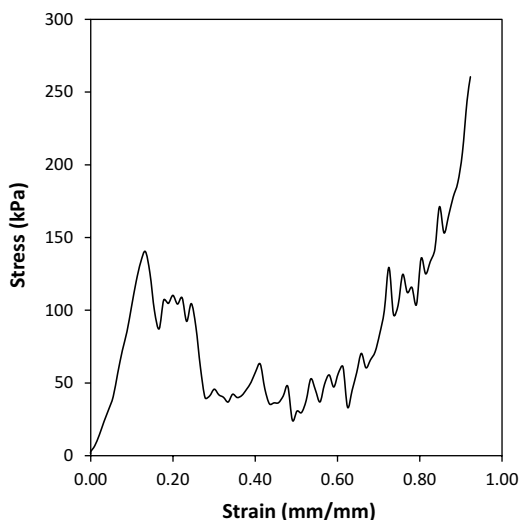
Fig. 11.5 Effect of (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) carrot concentration ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (c) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (d) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) on expansion ratio of extrudates

degradation of starch during extrusion at higher temperatures (Hagenimana et al. 2006). At high temperatures, the molten mass, when exiting the die, cannot retain the vapor generated, due to starch dextrinization and weakening of the structure (Ferreira et al. 2011; Lue et al. 1990). Ferreira et al. (2011) also observed that high temperature combined to low moisture content produced snacks with large cells due to reduced viscosity. The increase of porosity and decrease of expansion ratio with temperature were observed by other authors (Hagenimana et al. 2006; Jyothi et al. 2009).

11.3.2 Textural Properties

Figure 11.6 presents a typical stress–strain curve. Stress increases linearly with strain until the first rupture. After the first breakpoint, the curve is characterized by a crushing region with several peaks. During compression, the samples collapsed and broke

Fig. 11.6 Typical stress–strain curve



into numerous pieces. Maximum stress and elasticity modulus are indicative of material hardness. Figures 11.7, 11.8, and 11.9 show the plot of textural properties (maximum stress, maximum strain, and elasticity modulus) of extrudates correlated with process conditions and material characteristics. As it can be seen, the selected models describe well the experimental data. The values of parameter estimation of textural properties and the statistical results are shown in Table 11.5. According to the experimental data, maximum stress ranged from 85 to 430 kPa and elasticity modulus changed from 363 to 1210 kPa. The values of textural properties are in accordance with literature (Agbisit et al. 2007). Elasticity modulus and maximum stress were also plotted against extrudates' expansion (Fig. 11.10) and decreased with expansion's increment. Textural properties change with process conditions and material characteristics due to complex reactions and phase changes that occur during processing (Gogoi et al. 2000). In addition, texture is controlled by density, expansion, distribution of cell sizes, and width of cell walls (Agbisit et al. 2007). Higher wall thickness or lower density result in an increase in stress and elasticity modulus.

Regression analysis showed that maximum stress, maximum strain, and elasticity modulus were affected positively by moisture content and extrusion temperature, while screw speed had the opposite impact. Similar results were found by Chakraborty et al. (2009). Higher screw speed resulted in products with higher porosity and decreased rupture point and elasticity modulus (Hayter and Smith 1988). Increased initial moisture content formed products with high apparent density and high values of textural properties. These results indicate the effect of structure on products' hardness, revealing the reduction of textural properties with porosity. The increment of moisture content reduces dough viscosity and results in

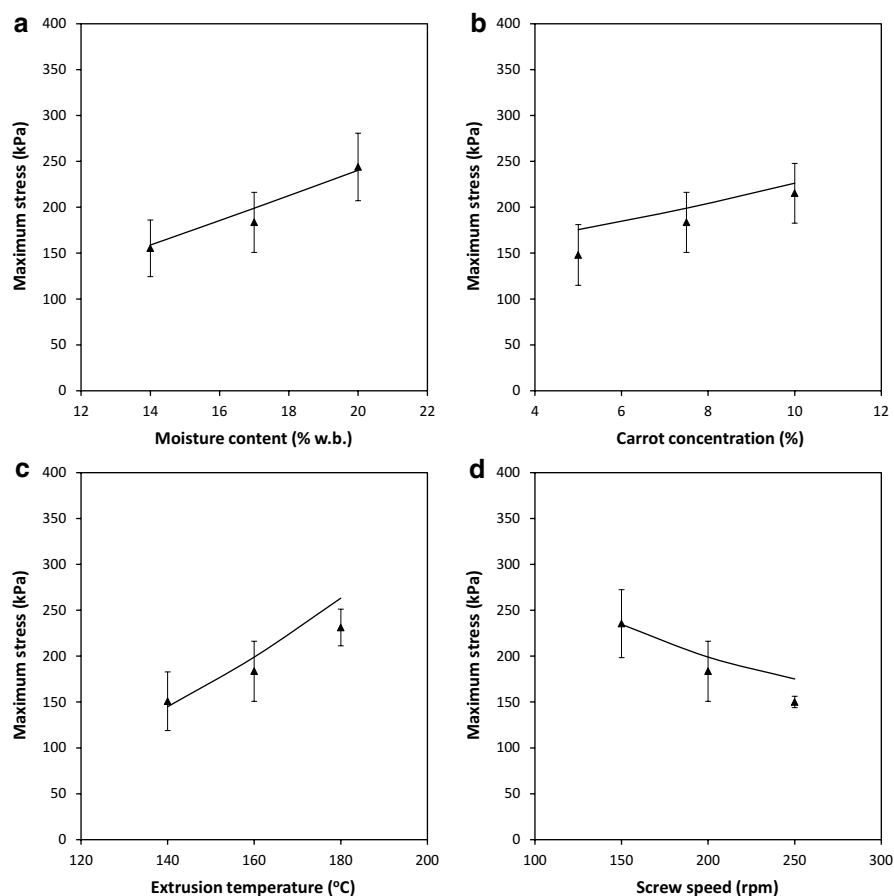


Fig. 11.7 Effect of (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) carrot concentration ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (c) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (d) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) on maximum stress of extrudates

products with increased density and higher cell wall thickness (Karkle et al. 2012). As a consequence, the hardness of extrudates is increased. Both elasticity modulus and stress decreased with increasing expansion, indicating a general weakening of the greatest expanded foam structure. On the other hand, although temperature increment led to less dense extrudates, due to the increased bubble growth of the molten dough, it caused the increment of maximum stress and elasticity modulus. This may be attributed to the lower expansion ratio of the extrudates. Jozinovic et al. (2012) reported that extrudates with lower expansion ratio showed higher hardness. The addition of carrot in the initial mixture made the extrudates tougher and caused the increment of maximum stress and elasticity modulus. This fact can be attributed to the increased density, since proteins and fibers reduced the elasticity of the dough and prevented air bubbles from growing. According to regression analysis, carrot concentration did not significantly ($p>0.05$) affect maximum strain.

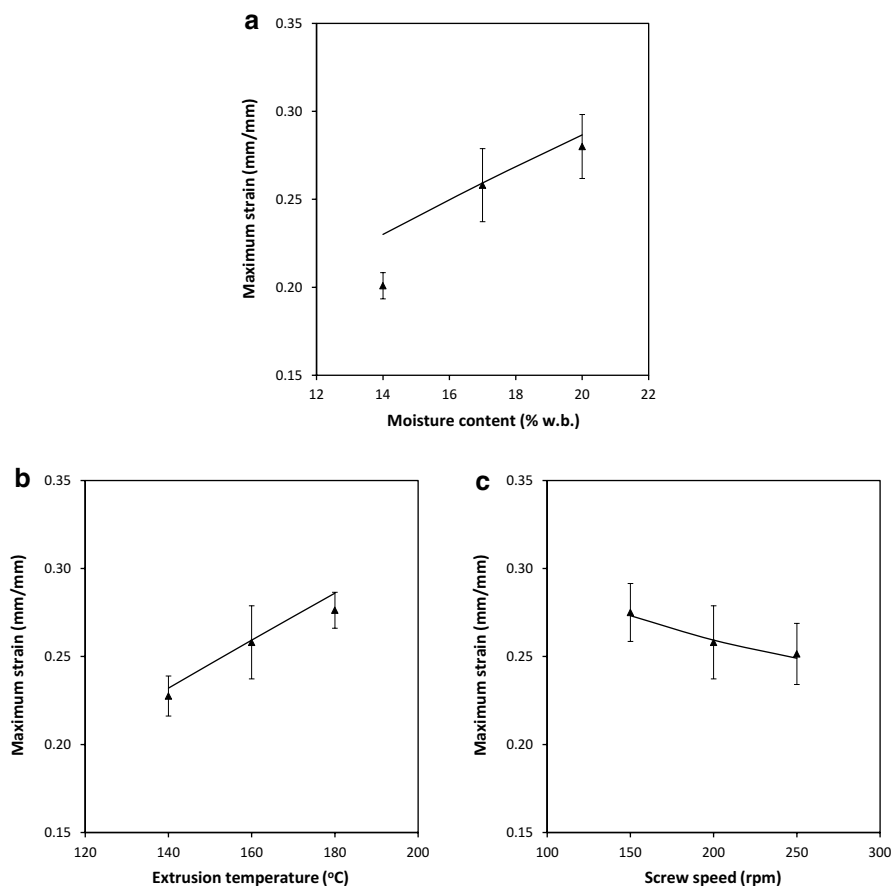


Fig. 11.8 Effect of (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (c) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) on maximum strain of extrudates

In addition, as it can be seen from SEM images, the increment of moisture content and material concentration and the decrement of screw speed led to the development of thicker cell walls, enhancing as a consequence, the rigidity and the resistance of the snacks' structure. The decrement of carrot concentration and the increment of screw speed resulted in the formation of extrudates with thinner walls (Figs. 11.4b, d) that reached earlier the rupture point. Finally, the thickness of cell walls decreased with increasing extrusion temperature (Fig. 11.4c) (Saeleaw et al. 2012).

As shown in Fig. 11.10, elasticity modulus and maximum stress decreased with expansion's increment, indicating the reduction of hardness of the most expanded extrudates. The application of Eq. 11.9 to the experimental data was considered satisfactory. The values of parameter estimation are presented in Table 11.6.

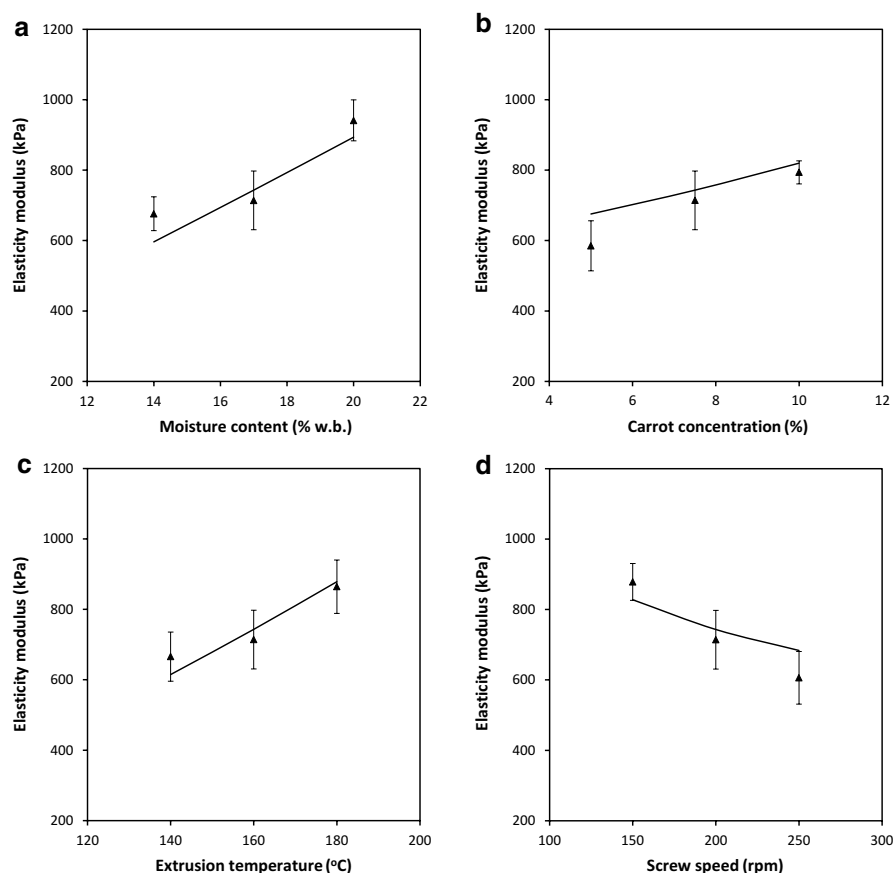


Fig. 11.9 Effect of (a) moisture content ($C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (b) carrot concentration ($X=17\%$, $T=160\text{ }^{\circ}\text{C}$, $N=200\text{ rpm}$), (c) extrusion temperature ($X=17\%$, $C=7.5\%$, $N=200\text{ rpm}$), (d) screw speed ($X=17\%$, $C=7.5\%$, $T=160\text{ }^{\circ}\text{C}$) on elasticity modulus of extrudates

11.3.3 Sensory Evaluation

Rice extrudates were evaluated concerning their appearance and texture and significant differences were observed for each attribute. Table 11.7 presents the effect of extrusion conditions and feed composition on sensory characteristics of produced extrudates. The central points of the experimental design are presented. Extrudates' appearance was influenced by process conditions (extrusion conditions and raw materials characteristics). The uniformity of the products decreased with the increment of initial moisture content, while it increased with the increment of carrot concentration and extrusion temperature. In addition, as it can be seen in the table, the extrudates' expansion significantly decreased with the increment of

Table 11.5 Parameter estimation for textural properties

<i>Mathematical models</i>						
Maximum stress						
$\sigma_{\max} = \sigma_o \left(\frac{X}{X_o} \right)^{\sigma_X} \left(\frac{100 - C}{100 - C_o} \right)^{\sigma_C} \left(\frac{T}{T_o} \right)^{\sigma_T} \left(\frac{N}{N_o} \right)^{\sigma_N}$						
Maximum strain						
$\varepsilon_{\max} = \varepsilon_o \left(\frac{X}{X_o} \right)^{\varepsilon_X} \left(\frac{100 - C}{100 - C_o} \right)^{\varepsilon_C} \left(\frac{T}{T_o} \right)^{\varepsilon_T} \left(\frac{N}{N_o} \right)^{\varepsilon_N}$						
Elasticity modulus						
$E = E_o \left(\frac{X}{X_o} \right)^{E_X} \left(\frac{100 - C}{100 - C_o} \right)^{E_C} \left(\frac{T}{T_o} \right)^{E_T} \left(\frac{N}{N_o} \right)^{E_N}$						
<i>Parameter estimation</i>						
Maximum stress						
	σ_o	σ_X	σ_C	σ_T	σ_N	R^2
	198.96	1.16	-4.68	2.37	-0.57	0.752
<i>p</i> -Value	0.000	0.000	0.000	0.000	0.000	
Maximum strain						
	ε_o	ε_X	ε_C	ε_T	ε_N	R^2
	0.26	0.62	0.21	0.83	-0.18	0.794
<i>p</i> -Value	0.000	0.000	0.537	0.000	0.000	
Elasticity modulus						
	E_o	E_X	E_C	E_T	E_N	R^2
	743.17	1.13	-3.58	1.42	-0.37	0.710
<i>p</i> -Value	0.000	0.000	0.000	0.000	0.000	

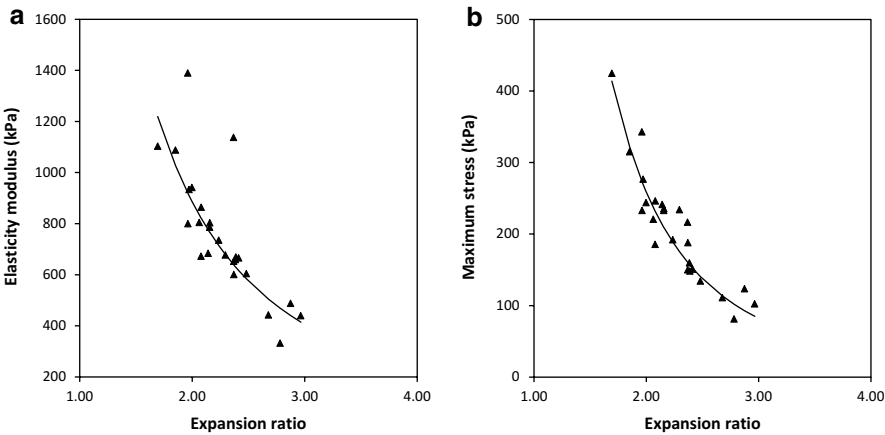


Fig. 11.10 Correlation of (a) elasticity modulus and (b) maximum stress with expansion ratio of extrudates

Table 11.6 Correlation of textural properties with expansion ratio

<i>Mathematical models</i>			
Maximum stress			
$\sigma_{\max} = \sigma_{\max o} \left(\frac{\text{Exp}}{\text{Exp}_o} \right)^a$			
Elasticity modulus			
$E = E_{o1} \left(\frac{\text{Exp}}{\text{Exp}_o} \right)^a$			
<i>Parameter estimation</i>			
Maximum stress		Elasticity modulus	
$\sigma_{\max o}$	a	E_{o1}	a
192.07	−2.60	723.51	−1.84

moisture content, carrot concentration, and extrusion temperature, while screw speed rise caused expansion increase. Initial moisture content, carrot concentration, and screw speed influenced extrudates’ porosity similar to expansion, while extrusion temperature caused the opposite effect. Extruded products presented higher porosity with temperature increment. These results are in accordance with the results obtained with the instrumental methods. Temperature increment favors bubble growth in the dough, resulting in the production of extrudates with higher porosity. Porosity and expansion reduction with the increment of carrot concentration is attributed to the increased fiber composition that disrupts the cell walls before the overall expansion of the air bubbles (Altan et al. 2008). Screw speed rise leads to increased starch gelatinization, creating products with increased volume and lower density (Hagenimana et al. 2006). Low initial moisture content restricts the flow of the dough in the barrel, resulting in increased shear rate and residence time, causing high starch gelatinization and increased porosity (Chinnaswamy and Hanna 1988; Jyothi et al. 2009).

Extrudates’ texture was also significantly influenced by extrusion conditions and raw materials’ characteristics. Extrudates that were produced in lower temperatures and lower screw speeds were characterized by increased hardness. Lower carrot concentration and higher moisture content also caused similar effect. In addition, raw materials characteristics negatively influenced crunchiness of extruded products, while extrusion temperature showed a positive effect. Screw speed had no significant influence on extrudates’ crunchiness. Extrudates with high initial moisture content that were produced in low process temperatures and screw speeds presented reduced porosity with limited air volume and were characterized by increased sensorial hardness (Barrett and Kaletunc 1998).

Hydration of the extruded products significantly increased with extrusion temperature, carrot concentration, and screw speed increment, while it decreased with moisture content rise. High extrusion temperatures increase the degree of starch gelatinization, promoting the hydration rate of the products (Solomon 2008).

Table 11.7 Mean values for sensory characteristics of rice extrudates

X (% w.b.)	C (%)	T (°C)	N (rpm)	Uniformity	Porosity	Expansion	Crunchiness	Hardness	Adhesiveness	Cohesiveness	Hydration
14	7.5	160	200	8.00a	5.75a,b	5.94a,d	4.75a	2.33a	5.88a	6.20a	6.60a
17	7.5	160	200	5.99b	5.86a,b	4.00b	5.65a	4.54b,e,f	5.13a	4.50b,c	4.20b
20	7.5	160	200	4.78c,d	5.10a	3.61b	3.33a	7.00c,d	6.00a	2.75b,d	2.00c
17	5	160	200	5.25b,c	6.10a,b	7.63c	5.10a	5.50b,c	4.63a,b	4.00b,c,d	3.17b,c
17	7.5	160	200	5.99b	5.86a,b	4.00b	5.65a	4.54b,e,f	5.13a	4.50b,c	4.20b
17	10	160	200	6.50b,e	4.89a,b	3.38b	4.30a	3.30a,b	3.80b,c	3.63b,d	4.33b,d
17	7.5	140	200	3.50d	5.00a	7.00a,c	3.25a	7.38d	4.58a,d	3.00b,d	3.20b,c
17	7.5	160	200	5.99b	5.86a,b	4.00b	5.65a	4.54b,e,f	5.13a	4.50b,c	4.20b
17	7.5	180	200	6.00b,e	6.00a,b	4.43b,d	5.60a	3.63a,e	5.00a,c	6.00,c	6.40a,d,e
17	7.5	160	150	7.00a,e	5.22a,b	3.86b	4.38a	5.70f,c	3.88a,c	2.50d	3.30b,c
17	7.5	160	200	5.99b	5.86a,b	4.00b	5.65a	4.54b,e,f	5.13a	4.50b,c	4.20b
17	7.5	160	250	7.25a,e	7.23b	7.50a,c	6.25a	4.67b,e,f	2.88b,c,d	4.43b,d	4.33b,e

Mean values in the same column with different letter differ significantly ($p < 0.05$)

In addition, the increased operating conditions, as well as low initial moisture content, led to products with higher porosity and more open air cells, resulting in increased hydration ability during mastication.

Extrudates' adhesiveness increased with moisture content and extrusion temperature increment. On the other hand, the increment of carrot concentration and screw speed resulted in reduced adhesiveness. Extrudates that were produced in higher screw speeds presented increased cohesiveness during mastication. Similar behavior was observed by other authors (Liu et al. 2000). Extrusion temperature did not significantly influence cohesiveness. The rise of initial moisture content caused decrement of cohesiveness, while carrot concentration had no significant influence on that attribute.

11.4 Conclusions

Extrusion operating conditions affect the properties of final products and consequently influence their acceptance by consumers. In the present study, structural, textural, and sensory properties of rice extrudates fortified with freeze dried carrot powder were correlated with process conditions and materials characteristics. The independent variables that were examined were extrusion temperature, screw speed, feed moisture content, and carrot concentration. The effect of process parameters on cellular architecture of produced extruded snacks was studied. Food texture was related to the produced snacks' structure. An increase in moisture content, carrot ratio, and temperature resulted in extrudates with lower expansion and increased hardness, while a screw speed rise increased puffing and lowered products' toughness. Sensorial characteristics were highly influenced by process parameters. Products that were produced in high temperatures and contained less initial moisture presented higher sensorial porosity and were crunchier. According to regression and sensory analysis, the most desirable extrudates, characterized by high expansion and acceptable hardness, were produced at 250 rpm, intermediate temperatures and contained 14 % initial moisture content. Knowledge on the effect of extrusion on structural, textural, and sensory properties is of utmost importance for the development of food products with superior quality, advanced characteristics, and increased acceptability by consumers.

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Chapter 12

Quality Standardization of Traditional Dry Fermented Sausages: Case of Petrovská klobása

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12.1 Introduction

The European market of dry-cured meat products is characterized by a wide range of traditional fermented sausages, which come from different parts of the continent, primarily from the Mediterranean. Production of these products has lasted for centuries and when the first sausage was produced remains uncertain, because its production dates from the period before written history. Traditional dry-fermented sausages, originating from different countries and regions, are characterized by specific sensory and physicochemical properties affected by the environmental conditions, raw materials, production method, activity and composition of present microflora, biochemical and physicochemical changes occurring during the smoking, fermentation, drying, and ripening process, and specificities of the local population (customs, habits, traditions, religion, etc.). Diversity in appearance, color, taste, smell, and texture of these products can contribute to the preservation of gastronomic heritage and culture, as well as economic development of rural areas by increasing consumption and production volume (Santos et al. 1998; Casaburi et al. 2007; Aquilanti et al. 2007; Talon et al. 2007; Latorre-Moratalla et al. 2008; Roseiro et al. 2008; Settanni and Moschetti 2014).

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The Republic of Serbia also has a long tradition of dry-fermented sausages production. Mostly, it takes place in small scale processing units and rural households, according to experience and traditional technology, during the winter period (Petrović et al. 2007, 2010, 2011; Ikonić et al. 2010, 2012).

12.2 *Petrovská klobása*: Traditional Dry-Fermented Sausage from Northern Serbia

Petrovská klobása is a traditional dry fermented sausage, which has been produced for over 250 years in the area nearby town of Bački Petrovac in the Autonomous Province of Vojvodina, Republic of Serbia, as a part of Slovaks' gastronomic heritage. In rural households, this sausage is made at the end of November and in December, when temperatures are around 0 °C or lower, according to the experience and traditional technology. It is produced exclusively from pork meat and fat, red hot paprika powder, salt, crushed garlic, caraway, and sugar, without chemical additives (nitrate/nitrite, glucono delta-lactone, etc.) or microbial starter cultures (Petrović et al. 2007, 2011; Ikonić et al. 2010).

Pork meat and fat are minced to a 10 mm particle size and all ingredients are mixed by traditional technique, until homogenous mass is obtained. Well mixed compound is stuffed into natural casing, back part of pig large intestines (colon), in 35–45 cm long units. After stuffing it undergoes smoking process for about 10 days with pauses, *using specific kinds of wood*. Afterwards it is left to dry and ripen for a period of up to 4 months, until it achieves optimum quality. At the end of ripening *Petrovská klobása* is characterized by specific savory taste, aromatic and spicy-hot flavor, dark red color and hard consistency (Petrović et al. 2007, 2010, 2011; Ikonić et al. 2010, 2013; Danilović et al. 2011; Tasic et al. 2012; Šojić et al. 2013; Škaljac et al. 2014).

12.3 Quality Parameters and Criteria Defined during the Protection of *Petrovská klobása* **Designation of Origin**

Serbia, as well as European countries, has recognized the importance of geographical indications and quality standardization of traditional food products. In our country the protection of designations of origin and geographical indications is regulated by national legislation "Official Journal of RS," No. 18/2010 and in the European Union by Council Regulation (EU) No. 1151/2012 (Table 12.1).

Production of *Petrovská klobása* in traditional conditions leads to a significant heterogeneity of final products. Aiming to establish quality standard and to protect designation of origin, the traditional process of manufacturing and gross characteristics

Table 12.1 Physicochemical and biochemical properties of traditionally produced *Petrovská klobása* by different producers (A, B, C, D, E) at the end of drying and ripening (120th day) (Ikonić et al. 2010; Petrović et al. 2011)

Property	Manufacturer				
	A	B	C	D	E
Protein content (%)	29.79±0.30 ^{ab}	29.62±0.57 ^a	23.36±0.42 ^c	27.52±0.58 ^d	30.43±0.62 ^b
Fat content (%)	41.39±1.19 ^a	41.48±0.79 ^a	46.01±0.56 ^d	37.71±0.52 ^c	34.09±1.15 ^b
Moisture content (%)	22.14±1.00 ^a	23.19±0.47 ^b	25.05±0.72 ^c	27.53±0.49 ^d	29.11±0.47 ^c
Ash content (%)	4.87±0.13 ^b	3.87±0.21 ^a	3.99±0.13 ^a	5.26±0.13 ^c	4.74±0.15 ^b
NaCl content (%)	3.01±0.10 ^a	3.28±0.14 ^a	3.01±0.29 ^a	3.15±0.14 ^a	2.99±0.20 ^a
RCCTP ¹ (%)	4.74	5.85	7.88	7.27	6.70
Weight loss (%)	45.71	40.83	44.82	42.92	40.33
pH	5.31	5.36	5.09	5.14	5.42
Lightness (L*)	31.78±3.51 ^a	34.09±1.64 ^{ab}	36.38±2.55 ^b	35.18±2.03 ^{ab}	34.37±1.76 ^{ab}
NPN (g/100 g)	0.64±0.01 ^b	0.67±0.01 ^c	0.62±0.01 ^a	0.72±0.01 ^d	0.74±0.01 ^c
NH ₂ -N (g/100 g)	0.239±0.045 ^b	0.353±0.04 ^a	0.278±0.04 ^b	0.372±0.02 ^a	0.362±0.00 ^a
%NPN in TN ²	13.43	14.14	16.59	16.35	15.20
Sensory quality	3.94	4.18	4.27	4.64	4.74
Firmness (N)	14.48±1.04 ^a	10.38±2.01 ^c	6.18±0.48 ^b	13.04±1.16 ^a	13.53±0.82 ^a

¹Relative content of connective tissue protein²Share of non-protein nitrogen (NPN) in the total nitrogen (TN)^{a-c}Means within the same row with different superscript letters are different ($p < 0.05$)

of *Petrovská klobása* have been investigated during three production seasons in five rural households (producers A, B, C, D, E).

Some physicochemical and sensory properties of sausages, as well as climate parameters were registered in production season when most of products achieved high overall quality. Based on the properties of the best rated sample sausages the parameters of optimal quality for this traditional dry-fermented sausage were defined (Petrović et al. 2007).

Compared to other dry-fermented sausages, *Petrovská klobása* is characterized by high protein content, usual moisture, fat and mineral substances contents, and lower NaCl content. It is also characterized by a high weight loss, and pH which corresponds to the published values for this parameter for other traditional fermented sausages. During ripening, *Petrovská klobása* undergoes significant proteolytic changes that have positive effects on sensory properties, primarily due to the

formation of characteristic taste and odor (Casiraghi et al. 1996; Lorenzo et al. 2000; Comi et al. 2005; Salgado et al. 2006; Spaziani et al. 2009).

Quality of sausage produced by manufacturer E during third season was recognized as quality of the typical traditional sausage, and adopted as standard which is protected. Defined quality parameters are: pH_k >5.4; moisture content <35 %; meat protein content >25 %; free fat content <35 %; relative content of connective tissue protein (RCCTP) <15 %; NaCl content <3.5 %; share of non-protein nitrogen in total nitrogen (NPN in TN) >15 %; lightness (L^*)=32–37; firmness=10–15 N; sensory quality >4.5 points.

12.4 Quality and Safety Standardization of *Petrovská klobása*

In the first step, quality parameters were defined based on the comparison of quality characteristics of sausages produced by five chosen manufacturers during three seasons. After the quality parameters were defined, second step was to define optimal production model in traditional conditions by analyzing complex interactions of various factors, and at the same time to start standardization of quality and safety during processing in controlled conditions. For that purpose 13 experimental sausage groups (models) during three seasons have been made according to traditional (protected) recipe, but with investigation of different variable factors: *post mortem* time of deboning, manual and mechanical mixing, type of casing, type of smoking and drying, duration of drying and ripening, addition of a commercial starter culture, type of packaging (vacuum and modified atmosphere (30 % CO₂ and 70 % N₂) packaging, as well as coated with chitozan film) (Table 12.2).

During quality and safety standardization, smoking, drying, and ripening in traditional and industrial conditions were investigated. Traditionally *Petrovská klobása* sausages are smoked using cool procedure for 10 days with pauses, while sausages processed in industrial conditions were smoked for 6 h (12×30 min), during 3 days. The ambient conditions in traditional room, which are highly dependent on outdoor climate conditions, were followed up regularly during the drying and ripening process (averagely around 8 °C and 80 % RH). Thermo-hygrometric conditions in the industrial room (10 °C and 75 % RH) were set to imitate conditions present in traditional practice (low temperature) and, at the same time, to enable faster drying, in order to shorten the production period of this sausage (Ikončić et al. 2012, 2013).

According to Serbian legislation (Serbian Regulations 2012) moisture content for dry fermented sausages has to be less than 35.0 %. Sausages dried in traditional room needed 90 days to reach required moisture content, while sausages dried in industrial ripening chamber reached this value after 45 or 60 days.

Table 12.2 Different factors examined in 13 experimental sausage groups (models)

Group	Time of deboning	Casing	Processing conditions	End of drying (day)
A1	Hot deboned meat (2 h <i>post mortem</i>)	Natural	Traditional room	90
A2		Collagen		
B1	Cold meat (24 h <i>post mortem</i>)	Natural	Industrial ripening chamber	45
B2		Collagen		
B3		Natural		
B4		Collagen		
Way of mixing				
C1	Manual	Natural	Industrial ripening chamber	60
C2		Collagen		
C3	Mechanical			
Commercial starter culture addition				
D1	Yes	Collagen	Traditional room	90
D2	No			
E1	Yes		Industrial ripening chamber	60
E2	No			

12.5 Optimal Fermentation, Drying, and Ripening Model for *Petrovská klobása* Production

Production of fermented sausages is a complex process which depends on a wide range of factors and despite the exceptional skills and experience of *Petrovská klobása* manufacturers, variations in product quality are possible. Based on the investigations on basic raw materials, production methods and overall quality of sausages, the optimal model of production in traditional conditions had been found. By using this model manufacturers are able to modify the production process properly in order to get high quality product in traditional and controlled conditions.

At the end of drying, as well as at the end of ripening period (120th day), both non-packaged and vacuum packaged sausages from B1 and B2 groups had the best sensory quality according to level requested by criteria in Code of Practice (Petrović et al. 2007). Based on total sensory quality analysis, as well as on analysis of numerous other physical-chemical and biochemical properties, the fermentation, drying, and ripening model used in two experimental groups is defined as optimal. None of the other tested models showed required quality of sausages at the end of the drying, ripening, and storage.

To achieve the optimal quality of *Petrovská klobása* it is essential to have a raw mixture with pH value lower than 5.8 (Petrović et al. 2010, 2011). After that, pH drops constantly until 60th day of production, when it reaches its minimum, being around 5.3. Faster decrease of pH and lower final value, results in some of the technological defects. After that period, pH should increase gradually, reaching, after

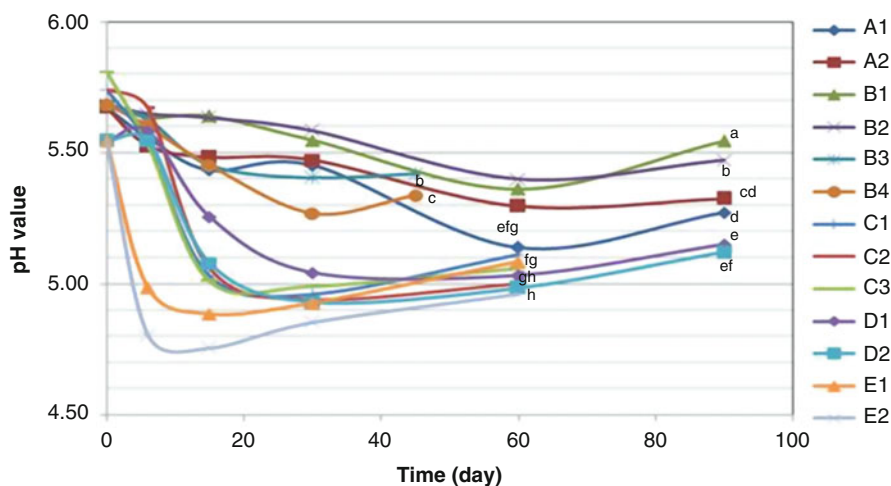


Fig. 12.1 Changes of pH value in 13 experimental sausage groups (models) produced in three seasons

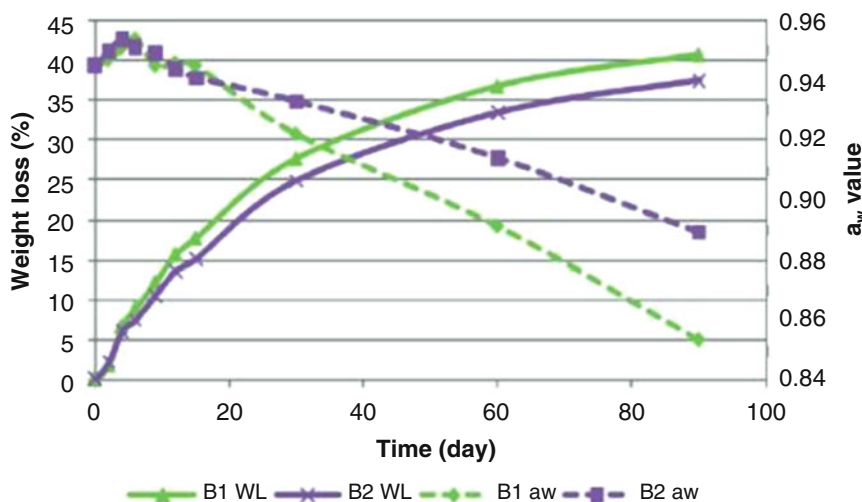


Fig. 12.2 Changes of weight loss and a_w value of *Petrovská klobása* in natural (B1) and collagen casing (B2) according to optimal model of fermentation, drying, and ripening (Petrović et al. 2011)

120 days, at the end of ripening, values higher than 5.4 (Ikonić et al. 2010; Petrović et al. 2011) (Fig. 12.1).

Optimal model of fermentation, drying, and ripening (Fig. 12.2) should result in approximately 28 % of weight loss until 30th day of production, 37 % until 60th day and 40 % after 90 days of production. Such drying behavior can be described adequately by Page's regression model (Ikonić et al. 2010). Water activity change

occurs following the dynamics: from 0.95 (raw mixture) to 0.93 during first 30 days (artificial casing), from 0.930 to 0.915 in next 30 days and at the end of drying process a_w should be lower than 0.89.

12.6 Selection of Pig Breed and Quality

Traditionally *Petrovská klobása* was produced from white breed pigs and their hybrids (150–180 kg, about 9 months old). Since the quality of meat originating from these pigs is very poor and it does not meet the requirements set in a code of practice (Petrović et al. 2007), it was concluded that pigs for *Petrovská klobása* production have to be exclusively Landrace breed and meat quality has to be “normal” (Petrović et al. 2009, 2011; Džinić et al. 2009; Tomović et al. 2011, 2014).

12.7 Determination of Traditional *Petrovská klobása* Microbial Profile

The knowledge of microflora present in *Petrovská klobása* may help in understanding a specific characteristic of the product and the selection of appropriate starter cultures for the industrial production of this product (Fig. 12.3).

As lactic acid bacteria (LAB) play a crucial role in the fermentation of sausages the analysis of LAB population in *Petrovská klobása* was done. A total of 404 LAB

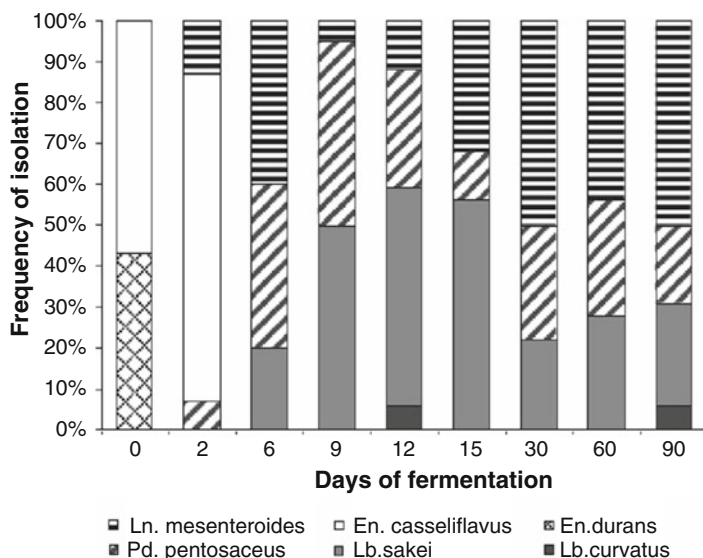


Fig. 12.3 Changes of microbial population during the fermentation of *Petrovská klobása* (Danilović et al. 2011)

strains were isolated during the fermentation of the sausages. The isolates were subjected to (GTG)5-PCR fingerprinting and the identification of representatives was performed by 16S rDNA sequencing. Obtained results showed that microbial population consists of four genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. The results showed that among the isolates, *Lactobacillus sakei* and *Leuconostoc mesenteroides* predominate with 36.4 % and 37.1 % of total LAB strains, respectively. *Pediococcus pentosaceus* was also isolated in high proportion (18.4 %) whereas *Enterococcus durans* and *Enterococcus caseliflavus* made only 1 % and 6 % of the total isolates, correspondingly. Further research should be done in order to determine technological properties of the strains so they can be used as starter cultures for the industrial production of *Petrovská klobása*.

After the identification of endogenous microflora profile throughout the first production season, in the third production season commercial starter culture, most similar to identified microflora (*Staphylococcus carnosus* 25 %, *Staphylococcus xylosum* 25 %, *Lactobacillus sakei* 25 %, and *Pediococcus pentosaceus* 25 %) were added.

The analysis of vacuum packed and modified atmosphere packed (MAP) samples showed higher presence of *L. mesenteroides* and *L. sakei* in the total microflora (Danilović et al. 2011).

12.8 Determination of *Petrovská klobása* Safety

In order to ensure the safety of sausages produced in traditional conditions, monitoring of microbiological (total count of aerobic mesophil, aerobic spore bacteria, *Micrococaceae*, *Enterobacteriaceae* and *E. coli*, *Streptococcus* spp., *Staphylococcus aureus*, *Pseudomonas* spp., UB *Clostridium* spp., *Salmonella*, *Listeria monocytogenes*, *Proteus* spp.) and physical-chemical parameters (presence of veterinary drugs, hormones, β -agonists, animal peptides, polycyclic aromatic hydrocarbons, products of lipid peroxidation-MDA values) were carried out according to current legislation. Based on the obtained results it can be concluded that sausages produced during research within mentioned project, over three seasons, were safe (Petrović et al. 2011).

Content of 13 polycyclic aromatic hydrocarbons (acenaphthylene, fluorene, phenanthrene, anthracene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, and benzo[ghi]perylene) from Environmental Protection Agency list (US-EPA PAHs) in traditional dry fermented sausage *Petrovská klobása* were determined (Table 12.3).

Acenaphthylene, fluorene, phenanthrene, anthracene, and pyrene were determined in all analyzed sausage groups at the end of drying and at the end of storage period, while other examined PAHs from EPA priority list were below the limit of detection.

Table 12.3 Content of polycyclic aromatic hydrocarbons ($\mu\text{g/kg}$) in dry fermented sausage *Petrovská klobása* at the end of drying and storage period (Škaljac et al. 2014)

Polycyclic aromatic hydrocarbons ($\mu\text{g/kg}$)		0 day of production	End of drying period			End of storage period		
		0	A1	A2	B1	B2	B3	B4
Acenaphthylene	Acy	nd	36.7 ^a ±3.00	38.5 ^b ±3.50	45.0 ^c ±4.00	27.9 ^d ±0.40	18.3 ^e ±0.20	7.80 ^b ±0.90
	Flu	1.70±0.10	26.7 ^e ±0.60	28.5 ^f ±1.50	25.0 ^d ±1.00	13.7 ^e ±0.40	7.90 ^b ±0.65	4.80 ^a ±0.05
Phenanthrene	Phe	3.15±0.25	120 ^a ±2.00	119 ^a ±10.5	40.5 ^b ±0.50	37.6 ^b ±4.00	11.3 ^a ±1.55	13.1 ^a ±0.55
	Ant	4.15±0.45	21.0 ^d ±0.90	16.5 ^c ±1.50	7.30 ^b ±0.25	7.50 ^b ±0.15	2.00 ^a ±0.10	2.00 ^a ±0.20
Pyrene	Pyr	nd	15.5 ^c ±0.20	11.8 ^d ±1.05	7.20 ^a ±0.65	7.20 ^a ±0.05	4.70 ^a ±0.60	3.60 ^b ±0.35
Benz[<i>a</i>]anthracene	BaA	nd	nd	nd	nd	nd	nd	nd
	CHR	nd	nd	nd	nd	nd	nd	nd
Benzo[<i>b</i>]fluoranthene	BbF	nd	nd	nd	nd	nd	nd	nd
	BkF	nd	nd	nd	nd	nd	nd	nd
Benzo[<i>k</i>]fluoranthene	BaP	nd	nd	nd	nd	nd	nd	nd
	IcP	nd	nd	nd	nd	nd	nd	nd
Indeno[1,2,3- <i>cd</i>]pyrene	DhA	nd	nd	nd	nd	nd	nd	nd
	BgP	nd	nd	nd	nd	nd	nd	nd
Σ EU PAH ^g		nd	nd	nd	nd	nd	nd	nd
	Σ EU PAH ^h	nd	nd	nd	nd	nd	nd	nd
Σ 6 IARC PAH ⁱ		nd	nd	nd	nd	nd	nd	nd
	Σ 7 US-EPA PAH ^j	nd	nd	nd	nd	nd	nd	nd
Σ 13 US-EPA PAHs		nd	nd	nd	nd	nd	nd	nd
		9.00±0.80	220 ^a ±6.30	214 ^a ±8.95	125 ^c ±6.40	93.9 ^d ±4.60	44.2 ^e ±3.10	31.3 ^b ±0.55

In the same row, different superscript letters (a, b, c, d, e, f) means that values are significantly different ($P < 0.05$); *nd* not detected

Results are expressed as means ± standard deviations

^gBaA, CHR, BbF, BkF, BaP, IcP, DhA, BgP

^hBaA, CHR, BbF, BaP

ⁱBaA, BbF, BkF, BaP, IcP and DhA

^jBaA, CHR, BbF, BkF, BaP, IcP, DhA

(continued)

Table 12.3 (continued)

Polycyclic aromatic hydrocarbons ($\mu\text{g/kg}$)	0 day of production	End of drying period				B2	B3	B4
	0	A1	A2	B1	B2			
Acenaphthylene	nd	142 ^c ±9.00	43.2 ^d ±4.25	31.6 ^a ±1.70	22.3 ^c ±1.50	31.5 ^a ±3.55		10.1 ^b ±0.82
Fluorene	1.70±0.10	102 ^c ±8.00	28.1 ^b ±2.25	23.5 ^b ±2.10	9.90 ^a ±0.00	10.4 ^a ±0.45		5.60 ^a ±0.53
Phenanthrene	3.15±0.25	201 ^c ±6.50	107 ^d ±10.0	30.5 ^a ±2.40	49.4 ^c ±0.70	38.7 ^a ±2.05		18.3 ^b ±1.27
Anthracene	4.15±0.45	40.0 ^a ±4.00	46.4 ^c ±4.55	34.4 ^a ±3.55	34.9 ^a ±3.70	23.0 ^b ±2.50		20.1 ^b ±1.63
Pyrene	nd	10.4 ^a ±1.15	16.5 ^d ±0.40	10.4 ^a ±0.30	12.8 ^c ±1.05	10.7 ^a ±0.55		nd ^b
Benz[<i>a</i>]anthracene	BaA	nd	nd	nd	nd	nd		nd
Chrysene	CHR	nd	nd	nd	nd	nd		nd
Benzo[<i>b</i>]fluoranthene	BbF	nd	nd	nd	nd	nd		nd
Benzo[<i>k</i>]fluoranthene	BkF	nd	nd	nd	nd	nd		nd
Benzo[<i>a</i>]pyrene	BaP	nd	nd	nd	nd	nd		nd
Indeno[1,2,3- <i>cd</i>]pyrene	IcP	nd	nd	nd	nd	nd		nd
Dibenz[<i>a,h</i>]anthracene	DhA	nd	nd	nd	nd	nd		nd
Benzo[<i>ghi</i>]perylene	BgP	nd	nd	nd	nd	nd		nd
Σ EU PAH ^g	nd	nd	nd	nd	nd	nd		nd
Σ EU PAH ^g	nd	nd	nd	nd	nd	nd		nd
Σ 6 IARC PAH ^h	nd	nd	nd	nd	nd	nd		nd
Σ 7 US-EPA PAH ⁱ	nd	nd	nd	nd	nd	nd		nd
Σ 13 US-EPA PAHs	9.00±0.80	495 ^c ±7.65	241 ^d ±7.10	130 ^a ±9.45	129 ^a ±3.45	114 ^c ±3.20		54.1 ^b ±4.25

In the same row, different superscript letters (a, b, c, d) means that values are significantly different ($P<0.05$); nd not detected

Results are expressed as means±standard deviations

^aBaA, CHR, BbF, BkF, BaP, IcP, DhA, BgP

^gBaA, CHR, BbF, BaP

^hBaA, BbF, BkF, BaP, IcP and DhA

ⁱBaA, CHR, BbF, BkF, BaP, IcP, DhA

Table 12.4 Biogenic amines content in traditional dry fermented sausage *Petrovská klobása* as possible indicator of good manufacturing practice (Tasic et al. 2012)

Source and criterion for good manufacturing practices evaluation (mg/kg)		Batch					
		A1	A2	B1	B2	B3	B4
Santos (1996) total	<1000	113	164	174	163	77.8	86.5
Shalaby (1996)							
Tyramine	100–800	ND	17.3	6.90	16.8	7.34	14.8
Histamine	50–100	ND	ND	ND	ND	ND	ND
Phenylethylamine	<30.0	51.6	33.2	28.6	29.4	ND	11.8
Eerola et al. (1998) sum of vasoactive	<200	66.4	103	73.6	121	36.1	52.8

Contents of BaP and PAH4 were below the limit of detection in all analyzed samples of *Petrovská klobása*, meeting the requirements of the European regulation regarding PAHs content. PAH8, 6 IARC PAH, and 7 US-EPA PAH were not detected in any analyzed sample.

According to the results obtained in this study, traditional dry fermented sausage *Petrovská klobása*, smoked in traditional and industrial conditions was safe for its consumers regarding European regulation on PAHs content (Škaljac et al. 2014).

Content of nine biogenic amines (tryptamine, phenylethylamine, putrescine, cadaverine, histamine, serotonin, tyramine, spermidine, and spermine) and total biogenic amines in traditional dry fermented sausage *Petrovská klobása* at the end of drying period, as possible indicators of good manufacturing practice (GMP) were determined.

Limit of 1000 mg/kg considered by Santos (1996) was not exceeded in any analyzed sample. Shalaby (1996) suggested levels of few amines as parameters for the evaluation of GMPs: tyramine 100–800 mg/kg, histamine 50.0–100 mg/kg, phenylethylamine <30 mg/kg. Proposed values of tyramine and histamine were not exceeded in any analyzed sample, while content of phenylethylamine was higher in sausages manufactured from hot deboned meat, with values of 51.6 and 33.2 mg/kg, respectively. The limit of 200 mg/kg for the sum of vasoactive biogenic amines proposed by Eerola et al. (1998) as an indicative criteria for good hygienic conditions and GMP was not exceeded in any sample at the end of drying (Table 12.4).

Influence of drying and ripening in traditional and industrial conditions on *Petrovská klobása* fatty-acid composition and oxidative changes in lipids, during 7 months of storage, was investigated (Table 12.5).

Conditions of slower drying and ripening at lower temperatures result in less lipid oxidative changes in traditional dry fermented sausage *Petrovská klobása* during prolonged storage period (7 months) (Šojić et al. 2013).

The effect of a chitosan coating on lipid oxidation was investigated during 5 months of storage. Chitosane caraway coated sausage showed lower intensity of lipid oxidative changes at the end of 5 months storage in comparison to control (Krkić et al. 2013).

Table 12.5 Lipid oxidation parameters in traditional dry fermented sausage *Petrovská klobása* during storage (Šojić et al. 2013)

Lipid oxidation parameters	Sausage	End of drying	Storage	
			2 months	7 months
TBARS (mg malondialdehyde/kg)	B2	0.79 ± 0.02 ^{ya}	0.36 ± 0.01 ^{yb}	0.16 ± 0.02 ^{yc}
	C2	1.25 ± 0.00 ^{xb}	1.63 ± 0.02 ^{xa}	0.93 ± 0.00 ^{xc}
Aldehydes content (µg/g)				
Propanal	B2	1.86 ± 0.53 ^b	0.94 ± 0.01 ^{yb}	32.59 ± 1.57 ^a
	C2	2.68 ± 0.26 ^b	4.80 ± 0.10 ^{xb}	44.32 ± 10.67 ^a
Pentanal	B2	1.16 ± 0.38 ^c	2.75 ± 0.12 ^{xb}	4.03 ± 0.25 ^{ya}
	C2	1.61 ± 0.08 ^b	0.89 ± 0.06 ^{yb}	9.52 ± 2.40 ^{xa}
Hexanal	B2	0.12 ± 0.08 ^b	0.05 ± 0.00 ^{yb}	1.67 ± 0.07 ^{ya}
	C2	0.05 ± 0.00 ^b	0.22 ± 0.01 ^{xb}	4.94 ± 1.29 ^{xa}
Heptanal	B2	0.24 ± 0.07 ^c	1.65 ± 0.09 ^{xa}	0.52 ± 0.02 ^b
	C2	0.30 ± 0.12 ^c	1.40 ± 0.05 ^{ya}	0.86 ± 0.21 ^b
Octanal	B2	3.24 ± 1.26 ^{xa}	0.22 ± 0.03 ^{yb}	0.35 ± 0.00 ^b
	C2	0.87 ± 0.11 ^{ya}	0.50 ± 0.03 ^{xb}	0.61 ± 0.17 ^b

^{xy}The values of the same column significantly differ with 95 % probability ($P < 0.05$)

^{abc}The values of the same row significantly differ with 95 % probability ($P < 0.05$)

Obtained results of microbial profile determination, fermentation, drying, and ripening processes and consequently proteolytic and lipolytic changes, sensory properties, as well as safety parameters indicates that in next experimental groups (models), outside of usual production period, isolated autochthonous microflora as starter culture consistent of 90 % of *Lactobacillus sakei* and 10 % of *Staphylococcus* spp., will be applied.

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Part III

Food Biotechnology

Chapter 13

Traditional and Emerging Technologies for Autochthonous Lactic Acid Bacteria Application

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13.1 Traditional Cheeses: Source of Autochthonous Lactic Acid Bacteria

White brined cheeses are the most widely produced and consumed traditional cheeses in Serbia (about 60 % of total cheese consumption). There are many different types of Serbian brined cheeses named according to their production regions as follows: Sjenica cheese, Zlatar cheese, Svrlijig cheese, Homolj cheese, etc.

Traditionally, white brined cheeses have been made from unpasteurized or medium heat-treated milk in small dairy plants and households without using starter cultures. This production is poorly organized and done in conditions which may involve substantial safety risks. Dominant microbiota constitutes of different lactic acid bacteria (LAB) strains which mainly originate from raw milk and the environment of the region where the cheese production takes place. The composition and metabolic activity of cheese autochthonous microbiota significantly affect the quality and flavor characteristics of the final product. Autochthonous LAB as very important agents of cheese ripening contribute to the maturation process either directly through their metabolic activity or indirectly through the release of enzymes into the cheese matrix through the lysis of bacterial cells. Their growth and activity are completely uncontrolled and unpredictable, resulting in less uniform sensory characteristics and composition than in cheeses made from pasteurized milk with starter cultures' addition (Karakuş and Alperden 1995). On the other side, the maturation process of cheeses manufactured in the traditional way is faster contributing

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in formation of specific and unique flavor (McSweeney et al. 1993; Grappin and Beuvier 1997).

Traditionally made cheeses represent a great natural source for the isolation of different strains of LAB with potential application in two directions: a) as starter cultures for standardization of procedures for the safety production and quality of autochthonous cheeses and b) as potential beneficial strains for production of functional “healthy” foods.

- (a) Autochthonous LAB represent a significant pool of different species of LAB. Isolation, selection, and better recognition of these microorganisms enable obtaining authentic starter cultures for standardization of white brined cheese production. Radulović et al. (2010) presented that among the all isolates, the most frequent species, subspecies or biovars in the traditional Sjenica cheese were *L. lactis* spp. *lactis* (35.85 %), *Lb. paracasei* (18.86 %), *Lb. plantarum* (16.99 %), *L. lactis* spp. *lactis* biovar. *diacetylactis* (7.55 %), *Leuconostoc* spp. (3.77 %), *Lb. curvatus* (1.89 %), *Lb. brevis* (1.89 %), and *Enterococcus* sp. (13.20 %). This diversity of different species offers a great potential for their combining and application as starter cultures in standardized cheese production (Radulović et al. 2011a). Improving milk quality and hygienic conditions, as well as using autochthonous starter cultures, represent an important step in establishing a standard production procedure which would contribute to obtaining a higher and more uniform cheese quality. Implementation of selected LAB isolated from natural microbiota of traditional cheeses, is necessary in order to avoid the loss of desirable characteristics of traditional products and to fulfill safety requirements. In addition, achievement of all the international requirements relating to quality and safety of Serbian dairy products would certainly provide an opportunity to their better position in domestic and international markets.
- (b) On the other hand, in the last decades, there is a significant interest in high quality foods with potential beneficial effects on the consumer’s health. Also cheese plays an important role in healthy nutrition and could be a suitable vehicle for the probiotic strains. Investigation of cheeses with additional health benefits represents a great opportunity for development of various types of cheeses belonging to functional foods group.

Incorporation of probiotic cultures in cheeses provides potential not only to improve the health status and quality of products, but also to increase the range of probiotic products. Selection of new autochthonous potential probiotic strains offers a great opportunity for development of cheeses with different desirable characteristics, considering that applications of probiotic strains could positively influence the flavor of cheese, especially in the production of low-fat and low-salt cheeses, goat cheeses, etc.

13.2 Probiotic Bacteria Properties

Development of new analytical techniques and the extensive clinical studies aimed to verify the positive impact on human health have led to an increased usage of probiotic cultures in various food products. The probiotic strains of lactobacilli and

bifidobacteria can be isolated from the human gastrointestinal tract (GIT) (Dimitrijevic et al. 2008), and are applied for food fortification or dietary supplement production intended to improve human health. However, in the past decades, it has been revealed that many *Lactobacillus* strains isolated from food sources also manifest probiotic properties. For example, a higher tolerance to high bile salt concentration was reported for autochthonous strains isolated from various fermented foods in comparison to some human derived strains (Petrović et al. 2012). The main sources of probiotic lactobacilli other than GIT are various traditional fermented products (Petrovic et al. 2012).

However, the criteria for their selection still remain within the framework that have been set up by Salminen et al. (1998) and by Charteris et al. (1998). According to them, there are three groups of properties that should be considered in probiotics' selection:

1. General aspects that include source of probiotic as well as their Generally Recognized as Safe (GRAS) status
2. Technological aspects that include strain stability and activity in products as well as survival ability in GIT conditions
3. Functional aspects that include in vitro and in vivo testing of their health effect

As it has already been mentioned, that various traditional fermented foods are good sources of probiotics (Radulović et al. 2010; Hashemi et al. 2014). Such probiotics can have a particular advantage compared to the human strains in relation to their diversity and easier way of isolation. Since traditional fermented foods are used for centuries in the human diet, the microorganisms isolated from such sources can be considered as safe for use. Moreover, such isolates are usually more sensitive to antibiotic drugs (Petrovic et al. 2012; Borriello et al. 2003; Radulović et al. 2012a). However, a potential disadvantage of these strains may be weak or lack of functionality in the human GIT. It is therefore very important to determine their ability to survive in the presence of bile salts and digestive enzymes and at low pH. On the other hand, given that they have been passed certain technological processes, it is possible that they need less adjustment to stressful conditions during food production (Hussain et al. 2013).

It is very important for the probiotic strains to retain their characteristics and viability during the production processes and storage in different conditions. The food matrix has a great impact on the viability and shelf life of probiotics (Forssten et al. 2011). Therefore, it is necessary to examine the conditions of survival of probiotics in a specific type of food. Stress conditions in cheese production, such as heat, the stationary growth phase, and starvation can significantly affect the viability and expression of physiological characteristics of probiotics in food. Generally, lactobacilli showed better stress adaptability over bifidobacteria (Hussain et al. 2013).

The viability of probiotics depends on the numerous factors as follows: type and characteristics of bacteria, food composition, temperature, pH values, salt and fat content, as well as presence of other bacteria (e.g., LAB used in cheese production as primary starter culture). The rate of tolerance to different environmental factors such as food and/or hostile conditions of the GIT, after consumption, varies among different strains of the same species and from species to species. Therefore, the

proper selection of probiotic strains highly depends on the product characteristics. Also, probiotics' stability and their viability may be enhanced in several ways as follows: optimization of growth medium, protective materials used, encapsulation techniques, addition of prebiotics, etc. (Vasiljevic and Shah 2008; Mattila-Sandholm et al. 2002).

Concerning the growing consumer demands for healthier, but still sensory acceptable foods, the impact of probiotics on the sensory properties of foods may be an important selection criterion. Recently, several investigations confirmed the ability of lactobacilli to detoxify some environmental contamination of food and thus reduce the risk of exposure on human health. For example, *Lb. plantarum* F14 completely inhibited the toxic protein hemolysin secretion in *B. cereus* found in meat (Reda 2014). Some strains of *Lb. plantarum* reduced the level of pirimiphos-methyl pesticide during wheat fermentation (Đorđević et al. 2013). Monachese et al. (2012) explained the role of probiotic on heavy metals reduction in food by probiotic bacteria. These capabilities can also be considered as potential criteria for selection of probiotics.

A number of health effects are associated with the use of probiotics. The beneficial effects of probiotics on disorders associated with GIT, cardiovascular diseases, and mucosal immunity are documented through some clinical outcomes (FAO/WHO 2006). Recently, some other health effects of probiotics have been investigated, including influence on diabetes and obesity, oral diseases as well as on mental health (Pande et al. 2012). It is believed that mode of action of probiotics includes gut pH modification, production of antimicrobial compounds, competition for available nutrients and growth factors with pathogenic bacteria, immunomodulatory cell stimulation, and lactase production (Plessas et al. 2012).

Although the human trials in testing of probiotics is strongly recommended (FAO/WHO 2006), the clinical studies are still limited on a small number of strains such as *Lactobacillus rhamnosus* LGG, *Lactobacillus plantarum* 299v, etc. Therefore, in vitro and in vivo animal model testing remains the most frequently applied approach to determine the potential health benefits of newly isolated probiotics (Ahrné et al. 2005). These include investigations of the ability of intestinal pathogen inhibition by plate techniques or animal models. Recently a small soil nematode that feeds on bacteria, *Caenorhabditis elegans* has been introduced as an experimental model system for studying bacterial infection and/or longevity extension by probiotics (Zhou et al. 2014; Lee et al. 2011).

Interactions of probiotic strains with human intestinal mucosa including adhesion properties, the competition against enteropathogens, and the modulation of IL-8 production, are often studied on intestinal epithelial cell models (Caco-2, HT-29, etc.) (Giraffa 2012). The immunomodulatory properties of probiotics can be measured by determination of immune cell proliferation or other specific immune responses such as IFN- γ secretion (Lee et al. 2011).

Despite a significant body of research on the beneficial effects of probiotics, to date, not a single probiotic health claim application has received a positive ruling from EFSA, except for standard yogurt and lactose maldigestion (Makinen et al. 2012). According to the recommendation of FAO/WHO (2006), it is necessary to establish the regulatory framework to allow specific health claims on probiotic food labels, in cases where scientific evidence exists. The further development of methods (in vitro and in vivo) to evaluate the functionality and safety of probiotics is also needed.

13.3 Selection of Autochthonous Potential Probiotic Bacteria

Considering the fast growing interest for application of probiotic strains in food production, the isolation of strains with potential probiotic ability, from the autochthonous microbiota of traditional products, has become an interesting challenge. Nowadays the criteria for selection of LAB strains from traditional products are extended and include besides technological and biochemical criteria, also the probiotic ability.

Radulović et al. (2010) isolated, identified, and characterized new strains with high potential for applications as probiotic cultures in foods and healthy products, from the microbiota of local high quality traditional products. New probiotic bacterial strains of *Lactobacillus* exhibiting antimicrobial properties were obtained after screening of isolates from traditional fermented products and local ecosystems in Serbia were targeted and screened. The tested autochthonous strains of *Lb. paracasei* demonstrated a good ability to grow in a wide range of temperature (15–45 °C) and high salt concentrations (4 and 6.5 % NaCl), which is important for their survival during technological processes during food production. Three tested *Lactobacillus paracasei* strains (08, 564, and 05) showed in vitro potential probiotic ability, comparable with the reference strain *Lactobacillus rhamnosus* GG (Table 13.1).

Klaenhammer and Kullen (1999) reported that probiotic bacteria vary considerably in their level of bile tolerance. The mechanism of tolerance, however, has not been well understood and the minimum acceptable level of bile tolerance for a candidate probiotic remains unknown.

Further, Radulović et al. (2010) demonstrated very good inhibition of pathogen microorganisms by autochthonous lactobacilli strains (Table 13.2).

13.4 Encapsulation Techniques for Protection of Probiotic Bacteria

In the food industry, probiotics are used in the production of fermented dairy products, mainly yogurt, cream cheese, semi-hard and hard cheeses, ice cream and frozen fermented dairy desserts (Ranadheera et al. 2010). Good viability and activity

Table 13.1 Survival of lactic acid bacteria, isolated from Sjenica cheeses, in simulated gastric and intestinal conditions (Radulović et al. 2010)

Strains	Initial number (CFU/mL)	Pepsin, pH 2.5		Pancreatin + bile salts	
		Number after exposure (CFU/mL)	Survival (%)	Number after exposure (CFU/mL)	Survival (%)
<i>Lb. paracasei</i> 08	6.03×10^8	5.69×10^8	94.36	4.85×10^8	80.43
<i>Lb. paracasei</i> 564	4.10×10^8	4.10×10^8	100	4.05×10^8	98.78
<i>Lb. paracasei</i> 05	2.50×10^8	2.30×10^8	92.00	2.00×10^8	80.00
<i>Lb. paracasei</i> 02	7.80×10^8	4.48×10^8	57.43	2.56×10^8	32.82
<i>Lb. rhamnosus</i> GG	1.50×10^9	1.50×10^9	100	6.90×10^8	46.00

Table 13.2 Antimicrobial activity of isolated lactic acid bacteria strains (Radulović et al. 2010)

Strains	Diameter of inhibition halo ^a (mm) against seven indicator pathogenic strains						
	<i>Listeria monocytog.</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Staph. aureus</i>	<i>Candida albicans</i>	<i>E. coli</i>	<i>Salmonellae enteritidis</i>
<i>Lb. paracasei</i> 08	21	15	17	24	17	25	26
<i>Lb. paracasei</i> 564	23	16	16	24	16	25	26
<i>Lb. paracasei</i> 05	21	16	17	22	16	24	25
<i>Lb. paracasei</i> 02	11	0	0	7	13	0	0
<i>Lb. rhamnosus</i> GG	23	NI	NI	25	12	26	27

^aDiameter of inhibition halo includes 5 mm diameter of culture spot; *NI* not investigated

of probiotic cultures is considered essential for their application, as well as their ability to survive and proliferate under very specific conditions in the GIT of the host (Gilliland 1989).

Therefore, different approaches for the protection of probiotics have been developed, such as the screening of strains for resistance to acidic conditions, the control of the acidification of dairy products, and the addition of cysteine or ascorbic acid. In addition, different encapsulation techniques of probiotics have been employed in order to enhance their viability and target delivery in the GIT (Petrović et al. 2007). Additional benefit for application of microencapsulated probiotics is their easier incorporation into the food products and higher viability and stability during storage.

Encapsulation process is defined as a “package” of sensitive material in a special semi-permeable polymer membrane, wherein the milli-, micro-, or nano-scaled particles are formed, which may release its content under controlled and specified conditions (Nedović et al. 2013). The capsules may vary in size from submicron to several millimeters and can have different shapes, which depend on the carrier material used as well as on the applied methods of their production. However, for food applications the size of the beads should be below 100 µm to prevent the negative influence of capsules on the sensorial properties of food products. The encapsulation techniques provide the higher viability of encapsulated probiotics during the product shelf life as well as target delivery in the GIT of the host. This method can also prevent the proliferation of probiotics in the food when this would negatively influence the sensory properties. Another requirement which have to be considered is that the carriers used for the production of protective shell must be of food-grade, biodegradable, and able to create a barrier between the active substances and the environment.

The active substances are released from the microcapsules by the action of elevated temperature or changing the pH, by dissolving, by osmotic shock, or by the action of elevated pressure (Zuidam and Nedović 2010).

In order to select the suitable techniques for probiotics' encapsulation, we should consider their size (generally, between 1 and 5 μm in diameter) which eliminate the possibility of using nanotechnologies and the fact that they must retain the viability to ensure the health benefits for consumers.

The most commonly used methods for the encapsulation of probiotics are based on the entrapment of probiotics in a polymer gel matrix by formation of capsules, using the emulsion or extrusion techniques. The most commonly used carriers for the encapsulation of probiotics by these techniques are: kappa-Carrageenan, gellan, alginate, chitosan, Carrageenan, starch, pectin, xanthan, and gelatin (Nedović et al. 2013).

Extrusion is a simple and inexpensive technique that uses a mild operation that has no harmful effect on probiotic cells and thus maintain high probiotic viability. The emulsion can be easily scaled up and the diameter of produced beads is in the range of 25 μm to 2 mm, but the main disadvantage of this method is the large difference in the beads size and shape (Krasaekoopt et al. 2003). However, residual oil in capsules might not be suitable for applications in low-fat functional foods (Burgain et al. 2011).

Nowadays, the equipment available for the encapsulation by extrusion and emulsion techniques enables the production of a large amount of micro- and nano-capsules. However, the introduction of spray-drying and spray-coating techniques has resulted in more effective production of the capsule particles which can be easily applied on an industrial scale.

The encapsulation by freeze-drying is performed by freezing probiotics in the presence of carrier material at low temperatures, followed by sublimation of the water under vacuum (Chávarri et al. 2012). Although freeze-drying is the conventional drying technique used commercially by the starter culture manufactures, its major disadvantages are the high energy input and high operating cost as well as long processing time compared to the other drying processes (Morgan et al. 2006).

Spray drying is an economical process of conservation of probiotic cultures, which ensures high productivity rate and relatively low operating costs (Chávarri et al. 2012). Microencapsulation by spray drying involves the formation of the emulsion or suspension by mixing the cells with a solution of a polymeric carrier and spray drying in a cyclone. The resulting powder is stable and suitable for industrial applications. The use of spray drying for the preservation of probiotic cultures has been attracting considerable attention (Gardiner et al. 2002; Lian et al. 2002). The process of spray drying is controlled by the flow of substrate, the gas flow rate, and temperature. A potential disadvantage of spray drying is often a poor survival of probiotic cells during drying and the lower stability during storage compared with the particles obtained by emulsion and extrusion technique. This is a result of the exposure of cells to high temperatures and simultaneous dehydration during the process, leading to thermal and oxygen stresses. It has been shown that spray-dried cells always have a slightly longer lag phase and need more time to start the production

of lactic acid compared to the cells that are preserved by freeze-drying (Champagne et al. 1991). The loss of viability during drying is related to the damage that can occur in the cell membrane, causing the dried cells to become more sensitive. In order to improve probiotic survival, protectants can be added to the media prior to drying. Substrates for encapsulation by spray drying may be from the group of polysaccharides, proteins, peptides, etc. Prebiotics are the substrates that are commonly used as a carrier for the spray drying of probiotics. Prebiotics are indigestible food ingredients (typically polysaccharides) which can selectively stimulate the growth of beneficial microbiota (bifidobacteria, lactobacilli, etc.) in the gut, thereby increasing resistance of the host to the pathogenic bacteria.

The difference in the coefficient of thermal conductivity of different carriers can influence the survival of probiotics in the obtained powder (Lian et al. 2002). Also, the chemical composition of the carrier can influence the survival of probiotics. Biopolymers, such as natural gums (acacia, k-Carrageenan, alginates etc.), can be successfully used as carriers. Besides polysaccharides, protein carriers (skim milk, whey, protein, gelatin, etc.) are generally considered as good wall materials (Chávarri et al. 2012). However, skim milk has proved to be a better wall material than gelatin (Lian et al. 2002; Hsiao et al. 2004). In addition, in the study of Petrović et al. (2012), reconstituted skim milk used as a feed media showed significantly higher protection of potential probiotic from cell damage associated with spray drying, compared to inulin and maltodextrin. Skim milk proteins may form a protective layer around the bacterial cells, whereas calcium enhances survival of cells after the dehydration. For the encapsulation of probiotics by spray drying, a physiologically inert cellulose derivative, cellulose acetate phthalate has also been demonstrated as a good carrier, as the encapsulated probiotic cells survived better the exposing to low pH 2 and pH 1 and the high concentration of the bile salts, compared to the free cells (Fávaro-Trindade and Grosso 2002).

Generally, spray drying presents a suitable way to preserve large amounts of probiotic cultures. The microencapsulated probiotics with skim milk or inulin as a carrier are a useful ingredient in the production of functional food.

13.5 Methods for Assessing the Viability of Probiotic Bacteria after Spray Drying

Viability is generally considered as a prerequisite for optimal probiotic functionality (Maukonen et al. 2006). Many studies have shown that the viability of bacteria after encapsulation is not a simple question whether the cells are dead or alive (Nyström 2001; Bogovic-Matijasic and Rogelj 2006).

Survival of probiotic strains can be determined by the conventional plate-counting technique, as well as with modern methods such as viability real-time PCR for selective quantification of cultivable or viable bacteria, respectively.

Plate counting is one of the most widespread techniques for determination of the viability of spray-dried bacteria, which has obvious disadvantages, such as limited

microbial recovery, sensitivity and relatively long period for the colony growth. Therefore, during the recent years there has been an increasing interest in the development of culture-independent molecular methods for quantification of probiotic bacteria. Quantitative real-time polymerase chain reaction (real-time PCR) is among the most widely applied techniques for direct quantification of LAB in food or lyophilised dairy starter cultures (Justé et al. 2008). The use of this method for the quantification of probiotic bacteria has also been reported in several studies, however, most often in different foods, such as fermented milk products, while studies reporting the application in probiotic food supplements are scarce (Monnet and Matijasic 2012).

However, at the moment, there are still some limitations to overcome before the introduction of such methods in routine analysis. Conventional real-time PCR for instance does not enable the distinction between DNA arising from dead or alive cells, therefore the DNA from dead cells affects the accuracy of the results.

A possible way to distinguish between alive and dead cells is by determination of the integrity of the membrane. Viable cells with intact membranes are impermeable to the passage of certain intercalating DNA agents, which, however, selectively penetrate dead cells. Propidium monoazide (PMA) has been shown to be useful for differentiating between live and dead Gram-positive and Gram-negative bacteria (Nocker et al. 2006). PMA is a DNA-intercalating dye with the azide group, which enables covalent binding to DNA under bright visible light and, consequently, strongly inhibits PCR amplification.

Radulović et al. (2012b) compared survival of *Lb. paracasei* 08 and *Lb. plantarum* 564 strains, isolates from Sjenica cheese, previously characterized as potential probiotic bacteria, during spray drying (Table 13.3).

Both tested strains have shown a very good survival during the process of spray drying. The results indicated that microencapsulation by spray drying can offer a protection of the tested strains. Regarding the complexity of the bacterial population, such as their physiological states, the combination of different methods would represent a significant improvement in the analysis of the bacterial viability after spray drying.

Table 13.3 The number of tested strains performed by the plate count method, real-time polymerase chain reaction (PCR), and real-time PCR combined with pretreatment of the samples with propidium monoazide (PMA) (Radulović et al. 2012b)

Strains	Plate count ^a		Real-time PCR without PMA pretreatment ^b	Real-time PCR with PMA pretreatment ^b
	Before spray drying	After spray drying		
<i>Lb. plantarum</i> 564	9.45 ± 0.09	9.44 ± 0.05	9.31 ± 1.11	9.20 ± 0.98
<i>Lb. paracasei</i> 08	10.52 ± 0.02	10.42 ± 0.02	10.42 ± 0.4	10.35 ± 0.50

^aMean values (log CFU/g) and standard deviation were calculated from three parallel plate count analyses

^bMean values (log CFU/g) and standard deviation calculated from Ct values; based on two parallel DNA extracts from which two real-time analyses were run

Treatment of the spray-dried strains with PMA, followed by real-time PCR analysis, appeared to be a promising approach for the routine determination of viable cells in spray-dried powders.

13.6 Application of Probiotic Bacteria in Cheese Production

The numerous modern studies have been based on the development of new technologies and functional foods which may have benefits for a consumer's health. Functional foods may be considered as products which are between food and medicaments.

Dairy products, according to their composition and properties, present a good base for the development of new food with functional and dietetic properties. Probiotics are considered among the best known functional food ingredients. Dominant position within functional dairy products belongs to fermented dairy drinks such as yogurt. However, the increasing consumer demands for the products with functional properties have contributed to the expansion of assortment of these products. In the last decades, numerous researches have been done in order to investigate the possibility of incorporation of probiotic bacteria in different kind of cheeses and maintenance of their viability during the cheese production, ripening, and storage until consumption. In order to achieve therapeutic effect, it is required that probiotics incorporated into cheeses remain viable at $>10^7$ CFU/g throughout the cheese making process and storage.

The probiotic strains should be compatible with technological process of cheese production. Hence, it is important that these microorganisms have high cell density in inoculums, as well as ability to survive and/or grow in the production conditions, ripening, and storage of cheeses. The critical factors affecting survival of probiotic strains during cheese production include the aerobic conditions, the cooking process for hard and semi-hard cheeses, the presence of fast growing starter bacteria, the low pH of the curd, and the low temperatures during ripening and storage (Mattila-Sandholm et al. 2002; Ross et al. 2002).

It is assumed that the cheese may represent an even better probiotic carrier than fermented dairy beverages. Sharp et al. (2008) found that low-fat Cheddar cheese represents better delivery medium for probiotic *Lactobacillus* strain compared to yogurt, as it enhanced the survival under acidic conditions, which are present during stomach transit. Cheeses have a relatively high buffering capacity, high fat content, and a tight structure which may enable stabilization and better survival of probiotic bacteria during passage through GIT after consumption (Gomes and Malcata 1999; Ross et al. 2002).

On the other hand, the high fat content in cheese influences the high caloric value of the product which may negatively affect the consumer's health. Since the cheeses with incorporated probiotics are usually considered as a food with health claims aspect, it is recommended that those products are produced with reduced fat content. However, a reduced milk fat content in a numerous cheeses causes altered,

weakly expressed, and less acceptable sensory properties. For these reasons, it is of particular importance to perform proper selection of starter and probiotic culture, type of cheese and technology, in order to obtain high quality and sensory acceptable products that can be classified in the group of functional products.

A great number of probiotic bacteria strains are not resistant to high salt content (Yilmaztekin et al. 2004). For these reasons, it is not recommended to select cheeses characterized with a high salt content (such as white brined cheeses etc) as a carrier of these bacteria. In this case, it is necessary to modify the technological procedures in order to obtain products with a lower salt content which may belong to functional foods group.

Miočinović et al. (2014) investigated the properties of low-fat UF cheeses produced with adjunct commercial probiotics (*L. acidophilus* LAFTI®L10 i *B. lactis* LAFTI®B94, DSM Netherlands). They found that the viability of probiotic bacteria in both cheeses was maintained at the high level during the overall ripening periods (12 and 5 °C during 7 days and 7 weeks, respectively). However, the significant reduction of bifidobacteria number was found at the end of investigated ripening period ($<10^7$ CFU/g). Mc Brearty et al. (2001) indicated that the viability of bifidobacteria is significantly dependent on the type of the used strains. The viability of probiotic bacteria during cheese ripening, especially of very sensitive bifidobacteria, can be improved by microencapsulation techniques (Boylston et al. 2004; Özer et al. 2009).

Fresh cheeses may be more attractive carriers of probiotic strains, concerning that during their production there is no ripening period, storage is at refrigerated temperatures and the shelf life is limited compared with ripened cheeses. There have been several studies describing the development of fresh cheeses containing suitable doses of probiotic bacteria (Roy et al. 1997; Vinderola et al. 2000).

Radulović et al. (2011b, 2012c) have performed a couple of studies to investigate the possibility of probiotics incorporation during production and storage of soft fresh acid coagulated cheeses made from goat milk. The authors have chosen goat milk as raw material due to its already known functional properties. The improvement of nutritional benefits of goat dairy products, including cheeses, by probiotic strains could contribute to human health. It has been reported that goat milk possesses a higher digestibility and lower allergenic properties than cow milk, due to the higher proportion of smaller size of casein micelles and fat globules. Because of its anti-allergenic properties, potential application of goat's milk as a substitute for cow's milk is of importance for both infants and adults. Goat's milk has been recommended for the persons who suffer from malabsorption syndrome (Antunac et al. 2000).

In Fig. 13.1 is shown the viability of potential probiotic strains *Lb. plantarum* 564 and *Lb. paracasei* 08 as well as of commercial probiotic *Lb. acidophilus* LA-5 (Chr. Hansen) during storage of fresh goat cheeses (Radulović et al. 2011b, 2012c).

The CFU number of potential probiotics *Lb. plantarum* 564 and *Lb. paracasei* 08 was maintained above and around 7 log CFU/g, immediately after production and after 56 days of cheese storage, respectively. These results are similar to the results obtained by Kılıç et al. (2009) who found high viability (10^8 CFU/g) of potential

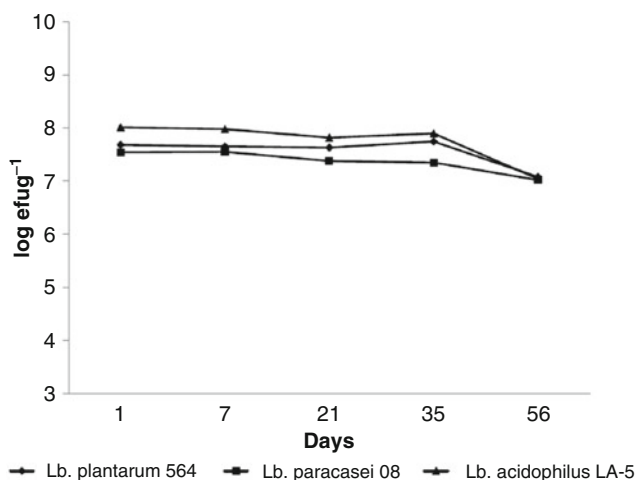


Fig. 13.1 The viability of commercial probiotic strains and autochthonous isolates with probiotic potential during storage of goat fresh cheeses (Radulović et al. 2011b, 2012c)

probiotic strains *Lb. plantarum* (AB16-65 and AC18-82) and *Lb. fermentum* (AB5-18 and AK4-120) in a Turkish Beyaz cheese after 90 ripening days. Similarly, Buriti et al. (2007) reported the high level of probiotic strain *Lb. paracasei* during 21 days of storage of fresh cream cheeses.

13.7 Application of Encapsulated Probiotic Bacteria in Cheese Production

The biggest challenge in the production of functional foods with probiotic strains is to obtain the desired probiotic bacteria counts in the food products, including cheese, and target delivery to the GIT of the host. Therefore, some studies have been performed in order to investigate the different encapsulating and coating techniques to maintain and enhance the viability and biological functionality of probiotics during dairy fermentation and storage as well as after consumption (Özer et al. 2009; Ortakci et al. 2012).

The survival of potential probiotic strains *Lb. paracasei* 08, *Lb. plantarum* 564 encapsulated by spray drying and commercial probiotic bacteria *Lb. acidophilus* LA-5 (Chr. Hansen) and their effect on the soft goat cheese properties were investigated (Radulović et al. 2011c, 2012d).

The count numbers of encapsulated potential probiotic strains during the storage of fresh goat cheeses are shown in Fig. 13.2.

After the fresh goat cheese production, the counts of potential spray-dried probiotic bacteria *Lb. paracasei* 08 and *Lb. plantarum* 564 were 7 log CFU/g and 7.76 log CFU/g, respectively. The high levels were maintained during storage. Similar results

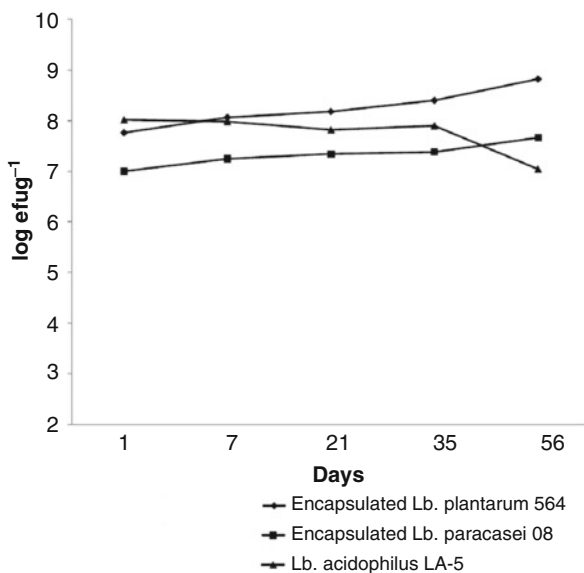


Fig. 13.2 The viability of encapsulated potential probiotic isolates and commercial probiotics during storage of goat fresh cheeses (Radulović et al. 2011c, 2012d)

were found in a study of Gardiner et al. (2002), who applied spray-dried *Lactobacillus paracasei* NFBC 338 in Cheddar cheese production.

From the data presented in Fig. 13.1, it can be observed that encapsulation by spray drying contributed to slightly higher counts of potential probiotics in fresh goat cheeses during storage. Özer et al. (2009) found that the counts of probiotic bacteria decreased for approximately three logs in the white brined cheeses produced by probiotic free cells, while the loss of viability was more limited in the cheeses containing microencapsulated cells (app. 1 log). Ortakci et al. (2012) demonstrated slightly better survival of alginate-microencapsulated *Lactobacillus paracasei* spp. *paracasei* LBC-1 (LBC-1e) during the heating and stretching processes used in the production of partly skim Mozzarella cheese. Hence, it can be concluded that encapsulation could provide certain protection to probiotic bacteria.

13.8 Effect of Probiotic Bacteria on the Composition, Proteolysis, and Sensory Quality of Cheeses

The significant influence of probiotic strains on the composition of different cheeses has not been reported. Commonly, the addition of probiotic strains has not shown an effect on the pH value of cheeses (Ong et al. 2006; Milesi et al. 2009). However, this strongly depends on the type of cheese produced as well as on the characteristics of probiotic strains.

Some probiotic lactobacilli strains have a proteolytic system and thus could potentially influence the proteolysis rate and the amount of small peptides and free amino acids in cheese. The effect of probiotic lactobacilli on proteolysis was studied in different cheese varieties (Bergamini et al. 2006; Ong et al. 2006; Milesi et al. 2009). Miočinović (2010) examined the influence of different probiotics, commercial and potential autochthonous *Lb. paracasei* 08, on the proteolysis rate of low-fat UF cheeses during ripening. Addition of adjunct probiotic culture showed no significant influence ($P > 0.05$) on the rate of primary proteolysis, but the higher rate of secondary proteolysis due to probiotic addition was found. Similar results were also presented by other researchers (Bergamini et al. 2006; Ong et al. 2006).

These results were expected due to the fact that primary proteolysis of the most cheeses takes place under the action of residual rennet and starter proteinases. Therefore, adjunct starter cultures, including probiotics, usually possess strong peptidase systems which don't have a significant role during primary proteolysis, but influence the secondary proteolytic changes (Sousa et al. 2001). However, Miočinović et al. (2012) observe an increase in both primary and secondary proteolysis during ripening of white brined cheeses produced with autochthonous starter and potential probiotics compared to control cheese (commercial starter and probiotic). These results were probably a consequence of the proteinase as well as peptidase activity of autochthonous LAB. Milesi et al. (2009) reported that effect of potential probiotic strains (*Lb. rhamnosus*, *Lb. plantarum* and *Lb. casei*) on proteolysis and the sensory characteristics of soft and semi-hard cheeses was strongly strain depended.

Sensory properties of cheeses are one of the most important attributes, especially from the consumer's point of view. Hence, a lot of studies investigate the influence of probiotics on sensory quality of different cheeses. Commonly appearance, consistency, and texture are not influenced by adjunct probiotic bacteria, since the bacteria have no effect on composition. On the other side, many studies indicated that addition of probiotics showed a positive influence on the flavor of cheeses (Miočinović 2010). Improving of probiotic cheeses flavor could be a consequence of an increased proteolysis rate due to probiotic addition (Ong et al. 2006; Sabbagh et al. 2010; Kılıç et al. 2009).

Radulović et al. (2011b, 2012c) also presented that fresh goat cheeses produced with free and spray-dried potential probiotic strains (*Lb. plantarum* 564, *Lb. paracasei* 08) were evaluated with high sensory score, while those made with commercial probiotic strains (*Lb. acidophilus* LA-5) had acceptable sensory quality (Table 13.4).

Gobbetti et al. (1998) found that the flavor intensity score of Crescenza cheeses with *B. bifidum* and *B. longum* were slightly higher than those produced by conventional methods, which was probably due to the combination of the higher concentrations of lactic and acetic acids and free amino acids and soluble peptides.

The results presented in this work indicate that autochthonous potential probiotic strains could be suitable in the development of new probiotic cultures and probiotic cheeses as new functional food products.

Table 13.4 The sensory evaluation of cheeses (Radulović et al. 2011b, 2012c)

Sensory attributes ^a	Time (days)	Exterior and interior appearance		Body and consistency		Flavor and odor		% of Max. quality
		R ^a	W ^a	R ^a	W ^a	R ^a	W ^a	
Control cheese	7	4.90	14.70	4.9	34.30	4.50	45.00	94.00
	21	5.00	15.00	5.0	35.00	4.20	42.00	92.00
Cheese	7	4.90	14.70	4.9	34.30	4.50	45.00	94.00
<i>Lb. paracasei</i> 08	21	5.00	15.00	4.70	32.90	3.50	35.00	82.90
Cheese	7	4.88	14.63	5.00	35.00	5.00	50.00	99.63
SD <i>Lb. paracasei</i> 08	21	5.00	15.00	4.80	33.60	4.10	41.00	89.60
Cheese	7	4.88	14.63	5.0	35.00	4.75	47.50	97.13
<i>Lb. plantarum</i> 564	21	5.00	15.00	5.0	35.00	4.90	49.00	99.00
Cheese	7	4.88	14.63	5.00	35.00	5.00	50.00	99.63
SD <i>Lb. plantarum</i> 564	21	5.00	15.00	4.80	33.60	4.10	41.00	89.60
Cheese	7	4.88	14.64	4.88	34.16	3.75	37.50	86.30
<i>Lb. acidophilus</i> LA-05	21	5.00	15.00	4.80	33.60	2.80	28.00	76.60

^aR-score (mean value), W weighted (mean value multiplied with coefficient): Appearance 3; Body and texture 7; Flavor and odor 10; SD spray-dried

Conclusion

Traditionally made cheeses represent a great natural source for selection of new LAB strains for different application possibilities. Apart their application as starter cultures in standardized traditional cheese production, they could be used as potential probiotic bacteria for production of functional foods.

The autochthonous strains of lactobacilli (*Lactobacillus paracasei* 08, *Lactobacillus plantarum* 564 and *Lactobacillus paracasei* 05) isolated from traditional cheeses in Serbia showed a good ability to grow in a wide range of temperature and high salt concentrations, antimicrobial properties, and potential probiotic ability in vitro. Two potential probiotic strains (*Lb. paracasei* 08 and *Lb. plantarum* 564) survived the spray-drying process well, indicating that this microencapsulation technique could be a cost-effective way in protection of these strains. Treatment of the spray-dried strains with PMA, followed by real-time PCR analysis, appeared to be a promising approach for the routine determination of viable cells in spray-dried powders.

The application of free and spray-dried potential probiotic cells of strains *Lb. plantarum* 564 and *Lb. paracasei* 08 in soft fresh goat cheeses resulted in their high viability ($\geq 7 \log$ CFU/g) during production and storage period, with slightly higher counts of spray-dried cells. The fresh goat cheeses produced with free and spray-dried potential probiotic strains were very acceptable and evaluated with high sensory score.

Incorporation of new autochthonous potential probiotic strains in different cheeses provides an opportunity to improve the healthy status and quality of the final products with desirable properties.

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Chapter 14

Effect of Cell Immobilization on Properties of Presumptive Probiotics

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14.1 Introduction

Nowadays there is an upsurge of interest in developing novel foods containing probiotic microorganisms, such as bifidobacteria and lactic acid bacteria (LAB). According to World Health Organization (WHO), probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host.” Many beneficial effects are associated with consumption of probiotics, such as prevention of pathogenic infections, stabilization of the gastrointestinal (GI) barrier function, maintenance of the intestinal microbial homeostasis, and production of anticarcinogenic and antimutagenic compounds (Kopp-Hoolihan 2001; Mitropoulou et al. 2013; Penner et al. 2005).

GI survival, adhesion, and colonization to the intestine are crucial for providing the beneficial effects of probiotics, since they may influence interaction with the host and the other bacteria present, affect the local microbial composition, and/or stimulate the host’s immune system. For many probiotics, the aim is to achieve at least transient colonization, in which case they grow or at least metabolize in the intestine (Tuomola et al. 2001).

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In order to deliver the health benefits, probiotic foods need to contain an adequate amount of live bacteria (at least 10^6 – 10^7 cfu/g) (Boylston et al. 2004; Oliveira et al. 2002), able to survive the acidic conditions of the upper GI tract and proliferate in the intestine, a requirement that is not always fulfilled. Thus, a daily intake of at least 10^8 – 10^9 viable cells, which could be achieved with a daily consumption of at least 100 g of probiotic food, has been suggested as the minimum intake to provide a probiotic effect.

A properly designed strategy for incorporation of probiotic microorganisms into foods (formulation strategies, processing, stability, and organoleptic quality issues) is a key factor in the development of functional products. Although encapsulation systems have largely been exploited in the pharmaceutical (e.g., drug and vaccine delivery) and agricultural sector (e.g., fertilizers), the food industry has only recently become aware of the immense benefits that these technologies are able to offer (Champagne et al. 2010). Insertion of beneficial bacteria into a food matrix presents a fully new challenge, not only because of their interactions with other constituents, but also because of the severe conditions often employed during food processing and storage, which might lead to important losses in viability, as probiotic strains are very often thermally labile (on heating and/or freezing) and sensitive to acidity, oxygen, or to other food constituents (e.g., salts). To overcome such deficiencies, immobilization techniques are usually applied in order to maintain cell viability, activity, and functionality. Thus, many studies have focused on immobilization of probiotic bacteria on various supports (Doherty et al. 2011; Mitropoulou et al. 2013). Starch (Mattila-Sandholm et al. 2002), wheat grains (Bosnea et al. 2009), casein (Dimitrellou et al. 2008, 2009), and fruit pieces (Kopsahelis et al. 2007; Kourkoutas et al. 2005, 2006) were among the most promising immobilization supports suitable for the food industry.

The controlled and continuous delivery of probiotics in the gut is a fascinating approach. Potential benefits of such a strategy include maintenance of greater cell viability during passage through the stomach acidic environment.

Selecting the immobilization support and technology is a very crucial matter and a number of issues, such as physicochemical properties (chemical composition, morphology, mechanical strength), stability in gastric and intestinal fluids, food grade and toxicology assays, and manufacturing and sterilization processes, should be very seriously considered.

Hence, the overall objective of this chapter is to analyze and assess the data on the effect of cell immobilization on the properties of presumptive probiotic strains. Emphasis is provided on tolerance to simulated GI tract conditions, adhesion attributes, and modulation of microbial intestinal flora. Specific research results from both *in vitro* and *in vivo* assays are below presented and assessed.

14.2 In Vitro Assays

Difficulties experienced in studying probiotic properties *in vivo* have stimulated interest in the development of *in vitro* models for preliminary screening of potentially probiotic strains. These models mainly focus on tolerance to simulated GI tract conditions and on adhesion properties.

14.2.1 Tolerance to Simulated GI Tract Conditions

Cell survival in acidic and GI tract simulated conditions is considered among the most important prerequisites for probiotic strains. To predict tolerance to upper GI transit, Sidira et al. (2010) exposed immobilized cells of *L. casei* ATCC 393 on apple pieces to in vitro conditions simulating acidic environment, gastric and pancreatic juices, and bile salts. For comparison reasons, tolerance to GI transit was also tested for free cells. Although acidic conditions resulted in a significant reduction of *L. casei* ATCC 393 levels in both immobilized and free cells, counts of immobilized cells were significantly higher after 120 min at pH 2.0 and after 30, 60, 90, and 120 min at pH 1.5 compared to free cells (Sidira et al. 2010). Likewise, simulated gastric juices, pancreatic juices supplemented with bile salts, and bile salts caused a significant reduction on levels of both immobilized and free cells (Sidira et al. 2010). In contrast, pancreatic juices had no effect on the survival of immobilized cells but affected significantly viability of free cells. Of note, cell immobilization resulted in significantly higher survival rates in pancreatic juices supplemented with 0.45 % bile salts after 240 min and in bile salts after 120 min compared to free cells (Sidira et al. 2010).

Immobilization of probiotic cultures in whey protein-based microcapsules can increase cell survival when subjected to extreme conditions, making this approach potentially useful for delivery of viable bacteria to the GI tract of humans via dairy fermented products. *Lactobacillus paracasei* ssp. *paracasei* F19 and *Bifidobacterium lactis* Bb12 were encapsulated in milk protein matrices by means of an enzymatic induced gelation with rennet (Heidebach et al. 2009a) and in food grade casein microcapsules based on a transglutaminase-catalyzed gelation of casein suspensions (Heidebach et al. 2009b). Analysis of living cell numbers after incubation of free and encapsulated probiotics at low pH values and in simulated gastric juice without pepsin at pH 2.5 and pH 3.6 (37 °C, 90 min) showed a protective effect due to microencapsulation under all conditions tested. Both studies indicated that the microencapsulation of probiotic cells can be a suitable alternative to current available technologies and can protect probiotic cells from damage, due to pH levels similar to those in the human stomach. Similarly, Khater et al. (2010) declared that encapsulation effectively protected probiotics from the hostile environment in the GI tract and prevented cell loss.

The efficacy of whey protein isolate as an encapsulation matrix for the maintenance of *Lactobacillus rhamnosus* GG viability was also previously evaluated (Doherty et al. 2011). Following 3 h in vitro stomach incubation (pH 1.8; 37 °C), micro-beads laden with 10¹⁰ cfu demonstrated acid stability and peptic resistance, characteristics required for optimum probiotic refuge. However, enzyme-activated intestinal conditions catalyzed a synergistic response engaging rapid matrix disintegration and controlled probiotic release. Overall, the study led to the development and design of a protein encapsulation polymer based on congruent matrix interactions for reinforced probiotic protection during challenging situations for their targeted delivery to intestinal adsorption sites.

On the other hand, although immobilized cells of *Streptococcus thermophilus* A21 and W22 on alginates were more resistant to low pH and to bile salts than free cells, no significant differences in the viability was observed after a 2-week storage period at refrigerator and room temperatures (Aslim and Alp 2009). Additionally, L(+) lactic acid and exopolysaccharides production was not affected by the immobilization process. Nevertheless, the immobilized cells were significantly less affected by nisin and penicillin-G. On the whole, the study concluded that cell immobilization did not impair, or even improved, the probiotic and technological properties of the tested strains.

14.2.2 Evaluation of Adhesion Properties

Since many factors affect cell binding to mucus in vitro, experimental protocols should be carefully designed when investigating adhesion properties of probiotics, due to the direct effects that they may have. Adhesion to the intestinal mucosa has been studied mainly using in vitro model systems, such as the Caco-2 and HT-29 human epithelial cell lines. In this vein, the adhesion characteristics of both immobilized and free *L. casei* ATCC 393 to Caco-2 cells and *n*-hexadecane were evaluated and compared to *Lactobacillus plantarum* NCIMB 8826 strain, which is considered a strain of high adhesion ability.

Adhesion to Caco-2 cell monolayers grown on glass coverslips (Deepika et al. 2009) was expressed as the number of bacteria adhering to 100 Caco-2 cells. Although cell immobilization of *L. casei* ATCC 393 on apple pieces resulted in significantly reduced number of adhered cells to a monolayer of Caco-2 cells compared to free *L. casei* cells (Fig. 14.1), a high adhesion ability was observed, similar to other species, such as *L. rhamnosus* and *L. acidophilus* (Ren et al. 2012).

The interaction between *L. casei* ATCC 393 cells and the surface of Caco-2 cells was illustrated by scanning electron microscopy (SEM) micrographs (Fig. 14.2). It was noted that both immobilized and free cells adhered to the brush border microvilli of the Caco-2 cell monolayer. The starch granules of apple pieces interacted with the monolayer, indicating that the immobilization support interacted with the Caco-2 cells and more specifically adhered to the brush border microvilli (Fig. 14.2).

To estimate cell surface properties, microbial adhesion to *n*-hexadecane (MATH) in a two-phase system (Harty et al. 1993; Kmet and Lucchini 1997; Reid et al. 1992; Pelletier et al. 1997) is often used because cell surface hydrophobicity has been associated with bacterial adhesion to a variety of surfaces (Marin et al. 1997). According to Reid et al. (1992), the surface hydrophobicity of *Lactobacillus* strains varies greatly. Application of the above methodology indicated that *L. casei* ATCC 393 strain was relatively hydrophilic (Fig. 14.3), confirming the results previously reported by Harty et al. (1993) and Pelletier et al. (1997). Nevertheless, cell immobilization had a positive effect on the adhesion properties although not significant.

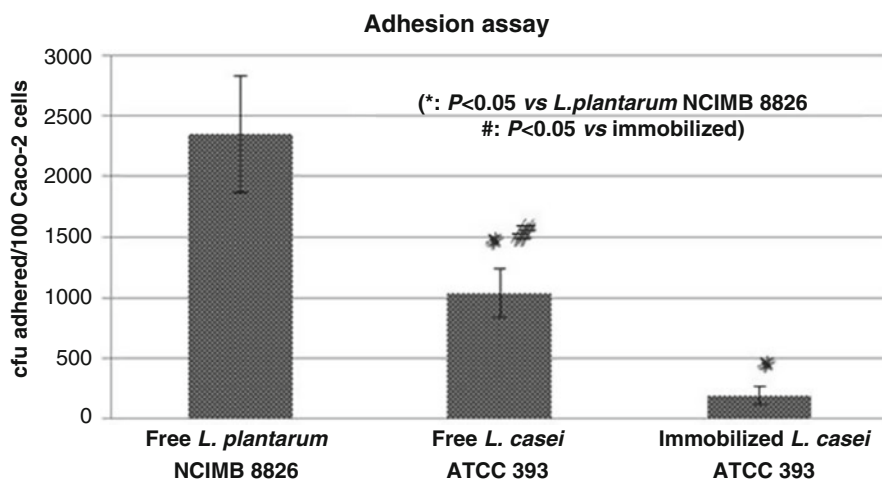


Fig. 14.1 Adhesion of free and immobilized *L. casei* ATCC 393 on apple pieces to a monolayer of Caco-2 cells, in comparison to free *L. plantarum* NCIMB 8826. All treatments were carried out in 10 repetitions. The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple range test was used to determine significant differences among results (coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica v.5.0)

However, no correlation was found for *L. casei* ATCC 393 between the adhesion ability to Caco-2 monolayer and hydrophobicity, in contrast to *L. plantarum* NCIMB 8826. A high hydrophobicity frequently results in greater attractive forces and higher levels of adhesion, whereas a low hydrophobicity usually results in lower levels of adhesion (Del Re et al. 2000; Ehrmann et al. 2002). On the other hand, other studies indicated that there is no correlation between cell surface hydrophobicity and adhesion to intestinal mucus. As highly adhesive bacteria may demonstrate fairly low surface hydrophobicities (Ouwehand et al. 1999; Van Tassell and Miller 2011), such properties should not be considered as an accurate measure of adhesive potential.

Trans-Epithelial Electrical Resistance (TEER) assay is usually applied to assess the effect of probiotics on the monolayer integrity and tight-junction function of the Caco-2 cells. When TEER increases, the tight junctions between the cells become stronger and the permeability of the monolayer decreases (Mattar et al. 2001). Addition of immobilized *L. casei* ATCC 393 to a Caco-2 cell monolayer cultured for 16 days resulted in an increase of TEER values after 17 h although not significant. On the contrary, the TEER values increased significantly when free cells of *L. casei* ATCC 393 or *L. plantarum* NCIMB 8826 were added (Fig. 14.4). Several studies have shown that *Lactobacillus* cells prevent or counteract the permeability of the intestinal barrier (García-Lafuente et al. 2001; Isolauri et al. 1993; Mangell et al. 2002; Mattar et al. 2001).

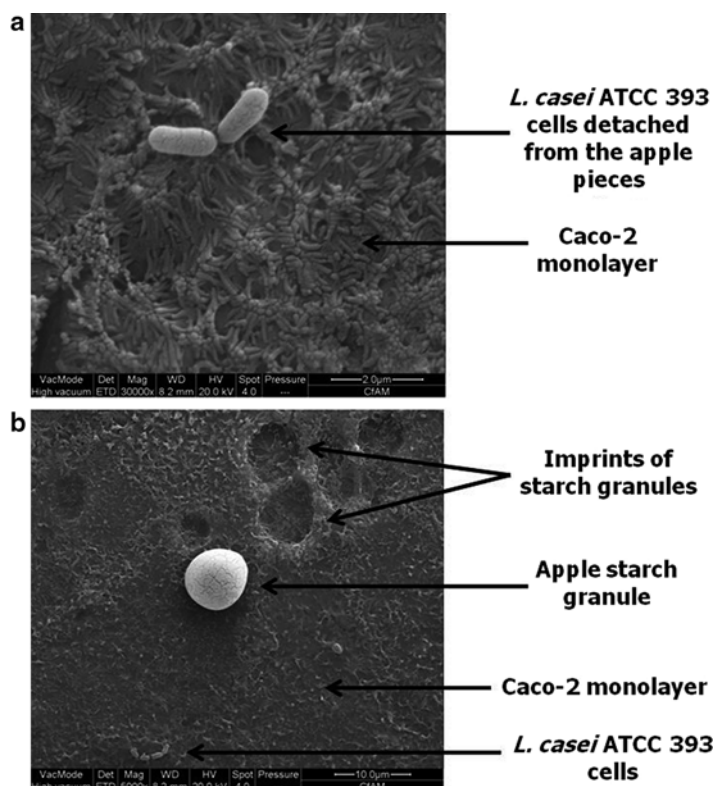


Fig. 14.2 SEM micrographs showing (a) Caco-2 and *L. casei* ATCC 393 cells detached from the apple pieces, (b) Caco-2 cells, the starch granules of apple pieces and *L. casei* ATCC 393 cells interacting with the monolayer. The imprints of apple starch granules were observed on the surface of the monolayer. The samples were imaged using CfAM's FEI Quanta 600 FEG scanning electron microscope

14.3 In Vivo Assessments

Although in vitro models offer great help in selecting probiotic candidates, most of them represent simplifications of the in vivo conditions and hence it is difficult to extrapolate in vitro results to the in vivo situation. Therefore, in vivo studies are necessary for validating indications deriving from the in vitro tests.

14.3.1 Survival During Passage Through the GI Tract

Fecal microbial analysis is usually applied to investigate probiotic survival during passage through the GI tract. In vivo assays using Wistar rats as animal model carried out by Sidira et al. (2010) aimed at the assessment of survival of both free and

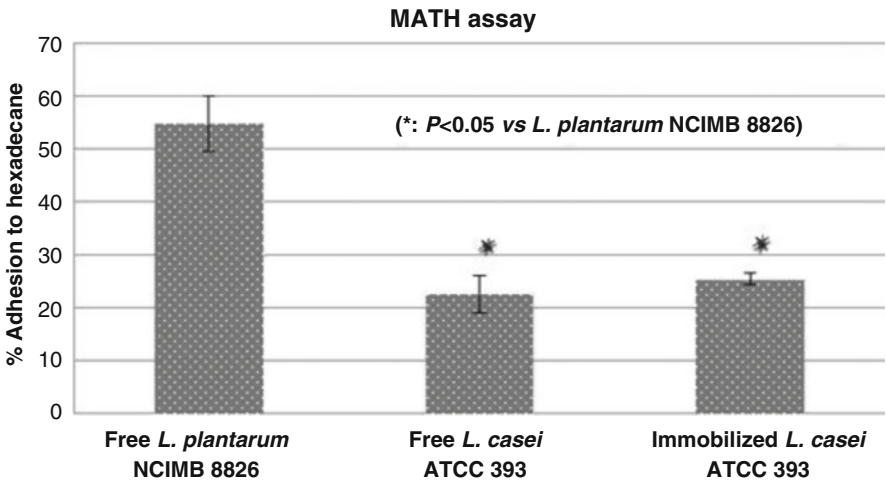


Fig. 14.3 Attachment of free and immobilized *L. casei* ATCC 393 on apple pieces to *n*-hexadecane, in comparison to free *L. plantarum* NCIMB 8826. All treatments were carried out in triplicate. The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple range test was used to determine significant differences among results (coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica v.5.0)

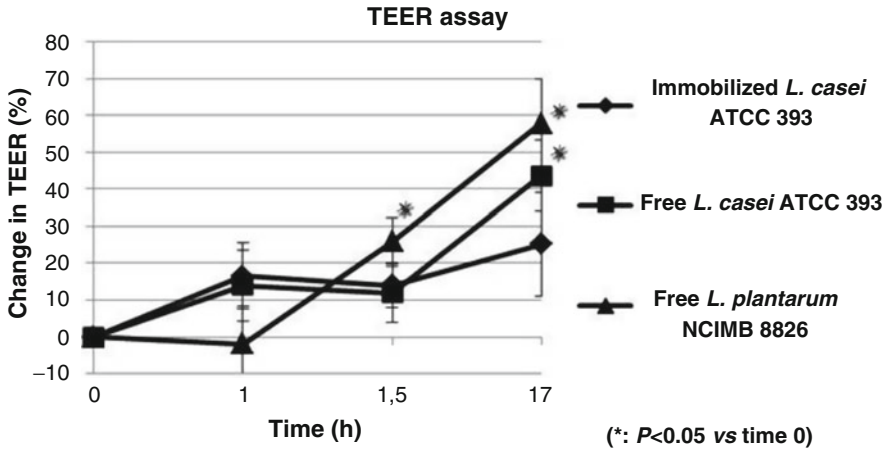


Fig. 14.4 Changes in trans-epithelial electrical resistance (TEER) values of Caco-2 cells in the presence of free or immobilized *L. casei* ATCC 393 on apple pieces, in comparison to free *L. plantarum* NCIMB 8826. All treatments were carried out in triplicate. The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple range test was used to determine significant differences among results (coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica v.5.0)

immobilized *L. casei* ATCC 393 on apple pieces during passage through the GI tract. The effect of single dose administration of probiotic fermented milk on GI survival was initially investigated. In both groups of rats administered a single dose of fermented milk containing either free or immobilized *L. casei* ATCC 393 cells on apple pieces (Kourkoutas et al. 2005), the above strain was detected at levels of ≥ 6 log cfu/g of feces at 12 and 24 h post-administration by combining microbiological and strain-specific multiplex PCR analysis (Karapetsas et al. 2010), while it was undetectable at 0 h, as expected. Levels were reduced to 4 log cfu/g at 36 h and *L. casei* ATCC 393 was not detected at 48 h. The fact that the probiotic strain was identified after such a short period following administration of the products and remained at suitable levels for 24 h, could be of crucial importance in the use of probiotic foods, not only as preventing agents, but also as an alternative or complementary therapy in a number of intestinal and non-intestinal clinical applications. However, it seems that daily consumption of probiotics is essential for conferring a probiotic effect.

14.3.2 Adhesion and Colonization to the Intestinal Mucosa

Although studies on the survival of administered probiotic bacteria by analyzing fecal samples offer an indication that the presumptive probiotic strains survived the harsh conditions of the intestine, they do not reveal the number of cells that remain attached to different sites of the intestinal tract. The use of biopsies from the intestinal mucosa is considered the most accurate means of determining adhesion and colonization.

The adhesion ability of *L. casei* ATCC 393 to the GI tract of Wistar rats was examined after single or daily administration of fermented milk containing either free or immobilized cells on apple pieces (Saxami et al. 2012). The adhesion of the probiotic cells at the large intestine (cecum and colon) was recorded at levels ≥ 6 log cfu/g (suggested minimum levels for conferring a probiotic effect) following daily administration for 7 days. Single dose administration resulted in slightly reduced counts (5 log cfu/g), while they were lower at the small intestine (duodenum, jejunum, ileum) (≤ 3 log cfu/g) in both cases, indicating that adhesion was a targeted process. These findings are probably due to considerable differences between the two organs, arising from differences in physiology. For example, thickness of the mucus layer tends to increase from the jejunum to the colon, providing a more extensive mucosal habitat for microbes in the colon compared with the upper parts of the small intestine. Noticeably, the levels of *L. casei* ATCC 393 were enhanced in the cecal and colonic content both after single or daily administration of immobilized cells (6 and 7 log cfu/g, respectively) (Saxami et al. 2012). The adhesion on the GI tract was transient and thus daily consumption of probiotic products containing the specific strain is suggested as an important prerequisite for retaining its levels at an effective concentration.

Many clinical studies of probiotic persistence and colonization show that probiotic microorganisms may not permanently colonize the GI tract and provide the

benefits only for short periods after the end of administration (Garrido et al. 2005; Tannock et al. 2000). On the other hand, good adhesion to the host tissue may have detrimental effects and could be associated to potentially negative properties. Adhesion to tissues, especially damaged tissues, is often the first step in pathogenesis and thus selection of extremely well-adhering strains could lead to isolation of potential pathogens. However, most probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, which are “generally regarded as safe” and are very rarely involved in disease. Taking into account that adhesive characteristics of probiotics vary considerably among strains and species and very little is known about the reasons of transient colonization of many probiotics compared to commensals, it is important to define the factors that influence adhesion ability.

14.3.3 Modulation of Intestinal Microbial Flora

There is growing scientific evidence to support the concept that the maintenance of healthy gut microbiota may provide protection against GI disorders, such as GI infections and inflammatory bowel diseases (Kopp-Hoolihan 2001; Mitropoulou et al. 2013; Penner et al. 2005; Salminen et al. 1998). The use of probiotic bacterial cultures may stimulate the growth of preferred microorganisms, crowd out potentially harmful bacteria, and reinforce the body’s natural defense mechanisms (Kopp-Hoolihan 2001; Salminen et al. 1998; Serban 2014).

The impact of *L. casei* ATCC 393 on modulating fecal microbial flora was evaluated in the survey of Sidira et al. (2010). For this reason, fermented milk containing either free or immobilized *L. casei* ATCC 393 on apple pieces was daily administered orally to Wistar rats for 9 days. For comparison reasons, administration of milk with apple pieces or acidified milk produced by addition of lactic acid was also tested. *L. casei* ATCC 393 was detected at levels ≥ 6 log cfu/g in rat feces at all time points (1–9 days) after administration of fermented milk containing free or immobilized cells (Sidira et al. 2010). Administration of milk with apple pieces or acidified milk had no effect on microbial counts in all cases. On the contrary, staphylococci counts were significantly reduced in both groups of rats administered fermented milk containing immobilized or free *L. casei* ATCC 393 by day 1 and 2, respectively. Likewise, coliforms and enterobacteria counts were significantly reduced in rats administered fermented milk containing immobilized cells by day 7, while in rats administered fermented milk with free cells at day 9. A decrease in streptococci counts was noted at days 1, 2, 7, and 9 in rats administered fermented milk containing immobilized cells and only at day 9 in rats administered fermented milk with free cells. No differences were observed in lactobacilli counts. The above results revealed regulation of intestinal content microbiota and hence it is not unreasonable to suggest the development of a microbial association, due to *L. casei* ATCC 393 cells, leading to repression of facultative pathogens although pathogenesis has not been considered for all strains of the above microbial species.

14.4 Conclusions and Perspectives

Despite the plethora of probiotic products and the immobilization supports proposed by several researchers, the immobilized cell technology has not yet been widely adopted by the industrial sector, mainly due to safety issues related to the immobilization agents, confirmation of the stability and functionality of probiotic cultures, the lack of processes that can be readily scaled up, and cost limitations. As a consequence, assays concerning evaluation of probiotic properties including immobilized strains, especially employing in vivo models, are limited in literature.

It is thus obvious that more research is still required for the selection of immobilization supports that can trigger successful adhesion to specific intestinal cells, therefore achieving targeted delivery of probiotic bacteria to various sites within the GI tract. It may be advisable to assess the adhesion of potential probiotics in more than one model, each supplementing the other and due to host-specific physiology, anatomy, and intestinal microbiota factors, experiments should be performed in the host for which the probiotic is designated. Additionally, the mechanisms of action of probiotics should be clarified so that their activity in the balance of intestinal ecosystem can be appreciated. Likewise, more evidence on the beneficial effects is still required to consolidate their role not only in GI disorders, but in many infectious diseases.

Finally, the development of novel functional foods is a major challenge to address the expectation of consumers for healthy and beneficial food products. The research of novel probiotic strains is important in order to satisfy the increasing request of the market. It is evident that the probiotic market has a strong future, as the benefits provided by probiotics consumption are now well documented, and thus consumer requirements are expected to increase.

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Chapter 15

The Biocide and Antibiotic Resistance in *Campylobacter jejuni* and *Campylobacter coli*

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Abbreviations

BC	Benzalkonium chloride
CCCP	Cyanide 3-chlorophenylhydrazone
CHA	Chlorhexidine diacetate
CPC	Cetylpyridinium chloride
EPI	Efflux pump inhibitor
MDR	Multidrug resistance
MIC	Minimal inhibitory concentrations
NMP	1-(1-Naphthylmethyl)-piperazine
OMP	Outer membrane protein
PAβN	Phenylalanine-arginine beta-naphthylamide
QRDR	Quinolone resistance-determining region
RND	Resistance-nodulation cell division
SDS	Sodium dodecyl sulphate
TLN	Triclosan
TSP	Trisodium phosphate.

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15.1 Introduction

The extended and intensive use of antibiotics and biocides in recent decades has become indispensable in protection of human and animal health but has also provoked the development and spread of highly resistant and also multidrug-resistant microorganisms. Antibiotics exist in many types and can be grouped on their chemical structure and also precise targets and mechanisms of action in microbial cells. Biocides also comprise various chemical agents that can efficiently inactivate microorganisms and are regularly used in the food industry and in housekeeping to prevent bacterial contamination during food processing, to disinfect, sanitise and/or sterilise objects and surfaces and to preserve materials or processes from microbiological degradation (Meyer 2006). A term “microbicide”, instead of biocide, which is widely used, has been recently suggested (Maillard et al. 2013) to point more directly on microorganisms as targets. Unlike antibiotics which generally affect a specific physiological process, biocides usually have more than just one target site. Basic mechanisms of action by biocides may be defined according to the structure of microbial cell against which it has its main activity. The interactions with outer cellular components, cytoplasmic membrane and cytoplasmic constituents have been described (Morente et al. 2013).

The extensive and especially inappropriate use of biocides and antibiotics may generate a selective pressure on bacteria, which can result in adaptation and resistant microorganisms. Resistance can be developed through by mutations of the antimicrobial target, changes in cell permeability and efflux of toxic substance and transfer of resistance genes. In comparison to antibiotic resistance, the mechanisms of bacterial resistance to biocides have been described more recently and have been less studied overall. They can be intrinsic, defined as a natural property of bacteria, like sporulation, biofilm formation or different cell wall structures. Acquired resistance is developed due to mutations and/or acquisitions of plasmids/transposons (McDonnell and Burke 2011).

Microorganisms are capable of adaption to almost any environment. Their response to environmental stress is regulated through many cell mechanisms. As an example, Gram-negative bacteria can control the intracellular concentration of antibacterial agents just by regulating membrane permeability with a decreased influx (uptake) by reducing the expression of active porins in the outer membrane or by altering the lipopolysaccharide structure, or by the expression/overexpression of the efflux pump (Davin-Regli and Pagès 2012). Efflux systems expel many antimicrobials and decrease their concentration inside the cell.

Under-dosing of biocide, ineffective cleaning before biocide application and/or insufficient applying of biocide may lead to reduced mechanism of biocide action. Exposure time, actual residual concentration over time, frequency of application, dilution on application, amount and type of exogenous organic matter present, the nature of the surface treated (i.e. porous or non-porous) and also pH, temperature and water hardness are parameters that will impact efficacy of biocide (Maillard et al. 2013). Regular exposure to sub-lethal concentrations of biocide due to

insufficient dose, inadequate distribution of the agent or presence of excessive amounts of organic matter may increase the resistance to this biocide by different mechanisms described above (Alonso-Hernando et al. 2009).

Resistance to one antimicrobial agent may provide cross-protection against another. A potential concern is the possibility that mechanisms providing resistance to biocides may also provide cross-protection to the activity of antibiotics as well as antibiotic resistance mechanisms such as reduced cellular uptake, drug efflux, drug inactivation and mutation at the target site can also apply to biocides (Thorrold et al. 2007; Meyer and Cookson 2010). A possible linkage of biocide and antibiotic resistance in bacteria has been reported by several authors (Russell 2002; Braoudaki and Hilton 2005; Thorrold et al. 2007; Karatzas et al. 2008) and recently reviewed (Davin-Regli and Pagès 2012; Gnanadhas et al. 2013). Still contrary evidences appear in the literature suggesting that this phenomenon has not caused a real problem in practice (Lear et al. 2006; Fernández-Fuentes et al. 2012).

In this work, we review the recent studies about antibiotic and biocide resistance and possible cross-resistance in *Campylobacter jejuni* and *C. coli*. Despite their widespread occurrence and increasing antibiotic resistance, the data have been collected just recently (Mavri 2013).

15.2 Antibiotic Resistance of *C. jejuni* and *C. coli*

Gram-negative, spiral-shaped bacteria of *C. jejuni* and *C. coli* are the major cause of human bacterial gastroenteritis. *Campylobacter jejuni* is the most prevalent species of its genus in poultry and its transmission via food chain to humans is a serious public health concern (Thakur et al. 2013). Although *Campylobacter* species lack many of the adaptive responses to the environmental stress, they can dynamically adapt to and survive transmission to another host (Park 2002). Besides incomplete understanding of bacterial physiology, population dynamics and pathogenesis, an increasing antibiotic resistance represents a threat for public health.

Campylobacter have become increasingly resistant to antibiotics. The highest frequency of resistance in *C. jejuni* and *C. coli* appears for fluoroquinolones, such as ciprofloxacin. In general, *C. coli* tends to be more resistant than *C. jejuni* (EFSA 2013; Mavri et al. 2012). The major concern is the development and spread of the multidrug-resistant *Campylobacter* strains (Kurinčič et al. 2005; Moore et al. 2006). The problem of highly prevalent multiresistant strains was observed in the food chain, especially in meat isolates (Smole Možina et al. 2011).

Numerous studies have been conducted to better understand the molecular mechanisms of bacterial resistance to antimicrobial agents, and various drug specific as well as multidrug resistance mechanisms were observed. In addition to target mutations, the resistance can be conferred by efflux pumps, limiting the access of agents to their targets by actively pumping them out of the cells and thereby preventing the intracellular accumulation necessary for lethality.

15.2.1 The Role of Efflux Mechanism in Antibiotic Resistance in *Campylobacter*

In *C. jejuni* and *C. coli*, the presence of the major efflux system, known as the CmeABC efflux pump, has been demonstrated to contribute to resistance to a broad range of antimicrobials (Lin et al. 2002; Martinez and Lin 2006). The expression of this efflux pump is under the control of transcriptional repressor that binds directly to the *cmeABC* promoter and is induced by the natural substrates of this efflux pump, bile salts (Lin et al. 2005). Functionally distinct efflux system, CmeDEF, has been identified to belong as well to the resistance-nodulation cell division (RND) family of transporters (Akiba et al. 2006). The CmeGH multidrug efflux transporter from the major facilitator family has been recently shown to additionally contribute to antibiotic resistance in *C. jejuni* (Jeon et al. 2011).

The involvement of efflux mechanisms in bacterial intrinsic and acquired resistance has been mostly demonstrated by the use of efflux pump inhibitors (EPIs), which enhance drug accumulation inside the bacterial cell, thereby increasing the bacterial susceptibility to antimicrobials (Kern et al. 2006; Martins et al. 2009b). In the study of Mavri and Smole Možina (2012), phenylalanine-arginine beta-naphthylamide (PAβN) and 1-(1-naphthylmethyl)-piperazine (NMP) significantly reduced minimal inhibitory concentrations (MICs) of erythromycin in high portion of tested *Campylobacter* strains from different sources, wherein PAβN had much higher effect in *C. coli* compared to *C. jejuni*. This finding might explain the lower occurrence of antimicrobial resistance among *C. jejuni* than *C. coli*. Moreover, PAβN had the greatest effects in erythromycin-resistant *C. coli* strains, with no A2075G mutation in the 23S rRNA and unstable erythromycin resistance phenotypes. These data suggest erythromycin resistance that is not mediated by the A2075G mutation in the 23S rRNA gene but by other mechanism(s), and that this might be conditionally (temporarily) induced to provide the bacterial pathogens with rapid adaptation to environmental changes. Other studies have reported unstable phenotypes in *Campylobacter* which had easily lost their resistance phenotype in the absence of macrolide antibiotics (Caldwell et al. 2008; Kim et al. 2006; Luangtongkum et al. 2009).

Similarly as the use of EPIs, the involvement of efflux mechanisms in bacterial resistance was demonstrated with the use of the efflux pump inducers, such as the bile salts (Lin et al. 2005). Different effects of bile salts related to resistance to structurally diverse antimicrobials were also reported by Hannula and Hanninen (2008) and Mavri and Smole Možina (2013c) although the reasons for these effects are as yet unclear. Studies with the use of EPIs and efflux pump inducers can additionally be confirmed using genetic approaches, with mutants lacking functional genes that code for the efflux pump proteins (Mavri and Smole Možina 2012; Pumbwe et al. 2005). The investigation of the mutants of *C. jejuni* NCTC 11168 that lacked *cmeB* and *cmeF* genes, which code the inner membrane efflux transporters, as well as *cmeR* mutant (Klančnik et al. 2012), showed that the CmeABC efflux pump has a predominant role in erythromycin resistance. However, this was not shown for ciprofloxacin resistance (Mavri and Smole Možina 2012).

15.3 Biocide Resistance in *Campylobacter jejuni* and *Campylobacter coli*

In the last two decades, many data about biocide resistance have been published for *Salmonella*, *Listeria*, *Pseudomonas* and *Escherichia coli* (Karatzas et al. 2008; Sheridan et al. 2012; To et al. 2002; Tattawasart et al. 1999). However, biocide resistance in *Campylobacter* spp. was studied only recently. Mavri et al. (2012) examined the prevalence of resistance among *C. jejuni* and *C. coli* isolates from food, animal, human and environmental water sources to a broad range of biocides: triclosan (TLN), benzalkonium chloride (BC), cetylpyridinium chloride (CPC), chlorhexidine diacetate (CHA) and trisodium phosphate (TSP). The anionic surfactant sodium dodecyl sulphate (SDS), the microbicide with protein denaturing potency that is added to a large class of cleaning agents to assist in the cleaning (Smulders et al. 2002), was also included. The results of this study demonstrated biocide resistance among thermotolerant *Campylobacter* spp.. In contrast to antibiotic resistance, there are no literature data about breakpoints of resistance levels to biocides. Some authors observed and classified distinct biocide resistance phenotypes in different bacteria, like *Salmonella*, *Pseudomonas* and *Listeria monocytogenes* (Braoudaki and Hilton 2005; Copitch et al. 2010; Webber et al. 2008; Aase et al. 2000; To et al. 2002; Lear et al. 2006). However, the MICs differ among different bacteria, as well as among different testing methods. The range of MICs for antibiotics and biocides (in twofold increases) for tested *Campylobacter* isolates (Mavri et al. 2012) and the breakpoints for antibiotics according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI 2007) are shown in Table 15.1. Similar as for erythromycin resistance, *C. coli* strains were in general more resistant to TLN than *C. jejuni* strains. Conversely, the level of BC resistance was higher in *C. jejuni* than in *C. coli*. There were no significant differences among *C. coli* and *C. jejuni* for other tested biocides.

Due to the possibility that biocide resistance is linked to antibiotic resistance in bacteria, Mavri et al. (2012) investigated cross-resistance to antibiotics and biocides among tested *Campylobacter* strains. The overall level of biocide resistance was not significantly different in strains with different erythromycin and ciprofloxacin

Table 15.1 The range of MICs of biocides and antibiotics for tested *Campylobacter* strains (Mavri et al. 2012) and breakpoints for antibiotics (according to CLSI 2007)

Biocide/antibiotic	Concentration	Resistance breakpoint
Triclosan (µg/mL)	8–64	
Benzalkonium chloride (BC) (µg/mL)	0.016–4	
Cetylpyridinium chloride (CPC) (µg/mL)	0.25–4	
Chlorhexidine diacetate (CHA) (µg/mL)	0.063–2	
Trisodium phosphate (TSP) (mg/mL)	2–16	
Sodium dodecyl sulphate (SDS) (µg/mL)	128–1024	
Erythromycin (µg/mL)	0.125–1024	≥32
Ciprofloxacin (µg/mL)	0.125–128	>4

resistance levels, with an exception of SDS. Interestingly, a higher level of SDS resistance was found in erythromycin-sensitive than in erythromycin-resistant *Campylobacter* isolates. Similar situation was observed for bile salts; erythromycin-resistant *Campylobacter* strains showed lower tolerance to bile salts, compared to erythromycin-sensitive isolates (Mavri and Smole Možina 2013c). This might be related to the impaired fitness in macrolide-resistant *Campylobacter* isolates.

However, the study of Mavri et al. (2012) does not provide evidence to confirm that tolerance to biocides is connected to antibiotic resistance in *Campylobacter* spp..

15.3.1 The Involvement of Efflux Mechanism in Biocide Resistance

Since the efflux mechanisms are responsible for the transportation of a wide range of unrelated toxic compounds, these can be the link between biocide and antibiotic resistance. It has been demonstrated on the basis of the effects of putative EPIs, PA β N and NMP, as well as of the efflux pump inducers, bile salts and sodium deoxycholate, that efflux mechanisms have important roles in the resistances of *C. jejuni* and *C. coli* to a broad range of biocides, TLN, BC, CPC, CHA, TSP and SDS, as well (Mavri and Smole Možina 2012, 2013b). Both of tested EPIs partially reversed the resistances to all of these biocides, wherein different PA β N and NMP target preferences were seen, with similar situations reported previously (Kern et al. 2006; Pannek et al. 2006; Hannula and Hanninen 2008). This might be related to distinct mechanisms of these EPIs in their inhibition of the efflux systems. Another explanation is that PA β N and NMP have different activities towards the different efflux pumps, which was also demonstrated through the comparisons of the effects of these EPIs using the mutants lacking functional genes that code for the efflux pump proteins.

In addition, Mavri and Smole Možina (2013b) highlighted the role of efflux mechanisms in biocide resistance of *Campylobacter* through the use of efflux pump inducers, bile salts and sodium deoxycholate. Both of these increased the biocide resistance in a high proportion of the tested strains although this was not the case for TLN and TSP resistance. Active efflux mechanisms have therefore distinct roles in susceptibility to antimicrobials from diverse classes, which might be related to their different mechanisms of action. On the basis of restored sensitivity in the presence of EPIs that block different types of efflux pumps, it has been shown previously that more than one type of active efflux is involved in bacterial resistance to dissimilar biocides (Braoudaki and Hilton 2005). Another possible explanation is that different antimicrobials might compete with each other for the binding sites on the efflux pumps (Kern et al. 2006; Martins et al. 2009b). The antimicrobial agent, like bile salts that are presented in campylobacter's natural habitat, might bind to the efflux pump more successfully than another and therefore lead to increased susceptibility to other antimicrobials.

To investigate the contributions of the CmeABC and CmeDEF efflux pumps to biocide resistance, Mavri and Smole Možina (2012, 2013b) analysed the *cmeB*, *cmeF* and *cmeR* mutants of *C. jejuni* NCTC 11168, for susceptibility to the biocides in the presence and absence of the two EPIs (PA β N and NMP) and two efflux pump inducers (bile salts and sodium deoxycholate). In contrast to erythromycin resistance, where the CmeABC pump plays a predominant role, this efflux pump appeared to function interactive with at least one other non-CmeABC efflux systems. The decreases in the biocide resistances with these *cmeB* and *cmeF* mutations, the additional effect of both EPIs and efflux pump inducers, and the increases of the biocide resistances upon *cmeR* inactivation, all suggest that at least two of the efflux systems, CmeABC and CmeDEF, are involved in biocide resistance. Indeed, the exclusion of one of these efflux systems can lead to increased activity of another efflux system, or even the activation of other resistance mechanisms. These findings coincide with a previous finding on the interactive involvement of the CmeABC and CmeDEF efflux pumps in the extrusion of toxic compounds in *C. jejuni*. Inactivation of *cmeDEF* has led to increased expression of *cmeABC*, whereby CmeDEF might be primarily responsive to certain conditions (Akiba et al. 2006).

15.3.2 The Instability of Biocide Resistance in *Campylobacter*

The instability of erythromycin resistance phenotype in *Campylobacter* has been reported by several authors (Caldwell et al. 2008; Luangtongkum et al. 2009; Mavri et al. 2012). Besides unstable erythromycin resistance, Mavri and Smole Možina (2013b) observed also a large instability of biocide resistance among tested *Campylobacter* strains. The reasons for this appearance of reduced or enhanced tolerance to biocides are as yet not clear. *Campylobacter* lack many of the adaptive responses to environmental stresses (Parkhill et al. 2000; Park 2002; Zhang et al. 2006). Despite these defects, it can dynamically adapt and survive environmental stress (Klančnik et al. 2006, 2009; Zhang et al. 2006). Moreover, *Campylobacter* also lack many genes that encode components of the DNA repair systems that are present in other bacteria (Parkhill et al. 2000). This would explain the high mutation rates in this bacterium, which is also linked to its ability to acquire exogenous DNA by natural transformation and to easily undergo genetic recombination (Dorrell et al. 2001). This high genetic heterogeneity is believed to be involved in the survival strategy that helps the organism to adapt to sudden and unforeseen stress conditions (Jayaraman 2011). It has been demonstrated that bacteria are capable to adapt to environment that remains constantly noxious by the activation of a mutator system, which results in the accumulation of mutations that render the organism MDR (Chopra et al. 2003; Martins et al. 2009a). The accumulation of these mutations was accompanied with restoration of efflux activity, which might also be an explanation for the appearance of enhanced tolerance to biocides and acquired erythromycin resistance, not mediated by chromosomal mutation.

15.4 The Adaptive Resistance to Sub-inhibitory Concentrations of Biocides

Biocides are widely used to prevent bacterial contamination in food-processing and/or in clinical environments (McDonnell and Russell 1999; Møretrø et al. 2011). However, elimination of bacteria has proven difficult for several reasons. Underdosing of applied disinfectants and insufficient cleaning before disinfection can significantly reduce the efficacy of disinfectants. Under such conditions, bacteria are regularly exposed to sub-lethal concentrations of disinfectants, and this can lead to adaption of initially susceptible bacteria (Davidson and Harrison 2002).

The ability of bacteria, such as *Pseudomonas*, *Salmonella*, *E. coli* and *L. monocytogenes*, to survive in increasing concentrations of biocides is well known. Adaptation to biocides by serial passage in increasing sub-inhibitory concentrations of biocides leads to reductions in the susceptibilities to dissimilar disinfectants or antimicrobial compounds (Abdel Malek and Badran 2010; Braoudaki and Hilton 2005; Langsrud et al. 2004; Loughlin et al. 2002; Tattawasart et al. 1999; Thomas et al. 2000). It has also been demonstrated that *Campylobacter* is able to survive cleaning and disinfection procedures (Peyrat et al. 2008a) and that specific genotypes, recovered after cleaning and disinfection, may be particularly adapted to survive such conditions (Peyrat et al. 2008b). However, those strains did not show an increased resistance to antimicrobial compounds compared to isolates collected before cleaning and disinfection. The ability of *C. jejuni* and *C. coli* to adapt to sub-inhibitory concentrations of biocides was recently demonstrated by Mavri and Smole Možina (2013a) (see the flow chart of experiment in Fig. 15.1). Repeated stepwise exposure to gradually

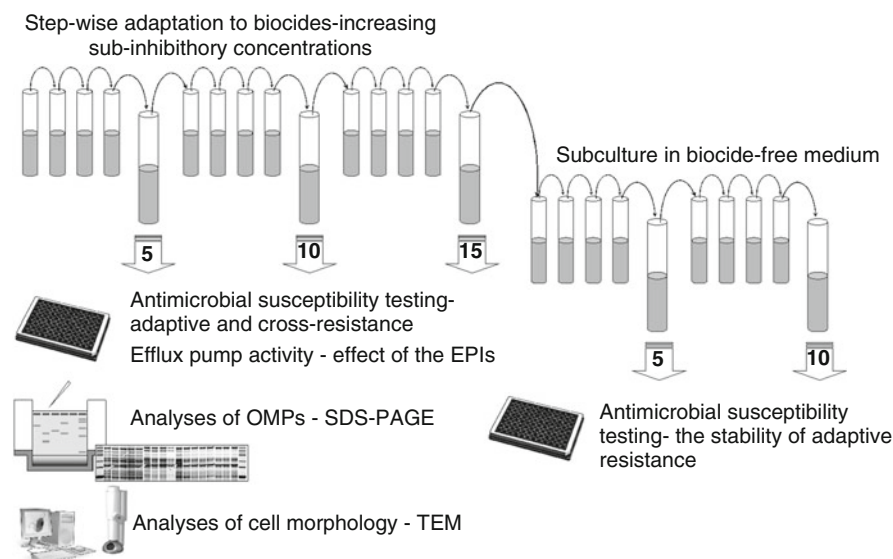


Fig. 15.1 The flow chart of adaptation experiment—stepwise exposure of *Campylobacter jejuni* and *C. coli* strains in increasing concentrations of different biocides

increasing concentrations of five biocides, as TLN, BC, CPC, CHA and TSP, over 15 days resulted in partially enhanced tolerance to biocides itself, and to other biocides and antibiotics. However, in some of adapted *Campylobacter* strains, this was not the case, as these strains were actually more susceptible to the antimicrobials tested after stepwise exposure to biocides than the parent strains. A rapid decrease in antimicrobial resistance following exposure to CHA has also been reported for *Pseudomonas aeruginosa* (Thomas et al. 2000). The reasons for these susceptibilities are as yet not clear. A possible explanation is that these susceptibilities might result from the alterations in the cell envelope, which promote the uptake of biocides across the outer membrane (Tattawasart et al. 2000a).

15.4.1 Contribution of Efflux Mechanism in Adaptive Resistance

Active efflux is one of the mechanisms involved in establishment of adaptive resistance to a variety of antimicrobials in bacteria (Braoudaki and Hilton 2005; Li and Nikaido 2004; Langsrud et al. 2004; Marquez 2005). In the study of Mavri and Smole Možina (2013a), the role of active efflux as an adaptive resistance mechanism to biocides was assessed according to the inhibitory effects of five different EPIs, PA β N, NMP, cyanide 3-chlorophenylhydrazine (CCCP), verapamil and reserpine that block different types of efflux pump systems. These EPIs partially restored the susceptibility to all of the tested biocides in adapted strains, with exception of reserpine that showed no effect to these susceptibilities. Active efflux is thus one of the mechanisms involved in adaptation to biocides in *C. jejuni* and *C. coli*. This enhanced tolerance to biocide is mediated by more than one type of active efflux mechanism, wherein the RND efflux system has the most important role. However, the addition of three EPIs, verapamil, CCCP and reserpine, had unexpected effects for TLN, CHA and TSP resistance (MICs increased, instead of reduced). This indicates that the exclusion of one of these efflux systems can lead to increased activity of another efflux system, or even to the activation of other resistance mechanisms. It has been shown here that active efflux mechanisms have a distinct role in the susceptibilities to biocides from diverse classes, which might also be related to their different mechanisms of action. In spite of their diverse activities, they disrupt the same target, the cell membrane. The resistance to these biocides might be due to membrane structure alterations, as has been observed previously (Tattawasart et al. 2000a; Yuk and Marshall 2006).

15.4.2 Outer Membrane Protein Alterations

It has been demonstrated by several authors that alterations in the outer membrane are involved in establishment of adaptive resistance of bacteria, including the content of lipopolysaccharide and outer membrane proteins (OMPs) (Loughlin et al. 2002;

Tattawasart et al. 2000a; Yuk and Marshall 2006). Similar situation was reported also for *Campylobacter* (Mavri and Smole Možina 2013a). A decrease and loss of the content of OMP was observed after repeated exposure of *C. jejuni* and *C. coli* strains to biocides. All of the tested *Campylobacter* strains that partially or completely lost their OMP content showed no adaptive resistance to the tested biocides, or were even more susceptible than the parent strains. Moreover, some adapted strains, that showed increased tolerance to biocides and/or antibiotics, showed some particular changes in their OMP: lost the 66-kDa protein band and increased proportion of 96 kDa protein band. According to these noted changes in the OMP profiles and their content in the adapted strains, this could be an additional mechanism involved in adaptation of *Campylobacter*. However, each of the adapted strains showed different alterations in their OMP profile.

15.4.3 Morphological Changes of Bacterial Cells

It has been demonstrated for *Campylobacter* that the acquisition of biocide resistance was associated with alterations in cell morphology and with the integrity of the cell membrane (Mavri and Smole Možina 2013a). Electron micrographs revealed several morphological changes in the adapted strains (Fig. 15.2). The adapted cells were elongated (Fig. 15.2b, d) compared to their parent strains (Fig. 15.2a). Some adapted cells had thicker cell envelope (Fig. 15.2f). The loss of the spiral form was noted (Fig. 15.2c, e), and one or both bipolar flagella also disappeared after exposure (Fig. 15.2c–e). It has been demonstrated previously in *Pseudomonas stutzeri* and *L. monocytogenes* that the adaptive resistance is directly related to the length of the cells and to the roughness of the cell surface (Tattawasart et al. 2000b; To et al. 2002). Previous studies have also shown that the cell envelope is thicker in adapted *P. stutzeri* than in its sensitive parent isolate (Tattawasart et al. 2000b). Moreover, Almofti et al. (2011) have recently shown that *C. jejuni* strains exposed to macrolides lost their flagellar filaments and motility. In addition, the exposure to biocides resulted in the coccoid form of cells (Fig. 15.2g). Exposure to biocides also produced progressive cellular damage, seen as blebbing of the outer membrane (Fig. 15.2e, i, j), with an occasional larger break that resulted in cytoplasmic leakage (Fig. 15.2h, k, l), which was associated with the increased susceptibility after exposure to these two biocides. However, these morphological changes differ between the different biocides, as well as between the individual strains. Adaptation is unique for each strain of *Campylobacter* and does not result from a single mechanism that is shared by the whole species.

Conclusion

Efflux was confirmed as an important mechanism for biocide and antibiotic resistance in *Campylobacter*, but more than one type of efflux pump was involved and specific differences were found when different antibiotics (e.g. erythromycin and

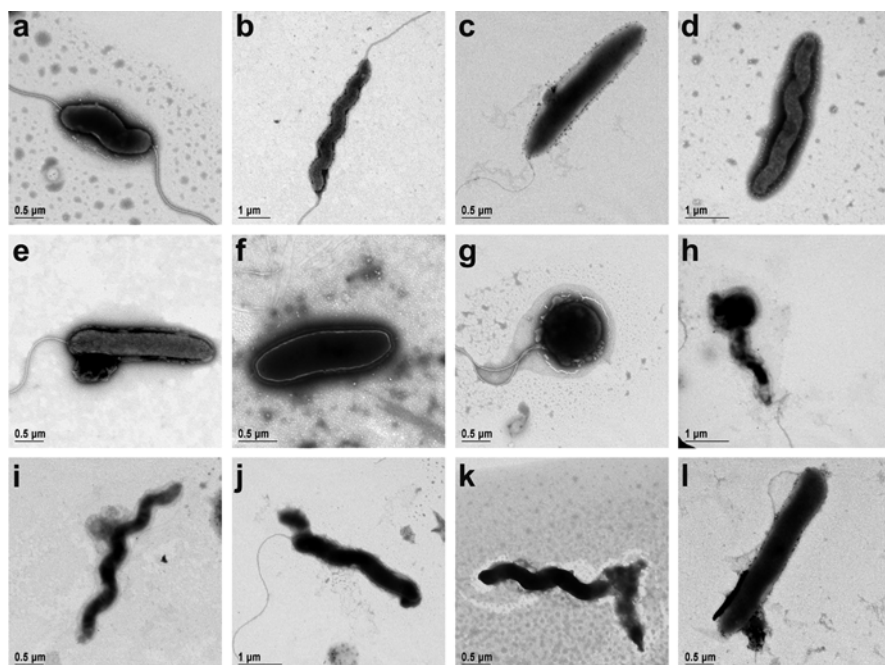


Fig. 15.2 Representative transmission electron micrographs of control and adapted *C. jejuni* and *C. coli* strains. (a) *C. jejuni* control strain; (b) CHA-adapted *C. jejuni*; (c) TLN-adapted *C. coli*; (d) CHA-adapted *C. jejuni*; (e) TLN-adapted *C. coli*; (f) CPC-adapted *C. jejuni*; (g) CPC-adapted *C. jejuni*; (h) CPC-adapted *C. jejuni*; (i) CPC-adapted *C. jejuni*; (j) TSP-adapted *C. jejuni*; (k) CHA-adapted *C. jejuni*; (l) CHA-adapted *C. coli*

ciprofloxacin) were tested. Repeated exposure of *C. jejuni* and *C. coli* to biocides resulted in partial increases in tolerance to biocides and antibiotics. Different mechanisms were found to be involved in adaptation to biocides including active efflux, alterations in the outer membrane protein profiles and morphological changes. It was shown that this adaptation does not result from a single species-specific mechanism. More specific studies still remain a challenge for the future since the involvement of biocides as selectors and/or inducers of mechanisms responsible for the emergence of strains with reduced susceptibility to biocides and antibiotics has been shown in several recent studies, mainly of Gram-negative bacteria (Davin-Regli and Pagès 2012; Lerma et al. 2013; Sothill et al. 2013). In practice, the driving force behind selection of resistant bacterial populations are sub-lethal concentrations of the antimicrobial agents in the environment, so their prudent use is an urgent need to prevent microbial resistance development and preservation of the antimicrobial efficacy of the agents, which are currently in use.

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Chapter 16

Food Cold Chain Management and Optimization

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16.1 Introduction

Effective control and management of the cold chain of chilled and frozen food products is important for their commercial viability, since deviations from specifications with regard to the temperature conditions that often occur can be detrimental to their quality and safety (Gwanpua et al. 2014; Taoukis et al. 2012). The cold chain is defined as the set of refrigeration steps that maintain the quality and safety of the food product from production to consumption by the consumers. FRISBEE (<http://frisbee-project.eu>) is a Food Refrigeration Innovation for Cold Chain research IP European project. Within FRISBEE, a web-based platform (hosted in the link <http://www.frisbee-project.eu/coldchaindb.html/>) has been built for data collection. Data from industry, cold chain parties (distributors, retailers) and consumer surveys, including all stages of the cold chain (from production to consumption), were collected. More than 14,000 actual t – T profiles have been contributed to the database. The use of the Cold Chain Database (CCD) temperature profiles allows the calculation of the remaining shelf life of a specific food product at different stages of the cold chain corresponding to a specific t – T profile, based on known shelf-life kinetic data.

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16.2 Monitoring the Cold Chain and Shelf Life

It is important that the shelf life of any food product is monitored on a regular basis and in such a way that is compatible with the shelf life in question, production volume and environmental conditions to which the product is exposed (or even abused) up to the point of consumption (Fu and Labuza 1997). It has been reported that a substantial portion of frozen and chilled products are exposed to temperatures that deviate significantly from the recommended range (Fig. 16.1).

Taking into account the multiple parameters that may affect the efficiency of the real cold chain and the importance of an adequately cold logistic path for product safety and quality, it becomes evident that effective monitoring and control of the cold chain is a prerequisite for reliable quality management and optimization. Effective temperature control is essential in all stages of the food cold chain and can be achieved through improved equipment, quality assurance systems and increased operator awareness.

The current philosophy for food quality optimization is based on the introduction of temperature monitoring in an integrated, structured quality assurance system, based mostly on prevention, through the entire life cycle of the product (Taoukis et al. 2012). Ideally, what would be needed is a cost-effective way to either maintain temperature in recommended ranges or to individually monitor the temperature conditions of food products throughout distribution (e.g. by Time–Temperature Integrators, TTIs) in order to indicate their real quality state (Taoukis 2010). This could lead to an effective control of the distribution, optimized stock rotation and waste reduction, giving at the same time some meaningful information on the product remaining shelf life.

The main shelf-life determining post-processing parameter in the cold chain of foods is temperature. An efficient quality and safety assurance system should rely on prevention through monitoring, recording and controlling of critical parameters during the life cycle of a particular product from production to the point of consumption. Temperature conditions in the chilled distribution chain

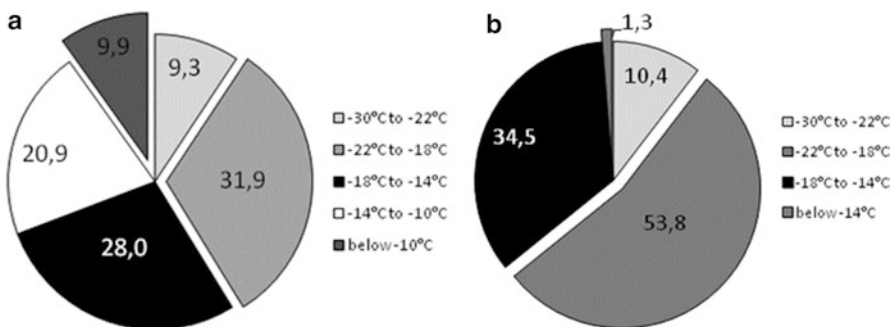


Fig. 16.1 Temperature distribution in: (a) domestic freezers and (b) retail display (source: Cold Chain Database (2013), FRISBEE project)

determine the risk potential, the shelf life and final quality of chilled products processed and packed under Good Manufacturing Practices and Good Hygiene Practices. Since in practice significant deviations from specified conditions often occur, temperature variability has to be taken into account for cold chain control and any logistics management system that aims on product quality optimization at the consumer's end.

16.2.1 Cold Chain Database

The development of a *Cold Chain Database (CCD)* web-based tool could significantly contribute to the determination of the weak links of the cold chain for a significant number of food products. Stage and product specific t - T information was used for the quantitative description of cold chains within the FRISBEE project (Food Refrigeration Innovations for Safety, consumers Benefit, Environmental impact and Energy optimization along the cold chain in Europe). The contributed data of the cold chain allows simulation of realistic cold chain scenarios based on actual cold chain data and can lead to corrective actions aimed to optimizing efficiency and commercial shelf life.

A systematic data collection for identification and evaluation of the weak links of the cold chain for different types of chilled and frozen products is considered as a necessity from food producers and retailers. Although it has potential positive effect on recognizing the weak links of the CCD, there is no such a systematic data collection performed up to now. Within FRISBEE project, one of the objectives was the development of the first European CCD for time-Temperature (t - T) data collection, maximizing information retrieval with user-friendliness hosted in the link <http://www.frisbee-project.eu/coldchaindb.html>. The database was developed during the first of the 4 years of the project and is dynamically enriched with data. Priority is the collection of as much data as possible. To allow for maximum data exploitation and evaluation, specifications for input data are introduced but in such a way that it does not discourage prospective contributors. A menu-driven web-based questionnaire accompanies or incorporates the basic information that in all cases has the form of t - T data. Additionally, some basic information apart from t - T profile are specified to the extent it is available by the data provider such as the type of food product, the packaging, the recommended storage conditions, the stage (step) of the cold chain, geographical and seasonal information, information concerning the storage and distribution equipment, specifications of data collecting equipment, the position of the data collecting equipment and the format the contributed data should have. The processing of these data addresses the needs of the FRISBEE project and the needs of the users. Therefore, the web-based database provides information (output) useful and usable for the users (including data providers and ultimately European researchers, regulators, industry and even the consumer). In the developed CCD, all contributed t - T profiles were organized. In this platform, all t - T files were correlated to the meta-data of the original contributed t - T profile.

Better consumer protection regulation, better monitoring of consumer markets and enhancing product safety through the development of market monitoring tools are some of the targets of the FRISBEE project to be performed. The database is also a valuable source of information for European industry to be able to benchmark and compare their process efficiency with others. The developed database is easily and freely accessible to users including research institutes, industries and food stakeholders.

The work within FRISBEE directly addresses and contributes to consumer needs for:

- Increased safety of food through enhanced temperature control throughout the food cold chain.
- Enhanced food quality and nutritional content of chilled and frozen foods by enhanced temperature control.
- Enhanced quality control systems for the cold chain.
- A more sustainable cold chain.
- Economic but high-quality foods.

16.2.2 Use of the Cold Chain Database

CCD (hosted in the link <http://www.frisbee-project.eu/coldchaindb.html>) has been constructed in order to develop a user-friendly online platform where collected data from all cold chain stages can be retrievable and available to be used from candidate users. The CCD is hosted and linked to FRISBEE public website (www.frisbee-project.eu) as depicted in Fig. 16.2a. One is able to retrieve t - T profiles of specific products along the cold chain using search criteria such as stage/step of the cold chain, food storage temperature range, characterization of food, food product, etc. (Fig. 16.2b). He may also retrieve all his submitted profiles. All received data in the Cold Chain Data Collection platform were further processed in such a way that the output of the web-based database includes:

- Actual t - T profiles
- Temperature distribution for the selected profile
- Mean, min and max value of temperature for the whole t - T profile
- Effective temperature of the t - T profile: To demonstrate the integrated effect of the temperature variability on product quality, the term of the effective temperature T_{eff} is introduced. Effective temperature is defined as the constant temperature that results in the same quality value as the variable temperature distribution over the same time period and is based on the Arrhenius model and integrates, in a single value, the effect of any variable temperature profile $T(t)$ (Eq. 16.1):

$$T_{\text{eff}} = \int_0^t - \frac{E_a \cdot T_{\text{ref}}}{R \cdot T_{\text{ref}} \cdot \ln \frac{k(T(t))}{k_{T_{\text{ref}}}} - E_a} \cdot dt \quad (16.1)$$

Up to August 2014, the CCD consists of more than 14,000 t - T profiles and is being continuously updated with new data uploaded from an expanding network of contributors. In this database, the user can build a specific sequence of cold chain stages for specific food product based on user defined search criteria.

16.2.3 Cold Chain Predictor Software

Application of an optimized management for food products distribution requires either continuous monitoring and control of storage conditions from production to consumption (TTIs application, RFID monitoring) or knowledge of the weak links of the cold chain (FRISBEE CCM tools) so as corrective actions to be conducted.

Cold Chain Predictor (CCP) is a software tool designed in the framework of FRISBEE project. It is a stand-alone tool that the user may download from FRISBEE CCD. The purpose of this tool is to simulate a cold chain by building a t - T history from the contributed profiles in the CCD. This tool is based on all CCD contributed t - T profiles obtained for different food products along the European cold chain. The FRISBEE CCP (FRISBEE_CCP) software allows the user to estimate the distribution graph of (effective) temperature for a specific stage of a selected food product and to calculate the remaining shelf life (SL_R) of the food product at different stages of the cold chain if quality decay data are known.

The FRISBEE CCP software was designed to reproduce by Monte Carlo simulation the most likely time/temperature distribution for each defined stage of the cold chain and to estimate for a selected food product, going through the cold chain, the SLR after each individual stage.

Prior to the use of the software, the user should login to the FRISBEE CCD and build a specific sequence of cold chain stages for specific food product. In the next step, the user can define the number of iterations for the Monte Carlo simulation in order to build the representative profile. Monte Carlo simulation (Metropolis and Ulam 1949) is a very useful technique to facilitate data from the CCD. This numerical approach is based on the generation of hypothetical scenarios in terms of the temperature values reported during all the segments of the cold chain by substituting the entire range of values. The default (suggested) number of iterations is 10,000. The Monte Carlo simulation is performed using the file retrieved from the online platform of the CCD. The simulation generates a representative time-temperature profile where each cold chain stage is represented by an isothermal step. The temperature of this step (cold chain stage) represents the most probable effective temperature of the t - T profiles (pre-estimated for each profile when building the cold chain in the CCD) for the specific stage of cold chain. The Standard Deviation—to represent the most probable range of deviation above and below the effective temperature—is also calculated and presented in the graph for each stage of the representative profile. The duration of the step is also estimated applying Monte Carlo simulation, as the most probable duration for each step. The total duration of the representative profile can be defined by the user when changing the value

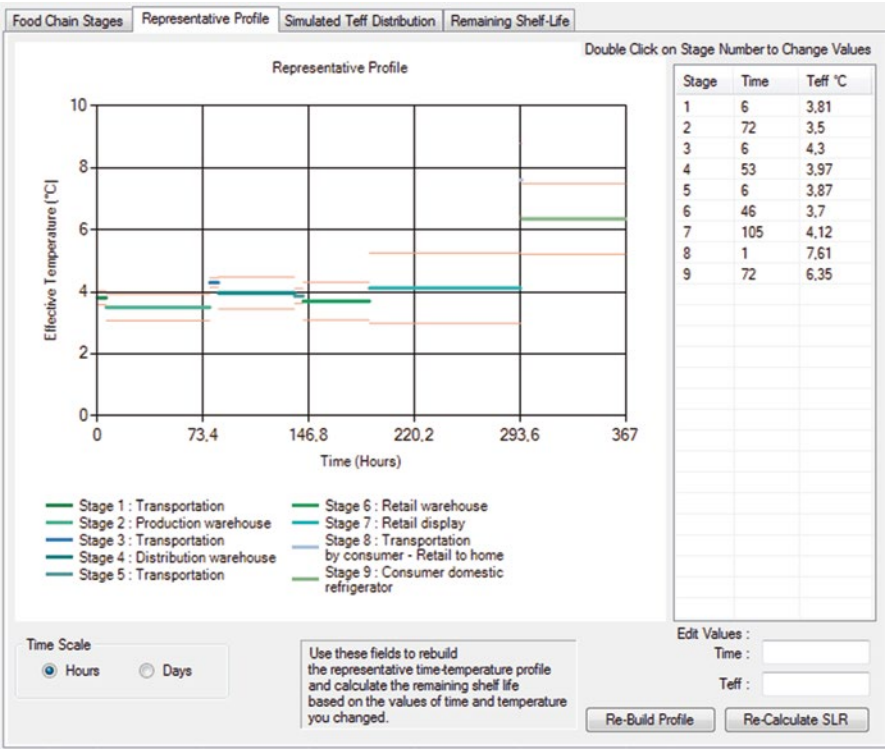


Fig. 16.3 Representative time–temperature profile generated by Monte Carlo simulation through the Cold Chain Predictor Software using the cold chain profiles for milk and milk products retrieved from the CCD for nine successive cold chain stages

in the time box. The duration of each stage of the profile is accordingly modified (modification of time and temperature for each of the stages is possible).

After the representative profile has been built (Fig. 16.3), the user can determine the product quality status, in terms of SL_R , at the different stages of the cold chain (Fig. 16.4). The quality status determination can be performed by using either (a) available kinetic data or (b) user’s own shelf-life data.

(a) Using kinetic data

The user can select among different food product types that are available within the software. When selecting a food product, automatically the software recalls the kinetic characteristics of a typical quality index concerning the selected food product. The kinetic characteristics are retrieved from the corresponding kinetic models that have been published at peer reviewed scientific journals. Once the user selects the Food Product Type, the Quality Index Type is automatically filled in the next tab.

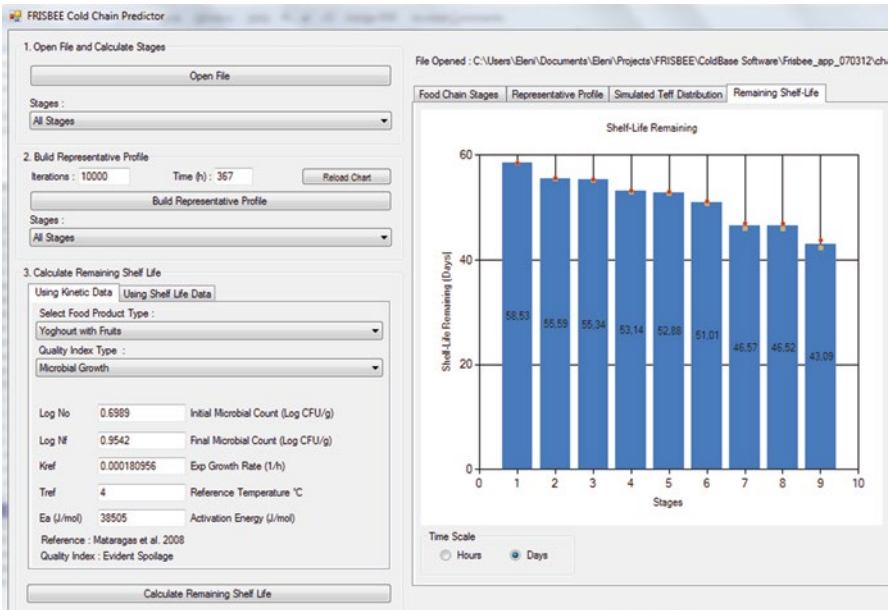


Fig. 16.4 Calculated remaining shelf life of milk product (yoghurt with fruits) at each stage of the cold chain based on the built representative profile using kinetic data

The decision for corrective actions may be applied after the estimation of the SL_R of any food product at the end of any cold chain stage. Prerequisite is the knowledge of the kinetic characteristics of the dominant deterioration factors for any food. Based on the above kinetic characteristics, the SL_R of any food product may be calculated according to Eqs. 16.2–16.4, taking into account the three types of quality index: microbial growth, chemical index, sensory index (Labuza 1984).

Microbial Growth

The SL_R at different cold chain stages is calculated considering that microbial growth follows first-order kinetics, using Eq. 16.2:

$$SL_R = \frac{\log N_F - \log N_o - k_{ref} \cdot \exp \left[-\frac{E_a}{R} \cdot \left(\frac{1}{(T_{eff} + 273.16)} - \frac{1}{(T_{ref} + 273.16)} \right) \right] \cdot t}{k_{ref}} \quad (16.2)$$

where SL_R : Shelf life remaining at reference temperature (h); $\log N_F$: Log of acceptable cell concentration at the end of product shelf life; $\log N_o$: Log of cell concentration at time of entering the cold chain; k_{ref} : Rate constant of microbial growth (h^{-1}); E_a : Activation energy (J/mol); R : Universal constant

(8.314 J/mol K); T_{eff} : Effective temperature of the cold chain stage (°C); T_{ref} : Reference temperature (°C); t : time spent at the cold chain (h).

Chemical Index

The SL_R at different cold chain stages is calculated considering that chemical index either increase or loss follows first-order kinetics, using Eq. 16.3:

$$SL_R = \frac{\ln \left(V_o \cdot \exp \left(-k_{\text{ref}} \cdot \exp \left(-\frac{E_a}{R} \cdot \left(\frac{1}{(T_{\text{eff}} + 273.16)} - \frac{1}{(T_{\text{ref}} + 273.16)} \right) \right) \cdot t \right) - \ln \left(\frac{100 \pm a}{100} \cdot V_o \right) \right)}{k_{\text{ref}}} \quad (16.3)$$

where, SL_R : Shelf life remaining at reference temperature (h); V_o : Concentration of a quality index (i.e. vitamin, enzyme, chemical compound) at the time of entering the cold chain; a : % of acceptable increase or loss of a specific quality index at the end of product shelf life; k_{ref} : Rate constant of increase/loss of the specific quality index at the reference temperature (h^{-1}); E_a : Activation energy (J/mol); R : Universal constant, (8.314 J/mol K); T_{eff} : Effective temperature of the cold chain stage (°C); T_{ref} : Reference temperature (°C) and t : time spent at the cold chain (h).

Sensory Index

The SL_R at different cold chain stages is calculated considering that sensory index loss follows zero-order kinetics, using Eq. 16.4:

$$SL_R = \frac{S_o - k_{\text{ref}} \cdot \exp \left[-\frac{E_a}{R} \cdot \left(\frac{1}{T_{\text{eff}} + 273.16} - \frac{1}{T_{\text{ref}} + 273.16} \right) \right] \cdot t - S_F}{k_{\text{ref}}} \quad (16.4)$$

where SL_R : Shelf life remaining at reference temperature (h); S_o : Score of a sensory index at the time of entering the cold chain; S_F : Lower acceptable score of a sensory index corresponding to the end of product shelf life; k_{ref} : Rate constant of sensory index decrease (h^{-1}); E_a : Activation energy (J/mol); R : Universal constant (8.314 J/mol K); T_{eff} : Effective temperature of the cold chain (°C); T_{ref} : Reference temperature (°C) and t : time spent at the cold chain (h).

(b) Using shelf-life data

In most cases, especially in food industries, the knowledge of the kinetic characteristics of their food products is not sufficiently available or retrievable. The option of using own shelf-life data for their products is more realistic, and it is allowed from the software (FRISBEE CCP). The information required is the product shelf life at least at two different storage temperatures.

The calculation of SL_R based on shelf-life data is performed using Eq. 16.5:

$$SL_R = \exp \left[\frac{\ln(SL_B) - \ln(SL_A)}{\frac{1}{T_B + 273.16} - \frac{1}{T_A + 273.16}} \cdot \frac{1}{T_{ref} + 273.16} \right] \quad (16.5)$$

where SL_R : Shelf life remaining at reference temperature (d); SL_A : Shelf life (days) at storage temperature A ($^{\circ}\text{C}$); SL_B : Shelf life (days) at storage temperature B; T_A : Storage temperature A ($^{\circ}\text{C}$); T_B : Storage temperature B ($^{\circ}\text{C}$) and T_{ref} : Reference temperature ($^{\circ}\text{C}$).

Overall useful information from the CCD may be retrieved and used to run realistic scenario for the behaviour of food products along the cold chain avoiding costly and time-consuming field tests. The FRISBEE CCP offers thus the potential to effectively manage and improve cold chain weak links using appropriate shelf-life decision systems leading to an optimized handling of products in terms of both safety and quality. Using this tool, one can efficiently manage the food cold chain and undertake corrective actions at predetermined important stages of the cold chain, while simultaneously it is a cost-efficient approach since no consumables are needed. The described approach (FRISBEE tools) could be used in combination to monitoring tools such as TTI smart labels (described in the next section) combining the advantages of each approach such as corrective actions before product distribution and storage (FRISBEE tools) and during complete cold chain, resulting in optimized shelf-life management during the cold chain.

16.3 Application of a TTI-Based Cold Chain Management System on Shelf-Life Monitoring of Food

Current packaging technology aims to provide more than the protective functionality required for ensuring the safety and integrity of food products. Intelligent packaging imparts passive protection, contributes to shelf-life extension and provides valuable information about the quality of food products for better management of the food chain, reduction of food waste and increased protection of the consumer. The “intelligence” of packaging refers to its ability to communicate information about the requirements known to ensure product quality, like package integrity (leak indicators) and time–temperature history of the product (Taoukis 2010). A cost-efficient way to monitor and continuously communicate the temperature conditions of individual food products throughout distribution would be required in order to indirectly indicate actual state in terms of quality. TTIs could be effective tools to fulfil this requirement. TTI can show an easily measurable, time–temperature dependent change that cumulatively reflects the time–temperature history of the food product. Prerequisite for application of TTI is the systematic kinetic modelling of the temperature dependence of shelf life of the target food products. Similar kinetic study

is needed for the TTI response (Taoukis and Labuza 2003). Based on having available reliable models of food product shelf-life and information on the kinetics of a TTI's response, temperature can be monitored, recorded and translated into its effect on quality, all the way from production to the consumer's table.

Despite the potential of TTIs to substantially contribute to improving food distribution, reducing food waste and benefiting the consumer with more meaningful shelf-life labelling, their applications up to now have not lived up to initial expectations. Suppliers and earlier studies have been ineffective in establishing a clear methodology correlating TTI responses as measures of food quality throughout the cold chain. The most often underestimated requirement when developing and applying a TTI has been the need for acquiring systematic knowledge of the loss of quality during shelf life of the food system to be monitored, and a method for expressing quantitatively as accurately as possible with kinetic models the important quality-determining phenomena (Taoukis 2010).

In the IQ-Freshlabel (www.iq-freshlabel.eu), European project enzymatic and photochromic TTI were developed and tested for frozen products. Methodology was developed for selection of the optimum TTI design of specific frozen seafood products and their application was validated in cold chain simulating trials and in pilot studies. The selection and use of the optimum TTI for a particular product could lead to realistic control of the cold chain, while reliable estimation of the quality status and the remaining shelf life could be performed, allowing better management and optimization from production to the point of consumption.

16.4 Case Studies: Application and Management of Frozen Food Products

16.4.1 Shelf-Life Management of Frozen Spinach Leaves

The quality deterioration of frozen spinach leaves is studied. Vitamin C, chlorophyll (a, b and total), texture and sensory attributes are measured as frozen spinach quality indices (Dermesonluoglu et al. 2014; Giannakourou and Taoukis 2003; Reid et al. 2003; Martins and Silva 2002). Kinetic models of the selected quality indices were developed to determine the frozen spinach quality loss and calculate product remaining shelf life. Validated kinetic models of quality loss are then applied in the evaluation, control and management of frozen spinach in the cold chain. Storage experiments are conducted at 4 temperature-controlled incubators (isothermal conditions: -5 , -8 , -12 , -18 °C) and at variable temperature conditions (non-isothermal temperature conditions: 12 h at -5 °C, 12 h at -8 °C and 12 h at -10 °C, $T_{\text{eff},1} = -6.9$ °C) to validate proposed shelf-life models.

Vitamin C determination: Vitamin C (L-ascorbic acid) was determined using a high performance liquid chromatography method (HPLC) (Giannakourou and Taoukis 2003).

Chlorophylls determination: A spectrometric system UV-Vis was used to determine the concentration of the chlorophylls a and b in spinach according to methodology by Olivera et al. (2008) and Mazzeo et al. (2011). The measurement absorbance values, at 664 and 647 nm, at the absorbance maxima for chlorophylls a and b, respectively, were used to calculate total chlorophyll (Porra et al 1989), and chlorophyll a and chlorophyll b contents (Linchtenthaler and Buschmann 2001).

Texture measurement: The texture attributes of thawed spinach leaves were expressed by the burst strength value measured as the maximum force to rupture the product during testing in texture analyzer (MODEL TA-XT2i, Stable Micro Systems, Godalming, Surrey, UK).

Sensory evaluation: Sensory evaluation of the spinach samples was conducted by ten panellists of the accredited (according to ISO 17025) sensory laboratory of NTUA. Spinach samples (leaves) were portioned (approximately 150 g), repacked and tempered at -18°C for 24 h before their sensory assessment. The sensory characteristics of frozen spinach were evaluated after cooking (2 min in boiled water). Cooked spinach samples were provided on white plastic plates coded with random 3-digit numbers, in individual booths and evaluated twice. Overall impression including all perceived sensory attributes (overall visual acceptability, colour, off-odour, texture (in mouth, expressed as juiciness), aroma, flavour/taste, freshness) was rated based on the preference using a 9-point hedonic scale (1: extremely dislike to 9: extremely like).

Methodology: The average retention of vitamin C was expressed relatively to an initial, average value of day 0 of the experiment, where C represents the concentration of L-ascorbic acid in 100 g of frozen raw material. In all cases, vitamin C loss was found to be adequately described by an apparent first-order reaction (Eq. 16.6, Table 16.1):

$$C_{\text{vitC}} = C_{0,\text{vitC}} e^{-k_{\text{vitC}} t} \quad \text{or} \quad \ln \frac{C_{\text{vitC}}}{C_{0,\text{vitC}}} = -k_{\text{vitC}} t \quad (16.6)$$

where C_{vitC} and $C_{0,\text{vitC}}$ are the concentrations of L-ascorbic acid at time t and zero, respectively, and k_{vitC} is the apparent reaction rate of vitamin C loss, estimated by the slope of the linearized plot of $\ln(C_{\text{vitC}}/C_{0,\text{vitC}})$ vs. t . Arrhenius equation expressed adequately the temperature dependence of reaction rate of vitamin C, k_{vitC} (Eq. 16.7):

$$k_{\text{vitC}} = k_{\text{ref,vitC}} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right] \quad \text{or} \quad \ln k_{\text{vitC}} = \ln k_{\text{ref,vitC}} - \frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \quad (16.7)$$

where $k_{\text{ref,vitC}}$ is the reaction rate of the vitamin C loss at a reference temperature $T_{\text{ref}} (-18^{\circ}\text{C})$, E_a is the activation energy of the chemical reaction and R is the universal gas constant (Taoukis et al. 1997). By linearly correlating $\ln k_{\text{vitC}}$ vs. $(1/T_{\text{ref}} - 1/T)$ (Arrhenius plot), the E_a of L-ascorbic oxidation was estimated from the slope of the fitted line. The estimated activation energies E_a , the 95 % confidence range as well

Table 16.1 Kinetic models and parameters used for quality deterioration of frozen spinach leaves (Dermesonluoglu et al. 2014)

Quality index	Kinetic model structure	Model dependent variables	Model parameters
Vitamin C (L-ascorbic acid)	$C_{vitC} = C_{0,vitC} e^{-k_{vitC} t}$	C_{vitC} = L-ascorbic acid content (mg/100 g)	$C_{0,vitC}$ = 20.03 ± 1.77 mg/g of frozen vegetable (18–22)
	$k_{vitC} = k_{ref,vitC} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right]$	k_{vitC} = The reaction rate of vitamin C loss (d ⁻¹)	$C_{i,vitC}$ = 6.01 (70 % vitamin C loss) E_a = 132.0 ± 5.8 kJ/mol k_{ref} (−18 °C) = 0.0029 ± 0.0004 d ⁻¹
Chlorophyll, total	$C_{chl} = C_{0,chl} e^{-k_{chl} t}$	C = Total chlorophyll content (mg/100 g)	C_i = 28.7 ± 3.0 mg/g of frozen vegetable
	$k_{chl} = k_{ref,chl} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right]$	k_{chl} = The reaction rate of chlorophyll loss (d ⁻¹)	C_i = 17.22 (60 % chlorophyll retention) E_a = 70.3 ± 11.3 kJ/mol k_{ref} (−18 °C) = 0.0011 ± 0.0004 d ⁻¹
	$F = F_0 + (k_{texture} * t)$	F = Burst strength (g)	F_i = 60 g
	$k_{texture} = k_{ref,texture} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right]$	$k_{texture}$ = The reaction rate (d ⁻¹)	F_i = 180 g E_a = 43.38 ± 4.81 kJ/mol k_{ref} (−18 °C) = 0.2872 d ⁻¹
Sensory evaluation, overall impression	$S_{overall} = S_{0,overall} - k_{overall} * t$	S = Sensory score (1–9)	S_i = 8.84 ± 0.05
	$k_{overall} = k_{ref,overall} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right]$		$k_{sensory}$ = The reaction rate (d ⁻¹) S_f = 5
			E_a = 61.26 ± 3.36 kJ/mol
			k_{ref} (−18 °C) = 0.0077 ± 0.0007 d ⁻¹

Table 16.2 Shelf life (SL, days) calculation for frozen leafy spinach samples based on different quality indicators (70 % vitamin C loss, 60 % total chlorophyll retention, score for acceptability 5/9 during sensory evaluation, final value for burst strength 180) (temperature range=−20 to −5 °C) (Dermesonluoglu et al. 2014)

Criterion	Kinetic model structure	Shelf life (SL, days) calculation for leafy frozen spinach					
		−5 °C	−8 °C	−12 °C	−18 °C	−20 °C	
70 % Vitamin C loss	$SL = \frac{\ln\left(\frac{C_{0,vitC}}{C_{vitC}}\right)}{k_{vitC}}$	23	66	100	401	641	
60 % Total chlorophyll content retention	$SL = \frac{\ln\left(\frac{C_{0,vitC}}{C_{vitC}}\right)}{k_{vitC}}$	27	50	119	464	740	
Texture (final value for burst strength = 180)	$SL = (F_0 - F_t) / k_{texture}$	155	193	261	418	491	
Sensory evaluation (final score for overall impression = 5/9)	$SL = (S_{0,overall} - S_{t,overall}) / k_{overall}$	148	195	283	506	618	

as the goodness of fit (R^2) for frozen spinach are shown in Table 16.1. The activation energy E_a for vitamin C loss was calculated as 132.0 ± 5.8 kJ/mol.

Seventy percent of vitamin C loss was used as the acceptability level for frozen spinach samples (Table 16.2) in accordance with the level of sensory acceptability. Since the vitamin C loss follows first-order kinetic, the shelf life (SL) can be predicted according to Eq. 16.8. The shelf life is calculated as 23, 66, 100 and 401 days at storage temperatures -5 , -8 , -12 and -18 °C, respectively (Table 16.2).

$$SL = \frac{\ln \left(\frac{C_{0,\text{vitC}}}{C_{\text{vitC}}} \right)}{k_{\text{vitC}}} \quad (16.8)$$

The loss of chlorophyll a and chlorophyll b with storage time and temperature followed the same pattern. Total chlorophyll content calculated was used as quality indicator, exhibiting a good correlation coefficient between the fitted model and experimental data. In all cases, total chlorophyll loss was found to be adequately described by first-order reaction kinetics. The temperature-dependence is adequately modelled by the Arrhenius equation (average, 70.30 kJ/mol). The shelf life of frozen spinach was calculated based on 60 % retention of total chlorophyll taking into account the sensory limit of acceptance based on the overall impression scores. The shelf life is calculated to be 27, 50, 119 and 464 days at storage temperatures -5 , -8 , -12 and -18 °C, respectively.

The increase of burst strength values F was described by a linear equation. The temperature-dependence is adequately modelled by the Arrhenius equation (average, 43.38 kJ/mol). The shelf life of frozen spinach was calculated based on measured burst strength value of 180 g as limit of acceptance for the texture parameter taking into account the sensory evaluation results.

Validation: Variable temperature profiles were described using the effective temperature concept. The effective temperature value, T_{eff} , is the calculated constant temperature of a variable temperature profile that results in the same quality deterioration in the same time period and depends on the activation energy (E_a) value (Taoukis and Labuza 1989) as defined in Eq. 16.1.

In Fig. 16.5, measurements of vitamin C loss and the corresponding exponential fit are shown and compared to predictions at the corresponding T_{eff} , with the dotted lines representing the limits of 95 % confidence range of the quality prediction for frozen spinach leaves. Repeated temperature cycles included three step changes: first step = 12 h at -5 °C, second step = 12 h at -8 °C and third step = 12 h at -10 °C with an effective temperature of -6.9 °C. Predicted rate of loss ($k_{\text{eff,pre}}$) is in good agreement with the experimentally estimated value (Dermesonluoglu et al. 2014).

Real Time–Temperature Scenarios via CCD: To fully understand the need to take into account the real time–temperature history of the product, and not assume a common scenario, two alternative scenarios were applied in the case of frozen spinach. Therefore, using Eqs. 16.3 and 16.4, and the kinetic results of Tables 16.1 and

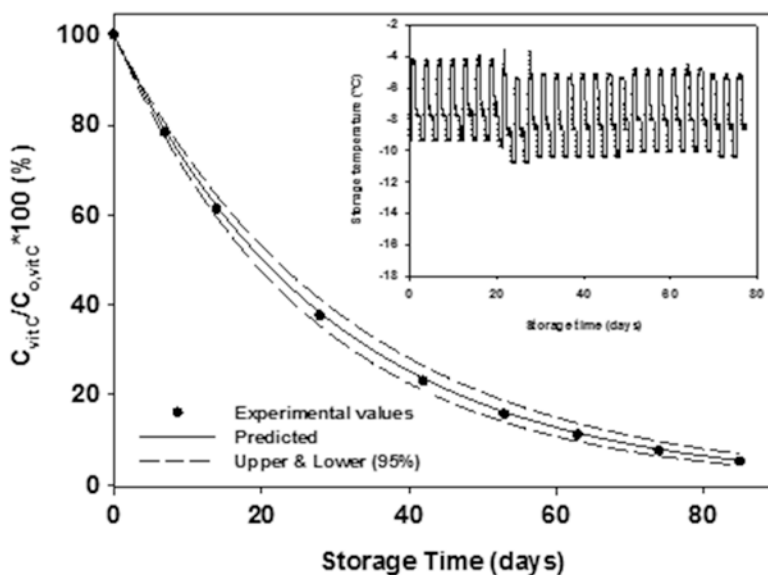


Fig. 16.5 Comparison of experimental and predicted results of vitamin C loss of frozen spinach leaves for exposure at the shown variable temperature profile. The *solid line* represents the exponential fit of the quality measurements and *dotted lines* depict the upper and lower 95 % confidence range of quality predicted for T_{eff} (-6.9°C)

16.2 (concerning Vitamin C and overall sensory impression, respectively), the shelf life consumed at the end of each stage was estimated. To illustrate the importance of assessing the effect of the real temperature conditions during transport and storage on food quality, a realistic scenario of 240 days distribution was studied, based on FRISBEE database. It included an initial stage of 40 days storage in the packing plant, followed by transportation and storage in a distribution centre for 40 days. Subsequently, packages of frozen spinach were kept at the retail display for 60 days, before being purchased by the final consumers that store them in their domestic freezer for 100 days before consumption. At the end of the assumed cycle, i.e. the time of consumption, the remaining shelf life of frozen spinach (at -18°C) according to vitamin C and overall impression values was estimated.

The first scenario of 240 days distribution cycle, scenario (a) (Fig. 16.6a) was one, with very low temperatures at the first two stages of the chain (warehouse and distribution centre) and appropriate storage conditions during retail display and consumer storage (corresponding to almost 40 % of Fig. 16.6a). The second scenario (b) (Fig. 16.6a) was the same one, regarding the first three stages, but took into account the possibility of abusive storage at the final crucial stage of domestic storage (corresponding to almost 10 % of Fig. 16.6a). In Fig. 16.6b, remaining shelf life

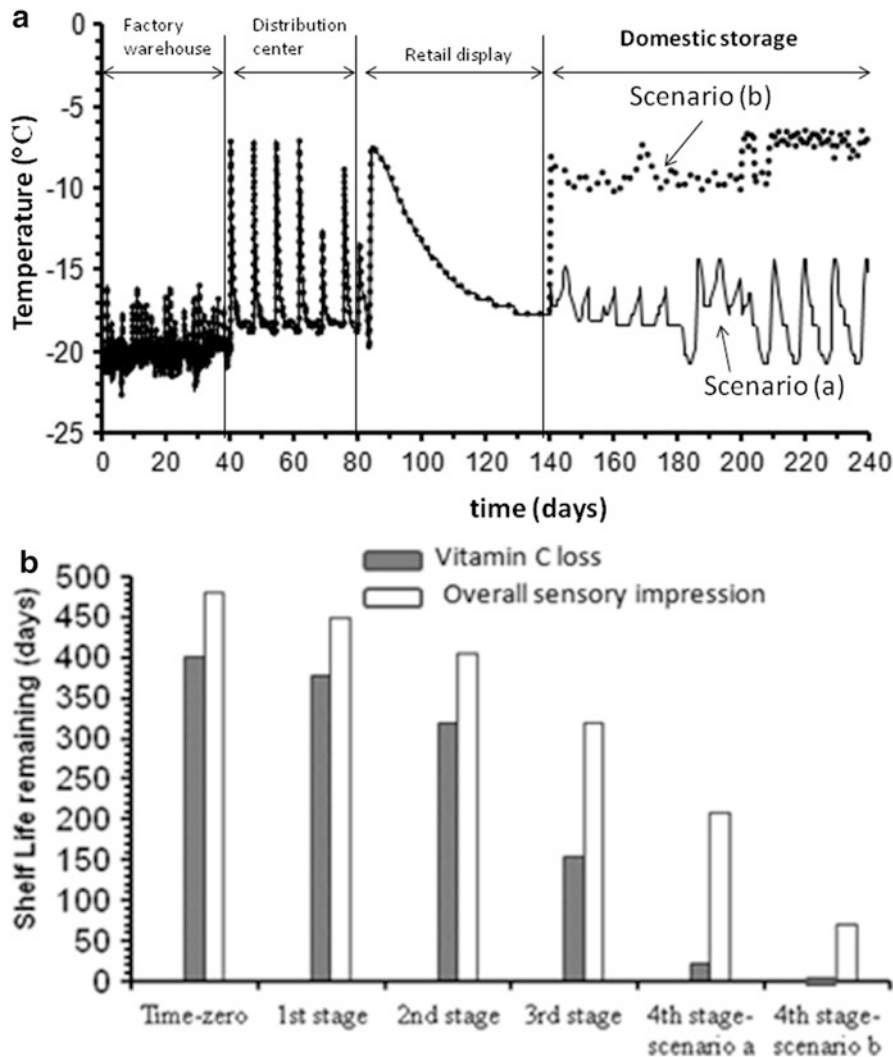


Fig. 16.6 (a) Cold chain conditions for frozen spinach (*source*: FRISBEE project database (2013)). (b) Remaining shelf life of frozen spinach at the end of each stage of the cold chain, for scenario (a) and (b)

(estimated assuming an isothermal storage at -18°C) was plotted at the end of each stage for both scenarios. It was observed that for scenario (a), at the end of the cycle, the frozen product was still of more than acceptable quality, based on both criteria (30 and 200 days for vitamin C and sensory scoring respectively of remaining shelf life if stored at -18°C for the remaining time). On the other hand, for the abusive

scenario (b), the product reached the threshold of Vitamin C loss a month after its storage in the domestic freezer but was still sensorially of good quality (60 days of remaining shelf life) (Fig. 16.6b)

16.4.2 *Field Evaluation of a TTI-Based Cold Chain Management System on Shelf-Life Monitoring of Frozen Shrimp*

In this section, the applicability of selected enzymatic and photochromic TTI smart labels for monitoring the quality of frozen shrimp in real conditions is described. Two different TTI technologies were used, an enzymatic and a solid state photochromic TTI. The enzymatic indicators are based on a colour change caused by a pH decrease which is the result of a controlled enzymatic hydrolysis by a microbial lipase of a lipid substrate like methyl myristate. The M-type enzymatic TTI (VITSAB, Malmo, Sweden) starts from initial green colour, becomes progressively yellow/orange and reaches a final red colour. Different enzyme concentrations (4–44U) were used and kinetically modelled. The OnVu™ TTI (Bizerba, Germany) is based on the inherent reproducibility of reactions in crystal phase (Patent EP 1049930 B1). Photosensitive compounds, such as spiropyran, are excited and coloured (dark blue) by exposure to low wavelength light. By controlling the type of the photochromic compound and the length of UV light exposure during activation, the length and the temperature sensitivity of the TTI can be set. The B1 TTI was charged (Bizerba Desktop Charger, Bizerba GmbH & Co. KG, Balingen, Germany) for 0.1–1 s and subsequently laminated with an optical filter to protect TTI from light exposure and recharging (Giannoglou et al. 2014).

Kinetic modelling of response was based on measurements, at appropriate time intervals, of the response of five TTI tags isothermally stored in high-precision low-temperature incubators (−15 to −5 °C). For the modelling of the response rate constant of the photochromic TTI as a function of degree of initial activation and storage temperature, a composite mathematical equation was developed (Giannoglou et al. 2013; Giannoglou et al. 2014)

$$\Delta E = \Delta E_{t_c=1s} \cdot t_c^b \cdot \exp \left(- \left(k_{ref} (T_{ref}, t_c=1s) \cdot t_c^{-a} \cdot \exp \left(\frac{-E_a}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right) \right) \cdot t \right) \quad (16.9)$$

where $\Delta E_{(t_c=1s)} = 34.7$, the initial response ($t=0$) of the B1-type photochromic TTI exposed under UV radiation for 1 s, t_c is the charging time, E_a is the activation energy (123 kJ/mol), R is the universal gas constant, T_{ref} is a reference temperature (−5 °C), $k_{ref, Tref, 1s} = 0.008 \text{ d}^{-1}$ is the TTI response rate constant at T_{ref} (with charging time $t_c = 1 \text{ s}$), a and b are constants ($a=0.416$ and $b=0.394$) and t , T are the storage time and temperature, respectively.

A mathematical model which describes the effect of the enzyme concentration and the storage temperature on the response of the enzymatic TTI was also developed (Giannoglou et al. 2013; Giannoglou et al. 2014)

$$X = F(X_C) = \frac{1}{1 + \exp \left(\frac{k_{1,\text{ref}} * C^{-B_1} * \exp \left[\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right] - t}{k_{2,\text{ref}} * C^{-B_2} * \exp \left[\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right]} \right)} \quad (16.10)$$

where T is the storage temperature (K), E_a is the activation energy (112 kJ/mol), R is the universal gas constant, T_{ref} is a reference temperature (-5°C), C is the enzyme concentration (4–44U) and $B_{1,2}$ are constants ($B_1 = 1.041$, $B_2 = 1.001$), $k_{1\text{ref}} = 663.5 \text{ d}^{-1}$ and $k_{2\text{ref}} = 158.6 \text{ d}^{-1}$.

The following equations for shelf-life calculation for frozen shrimp have been developed and used

$$t_{\text{SL}} = \frac{\ln C_{\text{TVB-N},1} - \ln C_{\text{TVB-N},0}}{k_{\text{ref,TVB-N}} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right]} \quad (16.11)$$

$$t_{\text{SL}} = \frac{s_0 - s_1}{k_{\text{ref},s} \exp \left[\frac{-E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{\text{ref}}} \right) \right]} \quad (16.12)$$

where t_{SL} is the shelf life (d) of frozen shrimp, $C_{\text{TVB-N},1}$ and s_1 are the limits TVB-N (25 mgN/100 g) and overall impression (5 score for overall impression), respectively, $C_{\text{TVB-N},0}$ and s_0 are the initial TVB-N and overall impression, respectively, k_{ref} is the rate constant of change of each quality index at $T_{\text{ref}} = -15^\circ\text{C}$ ($k_{\text{ref,TVB-N}} = 0.0038 \text{ d}^{-1}$ and $k_{\text{ref},s} = 0.0114 \text{ d}^{-1}$), E_a is the activation energy of the respective quality index ($E_{a,\text{TVB-N}} = 119 \text{ kJ/mol}$ and $E_{a,s} = 111 \text{ kJ/mol}$) and R is the universal gas constant (Tsironi et al. 2009).

Based on the above kinetic models, B1-0.3 s photochromic and M-15u enzymatic labels were well correlated with the shelf life of frozen shrimp, showing the potential of using these TTI as reliable tools for quality assessment of frozen shrimp at a given point of the frozen distribution chain. This could allow better management and optimization of the cold chain from manufacture to the point of consumption.

In order to evaluate the applicability of the selected TTI in the real chill chain, an application in the real cold chain was performed. Frozen whole, unpeeled shrimp (*Penaeus notialis*) were packed in their commercial packages by the seafood processing company. Temperature was continuously monitored by electronic,



Fig. 16.7 Field test design

programmable miniature data loggers (mini NOMAD RFID Temperature Logger, OM-84-TMP, OMEGA Engineering Inc., US) placed inside each package. Appropriate photochromic and enzymatic TTIs were selected and attached on each frozen food based on the shelf-life tests and mathematical models. Thirty five packages were stored for 2 months in the production warehouse, before transportation and storage in the distribution centre (for 2 months). Afterwards, samples were distributed to eight retail outlets in collaboration with a leading supermarket chain. Samples were collected and transported to the laboratory to simulate the storage conditions of domestic freezers, in high-precision low-temperature incubators (Sanyo MIR, Sanyo Electric Co., Ora-Gun, Gunma, Japan) at two constant sub-frozen temperatures (-10 and -18 °C). The design of the field test is illustrated in Fig. 16.7.

At each measured point of the cold chain, the quality level and remaining shelf life were (1) estimated experimentally by the actual measured values of TVB-N and sensory scoring, (2) calculated from the food kinetic models using the real T_{eff} from the electronic data loggers, (3) calculated from the food kinetic models using the real T_{eff} from the TTI response and (4) directly correlated from the TTI reading.

The comparison between the experimental (actual) and predicted (calculated by the mathematical food kinetic models or the TTI measurement) was based on the accuracy factors (Eq. 16.13).

$$\text{accuracy factor} = 10^{\frac{\sum \left| \log(SL_{R,\text{predicted}} / SL_{R,\text{experimental}}) \right|}{n}} \quad (16.13)$$

where n is the number of observations. An accuracy factor of 1 represents perfect agreement between observed and predicted values. The larger than 1 the value is, the less accurate the average estimate is between observed and predicted values (Ross 1996).

The applicability of the validated mathematical models for the chemical and sensory quality indices of frozen shrimp was verified by comparing the predicted values with the experimental. Based on the experimental and the respective calculated by the mathematical models TVBN and sensory scoring values and using the actual cold chain conditions, the remaining shelf life (SL_R) at a reference temperature ($T_{\text{ref}} = -15$ °C) was determined (see Figs. 16.8 and 16.9).

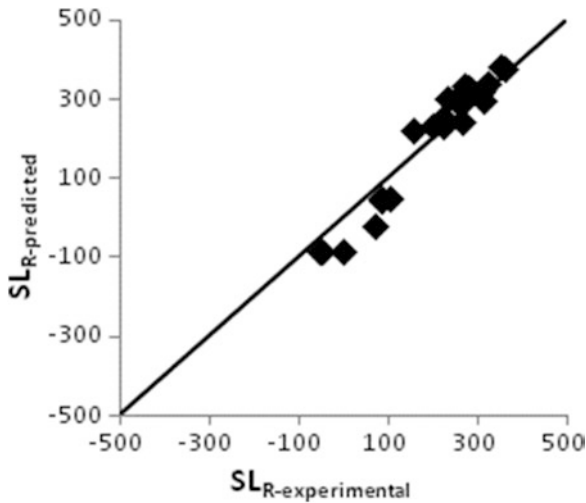


Fig. 16.8 Remaining shelf life in days ($SL_{R-experimental}$) of frozen shrimp at $T_{ref}=-15\text{ }^{\circ}\text{C}$ measured (via TVBN index) and $SL_{R-predicted}$ calculated by the food kinetic mathematical model and the actual time–temperature history of the product (accuracy factor=1.002)

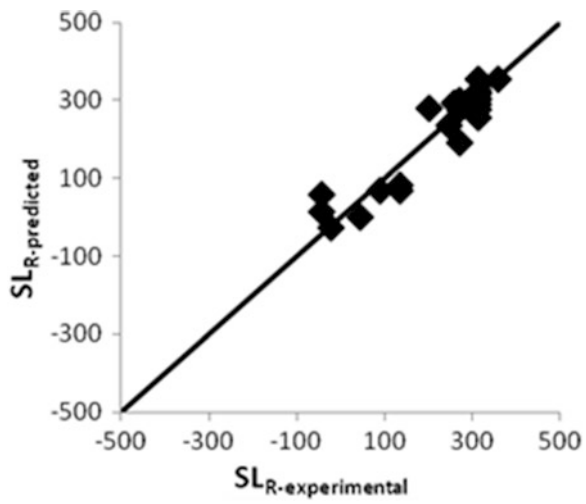


Fig. 16.9 Remaining shelf life in days ($SL_{R-experimental}$) of frozen shrimp at $T_{ref}=-15\text{ }^{\circ}\text{C}$ measured (via sensory evaluation) and $SL_{R-predicted}$ calculated by the food kinetic mathematical model and the T_{eff} determined by the M-15 response (pred-TTI) (accuracy factor=1.001)

SL_R was also estimated based on the effective temperature (T_{eff}) of the cold chain calculated via the TTI measurement and compared with the $SL_{R,experimental}$. The accuracy factors demonstrated a good agreement between TTI predicted and observed SL_R values.

Finally, the actual remaining shelf life ($SL_{R,experimental}$) of frozen shrimp at each point of the cold chain (assuming consequent isothermal storage at $-15\text{ }^{\circ}\text{C}$) was compared with the remaining shelf life directly obtained by the TTIs (SL_{R-TTI}), i.e. the time needed for each label to reach their end point. Based on this comparison, the applicability of the appropriate TTIs was established at the real conditions of the cold chain. A very good agreement at all temperatures is achieved if the temperature sensitivity of the TTI response expressed via the E_a value is very close to the respective E_a value of the food kinetics (less than 20 kJ/mol difference). The agreement between the TTI-based SL predictions and the actual remaining shelf life is depicted in Figs. 16.10 and 16.11 for the two different TTIs used: M-15u enzymatic and B1-0.3 s photochromic TTI labels, respectively. As a practical alternative, when TTIs with larger E_a are only available, the selection of the suitable TTI can be based on matching TTI response and food kinetics at one reference temperature (e.g. $-12\text{ }^{\circ}\text{C}$). Based on this selection, if the products are stored at very low temperatures (i.e. below $-15\text{ }^{\circ}\text{C}$), the end of shelf life will be determined and limited by the expiration date on the food package. On the other hand, if abuse temperatures prevail, then the TTI will conservatively signal poor quality products slightly before the end of shelf life.

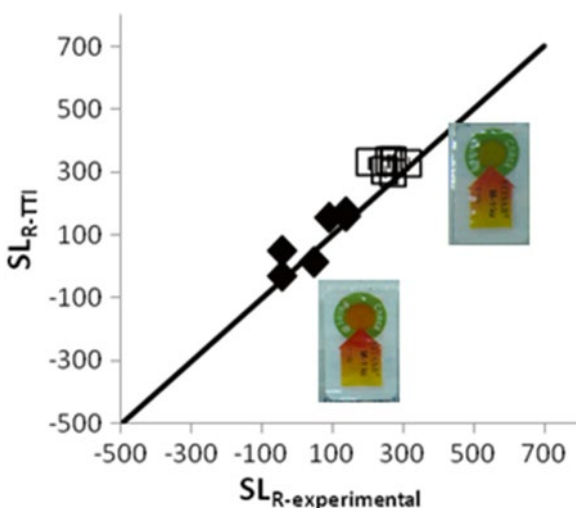


Fig. 16.10 Remaining shelf life in days ($SL_{R,experimental}$) at different points of the cold chain (measured via sensory evaluation) and shelf life directly based on the M-15u reading (SL_{R-TTI}) (open square domestic storage study at $-18\text{ }^{\circ}\text{C}$ (accuracy factor = 1.005), filled diamond domestic storage at $-10\text{ }^{\circ}\text{C}$ (accuracy factor = 1.008))

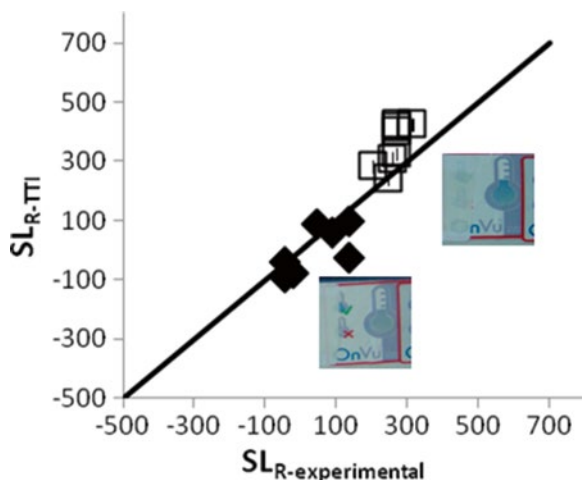


Fig. 16.11 Remaining shelf life in days ($SL_{R-experimental}$) at different points of the cold chain (measured via sensory evaluation) and shelf life directly based on the B1-0.3 s reading (SL_{R-TTI}) (*open square* domestic storage study at -18°C (accuracy factor=1.038), *filled diamond* domestic storage at -10°C (accuracy factor=1.014))

16.5 Conclusions

Two different approaches are proposed for the management of the cold chain of food products. In the first approach, the use of appropriate developed tools developed within FRISBEE project is described. The aim is the estimation of the remaining shelf life of food products after each stage of the cold chain, based on $t-T$ profiles using the CCD. The contributed data of the cold chain will allow one to run simulations and distribution scenarios based on real cold chain data. Corrective actions could be applied for optimizing remaining shelf life. The other approach is the application of TTIs which allow such control down to product unit level. The selection and use of the optimum TTIs for a particular product, with regard its visual response characteristics and temperature sensitivity, could lead to realistic control of the cold chain, reduction of waste and efficient shelf-life management. If the temperature conditions of the products could be continuously monitored by TTIs, reliable estimation of the quality status and the remaining shelf life could be performed, allowing better management and optimization of the cold chain from production to the point of consumption.

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Part IV

Food Engineering

Chapter 17

Microbial Polysaccharides: Between Oil Wells, Food and Drugs

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17.1 Introduction

The ability to produce polysaccharides is widely found among different species, but despite many sources of these biopolymers, those from algae and higher plants are dominant on the world market. Polysaccharides derived from microorganisms, including bacteria, yeasts and moulds, have still not been exploited enough. The main reasons for that are associated with costs of production, because of specific substrate requirements in certain cases, bioreactors demands or provision of aseptic conditions. Nevertheless, polysaccharide production from microorganisms has many advantages: it takes significantly less time compared to plants; in case of some algae species, it is more energy efficient because of the use of solar energy for production, and a lot of industrial waste and raw materials can be used as carbon sources, which is probably the greatest advantage of all (Donot et al. 2012). MPSs are synthesized and accumulated mostly after the growth phase, and, in regard to their location in the cell, they can be divided into three main groups. Inside the cell, carbon and energy sources are cytosolic endopolysaccharides. The second group is made of those that make up the cell wall. Polysaccharides exuded into the extracellular environment are known as exopolysaccharides (EPSs), and they appear in the form of capsules or slime. They are also involved in biofilm formation, where they have many significant roles: participation in attachment to a surface, formation and stabilization of biofilm structure, enhancement of resistance to environmental biotic

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and abiotic stresses and antimicrobial agents, preventing of desiccation and assumption of nutrients (Shia and Zhua 2009). MPSs are divided into two groups: homopolysaccharides, made up of a single type of monosaccharide (e.g. pullulan, dextran or levan) and heteropolysaccharides, made up of several types of monosaccharide, with complex structures (e.g. xanthan or gellan). MPSs are also classified on the basis of their microbiological origin on bacterial and fungal polysaccharides. Commercially, the most important are following glycans: from bacteria—heteropolysaccharides (xanthan, alginate, hyaluronan, gellan); homopolysaccharides (dextran, cellulose, curdlan, levan) and from fungi—homopolysaccharides (beta glucan, pullulan, scleroglucan). Structures of some MPSs are shown in Fig. 17.1. In many cases, they are mainly composed of glucose, galactose and mannose but many other neutral amino sugars and uronic acids are often present, too. Also they can contain some organic ester-linked substituents and pyruvate ketals, which give them anionic character and increase their lipophilicity (Freitas et al. 2011).

Despite differences in monosaccharide composition, they differ in charge, type and configuration of glycosidic linkages (which affects the rigidity of molecule),

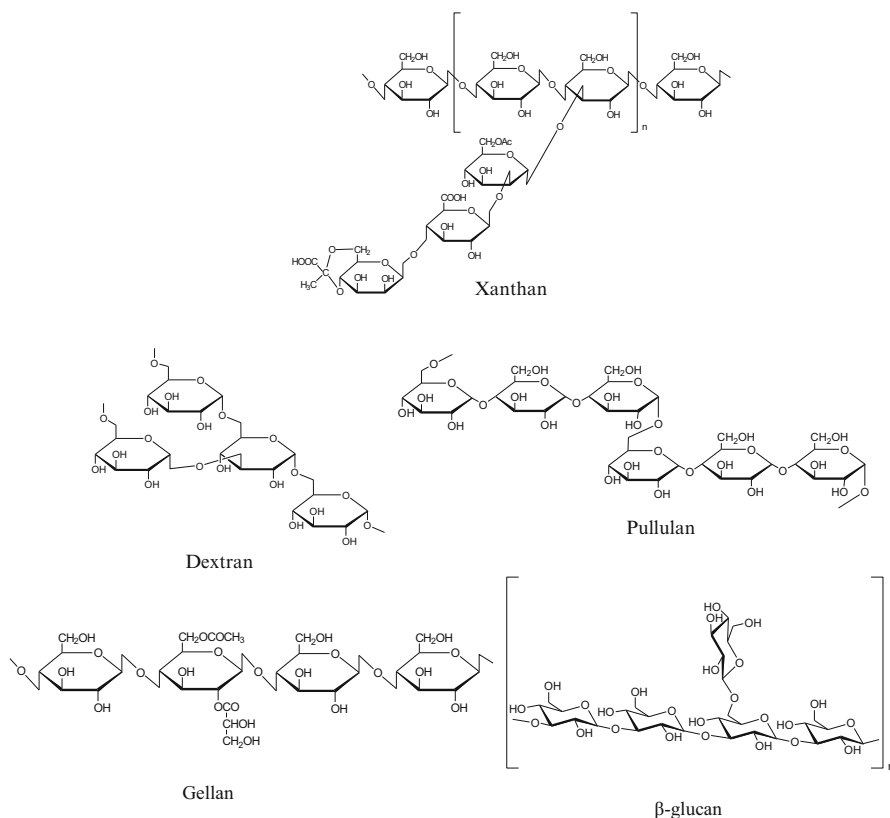


Fig. 17.1 Structure of some widespread MPSs. (a) Xanthan; (b) Dextran; (c) Pullulan; (d) Gellan and (e) β -glucan (frequently component of cell wall-endopolysaccharide)

molecular weight, length and frequency of branches (from which rheological properties depend) (Duboc and Mollet 2001). Some classes of industrially important microbial polysaccharides and their characteristics are summarized in Table 17.1.

17.2 Structure and Application of Some of the Most Important Microbial Polysaccharides

Xanthan. Xanthan is a microbial polysaccharide which is most often applied in industry. This heteropolysaccharide arises as a product of the microorganism *Xanthomonas campestris*. The main chain of this glycan (Fig. 17.1a) consists of (1,4)-linked β -D-glucopyranose units. The side chains constitute two mannopyranoses and one glucuronic acid linked to the O-3 position of every second glucose residue in the basic chain. About 50 % of terminal mannose units contain the pyruvate residue that is attached via a keto group in positions C-4 and C-6. Nonterminal mannose residues contain acetyl groups at the position C-6. Xanthan is easily soluble in hot and cold water giving viscous solutions at low concentrations and they show a special rheological properties and pseudoplastic behaviour (García-Ochoa et al. 2000). Its solutions are insensitive to a broad range of pH, temperature and electrolyte concentrations. Because of these properties, xanthan has a great application in the food industry as a thickener and emulsifier which improves the viscosity and texture of food (Freitas et al. 2011; Desplanques et al. 2012).

It is also widely used in the ceramic industry, emulsions, gels, textiles and cosmetics (Sutherland 2001; Rehm 2010; Badwaik et al. 2013). The special rheological properties of xanthan make this polysaccharide very suitable for application especially in oil industry, for “enhanced oil recovery” applications. In petroleum industry, it is used for oil drilling, fracturing, pipeline cleaning, and also in micellar—polymer flooding as a tertiary recovery operation (Palaniraj and Jayaraman 2011).

Dextran. Dextran is a polysaccharide with the great industrial application. Dextran is a collective name for a large group of bacterial extracellular polysaccharides. Some dextran are composed almost exclusively of (1,6)- α -D glucopyranosidic linkages within the main chain, whereas others may contain as little as 50 % of α -(1,6)-linkages with different content of α -(1,2), α -(1,3) or α -(1,4)-linkages (Sidebotham 1974; Lazić et al. 1993; Naessens et al. 2005). Such a distribution of glycosidic bonds created the possibility of significant variability in the structure, which is another characteristic of these microbial biopolymers. Figure 17.1b shows the structure of dextran produced by *L. mesenteroides* B 512-F. The high proportion of (1,6)-linkages gives it an unusually flexible backbone on which the 3-hydroxyl groups in consecutive positions are available for complexing metal ions. Almost all producers of industrial dextran use the NRRL512(F) strain of *Leuconostoc mesenteroides* or a similar organism (Sanford 1979). Most dextrans have high molecular weights (in the millions). There are developed methods for the depolymerization of this glucan as well as synthesis of oligodextrans (Goulas et al. 2004). Obtained fractions are used in medicine as substitute for blood plasma. Dextran is widely applied in pharmacy (as a prodrug)

Table 17.1 Principal microbial polysaccharides: overview of the most relevant properties and applications

MPS	Components	Charge	Main Properties	Main applications	Organism
Bacteria	Guluronic acid Mannuronic acid Acetate	Anionic	Hydrocolloid Gelling capacity Thermoreversible gels	Food hydrocolloid; Medicine: Surgical bandages, Wound treatment, Controlled drug release	<i>Pseudomonas</i> sp.; <i>Azotobacter</i> sp.
Cellulose	Glucose	Neutral	High crystallinity	Foods (indigestible fiber)	<i>Acetobacter</i> sp.
			Insolubility in most solvents	Biomedical: Wound healing	
			High tensile strength	Tissue engineering	
Curdlan	Glucose	Neutral	Moldability	Audio speaker diaphragms	<i>Agrobacterium</i> sp.
			Gel-forming ability; Water insolubility; Edible and non-toxic; Biological activity	Foods; Pharmaceutical industry	
				Heavy metal removal	
Dextran	Glucose	Neutral	Non-ionic; Good stability	Concrete additive	<i>Leuconostoc</i> sp.
			Newtonian fluid behaviour	Foods; Pharmaceutical industry; Chromatographic media	
Gellan	Glucose, Rhamnose, Glucuronic acid Acetate, Glycerate	Anionic	Hydrocolloid; Gelling capacity; Thermoreversible gels	Foods; Pharmaceuticals	<i>Sphingomonas</i> sp.
				Research: agar substitute and gel electrophoresis	
Hyaluronan	Glucuronic acid Acetylglucosamine	Anionic	Biological activity	Medicine	<i>Pseudomonas</i> sp.
			Highly Hydrophilic; Biocompatible	Solid culture media	

Levan	Fructose	Neutral	Low viscosity; High water solubility; Biological activity Film forming capacity	Food (prebiotic) Medicines; Cosmetics	<i>Bacillus</i> sp. <i>Pseudomonas</i> sp.
Xanthan	Glucose, Mannose Glucuronic acid	Anionic	Hydrocolloid; Stability over wide temperature, pH range	Foods; Petroleum industry; Pharmaceuticals; Cosmetics; Agriculture	<i>Xanthomonas</i> sp.
Fungi					
Pullulan	Glucose	Neutral	High water solubility; Biological activity; Film forming capacity	Foods; Pharmaceutical industry; Cosmetics	<i>Aureobasidium</i> sp.
Scleroglucan	Glucose	Neutral	Hydrocolloid; Stability over pH range; Biological activity	Foods; Pharmaceuticals Petroleum industry	<i>Sclerotium</i> sp.
Beta-glucan	Glucose	Neutral	Biological activity: Antitumour, Immunomodulatory, Antioxidative activity	Foods; Medicines	<i>Saccharomyces</i> sp.; <i>Basidiomycetes</i>

biochemistry, biotechnology and in the food industry (Bhavani and Nisha 2010). The ability of dextran to form inert, hydrophilic supports of well-defined dimensions has been exploited on both small and industrial scales for the separation and purification of various biological and other molecules (Freitas et al. 2011).

Pullulan. Pullulan is the main product of the yeast-like fungi *Aureobasidium pullulans*. It is a neutral homopolymer (Fig. 17.1c), that consists of linear chain of α -D-glucopyranoses with regular repeating of two (1,4)-linkages and one (1,6)-connection and is often described as an α -(1,6)-linked polymer of maltotriose subunits (Leathers 2003). Some strains of *A. pullulans* produce pullulan containing less content of tetrasaccharide fragments (Jakovljević et al. 2001; Leathers 2003), while other pullulans have a few (1,3)-linkages (Singh et al. 2008). Unique connection pattern results in many distinctive physical traits of pullulan. This glucan is easily soluble in water forming a viscous, adhesive solution. Films obtained by drying pullulan solutions are clear, non-toxic, highly impermeable to oxygen and have excellent mechanical properties; therefore, they are used commercially as a coating for keeping food and drugs (Cheng et al. 2011). Pullulan is non-digestible polysaccharide which is treated as a dietary fibre with the role of a prebiotic, and it is used as partial replacement for starch in dietary foods (Rekha and Sharma 2007; Maathuis et al. 2009). This glucan has application in medicine, cosmetics and industrial adhesives, and it was used as a flocculant and a remediation agent in the environmental protection (Iyer et al. 2005; Radulović et al. 2008). Pullulan is a photosensitive material that has excellent holographic characteristics (Pantelić et al. 1998; Savić et al. 2002).

Gellan. Gellan represents a series of eight structurally similar high molecular anionic heteropolysaccharides that synthesizes a group of bacteria of the genus *Sphingomonas* (Pollock 2002; Prajapati et al. 2013). In its native form, gellan contains *O*-acetyl groups and glyceryl substituents linked to the linear polymer consisting of repetitive units composed of (1,4)- β -D-glucuronic acid, (1,3)- β -D-glucose and (1,4)- α -L-rhamnose in ratio 1:2:1 (Bajaj et al. 2007) (Fig. 17.1d). The main difference between these polymers is the nature and location of the side groups, and the degree of acetylation. The native gellan under suitable conditions forms weak elastic gels, while the deacetylated polysaccharide forms strong intermolecular association that affects the formation of the brittle gel in the presence of different cations (Banik et al. 2000). Thermoreversible gels with different properties, stable at a wide pH range, were obtained by controlled deacetylation. The similarities between gellan gels and other gels in foods enable gellan to be used as a partial or total replacement for current commercial gelling agent, a stabilizer and suspending agent, and it is often used in combination with other hydrocolloids (Bajaj et al. 2007; Freitas et al. 2011). Thermostable gellan is a substitute for agar in microbiological media for cultivation of various cell cultures (Sutherland 1998).

Bacterial cellulose. This polysaccharide produces *Acetobacter xylinum*, and other types of predominantly Gram-negative bacteria. Bacterial cellulose is a linear polysaccharide with (1,4)-linked β -D-glucopyranosyl units. This polymer is excreted into the ground where rapidly aggregate in microfibrils with excellent mechanical

properties, crystallinity and biocompatibility, and a diameter which is approximately one hundred times smaller than diameter of plant cellulose fibrils (Khan et al. 2007; Putra et al. 2008). It is produced commercially as a source of high-purity cellulose. This glycan is used as a component of high quality audio membranes, electronic paper, membranes of fuel cells and biomedical material (Czaja et al. 2006; Weia et al. 2011). One of the most interesting uses is in the treatment of chronic wounds, such as venous leg and diabetic ulcers, bedsores and burns, where this polysaccharide, in the form of film, provides necessary moist environment. It also helps in eliminating pain symptoms by isolating the nerve ending, provides a good barrier against infection and decreases the healing time (Czaja et al. 2007). There are also promising applications of bacterial cellulose in food industry (Shi et al. 2014).

Curdlan. Some genera of bacteria, among them *Agrobacterium* and *Rhizobium*, produce several EPSs, including curdlan. The main chain of this glycan is composed of (1,3)- β -D-linked glucopyranoses and has a relatively low molecular weight, about 74000. Curdlan forms a weak gel on heating above 55 °C, and stronger gels are produced with further heating to 100 °C, while autoclaving at 120 °C converts the molecular structure to a triple helix. The gel obtained at this high temperature no longer melts when heated (Sutherland 1998). Curdlan today is still not used as extensively as xanthan or dextran but predicts its extensive use in food industry as a gelling agent, for coating, and also for the production of suspensions (Laroche and Michaud 2007; Freitas et al. 2011). As studies indicated that this glucan has no caloric value, it should be useful in low-calorie food. Curdlan and its derivatives were also modulators of the biological responses with many potential applications (Goodridge et al. 2009; Zhan et al. 2012).

Scleroglucan. Scleroglucan is the name for a large number of extracellular polysaccharides produced by both the different molds, mainly of the genus *Sclerotium*. It is a linear polysaccharide of β -(1,3)-linked glucopyranoses of which, on average, every third unit substituted via positions O-6 with one β -D-glucose unit (Survase et al. 2007). Solutions of scleroglucan show pseudoplastic behaviour, and their viscosity does not change significantly with the increasing temperature. This polysaccharide has a wide application in the food industry where it is used as a thickener, gelling agent and a stabilizer as well as in the pharmaceutical industry, where it is most often used as a matrix for drugs (Giavasis et al. 2002). In addition, because of its good stability in a wide range of pH and temperature, raw scleroglucan is widely used in the oil industry as an additive for increasing the viscosity of the mixture used for oil exploration (McNeil and Harvey 1993). When the problem of high cost of its production is resolved, this polysaccharide shall successfully replace xanthan in the food industry, as it has been the case in Japan since 1982. One of the significant roles of the β -D-glucans is in their application against viral and bacterial infections, as well as their antitumour activities. It has been found that scleroglucan has antiviral and immunomodulatory effects (Marchetti et al. 1996; Huang et al. 2009). Scleroglucan and curdlan are modulators of the biological responses through a mechanism dependent on activation of the immune system, comprising non-specific stimulation functions (Survase et al. 2007).

Levan. Levans are a group of fructose polysaccharides produced by bacteria of the genus *Bacillus*, *Rahnella*, *Aerobacter*, *Erwinia*, *Streptococcus*, *Pseudomonas* and *Zymomonas* (Freitas et al. 2011). The characteristic of these polymers is a linear chain consisting of β -D-fructofuranoses residues predominantly connected by β -(2,6)-glycosidic bonds and a D-glucose residue on the non-reduced end of the chain. Branchings that occur through β -(2,1)-linkages are often short and sometimes consisting of a single fructose residue (Kang et al. 2009). Compared with other molecules with the similar molecular weight, levans show unusual behaviour because they have relatively low intrinsic viscosity and do not swell in water. Fructose-based polymers can be potential bioactive polysaccharides, wherein branched structures could have a crucial role in their antitumour activity (Yoon et al. 2004). Some of these polysaccharides exhibit prebiotic effect (Korakli et al. 2003). Levans have the great potential for being applied in food, pharmaceutical, medical, cosmetic and chemical industries (Kang et al. 2009; Patel et al. 2012).

Alginate. Alginates are linear polysaccharides consisting of mannuronic acid and its C-5 epimer, guluronic acid, that form the block structure of sequentially linked β -(1,4)-D-polymannuronic or α -(1,4)-L-polyguluronic acid and mixed blocks composed of both acids. These EPSs produced by bacteria genera *Azotobacter* and *Pseudomonas*. Bacterial alginates, unlike algal, contain in their structure *O*-acetyl groups in varying ratios (Sabra et al. 2001). The high level of acetylation significantly increases the viscosity of the polymer and pseudoplastic behaviour (Donati and Paoletti 2009). Commercially, the most important is alginic acid synthesized by the *Azotobacter vinelandii*, due to the fact that the *Pseudomonas aeruginosa* is opportunistic pathogen. Currently, alginates are more commonly used to obtain hydrocolloids, widely used in the food industry, and due to the formation of high-capacity gels they are applied in medicine as a surgical dressing, in the treatment of wounds and in the synthesis of drugs with the controlled release of active substances, and as one of the most commonly used dental materials, too (Nandini et al. 2008; Freitas et al. 2011; Goh et al. 2012; Hay et al. 2013).

Hyaluronic acid. The bacterial hyaluronic acid produces *Streptococcus* groups A and C (*Streptococcus equi* and *Streptococcus zooepidemicus*). This polysaccharide, composed of disaccharide as repeating units consisting of β -(1,3)-*N*-acetyl-D-GLUCOSAMINE and β -(1,4)-glucuronic acid, apparently is the identical chemical structure to that obtained from eukaryotic material (Garg and Hales 2004). Viscous and elastic properties, a significant capacity to hold moisture and high biocompatibility resulted in the wide application of this polymer (Kogan et al. 2007). Bacterial hyaluronic acid was commercialized as an alternative material, obtained from human or animal sources, for use in surgical, pharmaceutical and cosmetic products, and also as dietary supplement products (Jin et al. 2010; Bildstein et al. 2011; Liu et al. 2011; Boeriu et al. 2013).

β -glucans. β -glucans are structural polysaccharides of the cell wall of fungi, yeast, bacteria and some cereals. One of the most studied β -glucan of the microbial origin is the β -glucan isolated from the cell wall of *Saccharomyces cerevisiae*. The main chain of this glycan composed of (1,3)-linked β -glucopyranoses of which a part is substituted through O-6 position with single glucopyranosyl residues, as

shown in Fig. 17.1e (Stone and Clarke 1992; Zlatković et al. 2003). It is known that glucan from *S. cerevisiae* participates in the activation of macrophages, neutrophils, natural killer cells and lymphocytes (Zeković et al. 2005; Petravić-Tominac et al. 2010; Goodridge et al. 2011). This β -glucan is not digestible and can be, in some degree, fermented by intestinal microbial flora (Laugier et al. 2012). Especially interesting are the β -D-glucans isolated from medically important basidiomycetes. These polysaccharides with chitin, cellulose and glycoproteins build the cell walls of higher fungi (Wasser 2002). These polymers are very much currently applied due to a number of their biological properties, such as anticancer, antiviral and hypolipidic effect as well as the immunomodulatory and immunostimulant activity, resulting in many applications, especially in biomedical and food industries, as well as in agriculture, environmental protection and waste water treatment (Zhang et al. 2007; Chan et al. 2009; Rahar et al. 2011; Kozarski et al. 2013). Also, polysaccharides from different species of mushrooms are potential sources of prebiotics (Aida et al. 2009). In Japan, lentinan, β -glucan isolated from the basidiomycete *Lentinula edodes* is registered as a cure for cancer and is in the top 10 of antitumour preparations in this market (Mizuno 1999).

The main advantage of supplements containing β -D-glucans over conventional immune therapies is in the possibility of oral administration, less costs and fewer side effects, but their independent use as therapeutics is still not sufficient (Chan et al. 2009; Murphy et al. 2010).

17.3 Specificity of Microbial Polysaccharides Important for the Use in the Food System

In food industry, microbial polysaccharides are mostly used as thickening, stabilizing, emulsifying, binding, creating structure and gelling agents, because of their high viscosity in aqueous media (Freitas et al. 2011). They need to have physico-chemical properties that can satisfy some food processing conditions—variations in pH, temperature, ionic strength, influence of other food components, etc. Many of MPSs have such properties; however, only two are allowed to be used as additives in the food industry in Europe and in the United States: xanthan and gellan (Donot et al. 2012). Some potential food applications of certain microbial polysaccharides are shown in Table 17.1.

Certain strains of lactic acid bacteria synthesize EPSs, which participate in the production of fermented milk products: yoghurt, cheese, fermented cream, kefir, etc. They are very important for the final texture (as biothickening agents they improve the rheology of the product—viscosity which makes them slimy and fluid, and elasticity which gives them firmness and gum-like properties), taste and stability of products (they bind water and limit syneresis). EPSs from lactic acid bacteria are not used only in technology, but also they have some health benefits for consumers. Their viscosity increases as that fermented product stays in the gastrointestinal tract, which helps its colonization with probiotic bacteria. Also they can be metabolized

by microorganisms of the colon to short-chain fatty acids (acetate, propionate, butyrate), and those cannot only provide energy to the epithelial cells, but also play roles in the prevention of colon cancer (Duboc and Mollet 2001). Because of this property, some microbial polysaccharides are defined as prebiotics—“non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve the host health” (Scantlebury-Manning and Gibson 2004).

Polysaccharide extracts of some higher fungi have shown a significant antioxidant activity (Wasser 2002). Recently, results are published on determining antioxidant and immunomodulatory activities of polysaccharide extracts on some medicinal mushrooms, among them *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*, and chemical characterization and antioxidant properties of polysaccharide extracts obtained by the alkaline treatment of fruiting body *Schizophyllum commune*, and also antioxidant activities of polysaccharide extracts of mushrooms *Ganoderma applanatum*, *Ganoderma lucidum*, *Lentinus edodes*, *Trametes versicolor* and *Laetiporus sulphureus* (Kozarski et al. 2011, 2012; Klaus et al. 2011, 2013). Investigations of glycans of different species of higher fungi resulted in a review of chemical characteristics and antioxidant properties of polysaccharides extracts isolated from basidiomycetes (Kozarski et al. 2013).

17.4 Modified Polysaccharides

With the increased commercial use of polysaccharides, increased are the requirements of synthetic methods that would selectively structurally modify these polymers using procedures that could be easily controlled and simplified. This primarily applies to those modifications that would affect the improvements of properties of a product, such as, for example, its viscosity, hydrophilicity/hydrophobicity, polyelectrolytic effect or capacity of the gel to form metal chelates, and the incorporation of antimicrobial agents or nanoparticles of silver and copper, and among many applications, increases the function of polymer films, especially in food packaging, or in the form of coatings for food, or as a package (Vijayendra and Shamala 2014; Hu et al. 2014).

Microbial exopolysaccharides can be shaped into micro/nanoparticles, scaffolds and hydrogels, that can be applied in biomedicine for drug delivery, encapsulation of bioactive compounds, imaging, tissue engineering and wound dressing (Gupta and Gupta 2004; Lévesque and Shoichet 2007; Kanmani and Lim 2013; Collins and Birkinshaw 2013). Some of those MPSs (xanthan, sulphated dextran, sulphated curdlan) possess some biological activity, too, so their use is very important for the design of such pharmaceuticals (Huynh et al. 2001; Delair 2011; Lehtovaara et al. 2012). Also, some formulations that contain fucose and oligosaccharides obtained by its hydrolysis are known as anti-carcinogenic, anti-inflammatory and anti-aging agents (Freitas et al. 2011). After adequate chemical modifications, they may serve as a covalent carrier for drugs, e.g. antibiotics, facilitating their solubility in water

while decreasing cytotoxicity and retaining the drug activity (Zeković et al. 2006; Rekha and Sharma 2011; Vetvicka and Vetvickova 2012; Li et al. 2013; Goodarzi et al. 2013). The main advantages for the use of such polymers in this kind of formulations are their biocompatibility, non-toxicity and biodegradability (Fu and Kao 2010; Zhang et al. 2011; Jana et al. 2013; Sun and Tan 2013).

17.5 Outlook and Perspectives

Up to date, polysaccharides isolated from microorganisms are of great interest in the overall hydrocolloid market, even though they are not sufficiently represented. Research interests in its production are exponentially growing, especially because of the possibilities of using low-cost substrates in improving their production and downstream processing, as well as the possibility of metabolic engineering which allows a controlled production of polymers with exact, fine-tuned properties. By altering conditions of some biotechnological processes for obtaining microbial polysaccharides, such as nutrient media for the growth of polysaccharide producing microorganism, carbon and nitrogen content, temperature, pH, aeration, stress conditions, polymers with various chemical composition, structure and properties can be obtained consequently. In the next few years, a significant increase in the number of different products and technologies based on microbial polysaccharides may be expected.

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Chapter 18

Encapsulation Technologies for Food Industry

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18.1 The Rising Interest for Encapsulation Technologies in Industrial Applications

The food processing industry is one of the largest manufacturing industries world-wide. This industry handles and processes numerous raw materials and finished products in powdered and particulate forms. New trends of living impose food which fulfill many criteria (tasteful, healthy, of nice appearance). Therefore, the improvement of the existing technologies and development of the new ones is inevitable. In this sense, future competitiveness may be critically dependent on the knowledge obtained by research activities in the field of encapsulation technologies. Encapsulation has a large impact on different aspects of food industry as it is evidenced from the huge number of published scientific papers, patents, and reports. Driven by the increasing consumers' demand for more healthy, tasty, and safe food products, the need for edible systems able to protect and release functional

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compounds and the necessity for creation of a more sustainable industry, encapsulation has covered many issues relevant to food and nutrition.

Encapsulation is defined as a technology for the packaging ingredients or cells with the help of protective membranes, also called shell or coating. Basically, any compound that needs to be protected (from heat, light, or oxygen), isolated (from the surrounding or other food ingredients) or slowly released can be encapsulated in the form of nano- and microstructures. The entrapped or coated compound is usually a liquid but it can also be a solid or a gas. In the food industry, encapsulated compounds include acids, lipids, enzymes, microorganisms, flavors, artificial sweeteners, nutraceutical and therapeutic actives, nutrients, antioxidants, vitamins, minerals, water, chemical leavening agents, sweeteners, colorants and salts, and antimicrobial agents (Schrooyen et al. 2001; Gouin 2004; De Vos et al. 2010; Nedović et al. 2013).

It is well known that microorganisms are able to produce a wide array of valuable products: amino acids, organic acids, vitamins, and pigments. For over 40 years much effort has been expended on examination of the possibility of improving the efficiency of fermentation processes by using encapsulated or immobilized enzymes or even whole microbial cells. Immobilized cells/enzymes refer to those which are attached onto a solid carrier material, or are able to penetrate through the pores of a matrix material, or are only self-aggregated (flocculation). In a broader sense of meaning, immobilization covers encapsulation as well. Several companies worldwide (NOVO Industry and Gist Brocades are the largest) have patented immobilized enzymes and have been producing them for industrial applications for some 50 years (Sweigart 1979). Also, immobilization technology has been attracting the attention of the fermentation industry of beverages including continuous primary fermentation, low alcohol beer production, and secondary maturation. Immobilization of cells as a tool for improvement of efficiency of biotechnological processes offers series of advantages over conventional processes: possibility of continuous operation with immobilized cells, greater cells stability/extended viability and higher cell density during the process, as well as easier product separation (Rathore et al. 2013). Additional benefits upon cell encapsulation reported in literature are improvement of metabolic activity when compared to free cells, as well as simplified process downstream (for secreted products) (Murthy et al. 2014). Immobilization of cells provides reduced shear stress, higher flow rates, and better mass transfer in comparison to cell-free systems (Murthy et al. 2014). Finally, the use of reactor-immobilized cell system results in the increased productivity and higher fermentation efficiency (Willaert and Nedovic 2006). However, only a limited number of beer fermentation, maturation, and alcohol-free beer production processes utilizing immobilized cell technology have found their way into the industry: Kirin Brewery Company in Japan, Labatt Breweries (InBev) in Canada, Meura Delta in Belgium, Sinebrychoff's Helsinki brewery in 1990, and later at Sinebrychoff's Kerava brewery and at Hartwall Plc's Lahti brewery, Alfa Laval and Schott Engineering and Sinebrychoff Brewery in Finland, Sapporo Breweries Ltd. in Japan, Bavaria in the Netherlands (Narziss 1997; Virkajärvi and Linko 1999; Brányik et al. 2005; Verbelen et al. 2006; Willaert and Nedovic 2006). Namely, pilot-plant and full

industrial-scale processes have encountered numerous engineering problems (carrier choice, reactor design, risk of contamination), which have had, along with the effect of immobilization on the yeast physiology, a very unpredictable impact on the flavor profile of the beer produced. A number of technical challenges still require resolution: removal of excess yeast and carrier regeneration, removal of carbon dioxide, sustaining yeast viability, optimization of oxygen (air) feed, prevention of microbial contamination and prevention of clogging or channelling of the reactors, just to name a few.

The main restriction for wider use of encapsulation technology in food industry is high cost of encapsulation technology. Although there are many literature data on available encapsulation technologies, and enormous number of scientific papers describing many different types of encapsulates, the papers dealing with evaluation of encapsulation costs are difficult to find. Actually, precise information about the costs of encapsulation technologies is not available because a lot of developed applications are keeping as secret. One of the very few publication, written by Brandau (2002), reports manufacturing costs of patented BRACE-Processes (based on vibrating nozzle extrusion techniques) from 0.31 to 2.66 €/kg depending on a manufacturing capacity from 1000 to 50 tons/year, respectively. On the other hand, Gouin (2004) roughly estimated €0.1/kg as the maximum cost for microencapsulation process acceptable for the food industry. Therefore, many, if not most of encapsulation technologies today accessible, no matter how scientifically impressive they are, supported by strong theoretical treatments, and even utilizable in cosmetic and pharmaceutical industry, actually are not appropriate for food industry. This chapter is focused on those which are scalable and thus acceptable for the industry.

18.2 Benefits of Microencapsulated Ingredients in the Food Industry

Technological advantages achieved by encapsulation are protection of food/feed ingredients against chemical degradation (caused by oxidation or hydrolysis), or reaction with other ingredients or any of the unwanted changes caused by environmental variations of enzymatic, pH, temperature, and ionic strength conditions (McClements et al. 2007; Wandrey et al. 2010; Fang and Bhandary 2010). Reduced evaporation of volatile compounds (such as aroma compounds) is preferable in thermally processed food products. Then, many flavoring compounds are highly susceptible to oxidative degradations during storage. For example, citral (lemon aroma), one of the most important flavors (widely used as an additive in foods, beverages and cosmetics) is a good example of such compounds. It decomposes rapidly during storage by a series of cyclization and oxidation reactions. Acid-catalyzed cyclization of citral reduces the intensity of the fresh lemon flavor due to its decreased levels and hence results in the formation of variety of undesirable compounds creating off-flavors that limit the shelf-life of acidic citrus flavored foods and beverages (Maswal and Dar 2014). Consumer demand for natural ingredients as well as for more

complex and authentic aroma profiles have resulted in an increased demand for the incorporation of the compounds, such as citrus oil and citral, into different food and beverage products. Food products with incorporated encapsulated flavors will have increased stability during processing, storage and consumption and longer shelf-life in comparison to those with free compounds.

Encapsulation technology is particularly suited for the production of systems suitable for delivery of functional compounds. Bioactive components and micronutrients, such as vitamins, polyphenols, carotenoids, phytosterols, essential polyunsaturated fatty acids, and peptides, exhibit a wide range of biological activities, such as antioxidant, anti-inflammatory, antibacterial, and antiviral, but are only hardly soluble in water and susceptible to deterioration during processing or exposure to light, oxygen, moisture, or temperature (Manach et al. 2005). The encapsulated systems should prevent degradation of bioactive compounds and improve their bioavailability.

Many ingredients used in the food industry are unpalatable, and their unwanted odor or taste needs to be masked, e.g., polyphenols, vitamins, and minerals, added to improve nutritional properties of the food product. Particularly, plant polyphenols (natural antioxidants) exhibit a wide array of positive health effects and therefore, they are highly recommended for consumption. However, polyphenols have bitter taste which limits their consumption in high amounts. Encapsulated formulations of plant extracts (rich in polyphenols) have a potential to be used as additives in new functional food products; upon intake of such products it is possible to achieve a synergistic action of different polyphenolic compounds. Conversion of liquid ingredients to solid forms by encapsulation has been used as a strategy for easier handling with them (Brownlie 2007; Nedović et al. 2013). For example, many flavors occur at liquid state at room temperature, and easily evaporate. Encapsulation may have a purpose to separate components from each other within a food system; e.g., oils from egg whites so that the egg whites will yield a larger foam volume when whipped. Other possible benefits sometimes actually arise as side effects of encapsulation. For example, volatiles are primarily encapsulated with an aim of their stabilization, but the side effect is improved safety of processing with them due to reduced flammability of such aroma compounds.

One of the goals of encapsulation, the one which is most difficult to accomplish, is a timely and targeted release of bioactive molecules and living cells (e.g., probiotics) at a controlled dynamics (often referred as controlled delivery). In this way, a proper delay of release of an active compound is achieved. Also, the rate of release is reduced so that encapsulated flavors and sweeteners maintain a desired flavor effect throughout the time of consuming the product. This manner of delivery of active substances enables the application of a wider range of food ingredients and ensures optimal dosage with those. The common triggers used to prompt the release of encapsulated ingredients are pH change, mechanical stress, temperature, enzymatic activity, time, and osmotic force. Herein we should give some examples. A pH decrease to a stomach acidic medium of pH 2.2 will trigger release of actives from chitosan microbeads (Trifković et al. 2014), while pH increase in the small intestine will trigger release from the multilayer emulsion stabilized with the pectin layers (Benjamin et al. 2012). The liposome bilayer is broken down at certain

temperature, the process followed by release of the content; the trigger temperature is the transition temperature of the phospholipids from which the liposomal bilayer is made; most phospholipids have phase transition temperature between 42 and 50 °C, so they will release actives upon heating. Most liposomes will also release actives upon being attacked by lipase activity, thus at the specific site of the gut, precisely in the duodenum (Woodley 1986; Chakraborty et al. 2009). Hydrogel-based systems are examples of those influenced by osmotic changes (Gupta et al. 2002).

From the aspect of release properties, i.e., mass transfer properties, particle size plays an important role. Depending on the method of microencapsulation, the size of the produced microparticles ranges in wide interval, usually from 5 µm to 3 mm. The release from large particles is slower than that of smaller particles of the same material (if the release is governed by diffusion rate). Furthermore, large particles move faster through the colon and come rapidly in the descending colon prior defecation (Washington et al. 2001). On the other hand, the size of encapsulates influence rheological, textural and sensory properties, quality and stability of the food products to which they are added. The size of probiotic-encapsulating particles affects survival rate and colonization of encapsulated bacteria. The large particles can negatively affect the textural and sensorial properties of the food products in which they are added (Burgain et al. 2011; Sandoval-Castilla et al. 2010). Thus, it is preferable for solid encapsulates to be smaller than 22 µm in order not to be sensed (Heath and Prinz 1999). Small and controlled size particles are more convenient for incorporation into food products due to the easier managing and higher stability (Cui et al. 2001). On the other hand, large solid particles can create certain visible and texture characteristics (e.g., increased brittleness) of final products, desirable for some special applications, (e.g., crunchy products), while smaller particles are better for achieving successful adhesion and probiotic colonization in the gut.

18.3 Encapsulation Technologies

This section aims to provide a short overview of commonly used processes to encapsulate food active agent. Table 18.1 summarizes the basic principles of these processes with advantages and limitations.

18.3.1 *Spray-Drying*

Spray-drying is the most accepted encapsulation technology in the food industry, traditionally used since the late 1950s. After being spray-dried, liquids (such as flavors, unsaturated oils, vitamins, minerals, and enzymes) are converted to powders and protected from degradation. Spray-drying is typically used to produce food additives and powdered flavors. Both, hydrophilic and hydrophobic bioactive compounds can be used as a core material, but hydrophobic molecules are usually first

Table 18.1 Overview of some conventional encapsulation methods applicable in food industry

Encapsulation technologies		Principle	Advantages	Disadvantages	Product
Spray-drying		Atomization of core/wall materials mixture into a hot chamber where evaporation of water occurs.	Hydrophilic and hydrophobic core materials; fast, flexible, and highly reproducible operation; low cost; large-scale production in continuous mode	High operation temperature; difficult to control particle size; yields for small batches are moderate	Powder of spherical particles with diameters in the range 10–100 μm
Production of gel microspheres via	Extrusion	Forcing of a polymer solution through a syringe and formation of droplets which solidify into gel microspheres	The active compound is totally surrounded by the wall material; relatively low-temperature operation	Difficult to scale-up; low production capacity; the gel beads must be separated from the liquid bath; limited to low viscous solutions of carrier material	Gel beads (matrix type) with diameters in the range 0.2–2000 μm
	Emulsification	Gelling agent is added under shear to an emulsion of biopolymer-with-water in oil	The compound is totally surrounded by the wall material; easier to scale-up than extrusion	The gel beads must be separated from the liquid bath; more expensive than extrusion; more difficult to control the process	Gel beads (matrix type) with diameters in the range 10–1000 μm
Fluid-bed coating		Spraying the coating material onto solid particles which are fluidized by air	Uniform layer of shell; lower operation temperatures than in spray-drying; possibility to control capsule size distribution; controlled release of an active compound	Complex technology; high energy input; difficult to process submicron particles; degradation of highly temperature-sensitive compounds	Coated particles with diameters in the range 100–5000 μm
Melt extrusion		Extrusion of carbohydrate molt mixed with an active compound through one or more orifices	Cost-effective and environmentally friendly technology; long shelf-life of encapsulates	Complex equipment; high energy input; low loads of actives	Glassy carbohydrate particles with diameters in the range 300–5000 μm
Spray cooling/chilling		A molten matrix/core compound is sprayed into a cold chamber where solidification of particles occurs	Economical processing; suitable for heat-sensitive compounds	Low encapsulation efficiency; difficult to control particle size; rapid release of the active; hydrophobic character of the particles	Matrix-type particles with diameters in the range 20–200 μm

Oil-in-water emulsions	The core material is added in the polar or apolar layer of an O/W emulsion or W/O emulsion. One liquid (the dispersed phase) is dispersed by high-shear mixing in the other (the continuous phase) in the presence of surfactant(s) and biopolymers	Hydrophilic, hydrophobic and amphiphilic actives can be incorporated	Scale-up is difficult; limited choice of food-grade surfactants; weak physical stability; poor protection capacity; only spray-dried or freeze-dried forms are stable	The size dispersion of the emulsion particles is 0.2–5000 μm
Liposomes	Phospholipids are dispersed in an aqueous phase	Polar, apolar and amphiphilic actives can be incorporated; controlled delivery	Scale-up is difficult; high costs of raw materials; limited physical and chemical stability	Liposomes in the range 10–1000 μm
Complex coacervation	The oppositely charged biopolymer molecules are mixed at a pH below the protein isoelectric point (pI) leading to separation of the polymer-rich phase and the formation of a complex, i.e., coacervate containing active, which precipitates	High loads of actives; suitable for heat-sensitive compounds; heat-resistant capsules; controlled-release properties	Complex and time-consuming method, sensitive to pH and temperature; burst release effect, aggregation and conglutination problems; toxic chemical agents are used; residual solvents and coacervating agents on the capsules surfaces	Particles in the range 10–800 μm
Inclusion complexation	Co-precipitation occurs after addition of carrier (guest molecule) in active (host) aqueous solution followed by stirring/heating	Efficient protection; high temperature stability of capsules; increased solubility of actives; controlled release of actives	Limited to apolar compounds with a suitable molecular dimensions; host compounds are expensive	Particles with diameters 0.001–0.01 μm
Physical/chemical adsorption/adhesion	Physical or chemical adsorption on organic and inorganic solid materials	Low costs; suitable for microbial cells; high volumetric cell densities	Technical difficulties; changed metabolic activity of immobilized cells	Particles from several hundred microns to few millimeters

dissolved in an oil phase to create oil-in-water emulsion which is then spray-dried. Also, living probiotics have been spray-dried in order to ensure their survival in foodstuffs. Actually, probiotic cultures are frequently supplied as spray-dried powders to food producers (Holzapfel et al. 2001). The successful spray-drying of *Lactobacilli* and *Bifidobacteria* has previously been reported for a number of different strains, including *L. paracasei* (Gardiner et al. 2000; Desmond et al. 2002; Poddar et al. 2014), *L. curvatus* (Mauriello et al. 1999), *L. acidophilus* (Prajapati et al. 1987; Maciel et al. 2014; Yonekura et al. 2014), *L. rhamnosus* (Corcoran et al. 2004; Avila-Reyes et al. 2014), *L. plantarum* (Perdana et al. 2014), and *Bifidobacterium ruminantium* (O’Riordan et al. 2001).

The main principle of spray-drying is dissolving of an active compound (core) in a dispersion of a matrix (wall) material. This dispersion is atomized with a nozzle or a spinning wheel in a hot chamber where fast evaporation of water occurs under the stream of hot air. The dry particles fall to the bottom of the tower where they are collected. Alternatively, a cyclone is used for powder separation. The particles obtained are spherical with diameters in the range 10–100 μm .

Spray-drying is a fast, flexible, continuous, and highly reproducible operation. From the point of industrial application, the most important is that spray-drying can produce large amounts of encapsulates. Still, there are several limiting factors. One is the limited number of materials used for a matrix or shell. The carrier material must be soluble in water. Typical shell materials used in spray-drying processes include carboxydratyes (such as gums, maltodextrins, modified starches, alginates, and carboxymethylcellulose) and proteins (e.g., whey proteins, soy proteins, sodium caseinate). The process is as more expensive as the material is less soluble in water, since the amount of water which needs to be evaporated during processing increases. Another disadvantage of spray-drying is high operation temperature. Firstly, safety measurements must be considered to avoid explosion risks. Secondly, there are many bioactive compounds sensitive to heating. For example, high inlet temperature of air ($>160\text{ }^{\circ}\text{C}$) may cause a loss of certain polyphenolic compounds (Fang and Bhandary 2010). To illustrate, the inlet gas temperature had significant effect on the total polyphenol, protein, and genistein contents of the dried extracts of soybean (Georgetti et al. 2008). Some aromas with low boiling pint (e.g., acetaldehyde, diacetyl, dimethylsulfide) can be partially lost during processing (Porzio 2008). Also, simultaneous dehydration and phase changes, which are accompanying effects of spray-drying, often lead to cell membrane damages and denaturation of associated proteins (Gardiner et al. 2000; Morgan et al. 2006; Silva et al. 2011; Rathore et al. 2013). Therefore, thermal inactivation and even cell death were the end-results of the spray-drying process in case of many probiotic cultures (Anal and Singh 2007). Even those which survive typically loose activity after a few weeks of storage at room temperature. However, many studies showed that with the correct selection of the drying excipients (e.g., gum arabic, gelatin, and pectin) it is possible to increase stability of sensitive compounds during processing and to improve quality of the finished product (Georgetti et al. 2008; Burgain et al. 2011; Salar-Behzadi et al. 2013). Similarly, there have been some trials to improve culture viability during processing and storage, based on the addition of some agents prior to

drying. The agents which acted as thermoprotectants were trehalose (Conrad et al. 2000), nonfat milk solids and/or adnitol (Selmer-Olsen et al. 1999), growth promoting factors including various probiotic/prebiotic combinations (Desmond et al. 2002; Corcoran et al. 2004; Avila-Reyes et al. 2014), granular starch (Crittenden et al. 2001), and soluble fibers (gum acacia) (Desmond et al. 2002). Another approach has been proposed by Picot and Lacroix (2003) who applied coating of milk fat droplets containing powder particles of freeze-dried bacteria with whey protein polymers using emulsification; thus obtained emulsions were subsequently subjected to spray-drying. According to the authors, the developed two-step continuous process can be easily scaled up and could also be suitable for stabilization and/or controlled release of numerous food ingredients or supplements, including hydrosoluble (minerals, vitamins, and so on) and liposoluble (essential oils, flavoring oils, and so on) components. The use of freeze-dried probiotic powder is probably a more practical solution in the food industry because of the stability of the freeze-dried product during transport and storage (Rokka and Rantamäki 2010). Spray freeze drying method is perhaps the best solution for production of probiotic encapsulates. This technology combines processing steps that are common to freeze-drying and to spray-drying. The first step is atomization of probiotic cells into a cold vapor phase of a cryogenic liquid such as liquid nitrogen. Thus obtained frozen droplets are then dried in a freeze dryer (Wang et al. 2006; Semyonov et al. 2010).

Many factors determine retention of an active compound and formation of a stable final product, such as composition of matrix and its properties, portion of a carrier material of spray-dried encapsulates (Rosenberg et al. 1990; Charve and Reineccius 2009), inlet and outlet air temperatures (Jafari et al. 2008a; Wang et al. 2009; Bringas-Lantigua et al. 2012) and characteristics of emulsion preparation prior to spray-drying of hydrophobic compounds (Soottitantawat et al. 2003, 2005; Jafari et al. 2008a, b; Frascareli et al. 2012). In most cases, a large portion of an active compound actually stays on the surface of microparticles, so that it becomes directly exposed to the environment, easily prone to oxidation and loss. The non-uniformity of the microparticles is another unfavorable feature of the end powder product (Reineccius 1989).

18.3.2 Extrusion and Emulsification

Hydrogel microspheres have been used to encapsulate a wide variety of active agent, such as oil droplets containing aroma, cells, yeast, probiotics, and enzymes. The basic principle is forcing of a polymer solution through a syringe and formation of droplets which solidify (usually via ion exchange) into gel microspheres. In order to produce microsize particles, some force (in addition to gravity) must participate in droplet formation. In this purpose, an additional air-flow through an outer concentric nozzle, electric field, vibrations on a laminar fluid jet which breaks apart, or mechanical cutting of a liquid jet have been used to enhance dropping in case of

extrusion with coaxial air flow, electrostatic extrusion, vibration technology, and jet-cutting technology, respectively (Pruesse et al. 2008). Although promising on a laboratory scale, the technologies used for gel bead formation present serious difficulties for large-scale production. Low production capacity (e.g., production rate of ~ 800 μm -sized beads is between 0.1 and 8 mg s^{-1} depending on the solution viscosity and type of extrusion devices) and large bead diameter (from 0.2 to 2000 μm) are the main reasons behind small interest of industries for the droplet extrusion methods. Still, the application of the Jet Cutter technology for industrial purposes has been well established by geniaLab[®], which in 2003 produced more than 40 tons of hydrogel microbeads (Pruesse et al. 2003).

Small hydrogel beads are able to produce by utilizing emulsions. In short, calcium chloride (gelling agent for sodium alginate) is added to an emulsion of water droplets of a sodium alginate solution and active compound in vegetable oil. There are also some variations of this preparation. One is that both alginate and gelation agent are present in the water phase of the emulsion. Upon addition of an oil-soluble acid (acetic acid) calcium ions become released due to pH decrease and they start with networking with alginate. Gelling materials other than alginate can also be used such as kappa-carrageenan (gelation upon cooling with potassium ions), chitosan (crosslinking by addition of anions), gelatin (crosslinking with anionic polysaccharides, such as gellan gel at neutral pH), and pectin (chemically or physically crosslinked) (Zuidam and Shimoni 2010). The end products of emulsification are smaller microbeads (10 μm –1 mm) than that of extrusion, and the process is even easier for scaling-up. However, processing costs seem to be higher than those of extrusion since vegetable oil has to be completely removed and recycled. The new emulsion-based preparation methods have been developing for production of nano-sized particles from food-grade materials (Paques et al. 2013, 2014a, b).

18.3.3 *Fluid-Bed Coating*

If increased stability or controlled release of an active compound is required, a second spray-coating may be performed to secure an additional coating layer. The coating at industrial scale is commonly performed by fluid-bed coating. The use of well-known “wurster” fluid bed coating system in the food industries is problematic because the actual cost of the final coated powder is too high (Teunou and Poncelet 2002). The continuous process appears to be a cost-effective solution for food powder coating. In a fluid-bed tower, the matrix molecules are sprayed onto a pre-formed encapsulated bioactive food products or onto solid food particles which are fluidized by air. In this way, a uniform layer of shell material is created onto solid particles. The choice for matrix material is much wider compared to spray-drying technology. It includes fats, waxes, polysaccharides, proteins, emulsifiers, and complex formulations. The use of a molten lipid (hydrogenated vegetable oils, fatty acids, emulsifiers, or waxes) as a coating material (supplied either from the bottom or the top of the chamber) is particularly favorable from an industrial point of view,

since the coating formulation is concentrated (and not an aqueous-based), which means dramatically shorter processing times and lower energy input. Due to an optimal heat and mass transport, drying and granulation processes in a fluidized bed dryer can be carried out at lower temperatures compared to spray-drying. This is particularly important for survival of encapsulated bacteria (Schell and Beermann 2014). Further, granulation and coating procedures can be combined within one fluidized bed drying process. However, this technology is rather complex consisting of three major operations (fluidization, atomization, and drying) and involving as many as 20 different variables (Werner et al. 2007). An innovative hot melt fluidized bed coating is the one with the help of supercritical carbon dioxide for dissolving of a coating material (Schreiber et al. 2002; Wu et al. 2000).

The main disadvantage of this technology is high energy input needed for fluidization of solid particles. Namely, gravity force experienced by the particles has to be exceeded by an upward moving air flow. Conventional fluidized bed devices can efficiently process particles from 100 μm to few millimeters (Gouin 2004). However, processing smaller particles is rather difficult to achieve, especially submicron particles. There are two main engineering problems still needed to be solved: how to maintain fluidized bed of such small core particles, and even more complicate, how to atomize coating droplets as they should be one order smaller than particles. Fluid-bed coating have been commonly used to produce encapsulates aimed at controlled release applications.

18.3.4 Melt Extrusion

The basic principle of this technology is extrusion of carbohydrate molt mixed with an active compound through one or more orifices. The mixture is then quenched to form a carbohydrate glass containing an active compound, which is in most cases a volatile and unstable flavor. Encapsulates produced by melt extrusion have typical glass transition temperature between 30 and 70 $^{\circ}\text{C}$ (Zuidam and Shimoni 2010). The concept of this process is relatively simple, but the design and application of extruders is a complex field where a combination of different expertise is required. The extruders consist of thermomechanical mixers with one or more screws in a barrel. The material transfer is performed by rotation of the sinusoidal screws (self-wiping), which is sometimes combined with oscillatory movement of the screws. One of disadvantages is high energy input—mechanical energy via shearing and heat energy through the barrel wall. The horizontal extruders consist of five integrated processes and zones—(1) the feeding zone, (2) the melting, dissolving/conveying zone, (3) the mixing/dispersing zone, (4) the conveying/compression zone, and (5) the end zone or the die. An active compound is usually mixed with the matrix material within the feeding zone, but alternatively, the mixing can be performed in one of the subsequent sections, in order to reduce the time exposure of active compounds at high temperatures. Processing conditions such as temperature, pressure, and shear should be carefully controlled. The pressure gradually increases

from the beginning of the barrel (the feeding section) toward the end zone where the material is chopped to give final shapes, e.g., sheets, ropes, threads, or granular extrudes. Extrudes have size from several hundred microns to 2 mm. Starch, thermoplastic starch and modified starches, maltodextrins and celluloses have been commonly used for production of extruded encapsulates. In order to reduce water solubility of encapsulates, plasticizers and other constituents have been added to formulations. For the encapsulation of hydrophobic substances, emulsifiers are often used to stabilize the morphology of the two-phase system.

Melt extrusion is a cost-effective and environmentally friendly technology employing continuous processing. Therefore, extrusion processing is an attractive method to encapsulate a great variety of bioactive substances, mainly in starch based matrices (e.g., oils, Yilmaz et al. 2001; micronutrients, Li et al. 2011a, b). The compact carbohydrate glassy matrix provides good barrier to diffusion of oxygen and other molecules, providing in this way a long shelf-life for encapsulates, up to several years (Gouin 2004). The main restriction of this technology is a low load of actives (typically less than 10 %) which reflects on their cost-in-use (Zuidam and Shimoni 2010).

18.3.5 Spray Cooling/Chilling

Spray cooling/chilling has been used to conserve a number of flavors, enzymes, functional and textural ingredients such as minerals, proteins, and vitamins (Oxley 2012; De Vos et al. 2010; Zoet et al. 2011). About 10 % of all aroma encapsulates are produced by spray cooling. Spray cooling/chilling is considered as economical processing suitable for encapsulation of heat-sensitive compounds. This technology produced solid lipid microparticles that were efficient in protecting the probiotics against the passage through gastric and intestinal fluids (Pedroso et al. 2012). A molten matrix (usually fats) with a low melting point (32–42 °C) containing the bioactive compounds is sprayed through a nozzle into a vessel. Solidification of particles is induced by cold air injected into the vessel. The process parameters known to have impact on microparticles characteristics are heating temperature, feed flow rate, compressed air pressure (or wheel speed), air flow rate, but also the relationship between all these variables (Gamboa et al. 2011).

Spray chilling encapsulation presents some technological disadvantages, such as low encapsulation efficiency and the possibility of expulsion of the active ingredient during storage (Okuro et al. 2013). Encapsulates thus obtained are of matrix type and often, a high portion of the active compounds is actually located at the surface of microcapsules. Therefore, the possible negative of this technology is a rapid release of the content (within a few minutes after being incorporated in the foodstuff, Gouin 2004), since active compounds are in direct contact with the environment. Another disadvantage of spray chilling encapsulation is related to the hydrophobic character of the particles, which can make some applications difficult (Okuro et al. 2013).

18.3.6 *Oil-in-Water Emulsions*

Conventional oil-in-water emulsions have been largely used to encapsulate flavors. The major disadvantage of oil-in-water emulsions is that they are only kinetically stable. Namely, emulsions have weak physical stability when exposed to environmental stresses such as heating, refrigeration, freezing, drying, pH, and ionic strength changes and high mineral concentrations during transport, storage, or utilization. Nanoemulsions containing smaller oil droplets ($r < 100$ nm) are more resistant to oxidation than microemulsions (Maswal and Dar 2014), but, at the same time, they are prone to growth in particle size (by Oswald ripening) (Sagalowicz and Leser 2010). Then, emulsions have poor capacity to protect the encapsulated actives from release, due to the low thickness of the interfacial membranes and consequently, extremely high rate of molecular diffusion of the encapsulated compounds. From these raised a need for engineering the interface of oil-in-water emulsion droplets with biopolymers that modify its permeability and improve retention of volatile actives such as flavors. Thus, multilayer emulsions have become subject of many scientific works (e.g., Caruso 2001; Moreau et al. 2003; Ogawa et al. 2004; Grigoriev et al. 2008; Benjamin et al. 2012; Fioramonti et al. 2014). Still, emulsions as weakly stable encapsulates are often spray-dried or freeze-dried to provide a stable powder. Such dry emulsions can be used as instant formulations in food and beverages. Despite a countless number of scientific publications on emulsions as delivery tools for food ingredients, industry has not found yet enough interest in them. Probably, this technique is quite laborious at large scales, and, at the end, the size dispersion of the emulsion particles is too large.

18.3.7 *Liposomes*

The ability of liposomes to entrap water-soluble, lipid-soluble, and amphiphilic materials makes them attractive carriers for food industry. Phospholipids are primarily used as the encapsulating agents in liposome based microencapsulation processes. In recent years liposomes have been investigated as delivery systems for enzymes (Mozafari et al. 2008; Smith et al. 2010; Jahadi et al. 2012), proteins (Sun-Waterhouse and Wadhwa 2013), vitamins (Loveday and Singh 2008; Gonnet et al. 2010), flavors (Yoshida et al. 2010; Nedović et al. 2013), minerals (Ding et al. 2009; Evens et al. 2012), antioxidants (Isailović et al. 2013; Kerdudo et al. 2014; Peng et al. 2014; Rashidinejad et al. 2014), and antimicrobials (Malheiros et al. 2012; Zou et al. 2012; Boualem et al. 2013). Liposomes can stabilize the encapsulated material against the changes in the environment (temperature, moisture, pH, ionic strength) during processing and storage (Augustin and Hemar 2009). Perhaps liposome entrapment has experienced the most rapid growth in interest of scientists and food technologists. The unique characteristic of these delivery systems is targetability which makes them attractive in pharmacy but also in the food sector (Fathi et al. 2012).

Besides, the fact that liposomes can be prepared using natural components (biocompatible, biodegradable, and nontoxic) enables overcoming of regulatory barriers and thus, faster and easier implementation of liposomes in food systems (da Silva Malheiros et al. 2010). On the other hand, the costs of raw materials are rather high and liposomal encapsulates have quite limited physical and chemical stability. Moreover, the most methods used for liposomes preparation have never been scaled-up because they are time-consuming and complicated (thin lipid film hydration, extrusion through membranes), some of them imply forces which have detrimental effect on sensitive actives (sonication, high pressure homogenization, microfluidization), some of them include the use of organic solvents (thin lipid film hydration). Only solvent injection and proliposome methods can be adopted by industry. In food applications liposomes encapsulating enzymes have been used to enhance cheese ripening (Taylor et al. 2005).

18.3.8 *Complex Coacervation*

Coacervation, often called “phase separation,” is considered as a true microencapsulation technique because the core material is completely entrapped in the matrix. Coacervation is based on liquid-liquid phase separation of an aqueous solution into a polymer-rich phase (coacervate) and a polymer-poor phase. In complex coacervation two or more types of polymers participate. The oppositely charged biopolymer molecules are mixed at a pH below the protein isoelectric point (pI) leading to separation of the polymer-rich phase and the formation of a complex, i.e., coacervate, which precipitates. At the same time the active compound is retained at the coacervate phase. The process is governed by pH and temperature manipulation.

Microcapsules made by complex coacervation have high payloads and a series of distinct strengths, including oxidation retarding, heat resistance, and controlled release. Therefore, this technology has been applied in encapsulating flavor oils (Xing et al. 2004; Weinbreck et al. 2004; Yeo et al. 2005; Prata et al. 2008; Leclercq et al. 2009; Jun-xia et al. 2011; Zhang et al. 2011; Ades et al. 2012; Koupantsis et al. 2014; Yang et al. 2014) and nutritional ingredients (Drusch and Mannino 2009; Junyaprasert et al. 2001; Barrow et al. 2007; Augustin and Hemar 2009; Zhang et al. 2009, 2012). Various combinations of proteins (including gelatine or soybean protein isolate) with gum arabic, xanthan, pectin, and carboxymethyl cellulose (CMC) have been used as raw materials. A challenge issue with complex coacervation is the burst release effect, aggregation and conglutination problems, which is not desirable in applications of most microcapsules. Current technology works as a batch and time-consuming process. Since coacervates are stable at a very narrow range of pH and ionic strength, a careful monitoring of processing conditions is required even at production levels (Zhang et al. 2009; Kaushik et al. 2014). And yet, complex coacervation is one of the commercially successful methods for microencapsulation of flavor oils and oils rich in omega-3 fatty acids (Barrow et al. 2007; Barrow 2010; Gouin 2004).

18.3.9 Inclusion Complexation

Molecular inclusion is the association of the active in a cavity of the host material's molecules. The best known host material is cyclodextrin. Most of literature data refer to cyclodextrin inclusion complexes with volatile and sensitive flavors and other food additives (the most recent are those from Ponce Cevallos et al. 2010; Del Toro-Sánchez et al. 2010; García-Segovia et al. 2011; Wang et al. 2011; Liang et al. 2012; Mantegna et al. 2012; Ciobanu et al. 2013a, b; Yuan et al. 2014; Gomes et al. 2014). About 1 % of all aroma encapsulates are based on the use of β -cyclodextrins (Porzio 2004). Cyclodextrin-flavor inclusion complexes provide prolonged shelf-life and high temperature stability of the flavor (up to 200 °C). However, the load of cyclodextrins with aromas is low, between 8 and 10 % (Szente and Szejtli 2004).

18.3.10 Physical/Chemical Adsorption/Adhesion

Physical or chemical adsorption is commonly applied to immobilize microorganisms. They adsorb even spontaneously on a wide variety of organic and inorganic solid materials, so-called supports. Binding of cells occurs via Van der Waals forces, ionic bonds, hydrogen bridges, or covalent interactions. Microbial cells exhibit a dipolar character and behave as cations or anions, depending on the cell type and environmental conditions. Diethylaminoethyl (DEAE)-cellulose (beads), kieselguhr (diatomaceous earth), clay, zeolites, kissiris, wood (chips), silicon carbide (rods), glass (beads), gluten pellets, stainless steel, and fruit pieces have been used for cells to adhere on the surface, to flocculate, or settle in the pores of the framework. Thus obtained immobilized biocatalyst can be used for bioprocess intensification, for example, of beer, wine, or cider fermentation (Verbelen et al. 2010; Kourkoutas et al. 2010), and production of amino acids, vitamins, organic acids, carotenoids, and other microbial metabolites (Breguet et al. 2010). Flavors also have tendency to adsorb to some microporous materials (Veith et al. 2004a, b).

18.4 Materials for Encapsulation

A lot of substances can be used to encapsulate solids, liquids, or gases of different types. Compounds used in the food industry undergo a rigorous scientific safety evaluation before they become approved for use and they have to be certified for food applications as “generally recognized as safe” (GRAS) materials (Barlow 2013). Actually, the whole food process should be designed to meet the safety requirements of the governmental agencies such as the European Food Safety Authority (EFSA), Food and Agriculture Organization (FAO) and the World Health Organization (WHO) or Food and Drug Administration (FDA) in the USA (Wandrey et al. 2010).

It is important to recommend Acceptable Daily Intake (ADI) level for the consumption of additives to food and so reduce human health risk. Regulatory information associated with materials, which can be used for food is available in standard bibliographies (e.g., the Codex General Standard for Food Additives or the *Codex Alimentarius*).

A lot of publications are related to the materials used for microencapsulation in the food industry (e.g., Werner et al. 2007; Nedović et al. 2013; Wandrey et al. 2010; Fang and Bhandary 2010; Serna-Cock and Vallejo-Castillo 2013; Jackson and Lee 1991; Gibbs et al. 1999; Desai and Park 2005). Before selecting an encapsulation material, it is important to consider some criteria such as functionality that encapsulates should provide to the final product, potential restrictions for the coating material, concentration of encapsulates, the required type of release and stability requirements (Wandrey et al. 2010; Desai and Park 2005). The fundamental knowledge of the chemistry and physicochemical properties of the materials is necessary for a successful product development. Materials used for a design of the protective shell (coating, membrane, capsule, carrier material, external phase, or matrix) of encapsulates must be food-grade, biodegradable, and able to form a barrier between the internal phase and its surroundings (Wandrey et al. 2010; Fang and Bhandary 2010). From the functionality point of view, ideally encapsulating materials have to provide mild conditions for encapsulation, be biocompatible, nontoxic to the host (and to the cells if cells are ought to be encapsulated), to hold actives within capsules structure during processing or storage under various conditions, not to react with the encapsulated material, to have good rheological characteristics, to have easy work ability during the encapsulation process, to be impermeable for antibody-sized molecules, have sufficient membrane permeability and ability to overcome the acidic and enzymatic environment of the stomach and to increase adherence capacity and residence time of the actives in certain segments of the gastrointestinal tract. Because one material could not possess all these properties, combinations of materials with different properties are often used. From the industrial point of view, the price plays an important role in a selection of the encapsulating material. The majority of materials used for encapsulation in the food sector are carbohydrate polymers, proteins, lipids, and other organic and inorganic materials. In the following section we well described some of the most frequently used materials.

18.4.1 Carbohydrate Polymers

Among all materials, the most widely used for encapsulation in food applications are carbohydrates (Nedović et al. 2011; Serna-Cock and Vallejo-Castillo 2013). Native polysaccharides are of enormous varieties. The names carbohydrate polymer and polysaccharide refer to the chemical structure; these materials are composed of sugar residues and/or their derivatives. Carbohydrate polymers include gums/hydrocolloids obtained by chemical modifications of native polysaccharides and others natural compounds, such as starch and cellulose and their derivatives and numerous complexes of macromolecular structures, plant and marine extracts.

Starch and derivatives. Starch is the second most abundant polysaccharide, after cellulose (Vorwerk et al. 2002), supplied as a low cost product. Starch is a polysaccharide consisting of a large number of glucose units linked by glucosidic bonds. It consists mainly of amylose, a linear polymer of D-glucopyranose joined by α -1-4 glucosidic bond and amylopectin, a branched polymer of glucose joined by α -1-4 glucosidic bond and α -1-6 glycosidic bond (Sajilata et al. 2006). Starch is normally a white powder, insoluble in cold water, ethanol, and common solvents. Natural starches do not have the emulsifying property and they present an ideal surface for the adherence of the probiotic cells to the starch granules (Anal and Singh 2007). The *modified starches* have been put in a practice at commercial scale for designing of encapsulates. The aim of modification (by introducing bulky groups) is to alter the structure in order to enhance and extend the industrial applicability. The chemically modified starches are found to be superior in emulsification processes of volatile flavors during spray-drying (Varavinit et al. 2001). Hydrolyzed starches offer good protection against oxidation. The disadvantage is a lack of emulsifying properties so they have to be used in a combination with gum acacia or other emulsifying agents like whey proteins (Poshadri and Aparna 2010). Starches have been used for coating and encapsulation by several technologies including spray-drying, fluidized bed coating, freeze-drying, microwave-assisted heating technologies, and extrusion. *Syrups* are starch hydrolysates with a dextrose equivalent, DE > 20 (Critical Data Tables 1975). They are suitable for fluidized bed coating, extrusion, and co-crystallization of biomolecules in the food industry (Wandrey et al. 2010).

Maltodextrin is a corn flour partially hydrolyzed with acids or enzymes. It is relatively cheap material with neutral aroma and taste and with high water solubility, low viscosity at high solid concentrations, and good antioxidative property (Turchiuli et al. 2005). Maltodextrin provides structural integrity to the final product, and possesses functions such as film formation and thermal resistance. Nowadays, maltodextrin is commonly mixed with gum Arabic and used for encapsulation of various compounds, for binding of flavors and fat compounds, and reducing oxygen permeability of the wall matrix (Sansone et al. 2011; Zhang et al. 2007; Madene et al. 2006).

Dextrins (starch gums) may refer to any product obtained by any method (e.g., heat, acid, enzyme) for degrading the starch. Dextrin formulations can be prepared at higher concentrations than unmodified starch. Yellow corn dextrin is used to encapsulate water-insoluble flavors and oils by spray-drying technology (Wurzburg 2006).

Cyclodextrins are a family of cyclic oligosaccharides composed of 6–8 glucose molecules (α -(1 $\rightarrow$ 4)D-glucopyranoside units). The three most common cyclodextrins used are α -cyclodextrins, β -cyclodextrins, γ -cyclodextrins having 6, 7, and 8 glucopyranose units in the cyclic structure, respectively (Del Valle 2004). Astray et al. (2009) nicely reviewed applications of cyclodextrins in food processing and as food additives with a variety of aims: (1) to protect lipophilic food components that are sensitive to oxygen and light- or heat-induced degradation; (2) to solubilize food colorings and vitamins; (3) to stabilize fragrances, flavors, vitamins, and essential oils against unwanted changes; (4) to suppress unpleasant odors or tastes; and (5) to achieve a controlled release of certain food constituents. Cyclodextrins cannot be adsorbed in the upper gastrointestinal tract, but they are completely metabolized by the colon microflora (Szente and Szejtli 2004).

Polydextrose is a synthetic, highly branched polymer with many types of glycosidic linkages created by heating dextrose with an acid catalyst and purifying the resulting water-soluble polymer (Elias 1992). Polydextrose is tasteless and similar to fiber in terms of its resistance to digestion.

Celluloses and derivatives. Cellulose is a polymer of β -D-glucose linked by β -(1 $\rightarrow$ 4)-glycosidic bonds. It is insoluble in water and other common solvents. The primary cell wall of green plants is made of cellulose. This material requires 320 °C and high pressure to become amorphous in water (Deguchi et al. 2006). The hydroxyl groups of cellulose can partially or completely react with various reagents to produce derivatives with properties desired for food applications. In general, aqueous solutions of modified celluloses are odorless, colorless, and clear. Cellulose and derivatives are suitable materials for spray-drying, fluidized bed coating, extrusion, and emulsification technologies (Wandrey et al. 2010). *Delignified cellulosic materials (DCM)* are supports of food grade purity, low cost, and easy to prepare in industry. It has been used as immobilization supports of yeasts and bacteria for beer and wine making (Mallouchos et al. 2003a, b; Agouridis et al. 2005). The possible commercialization of yeast cells immobilized on these materials was evaluated through freeze-drying experiments (Iconomopoulou et al. 2000). The safety, low cost, and consumer acceptance of these types of supports are unquestionable. *Carboxymethylcellulose* is produced by treating cellulose with aqueous sodium hydroxide followed by reaction with monochloroacetic acid. It is an anionic polysaccharide widely used in the food industry as a functional ingredient due to its water solubility and pseudoplastic nature of its solutions (Zecher and Gerrish 1999). *Methylcellulose* has good film forming properties and surface activity, but it is not digestible. *Hydroxypropyl methyl cellulose* is supplied as a white powder or as granules, which form a viscous solution in the water as well as in the most polar solvents. Aqueous solutions undergo reversible transformation from sol to gel upon heating and cooling (Wandrey et al. 2010). *Hydroxypropyl cellulose (HPC)* is non-ionic cellulose, soluble in cold water but it becomes insoluble at temperatures above 45 °C. HPC has good film formation ability, and the high surface activity (Murray 2000). It is compatible with the most of water-soluble gums and it yields homogeneous solutions with CMC, hydroxyethyl cellulose, gelatin, sodium caseinate, poly(ethylene oxide), carbowax, guar, alginate, and locust bean gum (LBG). The addition of HPC to wax or oil systems will increase the system viscosity (Butler and Klug 1980). *Cellulose acetate phthalate* is composed of cellulose polymer where 50 % of hydroxyl groups were esterified with acetyls and 25 % were esterified with one or two carboxyls of a phthalic acid. It is reusable, sterilizable, biologically, mechanically, and chemically stable material under fermentation conditions, neutral in taste, food approved (Linko et al. 1997; Branyik et al. 2001). Spray-drying of probiotic bacteria using this material as a shell provides good protection for microorganisms (Fávaro-Trindade and Grosso 2002).

Gums. In general, plant gums are plant products of gummosis, which is extruded and deposited on the bark of trees. Plant species that belong to the Leguminosae family are cultivated to provide gums for the food industry (Orozco-Villafuerte et al. 2005). Plant exudates (gum arabic, gum karaya, gum tragacanth) have been

used for the purpose of spray-drying, fluidized bed coating, extrusion, coacervation, and freeze-drying technologies (Wandrey et al. 2010). Gum arabic (*Acacia*) is a dried plant exudate obtained from *Acacia* species (Poshadri and Aparna 2010). It is the most widely used encapsulating material in the food industry, mainly for spray-drying, due to its good solubility, emulsification characteristics, and low viscosity in an aqueous solution (Gharsallaoui et al. 2007). *Acacia* is a nontoxic, odorless, and tasteless material, compatible with the most of other plant hydrocolloids, proteins, carbohydrates, and modified starches. In addition, the wall material made from gum arabic is ideally suited for encapsulation of lipid droplets, creating a strong protective film around them (Krishnan et al. 2005). Also, it is the most used gum for a flavor encapsulation forming a thin film and protecting the coated compound from oxidation, evaporation, and moisture from the air (Buffo and Reineccius 2000). To lower the cost, the mixtures of gum arabic and maltodextrin have been proposed for encapsulation of sensitive compounds, e.g., oil, flavor, fatty acids, and micronutrients (Kanakdande et al. 2007; Jafari et al. 2008a, b; Gomes et al. 2010; Gupta et al. 2014; Fernandes et al. 2014). It was also found to be a useful prebiotic (Phillips et al. 2008).

Gum tragacanth is dried exudates obtained from species of *Astragalus* (FAO 1992b). In nature, it can be found as acidic calcium, magnesium, or potassium salt. This polysaccharide is formed by (1→4)-linked D-galactose residues with side chains of (1→3)-linked D-xylose units. Gum tragacanth consists of two components: a water-swellable component, which contains the tragacanthic acid polymer, and a water-soluble component, a colloidal hydrosol tragacanthin (Elias 1992). It is one of the most acid-resistant gums and it lowers the interfacial tension between the oil-in-water emulsions.

Gum karaya is dried exudates from *Sterculia* or *Cochlospermum* species (FAO 1992a). It is a partially acetylated polysaccharide obtained as calcium or magnesium salt formed of α-D-galacturonic acid and α-L-rhamnose residues. Side chains are attached by (1→2)-linkage of β-D galactose or by (1→3)-linkage of β-D-guluronic acid to the galacturonic acid of the main chain. One part of the rhamnose residues of the main chain are (1→4)-linked to β-D-galactose units (Weiping 2000). Gum karaya is compatible with other plant hydrocolloids, proteins, and carbohydrates.

Galactomannans. LBG, tara, and guar are isolated from the carob tree (*Ceratonia siliqua*), the tara shrub (*Cesalpinia spinosa*), and the guar plant (*Cyamopsis tetragonoloba*), respectively. They consist of linearly (1→4)-linked β-D-mannopyranosyl units with single α-D-galactopyranosyl units connected by (1→6) linkages as side branches. The three gum types differ in the ratio of D-mannosyl to D-galactosyl and in their solubility which increases with increasing number of side units. While guar is fully water soluble at room temperature, the solubility of tara is about 70 % under these conditions, but is completely soluble above 70 °C (Hoefer 2004; Seaman 1980). All galactomannans have been used for extrusion and phase separation technologies in the food industry (Wandrey et al. 2010).

Pectins form complexes with divalent ions, such as Ca²⁺, Ba²⁺, and Sr²⁺ in an aqueous solution (Chávarri et al. 2010). Pectins are hetero-polysaccharides with at least 65 % of α-(1→4)-linked D-galacturonic acid-based units. These units may be

present as free acid, salt, or naturally esterified with methanol. Pectins are soluble in water and in the most organic solvents whereas the gel strength increases with decreasing pH. Furthermore, consumer acceptance of pectins is not under the question (Mallouchos et al. 2002). Apple, grape skin, and quince pieces (rich in pectins) were considered as cheap supports for cell immobilization, suitable for continuous processes (Kourkoutas et al. 2003; Genisheva et al. 2012, 2014a, b).

Soluble soybean polysaccharide (SSPS) is mainly composed of galactose, arabinose, and galacturonic acid, but the chain also contains rhamnose, glucose, xylose, and fructose. The adhesive and film forming properties are excellent. The films are suitable for coating the surfaces of food ingredients. SSPS has the ability to stabilize protein particles at low pH without increasing the viscosity (Nobuhara et al. 2014). It can be used as an emulsifier and to protect oils from oxidation (Furuta and Maeda 1999; Wu et al. 2014a). Soybean polysaccharide has been used as a material for spray-drying and freeze-drying technologies (Wandrey et al. 2010).

Carrageenans are natural polysaccharides obtained from the red seaweeds *Rhodophyceae*. Carrageenans are commonly used in the food industry as a safe material approved by FDA, Codex Alimentarius, and the Joint FAO/WHO Food Additives (Sarett 1981). They are composed of high molecular weight linear polysaccharide with repeating of galactose units and 3,6-anhydrogalactose, both sulfated and non-sulfated. Carrageenans naturally exist as kappa (κ), iota (ι), and lambda (λ) (Thomas 1997). The gel strength, pseudoplastic properties, texture, solubility, and melting temperature vary due to the different components (Wu et al. 2014b). Carrageenan-based coatings have been applied for a long time to a variety of foods due to antimicrobial or antioxidant nature, and ability of reducing moisture loss, oxidation, or disintegration (Lacroix and Le Tien 2005). Edible films made of i-carrageenans possess good mechanical properties, enable stabilization of emulsions and reduction of oxygen transfers, but their highly hydrophilic nature limits the ability to provide a significant moisture barrier. This can be overcome by including lipidic materials in their formulation, such as fatty acids or waxes (Fabra et al. 2009). Spray-drying, extrusion, coacervation, and emulsification are typical technologies where carrageenan has been used frequently (Wandrey et al. 2010). The encapsulation of microbial cells in k-carrageenan beads keeps them in a viable state (Chen and Chen 2007).

Alginate is a naturally derived polysaccharide extracted from various species of algae. For the encapsulation purposes, calcium-alginate is preferable because it is simple to produce Ca-alginate. This material is relatively cheap, nontoxic, and biocompatible with the cells (Krasaekoopt et al. 2003, 2006). Calcium-alginate forms a strong heat-stable gel matrix which can develop and set at the room temperature, but physicochemical properties of gel can vary depending on the concentration of alginate and CaCl_2 . Co-encapsulation of probiotic bacteria with prebiotics in calcium alginate increases the survival of probiotics, even 1000 times in comparison to alginate alone (Dong et al. 2013). Calcium alginate gels are the most extensively tested supports for yeast cells in alcoholic fermentation, such as wine and beer making (e.g., Ferraro et al. 2000; Silva et al. 2002; Reddy et al. 2008). Ca-alginate gel has been mainly used in the form of spherical (micro)particles, i.e., (micro)beads. To keep stability of sensitive compounds (e.g., antioxidants, vitamins), and ensure

a prolonged release, alginate have been used in blends with other polysaccharides, such as chitosan, pectin, carrageenan, and psyllium (Belščak-Cvitanović et al. 2011, 2015; Azevedo et al. 2014). It is also a promising material for protein and peptide encapsulation (Dai et al. 2005; Gao et al. 2013). Some disadvantages of alginate beads are sensitivity to the acidic environment, the scaling-up of the production process (extrusion process in particular) that is very difficult, and very porous microparticles cannot protect the cells from its environment (Chen et al. 2005; Mortazavian et al. 2008; Gouin 2004). The last one can be overcome by mixing alginates with other polymers such as starch or applying structural modification of the alginate by using different additives (Sultana et al. 2000; Krasaekoopt et al. 2003; Mansouripour et al. 2013).

Xanthan gum is an exopolysaccharide produced by *Xanthomonas campestris* fermentation of glucose medium. It occurs as the mixed salt of sodium, potassium, and calcium. Xanthan may contain cellulases, which prevents its use with cellulose derivatives. It is soluble in cold water and the solution viscosity is stable over a wide range of pH (2–12) and temperature. The xanthan gum has been used for encapsulation of probiotics (Gbassi and Vandamme 2012) or as a carrier of bioactive compounds, such as antioxidants or phenolic compounds (Da Rosa et al. 2014).

Gellan gum is a microbial polysaccharide derived from *Pseudomonas elodea*, constituted of one molecule of rhamnose, one molecule of glucuronic acid, and two molecules of glucose (Chen and Chen 2007). Gellan gum forms hard gel through chemical reaction with cations such as sodium, potassium, calcium, and magnesium. The solubility and solution properties depend on the degree of substitution and the type and concentration of ions present in the solution. Gellan is able to withstand heating to 120 °C, which is a preferable characteristic from the aspect of sterilization. Since gellan gum has a low acid resistance, a mixture of xanthan and gellan gums has been used to encapsulate probiotic cells (Sultana et al. 2000; Sun and Griffiths 2000). Since the viscosity of gellan increases with the degree of acetylation, both high acyl gellans and low acyl (deacetylated) gellans are commercially available.

Chitosan is a linear polysaccharide composed of glucosamine units. It has been used in the food industry for the encapsulation of probiotics and prebiotics, aromatic compounds, enzymes, antioxidants, vitamins, minerals, catechins, and proteins due to its filmogenicity and nontoxicity (Chávarri et al. 2010; Higuera-Ciupara et al. 2003; Žuža et al. 2011; Belščak-Cvitanović et al. 2011; Agnihotri et al. 2004; Tang et al. 2012; Papadimitriou et al. 2012; Trifković et al. 2014). Chitosan has been preferably used as a coating material; thus, a chitosan coat improved the mechanical and chemical stabilities of the double layer alginate–chitosan beads during wine fermentation and prevented cell leakage from the beads into the medium (Drichoutis et al. 2007). Chitosan has not shown very good results in respect to probiotic cell viability (Mortazavian et al. 2008). Double-layer alginate–chitosan beads have been also used for the entrapment of bacterial and yeast cells in batch and continuous fermentation processes (Klinkenberg et al. 2001; Liouni et al. 2008).

Dextran is obtained from microbial fermentation processes of sucrose. It is mainly linear neutral polymers of α -D-glucose linked mainly by α -(1→6) glycosidic bonds, which can have variable amounts of α -(1→3) branches (Elias 1992). Dextrans are well soluble in water.

18.4.2 Proteins

Food proteins including soy proteins, milk proteins—caseins and whey proteins, egg proteins, zein, or hydrolysates of these proteins are commonly used as encapsulant matrices. The properties of proteins are influenced by the amino acid composition, conformation and charge, as well as their denaturation temperature. Proteins, because of their amphiphilic nature, are prone to self-assembly (Augustin and Hemar 2009). Aggregation and gelation of proteins enable the development of networks with embedded ingredients. Proteins are an effective transporter of bioactive molecules due to the ligand binding properties. Proteins have been used for the purposes of encapsulating fats, oils, fatty acids, and flavors (Chen et al. 2006).

Gluten is a complex mixture of gliadins, monomeric gluten proteins and glutenins, polymeric gluten proteins (MacRitchie et al. 1990). They exist conjoined with starch in wheat, rye, and barley. Gliadins and glutenins have unusually high levels of proline and glutamine and are therefore designated as “prolamins” (Shewry et al. 1986). Gluten proteins are strongly associated with baked products (Örnebro et al. 2000). They can be used for yeast cells immobilization (Bardi et al. 1996a, b) by using spray-drying, coacervation, and emulsification technologies (Wandrey et al. 2010).

Milk proteins have an excellent functional (emulsion preparation and stabilization, water and fat binding, thickening, gelation) and nutritional properties. Thanks to their structural and physicochemical properties, they can be used as a delivery system for probiotics cells (Livney 2010). Thus, milk proteins encapsulating probiotic cells have been implemented in biscuits, vegetable and frozen cranberry juice (Heidebach et al. 2009a, b). The proteins in milk also have the ability of delivering functional ingredients, binding small molecules and interacting with other polymers to form complexes (Livney 2010). Milk proteins have been successfully used in combination with polysaccharides, such as gum arabic, xanthan, and carboxymethylcellulose, in the formation and stabilization of food emulsion systems. Depending on pH, they interact with polysaccharides forming soluble or insoluble complex coacervates, nanoparticles or precipitates (Koupantsis et al. 2014).

Caseins are proteins that contain both hydrophilic and hydrophobic parts. Gelling of caseins by rennet enzyme makes a water-insoluble matrix suitable for probiotics encapsulation. High pH within the casein gel matrices, caused by buffering capacity of casein, protects the cells from the severe condition of simulated gastric juice at low pH (Krasaekoopt 2013). Sodium caseinate has been used to stabilize emulsions (Dwyer et al. 2013), such as fish oil emulsions (Day et al. 2007). A lot of work has been carried out on the encapsulation properties of sodium caseinate with respect to spray-dried emulsions containing functional lipophilic food ingredients (Drusch et al. 2012).

Whey proteins are composed of α -lactalbumin, β -lactalbumin, immunoglobins, and serum protein. For microcapsule formation, pH is the most important factor to control heat-induced whey protein aggregation. Whey proteins microcapsules can be formed by various methods, such as spray-drying, emulsifying–crosslinking, coacervation and from recent, electrospraying (López-Rubio and Lagaron 2012).

and desolvation technique for nano-sized particles (Bagheri et al. 2013). Whey proteins have been successfully applied for probiotic encapsulation by using extrusion method (Krasaekoopt 2013). Also, they have been used as the wall material to protect fish oil or volatile compounds in spray-dried formulations for a long time (Sheu and Rosenberg 1993; Park et al. 2014). Whey protein in combination with maltodextrins and corn syrup solids are reported to be the most effective encapsulation material during spray-drying (Kenyon and Anderson 1998).

Gelatin is a protein gum derived from collagen, the main protein component of animal connective tissues. Commercial gelatins can be divided into two groups, type A (acid pre-treatment) and type B (basic pre-treatment) (Poppe 1997). Gelatin thermo reversible gel exists only in a small temperature range depending on gelatin grade and concentration. The amphoteric hydrocolloid gelatin forms complex coacervates at low pH with anionic polysaccharides, such as gum arabic when it becomes positively charged (Madene et al. 2006). Mammalian gelatins dissolve in hot water forming solutions of high viscosity, which gel below 35–40 °C (Bigi et al. 2004; Chaplin 2007). Gelatin has been used for probiotic encapsulation, alone or in combination with other compounds (Annan et al. 2008). Also, it has been used as the wall material for extrusion, coacervation and freeze-drying of fish oil, flavors and volatile compounds (Liu et al. 2014; Nakagawa and Nagao 2012; Boland et al. 2004).

18.4.3 Lipids

A wide range of lipids (natural fats and oils, mono-, di-, and tri-glycerides, phospholipids, glycolipids, and waxes) can be used for encapsulation due to the fact that most food products actually contain fats. Polar lipids (monoglycerides, phospholipids, glycolipids) are surface active compounds and they can be used for stabilization, protection, and release control of emulsions containing active food ingredients. *Triglycerides* are the main constituents in animal fats and vegetable oils. They are not soluble in water. In a triglyceride molecule, the three fatty acids can be saturated or unsaturated. Glycerides have been processed in fluidized bed coating, extrusion, and emulsification processes (Wandrey et al. 2010). Lipids have been used to form emulsified films encapsulating active molecules or aroma compounds (Hambleton et al. 2008; Fabra et al. 2008; Marcuzzo et al. 2010); in the case of emulsified film, incorporated lipid globules act as carriers of active molecules, such as aroma compounds. Lipid-based nanosystems (made from lipids which are solid at room temperature), such as solid lipid nanoparticles (SLNs), have drawn much attention for enhancing the oral bioavailability of lipophilic compounds and control release properties (Ghosh et al. 2008; Salminen et al. 2013; Pandita et al. 2014; Cortés-Rojas et al. 2014). *Fatty acids* are produced by the hydrolysis of the ester linkage of naturally occurring fats and oils, which are in general triglycerides. Reduction of fatty acids yields to fatty alcohols. The water solubility of fatty acids rapidly decreases with increasing chain length. In fatty alcohols, a hydroxyl group has replaced the carboxylic group. Both, fatty acid and alcohols have been used for encapsulation by

fluidized bed coating, spray-cooling, and extrusion (Wandrey et al. 2010). *Waxes* are efficient substances to reduce moisture permeability because of their high hydrophobicity (Fabra et al. 2008). Natural waxes are considered as stable, inert, and safe materials. Natural waxes, especially carnauba wax and bees wax, seem suitable for aroma encapsulation (Milanovic et al. 2010, 2011). They have been used for fluidized bed coating, emulsification, and extrusion (Wandrey et al. 2010). Since natural waxes are of food grade purity (insect waxes like bees wax and plant waxes), they are permitted additives in the European Union (E901-903).

18.4.4 Inorganic Materials

There are several food-grade inorganic materials, which have been described as useful for coatings or encapsulation in the food applications. They include tripolyphosphate, silicon oxides, or aluminum oxides (Desai and Park 2006; Amberg-Schwab et al. 2006). The natural adsorption of cells on inorganic supports creating a biofilm, is a good choice for the biomass retention in the packed bed and fluidized bed fermenters. Inorganic supports can be utilized alone or in combination with other materials. They include tripolyphosphate, silicon oxides (silica), or aluminum oxides (Desai and Park 2006; Amberg-Schwab et al. 2006). These carriers are usually cheap, with a high mechanical stability (Baptista et al. 2007). They are micropores materials which have a significant portion of mesopores (with openings 20–500 Å in diameter) in which capillary condensation of volatile flavors may occur apart from physical adsorption. The examples are activated carbons (surface 500–1400 m²/g) and silicas (surface are of 100–1000 m²/g), but their use is limited in food. Inorganic materials are also used for encapsulation based on supercritical fluid technology (Wandrey et al. 2010). Immobilization supports as porous ceramics, diatomaceous silica (kieselguhr), porous bricks pieces, and porous glass have been also put in practice for brewing. Silicon carbide is inert, durable, reusable, and steam sterilizable material, thus suitable for CIP cleaning at industrial applications. Rods rather than beads have been used by industry for yeast immobilization in order to maximize mass transfer between the cells and the liquid (Pilkington et al. 1998).

18.5 Food Applications for the Production of Value-Added Products

The application of encapsulation has been introduced to the food industry, typically for controlling the release of flavorings and the production of foods containing functional ingredients (e.g., bioactive ingredients and probiotics). Here we will give some recent examples of encapsulates aimed at production of value-added products. The encapsulation of fennel oleoresin, a well-known Mediterranean aromatic plant

that has been long considered as a medicinal and spice herb, by freeze-drying method using binary mixtures of modified starch, maltodextrin and chitosan resulted in great protection in terms of storage stability for 12 weeks (Chranioti and Tzia 2013). In another study, a novel flavor microcapsule containing vanilla oil, developed by Yang et al. (2014) using complex coacervation approach (with chitosan and gum arabic), was found to retain about 60 % of the vanilla oil in the microcapsules after 30 days, indicating a good potential to serve as a high quality food spice with long residual action and high thermostability. Complex coacervation between sodium caseinate or whey protein isolate with carboxymethylcellulose was also applied for β -pinene encapsulation (Koupantsis et al. 2014).

The entrapment of functional compounds within biopolymer-structured networks constitutes a means to improve their processability (i.e., solubility, dispersibility, flowability), enhance their stability against chemical or physical degradation, allow their controlled, sustained, or targeted release under desired conditions, promote easier handling, and mask unpleasant feelings during eating (Sagalowicz and Leser 2010). Many protective mechanisms that can maintain the active ingredients until the time of consumption have been provided through the design of a great number of formulations, thus enabling their incorporation into functional foods and delivery at the appropriate site of action within the gastrointestinal tract after ingestion. For example, the utilization of encapsulated polyphenols instead of free compounds can lead to improvements in both the stability and bioavailability of the compounds in vivo and in vitro, while their unpleasant bitter taste and astringency can be minimized (Nedović et al. 2011). Liposomes, cyclodextrins, and chitosan–sodium tripolyphosphate microparticles have been shown to be convenient carriers for resveratrol, since they were physically stable and provided its prolonged release (Isailović et al. 2013; Lucas-Abellán et al. 2007; Cho et al. 2014). Efficient stabilization and elevation of curcumin's bioavailability was also succeeded via encapsulation into reconstituted natural or artificial oil bodies (Bettini et al. 2013; Chang et al. 2013), while encapsulation is a promising method to improve the stability and bioavailability of tea polyphenols (Zhou et al. 2012; Zhang et al. 2013). Flavonoids, such as quercetin, catechin, and rutin, have been also successfully incorporated in nanoemulsions or w/o/w multiple emulsion based delivery systems (Pool et al. 2013; Akhtar et al. 2014). Accordingly, in the case of carotenoids (e.g., β -carotene, lycopene, lutein, and zeaxanthin), several effective delivery systems have been developed to improve their dispersibility in water and coloring strength potential and to increase the bioavailability, e.g., nanoemulsions stabilized by globular proteins or nonionic surfactants using a high pressure microfluidizer (Qian et al. 2012), micellar formulations by ultrasound emulsification, high-shear emulsification and precipitation from a pressurized emulsion (De Paz et al. 2013), nanodispersions containing β -carotene by solvent displacement method (Ribeiro et al. 2008), lycopene micelles and chylomicrons by a microemulsion technique (Chen et al. 2014). A number of different α -tocopherol delivery systems have been investigated to limit its exposure to high temperature, light, or oxygen, including oil-in-water nanoemulsions and emulsions (Yang et al. 2012), nanodispersions (Cheong et al. 2008), liposomes (Nacka et al. 2001), and biopolymer-based nanoparticles (Luo et al. 2011).

Similarly, encapsulated natural oils, rich in biologically active polyunsaturated fatty acids (i.e., bioactive oils), are promising systems for the formulation of new preparations for the food industry and the delivery of nutraceuticals (Averina and Allémann 2013).

Aside from the development of delivery systems to encapsulate, protect, and deliver lipophilic bioactive components within the human body, structured emulsion-based systems, fabricated from natural lipids and polymers, with controlled stability and digestibility within the gastrointestinal tract have been designed. These systems are considered particularly suitable for use within the food industry since they control the rate of digestibility and release of encapsulated lipids and hence modulate satiety/satiation and fight obesity. To this direction, hydrogel particles can be constructed entirely from edible biopolymers, such as proteins and polysaccharides, using a number of different assembly principles, such as aggregation/gelation, segregation/gelation, injection/gelation, and macro-gel disruption (McClements et al. 2009). In their study, Li et al. (2011a, b) focused on the development of filled hydrogel beads by trapping submicron lipid droplets dispersed within calcium alginate beads and suggested that the rate and extent of lipid digestion could be greatly decreased and manipulated by controlling bead size, bead composition (calcium/alginate), or lipid type.

Encapsulation of probiotics is a field of food technology that has emerged and developed rapidly in the past decade. The probiotic market exhibits a strong future, since the benefits provided by probiotics consumption are well documented, which in turn explains the demand of food industry for technologies ensuring their stability in foods. Given that high cell survival is important for both economical and health effects in addition to the fact that probiotic microorganisms are also very sensitive to environmental parameters, their encapsulation is considered as a successful way of protecting and improving cell viability. It can be characterized as a “therapeutic strategy” that contributes to the prolongation of cell viability, despite stomach’s acidity, as well as to the controlled and continuous delivery of cells in the gut. Many reports exist regarding the addition of encapsulated probiotics in fermented dairy products like yogurt, cheese, ice cream, fruit juices, and chocolate (Burgain et al. 2011; Mitropoulou et al. 2013). For instance, encapsulated *Bifidobacteria* in acid-stable beads enhanced cells survival in pasteurized yogurt in comparison to free cells (Sun and Griffiths 2000; Sandoval-Castilla et al. 2010). Probiotic cells entrapped in alginate particles were also found to maintain their viability over ripening period of cheese (Darukaradhya et al. 2013; Amine et al. 2014). Homayouni et al. (2008) reported that in ice cream the survival of *Lactobacillus casei* and *Bifidobacterium lactis* increased up to 30 % if the cells were protected within calcium alginate matrix compared to the free cells at the same conditions. There are also some reports about the addition of encapsulated bifidobacteria in mayonnaise (e.g., Khalil and Mansour 1998) aiming at cell protection from vinegar, in sausages resulting in satisfying sensorial quality (Muthukumarasamy and Holley 2006), in fruit juices (Petreska-Ivanovska et al. 2014; Krasaekoopt and Watcharapoka 2014) resulting in high cell viability and other.

18.6 Bioprocess Intensification Using Encapsulation Technology

In addition to the above, the introduction of cell or enzyme encapsulation technology in food processing applications, such as fermentation (e.g., beer, wine, cider, dairy, and meat fermentation), and metabolite production processes offers many advantages in comparison to traditional ones. Among several different approaches described in literature (Nedović et al. 2010), the most useful for food processing is entrapment of cells within matrix of natural polymers like alginate, agarose, carrageenan, chitosan, and pectin. Such natural gelling polysaccharides represent an emerging group due to their advantage of being nontoxic, biocompatible, and cheap (Nedović et al. 2010, 2011). Considering beer production, the implementation of cell encapsulation technology is related to the development of a continuous fermentation process and the design of proper fermenters, in view of achieving higher fermentation rates and increasing system productivity without affecting flavor formation (Willaert and Nedovic 2006). Traditional beer fermentation systems uses freely suspended yeast cells to ferment wort in an unstirred batch reactor and lasts few weeks. Immobilized cell technology is able to produce lager beer in a much shorter period, usually 1–3 days, and the ultimate goal is the production of beer with satisfied final quality in that period of time (Verbelen et al. 2010; Willaert and Nedovic 2006). In general, encapsulation in beer production offers higher degree of fermentation and increased volumetric and ethanol productivity mainly at maturation stage, while in alcohol-free (≤ 0.5 %, v/v) and low-alcohol (≤ 1.2 %, v/v) beer production encapsulation technology has been well established (Brányik et al. 2012). However, regardless of the extensive research performed during the last 30 years, the industrial applications are still limited due to major shortcomings (e.g., unbalanced beer flavor, cost of encapsulation systems). Actually, despite the extensive research carried out in the last few decades, immobilized yeast beer fermentation has not yet managed to outperform the traditional batch technology. Further work towards the utilization of new materials as cell potential carriers and characterization of encapsulated cell physiology and metabolism will considerably assist in the production of a stable beer with enhanced aroma profile.

Encapsulated cell technology has introduced many technological advances in the field of wine and cider fermentation, i.e., greater protection against inhibitory substances already present in the substrate or produced during yeast metabolism, better yeast performance even at low temperatures, acceleration of the process (prospect of enabling a continuous operation), ability to separate and reuse the encapsulated cells, improved efficiency of malolactic fermentation, enhancement of the aroma profile of the end product, possibility of using a variety of yeast strains and carrier materials, most of which coincide with the requirement for cost decrease (Kourkoutas et al. 2004, 2010; Nedović et al. 2010, 2011, 2013). For example, the use of sodium alginate and carrageenan entrapped yeast cells led to the production of rose sparkling wine with sensory characteristics similar to those of the traditional products, as well as to faster fermentation rates and lower cost since the removal of beads

(riddling) from the bottles was much easier (Tataridis et al. 2005). In another work, *Lactobacillus casei* cells encapsulated in calcium pectate gel increased the fermentation rate in Chardonnay wine production and operational stability (up to 6 months), while the degree of conversion of malic acid by the encapsulated cells was twice as high as that obtained with the free cells (Kosseva and Kennedy 2004). Additionally, rapid fermentations, great stability, suitability for continuous processes, as well as enhanced products' flavor characteristics have been reported when yeast cells were immobilized on pieces of various fruits (e.g., apple, quince, pear, fig, raisin berries, grape berries, grape stems and skins, orange, and watermelon) or other natural supports (e.g., DCM, gluten pellets, brewer's spent grains) (Kourkoutas et al. 2004; Kandyliis et al. 2012a, b; Kopsahelis et al. 2012; Genisheva et al. 2014a, b; Sroka et al. 2013). Yeast immobilized in sodium alginate beads was also found to be a suitable biocatalyst in the fermentation of diluted honey for mead production (Pereira et al. 2014), in pomegranate wine making at ambient temperatures (Sevda and Rodrigues 2011), in raspberry and mango wine fermentation (Kalušević et al. 2012; Varakumar et al. 2012), as well as in wine made from the tropical fruit cagaita (Oliveira et al. 2011). In general, fruit wine production is another area where cell encapsulation technology has only recently been implicated, probably due to increased consumer interest for their potential health benefits. Likewise, in cider fermentation, which also combines two steps, i.e., alcoholic and malolactic fermentation, cell encapsulation aims at accelerating the time-consuming step of malolactic fermentation, increasing productivity and producing a well-balanced alcoholic beverage with respect to aroma, taste, and overall quality. In their approach, Nedovic et al. (2000) attempted the simultaneous performance of alcoholic and malolactic fermentation for apple juice by co-immobilizing *S. bayanus* and *L. oenos* in calcium alginate matrix, resulting in reduced maturation time in comparison to the traditional cider fermentation process, increased productivity and development of a quite acceptable end product.

Apart from beverage production, cell encapsulation technology has been applied in dairy and meat fermentation. Dairy fermented products, such as cheese and yoghurt, have been reported to have benefited from lactic acid bacteria encapsulation in terms of enhanced cell survival, during processing (heating or freezing), protection against bacteriophages, inhibition of undesirable flora, acceleration of flavor development, and improvement of cells' stability during storage (Nedović et al. 2011). In the case of sausages, which are the most known fermented products, a noteworthy reduction in the fermentation time can be achieved by the use of cell encapsulation technology (e.g., from 45 to 28 h with encapsulated starter cultures of *L. plantarum* and *Pediococcus pentosaceus* in calcium alginate beads) (Kearney et al. 1990), while the survival of encapsulated probiotic bacteria can be enhanced during drying process (Muthukumarasamy and Holley 2006).

Cell encapsulation technology also constitutes a tool for microbial production of high-value food ingredients, such as vitamins, carotenoids, organic acids, and amino acids. As conventional processes for the production of these ingredients usually involve either chemical synthesis, extraction from plants, petrochemical processes, or enzymatic conversions, hydrolysis of proteins, fermentation and chemical

methods, their biotechnological production has gained growing interest, being sustainable, environmentally friendly and usually employing renewable resources or waste materials (Stahmann et al. 2000; Wendisch 2014). As regards vitamins, there have been only some trials to improve efficiency of their production by using immobilized cells as biocatalysts. For example, Yang et al. (2004) achieved a final concentration of vitamin B₁₂ ten times higher than that of chemical synthesis by using loofah (sponge) as carrier for immobilization of methanogens. Similarly, in terms of cost-efficiency and sustainability, Garbayo et al. (2003) reported great increase in carotenoid biosynthesis by calcium-alginate immobilized *Gibberella fujikuroi* mycelia, while Leng et al. (2006) proposed the process of production of L-phenylalanine using encapsulated *Escherichia coli* within κ -carrageenan beads with the yield reaching approximately 90 %.

Over the last years, there has been an increasing trend towards the exploitation of agro-industrial wastes via microbial fermentation processes for the production of primary or secondary desired metabolites (Galanakis 2012; Thomas et al. 2013; Uçkun Kiran et al. 2014). The main obstacle of this type of wastes is the presence of extremely toxic to microbial cells compounds (weak acids, furan derivatives, phenolic compounds)—present in the raw material or derive after appropriate pretreatment—that are expected to cause negative interference in the bioprocess performance (Palmqvist and Hahn-Hägerdal 2000). An effective way to address the substrate toxicity is the use of immobilized cells technology as indicated in the following selected examples.

Ethanol production. Numerous are the studies concerning ethanol production since it is currently the most popular biofuel available in the world market (68 % of total ethanol production in 2009–2010) (Vohra et al. 2014). In a recent review (Westman et al. 2012), encapsulated yeast was proposed as a promising tool for the production of second-generation bioethanol due to the cells' ability to ferment rapidly toxic media. It is mainly produced by biodegradation of molasses, a waste product after the refining of sugar (Dodić et al. 2009; Ghorbani et al. 2011; Razmovski and Vučurović 2011, 2012). After optimization, the use of immobilized/encapsulated cells resulted in increased ethanol productivity related to that of free counterparts. In a more recent study (Singh et al. 2013), immobilized *S. cerevisiae* on sugarcane bagasse was also found to be a useful tool for ethanol production from concentrated sugarcane bagasse hydrolyzate having several advantages, such as ease of handling, good operational stability, high mechanical strength, and low cost raw materials.

Enzyme production. Numerous agro-industrial wastes (wheat bran, rice straw, apple pomace, bagasse, corn cobs) have been widely used as immobilization carriers and nutrient-rich sources, at the same time, in the production of microbial enzymes, such as proteases, α - and β -glycosidases, pectinases and lipases, via solid state fermentation (SSF) (Chatzipavlidis et al. 2013). SSF of agro-industrial wastes has been found to simulate the natural environment of many microorganisms offering high productivity rates, higher product stability, and lower extent of catabolite repression (Singhania et al. 2009).

Aroma compound production. The utilization of non-conventional media for the production of flavor-active compounds has been studied by many researchers (Couto and Sanroman 2006; Bicas et al. 2010; Mussatto et al. 2012; Mantzouridou and Paraskevopoulou 2013). Among the various types of microbial processes to convert solid wastes into value-added compounds, the use of SSF as a means to improve cost effectiveness of these processes and its application for the production of aroma compounds has been recommended. Among others, cassava bagasse, sugarcane bagasse, apple pomace, soybean and coffee husk have been evaluated for this purpose by cultivating different microorganisms (Longo and Sanromán 2006; Dastager 2009; Bicas et al. 2010). These residues have been proposed to have a double action during the fermentation process, i.e., as physical support, as well as source of nutrients.

The positive impact of encapsulated yeast in sodium alginate beads for the valorization of orange peel hydrolysate towards volatile ester production was highlighted in the recent work of Lalou et al. (2013). The economic feasibility of the proposed bioprocess was strengthened by the capability of the biocatalyst to perform repeated batch fermentations of hydrolysate of a total period of 240 h.

More recently, the treatment of mixed solid and liquid food industry wastes, i.e., cheese whey, molasses, brewer's spent grains, malt spent rootlets, orange, and potato pulp, using selected *S. cerevisiae* and *K. marxianus* strains and the natural mixed culture *kefir*, led to the production of a significant amount of the aroma compound ϵ -pinene (Aggelopoulos et al. 2014).

Organic acid production. The use of immobilized cells in the production of organic acids (acetic, butyric, lactic, and citric acid) using different agro-industrial wastes (corn by-products, pineapple wastes, dairy waste water) was found to improve yield and productivity of the processes (Huang et al. 1998; Kim et al. 2002; Zhu et al. 2002; Idris and Suzana 2006). The positive effect of immobilization technology on lactic acid production from different substrates, including also food wastes, has been recently reviewed by Abdel-Rahman et al. (2013).

Other secondary metabolites production. Pérez-Bibbins et al. (2013) exploited corn-cob hydrolyzates and vinasses for xylitol production by *Debaryomyces hansenii* encapsulated in alginate beads (reaching up to 13.7 g/L after optimization). Khanna et al. (2013) explored the potential of glycerol valorization (in pure and crude form, i.e., the one derived from bioethanol production) for the production of ethanol, butanol, and 1,3-propanediol by immobilized *Clostridium pasteurianum*. Immobilized cells exhibited better bioconversion potential and an increased tolerance toward inhibitors present in crude glycerol.

Wine production. Several studies have been conducted over the last years trying to exploit different lignocellulosic byproducts/wastes of food industry in wine making process taking advantage the encapsulation technology. Genisheva et al. (2012a, b) produced white wine by immobilized *S. cerevisiae* on grape pomace, consisted of skins, seeds, and stems. Overall, a more rapid and efficient process was recorded when immobilized cells were used, especially when large amounts of SO₂ were present in the must. Furthermore, the final product possessed improved sensory properties in terms of color and flavor. The potential of potato pieces to serve as immobilization matrix for freeze-dried *S. cerevisiae* in wine making at low temperatures was investigated recently by Kandyliis et al. (2014). The final product possessed

similar aromatic profile, but higher content of flavor-active esters compared to that from free cells. Similar attempts in winemaking, especially at low temperatures, using a variety of agro-industrial wastes or even mixtures of them has been recorded (Mallouchos et al. 2003a, b; Plessas et al. 2007; Kandyliis et al. 2010, 2012a, b; Tsaousi et al. 2011).

Beer production. Bardi et al. (1996a, b) exploited delignified cellulosic material as an immobilization carrier for *S. cerevisiae* in wort fermentation using batch and continuous systems. According to findings, the continuous system was able to operate for 3 months with high fermentation rate. Nevertheless, the derived product was found to contain high levels of polyphenols affecting its taste. Also, spent grain particles and corn cobs were used as immobilization matrices in high gravity batch and continuous brewing achieving very high productivity and yield under continuous operation for 2 months (Silva et al. 2008). According to the authors, the carriers used are natural, abundant, and easily available cellulosic materials, fully compatible with beer.

Vinegar production. Limited research has been undertaken concerning the application of immobilization technology in vinegar production (De Ory et al. 2004). In the case of wine vinegar, siran (Sintered glass), oak chips, and polyurethane foam have been exploited. In the production of tea vinegar, sugarcane bagasse and corn cobs were used as immobilization matrices for *Acetobacter aceti* (Kaur et al. 2011). Immobilized *A. aceti* cells in both carriers resulted in similar products with total acidity of 4.6 % (w/v) at 42.7 % fermentation efficiency (the percentage of ethanol oxidized to acetic acid). In the semi-continuous system, 9 flow cycles of 12 h were carried out successfully (total fermentation period 108 h).

18.7 Functionality of Immobilized Yeast Cells

During cell encapsulation, desirable and undesirable alterations in cell growth, physiology, and metabolic activity may take place (Melzoch et al. 1994; Norton and D'Amore 1994; Walsh and Malone 1995; Willaert and Nedovic 2006). Mass transfer limitations and nutrient availability, modified surface tension and osmotic pressure, altered cell morphology and membrane permeability and reduced water activity have been considered as key parameters that cause the above changes (Kourkoutas et al. 2004; Rathore et al. 2013). The effects of encapsulation technology on functionality of yeast cells become obvious through comparison of immobilized vs. suspended yeast cell physiological activity. The most important effects are presented below:

18.7.1 Effects on Growth Rate and Physiology

Growth rate of immobilized yeast cells is the most studied metabolic phenotype but also the one with the most contradictory results since researchers have recorded increased, unchanged or decreased growth rate for immobilized yeast compared to

that for free cells (Junter et al. 2002; Junter and Jouenne 2004). For example, yeast cells immobilized within calcium alginate beads exhibited reduced growth rate during the fermentation of wort with regard to that of free cells. This phenomenon was more pronounced at high populations of yeast cells within the alginate beads (Ryder and Masschelein 1985). Unlike free cells, encapsulated ones have to overcome mass transfer limitations that can explain decreased or unchanged rates. Nutrient or oxygen deficiency, limited diffusion rates and osmotic pressure could affect negatively the growth rate. On the other hand, the increased growth rates can be justified by the protective effect of immobilization against substrate inhibitors.

An interesting observation has been reported by Talebnia and Taherzadeh (2007), showing that, unlike free cells for which the specific growth rate and the RNA/protein ratio are linearly proportional these two variables for encapsulated cells are not proportionally decreased.

Melzoch et al. (1994) recorded a considerable size diversity of encapsulated yeast cells in combination with irregular shape, attributed to the endeavor of yeast cells to occupy all the available free space inside the encapsulation matrix. The yeast cells grow heterogeneously inside the encapsulation matrix and tend to attach to the membrane from one side of the capsule (Talebnia and Taherzadeh 2007). In continuous processes, cell physiology can change significantly relatively to free cells, due to continuous mode of reactor operation, internal and external mass transfer limitations and aging of immobilized biomass (Berlowska et al. 2013). Knowing that the physiology of immobilized cells is affected by the microenvironment inside the beads, a main challenge its control by appropriate process design to maintain maximum viability and metabolic activity of the cells.

18.7.2 *Effects on Metabolic Activity*

The metabolic activity of encapsulated cells can be rather different from that of free cells. In many cases, it is the immobilization procedure itself that causes modification to the metabolism of the cells (Melzoch et al. 1994; Talebnia and Taherzadeh 2007). Borovikova et al. (2013) reported increased metabolic rate as a results of increased respiratory rate and reduced lag period in relation to those of free cells. Also, Ma et al. (2007), based on microcalorimetric and metabolic measurements, suggested that more substrate can be used by encapsulated *S. cerevisiae* cells to produce biomass, resulting in higher biomass yield. Higher conversion efficiency and production rates of immobilized cells offer great advantages for the implementation of this technology in industry. Higher rates of substrate uptake (usually glucose) and product excretion (especially ethanol) are widely recorded in many studies for a variety of fermented products, such as wine, beer, and cider (Van Iersel et al. 2000; Kourkoutas et al. 2003; Divies and Cachon 2005; Bezbradica et al. 2007). Reduced metabolic rates in immobilized cells have also been reported and are usually attributed to the mass transfer limitations (Junter and Jouenne 2004; Berlowska et al. 2013).

In general, increased enzyme activity and thus productivity using immobilized cells has been linked to reduced intracellular pH. This promotes the membrane permeability to protons and ATP use, stimulating glucolytic activity and glucose uptake (Westman et al. 2012; Berlowska et al. 2013). *S. cerevisiae* immobilized in DEAE-cellulose showed increased activity of hexokinase, glucose dehydrogenase, pyruvate decarboxylase and alcohol dehydrogenase and an increasing flux of glucose carbon towards the main fermentation products, at the expense of biomass formation (Van Iersel et al. 2000). In the case of invertase, a cell wall enzyme that catalyzes the conversion of sucrose to glucose and fructose, encapsulated yeast cells contained a significant amount of this enzyme despite the initial glucose concentration, which acts as a regulator of the enzyme synthesis. The ability of encapsulated cells to channel greater quantities of glucose into storage carbohydrates (e.g., glycogen and trehalose), the increased glycosylation or even the high glycoprotein content in the periplasmic space between the cells could explain this increased invertase activity (Norton and D'Amore 1994). Increased synthesis of superoxide dismutase has been reported in immobilized cells of *Aspergillus niger* in beads as compared to free counterparts (Angelova et al. 2000).

Adsorption of *S. carlsbergensis* onto porous glass beads and *S. cerevisiae* on ceramics resulted in increased ethanol yield from glucose with the concomitant decrease of carbon dioxide yield (Kourkoutas et al. 2004). According to Westman et al. (2012), higher ethanol production from encapsulated *S. cerevisiae* has been attributed to the fact that, due to growth reduction, the larger proportion of the cell population's catabolism is used for the maintenance of energy as the growth decreases.

Norton and D'Amore (1994) reported an increased content of structural polysaccharides (mainly glucan and mannan) in immobilized cells, in comparison to free cells, stimulated by the change on the microenvironment within the beads and the abnormal longitudinal growth of cells. This trend was not followed in prolonged cultivation of encapsulated yeast cells (Talebnia and Taherzadeh 2007). On the other hand, the metabolic shift towards the production of storage polysaccharides (glycogen and trehalose) in encapsulated cells has been justified by the very dense cell populations inside the encapsulation matrix (overgrowth), whose growth has been suppressed due to cell attachment and lack of nutrients (Norton and D'Amore 1994; Talebnia and Taherzadeh 2007).

18.7.3 Effect on Stress Tolerance

One of the major reasons for the preparation and use of immobilized cells is the protection of cells against inhibitory compounds. A crucial parameter in the performance of alcoholic fermentation is the tolerance of yeast cells to ethanol (Junter et al. 2002). Several studies have tried to develop a fundamental understanding of the encapsulation effect on cell tolerance to this stressful parameter. Immobilized cell resistance against ethanol has been attributed either to the protective layer of the

gel material, i.e., the encapsulation material, or to modified composition of the cell wall and plasma membrane influencing cell permeability (Junter et al. 2002; Junter and Jouenne 2004; Kourkoutas et al. 2004). Parascandola et al. (1997) suggested that immobilization, as well as ethanol stress, influence the cell wall organization by inducing the synthesis of mannoproteins, which are covalently linked to the glycan network and implicated in cell wall porosity. The lipid composition of cell membrane is also affected by immobilization by an increase in the content of saturated fatty acids, phospholipids and ergosterol (Hilge-Rotmann and Rehm 1991; Jirku 1999). Another possible explanation for ethanol tolerance of immobilized cells was proposed by Sun et al. (2007a). It was suggested that the non-gelling liquid alginate matrix may contribute to the cell protection against ethanol and other polar solvents by enhancing the stability of the hydration layer around the cells.

Encapsulation has been shown to increase tolerance of cells against other adverse conditions such as osmotic stress, nutrient deficiencies, or antimicrobial compounds (e.g., phenolic compounds). Regarding osmotic stress resistance, studies have demonstrated an increased intracellular pressure in immobilized cells via the induction of the synthesis of regulating factors such as glycerol, trehalose, and the enzyme superoxide dismutase (Hohmann 2002; Balli et al. 2003; Kourkoutas et al. 2004; Talebnia and Taherzadeh 2007; Sun et al. 2007b). According to findings of Sun et al. (2007b), both solid and liquid alginate matrix of microcapsules contribute significantly to the cell domestication against hyper-osmotic stress. Moreover, encapsulated cells can successfully perform alcoholic fermentation in other stressful conditions such as low temperatures or high gravity substrates (Iconomopoulou et al. 2002; Dragone et al. 2007).

18.7.4 Flavor Formation

Food industry aims at producing high yield low cost products with desirable organoleptic characteristics. In fermented products, the latter are closely linked with the metabolic activity of the microbial cells. More specific, in alcoholic fermentation, the amino acid metabolism of yeast cells affects directly the production of many flavor compounds such as esters, higher alcohols, fatty acids, and diketones (Kourkoutas et al. 2004).

So far, studies concerning the effect of immobilization on flavor formation highlight the complex nature of the occurring phenomena and the difficulties to control them. In most studies (Reddy et al. 2008; Mantzouridou and Paraskevopoulou 2012; Lalou et al. 2013; Servetas et al. 2013), cell encapsulation has been found to promote aroma formation and mainly ester and alcohol synthesis with a concomitant reduction of off-flavors (Van Iersel et al. 2000; Kourkoutas et al. 2004). In wine fermentation, previous findings indicate the combined positive impact of low temperature and immobilized cells on the fruity character of the final products due to the improved ratio of esters to alcohols and better balance of primary and fermentation acids (Iconomopoulou et al. 2002; Kourkoutas et al. 2004; Tsaousi et al. 2011; Kandyliis et al. 2012a, b). Moreover, approaches using immobilized yeast cells in

brewing have been proven to be very promising for controlling flavor by the removal of diacetyl, leading to reduced maturation time and production costs (Kourkoutas et al. 2004).

18.8 Trends

Today scientists seek for new materials which will provide unique protection and delay release properties of encapsulates. The new materials based on Maillard Reaction Products (Augustin et al. 2006), polymer mixtures (Millqvist-Fureby et al. 2000; Chranioti and Tzia 2013), multiple emulsions (Edris and Benrgstahl 2001; Rodríguez-Huezo et al. 2014) are proved to provide better protection of sensitive ingredients, but the key question is whether they are too complex and/or not efficient enough to be used in practice. One of the trends is to replace a common matrix material with an encapsulating agent which is per se a functional food. For example, Chiou and Langrish (2007) combined two products (fruit fibers and polyphenols) into one multipurpose functional food, creating a novel nutraceutical product. Moreover, substances which are able to improve matrix properties and at the same time, increase nutritive and health values of food or have other beneficial effects become more and more popular. For example, by using inulin or saccharose as fillers, it is able to stabilize cellular structure of alginate microbeads exposed to drying which otherwise collapse during this process (Stojanovic et al. 2012); at the same time, inulin is a source of dietary fibers; in the case of sucrose, its sweet sugar flavor masks the unpleasant taste of polyphenolic compounds. Then, common crosslinkers can be replaced with plant bioactive compounds (Montero et al. 2005; Peng et al. 2010). Also, acids commonly used in preparations can be replaced by those which have other functions (beside simple pH adjustment), e.g., to act as a powerful anti-oxidant agent, such as in the study of Belščak-Cvitanović et al. (2011) who encapsulated naturally derived antioxidants from herbal extracts, in chitosan-alginate containing vitamin C. There is a trend to replace animal proteins by plant proteins in emulsion- or coacervate-based formulations (Ducel et al. 2004; Wang et al. 2010).

Food consumers expect from food many attributes. Consequently, industry has to find a way to produce encapsulates fulfilling many demands, and this is often impossible to achieve with only one of the existing technologies. Therefore, food companies are pressured to develop new technologies involving two or more processing steps in order to produce encapsulates with superior characteristics or to produce multi-encapsulated formulations. Spray-drying of flavor-cyclodextrin inclusion complexes (to diminish effect of high temperatures during spray-drying) and liposomes- or inclusion complexes-production with the help of supercritical fluids (to increase solubility of hardly soluble actives) are some of the possibilities derived from researchers. Increasing demands for production of functional food with higher nutritional value, lower dose of synthetic preservatives and better organoleptic features lead to innumerable applications of nano-encapsulation in food processing (Fathi et al. 2014; Joye and McClements 2014). Also, using yeast cells (*Saccharomyces*

cerevisiae) as wall materials have proven to be a safe, low cost, and high volume process. This technology was typically used in the past for encapsulation of small lipophilic molecules, such as essential oils (Bishop et al. 1998), and more recently for water-soluble polyphenols (Shi et al. 2007; Blanquet et al. 2005), but in future it should be considered for encapsulation of other ingredients, too. Even though the number of emerging encapsulation technologies is growing, in the close future, conventional technologies based on encapsulation in glassy state of amorphous carbohydrates are expected to dominate the food industry.

In the field of biocatalyst processing, an industrial breakthrough could be expected only upon achieving the following process characteristics: simple design, low investment costs (application of cheap carrier materials), flexible operation, effective process control, and good product quality. Also, searching for new materials that are environmentally friendly and suited for immobilizing microbial cells is necessary. For example, bacterial cellulose membrane can serve as a support material for yeast immobilization in fermentation processes (Yao et al. 2011). The role of the R&D will be also an important one, in better understanding of industrial constraints and requirements to make a process industrially accepted and in better implementation of multi-disciplinary-based research concept.

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Chapter 19

Innovations in Food Packaging Materials

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19.1 Introduction

Food packaging was originally perceived to secure proper preservation of food in a container by protecting it from the influence of the various external and internal environmental conditions. The days when food packaging was used simply to provide information about packed product, protect and provide convenient method of transportation are long gone. Current innovations of food packaging technology are focused on following functions: better protection, more efficient and long-lasting quality preservation, and enhanced safety in general for both consumer and environment. In addition nowadays, active and smart packaging facilitates increased functionality of packed product. Now a food package is expected to be informative and fit to existing specific product requirements including production, logistic and storage, and finally should be easy-to-handle and disposal-friendly to fulfil all existing ecological regulations.

The most recent packaging innovations on the market include unique features that capture the imagination of consumers providing an interactive communication experience that lasts long after a consumer has discarded the packaging. Most of such innovations lead to extending shelf life, and even further providing useful information that updates during the product's lifetime.

To fulfil all these requirements and challenges the new tailored-made materials and technologies are recently developed, which potentially could be used in the future in food packaging.

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19.2 Barrier Packaging Materials for Food Application

Many foodstuffs require specific atmosphere quality during storage. To ensure a constant gas composition and balance inside the package, the packaging material needs to have a certain gas barrier, where some kinds of food are sensitive to the presence and level of oxygen, nitrogen, water vapour and other gases. One of the conventional approaches is to produce high-barrier films for packaging of food to storage items in protective atmosphere. In this case, one uses the composite multi-layers of packaging materials or modified bio-based materials to obtain the required properties. The design of effective packaging system, which is dedicated for specific kinds of food, enables maximal protection of food and the prolongation of its shelf-life (Labuza and Breene 1989; Day 2003; Rhim et al. 2013; Guillaume et al. 2010; Guinault et al. 2010; Johansson et al. 2012; Aday and Caner 2013).

High-barrier properties depend on type of packaging materials, composition, their structure, and finally the thickness; sometimes to obtain the high efficient materials the combination of functional layers is recommended (for example the high oxygen barrier layer + water vapour barrier layer + grease barrier layer + mechanical protection layer). Also some requirements concerning the foodstuffs should be taken into the consideration; for example the grease protection is not necessary required for packaging of fruits, but is strongly desired for products with high content of oil/grease—e.g. butter (Han and Krochta 1999; Ham-Pichavant et al. 2005; Colomines et al. 2008; Abdillalia et al. 2013; Consuelo et al. 2013).

19.2.1 Bioplastics and Combined Biomaterials

Different biopolymers exist on the food packaging materials market, but their application is limited, because their barrier properties are not sufficient for effective food protection. Additionally, their price, especially price of bioplastics with required properties and good processability using conventional converting processes, is still too high in comparison to conventional synthetic polymers. Even though, the ecological consciousness of customers increases, the economical aspects are still a main barrier for wide application of bioplastics on food packaging market. On the other hand, to achieve similar barrier properties to conventional packaging materials additional effort is necessary. However, for some food products high-barrier properties are not a key factor (for example some fruits or vegetables), and therefore bioplastic packaging materials are strongly recommended for such application (they allow to “breathe” for post-harvest food products; and they keep the required balance of humidity between inside/outside of packaging). There are also different methods to improve barrier properties and to include biopolymers into high quality packaging for food application.

Petersen et al. (1999) and van Tuil et al. (2000) had grouped biopolymers into three main categories in accordance with their origin:

1. Polymers directly extracted from natural materials such as polysaccharides (e.g. cellulose and starch), proteins (e.g. whey protein, soy protein, casein and gluten) and lipids (e.g. wax, fatty acids).
2. Polymers produced from renewable bio-derived monomers as a result of classical chemical synthesis (e.g. poly(L-lactide) (PLA), poly(glycolic acid) (PGA), poly(ϵ -caprolactone) (PCL), poly(butylenes succinate) (PBS), poly(vinyl alcohol) (PVA)).
3. Polymers produced by microorganisms or genetically transformed by bacteria, for example polyhydroxyalkanoates (PHAs).

Different parameters could influence the gas-barrier properties of packaging materials especially in case of biopolymeric materials. For example the crystallinity is well known to have major effects on the gas permeability. Unfortunately, this is difficult to estimate because two aspects of crystallinity have to be considered: the crystallinity degree and the crystalline morphology (Colomines et al. 2008). Some biopolymers such as for example PLA are known to recrystallise when heated at a temperature higher than their T_g , which influence significantly the gas-barrier properties (Guinault et al. 2010).

Other biopolymers, such as PHAs show intermediate to high oxygen barrier properties depending on the considered grade and chemical composition. Comparing PHAs to PLA, PHA features an oxygen barrier capacity up to more than ten times that of PLA, depending on the grade of PHA. The crystallinity ratio and the presence of additives such as plasticisers or inorganic fillers can influence the oxygen permeability. For example the commercial product Enmat Y1000P (TianAn Biopolymer, China) due to high crystallinity has higher than other PHAs oxygen barrier properties. Other commercial products such as for example Mirel F1006 and Mirel F3002 (Metabolix, USA) have intermediate water vapour barrier properties, but higher than PLA 7001D (NatureWorks LLC, USA). The presence of plasticisers in most cases due to their mobility increases the water vapour permeability (WVP). In general, PHA compared to PLA as competing biopolyesters seems to be more attractive in term of water vapour barrier properties for food packaging application (Corre et al. 2012).

There are known different methods to improve barrier properties of packaging materials, including addition of different modifiers or development of new technological processes of production. Recently to modify barrier properties various nanotechnological processes have been investigated, including addition of nanoclays, nanowhiskers or nanofibres (e.g. nanocellulose).

So far the PHB nanocomposites prepared by melt mixing possess the lowest oxygen permeability value of all biodegradable materials, becoming close to the value of the poly(ethylene terephthalate) (PET) nanocomposite. Significant oxygen barrier improvements have been achieved for all the biodegradable materials upon addition of just 5 % of dispersed nanoclays resulting in more competitive materials for packaging of oxygen-sensitive products. Specifically, reductions between 20 and 55 % were observed for the nanocomposites with 5 wt% of nanoclay or cellulose nanowhiskers. The reason why different nanoparticles render different barrier performance per a

given filler loading is related to a number of factors such as changes in permeate solubility, crystallinity alterations, interfacial adhesion, dispersion etc. They all have to be considered and analysed. Similarly to the oxygen permeability, addition of nanoclays to the biopolyester matrices results in significant reductions of water permeability (between 10 and 76 %) in the comparison to unmodified biopolymers (Sanchez-Garcia et al. 2010). The addition of nanocellulose to nano-biocomposites of PLA (1 wt% (PLA/1s-CNC) or 5 wt% (PLA/5s-CNC) of s-CNC) has positive effect on the increase in barrier properties (Fortunatia et al. 2012). This effect is also confirmed for the PLA/5s-CNC films that showed the highest reduction in OTR values (ca. 48 %). Nano-biocomposites containing between 2 and 3 wt% of nanocrystals of cellulose possess the highest water and oxygen barrier properties.

The amorphous nanocomposite films from blends of polylactide (PLA) and an organomodified montmorillonite (O-MMT) have lower gas permeability than pure PLA (Picard et al. 2011). The decrease of gas diffusion was the sole effect of their PLA modification. The hydrogenated amorphous carbon (a-C:H) films that grown on a PLA substrate by means of a radiofrequency plasma enhanced chemical vapour deposition (rf-PECVD) technique with different deposition times (5, 20 and 40 min) could significantly increase the barrier properties of PLA (Mattioli et al. 2013). The oxygen transmission rate and the WVP coefficients of PLA/a-C:H films are lower than untreated PLA. The gas-barrier properties of PLA films are significantly enhanced by the presence of a thin a-C:H film obtained after only 5 min. treatment with reductions in OTR values of ca. 59 %; and water permeability of ca. 68 %.

To modify barrier properties, some special blends have been suggested; for example the blends of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and PLA were examined by Zembouai et al. (2013). Blend compositions based on PHBV/PLA, prepared by melt mixing, have been investigated according to the following weight ratios, i.e. 100/0, 75/25, 50/50, 25/75 and 0/100 wt%. The water and oxygen barrier properties of PHBV/PLA blends are significantly improved with increasing the PHBV content. In contrast, the value of oxygen permeability coefficient of PLA decreases by about 35.3, 43.2 and 81.5 % with the addition of 25, 50 and 75 wt% of PHBV in the blend, respectively. This shows clearly the important role of PHBV as an efficient barrier promoter for PLA, even at low content. Crystals in a polymer matrix reduce the water transmission due to their small cross-sectional and low permeability restricting chain mobility, thus lowering water permeability. The increase of content of PHBV has a positive effect on water vapour barrier properties. The decrease of the gas permeability with crystallinity increase is generally attributed to two factors. The first is the inclusion of impermeable crystallites, which decreases the content of amorphous phase with high gas permeation. The second one is related to increase of the tortuosity of the transport path of gas molecules due to high content of small impermeable crystals.

Furthermore, the effect of different additives on barrier properties should be considered as another important factor. The role of citric acid in wheat flour/glycerol/poly(lactic) acid (PLA) blends prepared by one-step twin-screw extrusion has been studied in relation to barrier properties of starch-based materials (Abdillahia et al. 2013). The low citric acid content (10 parts of CA) significantly reduced the WVP up to 80 % and around 90 % the oxygen permeability.

Alternative option, which should be taken into the consideration to improve barrier properties of different materials, is multilayer composite structures. So called “sandwich” materials can differ depending on the final purposes of application. For example innovative coating can be applied on conventional material (e.g. paper or paper board), as well as various bioplastics can be coated for some specific purposes including improvement of barrier properties. For example the addition of essential oils such as *Zataria multiflora* Boiss (ZEO) and *Mentha pulegium* (MEO) at three levels (1, 2 and 3 % (v/v)), incorporated into starch films using a solution casting method (Ghasemloua et al. 2013) improves water vapour barrier properties. WVP and oxygen permeability coefficient of starch films varied significantly with content of cinnamon essential oil incorporated (Souza et al. 2013).

Some biopolymers can be applied directly on the surface of foodstuffs—this is an innovative solution, which recently have gained more attention. Direct protection of food enables the reduction of external packaging costs; also some other packaging materials (e.g. lightweight, with lower barrier properties) can be applied to protect the food still in an effective way. Barrier properties of edible films based on blends of Pea starch (PS) and Peanut protein isolate (PPI) plasticised with glycerol (30 %, w/w) were investigated by Sun et al. (2013). Their results showed that when PPI was added into PS film at 40 % level, the WVP of the films markedly dropped from 11.18 to 4.19 % respectively. Composites films were prepared by Belibi et al. (2013) by the casting method using native cassava starch plasticised with glycerol and 3D or 2D synthetic fillers, i.e. beta zeolite and Na-beidellite type 2:1 phyllosilicate. Special attention was paid to the effect of the filler type and its content on barrier properties. Na-beidellite was the most effective in decreasing the WVP, followed by non-lyophilised Beta nanocrystals. To improve WVP of starch films the addition of lipids such as stearic acid to starch films has been studied (Consuelo et al. 2013). It was found that films elaborated with 4 g stearic acid and 24 g glycerol/100 g starch presented reductions in the water vapour permeability ($WVP = 1.93 \times 10^{-7}$ g m/m² Pa h, when compared to $WVP = 5.81 \times 10^{-7}$ g m/m² Pa h “in nature” starch films). The results for films obtained by cast method (Prakash Marana et al. 2013) have showed that hydrophilic nature and plasticising effect of glycerol increased both water vapour and oxygen permeability, where addition of surfactant (Span80) to such composition decreased the barrier properties of the films.

As summary, one could conclude that biopolymers and bioplastics can be used as food packaging materials; some of them require the additional improvement to gain barrier properties suitable for food application. However, it is also important to select the proper packaging material for specific foodstuff.

19.2.2 Biopolymer Coatings on Cellulosic Materials

Flexibility of paper and paperboard as food packaging materials has always been widely appreciated. Moreover, the fact that they are manufactured from natural and renewable materials provides them with additional benefits of being recyclable and

biodegradable. However, to make a full use of their functional properties such as stiffness they often have to be associated with other materials of good functional barrier properties and low moisture sensitivity, such as plastic materials or aluminium (Gastaldi et al. 2007). The aim of such modification is to improve the surface strength of the paper material, enhance its resistance to moisture, and reduce its weight, while still maintaining its original functionality and barrier properties.

The association of biopolymers to paper and paperboard adds remarkable functionalities whereas the eco-friendly characteristics of the material can be maintained. If renewable biopolymer coatings are used as gas and solute barriers the shelf life of degradable products can be prolonged and food quality deterioration can be reduced (Miller and Krochta 1997; Andersson 2008; Khwaldia et al. 2010). The function determines the choice of materials for coating. To improve its performance such materials as plasticisers, regular paper pigments, antioxidants or antimicrobial agents can be added to paper coating formulations.

Different coating techniques such as surface sizing, solution coating, compression moulding and curtain coating can be used to apply bio-based polymers on surface of paper or paperboard. The selection of appropriate method depends on the properties of suitable coating material and type of cellulosic material. Among them surface sizing is the most popular for applying an aqueous coating to a paper substrate. The solid content of the coating in this technique is normally lower than 10–15 wt% (Vartiainen et al. 2004), which does not require a fully continuous coating but, on the other hand, increases the cost of drying process. A thick and continuous coating, sometimes necessary, cannot be obtained by solution coating, but this coating technique provides additional mechanical properties (Gällstedt et al. 2005). The compression-moulding technique can be used if complete coverage and thick coatings are necessary, and therefore, significantly more coating material is required. Finally, curtain-coating technique has attracted recently huge interest from the industry, especially in special paper sector because it allows obtaining a higher coating weight of multilayer films and better gas-barrier properties (Kjellgren et al. 2006).

The improvements of the paper and paperboard packaging barrier properties are usually concentrated on oxygen, water vapour and aroma permeability, and are essential to prolong the shelf life of fresh foods. Grease resistance and hydrophobicity are also vital for many applications of paper and paperboard packaging. Many functional materials have been tested with the aim to increase paper barrier properties. Some of them have been already used in the packaging and paper industries. Others, however, have become principally attractive only recently due to their intrinsic sustainability and their abundance as by-products from other processes (Johansson et al. 2012).

Whey protein as well as wheat gluten, soy proteins and zein were tested extensively as barrier layers on paper and paperboard. Krochta with different partners studied whey protein as a paper coating—barrier properties and grease resistance of coated paper and paperboard were analysed (Han and Krochta 1999; Chan and Krochta 2001; Lin and Krochta 2003). Composites of wheat gluten and paper or paperboard produced by compression moulding showed oxygen barrier properties comparable to those of paper and paperboard coated with commercial barrier

materials such as oriented PET (Gällstedt et al. 2005). At intermediate and high humidity levels, gluten-coated papers were found to have better aroma barrier properties than Low Density Polyethylene (LDPE) films (Chalier et al. 2007). Guillaume et al. (2010) show a decrease of as much as 56 % in the water vapour transmission rate (WVTR) and a 400-fold reduction in oil wettability for wheat gluten-coated paper when compared to uncoated paper. Based on own presented studies and according to results obtained on soy protein isolate (Park et al. 2000), whey protein isolate (Han and Krochta 2001 Lin and Krochta 2003) and corn zein (Trezza and Vergano 1994) Guillaume et al. (2010) stated that an important improvement of grease resistance could only be achieved when the layer is homogenous and thick enough to fill the porous structure of paper and the coating weight is higher than a threshold value around 15 g/m². Trezza et al. (1998) reported a reduction in the oxygen permeability of paper coated with corn zein.

Among the polysaccharides, chitosan (Ham-Pichavant et al. 2005; Kjellgren et al. 2006; Bordenave et al. 2007, 2010) and alginate (Rhim et al. 2006) were studied as a barrier coating, either used alone or in combination with each other (Ham-Pichavant et al. 2005). Chitosan is very useful for coating purposes because it possesses good film-forming, antimicrobial properties and high surface compatibility with paper and also results in increase of water and grease resistance and oxygen barrier of coated paper (Johansson et al. 2012). Rhim et al. (2006) showed that as far as water resistance and moisture barrier are concerned alginate films have worse properties with respect to soy protein coatings.

Starch is a widely available, renewable, biodegradable, inexpensive agricultural raw material (Teixeira et al. 2009; Khwaldia et al. 2010). The surface sizing treatment uses native starch and modified starches to improve paper and paperboard properties, including oil/grease resistance, physical strength, and optical properties (Khwaldia et al. 2010). One method to decrease starch hygroscopicity is the acetylation reaction, which allows obtain thermoplastic starch-based materials with an increased hydrophobicity (Fringant et al. 1996; Larotonda et al. 2005).

Among the cellulose derivatives materials, methylcellulose (Debeaufort et al. 2000; Quezada Gallo et al. 2000) and nanofibrillated cellulose (Syverud and Stenius 2009) have shown very interesting barrier properties.

Lipid coatings provide good moisture barrier, but they have certain disadvantages such as brittleness, lack of homogeneity, and presence of pinholes and cracks on the surface of the coating (Khwaldia et al. 2010). Taking into account the properties of lipids, waxes are the most efficient substances to reduce moisture permeability. A high hydrophobicity of waxes is a result of a high content in esters of long-chain aliphatic alcohols and acids as well as long-chain alkanes (Morillon et al. 2002).

Multicomponent films, applied either in the form of an emulsion (composite coating) or in subsequent layers (multilayer coating), make it possible to combine the advantages associated with different components. Hydrophobic substances can create effective moisture barrier, and hydrocolloids provide structural support, as well as limit oxygen and carbon dioxide transfer (Bravin et al. 2004). Comparative studies of the properties of the emulsion film (Quezada Gallo et al. 2000) and two-layer films (Debeaufort et al. 2000) were carried out using the same film components

(methylcellulose and lipids—alkanes or triglycerides). WVP was mainly influenced by the nature of lipid (hydrophobicity and crystal arrangement): high content of alkanes, more hydrophobic and denser lipids are superior as WV barrier in comparison to typical triglycerides. Compared to bilayer films, emulsified films have inferior moisture barrier properties but enhanced mechanical properties (Debeaufort et al. 2000; Quezada Gallo et al. 2000).

Rhim et al. (2007) carried out water barrier properties tests of PLA-coated paper, such as WVP, water absorptiveness and contact angle. Based on the obtained results they stated that water resistance of paperboard was improved through surface coating with PLA. Krook et al. (2000) examined properties of paperboard laminates coated with two grades of poly(ϵ -caprolactone) (PCL), poly(hydroxy butyrate-co-valerate) (PHBV) or a liquid crystalline copolyester (LCP) prepared by compression moulding. WVTRs were ranged between 1 and 300 times higher than polyethylene and were lowest for the LCP-coated paperboard (Krook et al. 2000).

From an environmental viewpoint bio-based polymers or polymers in the form of aqueous dispersions should have much wider application in the packaging industry. However, bio-based polymers usually have both higher values of water vapour and oxygen permeability than those of conventional polymers. There is still further research needed for significant improvement in the barrier properties, therefore future functional surface treatment of cellulosic materials will most likely involve multilayer coatings. Important is also to fulfil all types of functional properties as well as material availability, good processability, suitability for food contact, price etc. (Andersson 2008).

19.2.3 Nanocellulose

The most common renewable polymer in the world is cellulose. It has been estimated that globally approximately 10^{10} - 10^{11} tons of cellulose are synthesised and also destroyed every year (Hon 1994). According to Heux et al. (1999), a number of living organisms, such as plants, some amoebae, sea animals (e.g. tunicates), bacteria and fungi, can biosynthesise it. Cellulose consists of a linear homopolysaccharide, a chain of β -D-glucopyranose units joint by β -1-4-linkages (Hon 1994). Each monomer unit has three hydroxyl groups with aptitude to form intermolecular hydrogen bonds, which play an important role in directing the crystalline packing and setting physical properties of cellulose (John and Thomas 2008).

Nowadays, the isolation, characterisation, and search for application of new forms of nanocellulose, also known as crystallites, nanocrystals, whiskers, nanofibrils, and nanofibres, become a hot research topic. They are produced using top-down methods with enzymatic/chemical/physical methodologies of their isolation from wood and forest/agricultural residues to the bottom-up production of cellulose nanofibrils from glucose by bacteria (Klemm et al. 2011). Nanocellulose, a material composed of nanosized cellulose fibrils with a high aspect ratio, may be classified into three main subcategories: microfibrillated cellulose (MFC), nanocrystalline

celluloses (NCCs) and bacterial nanocellulose (BNC) (Klemm et al. 2011) on the basis of their dimensions, functions and preparation methods.

Sandberg et al. at ITT Rayonier began to manufacture MFC in the late 1970s and early 1980s (Turbak et al. 1983; Herrick et al. 1983). These days to prepare MFC a combination of methods based on high mechanical shearing forces and mild acid and/or enzymatic hydrolysis can also be used (Henriksson et al. 2007). Henriksson et al. 2007 have discovered that enzyme-treatment facilitated disintegration, and the MFC nanofibres produced showed higher average molar mass and larger aspect ratio than nanofibres obtained by acidic pre-treatment.

NCCs, also known as whiskers, consist of rod-like cellulose crystals with widths and lengths of 5–70 nm and between 100 nm and several micrometres, respectively. They are generated by the removal of amorphous sections of a purified cellulose source by acid hydrolysis, often followed by ultrasonic treatment and their suspensions have liquid-crystalline properties (Klemm et al. 2011). Their dimensions depend on a couple of factors, including the source of the cellulose, the exact hydrolysis conditions, and ionic strength (Ramires and Dufresne 2011). NCCs from wood (Araki et al. 1998; Beck-Candanedo et al. 2005), cotton (Araki et al. 2000; Podsiadlo et al. 2005; Teixeira et al. 2010) have width of 3–20 nm and length of 100–300 nm, whereas other cellulose sources, such as tunicates (Favier et al. 1995) bacteria (Grunert and Winter 2002; Roman and Winter 2004) and algae (Revol 1982; Hanley et al. 1992), generate crystals with larger polydispersities and dimensions comparable to those of MFC.

BNC is also called bacterial cellulose, microbial cellulose or biocellulose. BNC is formed as a pure component of biofilms produced by aerobic, acetic acid bacteria of the genus *Gluconacetobacter*. In biotechnological processes the bacteria are cultivated in common aqueous nutrient media, and the BNC is excreted as exopolysaccharide at the interface between liquid and air. The resulting form-stable BNC hydrogel is composed of a nanofibre network containing up to 99 % water. The fibre diameter ranges from 20 to 100 nm. The BNC is a polymer with a high weight-average molecular weight, high crystallinity and good mechanical stability (Klemm et al. 2005, 2011).

During the last decade an increasing number of research papers and information in the area of cellulose nanoparticles have been published. The possibility of surface chemical modification and nanosized dimensions of cellulose nanoparticles have been extensively used in a wide variety of application, e.g. food additives (Dong et al. 1998), stabilisers in emulsions and dispersions (Andresen and Stenius 2007; Khanari et al. 2011), paper and nanocomposites (Nogi et al. 2009; Siqueira et al. 2010), additives in the cosmetics and pharmaceutical industries (Turbak et al. 1984), medical devices applications—BNC as wound dressings (Czaja et al. 2006; Fu et al. 2013) and implants (Nimeskern et al. 2013).

Among the most important potential applications of the MFC associated with the packaging industry one should mention an agent to enhance the mechanical strength of pulps produced by thermomechanical processing—the addition of 4 % of MFCs obtained from Kraft pulp to thermo-mechanical pulp (TMP) resulted in a 10 % increase in tensile index (Eriksen et al. 2008). Another potential application of MFC

is as a component of nano-paper, which is a very interesting packaging material due to its high toughness, good oxygen barrier and transparency (Henriksson et al. 2008; Nogi et al. 2009). MCF can be applied also to prepare films, for example from wood pulp (Taniguchi 1998), which has tensile strength approximately 2.5 times higher than the printing paper. Syverud and Stenius (2009) have examined strength and barrier properties of microfibrils from wood cellulose fibres prepared as pure MFC films and as coating on base paper. MFC films have comparable properties to the best synthetic polymers (like polyvinylidene chloride (PVDC)-coated oriented polyester) with respect to oxygen transmission rate, while the strength of base paper increased and its air permeability decreased significantly upon coverage with a layer containing less than 10 % MFC (Syverud and Stenius 2009).

The nonwood and wood-based MFC (from the terms sulfite and kraft pulp) and NCCs isolated from different sources have been examined as nano-fillers with various natural and synthetic polymers and latexes, which were used as matrix materials. Among natural matrix the following materials were tested: starch (Dufresne and Vignon 1998; Dufresne 2003; Chen et al. 2009), PLA (Iwatake et al. 2008; Nakagaito et al. 2009; Lin et al. 2009), PVA (Zimmermann et al. 2004; Garcia de Rodriguez et al. 2006; Lu et al. 2008; Roohani et al. 2008), natural rubber (Bendahou et al. 2009), PHA (Dufresne et al. 1999), soy protein (Wang et al. 2006) and chitosan (Li et al. 2009).

According to Siqueira et al. (2010), using cellulosic particles as a reinforcing phase in nanocomposites has many recognised advantages such as: “low density; renewable nature; wide variety of biological origin of filler available; low energy consumption; high specific properties; modest abrasivity during processing; biodegradability; relatively reactive surface, which can be used for grafting specific groups and almost unlimited availability”. However, there are also some disadvantages of cellulose nanoparticles such as “high moisture absorption, poor wettability, incompatibility with most of polymeric matrices and limitation of processing temperature” (Siqueira et al. 2010).

Cellulose nanoparticles are nature-based materials with unique and potentially useful features, which extend the application scope of cellulose. These novel nanocellulosic additives strongly expand fields of sustainable materials and nanocomposites for packaging sector (Klemm et al. 2011).

19.3 Special Functions of New Packaging Materials

In order to keep food quality and freshness, it is necessary to select suitable packaging materials and also packaging technologies. In this way, current tendencies include the development of novel packaging materials that interact with the product. One of the several possibilities, which are being extensively studied, is the incorporation of active substances within the package material. Active packaging provides among many features microbial safety for consumers, reducing, inhibiting or retarding the growth of microorganisms, thermo-protection during transport and storage,

indication of packaging damages, also self-repairing/healing of packages after damaging, and therefore could extend the shelf life and safety of the packaged food (Souza et al. 2013).

19.3.1 Antimicrobial Properties

To confer antimicrobial activity, antimicrobial agents may be coated, incorporated, immobilised or surface modified onto package materials. It should be underlined, that mostly all antimicrobials are temperature sensitive. Their incorporation into the structure the packaging materials should be performed at mild conditions, for example by grafting or coating of packaging material surface most preferable at the last step of technological processes (Aikio et al. 2006). The classes of antimicrobials cover broad range of chemical structures from acid anhydride, alcohol, bacteriocins, chelators, enzymes, organic acids and polysaccharides. Antimicrobial films and or/ packaging materials can be classified into two types: those that contain an antimicrobial agent that migrates to the surface of the food, and those that are effective against surface growth of microorganisms without migration. The direct incorporation of antimicrobial additives in packaging films is a convenient method by which antimicrobial activity can be achieved. Some antimicrobial packaging systems utilise covalently immobilised antimicrobial substances that suppress microbial growth. However, this is strongly related to type of bioactive agent, where for example PE/polyamide (70:30) formed a stable bond with nisin, in contrast to lacticin 3147. It was observed that antimicrobial packaging reduces the population of lactic acid bacteria in ham stored in modified atmosphere packaging (MAP) (60 % N₂:40 % CO₂) thereby extending product shelf life. Also nisin-adsorbed within bioactive inserts reduces the level of *L. innocua* and *S. aureus* in hams (Kerry et al. 2006). Essential oils (EOs) are aromatic oily liquids obtained from plant material. It has been recognised that some EOs possess antimicrobial properties. These have been reviewed in the past, but the relatively recent enhancement of interest in “green” consumerism has led to a renewal of scientific interest in these substances. Besides antibacterial properties, EOs or their components have been shown to exhibit antiviral, antimycotic, antitoxigenic, antiparasitic and insecticidal properties. A number of EOs has been identified as effective antibacterials, e.g. carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde, cinnamic acid (Burt 2004), oregano, cinnamon, clove (Fabian et al. 2006; Kaloustian et al. 2008), lemon balm and marjoram (Gutierrez et al. 2009).

Based on results presented by Souza et al. (2013), cinnamon essential oil can be chosen as antimicrobial substance for design biopolymer-based films that can be applied in food packaging. The results of their study indicate that there were significant differences among antimicrobial activity of cassava starch films with different cinnamon essential oil contents. Even at minimum concentration applied into the film formulation, cinnamon essential oil shows the inhibition against microorganisms such as *Eurotium amstelodami* and *Polytrichum commune*, that was considered

an important result since that higher concentrations could imply a sensorial impact, altering the natural taste of the food packaged by exceeding the acceptable flavour thresholds. As expected, a better inhibition was observed with higher content of cinnamon essential oil.

EOs combinations were also investigated against microorganisms. Combining lemon balm with thyme has yielded additive activity against *Listeria* strains. The effect of simple sugars and pH on antimicrobial efficacy was also studied. EOs retained greater efficacy at pH 5 and at 2.32 % concentration of sugar, where concentration above 5 % did not negatively impact EOs efficacy. EOs might be more effective against food-borne pathogens and spoilage bacteria when applied to coated package of food containing a high protein level at acidic pH, as well as moderate levels of simple sugars (Gutierrez et al. 2009). It is also important to envision the use of vacuum packaging (VP) with or without addition of essential oil (EOs), as an antimicrobial treatment for shelf-life extension of fresh food (Atrea et al. 2009). VP with addition of oregano oil can prolong the shelf life of food. In conclusion, based primarily on sensory data the shelf life of fresh food was 6 days (under aerobic storage), 9 days (under vacuum), 17 and 23 days treated with oregano oil (0.2 and 0.4 %, v/w) under VP, respectively. The antimicrobial effect of thyme essential oil at 0.3, 0.6, or 0.9 % concentration, nisin at 500 or 1000 IU/g, and their combination against *L. monocytogenes* was also studied (Solomakos et al. 2008). The results demonstrate that nisin at 500 or 1000 IU/g shows antibacterial activity against *L. monocytogenes*; the efficacy depends on the concentration of nisin and the type of microorganism. It should be underlined that addition of thyme oil (even on low level 0.6 % wt.) shows stronger inhibitory activity against *L. monocytogenes* than nisin at 500 or 1000 IU/g. The combined addition of EO at 0.6 % and nisin at 500 or 1000 IU/g shows a synergistic effect against the pathogen. Most efficient combination is EO at 0.6 % with nisin at 1000 IU/g, which decreases the population of *L. monocytogenes* below the official limit of the European Union, recently set at 2 log cfu/g, during storage at 4 °C (Solomakos et al. 2008).

Also chitosan can be used as bioactive additive of bio-based packaging films (Bonilla et al. 2013). The films based on polylactic acid (PLA) and different amounts of chitosan powder (CH) have been prepared by extrusion. The effects of CH particle size and the amount of chitosan incorporated in the PLA matrix (5 or 10 % on PLA basis) were investigated in terms of antimicrobial activity of the films. All films show an antimicrobial activity against aerobic mesophilic and coliform microorganisms as compared to non-coated samples. In pure PLA films the slight reduction in bacterial counts has been detected that can be related to their reduced oxygen permeability. In the case of PLA/CH film the reduction about 2 log cfu/g for coliform microorganisms has been observed, which is associated to the antibacterial activity of CH. These antimicrobial properties of CH have been explained by the interaction of the positive charges of protonated amino groups of chitosan with the negative charges from the residues of macromolecules on the membrane surface of microbial cells. This can directly interfere with the nutrient exchange between the exterior and interior of the cell. Previous researches have shown that chitosan was efficiently incorporated into PLA matrices by extrusion at

5 and 10 % of the matrix. The antimicrobial effect of chitosan has been proven in the composite films, which show a greater reduction of the microbial counts. PLA-based composite films with different types of nanoclays, such as Cloisite Na⁺, Cloisite 30B and Cloisite 20A, have been prepared using a solvent casting method and their antimicrobial properties were tested (Rhim et al. 2009). Four typical food pathogens including two Gram-positive bacteria, *L. monocytogenes* ATCC-19111, *S. aureus* ATCC-14458, and two Gram-negative bacteria, *Salmonella typhimurium* ATCC-14028 and *Escherichia coli* O157:H7 ATCC-11775 have been used. In the preliminary test, organically modified clay powders, substituted with quaternary ammonium ions, especially Cloisite 30B, showed strong antimicrobial activity against both Gram-positive and Gram-negative bacteria with small amounts of clay. However, when compounded with PLA, even low noticeable antimicrobial activity was not observed against all of the test microorganisms, regardless of clay types, except PLA/Cloisite 30B composite film. As expected, pure PLA and PLA/Cloisite Na⁺ films did not show any antimicrobial activity. Only PLA composite films compounded with Cloisite 30B showed bacteriostatic activity against *L. monocytogenes*. In this study the authors postulated that the antimicrobial action of the composite film was attributable to the polymer matrix itself (chitosan), solvent used for the preparation of the film (acetic acid) and the quaternary ammonium ions in the organically modified montmorillonite (Cloisite 30B). The main difference in antimicrobial activity between the chitosan/organoclay and the PLA/organoclay composite films might be also attributed to the hydrophilicity or hydrophobicity of the polymer matrix, as well as the type of organoclay used. Due to the hydrophobic nature of PLA, microorganisms cannot easily access the surface of the film; in consequence, no antimicrobial activity was observed in the PLA/clay composite films.

The antimicrobial function of food packaging is still questionable. Some countries, such as USA, Japan or Australia have been accepted the antimicrobial packaging on the market; in Europe such packaging become recently to be more popular, but still has to gain the market. Selected producers offer active antimicrobial packaging for daily application, such as for example wrapping cheese and ham (e.g. Sanocoat, Mondi, Austria), but in the comparison to conventional products, the antimicrobial packaging market in Europe is still on the developing stage.

19.3.2 Self-Healing Properties

Polymers and structural composites used in various applications including packaging are susceptible to damage induced by mechanical, chemical, thermal, UV radiation, or a combination of these factors (Osswald and Menges 2003). These factors can lead to the formation of microcracks deep in the structure, which are difficult to detect and repair. The presence of the microcracks in the polymer matrix can affect both the fibre- and matrix-dominated properties of a composite (Wu et al. 2008).

Researches have shown that matrix damage can cause reductions properties such as tensile and compressive strength or delamination and eventually fibre fracture (Wilson et al. 1986; Jang et al. 1990).

The conventional repair methods are not effective for healing invisible microcracks within the material structure during its service life (Wu et al. 2008). Therefore, Jud et al. (1981) proposed the concept of self-healing polymeric materials for extending the working life and safety of the polymeric components by healing invisible microcracks. During the last years various self-healing/self-repairing concepts for polymeric materials have been presented (Wu et al. 2008).

The concept of releasing healing chemicals incorporated within hollow fibres to repair damage was proposed by Dry (1992, 1996), it was a single-component system. Two-component concept of self-repair system was first presented by White et al. (2001); in this system healing is accomplished by incorporating a microencapsulated healing agent and a catalytic chemical trigger (hardener) within an epoxy matrix. Damages of matrix cause rupture the microcapsules and releasing the healing agent into the crack plane through capillary action. Polymerisation of the healing agent is triggered by contact with the embedded catalyst, bonding the crack surfaces (White et al. 2001).

Andersson et al. (2009) examined the possibility of using a particular class of microcapsules as self-healing agents in paperboard coatings. The microcapsules with a hydrophobic core surrounded by a hydrophobically modified polysaccharide membrane in aqueous suspension were coated on surface of paperboard to increase the packaging functionality. The self-healing mechanism involves the rupture of microcapsules and release of the core material during applied stress induced, e.g. during creasing and folding operations. Crack propagation is hindered by plasticisation of the coating layer, while the barrier properties are maintained by the increased hydrophobicity due to the release of hydrophobic compound from capsule core (Andersson et al. 2009).

Chen et al. (2002, 2003) have proposed the application of a thermally reversible reaction such as the Diels-Alder reaction for self-healing. They synthesised remediable cross-linked polymeric materials, which allow to heal crack effectively with a simple thermal healing procedure and this process could be repeated multiple times (Chen et al. 2002, 2003).

A number of other approaches have been suggested during the development of self-healing materials including the thermoplastic additive (Zako and Takano 1999), chain rearrangement via interdiffusion of dangling chains (Yamaguchi et al. 2007) or chain slippage in the polymer network (Ho 1996), repair of lightly cross-linked hydrophilic polymer gels via metal-ion-mediated reactions (Varghese et al. 2001).

The self-healing polymeric materials have been based in large part on mimicking of biological healing, which is called biomimetics (Wu et al. 2008), e.g. White et al. (2001) have developed self-healing system which mimics a circulatory system that continuously transports the necessary chemicals and building blocks of healing to the site of damage. There is still much to be done before even the simplest biological healing mechanism can be copied in these synthetic materials (Wu et al. 2008).

19.3.3 Thermo-Protection

In order to keep food at the proper temperature two physical phenomenon: low thermal conductivity of package resulting from the ventricular wall construction (packaging foam and corrugated cardboard) and the ability to reflect radiant heat (package with metallic coatings) are currently used.

In modern food packaging the conventional foam materials such as polystyrene are increasingly replaced by their biodegradable equivalents. Still the main raw material applied in processing of such eco-foam packaging is usually starch enriched with plasticisers, plant fibres (Lawton et al. 2004; Salgado et al. 2008), natural rubber latex (Shey et al. 2006), mineral fillers (Vercelheze et al. 2012) or hydrophobic additives such as PCL (Preechawong et al. 2004) and PLA (Preechawong et al. 2005).

In case of the first commercially available biodegradable packagings based on foamed starch various protective coatings were often used, where the primary purpose was the protection of hygroscopic foam material from moisture and improvement of mechanical properties (Glenn and Ortr 2001a, b; Doe 2009). On the world market, there are also first foam products from biodegradable plastic such as PLA—for example Natural Box trays produced by Coopbox Italia Sri (Doe 2013a). At the same time BASF company is working on the introduction of biodegradable packaging foam based on the Bionovio material, which is a mixture of petroleum-based biodegradable plastic Ecoflex and PLA. This could be an eco-alternative to popular Schellpac packagings. Addition of Ecoflex increases the mechanical strength and resistance to liquids of foamed materials made of other biodegradable polymers such as starch and PLA (Siracusa et al. 2008; Schut 2007; Doe 2013b).

In case of foaming technologies the classical extrusion method is often replaced by the baking method, similarly to one used in the production of wafers (Shogren et al. 2002) or compression/explosion processes (Glenn and Ortr 2001b).

19.4 Intelligent Solutions for Packaging Systems

19.4.1 Barcodes and RFID Systems

Barcodes and RFID (Radio Frequency Identification) systems are very close related technologies. Both allow for tracking the product in the supply chain. The main difference lies in a method of information readout, which in case of barcodes is carried out by an optic or laser reader and wireless radio devices in case of RFID (Rhim et al. 2013).

Barcodes are currently widely used in the food industry. There are three basic readers used to read the codes: fixed-beams (inexpensive manual scanners), moving-beams (using mirrors leading beam in multiple axis) and imaging devices (operating similarly to digital cameras where picture is being decoded by a microprocessor).

Traditional codes (1D) are increasingly replaced by the much more modern matrix (2D) codes which have much higher capacity compared to the linear barcode. The information encoded in a 2D barcode can be scanned and encoded by mobile phones equipped with built-in cameras and the appropriate software. The encoded information can be URL, text, or series of alphanumerical characters such as a phone number or an SMS (Ozcelik and Akarturk 2011).

Construction of the Quick Response (QR) codes allows them to be quickly scanned during the quick movement of the packaging on the carriers. Depending on the model (I or II) it is possible to encode 7089 numeric or 4296 alphanumeric characters.

There is also an interesting solution where the barcodes are integrated with the smart label technology. Barcodes made in this technology are becoming useless for readers when the safety of the consumer is threatened due to too long or bad packed product storage. The two main drawbacks of the barcode are: that it is a ready-only technology—meaning that once printed the data cannot be changed and second that the scanner needs a relatively short direct distance to the barcode (Musa et al. 2014).

Tag barcodes are the latest development in the field of codes. Its content refers to data stored on a server and thus can be quickly updated making it an effective tool for on-line information and promotion. Unlike QR codes the HCCB (High Capacity Color Barcode) codes, developed by Microsoft Research, can be multi-coloured and contain graphic images such as company logos. They are already used in the promotion of many products such as: Meyer Natural Beef, Coca-Cola, Dr Pepper, Skky Vodka (Doe 2013c).

RFID system allows to track product using radio frequency waves. Construction of the system is mostly based on three main elements: transponders (tags), readers and computer system. RFID systems are based on two types of transponders: passive and active. Active transponders usually have power source (batteries). They have silicon chip and antenna plus additional AC/DC converter. Due to the applied frequency RFID systems can be divided into operating at: low (LF), high (HF) and ultra high (UHF) frequencies (Wu et al. 2006; Tajima 2007).

To make RFID system applicable in the food industry it needs to be less expensive so it became more competitive to much cheaper solutions based on barcodes 1D and 2D. It is also important to increase the measurement capabilities of RFID systems particularly those with very low electricity demand (ULP ultra-low-power-lower than 10 mW) (Elmi et al. 2008). There are commercial solutions currently available for the measurement of physical parameters such as temperature, humidity, mechanical shock and light (Kassal et al. 2013; Musa et al. 2014). From the standpoint of food packages there is particular interest in the possibility of implementation of ultra-low-power Metal Oxide (MOX) semiconductor gas sensor (Abad et al. 2007; Zampolli et al. 2008; Briand et al. 2011; Potyrailo and Surman 2013).

The widespread use of RFID in the food packaging brings certain risks. As with all non-edible components connected with the package there is a risk of swallowing. RFID systems mounted on the packages will also hinder the recycling process of the dispose packaging (Dainelli et al. 2008; Aliaga et al. 2011; Angerer et al. 2012).

19.4.2 Self-Heating and Self-Cooling Package

Innovative packaging should allow for a quick cooling or heating of its content immediately before serving. Food products in self-heating containers are currently offered only by a few companies in the world. Heating of the packaged ready meals is possible due to limestone placed in a separate part of the package, which release large amounts of heat after the hydration (Mahalik and Nambiar 2010). An acid–base exothermic neutralisation process could also be an alternative solution. The significant drawbacks of this technology are the health and environmental hazards. Also, as the process is carried out in water, it limits the temperature to which the beverage or food can be heated. Another chemical process for heating is to oxidise solids. In this case the major drawbacks are difficulties during initiation, significant safety risks and also economical aspects.

One of the latest achievements in the field of self-heating packages is the innovative system for self-propagating high-temperature synthesis (SHS). The most common SHS processes are based on redox reactions between metals or semiconductors and metal oxides that are carried out in the solid state. The temperature of SHS reactions exceeds 1000 °C and as a result it requires good heat transfer and also special precautions (Gluch and Mandler 2001). Another system is based on the Flameless Ration Heater (FRH). In this technology the heater, which contains a magnesium-iron compound, can be activated by a saline solution to initiate an exothermic chemical reaction (Ho et al. 2010).

Self-cooling packaging, using zeolite-technology, have been applied in brewing (Mahalik and Nambiar 2010). It is based on simple water evaporation for self-refrigeration and it proved to lower the temperature to a minimum of 16.7 °C in just 3 min. There are also other technologies using hydrofluorocarbon (HFC) expansion phenomenon or endoenergetic reaction of ammonium nitrate (Day 2001, 2003).

19.4.3 Indicators of Temperature, Freshness and Humidity

An indicator may be defined as a substance/material that indicates the presence or absence of another substance, external conditions or the degree of reaction between two or more substances by means of a characteristic change, especially in colour. In contrast with sensors, indicators do not comprise receptor and transducer components and communicate information through direct visual change (Kerry et al. 2006).

Temperature is a crucial external factor affecting the quality and safety of food products during both distribution and storage. The difficulty in controlling and monitoring the temperature history of packed products makes impossible the precise prediction of shelf-life of food product. Time–temperature indicators (TTIs) provide a visual summary of a product's accumulated chill-chain history, recording the effects of both time and temperature (Han et al. 2012; Wanihsuksombat et al. 2010;

Kim et al. 2012a; Jung et al. 2012). The (lactic acid)-based TTIs change their colour that is associated with the vapour diffusion of lactic acid (Wanihsuksombat et al. 2010). An irreversible colour change of a chemical chromatic indicator (from green to red) clearly and progressively occurs due to the pH reduction. The indicators can be applied to demonstrate the time–temperature history to indicate food quality associated with time–temperature exposure in the range of 4–45 °C.

An enzyme-based TTI prototype was developed using an advantageous enzyme—laccase (EC1.10.3.2) that has simple discoloration kinetics and is widely available (Kim et al. 2012b). Commercial enzymatic TTIs use synthetic indicators based on rather expensive chromogenic substances, which during hydrolysis change their colour. Recently more frequently natural pigments or dyes as colour substrate in such enzymatic indicators have been introduced, provide several advantages including improved visibility, lower cost, and wider use of colour coding.

The other intelligent solutions, which can be incorporated as effective protection of food products, are freshness indicators. The information provided by intelligent packaging systems on the quality of products may be either indirect or direct. Freshness indicators provide direct product quality information resulting from microbial growth or chemical changes within a food product. Microbiological quality may be determined through reactions between indicators included within the package and microbial growth metabolites. The formation of different potential indicator metabolites in food products is dependent on the product type, associated spoilage flora, storage conditions and type of packaging system (Kerry et al. 2006). The principles for a novel intelligent packaging concept, which indicates specifically the spoilage or the lack of freshness of a food product, have been described by Smolander et al. (2002). For example freshness indicators for modified atmosphere packed poultry meat are based on the indication of the presence of hydrogen sulphide (H_2S), which is produced in considerable amounts during the ageing of packed poultry. A chromogenic array for monitoring boiled marinated turkey meat freshness in a modified packaging atmosphere (30 % CO_2 , 70 % N_2) has also been developed (Salinas et al. 2014). The array is composed of 16 sensing materials prepared through the combination of 13 indicators (including pH indicators, nucleophilic sensing dyes, etc.) and three different inorganic supports (i.e. UVM-7, alumina and silica gel). Colour differences from the chromogenic array have been also employed to create PLS models for aerobic mesophilic bacteria count, sensory score and storage time.

A non-destructive method for monitoring headspace ammonium as an indicator for changes in the freshness status of packed fish has been developed (Heising et al. 2012). Electrodes in an aqueous phase in the package monitor change in the concentration of ammonia produced in/on the packed fish and released in the headspace. The outputs of an ammonium ion-selective electrode (NH_4^+ -ISE) are compared with the volatile amines content of the fish fillets. The changes in the NH_4^+ -ISE signal correlated with the content of volatile amines (TVB N) in the cod fillets. This non-destructive method might be the basis for the development of an intelligent packaging for monitoring freshness of packed fish.

To monitor the changes of humidity and temperature during transport and storage of foodstuffs also some loggers (e.g. Ecotone, USA) can be applied. They collect the data during the whole chain, which can be transferred into the logistic system. Also colour-coded sensor labels are available on the market to indicate the state of the foodstuffs (e.g. RipeSense for pears show the ripeness of fruits; they react on the aroma that are released during ripening process). From customer's point of view the indicators should be readable and the result should be easy to interpret. They should help to buy fresh products with the high quality, or in the state that they require.

19.4.4 Scavengers, Absorbers and Emitters

19.4.4.1 Oxygen Scavengers

Oxygen can have considerable detrimental effects on foods. Oxygen scavengers can therefore help maintain food product quality by decreasing food metabolism, reducing oxidative rancidity, inhibiting undesirable oxidation of labile pigments and vitamins, controlling enzymatic discoloration and inhibiting the growth of aerobic microorganisms (Day 2001; Rooney 2005). Oxygen scavengers are the most commercially important sub-category of active packaging for food products and the most well known take the form of small sachets containing various iron-based powders containing an assortment of catalysts. The main advantage of using oxygen scavengers is that they are capable to reduce oxygen levels to less than 0.01 % which is much lower than the typical 0.3–3.0 % residual oxygen levels achievable by MAP. Oxygen scavengers can be used alone or in combination with MAP. Non-metallic oxygen scavengers have also been developed to alleviate the potential for metallic taints being imparted to food products. Non-metallic scavengers include those that use organic reducing agents such as ascorbic acid, ascorbate salts or catechol. They also include enzymatic oxygen scavenger systems using either glucose oxidase or ethanol oxidase, which could be incorporated into sachets, adhesive labels or immobilised onto packaging film surfaces (Day 2003). There are many commercial oxygen scavengers dedicated to food applications: the PureSeal™ oxygen scavenging bottle crowns (produced by W.R. Grace Co., Inc. USA), oxygen scavenging plastic (PET) beer bottles (manufactured by Continental PET Technologies, USA), OS2000® cobalt catalysed oxygen scavenger films (produced by Cryovac Sealed Air Corporation, USA) and light activated ZerO2® oxygen scavenger materials (developed by Food Science Australia, North Ryde, NSW, Australia) (Rooney 2000, 2005; Scully and Horsham 2005). For example there are commercial oxygen scavenger sachets (ATCO-100 and ATCO-210 supplied by Standa Industries, Caen, France). ATCO-100 and ATCO-210 sachets have an oxygen absorbing capacity of 100 and 210 mL, respectively (Aday and Caner 2013). O₂ absorbers can be used to slow down food metabolism and mould growth. They maintain the quality better than in case of control group that was kept at refrigerator temperatures.

The effectiveness of O₂ absorbers on fresh strawberry quality during 21 days of storage also has been tested.

Oxygen scavengers are still to be improved. One of the inventions is oxygen scavenger that is dispersed in a low-density foam, wherein the oxygen scavenger has a particle size of less than 25 µm (Chieh-Chun et al. WO2011112304). To increase air permeability and oxygen scavenging capacity also a perforated plastic film laminated to a microporous film composed of water-resistant and oil-resistant processed paper by a specific adhesive and making the perfected plastic film outside to seal an oxygen scavenger has been developed (Kiyoshi and Masichi JPH03238043). As an embodiment of the oxygen scavenger one can use sulphite, dithionite and hydroquinone.

A free-oxygen absorber package with an oxygen detecting function has been also proposed (Teruyoshi et al. JP2003252374). This new packaging system comprises a free-oxygen absorber bag packaged with a multi-layered packaging material. The bag has an outermost layer formed of a film having at least one or more transparent portions from which the inside can be viewed. A colour reaction tape that varies in hue is fixedly sandwiched between a rear face of the film and a subsequent packaging material in contact with at least a part of the transparent portion of the film.

19.4.4.2 Ethanol Emitters

Ethanol emitters are preservative releasing products that are usually in sachet forms but can be used also as impregnated preservative releasing films. The ethanol is used as an antimicrobial agent. Many applications of ethanol emitting sachets had been patented, primarily by Japanese manufacturers. These include Ethicap™, Antimould 102™ and Negamould™ (Freund Industrial Co. Ltd), Oitech™ (Nippon Kayaku Co. Ltd), ET Pack™ (Ueno Seiyaku Co. Ltd), Oytech L (Ohe Chemicals Co. Ltd) and Ageless™ type SE (Mitsubishi Gas Chemical Co. Ltd). All of these films and sachets contain absorbed or encapsulated ethanol in a carrier material that allows for controlled release of ethanol vapour (Rooney 1995; Day 2003).

19.4.4.3 Ethylene Scavengers

Ethylene accelerates the respiration rate and subsequent senescence of packaged post-harvest and fresh products (especially, fruits and vegetables). In some situations (e.g. for long distance transport) it is necessary to remove ethylene or to suppress its effects. It is the main reason why many researches have been undertaken many efforts to incorporate ethylene scavengers into packaging of fresh products (Day 2003; Scully and Horsham 2005). Effective systems that adsorb ethylene are based on potassium permanganate immobilised on mineral substrate such as alumina or silica gel. These scavengers are available in sachets form to be placed inside blankets or tubes (Labuza and Breene 1989; Day 2003). Activated carbon-based

scavengers (incorporated into sachets) with various metal catalysts can also remove ethylene. There are also activated minerals including clays, pumice, zeolites, coral, ceramics and even Japanese Oya stone that are also very effective (Labuza and Breene 1989; Day 2003).

19.4.4.4 Carbon Dioxide Scavengers/Emitters

Many commercial available sachets and labels can be used either to scavenge or emit carbon dioxide. One of the solutions is to use a carbon dioxide scavenger or a dual-action oxygen and carbon dioxide scavenger system (Day 2003). These dual-action sachets and labels typically contain iron powder for scavenging oxygen and calcium hydroxide, which scavenges carbon dioxide when it is converted to calcium carbonate at sufficiently high humidity conditions (Rooney 1995). Carbon dioxide emitting sachet and label devices can either be used alone or combined with an oxygen scavenger. An example of the former is the Verifrais™ package that has been manufactured by SARL Codimer (Paris, France) and used for extending the shelf life of fresh meats and fish (Rooney 1995).

19.4.4.5 Flavour/Odour Absorbers and Releasers

The commercial use of flavour/odour absorbers and releasers is controversial due to concerns arising from their ability to mask natural spoilage reactions and hence mislead consumers about the condition of packaged food (Anon 2005a, b). Two types of taints amenable to removal by active packaging are amines and aldehydes. Unpleasant smelling volatile amines, such as trimethylamine, associated with fish protein breakdown, are alkaline and can be neutralised by various acidic compounds. In Japan, Anico Co. Ltd has marketed Anico™ bags that are made from film containing a ferrous salt and an organic acid such as citrate or ascorbate. These bags are claimed to oxidise amines (Rooney 1995). Removal of aldehydes such as hexanal and heptanal from package headspaces is claimed by Dupont's Odour and Taste Control (OTC) technology that is based upon a molecular sieve with pore sizes of around 5 nm. Dupont claims that their OTC technology removes or neutralises aldehydes, although clear evidence for this is lacking. The claimed food applications for this technology are snack foods, cereals, dairy products, poultry and fish (Day 2003).

19.5 Innovative Design and Eco-Design Aspects

Innovative packaging with enhanced functions is constantly being studied in response to consumer demands for minimally processed foods with fewer preservatives, increase in regulatory requirements and market globalisation (Yoshida et al.

2014). Due the globalisation the transport of food on long distances should be considered; also the minimal processed foodstuffs demand higher protection and extending of shelf-life. To reduce the amount of wastage, the environmental impact of packaging should be taken into account. Eco-design is an idea, which is focusing on the whole chain life of packaging. Ecological aspects are important starting from the innovative design and development of new, innovative (intelligent and/or active) packaging, through the usage, until the end of life of packaging. On every stage ecological and sustainability aspects should be considered. However for example the biodegradability not always is desired, some foodstuffs demand high level of protection and during the usage the packaging has to fulfil the high requirements to storage at environmental conditions that are not suitable for biodegradable packaging. The most important is to select the suitable packaging for specific food requirements. For example, the spices should be packed into the materials that are resistant for their active (even volatile) compounds. The packaging should keep the aroma and all properties of spices as long as possible at the same state. For some application the biopolymers and bioplastics are not suitable, and there is no sense to use them at such application; but for some purposes they are ideal solutions and can replace conventional polymers very easily. The most important is to consider environmental impact at every stage of design process of packaging: starting from research and development, through the production and usage, until the end of life of packaging material.

Also other aspects should be taken into the account. Recently, the environmental impact caused increased demand for biodegradable packaging materials from renewable resources (biopolymers) as an alternative to synthetic packaging plastics. Unfortunately, they possess in most cases relatively poor mechanical and barrier properties, which currently limit their industrial application (Rhim et al. 2013). Nanoreinforcement of these biodegradable polymers to prepare nanocomposites has already been proven to be an effective way to enhance these properties concurrently. So, these newly developed biodegradable polymer-based nanocomposites, that is, green nanocomposites, are the wave of the future and considered as the next generation materials (Sanchez-Garcia et al. 2010). Bio-nanocomposites consist of a biopolymer matrix reinforced with nanoparticles having at least one dimension in the nanometer range. They have improved mechanical, barrier, rheological and thermal properties in the comparison to biopolymer packaging products. Unfortunately, nowadays nanocomposites are not considered as “environmentally safe”, “environmentally friendly” or “eco-friendly” especially if one considers to apply them as food packaging additives. Though there is limited scientific data about migration of nanoparticles from packaging materials into food, it is reasonable to assume that migration may occur because of their tiny dimensions. It is prudent to consider that, once present in the food packaging material, nanoparticles might eventually migrate into food. So it is mandatory to verify the extent of migration of nanoparticles from the package into the food and to develop a method to prevent such migration before applying the nanocomposite into the food packaging. The possible options for hazard reduction of migration of nanoparticles from nanocomposite materials are: better fixation of nanoparticles in nanocomposites, including persistent suppression of

oxidative damages to polymer by nanoparticles, changes of nanoparticle surface, structure or composition and design changes leading to the release of relatively large particles (Rhim et al. 2013). These newly developed nano-biocomposites could have some potential applications in food packaging, for instance, in micro-wavable packaging, in anti-static packaging or in intelligent packaging designs, due to their additional functionalities that include electrical and thermal conductivity.

Similarly, reinforcement of biopolymers with organically modified layered nanoclays has also been observed to result in significant improvements in their properties. It is known that the addition of low contents of nanoclays (less than 10 % wt.) leads to a remarkable increase in rigidity (elastic modulus), thermal and dimensional stability, and in the barrier properties to gases and vapours without compromising other properties like toughness and transparency. Nevertheless, most of the works carried out using nanoclays have been related to the use of montmorillonite (MMT) type of clays. Moreover, some nanoclays have been reported to be able to disperse UV visible radiation due to the inherent enhanced scattering and reflection and reduced absorbance caused by the highly dispersed clay nanolayers. This property is highly important for food packaging as protection against light as basic requirement to preserve the quality of many food products. An additional functionality that nano-biocomposites based on nanoclays can maintain is controlled release of substances for the development of active packaging technologies. It was proved that the addition of different types of clays, biofibres and their nanowhiskers, carbon nanotubes and carbon nanofibres to biopolyester matrices (PLA, PCL, PHB and PHBV) leads not only to barrier improvements but also to novel functionalities such as UV visible protection and controlled release of antimicrobial substances (Sanchez-Garcia et al. 2010).

There are still many challenges in front of innovative food packaging design; there are many ideas that can be applied in effective way for maximal protection of food, maintaining their high quality, to extend their shelf life, to limit applying various preservatives and other chemicals and finally to reduce the wastage during processing. However the most important issue is to make all such technologies as “green” as possible and furthermore implement only substances, which are allowed to be use as direct or indirect food contact materials. Finally one has to remember that during commercialisation of innovative packaging materials “sometimes the simplest designs and solutions are the most useful and effective.”

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Chapter 20

Food Processing Using Supercritical Fluids

Željko Knez

Nomenclature

a, b, u, w	Parameters of cubic equations of state
p_c	Critical pressure
R	Gas constant
T_c	Critical temperature
V_c	Critical volume

20.1 Introduction

Design of new products with special characteristics or design of new processes, which are environmental friendly and have an impact to sustainable processes are a great challenge for chemical engineers.

In the man's living environment the pressure ranges between 0.25 bar at the highest mountain up to 1000 bar at the bottom of deepest ocean. Therefore the first technologies for production of various substances were operated at atmospheric pressure. Demand on new products like ammonia shifts the technological processes towards the high pressure.

In high pressure industrial processes the pressures ranges from about 50 bar (in particle formation processes) up to over 200 kbar (conversion of graphite to diamonds).

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High pressure as a relatively new tool gave in several processes a completely new products with special characteristics. Several new processes are environmental friendly, of low costs and sustainable.

The advantages of use of supercritical fluid (SCF) as solvents in chemical synthesis have environmental benefits, health and safety benefits, and chemical benefits (Jessop and Leitner 1999; Knez et al. 2010).

Environmental benefits of most SCFs in industrial processes are in replacement of environmentally far more damaging conventional organic solvents. An environmental impact is also low energy consumption during operation.

Health and safety benefits include the fact that the most important SCFs (SC CO₂ and SC H₂O) are non-carcinogenic, nontoxic, non-mutagenic, nonflammable, and thermodynamically stable.

One of the major process benefits is derived from thermophysical properties of SCFs, high diffusivity, low viscosity, the density, dielectric constant of SCF, which can be fine-tuned by changes of operating pressure and/or temperature.

As in all extraction processes also in supercritical extraction of solid and liquid mixtures the solubility of single component or mixture of components in SCF is the basic data for design of separation processes. The components or mixture of compounds which have to be extracted have to be soluble in SCF/dense gas.

As known from thermodynamics the solubility of compounds in SCF/dense gases depends on the density of SCF/dense gas which depends on pressure and temperature of SCF. Another very important parameter which influences the solubility of compounds in SCF is dielectric constant of SCF, which is influenced by temperature and/or pressure of SCF.

The motivation for use high pressure in a large range of high pressure technologies and processes is based on chemical physicochemical, physico(bio)-chemical, physico-hydrodynamical and physico-hydraulic effects (Bertuco and Vetter 2001).

20.2 Solubility and Phase Equilibrium

For the design of high pressure process, data are required on the operating parameters, the type and quantity of the solvent, the recirculation rate, and energy consumption. This information can be obtained from phase equilibrium and mass transfer measurements.

In order to determine the hold-up times transfer studies are performed, and for determining mass transport coefficients different mathematical models were tested.

For determination of equilibrium solubilities and phase equilibria, sophisticated experimental methods are available.

For the vapor–liquid equilibria, static or recirculating methods are used for the composition measurements of coexisting phases (Dohn and Brunner 1995; Fornari et al. 1990), while for the solid–liquid equilibria static and dynamic methods are used (Bruno and Ely 1991; Kikic and de Loos 2002).

Different experimental techniques are known in the literature for determination of the three-phase solid–liquid–gas (S–L–G) line (Cheong et al. 1986; Fukne-Kokot et al. 2000; Fukne-Kokot et al. 2003; McHugh and Yogan 1984; Weidner et al. 1997). The knowledge of S–L–G equilibria at high pressures is very important not only for understanding and design of high pressure separation processes in the food, pharmaceutical, and chemical industries but also for predicting the applicability of high pressure micronization and high pressure crystallization processes—i.e., the processes which were intensively researched in the present time.

Methods employed to deduce the p–T projection of the S–L–G curve as well as the compositions of the liquid and the gas phase along the S–L–G curve are:

- Static solubility measurements using known quantities of the solid and the gas.
- Modified freezing method (Fukne-Kokot et al. 2000), where the cell is filled with melt of the substance and a cooling pipe attached horizontally in the view cell is used to initiate crystallization in the middle of the cell.

In another approach, only the p–T projection of the S–L–G curve is determined. Methods described in the literature are:

- “First melting point” method, where the temperature of an initial S–G condition is slowly increased until the solid begins to melt (Cheong et al. 1986).
- “First freezing point” method (Cheong et al. 1986), where the initial appearance of the solid phase is observed.
- Modified capillary method (Fukne-Kokot et al. 2000; Weidner et al. 1997), where the substance is introduced into the glass capillary and placed with the thermocouple in the high-pressure view cell.

The behavior of pure compressed gases near and over the critical region is relatively well known and therefore the pVT data can be computed with different equations.

Empirical equations use a lot of parameters for describing phase behavior in a wide range of temperature and pressure.

The drawback of empirical equations is that it needs a lot of experimental data, which are necessary for fitting parameters in equation. Bender’s equation (Bender 1975) includes 20 parameters.

On the other hand cubic equations of state (EOS) based on attractive and repulsive forces in combination with mixing rules are currently the most widely used. Several types/modifications of cubic EOS exist: Van der Waals (Anderko 2000), Redlich-Kwong (RK) (Redlich and Kwong 1949), Soave-Redlich-Kwong (SRK) (Soave 1972), and Peng-Robinson (PR) (Peng and Robinson 1976).

General form of cubic EOS is

$$p = \frac{RT}{V - b} - \frac{a}{V^2 + ubV + wb^2}$$

using different values for u and w , which are presented in Table 20.1.

Advantage of use of cubic EOS is that thermodynamic properties like enthalpy, internal energy, and entropy can be calculated.

Table 20.1 Parameters a , b , u , and w for cubic EOS

	a	b	u	w
Van der Waals	$\frac{27}{64} \frac{R^2 T_c^2}{P_c}$	$\frac{RT_c}{8P_c}$	0	0
RK	$\frac{0.42748 R^2 T_c^{2.5}}{P_c}$	$\frac{0.08664 RT_c}{P_c}$	1	0
SRK	$\frac{0.42748 R^2 T_c^2}{P_c} \left(1 + f_w (1 - T_r^{0.5}) \right)^2$, $f_w = 0.48 + 1.574w - 0.176w^2$	$\frac{0.08664 RT_c}{P_c}$	1	0
PR	$\frac{0.45724 R^2 T_c^2}{P_c} \left(1 + f_w (1 - T_r^{0.5}) \right)^2$, $f_w = 0.37464 + 1.54226w - 0.26992w^2$	$\frac{0.0778 RT_c}{P_c}$	2	-1

Cubic EOS can be used for predicting thermodynamic properties of mixtures, and parameters a and b are calculated by mixing rules. By this procedure the mixture is reduced to a hypothetical “pure substance” and therefore phase behavior can be described as for pure substance. The form of the mixing rules that extend the use of EOS developed for pure fluids to mixtures is, as reported (Anderko 2000), more important than the particular P - V - T relationship embodied in EOS. The adjustable binary parameters of combining rules have to be obtained by fitting the EOS to experimental data.

Calculation of phase equilibria solid/compressed gases is problematic when critical data and acentric factor (w) of solids are not known. Different group contribution methods are usually used to estimate physicochemical properties of pure substances.

For same systems, solubility of solids in compressed gases could be relatively well described with different equations, where the solubility is a function of fluid density (Adachi and Lu 1983; Kumar and Johnston 1988).

There is no method to predict phase equilibria behavior or solubility without experimental data, either using cubic EOS (determination of k_{ij}) or density based models.

The problem, which persists in dimensioning SCF-processes, where systems are highly nonideal due to high pressures involved, is that usually physicochemical and transport data of the investigated components are not available in the literature. It is difficult and time-consuming task to measure them experimentally: therefore they are usually estimated with different thermodynamical or empirical mathematical models. In most cases conventional models for modeling phase equilibria in dependence of pressure do not fit the experimental points equally well at all temperatures and pressures and can give misleading results. To solve the problems, conventional thermodynamical models have been modified or new thermodynamical or empirical models have been and will be developed. However, the problem has not been completely solved yet.

A review of published data on high pressure fluid phase equilibria-experimental methods and investigated systems are well presented by Christov, Dorn, and Fonseca (Christov and Dohrn 2002; Dohrn et al. 2010; Fonseca et al. 2011; Dohrn et al. 2012)

20.3 Extraction of Solids and Liquids Using Dense Gases

20.3.1 Introduction

The advantages of use of SCF as solvents in chemical synthesis have environmental benefits, health and safety benefits, and chemical benefits (Jessop and Leitner 1999).

Environmental benefits of most SCFs in industrial processes are in replacement of environmentally far more damaging conventional organic solvents. An environmental impact is also low energy consumption during operation.

Health and safety benefits include the fact that the most important SCFs (SC CO₂ and SC H₂O) are non-carcinogenic, nontoxic, non-mutagenic, nonflammable, and thermodynamically stable.

One of the major process benefits is derived from thermophysical properties of SCFs, high diffusivity, low viscosity, the density, dielectric constant of SCF, which can be fine-tuned by changes of operating pressure and/or temperature.

Extraction of hop constituents, decaffeination of tea and coffee are the largest scale processes, which are mostly performed in industrial scale.

The advantages of use of SCFs for isolation of natural products is well described in the literature (Marr and Gamse 2000; Knez et al. 2010) (solvent free products, no side-products, low temperature, etc.). One of the most important advantages of use of SCFs is selective extraction of components or fractionation of total extracts.

This is possible by use of different gases for isolation/fractionation of components and/or changing process parameters. The limitation of further application of extracts obtained by high pressure technology is in the price of the product, which is in comparison with conventionally obtained products relatively high. The legal limitations of solvent residues and solvents (for the products for human applications) and isolation/fractionation of special components from total extracts in combination with different formulation and sterilization processes (controlled release for example) will increase the use of dense gases for extraction applications.

Design of process parameters has very important influence on the investment cost for the high pressure plant and subsequently on the economy of the process.

Beside the solubility data of solute in SCF mass transfer have also enormous influence on the economy of extraction process. Mass transfer models usually describe extraction yield vs. extraction time, but better presentation for the design of extraction apparatuses is yield vs. S/F (mass of SC solvent /to solid material).

20.3.2 *Extraction of Solids*

Extraction of hop constituents, decaffeination of tea and coffee are the largest scale processes, which are mostly performed in industrial scale. Several industrial plants are in operation also for extraction of spices for food industry and natural substances for use in cosmetics.

General flow sheet of extraction process is presented in Fig. 20.1.

In extraction step the solubility of compound or mixture of compounds has to be the highest while in separation step the solubility of compound in SCF has to be the lowest.

Therefore the phase equilibrium data are the most important data for the design of operating pressure and operating temperature of SCF in extraction plant. Based on phase equilibrium data the theoretical amount of SCF necessary for separation of compound from solid or liquid mixture could be calculated.

Design of process parameters has very important influence on the investment cost for the high pressure plant and subsequently on the economy of the process.

Beside the solubility data of solute in SCF mass transfer have also enormous influence on the economy of extraction process.

Mass transfer models usually describe extraction yield vs. extraction time, but better presentation for the design of extraction apparatuses is yield vs. S/F (mass of SC solvent /to solid material).

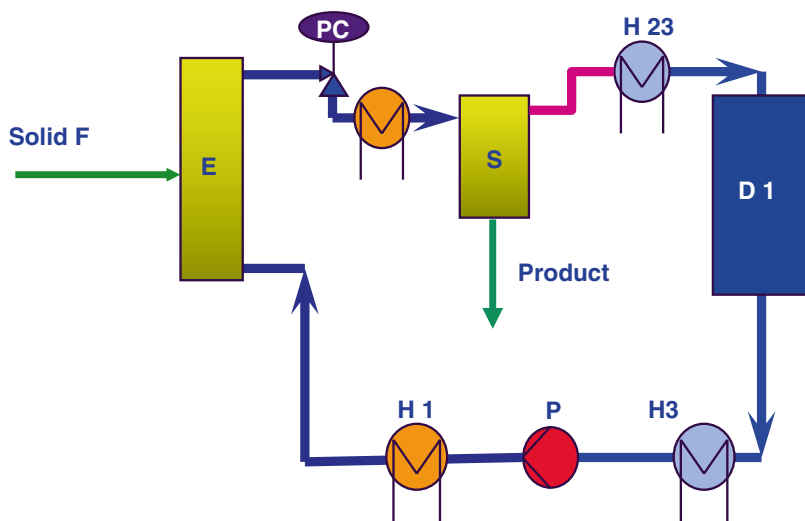


Fig. 20.1 General flow sheet of SCF extraction plant

Cascade operation is used in industrial scale plants to increase the economy of the solid—SC solvent extraction process. Till now, there were some research on continuous operation of plants for extraction of solids with SC solvent (Eggers et al. 1996) but till today no application for continuous feed of solids were applied on industrial scale.

In industrial scale usually more extractors are combined in series. By cycle operation of battery of extractors a quasi-continuous solid flow could be achieved. Such mode of operation gives extremely high extraction yields because pure solvent is contacted with pre-extracted material and so the solvent is loaded to maximal solvent capacity.

One of the major advantages of SC fluid extraction processes is fractionation of extracts. Multi-step separation could be performed in several separators with decreasing the solvent power.

Decreased solvent power could be obtained by reduction of pressure and temperature increase, separation by expansion, separation of solute and solvent by a mass separating agent (absorption, adsorption, membranes, adding a substance of low solvent power).

20.3.3 Design of Extraction Plant

For successful engineering and/or design of a SC extraction process following parameters should be defined:

- Specific basic data
- Thermodynamic conditions for operation of extraction and separation process
- Mass transfer data for the system
- Energy consumption by means of TS diagram

For the design of industrial plant following data are essential:

- Raw material specification
- Final product specification
- Desired plant size
- Plant location sometimes with prevailing local conditions

Raw material specification influences the quality of obtained extracts and overall economy of process. When raw material is contaminated either with degradation products or with plant protection materials usually the specification of extract on the purity could not be achieved. On the other hand when raw material contains a low concentration of substances to be extracted, the economy of the process is questionable.

The final product specification is usually based on customer needs, expectations, and specifications and extremely influences the costs of extraction process.

The process conditions in extraction step should be determined, so that maximal yield and high selectivity for desired substance at minimal separation costs should be obtained. On the other hand several times multi-step separation is applied for fractionation of extracts.

From the literature (Gamse and Marr 2001) it is known that extractor volume influences the investment costs for extraction unit (investment costs are a logarithmic formation of extractor volume).

Plant location determines the mechanical construction of extraction plant (location in GMP area or on the other hand in earthquake areas). Climate conditions also influences the design of heating and cooling devices and electrical drives.

20.3.4 Application on Extraction of Solids Using SCF

Application of extraction of solids using SCFs is numerous. In the literature several overviews could be found (Lack et al. 2000; Brunner 1994; Stahl et al. 1987; Temelli 2009; Catchpole et al. 2009; King and Srinivas 2009; Temelli et al. 2007; Fang et al. 2007; Eltringham et al. 2007; Mendes 2007; Li 2007; Meireles 2007; Diaz-Reinoso et al. 2007; Reverchon and De Marco 2007; Mukhopadhyay 2007; Gardner 1993; Lack and Seidlitz 1993; Moyler 1993). An overview of literature data related to food products is given in Table 20.2.

On internet pages of equipment producers (Uhde HPT, Natex, Sitec, Nova Swiss) references to their plants are given.

From this data it could be seen that the highest capacities are installed for coffee and tea decaffeination. The second largest application is extraction of hop.

Extraction of spices for production of oleoresins and extraction of bioactive compounds from plants are very widely used applications of SC fluids for extraction of solids.

One of the latest applications is extraction of oil from degumming residue to obtain highly concentrated and very pure lecithin plant designed, manufactured, and erected by Uhde HPT.

In the future further limitations on use of organic solvents and new application demands will be governing force for processing in sustainable manner.

Table 20.2 Mostly research applications of SFE for the extraction of different compounds from plants

Plant material (<i>Biological name</i>)	Compounds	Method/solvent	Year	Ref.
Abajeru (<i>Chrysobalanus icaco</i>)	Hentriacontane, nonacosane	SE; SFE/ethanol; CO ₂	2010	Vargas et al.
Agarwood (<i>Aquilaria crassna</i>)	Triglycerides	SFE/CO ₂	2010	Chen et al.
Annual herbs (<i>Nepeta persica</i>)	Nepetalactone, farnesene	SFE/CO ₂ + methanol	2010	Khajeh et al.
Berries	Catechin	SFE/CO ₂	2010	Laroze et al.
Black cumin (<i>Nigella sativa</i> L.)	4-Terpineol, carvacrol, P-Cymene, thymoquinone, α -thujene	SFE/CO ₂	2010	Venkatachallam et al.
Blueberry (<i>Vaccinium corymbosum</i>)	Anthocyanins	SFE/CO ₂	2010	Laroze et al.
Brazilian propolis	Artepillin C, coumaric acid	SFE/CO ₂	2010	Paviani et al.
Cranberry (<i>Vaccinium oxycoccos</i>)	Anthocyanins	SFE/CO ₂	2010	Laroze et al.
Canola (<i>Brassica napus</i> L.)	Tannin, linoleic acid linolenic acid, oleic acid	SFE; SE/CO ₂	2010	Li et al.
Cantaloupe (<i>Cucumis melo</i>)	Linoleic acid, oleic acid, palmitic acid, stearic acid	SFE/CO ₂	2010	Ismail et al.
Chia (<i>Salvia hispanica</i>)	α -linolenic acid, γ -linolenic acid	SFE/CO ₂	2010	Ixtaina et al.
Cinnamon (<i>Cinnamomum verum</i>)	Cinnamonaldehyde	SFE/CO ₂	2010	Baseri et al.
Gardenia (<i>Gardenia jasminoides</i>)	Linoleic acid, palmitic acid, tocopherols	SFE/CO ₂	2010	He et al.
Grape	Catechin	SE; SFE/ethanol + water; CO ₂ + ethanol	2010	Xia et al.
Grape	Genistein	SFE/CO ₂ + ethanol, + methanol	2010	Floris et al. Ghafoor et al.
Guava (<i>Psidium guajava</i>)	Lycopene	SFE/CO ₂	2010	Kong et al.
Macela (<i>Achyrocline satureioides</i>)	Camellianin A, cinnamic acid	SFE/CO ₂ , CO ₂ + ethanol	2010	Takeuchi et al.
<i>Marchantia convoluta</i>	Apigenin	SFE/CO ₂ + ethanol	2010	Xia et al.

(continued)

Table 20.2 (continued)

Plant material (Biological name)	Compounds	Method/solvent	Year	Ref.
Marigold (<i>Calendula officinalis</i>)	β -cadinene, cadinol	SFE/CO ₂	2010	Petrović et al.
Marigold (<i>Tagetes erecta</i>)	Lutein	SFE/CO ₂	2010	Fernandez et al.
Microalgae (<i>Paracoccuszeaxanthinifaciens</i>)	Zeaxanthin	SFE/CO ₂ + ethanol	2010	Sajilata et al.
Oregano (<i>Origanum vulgare</i>)	Carvacrol, caryophyllene, limonene, ocimene, thymol, α -pinnene	SFE/CO ₂	2010	Ocana et al.
Peach kernel (<i>Prunus persica</i>)	Linoleic acid, oleic acid	SE; HD; SFE/ethanol, water, DCM; CO ₂ , +ethanol	2010	Mezzomo et al.
Pigeon pea (<i>Cajanus cajan</i> L.)	Orientin, pinostrobin, vitexin stilbenes;	SFE/CO ₂	2010	Zu et al.
Pumpkin (<i>Curcubita moschata</i>)	Lutein, lycopene	SE, SFE/CO ₂	2010	Shi et al.
Raspberry (<i>Rubus idaeus</i>)	Anthocyanins	SFE/CO ₂	2010	Laroze et al.
Rice (<i>Oryza sativa</i>)	Coumarins, gallic acid, Fatty acid, γ -linolenic acid	SFE/CO ₂ + ethanol	2010	Mansroi et al.
Rosemary (<i>Rosmarinus officinalis</i> L.)	Verbenone, camosic acid, carnosol	SFE/CO ₂ + ethanol PLE/CO ₂ ; H ₂ O, ethanol	2010	Rubiolo et al., Soler et al.
Tomato (<i>Lycopersicum esculentum</i> L.)	Lycopene, β -carotene	SFE/CO ₂	2010	Egydio et al.
Sesame (<i>Sesamum indicum</i>)	N.D.	SFE/propane, CO ₂	2010	Corso et al.
Sikkim microula (<i>Microalastikkimenis</i>)	Fatty acid (unsaturated)	SFE/CO ₂	2010	Yang et al.
Soybean (<i>Glycine max</i>)	Linoleic acid, linolenic acid, oleic acid, phytosterol esters, tocopherols, vitamin E	SFE/CO ₂	2010	Jokić et al.
<i>Srobilanthes crispus</i>	Apigenin, catechin	SFE/CO ₂ + ethanol	2010	Liza et al.
Sweet bay (<i>Laurus nobilis</i> L.)	1,8-Cineole, linalool ocimene, sabinene α -pinnene	SFE/CO ₂	2010	Ivanović et al.
Yellowhorn (<i>Xanthoceras sorbifolia</i>)	N.D.	SFE/CO ₂	2010	Zang et al.
Argyle apple	1,8-Cineole	HD; SFE/CO ₂	2011	Mann et al.

Barbados nut (<i>Jatropha curcas</i>)	Corilagin, gallic acid, ellagic acid	SFE/CO ₂ + methanol	2011	Manpong et al.
Borage (<i>Borago officinalis</i>)	Camphor, α -pinene, linoleic acid, palmitic acid, γ -linolenic acid	HD; SFE/CO ₂ + methanol	2011	Ramandi et al.
Brazilian cherry (<i>Hymenaea</i>)	Lycopene, β -carotene	SFE/CO ₂	2011	Malaman et al.
Broccoli (<i>Brassica oleracea</i>)	Linoleic acid, linolenic acid, palmitic acid	SFE/CO ₂	2011	Araniz et al.
Canola (<i>Brassica napus</i>)	Linoleic acid, linolenic acid, oleic acid	SFE/CO ₂ ; propane	2011	Pederssetti et al.
Chamomile (<i>Chamomilla recutita</i>)	Apigenin	SFE/CO ₂ + ethanol	2011	Rahimi et al.
Chia (<i>Salvia hispanica</i>)	α -linolenic, γ -linolenic acid	SFE/CO ₂	2011	Uribe et al.
Cinnamon (<i>Cinnamomum verum</i>)	Cinnamonaldehyde	HD; SFE/CO ₂	2011	Baseri et al.
Clove (<i>Syzygium aromaticum</i>)	Eugenyl acetate	SFE/CO ₂	2011	Ivanović et al.
Guava (<i>Psidium guajava</i>)	N.D.	SFE/CO ₂ + ethanol	2011	Castro-Vargas et al.
Indian dill (<i>Anethum sowa</i>)	Carvone, dihydrocarvone, dillapiol, limonene	HD; SFE/CO ₂	2011	Nautiyal et al.
Kenaf (<i>Hibiscus cannabinus</i>)	Linoleic acid, oleic acid, tocopherols, β -sitosterol	SFE/CO ₂	2011	Mariod et al.
Khoa (<i>Satureja boliviana</i>)	Caryophyllene, P-Cymene, thymol	SFE/CO ₂ + ethanol	2011	Hatami et al.
Lotus (<i>Nelumbo nucifera Gaertn</i>)	N.D., linolenic acid, squalene, β -sitosterol	SFE/CO ₂	2011	Xu et al.
Madeira mahogany (<i>Persea indica</i>)	Caryophyllene, δ -cadinene	SFE/CO ₂ + ethanol	2011	Martin et al.
Marigold (<i>Tagetes minuta</i>)	E-ocimenone, lutein	SFE/CO ₂	2011	Ganan et al., Palumpitag et al.
Microalgae (<i>Nannochloropsis oculata</i>)	Zeaxanthin	SFE/CO ₂	2011	Cho et al., Liao et al.
Microalgae (<i>Schizochytrium limacinum</i>)	PUFA	SFE/CO ₂ + ethanol	2011	Tang et al.
Palm oil (<i>Elaeis guineensis</i>)	β -carotene	SFE/R134a	2011	Mustapa et al.
Olive (<i>Olea europaea</i>)	Apigenin, tocopherols, rutin, linolenic acid catechin	SFE/CO ₂	2011	Jimenez et al., Lafka et al.

(continued)

Table 20.2 (continued)

Plant material (Biological name)	Compounds	Method/solvent	Year	Ref.
Olive oil residues (<i>Olea europaea</i>)	Cinnamic acid, coumaric acid;	SFE; SE/CO ₂	2011	Lafka et al.
Oregano (<i>Origanum vulgare</i>)	Carvacrol Caryophyllene Limonene Ocimene Thymol α -pinene	SFE/CO ₂	2011	Ivanović et al.
Peppermint (<i>Mentha piperita</i>)	Isomenthyl acetate, menthol	SFE/CO ₂	2011	Ganan et al.
Peruvian pepper (<i>Schinus molle</i>)	Bicyclogermacrene, caryophyllene, limonene	SFE/CO ₂	2011	Barroso et al.
Sage (<i>Salvia officinalis</i> L.)	1,8-Cineole, camphor; myrcene, ocimene, valerenic acid, α -pinene, α -thujone	HD; SFE/CO ₂	2011	Ganan et al., Mann et al.
Southern cone marigold (<i>Tagetes minuta</i>)	E- β -ocimene	SFE/CO ₂	2011	Ganan et al.
Spearmint (<i>Mentha spicata</i> L.)	Apigenin, catechin, myricetin, naringenin, rutin	SFE/CO ₂	2011	Bimakr et al.
Summer savory (<i>Satureja hortensis</i>)	Carvacrol, thymol, γ -terpinene	HD, SFE/CO ₂ + methanol	2011	Khajeh et al.
Sunflower (<i>Helianthus annuus</i>)	Linoleic acid, oleic acid, palmitic acid, α -tocopherol	SFE/CO ₂ ; propane	2011	Nimet et al.
Viper's bugloss (<i>Echium amoenum</i>)	Linoleic acid, PUFA, γ -linolenic acid	SFE/CO ₂	2011	Ghoreishi et al.
Wormwood (<i>Artemisia absinthium</i> L.)	Epoxyocimene	SFE/CO ₂ + ethanol	2011	Martin et al.
<i>Agaricus brasiliensis</i>	Linoleic acid, palmitic acid	SFE/CO ₂ + ethanol	2012	Mazzutti et al.
Bitter melon (<i>Momordica charantia</i>)	N.D.	SFE/CO ₂ + ethanol	2012	Shan et al.
Canola (<i>Brassica napus</i>)	Erucic acid, eicosenoic acid, linoleic acid, linolenic acid oleic acid	SFE/CO ₂	2012	Yu et al.
<i>Diplotaeniacaerydifolia</i>	Limonene, dillapiol, α -calacorene	SFE/CO ₂ + ethanol	2012	Khajeh, Khajeh et al.
Grasses (<i>Capillipedium parviflorum</i>)	4-Nonanone, 4-Nonanol, 4-Undecanone, 4-Undecanol	HD; SFE/CO ₂	2012	Saini et al.
Guava (<i>Psidium guajava</i>)	α -selinene, β -caryophyllene, β -selinene	SFE/CO ₂ + ethanol, + isopropanol	2012	Moura et al.

Honeyherb (<i>Lippia dulcis</i>)	Caryophyllene, vopaene hernandulcin, δ -cadinene	HD; SFE/CO ₂	2012	De Olivera et al.
Jelly fig (<i>Ficus avicatsang M.</i>)	Triglycerides, α -linolenic	SFE/CO ₂	2012	Cheng et al.
Lavender (<i>Lavandula angustifolia</i>)	Linalyl acetate, fenchon linalool, camphor	SFE/CO ₂	2012	Danh et al, Ghoreishi et al.
Lavender (<i>Lavandula viridis</i>)	Camphor, myrtenol, verbenone	HD; SFE/CO ₂	2012	Costa (b) et al.
Macela (<i>Achyrocline satureioides</i>)	Galic acid, quercetin	SFE/CO ₂ + ethanol	2012	Hatami et al.
Microalgae (<i>Haematococcus pluvialis</i>)	Astaxanthin	SFE/CO ₂ + ethanol	2012	Pan et al., Wang et al.
Microalgae (<i>Chlorella vulgaris</i>)	Lutein	SFE/CO ₂	2012	Ruenngam et al.
Oregano (<i>Origanum vulgare</i>)	Caryophyllene, thymol γ -terpinene	SubWE/H ₂ O	2012	Missopolinou et al.
Pumpkin (<i>Cucurbita pepo</i>)	Phytosterol	SFE/CO ₂	2012	Habovski et al.
Purple amaranthus (<i>Amaranthus paniculatus</i>)	Squalene	SFE/CO ₂	2012	Bhattacharjee et al.
Red pepper (<i>Capsicum annuum</i> L.)	β -carotene	SFE/CO ₂	2012	Romo-Hualde et al.
Seabuckthorn (<i>Hippophae rhamnoides</i> L.)	Carotenes	SFE/CO ₂	2012	Kagliwal et al.
Spearmint (<i>Mentha spicata</i> L.)	Apigenin, myricetin catechin naringenin, rutin; Carvone, β -caryophyllene	SFE/CO ₂ ; SE; SFE/hexane, DCM, ethanol; CO ₂	2012	Bimkr et al., Almeida et al
Tasmanian blue gum (<i>Eucalyptus globulus</i>)	Triterpenoid	SFE/CO ₂ + ethanol	2012	Domingues et al
Tomato (<i>Lycopersicon esculentum</i> L.)	Lycopene	SFE/CO ₂	2012	Machmudah et al.
<i>Thymus lotocephalus</i> G. Lopez & R. Morales	Camphor, linalool	SFE/CO ₂	2012	Costa (a) et al.

20.3.5 *Extraction of Liquids*

There are less industrial units for separation of components from liquid mixtures using SCFs. Literature search shows some laboratory scale studies on extractions in the systems liquid/SCF. Several data on binary systems liquid/SCF could be found, but there are less data on systems liquid/liquid/SCF (Gupta and Shim 2007).

(Retyped first sentence) Extraction of liquid mixtures with SCFs is comparable to liquid–liquid extraction, where compressed gas is used instead of an organic solvent. In liquid–SCF extraction processes the pressure plays an important role. Changing pressure and/or temperature, the physicochemical properties of the SCF, like density, viscosity, surface tension, and dielectric constant, are changed. Selective extraction of components or fractionation of total extracts is possible by use of different gases for isolation/fractionation of components and/or changing process parameters. Another advantage is that depending on the feed material the density difference between the two countercurrent flowing phases can be adjusted.

One of the most important advantages of use of SCFs is simple solvent regeneration in comparison with liquid–liquid extraction solvent regeneration includes, where in the most cases, a re-extraction or distillation step, which is energy consuming and therefore cost intensive is necessary. Heat treatment of extract or raffinate phase may causes degradation of heat sensitive substances. For extraction plant where SCFs are used the solvent regeneration is achieved by changing pressure and/or temperature after the extraction step, thus changing the density and by it the solvent power of gas, which can be later easily recycled after separation of solute.

Compared to extraction of solids with SCF, liquids could be continuously introduced in and withdrawn from the high pressure extraction unit. This gives the benefit of higher throughputs in continuous operating counter current processes.

Literature search shows some laboratory scale studies on extractions in the systems liquid/SCF.

Applications of liquid/sub- or supercritical fluid extraction are numerous and were used for separation of ethanol from water (Knez et al. 1994; Hsu and Tan 1994), separation of aromas from different alcoholic beverages (Gamse et al. 1999), separation of components from citrus oils (Knez 1989), for purification of tocopherols (Fleck et al. 2000). Separation of caffeine from CO₂ with water is used widely in decaffeination process.

20.4 **Chemical and Biochemical Reactions in/and with Supercritical Fluids**

Reactions under supercritical conditions are applied on large-scale production in the twentieth century, but the use of supercritical solvents for synthesis of complex organic molecules is under development. SCFs may be an alternative to conventional

solvents, reactant, catalyst and/or an aid to separation in food processing (Primozic et al. 2003). There are several advantages using the SCFs as solvents in chemical synthesis, where all are based on unique thermophysical properties of SCFs or their mixtures with reactants.

The application of SCFs enables also design of integrated reaction and separation processes.

The miscibility of SCFs with other gases is high which can lead to high rates of reaction if the kinetics is first order or higher in the concentration of dissolved gas. In mass transfer limited processes the reaction rate can be increased if SCFs are applied due to higher diffusivity and lower viscosity.

Other chemical benefit can be related to selectivity changes.

An overview for esterification and transesterification of fatty acids is given by Knez (Knez and Habulin 2002; Knez 2009).

20.5 Particle Formation

Particle formation and design of solid particles and powdery composites with unique properties is at the moment one of major development of SCFs applications (Knez et al. 2014).

Conventional well-known processes for particle-size redistribution of solid materials are crushing and grinding (which for some compounds are carried out at cryogenic temperatures), air micronization, sublimation, and recrystallization from solution. There are several problems associated with the abovementioned processes. Some substances are unstable under conventional milling conditions, in recrystallization processes the product is contaminated with solvent, and waste solvent streams are produced. Applying SCFs may overcome the drawbacks of conventional processes, and powders and composites with special characteristics can be produced. Several processes for formation and design of solid particles using dense gases are studied intensively.

The unique thermodynamic and fluid-dynamic properties of SCFs can be used also for impregnation of solid particles, for formation of solid powderous emulsions, particle coating, e.g., for formation of solids with unique properties for the use in different applications.

Applying SCFs may overcome the drawbacks of conventional processes, and powders and composites with special characteristics can be produced. Several processes for formation and design of solid particles using dense gases are studied intensively (Jung and Perrut 2001; Knez and Weidner 2001; Knez and Weidner 2003)

20.5.1 *Rapid Expansion of Supercritical Solutions*

Crystallization from supercritical solutions (CSS) is a special process where the fine particles are formed from the substances, which are soluble in supercritical solvents.

Table 20.3 Application of rapid expansion of supercritical solutions (REES) in food

Substance	Solvent	Year	Ref.
Lipase (from <i>Pseudomonas cepacia</i>) and lysozyme (from chicken egg white)	CO ₂ + low molecular weight alcohols	2001	Matsuyama et al.
Cholesterol	CO ₂	2004	Subra et al.
Hydrogenated palm oil (HPO)	CO ₂	2005	Li et al.
Caffeine	CO ₂	2006	Ksibi et al.
Poly(L-lactic acid) and poly(methyl methacrylate)	CO ₂ into neat water and an aqueous NaCl solution	2006	Meziani et al.
Benzoic acid, cholesterol, and aspirin	CO ₂ + methanol, ethanol, and isopropanol	2007	Harrison et al.
Green tea	CO ₂ + ethyl alcohol	2008	Sheu et al.
Chitin	CO ₂	2009	Salinas-Hernandez et al.
Quercetin and astaxanthin	CO ₂	2009	Quan et al.
Benzoic acid	CO ₂	2010	Carneiro et al.
Magnolia Bark Extract	CO ₂	2010	He et al.
Creatine monohydrate	CO ₂	2010	Hezave et al.
Cholesterol	CO ₂	2011	Satvati and Lotfollahi
Caffeine	CO ₂	2012	Sarfraz et al.

When the solute-laden solution is a SCF, supersaturation may be induced not only by varying the temperature but also by pressure variation. Thus pressure and pressure-gradients would be additional means for generation of particles with the desired size, form, and morphology (Tavana and Randolph 1989).

Rapid expansion of supercritical solutions (REES) is another way of performing the crystallization from SCF, where small-size particles can be produced. In this process, a solid is dissolved in a pressurized SCF and the solution is rapidly expanded to some lower pressure level, which causes the solid to precipitate (Table 20.3). This concept has been demonstrated for a wide variety of materials including polymers, dyes, pharmaceuticals, and inorganic substances (Debenedetti et al. 1993; Tom et al. 1994).

As special modification of RESS process in crystallization from SCF. An overview is given in Table 20.4

20.5.2 Gas Anti-solvent Processes (GASR, GASP, SAS, PCA, SEDS)

The application of SCFs as anti-solvents is an alternative recrystallization technique for processing solids that are insoluble in SCF. This method exploits the ability of gases to dissolve in organic liquids and to lower the “solvent power” of the liquid for the compounds in solution, thus causing the solids to precipitate.

Gas anti-solvent processes (GASR, gas anti-solvent recrystallization; GASP, gas anti-solvent precipitation; SAS, supercritical anti-solvent fractionation; PCA, precipitation with a compressed fluid anti-solvent; SEDS, solution-enhanced dispersion

Table 20.4 Application of crystallization from supercritical solutions (CSS) in food

Substance	Solvent	Year	Ref.
Beta-carotene	Ethyl acetate and dichloromethane/CO ₂	2002	Cocero and Ferrero
Poly(ethylene glycol) MW = 1000 diacrylate and poly(ethylene glycol) MW = 200 diacrylate	Methylene chloride	2003	Owens et al.
Ibuprofen, sodium benzoate	Water	2003	Kazarian and Chan
Hydrogenated palm oil (HPO)	CO ₂	2004	Li et al.
<i>p</i> -Acetamido phenol + ethanol + CO ₂ and cholesterol + diethyl ether + CO ₂	Ethanol, diethyl ether /CO ₂	2005	Wubbolts et al.
Hydrogenated palm oil (HPO)	CO ₂	2005	Li, J et al.
Lycopene	Dichloromethane/CO ₂	2006	Miguel et al.
Carotene	Dichloromethane/CO ₂	2006	He et al.
Alpha-glycine, phenylalanine monohydrate and lysozyme	Methanol, ethanol, 2-propanol and acetone/CO ₂	2007	Bouchard et al.
Lutein and poly-lactic acid	Ethyl acetate solutions/CO ₂	2008	Miguel et al.
Adipic acid	Urea solution/CO ₂	2008	Caputo et al.
Caffeine	Chloroform and methylene/CO ₂	2008	Park and Yeo
All-trans-beta-carotene	Tetrahydrofuran + CO ₂	2009	Cardoso et al.
Egg yolk phospholipids	Ethanol solution + CO ₂	2009	Aro et al.
Cholesterol	CO ₂ + acetone, ethanol	2009	Dalvi and Mukhopadhyay
Glyceryl monostearate (Lumulse (R) GMS-K). a waxy triglyceride (Cutina (R) HR), silanized TiO ₂ and caffeine	CO ₂	2010	Garcia-Gonzalez et al.
Stearic acid	CO ₂ -expanded ethyl acetate	2010	Sala et al.
Vitamin B ₆	Ethanol solution in CO ₂	2011	Kikic et al.
Emodin-polyethylene glycol (PEG)	Dichloromethane-methanol	2012	Lang et al.
Glycine phases	Aqueous solutions, N ₂	2012	Surovtsev et al.

by SCFs) differ in the way the contact between solution and anti-solvent is achieved (Jung and Perrut 2001; Knez and Weidner 2001; Knez and Weidner 2003) (Table 20.5).

20.5.3 Particles from Gas-Saturated Solutions (PGSS™)

This process allows to form particles from substances that are non-soluble in SCF, but absorb a large amount of gas that either swell the substance or decrease the melting (for polymers glass transition temperature). This process can also be used for micronization of liquids, suspensions, and emulsions.

Table 20.5 Application of gas anti-solvent processes in food

Substance	Solvent	Year	Ref.
Nicotinic acid	Methanol, CO ₂ antisolvent	2001	Rehman et al.
Ginkgo ginkgolides	Ethanol, CO ₂ antisolvent	2005	Chen et al.
Caffeine	Chloroform, methylene chloride, CO ₂ antisolvent	2008	Park and Yeo
Cholesterol	Acetone, ethanol, CO ₂ antisolvent	2009	Dalvi and Mukhopadhyay
Rosemary extract	Aqueous medium, CO ₂ antisolvent	2012	Yesil-Celiktas et al.

In PGSSTM process the substance or the mixture of substances to be powderized must be converted into a sprayable form by liquefaction/dissolution. This can be achieved by melting or/and dissolving of the substance or mixture of substances in a liquid solvent, or by dispersing solids or liquids in a melt or solution, and saturation of the melt/solution/dispersion with the gas (Weidner et al. 1995). Then the gas-containing solution is rapidly expanded in an expansion unit and the gas is evaporated. Due to the Joule-Thomson effect and/or the evaporation and the volume-expansion of the gas, the solution cools down below the solidification temperature of the solute, and fine particles are formed. The solute is separated and fractionated from the gas stream by a cyclone and electro-filter.

When the liquefaction is achieved by melting, the knowledge of the P-T trace of the S-L-V equilibrium gives information on the pressure needed to melt the substance to be micronized and form a liquid phase at a given temperature, and to calculate its composition (Fukne-Kokot et al. 2000; Fukne-Kokot et al. 2003; Skerget et al. 2002).

The PGSSTM process was tested in the pilot- and technical size on various classes of substances. Up to the present time the application of the PGSSTM process has been investigated for polymers, waxes and resins, natural products, fats and fat derivatives, pharmaceuticals, synthetic and natural antioxidants, surface-active compounds, UV-stabilizers, composites of the mentioned substances, etc. (Jung and Perrut 2001; Knez and Weidner 2001; Weidner et al. 1995). And what about food application?

The highly compressible fluids which have been used were carbon dioxide, propane, butane, dimethyl ether, freons, nitrogen, alcohols, esters, ethers, ketones and mixtures of abovementioned gases and solvents.

The powders produced show narrow particle-size distributions, and have improved properties compared to the conventional produced powders.

The material structure of the substance to be micronized (crystalline-amorphous, pure or composite), the process parameters (pre-expansion pressure, temperature, gas to substance ratio (GSR), viscosity of melt/solution/dispersion) of the PGSSTM process and geometry of the process equipment influences particle size, particles size distribution, bulk density, the morphology (particle shape) and ratios crystalline/amorphous of the processed substances.

The removal of solvent is a problem of conventional coprecipitation or co-evaporation techniques where large amounts of organic solvents are needed and in which complete removal is often a long and difficult process. Applications of PGSS process are presented in Table 20.6.

Table 20.6 Application of particles from rapid gas-saturated solutions (PGSS™)

Substance	Solvent	Year	Reference
Theophylline prepared with hydrogenated palm oil (HPO)	CO ₂	2004	Li et al.
Hydrogenated palm oil (HPO)	CO ₂	2004	Rodrigues et al.
Hydrogenated palm oil (HPO)	CO ₂	2005	Li et al.
Rapeseed 70 (RP70)	CO ₂	2007	Munuklu and Jansens
Edible fat, rapeseed 70 (RP70)	CO ₂	2007	Munuklu and Jansens
Poly(ethylene glycol) (PEG) of different molecular weights	CO ₂	2007	Nalawade et al.
Green tea antioxidants	CO ₂	2007	Meterc et al.
Cocoa butter	CO ₂	2008	Perva-Uzunalic et al.
Green tea (caffeine, catechins), chamomile (wax, alpha-bisabolol, chamazulene, and matricine), borage and evening primrose (free fatty acids) and grape mare and elder berry (phenolic compounds and anthocyanins)	CO ₂	2008	Perva-Uzunalic et al., improvement
Green tea antioxidants	CO ₂	2008	Meterc et al.
Mixtures of ceramide 3A and cholesterol	CO ₂	2008	Vezzu et al.
Triterpene saponin, protopanaxadiol saponin (Rb group) and protopanaxatriol saponin (Rg group)	CO ₂	2009	Kim et al.
Lipid/PEG particles	CO ₂	2010	Vezzu et al.
Lavandin essential oil in liposomes, soy lecithin particles	Water	2011	Varona et al.
Microparticles from anhydrous milk fat (AMP) and a diacylglycerol-based modified milk fat (D-AMF)	CO ₂	2011	Lubary et al.
Fucoxanthin and astaxanthin	CO ₂	2011	Kwon et al.
Coenzyme Q ₁₀ (CoQ ₁₀)	Water	2011	Hu et al.
Beta-carotene	CO ₂	2012	de Paz et al.
Hydrogenated Castor oil	Water	2012	Hanu et al.
Chocolate	CO ₂	2006	Weidner et al
Castor fat	CO ₂	2007	Wendt et al
Spherical PEG particles	Aqueous solution and CO ₂	2010	Martin et al
Spherical PEG particles	CO ₂	2010	Martin and Weidner
Edible fat powders	CO ₂	2011	Flöter et al
β-carotene + soybean lecithin	CO ₂	2012	De Paz et al
Poly(D,L-lactic acid) (PDLLA) and poly(ethylene glycol) (PEG)	CO ₂	2012	Kelly et al

Through the choice of the appropriate combination of solvent and operating conditions for a particular compound, PGSSTM can eliminate some of the disadvantages of traditional methods of particle-size redistribution in material processing. Solids formation by PGSSTM therefore shows potential for the production of crystalline and amorphous powders with a narrow and controllable size-distribution, thin films, and mixtures of amorphous materials. Due to the low processing costs PGSSTM can be used not only for highly valuable but also for commodity products.

20.6 Conclusions

The chapter contains a limited overview of SCF based technologies which offer important advantages over organic solvent technology, such as ecological friendliness and ease of product fractionation.

Extraction of hop components, decaffeination of tea and coffee are the largest scale extraction processes using sub- or supercritical solvents, which are realized on industrial scale. Several industrial plants are in operation also for extraction of spices for food industry and natural substances for use in cosmetics. There are less industrial units for separation of components from liquid mixtures using sub- or supercritical fluids.

The main advantages of using SCFs for isolation of natural products are solvent free products, no coproducts, low temperature in separation process. In addition the processes can easily be linked with direct micronization and crystallization from SC CO₂ by fluid expansion.

But the most important advantage of the use of SCFs is selective extraction of components or fractionation of total extracts. This is possible by the use of different gases for isolation/fractionation of components and/or by changing the process parameters. Beside the mostly used gas for sub- or supercritical extraction, i.e., carbon dioxide also other sub- or supercritical solvents are used. Sub- and supercritical CO₂ and supercritical H₂O are non-carcinogenic, nontoxic, non-mutagenic, nonflammable, and thermodynamically stable. In addition, CO₂ does not usually oxidize substrates and products, allowing the process to be operated at low temperatures. Water is at the moment the cheapest solvent and several substances are highly soluble in water. Therefore, more and more research on the use of sub- or supercritical water for isolation and fractionation of substances is under investigation.

One of the major process benefits is derived from thermophysical properties of SCFs, high diffusivity, low viscosity, the density, dielectric constant of SCF, which can be fine-tuned by changes of operating pressure and/or temperature.

The limitation of further application of extracts obtained by high pressure technology is in the price of the product, which is in comparison with conventionally obtained products relatively high. The legal limitations of solvent residues and solvents (for products used in human applications) and isolation/fractionation of special components from total extracts in combination with different formulation (for example controlled release of aromas) (Weidner 2009), chromatography (Taylor 2009), and sterilization processes will increase the use of dense gases for extraction applications.

It is evident that the number of extraction units (Lütge et al. 2007) increase with time and the most “dense” areas are Europe and Asia where increased number of installed new SCF extraction units also in recent time could be observed.

There are several new topics on use of SCFs which are already developed to the commercial scale, like dry cleaning, high pressure sterilization, jet cutting, thin-film deposition for microelectronics, separations of value-added products from fermentation broths in biotechnology fields, and as the solvent in a broad range of synthesis including transition metal. All of these applications lead to sustainable manufacturing methods that are not only ecologically preferable, but in themselves are enabled by working with a unique solvent that has the density of common liquids but the transport properties of a gas.

In the future, further limitation on the use of organic solvents, new applications of several substances, new customer desired properties of products, sustainable production, and processing of substances will open new applications of high pressure processing. We can be sure that advances in the field of high pressure research in cheap and environmental friendly solvents, like CO₂ and some other gases and sub- or supercritical water will in the future open up new pathways for substances and products produced at high pressure.

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Chapter 21

Aqueous Fractionation of Dry-Milled Corn Germ for Food Protein Production

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21.1 Introduction

Ninety-seven percent of domestic ethanol production uses corn as the feedstock, and an estimated 43 % of United States' corn crop is converted to ethanol (USDA Economic Research Service 2013). Based on the Renewable Fuels Association estimates (2013), ethanol demand averaged 893,000 barrels per day in 2012, for a total of 13.3 billion gallons, which reduced the need of imported oil by 462 million barrels. New demand from higher level ethanol blends like E10, E15, E20, and E85 indicates that starch-based ethanol use may exceed the 2013 estimated production capacity of 13.9 billion gallons (US Energy Information Administration 2013).

Nearly 80 % of the corn ethanol plants in operation use a dry-grind process (Erickson and Carr 2009). In the dry-grind process, the entire kernel is ground into coarse flour prior to saccharification and fermentation. During fermentation, the starch and sugar components are converted to ethanol and the residuals (protein, fiber, oil, ash, and minerals) are marketed as distillers dried grain with solubles (DDGS) or wet distillers' grains (Kim et al. 2008). DDGS is used for cattle, poultry, swine, aquaculture, and pet foods (Davis 2001). However, the use and value of DDGS is limited by the presence of various undesirable components such as fiber, phosphorus, and unsaturated fats. Coproducts of the traditional ethanol fermentation from dry grind corn are typically used for lower value applications compared to wet milling coproducts (Murthy et al. 2009). As the production of ethanol continues

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to increase, the value of DDGS is projected to decrease and market saturation is expected to occur for the current applications of DDGS (Wyant 2006). Therefore, various other options are being explored to improve the long-term outlook and economic stability of corn processing plants.

Since the overall process economics for a corn-to-ethanol plant depends on composition, quality, and yield of each coproduct, various wet- and dry-milling (fractionation) alternatives have been proposed to make corn (endosperm starch) a more cost-competitive feedstock for biofuel production. One of the currently explored options is the removal of non-fermentable seed fractions (germ and fiber) to increase fermentation efficiency (Ponnampalam et al. 2004; Wahjudi et al. 2000) and the development of higher value coproducts from fiber and germ. Fiber oil and gums are potential coproducts that would require further attention and development. For example, recent studies suggest fiber oil can be used for nutraceutical applications (Hicks 1998; Moreau et al. 1996) and/or biofuels (Dien et al. 2005) and fiber gum for food and industrial applications (Singh et al. 1999; Yadav et al. 2007; Rose et al. 2010).

Corn oil and germ meal are traditional coproducts from wet-milled germ with a market value dictated by the price of corn oil and to a lesser extent, corn germ meal (Johnston et al. 2005). Refined corn oil is typically sold for food applications at \$800–1000 per ton whereas germ meal (residues after oil extraction containing the germ protein) is sold for animal feed at \$90–100 per ton. There is an opportunity to further improve corn-to-ethanol economics by producing protein coproducts for food applications instead of allowing the protein to remain in the germ and be sold as animal feed at a relatively low cost. Based on the protein composition, amino acid distribution, and functional properties, corn germ is a good source of protein for products aimed at food applications. Although the majority of corn protein is contained in the endosperm, the low water solubility of endosperm proteins (zeins) makes them unsuitable for aqueous food applications. In addition, the protein concentration (g protein per g fraction) in the germ is higher than that in the endosperm (Inglett and Blessin 1979). The germ represents 11 % (dry basis) of the corn kernel (Watson 1994) and contains most of the water- and salt-soluble proteins. Corn germ protein is nutritionally superior to endosperm protein based on the amino acid distribution (Landry and Moureaux 1980) with a protein efficiency ratio similar to soybeans (Lusas et al. 1989).

Although numerous studies on the composition, functionality, and applications of corn germ flours are available, relatively little work has been devoted to producing protein-rich concentrates from the germ, which would have high protein dispersibility indexes (PDI) suitable for food applications. One reason for lack of activities in this area is that corn germ obtained by traditional wet- or dry-milling processing is not an adequate starting material for extracting functional germ protein. Wet-milling yields high purity germ, but process conditions (pH and reducing agents) denature most of soluble germ proteins (Parris et al. 2006). Modified wet milling processes (enzymatic milling, quick germ, quick germ/quick fiber, HydroMilling) that were developed to produce high oil and higher protein germ (Johnston et al. 2005; Johnston and Singh 2001, 2004, 2005; Lohrmann 2006; Puastian et al. 2007; Singh and Eckhoff 1996; Wahjudi et al. 2000) seem to have milder processing conditions, but protein extraction and/or quality of germ proteins (water solubility/dispersibility) have not been reported. Dry milling yields relatively

low purity germ (≤ 23 % oil and 15 % protein) with significant residual endosperm starch attached to the germ. Direct germ protein extraction of dry-milled germ would not be efficient and cost effective unless attached endosperm starch is removed prior to extraction.

Public objection to using corn for bioenergy rather than food is forcing many corn-to-ethanol plants to consider using dry-milling instead of the traditional dry grinding process. Such transition is expected to significantly expand the dry-milled germ supply and depress the price of germ meal. Thus, the challenge facing ethanol producers today is to extract additional value from dry-milled germ while maintaining a competitive processing cost for ethanol. One way to achieve this is to upgrade germ protein value by extracting functional protein suitable for food applications. To achieve this objective, as mentioned above, the purity of dry-milled germ has to be significantly improved.

The goal of this study was to evaluate the capability of a wet germ processing method, proposed by Lohrmann et al. (2015), to increase the purity of dry-milled corn germ. Specifically, the effect of germ fractionation conditions, such as water soaking pH, temperature, and time, on the production of high protein and high PDI germ was evaluated. The effect of the latter two (initial protein concentration and PDI value) on the germ protein extraction yield was also considered.

21.2 Materials and Methods

21.2.1 Materials

Dry-milled corn germ was provided by Quality Technology International, Inc. (Chicago, IL) from either SunRich Food (Cresco, IA) or Didion Milling (Cambria, WI). The germ was prepared from field-dried corn to maintain a high PDI.

21.2.2 Analytical Methods

21.2.2.1 Total Soluble Protein Quantification

Total soluble protein (TSP) was determined using the Bradford method (Bradford 1976) with bovine serum albumin as a standard. Samples and standards were run in duplicates on each microtiter plate. Assay reagents were purchased from Bio-Rad (Hercules, CA).

21.2.2.2 Protein Analysis by SDS-PAGE

The protein profiles of soak water samples were evaluated by electrophoresis as described by Laemmli (1970) and according to manufacturer's instructions (Life Technologies, Carlsbad, CA). Samples were loaded on 8–16 or 4–20 % tris-glycine

gels under non-reducing conditions. Mark 12 was used as the standards and proteins were visualized by Coomassie® G-250 SimplyBlue Safe Stain. All supplies were purchased from Life Technologies.

21.2.2.3 Phytic Acid Quantification

Phytic acid concentrations in soak water samples were determined since this compound affects protein solubility and is an antinutritional factor (Maga 1982). The phytic acid concentration in samples was determined using an indirect detection method, which is based on the strong iron chelating ability of phytic acid and measures the amount free iron in solution (Haug and Lantzsch 1983; Reichwald and Hatzack 2008). The method was adapted for use in a microplate assay format using sodium phytate (Sigma-Aldrich, St. Louis, MO) as a standard with concentrations ranging from 5 to 75 µg/mL. Ferric ammonium sulfate, bipyridine, and thioglycolic acid were purchased from Sigma-Aldrich.

21.2.2.4 Composition Analysis

Protein by combustion, moisture by forced draft oven, ash, starch, PDI, crude fiber, and amino acid composition was analyzed by Eurofins Scientific, Inc. (Des Moines, IA). Crude fat (by acid hydrolysis) was determined by Dairyland Laboratories, Inc. (Arcadia, WI).

21.2.3 Experimental Methods

21.2.3.1 Lab-Scale Germ Soaking: Soak Water Analysis

The amount of total protein and the protein profiles of soak water samples were determined over 10 h for 50 °C soak water and for 24 h for 25 °C soak water at pH 4.5 ($4.5 \leq \text{pH} \leq 5.0$) and pH 7.5 ($7.0 \leq \text{pH} \leq 7.5$). For each experiment, 100 g of dry-milled full-fat corn germ was added to 200 mL of RO water. The soak water pH was maintained throughout the experiment using 1 M HCl or 1 M NaOH. At each time point, a 1 mL soak water sample was taken, centrifuged, and the supernatant was removed and analyzed for TSP and by SDS-PAGE analysis. The volume of soak water remaining at each time point was also measured to calculate the amount of protein lost (g per 100 g germ).

21.2.3.2 Lab-Scale Germ Soaking: Germ Analysis

Lab-scale soaking experiments were conducted at the Bioseparations Lab at Texas A&M University. Dry-milled germ (SunRich Food, Cresco, IA) with 33 % starch, 15 % protein (PDI of 56 %), 16 % oil, and 3.8 % fiber (% dry basis) was used as the

starting material for all experiments. The effect of three different soak water pHs (pH 3, 6, 9), two temperatures (25, 60 °C), and four soaking times (0.5, 2, 4, and 8 h) on the composition of the soaked germ was determined. Another set of experiments was conducted at pH 6 and 60 °C with 0.01 % α -amylase to determine if the addition of this enzyme would reduce starch.

For each experiment, 200 g of the dry-milled germ was added to a 1 L Erlenmeyer flask and either 400 mL (25 °C soaking) or 425 mL (60 °C soaking) of tap water was added to the flask and placed in a water bath. The pH of the soak water was adjusted with either 1 M NaOH or 2 M HCl and once the desired pH was reached, the soak time started. The suspension was mixed periodically by shaking and the pH was maintained at the respective value throughout soaking. After soaking, the wet germ mass and soak water mass were individually measured. The germ was rinsed with tap water and then fresh water was added for grinding (sufficient water to cover the germ in the flask). The germ was mixed with an overhead mixer (Arrow 850, Arrow Engineering Co.) to knock off any loosened starch. After grinding, the germ was removed with a mesh strainer and placed in a starch solution (8 Bé) to separate the clean germ from the low purity germ. The clean germ (floaters) was recovered with a strainer, rinsed with water, and weighed. The clean germ was placed in an aluminum dish and dried at a temperature of 37–43 °C to avoid protein denaturation. The mass of the germ was measured throughout drying and once the mass was constant, the germ was removed and stored at 4 °C until analysis. The germ samples were analyzed for protein, PDI, starch, ash, and crude fat.

21.2.3.3 Pilot-Scale Germ Soaking: Soak Water Analysis

Pilot plant trials were conducted at the Food Protein Research & Development Center at Texas A&M University (College Station, TX). Dry-milled germ, prepared by Didion Milling (Cambria, WI), was used as the starting material and had 16.4 % protein (58.3 % PDI), 26.9 % starch, 22.3 % oil, and 4.9 % crude fiber (% dry basis).

For each pH 4.5 and 25 °C soaking experiment, 10 kg of germ was added to 33 L of water (with 0.01 % α -amylase) in a stainless steel tank equipped with a pump and circulating loop. The pH was adjusted to 4.5 with 1.5 M phosphoric acid and soak water circulation started immediately. For pH 7.5 and 60 °C (50 °C $\leq T \leq$ 60 °C) experiments, the same soaking procedure was used except the germ was added to 39 L of preheated water. The additional 6 L of water was required at the higher temperature to completely submerge the germ. Soak water samples were taken at 1, 2, 4, and 6 h for TSP, SDS-PAGE, and phytic acid analyses.

21.2.3.4 Pilot-Scale Germ Soaking: Germ Analysis

After soaking, the steep water and germ were transferred to a Bauer hammer mill. After grinding, the Baumé level of the steep water was checked and then a hydroclone (Dorr-Oliver) was used to separate the clean germ (low-density) from the

high-density fraction (lower oil purity germ and germ fractions). The high-density fraction was passed through the Bauer hammer mill until no additional clean germ was recovered. The clean germ and high-density fraction were rinsed, dried, and weighed. After drying, the clean germ was aspirated to remove fiber and reweighed.

21.2.3.5 Extraction of Defatted Corn Germ Flour

Germ protein extraction and precipitation methods were based on those proposed by Nielsen et al. (1973). Defatted corn germ (DCG) flour (30 g) was gradually added to 300 mL pre-heated water (temperature maintained at or below 50 °C) as pH was adjusted to 8.7 with 1 N NaOH. The mixture was blended with a Silverson L4RT high shear mixer at 4500 rpm until the temperature reached 50 °C. Once this temperature was reached, the extraction time of 30 min was started. The required temperature and pH were maintained throughout the extraction. The extract was then clarified by centrifugation (Beckman Coulter Allegra 25R) at 10,000×g and 20 °C for 15 min. In this study, only a single stage extraction was used because the main objective was to determine the effect of multiple processing steps and conditions on protein recovery.

21.2.3.6 Preparation of Corn Protein Concentrates by Precipitation

The protein was precipitated from the clarified extract by adjusting the pH to 4.7 with 1.5 M phosphoric acid or 1 M HCl and mixing slowly for 1 h. The protein precipitated was removed by centrifugation at 10,000×g and 20 °C for 15 min and then washed with 50 mL of water. After 15 min centrifugation (10,000×g at 20 °C), the wash water was decanted, and the precipitate washing procedure was repeated. To resolubilize the precipitate, RO water was added and the pH was adjusted to 7 with 1 M NaOH. The slurry was added to a glass baking dish, placed in a freezer overnight, and freeze dried (LabconcoFreeze Dry 5) for 72 h. The clarified extract, the precipitate supernatant, and the wash supernatants were analyzed for TSP and by SDS-PAGE.

21.3 Results and Discussion

Germ wet milling (Lohrmann et al. 2008) was used to produce high purity germ in this study. The germ wet milling method includes soaking dry-milled germ in water to enhance protein and oil content (% basis) by reducing starch content. The primary objective of germ soaking is to increase germ purity by releasing or leaching undesirable impurities such as phytic acid, starch, and salts while retaining both the quantity and quality of germ oil and protein. One initial concern was the potential for excessive protein loss during soaking on a pilot-scale since the feasibility of

soaking dry-milled germ had not been previously reported. For this reason, the effect of soak water temperature, time, and pH on protein content in the soak water was investigated. Leaching kinetics were determined to further optimize the soaking conditions, and the molecular weight distribution of leached protein was analyzed to evaluate the potential use of soak water for other applications including as a nitrogen source for ethanol fermentation.

21.3.1 Lab-Scale Germ Soaking: Soak Water Analysis

The amount of germ protein leached into the soak water at two temperatures (25, 50 °C) and pHs (4.5 and 7.5) was determined over a 10 h period. The amount of protein leached was the lowest in the pH 4.5 and 25 °C soak and highest in the pH 7.5 and 50 °C soak water (Fig. 21.1). For all soak conditions, the amount of protein leached over the first 2 h increased linearly with time and leveled off by 6 h of soaking. After 6 h, no additional protein was leached for all conditions except for pH 4.5 and 25 °C. At pH 4.5 and 25 °C (Fig. 21.2a), the soak water contained primarily low molecular weight proteins (6–14 kDa). Some higher molecular weight proteins can be seen in the 24 h sample, but they only represent a small fraction of the total protein present in the soak water. Increasing the soak temperature to 50 °C increased the amount of higher molecular weight proteins and total protein lost in the soak water

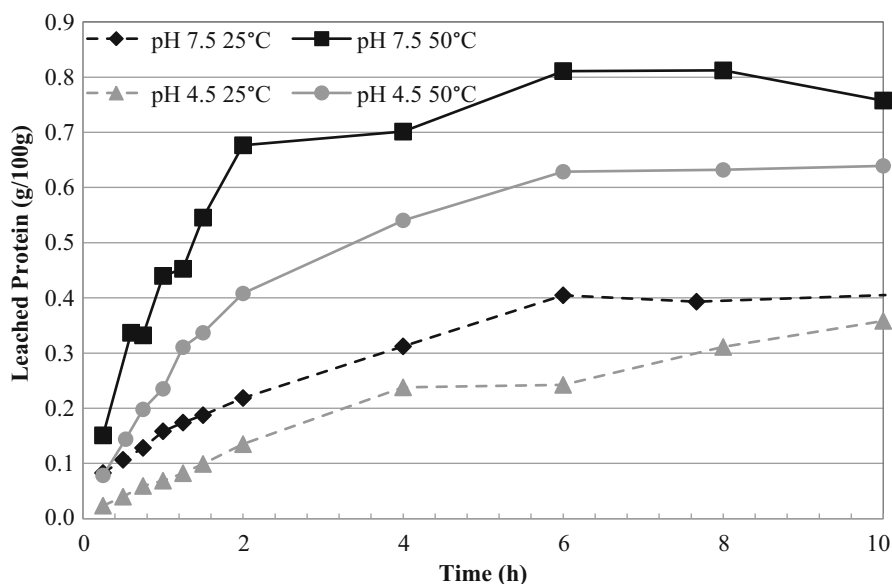


Fig. 21.1 Lab-scale soak water analysis: effect of temperature, time, and pH on total protein loss from dry-milled corn germ. Leached protein reported as g total soluble protein (TSP) lost in soak water per 100 g of dry-milled germ

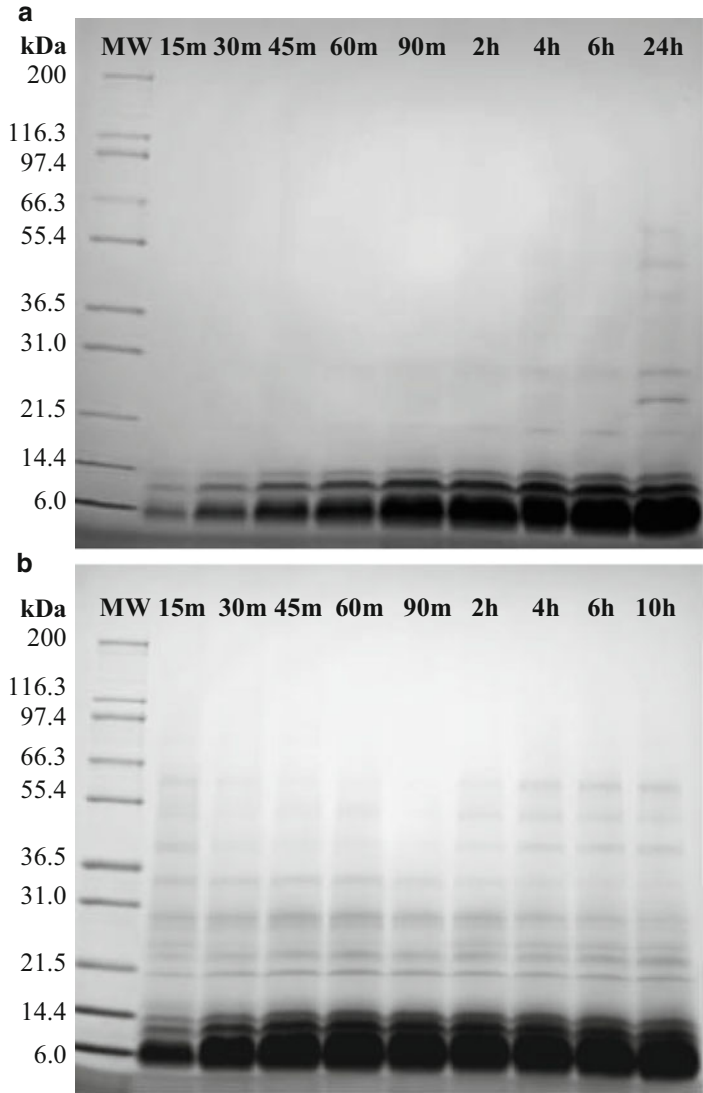


Fig. 21.2 Non-reduced SDS-PAGE images showing protein profiles of selected (a) pH 4.5 and 25 °C soak water samples from 15 min (15 m) to 24 h and (b) pH 4.5 and 50 °C samples from 15 m to 10 h. Samples were loaded based on protein content (12.5 µg per well)

(Fig. 21.2b). Based on Bradford assays, leached protein was 65 % higher at 50 °C than 25 °C (average difference over 10 h) at pH 4.5. Soaking at pH 7.5 resulted in the loss of proteins with molecular weights from 6 to 60 kDa in the 25 °C soak (Fig. 21.3a) and 50 °C soak (Fig. 21.3b). Loss of lower molecular weight proteins into the soak water increased with soak temperature and time. The amount of protein

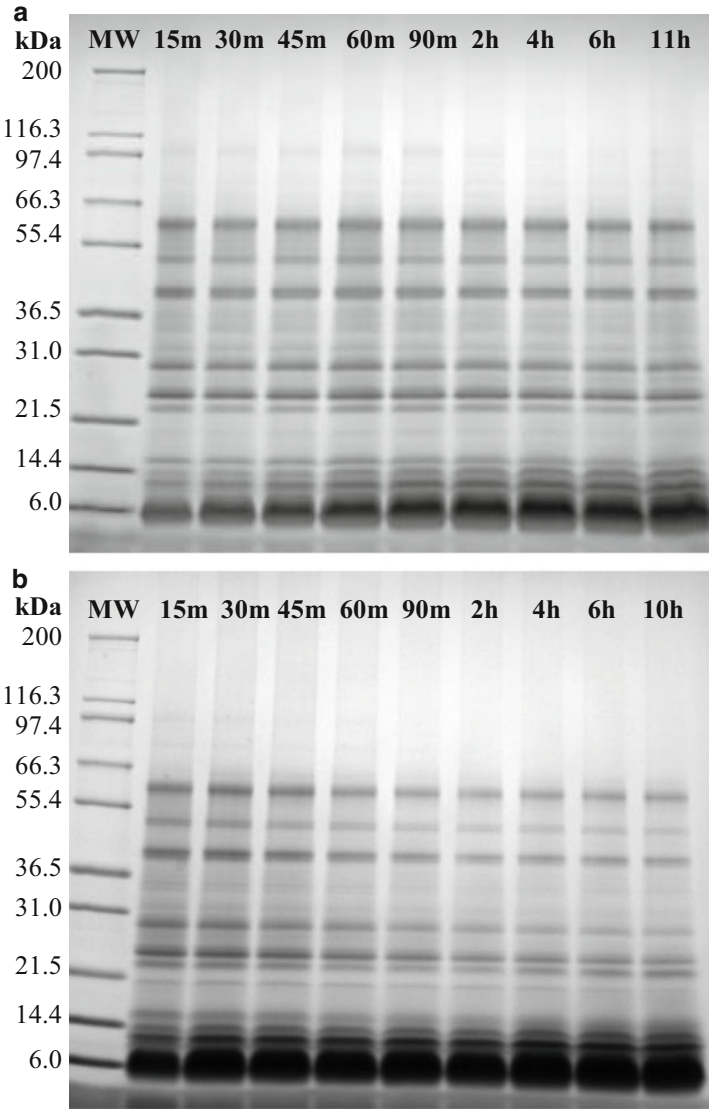


Fig. 21.3 Non-reduced SDS-PAGE images showing protein molecular weight profiles of selected pH 7.5 **(a)** 25 °C soak water samples and **(b)** 50 °C soak water samples from 15 min (15 m) to 11 h. Samples were loaded based on protein content (12.5 µg per well). The gel shows the distribution of proteins by molecular weight and not the amount extracted over time

solubilized in the soak water was almost 60 % higher at 50 °C than at 25 °C (average difference over 10 h). The increase of protein leached by soaking at a higher temperature was similar to pH 4.5 results. The amount of leached protein as a function of pH reflects the isoelectric protein properties of germ proteins. Most of germ

proteins are acidic and their solubility in water at pH 4.5 is expected to be lower than at pH 7.5 (Gu and Glatz 2007). Using a higher soak temperature apparently increases cell wall porosity allowing larger germ proteins to diffuse out of individual cells; nevertheless, protein solubility was a determining factor in protein leaching.

21.3.2 Pilot-Scale Studies: Soak Water Analysis

Based on lab-scale studies, two soaking conditions were selected for scale-up to confirm the effect of the soaking conditions on protein and phytic acid leaching into the soak water. Soaking conditions that minimized protein leaching (pH 4.5, 25 °C) and maximized protein and phytic acid leaching (pH 7.0, 60 °C) in the lab-scale studies were further evaluated.

Protein leaching kinetics (Fig. 21.4) in the pilot-scale studies showed similar trends when compared to lab-scale data at the same pH and temperature. Loss of germ protein (as % of initial germ protein) due to leaching in the soak water ranged from 0.3 % at 0.5 h to 1.3 % at 6 h for pH 4.5 and from 3 to 6 % for pH 7.0 soaking. The percent of germ protein leached at pH 7.0 and 60 °C was 83 % higher than that at pH 4.5 and 25 °C in the pilot-scale soaking experiments and similar to the 75 % difference measured in the lab-scale experiments. The proteins present in the soak water were predominately 6–14 kDa (Fig. 21.5). However, higher molecular weight proteins were observed in the pilot-scale pH 4.5 soak water samples (Fig. 21.5a) but not in lab-scale samples.

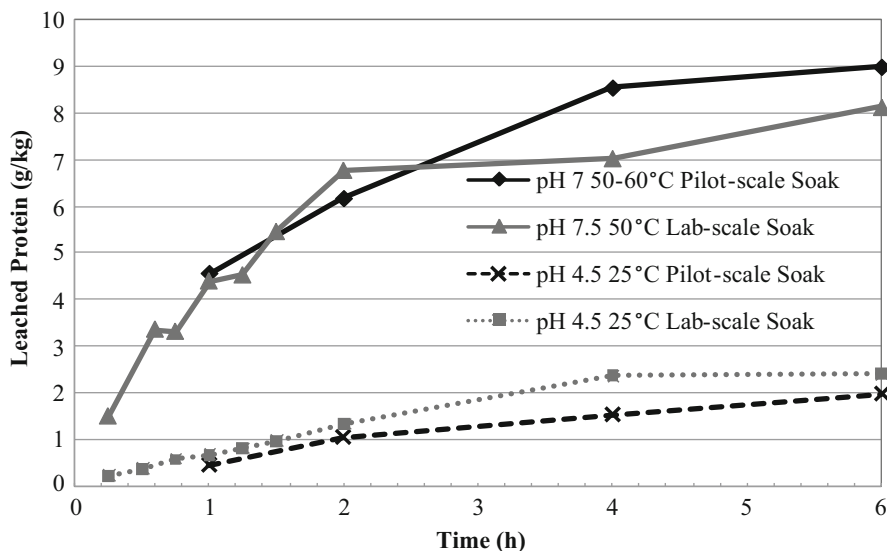


Fig. 21.4 Comparison of pilot-scale and lab-scale data for protein leached into soak water throughout the soaking of dry-milled germ. Data reported as g TSP per kg of germ soaked

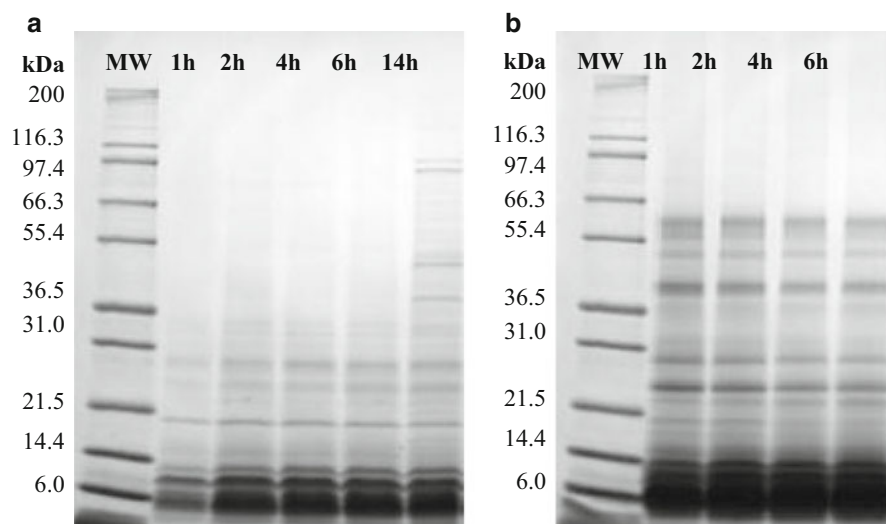


Fig. 21.5 Pilot-scale soak water analysis: non-reduced SDS-PAGE image showing protein molecular weight profiles of selected (a) pH 4.5 and 25 °C soak water samples from 1 to 14 h and (b) pH 7 and 60 °C soak water samples from 1 to 6 h. Samples were loaded based on equal protein content with (a) 8.5 µg protein and (b) 12.5 µg protein. Compared to the lab-scale experiments, higher molecular weight proteins were leached during pH 4.5 soaking for the pilot-scale experiments

The amount of phytic acid (undesirable anti-nutritional factor) in the soak water as a function of soak pH and temperature was also determined. The removal of phytic acid during soaking would be an important benefit of soaking germ because it reduces the amount of phytic acid that would be co-extracted during the subsequent protein extraction step. The amount of initial phytic acid content of germ leached ranged from 0.3 % (0.1 g/kg germ) after 0.5 h to 23 % (8.3 g/kg) after 6 h for the pH 4.5 soak and from 17 % (6.3 g/kg) to 20 % (7.5 g/kg) of total phytic acid present in dry-milled corn germ (O'Dell et al. 1972) during the pH 7.0 soak. The amount of phytic acid leached was higher at pH 7.0 than pH 4.5 for the shorter soak times (1, 2, and 4 h) but after 6 h, values were similar (~20 %) for both pHs. Because acidic conditions favor solubilization of undissociated phytic acid (Cheryan and Rackis 1980), a higher temperature or more soak time was required to extract the same amount of phytic acid at pH 7.0 compared to pH 4.5 soaking. The ratio of phytic acid to TSP in the soak water was also determined for both soak conditions (Fig. 21.6). The ratio could be used as an indirect measurement of the effectiveness of the soaking conditions. To obtain the highest germ protein purity, the ratio of phytic acid to TSP in the soak water should be maximized. Figure 21.6 shows that increasing the soaking time increased the phytic acid-to-TSP ratio for soaking at pH 4.5 but decreased the ratio at pH 7.0. The highest ratio of leached phytic acid to TSP was 4.2, which was obtained after soaking for 6 h at pH 4.5 and 25 °C. Thus, soaking at pH 4.5 appears to be a better choice unless soak time is limited to 1.5 h or less. In summary, the pilot-scale soaking studies indicated that the lab-scale experiments adequately reflected the results expected for larger scale processing.

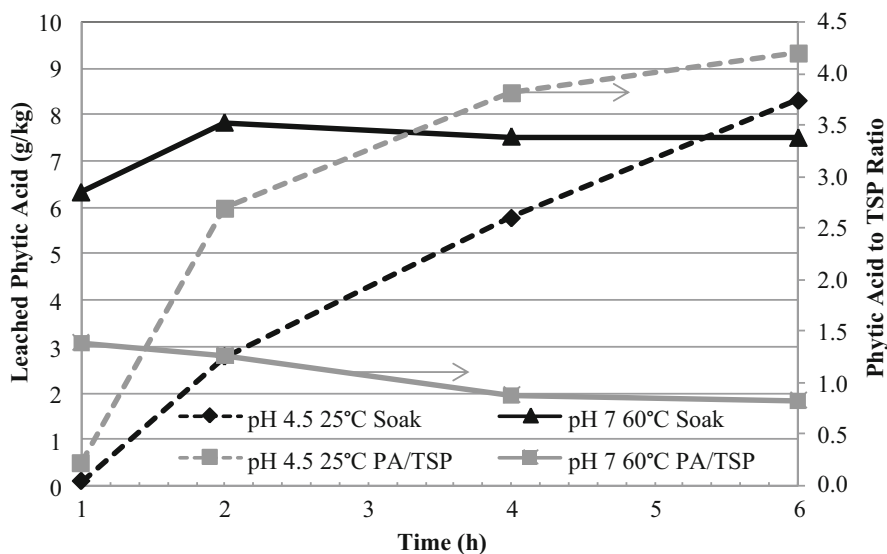


Fig. 21.6 Phytic acid and the ratio of phytic acid to total soluble protein (PA/TSP) leached into soak water as a function of time

21.3.3 Lab-Scale Studies: Germ Composition

As mentioned before, one of the desirable outcomes of germ soaking is to increase germ purity. The increase of germ purity is reflected in increased oil and total protein content and these two values were used throughout this study as process performance criteria. A set of screening experiments were performed to assess the effect of broader pH range (3.0, 6.0, and 9.0) for soaking at 25 and 60 °C on protein, starch, and oil content of the clean germ.

The percent increase in germ protein content varied from 30 to about 35 % for all pHs at 25 °C. Protein contents of samples soaked at 60 °C decreased over time irrespective of pH reflecting previously discussed leaching kinetics. Final protein content on a moisture-free and oil-free basis varied between 27 and 31 % for all soaking conditions.

The starch content of the germ was reduced from 33 % to an average of 9 % for all conditions and was independent of soak pH, temperature, and time. The final starch content was within the range of 5–10 % expected for clean germ (Watson 1994). The high starch content in the starting material indicates that a significant amount of endosperm remained attached to the germ after degermination, which is characteristic of the dry milling process. The addition of amylase enzyme to the soak water did not further reduce starch content in the germ suggesting that soaking alone was an effective way to remove attached starch even after 0.5 h of soak time. The two potential benefits of reducing starch content of dry-milled germ by soaking

is that it (1) improves hexane extraction efficiency and (2) increases overall corn-to-ethanol yield production since starch released into the soak water can be recovered with leached protein and salt and used to supplement fermentation media.

Soaking the germ increased oil content from 16 % to as much as 39 %, which depended on applied temperature and time. In general, germ oil content increased with temperature and time at all three pHs. Using a higher temperature soaking was more effective for producing higher oil germ with an average increase of 120 % at 60 °C compared to 94 % at 25 °C. Achieving higher oil content in the germ is desirable because higher oil content improves the efficiency of the subsequent oil extraction step and increases protein purity of the defatted germ.

21.3.4 Pilot-Scale Studies: Germ Composition

Two soaking conditions (pH 4.5 at 25 °C and pH 7.0 at 60 °C) were selected for scale-up to confirm the effect of the soaking conditions on germ protein and oil content and to produce germ for subsequent corn protein concentrate (CPC) development. Dry-milled germ was soaked at neutral pH (pH 7.0) and high temperature (60 °C) for either 2 or 6 h and compared to the pH 4.5 soaking experiments. Two different soaking periods (2 and 6 h) were evaluated because we wanted to reduce the total processing time and still get high germ protein content.

The composition of the dry-milled germ used as the starting material and soaked germ are given in Table 21.1. Soaking improved the germ protein content from 16 to 19–20 % protein (% dry basis) and was independent of soak time, temperature, and pH. Although pH 7.0 and 60 °C soaking conditions resulted in 75 % more protein leached than at pH 4.5 and 25 °C, the loss was not sufficient to reduce final protein content (20 vs. 19 %) of the soaked germ. However, differences could be seen if protein content was expressed on an oil-free basis. Since DCG flour would be used for preparing CPC, protein content calculated on an oil-free basis is good way to determine the starting material with the highest protein content. Germ protein content on oil-free basis was 31 % (2 h) and 32 % (6 h) for pH 4.5 and 25 °C samples and 35 % (2 h) and 37 % (6 h) for pH 7.0 and 60 °C samples (Table 21.1).

Table 21.1 Comparison of the composition of the starting material (dry-milled germ) to the clean germ (soaked at specified conditions, separated, and dried) from pilot-scale experiments

Sample	Soaking conditions			Composition (% dry basis)			
	pH	Temp (°C)	Time (h)	Protein	Protein (oil-free basis)	Oil	Starch
Dry-milled germ	NA	NA	NA	16	21	22	27
Clean germ	4.5	25	2	20	31	36	13
Clean germ	4.5	25	6	20	32	38	12
Clean germ	7.0	60	2	19	37	46	11
Clean germ	7.0	60	6	19	35	45	11

Table 21.2 Germ composition from pilot-scale dry-milled germ soaking at pH 7 for either 0.5 h at 20 °C or 16 h at 60 °C

Sample	Soaking conditions			Composition (% dry basis)			
	pH	Temp (°C)	Time (h)	Protein	Protein (oil-free basis)	Oil	Starch
Dry-milled germ	NA	NA	NA	14	17	18	41
Clean germ	7.0	20	0.5	20	30	35	11
Clean germ	7.0	60	16	19	33	42	7

Clean germ was analyzed after soaking, separation, and drying

The higher protein content (oil-free basis) at pH 7.0 and 60 °C was primarily a reflection of the higher germ oil content which was 46 % compared to 36 % for germ soaked at pH 4.5 and 25 °C.

Pilot-scale data were also similar to lab-scale in terms of starch content since soaking was very effective in reducing initial starch concentration, even at the shorter soak times. Starch content was reduced by 50–60 % for all soak conditions (Table 21.1).

An additional set of pilot-scale experiments was completed to determine germ composition after soaking at pH 7.0 and 20 °C, conditions that can be maintained with little or no adjustment. Germ composition was compared after soaking one batch at room temperature for only 0.5 h and the other batch for 16 h at 60 °C. Even at these two extremes, the protein contents (Table 21.2) were comparable and the slight improvement in oil purity and decrease in starch content probably would not justify using the longer soaking time (16 h) from an economical or operational point of view.

21.3.5 Corn Protein Concentrates

Dry-milled germ goes through several processing steps to make defatted germ flour (Fig. 21.7) and each step warrants consideration. Since the goal is to use defatted germ as the starting material for developing protein concentrates, the effect of processing conditions on germ protein quality was evaluated. Protein quality in this work was evaluated by measuring the PDI. PDI is one of the main methods used to determine protein solubility (Dubois and Hoover 1981) and one factor known to have a significant effect on protein functionality (Heywood et al. 2002; Zayas 1997; Zayas and Lin 1989).

Lab-scale and pilot-scale studies resulted in a similar protein content of soaked germ for all soaking conditions evaluated. Data from lab-scale soaking experiments at pH 3.0, 6.0, and 9.0 were used to better understand the impact of pH and temperature on PDI. PDI estimates summarized in Table 21.3 indicated that the PDI depended on soaking time, temperature, and pH. PDI decreased as the soaking time increased under most conditions. In addition, germ PDI was generally greater when soaked at 25 °C instead of 60 °C, unless soaking time at 60 °C was limited to 0.5 h, in which case the PDI did not change. The soaking time was particularly detrimental

Fig. 21.7 Process diagram for preparing high protein defatted germ flour from dry-milled germ

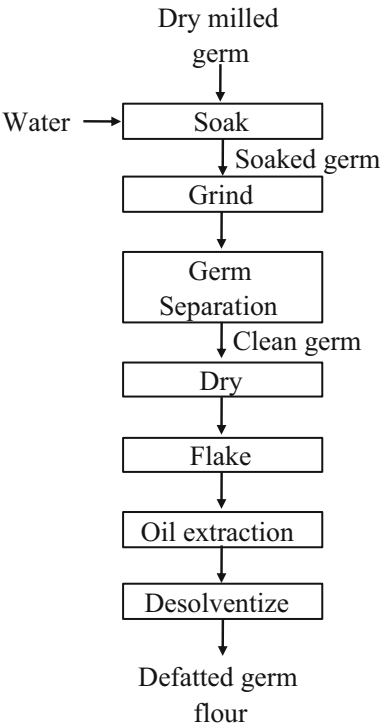


Table 21.3 Protein dispersibility index (PDI) of soaked dry-milled germ as a function of soak pH, temperature, and time

Soak time (h)	pH 3.0		pH 6.0		pH 9.0	
	25 °C	60 °C	25 °C	60 °C	25 °C	60 °C
0.5	61	53	62	63	65	70
2.0	53	27	62	44	70	60
4.0	54	16	61	43	71	61
8.0	47	13	58	43	69	58

at pH 3.0 and 60 °C due to protein denaturation since the PDI value was reduced 77 % when germ was soaked 8 h instead of 0.5 h. Based on the germ PDI value, soaking at 25 °C at either pH 6.0 or pH 9.0 appeared to be the best conditions for improving germ protein content and maintaining protein solubility.

Pilot-scale experiments confirmed the effect of processing on PDI. The PDI values were reduced 19–28 % during soaking, grinding, separation, and drying of the wet germ (data not shown). The PDI was further reduced 28–35 % after hexane defatting and drying. The drying temperature and time as well as the hexane temperature during defatting likely caused the decrease in PDI. Malumba et al. (2008) also found that high drying temperatures (54–130 °C) adversely affected protein solubility, with the water-soluble albumins being the most heat-denatured class of

protein. Oil extraction with hexane has also been shown to affect the PDI of soy-bean flakes (Milligan and Suriano 1974). To differentiate temperature and organic solvent effects on PDI, oil extraction with hexane at room temperature was performed. The PDI of defatted germ was only reduced 8 % indicating that reducing solvent temperature was effective for maintaining germ PDI.

The effect of PDI was evaluated further by comparing the yields (by mass) and protein contents of CPCs prepared from DCG flours with different PDI values. CPC yield ranged from 7.0 g per 100 g DCG to 16 g per 100 g DCG (Table 21.4). The amount of protein recovered ranged from 3.7 to 11 g. The differences in CPC yield and protein content are probably a reflection of the differences in the amount of water soluble protein among the different DCG flours. Further evidence of the importance of PDI on protein yield (% of protein present in DCG recovered in CPC) was demonstrated as CPC prepared from DCG flour with protein contents of 25–26 % had very different protein recovery yields of 27 and 46 %. The higher protein yield was prepared from DCG with a 52 % PDI and the lower yield with 26 % PDI white flakes (Table 21.4).

To confirm these results and separate the effect of initial germ protein content from PDI, two batches of defatted germ flour with similar protein contents but different PDIs were used to prepare CPC. CPC was prepared from high protein, high PDI germ (HHPHG) and high protein, lower PDI germ (HPLPG) to determine if a high PDI would give higher protein yield. The starting material (corn germ) and soak conditions were identical for both cases.

CPC prepared by isoelectric precipitation from the defatted HHPHG flour had a yield of 16 g CPC per 100 g flour compared to 10 g per 100 g for the HPLPG flour (Table 21.5). The fraction of protein recovered from the higher PDI germ flour was 70 % higher than that from the lower PDI germ flour.

Table 21.4 Effect of defatted corn germ (DCG)PDI on the amount of protein recovered in corn protein concentrate (CPC)

Defatted germ flour PDI (%)	DCG protein (%)	Water soluble protein (g) in 100 g DCG flour	CPC (g) per 100 g DCG flour	Protein (g) in CPC from 100 g DCG flour	Protein yield (%)
15	25	3.7	7.0	3.7	15
26	26	6.9	15	7.2	27
52	25	13	16	11	46

The amount of water soluble protein was calculated by multiplying protein content by PDI of DCG flour. Protein yield was calculated by dividing the total protein in CPC by the total protein in DCG

Table 21.5 Effect of germ flour PDI on CPC yield (by mass), protein content, and protein yield

DCG PDI (%)	DCG protein (%)	Water soluble protein (g) in 100 g DCG flour	CPC (g) per 100 g DCG flour	Protein (g) in CPC from 100 g DCG flour	Protein yield (%)
37	30	11	10	8.0	27
52	25	13	16	11	46

Protein yield represents the amount of protein in CPC as a % of the total protein in the DCG

21.4 Conclusions

Soaking dry-milled germ was an effective method for improving germ protein and oil contents (criteria for germ purity) and reducing attached endosperm starch. In the lab-scale experiments, starch was reduced from 33 to 9 % and protein content (moisture-free, oil-free basis) was increased from 18 % to an average of 29 % for all soak conditions. Oil content increased from the starting composition of 16 % to as much as 39 % for the clean (soaked and separated) germ. Pilot-scale experiments were consistent with the lab-scale studies with little variation in final protein and starch composition and higher oil content with higher temperature soaking. Highest protein and oil contents were reached after only 2 h of soaking at 60 °C. The oil content of the clean germ (46 %) was within the 42–50 % (dry basis) range typically seen in traditional wet milling (Johnson and May 2003), which uses 24 h soaking of whole kernels. Clearly, soaking germ instead of the entire kernel was advantageous since the same oil content could be achieved in only 2 h.

PDI was affected by soaking temperature, pH, and time. High temperature (60 °C) and longer soaking times (≥ 2 h) reduced PDI values as much as 75 %. Germ soaked using these conditions would be undesirable for CPC preparation because CPC yield was proportional to the PDI of defatted germ flour. Based on the germ PDI value, soaking at 25 °C at either pH 6.0 or pH 9.0 appear to be the best conditions for improving germ protein content and maintaining protein solubility. Controlling the temperature during the germ drying and defatting processes was also important for maintaining the PDI throughout processing.

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