KAUNAS UNIVERSITY OF TECHNOLOGY

IEVA RAČKAUSKIENĖ

BEETROOT (*BETA VULGARIS*) AND LINGONBERRY (*VACCINIUM VITIS-IDAEA* L.) LEAVES PREPARATIONS ANTIOXIDATIVE CHARACTERISTICS AND IMPACT ON THE FORMATION OF TOXIC MAILLARD REACTION PRODUCTS IN FOOD MODEL SYSTEMS

Doctoral dissertation
Natural Sciences, Chemistry (N 003)

2020, Kaunas

This doctoral dissertation was prepared at Kaunas University of Technology, Faculty of Chemical Technology, Department of Food Science and Technology during the period of 2011–2019. The studies were supported by the State Studies Foundation and the Research Council of Lithuania. Part of the research was performed at University of Naples Federico II, Department of Agricultural Science.

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This doctoral dissertation has been published at:
http://ktu.edu

Editor:

Armandas Rumšas (Publishing Office “Technologija”)

KAUNO TECHNOLOGIJOS UNIVERSITETAS

IEVA RAČKAUSKIENĖ

BUROKĖLIŲ *(BETA VULGARIS)* IR BRUKNIŲ (*VACCINIUM VITIS-IDAEA L.*) LAPŲ PREPARATŲ ANTIOKSIDACINĖS SAVYBĖS IR ĮTAKA TOKSINIŲ MEJARO REAKCIJOS PRODUKTŲ SUSIDARYMUI MAISTO MODELINĖSE SISTEMOSE

Daktaro disertacija
Gamtos mokslai, chemija (N 003)

2020, Kaunas

Disertacija rengta 2011-2019 metais Kauno technologijos universiteto, Cheminės technologijos fakultete, Maisto mokslo ir technologijos katedroje. Mokslinius tyrimus rėmė Valstybinis studijų fondas ir Lietuvos mokslo taryba. Dalis tyrimų atlikta Neapolio Frederiko II universitete.

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Interneto svetainės, kurioje skelbiama disertacija, adresas:
http://ktu.edu

Redagavo:

Armandas Rumšas (leidykla “Technologija”)

ACKNOWLEDGEMENTS

I wish to express my gratitude to my scientific supervisor assoc. prof. dr. Audrius Pukalskas and prof. dr. Petras Rimantas Venskutonis for the idea and subject of this doctoral dissertation, valuable scientific consultations and discussions as well as help in preparing publications, encouragement and support during the doctoral studies at Kaunas University of Technology.

I am highly thankful to Prof. dr. Vincenzo Fogliano for the possibility to perform internship at University of Naples Federico II, scientific ideas and consultations and to my dear colleagues from University of Naples Federico II Alberto Fiore and Antonio Dario Troise for technical help during the experiments and help in preparing publications.

I am grateful to all of my colleagues from the Department of Food Science and Technology at Kaunas University of Technology for technical help during lab experiments for the friendly and creative environment.

My special gratitude goes to my husband Justinas, my son Izidorius, parents and friends for their patience, encouragement and support.

Thanks to everyone who contributed to the success of this work!

Ieva Račkauskienė

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ABBREVIATIONS

4,8-DiMeIQx – 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
AαC – 2-amino-9H-pyrido[2,3-*b*]indole
$ABTS^{\bullet+}$ – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AE – lingonberry leaves acetone extract
AGEs – advanced glycation end products
a_w – water activity
BJP – beetroot juice powder
BnF – betanin fraction
BRP – beetroot powder
BtF – betalains fraction
CML – N^{ε}-(carboxymethyl)-l-lysine
DAE – lingonberry leaves deodorized acetone extract
DME – lingonberry leaves deodorized methanol extract
$DPPH^{\bullet}$ – 2,2-diphenyl-1-picrylhydrazyl, free radical
dw – dry weight
EC_{50} – effective concentration
edw – extract dry weight
EGCG – epigallocatechin gallate
Furosine – N^{ε}-(2-furoylmethyl)-L-lysine
Glu-P-1 – 2-amino-6-methyldipyrido[1,2-a:3', 2'-*d*]imidazole
Glu-P-2 – 2-aminodipyrido[1,2-a:3', 2'-*d*]imidazole
Harman – 1-methyl-9H-pyrido[3,4-*b*]indole
HAs – heterocyclic amines
HMF – 5-hydroxymethylfurfural
HPLC – high-performance liquid chromatography
IQ – 2-amino-3-methylimidazo[4,5-*f*]quinolone
IQx – 2-amino-3-methylimidazo[4,5-*f*]quinoxaline
IARC – Iternational Agency for Research on Cancer
LC/MS/MS – liquid chromatography-tandem mass spectrometry
LL – lingonberry leaves
LOD – limit of detection
LOQ – limit of quantification
m/z – molecular mass and charge ratio
ME – lingonberry leaves methanol extract
MeIQ – 2-amino-3,4-dimethylimidazo[4,5-f]quinolone
MeIQx – 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
MMS – meat model system
MPMS – meat-protein model system (phenylalanine)
MR – Maillard reaction
MRI – Maillard reaction intensity
MRM – multiple reaction monitoring
MRPs – Maillard reaction products
MS – milk model system

MS/MS – tandem mass spectrometry
Norharman – 9H-pyrido[3,4-*b*]indole
ORAC – oxygen radical absorbance capacity
PhIP – 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
Q-TOF – quadrupole time-of-flight analyzer
R^2 – coefficient of determination
TE – Trolox equivalents
TEAC – Trolox antioxidant activity coefficient
TPC – total phenolic content
t_R – retention time
Trp-P-1 – 3-amino-1,4-dimethyl-5H-pyrido[4,3-*b*]indole
Trp-P-2 – 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole
UHT – ultrahigh temperature
UPLC – ultra performance liquid chromatography
UV– ultraviolet
VxF – vulgaxanthin I fraction
WE – lingonberry leaves water extract

1. INTRODUCTION

Relevance of the research. Thermal processing of foods is an essential and highly beneficial step which inactivates foodborn pathogens, extends the shelf life and forms the desired sensorial (e.g., color, texture, aroma, and taste) and technological (protein solubility and emulsifying ability) properties. On the other hand, chemical reactions take place between food components, and consequences in the formation of toxic molecules may be manifested. The majority of those reactions are linked to the Maillard reaction (MR) occurring between the carbonyl groups of reducing sugars and the free amino groups of amino acids. In addition, other molecules, such as lipids, antioxidant, vitamins, and creatinine, may also interact and contribute to Maillard chemistry. Dietary MRPs (Maillard reaction products), such as advanced glycation end products (AGEs), heterocyclic amines (HAs), polycyclic aromatic hydrocarbons, acrylamide, furan and furfural products, are an emerging concern due to the evidence of the negative impact on human health. Most of them stem from proteinous foods. For example, IARC in 2015 assigned red meat to the status of "probably carcinogenic to humans" (Group 2A), while processed meat was assigned to "carcinogenic to humans" (Group 1). Nowadays, there is available evidence that consumption of thermally treated foods has been increasing, therefore, there is emerging demand for tools to control MR, especially in order to reduce the potentially toxic MRPs formation. One of the tools is the interaction between MR and phenolic compounds. It is known that phenolic compounds can react with sugar fragmentation products and amino acids/proteins to form adduct reaction products. However, there is lack of information on the impact of natural substances or the effects of phenolic compounds on toxic MRPs formation. Phenolic compounds have been demonstrated to possess the ability to moderate MR in foods and in model systems. Several studies revealed that green tea catechins are effective inhibitors of the formation of MRPs. As leaves of *Vaccinium vitis-idaea* L. were proved to be a rich source of flavonoids including catechins, they are a natural source with promising properties. Also, so far, no research has been made on how betalains from *Beta vulgaris* can influence MRP formation, while betalains under E162 food additive number are broadly used in many foods for coloring purposes. One of expectations to inhibit MRP formation of betalains is the competition of amino acids in the case of the action of betaxanthins; the other case is quinone methide formation and trapping of dicarbonyls and amino acids in the case of the action of betacyanins or phenolic compounds. Thus, the study of the influence of bioactive phytochemicals on the toxic MRP formation remains an essential prerequisite. Therefore, the main goal of this thesis is to provide potential application of some natural ingredients extracted from plants as possible inhibitors for the formation of toxic MRPs in order to enhance the quality of processed foods.

Aim and tasks of this research. The main aim of this work was to investigate the effect of betalains isolated from beetroot (B*eta vulgaris*) and phenolic compounds isolated from lingonberry (*Vaccinium vitis-idaea* L.) leaves on toxic Maillard reaction products formation in food model systems. To achieve this aim, the following objectives were outlined for this research work:

1. to purify betanin and vulgaxanthin I from beetroot by using gel chromatography and preparative high-performance liquid chromatography methods and to determine the antioxidative properties and chemical composition of various betalains preparations.

2. to prepare various polarity extracts from deodorized and non-deodorized lingonberry leaves and to determine their antioxidative properties and the composition of phenolic compounds.

3. to evaluate the effects of beetroot preparations on the formation of toxic MRPs in milk and meat model systems.

4. to evaluate the effect of various extracts of lingonberry leaves on the formation of toxic MRPs in milk and meat model systems.

5. to evaluate the effects of beetroot preparations and lingonberry leaves extracts on MR intensity parameters in the phenylalanine model system.

Scientific novelty of the research. The scientific novelty of the performed research may be summarized by the following main points:

1. The effects of betalains from beetroot on the formation of toxic Maillard reaction products in food models have been evaluated for the first time.

2. The effects of lingonberry leaves extracts on the formation of toxic Maillard reaction products in food models have been evaluated for the first time.

3. Systematic evaluation of the antioxidant properties and the content of phenolic compounds of various polarity extracts form lingonberry leaves has been performed for the first time.

4. This is the first study showing that various polarity extracts from the same material can exert the Maillard reaction in different ways.

Practical significance of the research. Results showed that the antioxidant activity of betanin depends on its purity and is 21 and 300 times higher than the values of BJP and VnF, respectively. It was observed that beetroot and lingonberry leaves preparations could suppress MRP formation in the milk model system. Quinic acid and catechin were attributed to be the compounds responsible for the inhibition action in WE. It was determined that beetroot and lingonberry leaves preparations could inhibit and promote HAs formation depending on their concentration in meat model systems. The inhibitory effect of various beetroot preparations on HAs formation was more pronounced at lower concentrations, whereas higher concentrations intensified their formation and increased the intensity of MR. The inhibitory effect of lingonberry extracts on HAs formation and the total MR intensity was more pronounced at higher concentrations, whereas the lower concentrations of water extract enhanced their formation, and acetone extract did not exert any effect. The obtained results indicate that the different action of lingonberry leaves extracts on HAs formation was due to the different composition of phenolic compounds. Hydroxycinnamic acids were attributed to be the promoters of HAs formation, whereas catechin and arbutin derivatives were assigned to be inhibitors. The results of this work can be used to develop functional ingredients for processed foods in order to control the MR due to the potentially toxic MRPs formation; they thus provide an opportunity to make better quality food.

Systematic evaluation of antioxidant properties and the composition of phenolic compounds of various polarity extracts form lingonberry leaves was performed for the first time. From this point of view, the results might be useful for preparing efficient natural functional ingredients for food, pharmaceutical, nutraceutical and cosmetics industries.

Structure and outline of the dissertation. This dissertation is divided into the following parts: a list of abbreviations, an introduction, literature review, materials and methods, results and discussion, conclusions, a summary, a list of 263 references, and a list of publications on the dissertation topic. The literature survey and results of the research are presented in 114 pages including 15 tables and 51 figures.

Publication of the research results. The results of this research have been presented in 2 publications included in the list of the *Institute of Science Information* (ISI) database and have been presented at 5 international conferences.

Statements presented for the defence.

1. Betalains can inhibit or promote the formation of toxic MRPs depending on their concentration.

2. Lingonberry leaves extracts mitigate MR and thus inhibit the formation of MPRs. The action of lingonberry leaves extracts depends on their concentration and the composition of phenolic compounds.

2. LITERATURE REVIEW

2.1. MAILLARD REACTIONS IN FOODS

Thermal treatment of foods results in the development of delicate organoleptic properties and the elimination of the risk of foodborne diseases due to microbiological contamination. Heat processing of foods leads to the formation of favorable effects of MR, such as formation of color, aroma and taste compounds. MR compounds such as volatile compounds and brown pigments are the most desire compounds among consumers because they give delicate sensory properties for food. In addition, during the heating of foods (e.g., coffee, bread and soybeans), MR can lead to the formation in such compounds as melanoidins possessing antioxidative, antimicrobial and chemopreventive activities (Morales, Somoza, & Fogliano, 2012; Vignoli, Bassoli, & Benassi, 2011). Heat treatment of foods also produces negative effects: the formation of undesirable molecules, such as toxic compounds (acrylamide, AGEs and HAs), off-flavors (e.g., ketones and aldehydes in milk and fruit juices), and the loss of nutrients or digestibility/bioavailability of essential amino acids (Budhathoki *et al*., 2015; Chao *et al*., 2005; Dumont *et al*., 2010; Friedman, 1996; Malec, Pereyra Gonzales, Naranjo, & Vigo, 2002; Nguyen, van der Fels-Klerx, & van Boekel, 2014; Ramírez-Jiménez, García-Villanova, & Guerra-Hernández, 2004; Scheijen *et al*., 2018; Shirai *et al*., 1997; Zhao *et al*., 2017). Depending on the food, both negative and positive effects of MR can be stronger in some types of foods than in others. For example, roasting, baking or frying of meat products leads to the formation of delicate flavors and undesired toxic compounds (e.g., HAs). However, pasteurization, sterilization or drying of milk leads to the formation of off-flavors and toxic compounds (e.g., CML). In other words, the MR is desired in some products (e.g., meat, bakery items and coffee), but, in other products, it is not desired at all (e.g., UHT treated milk, infant milk and fruit juices). Taking into account the nutritional aspects, MR reduces protein digestibility/bioavailability of essential amino acids (Friedman, 1996). Among the amino acids, lysine is the most susceptible to glycation due to its two reactive amino groups (α- and ε-). Therefore, products, such as milk and cereal products which contain higher amounts of lysine, suffer higher loss of the nutritional value due to heat processing. MRs decrease the availability of some nutrients, such as Vitamin C and minerals (calcium, magnesium, zinc, iron and copper), by involving them in the reactions and modifications which can change their absorption in the organism (Ramonaitytė, Keršienė, Adams, Tehrani, & Kimpe, 2009; Smuda & Glomb, 2013). In order to obtain the equilibrium between the positive and negative outcomes of MRs, we need to search for ways to control MRs.

2.1.1. Chemistry of Maillard reactions

Maillard reaction is a succession of nonenzymatic browning reactions produced during heat treatment of foods which was discovered in 1912 by food chemist Louis-Camille Maillard. The reaction occurs between the carbonyl groups of reducing sugars and the amino group of free amino acids, the side chain of the amino acid in protein,

the last amino acid in the protein (Fig. 2.1). MR is divided into three steps (Nursten, 2005).

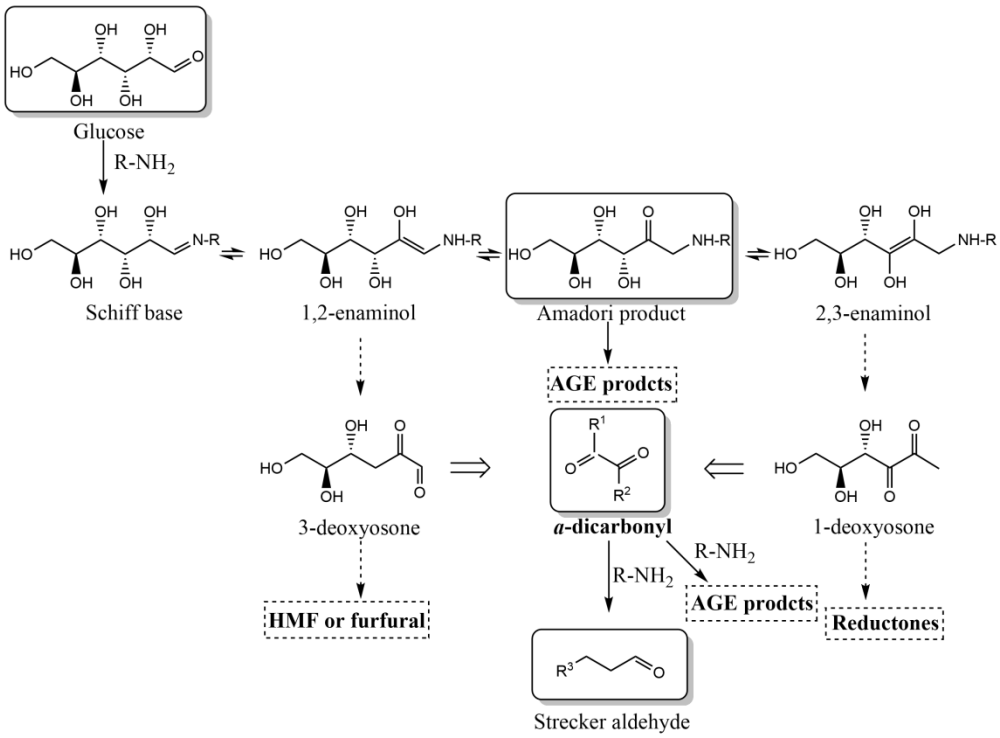


Fig. 2.1. Simplified scheme of Maillard reaction (adapted from Nursten, 2005; Zamora and Hidalgo, 2005)

The first step (the initial stage) begins with the condensation of carbonyl and amino groups and the formation of labile *N*-substituted-glycosylamine. The products of the initial stage are colorless and show no absorption in the ultraviolet light. During the second step, when the molecule is *N*-substituted-aldosylamine, the respective 1-amino-1-deoxy-2-ketose is formed by means of Amadori rearrangement. In the case when the molecule is *N*-substituted-ketosylamine, by means of Heyns rearrangement, 2-amine-2-deoxy-2-ketose is formed. Amadori and Heyns products are the main products of the initial stage of MR and, depending on the pH and temperature, they decompose into different intermediate compounds. A low pH, favors 1,2-enolisation via 3-deoxy-1,2-dicarbonyls and gives rise to the formation of furfural and HMF (5-hydroxymethylfurfural). Basic pH favors 2,3-enolisation via 1-deoxy-2,3-dicarbonyls and leads to the formation of reductones (e.g., furanone). Amadori compounds can also decompose into dicarbonyl compounds (fission products), such as acetal or acetaldehyde. The interaction of amino acids with dicarbonyl compounds leads to the formation of aldehydes through Strecker degradation. The resulting products of the intermediate stage are colorless or slightly yellow and exhibit strong absorption in the ultraviolet light. They also contribute to the aroma of foodstuffs. During the third step, namely, the advanced stage, aldol condensation, aldehyde-amine condensation and

the formation of heterocyclic nitrogen compounds occurs. The condensation of aminated products produces brown polymeric compounds denoted by a high molecular mass called melanoidins. Melanoidins are colored molecules with various absorbance spectra in the ultraviolet (λ_{max} = 280 nm) and visible (λ_{max} = 420 nm) ranges (Nursten, 2005; Rufián-Henares & Delgado-Andrade, 2009).

The extent of MR is influenced by a number of factors, such as pH, water activity (a_w), temperature, substrates and interacting molecules. At a low pH (<3), the rate of MR is low, and it in increases when pH increases up to 10. The highest rate of MR is achieved when a_w is between 0.67 and 0.70, which means that the water content is between 10–15%. The temperature is the most decisive factor regarding the extent of MR. The development of MR intensifies when the temperature is increasing. However, the key factors are both the degree of temperature and the heating time. The same extent of MR can be obtained with a product which is heated at a high temperature for a short time and with a product which is heated at a low temperature for a long time. Moreover, MR can proceed at a very low temperature (37 °C) and even during the product storage (25 °C). The main substrates of MR are carbonyl groups and amino groups. Monosaccharides are more reactive than disaccharides, while pentoses are more reactive than hexoses. The interacting molecules, such as lipids, their oxidation products, antioxidants (e.g., phenolic acids and flavonoids) and vitamins, can promote or increase the development of MR (Nursten, 2005; Rufián-Henares & Delgado-Andrade, 2009).

2.1.2. Brief overview of toxic Maillard reaction products

Up to date, many toxic compounds in heat processed foods have been identified in which thermal reactions occur, including protein pyrolysis, carbohydrate caramelization, amino-carbonyl reactions, and amino-creatinine (Friedman, 1996). The main toxic compounds in foods known so far are acrylamine, HMF, AGEs, HAs, N-nitroso compounds, polycyclic aromatic hydrocarbons and their distribution in foodstuffs as shown in Table 2.1. Several excellent reviews describing Maillard reaction products and their formation mechanisms in detail have been published (Poulsen *et al.*, 2013; Vistoli *et al.*, 2013). Acrylamides are the most important contaminants found in potatoes, bakery products and coffee. Acrylamide is considered to be a potentially carcinogenic compound (EFSA, 2015a; El-Zakhem Naous, Merhi, Abboud, Mroueh, & Taleb, 2018). Acrylamide is formed from asparagine degradation in the presence of reducing sugars through such intermediates as acrolein or 3-aminopropionamide (Granvogl & Schieberle, 2006; Yaylayan, Wnorowski, & Perez Locas, 2003). Since heat processed carbohydrate rich foods (potatoes and bakery items) have been found to contain high levels of acrylamide, a concern has emerged regarding the consumption of this type of foods. In 2007, the European Commission recommended monitoring acrylamide levels in food products, such as potatoes and cereal products. Besides, in 2013, a toolbox was developed to help the food industry lower the levels of acrylamide (EFSA, 2015a).

Table 2.1. MRPs possessing negative effect on human health found in foods

Food category	Harmful compounds
Dairy products	CML, CEL, pyrraline, pentosidine, methylglyoxallysine dimer (MOLD), glyoxallysine dimer (GOLD), hydroxycarbonyls, dicarbonyls, 2-acetyl-2-thiazoline
Meat and fish products	HAs, N-Nitroso compounds, Polycyclic aromatic hydrocarbons
Bakery products	HMF, Acrylamide, Furan
Potato products	Acrylamide
Fruit juices	HMF
Coffee, cacao and tea	Furan, acrylamide

Another contaminant in foods is HMF which is a furanic compound that can be formed from MR as well as sugar dehydration (caramelization). HMF is formed during the intermediate stage of MR from Amadori compound degradation. HMF is widely found in such foodstuffs as bakery and cereal products, honey, fruit juices and coffee (Capuano & Fogliano, 2011). HMF and its metabolite (5-sulfooxymethylfurfural) have been found to possess cytotoxic effects, but there are also several contradictory results (Rosatella, Simeonov, Frade, & Afonso, 2011; Zhao *et al*., 2017).

Other compounds related with the risk to human health are furans and methylfurans. Furan is classified as "possibly carcinogenic to humans" by the *International Agency for Research on Cancer* (IARC). They are formed in various foods, e.g., during the thermal processing of coffee, canned and jarred foods. Several ways of formation of furans as well as of HMF explain their wide occurrence in foods and consequently raise the risk on human health. Furans can be produced by ascorbic acid, amino acids, carbohydrates, polyunsaturated fatty acids, carotenoids degradation and MR (EFSA, 2017).

HAs are formed in meat and fish as MRPs of amino acids, creatinine and glucose. HAs are known as carcinogenic and mutagenic compounds which increase the risk of various types of cancers (Abel & DiGiovanni, 2008; Choi & Mason, 2003; Tamanna & Mahmood, 2015). The most abundant HAs in meat is PhIP which manifest the carcinogenic effect on the rat colon, prostate, breast and mammary gland (Shirai *et al*., 1997). Also, significant amounts of IQ, IQx, MeIQ, MeIQx, harman and norharman are found in meat. The carcinogenic/mutagenic/genotoxic effects of HAs *in vitro* (Dumont *et al*., 2010) and *in vivo* (Chao *et al*., 2005; Shirai *et al*., 1997) have been demonstrated in many studies. Recently, positive association of HAs has been found with the risk of colorectal adenoma (Budhathoki *et al*., 2015) and altered oxidative stress in humans (Carvalho *et al*., 2015).

Dietary AGEs are similar to those found in the human body during the aging process. Glycation of proteins in the human body was discovered 65 years ago, while first observed glycated protein was hemoglobin. Since then, increasing attention has been paid to their role on human health, while, in the last two decades, great attention has been paid to the tools to control or inhibit their formation. In many studies, AGEs

showed contribution to pathogenic effects, such as diabetes (Vlassara, 2001), cardiovascular issues (Hartog *et al*., 2007), renal failure (Uribarri et al., 2003) diseases and neurological disorders (Takeuchi *et al*., 2007). Dietary AGEs consumed with food also showed contribution to the total body AGEs load; consequently, they affected diabetes and cardiovascular diseases (Scheijen *et al*., 2018). AGEs can be produced via various pathways, such as glucose autoxidation, lipid peroxidation, polyol pathway and glycolysis (Vistoli *et al*., 2013). In most studies, the researched AGEs were CML, CEL (N^{ε}-(carboxyethyl)-l-lysine), GOLD, MOLD, pyrraline and pentosidine. Such AGEs as CML, CEL and N^{δ}-5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine have been determined in various food categories, and the obtained results revealed that high-heat processed foods were contained high AGE levels (Scheijen *et al*., 2016). AGEs are formed between amino acids, peptides, proteins and reducing sugar or carbonyl compounds in heated foods, such as milk products and bread products. In the human body, glycation of proteins influences the loss of the physiological functions due to AGEs formation whose consequences are observed in diabetic complications including, retinopathy, nephropathy and neuropathy, and cardiovascular diseases.

In the course of meat or milk processing at both household and industrial levels, toxic compounds are formed during MR. These toxic MRPs have such implications for human health as obesity and cardiovascular disease as well as various cancers, which has been demonstrated by epidemiological studies. Meat and dairy products are one of the most consumed food categories in various countries, and the future patterns show their increasing consumption (Kearney, 2010). Moreover, the changing lifestyle and trade policies lead to the growth of the consumption of processed foods. Therefore, there is emerging need to search for ways to reduce the amounts of MRPs in foods. Therefore, frequent consumers of processed meat and dairy products are at high risk of being exposed to toxic MRPs. This thesis focuses on the toxic compounds produced in meat and milk, and the following subsections deal with the toxic compounds formed in these product categories.

2.1.3. Toxic Maillard reaction products in milk

In dairy products, lactose and lysine residues, mainly from casein, are the dominant reactants in MR. The dominant MRPs arising from the reaction of these reactants during thermal treatment are AGEs, such as CML, CEL, pyrraline and pentosidine, and intermediate reactants, such as furosine (Fig. 2.2). CML is one of the most studied AGEs in dairy products (Nguyen *et al*., 2014). CML is formed by the oxidation of Amadori compounds or by the reaction of amino acids/peptides/proteins with dicarbonyl compounds (glyoxal, methylglyoxal, 3-deoxyglucosone) produced by degradation/oxidation of sugars during thermal treatment and lipid peroxidation (Fig 2.3). In addition, the direct reaction of lysine and ascorbate was also shown to lead to CML formation (Dunn *et al*., 1990). Furosine is another well-known marker showing the extent of protein damage in milk. It is an indirect marker of AGEs formation, as it indicates the levels of Amadori compounds which are further oxidized to AGEs.

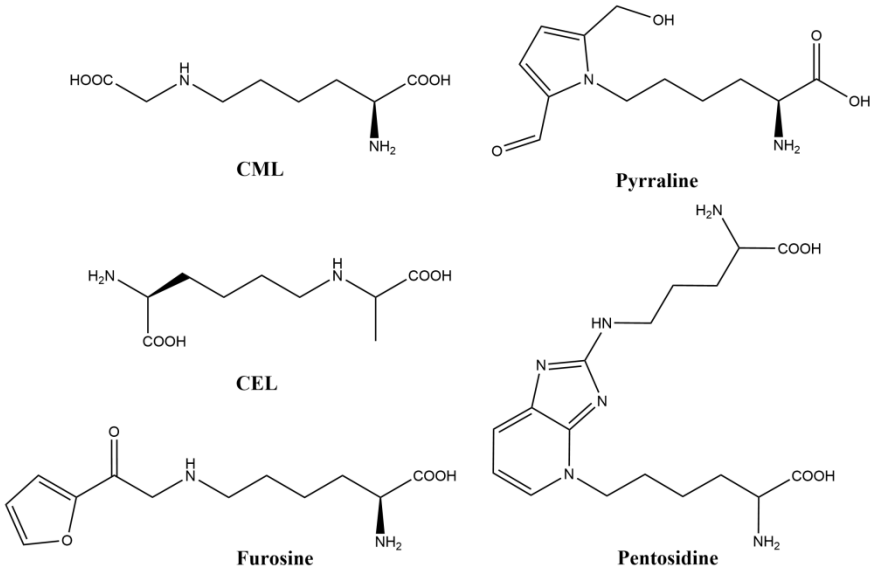


Fig. 2.2. Most common MRPs in milk

Furosine is formed after acid hydrolysis of Amadori compounds, such as fructose-lysine, lactulose-lysine, and maltuloselysine (Fig. 2.4). Usually, milk is subjected to heat treatment via various processing steps, such as milk separation (approx. 40–45 °C), then follows normalization (approx. 45–50 °C), homogenization (approx. 60 °C), and, finally, pasteurization (minimum 71.7 °C, 15 s), sterilization (110–120 °C, 10–20 min), or ultra-heat treatment (minimum 135 °C, 1 s).

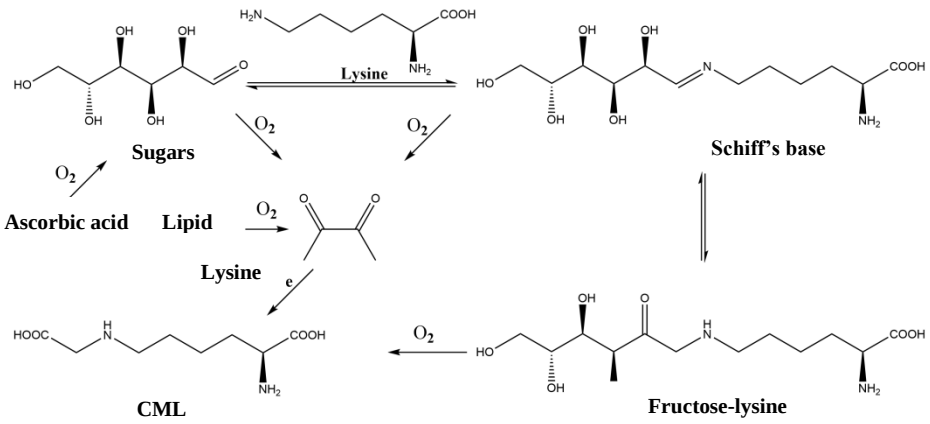


Fig. 2.3. CML and Amadori product formation pathways (adapted from Nguyen *et al*., 2014; Dunn *et al*., 1990)

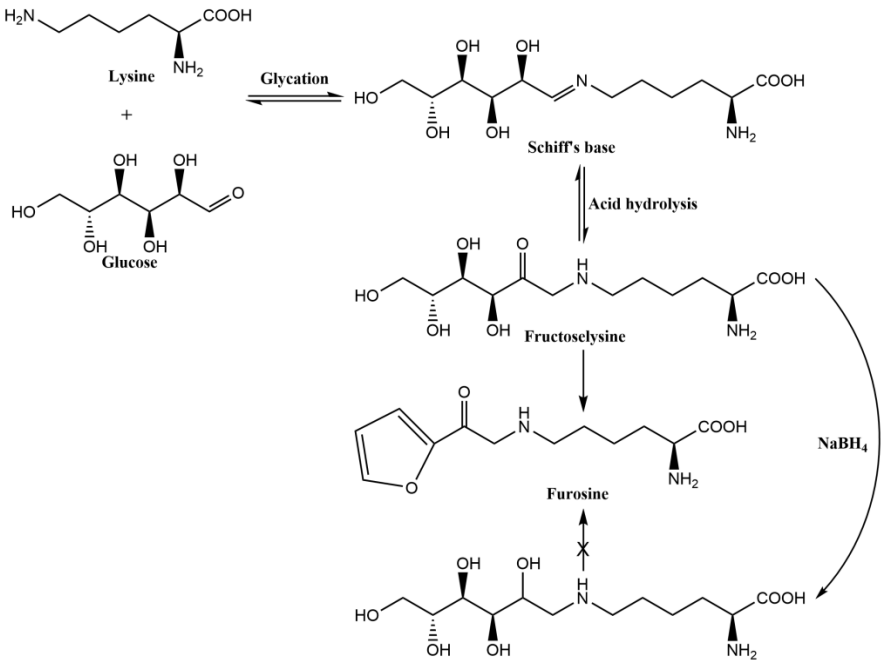


Fig. 2.4. Furosine formation pathway (adapted from Rufián-Henares & Delgado-Andrade, 2009)

All of these steps are susceptible to protein damage, and the level of dietary AGEs increases with the increased heating temperature and time; moreover, their formation continues during the subsequent storage of products (Sunds, Rauh, Sørensen, & Larsen, 2018). For example, the CML content in raw, pasteurized, and UHT cow milk was 42.0±49.3, 62.8±17.9 and 293±232 ng/mL, respectively.

2.1.4. Toxic Maillard reaction products in meat

Currently, the main compounds which are known to be present in meat and are toxic to human health are HAs. In 1980, HAs were discovered as mutagenic/carcenogenic compounds from broiled and grilled meat and fish products. The cancerogenic effect of PhIP as well as the mutagenic effects of Trp-P-1, MeIQx, and MeIQ have been observed in several studies (Nagao & Sugimura, 1993; Stavric, Matula, Klassen, & Downie, 1993; van Herwaarden et al., 2003). Animal studies showed that the diet with HAs in the long term resulted in colon, breast and prostate cancers in rodents, while some of them resulted in liver cancer development in monkeys (Sugimura, Wakabayashi, Nakagama, & Nagao, 2004). After the carcinogenic effect of HAs has been proven in animal studies, the IARC has assigned IQ to the status of possible carcinogens, whereas MeIQ, MeIQx, PhIP, AαAC, MeAαAC, Trp-P-1, Trp-P-2 and Glu-P-2 were listed as probable carcinogens (Kujawa, 1994). However, later on, there were many divergent studies showing different results regarding their carcinogenicity (Sinha *et al*., 2000). Recently, the *International Agency for Research on Cancer* (IARC, 2015) assigned red meat to

"probably carcinogenic to humans" (Group 2A) based on the evidence that the diet in red meat causes cancer in humans (associations were also seen for pancreatic and prostate cancers development), and strong mechanistic evidence of carcinogenic effect was observed. Meanwhile, processed meat has been assigned as "carcinogenic to humans" (Group 1) based on clear evidence that it causes colorectal cancer (Bouvard *et al*., 2015). The meat that was transformed by salting, fermentation, smoking, curing, or any other processes to enhance flavor or improve preservation is referred to as *processed meat*. Also, the IARC Working Group concluded that the risk of colorectal cancer is increased by 18% when a human's daily diet includes 50 grams of processed meat.

The composition of red meat is as follows: 75% water, 19% protein, 2.5% lipids and 2.5% soluble non-protein substances. The chemical composition varies between the types of meat, and this favors the formation of different HAs. HAs are formed at elevated temperatures, and the main source of human exposure to HAs is via the consumption of cooked proteinous foods. The total per capita meat consumption of 208 g/day in Europe was determined in (EFSA, 2011), while the mean consumption of processed meat for adults in Europe was calculated to range from 10 to 80 g/day. The dietary exposure to HAs is difficult to evaluate because their formation depends on many factors (the cooking technique, time, temperature, and the meat type). Therefore, there is no available international dietary exposure assessment. However, there are several available studies that make the relevant estimations. In one study, the results show that the total median exposure to HAs was 30.6 ng/day (13–71.3 ng/day) when the estimation was based on the total meat consumption.

Fig. 2.5 shows two types of HAs aminoimidazoazaarenes which contain the 2-aminoimidazole group, and carbolines which contain 2-aminopyridine. The first type of HAs is produced from the reaction of amino acid, creatinine and sugars, while the second type could be produced through the pyrolytic reaction of amino acids (Skog, Johansson, & Jägerstad, 1998). In the formation of IQ type compounds, pyridines and pyrazines are formed from hexoses and amino acids, respectively, in the MR via Strecker degradation. Then, pyridines and pyrazines react with creatinine via aldol reaction, and imidazoquinolines and imidazoquinoxalines are produced, respectively (Skog *et al*., 1998). Which HAs are formed depends on the available amino acid (Johansson, Fay, Gross, Olsson, & Jägerstad, 1995). For, example, PhIP is mainly produced from phenylalanine, MeIQx is produced from glycine, threonine, alanine and lysine, 4,8-DiMeIQx from threonine, alanine and lysine, MeIQ from alanine. The formation of PhIP was the most studied from all HAs. It has been shown that phenylalanine oxidizes to phenylacetaldehyde via Strecker degradation in the presence of reducing sugars (e.g., glucose, fructose), ascorbic acid, *a*-dicarbonyls and lipids (Adamiec, Rössner, Velíšek, Cejpek, & Šavel, 2001; Gallardo *et al*., 2008). Phenylacetaldehyde further reacts via the aldol reaction with creatinine and forms an intermediate, which subsequently produces PhIP through the condensation reaction (Fig. 2.6) (Zöchling & Murkovic, 2002). Reducing sugars have a strong impact on the formation of HAs.

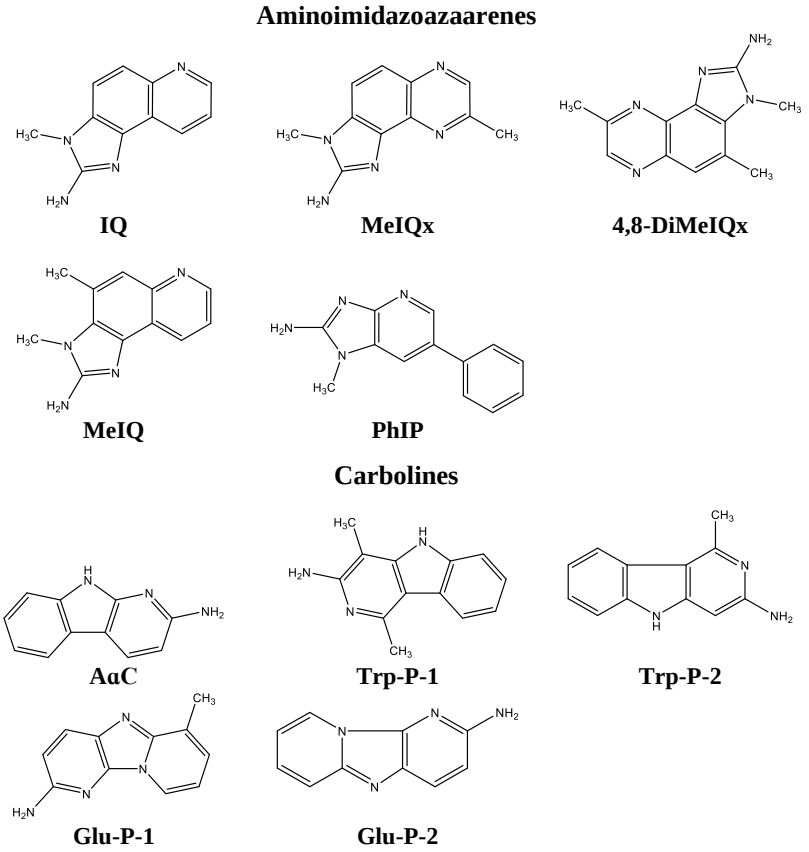


Fig. 2.5. Structure of the studied heterocyclic amines

The linear correlation between glucose concentration an HAs formation was found (Gibis & Weiss, 2015). Carbolines, or non-IQ type compounds, are mainly formed from the pyrolysis of amino acids at temperatures above 300 °C. Harman and nonharm are exceptional HAs formed through the reactions of amino acids and reducing sugars at low temperatures.

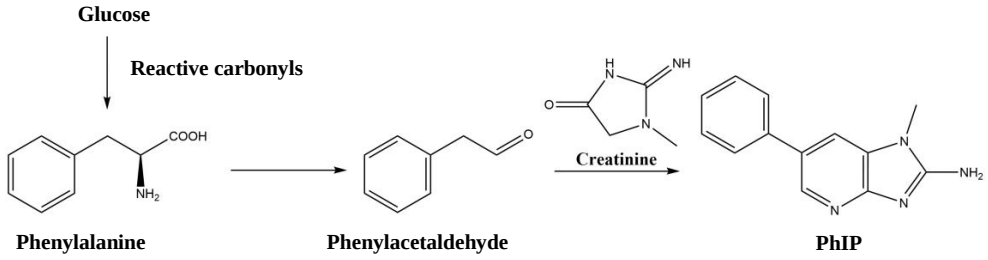


Fig. 2.6. PhIP formation pathway (adapted from Zöchling & Murkovic, 2002)

Both compounds do not possess mutagenicity as it was observed by Ames test, and this was related to the absence of free amino groups (Pfau & Skog, 2004). Currently,

over 25 different HAs have been identified in cooked meat. The kind of HAs to be produced depends on many factors, such as the type of meat, its cooking method and time, temperature, water activity, pH, and the content of precursors (Gibis & Weiss, 2015).

In meat, the most dominant mutagenic HA is PhIP, while chicken was found to be the most susceptible type of meat for the formation of this HA (Gibis & Weiss, 2015). Other HAs commonly occurring in meat are MeIQx, 4,8-DiMeIQx, MeIQ, AαC IQ, and IQx (Skog *et al*., 1998). Usually, meat is processed with various techniques, such as boiling (100 °C), broiling (225 °C), deep-frying (180 °C), oven-frying (230 °C), roasting (177 °C), grilling (260 °C), searing (100 °C), and smoking (80–85 °C), which exerts influence on the amount and type of HA to be produced. For example, oven-roasting yields fewer HAs compared to pan-frying due to the less efficient heat transfer to the surface of the product (Skog *et al*., 1998).

HAs in meat are found at nanogram levels: in fried ground beef patties, the PhIP content ranged from 4.0 to 25.4 ng/g, in pan-fried pork, it ranged from 0.3 to10.5 ng/g, while in oven-broiled chicken breast, it ranged from 5.6 to 72 ng/g (Balogh, Gray, Gomaa, & Booren, 2000; Gibis, 2016; Ni, McNaughton, LeMaster, Sinha, & Turesky, 2008). However, the concentrations of HAs vary between the studies, in which lower or many times higher amounts of HAs were also detected (Gibis, 2016; Johansson, Fredholm, Bjerne, & Jägerstad, 1995; Knize, Salmon, Pais, & Felton, 1999; Sinha *et al*., 1995).

AGEs (e.g., CML, CEL and N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine were also determined in heated meat; however, they were less analyzed if compared to HAs.

2.1.5. Food models to study regarding Maillard reaction

A range of natural phenolic antioxidants have been reported because of their inhibitory effects against dietary AGEs and HAs formation in foods, food model systems and chemical model systems. In order to test the effect of functional ingredients on MRPs formation, real food matrixes are used, but the model systems are also conventional and more convenient tools to study MR. There are many formulations to simulate MR, and the choice depends on the aim of the study. The AGEs, such as CML (including Amadori compounds) formation is observed in various foods (Sheng *et al*., 2016) or food model systems (Zhang, Chen, & Wang, 2014a). Many chemical model systems are also possible: bovine serum albumin/glucose or fructose (Kang, Zhao, Yue, & Liu, 2017; Kim & Kim, 2003), lysine/glucose or sucrose or lactose (Li *et al*., 2012; Wu *et al*., 2015), casein/lactose or glucose (Akillioglu & Gokmen, 2014; Liu *et al*., 2017), soy protein glycinin or bovine/fructose (Silván, Assar, Srey, Dolores del Castillo, & Ames, 2011). Testing of the effect of functional ingredients on the formation of HAs in the real meat matrix is usually performed when using meat patties or meatballs. Chemical model systems usually consist of glucose, creatinine and various amino acids: phenylalanine is used for PhIP formation (Shin, Strasburg, & Gray, 2002; Zhu, Zhang, Wang, Chen, & Zheng, 2016), and glycine for MeIQx, IQx, 7,8-DiMeIQx (Monti *et al*., 2001; Wong, Cheng, & Wang, 2012). Also, chemicals can be suspended in various solutions, such

as water, diethylene glycol, diethylene glycol-water (86/14, v/v), and phosphate buffer. Diethylene glycol is the preferred solvent because it assures even heat transfer and has a high boiling point. The meat model system which is the closest to the real meat system usually consists of lyophilized meat (beet, pork, chicken) suspended in water, diethylene glycol or diethylene glycol/water (Messner & Murkovic, 2004). According to the water loss during cooking, when lyophilized meat is suspended in diethylene glycol, the process is similar to the conditions of pan-frying. During lyophilization, the weight loss of beet meat is around 60%, while humidity varies between 10% and 6%. Raw beef meat contains 23.2% of proteins, 2.4% of fats, 0.8% of collagen, 73.5% of moisture, 1.15% of minerals and 2.60% of nonprotein nitrogen substances. The pH raw beef meat has around 5.6%. The total free amino acids in pork, beef and chicken raw meat are approximately around 16.53, 18.68 and 28.06 mmol/kg, respectively. The amino acids which are most common in beef meat are alanine, glycine and glutamine.

In addition, PhIP formation was also observed in creatinine/phenylalanine (Zöchling, Murkovic, & Pfannhauser, 2002) and phenylalanine/creatinine/oxidized lipid reaction mixtures (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014; Zamora, Alcón, & Hidalgo, 2013) andcreatinine/phenylacetaldehyde (Chen & Yu, 2016). The environment conditions (e.g., a_w, pH) of the model system are also an important factor which is usually taken into account when modeling the food.

2.2. STRATEGIES FOR CONTROL OF MAILLARD REACTIONS IN FOODS

Various strategies for controling MR in foods have been investigated up to date ranging from marinating or soaking to optimizing the heating process or choosing an alternative process. Adding the competing substances to the MR reactants in foods was one of the most commonly analyzed strategies. There are many substances which can inhibit MR but the most analyzed compounds were phenolics or natural extract from various plant origins. The antiglycation activity of phenolic antioxidants was related to the conjugation to glycation sites of the protein structure, their antioxidant character, and the trapping of dicarbonyl intermediates (Silvan, Srey, Ames, & del Castillo, 2014). For instance, the trapping of dicarbonyl compounds by catechins, namely (–)-epicatechin (Totlani & Peterson, 2006), (–)-epigallocatechin-3-gallate and (+)-catechin (Bin, Peterson, & Elias, 2012) was reported. Moreover, the suppressing effect of catechin against the development of dicarbonyls through the formation of phenolic-reactive carbonyl species in milk processed at an ultrahigh temperature was later shown (Kokkinidou & Peterson, 2014). Various mechanisms were suggested to explain the ability of natural plant antioxidants to inhibit the formation of HAs. Firstly, the inhibiting activity was related to their free radical scavenging capacity: it was shown that phenolic antioxidants inhibit the generation of free radical intermediates which are involved in the formation of HAs (Kikugawa, 1999). A decade later, it was demonstrated that epigallocatechin might scavenge phenylacetaldehyde intermediate in the formation of PhIP through the formation of adducts (Cheng *et al*., 2009). Furthermore, the structure of phenolic compounds was related to the inhibition effect

in the mechanism of PhIP formation (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014). Therefore, phenolic antioxidant-rich natural plant extracts may be useful additives in proteinous foods for the inhibition of the formation of toxic Maillard compounds.

2.2.1. Brief overview of mitigation strategies of Maillard reaction products

The preventive or protective strategies to control MR throughout the food process can be divided as follows:

- pre-processing procedures of the foodstuff;
  - addition of microbial organisms and enzymes;
  - soaking;
  - blanching;
  - marinating;
- optimization of the heating process;
  - heating time;
  - heating temperature;
  - pressure/temperature ratio;
- addition of functional ingredients;
  - salts;
  - sulfhydryl compounds;
  - competitive amino acid group;
  - phenolic compounds;
- encapsulation of reactants;
  - metals;
  - salts;
  - ascorbic acid;
- location of reactants in microemulsion;
- choosing of alternative processing;
  - ohmic heating;
  - pulsed electrical fields;
  - high-pressure processing

Pre-processing procedures of food before heat treatment is one of the ways to mitigate MR. This strategy is partly based on the removal of precursors. By using various enzymes or microbial organisms, such reactants as amino acids or reducing sugars can be removed thus lowering the extent of Maillard reaction. For example, enzyme asparaginase was effective in lowering the acrylamide level in cereal and potato products by hydrolyzing asparagine which is the main precursor of its formation (Xu, Oruna-Concha, & Elmore, 2016). Another study showed that glucoamylase, by subtracting the reducing sugars, lowered the levels of acrylamide in bakery products (Bartkiene *et al*., 2013). Soaking and blanching techniques were also related to the changed amounts of reactants (Pedreschi, Kaack, & Granby, 2004).

Optimization of heat processing is another way to reduce the formation of MRPs. The extent of MR depends on the temperature of heating and time. Therefore, reduction of the time and temperature during food processing can minimize the

amount of the formed MRPs (Pedreschi *et al*., 2004; Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012).

The addition of functional ingredients is one more way to tune MR. Salts can both promote and inhibit MR depending on their nature, amount, and the food matrix. For example, sodium salt at a low concentration inhibited CML formation, while, at a higher (1.5–5%) concentration, it promoted CML formation in pork during heating. Sodium nitrite (50–150 ppm) showed a great reducing effect on CML and CEL formation when added in raw pork prior to heating (Niu *et al*., 2018). In biscuits, the amount of acrylamide was strongly reduced with $CaCl_2$ and $MgCl_2$ additions (Quarta & Anese, 2010). In many studies, it was shown that sulphite-containing compounds are effective in the inhibition of MR (Nursten, 2005). They are commonly used to inhibit browning reactions in foods. Also, reduction of toxic MRPs, such as acrylamide (Casado, Sánchez, & Montaño, 2010), was observed.

The addition of a competing amino acid to the amino acid for the precursor of MRP is another technique to reduce the formation of undesirable compounds. This technique is of great interest because the source of amino acids could be naturally found in foods. For example, the inhibition effect of soy protein hydrolyzates and amaranth flour protein isolate on acrylamide formation have been showed (Cook & Taylor, 2005; Salazar, Arámbula-Villa, Vázquez-Landaverde, Hidalgo, & Zamora, 2012).

The mitigation of MR with phenolic compounds is also of great interest because they are from natural sources and possess antioxidant and biological properties that correspond to the desire of consumers. Many phenolic compounds have been tested to inhibit MR, and their action leads to controversial results showing positive, negative or zero effects depending on their nature and the food matrix. The impact and inhibitory effects of phenolic compounds shall be described in detail in the subsection below. Such substances as metals, ascorbic acid and salts have been shown to be able to strongly increase MR in foods.

The encapsulation technique is an attractive strategy which is based on the limitation of the reactive substances which participate in MR (Troise & Fogliano, 2013). However, this technique is under investigation because the matrix of its capsule can also release reactants which participate in MR.

Microemulsions are of special interest strategy in terms of tuning MR. It is as if a vehicle for controling the reactivity and orientation of reactants and water activity and thus tuning MR (Lutz, Aserin, & Garti, 2005).

Replacing the classic thermal treatments with new technologies, such as infrared heating, supercritical carbon dioxide technology, high hydrostatic pressure, pulsed electric field ohmic heating, the extent of MR could be better controlled (Casal, Ramírez, Ibañez, Corzo, & Olano, 2006; Fillaudeau *et al*., 2006; Jaeger, Janositz, & Knorr, 2010)

2.2.2. Inhibitory mechanism of phenolic compounds on Maillard reaction

Phenolic compounds from natural sources have gained an increasing focus in foods regarding the mitigation of toxic MRPs formation because they are considered to be natural and are more acceptable that synthetic compounds. Also, other natural

compounds, such as amino acids and vitamins, have shown potential to inhibit MR by similar pathways to phenolic compounds. There are several major mechanisms by which polyphenolic compounds inhibit the Maillard reaction products formation, and they can be as follows (Fig. 2.7):

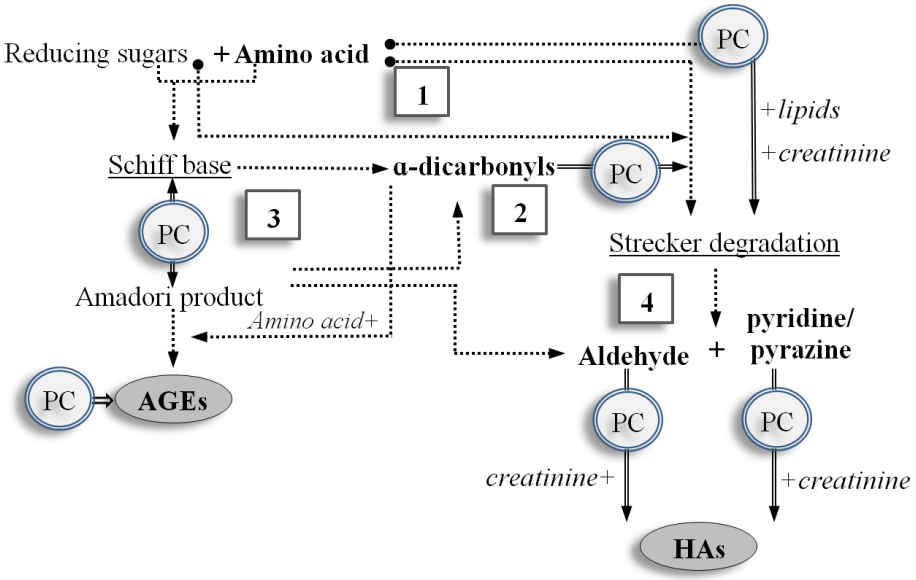


Fig. 2.7. Possible pathways for toxic MRP formation and mitigation strategies by phenolic compounds (adapted from Friedman, 1996; Gallardo *et al*., 2008; Li *et al*., 2018; Totlani & Peterson, 2006; Wu, Huang, Lin, & Yen, 2011; Zöchling & Murkovic, 2002). The squared numbers indicate: 1, strategy by blocking or modification of amines; 2, strategy by trapping of α-dicarbonyls; 3, strategy by scavenging Maillard-derived radicals; 4, strategy by trapping Strecker aldehydes. Abbreviations: PC, phenolic compounds; HAs, heterocyclic amines; AGEs, advanced glycation end products

1) *Inhibition by blocking or modification of amines.* This approach is based on blocking the amine group of amino acid by oxidized polyphenols, quinones. Polyphenols oxidize on the B-ring to quinones, which, during the processing of foods and storage, react with thiols and amines and form with the latter benzoquinone imines or amine-quinone adducts (which is called the Michael addition or 'Strecker like' reaction, Fig. 2.8).

2) *Inhibition by trapping α-dicarbonyls.* Trapping of reactive intermediates is one of the main mechanisms by which phenolic compounds inhibit the formation of Maillard reaction products. Flavonoids trap *α*-dicarbonyls by electrophilic aromatic substitution reaction occurring at highly activated A-ring forming adducts. The structure of flavonoids strongly contributes to the trapping ability of *α*-dicarbonyls (Shao *et al*., 2014). Phenolic compounds having hydroxyl groups at meta configuration in the aromatic ring have strong reactivity to the ortho configuration, their trapping efficiency enhances when the hydroxyl groups are at C-5 on the A-ring,

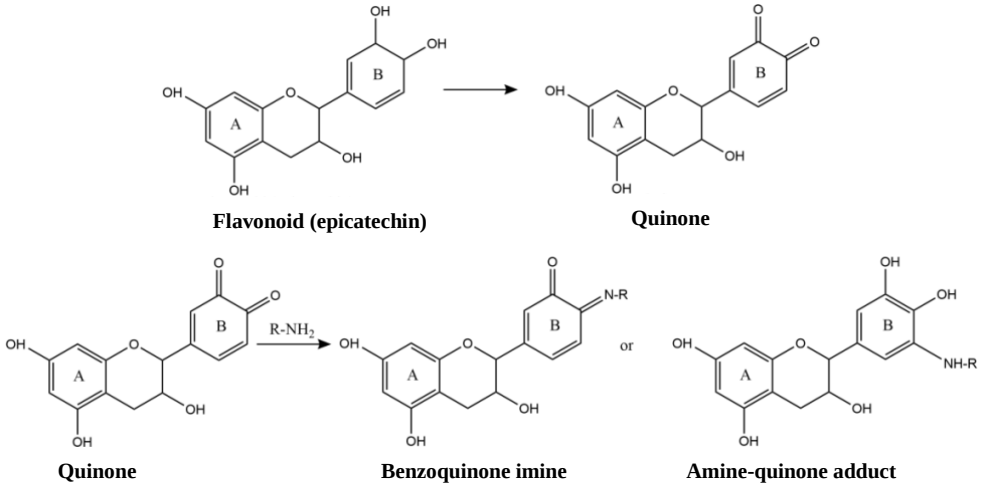


Fig. 2.8. Blocking of amine groups by quinones via Michael addition (adapted from Pierpoint, 1969; Porter *et al*., 2006; Yin, Hedegaard, Skibsted, & Andersen, 2014)

and the double bond between C-2 and C-3 on C-ring could contribute to the enhanced efficiency (Shao *et al*., 2014). *α*-dicarbonyls, such as glyoxal, methylglyoxal and 3-deoxyglucosone, are reactive intermediates in MR thus accelerating the rate of this reaction. They can be formed through the fragmentation of Amadori and Heyns products, carbohydrates autoxidation and lipid peroxidation. Many phenolic compounds have been shown to trap *α*-dicarbonyls such as phenolic acids (Lo, Hsiao, & Chen, 2011; Totlani & Peterson, 2006) and flavonoids (Es-Safi, Le Guernevé, Cheynier, & Moutounet, 2000; Silván, Srey, Ames, & del Castillo, 2014) (Fig 2.9).

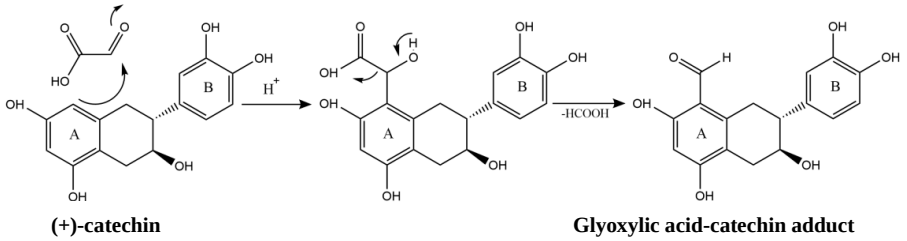


Fig. 2.9. Reactive dicarbonyls trapping by (+)-catechin pathway leading to the formation of dicarbonyl-catechin adduct (adapted from Es-Safi *et al*., 2000)

3) *Inhibition by scavenging Maillard-derived radicals*. MR also generates stable radicals, and it was proved in foods and model systems (Jehle, Lund, Øgendal, & Andersen, 2011; Namiki & Hayashi, 1983). It was proposed that the formation of free radicals occurs at the early stage of MR before the Amadori product rearrangement, and their amount strongly contributes to the browning and MRPs formation (Namiki & Hayashi, 1983).The addition of phenolic compounds, such as butylated hydroxyanisole, sesamol and epigallocatechin gallate (EGCG), inhibited the formation of unstable pyrazine cation radicals and unstable carbon-centred radicals in the model systems thus inhibiting the formation of imidazoquinoxaline-type HAs

(Kato, Harashima, Moriya, Kikugawa, & Hiramoto, 1996). In another study, catechin and EGCG showed an enhancing or suppressing effect on pyrazinium radical formation, which depended on their concentration (Bin, Peterson, & Elias, 2012). In several studies, it was found that free radical intermediates, pyrazine cation radicals and carbon-centered radicals are involved in the formation of MRPs, such as HAs (Kikugawa, 1999), but in other studies it was proposed that they are not the key intermediates in their formation (Zöchling & Murkovic, 2002). Therefore, the reduction of MRPs by phenolic compounds through free radicals scavenging is one of the minor pathways by which their can act as inhibitors. Nevertheless, many studies demonstrated lack of significant positive correlations between the radical scavenging capacity and MRPs inhibition (Cheng, Chen, & Wang, 2007; Oguri, Suda, Totsuka, Sugimura, & Wakabayashi, 1998).

4) Inhibition by trapping Strecker aldehydes. MR generates aldehydes through Strecker degradation of amino acids, secondary lipids oxidation products and furfurals. The mechanism by which flavonoids scavenge aldehydes is similar to that of α-dicarbonyls. The A-ring of flavonoids or phenylpropanoids, due to the activating hydroxyl groups, bears binding sites for electrophiles (aldehydes). The Strecker aldehyde, such as phenylacetaldehyde, which is the main precursor for PhIP generation (HAs), was trapped by various flavonoids by the formation of adducts (Fig. 2.10).

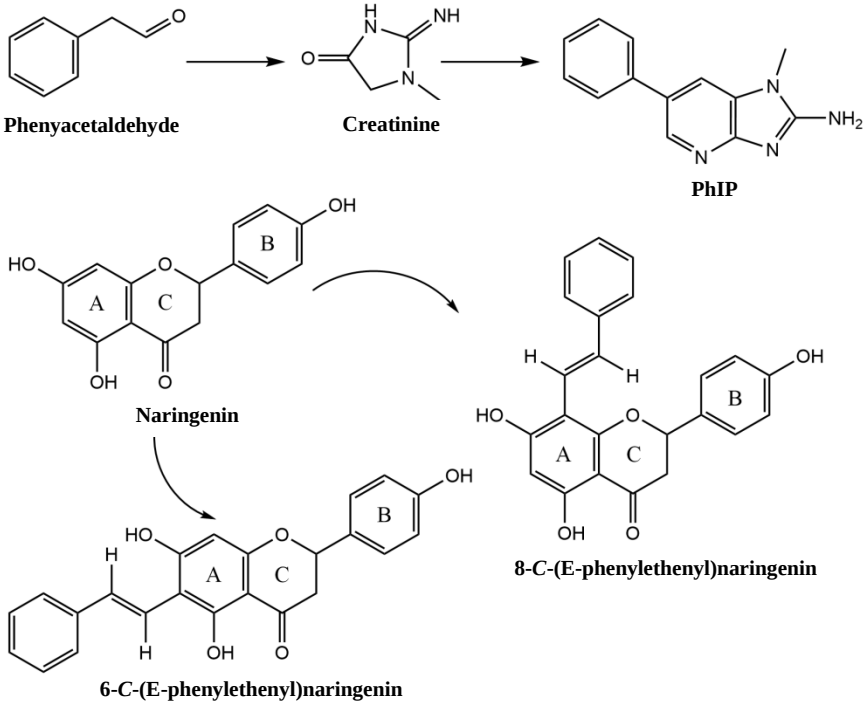


Fig. 2.10. Inhibitory mechanism of naringenin in PhIP formation (Cheng *et al*., 2008)

Favonoids, such as naringenin (Cheng *et al*., 2008), dihydromyricetin (Zhou *et al*., 2018), epigallocatechin (Cheng *et al*., 2009), showed a potential to inhibit

phenylacetaldehyde formation and thus the PhIP level when tested in meat model systems. Acetaldehyde, which is a precursor for MeIQx formation, was shown to be scavenged by (+)-catechin in the wine model system (Peterson & Waterhouse, 2016). Recently, it was shown that the aldehyde-trapping ability of phenolics is selective, and the formation of aldehyde-phenol adducts depends on the structures of both phenol and aldehyde (Hidalgo, Aguilar, & Zamora, 2017).

All the mitigation strategies may involve unwanted side reactions caused by the phenolic compound, such as protein modification, discoloration, changes of taste and flavor. Phenolic compounds due to their ability to trap carbonyl compounds which are also flavor compounds may change the food flavor (Hidalgo *et al*., 2017), therefore, the concentration of phytochemicals can be limited due to the changed sensorial characteristics of the product. In addition, lipid-derived reactive carbonyls (2,4-hexadienal, 2,4-heptadienal, and 2,4-decadienal) were also trapped by phenolic compounds (Hidalgo & Zamora, 2018c).

2.2.3. Impact of phytochemicals on the formation of advanced glycation end-products in milk

In milk products, especially in infant formulas, in which, the content of substrates for MR is high, is challenged to maintain the nutritional value and the chemical safety after heat treatment. CML is the dominant AGEs product in milk, whose formation correlates well to the extent of lysine blockage (Leclère, Birlouez-Aragon, & Meli, 2002). Another negative factor of the heat treatment of milk is the formation of off-flavors, especially in UHT milk, because it leads to the reduced shelf life of the product. The addition of phytochemicals to milk or milk products seems to be one of the possible approaches towards the objective of avoiding the negative effects of heat treatment, while the same time the products could be enriched with bioactive constituents in the case of usage of plant extracts.

It was shown that olive mill waste water phenolic powder reduced CML and reactive carbonyl species formation in UHT treated milk (Troise *et al*., 2014). Phenolic compounds, such as catechin, genistein, and 1,3,5-trihydroxybenzene, suppressed the development of off-flavor and reactive carbonyl species in UHT-treated milk (Kokkinidou & Peterson, 2013; Kokkinidou & Peterson, 2014). The green tea extract inhibited the formation of Strecker aldehydes in lactose-hydrolyzed UHT treated milk (Jansson *et al*., 2017). The formation of CML and crosslinked AGEs was inhibited with *Aloe Vera* anthraquinones, such as aloe-emodin, emodin, and chrysophanol in the bovine serum albumin/glucose model system (Kang *et al*., 2017). Recently, it was also demonstrated that catechin quinones (epicatechin and EGCG) reduced the CML amount in coconut milk by reacting directly with free CML in the solution (Li *et al*., 2018).

2.2.4. Impact of phytochemicals on the formation of heterocyclic amines in meat

It is known that phenolic compounds can inhibit or promote HAs formation in meat. Many natural extracts and phenolic compounds showed the inhibition effect on HAs formation and have been described in several reviews (Rannou, Laroque,

Renault, Prost, & Sérot, 2016; Xiong, 2017). Firstly, the inhibition effect of HAs by antioxidants was postulated by free radical intermediates scavenging (Kikugawa, 1999). Later studies demonstrated inhibition of HAs through carbonyl-scavenging. Penylacetaldehyde, which is the intermediate in the formation of PhIP, was scavenged by EGCG (Cheng *et al*., 2007), naringenin (Cheng *et al*., 2008), phlorizin, epigallocatechin gallate and quercetin (Zhu *et al*., 2016) through the formation of adducts. Further studies showed that the structure of phenolic compounds is a very important factor, and different effects of phenolics on HAs formation have been partly explained by their structural characteristics (Salazar *et al*., 2014). Recently, it was observed that phenolics which have high carbonyl scavenging activity (*m*-diphenols) inhibited the formation of PhIP, while those phenolics that acted as radical scavengers (*o*- and *p*-diphenols) promoted the formation of PhIP (Hidalgo, Navarro, & Zamora, 2018b). Such spices as rosemary, thyme, sage and garlic, applied on the beef surface before frying reduced the formation of HAs (Murkovic, Steinberger, & Pfannhauser, 1998). Inhibition of HAs was also shown by water soluble vitamins, while pyridoxamine, niacin and ascorbic acid showed the greatest reducing effect both in model systems and in beef patties (Wong et al., 2012). In the recent papers, it was shown that Sichuan pepper, sanshoamide extract (Zeng *et al*., 2018), black pepper, piperine (Zeng *et al*., 2017) reduced the formation of HAs in beef patties. Marinated meat with different ingredients (tea extract, wine, beer, hibiscus, grape seed and rosemary extracts) looked promising regarding the reduction of HAs (Gibis & Weiss, 2012; Quelhas *et al*., 2010). The amido acids that were also found could inhibit HAs formation (Zamora *et al*., 2013). In a recent study, it was found that tryptophan, lysine and proline amino acids were the best inhibitors of PhIP formation, while phenylalanine and asparagine showed the lowest inhibitory effect (Linghu, Karim, & Smith, 2017). The inhibition mechanism was explained through the reaction between penylacetaldehyde with the amino acid ‘penylacetaldehyde-scavenging mechanism’. Also, it was found that the essential oil, e.g., from rosemary and marjoram, increased the PhIP formation (Zöchling *et al*., 2002).

2.3. NATURAL PHYTOCHEMICALS POSSESSING BIOLOGICAL ACTIVITIES

2.3.1. Characterization of betalains and *Beta vulgaris* roots

Previously, betaxanthins were called *flavonoids*, while betacyanins were addressed as nitrogenous anthocyanins. In 1968, both pigments were started to call *betalains*, and this name has been used until now. Betalains are water soluble nitrogen-containing pigments which are present in plant vacuoles of *Caryophyllales* (Brockington, Walker, Glover, Soltis, & Soltis, 2011) and in cells of higher fungi (*Amanita muscaria*, *Hygrocybe*, and *Hygrophorus*) (Babos et al., 2011; Musso, 1979). Red beets (*Beta vulgaris*) are one of the main plant which contains high amounts of betalains. There are two types of betalains: betacyanins, which exhibit red-violet coloration with the absorbance maximum at 536 nm (λ_m), and betaxanthins, which are yellow pigments, and their absorbance spectrum has a maximal wavelength at 480 nm. The main structural characteristic of betalains is the presence of betalamic acid, which, in the case of betacyanins, is condensed with *cyclo*-dihydroxyphenylalanine (*cyclo*-DOPA) or its glucosyl derivatives, while, in the case of betaxanthins, it is condensed with various amines and amino acids (Fig. 2.11).

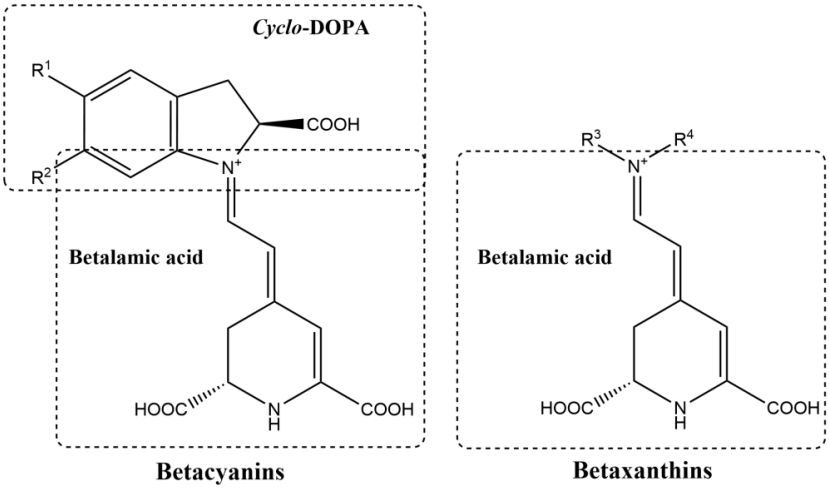


Fig. 2.11. Structures of betalains: Betanin: R^1, glucose, R^2, OH; Betanidin: R^1, OH, R^2, OH; Vulgaxanthin I: R^3, glutamine, R^4, H; Vulgaxanthin II: R^3, glutamic acid, R^4, H; Valine-Bx: R^3, valine, R^4, H; Proline-Bx: R^3, proline, R^4, H

The main betalains in red beet roots in the class of betacyanins are betanin and isobetanin, while in the class of betaxanthins, it is Vulgaxanthin I (Fig. 2.12) (Stintzing & Carle, 2007). Other betalains, such as betanidin, vulgaxanthin II, miraxanthin valine/proline-Bx, are in lower amounts. In beetroots, some of phenolic compounds were also found: phenolic acids, such as 5,5',6,6'-tetrahydroxy-3,3'-biindolyl, feruloylglucose and 4-hydroxybenzoic acid, caffeic acid and β-D-fructofuranosyl-α-D-(6-O-*E*-feruloylglucopyranoside) and phenolic amines such as *N*-trans-feruloyltyramine and *N*-trans-feruloylhomovanillylamine, and flavonoids,

such as betagarin, betavulgarin, cochliophilin A, dihydroisorhamnetin and rutin, flavan-3-ols such as catechnin and epicatechin (Georgiev *et al*., 2010; Kujala, Vienola, Klika, Loponen, & Pihlaja, 2002). When comparing their amounts which are present in roots, betalains comprise 50 times higher amounts than phenolic compounds.

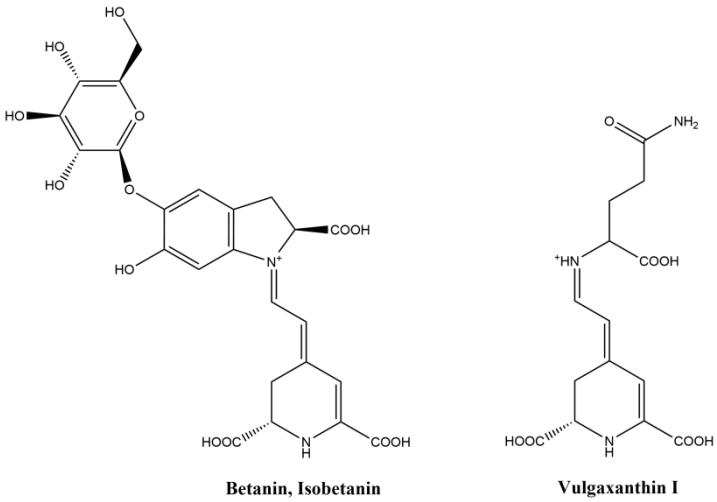


Fig. 2.12. Structures of the main betalains in the roots of *Beta vulgaris*: A, betanin, isobetanin; B, Vulgaxanthin I

In the recent years, beetroot has been attracting great attention as natural colorants and as a health promoting functional ingredient. Interest to betalains has grown since their antiradical activity was determined by Escribano *et al*. (1998). Later on, many studies have proved very high antiradical properties of betalains *in vitro* (Gandía-Herrero, Escribano, & García-Carmona, 2009; Gliszczyńska-Świgło, Szymusiak, & Malinowska, 2006; Kanner, Harel, & Granit, 2001; Pedreño & Escribano, 2001; Stintzing *et al*., 2005; Tesoriere, Allegra, Gentile, & Livrea, 2009; Vulić *et al*., 2012). It was reported that the 'basic antiradical activity' of betalains was not linked to the presecnce of hydroxyl groups and aromaticity in their structure and was more associated with the electronic resonance system held between two nitrogen atoms. However, the antiradical activity of betalains is strongly increased with the number of hydroxyl and imino groups (Gandía-Herrero, Escribano, & García-Carmona, 2010). The antiradical capacity of betalains depends on their structure. For example, for the simplest betalains without aromatic resonance, the charge and hydroxyl groups TEAC value was determined to be around 2.4 (Gandía-Herrero *et al*., 2010). In betalains with charge and no aromatic resonance, this value decreases to 1.8 yet increases to 2.8 for the compounds carrying the aromatic ring in the structure. For betalains having indoline-like substructures, this value goes up to 4 units (Gandía-Herrero *et al*., 2010). When phenolic hydroxyl groups are present in the betaxanthin and betacyanin structure, it strongly enhances their radical scavenging capacity. The increase of 1.6 and 3.4 units in the TEAC values was determined when one and two hydroxyl groups was/were present in the structure of betaxhanthins, respectively

(Gandía-Herrero *et al*., 2009). Meanwhile, the increase of 2.5 and 4.1 units in the TEAC values was determined when one and two hydroxyl groups were present in the betacyanin structure, respectively (Gandía-Herrero *et al*., 2010). Moreover, it was shown that glycosylation of betalains reduces their antiradical activity, and 6-O-glycosylated betacyanins are more active radical scavengers than 5-O-glycosylated ones. Contrary to glycosylation, acylation enhanced the antiradical activity of betacyanins. In general, betacyanins are stronger scavengers of free radicals than betaxanthins, and antiradical activity is strongly dependent on their structure. Different TEAC values according to the research of Gandía-Herrero *et al*. (2009) and Gandía-Herrero *et al*. (2010) were found for different structures of betalains: 4.5 for betanin, 6.5 for betanidin and dopaxanthin, 4 for indoline, (S)-indoline-2-carboxylic acid, and 3- or 4-hydroxyphenethylamine, 2.5 for 1-aminopropane, ethylamine, aniline, (S)-phenylalanine and 2-phenylethylamine. The antioxidant activity of betalains was found to be pH dependent and was related to their hydrogen and electron donation ability (Gliszczyńska-Świgło *et al*., 2006). It was shown that the antiradical activity increases at higher pH values, and it was associated with the deprotonation of the NH group which is present in all the betalains (Gandía-Herrero *et al*., 2010). For example, betanin at pH>4 was 1.5–2.0-times more active than in some anthocyanins (Gliszczyńska-Świgło *et al*., 2006).

Previous studies showed that, after *in vitro* digestion of beetroot juice, antioxidive propertieswas increased. Other *in vitro* studies showed the capacity of betanin and betanidin to inhibit lipid peroxidation and heme decomposition (Kanner *et al*., 2001; Tesoriere *et al*., 2009), while betanin and indicaxanthin showed ability to scavenge hypoclorous acid (Allegra *et al*., 2005). Besides, antimicrobial and cytotoxic activities were demonstrated by beetroot pomace (Vulić *et al*., 2013).

The bioavailability of betanin has not been fully clarified yet. Some studies showed that, after consumption of beetroot juice or beetroots, betanin is primarily excreted via urine (Frank *et al*., 2005; Wiczkowski, Romaszko, Szawara-Nowak, & Piskula, 2018). However, in plasma, poor bioavailability of betanin was determined (Clifford *et al*., 2017). Nevertheless, the elevated antioxidative and nitric oxide levels in plasma (Frank *et al*., 2005) and antioxidant compounds (including phenolics and betalains) in excreted urine (Netzel *et al*., 2005) were determined with the oral consumption of red beet juice. There are many studies showing the health benefits of betalains *in vivo* (Clifford, Howatson, West, & Stevenson, 2015). Betalains (mainly betanin) suppressed the formation of skin and lung cancer (Kapadia, Tokuda, Konoshima, & Nishino, 1996), skin and liver tumors (Kapadia *et al*., 2003) in mice, protected red blood cells from oxidative hemolysis (Tesoriere, Butera, Allegra, Fazzari, & Livrea, 2005), showed antihyperglycemic activity against the STZ-NA induced diabetic rats (Dhananjayan, Kathiroli, Subramani, & Veerasamy, 2017). Besides, betalains do not provide any toxic effects on the human body even when consumed at high doses (5 g/kg body weight) (Reynoso, Giner, & de Mejia, 1999).

As betalains are released from their protecting compartment, there are multiple exogenous factors which affect their stability. The factors affecting betalains stability are pH, light, oxygen, water activity, metal ions, temperature and enzymatic reactions (Azeredo, 2009). It was determined that betalains are more stable in the matrix of juice

which is constituted mainly of sugars, pectic substances and proteins. The main pathways of betalains degradation are via isomerization, decarboxylation, dehydrogenation, deglycosylation and hydrolytic cleavage reactions. The fast degradation rate of betanin was associated with its high ability to quench singlet oxygen and to support photo-protective role *in vivo* (Wendel *et al*., 2016). The degradation of betalains can also occur in plants through enzymatic reactions induced by the so-called decolorizing enzymes (Strack, Vogt, & Schliemann, 2003), such as β-glucosidases, polyphenol oxidases and peroxidases. The degradation products of enzymatic reactions are similar to those of exogenous factors, such as pH or thermal heating. During purification process of betalains these decolorizing enzymes are also active and induce betalains degradation. It has been found that the activity of polyphenol oxidases and *β*-glucosidase enzymes was suppressed by ascorbic acid (Strack *et al*., 2003). Therefore, it was strongly recommended to use 50 mM of ascorbic acid during purification of betalains (Schliemann, Kobayashi, & Strack, 1999). Moreover, betacyanins were shown to be more stable than betaxanthins (Slimen, Najar, & Abderrabba, 2017). As betalains react with singlet oxygen, the presence of high oxygen level favors their degradation rate, while, in the presence of light, degradation depends on the oxygen level. In the absence of oxygen, light exposure is negligible. Metal ions also accelerate the degradation of betalains. However, temperature is the most decisive factor on betalain stability. Betalain degradation follows the first-order reaction kinetics during thermal heating (Herbach, Stintzing, & Carle, 2004a). Decarbocylation and dehydrogenation of betanin is favored during thermal treatment, but the predominant pathway is hydrolytic cleavage (Herbach, Stintzing, & Reinhold, 2005). The proposed degradation pathway of betanin during heating is showed in Fig. 2.13, while other products formed after betanin heating are listed in Table. 2.2. The structures of the main degradation products are presented in Fig. 2.14. During thermal treatment, betanin decomposes into betalamic acid and *cyclo*-dopa-5-O-β-glucoside and its acyl derivatives through the hydrolytic cleavage of the aldimine bond. After thermal treatment, hydrolyzed betanin can be regenerated by the cooling solution and pH adjustment (Han, Kim, Kim, & Kim, 1998). During the regeneration of betanin, the amine group of *cyclo*-dopa-5-O-*β*-glucoside condenses to the aldehyde group of betalamic acid. Betalamic acid was found to be stable during thermal treatment and is found in a traceable amount. In contrast, *cyclo*-dopa-5-O-*β*-glucoside is less stable and decomposes to dopamine after prolonged thermal treatment, thus preventing the regeneration of betanin (Herbach *et al*., 2005). The main monosaccharides of betacyanidins are glucose, glucuronic acid, and apiose, while the main substituents are ferulic acid, *ρ*-coumaric acid, caffeic acid and malonic acid (Slimen *et al*., 2017). The oxidation pathways of betacyanins and their degradation products were also investigated. It was found that enzymatic and nonenzymatic oxidation of betalains yields in similar degradation products which strongly depend on pH. Studies on the enzymatic oxidation of betacyanidins revealed the formation of 5,6-hidydroxyindole derivatives. The dominant products of enzymatic oxidation (pH 3) of betanidin were 2-decarboxy-2,3-dehydrobetanidin and 2,17-bidecarboxy-2,3-dehyrobetanidin, which were

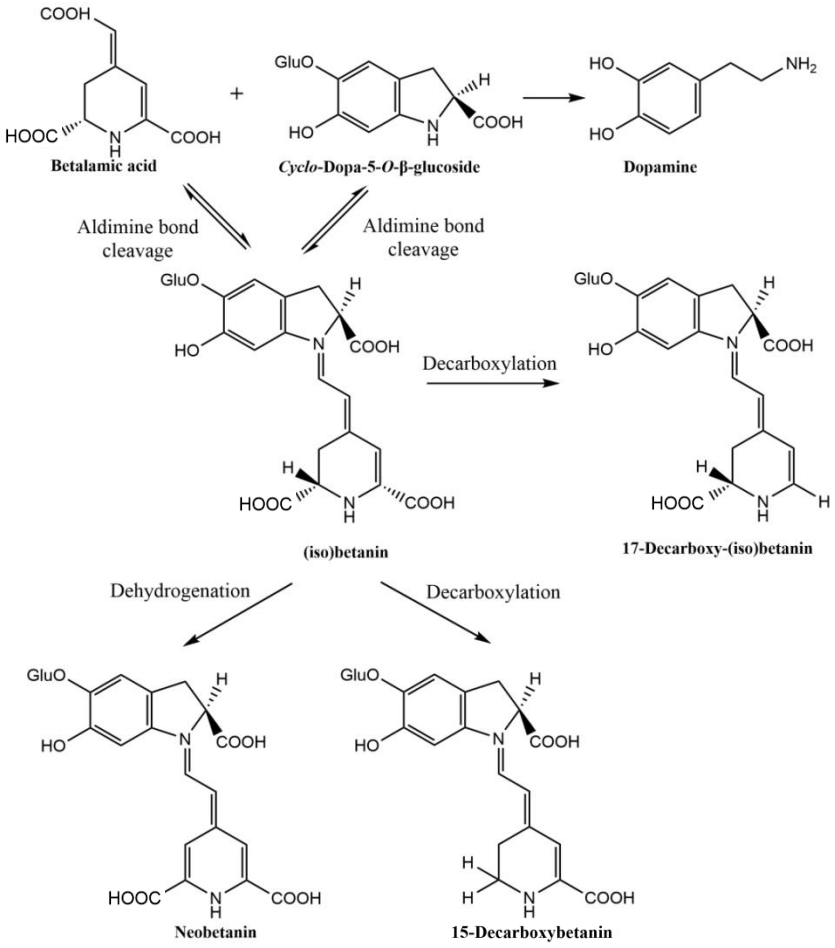
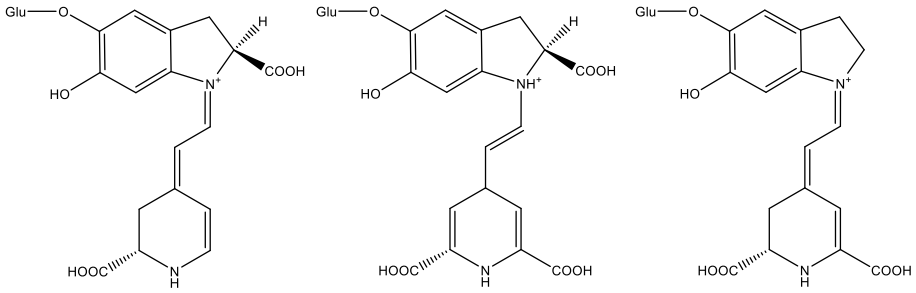
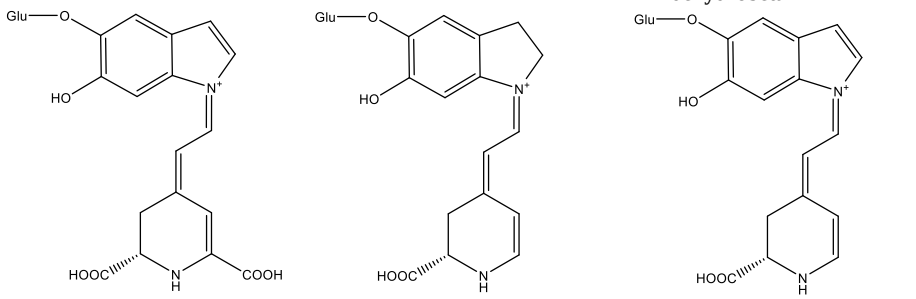


Fig. 2.13. Thermal degradation of betanin pathway (adapted from Herbach *et al*. 2004; Herbach *et al*. 2005)

plausible to be produced through quinonoid intermediates such as formation of dopachrome and quinone methide. Within the pH range of 4–8, betanidin oxidation yielded mainly betanidin quinonoid and 2-decarboxy-2,3-dehydrobetanidin. Meanwhile, betanin was mainly oxidized to 2,3-dehydro- or neoderivatives through quinone methide intermediate formation (Wybraniec & Michałowski, 2011). Recent studies on nonenzymatic oxidation of betacyanidins showed betanin oxidase to 2-decarboxy-2,3-dehydrobetanin through the formation of quinone methide intermediate. In contrast, oxidation of 2-decarboxy-betanin resulted in the formation of 2-decarboxy-neobetanin, 2-decarboxy-2,3-dehydrobetanin and 2-decarboxy-2,3-dehydroneobetanin.



17-dehydrobetanin **Neobetanin** **2-decarboxy-2,3-dehydrobetanin**



2-decarboxybetanin **2,17-bidecarboxybetanin** **2,17-bidecarboxy-2,3-dehydrobetanin**

Fig. 2.14. Structures of degradation products of betalains

Still further, those compounds can undergo decarboxylation at carbon C-17 and yield 2,17-bidecarboxy-2,3-dehydro-betanin, 2,15,17-tricarboxy-2,3-dehydro-neobetanin and 2,17-bidecarboxy-neobetanin. In general, the formation of the above mentioned oxidation products of betacyanidins was related to the formation of quinonoid derivatives intermediates. Red beet extracts (E162) are used for coloring purposes in the food industry. E 162 approximately contains 80% of betacyanins and 20% of betaxanthins.

The main part constitutes betacyanins, of which, betanin amounts up to 75–95%, and isobetanin up to 15–45%, whereas the lower part constitutes betaxanthins, of which, Vulgaxanthin I amounts up to 25–70% and vulgaxanthin II up to 5–15%. Besides, beetroot red contains sugars, salts and proteins which are natural components in red beets. The purity of the commercial material of red beets should be not less than 0.4% of betanin. The other 99.6% is calculated for proteins, sugars and salts which are naturally present in red beets (EFSA, 2015b). The main concern assosiated with the use of beetroot red is the nitrate content. According to EU specification (Commision Directive 95/36/EC), there has to be no more than 2 gram of nitrate anion in 1 gram of red color. Usually, betanin (E162) is made from beet juice concentrate or dehydrated beet powder. The ideal products for coloring with betalain-based preparations are low acid foods due to their pH stability between 3 and 7 (Pedreño & Escribano, 2001) and products which are moderately heat-treated. For example, such common products are milk, yogurt, ice-cream and sausages (Kumar, Manoj, Shetty, Prakash, & Giridhar, 2015; Martínez, Cilla, Beltrán, & Roncalés, 2006).

Table 2.2. Betalains and their degradation products[1]

No.	Name	$[M+H]^+$ *m/z*	Reference
Main betalains			
1.	Betanin	551	1
2.	Isobetanin	551	1
3.	Betanidin	389	1
4.	Neobetanin	549	1
5.	Vulgaxanthin I	340	1
6.	Vulgaxanthin II	341	1
7.	Miraxanthin V	347	1
8.	Portulacaxanthin II	375	1
9.	Portulacaxanthin III	269	1
10.	Histamine-Bx	305	1
11.	Valine-Bx	311	1
12.	Serine-Bx	299	1
13.	Phenylalanine-Bx	359	1
14.	Isoleucine-Bx	325	1
Degradation products of thermal treatment and oxidation			
15.	cyclo-Dopa-5-O-*β*-glucoside	358	2
16.	2-(2,3-Dihydroxyphenyl)ethylamine (Domamine)	137	2
17.	15-Hydroxybetanidin 5-O-*β*-glucoside	567	2
18.	Betanidin 5-O-*β*-glucoside, decarboxylated	507	2
19.	15-Hydroxyisobetanidin 5-O-β-glucoside	567	2
20.	2-(2,3-Dihydroxyphenyl 3-O-*β*-glucoside)-2-amino-1-propanal	182	2
21.	5,6-Dihydroxyindole 5-O-*β*-glucoside	312	2
22.	Betalamic acid	212	2
23.	2-decarboxybetanin	507	2
24.	Neobetanidin, bi-decarboxylated, dehydrogenated	297	2
25.	2,17-bidecarboxy-2,3dehydrobetanin	461	3
26.	2-decarboxy-2,3-dehydrobetanin	505	3
27.	2,15,17-tridecarboxy-2,3-dehydroneobetanin	415	3
28.	2,17-bidecarboxy-2,3-dehydroneobetanin	459	3
29.	2-decarboxy-2,3-dehydroneobetanin	503	3
30.	2-decarboxybetanin	507	3

References: 1, (Slimen *et al*., 2017); 2, (Herbach *et al*., 2005); 3, (Wybraniec *et al*., 2013)

A wider application of red beet has increased since their stability was enhanced by using encapsulation approaches, or they were incorporated in W/O/W emulsions (Gandía-Herrero, Cabanes, Escribano, García-Carmona, & Jiménez-Atiénzar, 2013; Pagano *et al*., 2018; Serris & Biliaderis, 2001). Recent studies showed other possible application areas of beetroot: beetroot extracts were incorporated in the chewing gum system (Chranioti, Nikoloudaki, & Tzia, 2015), beetroot juice was stabilized in double emulsion and used in meat systems both for coloring and for animal fat replacement (Eisinaite, Juraite, Schroën, & Leskauskaite, 2017), beetroot pomace extracts showed potential usage in pseudocereals-enriched einkorn water biscuits (Hidalgo,

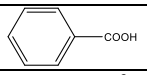
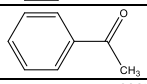
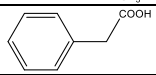
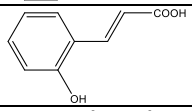
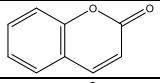
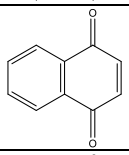
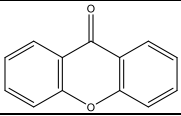
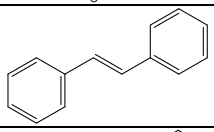
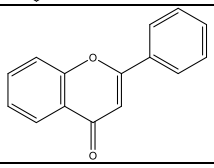
Brandolini, Čanadanović-Brunet, Ćetković, & Tumbas Šaponjac, 2018a), beetroot extract was used for impregnation of potato snacks in order to obtain healthier functional snacks (Moreira & Almohaimeed, 2018), beetroot powder as a source of dietary fibre was used in wheat dough (Kohajdová, Karovičová, Kuchtová, & Lauková, 2018).

2.3.2. Characterization of phenolic compounds and *Vaccinium vitis-idaea* L. leaves

Plants are the main sources of phenolic compounds that may occur in all parts of plants, such as fruits, nuts, seeds, leaves, roots and barks. Plants contain various groups of phenolic and polyphenolic compounds, such as phenolic acids, flavonoids, stilbenes, hydrocinnamic acids and other compounds listed in Table 2.3. The main role of polyphenols and essential oils which are synthetized in plants is to prevent insect, fungal and microbial infections of the plant tissues. Not surprisingly, plant antioxidants have been showed to exhibit physiological activities, such as antibacterial (Bajpai, Al-Reza, Choi, Lee, & Kang, 2009; Huang, Cai, & Zhang, 2009; Martins, Barros, & Ferreira, 2016), antiviral (Suárez *et al*., 2010), antimutagenic (Zahin, Ahmad, & Aqil, 2010). Flavonoids constitute the largest group of plants phenolics such as flavones, flavonols, isoflavones, flavanones, flavanonols, and chalcones. These compounds occur in almost all types of plant tissues. Usually, flavonoids in plants occur as glycosides. Flavonoids may act as antioxidants by scavenging radicals, quenching singlet oxygen, inhibiting lipoxygenases and chelating metals. Flavonoids are responsible for the antioxidant activity of many plant and spice extracts. Phenolic acids, such as hydroxycinnamic (caffeic, ferullic and sinapic) acids, hydroxybenzoic acids (ellagic, gallic, salicylic and vanillic) and hydroxycoumarin (scopoletin) are also frequently found in plants. Phenolic acids usually occur in plants as esters of organic acids or glycosides. Their antioxidant activity depends on the molecular structure and substitutions. It has been observed that monophenols were less efficient than polyphenols. The antioxidant activity of monophenols is increasing by one or two methoxy substitutions. It was reported that the antioxidative effect of cinnamic acid derivatives is higher than that of benzoic acid derivatives (Pokorný, Yanishlieva, & Gordon, 2001). The antioxidant activity of phenolic compounds depends on the position and degree of hydroxylation, polarity, solubility, reducing potential, and the stability of the phenolic compounds during food processing operations, as well as the stability of phenolic radicals (Crozier, Jaganath, Jaganath, Clifford, & Clifford, 2009; Kumar & Pandey, 2013). Food antioxidants are compounds inhibiting oxidative processes which cause the deterioration of the food lipid quality (Decker, 2010). Antioxidants may occur as natural constituents of foods and can also be purposely added to products or formed during processing (Reische, Lillard, & Eitenmiller, 2002). Their main role is to maintain the food quality and extend their shelf-life. Antioxidants used in food industry are classified into synthetic and natural ones.

The plant leaves of the *Ericaceae* family and the *Vaccinium* genera are rich in secondary metabolites. LL as several other berry leaves including cranberry, blueberry, and bilberry contains phenolic acids (hydroxybenzoic and hydroxycinnamic acids and their derivatives), flavonoids, such as flavonols and

Table 2.3. Basic structure of phenolic and polyphenolic compounds

Classification	Basic structure
Phenolic acids	
Acetophenones	
Phenylacetic acid	
Hydroxycinnamic acids	
Coumarins	
Naphthoquinones	
Xanthones	
Stilbenes	
Flavonoids	

flavanols, and tannins, such as proanthocyanidins (condensed tannins) (Kähkönen, Hopia, & Heinonen, 2001). All of these plants are rich in bioactive phytochemicals which contribute to their biological activities and hence are beneficial for health; therefore, bioactive compounds from leaves could be used to lower the harmful effects (lipid oxidation, microbial growth and toxic compounds) of food which arise due to processing or storage. Moreover, food products could be enriched with bioactive compounds and labeled as functional foods or 'superfoods'.

Lingonberry (*Vaccinium vitis-idaea* L., Ericaceae) is one of the most important Nordic wild berry plants. The leaves have a waxy surface and are green on top and light green underneath; they contain a low yield of essential oils (0.06%). Terpenoids (49.5%) and fatty acid derivatives (34.1%) were predominant in leaves. The main compounds detected in oil were *a*-terpineol (17%), pentacosone (6.4%), (*E,E*)-*a*-farnesene (4.9%), linalool (7.3%) and (*Z*)-hex-3-en-1-ol (4.4%) (Radulović, Blagojević, & Palić, 2010). The composition of phenolic compounds of berries (Bujor, Ginies, Popa, & Dufour, 2018; Dróżdż, Šėžienė, & Pyrzynska, 2017; Ek,

Kartimo, Mattila, & Tolonen, 2006; Häkkinen & Auriola, 1998; Kähkönen *et al*., 2001; Määttä-Riihinen, Kamal-Eldin, Mattila, González-Paramás, & Törrönen, 2004; Teleszko & Wojdyło, 2015; Tian *et al*., 2018; Zheng & Wang, 2003) and leaves (Bujor *et al*., 2018; Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen, 2009; Ieri, Martini, Innocenti, & Mulinacci, 2013; Liu *et al*., 2014; Teleszko & Wojdyło, 2015; Tian *et al*., 2017; Vyas, Kalidindi, Chibrikova, Igamberdiev, & Weber, 2013) of *Vaccinium vitis idaea* have already been extensively studied previously. In general, the composition of phenolic compounds of berries and leaves was found to be quite similar, except that berries contain anthocyanins (hexoside derivatives of cyanidin, peonidin, petunidin, malvidin and delphinidin) and hexose sugar conjugates of ferulic acid (ferulic acid-hexosides) (Ek *et al*., 2006; Mane, Loonis, Juhel, Dufour, & Malien-Aubert, 2011). In addition, it was reported that leaves are richer in phenolics than berries (Bujor *et al*., 2018).

The most abundant phenolic classes in leaves are flavan-3-ols (catechins), flavanols (quercetin and kaemferol glycosides), hydroxycinnamic acids (ferulic acid, *p*-coumaric acid and caffeic acid) and proanthocyanidins (Hokkanen *et al*., 2009; Ieri *et al*., 2013; Liu *et al*., 2014; Mane *et al*., 2011) (Fig. 2.15). *Vaccinium vitis-idaea* L. leaves differ from its berries and leaves of other berries as they contain a high amount of glucoside such as arbutin and its derivatives (2-*O*-caffeoylarbutin and acetyl arbutin).

The amount of arbutin in LL was reported to be around 31–50% of the total phenols (approximately 2711 mg/100 g fresh leaves) (Jurica *et al*., 2018; Tian *et al*., 2017). Furthermore, pears also contain arbutin which can be found in their leaves, peel, flesh and stems (Cho *et al*., 2011); however, the highest concentration is found in the peel, which could range from 0.46 to 1.01 mg/g of fresh weight (Sasaki, Ichitani, Kunimoto, Asada, & Nakamura, 2014). As it is already known, arbutin is the key component in plant leaves (*Arbutus unedo* L., *Arctostaphylos uva ursi* L., *Bruckentalia spiculifolia Rchb*., *Calluna vulgaris Salisb*., *Erica arborea* L. and *Erica carnea* L. and others) which are used to treat bladder and urinary tract infections (Pavlović, Lakušić, Došlov-Kokoruš, & Kovačević, 2009; Schindler *et al*., 2002). It was determined that arbutin and its hydroquinone form possess strong radical scavenging activities (Tai, Ohno, & Ito, 2016; Takebayashi *et al*., 2010), antiinflammatory effects (Lee & Kim, 2012) and do not exhibit cytotoxic or DNA damage in *in vitro* assays (the lymphocytes model system) (Jurica *et al*., 2018).

Furthermore, the triterpenoid profile of leaves was also investigated (Szakiel, Pączkowski, Koivuniemi, & Huttunen, 2012). There have been numerous reports on the health-promoting and nutritional beneficial properties of lingonberry berries (Jepson & Craig, 2008; Kivimäki *et al*., 2012; Kivimäki, Siltari, Ehlers, Korpela, & Vapaatalo, 2013; Kylli *et al*., 2011; Toivanen *et al*., 2009; Wong *et al*., 2012); however, leaves have been scarcely investigated. It has been known for centuries that lingonberry leaves possess diuretic, astringent, and antiseptic activities, and therefore they were used to treat urinary tract infections in the traditional medicine (Davidson, Zimmermann, Jungfer, & Chrubasik-Hausmann, 2014). Recently, it was reported that LL is denoted by strong antioxidative (Vyas *et al*., 2013), antibacterial (Tian *et al*., 2018), anti-inflammatory activities and a neuroprotective effect against

glutamate-mediated excitotoxicity (Vyas *et al*., 2013). Ethanol extracts of LL and stems have shown anti-inflammatory, anti-coughing and phlegm-removing effects *in vivo*, while arbutin and fraxin were assigned to these effects because they were the main absorbed compounds into the rat blood (Wang *et al*., 2005). In addition, the sheep diet supplemented with dried LL slightly stimulated pancreas exocrine activity (Majewska, Pająk, Skomiał, Miltko, & Kowalik, 2017).

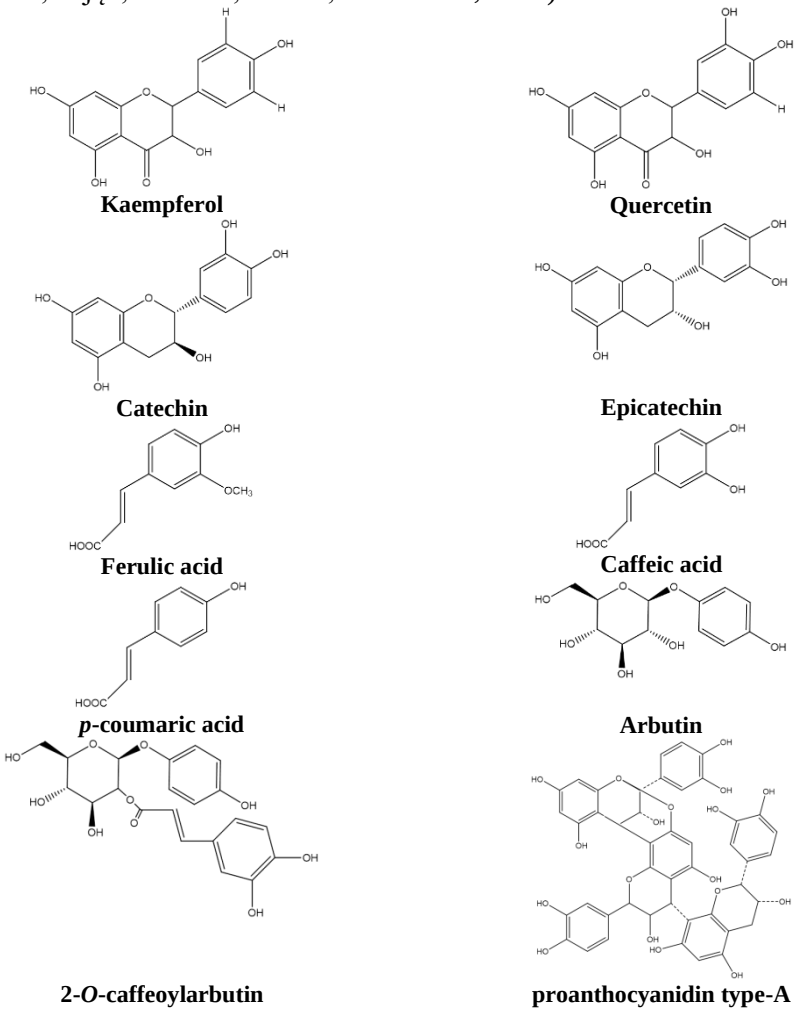


Fig. 2.15. Chemical structures of the main phenolic compounds found in lingonberry leaves

As berry leaves have been reported to be a rich source of phytochemicals which possess various biological activities, scientific interest is strongly growing regarding their possible use in the nutrition and medicine areas in recent years (Ferlemi & Lamari, 2016). Moreover, according to the *Novel Food Catalogue* in the European Commission, the application of *Vaccinium vitis-idaea* L. leaves has been authorized as food supplements; therefore, it is considered as an attractive source of phytochemicals for further applications in foods. The antiglycation activity of extracts

from *Vaccinium vitis-idaea* berries have been shown (Beaulieu *et al*., 2010), while there have been no reports on antiglycation properties or HAs inhibition of extracts from *Vaccinium vitis-idaea* L. leaves. Leaves were reported to be a rich source of flavonoids, especially in catechins, which was proven in many authors' works as a highly effective inhibitor in AGEs and HAs formation. Betalains from *Beta vulgaris* under E162 food additive number are broadly used in many foods for coloring purposes. Therefore, the objective to know how betalains can turn MR is valid, while, up to date, there has been no research performed on their effects on AGEs or HAs formation. The pure structures of betalains were shown to have very good antioxidant properties. Nevertheless, betalains are unstable and degrade during heating. One of the expectations to inhibit the MRPs formation of betalains is through the competition of amino acids. The heat unstable betalains degrade and produce amine-substances which might compete with the amino acid. In the case of betanin degradation, dopamine formation during heating was reported. Dopamine can oxidize to the quinone form and react with other amino acids.

3. MATERIALS AND METHODS

This chapter quotes some passages from the article Effects of beetroot (*Beta vulgaris*) preparations on the Maillard reaction products in milk and meat-protein model systems (10.1016/j.foodres.2015.01.026) with permission of *Elsevier* including the following subchapters: 3.2.1.2; 3.3.3; 3.4.2; 3.4.3; 3.4.4; 3.5.1; 3.6.1; 3.6.2; 3.7.1; 3.7.2.

3.1. MATERIALS

3.1.1. Research objects

Beetroots (*Beta vulgaris*) were collected from the Institute of Horticulture of the Lithuanian Research Centre for Agriculture and Forestry (Kaunas, Lithuania).

Leaf samples of lingonberry (*Vaccinium vitis-idaea* L.) were collected in the forests of Telšiai District (Mažeikiai, Lithuania) in 2010 (June to early-September). Dried (at 40 °C) leaves were milled to a fine powder with a grinder, and the ground herb was used for analysis.

3.1.2. Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$, 95%), 2,2'-azinobis-(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8,-tetramethylchromane-2-carboxylic acid (Trolox, 97%), 2,2'-azobis(2-methylpropionamidine) dihydrochloride (97%), diethylene glycol (≥99%), potassium persulfate ($K_2S_2O_8$) (≥99.%), sodium phosphate salts (99%), formic acid, sodium borohydride (98.0%), boric acid (≥99.5%), nonafluoropentanoic acid (97%), trichloroacetic acid, D (+)-lactose monohydrate (99%), rutin, quercetin-3-O-glucoside, quinic acid, *p*-coumaric, caffeic and gallic acids, Folin-Ciocalteu phenol reagent (2 M, 99%), NaCl, KCl, KH_2PO_4 were from *Sigma-Aldrich* (St. Louis, MO, USA). Milli-Q water and acetonitrile (high-performance liquid chromatography (HPLC) grade) were from *Merck KGaA* (Darmstadt, Germany). (+)-Catechin reference (98.3%) was purchased from *Chromadex* (Irvine, California, USA), chlorogenic acid (97%) from *Roth* (Karlsruhe, Germany); fluorescein sodium salt (FL) from *Fluka Chemicals* (Steinheim, Germany); solvents acetone and methanol were acquired from *Lachema* (Brno, Czech Republic). HAs as MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinolone, 98.0%), MeIQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, 98.0%), Trp-P-1 (3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, 98.0%) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 98.0%) were purchased from *Toronto Research Chemicals Inc.* (Toronto, Canada). CML, d_2-CML and furosine were purchased from *PolyPeptide Laboratories* (Strasbourg, France). L-phenylalanine (98.5%) and glucose (99.5%) were sourced from *Roth* (Karlsruhe, Germany). Creatinine (98.0%) was purchased from *Alfa Aesar* (Karlsruhe, Germany). D-(+)-maltose monohydrate (95%) and D-(+)-glucose anhydrous (99.9%) were obtained from *Sigma-Aldrich* (St. Louis, MO, USA). Skimmed milk powder and whey protein were *Prolacta* from Lactalis Ingredients (Bourgbarré, France). Palm oil was purchased from *Kerfoot Group* (Yorkshire, UK). Soy lecithin was Solec from *DuPont*

(Dangé Saint Romain, France). Beef meat was purchased from the local supermarket. Deionized water was prepared in a *Millipore Simplicity* 185 purification system (Millipore Molsheim, France); *Bond-Elut* PRS LRC cartridges (500 mg, 10 mL) and *Bond-Elut* C18 cartridges (100 mg, 3 mL) were purchased from *Agilent Technologies* (Lake Forest, CA, USA); *Chromabond* XTR cartridges (70 mL, 14.5 g of kieselguhr) were obtained from *Macherey-Nagel GmbH & Co.* (Bethlehem, PA, USA); *Oasis* HLB cartridges for CML and furosine stable isotope dilution assay were purchased from *Waters* (Milford, CA, USA). Gel chromatography media *Sephadex-LH20* was acquired from *Sigma-Aldrich* (St. Louis, MO, USA).

3.2. PLANT PREPARATIONS

3.2.1. Preparations of beetroots

3.2.1.1. Beetroot juice preparation

First, beetroots were washed, dried and crushed with a juicer (*Stollar JE 800*, Australia). Then, juice was filtered through cotton and additionally centrifuged at 10000 rpm for 15 min. For protein and pectic-like polysaccharides removal, juice was mixed with acetone (3:1) and kept at −18 °C overnight. Then, the solution was spinned at 10000 rpm for 15 min, and the supernatant was carefully poured off. The precipitates amounted to 9% of the total juice weight. Then, acetone from the solution was removed by evaporation in a Büchi rotary vacuum evaporator (Flawil, Switzerland) at 40 °C, and the remaining liquid was lyophilized. Finally, the obtained beetroot juice powder (BJP) was kept at 4 °C until use. The yield of beetroot juice was expressed in mg/100 g fresh of beetroot.

3.2.1.2. Purification of betalains by gel chromatography

Beetroots were washed, dried, cut into small slices, freeze dried, powdered in a grinder and stored in dark glass containers at 4 °C temperature. For fractionation, 1 g of beetroot powder was dissolved in 25 mL of deionized water, centrifuged and filtered through cotton. The residue was additionally washed with 15 mL of deionized water in order to obtain 40 mL of total volume. The supernatant was purified by gel filtration on *Sephadex-LH20* in a glass column (40×3.0 cm) using methanol as elution solvent. Elution was performed with deionized water as the mobile phase. After complete elution, the column was regenerated by washing with 5 column volumes of deionized water. Cleaning and re-equilibration steps were performed before each elution. Three fractions were obtained by visual color difference as shown in Fig. 3.1. The elution was in the following order: BtFI (light red), BtFII (dark red) and BtFIII (yellow). The fractions were freeze dried and used as dry beetroot fractions. All the fractions were stored in the dark at 4 °C until use. The yield of beetroot fractions was expressed in mg/g dry beetroot weight.

3.2.1.3. Purification of betalains by preparative HPLC

One part of fresh juice was diluted with methanol to 1:1 ratio. Then, the methanol-beetroot juice mixture was kept at –18 °C overnight. After thawing, the supernatants were collected from the precipitate. Methanol was removed from the mixture by evaporation at 40 °C under vacuum, and the obtained solution was freeze dried. Then, the freeze dried powder was dissolved in methanol (0.1%) formic acid

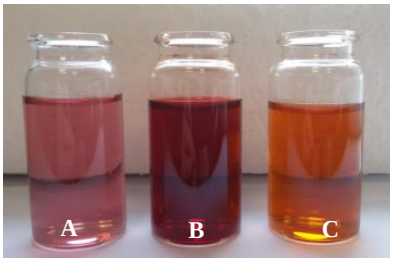


Fig. 3.1. Collected BtFI (A), BtFII (B), BtFIII (C) fractions after separation of beetroot powder on *Sephadex-LH20*

(20/80, w/v) solution at a concentration of 200 mg/mL before injection (200 μL) into the HPLC system (Shimadzu, Tokyo, Japan). The system consisted of a CBM-20A control unit, two LC-20AP compact pumps connected to a UV-visible spectroscopy detector SPD-20AV (Shimadzu, Tokyo, Japan), a fraction collector FRC-10A (Shimadzu, Tokyo, Japan), and a column oven and an auto sampler. Isolation and separation of betanin and vulgaxanthin were achieved on a C18 Altima column (22×150 mm, i.d., 10 μM, Alltech) operated at room temperature, at a flow rate of 15 mL/min and a pressure of 52 bar. The mobile phase consisted of deionized water with 0.1% formic acid (eluent A) and methanol (eluent B). The elution was performed by using isocratic flow: 80% of solvent A and 20% of solvent B. The effluents collected at 2.15 min for vulgaxanthin and at 4.8 min for betanin are shown in Fig. 3.2. Monitoring was performed by checking wavelength absorbance at 476 nm for vulgaxanthin and 538 for betanin of each tube in the fraction collector. The same fractions of vulgaxanthin and betanin were combined. Complete removal of water was obtained by lyophilization. The resulting powders were stored in sealed vials at –18 °C until use.

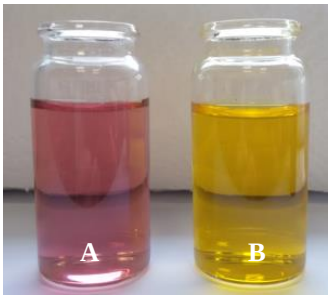


Fig. 3.2. Collected BnF (A) and VxF (B) fractions after separation of beetroot juice by HPLC system

3.2.2. Preparation of lingonberry leaves extracts

The water extract (WE) was obtained by freeze-drying liquid residues after hydro-distillation of essential oil form 200 g ground LL in a Clevenger-type apparatus (heated for 3 h). The solid residues (deodorized LL) were dried at 40 °C in a drying oven and further used for pressurized liquid extraction with methanol and acetone. Pressurized liquid extraction was performed on a Dionex ASE 350 system (*Dionex*, Sunnyvale, CA, USA) in 34 mL Dionex stainless-steel vessels. The ground powder of leaves (10 g) was mixed with 1 g of diatomaceous earth and extracted with methanol and acetone by using three cycles, 5 min each (i.e., 15 min total time), a flush volume of 100%, and purge with nitrogen gas of 60 s at the end of each extraction. Extractions were carried out at 70 °C and under 10 MPa pressure. The liquid extract was then evaporated in a rotary vacuum evaporator at 40 °C and stored at –18 °C until analysis. Methanol extract (ME) and acetone extract (AE) were obtained after extraction of raw LL, while deodorized methanol extract (DME) and deodorized acetone extract (DAE) were obtained after extraction of deodorized LL. All the extractions were done in triplicate.

3.3. COMPOSITIONAL ANALYSIS

3.3.1. Chromatographic sugar analysis of beetroot preparations

Fructose, glucose and saccharose determination was performed as described by Hernández, González-Castro, Naya Alba, & de la Cruz García (1998) with some modifications. Powders of beetroot samples were dissolved in deionized water at a concentration of 2 mg/mL. Chromatographic analysis was performed by using a Waters Acquity UPLC system (*Waters Corporation*, Milford, MA, USA) consisting of a quaternary pump, an autosampler, a column temperature controller and a PDA detector. The effluent from the PDA detector was introduced directly into quadrupole and time-of-flight mass spectrometer (Q-TOF) (*Brüker Daltonics*, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The Acquity UPLC system was equipped with a binary solvent delivery system, an autosampler, a photodiode array detector, and a column manager. The mobile phase consisted of acetonitrile and water (75:25, v/v). The separation was carried out by injecting 2 μL of sample on ACQUITY BEH Amide column (100×2.1 mm i.d., 1.7 μM) (*Waters*, Milford, MA, USA) at a flow rate of 0.4 mL/min set at the isocratic mode. The MS settings were as follows: capillary voltage, 4000 V; nebulizer, 1.5 bar; dry gas, 8 L/min; dry gas temperature, 200 °C; acquisition range, *m/z* 80–700; spectra acquisition rate, 1 Hz. Negative ESI-mass spectrometry and MS/MS spectra were used for mass analysis. Fructose and saccharose were identified by comparing the data of t_R (retention time), the elution order and mass spectrometry with the values provided in literature sources. Glucose was identified by standard. Also, the *Smart Formula* software of Data Analysis 4.0 SP4 was used for elemental formula analysis. The fructose and glucose content was expressed by using the glucose standard calibration curve, while sucrose was expressed by using the maltose calibration curve. The calibration curves of

glucose and maltose ranged from 1 to 100 mg/mL. The results were expressed as mg/g on the dry weight basis.

3.3.2. Spectrophotometric quantification of the content of betalains in fresh beetroot juice

The spectrophotometric method developed by Nilsson (1970) was used to measure the content of betacyanins (violet pigments) and betaxanthins (yellow pigments) in beetroot juice. The content of betalains was calculated in terms of betanin and vulgaxanthin-I for betacyanins and betaxanthins, respectively. For this purpose, 1 mL of fresh beetroot juice was diluted with 0.05 M phosphate buffer (pH 6.5) to obtain absorption values between 0.4 and 0.5 AU. Concentrations of betalains were calculated by taking into account the absorptivity values $A^{1\%}$ by using a 1-cm pathlength quartz cuvette. The measurements were carried out in triplicate. The content of betalains was calculated in mg per 100 mL juice by using the following equations:

$$Btcn = [(1.095 \times (A - C) \times DF)/\varepsilon_b) \times 1000]; \quad (1)$$

$$Btxn = [((B - (A - Btcn) - (Btcn/3.1))/\varepsilon_v) \times 1000]; \quad (2)$$

where A – absorption at 538 nm, B – absorption at 476 nm, C – absorption at 600 nm, DF – dilution factor, ε_b – extinction coefficient of betanin (1120) ε_v – extinction coefficient of vulgaxanthin-I (750).

3.3.3. Chromatographic analysis of betalains of beetroot preparations

Betalains identification was performed according to the method of Kujala, Loponen, Klika, & Pihlaja (2000). Chromatographic separation of beetroot preparations was performed on a Waters Acquity UPLC system. The mobile phase consisted of solvent A: 0.4% formic acid in deionized water and solvent B: acetonitrile. The gradient elution program was 0–0.5 min, 0% B; 0.5–6.50 min, 0–13% B; 6.50–7.50 min, 13% B; 7.50–8.50 min, 13–100% B and then 8.50–10 min, 100% B. The separation was carried out on ACQUITY BEH C18 column (50×2.1 mm i.d., 1.7 μM) (*Waters*, Milford, MA, USA) at a flow rate of 0.4 mL/min. All the beetroot preparations were dissolved in water at 1 mg/mL concentration and filtered through a (0.22 μM) filtre prior to injection (2 μL) into the column. The UPLC system was coupled to a Maxis 4G mass spectrometer (*Bruker Daltonics*, Bremen, Germany) equipped with an electrospray ion source. The instrument control, data acquisition and processing were performed by using the *Compass 1.3 SR3* software (*Bruker Daltonics*, Bremen, Germany). Positive ESI- mass spectrometry and MS/MS spectra were used for mass analysis. Instrument settings were capillary voltage: 4500 V, nebulizer: 1.5 bar, dry gas: 8 L/min, dry gas temperature 180 °C, and acquisition range: *m/z* 50–1500, spectra acquisition rate: 1 Hz. MS/MS was performed in the auto mode. The *Smart Formula* software of Data Analysis 4.0 SP4 was used for elemental formula analysis. For tentative identification of betalains, mass spectrometry and literature data were used.

3.3.4. Analysis of chromatographic phenolic compounds in lingonberry leaves extracts

Chromatographic separation of phenolic compounds was performed on a Waters Acquity UPLC system. The analytical column was Waters Acquity BEH C18 (50×2.1 mm i.d., 1.7 µm) (*Waters Corporation*, Milford, MA, USA). The eluents were 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient elution started with 10% of B and changed to 22% of B in 2 min, then reached 50% of B in 7 min. After each run, the chromatographic system was set to 10% of B in 10 min and equilibrated for 2 min (the column was also equilibrated for 1 min before each run). The flow rate was 0.4 mL/min, and the injection volume was 2 µl. UV spectral data for all the peaks was recorded in the range of 220–500 nm. The mass spectra were acquired by using negative (ESI-) ionization by full scan acquisition covering a range of *m/z* 100–1800. The capillary voltage was maintained at +4000 V with the end plate offset at −500 V. The collision cell energy was set at 8 eV. Nitrogen was used as the drying and nebulizing gas at a flow rate of 10.0 L/min and a pressure of 2.0 bar, respectively. Nitrogen was introduced into the collision cell as the collision gas. The identification of peaks was carried out by comparing t_R with the values of the corresponding peaks in chromatograms of standards or by the characteristic mass spectrometric fragmentation patterns and accurate masses. The fragmentation study data-dependent scan was performed by deploying collision-induced dissociation while using nitrogen as a collision gas where the collision energy was set at 35 eV.

3.4. ANTIOXIDANT ANALYSIS

3.4.1. DPPH$^{\bullet}$ scavenging capacity

The DPPH assay was determined by using the method described by Brand-Williams, Cuvelier, & Berset (1995). 7.5 µL of the extract or TE solution was mixed in microplate wells with 300 µL of a methanolic solution containing 60 µM DPPH$^{\bullet}$. The absorbance reduction was recorded at 517 nm in a FLUOstar Omega reader (BMG Labtech, Durham, NC) at every minute until the absorption curve had reached a plateau (a total of 35 min). The different concentration ranges of LL extracts were used: 0.05–0.5% for WE, ME, AE and DME; 0.05–1% for DAE. Five replicate absorbance measurements were done for each dilution. The final results were expressed as effective concentrations EC_{50} (mg/mL, edw (extract dry weight)) showing the concentration of the extract required to decrease the initial DPPH$^{\bullet}$ concentration in the reaction mixture by 50%.

3.4.2. ABTS$^{\bullet+}$ scavenging capacity

The method used ABTS$^{\bullet+}$ which is a radical cation generating substance widely used to monitor antioxidant abilities of various compounds (Re *et al*., 1999). Stock solution of 2 mM ABTS was prepared by dissolving it in 50 mL of phosphate buffered saline solution at pH 7.4. The ABTS$^{\bullet+}$ solution was produced by reacting 50 mL of ABTS stock solution with 200 µL of 70 mM $K_2S_2O_8$ solution and storing the mixture in the dark at room temperature for 15 h. The solution was then diluted with phosphate

buffered saline to give absorbance of 0.70±0.02 at 734 nm. The assay was adapted for spectrophotometric measurements in 96-well microplates. Three microliters of the sample solution was mixed with 297 µL of the $ABTS^{\bullet+}$ solution, and absorbance was measured in a microplate reader FLUOstar Omega (*BMG Labtech*, Durham, NC). 300 µL of PPB solution was used as a blank sample. The calibration curve was made by using different Trolox concentrations (0.5–2 mM) in phosphate buffered saline. The Trolox equivalent antioxidant capacity (TEAC) value represents the ratio between the slope of this linear plot for scavenging $ABTS^{\bullet+}$ by the sample compared to the slope of this plot for $ABTS^{\bullet+}$ scavenging by Trolox used as the antioxidant standard. Sample concentration providing 50% inhibition of $ABTS^{\bullet+}$ (EC_{50}) was also calculated from the graph plotting inhibition percentage against the concentrations of beetroot preparations or LL extracts. The scavenging activity was also expressed as µM Trolox equivalents (TE)/g (mM TE/g) *dw* (dry weight) of beetroot preparations, or *edw* (extract dry weight) of LL extracts, or pdw (plant dry weight) of LL raw material.

3.4.3. Oxygen radical absorbance capacity

ORAC assay measures the oxidative degradation of fluorescent compounds (fluorescein sodium salt) after being exposed to oxygen radical initiator, 2,2'-azobis(2-methylpropionamidine) (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002). All the solutions were prepared in phosphate buffer (75 mM, pH 7.4). The measurements were carried out in 96-well microplates by mixing 25 µL of the sample with 150 µL of 96 mM fluorescein and incubating for 15 min at 37 °C. Afterwards, 26 µL of 240 mM 2,2'-azobis(2-methylpropionamidine) solution was added, and the microplate was shaken for 30 s. The fluorescence was recorded (λ_{ex} = 493 nm, λ_{em} = 515 nm) every 66 s for 90 min in a fluorimeter microplate reader FLUOstar Omega. Trolox solutions in a concentration range of 0.5–200 µM were prepared for the standard calibration curve. The ORAC values were calculated based upon the differences in areas under the fluorescence decay curve among the blank, samples and standards. Finally, the ORAC values were expressed as µM TE/g (mM TE/g) dw of beetroot preparations or edw of LL extracts, or pdw of LL raw material.

3.4.4. Determination of total phenolic content

The total phenolic content (TPC) was determined according to the Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). The powders of beetroot preparations were dissolved in deionized water at 10 mg/mL concentrations, while LL extracts were dissolved at 1 mg/mL. Aliquots of the samples (0.2 mL) were mixed with 1.0 mL of 10-fold diluted Folin-Ciocalteu reagent, and, after 5 min shaking, 0.8 mL of 7.5% sodium carbonate solution was added. The absorbance was measured at 765 nm after 1.5 h using a HALO RB-100 UV–Vis spectrophotometer (*Dynamica*, Switzerland). Ethanolic solutions of gallic acid (0.05–0.25 mg/mL) were used for the calibration curve. The results are expressed as mg gallic acid equivalent (GAE)/g dw of beetroot preparations or edw of LL extracts or pdw of LL raw material.

3.4.5. Determination of active radical scavengers by HPLC-DPPH˙ online screening

This method was performed by using Waters HPLC system equipped with a 1525 binary pump (*Waters*, Milford, MA, USA), a 7125 manual injector (*Rheodyne*, Rohnert Park, CA, USA), and a 20 μL injection loop. The separation was performed by using a Discovery HS C18 column (250×0.46 cm i.d., 5 μM) (*Supelco Analytical*, Bellefonte, PA, USA). The linear binary gradient was set at a flow rate of 0.6 mL/min by using 0.1%, v/v, formic acid in deionized water (solvent A) and acetonitrile (solvent B). The gradient elution program was: 0–25 min 25% B, 25–40 min 25–40% B, 40–45 min 100% B, 45–50 min 100% B, 56–60 min 25% B. Detection of the compounds was performed by using a 996 photodiode array detector (*Waters*, Milford, MA, USA) at 210–450 nm. After that, 6×10^{5} M of DPPH methanol solution was added to the eluent stream at a flow rate of 0.6 mL/min by using a 1100 series quaternary pump (*Agilent Technologies, Inc.*, Santa Clara, CA, USA). The reaction between radical scavengers and DPPH radical was taken into a reaction coil (15 m×0.25 mm i.d.) made of PEEK (polyetheretherketone) tubing (*Interchim*, Frankfurt, Germany). Decrease of absorbance was detected photometrically as a negative peak at 515 nm with a SPD-20A UV detector (*Shimadzu*, Kyoto, Japan). For the identification of phenolic compounds, the HPLC system was coupled to a quadrupole mass detector Micromass ZQ (*Waters*, Milford, MA, USA) operating in negative (ESI^-) ionization at the following settings: scanning range 100–1000 *m/z*; capillary voltage 3 kV; source temperature 120 °C; cone voltage 30 V; cone gas flow 80 L/h; desolvation temperature 350 °C; desolvation gas flow 400 L/h. The flow rate was 0.6 mL/ min, the injection volume of 10 mg/mL extract solutions was 20 μL, and the gradient program was as described above.

3.5. PREPARATION OF FOOD MODEL SYSTEMS

3.5.1. Milk model system

A milk model system was prepared by first dissolving one-by-one 1.2% (w/w) skim milk powder, 2.5% (w/w) whey protein, and 5% (w/w) lactose monohydrate in 87.9% (w/w) Milli-Q water. The lipid mixture was prepared separately by melting 3.3% (w/w) palm oil and 0.1% (w/w) soy lecithin at 60 °C temperature. The lipid mixture set at 50 °C was added to the ingredient mixture (maintained at 50 °C), and this mixture was primarily homogenized 3 times (30 s) by using an Ultraturrax T25 homogenizer (*IKA*, Stockholm, Sweden) operating at 22000 rpm. The coarse milk was homogenized under pressure (160 bar, 3 passes) in a bench-top homogenizer (*GEA-Niro Soavi*, Italy). The milk model was stored in the dark at 4 °C until use. Beetroot-milks were made up by mixing equal volumes of the milk preparations (4 mL) with 0.01% (w/v) and 0.03% (w/v) of beetroot juice (0.1 and 0.3 mg/mL) before heat treatment. WE, quinic acid, and catechin powders were added at 0.05, 0.1 and 0.3 mg/mL concentrations in the milk model system. Milk controls (4 mL of milk) without any additions were also prepared. All the samples were aliquoted in headspace vials (10 mL) with a crimp seal with PTFE/silicone septa (*Phenomenex*, Torrance, CA) and

heat processed for 30 and 45 s. The control sample was without thermal processing and labeled as 0 s heat treatment.

The heating system was adapted according to the Kokkinidou & Peterson (2013) method. It consisted of two glass beakers filled with peanut oil that were heated on different hot plates (Fig. 3.3). The first hot plate was set at 180 °C, and the second one was set at 140 °C, and the temperature was additionally controlled by external thermometers with thermocouples (TM-906A, *Tersid*, Lutron, Milan, Italy). The temperature of the milk inside the headspace vial was also controlled with an inserted thermocouple (K-type thermometer, *Hanna Instruments*) through the septa. The samples were first heated up to 140 °C in a first oil-bath (the temperature of the external batch: 180 °C), and then immediately transferred to the second oil bath (at 140 °C) and heated for 30 and 45 s. The high temperature of the first tank allowed a rapid increase of the temperature in the vial; instead, the second tank stabilized the temperature at 140 °C for 30 and 45 s. The thermal treatment was performed one-by-one separately for each sample in order to monitor the temperature of each sample. At the end of the thermal process, the vial was rapidly moved into the cold water tank and kept until the sample temperature had reached 8 °C. Then, it was rapidly frozen in dry ice in ethanol and stored at −18 °C until the analysis.

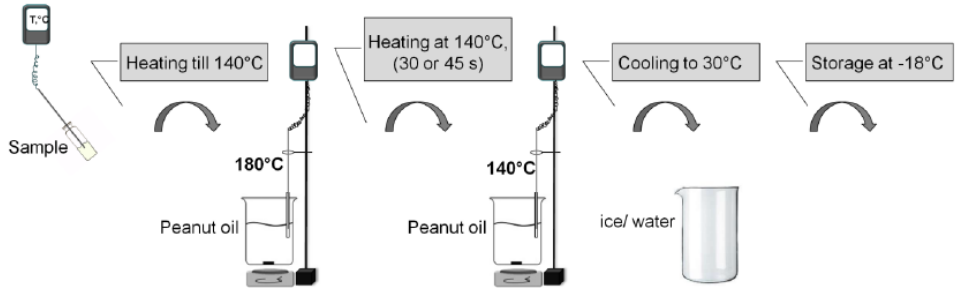


Fig. 3.3. Heating system used to treat milk samples

3.5.2. Meat protein model system

A meat-protein model system which mainly produced PhIP formation was used (Wong *et al*., 2012). For this model system, 0.4 mM phenylalanine, 0.4 mM creatinine and 0.2 mM glucose were weighed in test vials, and 3 mL of diethylene glycol (containing 14% of distilled water) was added. Then, in reaction mixture was added different beetroot preparations: beetroot fractions (BtFI, BtFII and BtFII) were added at 30 mg/mL, BJP was added at 5, 15 and 35 mg/mL (E162 was tested at 15 mg/mL), BnF was added at 0.55, 2.2 and 5.5 mg/mL, VxF was added at 5, 15 and 50 mg/mL, while WE and AE powders were added at 0.1, 1, 5, 10 mg/mL concentrations. The controls were without any additives. The reaction mixtures were placed in screw-cap stainless steel vials which were inserted in the aluminum steel block. The temperature was controled with an electronic thermometer (*Mastech*, China) which was coupled to the block. The block was preheated for 2 h in an oven before the vials were inserted into the holes. The samples were heated at 128±2 °C for 120 min, after that, they were

immediately cooled in an ice-water bath for 10 min. The samples were kept at −18 °C temperature before the purification process.

3.5.3. Meat model system

A meat model system, prepared as described by Messner & Murkovic (2004), was used. 0.1 g of homogenized freeze-dried beef meat powder (8.5% of humidity) with various amounts of WE and AE (0, 0.1, 1, 5 and 10 mg/mL) were weighed and suspended in 1 mL of diethylene glycol (14% water) in screw-cap steel vessels, and the vials were heated at 180 °C for 30 min. After the heating, vials were cooled on ice (10 min) in order to terminate the reaction, and the samples were stored at −18 °C prior to purification.

3.6. PURIFICATION PROCEDURES

3.6.1. Purification of CML and furosine

CML and furosine purification were performed according to Fenaille *et al*. (2006) and Delatour *et al*. (2009) with slight modifications. Briefly, 100 µL of each milk sample was diluted with 450 µL of sodium borate (0.2 M, pH 9.2), and 500 µL of sodium borohydride (1 M in 0.1 M sodium hydroxide) were added to the mixture. Then, the solution was incubated at room temperature (26 °C) for 4 h. After incubation, 1 mL of TCA (30%, w/v) was added in order to precipitate proteins. The samples were centrifuged at 4000 rpm for 10 min (4 °C), and supernatants were removed with a Pasteur pipet. Then, the precipitated proteins were hydrolyzed with 2 mL of hydrochloric acid (6 M, HCl) at 110 °C for 24 h in an air forced circulating oven (*Memmert*, Germany). The hydrolysates were further filtered through a polyvinylidene fluoride filtre (0.45 µM), and 1 mL of solution was evaporated to dryness under a steam of nitrogen gas. Dried acid hydrolysates were reconstituted in 390 µL of Milli-Q water and spiked with 10 µL of internal standard d_2-CML at a final concentration of 50 ng/mL. Then, reconstituted acid hydrolysates were subjected to solid phase extraction, which was performed in 30 mg Oasis HLB cartridges (*Waters*, Wexford, Ireland) which were first preequilibrated with 1 mL of methanol, then with 1 mL of nonafluoropentanoic acid (20 mM in Milli-Q water), and finally with 1 mL of methanol/20 mM nonafluoropentanoic acid (5/95, v/v). 400 µL of reconstituted acid hydrolysates was passed through the preconditioned cartridge. Then, CML, d_2-CML and furosine were eluted with 1 mL of methanol/20 mM NPFA (50/50, v/v). The eluent was dried under a gentle flow of nitrogen gas and redissolved in 400 µL of Milli-Q water. 20 µL of the final solutions was injected into the liquid chromatography tandem mass spectrometry (LC/MS/MS) system.

3.6.2. Purification of heterocyclic amines

For the purification of HAs, a method described by Wong *et al*. (2012) was employed. Samples of the meat protein model system were diluted with 47 mL of 2 M sodium hydroxide solution, while samples of the meat model system were homogenized by magnetic stirring for 60 min at 500 rpm with 15 mL of 1 M sodium hydroxide in 50 mL glass beakers. Then, 5 mL of prepared aliquots were mixed with

diatomaceous earth in a Chromabond XTR column. The elution was performed with 48 mL of dichloromethane or ethylacetate directly into an attached PRS Bond-Elut cartridge which was in advance conditioned with 4 mL of dichloromethane. The PRS cartridge was dried under maximum vacuum (Visiprep Vacuum Manifold 24 DL, *Supelco*) for 5 min and sequentially washed with 6 mL of 0.1 M HCl, then with 15 mL of methanol/0.1 M HCl, (40/60, v/v), and finally 2 mL of deionized water. A Bond-Elut C18 cartridge was conditioned with 1 mL of methanol and 10 mL of water. HAs adsorbed on the PRS cartridge were then eluted into an attached Bond-Elut C18 cartridge with 20 mL of 0.5 M ammonium acetate solution (pH 8, adjusted with 28% of ammonium hydroxide). The C18 cartridge was washed with 2 mL of deionized water and dried under positive pressure. The final elution was carried out with 1.2 mL of methanol-concentrated ammonia (9:1, v/v) into the vial. The eluates were dried under nitrogen gas. The residue of the sample from the meat-protein model system was re-dissolved in 400 μL of methanol, while the residue of the sample from the meat model system was re-dissolved in 100 μL of methanol before chromatographic analysis. The quantitation of HAs was performed by using the below described chromatographic techniques.

3.7. DETERMINATION OF MAILLARD REACTION PRODUCTS

3.7.1. Quantitative analysis of CML and furosine by LC-MS/MS

Analysis was performed by using an API 2000 triple quadrupole mass spectrometer (*Applied Biosystems*, Carlsbad, CA, USA) with an electrospray interface coupled to HPLC binary micropumps series 200 (*Perkin-Elmer*, USA). The separation was performed on a Mediterranea Sea18 (C18) column (150×2.1 mm, i.d., 5 μM, *Teknokroma*, Barcelona, Spain) by using the following mobile phases: A, 20 mM nonafluoropentanoic acid in Milli-Q water and B, 20 mM nonafluoropentanoic acid in acetonitrile. The flow rate was 0.2 mL/min with the following gradient for the solvent B: 10% 0–3 min, 70% 8 min, 70% 9 min, 10% 12 min and 10% 15 min. With the above-described chromatographic conditions, the typical retention time of CML and d_2-CML was 6.80 min, whereas, for furosine, it was 7.22 min. Positive electrospray ionization was used for the detection, and the source parameters were selected as follows: spray voltage: 5.0 kV; capillary temperature: 350 °C, dwell time: 100 ms. The quantitation was carried out in multiple reaction monitoring (MRM) the *m/z* ratio of 205 for CML and 207 for d_2-CML, respectively. Specific molecular fragments corresponding to *m/z* 84 and 130 for CML and *m/z* 84 and 144 for d_2-CML were also monitored. CML was quantified by using a linear calibration curve made with a specific solution of CML spiked with d_2-CML (internal standard final concentration 50 ng/mL) dissolved in water (5 to 500 ng/mL). The limit of detection (LOD) and the limit of quantification (LOQ) were 5 (ng/mL) for CML, and the coefficient of determination R^2 was higher than 0.999. The internal standard ratio was used for the quantification, and the relative standard deviation of the intraday and interday assay was less than 7%. The results are expressed as ng/mL of the sample. Furosine was quantified through an external standard calibration curve in the range of

5–1000 ng/mL (R^2 higher than 0.999). The LOD and LOQ were, respectively, 1 and 5 ng/mL for furosine. The furosine recovery and precision performances were validated through the recovery test and the intraday and interday assay (relative standard deviation <5%). For the recovery test, three UHT treated milk samples were used. After acidic hydrolysis, 1 mL of each sample was dried under nitrogen flow. Each sample was analyzed with and without spiked furosine (500 ng), and the same procedure as mentioned above was performed. The furosine concentration was calculated by subtracting the concentration of the non-spiked samples to the spiked ones. The recovery ranged from 89% to 110%.

3.7.2. Quantitative analysis of PhIP by HPLC

PhIP determination of the meat protein model system with beetroot fractions (BtFI, BtFII and BtFIII) was performed by using an HPLC system equipped with a Waters 1525 binary pump and a Waters Micromass ZQ-2000 mass detector by using a reversed phase analytical column TSK ODS 80TS (250×2.0 mm i.d., 5 μM, *Tosoh Bioscience*, Japan) operating at 40 °C. The separation was performed at a flow rate of 0.3 mL/min by gradient elution with methanol/acetonitrile/water/acetic acid (8/14/76/2, v/v/v/v) at pH 5.0 (adjusted with 25% ammonium hydroxide) as solvent A and acetonitrile as solvent B. The gradient for solvent B was: 30% 0–12 min, 30% 12–20 min, 100% 20–35 min, 100% 35–40 min, and 0% 40–50 min. The injection volume was 10 μL. Analytes were ionized with ESI in the positive mode by using the following parameters: desolvation temperature 300 °C, source temperature 120 °C, capillary voltage 3 kV, cone voltage 30 V, desolvation gas flow 350 L/h and cone gas flow at 80 L/h. The chromatograms were recorded in a selected ion recording mode (*m/z* 225 for PhIP) with a quadrupole mass spectrometer. The PhIP was identified by *m/z* data and by retention time comparison with authentic reference standards. Quantification was performed by using an external calibration curve in the range of 1000–4000 ng/mL for PhIP. The LOD and LOQ concentrations for standard solutions were calculated based on signal-to-noise ratios of 3:1 and 10:1, respectively. The LOD and LOQ values for PhIP were 0.09 and 0.26 in nM/mM creatinine, respectively. The recovery rate for PhIP was measured by, firstly, determining the concentrations of PhIP in various heated samples, and, secondly, by adding sufficient amounts of PhIP to these samples in order to double their concentrations. The difference between the first and the second measurements was divided by the added amount so that to calculate the recovery rates. Thus, the recovery of PhIP was 67%.

3.7.3. Quantitative analysis of PhIP by UPLC-mass spectrometry

PhIP determination in the meat protein model system with LL extracts (WE and AE) and betalain preparations (FB and FV) was performed by using an Acquity UPLC system and a BEH C18 column (50×2.1 mm, i.d.; 1.7 μM, Acquity, *Waters*, USA) maintained at 40 °C. The mobile phase was composed of 30 mM formic acid/ammonium formate (pH 4.75) in deionized water (solvent A) and acetonitrile (solvent B) delivered at 0.4 mL/min at the following gradient: 0–0.15 min, 5% B; 0.15–2.5 min, 5–30% B; 2.5–3.0 min, 30–60% B; 3.0–5.0 min, 60% B and 5.0–7.0 min 60–100% B. The post run time was 1 min, while the column was also equilibrated

for 1 min before each run. Positive ESI ionization was used for mass analysis at the following settings: collision energy 8 eV, capillary voltage – +4000 V with the end plate off set at -500 V, drying and nebulizing gas – nitrogen, nebulizer – 2 bar, dry gas – 10 L/min, dry gas temperature 200 °C. The sample injection volume was 5 μL. The ions were monitored from 100 to 800 *m/z*. Quantification of PhIP was carried out by preparing three standard curves of this compound in methanol and following the whole procedure described above. For each curve, seven different concentrations levels of PhIP (0.1–800 ng/mL) were used. The LOD detected with a signal-to-noise ratio greater than three was 0.03 nM/mM creatinine (1 ng/mL) and LOQ was 0.09 nM/mM creatinine (3 ng/mL). The PhIP recovery ranged from 67% to 86%.

3.7.4. Quantitative analysis of heterocyclic amines UPLC-MS/MS

HAs determination of the samples from the meat model system was carried out on a Waters Acquity UPLC H-Class system equipped with a triple quadrupole mass spectrometer Xevo TQ-S (*Waters Corporation*, Milford, MA, USA) by using the positive electrospray ionization (ESI) mode. The chromatographic separation of HAs was carried out by using an Acquity BEH C18 column (50×2.1 mm i.d., 1.7 μM) (*Waters Corporation*, Milford, MA, USA). The mobile phase consisted of a binary gradient: acetonitrile (solvent A) and 30 mM formic acid-ammonium formate buffer (pH 4.75) (solvent B) at a flow rate of 0.8 mL/min. The elution gradient program was 0–0.1 min, 5% A; 0.1–1.5 min, 5–30% A; 1.5–1.8 min, 30–60% A; 1.8–1.85 min, 60% A; 1.85–2.4 min, 60–95% A; 2.4–2.9 min, return to initial conditions; 3 min post-run delay. The sample injection volume was 2 μL. Data acquisition (MassLynx 4.1 software) was carried out in the MRM mode by using the protonated molecular ion of each compound as a precursor ion. The MRM conditions were automatically optimized with 100 ng/mL HAs by using the Intellistart function. Four MRM functions were used for recording transitions of all the four compounds (Table 3.1).

Table 3.1. MRM parameters used with a triple quadrupole instrument (Waters Acquity UPLC-Xevo TQ-S)

Time interval (min)	Analyte	MRM function		
		Precursor ion→ product ion (*m/z*)	Collision energy (eV)	Cone voltage (V)
0.8–1.2	MeIQ	213→198	35	52
0.8–1.2	MeIQx	214→199	35	50
1.5–1.7	Trp-P-1	212→195	35	52
1.7–2.0	PhIP	225→210	35	54

The optimal ionization source working parameters were: source temperature, 150 °C; capillary voltage, 3.0 kV; desolvation temperature, 350 °C; desolvation gas flow rate, 650 L/h (Nitrogen); cone gas flow rate, 150 L/h (Nitrogen); collision gas flow rate, 0.13 mL/min (Argon); The quantitative analysis was performed by using Waters TargetLynxTM software. HAs were quantified with a calibration curve which has a 1–500 ng/mL range. The LOD and LOQ values for each compound within the sample were 1 and 2 ng/mL for PhIP, 2 and 5 ng/mL for MeIQ, 2 and 5 ng/mL for

MeIQx, and 2 and 6 ng/mL for Trp-P-1, respectively. The recovery ranged from 67% to 86%.

3.8. CHARACTERIZATION OF MEAT PROTEIN MODEL SYSTEM

3.8.1. Sample preparation

Different solutions (water, methanol and acetone) and their ratios were tested in order to dissolve the reaction mixture so that to obtain a transparent solution. The most suitable solvent was found to be 100% methanol. Then, 100 μL out of 3 mL of each reaction mixture was diluted with 900 μL of methanol and syringe-driven filtered through a 0.45 μM filter. The prepared methanolic reaction solution was kept at – 18 °C before analysis.

3.8.2. Determination of browning intensity

The final MRPs formed in the meat protein model system during heating were monitored by absorbance measurements at 420 nm. The absorbance was measured with a microplate reader FLUOstarOmega (BMG Labtech, Durham, NC) by using 96-well microplates. The methanolic reaction solution was 100-fold diluted with methanol. For color measurement, 250 μL of each diluted mixture was directly taken for absorbance readings at 420 nm. Methanol was used for blank adjustment. The analysis was performed with six replicates.

3.8.3. Determination of antioxidant capacity

The antioxidant capacity was estimated in terms of radical scavenging activity in a methanolic medium (DPPH assay) and an aqueous medium (ABTS assay). The DPPH and ABTS assays were performed by using procedures and equipment as described above in Section 3.4.1 and Section 3.4.2, with some modifications.

In the ABTS assay, 5 μL of the sample solution (100-fold diluted in methanol) was mixed with 300 μL of the $ABTS^{\bullet+}$ solution and then taken for absorbance reading at 734 nm in a microplate reader. The phosphate buffered saline solution (300 μL) with 5 μL of methanol was used as a blank sample. The capability to scavenge $ABTS^{\bullet+}$ was expressed as percentage inhibition, and was calculated according to the following formula:

$$\text{Inhibition, \%} = 100 \times (A_B - A_X)/A_B; \quad (3)$$

where A_B − the absorption of the blank sample (t = 0); A_X − the absorption of the reaction solution at t = 30 min.

The analysis was performed with four replicates.

In the DPPH assay, 200 μL aliquot of $DPPH^{\bullet}$ solution was mixed with a 40 μL of the model mixture solution (100-fold diluted in methanol) and then was taken for absorbance reading at 517 nm in a microplate reader. The same amount of methanol and the $DPPH^{\bullet}$ solution was used as a blank sample. The capability to scavenge the $DPPH^{\bullet}$ expressed as percentage inhibition was calculated by using Equation (3). The analysis was performed with four replicates.

3.8.4. Determination of 2-phenylethenamine and phenylalanine

For 2-phenylethenamine and phenylalanine quantification, methanolic reaction solutions were subjected to UPLC analysis (the protocol was the same as described above) without further sample processing. Identification of phenylalanine was performed by comparing their mass spectrum and t_R to those of the authentic standards. The quantitation of 2-phenylethenamine and phenylalanine was performed by calculating their BC peak area (AU) in percentages.

3.9. STATISTICAL ANALYSIS

The mean values and standard deviations (SD) of compositional and antioxidative analyses were performed on at least three replications (n=3), unless otherwise metioned in the relevant sections. Milk, meat protein and meat model systems were prepared in duplicate, while the extraction procedure was performed in duplicate for each replicate. Each replicate was analyzed in triplicate in LC systems. Statistical comparisons among different groups were tested by multivariance analysis (the Turkey test as a post hoc analysis), the differences between samples showed significant variation ($P<0.05$). Correlation coefficients (R) were calculated by using Microsoft Excel 2010 software (CORREL statistical function).

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF BEETROOT PREPARATIONS

4.1.1. Yields and sugars content

The final BJP was deep-red colored highly hygroscopic granulates which tended to clog over time. The final betanin fraction was a deep red-colored friable powder, while the vulgaxanthin fraction was orange-brown colored highly hygroscopic granulates which also had differences in color and texture. BtFI was a light-red colored friable powder, while BtFII and BtFIII were highly hygroscopic deep-red and light-brown granulates, respectively. The yield and the sugar content in different beetroot preparations are shown in Table 4.1.

Table 4.1. The yield and sugar content in different beetroot preparations

Preparations	Yield, g/100 g	Fructose, mg/g dw	Glucose, mg/g dw	Sucrose, mg/g dw	Total sugars, mg/g dw
BJP	6.43±0.40	83.83±0.33b	35.01±1.55b	34.96±0.90e	153.80±2.12e
BnF	0.08±0.01	N.D.	N.D.	0.74±0.20a	0.74±0.20a
VxF	5.63±0.42	13.14±0.62a	11.50±2.82a	21.57±0.47	45.65±4.41
BtFI	0.02±0.01	TR	TR	1.41±0.06b	1.41±0.06b
BtFII	0.44±0.02	N.D.	N.D.	22.74±0.07b	22.74±0.07d
BtFIII	0.15±0.01	N.D.	N.D.	12.31±0.16c	12.31±0.16c

Carbohydrates (fructose, glucose, sucrose, total sugars) content is expressed in mg/g on a dry weight basis of preparation. The yields of BJP, BnF and VxF are expressed in g/100 g of fresh beetroot. The yields of BtFI, BtFII and BtFIII are expressed in mg/g dry weight basis of preparation. TR, traces; N.D.: not detected.

From 1 kg of fresh beetroot, around 0.5 kg of fresh juice was obtained. The amount of BJP from 100 g of fresh juice was 11.78±0.64 g. After the purification of a BJP solution with a preparative HPLC system, BnF and VxF yielded 0.13±0.01 and 8.94±0.67 g/100 g of fresh juice, respectively. When the amount was expressed in fresh beetroot weight, 6.43±0.40, 0.08±0.01 and 5.63±0.42 of BJP, BnF and VxF were obtained, respectively. The yields of BtFs after purification with HPLC or Sephadex-LH20 were quite low probably because of the degradation of betalains during the purification process. The decomposition of betalains could be prevented with the addition of ascorbic acid, but it would compromise the results of antioxidant properties and effects on MRPs formation. Precautions were taken in order to prevent decomposition of betalains from the light effect during the separation on Sephadex-LH20 resin, where the column was protected from the light with aluminum foil. The sugar content varied between the samples and is expressed in 1 g of beetroot preparation dry weight (Table 4.1). The highest concentration of the total sugars was found for BJP, then for VxF, BtFII, BtFIII, and, lastly, for BtF1. BnF was almost free from sugars, as it contained only sucrose representing less than 0.1%. In BJP, fructose was present in double amount in comparison to glucose or sucrose. During the chromatographic separation of the BJP solution, sugars were not bounded on a reserved phase-column and co-eluted at the first solvent movement, therefore, VxF collected at 2.15 min contained more carbohydrates than BnF which was collected at

4.8 min. In the column with Sephadex-LH20 resin, monosaccharides were co-eluted at first and did not remain in the BtFs, while disaccharide sucrose was co-eluted with betalains, and the highest concentration was collected in the second fraction (BtFII).

4.1.2. Total phenolic content and free radical scavenging properties

The TPC values and antioxidant capacity of beetroot preparations are shown in Table 4.2. TPC values were calculated for 1 g of beetroot preparations dry weight (pdw).

Table 4.2. Total phenolic content and antioxidant capacity of beetroot preparations

Samples	TPC, mg GAE/g dw	ABTS EC_{50}, mg/ml	ABTS, μM TE/g dw	ORAC, μM TE/g dw
BJP	12.46±0.17e	11.56±1.06c	67.44±5.99d	132.74±23.33b
BnF	4.01±0.43a	0.55±0.01a	1408.30±27.07f	N.T.
VxF	7.12±0.66b	151.45±7.09f	5.13±0.25a	N.T.
BRP	11.49±0.55d	18.35±0.51e	42.29±1.15b	70.31±16.95a
BtFI	20.26±0.16f	5.03±0.16b	154.19±4.92e	177.05±18.43c
BtFII	10.17±0.05c	15.34±0.70d	50.63±2.37c	87.50±12.96a
BtFIII	10.21±0.03c	19.52±1.05e	39.81±2.12b	97.49±9.74a

N.T.: not tested.

A two times higher TPC value was observed in BtFI than in sample (BRP) before fractionation and BJP. BtFI also features a two times higher TPC value than fractions BtFII and BtFIII, which means that phenolics were concentrated by using Sephadex-LH20 resin at the first solvent flow. TPC values in all the samples were in agreement with antioxidant capacity values except for samples obtained by HPLC purification. BnF showed the lowest TPC value but the highest radical scavenging capacity of all the samples. VxF possessed the lowest radical scavenging activity, while the TPC value was higher than that of BnF. The higher TPC value of VxF might stem from the reaction of the Folin-Ciocalteu reagent with other reducing ability possessing compounds, such as glucose and fructose (Huang, Ou, & Prior, 2005). The latter were present in VxF, however, they were absent in BnF. In our study, the TPC values of BJP are in accordance with previous investigations where the TPC content was 4.2 mg GAE/g dw (dry weight) in beetroot flesh, 15.5 GAE/g dw in beetroot peel (Kujala *et al*., 2000) and 1.87–11.98 mg/g dw in beetroot pomace extracts (Vulić *et al*., 2012). The antioxidant capacity of beetroot preparations was evaluated by ABTS and ORAC *in vitro* methods, and the obtained results are presented in Table 4.2. According to the decreasing antioxidant activity, the samples can be arranged in the following order: BnF, BtFI, BJP, BtFII, BRP, BtFIII and VxF. Given that BtFI and BnF showed a higher antioxidant activity than the samples before chromatographic purification (BJP and BRP), it might be assumed that betacyanins (mostly betanin) contributed with the antioxidant activity. Also, purification of betalains increased the antioxidant capacity. This is evident in the case of betanin purification by LC, where BnF manifested 21 times higher antioxidant activity than its primary material (BJP). Moreover, BnF showed a fairly low TPC value (4.01±0.43 mg GAE/g pdw) which was 3 times lower than BJP. These results are in agreement with previous reports where very strong antiradical properties of individual pure betalains were reported

(Gandía-Herrero *et al*., 2010). BtFI has a two times higher TPC value and a 3-times higher antioxidant activity than its primary material, while the content of betanin was 16% higher than in its primary material and quite similar to the values of BtFII. It should also be noted that the degradation products of betacyanins possess high antioxidant activity. BtFIII consisted mainly of the degradation products of betacyanins, while BtFIII contained a high content of the main betalains, their radical scavenging activities were fairly close to each other.

The EC_{50} and ORAC values of BtFIII were similar to those of BJP or BtFII while there was absence of the major betalains, except of betanin, whose content was negligible. Thus, 2-decarboxy-2,3-dehydro-neobetanin, neobetanin and unknown 2, which were found in BtFIII as the degradation products of betalains are the main contributors to the antioxidant activity. VxF has a 300-fold lower EC_{50} value than BnF as determined in the ABTS assay (Fig. 4.1). This indicates that betaxanthins (Vulgaxanthin I) possess low radical scavenging properties, and this is in agreement with the previous findings (Gandía-Herrero *et al*., 2010).

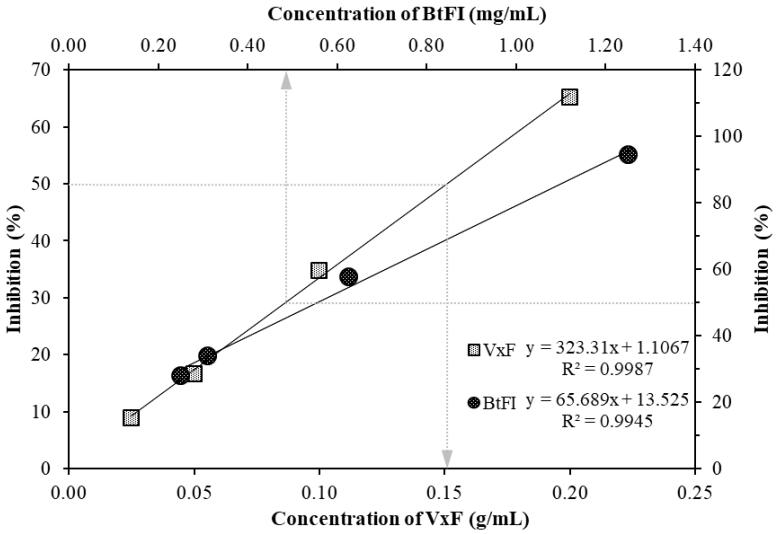


Fig. 4.1. Antioxidant activity of BtFI and VxF measured by ABTS assay

In general, the antiradical capacity of beetroot juice is considerably lower comparing to pure betalains (Table 4.2). The TEAC value of pure betanin was more than 10 times higher than that of BJP. Meanwhile, the EC_{50} values of BnF and Trolox were close to each other, which were 0.55±0.01 and 0.19±0.00, respectively. It was reported that the TEAC values may range from 2.4 to 7 (Gandía-Herrero *et al*., 2010) or reach 9 (Gliszczyńska-Świgło *et al*., 2006) for pure betalains. Betalains possess the strongest radical scavenger activity above pH 4 and showed better activity than some flavonoids (Gliszczyńska-Świgło *et al*., 2006). The high TEAC values of betalains such as dopaxanthin (around ~7) (Gliszczyńska-Świgło *et al*., 2006), betanin (4.9±0.7) (Gonçalves, Di Genova, Dörr, Pinto, & Bastos, 2013), miraxanthin V (5.5±0.5), betanidin (7.0±0.6) (Gandía-Herrero *et al*., 2013), betalamic acid (2.7±0.2)

(Gandía-Herrero, Escribano, & García-Carmona, 2012) were reported previously. The TEAC values are higher than those that have been reported for some of the phenolic compounds: ascorbic acid equaled 1.05 (Re *et al*., 1999), rutin 2.4, epicatechin 2.5, and quercetin 4.7 (Rice-Evans, Miller, & Paganga, 1996). The strong antiradical activity of pure betanin has been linked to its extended conjugate system, and it strongly depends on pH (Gliszczyńska-Świgło *et al*., 2006). With the increasing pH of betanin, the antiradical capacity increases due to the formation of its mono-, di-, tri-deprotonated forms. The same behavior for betanin structural unit betalamic acid was found (Gandía-Herrero *et al*., 2012). In the present study, the ORAC values of beetroot preparation ranged from 87.50 to 177.05 μM TE/g dw. Similar ORAC values were reported by Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer (2002) in dried beetroots (around 116 μM TE/g dw).

In general, the results indicate that pure betanin possesses a higher radical scavenging capacity than the whole mixture of betalains from beetroot. The degradation products of betanin also possess radical scavenging properties. The results also showed that a low radical scavenging activity of Vulgaxanthin I was observed.

4.1.3. Composition of Betalains

Betalains identification and quantitation was made by using UPLC-Q-TOF-MS/MS analysis. Individual pigments of beetroot preparations were identified by comparing the exact masses, fragmentation patterns, t_R and absorbance characteristics (UVmax) by using previously reported literature data (Herbach, Stintzing, & Carle, 2004b; Herbach *et al*., 2005; Kujala *et al*., 2000; Kumorkiewicz, Szmyr, Popenda, Pietrzkowski, & Wybraniec, 2019; Nemzer *et al*., 2011; Slatnar, Stampar, Veberic, & Jakopic, 2015; Wendel *et al*., 2016; Wybraniec, 2005). Also, MS/MS spectral data were checked at such databases as METLIN and PHYTOHUB. The basic chromatographic and MS qualitative results for all the analyzed betalains in different beetroot preparations are presented in Table 4.3. The main betacyanin peaks in BJP were identified as betanin and isobetanin (Fig. 4.2). According to their protonated molecular ions at *m/z* 551 and their fragmentation to ions of *m/z* 389, they were formed by deglucosylation. Both molecules differ only in the configuration of their C15 chiral center, and that gives a longer isobetanin t_R (Δt_R=0.3 min) value than that of betanin. Vulgaxanthin I was the major betaxanthin in BJP identified according to the absorption maxima at λ_{max}=471 nm, protonated molecular ions at *m/z* 340, and the fragmentation pattern (*m/z* 323=340–NH_3; *m/z* 277=340–NH_3–CO_2–2 H). Small peaks of other betaxanthins, specifically, Vulgaxanthin II and Valine-betaxanthin, were also observed. A small peak of neobetanin having a protonated molecular ion at *m/z* 549 was also detected. Neobetanin (14,15-dehydrobetanin) is commonly found in *Beta vulgaris* beetroots at low levels, but its amount increases during thermal treatment. It is formed via betanin or betanidin dehydrogenation which is enhanced through thermal treatment (Herbach *et al*., 2004b).

Table 4.3. Chromatographic and mass spectrometric data of tentatively identified betalains in beetroot preparations

No.	Compound	t_R, min	*m/z* $[M+H]^+$	Molecular ion formula	MS/MS fragments	Class of betalains
1.	Vulgaxanthin I	1.56	340.1140	$C_{14}H_{17}N_3O_7$	323, 277, 249, 231, 130	Btxn
2.	Unknown ($C_{21}H_{11}N_2O$)	1.76	307.0869	$C_{21}H_{11}N_2O$	291, 257, 231, 177, 130	Btxn
3.	Vulgaxanthin II	2.41	341.0980	$C_{14}H_{16}N_2O_8$	295, 249, 231, 148, 132	Btxn
4.	Unknown	2.84	345.1100	$C_{18}H_{13}N_6O_2$	164, 102	Btcn
5.	Proline-isobetaxanthin	3.32	309.1008	$C_{14}H_{16}N_2O_6$	291, 263, 217, 177, 150	Btxn
6.	Betanin	3.40	551.1508	$C_{24}H_{26}N_2O_{13}$	389	Btcn
7.	Isobetanin	3.80	551.1508	$C_{24}H_{26}N_2O_{13}$	389	Btcn
8.	Proline-betaxanthin	4.01	309.1010	$C_{14}H_{16}N_2O_6$	291, 263, 217, 177, 150	Btxn
9.	Betanidin	4.33	389.0986	$C_{18}H_{16}N_2O_8$	343, 297, 246, 194, 150	Btcn
10.	Neobetanin	4.65	549.1356	$C_{24}H_{24}N_2O_{13}$	387, 341	Btcn
11.	Decarboxy-neobetanin	4.71	505.1456	$C_{23}H_{25}N_2O_{11}$	343, 297, 255	Btcn
12.	Valine-betaxanthin	4.9	311.1260	$C_{14}H_{18}N_2O_6$	265, 219, 193, 175, 150	Btxn
13.	Decarboxy-bidehydro-neobetanin	6.5	503.1302	$C_{23}H_{23}N_2O_{11}$	341, 295, 277, 251	Btcn

Btcn, betacyanins; Btxn, betaxanthins

It has a longer t_R than betanin or isobetanin, and fragmentation to ions of *m/z* 387 and 341 is observed, representing the loss of glucose and glucose and CO_2, respectively.

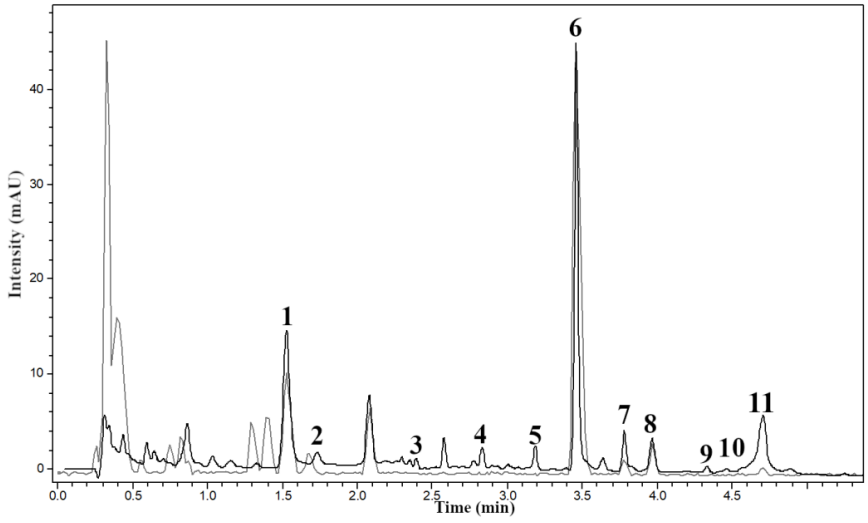


Fig. 4.2. UPLC chromatograms of BJP: Grey-line – All mass FullScan from 50 to 1500 *m/z*; Black-line – UV chromatogram at 254 nm

The results indicate that beetroot preparations prepared in a different way had a different profile of betalains. The changes in the betalains profile after purification with Sephadex-LH20 are shown in Fig. 4.3.

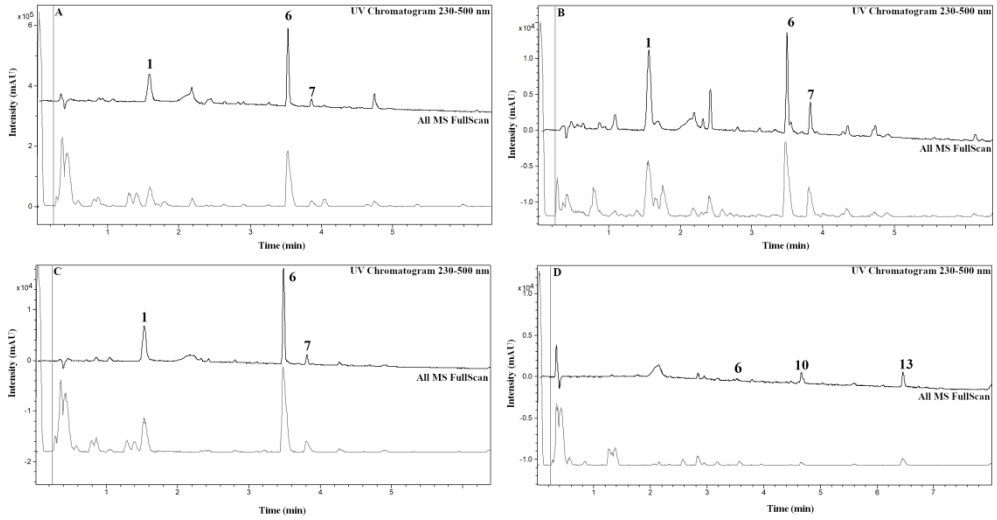


Fig. 4.3. PDA-Q-TOF- mass spectrometry chromatograms of *Beta vulgaris* before (A. – BRP) and after (B. – BtFI; C. – BtFII; D. – BtFIII) fractionation with Sephadex-LH20

Chromatograms show that the most dominant compounds in BtFI were vulgaxanthin I, vulgaxanthin II, betanin and isobetanin. Similarly, those compounds were found in other samples, but at a lower content, except for BtFIII. Again, betanin was the dominant compound from betacyanin, while vulgaxanthin I was the dominant compound from betaxanthin present in BRP, BtFI and BtFII. In BJP, BRP and BtFI, a compound was detected with a protonated molecular ion at *m/z* 307, but it was not been reported in literature sources. The fragmentation pattern of this molecule clearly indicates that this compound can be assigned to betaxanthins. The content of neobetanin was higher in BRP than in BJP, while, if comparing between the fractions, BtFIII has the highest level of the neobetanin content. This indicates betanin dehydrogenation which probably was favored by the basic conditions during the purification process. It also seems that, during the separation process, neobetanin was decarboxylated to decarboxy-neobetanin ***12***, and this compound was eluted in BtFI. Most likely, this compound could be 2-decarboxy-neobetanin as its protonated molecular ion at *m/z* 505 and fragment ions of *m/z* 343 (=505–162(glucose)), 297 (=505–162(glucose)–CO_2–2 H) and 255 (=505–162(glucose)–2 CO_2) fit well with a previous study (Kumorkiewicz *et al*., 2019). 2-Decarboxy-neobetanin is assigned to the degradation products of betanin, which strongly increases during thermal treatment (Herbach *et al*., 2004b). The decarbocylated and dehydrogenated form of betanin was found in BtFII and BtFIII; this shows a higher degradation rate of betanin. Compound ***14*** most likely is 2-decarboxy-2,3-dehydro-neobetanin as its protonated molecular ion at *m/z* 503 and fragment ions of *m/z* 341 (=503–162(glucose)), 295 (=503–162(glucose)–CO_2) and 253 (=503–162(glucose)–2 CO_2–2 H) also fit the

results of previous studies (Herbach *et al*., 2004b; Kumorkiewicz *et al*., 2019). Unknown compound ***4*** having a protonated ion at *m/z* 345 could be tentatively assigned to 17-decarboxy-isobetanin, as its mass spectrometry data fits with a previous study of Nemzer *et al*. (2011) who found this compound in a few dried extracts of beetroot. In our study, it was found only in the last fraction (BtFIII); therefore, this compound might be related with the stronger degradation of betanin. The emergence of 2-decarboxy-2,3-dehydro-neobetanin, 2-decarboxy-neobetanin and a compound with *m/z* 345 in the latter fractions indicates that decarbocylation of betalains was favored during the purification conditions. Also, the degradation products have a longer t_R, therefore, they can be partly separated from the main betalains. The highest diversity and total content of betalains was eluted in BtFI, while the highest content of betanin was obtained in BtFII. Betacyanins and betaxanthins were not separated by using Sephadex-LH20 equipment.

The aim of HPLC purification was to separate vulgaxanthin I and betanin and to obtain higher purity of those compounds. Therefore, monitoring of each fraction was performed with UPLC-PDA-UV, and only the fractions containing the highest amounts of these compounds were collected. The degree of purification depended on the calculations. The betanin content in BnF was 78% when calculated by the peak areas in a UV chromatogram at 230–500 nm and 57.99% when calculated by the peak areas in the mass chromatogram the (base peak). Vulgaxanthin I content in VxF was 100% when calculated by the peak areas in the UV chromatogram at 230–500 nm, and 1.00% when calculated by the peak areas in the mass chromatogram. The UPLC-mass spectrometry base peak chromatogram of BntF is shown in Fig. 4.4. UV chromatograms at different wavelengths (230–500 nm, 280 nm, 470 nm, 500 nm) are presented in Fig. 4.5.

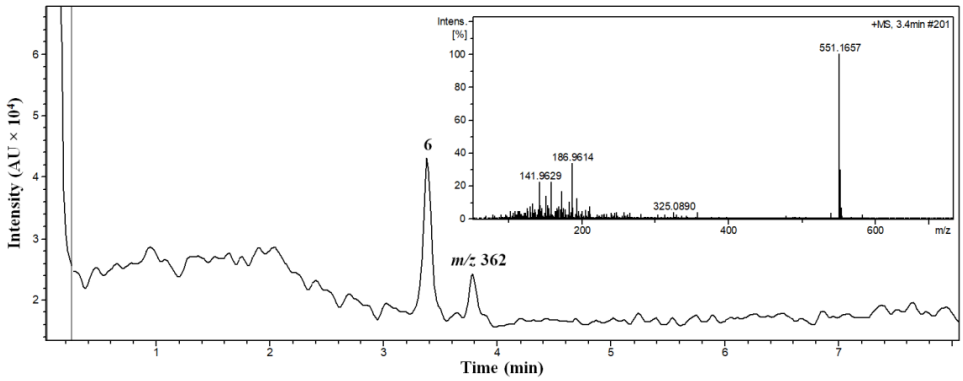


Fig. 4.4. Q-TOF-mass spectrometry base peak chromatogram of BnF (Insert: mass spectra of betanin)

Two main peaks were detected on the base peak chromatogram in BnF. The first peak was that of betanin, while the second peak was of the compound with *m/z* 362. The detailed MS/MS characterization of the compound with *m/z* 362 was not performed, but, possibly, it could be a different compound and not a variety of betalains as it shows no absorption at 470–500 nm, and it is not a phenolic compound because it

shows no absorption at 280 nm. The UV chromatogram at 280 nm indicates absence of phenolic compounds (Fig. 4.5B), while the UV chromatogram at 470 nm indicates absence of other betaxanthins (Fig. 4.5C).

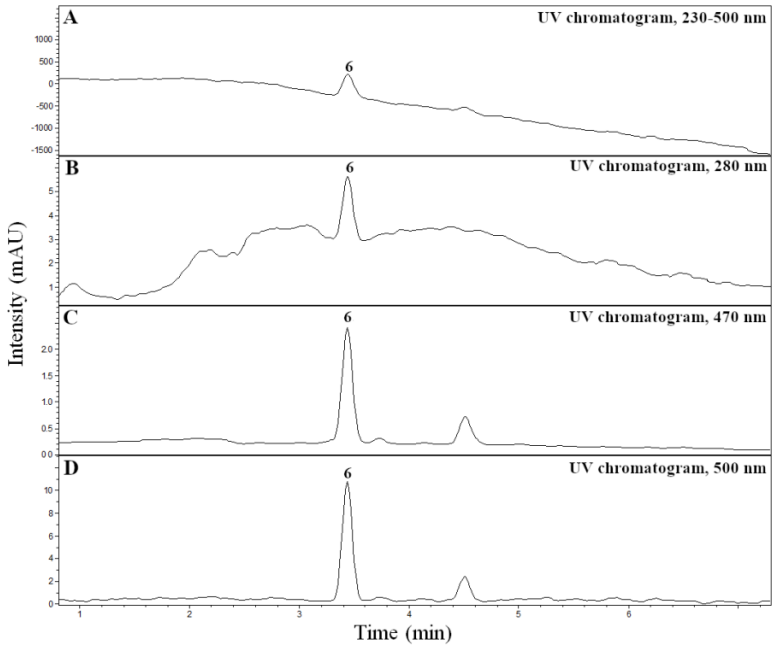


Fig. 4.5. PDA-UV chromatograms of BnF at different wavelengths

UV chromatograms at 230–500 nm, 470 nm and 500 nm revealed the presence of 17-decarboxy-betanin at t_R 4.52 which has a protonated molecular mass of *m/z* 507.1740 ($C_{23}H_{26}N_2O_{11}$), and a fragment of *m/z* 345. However, it was not detected in the mass base peak chromatogram. Meanwhile, 17-decarboxy-betanin is attributed to a heating degradation product of betanin (Herbach *et al*., 2004b; Wybraniec, 2005). The UPLC-mass spectrometry base peak chromatogram of VxF is shown in Fig. 4.6.

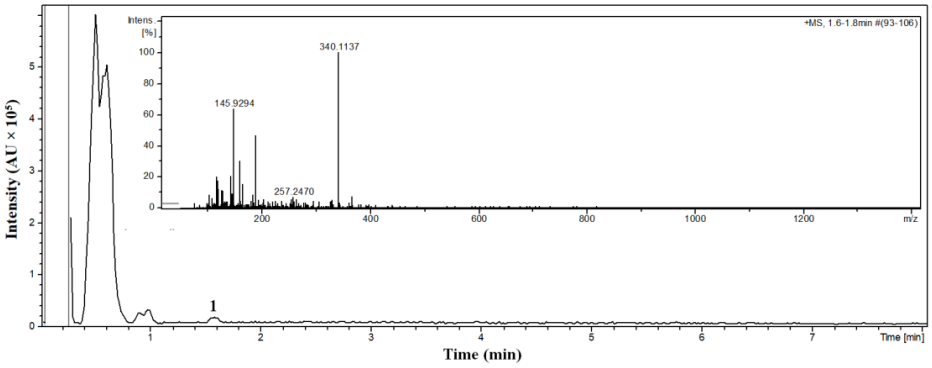


Fig. 4.6. Q-TOF-mass spectrometry base peak chromatogram of VxF (Insert: mass spectra of Vulgaxanthin I)

The chromatogram revealed the presence of various substances in VxF at t_R from 0.4 to 0.8 min and a low concentration of Vulgaxanthin I. UV chromatograms of various wavelengths (230–500 nm, 280 nm, 470 nm, 500 nm) are presented in Fig. 4.7. Monitoring of absorption at 230–500 nm revealed that Vulgaxanthin I was the main compound of betalains. Monitoring at 280 nm indicated absence of phenolic compounds (Fig. 4.7B), monitoring at 470 nm indicated absence of other betaxanthins (Fig. 3.7C). The UV chromatogram at 500 nm showed the presence of a traceable amount of betanin (Fig. 3.7D); however, it could not be detected at the mass chromatogram. Vulgaxanthin I was eluted at 1.7 min and had UV absorption maxima at 465 nm.

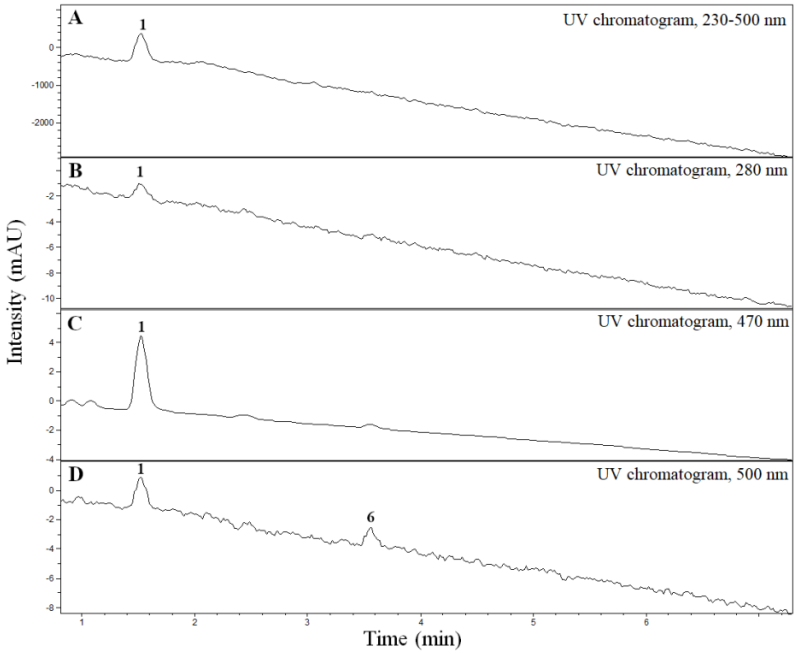


Fig. 4.7. PDA-UV chromatograms of VxF at various wavelengths: (A) 230–500 nm; (B) 280 nm; (C) 470 nm; (D) 500 nm

4.1.4. Content of betalains

UV-visible spectroscopy was used to calculate the content of betalains in fresh beetroot juice. Nilsson's method is the basic approach for betalain quantification recording betacyanins and betaxanthins in beetroot juice, in which, the main component is betanin, and vulgaxanthin I is a minor component. For betacyanins, the wavelength of maximum absorbance is 540 nm, whereas, for betaxanthins, it equals 480 nm; therefore, this methodology is basically used for the determination of pigment components in betalain-based foods. The absorbance was recorded immediately after fresh juice had been centrifuged. Impurities, such as browning substances (decarboxylated and oxidized derivatines of betanin), were corrected by measuring the absorption at 600 nm. The calculated betanin and vulgaxanthin I content in fresh

beetroot juice was 47.09±0.81 and 38.80±1.63 mg/100 mL, respectively. From the spectrophotometric results, the ratio of betanin to vulgaxanthin I (Bt:VI) in beetroot juice was 1:0.8. According to the analytical chromatographic method, the ratio of Bt:VI was close to 1:0.5, which is more consistent with most of the literature data (Georgiev *et al*., 2010; Nemzer *et al*., 2011). Therefore, a further analytical chromatographic technique was used for the calculations of the ratio of Bt:VI.

The comparison of the betalain content in various beetroot preparations was made by calculating the UPLC base peak areas and expressed as AU×10^5/mg pdw (Table 4.4). The betalain profile and content of BJP were similar to BRP. There was an increase up to a 2-fold level in BtFI and up to 1.4-fold level in BtFII in the total content of betalains after the purification of BRP by gel chromatography. The diversity and content of betacyanins were higher in BtFI comparing with BtFII, while BtFIII consisted mostly of the degradation products of betalains. The changes in the content of the major betalains during fractionation with Sephadex-LH20 are shown in Fig. 4.8. It is evident that BtFI contained the highest content of isobetanin, Vulgaxanthin I and Vulgaxanthin II, while BtFII contained the highest content of betanin. In BtFIII, only a minor amount of betanin was found. BnF contained 4-fold and 2-fold higher betanin content than BJP and BtFI, respectively. During HPLC purification of betalains from BJP, a high content and purity of betanin in BnF were obtained. The content of vulgaxanthin I in VxF was 2-times higher than in BJP and similar to BtFI.

In order to demonstrate the shifting proportions of the four major betalains in the beetroot fraction through the purification process, the ratios of isobetanin/betanin, vulgaxanthin II/vulgaxanthin I, vulgaxanthin I/betanin and vulgaxanthin II/betanin were calculated (Table 4.4).

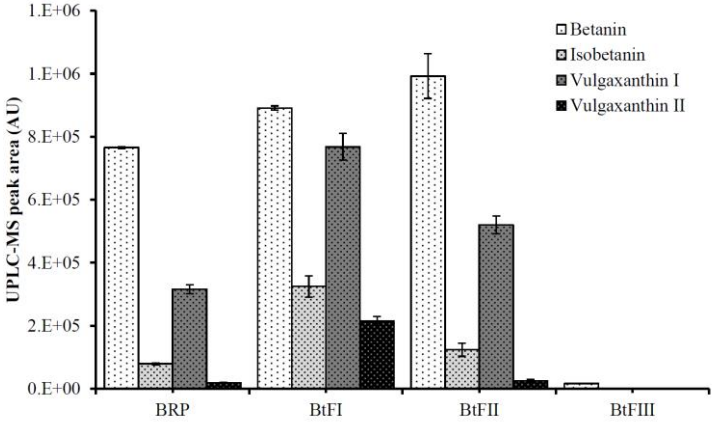


Fig. 4.8. Content of the major betalains in the samples before (BRP) and after (BtFI, BtFII and BtFIII) the purification process with Sephadex-LH20

Table 4.4. The content of identified betalains in various beetroot preparations

No.	Compound	Content of betalains in different beetroot preparations (AU×10^{-5}/mg pdw)						
		BJP	**BRP**	**BtFI**	**BtFII**	**BtFIII**	**BnF**	**VxF**
1.	Vulgaxanthin I	659.81±23.19	631.66±28.28	1535.65±84.85	1040.19±56.57	N.D.	N.D.	1419.56±13.58
2.	Unknown 1 ($C_{21}H_{11}N_2O$)	84.00±2.83	69.60±5.66	84.24±8.49	N.D.	N.D.	N.D.	N.D.
3.	Vulgaxanthin II	31.10±2.95	36.99±2.83	430.24±28.28	49.08±8.49	N.D.	N.D.	N.D.
4.	Unknown 2 ($C_{18}H_{13}N_6O_2$)	N.D.	N.D.	N.D.	N.D.	246.10±9.25	N.D.	N.D.
5.	Proline-isobetaxanthin	N.D.	N.D.	89.89±5.16	75.34±3.23	N.D.	N.D.	N.D.
6.	Betanin	1239.61±33.55	1530.94±6.67	1782.26±14.14	1984.97±141.42	31.47±1.41	4975.04±282.74	N.D.
7.	Isobetanin	193.54±5.66	158.37±5.66	649.37±67.88	247.08±42.43	N.D.	N.D.	N.D.
8.	Proline-betaxanthin	N.D.	N.D.	71.63±8.49	N.D.	N.D.	N.D.	N.D.
9.	Betanidin	N.D.	TR	187.81±11.31	N.D.	N.D.	N.D.	N.D.
10.	Neobetanin	N.D.	161.74±2.83	55.70±7.98	59.56±2.12	96.10±4.24	N.D.	N.D.
11.	2-Decarboxy-neobetanin	N.D.	TR	106.45±4.24	N.D.	N.D.	N.D.	N.D.
12.	Valine-betaxanthin	168.49±11.31	93.54±7.07	112.02±11.31	N.D.	N.D.	N.D.	N.D.
13.	2-Decarboxy-2,3-dehydro-neobetanin	N.D.	N.D.	N.D.	147.72±7.07	221.64±14.14	N.D.	N.D.
Total Content		**2376.55**	**2683.84**	**5105.25**	**3603.94**	**595.32**	**4975.04**	**1419.56**
Isobetanin/Betanin		1:0.16	1:0.10	1:0.36	1:0.12	0	0	0
Vulgaxanthin II/ Vulgaxanthin I		1:0.05	1:0.06	1:0.28	1:0.05	0	0	0
Vulgaxanthin I/Betanin		1:0.53	1:0.41	1:0.86	1:0.52	0	0	0
Vulgaxanthin II/Betanin		1:0.03	1:0.02	1:0.24	1:0.02	0	0	0

N.D.: not detected; TR, traces; N.T.: not tested.

The ratios of BJP were fairly similar to BRP. All the ratios reached double or even higher levels in BtFI. BtFII showed similar ratios of vulgaxanthin II/vulgaxanthin I and vulgaxanthin II/betanin to BJP and BRP, but higher ratios of isobetanin/betanin and vulgaxanthin I/betanin.This indicates that the content of Vulgaxanthin II did not change, while the content of isobetanin, betanin, and vulgaxanthin I increased in BtFII. The obtained results indicate that, through the gel chromatographic process, two aspects can be achieved: first, the concentration of betalains in the total amount, and, second, the concentration of isobetanin, betanin, vulgaxanthin I content. Moreover, the degradation of betanin proceeds during the purification process mainly through decarbocylation, and there is an increase in the content of decarboxylated derivatives, which could partly be separated from the main betalains. Dehydrogenation also increases, while dehydrogenated betanin derivatives are eluded with the main betalains and are not separated.

Various beetroot preparations were prepared and characterized in terms of their sugar and betalains content and antioxidant properties. BnF contained a high content of betanin and was pure from other betalains or other substances, such as sugars. VxF contained a high amount of Vulgaxanthin I pure from other betalains, but it had a high content of sugars. Taking into account our own and other previously published (Gandía-Herrero *et al*., 2010) results, it can be seen that purified betacyanidins possess much higher radical scavenging properties than the levels of properties that are present in such mixtures as beetroot juice. In our study, pure betanin showed a considerably higher (21 times) radical scavenging activity than BJP. In contrast, it can be observed that betaxhantins (Vulgaxanthin I) possess low radical scavenging activity. In our study, VxF possessed a 13 times lower radical scavenging activity than BJP and 300 times lower activity than BnF. This is in agreement with the previously published data where betaxanthins showed a low antiradical activity in comparison to betacyanins. Our study also revealed that the degradation products of betalains also possess radical scavenging activities.

The effect of beetroot preparations (BJP, BtFI, BtFII, BtFIII, BnF and VxF) on MPRs formation was further tested in food model systems.

4.2. CHARACTERIZATION OF LINGONBERRY LEAVES

4.2.1. Yields and total phenolic content

LL was obtained by using methanol and acetone from the raw and deodorized plant material. In addition, a water extract was obtained by freeze-drying the liquid phase remaining after hydro-distillation. In total, five extracts of LL were characterized: three extracts were obtained from raw LL (WE, ME, AE) and two extracts were obtained from deodorized LL (DME and DAE).

The extraction yields and TPC of different extracts of LL are presented in Table 4.5. In the case of raw LL, the highest and the lowest extraction yields were obtained with methanol and acetone, respectively, whereas deodorized LL gave 2-times lower extract yields than raw LL. In general, the yields of extracts decreased in the following

order: ME>WE>AE>DME>DAE. The differences between the yields of ME and WE were marginal; both solvents are high polarity compounds.

Table 4.5. The yield and TPC of different extracts of LL

LL Extract	Yield, g/100g pdw	TPC, mg GAE/g edw	TPC, mg GAE/g pdw
WE	32.96±1.90d	252.22±4.40c	83.12±1.45d
ME	36.38±2.51e	250.56±3.62c	91.15±1.31e
AE	23.69±2.60c	242.42±5.05c	57.42±1.19c
DME	14.09±0.60b	141.80±3.91b	19.98±0.55b
DAE	9.57±0.50a	96.52±8.53a	9.24±0.82a

Values represent means±SD; Values in the same column followed by different superscript letters are significantly different (P<0.05). WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract.

Methanol is known to be a very effective solvent for the extraction of low sugar content plant materials. Moreover, extraction with methanol was performed at the increased pressure, which facilitates the diffusion and solubilization processes. Meanwhile, acetone is not a highly effective solvent for higher polarity constituents, while DME and DAE were prepared from the solid hydrodistillation residue which had already been extracted by water. The TPC values of raw LL extracts were not significantly different; however, the highest amount of TPC expressed in mg GAE/g pdw was recovered with ME followed by WE, AE, DME, and DAE. More than 2-times lower TPC values were found for deodorized LL extracts than that of the raw LL material. Recent studies reported TPC values of *Vaccinium vitis-idaea* L. leaf in the range of 85.3–114.6 mg GAE/g DM (Bujor *et al*., 2018), which is in agreement with our data (57.42–91.15 mg GAE/g pdw).

4.2.2. Free radical scavenging properties

The antioxidant capacity of LL extracts was evaluated by three radical scavenging assays, including $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and ORAC. The linear fitting equations used to calculate effective $DPPH^{\bullet}$ scavenging concentration (EC_{50}) and expressions in TE from DPPH assay are shown in Table 4.6.

Table 4.6. $DPPH^{\bullet}$ radical scavenging activities of LL extracts

LL Extract	Linear fitting equation	R^2	DPPH EC_{50}, mg/mL	DPPH, µM TE/g edw	DPPH, µM TE/g pdw
WE	y = 16.883x+9.543	0.993	2.40±0.01a	187.57±0.90c	61.81±0.30d
ME	y = 16.952x+9.947	0.992	2.36±0.00a	190.24±0.19d	69.20±0.07e
AE	y = 17.410x+6.170	0.979	2.54±0.00a	178.54±0.36b	42.29±0.08c
DME	y = 17.314x+6.003	0.997	2.52±0.01a	176.89±0.33b	24.93±0.05b
DAE	y = 9.373x+2.386	0.999	5.11±0.58b	88.45±9.99a	8.47±0.96a

Values represent means±SD; Values in the same column followed by different superscript letters are significantly different (P<0.05). LL, lingonberry leaves; WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract; edw, extract dry weight; pdw, plant dry weight.

The weakest $DPPH^{\bullet}$ scavenger was acetone extract from deodorized leaves (DAE), while the differences between EC_{50} values of WE, ME, AE, and DME were

not significant in this assay. When radical scavenging activity was expressed in TE, the highest activity was shown by ME followed by WE, AE, DME, and DAE. In terms of the DPPH$^{\bullet}$ assay, LL extracts showed a slow kinetic behavior, whereas the reaction between the extracts and the radical was completed at around 35 min (Fadda *et al*., 2014).

In the ABTS$^{\bullet+}$ (Table 4.7) and ORAC (Table 4.8) assays, the antioxidant capacity of extracts was decreasing in the following order: WE > ME > AE > DME > DAE. Comparing our results with literature data is difficult because of the differences in the expression of antioxidative values and methods. Nevertheless, similar ABTS assay were found in a study by Raudone *et al*. (2019) where much higher values ranging from 620.49 to 2179.71 mg/g dw were reported in *Vaccinium vitis-idaea* L. leaves.

Table 4.7. ABTS$^{\bullet+}$ radical cation scavenging activities of LL extracts

LL extract	Linear fitting equation	R^2	ABTS EC_{50}, mg/mL	ABTS, mM TE/ g edw	ABTS, mM TE/g pdw
WE	y = 152.043x+14.388	0.982	0.23±0.01a	6.14±0.32e	2.02±0.11e
ME	y = 107.68x+15.589	0.870	0.32±0.01b	4.49±0.12d	1.63±0.05d
AE	y = 78.966x+12.557	0.963	0.47±0.01c	3.02±0.06c	0.72±0.02c
DME	y = 70.335x+6.2292	0.991	0.62±0.01d	2.30±0.03b	0.32±0.00b
DAE	y = 19.671x+15.719	0.987	1.74±0.02e	0.82±0.01a	0.08±0.00a

Values represent means±SD; Values in the same column followed by different superscript letters are significantly different (P<0.05). LL, lingonberry leaves; WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract; edw, extract dry weight; pdw, plant dry weight.

Significant correlations between TPC (mg GAE/g pdw) and DPPH$^{\bullet}$ scavenging (r = -0.70), TPC (mg GAE/g pdw) and ABTS$^{\bullet+}$ scavenging (r = 0.87), as well as ORAC values (r = 0.95) were determined.

Table 4.8. ORAC scavenging activities of LL extracts

LL Extract	ORAC, mM TE/g edw	ORAC, mM TE/g pdw
WE	10.63±0.88e	3.50±0.29e
ME	7.44±1.05d	2.71±0.38d
AE	4.43±0.23b	1.44±0.10c
DME	6.08±0.42c	0.62±0.03b
DAE	1.62±0.09a	0.16±0.00a

Values represent means±SD; Values in the same column followed by different superscript letters are significantly different (P<0.05). LL, lingonberry leaves; WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract; edw, extract dry weight; pdw, plant dry weight.

In general, methanol and water, which are high polarity protic solvents, gave higher yields, TPC and antioxidant capacity values than acetone, a lower polarity aprotic solvent. This indicates that LL contains higher amounts of polar polyphenolic compounds. Therefore, TPC, extraction yields and antioxidant activity values of extracts obtained from deodorized LL were remarkably lower in comparison to the extracts from raw LL; the major part of water soluble compounds remains in WE after

LL hydro-distillation. In addition, comprehensive *in vitro* evaluation of antioxidative properties of different LL extracts was performed for the first time.

4.2.3. Composition of phenolic compounds

Identification of phenolic compounds present in LL extracts was mainly performed by comparing their t_R, exact masses and fragmentation patterns with the data presented in such databases as METLIN and Human Metabolome (HMDB) (Benton, Wong, Trauger, & Siuzdak, 2008). Also, MS data was compared with the data that was previously reported for those compounds in LL (Ek *et al*., 2006; Liu *et al*., 2014) (Table 4.9).

Table 4.9. Compounds identified in extracts of leaves of *Vaccinium vitis-idaea* L.

No.	Compound	t_R (min)	$[M-H]^-$ (*m/z*)	Molecular ion formula	MS/MS (*m/z*)
14.	Catechin[1]	1.28	289.0719	$C_{15}H_{13}O_6$	245.0826(100), 179.0306(25)
15.	Epicatechin[1]	1.55	289.0716	$C_{15}H_{13}O_6$	245.0825(60), 179.0306(19)
Flavan-3-ols					
16.	Quinic acid[1]	0.35	191.0564	$C_7H_{11}O_6$	–
17.	Citric acid[1]	0.43	191.0201	$C_6H_7O_7$	111.0083(20)
18.	Caffeoyl-shikimic acid[2]	1.08	335.0775	$C_{20}H_{15}O_5$	179.0340(55), 161.0237(100)
19.	Chlorogenic acid[1]	1.33	353.0874	$C_{16}H_{17}O_9$	–
20.	Caffeic acid[1]	1.47	179.0353	$C_9H_7O_4$	–
21.	*p*-Coumaric acid[1]	1.87	163.0402	$C_9H_7O_3$	–
Hydroxycinnamic acids					
22.	Arbutin[2]	0.49	271.0824	$C_{12}H_{15}O_7$	108.0218(100), 109.0275(8)
23.	2-*O*-caffeoylarbutin[2,3]	1.70	433.1147	$C_{21}H_{21}O_{10}$	323.0778 (5), 179.0350 (30), 161.0245 (100), 135.0449 (10)
24.	Caffeoyl acetyl arbutin[3]	2.56	475.1242	$C_{23}H_{23}O_{11}$	179.0347(15), 161.0244(100)
Arbutin derivatives					
25.	B-type proanthocyanidin[2]	1.34	577.1336	$C_{30}H_{25}O_{12}$	407.0757(68), 289.0709(100), 245.0807(21), 161.0233(15), 125.0240(35)
26.	A-type proanthocyanidin[2,3]	1.95	575.1195	$C_{30}H_{23}O_{12}$	539.0998(65), 407.0791(48), 285.0387(100), 125.0245(58)
Proanthocyanidins					
27.	Rutin[1]	1.84	609.1459	$C_{27}H_{29}O_{16}$	300.0267(100), 301.0332(30)
28.	Quercetin-3-*O*-glucoside[1]	1.95	463.0881	$C_{21}H_{19}O_{12}$	301.0339(66), 300.0272(100)
29.	Quercetin 3-*O*-xyloside[2,3]	2.10	433.0773	$C_{20}H_{17}O_{11}$	301.0337(56), 300.0266(100)
30.	Quercetin-3-*O*-arabinoside[2,3]	2.25	433.0769	$C_{20}H_{17}O_{11}$	301.0345(58), 300.0274(100)
31.	Quercetin-3-*O*-*α*-rhamnoside[2,3]	2.28	447.0931	$C_{21}H_{19}O_{11}$	301.0338(23), 300.0252(100), 271.0227(86)
32.	Quercetin-3-*O*-(HMG)-rhamnoside[3]	2.67	591.1355	$C_{27}H_{27}O_{15}$	529.1334(27), 489.1028(100), 447.0924(72), 301.0269(65)
33.	Kaempferol-3-*O*-(HMG)-rhamnoside[3]	2.95	575.1392	$C_{27}H_{27}O_{14}$	515,1377(15), 473.1075(40), 431.0969(38), 285.0391(100)

[1]compounds identified by comparing to standard. [2]compounds identified by calculated molecular formula and fragmentation patterns comparing to METLIN and/or HMDB databases. [3]compounds identified by calculated molecular formula and fragmentation patterns comparing to literature data (according to Ek *et al*. and Liu *et al*.)

The UV chromatograms of phenolic compounds identified in different extracts are shown in Fig. 4.9, while the relative amounts of phenolic compounds based on UPLC-Q-TOF- mass spectrometry peak areas are presented in Table 4.10.

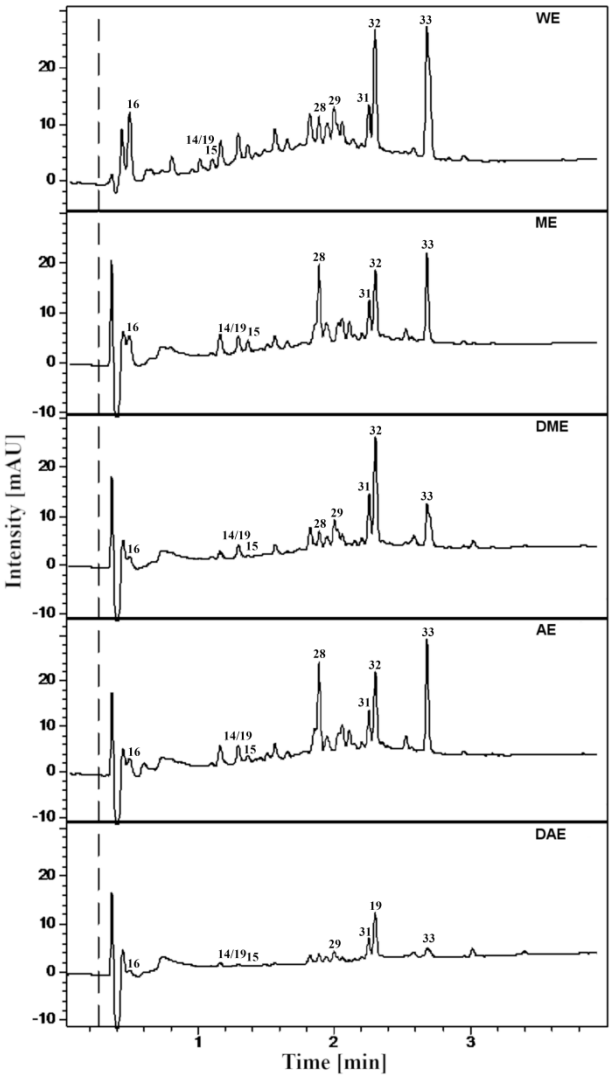


Fig. 4.9. UPLC-UV chromatograms at 254 nm of LL extracts: WE (A), ME (B), DME (C), AE (D) and DAE (E). Peak numbers correspond to the compounds listed in Table 4.9. Abbreviations: WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract

Both databases were used to retrieve additional information for supporting the identity of the compounds.Three compounds (caffeoyl acetyl arbutin ***24***, quercetin-3-O-(HMG)-rhamnoside ***32*** and kaempferol-3-O-(HMG)-rhamnoside ***33***) were not found in these databases; however, the same compounds were identified in the above

mentioned reports in LL by using NMR analysis, and their fragmentation patterns were well-fitting. Such MS data as exact masses and fragmentation patterns indicated that compounds ***29*** and ***30*** may be quercetin-3-*O*-xyloside and quercetin-3-*O*-arabinoside, respectively. Based on a previous study (Liu *et al*., 2014), their identity was additionally confirmed by the order of their elution. Catechin, quinic, chlorogenic, caffeic, citric and *p*-coumaric acids, rutin and quercetin-3-*O*-glucoside were identified by authentic standards. Twenty phenolic compounds were tentatively identified in LL extracts.

Table 4.10. Compounds tentatively identified in leaves of *Vaccinium vitis-idaea* L. and their LC/Q-TOF–MS/MS data and the content (AU×10^{-7}/mg edw).

No.	Compound	LL extracts				
		WE	**ME**	**AE**	**DME**	**DAE**
14.	Catechin[1]	31.85±0.23c	32.18±0.56c	36.48±0.31d	25.37±0.08b	5.93±0.15a
15.	Epicatechin[1]	14.62±0.35a	32.18±0.57b	14.33±0.16a	18.56±9.5ab	1.53±0.01a
Total flavan-3-ols		***46.47***	***64.36***	***50.81***	***43.93***	***7.46***
16.	Quinic acid[1]	42.81±2.52c	25.46±0.56b	2.00±0.02a	1.61±0.12a	TR
17.	Citric acid[1]	46.48±0.32	ND	ND	ND	ND
18.	Caffeoyl-shikimic acid[2]	2.85±0.02e	2.22±0.10d	1.96±0.02c	1.25±0.02b	0.72±0.05a
19.	Chlorogenic acid[1]	3.44±0.05a	TR	TR	TR	ND
20.	Caffeic acid[1]	2.62±0.04b	ND	ND	1.04±0.00a	0.98±0.08a
21.	p-Coumaric acid[1]	1.56±0.16b	ND	ND	0.92±0.02a	1.57±0.04b
Total hydroxycinnamic acids		***99.76***	***27.68***	***3.96***	***4.82***	***3.27***
22.	Arbutin2	33.18±0.03d	31.78±0.67d	23.00±0.43c	17.48±0.08b	9.61±0.05a
23.	2-O-caffeoylarbutin[2,3]	24.11±1.01c	29.85±0.35d	34.34±0.24e	21.92±0.07b	10.21±0.23a
24.	Caffeoyl acetyl arbutin[3]	8.51±0.14b	8.82±0.00b	10.97±0.22c	10.49±0.31c	6.05±0.08a
Total arbutin derivatives		***65.8***	***70.45***	***68.31***	***49.89***	***25.87***
25.	B-type proanthocyanidin[2]	8.35±0.35a	TR	TR	TR	ND
26.	A-type proanthocyanidin[2,3]	3.67±0.03c	0.66±0.03b	0.66±0.19b	0.49±0.00ab	0.27±0.07a
Total proanthocyanidins		***12.02***	***0.66***	***0.66***	***0.49***	***0.27***
27.	Rutin[1]	1.53±0.03a	TR	TR	TR	ND
28.	Quercetin-3-O-glucoside[1]	3.33±0.05b	3.31±0.05b	3.15±0.20b	3.26±0.11b	1.12±0.09a
29.	Quercetin-3-O-xyloside[2,3]	20.43±0.07c	ND	ND	18.20±0.32b	7.70±0.25a
30.	Quercetin-3-O-arabinoside[2,3]	7.71±0.28b	7.44±0.11b	7.68±0.16b	9.34±0.26c	4.17±0.17a
31.	Quercetin-3-O-α-rhamnoside[2,3]	15.95±0.08e	15.24±0.18d	12.38±0.14c	10.80±0.07b	7.70±0.00a
32.	Quercetin-3-O-(HMG)-rhamnoside[3]	29.80±0.10d	0.41±0.03b	0.61±0.02c	0.29±0.01a	0.30±0.01a
33.	Kaempferol-3-O-(HMG)-rhamnoside[3]	1.78±0.02d	1.07±0.02b	1.46±0.08c	1.02±0.06b	0.38±0.01a
Total flavonols		***80.53***	***27.47***	***25.28***	***42.91***	***21.37***
Total phenols		***304.58***	***190.62***	***149.02***	***142.04***	***58.24***

Data represents mean (AU×10^{-7}/mg edw) ± SD of triplicate analyses: different superscript letters ($^{a–e}$) within a row indicate significant differences between the extracts (P<0.05). Abbreviations: WE, water extract; ME, methanol extract; AE, acetone extract; DME, deodorized methanol extract; DAE, deodorized acetone extract; edw, extract dry weight; –, not performed; ND, not detected; TR, traces.

The indicative amount of the total phenols measured by UPLC was lower in ME and AE than in WE by 37% and 51%, respectively. Lower recovery of phenolics by acetone may be explained by the prevalence of higher polarity compounds in LL which are more soluble in more polar solvents. DME and DAE demonstrated lower recovery of phenolics than WE by 53% and 81%, respectively. The content of phenolics in deodorized plant extracts was considerably lower comparing with raw LL extracts because the main part of the compounds which had higher polarity remained in water during hydro-distillation. This indicates that, after water extraction of raw LL, phenolics compounds still remained there which were extractable with methanol and acetone in deodorized LL. Hydroxycinnamic acids and flavonols were the most abundant classes of phenolics in WE, while the dominating compounds in ME and AE were arbutin derivatives and flavan-3-ols. In agreement with previous studies, quercetin pentosides and hexosides were the dominant flavonols in all the extracts (Ek *et al*., 2006; Liu *et al*., 2014). Caffeic acid ***20***, *p*-coumaric acid ***21*** and quercetin-3-*O*-xyloside ***29*** were detected only in WE, DME and DAE indicating that detectable amounts of these compounds were released after hydro-distillation. Also, citric acid ***17***, chlorogenic acid ***19***, B-type proanthocyanidin ***25*** and rutin ***27*** were found only in WE. Previous studies found that lingonberry leaves of different cultivars contain 0.14–2.95 mg/g dw pf (+)-catechin, 0.026–0.7 mg/g dw of (-)-epicatechin and 7.07–56 mg/g dw of arbutin (Raudone *et al*., 2019).

4.2.4. Identification of active free radical scavengers

The HPLC-DPPH$^{\bullet}$ online assay was used to evaluate the radical scavenging profile of phenolics compounds in order to determine the main contributors to the antioxidant capacity in LL. The foundation of this method is the measurement of DPPH$^{\bullet}$ absorption decrease (at 515 nm) in the presence of compounds which reduce it to its hydrazine form (bleaching) by hydrogen or electron donation. The reaction takes place in the post-column loop, and the ability to donate hydrogen/electron is evaluated for HPLC separated compounds. Therefore, this method is a highly beneficial tool to detect compounds possessing radical scavenging capacity in a mixture of compounds. Fig. 4.10 shows the HPLC-DPPH$^{\bullet}$ chromatogram of WE, ME and AE form raw LL. The eight peaks that were detected at 254 nm appeared as negative peaks at 515 nm, which indicates their ability to quench the DPPH radicals. The negative peaks in all the extracts were found for catechin ***14*** and epicatechin ***15***, caffeoyl-hexose hydroxyphenol ***24***, quercetin-3-*O*-arabinoside ***30***, quercetin-3-*O*-α-rhamnoside ***31***, quercetin-3-*O*-(HMG)-rhamnoside ***32***, kaempferol-3-*O*-(HMG)-rhamnoside ***33***. Also, the negative peak of quercetin-3-*O*-xyloside ***29*** was found only in WE. The strongest DPPH$^{\bullet}$ quenchers in WE were catechin and epicatechin, while in ME and AE, these were catechin and caffeoyl-hexose hydroxyphenol. The screening of active compounds by HPLC-DPPH$^{\bullet}$ online assay revealed eight active radical scavengers in LL, which might contribute to the antioxidant activity of extracts.

The obtained results showed that water and methanol give higher yields and total phenolics values of LL extracts. The differences in the yields for the total phenols and antioxidant capacity for WE and ME were minor. However, in some assays, WE

showed stronger radical scavenging properties than ME. The most active radical scavengers were catechin and epicatechin in all extracts.

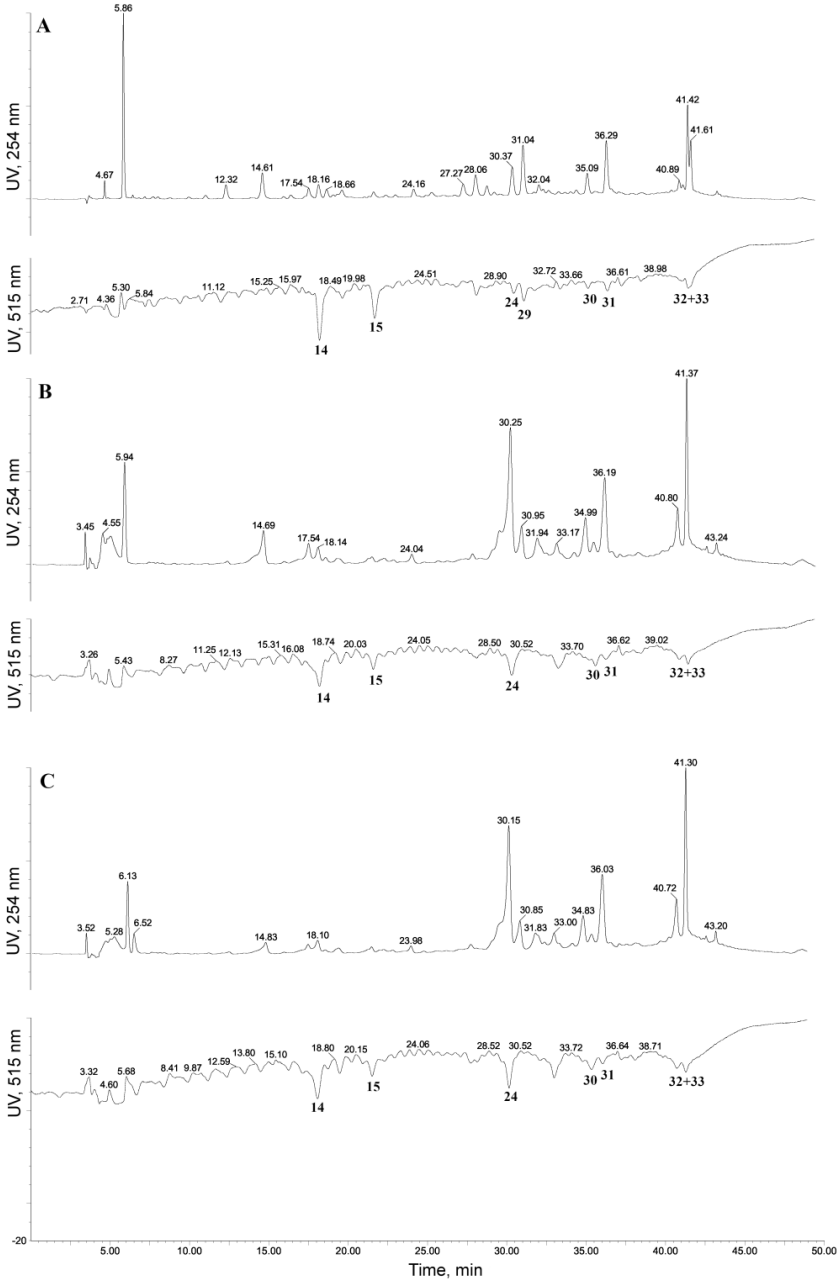


Fig. 4.10. HPLC chromatograms of WE (A), ME (B) and AE (C) detected at 254 nm and 515 nm. The negative peaks indicate DPPH radical scavenging activity, and their numbers correspond to the compounds listed in Table 3.9

The total identified value of phenolic compounds was considerably higher in WE than in ME or AE. However, the profile of phenols in WE comparing to the ME and AE was different, while, in the latters, it was quite similar. WE contained higher amounts of hydroxycinnamic acids and flavonols, while ME and AE contained higher amounts of arbutin derivatives and flavan-3-ols. ME and AE contained minor amounts of hydroxycinnamic acids which in WE were released during prolonged heating of LL in the hydro-distillation process. Extracts from deodorized leaves showed considerably lower values in yields, total phenolics and antioxidant activity, as it could have been expected because the major part of polar compounds remained in water during the hydro-distillation process. Therefore, additionally, less polar compounds could have been extracted from deodorized leaves.

In food model systems, AE was chosen for further evaluation of its possible inhibition of toxic Maillard reaction products. As WE showed the highest antioxidant potential, this was evaluated by *in vitro* antioxidant activity assays and contained the highest amount of the total phenols. AE had a different composition of phenolic compounds which matched the MRPs inhibitor properties: lower amounts of hydroxycinnamic acids and a higher content of catechin. In order to identify the possible active components in LE extracts, the standards of quinic acid and catechin were also chosen. Quinic acid was chosen because it was among the highest content from compounds in WE, while catechin possessed the strongest radical scavenging properties as it was determined in the on-line HPLC- DPPH$^{\bullet}$ scavenging assay.

4.3. IMPACT OF PLANT PREPARATIONS ON THE FORMATION OF MAILLARD REACTION PRODUCTS IN MILK MODEL SYSTEM

The antiglycation activity of plant preparations was evaluated through the determination of the changes of furosine and CML amounts in the milk model system. Furosine is an indicator of the early stages of MR arising from the acid hydrolysis of the Amadori products, such as fructosyl-lysine, lactulosyl-lysine, and maltulosyl-lysine produced by the reaction of lysine (ε-amino acid) with glucose, lactose and maltose, respectively (Guerra-Hernández, Corzo, & Garcia-Villanova, 1999). For dairy products, it is a quality index showing the type and intensity of food processing and storage conditions (Sunds, Rauh, Sørensen, & Larsen, 2018; Van Renterghem & De Block, 1996). CML is an indicator of the advanced stages of MR, and it is formed via different pathways including the fragmentation of the sugar moiety of fructose-lysine (Ahmed, Thorpe, & Baynes, 1986; Nguyen *et al*., 2014) and the direct reaction of glyoxal with the ε-amino group of lysine (Thornalley, Langborg, & Minhas, 1999).

The antiglycation activity of BJP was tested at 0.1 and 0.3 mg/mL concentrations in the milk model system. From LL, WE was chosen for this experiment. Since WE exhibited the highest radical scavenging capacity as well as the best solubility properties and made minor changes on the system color, it was chosen for further experiment. In order to assess the possible active components of LL, the standards of quinic acid and catechin were also tested as they were among the highest levels in WE. The antiglycation activity of LL WE, quinic acid and catechin were tested at 0.05, 0.1 and 0.3 mg/mL concentrations in the milk model system. The

extract concentrations were selected according to the literature data, and the color acceptability was also evaluated.

4.3.1. Effect of beetroot preparations on furosine and CML formation

After heating in the milk model system, the furosine content significantly increased in the control sample (Fig. 4.11A). Samples with added BJP contained lower levels of furosine at both heating times (30 and 45 s). After 30 s of heating, in the presence of 0.3 mg/mL BJP, the formation of furosine (84±6 ng/mL) was more lowered than in the presence of 0.1 mg/mL (102±7 ng/mL). The furosine level was reduced by more than 30% when 0.3 mg/mL of BJP was added. After 45 s of heating, the furosine level in the sample with 0.3 mg/mL BJP was lower (100±18 ng/mL), but no statistical difference was observed between these two researched concentrations. Advanced stages of MR were evaluated through the determination of CML changes in the milk samples during thermal heating (Fig. 4.11B). There were no significant differences between the control sample and the sample with added BJP at 0.1 mg/mL, after both heating times. The CML content was reduced by 17% when 0.3 mg/mL BJP was added in the milk sample and heated for 30 s. After 45 s of heating there were no significant differences in comparison with the control sample. These results indicate that the ability of beetroot phytochemicals to inhibit CML and furosine formation depend on the heating time and the concentration in use. We also observed that BJP was more effective in reducing furosine formation than CML in the milk model system during the heating.

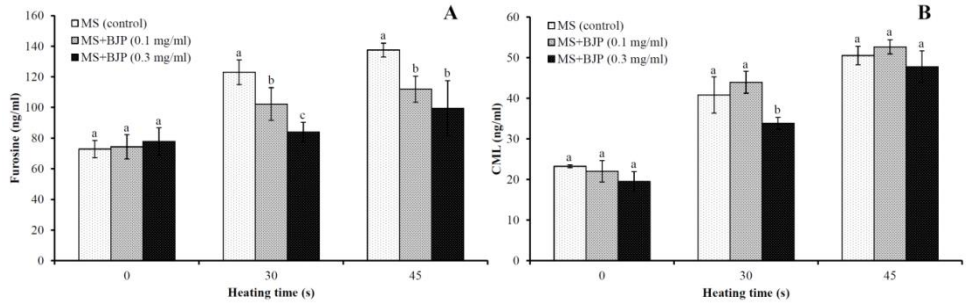


Fig. 4.11. Effects of BJP on furosine (A) and CML (B) formation in the milk model system at different heating times. The different letters on the columns at the same heating time indicate the significant differences (P<0.05). MS: milk model system, BJP: beetroot juice powder

4.3.2. Effect of lingonberry leaves extracts and some phenolic compounds on furosine and CML formation

The effect of WE on furosine and CML formation is shown in Fig. 4.12. The furosine level was reduced by 32, 21 and 17% after 30 s of heating with the addition of WE at 0.05, 0.1 and 0.3 mg/mL, respectively (Fig. 4.12A). After 45 s of heating, the reductions were 18% and 27% when 0.05 and 0.1 mg/mL of WE was added, respectively. There was no significant protective effect of WE at a concentration of 0.05 mg/mL on CML formation (Fig. 4.12B). The samples with added 0.1 and 0.3

mg/mL of WE contained lower amounts of CML by 38% and 19% after 30 s of heating, and 42% and 38% after 45 s of heating, respectively. The obtained results show that the formation of CML was more efficiently inhibited than that of furosine. Also, the lower concentrations of WE were more effective in reducing furosine formation, while higher concentrations of the extract was more effective in CML reduction. Overall, the results showed that the inhibition of WE on MR products in the milk model system depends on the heating time and the concentration in use.

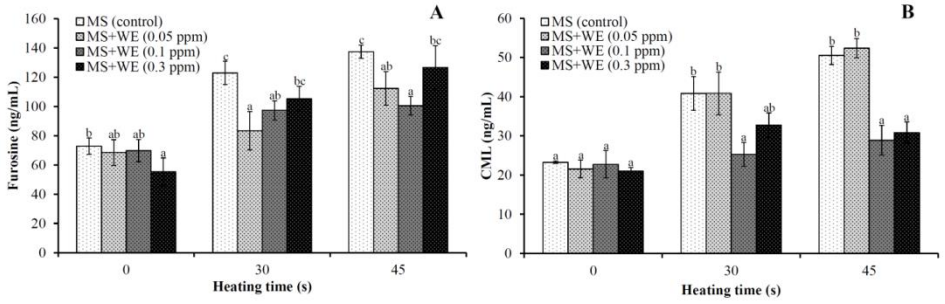


Fig. 4.12. Effects of WE on furosine (A) and CML (B) formation in the milk model system at various heating times. Different letters on the columns at the same heating time indicate significant differences (P<0.05). MS: milk model system, WE: water extract of LL

For comparison purposes, the standards of quinic acid and catechin were also tested for their antiglycation activities in the milk model system as they were among the highest levels in WE. Also, catechin was found to be the most active radical scavenger in WE. The effect of quinic acid on furosine and CML formation is shown in Fig. 4.13.

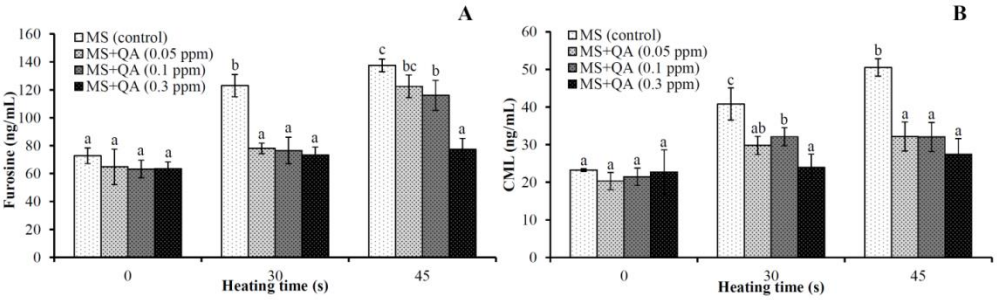


Fig. 4.13. Effects of quinic acid on furosine and CML formation in the milk model system at various heating times. Different letters on the columns at the same heating time indicate significant differences (P<0.05). MS: milk model system, QA: quinic acid

In all the samples, the concentrations of the two indicators increased after heating (30 and 45 s) in comparison with the non-heated samples (0 s). The addition of quinic acid to the milk model system resulted in significantly reduced levels of furosine, which were close to 40% after 30 s of heating with no statistical difference between the concentrations in use (Fig. 4.13A). Meanwhile, after 45 s of heating, the reducing effect was dependent on the concentration in use: a lower concentration (0.05 mg/mL)

showed less than 10%, while the highest (0.3 mg/mL) concentration showed around 46% of furosine reduction. In the samples with quinic acid addition, the reduction of CML was similar at both heating times; by approx. 31, 29, and 43% when 0.05, 0.1 and 0.3 mg/mL of quinic acid was added, respectively (Fig. 4.13B). The furosine level was similarly reduced with catechin addition as with quinic acid addition (Fig. 4.14A). The reduction was around 37% after 30 s of heating in all the samples with the addition of catechin, while, after 45 s of heating, reduction around 37% remained with the higher concentrations of catechin (0.1 and 0.3 mg/mL); the sample with the lowest concentration did not significantly differ from the control sample. In the samples with catechin, the formation of CML was concentration-dependent (Fig. 4.14B). Despite slight differences between the used concentrations, the highest concentration showed the best inhibition effect; the average decrease at both heating times was by 31, 39, and 51% when 0.05, 0.1 and 0.3 mg/mL of catechin was added, respectively. The results demonstrated that the formation of CML was more efficiently inhibited than furosine in the samples with quinic acid and catechin. Comparing to the effects of WE, quinic acid and catechin were more efficient in inhibiting the formation of furosine and CML.

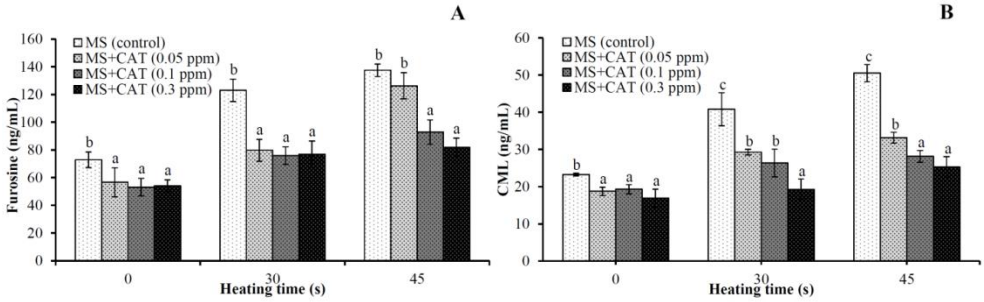


Fig. 4.14. Effects of catechin on furosine and CML formation in the milk model system at various heating times. Different letters on the columns at the same heating time indicate significant differences (P<0.05). MS: milk model system, CAT: catechin

Recent studies reported that phenolic compounds could undergo oxidation on the B ring and react with amino groups to form adducts (Guerra & Yaylayan, 2014). Accordingly, the reduction of catechin can be explained through the scavenging of lysine residue after its oxidation to the quinone form (Fig. 4.15). The inhibition effect of catechin on CML formation in the model bread system was also observed (Mildner-Szkudlarz *et al*., 2017). As CML formation is induced by the reactive carbonyl species deriving from sugar fragmentation, the suppressing effect of catechin can be explained through the trapping of the reactive carbonyl species by electrophilic aromatic substitution reaction occurring at the highly activated A ring (Kokkinidou & Peterson, 2014; Wang, Yagiz, Buran, Nunes, & Gu, 2011). No studies showed any influence of quinic acid on furosine and CML formation in milk. Quinic acid is a cyclic hydroxyl acid that is found in many fruits and vegetables. Several studies showed that quinic acid has no antioxidant capacity (Uranga, Podio, Wunderlin, & Santiago, 2016).

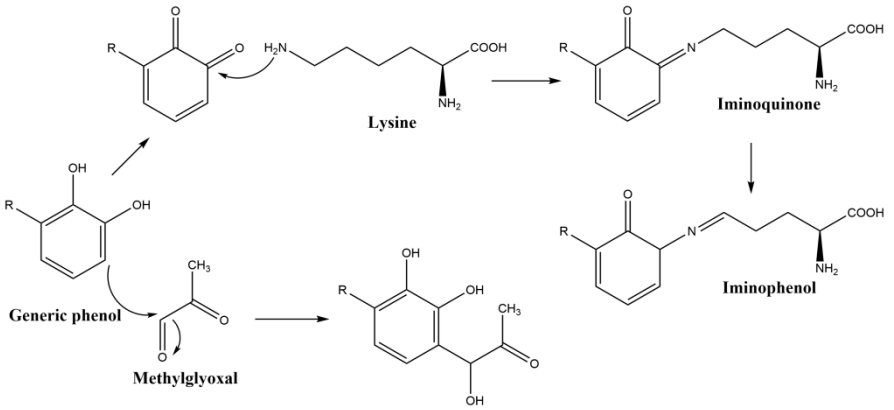


Fig. 4.15. Proposed mechanism of CML and furosine reduction by phenolic compounds in lingonberry leaves (adapted form Totlani and Peterson, 2006; Guerra and Yaylayan, 2014)

However, the protecting effect against protein carbonylation has been recently observed (Yoshimura *et al*., 2016). Our study shows that quinic acid is as effective as catechin in reducing MRPs formation in the milk model. We tentatively hypothesize that the reduction effect of quinic acid can be through the formation of adducts between the amino groups of lysine and quinic acid. As quinic acid has no phenol ring in the structure but has four hydroxyl groups, it can oxidize into quinone or any other structure and react with the amino groups of amino acids. The suppressing effect of quinic acid on furosine and CML formation is a novel finding. Moreover, additional studies are needed to clarify the reducing effect of quinic acid on the formation of MRP products. Moreover, CML formation was inhibited by using plant extracts, such as corolla of *Chrysanthemum* species (Tsuji-Naito, Saeki, & Hamano, 2009).

The reducing effect of WE on the formation of both MRPs can be associated with the above mentioned pathways. Catechin and quinic acid due to their antiglycation activity and relatively high abundance in leaves can be the main active compounds responsible for CML and furosine inhibition. Also, previous studies reported that catechin, quercetin-3-O-galactoside and cyaniding-3-O-glucoside were the main contributors to the antiglycation properties of *Vaccinium vitis-idaea* berry extract (Beaulieu *et al*., 2010).

4.4. IMPACT OF PLANT PREPARATIONS ON THE FORMATION OF HETEROCYCLIC AMINES IN MEAT PROTEIN MODEL SYSTEM

Two model systems – meat-protein and meat – were used to assess the effects of plant preparations on the formation of HAs. In the meat protein model system, the interference of meat matrix components (lipids, proteins, water) were eliminated by using only the substances necessary for the formation of HAs. In this system, amino acid phenylalanine was used; therefore, PhIP formation was generated, and the effect of plant preparations was evaluated on the formation of PhIP. Contrary to this, the meat model system had a meat-like matrix where only the water factor was eliminated, and the formation of several HAs found in beef was induced. Moreover, it is important

to mention that the formation of HAs does not take place in milk during its heating because the heating time is too short, and the main precursor for HAs formation, such as creatin(in)e, is not present in significant quantities. However, CML also forms in meat, but, in the present study, we investigated only the impact of plant preparations on HAs formation.

4.4.1. Effect of beetroot preparations on PhIP formation

In the meat model system, the effect of BJP was tested by adding 5, 15, 35 mg/mL concentrations. Besides, for comparison purposes, the effect of commercially used betanin concentrate under E number 162 was also tested at 15 mg/mL. BtFs (BtFI, BtFII, BtFIII) were tested at 30 mg/mL concentration. BnF was added at 0.55, 2.2, 5.5 mg/mL concentrations, while VxF was added at 5, 15, 50 mg/mL concentrations. Commonly, the betanin concentrate (which is commercially used in industry) contains ascorbic acid in the range of concentrations from 0.1% to 1%. Ascorbic acid is used as the stabilizing agent of betalains, which is added in beetroot preparations before the purification/concentration process of betanin. The addition of ascorbic acid reduces the degradation of betalains, which could occur due to purification conditions (light and pH) or thermal threatment (inactivation of enzymes and pasteurization). Therefore, the effect of ascorbic acid alone (0.18 and 1.8 mg/mL) and ascorbic acid (0.09 and 0.90 mg/mL) in combination with BnF (0.28 and 2.75 mg/mL) was also tested in the meat-protein model system.

4.4.1.1. Effect of beetroot juice powder on PhIP formation

First of all, this study analyzed the effect of BJP at different concentrations on the PhIP formation in the meat-protein model system. For comparison purposes, the commercial betanin or beetroot red (E162) was also tested. This food additive is made from beetroot (*Beta vulgaris*) concentrate and is used in meat products as a colorant. Therefore, testing the potential of beetroot preparations to inhibit the formation of HAs in meat is reasonable in order to find out other possible uses than only coloring. Fig. 4.16 shows the effect of BJP and E162 on PhIP formation.

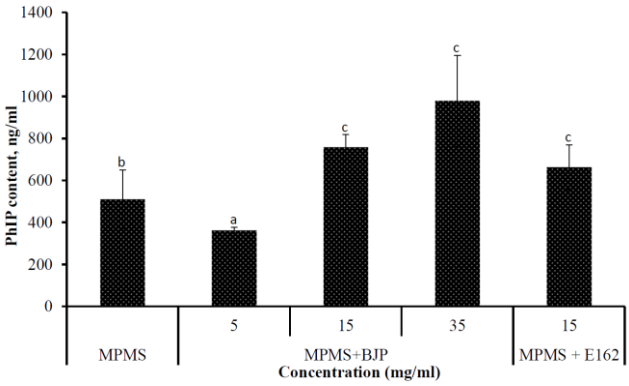


Fig. 4.16. Effect of BJP and beetroot red (E162) on the content of PhIP in the meat-protein model system. MPMS, meat-protein model system; BJP, beetroot juice powder; E162, beetroot red (food additive)

PhIP formation was reduced by ~30% with the lowest concentration (5 mg/mL) of BJP but increased by 48% and 91% with the addition of 15 and 35 mg/mL BJP. Commercial beetroot red E162 similarly to BJP promoted PhIP formation. The addition of 15 mg/mL E162 to the meat-protein model system increased the PhIP content by 30%. Also, the increase in the PhIP content was lower with E162 addition than with BJP.

4.4.1.2. Effect of betalain's fractions on PhIP formation

In the second set of this study, the effect of three BtFs obtained after gel chromatography was tested on PhIP formation in the meat-protein model system. Fig. 4.17 shows the effect of BtFI, BtFII and BtFIII on PhIP formation. The results showed that all BtFs significantly reduced PhIP formation. The most effective in inhibiting PhIP formation was BtFI, which reduced its content by 60%. The reducing effects of BtFII and BtFIII were 11% and 17%, respectively. Opposite effects of BtFs than BJP on PhIP formation were observed. There was no increase in the content of PhIP with the addition of 30 mg/mL BtFs, while the addition of 35 mg/mL BJP increased the PhIP content by 91%.

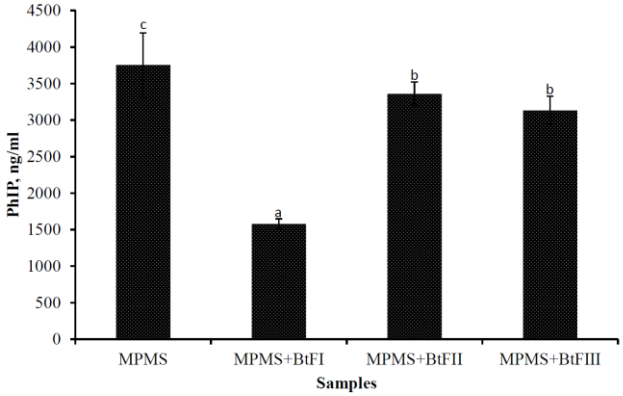


Fig. 4.17. Effect of BtFI, BtFII and BtFIII on the content of PhIP in the meat-protein model system. MPMS, meat-protein model system

This opposite effect might be due to the different composition of betalains and their ratio in BtFs and BJP. Comparing the antioxidant properties between BtFs, the highest radical scavenging capacity was observed in BtFI, then, BtFII followed, and BtFIII came last.

4.4.1.3. Effect of betanin and Vulgaxanthin I fractions on PhIP formation

In the next set of this study, the effect of BnF and VxF on HAs formation in the meat-protein model system was investigated (Fig. 4.18). The behavior of BnF in terms of PhIP formation was similar to BJP. A slight decrease in the PhIP content was observed when BnF was added at a low concentration. However, when the BnF concentration increased, the amount of PhIP increased as well. With the addition of 2.2 and 5.5 mg/mL of BnF, the amount of PhIP increased by 18% and 24%, respectively.

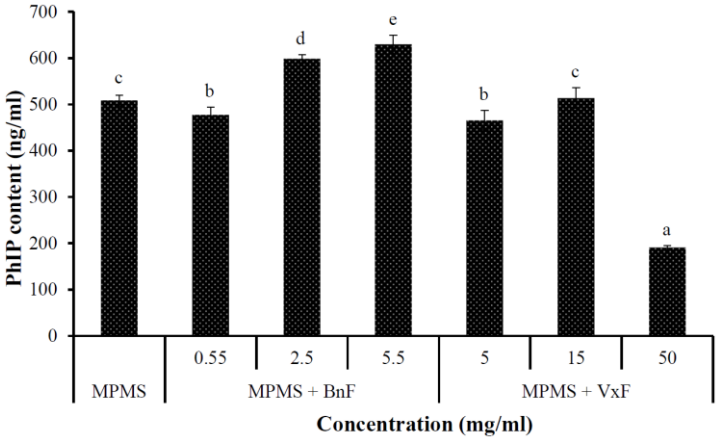


Fig. 4.18. Effect of BnF and VxF on the content of PhIP in the meat-protein model system. MPMS, meat-protein model system; BnF, betanin fraction; VxF, Vulgaxanthin I fraction

On the contrary, VxF reduced the amount of PhIP produced when added at low (by 8%) and high (62%) concentrations, while, when added at a medium concentration (15 mg/mL), the PhIP content was similar to the control sample.

In an attempt to determine the impact of ascorbic acid which usually comes in commercial betanin preparations, the effect of ascorbic acid and ascorbic acid in combination with BnF was also tested (Fig. 4.19). The results revealed that ascorbic acid added alone to the meat-protein model system increased the amount of the produced PhIP (increases were 12% and 24% when 0.18 and 1.80 mg/mL of ascorbic acid were added). However, no antagonistic effect of ascorbic acid in combination with BnF on PhIP formation was found.

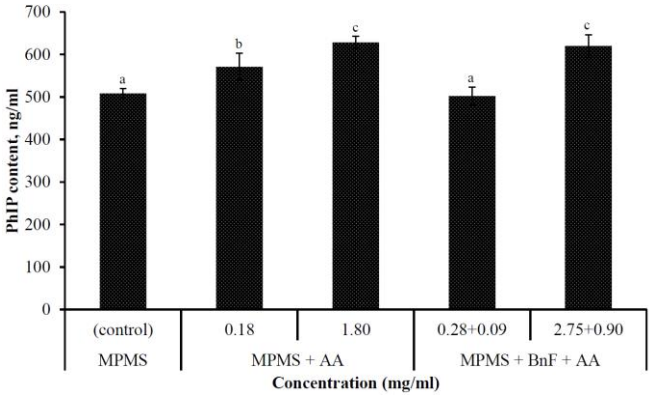


Fig. 4.19. Effect of ascorbic acid and BnF in combination with ascorbic acid on the content of PhIP in the meat-protein model system. MPMS, meat-protein model system; AA, ascorbic acid; BnF, betanin fraction

4.4.2. Effect of lingonberry leaves extracts on PhIP formation in meat protein model system

The influence of LL extracts on PhIP formation in the meat protein model system is shown in Fig. 4.20. The level of PhIP in the control sample was 12.06±1.64 nM/mM creatinine after heat processing at 128±2 °C for 120 min. The PhIP formation was significantly increased when WE was added in the range of 0.1–5 mg/mL and decreased by 20% with the addition of 10 mg/mL. The lower concentrations of AE (0.1–5 mg/mL) did not significantly affect the formation of PhIP, while the highest concentration (10 mg/mL) reduced the PhIP level by 40%, relative to the control. A better inhibitory effect observed with AE was somewhat surprising considering the stronger radical scavenging activity and the higher content of the total phenols in WE. In addition, WE promoted PhIP formation at lower concentrations. However, this observation is in agreement with a previous study that found the enhancing effects of phenolic compounds on PhIP formation in the model system despite their good radical scavenging properties (Cheng *et al*., 2007).

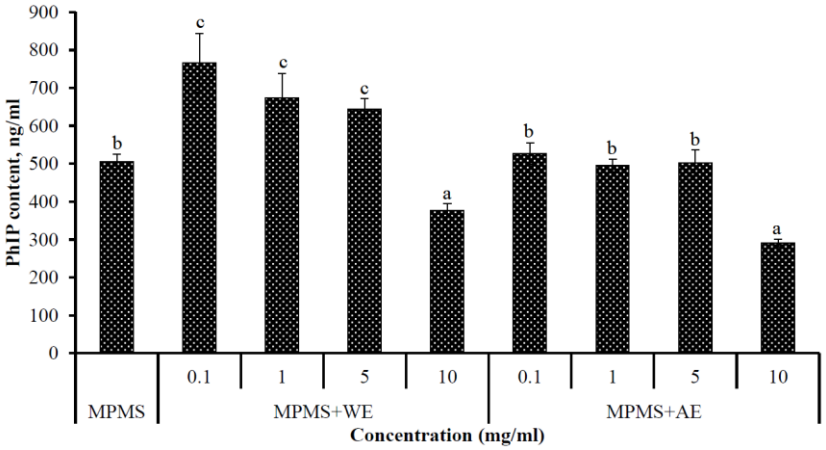


Fig. 4.20. Effect of WE and AE on the content of PhIP in the meat-protein model system. MPMS, meat-protein model system; WE, water extract of LL; AE, acetone extract of LL

Also, in Cheng's study, a weak free radical scavenger showed very good inhibitory activity towards PhIP formation. Therefore, it was suggested that a radical-involving reaction may not be an essential mechanism for the formation of PhIP. Also, free radical scavenging activity or the total phenolic content showed poor correlation with the inhibitory effect of phenolic compounds on the formation of HAs in many studies (Cheng *et al*., 2007; Damašius, Venskutonis, Ferracane, & Fogliano, 2011). Contrary to this, there were many reports showing a good correlation between the radical scavenging capacity and PhIP inhibition (Zhang, Luo, Shao, Yu, & Wang, 2014b). In order to understand these controversial behaviors, studies on structural characteristics of phenolic compounds that favor the inhibition of PhIP formation were performed, and it was proposed that flavonoids having hydroxyl groups at 3'-, 4'-, 5- and 7-positions are strong inhibitors of glycation (Matsuda, Wang, Managi, & Yoshikawa, 2003). Later, it was shown that phenolic compounds having two hydroxyl groups at

the *meta* position in the aromatic ring contribute to the inhibition effect on the PhIP formation in the model system. However, *ortho* and *para* dihydroxy derivatives at low concentrations increased the amount of PhIP and decreased at higher concentrations (Salazar *et al*., 2014). According to our results and observations of others studies, we may conclude that a different effect of WE and AE on PhIP formation was obtained due to the phenolic compounds of different compositions. The major difference between these extracts is that WE contained a higher content of flavonols, hydroxycinnamic acids and proanthocyanidins compared to AE. The amounts of hydroxycinnamic acids, such as quinic, protocatechuic, chlorogenic, caffeic and *p*-coumaric acids, were in trace amounts or were not detectable in AE. Several studies found that phenolic acids are ineffective inhibitors or even enhancers of PhIP formation (Oguri *et al*., 1998). For example, chlorogenic acid was reported as a very strong PhIP enhancer (Cheng *et al*., 2007). Moreover, in AE, higher amounts of flavan-3-ols, such as catechin and chinchonain 1, were present than in WE.
Many studies reported catechins as efficient inhibitors of PhIP formation (Cheng *et al*., 2007; Cheng *et al*., 2009; Salazar *et al*., 2014). Thus, the differences in the composition of phenolic compounds could be a reason for the different effect of LL extracts on the PhIP formation in the analyzed meat protein model system.

4.5. IMPACT OF PLANT PREPARATIONS ON THE FORMATION OF HETEROCYCLIC AMINES IN MEAT MODEL SYSTEM

4.5.1. Effect of betanin and Vulgaxanthin I fractions on formation of heterocyclic amines

In order to understand the effect of betalains on the formation of HAs, pure fractions of BnF and VxF were further tested in the meat model system. In this model, four HAs were found; the dominant compound was PhIP, whereas MeIQ, MeIQx and Trp-1 were found in lower amounts. In the control sample, PhIP amounted to 266.84±6.67 ng/g of beef dw. As shown in Fig. 4.21, the amount of PhIP increased in all the samples with BnF additions. The highest increase (75%) was observed with the lowest concentration of BnF. When the levels of BnF were increased, the amount of PhIP was further decreased, but still remained higher than in the control sample. The effect of BnF on the PhIP formation was contrary to the effect determined in the meat-protein model system when BnF was added at the same concentrations. Comparing to the control sample, the added BnF at the highest concentration (5 mg/mL) significantly increased the amounts of MeIQ, MeIQx and Trp-P-1. A different effect of VxF from that of BnF on the formation of HAs was observed. As the level of VxF increased, the amount of PhIP gradually decreased; the reductions were 66, 97 and 99% at 5, 15 and 50 mg/mL, respectively. The addition of VxF strongly promoted MeIQ, MeIQx and Trp-P-1 formation. The highest increase was with the lowest concentration of VxF. Considering that VxF contained sugars including the reducing ones, the reduction of PhIP could be explained through the generation of reactive oxygen species, which induced PhIP formation. Literature data shows that sugars, depending on their concentration, can promote or inhibit the formation of HAs. For

example, the inhibition action on PhIP formation was observed by glucose when its concentration was in equal or higher amounts of reactants. The action also depends on their type, for example, honey inhibited HAs formation more that table sugar.

The effect of beetroot preparations on the formation of HAs was different in phenylalanine and meat model systems. The different results probably were due to the interaction of lipids. Also, the formation of MeIQ and MeIQx increased, while their precursors are pyridine or pyrazine free radicals arising from MR between sugars and amino acids during Strecker degradation, respectively. Moreover, the formation of pyridines is particularly observed in the presence of lipids in the Maillard reaction pathway, where unsaturated aldehydes from lipid oxidation condense with ammonia and thus yield alkylpyridines (Kim and Ho, 1998). Besides, the higher amount of ammonia could stem from betalain degradation or amino acids in the samples with added BnF and VxF. The formation of pyrazines in the samples with BnF (5 mg/mL) and VxF was also favored. Formation of pyrazines is predominantly linked to Strecker degradation of amino acids.

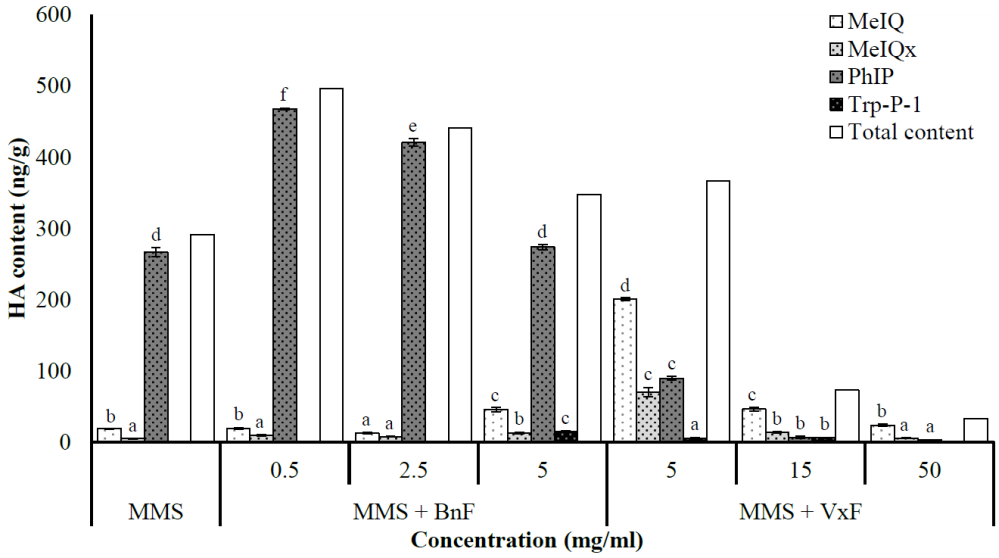


Fig. 4.21. Effect of BnF and VxF on the content of PhIP in the meat model system. MMS, meat model system; BnF, betanin fraction; VxF, Vulgaxanthin I fraction

The reaction starts from condensation of two α-aminocarbonyl compounds; dihydropyrazine is yielded which further reacts with Stecker aldehydes or α-dicarbonyls and produces various alkyl- and acyl- substituted pyrazine derivatives (Adams, Polizzi, van Boekel, & De Kimpe, 2008; Low, Parker, & Mottram, 2007).

Considering all the results, the reducing effect of beetroot preparations on the HAs formation could be due to reactive oxygen species generation. Han *et al*. (2017) reported that reducing sugars, such as glucose, fructose and honey, reduce PhIP formation through the generation of *a*-dicarbonyl compounds which react with the key precursors of PhIP, such as creatinine or itself. The proposed mechanism of

betanin to inhibit/promote the formation of PhIP is shown in Fig. 4.22. The inhibition/promotion action on HAs formation depended on the added concentration.

4.5.2. Effect of lingonberry leaves extracts on the formation of heterocyclic amines

The influence of WE and AE on the formation of HAs in the meat model system is shown in Fig. 4.22. A positive correlation between the levels of LL extracts and the reduction in PhIP generation was found; that is, the more WE or AE was added, the lower levels of PhIP were determined. The PhIP content was reduced by 3.24, 10.55, 39.29 and 14.42, 26.85, 37.43% compared with the control values when WE and AE levels increased, respectively.

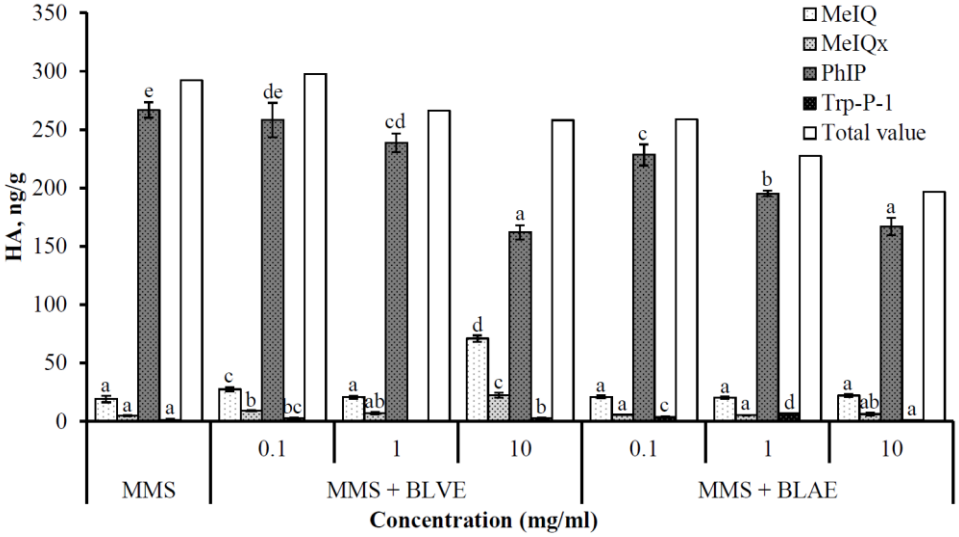


Fig. 4.22. Effect of WE and AE on the content of PhIP in the meat-protein model system. MPMS, meat-protein model system; WE, water extract of LL; AE, acetone extract of LL

Consequently, the opposite effects of WE addition on the formation of PhIP were observed in this sample, similar to the real meat model system; there was no promoting effect of WE on PhIP formation. This could be due to the interacting compounds with PhIP formation in the meat model system (Cheng *et al*., 2007). However, the levels of MeIQ, MeIQx and Trp-1 were increased in the samples with WE. The addition of 10 mg/mL of WE showed the highest promoting effect on the formation of MeIQ, MeIQx and Trp-1, and the highest reducing effect on PhIP, at the same time. The opposite effects of some phenolic compounds on MeIQx and PhIP formation was announced in earlier reports (Oguri *et al*., 1998). Recently, it was observed that such phenolic compounds as catechin or EGCG react with imine intermediates, thus influencing the formation of pyrazinum radicals. It was demonstrated that catechin and EGCG in the Maillard model with glyoxal and L-alanine increased the formation of the pirazinium radical at low concentrations (5–20 mM) and decreased at high concentrations (≥50 mM). Thus, the formation of MeIQ and MeIQx might be enhanced through the generation of pyridine and pyrazine

radicals. As PhIP belongs to the imidazopyridine type compounds, it is formed in a different way. It was reported that EGCG effectively inhibited the formation of PhIP in the model system through the trapping of phenylacetaldehyde, which is an intermediate product of PhIP formation (Cheng *et al*., 2009).
Based on the obtained data, we could consider that the WE extract acted as a generator of precursors for MeIQ and MeIQx formation, and as a scavenger of intermediates for the formation of PhIP in the meat model system. Also, it might be that the reduction of PhIP could be through the formation of MeIQ and MeIQx, when the precursors were acting as competitors. Besides, AE does not show pyridine and pyrazine generation the way it was observed with WE. Consequently, our study demonstrated that WE, although demonstrating stronger radical scavenging properties and a higher content of phenols, still possessed a lower inhibition effect on the formation of HAs compared to AE. These findings suggest that the radical scavenging capacity or the content of phenols are not reliable indicators of the potential to inhibit the formation of HAs. The results also suggest that the composition of individual phenolic compounds in the extracts might play a more important role. It may be assumed that acetone was a more effective solvent than water in the recovery of phenolic compounds with a higher potential to suppress HAs formation at the used conditions of extraction. For instance, by being an aprotic organic solvent, acetone better extracts more lipophilic compounds than water. Consequently, the phytochemical composition of extracts may be purposively tailored by using different organic solvents and extraction procedures.

4.6. IMPACT OF PLANT PREPARATIONS ON OVERALL MAILLARD REACTION INTENSITY IN MEAT-PROTEIN MODEL SYSTEM

Five parameters were chosen to assess the MR intensity (MRI) in the meat-protein model system. The extent of the final MR was estimated through the browning intensity (A_{420}) of the meat protein model system. In the progress of MR, high molecular weight brown products (melanoidins) are produced which have a characteristic absorbance maximum at 420 nm; therefore, it is a good indicator of the advance final MR products formation. As the development of the brown color is related to the increased antiradical capacity due to the formation of melanoidins (Kitrytė, Adams, Venskutonis, & De Kimpe, 2012; Morales, Somoza, & Fogliano, 2012; Vignoli, Bassoli, & Benassi, 2011; Yilmaz & Toledo, 2005), the antiradical activity of the meat-protein model system in the aqueous (ABTS) and methanolic (DPPH) medium was additionaly investigated. The percentage amount of 2-phenylethenamine (2-Peth) and phenylalanine in the reaction mixture after the heating are also good indicators of the unreacted amount of these compounds, and, accordingly, the higher percentage is remaining, the lower is MRI, the lower percentage is remaining, the higher is MRI.

A_{420}, antioxidant activity and the amounts of 2-Peth and phenylalanine in the meat model systems with and without addition of plant preparations are shown in Table 4.11. The results showed that all the plant preparations tailored the extent of MR as estimated by five parameters. BJP strongly increased the A_{420} value and

andioxidant activity, while, with increasing the concentration, the values also increased. Similarly, the amounts of 2-Peth and phenylalanine decreased thus indicating the higher MRI. Food additive E162 showed similar results to BJP when they were added at the same concentration of 15 mg/mL. All the MRI parameters well correlated to the formation of PhIP in the model system with BJP and E162 addition. The addition of BnF and VxF to the model system increased the values of A_{420} and antioxidant activity. Similarly, the consumption of 2-Peth and phenylalanine increased with the increasing BnF and VxF concentration. The additions of ascorbic acid and BnF with ascorbic acid showed small changes in the A_{420} values and antioxidant activity, while the consumption of 2-Peth and phenylalanine strongly increased.

In fact, the higher extent of MRI was observed in the samples with BJP and VxF additions. This can partly be related to the higher content of sugars, especially the reducing ones, which promote non-enzymatic browning (Naranjo, Malec, & Vigo, 1998). The addition of WE up to 5 mg/mL increased the development of the brown color, while, at 10 mg/mL, A_{420} values were lower than that of the control sample. The consumption of 2-Peth and phenylalanine showed the same results, while the antioxidant activity increased with the increasing WE concentration. Contrary to WE, AE showed a reducing effect on MRI at all concentrations. The addition of AE decreased the development of A_{420} while increasing its concentration. The antioxidant activity was lowered, and the consumption of 2-Peth and phenylalanine was increasing at all AE concentrations. All the parameters indicated a strong mitigation of MR. A different effect of different LL extracts was obtained on MRI. This might be related to their different chemical composition obtained by different extraction techniques. It is likely that WE promoted MRI due to the enhanced formation of melanoidins, which could be as a result of the interaction of Maillard-hydoxycinnamic acids. Our findings are in agreement with studies of other authors which showed that the effect of sugars, amino acids, polyphenol compounds, and ascorbic acid on the browning formation depended on the concentration in use: at a certain concentration, the browning reaction can increase, but continuous increasing of their concentration can decrease the browning reaction (Gao *et al*., 2017).

The MRI parameters correlated well with the formation of PhIP in all model systems with the exception of the model system with WE addition. Therefore, this confirms that PhIP formation is a part of MR. This agrees with the previous study findings where a good correlation between the browning reaction intensity and PhIP was observed in phenylalanine/creatinine/sugars model systems (Moon & Shin, 2013). Nevertheless, MRI parameters can give extra information on the effect of plant preparations on the progress of MR.

In general, the results showed that betalains from BJP enhanced the progress of MR as estimated by five parameters. Pure betanin, which is the main betalain in BJP, strongly increased MRI. VxF showed the highest enhancing impact on MRI. Extracts of LL differently affected the progress of MR. WE increased MRI up to 5 mg/mL, while, at 10 mg/mL, it showed reducing action. According to all MRI parameters, AE showed an inhibitory impact on MR development when it was added in the concentration range from 0.1 to 10 mg/mL.

Table 4.11. MRI parameters of meat protein model systems

Sample	A_{420}	DPPH inhibition, %	ABTS inhibition, %	2-Peth, %	Phe, %
MPMS (control)	0.19±0.02	38.44±2.15	12.78±0.93	16.36±0.54	11.15±0.55
MPMS + BJP (5 mg/mL)	0.22±0.03	40.00±2.22	14.75±0.86	13.11±0.64	10.12±0.37
MPMS + BJP (15 mg/mL)	0.36±0.05	48.59±2.70	15.55±0.89	12.64±0.39	8.96±0.23
MPMS + BJP (35 mg/mL)	0.56±0.04	55.53±5.43	18.27±2.33	7.59±1.49	5.13±0.86
MPMS + E162 (15 mg/mL)	0.41±0.05	44.26±2.47	12.85±1.19	15.16±1.77	10.88±1.34
MPMS (control)	0.20±0.01	39.48±1.48	10.65±0.52	15.40±0.19	14.17±0.71
MPMS + BnF (0.55 mg/mL)	0.19±0.02	39.60±0.71	10.61±0.86	12.01±0.70	11.58±0.44
MPMS + BnF (2.5 mg/mL)	0.24±0.03	40.46±0.62	11.81±0.62	6.76±0.19	5.15±0.18
MPMS + BnF (5.5 mg/mL)	0.27±0.05	43.74±2.01	12.29±1.32	6.98±0.29	5.43±0.15
MPMS + VxF (5 mg/mL)	0.20±0.04	28.99±5.82	7.28±1.22	8.94±0.69	6.40±0.43
MPMS + VxF (15 mg/mL)	0.39±0.03	42.32±5.30	15.93±1.62	2.67±0.07	2.00±0.12
MPMS + VxF (50 mg/mL)	1.21±0.07	76.39±9.02	95.22±4.48	0.36±0.19	0.36±0.12
MPMS + AA (0.18 mg/mL)	0.18±0.02	37.69±0.88	9.02±0.67	13.31±0.23	12.54±0.43
MPMS + AA (1.80 mg/mL)	0.24±0.04	39.00±0.57	10.63±0.61	7.18±0.11	5.48±0.20
MPMS + BnF + AA[1]	0.18±0.02	38.82±0.99	9.95±0.49	11.68±0.64	11.53±0.61
MPMS + BnF + AA[2]	0.24±0.02	39.50±0.50	11.10±0.55	7.16±0.44	5.35±0.28
MPMS (control)	0.17±0.01	36.67±0.52	9.93±0.63	15.88±0.31	10.76±0.62
MPMS + WE (0.1 mg/mL)	0.18±0.01	36.84±0.64	10.37±0.62	15.73±0.09	10.14±0.10
MPMS + WE (1 mg/mL)	0.20±0.01	38.03±0.47	11.17±0.69	15.07±0.52	9.30±0.41
MPMS + WE (5 mg/mL)	0.22±0.02	41.41±0.53	12.01±0.37	14.98±0.34	9.64±0.34
MPMS + WE (10 mg/mL)	0.16±0.01	37.28±0.96	10.86±0.32	17.62±1.27	12.28±0.71
MPMS + AE (0.1 mg/mL)	0.15±0.01	31.23±1.63	9.87±0.62	17.00±0.42	11.79±0.30
MPMS + AE (1 mg/mL)	0.15±0.01	29.65±0.90	9.40±0.35	18.52±0.25	12.02±0.20
MPMS + AE (5 mg/mL)	0.14±0.01	29.11±0.82	9.12±0.49	18.78±0.71	12.66±0.55
MPMS + AE (10 mg/mL)	0.11±0.01	26.88±0.94	8.26±0.33	23.01±0.95	16.36±0.85

[1] added at 0.28 mg/mL of BnF and 0.09 mg/mL of AA.[2] added at 2.75 mg/mL of BnF and 0.90 mg/mL of AA. Abbreviations: 2-Peth, 2-phenylethenamine; Phe, phenylalanine; MPMS, meat-protein model system; BJP; beetroot juice powder; BnF, betanin fraction; VxF, Vulgaxanthin I fraction; AA, ascorbic acid; WE, water extract of LL; AE, acetone extract of LL.

There is increasing interest currently observed in the polyphenolic compounds interaction in the MR. Especially, this interest is growing in studies searching for potential inhibitors on the formation of harmful MPRs (Teng, Hu, Tao, & Wang, 2018). Some studies demonstrated that phenolic compounds of plant extracts have a potential to reduce the formation of A_{420} in model systems: thyme leaves extract (Favre, dos Santos, López-Fernández, Mazzobre, & Buera, 2018), rosmarinic acid, epigallocatechin-3-gallate (Favreau-Farhadi, Pecukonis, & Barrett, 2015), sugarcane molasses extract (also reduced CML and CEL formation) (Yu, Xu, & Yu, 2017) and ferulic acid (also reduced CML formation) (Silván *et al*., 2011).

The above described results indicate that the effect of plant preparations on MRPs strongly depended on the tested model system and the added concentration. Betalains can promote or inhibit the formation of MRPs depending on their concentration in use. The obtained results show that betalains preparations used at low

concentrations have more potential to reduce MRPs formation. LL WE at a low concentration showed a promoting effect on MRPs formation, while, at a high concentration, WE has potential to reduce MPRs formation.

It seems that plant preparations can inhibit the formation of one HA but at the same time increase the formation of other(s). For instance, in the meat model system when WE (10 mg/mL), BnF (5 mg/mL) and VxF (5 mg/mL) showed a reducing effect on the PhIP formation, the amount of MeIQ and MeIQx strongly increased at the same time.

5. CONCLUSIONS

1. The total betalains content increased up to 34% (BtFII) by gel chromatography. The purity of betanin and vulgaxantin I fractions by preparative HPLC was 78% and 100%, respectively. The antioxidant activity of beetroot preparations did not depend on the total phenolic content, the total betalains content, and the betalains ratio. Purification of betanin increases its radical scavenging properties. Betanin fraction (EC_{50}, 0.55 mg/ml) showed 21 times higher radical scavenging activity than BJP (EC_{50}, 11.56 mg/ml) and 300 times higher than Vulgaxanthin I fraction (EC_{50}, 151.45). Betacyanins possess stronger radical scavenging properties than betaxanthins, while the degradation products of betanin were shown to manifest antioxidant activity.

2. The highest yields, antioxidant capacity and TPC of lingonberry leaves extracts were those of water and methanol extract, while the lowest yields were of acetone extract. The antioxidant activity of extracts well correlated with the content of phenolic compounds. The most abundant constituents in the water extract were hydroxycinnamic acids and flavonols, while in the methanol and acetone extracts these were arbutin derivatives and flavan-3-ols. Catechin and epicatechin were the main contributors to the antioxidant activity in the water extract, while catechin and caffeoyl-hexose hydroxyphenol were the main contributors in the methanol and acetone extracts.

3. The effect of beetroot preparations on the formation of Maillard reaction products depended on their concentration and the model system. BJP reduced furosine and CML formation in the milk model system: the highest reduction around 30% and 17% was with 0.3 mg/mL of addition, respectively. In the phenylalanine model system, BJP and BnF at a low concentration reduced PhIP formation, while, with the increasing concentrations, the amount of PhIP increased. In the meat model system, BnF showed a strong promoting effect on HAs formation. The addition of VxF in both model systems showed a reducing effect on HAs formation. It was observed that, at certain concentrations, VxF (5–15 mg/ml) and BnF (5 mg/mL) increased in amounts of these HAs whose precursors are pyridine and pyrazide radicals.

4. The effect of lingonberry leaves extracts on the formation of Maillard reaction products depended on their concentrations and the model system. WE addition reduced furosine and CML formation in the milk model system. The highest reduction of furosine and CML by 27% and 42% was with the concentration of 0.1 mg/mL. Quinic acid and catechin were attributed to be the compounds responsible for the inhibition action in WE. In the HAs model systems, it was determined that their effect on HAs formation also depended on the composition of their phenolic compounds. In the phenylalanine model system, the WE which contained a higher amount of hydroxycinnamic acids was less effective than AE which contained higher amounts of catechin and arbutin derivatives, and promoted PhIP formation in the samples up to 5 mg/ml of its concentration. The highest reductions by 20% and 40% were with 10 mg/mL of WE and AE, respectively. In the meat system, with the increasing WE and AE concentrations, the PhIP amount decreased. The highest reduction of the PhIP content by 40% was in samples with 10 mg/mL of extract additions. The significant

increase in MeIQx and MeIQ contents in the samples with WE additions at that concentration were observed.

5. In the phenylanine model system, the overall MR intensity increased in the samples with beetroot preparations. MR intensity increased with the increasing PhIP amount in the samples with BnF or BJP addition and when the amount of PhIP was decreasing in the samples with VxF addition. MR intensity (A420 and antioxidant activity) in the samples with LL extracts was lowered; it correlated well with the reducing amount of PhIP. The different action of beetroot preparations and LL extract on the MR intensity shows their different action mechanism(s). When beetroot preparations inhibited PhIP formation, the overall MR intensity was intensified, therefore, the reductive action might be through the reactive oxygen species generation at certain concentrations. LL extracts reduced the PhIP formation and the overall MR intensity, therefore, the reduction action might be through the trapping of reactive oxygen species and amino acids.

6. AUTHOR'S CONTRIBUTION

The author participated in all the experiments, data analysis as well as performed all manuscript writing on the results presented in Part **3 MATERIALS AND METHODS**. The results have previously been published in the following articles:

Effects of beetroot (*Beta vulgaris*) preparations on the Maillard reaction products in milk and meat-protein model systems;

"Phytochemical-rich antioxidant extracts of *Vaccinium vitis-idaea* L. leaves inhibit the formation of toxic Maillard reaction products in food models".

Subchapters such as 3.2.1.2, 3.3.3, 3.4.2, 3.4.3, 3.4.4, 3.5.1, 3.6.1, 3.6.2, 3.7.1 and 3.7.2. have been quoted verbatim from the article "Effects of beetroot (*Beta vulgaris*) preparations on the Maillard reaction products in milk and meat-protein model systems" (10.1016/j.foodres.2015.01.026) with permission of *Elsevier*.

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LIST OF PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

Publications in the list of the *Institute of Science Information* (ISI) database

1. Ieva Račkauskienė; Audrius Pukalskas; Petras Rimantas Venskutonis; Alberto Fiore; Antonio Dario Troise; Vincenzo Fogliano. Effects of beetroot (*Beta vulgaris*) preparations on the Maillard reaction products in milk and meat-protein model systems // Food Research International. ISSN: 0963-9969. 2015, vol. 70, p. 31-39 (I.F. 4.437).
2. Ieva Račkauskienė; Audrius Pukalskas; Alberto Fiore; Antonio Dario Troise; Petras Rimantas Venskutonis. Phytochemical-rich antioxidant extracts of *Vaccinium vitis-idaea* L. leaves inhibit the formation of toxic Maillard reaction products in food models // Journal of Food Science. ISSN: 0022-1147. 2019, vol. 84, No. 12, p. 3494-3503 (I.F. 2.081).

Abstracts of presentations at international conferences

1. Audrius Pukalskas; Ieva Račkauskienė, Petras Rimantas Venskutonis. Antioxidative capacity of beta vulgaris extracts and their effect on the formation of heterocyclic amines in A model system // Chemical Reactions in Foods VII, November 14-16, 2012, Prague, Czech Republic: international conference on new knowledge on chemical reactions during food processing and storage: book of abstracts. Prague: Institute of Chemical Technology, 2013. ISBN: 9788070808368. p. 10.
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SL344. 2020-04-16, 14,25 leidyb. apsk. l. Tiražas 12 egz.
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