UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

DEPARTMENT OF ANALYTICAL CHEMISTRY

MICHAELA FRÜHBAUEROVÁ

EVALUATION OF FUNCTIONAL FOOD INGREDIENTS WITH A FOCUS ON THEIR PHENOLIC CONTENT

THESES OF THE DOCTORAL DISSERTATION

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Author:	**Ing. Michaela Frühbauerová**
Supervisor:	**doc. Ing. Libor Červenka, Ph.D.**

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ANNOTATION

This doctoral thesis provides a comprehensive summary on the topic of phenolic compounds including chapters devoted to possibility of their determination using modern analytical techniques. The main part consists of a discussion of the results obtained from experiments with four selected matrices, carob (*Ceratonia siliqua* L.); black chokeberry (*Aronia melanocarpa* L.); grapevine (*Vitis vinifera* L.); and chocolate. Attention was paid mainly to the content of phenolic compounds during various treatment of original matrix. In case of chocolate, a simple analytical method for determination of antioxidant capacity and for possible verification of chocolate authenticity was developed. All presented results are supported by seven attached separate papers published in foreign and domestic scientific journals.

KEYWORDS

phenolic compounds, functional food, carob, grapevine, black chokeberry, cocoa, chocolate

ANOTACE

Tato disertační práce přináší ucelený souhrn na téma fenolických sloučenin včetně kapitol věnovaných možnosti jejich stanovení s využitím moderních analytických technik. Hlavní část je tvořena diskuzí výsledků získaných z experimentů se čtyřmi vybranými matricemi, karobem (*Ceratonia siliqua* L.); arónií černoplodou (*Aronia melanocarpa* L.); révou vinnou (*Vitis vinifera* L.) a čokoládou. Pozornost byla zaměřena zejména na sledování obsahu fenolických sloučenin při různé úpravě původní matrice. V případě čokolády byla vyvinuta jednoduchá analytická metoda pro stanovení antioxidační kapacity a případné ověření pravosti čokolád. Všechny prezentované výsledky jsou podloženy sedmi přiloženými separáty publikovanými v zahraničních i tuzemských odborných časopisech.

KLÍČOVÁ SLOVA

fenolické sloučeniny, funkční potraviny, karob, réva vinná, arónie černoplodá, kakao, čokoláda

GOALS OF THE DOCTORAL THESIS

Based on the doctoral thesis topic, following research goals were set to be accomplished:

- Summary of findings related to phenolic compounds and possibilities of their determination

Carob (*Ceratonia siliqua* L.)

- Evaluation of effect of roasting and different grinding methods on phenolic composition in carob powder
- Investigation of bioaccessibility of these phenolics during *in vitro* digestion steps
- Assessment of carob powder as a functional food ingredient

Black chokeberry (*Aronia melanocarpa* L.)

- Evaluation of black chokeberry phenolic content after osmodehydration treatment in different media and effect of ultrasonication
- Assessment of black chokeberry as a functional food ingredient

Grapevine (*Vitis vinifera* L.)

- Comparison of phenolic content present in freeze-dried and oven-dried grape skin powder
- Investigation of prepared processed cheese spread with addition of grape skin powder at different levels
- Assessment of grapevine by-products as a functional food ingredient

Cocoa (*Theobroma cacao* L.) and chocolate

- Development of a simple analytical method for determination of chocolate antioxidant activity
- Verification of the relationship between cocoa content and antioxidant capacity

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INTRODUCTION

Although, concept of functional food has been defined many times, there is no universally accepted definition of the term. In general, basic foods enriched by functional ingredients are referred to as functional foods. Mentioned functional ingredients are usually rich source of bioactive compounds, fiber, minerals, or probiotics. Also, by-products and wastes (peels, seeds, stems, shells, and many others) remaining from food production processes, which are generated in large quantities, can form promising functional food ingredients. Existence of functional food is connected to the improvement of food technology and beneficial health effects accompanied with food consumption. Due to this fact, functional food and potential functional food ingredients has significantly gained in popularity in the field of research over the last decades [1–3].

Biologically active compounds are substances with certain effect on the organism (usually associated with health improvement) [4]. They can be found in plants and animal products, and it is also possible to produce them synthetically. Examples of biologically active compounds occurring in animal products are vitamins (e.g., tocopherols in fish oil) [5], fatty acids [6] or bioactive proteins [7, 8]. As bioactive compounds coming from plants can be mentioned groups of antioxidants, oligosaccharides, carotenoids, phenolic compounds, glycosides, or phytosterols (mainly obtained from oils) [9]. It is known that consumption of fruit, vegetables and grains can be helpful for human health as well as for the prevention of chronic disease such as diabetes, cancer, or cardiovascular diseases [10]. Plants have been used for these purposes since the beginning of humanity. After discovering of its medicinal properties, plant material has become beneficial source of important compounds with role related to better health.

Based on the doctoral thesis research, phenolic compounds and their effects as antioxidants are described in this work. Various options of utilization of analytical techniques for identification and determination of phenolic compounds and antioxidants are discussed as well. Different materials concluded to be promising potential functional food ingredients were assessed and effects of their pretreatment was evaluated. Matrices chosen for experiments, carob (*Ceratonia siliqua* L.), black chokeberry (*Aronia melanocarpa* L.), grapevine (*Vitis vinifera* L.), and chocolate, are rich source of different kind of bioactive compounds with many beneficial properties. Parameters, such as phenolic content, antioxidant activity, or bioaccessibility of chosen phenolic compounds, were tested using different instrumental methods. For better orientation, each section has its own appendices with published manuscripts related to the topic.

For carob, effect of roasting (**Publication referred to as [11]**), utilization of different grinding method (**Publication referred to as [12]**), effect of vibratory grinding time (**Publication referred to as [13]**), and substitution of wheat flour by carob powder in preparation of muffins (**Publication referred to as [14]**) was investigated. Black chokeberry was subjected to osmo-dehydration using different osmotic agents and the effect of ultrasonication was also examined (**Publication referred to as [15]**). By-products of winemaking process, white grape skins, were used for enrichment of processed cheese spread and improvement of chosen characteristics was evaluated (**Publication referred to as [16]**). In **Publication referred to as [17]**, simple analytical method based on antioxidant capacity determination that offers rapid monitoring of cocoa content in commercial chocolates was introduced.

Chapter 1: PHENOLIC COMPOUNDS

Phenolic compounds (also known as phenolics or polyphenols, in case that structure contains more phenolic rings) are the most abundant and one of the most important secondary metabolites occurring in plants. Currently, more than 8000 different structures of plant phenolics are known. They arise biogenetically from shikimate or phenylpropanoid pathway, which provides phenylpropanoids directly [18, 19].

The presence of at least one aromatic ring with one or more hydroxyl substituents in the structure is common for all phenolic compounds [20, 21]. Based on the structure, phenolic compounds can be divided into two main classes – flavonoid compounds and non-flavonoid compounds. These groups can continue in subclasses of phenolic acids, lignans and stilbenes, and 6 main flavonoid subclasses, as can be seen in **Fig. 1**. Other two subclasses, tannins and lignins, occur mainly as complicated biopolymers. In this case, a defined carbon base is missing, and chemical structure is always unique to a particular polyphenol [22].

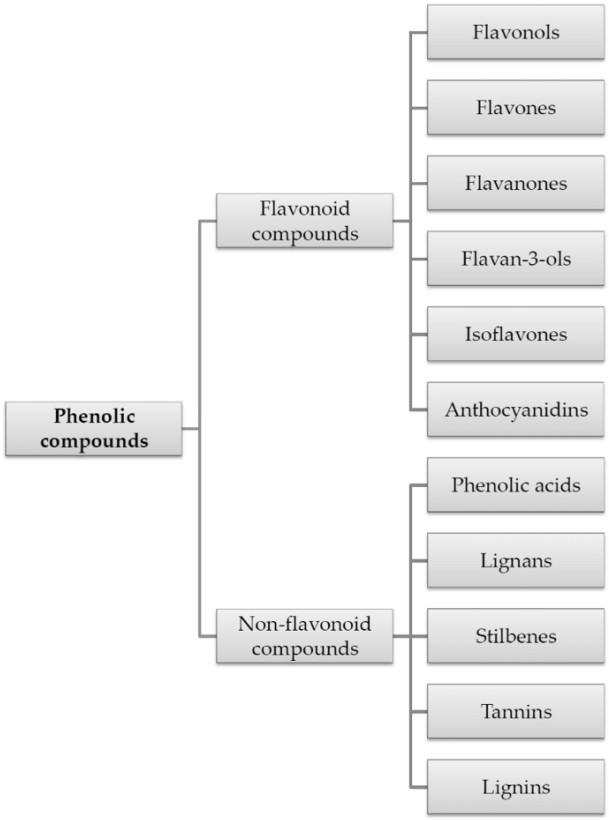

Fig. 1: Classification of phenolic compounds (edited) [22].

Various effects of these compounds on human health, e.g., antioxidant, anticarcinogenic, anti-inflammatory, anticholinergic, antimalarial, antileprosy, antidiabetic, antiproliferative, antiviral or antimicrobial and many others has been reported in a large number of studies. On the other hand, also some noxious effects of these compounds are known (e.g., neurotoxicity induced by myristicin) [23–26]. Polyphenols contribute to colour of vegetables and fruit and play an important role in terms of the aroma and taste of food [27–29]. In view of the above findings, the potential pharmacological effects of phenolic compounds as well as impact of phenolics on sensory characteristics of food are currently being studied by many research groups around the world.

1.1 Phenolic compounds as antioxidants

As already mentioned, phenolic compounds can be categorized as strong antioxidants. Substances with these properties prevent proteins, sugars, nucleic acids, and other biomolecules from undergoing oxidative damage by free radical-mediated reactions. Reactive oxygen (e.g., $O_2^{\bullet -}$ or •OH) and nitrogen (e.g., NO•) species are produced from normal cellular metabolism, and they cause potential biological damage known as oxidative or nitrosative stress. These conditions can occur after overproduction of discussed reactive species in biological systems or due to deficiency of enzymatic and non-enzymatic antioxidants [30, 31].

There are two possible mechanisms by which these compounds can provide antioxidant properties – hydrogen atom transfer or electron transfer mechanism (or their combination). In terms of antioxidant activity determination, a lot of methods already have been developed. Researchers divided these methods into two groups – *in vitro* and *in vivo* [32, 33].

In vitro methods (e.g., DPPH, ABTS, ORAC, FRAP, TRAP, CUPRAC, or reducing power assay) can show useful information about antioxidant effect of compounds extracted or isolated from plants. Methods belonging to this group are often relatively cheap, fast, and easy to perform. However, data obtained from these methods are difficult to apply on biological systems [33, 34].

In addition to the *in vitro* methods, which are widely used, there is also possibility to determine antioxidant activity with the use of other analytical methods (e.g., EPR, NMR, FTIR, CV, HPLC-DAD, or GC-FID) [33, 35–40].

1.2 Analytical methods used for determination of phenolics

Due to the beneficial effects of polyphenols on human health, these substances have become the subject of research by many scientific teams around the world.

Before analysis, sample preparation is one of the most important steps. The most used extraction media for phenolic compounds extraction are water and organic solvents such as methanol, ethanol, acetonitrile or acetone, and their mixtures. Sometimes also addition of small amount of acid (e.g., phosphoric, hydrochloric, or formic acid) can be helpful to obtain higher yield of extracted phenolics (due to destroying of cell membranes and stabilization of phenolic compounds). However, this procedure can cause change in original form of some phenolics (e.g., acylated anthocyanins are often labile under acidic conditions) and from this reason use of weak organic acids (acetic or formic) is preferable [41–43].

Nowadays, use of ionic liquids and deep eutectic solvents for highly effective and selective extraction of chosen compounds from samples is also very popular [44–46].

There are currently many publications describing possible ways for phenolic compounds analysis. The most common methods used to determine phenolics are shown in **Fig. 2**. It is important to say that there is no universal method applicable to all matrices and each sample needs its own optimization of the pre-treatment process as well as optimization of the instrumental analysis conditions.

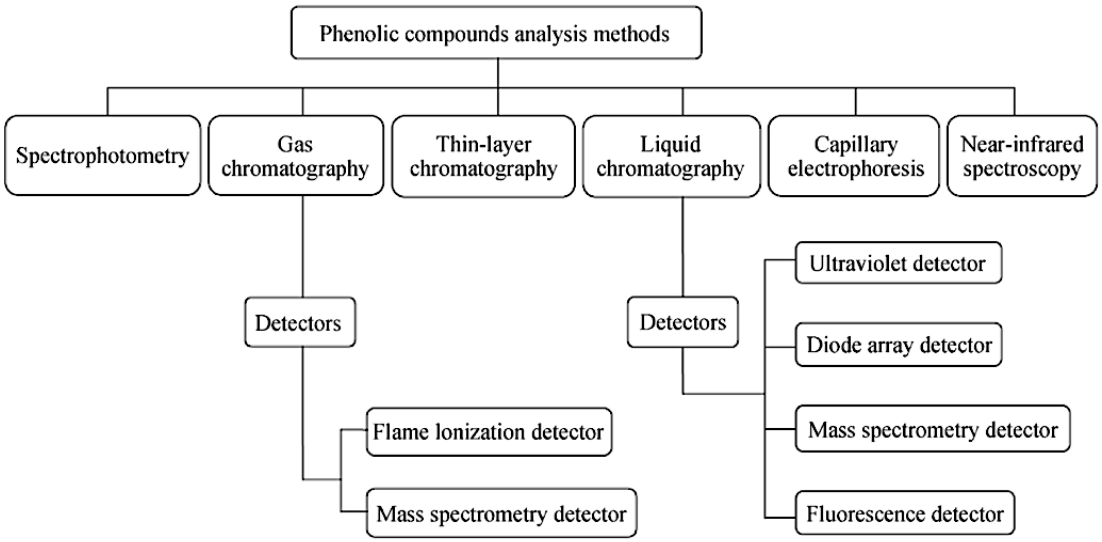

Fig. 2: Most common possibilities of phenolic compounds determination [20].

Summary of Chapter 1

Based on all facts discussed, phenolic compounds present in food are a topic worth paying attention to. Their beneficial effects (e.g., antimicrobial, antiproliferative, anti-inflammatory, antimalarial, antileprosy, hypoglycemic, or anticholinergic) have already been described in many studies. As an antioxidant agents, they have shown important ability to prevent appearance of oxidative stress. Thanks to their properties, they are also very significant factor in chronic disease prevention (e.g., diabetes, cardiovascular diseases, cancer, or obesity).

Due to their importance for human and animal organism, a lot of research focused on phenolic compounds measurements has been carried out till these days. Development of new methods for rapid analysis, determination, and characterization of phenolics in plant material and investigation on their effects and mechanisms of actions are very actual topic.

The most common technique used for phenolics identification and quantification is HPLC coupled with spectral techniques for detection (such as mass spectrometer or UV, DAD, PDA or fluorimetric detectors). But the application of other various analytical methods to phenolic compounds analysis is also wide. For example, data obtained from IR spectroscopy can distinguish different phenolic profiles (“fingerprint”) for matrices coming from different parts of the world. Methods such as NMR and ESR are also used as means to get information about complex samples.

Antioxidant activity of samples is often determined using *in vitro* methods, the method with DPPH radical is the most performed.

Chapter 2: CAROB (*Ceratonia siliqua* L.)

Carob (also known as St. John's bread or locust bean) is a fruit of an evergreen tree (*Ceratonia siliqua* L.) belonging to Leguminosae (~Fabaceae) plant family. Carob tree can be found mainly in Mediterranean area where it has been grown from antiquity (nowadays mainly Spain, Italy, Portugal, Morocco, Turkey, Greece, Algeria, and Cyprus). Fruit has appearance of 10–30 cm long, curved, or straight pod. When ripe, a carob pod has brown colour and comprises of sugary pulp (90%; rich in sucrose, fructose, glucose, cellulose, and tannins) and seeds (10%; rich in galactomannan and used mainly for locust bean gum (food additive; thickener E410) production). After grinding, roasting and final milling of seedless pods, a fine carob powder (flour) with taste very similar to cocoa is obtained. Based on temperature and time of roasting, different carob flours (lightly roasted, medium roasted, and highly roasted) can be gotten. While roasted, the carob flour undergoes significant changes due to important reactions such as Maillard reaction and sugar caramelization. These reactions affect the colour, taste, and aroma of final product. Carob powder; due to its content, taste, and the fact that it contains significantly lower amount (approximately 50 times) of theobromine and caffeine; can substitute addition of cocoa in food products [47–51].

2.1 Effect of ripening, roasting, and grinding on carob powder

The level of ripeness has significant effect on carob powder composition; while the sugar content increases at ripe stage; the phenolic profile, total phenolic content, and antioxidant activity decrease [52].

Important step in carob powder production is roasting. This process does not only result in better taste and aroma of final product, but mainly cause an increase in its antioxidant capacity and phenolic content (**Table 1 in [11]**). The increased values of these two parameters seems to be directly proportional to the increase in roasting temperature causing better solubility of phenolic compounds and higher formation of Maillard reaction products (MRP). Furfural and 5-(hydroxymethyl)furfural (HMF), the most plentiful MRP in procedure of carob roasting, are known for their toxic properties. Temperature of 130 °C and time of 30 min could be proposed as optimal for carob pod roasting (relatively low production of toxic MRP and high antioxidant activity); the combination of microwave/hot air roasting resulted in carob powder with the best sensory score, while 50% of energy consumption (compared to hot air roasting) was saved [48,

49, 53, 54]. Taste and aroma are also affected by the temperature and time of roasting; lower roasting temperature is accompanied by a sweeter, caramel-like taste and cocoa-like aroma; on the other hand, carob fruit roasted at higher temperature have a coffee-like aroma and an astringent taste [53].

Grinding method used to obtain fine carob powder, and final particle size, have also very significant effect on carob powder composition, especially on bioaccessibility of its bioactive compounds when digested *in vitro*. In comparison of vibratory and cryogenic grinding, higher phenolic content and antioxidant activity was found for powder ground by cryogenic grinder (**Table 3 in [12]**). This finding could be explained by the dependence of these parameters on the particle size of carob powder; the smaller the particles, the higher the antioxidant activity and the phenolic content (**Table 2 in [12]**). However, better bioaccessibility of phenolic compounds after *in vitro* gastrointestinal digestion was obtained for powders ground by vibratory grinder, probably due to higher temperature during grinding that could have helped to disrupt cell walls and have allowed better utilization of enzymes during process of *in vitro* digestion (**Chapter 3.3. in [12]**). When effect of vibratory grinding time was investigated in our study (**Publication referred to as [13]**), the obtained results showed that 30 seconds of vibratory grinding can be considered as the most suitable as good bioaccessibility of studied nutraceuticals was ensured at lower cost of energy. Temperature of metal parts of the grinder ranged between 22–24 °C and 37–39 °C when carob ground for 30 seconds and 180 seconds, respectively.

2.2 Bioaccessibility of carob phenolics

Bioaccessibility is parameter presenting amount of phenolics released from food matrix after digestion process in gastrointestinal tract. These compounds are then available for further absorption into the systematic circulation [55, 56]. Bioaccessibility of each phenolic compound can be influenced by food heat treatment (e.g., cooking, baking, or frying), interactions of these substances with food matrix, but also by digestive juices secreted by gastrointestinal tract [56, 57]. Bioaccessibility index (BA) of individual phenolics can be investigated by *in vitro* digestion procedures and calculated using equation $\mathbf{BA\ (\%) = (C_d/C_0) \times 100}$; where $\mathbf{C_d}$ and $\mathbf{C_0}$ indicates amount after digestion and before digestion (initial amount; obtained usually after extraction), respectively (**Chapter 2.8. in [12]**).

In our study (**Publication referred to as [12]**), for carob powder ground cryogenically, only ferulic acid bioaccessibility increased (BA ~ 108%), when digested *in vitro*; while carob powder prepared by vibratory grinding showed higher bioaccessibility for cinnamic acid, vanillic acid, quercitrin and naringenin. Total absence of luteolin, chrysoeriol (derivative of luteolin), and apigenin in digestive juices can be particularly attributed to thermal degradation and lower stability at different pH (**Chapter 3.3 in [12]**). Low bioaccessibility of luteolin and apigenin was also confirmed in our study when only vibratory grinding in different times was performed (**Table 3. in [13]**). Interestingly, process of *in vitro* digestion performed without enzymes (NED) showed high recovery for ferulic acid, cinnamic acid, and quercitrin, as well. This finding shows that water-soluble compounds may be extracted into digestive juice even without presence of enzymes. However, during NED, phenolic content in carob powder decreased by 67%, which was most likely caused by radical pH changes. The investigated phenolic acids showed high resistance during *in vitro* digestion, while flavones (apigenin and luteolin) appeared to be the least stable compounds.

2.3 Carob as a functional food ingredient

Due to the composition and all properties, carob represents interesting natural functional food ingredient. Carob flour can be used for the preparation of gluten-free and low-fat products, and to produce practically caffeine-free foods where cocoa is fully replaced by carob [48–50]. Flour-based food enriched by carob, such as cakes [58], pasta [59], and muffins (**Publication referred to as [14]**) were prepared.

Positive effects (e.g., low caffeine content; higher sugar, protein, and dietary fiber amount; higher antioxidant activity; higher level of essential minerals; positive effect on sensory analysis or better textural characteristics) were reported when using carob as a functional food ingredient (see **Table 2 and Table 3 in [14]**) [50, 58, 59].

Conclusion of Chapter 2

Ripe brown carob pods consist of pulp and seeds. Seeds of carob fruit are used mainly to produce locust bean gum (thickener E410). Pods are good source of sugars (up to 55% of total weight) as the pulp is rich in sucrose, glucose, and fructose. Besides sugars, carob pulp contains also significant amount of phenolic compounds with phenolic acids and flavonols being predominant. Due to the taste and aroma, roasted carob powder is promising component for cocoa substitution as it contains only trace concentration of significant cocoa methylxanthines (theobromine and caffeine), substantially higher amount of sugars and significantly lower fat content. When used in muffins as substitute for wheat flour, the higher the level of addition, the better the results of all parameters of phenolic content including increased antioxidant activity.

Roasting of carob is important step during which aroma and taste are developed, and also phenolic content and antioxidant capacity increases. While roasted at 130 °C for 30 minutes, results show high antioxidant activity of carob whereas formation of Maillard reactions products is low. Also, lower roasting temperature results in a sweeter taste and an astringent taste can be expected when carob is subjected to higher temperatures of roasting. In case of grinding, it was expected that utilization of cryogenic grinder would be more advantageous for the preparation of fine carob powder. It was confirmed that the level of phenolic compounds extracted from matrix after this grinding was higher compared to vibratory grinding. Also, cryogenic grinding provided carob powder of lighter colour which may be attributed to the smaller particle size. When different times of vibratory grinding was examined, grinding for 30 seconds seemed to be the most sufficient in terms of ratio of nutraceuticals released and energy costs.

However, better bioaccessibility results were obtained for phenolics in powder prepared by vibratory grinding (except ferulic acid). Low accessibility of apigenin and luteolin (or even their total absence) is attributed to their lability at different pH and thermal degradation. Phenolic acids investigated in our study showed good stability during digestive process while resistance of flavones was low. When non-enzymatic *in vitro* digestion was carried out, it was concluded that water-soluble phenolics are extracted into the digestive juices even in the absence of enzymes.

Chapter 3: BLACK CHOKEBERRY (*Aronia melanocarpa* L.)

Black chokeberry (*Aronia melanocarpa* (Michx.) Elliot.; AM) shrub belongs to the family of Rosaceae and originates from the eastern part of North America, however, today AM is also cultivated in Eastern Europe [60, 61]. Fruit of this bush has appearance of dark berries with diameter up to 18 mm. The dark purple to black colour of chokeberry is caused by presence of natural pigments anthocyanins in its outer skin which also allows the fruit to be used as a natural food and beverages colourant for example for tea, wine, or juice. Fresh berries are only rarely consumed directly due to their astringent and sour taste [60–62]. In the past, black chokeberry was used by the Potawatomi Native Americans as a treatment for cough, cold and fever [63].

3.1 Effect of pre-treatment on black chokeberry properties

In study of Cebulak and his colleagues [64], *Aronia melanocarpa* L. berries were exposed to the influence of microwave radiation, ultrasound, and UV-C (germicidal) radiation. When mentioned abiotic stress factors applied, values of phenolic content increase in all cases (levels of anthocyanins increased by 22%, phenolic acids by 20%, flavan-3-ols by 30%, and flavonols by 43%) [64].

Positive effect of ultrasound on phenolics released from chokeberry matrix was also observed in our study (**Publication referred to as [15]**), where osmotic dehydration with erythritol (ERT) and xylitol (XYL) solutions was applied. Results show that 30 min of sonication allowed to get AM powder with the highest content of bioactive substances. When ERT solution used, values of total phenolic content, total anthocyanin content and antioxidant capacity in AM powders were significantly higher than those for powders gotten after XYL solution exposure. Total content of flavonoids was similar for both osmotic dehydration agents (**Table 1 in [15]**).

Also, the powder prepared from oven-dried berries after 30 min exposure to ultrasonic-assisted osmo-dehydration in both, ERT and XYL solution, had darkest shade (lowest L^* values). Without effect of ultrasound, the use of XYL solution resulted in a lighter (higher L^* parameter), less red, and less yellow (lower a^* and b^* parameters) powder compared to that obtained when ERT solution was used (**Fig. 2A and 2B in [15]**).

In study published by Bae and colleagues [65], sucrose, glucose and xylitol solutions were examined for osmotic dehydration of AM berries followed by freeze-drying or hot-air drying

step. Sucrose and xylitol solutions as an osmo-dehydration agents in combination of freeze-drying provided higher values of total phenolic content and antioxidant activity than glucose solution. Aleksandrov et al. [66] describe in their paper that temperature of osmo-dehydration and concentration of osmotic solution were the most significant factors affecting water loss, while osmotic treatment temperature and ratio of fruit and solution had the highest impact on chokeberry antioxidant activity (higher the temperature, lower the values of antioxidant activity).

3.2 Black chokeberry as a functional food ingredient

Addition of *Aronia melanocarpa* L. has already been implemented in the preparation of various types of products such as cookies [67]; cakes [68]; shortcrust pastries [69]; extruded corn porridge enriched with AM [70]; milk, kefir and yoghurts supplemented with chokeberry powder or juice [71]; ice cream [72]; AM-based functional beverages [73]; AM infused beer [74]; or jelly candies where synthetic dye was substituted by AM extract [75].

Conclusion of Chapter 3

Dark berries of *Aronia melanocarpa* L. contain the highest amount of phenolic compounds amongst all berries. Colour ranging from dark purple to black is caused by anthocyanins occurring in black chokeberry skin. As level of anthocyanins is significant, AM is good natural colourant used in many beverages (e.g., tea, juice, or wine). Procyanidins, with (–)-epicatechin being the most frequent subunit, are another group of polyphenolics widespread in black chokeberry.

AM berries, juice, or black chokeberry pomace (by-product of AM juice production) have been used to fortify many food products and drinks. As skin of *Aronia melanocarpa* L. is rich in anthocyanins, their significant amount will remain in grape pomace. After enrichment, an increase if total phenolic content of prepared functional food can be expected. Addition may also contribute to colour change of final product and thus replace artificial food dyes.

In our study, effect of two osmotic dehydration solutions (erythritol and xylitol) was examined. In addition, ultrasonication was applied for different periods of time for disruption of cell walls and improvement of the whole process was expected. Both effect of ultrasound and effect of osmotic dehydration agent were significant. Combination of 30 min ultrasonication and erythritol used as osmodehydration solution was evaluated the most beneficial as obtained values of total phenolic content, total anthocyanin content, and antioxidant activity of AM powder were higher compared to results from other experiments in this study.

Chapter 4: GRAPEVINE (*Vitis vinifera* L.)

Grapes are one of the most favourite, delicious, and high-quality fruit. They grow in clusters with 15–300 individual berries. Each berry is oval shape and inside of fruit 2–3 seeds surrounded by soft greenish pulp can be found. Grapevine skin can differ in colour, from yellow, green, red to dark shades of purple. *Vitis vinifera* L. contain huge number of beneficial substances. Resveratrol, typical phenolic compound occurring in grapes, has documented potential cardioprotective effect. This relates to so-called "French Paradox" – lower appearance of cardiovascular disease in areas where red wine is consumed in higher amount [76–79].

Approximately 75% of all harvested grapes are used to produce wine. However, a large amount of residue remains during wine-making process. The most abundant by-product is grape pomace (GP) that consists mainly of skin, residual pulp, and seeds. These waste products represent promising ingredients for functional food preparation due to their high content of dietary fiber and phenolic compounds since they are not fully extracted during procedure. In fact, only about 30–40% of total phenolic content is present in final wine. The composition of each pomace differs with variety and strongly depends on the process of winemaking [80–83].

4.1 Effect of grapevine processing on grape pomace composition

The amount of phenolic compounds and other beneficial substances released into the final wine strongly depends on grapevine cultivar as well as on pressing step and fermentation process. GP is involved in fermentation step with pressed grape juice only in case of red wines. Thus, white GP is richer in pulp and residual sugars compared to red GP [77].

Grape pomace treatment steps after the vinification process are also very crucial. GP containing seeds, residual pulp, and skins (peels) is often later separated to seedless GP and other parts [77]. Probably the most critical operation is drying of GP before its reuse. A suitable drying procedure should be carried out carefully with regard to maintaining the highest possible stability of the bioactive compounds. Sokač and his colleagues [84] investigated effect of vacuum drying (at 35 °C for 12 hours, 50 °C for 5 hours, and 70 °C for 3 hours), open sun drying (for 26 hours) and conventional drying (at 70 °C for 7 hours) on bioactive compounds in Graševina cultivar GP. It concluded in findings that tannins were unstable when conventionally and open sun dried. Nevertheless, tannins showed less degradation trend while

vacuum dried (at 70 °C). Consequently, vacuum drying at 70 °C was evaluated as the most convenient drying method for GP [84].

Larrauri et al. [85] brought comparison of total polyphenols in freeze-dried red grape peels and those dried by hot air. Level of phenolic compounds decreased by 18.6% and 32.6% compared to freeze-dried samples when grape peels were dried at 100 °C and 140 °C, respectively [85]. As can be seen from our study (**Publication referred to as [16]**), type of drying process also plays important role in antioxidant properties of white grape skin powder production. Individual substances ((+)-catechin, (–)-epicatechin, and rutin) were found in higher amount when grape skin dried in oven at 46 °C for 24 hours. On the other hand, total phenolic content, as well as antioxidant activity, was significantly higher in case of freeze-dried grape skin powder (**Table 1 in [16]**).

4.2 Grapevine by-products as a functional food ingredients

Usage of by-products from wine-making process has allowed to produce many fortified food, such as yoghurt and salad dressing fortified with grape pomace powder [81], kefir [82], and processed cheese spread enriched with grape skin powder (**Publication referred to as [16]**). Besides dairy products, also wafers and biscuits enriched with grape pomace [80], chocolate where grape pomace is used as a bulking agent [86], or grape by-products ice cream [87]. Surprisingly, also preparation of meat products with content of parts of grapes was described. As examples can be mentioned low fat chicken meat balls with addition of grape pomace [88].

In case of processed cheese spread prepared in our study, usage of grape skin influenced all characteristics significantly. A drying procedure, as well as level of grape skin powder addition, played important role in final composition of spread (**Table 2 and Table 3 in [16]**). Fortification with freeze-dried grape skin powder at 2% (w/w) level allowed to achieve higher amount of individual phenolics (**Figure 3B in [16]**) and beneficial results of antioxidant capacity (**Figure 4 in [16]**).

Conclusion of Chapter 4

Grape wine can occur in colours ranging from yellow to deep purple. A typical compound found in grapes is resveratrol, which is attributed with effects supporting the prevention of cardiovascular diseases. Grape berries contain a significant amount of phenolic compounds (especially flavonols, stilbenes, flavan-3-ols, or phenolic acids), red grape varieties are rich in anthocyanins whereas they are absent in white varieties. Three quarters of harvested grapes are used to produce wine. This process is also associated with a high production of by-products. These residuals are very good source of dietary fiber and also contain high concentration of polyphenols (up to 70% of the original content). For this reason, many studies have already been published where these by-products (grape pomace and grape skin powder) were used to improve the properties of final food products and beverages. However, the implementation of red grape pomace has been described on a larger scale than that of white grape pomace. Due to this, white grape skin of variety Müller Thurgau was chosen to enrich processed cheese spread.

In our study, two types of drying (oven-drying and freeze-drying) were carried out to prepare grape skin powder. It was expected that freeze-dried grape skins will provide powder with higher amount of phenolic compounds. This was only partially confirmed as phenolic individuals ((+)-catechin, (–)-epicatechin, and rutin) were found in higher levels in oven-dried grape skin powder. Then, obtained grape skin powders were implemented to processed cheese spread at two different levels (1% and 2% w/w) and product with better nutritional values was expected. Based on results, it can be concluded that both chosen drying procedure and level of enrichment were evaluated as significant factor. Processed cheese spread with addition of 2% (w/w) of freeze-dried grape skin powder was found to be optimal choice due to higher levels of antioxidant activity as well as amounts of individual phenolics.

Chapter 5: COCOA (*Theobroma cacao* L.) AND CHOCOLATE

Cocoa beans, seeds extracted from a fruit of *Theobroma cacao* L. tree, are the key ingredient for production of chocolate. Production of cocoa beans is mostly located in Africa (over 76% of total world production). Trinitario, Criollo and Forastero are three basic varieties of cocoa beans which differ by organoleptic and textural characteristics as well as chemical composition. First two mentioned varieties have higher content of phenolic substances and sour–bitter flavour with slight acidity while dark brown Forastero beans have less pleasant aroma and taste. Forastero itself covers more than 90% of total world production of cocoa [89, 90, 91].

In ancient times, the Mayans considered chocolate (beverage consist of cocoa and hot water) the "food of Gods". Products from cocoa have been called potentially medicinal since the 17th century. Throughout history, chocolate was eaten to treat diseases such as angina or heart pain. Positive effect of chocolate on cardiovascular system (mainly due to presence of phenolic compounds in cocoa) has been published extensively. The perception of chocolate has changed rapidly over the last 40 years, it is currently considered more of a confectionery with negative effects on human health [90, 92, 93].

5.1 Instrumental verification of chocolate authenticity

Due to the presence of a relative high concentration of phenolic substances in cocoa, determination of antioxidant capacity can also be a useful tool for chocolate quality control. In our study (**Publication referred to as [17]**), a simple FIA with amperometric detection based on measurement of antioxidant activity of compounds present in chocolates was introduced. There is a positive correlation ($R = 0.9187$) between the amount of cocoa and values of antioxidant activity expressed as Trolox equivalent (TEAC) (**Fig. 6 in [17]**). For the experiment, BDDE was employed as an electrode, mixture of 0.1 M phosphate buffer (pH ~ 7) with methanol (30% v/v) was used as a working medium (**Fig. 3 in [17]**), detection potential was set at +1.3 V, and optimal flow rate of 1 mL/min was chosen. Brcanović with colleagues [94] compared usage of cyclic voltammetry and traditional spectrophotometric assays for determination of antioxidant capacity of dark and milk chocolates. They concluded that CV with GCE (in 0.1 M acetate buffer, pH ~ 4) is a reliable and comparable technique to commonly used methods.

Quality of chocolate and other cocoa products is usually controlled by chromatographic techniques (mainly HPLC) while complex analysis of samples and determination of individual substances is carried out. Risner [95] introduced RP-HPLC-UV method for simultaneous determination of chocolate alkaloids (theobromine, theophylline, and caffeine), (–)-epicatechin, and (+)-catechin with detection at 273 nm. Rýdlová et al. [96] described screening HPLC-MS method, that shows great potential to evaluate the authenticity and quality of chocolates by quantifying characteristic substances (theobromine, caffeine, and phenolic compounds) occurred in cocoa products. Rodríguez-Carrasco with colleagues [97] published results of UHPLC-MS/MS analysis of methylxanthines and phenolic profile in 80 chocolate samples with different cocoa content and prepared from different varieties and their combinations. LC-MS with PCA of large dataset can also be useful instrument to control origin of used cocoa [98]. As for GC, Oliveira et al. [99] published results showing significant differentiation in volatile profiles of cocoa nibs from different locations when comprehensive two-dimensional GC-FID analysis after headspace SPME was employed.

For the measurement of total antioxidant capacity or total phenolic content and thus also quality of chocolates can be also utilized FTIR-ATR when PLS or PCA applied on spectra [100]. Variety and geographical origin of used cocoa beans can also be verified using ^{1}H NMR techniques followed by PCA of obtained dataset [101].

5.2 Cocoa bean shell as a functional food ingredient

Hernández-Hernández and colleagues [102] prepared extra virgin olive oil jam fortified by encapsulated or freeze-dried CBS extract rich in polyphenols and theobromine. Produced jam with CBS extracts allowed to protect stability of bioactive compounds and antioxidant activity during storage. In study of Grassia et al. [103], chocolate bars enriched by microencapsulated phenolic extract from CBS were made and studied. Enriched chocolate bars offered increased value of total phenolic content (by 38%) without negative effect on sensory characteristics. Antun Jozinović and his team [104] introduced research describing production of extruded corn snacks (flips) with addition of CBS. Level of phenolics showed proportional increase to added CBS. Flips with 15% addition of CBS showed after extrusion promising retained values of total phenolic content (105.68 mg GAE per 100 g of DM) [104].

Conclusion of Chapter 5

Nowadays, chocolate is very popular confection all over the world. Cocoa, which is contained in chocolate products, gives it potential beneficial effects on human health (especially in the area of cardiovascular disease prevention). This fact is mainly contributed by the presence of phenolic compounds, of which the most abundant is flavan-3-ol (–)-epicatechin. The latter is also the main unit in procyanidin structures, which make up more than half of total polyphenols present in cocoa. During the process of chocolate production, these compounds can be lost up to 90% of the original content in used cocoa. However, a large part of cocoa polyphenols is also present in by-products from chocolate making process (e.g., cocoa bean shells) and it is therefore possible to consider them as an interesting functional food ingredients. Besides phenolics, cocoa bean shells can be also good source of fat and dietary fiber.

Dark chocolates contain more phenolic compounds than milk and white ones, which is due to the higher cocoa content in the final product. In our study, it was assumed that a higher cocoa content in chocolates would be associated with a higher antioxidant capacity of analyzed samples, which was confirmed based on the obtained results. Thus, the presented flow injection analysis (FIA) with amperometric detection performed using BDDE offers relatively elegant and easy way to control the percentage of cocoa in commercially available chocolates, which could in the future be an alternative to the usually used chromatographic procedures.

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