UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

DEPARTMENT OF ANALYTICAL CHEMISTRY

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

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DOCTORAL THESIS

2023

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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

NÁZEV PRÁCE

Hodnocení přísad funkčních potravin se zaměřením na obsah jejich fenolických sloučenin

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 osmotic dehydration 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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2D-PC	two-dimensional paper chromatography
2D-TLC	two-dimensional thin-layer chromatography
AAPH	2,2'-azobis(2-aminopropane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid
AdSV	adsorptive stripping voltammetry
AM	black chokeberry (*Aronia melanocarpa* L.)
AuNPs	gold nanoparticles
BA	bioaccessibility index
BDDE	boron-doped diamond electrode
C_0	initial amount / amount before *in vitro* digestion
C_d	amount after *in vitro* digestion
CAE	(+)-catechin equivalent
CBS	cocoa bean shell
CE	capillary electrophoresis
CNTs	carbon nanotubes
CTs	condensed tannins
CUPRAC	cupric ion reducing antioxidant capacity
CV	cyclic voltammetry
DAD	diode array detector
DHR/DMF	dihydroxyrhodamine in dimethylformamide
DM	dry mass
DMCA	4-(dimethylamino)-cinnamaldehyde
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPV	differential pulse voltammetry
ECD	electrochemical detector
EPR	electron paramagnetic resonance
ERT	erythritol
ESI-MS	electrospray ionization mass spectrometry
ESR	electron spin resonance
FAS	ferric ammonium sulphate
FC	Folin–Ciocalteu
FD	Folin–Denis

FLD	fluorimetric detector
Fe-C	Fe-catechol complex
Fe-G	Fe-galloyl complex
FIA	flow injection analysis
FID	flame ionization detector
FRAP	ferric-reducing ability of plasma / ferric-reducing antioxidant power
FTIR	Fourier transformed infrared spectroscopy
FTIR-ATR	Fourier transformed infrared attenuated total reflectance
FW	weight of fresh fruit (fresh weight)
GAE	gallic acid equivalent
GC	gas chromatography
GCE	glassy carbon electrode
GC-FID	gas chromatography with flame ionization detector
GC-MS	gas chromatography coupled with mass spectrometry
GP	grape pomace
GPx	glutathione peroxidase assay
GR	glutathione reductase assay
GSH	reduced glutathione assay
GSt	glutathione-S-transferase assay
HBAs	hydroxybenzoic acids
HCAs	hydroxycinnamic acids
HILIC	hydrophilic interaction liquid chromatography
HMF	5-(hydroxymethyl)furfural
HPLC	high performance liquid chromatography
HPLC-DAD	high performance liquid chromatography with diode array detector
HPLC-MS	high performance liquid chromatography coupled with mass spectrometry
HPLC-UV	high performance liquid chromatography with ultraviolet detector
HTs	hydrolysable tannins
IR	infrared
LC	liquid chromatography
LLE	liquid-liquid extraction
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MEKC	micellar electrokinetic chromatography
MRP	Maillard reaction products

MS or MS/MS	mass spectrometry or tandem mass spectrometry
MSPD	matrix solid-phase extraction
NBT	nitroblue tetrazolium
NED	non-enzymatic digestion / *in vitro* digestion performed without enzymes
NIR	near infrared
NMR	nuclear magnetic resonance
NP-LC	normal phase liquid chromatography
ORAC	oxygen radical absorbance capacity
PC	paper chromatography
PCA	principal component analysis
PDA	photodiode array
PLS	partial least squares
RP	reducing power
RP-HPLC	reversed-phase high performance liquid chromatography
SE	solvent extraction
SIA	sequential injection analysis
SOD	superoxide dismutase assay
SPE	solid-phase extraction
SPME	solid-phase microextraction
SWV	square wave voltammetry
TEAC	Trolox equivalent antioxidant capacity
TLC	thin-layer chromatography
TLC-MS	thin-layer chromatography coupled with mass spectrometry
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
TRAP	total peroxyl radical-trapping antioxidant parameter
UHPLC	ultrahigh performance liquid chromatography
UV	ultraviolet
VIS	visible
XYL	xylitol

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 pre-treatment 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 (**Appendix I.**), utilization of different grinding methods (**Appendix II.**), effect of vibratory grinding time (**Appendix III.**), and substitution of wheat flour by carob powder in preparation of muffins (**Appendix IV.**) was investigated. Black chokeberry was subjected to osmo-dehydration using different osmotic agents and the effect of ultrasonication was also examined (**Appendix V.**). By-products of winemaking process, white grape skins, were used for enrichment of processed cheese spread and improvement of chosen characteristics was evaluated (**Appendix VI.**). In **Appendix VII.**, a 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 [11, 12].

The presence of at least one aromatic ring with one or more hydroxyl substituents in the structure is common for all phenolic compounds [13, 14]. 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 [15].

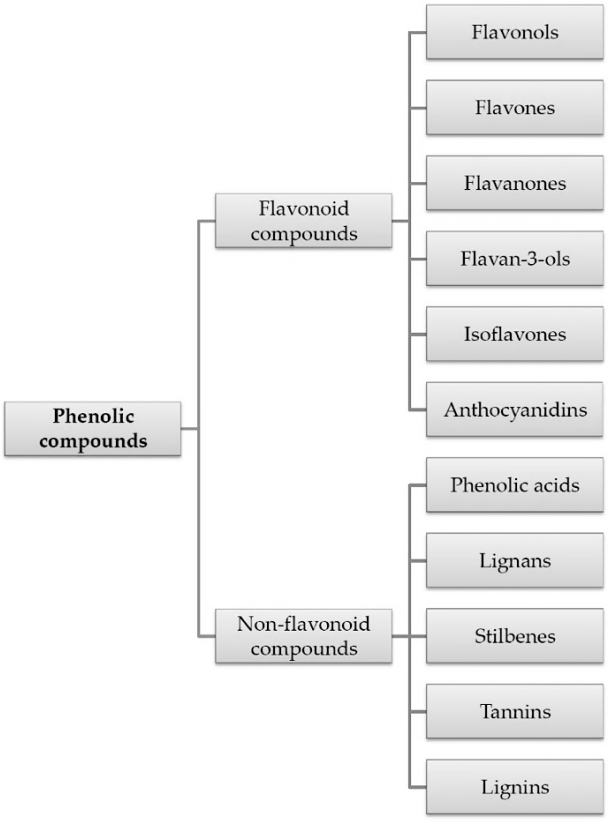


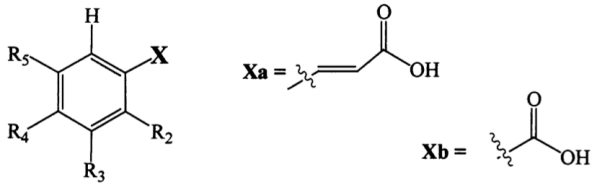
Fig. 1: Classification of phenolic compounds (edited) [15].

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 have 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) [16–19]. Polyphenols contribute to colour of vegetables and fruit and play an important role in terms of the aroma and taste of food [20–22]. 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 Classification of phenolic compounds

1.1.1 Phenolic acids

Phenolic acids are present in free or bound forms in almost all plant-derived foods. Group of phenolic acids consists of two main subgroups – hydroxycinnamic acids (HCAs) and hydroxybenzoic acids (HBAs), which are derived from cinnamic and benzoic acid, respectively (see **Fig. 2**) [23, 24].



R_2	R_3	R_4	R_5	X	common name
H	H	H	H	a	cinnamic acid
–OH	H	H	H	a	*o*–coumaric acid
H	H	–OH	H	a	*p*–coumaric acid
H	–OH	H	H	a	*m*–coumaric acid
H	$–OCH_3$	–OH	H	a	ferulic acid
H	$–OCH_3$	–OH	$–OCH_3$	a	sinapic acid
H	–OH	–OH	H	a	caffeic acid
H	H	H	H	b	benzoic acid
–OH	H	H	H	b	salicylic acid
H	H	–OH	H	b	*p*–hydroxybenzoic acid
H	$–OCH_3$	–OH	H	b	vanillic acid
H	$–OCH_3$	–OH	$–OCH_3$	b	syringic acid
H	–OH	–OH	H	b	protocatechuic acid
–OH	H	H	–OH	b	gentisic acid
–OH	–OH	–OH	–OH	b	gallic acid
H	$–OCH_3$	$–OCH_3$	H	b	veratric acid

Fig. 2: Structures of prominent naturally occurring phenolic acids (edited) [24].

HCAs are aromatic compounds with a side chain containing three carbons (C_6–C_3). Caffeic, ferulic, chlorogenic, *o*-, *m*- and *p*-coumaric, and sinapic acid belong to this group. Maybe the most widespread acid from this group is cinnamic acid, which can be found in coffee, wine, apples, potatoes, orange, olive oil, spinach, cabbage, and many others [15, 25]. Caffeic acid together with *p*-coumaric acid represent 75–100% of the total HCAs in fruits [26].

HBAs, with general structure C_6–C_1, include vanillic, protocatechuic, gallic, *p*-hydroxybenzoic, syringic, gentisic, ellagic, and salicylic acid. This group is present in plants mostly in the glycoside form [13, 23]. The main food sources of HBAs are vegetables, fruits, tea, and cereals. Salicylic acid is present in blueberries, apricot, and black tea. Gentisic acid can be found in tomato, pepper, cucumber, citrus, melon, and grapes. Gallic acid is part of teas such as Japanese and Chinese black and green tea [27].

Phenolic acids have been largely studied due to their beneficial effects on human health such as antioxidant (e.g., ferulic and caffeic acid), antitumor (e.g., cinnamic acid), antimicrobial (e.g., *p*-hydroxybenzoic acid), antimutagenic, chemoprotective and neuroprotective (e.g., protocatechuic acid), antineoplastic, hepatoprotective (e.g., syringic acid), antimelanogenic, bacteriostatic (e.g., gallic acid), anthelmintic (e.g., vanillic acid), antipyretic, antiseptic, anti-inflammatory and analgetic (e.g., salicylic acid), amongst others [25, 28–30].

1.1.2 Lignans

Lignans are produced by oxidative dimerization of two phenylpropanoid units. Although the base structure of lignans consists only of two phenylpropane (C_6–C_3) units, structural diversity of this group is enormous. These compounds can be subdivided to four major groups – lignans, lignolides, monoepoxylignans and biepoxylignans. The most important compounds belonging to this group are sesamin, sesamolin, secoisolariciresinol, lariciresinol, matairesinol, pinoresinol and two enterolignans – enterodiol and enterolactone (the most important mammalian lignans, both produced from precursors in food by the bacterial flora in the colon). Lignans can be found in a wide variety of plants, mostly in sunflower, sesame, and flax seeds (about 800 times higher lignan content than in any other plant), cereal grains, garlic, beans, lentils, or olive oil. This group of compounds appears in the nature mainly in free form, their glycoside derivates only form a minor part [14, 15, 26, 31–33].

Among the effects that lignans have on health, antimicrobial, immunosuppressive, anti-inflammatory, antifungal, antiviral, antioxidant, or cancer chemopreventive effects can be included [31, 34].

1.1.3 Stilbenes

This group of compounds (also known as vine phytoalexins) is associated with beneficial effects of wine drinking. The typical structure for stilbenes is C_6–C_2–C_6. Resveratrol is significant compound of stilbenes group that can occur as both *cis*- and *trans*-isomer. However, *trans*-isomer is known for its better biological activity related to protection against high pH and UV radiation. It is contained in nuts and popular fruit such as grapes and berries [13, 15, 35].

Due to their antioxidant and anti-inflammatory properties, stilbenes can be potentially used for prevention of diseases associated with oxidative stress or cancer. Studies have described resveratrol as a plant antibiotic with strong antifungal property. This compound has also recently been known to act against aging. Cardioprotective effect of resveratrol has been documented as well (low incidence of cardiovascular disease in areas with higher red wine consumption – phenomenon called "French Paradox") [36–38]. Interesting anticancer properties were described also for another compound from this group – pterostilbene. This compound, which is highly fungitoxic, has also antioxidant properties and is found in red wine and fruit, e.g., blueberries or grapes. Pterostilbene, dimethoxylated analogue of resveratrol, exhibits even higher effect as anticancer agent, concretely can be used as tool to reduce cancer cell growth or to cause cancer cell death (by affecting the permeabilization of the lysosomal membrane) [15, 35, 39, 40].

1.1.4 Tannins

Tannins are a type of water-soluble compounds of relatively high molecular weight (500–3000 g mol^{-1}) which can form insoluble complex with proteins and alkaloids. For tannins, presence of many hydroxyl (or other functional) groups is typical. These compounds are important for interaction of plant with its ecosystem (e.g., role of antimicrobial agent or acting against herbivores). Compounds of this group are very reactive and form hydrogen bridges (intra- and intermolecular) [13, 27, 41].

Chemically, they can be classified into two groups – hydrolyzable tannins (HTs) and condensed tannins (CTs). Hydrolyzable tannins can be subdivided to gallotannins and ellagitannins. Condensed tannins have more complex and uniform structure $(C_6–C_3–C_6)_n$, they are polymers or oligomers of flavan-3-ol monomers (also known as catechins) or/and flavan-3,4-diol monomers. The structures differ in stechiometry, degree of polymerization, hydroxylation, and type of interflavan linkage. Compounds belonging to this group are also called proanthocyanidins. Procyanidins and prodelphinidins can be mentioned as important representatives [11, 12, 41].

Condensed tannins are found in a wide variety of food such as vegetables, fruit, cocoa (they cause bitterness of chocolate), red wine and some grains and legumes. Important compounds that contribute to the quality of black tea, and belong to the group of condensed tannins, are thearubigins (main phenolic fraction of black tea, ~100 mg in typical cup of black tea). Ellagitannins from group of hydrolyzable tannins are present in nuts (e.g., cashew, pistachio, hazelnut), mango, peach, grape, pomegranate, and different kinds of berries (e.g., strawberry, raspberry) [11, 12, 26, 27].

1.1.5 Lignins

Lignin (structure $(C_6–C_3)_n$) is amorphous three-dimensional polyphenolic compound with high molecular weight, the second most abundant plant polymer. This compound consists of phenylpropanol units (monolignols) bound oxidatively via ether and carbon-carbon linkage. It is an important component in wood, where lignin's function is to ensure the woodiness of cell walls. Lignin can be found also in grains (grain bran contains approximately 8% of lignin). In addition to structural support and protection against pathogens, lignin acts as a hydrophobic component in plant water transport. Along with cellulose, it provides structural integrity necessary for the evolutionary emergence of self-supporting structures. Lignin lacks the regular structure and repeating units that can be found in other natural polymers such as cellulose. For this reason, lignin is not seen as a single compound, but as a mixture of heterogeneous substances forming the structure that can be interpreted by models [11, 41–44].

1.1.6 Flavonoids

Flavonoids represent the most extensively disseminated group of phenolic compounds. They comprise more than 6000 compounds among approximately 8000 total phenolic compounds found in plants. Their chemical structure is based on two aromatic rings (rings A and B) connected by a bridge from three carbons (common 15-carbon structure C_6-C_3-C_6) with bond to oxygen (ring C), as can be seen in **Fig. 3** [11, 15, 16]. Flavonoids are divided into 6 subclasses – flavan-3-ols (e.g., catechins and gallocatechins), flavonols (e.g., rutin and quercetin), flavanones (e.g., hesperidin), flavones (e.g., apigenin, chrysin and luteolin), isoflavones (e.g, daidzein and genistein) and anthocyanidins (e.g., cyanidins, delphinidins) [23, 45].

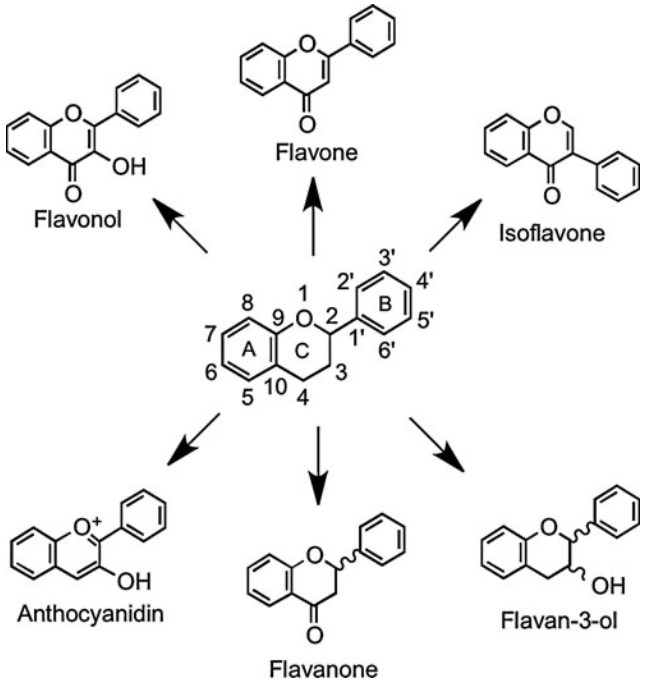


Fig. 3: Structure of flavonoid skeleton [11].

The main flavonoids sources are vegetables and fruit, rich for flavonoids are also seeds, grains, tea, coffee, and wine [15]. In the nature, they are often stored in plants in association with sugar(s) (so-called glycosides) as flavonoids are more stable in this form, however, when ingested, their bioavailability is relatively poor. Large part of these compounds has colour from yellow to red due to conjugated chromophores in their molecules and they are responsible for the colour of flowers and fruits. Flavones and flavonols are usually red, but on the other hand,

flavanones tend to be white, lightly brown, or colourless. Anthocyanidins such as cyanidin (colour from red to magenta), pelargonidin (orange to red), or delphinidin (magenta to purple) can be mentioned as other examples of colourful flavonoids [46–48].

Flavonols

Along with flavones, flavonols including their glycosides and other acylated products on all three rings present the largest subgroup among all polyphenols. The most common flavonols, kaempferol and quercetin, have together over 600 different glycosidic combinations. Other important compounds belonging to this group are rutin (quercetin-3-rutinoside), isorhamnetin, and myricetin. Biosynthesis of flavonols is simulated by light, that is why they accumulate in outer parts of fruits (differences in concentration can be found between fruit from the same tree or even between different sides of single piece of fruit, depending on the exposure to sunlight). The main sources of flavonols are red and yellow onions, curly kale, leek, broccoli, blueberries, red wine, or tea [11, 12, 26].

Beneficial properties in relation to human organism described for only quercetin itself include for example anticarcinogenic, antioxidative, antidiabetic, antiaging, vasodilating, or anti-inflammatory effect [38, 46].

Flavan-3-ols

Flavanols or also flavan-3-ols, commonly called catechins, represent four possible diastereoisomers (due to two chiral centres in the structure, at the C2 and C3 position of carbons), i.e., (+)-catechin; (–)-catechin and (+)-epicatechin; (–)-epicatechin. Significant compounds belonging to this group, which can be found in food plants, are (+)-catechin and (–)-epicatechin. Except these two, also their derivatives (+)-gallocatechin and (–)-epigallocatechin occur in food (e.g., tea leaves, chocolate, skin of grapes, apricots, cherries, blueberries, or apple). The level of flavanols in green tea leaves is decreasing during fermentation to obtain black tea, and there is a concomitant accumulation of other compounds from this group that contributes to the quality of black tea beverage – theaflavins. Unlike other classes of flavonoids, flavanols are not present in food in form of glycosides [11, 12, 26].

Studies describe positive effect of catechins extracted from green tea as a cardioprotective agents. For compounds of this group, anticancer, antiobesogenic, antidiabetic, and high antioxidant properties are also known [38, 49–53].

Flavanones

Compounds from this group are present in tomatoes or aromatic plants (e.g., mint) but the highest concentrations can be found in citrus fruits (naringenin in grapefruit, eriodictyol in lemons, and hesperetin in oranges). They occur mainly as hydroxyl, *O*-methylated and glycosylated derivatives (the most common flavanone glycoside is hesperetin-7-*O*-rutinoside also known as hesperidin). While flavanone rutinosides are tasteless, compounds such as naringin or neohesperidin have an intense bitter taste (presence in bitter oranges and grapefruits). A lot of attention has been paid to this group of compounds because of their anticarcinogenic properties. Hesperetin showed ability to inhibit carcinogenesis or inflammation. Together with naringenin, this substance also possesses antioxidant activity, however, compare to other polyphenols, this activity seems to be poorer [11, 26, 46].

Flavones

Flavones are fewer common flavonoids. Important representatives of flavones are apigenin and luteolin, which can be found in significant amounts in parsley, celery, or red peppers. Also, skin of many fruits contains a lot of flavones (e.g., mandarin skin). In form of glycosides, they appear also in rooibos tea [11, 26, 46].

As other flavonoids, compounds belonging to this group also has significant properties in relation to cancer (apigenin particularly in case of breast, skin, thyroid, colon, prostate or leukemia type of cancer, luteolin showed proliferative activity against breast cancer). Together with other compounds, apigenin and luteolin can be categorized as apoptotic flavonoids [49].

Isoflavones

These compounds are almost exclusively contained in leguminous plants (beans, soy etc.). Genistein and daidzein are two main isoflavones contained in soy, along with biochanin A,

glycitein, and formononetin. All these compounds occur in nature predominantly in association with sugar. This subgroup of flavonoids holds structural similarity to estrogens (sometimes referred to as "phytoestrogens"). Studies have described their effect against cancer (especially prostate and breast cancer), cardiovascular diseases, menopausal symptoms, osteoporosis, or atherosclerosis [12, 13, 54, 55].

Anthocyanidins

Anthocyanidins are main component of purple, blue and red pigments occurring in most flower petals (e.g., peonidin and petunidin), vegetables and fruit (especially berry fruit such as black currant, raspberries, or blueberries), and some special varieties of grains (black rice). The most known compounds of this group are cyanidin, pelargonidin, malvidin, and delphinidin. In plants, anthocyanidins mainly exist in glycoside form (commonly referred to as anthocyanins). In fact, 90% of all anthocyanins (more than 500 are currently known) are with base of cyanidin, pelargonidin, delphinidin, and their derivatives. These compounds may have antimicrobial, anticarcinogenic, antidiabetic, cardioprotective and antioxidant effects [12, 13, 35, 53].

The colour of anthocyanins is highly depending on pH values – in acidic conditions they have red colour, in basic conditions blue colour. Anthocyanins are chemically stable in acidic environment [12].

1.2 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•$^-$ or •OH) and nitrogen (e.g., NO•) species are produced from normal cellular metabolism, and they cause potential biological damage known as oxidative and nitrosative stress, respectively. These conditions can occur after overproduction of discussed reactive species in biological systems or due to deficiency of enzymatic and non-enzymatic antioxidants [25, 56].

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* methods [57, 58].

In vitro methods 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 [58, 59].

Compared to *in vitro* methods, *in vivo* methods are harder to carry out because of the problems related to cellular uptake and transport processes of compounds [59]. Assays belonging to this group are, e.g., reduced glutathione (GSH) assay [60, 61], glutathione peroxidase (GPx) assay [62], glutathione-S-transferase (GSt) assay [63], glutathione reductase (GR) assay [64] and superoxide dismutase (SOD) assay [65].

Another assay, which also can be used for antioxidant activity determination, is based on ability to form chelates with ferrous ion after reaction with ferrozine. Metal chelating ability is claimed as one of the important mechanisms of antioxidant activity [66].

1.2.1 Determination of antioxidant activity by *in vitro* methods

Evaluation of antioxidant properties of plant samples is mostly carried out using *in vitro* methods. The most common method belonging to this group is DPPH assay [58, 59].

DPPH assay

DPPH method was first described by Blois in 1958 [67] and has been modified by many researchers during years. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is characterized as a stable free radical used in the DPPH assay due to content of unpaired electron, delocalized over the entire molecule. Dissolved in methanol or ethanol, DPPH possess deep violet colour and principle of this method is to measure absorbance loss (spectrophotometrically, at 517 nm, colour change from violet to yellow) of this solution after reaction with potential antioxidants (substrate donates hydrogen atom and changing radical into inactive (yellow) form). Reaction is carried out at room temperature after optimization of reaction time. Scavenging capacity is then represented as the percentage of DPPH radical inhibition caused by antioxidant effect of

compounds present in sample. Strong antioxidants such as Trolox (water-soluble vitamin E analogue) are used as references, results are then expressed as their equivalents [57, 58, 68].

Advantage of DPPH assay is that it is easy, rapid, accurate, and cheap method to determine radical scavenging activity of non-enzymatic radicals. Due to lipophilic character of DPPH radical, this method has limited accessibility to hydrophilic components in samples. The major limitation is absorption maximum of DPPH as some compounds with antioxidant effect may have strong absorption in similar wavelengths (e.g., anthocyanins). Significant disadvantage is also narrow pH range where this test can be applied [57, 68, 69].

ABTS assay

This assay, also known as TEAC (Trolox Equivalent Antioxidant Capacity) method, was first described by Miller et al. in 1993 [70, 71] and later modified by Re et al. in 1999 [72]. ABTS assay is based on reaction of antioxidants with $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid cation radical) which is pre-generated in aqueous solution by mixing potassium persulfate ($K_2S_2O_8$) and ABTS. This oxidative reaction lasts from 12 up to 16 hours (colour change from almost colourless (ABTS) to deep blue/green ($ABTS^{\bullet+}$)). The next step is spectrophotometric measurement of the loss of absorbance at 734 nm (loss of colour intensity of dark blue/green solution). Results obtained from this method are also expressed as equivalent of referent antioxidant, in this case as TEAC (Trolox equivalent antioxidant capacity). The results from ABTS method should be comparable to the ones found in the DPPH test (can be considered as confirmation of DPPH assay), although, the values from ABTS assay are generally higher [57, 73, 74].

This method is fast and has one huge advantage over DPPH assay – it can be applied in a wide pH range. It is also possible to test both lipophilic and hydrophilic compounds [69].

ORAC assay

The ORAC (Oxygen Radical Absorbance Capacity) assay was originally described in 1999 by Cao and Prior [75]. *β*-Phycoerythrin (fluorescent oxidizable indicator protein), which is able to scavenge peroxyl radical generated by 2,2'-azobis(2-amininopropane) dihydrochloride (AAPH) at 37 °C, is used to determine antioxidant activity (non-fluorescent product formation).

After addition of antioxidant sample, which is protecting *β*-phycoerythrin from oxidation in presence of AAPH, reduced intensity of fluorescence is recorded, and the results are then compared to Trolox activity. This method is popularly used for evaluation of Maillard reactions model. Due to low photostability of *β*-phycoerythrin, there is also possibility to use fluorescein instead. Method using fluorescein is appliable for measurement of hydrophilic and lipophilic compounds [57, 58, 76].

ORAC assay has advantage compared to methods recording absorbance – it is possible to use it even for determination of coloured samples (wine, fruit etc.) [76].

FRAP assay

This method was developed by Benzie and Strain in 1996 [77] to control antioxidant effect of non-enzymatic defense in biological fluids (such as plasma) and further, in 2000, extended by Pulido et al. [78] for evaluation of antioxidant power of plant food.

FRAP (Ferric-Reducing Ability of Plasma or also Ferric-Reducing Antioxidant Power) assay is based on the ability of phenolics to reduce yellow Fe(III) complex with TPTZ (2,4,6-tris(2-pyridyl)-s-triazine to blue complex of Fe(II)-TPTZ at low pH (~3.6). Resulting blue colour is measured spectrophotometrically at 593 nm [59, 79].

FRAP method is rapid, cheap, simple, and robust, however, there is no possibility to measure glutathione (an important *in vivo* antioxidant) and problem can cause also presence of another species containing Fe(III) in the mixture [79].

Reducing power assay

Reducing power of the samples can be determined by direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$ due to antioxidants present in sample as introduced Oyaizu in 1986 [80]. Then, addition of free Fe(III) to the reduced product results to the formation of intense blue complex of $Fe_4[Fe(CN)_6]_3$ (Prussian blue) which can be monitored spectrophotometrically at 700 nm. Increasing values of absorbance will indicate higher reducing capacity of the sample [58, 68, 81].

TRAP method

TRAP (Total Peroxyl Radical-Trapping Antioxidant Parameter) method was developed by Wayner et al. in 1985 [82]. Principle of this method is measurement of oxygen consumption during controlled lipid oxidation after thermal decomposition of AAPH (2,2'-azobis(2-amininopropane) dihydrochloride) to peroxyl radicals. Previously, this oxygen consumption was detected by oxygen electrode, but during years this assay was modified to use chemiluminescence (enhanced by luminol) as a final step. Peroxyl radicals support chemiluminescent reaction, however, chemiluminescence is extinguished after addition of antioxidant sample and duration is directly proportional to radical trapping ability of the sample. Obtained results are then compared with Trolox as a reference [57, 59, 83].

β-Carotene test

This method is based on autoxidative bleaching of carotenoids induced by heat, light, or presence of peroxyl radicals (generated during linoleic acid degradation). Addition of antioxidant sample causes the inhibition of *β*-carotene bleaching which is then measured spectrophotometrically at 450–470 nm (strong antioxidants such as Trolox are used as references) [59, 79].

CUPRAC assay

This assay, devised by Apak et al. in 2004 [84], is based on reduction of Cu(II) to Cu(I). Neocuproine (2,9-dimethyl-1,10-phenantroline) in complex with Cu(II) is chromogenic oxidising reagent which is changing its colour (blue) after reduction to complex with Cu(I) (yellow-orange) due to contact with antioxidants. Colour change is measured spectrophotometrically at 450 nm and results are compared with data obtained for Trolox as reference. This method is easy, and it is possible to apply it on both lipophilic and hydrophilic antioxidants simultaneously [83, 84].

H_2O_2 scavenging assay

Hydrogen peroxide may come to human body through either inhalation of vapor or skin or eye contact. In organism, H_2O_2 is decomposed into water and oxygen by peroxidases (possible production of hydroxyl radicals OH• via Fenton or Haber-Weiss reaction when metal ions are present). Hydroxyl radical can cause DNA damage in the body due to lipid peroxidation [58, 68]. Hydrogen peroxide scavenging activity of antioxidants was established by Ruch et al. in 1989 [85]. Absorbance at 230 nm for mixture of H_2O_2 in phosphate buffer (pH 7.4) is measured to determine hydrogen peroxide concentration. After sample addition, obtained value of absorbance at 230 nm is expressed as percentage of hydrogen peroxide scavenging [58].

NO• scavenging assay

In this method described by Marcocci et al. in 1994 [86], nitric oxide is usually generated from sodium nitroprusside in aqueous solution at pH 7.2 (by decomposition) and its amount is determined after incubation by Griess reaction (by addition of Griess reagent containing 1% sulphanilamide, 2% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride). Curcumin can be used as standard (naturally occurring direct scavenger of NO) due to ability to reduce amount of stable products (nitrite or nitrate) formed from oxygen and nitric oxide generated from sodium nitroprusside. Further, absorbance of nitroprusside mixture after sample and Griess reagent addition is measured at 546 nm to obtain results for scavenging ability of antioxidants present in sample. Results are expressed as percentage of NO• inhibition [58, 87, 88].

Superoxide anion scavenging assay (NBT assay)

Superoxide radical anion ($O_2^{\bullet -}$), weak antioxidant producing ultimately more dangerous hydroxyl radical, is involved in this assay. Xanthine oxidase is enzyme responsible for conversion of hypoxanthine or xanthine into uric acid and superoxide radicals [58, 68, 89, 90].

This assay can be performed as described by Beauchamp and Fridovich in 1971 [91]. Scavenging activity of superoxide radicals is analysed with hypoxantine/xanthine oxidase system coupled with reduction of NBT (nitroblue tetrazolium). Results are obtained after

spectrophotometric measurement at 540 nm and expressed as percent of inhibition compared to blank (addition of KH_2PO_4/KOH buffer; pH 7.4 instead of sample) [81, 91].

Peroxynitrite radical (ONOO•) scavenging activity

Peroxynitrite (ONOO) is an oxidant and nitrating agent which can cause injury of neural cells and contribute pathogenesis of neurodegeneration. Radical of peroxynitrite has cytotoxic effect and strong antioxidant properties toward various cellular constituents (e.g., lipids, amino acids, or nucleotides), leading to carcinogenesis, aging, or lipid peroxidation [58, 68].

Method described by Kooy et al. in 1994 [92] involves use of dihydroxyrhodamine in dimethylformamide (DHR/DMF). Diluted working solution prepared from DHR/DMF stock is placed on ice at dark place before the experiment is carried out. Furthermore, phosphate buffer solution (pH 7.4) containing NaCl, KCl with diethylenetriaminepentaacetic acid is purged with nitrogen and placed on ice before assay. ONOO• radical oxidises dihydrorhodamine to rhodamine and ONOO• scavenging activity of antioxidants present in sample is carried out on microplate fluorescence spectrophotometer at 485 nm (excitation) and 530 nm (emission) at room temperature [58, 68].

1.2.2 Determination of antioxidant activity using other analytical techniques

In addition to the methods described in previous chapters which are widely used, there is also possibility to determine antioxidant activity with the use of other analytical techniques.

As example can be mentioned ESR (electron spin resonance, also known as electron paramagnetic resonance; EPR), only analytical technique able to specifically detect free radicals. Principle of this method is free radicals trapping. Unfortunately, ESR is not sensitive to detect reactive, short-lived free radicals (peroxyl or hydroxyl radical). Several techniques have been used to avoid this problem, besides pulse radiolysis and UV photolysis, also spin trapping. Spin trapping uses addition of compound (spin trap; usually nitroso- compounds) reacting with free radicals to form longer-lived radical adducts that can be detected by ESR method [57, 58, 93, 94].

Wang et al. [95] and Rivero-Cruz et al. [96] published studies where NMR (nuclear magnetic resonance) spectroscopy is used as an instrument to obtain information about antioxidant activity. Moreover, this method may also offer opportunity to explore antioxidative mechanism of natural products. There is also possibility to use NIR (near-infrared) spectroscopy [97] as well as FTIR-ATR (Fourier transformed infrared attenuated total reflectance) method to predict antioxidant capacity [98].

Electrochemical techniques such as cyclic voltammetry [99], flow injection analysis (FIA) with electrochemically generated $ABTS^{\bullet+}$ [100], flow injection biamperometric method [101], sequential injection analysis (SIA) with electrochemical detection [102], amperometric electronic tongue [103] or chronoamperometric method [104] can be also used for determination of antioxidant activity. The coupling of chromatographic techniques with electrochemical detector also offers a variant for antioxidant activity prediction [105, 106]. Most of these methods are based on measurement of current (generated by oxidation or reduction of electroactive analytes) that flows between working and referent electrode.

Method where DPPH or $ABTS^{\bullet+}$ is mixed with sample after HPLC–UV or HPLC–DAD (high-performance liquid chromatography with UV or diode array detection) separation followed by spectrophotometric measurements at 517 nm (DPPH) or at 734 nm ($ABTS^{\bullet+}$) was described by Koleva et al. [107, 108]. This technique offers possibility to monitor specific antioxidant activity of each compound or group of compounds, respectively.

Regoli and Winston [109] described procedure where peroxinitrite, hydroxyl, and peroxyl radicals are generated by Fenton reaction. Further, these oxidative radicals react with α-keto-γ-methiolbutyric acid which is oxidized and ethylene is produced. Then, ethylene is determined by gas chromatography with flame ionization detector (GC–FID) and results can provide information about antioxidant activity of samples (due to ability of antioxidants to inhibit ethylene formation) [109].

1.3 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 hierarchy of the procedure can be seen in **Fig. 4**. 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 addition of small amount of acid (e.g., phosphoric, hydrochloric, or formic acid) can also 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 [110–112].

Nowadays, use of ionic liquids and deep eutectic solvents for highly effective and selective extraction of chosen compounds from samples is also very popular [113–115].

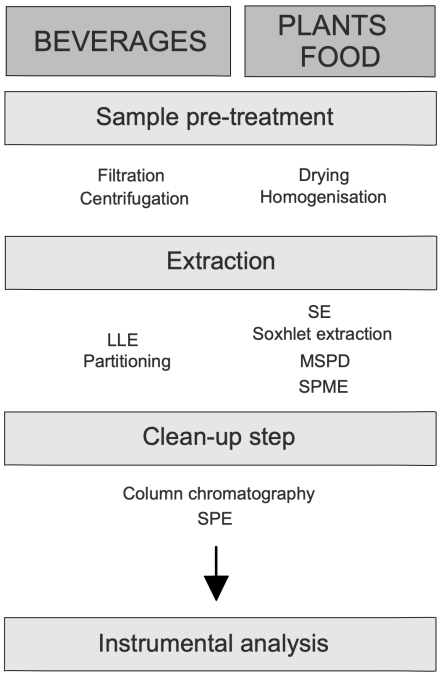


Fig. 4: Sample preparation before instrumental analysis (edited) [112].

LLE = liquid–liquid extraction; SE = solvent extraction; MSPD = matrix solid-phase extraction; SPME = solid-phase microextraction; SPE = solid-phase extraction [112].

There are currently many publications describing possible ways for phenolic compounds analysis. The most common methods used to determine phenolics are shown in **Fig. 5**. 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.

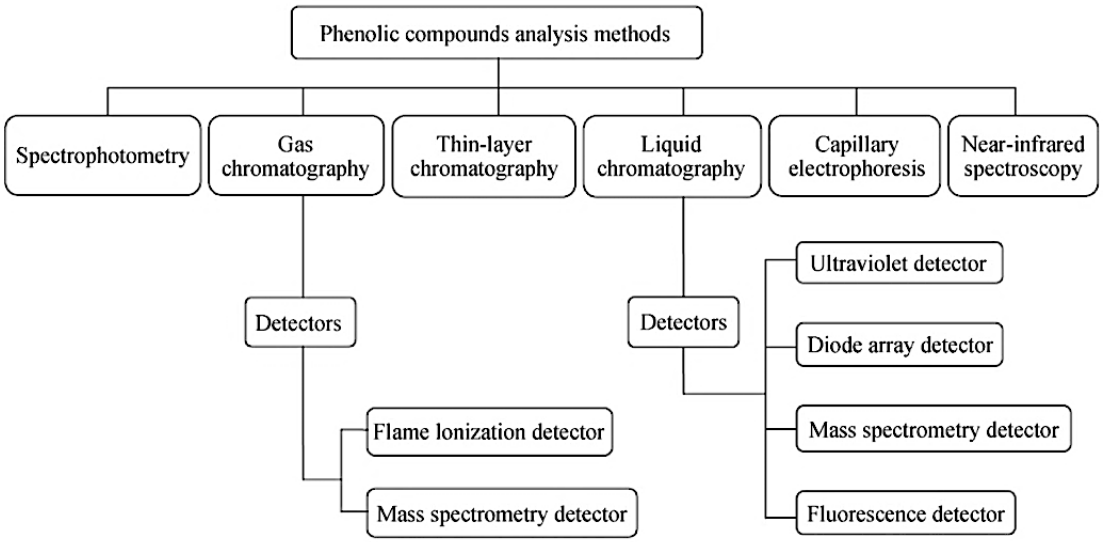


Fig. 5: Most common possibilities of phenolic compounds determination [13].

1.3.1 Spectral methods in determination of phenolic compounds

Large number of methods for determination of antioxidant activity, that is closely related to the presence of phenolics, was already described above. Chapters below discuss the most common methods for determination of individual groups of phenolic compounds (predominantly utilizing UV-VIS spectrometry).

For this purpose, traditional spectroscopic techniques (e.g., NMR, FTIR, or near IR spectroscopy) can be also used. However, these measurements can lead to overestimation of analysed phenolics. This complication can be prevented by use of chemometric techniques such as principal component analysis (PCA) or partial least squares (PLS) applied to obtained spectra [96–98].

Total phenolic content by Folin–Denis and Folin–Ciocalteu method

This well-known method [116], which is modification of Folin–Denis assay from 1912 [117], was originally designed in 1927 for analysis of proteins and later in 1999 [118] modified in order to analyse the phenolic components of food, beverages or plant extracts. Determination is based on reduction of Folin–Denis (FD) or Folin–Ciocalteu (FC) reagent in alkaline conditions by phenolic compounds present in sample. Composition of these intensively yellow reagents is not clearly defined, however, it is believed to contain mixture of phosphomolybdic and phosphotungstic acid complexes. From FD reagent, FC reagent differs basically in amount of molybdate (higher content to avoid formation of white precipitate). During the reduction, oxidation state of molybdenum and tungsten is decreased and colour of solution is changing into blue. This change is measured spectrophotometrically at 765 nm. Gallic acid is usually used as reference and results are expressed as gallic acid equivalent; GAE [67, 82, 118].

Total flavonoid content

Method for determination of total flavonoid content can be performed as described by Dewanto [119]. Extract of the sample is mixed with distilled water and 5% $NaNO_2$ solution in test tube. Further, $AlCl_3$ and NaOH are added. Spectrophotometric measurement at 510 nm follows. (+)-Catechin is used for standard curve construction and results are expressed as (+)-catechin equivalent (CAE) [119].

Another possibility is determination where the sample extract is directly mixed with 2%–10% solution of $AlCl_3$ (dissolved in water or methanol) [120, 121]. Absorbance is usually measured at wavelength range of 410–430 nm and total flavonoid content is expressed as rutin, quercetin, or catechin equivalent. Some studies also modified this procedure by acetate salts addition (CH_3COONa and CH_3COONH_4). Influence of mentioned salts on the aluminium complexation reaction of flavonoids was tested in research of Pękal and Pyrzynska. From their results can be seen that the presence of these modifiers is not affecting the reaction significantly [121].

While procedure with $NaNO_2$ and alkaline medium is probably specific for catechins, rutin and luteolin, method performed in neutral environment can be carried out only to obtain information about flavonols and luteolin content [121].

Determination of condensed tannins and related phenolics

First method belonging to this group is **vanillin assay** which is specific for some flavan-3-ols and proanthocyanins. It is based on reaction of methanolic solution of vanillin with proanthocyanins under acidic conditions (HCl or H_2SO_4) resulting into light pink to deep red coloured product measured spectrophotometrically at 500 nm. Catechin is often used as standard in this test. Vanillin assay can be described as useful, simple, sensitive, and specific method to determine condensed tannins in plant materials [122, 123]. **DMCA assay** is another one for determination of condensed tannins. This method was first described by Thies and Fischer in 1971 [124]. DMCA (4-(dimethylamino)-cinnamaldehyde) is compound able to react with proanthocyanidins in plants. However, DMCA is sensitive to both monomeric and polymeric units and, therefore, condensed tannin content can be overestimated [125]. **Proanthocyanidin assay** is being carried out in butanol–HCl solution (95:5, v/v). When this mixture is hot, condensed tannins are converted to anthocyanidins. Yield of this conversion depends on proanthocyanidin polymerization degree, presence of transition metals (Fe(II) and Fe(III) are the most effective calalysts), HCl concentration, water content (6% water in solution seems to be optimal), temperature, and time of reaction [126].

Hydrolyzable tannins content

Various approaches were already described for determination of hydrolyzable tannins present in plants. From all, the most widely used assay is based on method described by Haslam in 1965 [127], which was later modified by Bate-Smith [128] to be appliable for determination in plant material. The principle of method for determination of hydrolyzable tannins is their reaction with potassium iodate. Recently, Hartzfeld et al. added one more step to this assay, methanolysis (formation of methyl gallate) before reaction with potassium iodide. Product of the reaction is red chromophore that is then measured spectrophotometrically at 525 nm [129].

Determination of total chlorogenic acid content

This colorimetric technique can be performed as described by Clifford and Wright in 1976 [130]. Sodium metaperiodate is reagent able to provide simple and rapid means to measure total chlorogenic acid content (chlorogenic acid isomers: caffeoylquinic, feruloylquinic and

p-coumaroylquinic acids). Reaction is selective to *ortho*- and *para*-dihydroxyphenols and their monomethyl ethers. Resulting yellow-orange product is measured spectrophotometrically at 406 nm [130].

Anthocyanins determination

Quantification of anthocyanins takes advantage of their characteristic behaviour in acidic media. Anthocyanins exist in acidic media in equilibrium between coloured oxonium ion and colourless hemiketal form. At pH 3.9, oxonium form comprises about 15% of equilibrium mixture, while at pH 1.0 it is 100%. This method was first developed by Sondheimer and Kertesz in 1948 [131] and later modified by Swain and Hills [132]. They suggested to extend the pH differential to between 4.5 and 1.0. Thus, the difference when absorbance is measured at 520 nm is obvious. Results are expressed as equivalent of cyanidin-3-glucoside (the most common anthocyanin pigment found in nature) [133].

Assay for iron-binding phenolic compounds

Spectrophotometric assay developed by Brune et al. in 1991 [134] uses fact that creation of Fe-galloyl and Fe-catechol complexes is accompanied by the formation of colourful solutions. Ferric ions produce blue-coloured complex after reaction with compounds of galloyl group (presence of three adjacent hydroxyl groups) and green-coloured complexes with compounds from catechol group (presence of two adjacent hydroxyl groups). Procedure involves phenolics extraction into mixture of dimethylformamide with acetate buffer in the dark (16 hours) and addition of ferric ammonium sulfate (FAS) reagent to the supernatant follows. Absorption maximum for Fe-galloyl (Fe-G) complexes is approximately 587 nm (blue colour), for Fe-catechol (Fe-C) complexes around 680 nm (green colour). After standard curve measurement for tannic acid and catechin at both wavelengths, results are expressed as tannic acid equivalents (for Fe-G) and catechin equivalents (for Fe-C), respectively. This assay is not applicable for monohydroxy- and dihydroxy-phenolics with *para*- or *meta*-hydroxylation in the structure (missing chelating properties of these phenolics) [134, 135].

1.3.2 Determination of phenolic compounds by separation techniques

Identification and quantification of individual phenolic compounds is usually performed using separation techniques. These methods also offer options such as determining the phenolic profile or checking the authenticity of materials (e.g., geographical origin, species, or variety) [136–138].

Determination by high-performance liquid chromatography

High-performance liquid chromatography (HPLC) is technique which is most used for identification and quantification of phenolic compounds. The most common is instrumentation coupled with UV (HPLC–UV) and diode array (HPLC–DAD) detectors, or mass spectrometer (HPLC–MS) [13, 139, 140].

The mobile phases for HPLC analysis of group of phenolics are usually formed by water and acetonitrile or methanol (also their mixtures and their aqueous mixtures). Addition of acid (e.g., acetic, or formic) to mobile phase to avoid ionization of compounds during analysis (optimal pH range is 2–4) is also common. The use of phosphate or ammonium acetate buffer to lower the pH of mobile phase is also reported. Gradient elution is applied more often than isocratic elution [13, 141–144].

Temperature of analysis is very important factor affecting selectivity, retention, and viscosity of mobile phase. The most significant effect of higher temperature, reduction of the mobile phase viscosity, results in higher optimal flow rates and therefore also faster analysis [142, 145].

As for the stationary phases, many different columns are recently used. Proper chromatographic column selection is critical parameter for identification and quantification of food phenolics. Most of the time, column for RP-HPLC (reversed-phase) separation are chosen (to this group belong columns with C_8 or C_{18} phases). Nowadays, usage of new column types such as monolithic or columns with superficially porous particles is also very popular [142, 145].

Detection of phenolic compounds is often performed using UV-VIS, DAD, PDA (photodiode array), ECD (electrochemical) or fluorimetric (FLD) detectors. Also, application of mass spectrometry (MS) attached to HPLC system is very useful and sensitive for characterization of compounds from group of phenolics (especially ESI-MS (electrospray ionization mass

spectrometry) and MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry)) [140, 146, 147].

UHPLC (ultrahigh performance liquid chromatography) is advanced form of HPLC. Particles in columns for UHPLC are smaller than 2 μm and system is operating at high back pressures (600–1400 bar; higher temperature can be applied to lower the pressure). Advances of UHPLC over HPLC are mainly higher sensitivity (lower detection limits), shorter retention times and improved resolution. Also, UHPLC consumes about 80% less organic solvent (due to lower flow rates). Except classic C_{18} columns, other more polar stationary phases (e.g., with phenyl or cyanopropyl groups) can be used for analysis of phenolic compounds. UHPLC system is usually coupled with DAD or MS for detection [148, 149].

HILIC (hydrophilic interaction liquid chromatography) is another modification of HPLC technique for separation of polar and ionized compounds, which are too strongly retained on polar stationary phases used in normal-phase LC and/or badly separated in system with reversed-phase mode. HILIC method has been found to be very effective in analysis of phenolic compounds. This method can be categorized as a type of normal-phase liquid chromatography (NP-LC) but with more complex separation mechanism. Analyte retention in the system may be depending on the partition and intermolecular interactions between compound and solvent, and between compound and stationary phase. Usually, stationary phases used in HILIC consist of silica gels with modifications (e.g., amino, amide, or cyano groups). As mobile phase, aqueous mixtures of methanol and acetonitrile are commonly used [150, 151].

Determination using gas chromatography

Gas chromatography (GC) is another useful technique that can be applied in analysis of phenolic compounds (such as phenolic acids, flavonoids, and condensed tannins). However, main problem with using of this method is low volatility of phenolics and usually formation of more volatile derivatives is necessary (e.g., via methylation, acetylation, or silylation). Also, the need for many clean-up steps (lipid removal, release of compounds from ester and glycoside bonds etc.) before GC measurement can complicate and prolong the analysis. Frequently, helium is used as mobile phase (carrier gas) and stationary phase is formed by fused silica capillaries (i.d. 0.25–0.32 mm; length 30 m, with particles 0.25 μm). The most common instrumentation is GC coupled with flame ionization detector (GC–FID). In recent years, gas

chromatography coupled with mass spectrometry (GC–MS) is useful for quantification, especially due to its high sensitivity. Some studies describe even better resolution and minimal co-elution of separated standards compare to HPLC. Real advantage of GC–MS method is possibility to use mass spectral libraries [13, 139, 147, 152].

Determination by paper and thin-layer chromatography

Paper chromatography (PC) and thin-layer chromatography (TLC) are cheap and simple partitioning methods also used for isolation and purification of phenolic compounds in food (anthocyanins, condensed tannins, flavonols, and phenolic acids). Certain phenolics, such as anthocyanins, can be directly visualized on TLC plates coated with silica gel (stationary phase) due to their different colours. Some compounds appear under UV light as dark or absorbing spots, e.g., flavones or furanocoumarins (fluorescent spots). Furthermore, most absorbing compounds are giving green, yellow, or brown colour under UV light when plate is in the presence of ammonia vapor. Also, useful information about structure can be obtained after use of spray reagents (e.g., *p*-nitroaniline). As a mobile phase, system consisting of mixtures of different solvents (e.g., chloroform and methanol or methanol and water) is used for TLC. Due to impossibility to identify and characterize the analytes, interfaces that allow TLC combination with mass spectrometry were developed (TLC–MS). There is also possibility to perform two-dimensional PC (2D–PC) or TLC (2D–TLC) [147, 152–155].

Capillary electrophoresis in analysis of phenolics

Capillary electrophoresis (CE) is separation technique appliable for analysis (often faster and more efficient than HPLC) of polar or charged phenolic compounds with low to medium molecular weight. For example, there is a possibility to determine catechin, naringenin, apigenin, quercetin, luteolin, chlorogenic acid, cinnamic acid, vanillic acid, and syringic acid in different plant matrices, content of rutin in germinated buckwheat, or different phenolics in grapes, olives, broccoli, tea, and herbs. This technique has been also used for evaluation of red wine ageing (based on anthocyanins content). Main disadvantage of CE is that it lacks ability to distinguish compounds with close charge-to-mass ratios. However, addition of organic solvents to enhance the separation capability is possible. Coupling of CE with ESI-MS (electrospray ionization mass spectrometry) offers good opportunities for phenolics

determination in wide range of matrices. Micellar electrokinetic chromatography (MEKC), advanced modification of CE, has extended the utility of this technique to the analysis of neutral analytes under the influence of electric field [13, 155–159].

1.3.3 Electrochemical methods used in phenolics determination

These methods are used to characterize redox properties of analysed molecules. They provide information about electron transfer reactions and redox potentials. Electrochemical methods are associated with use of HPLC (electrochemical detectors), flow injection analysis systems (FIA), CE (electrochemical detectors), or (bio)sensor development [159–163].

Electroanalytical techniques are low-cost, sensitive, rapid, and very suitable for determination and characterization of electroactive species. They allow identification and quantification of phenolics even in complex matrices. Analyses can be carried out in techniques such as DPV (differential pulse voltammetry), CV (cyclic voltammetry), SWV (square wave voltammetry), AdSV (adsorptive stripping voltammetry), or with biosensors (based on enzyme catalysis). Redox behaviour of natural occurring phenolic compounds is determined mainly by electroactive –OH groups present in their structure. Also, other substituents such as methoxyl group, keto group, acetylesters, or glucosides may have impact on voltammetric behaviour. Substituents with large Hammett constants shift oxidation of phenolic groups to more positive potentials while electron-donor substituents shift this oxidation to more negative potentials. Usually, electrochemical measurements of these compounds are performed in three-electrode mode. Working electrode is often GCE (glassy carbon electrode), screen printed electrode, or BDDE (boron doped diamond electrode) due to wide potential window (from –1.0 V to +1.4 V; vs. Ag/AgCl). There is also possibility to modify surface of working electrode by graphene, carbon nanotubes (CNTs), gold nanoparticles (AuNPs), or ionic liquids. Experiments are done in aqueous solutions of electrochemically inert buffers (e.g., phosphate, acetate, or ammonia), however, aqueous mixtures of organic solvents such as methanol or ethanol are also used due to low solubility of some phenolics in aqueous media [163–168].

HPLC coupled with electrochemical detection (ECD) have an important place in analysis of phenolic compounds (especially flavonoids and phenolic acids) mainly due to high sensitivity, reproducibility, and selectivity (only for easily oxidized or reduced compounds at low potentials). The predominant electrochemical HPLC detector used for phenolic compounds

analysis is coulometric detector (measurement of electrical charge required for oxidation or reduction the total amount of compound) which can be improved in a multichannel system as well. This type of detection offers complete voltammetric differentiation according to reaction potential of analytes [164, 165].

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 individual 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 for 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 [169–173].

2.1 Chemical composition of carob pod

Carob fruit represents rich source of carbohydrates, proteins, fatty acids, dietary fibre, minerals, or polyphenolic substances. Constitution of carob pod is very variable based on the geographical origin, variety, gender, ripeness, and harvesting practices. In **Tab. 1**, typical composition of carob pulp with percentage representation is listed [170, 171, 174]. High level of total **sugar content** for carob cultivars ranging from 40 to 55% have been reported (predominantly sucrose; 30–64%, fructose; up to 18%, and glucose; up to 7%). Carob is also very significant source of D-pinitol, compound with strong antidiabetic effect. Compared to cocoa, carob pulp contains more sugars (cocoa bean sugars level ranges from 0.4 to 3.5%) but less proteins (about 2–7%). In case of **amino acids**, all seven essential ones (valine, leucine, isoleucine, methionine, threonine, lysine, and phenylalanine) can be found in carob, 57% of total amino acid content is comprised by aspartic acid, asparagine, leucine, valine, alanine, glutamic acid, and glutamine. For cysteine only low concentrations were found. **Fat content** in carob pod is very low (0.4–1.3%) compared to cocoa (up to 57%). Main carob fat components

are linolenic and linoleic acid (polyunsaturated), oleic acid (monounsaturated), and palmitic and stearic acid (saturated). Major **minerals** determined in carob are potassium (up to 9.70 mg per 1 g of DM), calcium, phosphorus, sodium, and magnesium, but also trace minerals such as iron, zinc, manganese, and copper can be found. Calcium content in 100 g of carob pulp (up to 480 mg) can be likened to intake of calcium from cup of cow milk. **Dietary fiber** of carob pod is largely insoluble. It constitutes up to 40% of pulp and consists of cellulose, hemicellulose, galactomannans, and lignin [170–172, 175].

Tab. 1: Composition of carob pulp (edited) [171].

COMPONENT	CAROB PULP COMPOSITION (%)
Moisture	6.0–11.0
Protein	2.0–7.6
Fat	0.4–1.3
Fiber	7.6–38.0
Ash	2.0–3.4
Sugars	40.7–54.7
Theobromine	Trace amount
Caffeine	Trace amount
Total polyphenols	1.2–7.0[a]

[a] Results are expressed as gallic acid equivalents (GAE); g per 100 g of dry mass (DM).

Polyphenols occurring in carob can be found mostly in pulp. Among the most represented phenolics are phenolic acids, flavonoids, and tannins. The most abundant polyphenolic compounds are phenolic acids, in particular, gallic acid (and its derivatives), ellagic, vanillic, syringic, gentisic or 4-hydroxybenzoic acid, in lower amount also coumaric, ferulic, cinnamic, caffeic, and chlorogenic acid. From group of flavonoids are present mainly flavonols (quercetin, kaempferol, myricetin, and their glycoside derivatives) and flavan-3-ol (+)-catechin. Naringenin (flavanones), luteolin, apigenin, chrysoeriol (flavones) and genistein (isoflavones) are also part of the carob pulp phenolics. Both groups of tannins (hydrolysable and condensed) are found in carob fruit as well, with CTs dominating [171, 174, 176].

2.2 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 [177].

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 Appendix I.**). 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 [171, 172, 178, 179]. 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 [178].

Grinding method used to obtain fine carob powder and final particle size of this powder have also very significant effect on its 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 Appendix II.**). 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 Appendix II.**). 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 Appendix II.**). When effect of vibratory grinding time was investigated in our study (**Appendix III.**), 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.

2.3 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 [180, 181]. 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 [181, 182]. Bioaccessibility index (BA) of individual phenolics can be investigated by *in vitro* digestion procedures and calculated using equation **BA (%) = (C_d/C_0) × 100**; where C_d and C_0 indicates amount after digestion and before digestion (initial amount; obtained usually after extraction), respectively (**Chapter 2.8. in Appendix II.**).

Chait et al. [180] concluded that the most bioaccessable phenolics from carob powder (particles ≤ 150 μm), measured after three-stage digestion (mouth, gastric, and intestinal phase), were gallic acid (BA ~ 647.4%), (+)-catechin (BA ~ 558.3%), chlorogenic acid (BA ~ 485.4%), and rutin (BA ~ 267.2%). In our study (**Appendix II.**), 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 Appendix II.**). Low bioaccessibility of luteolin and apigenin was also confirmed in our study when only vibratory grinding in different times was performed (**Table 3. in Appendix III.**). 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, phenolic content in carob powder decreased by 67% during NED which was most likely caused by radical pH changes. Research of Goulas and Hadjisolomou [183] described conclusion that most significant changes in the stability of chemical standards of phenolic compounds usually present in carob powder (gallic

acid, caffeic acid, syringic acid, rutin, quercetin, myricetin, catechin, and epigallocatechin gallate) seem to be induced mainly during gastric and intestinal steps as recovery of phenolics ranged between 40–87% and 16–57%, respectively. The investigated phenolic acids showed high resistance during *in vitro* digestion while flavones (apigenin and luteolin) appeared to be the least stable compounds. Ortega et al. [184] also described low stability of flavonoles, particularly quercetin that was totally degraded after *in vitro* digestion procedure in samples of carob flours.

2.4 Carob as a functional food ingredient

Due to the composition and all properties described in previous chapters, 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 [171–173]. Flour-based food enriched by carob, such as bread [185], cakes [186], cookies [187], pasta [188] and muffins (**Appendix IV.**), but also carob liqueurs [189], confectionery products [190], chocolates [191], carob flour fortified spreads [192], ice cream, yogurt [193] or milk beverages with carob addition [194] were prepared, and functional characteristics of these products were studied.

In mentioned applications, positive effects (e.g., higher sugar, protein, and dietary fiber amount, higher antioxidant activity, low caffeine content, higher level of essential minerals, positive effect on sensory analysis or better textural characteristics) were reported when using carob as a functional food ingredient (**Table 2 and Table 3 in Appendix IV.**) [173, 186–194].

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.

Appendices for Chapter 2

Appendix I.

Červenka, L.; Stępień, A.; Frühbauerová, M.; Velichová, H.; Witczak, M. Thermodynamic properties and glass transition temperature of roasted and unroasted carob (*Ceratonia siliqua* L.) powder. *Food Chemistry*, 2019, **300**, 125208.

Appendix II.

Frühbauerová, M.; Červenka, L.; Hájek, T.; Pouzar, M.; Palarčík, J. Bioaccessibility of phenolics from carob (*Ceratonia siliqua* L.) pod powder prepared by cryogenic and vibratory grinding. *Food Chemistry*, 2022, **377**, 131968.

Appendix III.

Červenka, L.; Frühbauerová, M.; Palarčík, J.; Muriqi, S.; Velichová, H. The effect of vibratory grinding time on moisture sorption, particle size distribution, and phenolic bioaccessibility of carob powder. *Molecules*, 2022, **27**(22), 7689.

Appendix IV.

Červenka, L.; Frühbauerová, M.; Velichová, H. Functional properties of muffin as affected by substituting wheat flour with carob powder. *Potravinarstvo Slovak Journal of Food Sciences*, 2019, **13**(1), 212–217.

Appendix III.

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The Effect of Vibratory Grinding Time on Moisture Sorption, Particle Size Distribution, and Phenolic Bioaccessibility of Carob Powder

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Abstract: Carob pod powder, an excellent source of health-promoting substances, has found its use in a wide range of food products. Grinding conditions affect the physical and chemical properties of the powder, but their influence on the bioaccessibility of phenolic compounds in carob pod powder has not yet been determined. The carob pods were ground for 30–180 s in a vibratory grinder. The median values (D_{50}) of particle size decreased after 60 s of grinding (87.9 µm), then increased to 135.1 µm. Lightness showed a negative correlation with D_{50} and a_w, while the values of redness and yellowness decreased with the reduction in particle size and water activity. The smaller the value of D_{50}, the higher the equilibrium moisture content of carob powder. Phenolic acids (vanillic, ferulic, cinnamic) and flavonoids (luteolin, naringenin, apigenin) were found in all samples of carob powder. The grinding time influenced their content in carob powder, with maximum values at 180 s. Similar observations were made when assessing antioxidant capacity. The in vitro digestion process only improved the bioaccessibility of catechin content in all samples. However, the bioaccessibility of the phenolic compounds and the total phenolic and flavonoid contents decreased with the increase in grinding time. Our findings revealed that the grinding of carob pods for 180 s improved the extractability of phenolics; however, their bioaccessibility was reduced. It is sufficient to ground the carob pod for 30 s, ensuring good availability of nutraceuticals and lower energy cost for grinding.

Keywords: phenolic; flavonoid; HPLC analysis; in vitro digestion; correlation

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1. Introduction

The carob pod is the fruit of *Ceratonia siliqua* L., a tree of the family Fabaceae. Carob pods serve as a valuable source of essential nutrients, including protein, essential fatty acids, and calcium [1]. It has a promising composition in relation to human health, containing various types of polyphenols (tannins, flavonoids, phenolic acids, etc.) and fibres [2,3]. In addition to the beneficial effect on metabolic health, carob cultivation in the Mediterranean area has a low carbon footprint, which is useful for achieving sustainable development goals [4]. Plant tissues are usually processed by drying to preserve the content of biologically active substances. Subsequently, the dried material is milled to a powder that can be used in various food formulations. As mentioned in a study by Issaoui et al. [5], the grinding of dried carob is easier compared to the grinding of wheat, resulting in low energy consumption. Their claim was supported by the development of a formula for manufacturing bread in which carob pod powder was used as a partial replacement for wheat flour. Since carob pod is naturally gluten-free, other products have been developed to reduce gluten content, such as muffins [6,7], pasta [8], and cookies [9]. In addition to

that, carob powder was added to chocolate [10] or Halva [11] to replace cocoa powder and obtain nutritionally more valuable confectionary products. In terms of the production of value-added food products, processes have been proposed to manufacture alcoholic beverages and carob-based drink powder [12,13]. Regarding the literature published in recent years and consumer demand for healthier food products, carob pod powder is at the forefront of the food industry.

Both drying and grinding techniques have an effect on particle size distribution, powder fluidity, colour, and hydration [14]. The influence of the particle size on the physicochemical properties of plant-based powders has been well documented [15–18]. However, in these studies, powder fractions with different particle size distributions were obtained by sieving. Several studies were conducted to elucidate the effect of grinding time on the functional properties of powder samples using a planetary ball mill [19–23]. Although particle size decreased with the increase in grinding time in all studies cited, the researchers concluded that some chemical properties of the powders were not proportional to the grinding time. For instance, onion peel powder had a maximum value of total phenolic content (TPC) after 18 h of milling, while a significant decrease was observed after 24 h [20]. Norhidayah et al. determined higher TPC values for the ground ginger rhizome after 2 and 4 h than those obtained after 6 or 8 h of grinding [21]. These findings indicate that a longer grinding time does not necessarily guarantee better powder properties. Therefore, it is advisable to find specific milling conditions for each particular material.

It is known that the decrease in particle size leads to an increase in the surface area of the particles, resulting in better extraction efficiency for various bioactive compounds [24–26]. However, the concept of chemical extraction alone is no longer sufficient, and the bioaccessibility of nutrients in relation to the structure of the food product must be examined [27]. A study of the bioaccessibility of phenolics from carob products has been published in only a few articles. Chait et al. [28] found that in vitro digestion affected the amount of free, bound, and conjugated phenolic compounds in carob powder. The three-step digestion process (oral, gastric, and intestinal) strongly reduced the phenolic content and antioxidant capacity of carob pulp [29]. In our recent work, the effect of cryogenic and vibratory grinding on the content of phenolic acids was studied [30]. It is unknown whether different grinding times affect the bioaccessibility of phenolic content and antioxidant potential using a vibratory grinder. We assume that the increase in grinding time positively affects carob powder's particle size distribution and moisture adsorption properties. Thus, a higher bioaccessibility of phenolics, flavonoids, and catechins is expected.

2. Results and Discussion

2.1. The Effect of Grinding Time on Particle Size, Colour, and Moisture Adsorption Properties of Carob Powder

Carob powder (CP) was prepared by vibratory grinding at various processing times (30, 60, 90, 120, and 180 s). In the text, these samples are referred to as CP30, CP60, CP90, CP120, and CP180, respectively. The physical parameters are shown in Table 1. The grinding time had a significant effect on the D_{50} values, decreasing significantly from 141.3 ± 2.5 to 87.9 ± 1.7 μm ($p < 0.001$) when ground for 30 and 60 s, respectively. A further increase in the grinding time resulted in a gradual increase in D_{50} values up to 135.1 ± 1.8 μm for CP180. A similar pattern was observed for the D_{10} values. However, the particle size in the upper percentile (D_{90}) showed a different behaviour: high D_{90} values of 527.7 ± 8.1 and 810.6 ± 21.3 μm were observed for CP60 and CP120, respectively. These findings also corresponded to high span values showing the lack of distribution uniformity (span 6.3 and 6.1, respectively). It was previously published that grinding time significantly influenced the particle size distribution of various food powders.

Table 1. Particle size (μm), water activity (a_w), and colour (CIEL*a*b*) of carob powder (CP) after vibratory grinding.

	Carob Samples					
	CP30	**CP60**	**CP90**	**CP120**	**CP180**	
D_{10}	17.8 ± 1.1^{b}	15.2 ± 0.5^{c}	16.3 ± 2.1^{bc}	21.4 ± 0.8^{a}	19.4 ± 0.3^{a}	**
D_{50}	141.3 ± 2.5^{a}	87.9 ± 1.7^{d}	110.1 ± 3.5^{c}	129.7 ± 2.9^{b}	135.1 ± 1.8^{b}	***
D_{90}	427.7 ± 12.5^{d}	572.7 ± 8.1^{b}	494.6 ± 7.9^{c}	810.6 ± 21.3^{a}	429.7 ± 5.6^{d}	**
Span	2.9	6.3	4.3	6.1	3.0	
a_w	0.380 ± 0.005^{d}	0.374 ± 0.002^{d}	0.403 ± 0.001^{c}	0.417 ± 0.001^{b}	0.433 ± 0.003^{a}	***
L*	47.1 ± 0.5^{d}	51.1 ± 0.4^{a}	49.4 ± 0.2^{b}	48.0 ± 0.3^{c}	47.0 ± 0.3^{d}	***
a*	6.3 ± 0.4^{bc}	5.8 ± 0.6^{c}	6.3 ± 0.3^{bc}	6.8 ± 0.4^{ab}	6.9 ± 0.2^{a}	***
b*	19.0 ± 0.5^{b}	17.9 ± 0.4^{c}	18.7 ± 0.3^{b}	20.1 ± 0.4^{a}	19.8 ± 0.3^{a}	***

Results are expressed as mean ± standard deviation (particle size N = 2; a_w N = 3; colour N = 5). Different letters in superscript indicate significant differences in a row according to Duncan's multiple pairwise test ($p < 0.05$). The significant effect of grinding time using ANOVA is marked as ** = $p < 0.01$, *** = $p < 0.001$. D_{10}, D_{90}, and D_{50} = lower decil, upper decil, and a median of the cumulative weight of the particle size distribution. CP30, CP60…CP180 represent carob powder ground for 30, 60…180 s, respectively.

Reduction in particle size was observed with the increase of ball milling time for onion peel powder [20], ginger rhizome powder [21], horseradish powder [22], or soybean protein isolate powder [23]. Longer grinding time resulted in an increase in the D_{90} value caused by the stickiness of the particles and the formation of agglomerates for horseradish powder [22]. The agglomeration process occurred during the entire grinding of dried mushrooms and was effectively disrupted by ultrasound treatment [19].

The grinding time of the carob pods significantly influenced all the values that describe the colour of the carob powder ($p < 0.01$). Lightness increased after 60 s of grinding (L* = 51.1 ± 0.4), then gradually decreased with the increase in processing time (Table 1). Carob powder ground for 120 s and 180 s had higher values of redness (a* = 6.8 ± 0.4 and 6.9 ± 0.2, respectively) and yellowness (b* = 20.1 ± 0.4 and 19.8 ± 0.3, respectively).

Different colours can be visually observed only for CP60 in our study. Correlation analysis revealed a strong association between the median particle size (D_{50}) and the colour in terms of L* ($r = -0.961$, $p < 0.001$), a* ($r = 0.634$, $p < 0.01$), and b* ($r = 0.764$, $p < 0.001$). Interestingly, all colour values exhibited an association with the water activity of the carob powder. Whereas the lightness showed a negative association with a_w ($r = -0.548$, $p < 0.05$), water activity positively correlated to a* ($r = 0.781$, $p < 0.01$) and b* values ($r = 0.795$, $p < 0.001$). The water activity of the carob powder was similar for CP30 and CP60, followed by its increase while increasing grinding time. It might seem that the smaller the particles, the lower the water activity, as can be seen in Table 1; however, the correlation coefficient indicates a weak and negligible association ($r = 0.464$, $p > 0.05$).

Colour changes have been observed during the grinding of flour [6], rose-myrtle powder [17], or black kidney bean powder [18]. Drakos et al. [16] found that the colour changes during grinding were product-specific, and different changes of a* and b* values were determined for rye flour and barley flour prepared by jet-milling. Various fractions of particle size of kidney bean powder exhibited a weak association with colour stimuli; for example, the values of b* decreased with the increase of particle size from 125 to 250 μm but increased significantly with a further reduction in particle diameter [18]. It should be noted that colour changes can also be affected by increasing temperature during milling. The colour of the carob powder was influenced by temperature treatment, including roasting [31] or spray-drying [32]. In this study, the temperature of the metal equipment after 30 and 180 s of grinding was 22–24 °C and 37–39 °C, respectively. According to the IUPAC classification, the shape of the moisture adsorption isotherms of all carob powder samples is type III [33], with an increase of the equilibrium moisture content (EMC) to ~25 mg/g in the range of 0–40% relative humidity (RH) followed by a steep increase to ~259–268 mg g^{-1} in the range of 40 to 80% RH. This is an obvious behaviour of high-sugar

food products [34]. The differences in the adsorption isotherm plots for the carob powder samples are barely visible; therefore, we compared the EMC for two levels of RH.

As can be seen in Figure 1, the EMC was significantly higher ($p < 0.05$) for the CP60 sample at 10% (7.1 mg g^{-1}) and 60% (79.3 mg g^{-1}) of RH compared to other grinding times. The EMC decreased with an increase in the grinding time from 60 to 180 s. These findings corresponded to the particle size distribution, where a strong association was found between D_{50} and EMC at 10% ($r = -0.883$, $p < 0.05$) and 60% ($r = -0.884$, $p < 0.05$) of RH. It was well documented that reducing the particle size distribution results in improved water adsorption measured by water holding capacity, i.e., the smaller the particles, the higher the surface area available for moisture uptake [15,24].

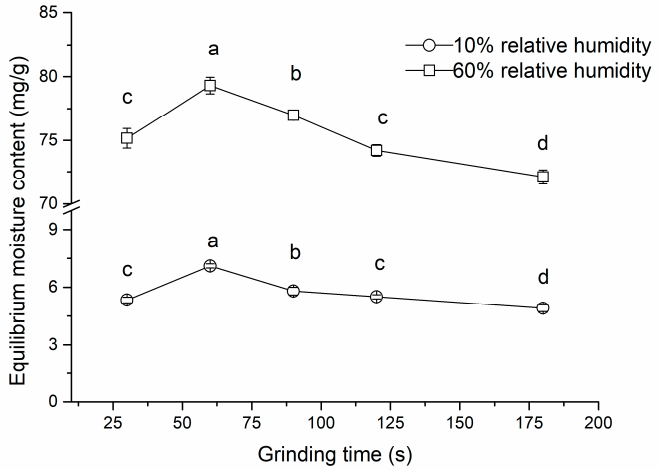

Figure 1. The effect of grinding time on the equilibrium moisture content of carob powder at two relative humidity (RH) levels. Mean ± standard deviation (N = 2). Different small letters indicate significant differences ($p < 0.05$) within the RH level.

2.2. Grinding Time as Affected Antioxidant Properties and Phenolic Content in Carob Powder

Twelve phenolic standards were involved in the HPLC analysis. Only 3 phenolic acids (vanillic, ferulic, and cinnamic), two flavons (luteolin and apigenin), and naringenin (flavanone) were identified in carob powder extract (Figure S1). The effect of grinding time on the phenolic content and antioxidant properties is presented in Table 2. ANOVA revealed that the grinding time had a significant effect on all variables ($p < 0.001$), excluding vanillic acid. Cinnamic acid was the most abundant phenolic constituent, with a maximum value of 54.28 ± 1.42 μg g^{-1} in the CP180 sample.

The carob powder ground for 180 s had a significantly higher content of all phenolics determined in this study compared to the CP120 sample. Total phenolic content (TPC) ranged from 4.73 ± 0.21 to 6.07 ± 0.15 GAE/(mg g^{-1}), with a maximum value for CP180. Total flavonoid content (TFC) was significantly higher for CP60 (0.33 ± 0.13 QUE/(mg g^{-1})) compared to CP30 ($p < 0.05$). The increase in grinding time from 90 to 120 s resulted in the same TFC values ($p > 0.05$). Although the catechin content (CC) appeared to increase with the increase in grinding time, the differences were not statistically significant ($p > 0.05$). DPPH values increased after 60 s of grinding to 11.91 ± 0.51 TEAC/(mg g^{-1}) ($p < 0.05$), then decreased significantly for CP90 ($p < 0.05$) followed by a further increase reaching its maximum value of 11.93 ± 0.74 TEAC/(mg g^{-1}) for CP180. The FRAP values followed the same pattern with the highest antioxidant capacity for the CP120 sample (16.95 ± 1.08 TEAC/(mg g^{-1})). As expected, positive associations were found between antioxidant properties in terms of the DPPH and FRAP assays and TPC, TFC, and catechin content ($r = 0.656$–0.836, $p < 0.05$). The particle size of chia seeds was positively associated

with the release of phenolic compounds during the extraction procedure [25]. On the contrary, some experiments did not confirm the correlation between particle size and phenolic content [26] or antioxidant capacity [16], probably due to different materials, grinding techniques, or particle size ranges. In our study, a weak negative association ($p > 0.05$) was observed between D_{50} and TPC ($r = -0.421$), ferulic acid ($r = -0.439$), and naringenin ($r = -0.460$) contents. The higher ability to eliminate the DPPH radical was significantly associated with lower D_{50} values ($r = -0.606$, $p < 0.05$). It suggests that small-sized carob powder can release more phenolics during extraction.

Table 2. Phenolic content and antioxidant properties of carob powder (CP) prepared by vibratory grinding at various processing times.

	Carob Samples					
	CP30	**CP60**	**CP90**	**CP120**	**CP180**	
$w/(\mu g\ g^{-1})$						
Vanillic acid	3.95 ± 0.40^{ab}	4.17 ± 0.40^{ab}	3.58 ± 0.43^{b}	4.08 ± 0.26^{ab}	4.41 ± 0.08^{a}	n.s.
Ferulic acid	8.64 ± 0.04^{d}	10.77 ± 0.22^{b}	10.85 ± 0.43^{b}	10.09 ± 0.51^{c}	11.28 ± 0.09^{ab}	***
Cinnamic acid	45.20 ± 1.20^{c}	50.30 ± 1.13^{b}	47.80 ± 1.64^{bc}	50.00 ± 2.15^{b}	54.28 ± 1.42^{a}	***
Luteolin	13.62 ± 1.82^{b}	16.11 ± 1.44^{b}	15.17 ± 1.74^{b}	16.97 ± 1.00^{b}	20.52 ± 0.57^{a}	**
Naringenin	2.76 ± 0.06^{c}	6.77 ± 0.25^{ab}	6.67 ± 0.44^{b}	7.28 ± 0.30^{a}	7.28 ± 0.13^{a}	***
Apigenin	1.29 ± 0.19^{d}	1.94 ± 0.05^{c}	2.17 ± 0.06^{ab}	1.97 ± 0.11^{bc}	2.27 ± 0.04^{a}	***
$w/(mg\ g^{-1})$						
TPC as GAE	4.73 ± 0.21^{d}	5.84 ± 3.98^{ab}	5.41 ± 2.19^{c}	5.72 ± 0.20^{b}	6.07 ± 0.15^{a}	***
TFC as QUE	0.19 ± 0.15^{b}	0.33 ± 0.13^{a}	0.27 ± 0.14^{a}	0.32 ± 0.07^{a}	0.39 ± 0.05^{a}	n.s.
CC as CAT	0.33 ± 0.13^{a}	0.36 ± 0.03^{a}	0.35 ± 0.12^{a}	0.44 ± 0.06^{a}	0.46 ± 0.19^{a}	n.s.
DPPH as TEAC	9.29 ± 3.16^{b}	11.91 ± 0.51^{a}	9.80 ± 0.53^{b}	9.59 ± 1.62^{b}	11.93 ± 0.74^{a}	*
FRAP as TEAC	13.78 ± 1.33^{c}	16.11 ± 1.80^{b}	13.12 ± 1.28^{c}	16.95 ± 1.08^{ab}	16.52 ± 0.65^{a}	***

All results were on a dry mass basis and expressed as mean ± standard deviation (N = 3). Different superscript letters indicate significant differences in a row according to Duncan's multiple pairwise test ($p < 0.05$). The significant effect of grinding time using ANOVA was marked as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant, TPC = total phenolic content, GAE = gallic acid equivalent, TFC = total flavonoid content, QUE = quercetin equivalent, CC = catechin content, CAT = catechin equivalent, DPPH = 1,1-diphenyl-2-picrylhydrazyl radical assay, FRAP = ferric reducing antioxidant capacity, TEAC = Trolox equivalent antioxidant capacity, CP30, CP60…CP180 represent carob powder ground for 30, 60…180 s, respectively.

2.3. The Effect of Grinding Time on the Bioaccessibility of Antioxidant Properties and Phenolic Content

The bioaccessibility of phenolic compounds from carob products was recently studied [28–30], but the effect of grinding time has not yet been determined. Chait et al. [28] observed an increase in free phenolic compounds, but the degradation of bound and conjugated compounds was determined after complete in vitro digestion. Pure phenolic acids and flavonoids were degraded during the oral, gastric, and intestinal digestion process, but the authors found that bioaccessibility depended on the product type [29]. For example, TPC retention was similar for carob powder, syrup, fibre, and extract. However, higher retention of DPPH was observed for carob powder than for carob extract. The digestion liquid was used to examine the effect of various grinding times on the release of phenolic substances from carob powder after a three-stage in vitro digestion process in our study. We assume that phenolics in digestion fluid are readily absorbed into the small intestine. As can be seen in Table 3, luteolin and apigenin were the flavonoids most affected by the digestion process of carob powder samples in this study. Luteolin content decreased by 12.5–19.5 $\mu g\ g^{-1}$, which is equivalent to 5–8% of its initial values. Apigenin content was reduced to 94–95%. Apigenin losses were similar to those observed in defatted lupin seeds during two-stage in vitro digestion [35]. Ferulic and cinnamic acid were the most stable during in vitro digestion showing 42–51% and 33–39% bioaccessibility. The grinding time strongly affected the content of vanillic acid ($p < 0.001$), cinnamic acid ($p < 0.001$), luteolin ($p < 0.01$), and apigenin ($p < 0.001$) after the three-stage digestion process, but

no trend can be identified. Although the vanillic acid content was the highest in CP30, the maximum content values for cinnamic acid, luteolin, and apigenin were observed in different CP samples. Particle size did not influence the phenolic content after in vitro digestion. When the bioaccessibility of substances in carob powder samples is compared, we get a clearer overview of their fate during digestion. Bioaccessibility was remarkably different in the CP30 and CP60 samples. For example, 23% and 14% vanillic acid was observed after the digestion of CP30 and CP60 samples, respectively. A further increase in the grinding time resulted in retention similar to that of the CP60 sample. The same pattern was observed for ferulic acid (decrease from 51% to 40%) and naringenin (decrease from 29 to 12%). It should be noted that the level of bioaccessibility is dependent on the experimental procedure applied to determine the initial content. The in vitro digestion process takes place in an aqueous environment, so the extraction of phenolics into a water solution is a better option [36,37]. However, water/organic solution mixtures were also used for the determination of the initial content of phenolic compounds [38,39], which were then used for the bioaccessibility calculation.

Table 3. Phenolic content and antioxidant properties of carob powder (CP) after in vitro digestion, and its bioaccessibility.

	Carob Samples					
	CP30	**CP60**	**CP90**	**CP120**	**CP180**	
w/(μg g^{-1})						
Vanillic acid	0.91 ± 0.01 ^{a} (23) †	0.57 ± 0.03 ^{b} (14)	0.56 ± 0.00 ^{b} (15)	0.55 ± 0.01 ^{b} (13)	0.67 ± 0.23 ^{b} (12)	***
Ferulic acid	4.45 ± 2.12 ^{ab} (51)	4.28 ± 0.17 ^{b} (40)	4.68 ± 0.28 ^{ab} (43)	4.65 ± 0.24 ^{ab} (46)	4.71 ± 0.18 ^{a} (42)	n.s.
Cinnamic acid	17.69 ± 0.37 ^{b} (39)	18.34 ± 0.38 ^{a} (36)	17.35 ± 0.35 ^{b} (36)	16.61 ± 0.28 ^{c} (33)	18.02 ± 0.14 ^{a} (33)	***
Luteolin	1.15 ± 0.03 ^{a} (8)	1.16 ± 0.04 ^{a} (7)	1.16 ± 0.03 ^{a} (8)	1.05 ± 0.04 ^{b} (6)	1.05 ± 0.07 ^{b} (5)	**
Naringenin	0.80 ± 0.02 ^{a} (29)	0.81 ± 0.02 ^{a} (12)	0.83 ± 0.01 ^{a} (12)	0.83 ± 0.03 ^{a} (11)	0.80 ± 0.03 ^{a} (11)	n.s.
Apigenin	0.08 ± 0.00 ^{c} (6)	0.09 ± 0.01 ^{c} (5)	0.12 ± 0.00 ^{a} (6)	0.10 ± 0.01 ^{b} (5)	0.10 ± 0.01 ^{b} (5)	***
w/(mg g^{-1})						
TPC as GAE	5.10 ± 0.10 ^{c} (109)	5.51 ± 0.27 ^{a} (94)	5.09 ± 0.12 ^{c} (94)	5.66 ± 0.33 ^{a} (99)	5.32 ± 0.29 ^{b} (88)	**
TFC as QUE	0.24 ± 0.02 ^{ab} (126)	0.28 ± 0.05 ^{a} (85)	0.20 ± 0.01 ^{b} (74)	0.23 ± 0.03 ^{b} (72)	0.19 ± 0.02 ^{b} (49)	*
CC as CAT	0.59 ± 0.23 ^{a} (148)	0.52 ± 0.18 ^{a} (141)	0.50 ± 0.13 ^{a} (143)	0.57 ± 0.18 ^{a} (129)	0.53 ± 0.16 ^{a} (116)	n.s.
DPPH as TEAC	10.24 ± 0.47 ^{b} (110)	12.17 ± 1.23 ^{a} (102)	11.21 ± 0.64 ^{ab} (114)	11.12 ± 1.16 ^{ab} (116)	11.09 ± 1.26 ^{ab} (93)	*
FRAP as TEAC	13.97 ± 0.26 (101) ^{c}	16.12 ± 1.43 ^{a} (100)	15.07 ± 0.47 ^{b} (115)	16.05 ± 0.69 ^{a} (95)	14.70 ± 0.31 ^{bc} (89)	***

† Bioaccessibility in % of the initial content (in brackets); different superscript letters indicate significant differences in a row according to Duncan's multiple pairwise test ($p < 0.05$); the significant effect of grinding time using ANOVA was marked as * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant. TPC = total phenolic content, GAE = gallic acid equivalent, TFC = total flavonoid content, QUE = quercetin equivalent, CC = catechin content, CAT = catechin equivalent, DPPH = 1,1-diphenyl-2-picrylhydrazyl radical assay, FRAP = ferric reducing antioxidant capacity, TEAC = Trolox equivalent antioxidant capacity, CP30, CP60…CP180 represent carob powder ground for 30, 60…180 s, respectively.

The total phenolic ($p < 0.01$) and flavonoid ($p < 0.05$) content of carob powder after in vitro digestion was affected by the grinding time. Although CP60 and CP120 have the highest TPC values (5.51 ± 0.27 and 5.66 ± 0.33 mg GAE g^{-1}, respectively), the highest TFC values were observed in the CP30 and CP60 samples. Catechin content was similar for all carob samples. Whereas bioaccessibility of phenolic individuals decreased after the digestion of carob powder ground for 30 s, meanwhile TPC, TFC, and CC increased to 109, 126, and 148%, respectively. This discrepancy can be explained by the subsequent release of other phenolic compounds after in vitro digestion process. An increase in total phenolic content and, at the same time, a decrease in some hydroxybenzoic acids after gastrointestinal digestion of soursop was reported [40]. Although a reduction in the content of vanillic acid, caffeic acid, and catechins in the *Matricaria recutita* flower was observed after duodenal digestion, the TPC value increased [41]. This was evidenced by increased rutin, quercitrin or quercetin content in their research. A further increase in grinding time resulted in a decrease in both TPC and TFC bioaccessibility, reaching their minimum level of 88% and 49% for the CP180 sample, respectively. The bioaccessibility of catechins also exhibited a gradual decrease with increased grinding time, but it was still improved in carob

powder ground for the longest time. Antioxidant properties of digestive liquid ranged from 10.24 ± 0.47 to 12.17 ± 1.23 mg Trolox g^{-1} using a DPPH assay and from 13.97 ± 0.26 to 16.12 ± 1.43 mg Trolox g^{-1} using a FRAP assay. The bioaccessible phenolic, flavonoid, and catechin contents were not affected by the particle size of carob powder. It is likely that the bioaccessibility of phenolics can be influenced by smaller particles than those used in our study. Li et al. [42] observed that the bioaccessible phenolic content and antiradical activity of wheat bran increased in the powder fraction smaller than 19.16 µm. Our findings suggest that carob powder ground for 30 s represents a good source of bioactive substances.

3. Materials and Methods

3.1. Chemicals

All enzymes, bile extract, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,4,6,-tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferric chloride, aluminium chloride, Folin-Ciocalteu reagent (2N), vanillin, gallic acid, quercetin, catechin, the analytical standards for UHPLC analysis (vanillic acid, ferulic acid, cinnamic acid, and naringenin, all with purity ≥ 97%), methanol Chromasolv®, and acetonitrile Chromasolv® were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin and apigenin standards were obtained from Thermo Fischer Scientific (Heysham, Lancashire, UK). Other inorganic salts, hydrochloric acid, and sodium hydroxide were analytical grade (Lach-Ner, Neratovice, Czech Republic).

3.2. Preparation of Carob Powder Sample

The dried carob pods were purchased in a store (Naturway, Czech Republic). A portion (42.0–43.0 g) was pulverised in a vibratory grinder BVM-2 (Brio, Hranice, Czech Republic) for 30, 60, 90, 120, and 180 s. The temperature of the metal parts of the grinder was checked with an infrared thermometer (830-T1, Testo, Titisee-Neustadt, Germany) to ensure the same initial grinding temperature. The temperature was measured in five random locations. The powder was prepared in duplicate, followed by mixing both batches. The carob powder was collected into plastic tubes and stored at room temperature. A portion (3.0 g) of powder was mixed with 10.0 mL of 90% (v/v) methanol and 30.0 µL of formic acid [30]. Extraction was supported by 20 min of sonication (RK106 ultrasound bath, Bandelin Electronics, Berlin, Germany) followed by centrifugation at 2440× g for 20 min (ST4R-Plus MD, Sorvall, Thermo Scientific, Waltham, MA, USA). The aliquots were stored at −25 °C until analysis.

3.3. In Vitro Digestion of Carob Powder

The static in vitro digestion process was adopted from the study by Swieca et al. [43], which comprises oral, gastric, and intestinal digestion stages. The in vitro digestion process has begun with a mixture of carob powder (3.0 g) and 7.0 mL of saliva juice [0.8% NaCl, 0.24% Na_2HPO_4, 0.02% KH_2PO_4, 4.0% α-amylase (≥10 U mg^{-1}), pH 6.75] under constant agitation (20/min) at 37 °C (BSK ET618, Lovibond, Amesbury, UK) for 10 min. The gastric phase was initiated by decreasing the pH to 1.2 (5.0 M HCl) and adding 5.0 mL of gastric juice [0.03 M HCl containing 0.012% pepsin (2500 U mg^{-1})]. After 120 min of agitation at 37 °C, the mixture was alkalised with 1.0 M NaOH to pH 7.0. The intestinal phase was started by adding 5.0 mL of intestinal digestion fluid [0.1 M $NaHCO_3$ containing 0.85% bile extract, 0.14% pancreatin (4 × USP)] and incubated at 37 °C for 120 min. The supernatant was obtained by centrifugation (2440× g, 15 min) and stored at −25 °C for further investigation. The same procedure was carried out without the presence of carob powder. The digestion experiment was carried out in triplicate.

The effect of the in vitro digestion process on the antioxidant properties and the content of phenolic substances was expressed as a percentage change compared to the methanolic extract.

3.4. Particle Size, Colour, Water Activity, and Moisture Sorption Determination

The particle size distribution of carob powder (CP) was measured by Morphologi 4 (Malvern Panalytical, Malvern, UK) by dispersing dry powder (3.0 mm^3) on a glass plate. Three magnifications (5×, 20×, 50×) were used for the measurement. The particle size was expressed as lower (D_{10}) and upper (D_{90}) decils and a median (D_{50}) of cumulative weight. It means that, for example, 10% of particles are smaller than the value of D_{10} (software Morphologi, v. 10.10). A span was calculated according to the equation:

$$\text{Span} = \frac{D_{0.9} - D_{0.1}}{D_{0.5}} \quad (1)$$

The span reflects the uniformity of the particle size distribution (value approaches 0 for an ideally uniform particle size distribution. The measurements of particle size were done in duplicate.

The colour was determined using a d/8° geometry spectrophotometer (HunterLab, Reston, VA, USA) and a small area view reflectance port (9.5 mm). A white tile was used as a standard. The colour was expressed as CIEL*a*b* where L* represents parameters of darkness (0)–lightness (100), a* red (+)–green (−) parameter, and b* yellow (+)–blue (−) parameter [44]. Five repetitions for each sample were performed.

The water activity of the powder samples was measured using the AquaLab TDL instrument (Meter Group, Pullman, WA, USA) at 25 °C immediately after grinding.

Moisture adsorption of powder was determined using a dynamic vapour sorption system (DVS Intrinsic Plus, Surface Measurement Systems, Wembley, UK) at 25 °C. A small amount of powder (15.0 mg) was distributed on an aluminium weighing plate and hung on the wire connected to the ultrasensitive analytical balance (± 0.1 µg) in a closed chamber. Before analysis, the sample was exposed to a dry airflow (200 ccm, moisture < 0.1%) for 4 h to obtain dry mass. Adsorption was carried out in relative humidity (RH) from 0 to 80% in 5% increments. Changes in the mass of the sample were monitored at 30 s intervals until the equilibrium was reached (<0.002% dm/dt for 20 min) for each RH level. The results were expressed as the equilibrium moisture content (EMC) on a dry mass basis (mg g^{-1}).

3.5. Antioxidant Properties of Carob Powder

Antioxidant activity was determined by absorbance changes in the methanolic solution of DPPH [45]. The DPPH assay was performed using 5.0 mL of DPPH methanolic solution (12.5 mg per 500 mL) and 300 µL of sample extract. After 10 min of incubation, a decrease in absorbance was observed at 517 nm (UV-2600, Shimadzu, Kyoto, Japan). The results were expressed as Trolox equivalent antioxidant capacity (TEAC) on a dry mass basis (mg g^{-1}. Ferric reducing antioxidant power (FRAP) assay was performed after the reaction of the sample extract with TPTZ solution and $FeCl_3$ in an acidic environment [46]. Briefly, the FRAP reagent mixture was prepared by mixing 20 mM $FeCl_3$, 10 mM of TPTZ (in 40 mM HCl), and 0.3 M acetic buffer solution (pH 3.6) in a respective ratio of 1:1:10. The results were expressed as TEAC (mg g^{-1}).

3.6. Phenolic Content in Carob Powder

3.6.1. Spectrophotometric Assays

Total phenolic content (TPC) was estimated using a mixture of sample extract (1.0 mL), 5.0 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent. After 5 min, 1.0 mL of 5% Na_2CO_3 was added, followed by 60 min incubation in a dark place. The increase in absorbance was monitored at 765 nm, and the results were expressed as gallic acid equivalent (GAE) per gram on a dry mass basis (mg g^{-1}) [6].

The determination of total flavonoid content (TFC) followed this procedure [47]: 2.0 mL of sample extract with 1.0 mL of 2.0% $AlCl_3$, distilled water, 1.0 M HCl and 1.0 M CH_3COONa. The absorbance (425 nm) was read after 10 min incubation at laboratory temperature in a dark place. Quercetin was used as the standard (QUE/(mg g^{-1})). For catechin content (CC), the reaction mixture consists of 1.0 mL of sample extract and 2.5 mL

of vanillin (4%) and sulfuric acid (both 25% methanolic solutions). Absorbance was read at 500 nm after 15 min of incubation. The result was expressed as catechin equivalent on a dry mass basis (CAT/(mg g^{-1})) [48].

3.6.2. HPLC Analysis of Phenolic Individuals

Analyses were performed using the Agilent 1290 Infinity liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) consisting of the degassing unit, high-pressure pump, autosampler, column thermostat, and diode array detector. Chromatographic column Kinetex XB-C18 100 Å (150 × 2.1 mm, particle size 1.7 µm) was chosen for separation. The conditions for the separation and quantification of target compounds were adopted from our previous study [30]. Briefly, the mobile phase was formed by water acidified with formic acid (pH ~ 3.1; solvent A) and acetonitrile (solvent B). Gradient elution (0 min—3% (B), 34 min—12% (B), 55 min—30% (B), 60–70 min—90% (B)) was applied. Conditions were set as follows; injection volume of 2 µL, mobile phase flow rate of 0.4 mL min^{-1}, a temperature of 40 °C, and detection wavelengths of 270, 290, and 320 nm according to the absorption maxima of separated compounds. Calibration standard solutions of phenolic acids (vanillic, ferulic, cinnamic, protocatechuic, caffeic, chlorogenic, syringic, p-coumaric) and flavonoids (rutin, luteolin, apigenin, naringenin) were prepared by dissolving analytical standards in 90% (*v*/*v*) methanol including 0.3% (*v*/*v*) of formic acid. Before analysis, all samples were filtered through a 0.22 µm PTFE syringe filter.

3.7. Statistical Analysis

The results were expressed as mean with standard deviation. The grinding time represents a factor in the 1-way analysis of variance (ANOVA) to determine its effect on the variables. Duncan's test was applied for multiple pairwise comparisons between means. The correlation coefficient (*r*) was calculated to find the association between the variables. All tests were performed at the probability level $p = 0.05$ using Statistica software (v. 12.0, StatSoft, Tulsa, OK, USA).

4. Conclusions

The carob powder prepared by vibratory grinding had different properties in relation to the grinding time. However, increasing the grinding time did not lead to a pronounced decrease in particle size. The carob powder contained small particles after 60 s of grinding; then, an increase was observed when ground for 180 s. It should be noted that large particles were also found in the carob powder ground for a longer time, probably because of their agglomeration. Moisture adsorption corresponded to the particle size. The colour changes of the carob powder were found to be important with respect to the grinding time, but small differences were observed. As expected, the extraction ability of phenolics to methanol/water solution has increased, particularly from carob powder ground for 180 s. In contrast, the bioaccessibility of the phenolic substances in that sample was low. This can be explained by the destruction of bioactive compounds in an alkaline environment during intestinal in vitro digestion. Based on the results, we have to admit that longer grinding time has a negative effect on the bioaccessibility of phenolics, particularly flavonoids. It is sufficient to grind the carob pod for 30 s, ensuring good availability of nutraceuticals and lower energy cost for grinding. Since the particle size did not significantly influence the bioaccessibility of the phenolics, the effect of temperature during grinding or the microstructure of the particles can be further studied.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27227689/s1, Figure S1: Chromatogram of vibratory ground carob powder (CP180) extract.

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Appendix IV.

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FUNCTIONAL PROPERTIES OF MUFFIN AS AFFECTED BY SUBSTITUING WHEAT FLOUR WITH CAROB POWDER

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ABSTRACT

Carob (*Cerationa soliqu*a L.) pod is the good source of dietary fiber, minerals and polyphenolic substances. The aim of this study was to prepare muffin where wheat flour was substituted with carob powder, and determine some physicochemical properties. Carob powder was prepared by milling dry carob pods to particles smaller than 600 µm. Then wheat flour in muffin dough was replaced by carob powder in 5, 10, 15 and 20% (w/w) and subsequently baked at 180 °C for 20 min. It was found that the height of the muffin fortified with carob powder decreased in comparison with that in control muffin sample. Although the height of muffins decreased with the increase in level of carob powder, the differences were not statistically significant. Weight loss was similar for all the muffin samples in this study. Moisture content of muffins with carob powder was significantly higher than that in control. Addition of carob powder had also effect on water activity of muffin. While 0.905 a_w was observed in control sample, significantly higher a_w values were determinated in fortified muffins (0.912 – 0.923 a_w). The antioxidant characteristics were determinated using spectrophotometric assays for total phenolics (TPC), total flavonoids (TFC), radical scavenging activities (DPPH, ABTS) and hydrogen peroxide scavenging (HPS). TPC values gradually increased with the increase in level of carob powder from 348.1 to 829.1 µg gallic acid.g^{-1} dry matter but TFC values significantly increased in muffin with 15 and 20% (w/w) of carob powder. All the antioxidant assays showed strong and positive association with the increase in level of carob powder. Addition of carob powder resulted in the increase of browning index and FAST index as a metrics of the formation of Maillard products.

Keywords: fortification; phenolics; antioxidant; browning

INTRODUCTION

Carob powder or flour is the product of the fruit of *Ceratonia siliqua L*. Carob powder is usually prepared from mature, dried carob pod (without seed) after milling to desired particle size. Carob powder is a good source of sucrose and other simple sugars (maltose, mannose), unsaturated fatty acids and minerals such as calcium, potassium and iron **(Ayaz et al., 2009)**. High content of dietary fibre, both soluble and non-soluble, is the most important parameter, which makes the carob powder applicable in various food products such as bread **(Durazzo et al., 2014)** and cookies **(Roman et al., 2017)**. Carob powder is also used as a replacer of cocoa in cocoa and chocolate-based products decreasing the content of caffeine and theobromine but keeps the cocoa-like aroma, particularly when roasting **(Loullis and Pinakoulaki, 2018)**. In addition, carob powder is the rich source of polyphenolic substances exhibited promising pharmacological actions such as antioxidant, antibacterial, anti-inflammatory and anti-diabetic activities **(Rtibi et al., 2017)**.

In a recent work of **Pawlowska et al. (2018)**, the effect of substitution of cocoa powder for carob powder at 5% (w/w) level in muffin dough was examined. Improved antiradical activity and higher content of phytosterols have been observed in their study. However, their research was focused on substitution of cocoa powder at a single level. Replacing wheat flour for carob flour/powder may lead to both cocoa-like aroma and gluten-free products **(Roman et al., 2017; Lauková, Kohajdová and Karovičová, 2016)**.

Scientific hypothesis

Increasing content of polyphenolic substances and increasing antioxidant status of muffins prepared by partial substitution of wheat flour for carob powder are expected.

MATERIAL AND METHODOLOGY

All the solvents (methanol, ethanol and acetone) and acids (sulphuric and acetic acids) were purchased from Lach-Ner s. r. o. (Neratovice, Czechia). Folin-Ciocalteau reagent solution, gallic acid, quercetin hydrate, (+)-catechin, vanillin, aluminium chloride, 2,2-diphenyl-1-

picrylhydrazyl (DPPH), 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), potassium persulphate, hydrogen peroxide, ferrous ammonium sulphate, 1,10-phenanthroline and (±)-6-hydroxy-2,5,7,8-ramethylchromane-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA). All the chemicals were of analytical grade.

Sample preparation and baking procedure

Dry carob pods (*Ceratonia silique* L.) without seed were purchased in local supplier. Pods were milled using knife mill Grindomix GM 200 (Retsch®, Haan, Germany) to obtain powder. Particles smaller than 600 μm were separated by passing through analytical sieve and used for subsequent experiments. Carob powder was stored in evacuated plastic bag at room temperature until used.

Muffin formulation dough was adopted from the study of **Ambigaipalan and Shahidi (2015)** The control sample (without carob powder) contains 65 g of wheat flour and 37.5 g of sugar (Castello, Lidl Stiftung, Germany), 3.0 g of baking powder (Dr. Oetker, Bielefeld, Germany), 0.62 g of salt, 60.0 mL of 2.5% fat milk (Pilos, Louny, Czechia), 20.0 mL of canola oil (Promienna, Lidl Stiftung, Germany) and 1 egg (large). A portion of wheat flour was substituted for carob powder at 5, 10, 15 and 20% (w/w) levels to obtain fortified muffin. When carob powder content had been 25% and 30% (w/w), unacceptable taste was observed by three panelists. A dough was prepared by mixing all the ingredients, a batter (30 g) was transferred into muffin paper cups and baked in pre-heated oven at 180 °C for 20 min. Muffins were baked in quadruplicate, cool to room temperature and moisture content, water activity, weight and height loss were immediately determined. Thereafter, muffins were mixed and stored in evacuated plastic bag at -20 °C.

General chemical and physical analysis

Moisture content of carob powder samples and muffins was determined in moisture analyser (Kern MLB 50-3, Kern & Sohn GmbH, Balingen, Germany) at 103 °C to a constant weight. Water activity was measured at 25 °C in Aw Sprint TH 500 (Novasina AG, Lachen, Switzerland). Weight loss was calculated from the weight difference between dough and muffin using balance (sensitivity 0.001 g). Height loss was measured using digital caliper (sensitivity 0.01 mm) in three random positions. For measurement of those parameters, muffin samples were cooled down after baking for 1 h at ambient temperature.

Preparation of extracts

Aqueous-organic extracts of muffin or carob powder were prepared according to **Durazzo et al. (2014)** with slight modification. About 3 – 4 g of muffin or carob powder was placed in plastic tube with 20 mL of methanol/water (1:1, v/v). The tubes were placed in vertical shaker for 60 min followed by centrifugation at 5000 rpm for 10 min. The supernatant was removed and 20 mL of acetone/water (70:30, v/v) was added to pellet. After shaking for another 60 min, both methanolic and acetonic extracts were combined, centrifuged (5000 rpm, 5 min) and stored at -20 °C.

Determination of phenolics

Total phenolic content (TPC) was determined using Folin-Ciocalteau procedure as was provided in our previous study **(Brožková et al., 2018)**. The methanol/acetone extract (1.0 mL) was mixed with 0.5 mL of Folin-Ciocalteau reagent and 5.0 mL of distilled water followed by addition of 1.0 mL of 5% Na_2CO_3. After 60 min of incubation in a dark cabinet, the absorbance was measured at 765 nm. The same volume of distilled water was added instead of sample extract to obtain control measurement. The results were expressed as gallic acid equivalent (mg GA.g^{-1}) of dried matter (DM).

Total flavonoid content (TFC) was measured after formation of flavonoids-$AlCl_3$ complex in acetate solution followed by recording absorbance at 415 nm **(Brožková et al., 2018)**. The results were expressed as quercetin equivalent (mg QE.g^{-1} DM).

Determination of antioxidant capacity

Three various methods were used to assess antioxidant capacity of carob powder and muffin. Two assays are based on both electron and hydrogen atom transfer mechanisms involving the reaction of antioxidants with stable chromogen radical DPPH or ABTS. The procedures were adopted from our previous study **(Brožková et al., 2018)**. Briefly, 5.0 mL of DPPH methanolic solution (25 mg.L^{-1}) was mixed with 0.5 mL of methanol/acetone extract and the decrease of absorbance was observed after 35 min at 517 nm. ABTS radical cation (ABTS•+) was prepared from ABTS solution (5.0 mL, 50 mg.L^{-1}) and 100 μL of potassium persulphate (64 mmol.L^{-1}). The mixture was stored in a dark for 12 – 16 hours at laboratory temperature before use. Diluted ABTS•+ solution (3.0 mL) was mixed with 0.5 mL of methanol/acetone extract and the decrease of absorbance was recorded at 734 nm after 50 min of incubation. For both DPPH and ABTS assays, the results were expressed as Trolox equivalent antioxidant capacity (TEAC) in μg Trolox.g^{-1} DM.

Hydrogen peroxide scavenging (HPS) assay represents the method describing the effect of antioxidant against reactive oxygen species **(Mukhopadhyay et al., 2016)**. Extract (0.5 mL) was mixed with 0.25 mL of 1.0 mM ferrous ammonium sulphate and 62.5 uL of 5.0 mM H_2O_2. After 5.0 min of incubation in a dark cabinet at laboratory temperature, 1.5 mL of 1.0 mM 1,10-phenanthroline was added and subsequently incubated for 10 min. The absorbance was red at 510 nm against blank. Distilled water was added to control sample instead of extract and H_2O_2 (total volume 1.562 mL). The increase in HPS activity was reflected by the decrease of absorbance. The % of H_2O_2 scavenging ability was calculated as $(A_{test}/A_{blank}).100$, where A_{test} is the absorbance of the solution containing sample extract, ferrous ammonium sulphate, H_2O_2, 1,10-phenanthroline and A_{blank} is the absorbance of solution containing only ferrous ammonium sulphate and 1,10-phenanthroline.

Determination of Maillard products

The reaction of reducing sugars and tryptophan during roasting of carob powder and baking of muffin may result in formation of advanced Maillard products. Those

products were determinated according to the FAST (Fluorescence of Advanced Maillard products and Soluble Tryptophan) method of **Birlouez-Aragon et al. (2001)**. Briefly, sample extract was prepared by sonication (30 min) of 1.0 g of sample in 40 mL 0.1 M borate buffer solution (pH 8.2). The extract was filtered through filter paper (Whatmann no. 2) and fluorescence of advanced Maillard products (F_{amp}) was recorded at excitation and emission wavelengths of 353 nm and 438 nm, respectively. The decrease of tryptophan amount (F_{trp}) was monitored at excitation and emission wavelengths of 353 nm and 438 nm, respectively. Acryl cuvettes and fluorimeter Fluorat® 02 Panorama (Lumex Instruments, Mission, Canada) were used. FAST index was calculated as (F_{amp}/F_{trp}).100 and the results were expressed in %.

The formation of brown pigment resulted in Maillard reaction was measured in 80% of ethanol at 420 nm against distilled water according to **Krishnan et al. (2010)**. The results were expressed as browning index (BI) in absorbance unit (A_{420}). UV/VIS Spectrophotometer DU 530 (Beckman Coulter Inc., Brea, CA, USA) was used for all colorimetric measurements.

Statistic analysis

The results were expressed as the average means with standard deviations of 4 replicates (n = 4). Non-parametric statistical tests were used throughout this study. *i.e.* Tukey´s multiply comparison method was to find differences between means. Association between carob powder levels and selected variables were determined using Spearman´s correlation coefficient (r). Statistical treatment was performed on the probability level of p = 0.05 (Statistica CZ, StatSoft CR s. r.o., Prague, Czechia).

RESULTS AND DISCUSSION

The main composition of carob powder was as followed (in g.100g^{-1} DM): crude protein (7.37 ±0.80), crude fat (0.43 ±0.05), reducing sugar (13.12 ±0.4), crude fiber (26.30 ±0.25), ash (3.29 ±0.22) and moisture (7.20 ±2.00), similarly to those found by **Mohamed, Hamed and Al-Okbi (2008)** for carob pods. Concerning fibre content, lower amount was found in our study (~26%) in comparison with carob powder dried in microwave oven (~50%) **(Tounsi et al., 2017)**. Those differences may be attributed to the different methods, which were used for fibre determination (enzymatic or chemical) or it may be caused by the variability in processing technology and particle size of powder **(Benković et al., 2017)**. Nevertheless, the crude fibre content found in this study fits to the range of 7.6 – 38% previously presented in a review article of **Loullis and Pinakoulaki (2018)** for carob pulp composition.

Weight loss and height of muffins fortified with carob powder

The height and weight loss of the control sample was 3.57 cm and 5.69 g, respectively. The general decrease in height was presented in Table 1. As can be seen, the addition of carob powder caused the reduction of height in comparison with the control. The height of muffin fortified with carob powder significantly decreased from 3.38 to 3.03 cm (p <0.05) with the increase of carob powder level. The decrease in height may be attributed to the dilution of gluten and disruption of gluten network as was previously described by **Lauková, Kohajdová and Karovičová (2016)**, who observed the decrease in diameter and volume of biscuits enriched with apple powder. Similarly, addition of chicory syrup to muffin dough significantly reduced the loaf volume of the products **(Zacharová et al., 2018)**. Incorporating of dietary fibre into dough formulation also exhibited reduction in volume and height of muffin fortified with red capsicum pomace powder **(Nath et al., 2018)**. Although the content of crude fibre in fortified muffins was not evaluated in this study, the content of carob powder with 25% of crude fibre should not be omitted and it requires further investigation. Weight losses of fortified muffins were not statistically significant for all the carob powder samples in comparison with the control muffin sample in the present study.

Moisture content and water activity of muffins fortified with carob powder

Weight loss occurs usually due to the evaporation of water during baking and reflects the changes in moisture content. As can be seen from Table 1, moisture content of muffin samples with carob powder (except of muffin with 15% (w/w)) was significantly higher than this in control. Some authors also observed small but significant increase in moisture content of bread fortified with 10% and 20% of carob flour **(Salinas et al., 2015)**. In a study of **Karaca, Saydam and Guven (2012)**, addition of carob molasses in yogurt significantly increased water-holding capacity of samples. The addition of carob powder to muffin formulation significantly increased water activity from 0.905 (control) to 0.912 – 0.923 a_w for muffins with carob powder (Table 2). Despite those findings, a_w values still fell within the safe water activity range.

Table 1 Weight loss, height, moisture content and water activity of muffin with different levels of carob powder.

Level (%, w/w)	Height (mm ±SD)	Weight loss (g ±SD)	Moisture content (g.100 g^{-1} ±SD)	Water activity
control	3.57 ±0.1^{d}	5.69 ±0.17^{a}	30.19 ±0.84^{a}	0.905 ±0.001^{a}
5	3.38 ±0.11^{bd}	5.71 ±0.18^{a}	31.52 ±0.55^{b}	0.914 ±0.002^{b}
10	3.23 ±0.16^{c}	5.62 ±0.15^{a}	32.41 ±0.19^{b}	0.923 ±0.001^{c}
15	3.22 ±0.05^{c}	5.51 ±0.14^{a}	29.76 ±0.43^{a}	0.918 ±0.001^{b}
20	3.03 ±0.13^{c}	5.58 ±0.16^{a}	31.12 ±0.41^{b}	0.912 ±0.002^{b}

Note: Average mean ± standard deviation (n = 4); significant difference between means in column is indicated by different small letters (p <0.05).

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Table 2 Antioxidant properties of muffin with different levels of carob powder.

Level (%, w/w)	TPC	TFC	$TEAC_{DPPH}$	$TEAC_{ABTS}$	HPS
control	193.2 ±18.1[a]	35.7 ±7.1[a]	ND	198.2 ±4.4[a]	5.5 ±0.4[a]
5	348.1 ±6.9[b]	49.6 ±3.3[a]	222.9 ±6.1[a]	1048.8 ±58.5[b]	19.8 ±0.3[b]
10	435.7 ±4.9[c]	49.1 ±2.0[a]	393.5 ±8.6[c]	1683.4 ±18.7[c]	30.6 ±0.2[c]
15	670.0 ±1.2[d]	73.0 ±7.8[b]	1099.5 ±23.9[e]	3137.5 ±4.9[d]	45.8 ±0.2[d]
20	829.1 ±8.8[e]	81.7 ±1.7[b]	1228.3 ±65.6[e]	3599.4 ±21.7[e]	74.6 ±0.2[e]

Note: TPC, Total phenolics content (μg gallic acid.g^{-1} DM); TFC, total flavonoids content (μg quercetin.g^{-1} DM); TEAC, Trolox equivalent antioxidant capacity (μg Trolox.g^{-1} DM); HPS hydrogen peroxide scavenging (%); significant difference between means in column is indicated by different small letters (p <0.05).

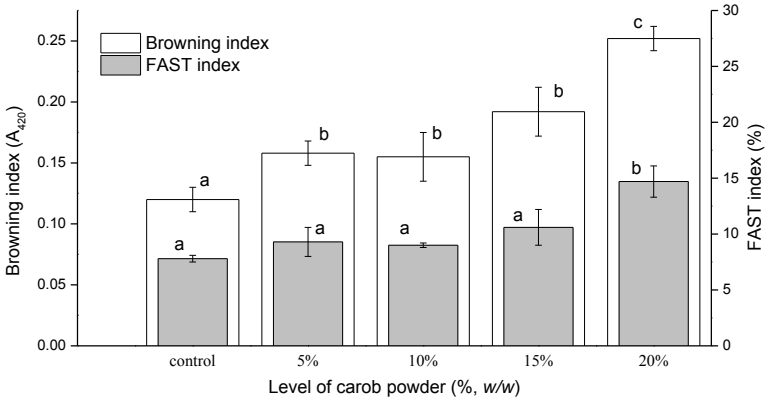

Figure 1 The effect of substitution of carob powder for wheat flour on browning index (white bars) and FAST index (gray bars) in muffin. Average mean ±standard deviation (n = 4).

Total phenolic, total flavonoid and condensed tannin contents in muffins fortified with carob powder

Total phenolic content of fortified muffins significantly increased in comparison with that in control (Table 2). While 193.2 μg GAE.g^{-1} DM was found in control sample, the substitution of wheat flour for carob powder resulted in gradual increase in TPC values from 348.1 to 829.1 μg GAE.g^{-1} DM with the increase of carob powder levels from 5% to 20% (w/w) (p <0.05). **Seczyk, Swieca and Gawlik-Dziki (2016)** also demonstrated that increase addition of carob flour to pasta resulted in the increase of total phenolic content. TFC values were significantly higher than in control (35.7 μg QE.g^{-1} DM; p <0.001) for all fortified muffins without regard of carob powder level. However, the increase in TFC values was not proportional to addition of carob powder. The addition of 5% and 10% (w/w) of carob powder led to the similar TFC values 49.6 – 49.1 μg QE.g^{-1} DM and to 73.1 – 81.7 μg QE.g^{-1} DM when 15% and 20% (w/w) of powders were added.

Antioxidant capacity of muffins fortified with carob powder

Antioxidant characteristics are presented in Table 3. While control muffin sample did not exhibit antioxidant capacity in terms of DPPH assay, the addition of carob powder at 5% (w/w) level to muffin recipe resulted in sharp increase to 222.9 μg Trolox.g^{-1} DM. Then, $TEAC_{DPPH}$ values increased with the increase in carob powder level. The highest $TEAC_{DPPH}$ values were obtained for muffin with 15% and 20% (w/w); *i.e.* 1099.5 and 1228.3 μg Trolox.g^{-1} DM (p <0.01), respectively. Antioxidant capacity measured by ABTS assay is based on similar principle as DPPH assay. Both radicals are able to accept hydrogen atoms or electrons provided by phenolic compounds in the extract. ABTS assay gave higher TEAC values than those in DPPH assay, probably due to the steric hindrance or different solubility of active substances in solutions, which were used for the preparation of stable radicals **(Apak et al, 2016)**. As can be seen from Table 3, ABTS assay always exhibited higher TEAC values at the same level of carob powder in comparison with those determined using DPPH assay (pairwise comparison test was not provided) in current study. It is known that each phenolic constituent contributes in different manner to total antioxidant capacity. We have found that rutin exhibited pro-oxidative effect during drying of buckwheat-based products **(Brožková et al., 2018)** and the same pattern was observed for quercetin, rutin or chlorogenic acid during processing of tomato paste **(Jacob et al., 2010)**. The addition of carob powder significantly increased the ability to scavenge H_2O_2 from initial 5.5% (control) to 19.8% followed by further increase with the increase of carob powder level (Table 3).

Table 3 The correlation analysis of increasing carob powder level *vs.* selected variables in muffin.

Parameters	*r*
Height	-0.756**
Weight loss	-0.006
Moisture content	-0.454
Water activity	-0.486
TPC	0.974***
TFC	0.824**
$TEAC_{DPPH}$	0.971***
$TEAC_{ABTS}$	0.971***
HPS	0.972***
Browning index	0.892***
FAST index	0.692**

Note: TPC, total phenolic content; TFC, total flavonoid content; TEAC, trolox equivalent antioxidant capacity; HPS hydrogen peroxide scavenging; * p <0.05, ** p <0.01, *** p <0.001.

Maillard reaction products in muffins fortified with carob powder

The higher content of reducing sugars and specific amino acids in food matrix may lead to the formation of various Maillard reaction products, which can be measured, in general, at specific wavelength or determination of compounds can be provided using HPLC techniques. Since carob powder is rich in reducing sugars (fructose, glucose) and reactive amino acids such as lysin or proline **(Ayaz et al., 2009; Benkovic et al., 2017)**, the formation of Maillard reaction products should be under control. With the increase in roasting temperatures and roasting times (130 – 150 °C for 5 – 30 min), the coloured Maillard reaction products, fluorescent compounds and hydroxymethylfurfural significantly increased in carob powder as was described in a study of **Cepo et al. (2014)**. The browning and FAST indices were determined in the present study. Addition of carob powder resulted in significantly higher BI when compared with the control muffin sample (p <0.05). BI values increased with the increase of the amount of carob powder up to 0.252 (A_{420}) for muffin with 20 % of carob powder (Figure 1). Surprisingly, FAST index did not differ in muffins fortified with carob powder from that found in control muffin sample (p >0.05) except in muffin with 20% level of carob powder, where significantly higher value was found (p <0.01).

The results of correlation analysis

The correlation analysis revealed that addition of carob powder instead of wheat flour to muffin recipe in the range from 5% to 20% (w/w) did not have effect on the weight loss, moisture content and water activity (Table 3). Strong and positive correlation coefficients ranged from 0.824 to 0.974 for antioxidant properties in terms of TPC, TFC, both TEAC values and hydrogen peroxide scavenging assay were obtained. While browning index showed strong positive correlation with the increase of carob powder level (r = 0.892, p <0.001), FAST index exhibited weak but significant association with the carob powder level (r = 0.692, p <0.01). However, this association is rather statistical than practical. It was evident from multiply comparison (see Figure 1) that FAST index values were not statistically different for control sample and muffins with 5 – 15% (w/w) of carob powder.

CONCLUSION

The additions of carob powder significantly affect the height of the muffins but not weight. Significant differences in moisture content of muffin samples were observed but it was not proportional to the level of carob powder. The addition of carob powder significantly increased water acitivity of muffins in comparison with the control sample. It was found that TPC gradually increased with the increase level of carob powder while TFC significantly increased in muffins fortified with higher levels of carob powder. Antioxidant capacity has increased with the increase level of carob powder in terms of DPPH, ABTS, and hydrogen peroxide scavenging assays. On the other hand, the addition of carob powder was associated with the formation of Maillard products. Browning index was significantly higher for muffin with the lowest carob powder level than that in control sample, but FAST index was significantly higher in muffin with the highest level of carob powder.

The results of this study showed that the replacement of wheat flour with carob powder increased the antioxidant status of muffins, however the higher the level of carob powder, the higher the potential to the formation of Maillard products.

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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 [195, 196]. 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 [195–197]. In the past, black chokeberry was used by the Potawatomi Native Americans as a treatment for cough, cold, and fever [198].

3.1 Typical content of black chokeberry fruit

Chokeberry phytochemical profile strongly depends on cultivar, climate, berries maturation, or harvesting time. **Phenolic content** is usually ranging from 2000 up to 8000 mg per 100 g of DM. Among the many berries, fruit of AM contents one of the highest amounts of phenolic compounds (see **Tab. 2**) and total anthocyanins (up to 1480 mg per 100 g of FW) [195–199].

Tab. 2: Comparison of phenolic content in different berries (edited) [199].

BERRIES AND THEIR PRODUCTS	PHENOLIC CONTENT
Aronia (*Aronia melanocarpa*) berries	2080 mg per 100 g of FW
Blackberries (*Rubus fruticosus*)	248 mg per 100 g of FW
Blueberries (*Vaccinium corymbosum*)	525 mg per 100 g of FW
Black currant (*Ribes nigrum*)	560 mg per 100 g of FW
Cranberries (*Vaccinium macrocarpon*)	120–315 mg per 100 g of FW
Red wines	1000–4000 mg per litre
White wines	about 250 mg per litre
Bilberries (*Vaccinium myrtillus*)	181–585 mg per 100 g of FW
Raspberries (*Rubus idaeus*)	126 mg per 100 g of FW
Strawberries (*Fragaria annassa*)	225 mg per 100 g of FW

FW = weight of fresh fruit.

As for anthocyanins, the most represented are cyanidin glycosides (namely cyanidin-3-glukoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, and cyanidin-3-xyloside), with cyanidin-3-galactoside being dominant phenolic substance found in black chokeberry [198]. Another significant group of phenolics present in AM fruits are condensed tannins, specifically procyanidins, where (–)-epicatechin figures as the main subunit in homogenous polymer structures. The values determined for the total procyanidin content range from 4000 to 5200 mg per 100 g of DM [197, 200]. The most common **sugars** present in AM are glucose and fructose, sum of them is determined to be between 13.0–17.6 g per 100 g of chokeberry FW. Sorbitol, a sugar alcohol used primarily as a sugar substitute in diet foods, is also represented relatively abundantly. Total content of reducing sugars in fresh berries is ranging from 16 to 18% [200, 201]. In case of **vitamins**, presence of ascorbic acid (14 mg per 100 g of FW), thiamine, riboflavin, pyridoxin, folate, vitamin K (all ranging from 18 to 28 μg per 100 g of FW), niacin, pantothenic acid (both about 300 μg per 100 g of FW), and tocopherols (2 μg per 100 g of FW) was confirmed. Besides vitamins, *β*-carotene was also quantified in high amounts [195, 200, 201]. Occurrence of **organic acids** in chokeberry, mainly quinic acid (293–591 mg of FW) and malic acid (308–350 mg per 100 g of FW), leads to its sour flavour [197]. The **mineral** that is contained in AM in the greatest quantity is potassium (218–498 mg per 100 g of FW), followed by calcium (32–117 mg per 100 g of FW), phosphorus, and magnesium [195, 200]. **Dietary fiber** in chokeberry pomace is within range 68–78% of DM, and overall value of fiber content in fruit reaches 5.6 g per 100 g of FW, with relatively low levels of pectin [195, 200]. AM berries **fat content** was analysed as very low, only 0.14 g per 100 g of FW [197].

3.2 Effect of pre-treatment on black chokeberry properties

In study of Cebulak and his colleagues [202], *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%) [202].

Positive effect of ultrasound on phenolics released from chokeberry matrix was also observed in our study (**Appendix V.**), 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 Appendix V.**).

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 Appendix V.**).

In study published by Bae and colleagues [203], 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. However, the results obtained after application of these conditions were still approximately two times lower compared to freeze-dried chokeberry powder without osmotic dehydration treatment. Also, application of freeze-drying resulted in higher phenolic content of final aronia products compared to hot-air drying [203]. Aleksandrov et al. [204] 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).

Kim and Mai [205] investigated an influence of dry and moist heat treatment on total phenolics and anthocyanins levels as well as on antioxidant activity of black chokeberry. Heating in water (20 min) caused increase of bioactive compounds and antioxidant activity with increasing temperature, nevertheless, non-significant loss of AM powder antioxidant activity for dry heat treatment was detected up to 160 °C and same heating duration [205]. In paper of Sadowska et al. [206], comparison of vacuum drying, freeze-drying, convection drying, and innovative method of fluidised bed jet drying was studied. The highest values of total anthocyanin content, total phenolic content, and antioxidant activity were obtained for chokeberry powders after freeze-drying and fluidised bed jet drying. Besides phenolics, also vitamin C level in AM powder was decreasing when convection drying and vacuum drying were carried out (loss of 24% and 59%, respectively) [206]. As can be seen, low stability of AM phenolics and vitamins

during drying treatment results in very significant loss when chokeberry powder is used to fortify bakery products requiring a higher temperature and longer baking time [205, 206].

3.3 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 [207, 208], cakes [209, 210, 211], shortcrust pastries [212], extruded corn porridge enriched with AM [213], milk, kefir and yoghurts supplemented with chokeberry powder or juice [214, 215], ice cream [216], AM-based functional beverages [217], juices and nectars [201, 218], tea [219], AM infused beer [220], chokeberry wine [221], jelly candies where synthetic dye was substituted by AM extract [222], or agar gel with the addition of AM juice concentrate which can be used to prepare many food products (also is suitable for vegetarians) [196]. In study of Tamkutė et al. [223], an inhibition of selected food pathogenic and spoilage bacteria (e.g., *Listeria monocytogenes*, *Pseudomonas putida*, or aerobic mesophilic bacteria), when extract of chokeberry pomace is added into processed pork products (cooked ham and pork meat burger), is described.

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 of total phenolic content in 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 osmo-dehydration 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.

Appendix for Chapter 3

Appendix V.

Frühbauerová, M.; Červenka, L.; Kosiorowska, A.; Królikowska, K.; Pająk, P. Colour, moisture adsorption, and antioxidant properties of oven-dried chokeberry powder obtained after ultrasound-assisted osmotic dehydration. *Scientific Papers of the University of Pardubice: Series A*, 2021, **27**, 21–32.

Appendix V.

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Colour, moisture adsorption, and antioxidant properties of oven-dried chokeberry powder obtained after ultrasound-assisted osmotic dehydration

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*Osmotic dehydration (OD) of chokeberry samples in erythritol (ERT) and xylitol (XYL) solutions enhanced by ultrasonication (US) has been examined in terms of moisture adsorption, colour and antioxidant properties. After air-forced drying, the powder subjected to OD in ERT solution exhibited higher equilibrium moisture contents (EMC) in 0.20–0.45 a_w than those in XYL solution. On the other hand, EMC values increased with the prolongation of US time from 5 to 30 min in the case of XYL solution. CIEL*a*b colour system was used for the determination of colour changes. While L* (the colour coordinate represents lightness (L* ~ 100) or darkness (L* ~ 0) of the sample) values decreased with the prolongation of US time from 5 to 30 min for both osmotic agents, only XYL solution caused the increase of a* (the colour coordinate represents green (−a*) or red (+a*) colour of the sample) and b* (the colour coordinate represents blue (−b*) or yellow (+b*) colour of the sample) to their maximum values at 30 min of sonication. A sample of powder subjected to OD in ERT solution has shown a higher total phenolic, total anthocyanin and antioxidant capacity. We may conclude that OD of chokeberries coupled with 30 min of sonication has resulted in chokeberry powder with the highest content of bioactive substances.*

Keywords: Aronia; Equilibrium moisture content; Sweetener; ANOVA

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Introduction

Black chokeberry (*Aronia melanocarpa* L.) is a perennial shrub of the *Rosaceae* family. The fruits are edible possessing many health-promoting effects on cardiovascular diseases, hyperlipidemia, hypertension, and diabetes [1]. Choke-berry fruits are an excellent source of various bioactive compounds including anthocyanins, flavonols, flavanols, proanthocyanidins, and phenolic acids [2].

To prepare the powder with extension stability, a few drying techniques have been examined. It was found that antioxidant activity, phenolic and anthocyanin contents decreased with the increasing of drying temperature from 50 °C to 70 °C [3]. Freeze-drying or spray drying appeared to be excellent in the retention of bioactive substances when compared with convective drying [4,5]. Osmotic dehydration (OD) is a nonthermal process, which allows water removal from plant tissue, and conversely, an impregnation of the tissue with the solutes presented in the osmotic solution. OD is usually applied as a pretreatment step prior to further drying process. Mass exchange occurs during the OD process, which changes the chemical composition of the dehydrated food. The water content decreases, dry matter increases, accompanied by the leakage of low-molecular compounds [6]. The application of ultrasound technology in the OD of plant tissues may cause both, water loss and water gain, with respect to the ultrasound parameters (sonication time, amplitude and ultrasound power) and products [7]. Ultrasound-assisted OD pretreatment was applied to various plant tissues, where both loss and gain of bioactive compounds have been observed together with the change of moisture sorption behaviour and colour [8–18]. There is scarce data available on the effect of sonication during osmotic dehydration of chokeberry fruits. Bae et al. examined the effect of OD with two osmotic agents followed by different drying techniques on the quality of chokeberry dried powder [11]. In this study, the effect of OD with the combination of various sonication times on the moisture adsorption properties, colour, and some antioxidant properties of chokeberry powder was investigated. In addition, xylitol and erythritol were used as osmotic agents during the OD process.

Materials and methods

Sample preparation

Fresh chokeberry fruits (*Aronia melanocarpa* L.) were harvested in Krakow (Poland) at processing maturity. The fruits were sorted, washed, and inedible parts removed. Then, the chokeberry fruits were stored at 8 ºC before further processing.

Ultrasonic pretreatment and osmotic dehydration

Osmotic dehydration was carried out in 30% (*w/w*) of xylitol (XYL) and erythritol (ERT) solutions according to the procedure of Nowacka et al. [19]. Solutions were prepared by dissolving the solutes in distilled water. Fruits (50 g) were placed in 250 mL beakers containing the osmotic solution. The weight ratio of osmotic medium to fruit sample was 4:1. The ultrasonic pretreatment was carried out at 40 ºC in an ultrasonic bath SONIC 14 (PolSonic, Warsaw, Poland) without mechanical agitation, using frequency 40 kHz and the total power of 400 W generated by sonotrodes, which corresponds to an intensity of 8 W/g. Samples were subjected to ultrasonic waves (US) for time periods of 5 to 45 min (US-5 to US-45). Afterwards, to continue the osmotic dehydration process for an additional 3 h, bakers with the tested samples were transferred to a rotary shaker at a speed of 120 rpm. After the treatment, the fruits were rinsed with distilled water for 10s and dried with absorbent paper. To evaluate the effect of ultrasound, the same procedure was carried out in the ultrasonic free environment (US-0). The treatment was conducted in three replicates for each osmotic solution.

Preparation of dried powder

Each berry was cut in four parts and dried in a forced-air oven for 22–23 hours at 45 °C. Dried material was manually homogenised in a mortar to obtain fine powder. Desiccator with freshly dried silica gel had been used for the storage of chokeberry powder until analysis was performed.

Moisture adsorption of chokeberry powder

Moisture adsorption in various relative humidities (0–80 %) was carried out in a device DVS Intrinsic Plus (Surface Measurement Systems Ltd., London, UK) monitoring the change in mass of the sample subjected to various levels of relative humidity (RH). Briefly, approximately 25 mg of dried sample was placed on an aluminium dish hanged on a sensitive analytical microbalance (mass resolution ±0.1 μg) in a closed chamber. Relative humidity of the surrounding space was controlled by the air stream (200 ccm) passing through the reservoir of re-distilled water at 10 % steps until the change in mass was lower than 0.002 mg/min within 10 min for each RH level. The results were expressed as equilibrium moisture content (EMC, mg/g of dry mass).

Colour determination

Transmission spectrum of chokeberry powder samples was measured in the reflectance mode using a benchtop UltraScan VIS spectrophotometer (HunterLab, Reston, USA) with a d/8° geometry and standard illuminant D65. The spectra were measured in the range from 400 to 700 nm (with 10 nm reporting interval) and the colour expressed in a CIELab three-dimensional colour system, where L^*-axis represents a lightness (0-black, 100-white), a^*-axis is extended from green ($-a^*$) to red ($+a^*$), and b^*-axis from blue ($-b^*$) to yellow ($+b^*$). White tile was used as a standard for colour measurement. Each value was measured in five replicates.

Determination of antioxidant properties

Extraction procedure

Chokeberry powder (0.5 g) was placed in a glass tube with 10 mL of 90% methanol solution and 30 μL of formic acid followed by extraction for 30 min in an ultrasonic bath [20]. Supernatant was removed from the pellet after centrifugation at 3000 rpm for 5 min and stored at −18 °C prior to analysis. Each extract was prepared in duplicate.

Spectrophotometric assays

The procedures for the determination of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity (TEAC) were adopted from our previous study [21]. Briefly, TPC was determined by the reaction of phenolics with Folin-Ciocalteau reagent. The product of the reaction was monitored at 765 nm and the results expressed as gallic acid equivalent in dry mass (mg GAE/g d.m.). Aluminium chloride assay was used for the determination of the total flavonoid content (TFC) where increase in absorbance at 425 nm was proportional to the increase of flavonoid content. The results were expressed as the quercetin equivalent (mg QRT/g d.m.). DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was applied to the determination of antioxidant capacity, where the discoloration of DPPH· methanol solution in the presence of compounds with antioxidant activity was monitored at 517 nm. Results were expressed as Trolox equivalent antioxidant capacity (TEAC) in mg/g d.m. Total anthocyanin content (TAC) was determined by the pH-differ method [22]. Briefly, the extract was mixed with two buffer solutions (1.0 and 4.5 pH) and the absorbance was observed at two wavelengths (510 and 700 nm). TAC values were calculated using the molar absorption coefficient of cyanidin-3-glucoside (C3G) and expressed as mg C3G/g d.m.

Statistical analysis

The normality of all variables was evaluated using Shapiro-Wilks test. The effect of osmotic agent (Factor A) and time of ultrasonic pretreatment (Factor B) was determined using one-way analysis of variance (ANOVA) for variables with the normal distribution. In other words, the null hypothesis assumes that the means are equal. For multiply comparison between the means, post hoc Duncan´s test was applied. The results were expressed as an arithmetic mean and standard deviation. If the normality of variables was not confirmed, nonparametric Kruskal-Wallis ANOVA was used to study the effect of osmotic agents and US pretreatment time. Median with its average absolute deviation (AD) was used for the estimation of the mean in case of non-normal distribution. All statistical analysis was performed with Statistica 12 (Tibco Software Inc., Palo Alto, CA, USA) at the probability level $p = 0.05$.

Results and discussion

Moisture adsorption of chokeberry powder

Moisture adsorption isotherms of all chokeberry powder samples (Figure 1) followed the type 3 according to BET classification [23]. The respective shape of the isotherm curve is characteristic for products with high soluble sugar content, such as osmotically dehydrated cambuci slices [8]. The gradual increase of EMC with the increase of a_w (absence of sigmoid shape) refers to the multilayer sorption of water molecules on the surface of solids. As seen, there were no differences among the equilibrium moisture content of chokeberry powder pretreated with erythritol in the whole a_w range, suggesting that ultrasound treatment did not affect the moisture adsorption properties. On the other hand, the moisture adsorption of powder previously dehydrated in xylitol solution increased with the increase of ultrasound treatment time from 5 to 30 min, particularly above 0.60 a_w. For example, the gradual increase of EMC from 191.5 to 228.7 mg/g d.m. at 0.70 a_w was observed with the prolongation of US time from 5 to 30 min. EMC was similar for chokeberry powder pretreated with OD-US for 45 min and those without US. In contrast to our findings, osmotic dehydration of quince in sucrose solution combined with sonication decreased the EMC in comparison with that of US untreated samples [9]. In the lower a_w region (0.20–0.45), apparently lower EMC for the powder pretreated with xylitol was observed without respect to the ultrasound treatment in our study. This is in agreement with a study by Cichowska-Bogusz et al., where ultrasound pretreatment of dried apples caused the lower moisture adsorption at 0.75 a_w, most intense when apples had previously been dehydrated in xylitol solution [10]. In our study, EMC of powders pretreated with erythritol (without respect of sonication) was at the same level as that for XYL-US-30 above 0.60 a_w.

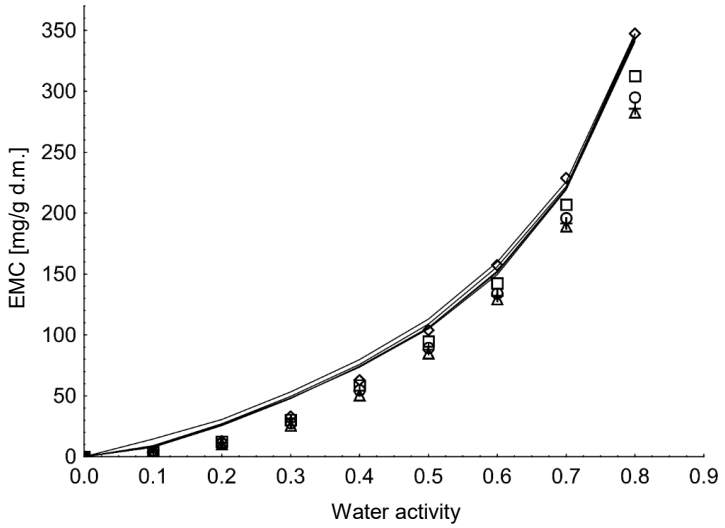

Fig. 1 Equilibrium moisture content (EMC) vs. a_w (at 25°C) of chokeberry powder pretreated by osmotic dehydration in erythritol (lines) and xylitol (symbols) with the assistance of ultrasound for 0 (+), 5 (○), 15 (□), 30 (◊), and 45 (Δ) min

The change of colour

Chokeberry powder pretreated with xylitol, not subjected to US treatment, showed higher L^* values, although not significant (p = 0.072), and lower a^* (p = 0.001) and b^* values (p = 0.007), which represents a lighter, less red, and less yellow colour in comparison with those of erythritol. The effect of glucose, sucrose, and xylitol during osmo-dehydration pretreatment of chokeberry fruits followed by air-forced drying was studied by Bae et al. [11]. They had found that lightness did not depend on the osmotic solution, but lower both a^* and b^* values were observed when xylitol was used as the osmotic agent.

One-way ANOVA indicated a significant impact of the US pretreatment on all L^* (p = 0.001), a^* (p = 0.028), and b^* (p = 0.025) values; however, a strong interaction between osmotic agent and US treatment time was identified for all colour coordinates (p < 0.01). As can be seen from Figure 2A, L^* values have decreased with the increase of US treatment time from 5 to 30 min for both erythritol and xylitol osmotic agents. Figure 2A also showed that lighter powder was obtained after osmotic dehydration in xylitol solution. The study of the effect of various osmotic agents on the lightness of dried apple slices revealed that the parameter L^* was significantly lower for US-treated samples, however, no differences among L^* values were obtained for erythritol, xylitol and sucrose [10].

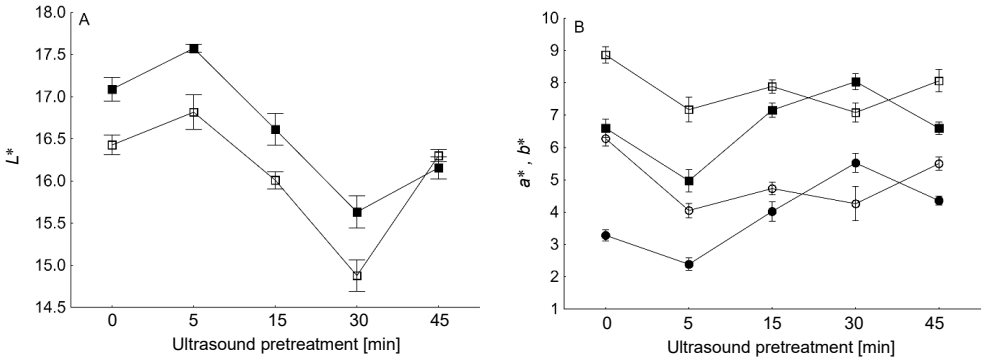

Fig. 2 The effect of ultrasonic time on A) lightness/darkness (L*) and B) green/red (a*, squares) and blue/yellow (b*, circles) colour coordinates of chokeberry powder during osmotic dehydration pretreatment process in erythritol (open symbols) and xylitol solution (closed symbols)

Increase of lightness was observed for freeze-dried strawberries previously treated by osmotic dehydration enhanced by ultrasound [12]. However, different intensity of ultrasound was applied in their study. It was recently described that the change in colour of dried plums was affected by various ultrasound intensities (0.45–1.35 W/g) [13]. Although a strong interaction between osmotic agent and US treatment time was identified for *a** and *b** colour coordinates ($p < 0.001$), different trends have been observed for samples pretreated with xylitol and erythritol (Figure 2B). Significant decrease of *a** and *b** values was determined after 5 min of US pretreatment, followed by their gradual increase to the maximum values for US-30 in xylitol solution. On the other hand, maximal values of *a** and *b** were determined for the powdered form using erythritol as a dehydrating solution, without ultrasound pretreatment prior oven-drying at 45 °C. In that case, we may conclude that application of ultrasound have just caused the decrease of both *a** and *b** values regardless of time of sonication pretreatment. Various effects on the overall colour changes of kiwi fruit after 30 min of ultrasound-assisted osmotic dehydration in XYL and ERT solutions for subsequent convective drying have been observed in the study of Kroehnke et al [14]. The sample subjected to OD-US pretreatment with ERT solution showed higher colour changes in comparison with that of OD without applying US waves. Using XYL, ultrasound caused smaller colour changes in kiwi fruit powder. In our study, the overall changes in colour did not significantly differ between chokeberry samples pretreated with XYL and ERT solutions (data not shown).

Antioxidant properties

Total phenolic, flavonoid and anthocyanin contents of chokeberry powder obtained by ultrasound-assisted osmotic dehydration with the subsequent air-forced drying at 45°C were in the range of 19.60–42.65 mg GAE/g d.m., 3.19–8.09 mg QRT/g d.m., and 0.20–0.57 mg C3G/g d.m., respectively. Those contents are in accordance with convectively dried chokeberry fruits at 50–70 °C [3], but being much lower than those obtained from chokeberry powder prepared by spray-drying or freeze-drying processes [4,5]. In the case of samples subjected to OD-US in erythritol and xylitol solutions, TFC values were similar as confirmed by Mann-Whitney test (Table 1). Therefore, we may say that chokeberry powder samples subjected to ultrasound-assisted osmotic dehydration in erythritol solution have exhibited significantly higher values for TPC ($p < 0.05$), TAC ($p < 0.05$) and TEAC ($p < 0.001$).

Table 1 The effect of erythritol (ERT) and xylitol (XYL), and ultrasound pretreatment on the total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and antioxidant capacity (TEAC) of chokeberry powder

	Effect of US pretreatment		Effect of osmotic agent	
	K-W ANOVA		M-W test	
	erythritol	xylitol		
TPC	rejected*	rejected**	rejected*	ERT > XYL
TFC	rejected**	confirmed	confirmed	
TAC	rejected**	rejected**	rejected*	ERT > XYL
TEAC	confirmed	rejected*	rejected***	ERT > XYL

Null hypothesis means that all means are equal against; US, ultrasound; K-W, Kruskal-Wallis; M-W, Mann-Whitney; * $p < 0.05$; ** $p < 0.01$, and *** $p < 0.001$

Similar results were obtained from US-assisted osmotic dehydration of kiwi fruit, where a higher retention of polyphenolic substances was achieved by applying erythritol and sorbitol in contrast to that of sucrose solution [14]. On the other hand, chokeberry fruit powder pretreated with sucrose, glucose or xylitol solutions did not significantly differ in TPC, but it has shown lower proantho-cyanidin content and antioxidant activity (evaluated using FRAP and ABTS assays) for the samples pretreated with xylitol [11]. It should be noted that the retention of bioactive compounds depends upon both osmotic solution and ultrasound time as was concluded for dried plums. While application of US for 30 min gave higher phenolic content for samples pretreated with glucose solution, the extension of US time resulted in the opposite effect; i.e., higher phenolics in samples pretreated with sucrose solution [15]. The effect of ultrasound applied

during OD on the antioxidant properties of chokeberry powder is illustrated in Table 1. The null hypothesis that all the means are equal was not confirmed for TPC ($p < 0.05$), TFC ($p < 0.01$), and TAC ($p < 0.01$) when ERT had been used as osmotic substance during ultrasound-assisted osmotic dehydration. It means that those antioxidant properties were affected by the time of ultrasound pretreatment. Multiplied comparison has revealed that there is no significant difference between TPC values, but the highest total flavonoid content was found for samples pretreated with ultrasound for 30 min (7.38 ± 0.03 mg QRT/g d.m.) in comparison with samples not subjected to ultrasound (6.36 ± 0.17 mg QRT/g d.m.; $p < 0.05$) or ultrasonicated for 5 min (6.12 ± 0.07 mg QRT/g d.m.; $p < 0.01$).

Similarly, chokeberry powder subjected to OD-US-30 showed significantly higher total anthocyanin content with a median 0.52 ± 0.01 mg C3G/g d.m. ($p < 0.05$) when compared to TAC value 0.38 ± 0.02 mg C3G/g d.m. after OD-US-5 process. Using xylitol as the osmotic agent, the effect of ultrasound pretreatment time was confirmed for TPC ($p < 0.01$), TAC ($p < 0.01$) and TEAC ($p < 0.05$) values. Similar trends were obtained for chokeberry powder samples pretreated by osmotic dehydration in xylitol solution showing the highest TPC (41.43 ± 3.36 mg GAE/g d.m.) and TAC (0.54 ± 0.01 mg C3G/g d.m.) values for ultrasound-assisted OD for 30 min. It was previously observed that ultrasonic treatment time during osmotic dehydration affected the release of phenolic substances from plant cells. For instance, sonication of cashew apple bagasse for 5 min increased the total phenolic content and antioxidant capacity in comparison with 2 min treatment [16]. Although our results are not consistent in all spectrophotometric assays, application of ultrasound for 30 min during OD of chokeberries seems to preserve more antocyanins when both xylitol and erythritol were used. Ultrasound may cause plasmolysis of cells; therefore, an enhanced release of phenolic compounds from plant cells can be observed in various products [16–18]. In addition, a formation of many microscopic channels by ultrasound during OD was responsible for the loss of structural integrity of pomegranate seeds [17]. We may hypothesize that US treatment for 45 min during OD of chokeberry caused a disruption of the cell walls, which facilitated the release of bioactive substances, exposing them for further oxidation. Similarly, a decrease of phenolic content and antioxidant capacity of Sanhua plum was observed with the increase of ultrasound power [13].

Conclusion

The osmotic dehydration coupled with the ultrasonication process was found to be suitable for the preparation of chokeberries prior to oven-air drying. OD in erythritol solution has resulted in chokeberry powder with higher EMC, particularly at the low a_w region. Various sonication times during OD were

reflected in different EMC for xylitol, but not for erythritol. Based on our results, OD in erythritol solution enhanced by sonication for 30 min can be recommended since the higher content of phenolic and anthocyanin substances has been observed. Finally, the powder samples exhibited also a lighter tone of the respective colour.

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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. contains 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 [37, 224–226].

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. Composition of each pomace differs with variety and strongly depends on the process of winemaking [227–230].

4.1 Typical content of *Vitis vinifera* L. and grape pomace

Chemical composition of grapes strongly depends on ripening time and different environmental parameters such as altitude, soil, humidity, temperature, sunlight, and UV radiation. Last two mentioned can, for example, affect levels of sugars, pigment, or total phenolics [231, 232].

Sugars typically found in this fruit are predominantly glucose (up to 16.47 g per 100 g of FW) and fructose (up to 15.55 g per 100 g of FW). Sucrose is usually determined only in trace amounts. **Organic acids** content is from more than 90% formed by tartaric acid and malic acid. When matured, berries contain significantly higher amount of tartaric acid than malic acid. Total **dietary fiber** makes up approximately 1% and **protein** 0.5–0.6% of fresh grape weight. Vitamin C is the **vitamin** present in the highest amount (3.68 g per 100 g of FW), niacin and other B complex vitamins, vitamin A, and vitamin E can be also found [225, 233–235]. Grape seeds are rich source of unsaturated fatty acids (predominantly linoleic, palmitic, and stearic acid). Total **fat** in grape berries is 0.3–0.4%, while only in seed oil is ranging from 7.23 to

7.98% [199, 225, 231]. **Minerals** that appear in grapevine in high levels are potassium (0.1–0.2%), phosphorus (0.08–0.1%, present mainly in seeds), calcium (0.01–0.02%), magnesium, and iron [225, 231, 236]. **Phenolic compounds** are in grapes located mainly in the seeds (up to 70%) and skin (up to 35%). Ghafoor with colleagues [237] described in their paper values for total phenolics in grape seed powder to be ranging from 11.21 to 34.85 mg of GAE per 1 g of seeds FW. Pulp only contains about 10% of total grapevine phenolic compounds. Main classes of these substances occurring in grape represent anthocyanins, flavonols, stilbenes, flavan-3-ols, and phenolic acids. First mentioned, anthocyanins, are responsible for typical colour of red grapes and organoleptic characteristics of wine. Total level of anthocyanins (results expressed as cyanidin-3-glukoside equivalent) is within range 16.1–29.5 mg per 100 g of FW, strongly depending on ripening time. As expected, over-matured red berries give higher values of anthocyanins than ones in veraison phase. Among main anthocyanins present almost exclusively in grape peel can be included pelargonidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, delphinidin-3-glucoside, and malvidin-3-glucoside. This category of compounds is also one of the parameters for grape varieties determination and evaluation of their ripening level. Resveratrol (in both *cis*- and *trans*- form) is main stilbene present in grape berries, followed by piceatannol, piceid, and resveratrol dimers, also known as viniferins. The beneficial effects associated with wine drinking are mostly attributed to this class of polyphenols. From group of flavonols, red berries typically contain quercetin, kaempferol, isorhamnetin, laricitrin, syringetin, and myricetin derivatives, whereas white grapevine flavonols content is reduced only on presence of derivatives of kaempferol, quercetin, and isorhamnetin. The most of flavonols can be found in grapevine seeds (up to 65% of total amount). Compounds from the category of flavan-3-ols, (+)-catechin, (+)-gallocatechin, (–)-epicatechin, and (–)-epicatechin gallate, are usually determined in significant quantities. Phenolic acids, such as gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, chlorogenic acid, and caffeic acid are represented in the highest amount [35–38, 199, 224, 231, 232, 236–239].

A residue after process of winemaking, grape pomace (GP), still contains high amount of dietary fiber, oils (unsaturated fatty acids occurred predominantly in seeds), vitamins, and phenolic compounds. Actually, 60–70% of total phenolic compounds retain in grape pomace after winemaking procedure. Research from Makris et al. [240] reports value of phenolic substances (predominantly flavonoids) in seeds of GP to be 11.1% of DM for white grape variety. White GP provides total phenolics in level of 4826 mg per 100 g of DM whereas

seedless GP contained only 970 mg of phenolics per 100 g of DM [240]. Kammerer et al. [239] found the amount of *trans*-resveratrol in skins of GP to be 1.11–12.3 mg per 100 g of DM (5.47 mg per 100 g of DM for white cultivar Müller Thurgau used in our study (**Appendix VI.**)). In the study of Gaafar et al. [241], red GP provided higher values for total flavonoid (by 36%), total phenolic (by 60%), and total tannin content (by 58%) than white GP. Red grape pomace is rich in anthocyanins while they are not found in white grape varieties. Proanthocyanidins and catechins are essentially located in grape skin and seed, only small amount can be found in the pulp. For this reason their presence is strongly expected also in the remaining GP. For example, catechin and epicatechin values corresponding to 1 mg of each per 1 kg were found in cold-pressed grape seed oil. Seeds of GP from Müller Thurgau variety showed presence of phenolic acids in level of 32.66 mg per 100 g of DM. Besides phenolic compounds, grape seeds contain approximately 40% of dietary fiber, up to 20% of lipids, 10% proteins, as well as significant amount of minerals and sugars [199, 224, 226, 232, 239, 242].

4.2 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 [224]. In study of Zhu et al. [230], after the fermentative maceration step of vinification process, a significant decrease in antioxidant activity was reported in grape skin, while relatively large amount of antioxidants remained in the seeds. This operation had also a significant effect on lower phenolic content in pomace when compared to fresh berries. In grape skin, there was a decrease of total phenolics, total flavonoids (both by 80%), and total flavan-3-ols (by 70%). However, grape seeds contained after this step still 75%, 72% and 65% of the original values of total phenolics, total flavonoids, and total flavan-3-ols, respectively. Acidic environment of alcoholic fermentation causes for anthocyanins from GP skin to be released and extracted into the red wine (up to 95.81% of original content in fresh berries skin) [230].

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 [224]. 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 [243] 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 [243].

Larrauri et al. [244] 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 [244]. As can be seen in our study (**Appendix VI.**), 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 Appendix VI.**). Khanal et al. [245] concluded that there is no significant loss in procyanidins level (489.4 mg per 100 g of DM) as grape pomace was heated in the forced air owen at 40 °C for 72 hours. However, total procyanidin content was gradually decreasing when temperature of 60 °C and higher was applied [245].

4.3 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 [228, 246], cheese [247], kefir [229], and processed cheese spread enriched with grape pomace [248] or grape skin powder (**Appendix VI.**). Besides dairy products, also cookies with grape skin and seeds [249], wafers and biscuits enriched with grape pomace [227, 250], muffins, cakes, brownies, bread and breadsticks where flour was supplemented with powder from grape pomace [251–254], pancakes, noodles and cereal bars with grape seed flour supplementation [255], chocolate where grape pomace is used as a bulking agent [256], grape by-products ice cream [257] or grape pomace pasta [258] were prepared. Surprisingly, also preparation of meat

products with content of parts of grapes was described. As examples can be mentioned low fat chicken meat balls and beef hamburger with addition of grape pomace [259, 260], chicken nuggets dipped into batter containing powder from grape seeds [261] or sausages and summer salami with grape seed powder content [262, 263]. Moreover, effect of white GP addition into both pasteurised and unpasteurised beers was studied by Gasiński et al., when beers were fortified by GP at two different levels (10% and 20% w/w) [264].

In all mentioned applications, addition of components from grapes resulted in increased levels of phenolic content and antioxidant activity compared to original product without enrichment. 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 Appendix VI.**). Fortification with freeze-dried grape skin powder at 2% (w/w) level allowed to achieve higher amount of individual phenolics (**Figure 3B in Appendix VI.**) and beneficial results of antioxidant capacity (**Figure 4 in Appendix VI.**).

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 processes (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 into 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.

Appendix for Chapter 4

Appendix VI.

Frühbauerová, M.; Červenka, L.; Hájek, T.; Salek, R. N.; Velichová, H.; Buňka, F. Antioxidant properties of processed cheese spread after freeze-dried and oven-dried grape skin powder addition. *Potravinarstvo Slovak Journal of Food Sciences*, 2020, **14**, 230–238.

Appendix VI.

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ANTIOXIDANT PROPERTIES OF PROCESSED CHEESE SPREAD AFTER FREEZE-DRIED AND OVEN-DRIED GRAPE SKIN POWDER ADDITION

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ABSTRACT

Processed cheese spread (PCS) is a popular product with high nutritional value and containing protein, fat and minerals. Grape skin is waste from winery processing plants that still has phenolic substances with significant antioxidant activity that could be used for valorisation of processed cheese and increasing the content of nutrients, phenolics and overall antioxidant properties. Both oven-dried (OD) and freeze-dried (FD) grape skin (GS) powder was characterised by the principal ingredients, the content of phenolic compounds and antioxidant capacity. Similarly, the influence of the addition of OD-GS and FD-GS powders on processed cheese spread (PCS) at 1% and 2% (w/w) levels were examined. The OD-GS and FD-GS powders were characterised by protein content, fat content, moisture and dietary fibre, thus showing that drying technique did not affect those parameters. The OD-GS powder exhibited higher content of rutin, (+)-catechin, (-)-epicatechin and total flavonoid content (TFC), while higher total phenolic content (TPC) and ABTS radical cation were observed for freeze-dried GS powder. Fortification of PCS with 1% and 2% (w/w) of GS powder increased protein content. An ANOVA procedure revealed that addition of FD-GS powder to processed cheese spread was superior to TPC values together with rutin, (+)-catechin, and (-)-epicatechin contents. The higher phenolic contents reflected the higher antioxidant capacity of PCS samples fortified with FD-GS powder. Freeze-dried gape skin powder was the better choice for valorisation of processed cheese spread.

Keywords: grape; valorisation; processed cheese; antioxidant; chromatography

INTRODUCTION

Processed cheese spread (PCS) is a multi-component mixture made from water, cheese, fat, and emulsifying salts (phosphates or citrates). This mix is processed by stirring and melting in temperatures ranging from 85 to 110 °C for up to 20 min **(Černíková, et al., 2018)**. The obtained hot mixture is poured into cups, cooled down and stored at refrigeration temperature. Processed cheeses are products with extended shelf life that deliver bioactive proteins, fats, minerals and vitamins to consumers **(Henning, et al., 2006)**. Despite the high nutritional value, various types of cheese have been enriched by addition of herb or medicinal plant extracts during their preparation, for instance, the addition of rosemary leaves to ripened semi-hard cheese **(Marinho, et al., 2015)**. The fortification of frequently used food products may enhance the consumption of various health-promoting substances and might be helpful for human health **(Rashidinejad, et al., 2015)**. The authors found that hard low-fat cheese fortified by catechin maintained its antioxidant activity after *in vitro* digestion experiment.

Grape berries (*Vitis vinifera L.*) are used in the winemaking industry to produce alcoholic beverages by pressing berries and subsequent fermentation of liquid. The press residues constitute 20% (w/w) of the total grapes used for wine production **(Teixeira, et al., 2014)**. Grape pomace from white grape varieties is an excellent source of phenolic compounds (for example gallic acid, catechin, epicatechin and procyanidins) **(Genova, Tosetti and Tonutti, 2016)**; therefore, it can be used for the valorisation of various food products. Grape skin powder or grape flour has been successfully incorporated into bread **(Šporin, et al., 2018)** or yoghurts **(Karnopp, et al., 2017)**. Only a limited number of studies regarding the enrichment of processed cheese, probably due to the higher processing temperature and high-fat content, exist. In our recent studies, we described the effect of processing parameters on the antioxidant properties of processed cheeses fortified by quercetin or/and rutin **(Přikryl, et al., 2018)**. Functional processed cheese spreads have been prepared with the addition of tomato paste **(Mehanna, et al., 2017)**, carrot paste **(Mohamed, Shalaby and Gafour,**

2016) and pulp from apricots **(Mohamed and Shalaby, 2016)**. In a study of **Torri et al. (2016)**, grape skin powder was added to cow's milk curd before ripening process to produce Robiola (soft-ripened cheese), focusing on optimisation of enrichment using sensory evaluation. The authors found that the amount of powders exceeding 0.8% and 1.6% (w/w) for Barbera and Chardonnay powder, respectively, negatively affected the acceptability of cheeses. In a recent study, various fruit and vegetable by-products were added into the curd to produce Primosale cheese **(Costa, et al., 2018)**. They found wine pomace to be an excellent source of polyphenolic compounds with antioxidant activity. The main aim of this study is to determine the effect of the addition of grape skin powder on principal ingredients and antioxidant properties of processed cheese. We assumed that the addition of freeze-dried grape skin powder would enhance the functional properties of enriched processed cheese spread.

Scientific hypothesis

Freeze-dried grape skin powder enhances the antioxidant status of fortified processed cheese spread more than the oven-dried grape skin powder does.

MATERIAL AND METHODOLOGY

All solvents for extraction, chromatographic analysis and chemicals used for determination of antioxidant activity were purchased from Sigma-Aldrich (Prague, Czech Republic).

Preparation of grape skin powder

Grapes of the white variety 'Müller Thurgau' were harvested from the Prostřední Hory (Bzenec, Czech Republic) vineyard track during September 2017. After pressing the grape berries to obtain a liquid for wine manufacturing, a portion of grape pomace was immediately stored at -20 °C in an evacuated plastic package. Before processing, grape pomace was thawed and the needles and seeds were removed using an analytical sieve (mesh size 0.5 × 0.5 cm). Grape skins (GS) were dried at the following conditions: oven-drying (OD) was performed in laboratory air-forced oven (HS62A, Chirana, Brno, Czech Republic) at 46 °C for 24 h. Freeze-dried (FD) samples were prepared at -40 °C (12 Pa) for 48 h (CoolSafe 100-4, Trigon Plus, Čestlice, Czech Republic). Dried grape skins were milled at 5000 rpm for 10 s with a Grindomix GM 200 (Retsch GmbH, Haan, Germany) and sieved to obtain particles <800 μm. Both GS-OD and GS-FD powders were stored in a tightly sealed plastic pack at -20 °C until use. The contents of crude protein (Method 960.52), fat (Method 920.39), moisture (Method 934.01), ash (Method 930.05) and total dietary fibre (Method 985.29) were determined according to AOAC **(Horwitz, 2000)** procedure in duplicate. The total content of saccharides was calculated from differences.

Processed cheese manufacturing

The composition of raw materials, including Eidam cheese (dry matter ≈50 g.100 g^{-1} and fat in dry matter ≈30 g.100 g^{-1}, 8-week maturity), butter (dry matter ≈84 g.100 g^{-1} and fat in dry matter ≈98 g.100 g^{-1}) and water, was adjusted to obtain processed cheese with dry matter ≈37 g.100 g^{-1} and fat in dry matter ≈50 g.100 g^{-1}. A ternary mixture of monosodium dihydrogenphosphate (19%), disodium hydrogen phosphate (37%), tetrasodium diphosphate (22%) and the sodium salt of polyphosphate (22%) was used in a total concentration of 2.8 g.100 g^{-1}. For the preparation of functional processed cheese spread (PCS), OD-GS and FD-GS powders were added to produce PCSOD-GS and PCSFD-GS samples. Both grape skin powders were added at 1.0 and 2.0% (w/w) levels. Processed cheese without grape skin powder served as a control. Model processed cheese was manufactured in Stephan UMC-5 (Stephan Machinery GmbH, Halmen, Germany) equipment with indirect heating as is described in the flow chart below (Figure 1). Eidam block cheese and butter were cut into small pieces, put into the kettle and minced for 30 s. Then water, emulsifying salts and GS powder were added into the blend. The mixture was heated at 90 °C for 13 min at a constant agitation of 1500 rpm. Samples were poured into 80 g polystyrene doses with caps. The packages were cooled down to 6 °C and stored at -20 °C to avoid deterioration. Dry matter (DM), fat and protein contents were evaluated according to Method 969.19, Method 2001.14 and Method 960.52 as described in **Horwitz (2000)**, respectively, in three repetitions.

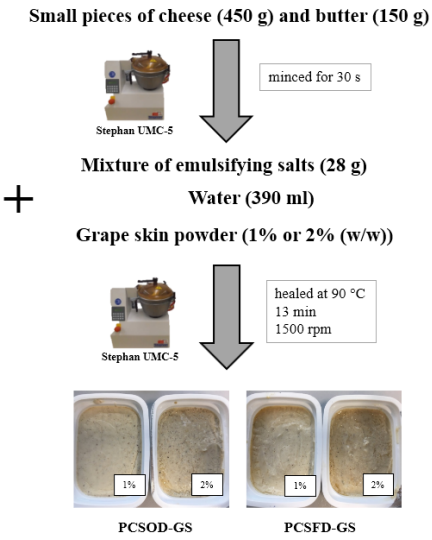

Figure 1 Flow chart of the cheese-making process. PCSOD-GS = processed cheese spread with oven-dried grape skin powder, PCSFD-GS = processed cheese spread with freeze-fried grape skin powder.

Extracts preparation

A glass test tube with 1.0 g of dried GS sample and 10.0 mL of 50% methanol solution was put in ultrasonic bath Sonorex TK52 (Bandelin Electronic, Berlin, Germany) for 30 min. A clear supernatant after centrifugation at 4100 rpm for 5 min (Universal 320, Hettich, Tuttlingen, Germany) was obtained.

Extract of PCS samples was obtained according to the procedure described in **Přikryl et al. (2018)**, i.e. 1.0 g of frozen PC sample was extracted into 10.0 mL of 50% methanol solution in an ultrasonic bath for 30 min. Subsequently, elimination of proteins and salts was performed using the procedure of **Khalifa, Omar and Mohamed (2017)** with a slight modification; the pH of extract was adjusted to 4.0 using HCl (2 M) and precipitated proteins were removed by centrifugation at 6000 rpm for 10 min. Then, the pH of clear supernatant was adjusted to 7.0 using NaOH (1 M) followed by centrifugation at 6000 rpm for 10 min to remove remaining proteins and salts. Supernatants were kept refrigerated and used for antioxidant assays and HPLC analysis. Two extracts per sample were prepared, and each extract was measured in duplicate, resulting in a sample size N = 4.

Chromatographic analysis of the extracts (HPLC analysis)

First, GS were screened for the presence of the following phenolic compounds: quercetin, rutin, (+)-catechin, (-)-epicatechin, resveratrol, caffeic acid, p-cumaric acid and ellagic acid. Secondly, phenolic substances identified in GS extracts were determined in PCSOD-GS and PCSFD-GS extracts. Before injection, each extract was filtered through a syringe filter (0.45 μm, Labicom, Olomouc, Czech Republic). Samples were injected into an Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA, USA) equipped with a degasser, an autosampler, a binary pump, a thermostated column compartment and DAD detector. A Zorbax Elipse Plus 1.8 μm C18 (50 × 2.1 mm; Agilent Technologies) column thermostated at 40 °C was used. For the analysis, 2 μL of the sample were injected. A mixture of 0.01 M ammonium acetate adjusted to pH 3.1 using formic acid (solution A) and acetonitrile (solution B) was used as mobile phase with a flow rate 0.6 $mL.min^{-1}$. The gradient for solution B was 0-3 min at 3%; 10 min at 20% and 20 min at 80%. The signal was detected at 280 nm. The identification of each peak in chromatograms of the extracts was carried out by comparing retention time and absorption spectrum against a pure standard. Quantitative determinations were done using calibration plots of a selected external standard.

Determination of antioxidant properties

Total phenolic content (TPC) was determined by measuring the complex of antioxidants with Folin-Ciocalteau reagent at 765 nm (DU 530, Beckman Coulter Inc., Brea, USA) using the procedure described in **Přikryl et al. (2018)**. The results were expressed as gallic acid (GAE) equivalents ($mg.g^{-1}$ of dry matter (DM)). Total flavonoid content (TFC) was determined using aluminium chloride assay **(Denni and Mammen, 2012)**. The increase of absorbance at 415 nm was proportional to the increase in the content of flavonoids. Results were reported as quercetin (QUE) equivalents ($mg.g^{-1}$ DM).

ABTS (2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging activity assay was adopted from our previous study **(Červenka, et al., 2018)**. The reaction between $ABTS^{\bullet+}$ and antioxidants was monitored at 734 nm, and the results were reported as Trolox equivalents antioxidant capacity (TEAC) in $mg.g^{-1}$ DM.

Reducing power (RP) of extracts was determined via the formation of Prussian blue at 700 nm (ferric-ferrous complex) according to the procedure of **Pavithra and Vadivukkarasi (2015)**.

Statistical analysis

Order statistic methods for small sample size were used throughout this study. The mean and its deviation were calculated according to Horn's procedure **(Horn, 1983)** and were expressed as pivot half sum (P_L) and pivot range (R), respectively. Nonparametric statistical methods were used in this study. The sign test was applied for pair-wise comparisons between means for grape powder samples. As far as processed cheese is concerned, the pair-wise comparison procedure was performed using Tukey's method. A two-factor Kruskal-Wallis analysis of variance (ANOVA) was applied to determine whether the drying method of GS powder (factor A) and the amount added to PCS sample (factor B) influenced the antioxidant properties in fortified PCS samples. To determine associations among variables, Spearman rank-order correlation coefficients (r) were assessed. All the statistical treatments were done at the probability P = 95%) (Statistica CZ, 12.0, StatSoft CR s.r.o., Prague).

RESULTS AND DISCUSSION

Composition of grape skin powder

As can be seen from Table 1, there were no statistical differences between the contents of crude protein, fat, total dietary fibre and ash for oven-dried and freeze-dried GS powders. On the other hand, the type of drying process affected the TPC and antioxidant properties of samples. Freeze-dried GS powder has a higher level of TPC (19.97 ±1.60 mg $GAE.g^{-1}$ DM) and more than twice as high $TEAC_{ABTS}$ value (127.10 ±27.28 mg $Trolox.g^{-1}$ DM) in comparison with the oven-dried GS samples. It has been previously concluded that the freeze-drying process is superior for the preservation of antioxidant compounds, and probably higher efficiency of extraction due to pronounced disruption of plant cells via the formation of ice crystals **(Kamiloglu, et al., 2016, Kamiloglu and Capanolgu, 2014)**. However, such a release of flavonoid compounds from plant cells may cause their pronounced exposure to oxygen. As was described in a study of **Nunes et al. (2016)**, oven drying of guava powder released more soluble flavonoids than the freeze-drying technique. In the chromatogram (Figure 2) of dried grape skin powder extract, fourteen well-resolved peaks were observed. However, from eight selected polyphenolic compounds, only the presences of (+)-catechin, (-)-epicatechin and rutin were confirmed in grape skin powder samples using retention time and absorption spectra in the current study. There was no difference in the number of peaks/compounds in oven-dried and freeze-dried GS powder extracts. The chromatograms differed in the heights of the peak showing that oven-dried GS powder extracts contained more polyphenolic substances.

Table 1 The main composition and antioxidant properties of grape skin powders (pivot half sum (P_L) ±pivot range (R), N = 2).

	Oven-dried	Freeze-dried
Main composition (g.kg^{-1})		
Protein	157.5 ±7.0^{A}	150.5 ±4.2^{A}
Fat	74.2 ±5.1^{A}	72.3 ±4.5^{A}
Moisture	55.0 ±4.3^{A}	59.5 ±6.4^{A}
Ash	13.2 ±2.0^{A}	14.6 ±3.1^{A}
Total saccharides	701.2	703.5
Total dietary fibre	228.8 ±8.5^{A}	231.7 ±9.1^{A}
Phenolic content (mg.g^{-1} DM)		
(+)-catechin	1712.5 ±1.2^{B}	1450.0 ±1.5^{A}
(-)-epicatechin	1383.3 ±3.0^{B}	1023.4 ±2.2^{A}
Rutin	221.8 ±1.0^{B}	112.0 ±1.2^{A}
Total phenolics (mg GAE.g^{-1} DM)	10.1 ±0.93^{A}	19.97 ±1.60^{B}
Total flavonoids (mg QUE.g^{-1} DM)	0.73 ±0.01^{B}	0.52 ±0.02^{A}
Antioxidant activity		
$TEAC_{ABTS}$ (mg Trolox.g^{-1} DM)	54.44 ±2.44^{A}	127.10 ±27.28^{B}
Reducing power (absorbance unit)	1.140 ±0.010^{A}	1.136 ±0.012^{A}

Note: DM, dry matter; GAE, gallic acid; QUE, quercetin; $TEAC_{ABTS}$, Trolox equivalent antioxidant capacity using 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid. Values sharing the same superscript letters in row $^{(A-B)}$ are not statistically significant different from each other (the sign test, p <0.05).

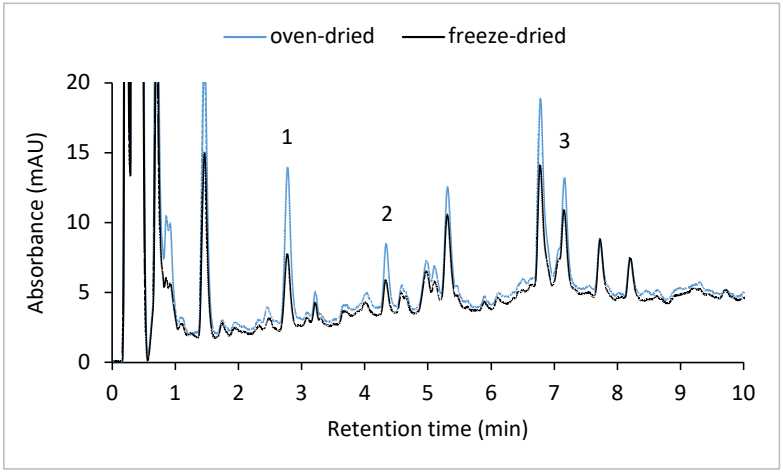

Figure 2 Separation of phenolic compounds in oven-dried and freeze-dried grape skin powder extracts using HPLC/DAD, detection at 280 nm; (+)-catechin (**1**), (-)-epicatechin (**2**) and rutin (**3**).

As shown in Table 1, significantly higher contents of (+)-catechin, (-)-epicatechin and rutin were observed in oven-dried GS powders, which corresponded to TFC values.

Properties of processed cheese spread containing grape skin powder

Table 2 shows the differences in the main composition of fortified processed cheese. The addition of grape skin powders significantly increased the protein content in processed cheese samples when added in 2% (w/w) levels, i.e. from 112.5 ±5.0 g.kg^{-1} (in control) to 128.4 ±5.7 and 129.7 ±2.3 g.kg^{-1} for processed cheese enriched with freeze-dried and oven-dried GS powders, respectively (p <0.05). Since we used the same amount of ingredients (cheese, butter, polyphosphate salts) for the preparation of all of the processed cheese samples, the variation in protein content was due to the addition of grape skin powder. Although the values of dry matter content increased linearly with the level of addition of grape skin powder, the effect of fortification was recognized as insignificant (p >0.05). On the contrary, **Khan et al. (2018)** found that fortification of Gouda cheese with the mango kernel did not significantly change the protein content.

Pair-wise comparison tests revealed that only processed cheese with 2% (w/w) of oven-dried GS powder had significantly higher dry matter content than that found in

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Table 2 The main composition of processed cheese spread (PCS) fortified with oven-dried (OD) and freeze-dried (FD) grape skin powder (GS). Pivot half sum (P_L) ±pivot range (R) (N = 3).

Ingredient (g.kg^{-1})	PCS control	PCS fortified with OD-GS powder (%, w/w)		PCS fortified with FD-GS powder (%, w/w)	
		1.0	2.0	1.0	2.0
Protein	112.5 ±5.0^{A}	120.3 ±4.3^{A}	129.7 ±2.3^{B}	118.0 ±3.8^{A}	128.4 ±5.7^{B}
Fat	191.0 ±3.1^{A}	188.5 ±2.7^{A}	187.4 ±3.0^{A}	189.3 ±4.2^{A}	186.9 ±1.8^{A}
Dry matter	368.2 ±14.0^{A}	394.6 ±6.2^{AB}	419.0 ±18.0^{B}	387.3 ±7.0^{AB}	395.9 ±4.8^{AB}
Ash content	39.5 ±1.8^{A}	40.8 ±2.2^{A}	42.1 ±1.4^{A}	40.9 ±1.1^{A}	42.4 ±2.0^{A}

Note: Values sharing the same superscript letters in row $^{(A-B)}$ are not statistically significant different from each other (Tukey's pair-wise comparison test, p <0.05).

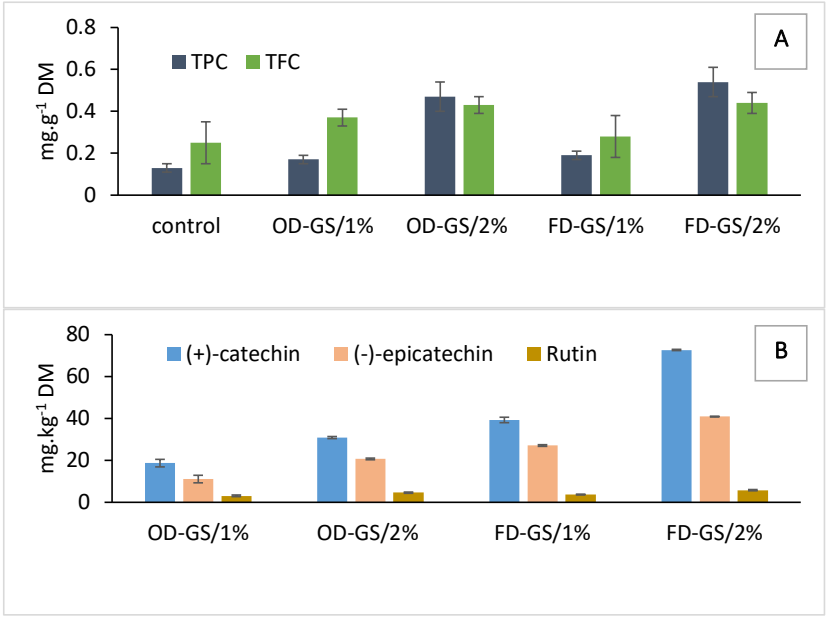

Figure 3 The content of **A**) total flavonoid (TFC), total phenolic contents (TPC), and **B**) phenolic individuals in processed cheese spread supplemented with oven-dried (OD) and freeze-dried (FD) grape skin (GS) powder at 1% and 2% (w/w) levels. Results are expressed as gallic acid and quercetin equivalents for TPC and TFC, respectively.

control (p <0.01). Fat and ash contents remained similar for all the processed cheese samples (p >0.05).

Antioxidant properties of processed cheese spread containing grape skin powder

The content of phenolic individuals is depicted in Figure 3B and show their higher content in processed cheese fortified with freeze-dried grape skin powder at both levels. Total phenolic content of processed cheese samples was determined using Folin-Ciocalteau's assay (Figure 3A). Significant increase of TPC values was obtained for processed cheese supplemented with GS powder at 2% (w/w) level from 0.13 ±0.02 mg GAE.g^{-1} DM (control sample) to 0.47 ±0.07 and 0.54 ±0.07 mg GAE.g^{-1} DM for oved-dried and freeze-dried GS powder, respectively. The addition of OD-GS powder to processed cheese spread resulted in the increase in TFC values from 0.25 ±0.10 mg QET.g^{-1} DM (control sample) to 0.37 – 0.43 mg QET.g^{-1} DM without respect to GS level. Freeze-dried GS powder enhanced processed cheese samples with flavonoids at a higher level (2%, w/w). It is interesting to note that even the control sample exhibited TFC value that then slightly increased with the addition GS powder. It has been reviewed that antioxidant properties of milk and milk products are due to the presence of sulphur-containing amino acids, vitamins, enzymes, peptides and oligosaccharides **(Khan, et al., 2019; Usta and Yilmaz-Ersan, 2013; Atmaca, 2004; Egger and Ménard, 2017; Alenisan, et al., 2017)**. Although oven-dried GS powder exhibited higher levels of (+)-catechin, (-)-epicatechin and rutin, their content in PCS samples with OD-GS powder decreased in comparison with PCS fortified with FD-GC powder. TPC values showed very high correlation with the content of all phenolic constituents (0.746 < r < 0.826, p <0.01) while low correlation coefficients have been

observed for TFC values showed values where (r = 0.610, p <0.05).

Antioxidant capacity of processed cheese samples in terms of $TEAC_{ABTS}$ significantly increased with the increase of GS level (p <0.05). Surprisingly, $TEAC_{ABTS}$ values were approximately tenfold higher (0.31 – 0.72 mg Trolox.g^{-1} DM) for processed cheese with 1% (w/w) of GS in comparison with that of the control, followed by an additional tenfold increase (5.22 – 6.69 mg Trolox.g^{-1} DM) when 2% (w/w) of GS powders were added (Figure 4). Such an increase may be attributed to the higher temperature used for melting the processed cheese in this study (90 °C for 13 min). It was previously published that pasteurization enhanced antioxidant properties of grape juice by releasing some polyphenolic compounds that were previously bound to other molecules **(Genova, Tosetti and Tonutti, 2016; Fuleki and Ricardo-da-Silva, 2003)**. For instant, general increase in ABTS scavenging was detected in fresh and technologically harvested grape juices followed by pasteriuzation at 78 °C for 30 min **(Genova, Tosetti and Tonutti, 2016)**.

In our previous work, the antioxidant properties of processed cheese fortified by rutin or quercetin increased with the increase of melting temperature as measured by ABTS$^{\bullet+}$ assay **(Přikryl, et al., 2018)**. In addition, polyphenolics contributed differently to antioxidant capacity measured by ABTS$^{\bullet+}$, as was observed in a study of **Lingua et al. (2016)**. They found that (-)-epicatechin, peonidin-3-glucoside and peonidin-3-acetylglucoside positively correlated with the assay, while pigment A and siringetin-3-glucoside had a negative effect. Similarly, strong positive correlation was observed for $TEAC_{ABTS}$ values and the content of (+)-catechin, (-)-epicactechin and rutin (r = 0.955, p <0.001; r = 0.739, p <0.01 and r = 0.951, p <0.001, respectively). Considering that high correlation coefficient reflects the strong association between variables, we may conclude that $TEAC_{ABTS}$ was mainly influenced by (+)-catechin and rutin contents followed by TPC values (r = 0.877, p <0.01).

The addition of GS powder had influence on reducing the power of processed cheese samples (Figure 4), where an increase was observed with the increase of GS powder level. Even though oven-dried and freeze-dried GS powders had similar RP values (see Table 1), processed cheese samples enriched with freeze-dried GS powder showed significantly higher RP values than in the case of incorporation of oven-dried GS powder, particularly at the 2% level (w/w) (p <0.05). Those discrepancies can be explained by the formation of new compounds, which may have enhanced the antioxidant capacity of the samples. Although the masking of antioxidant properties of various plant-based extracts by the addition of milk or whey proteins is common in literature, an interaction of protein and polyphenolic compounds may also increase antioxidant capacity. For instance, the mixing of α-casein or β-casein with epigallocatechin gallate led to the increase of inhibition of ABTS$^{\bullet+}$ during storage **(Almajano, Delgado and Gordon, 2007)**. In a study by **Sęczyk, Świeca and Gawlik-Dziki (2017)**, the addition of green coffee extract into soymilk significantly elevated ABTS radical scavenging ability (3.5-fold) and reducing power (13.8-fold). The study of interactions between polyphenolic compounds and β-conglycinin revealed that antioxidant capacity increased after the formation of the protein-phenolic complex **(Zhao, et al., 2018; Murray, 2002)**. The authors also demonstrated the increase of antioxidant capacity of the protein-phenolic mixture after heating at 90 °C for 30 min. Thus, we suppose a formation of new products during the preparation of enriched process cheese with enhanced antioxidant activity towards ABTS$^{\bullet+}$. Both TPC and TFC values had the greatest influence on reducing power. Strong positive associations have been found for RP vs. TPC (r = 0.97, p <0.001) and RP vs. TFC content (r = 0.944, p <0.001).

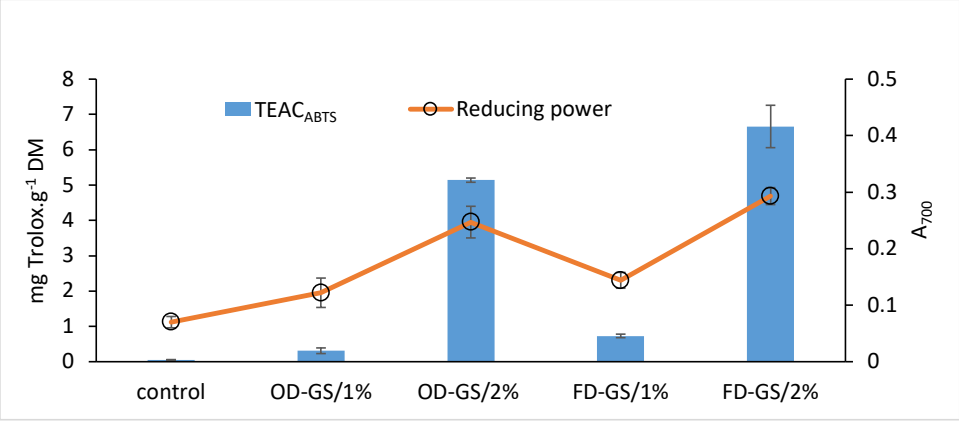

Figure 4 Trolox equivalent antioxidant capacity using ABTS ($TEAC_{ABTS}$, left y-axis) and reducing power (right y-axis) of processed cheese spread supplemented with oven-dried (OD) and freeze-dried (FD) grape skin (GS) powder at 1% and 2% (w/w) levels.

Rutin, (+)-catechin and (–)-epicatechin also showed strong positive correlations with reducing powder (p <0.001) of processed cheese enriched with GS powder, i.e. r = 0.859; 0.842 and 0.917, respectively.

Two-factor Kruskall-Wallis ANOVA was applied to study the effect of grape skin powder levels and type of drying used for their preparation (oven-dried vs. freeze-dried). As can be seen from Table 3, the drying technique has a significant effect on the TPC values, all the phenolic individuals, and reducing power (p <0.01) in processed cheese spreads where the addition of freeze-dried GS powder assured their higher values. Total flavonoids and $TEAC_{ABTS}$ values were not influenced by the drying technique. The effect of GS powder level was found to be significant for all parameters except for (-)-epicatechin content. As expected, the addition of GS powder at a higher level was reflected in higher values of TPC and TFC values, rutin and (+)-catechin contents, and antioxidant capacities in terms of $TEAC_{ABTS}$ and reducing power.

Table 3 The effect of drying technique (Factor A) for preparation of grape skin powder and its amount (Factor B) added to precessed cheese spread using two-factor Kruskall-Wallis ANOVA procedure.

	Factor A	Factor B
Total phenolics	p <0.05	p <0.001
Total flavonoids	p <0.05	p <0.01
(+)-catechin	p <0.01	p <0.001
(-)-epicatechin	p <0.01	p <0.05
Rutin	p <0.001	p <0.01
$TEAC_{ABTS}$	p <0.05	p <0.001
Reducing power	p <0.01	p <0.001

Note: $TEAC_{ABTS}$, Trolox equivalent antioxidant capacity using 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid.

CONCLUSION

Grape skin powder, a waste product from wine production, can be used as an ingredient for the production of processed cheese with enhanced properties. Grape skin powder samples have a high content of protein (150.5 – 157.5 $g.kg^{-1}$) and total dietary fibre (228.8 – 231.7 $g.kg^{-1}$). Freeze-dried grape skin powder possessed higher total phenolic content and the ability to scavenge $ABTS^{\bullet+}$, but lower total flavonoid content as well as the levels of rutin, (+)-catechin, and (-)-epicatechin. The incorporation of grape skin powder at 2 % (w/w) levels into the processed cheese significantly increased the protein content to 128.4 and 129.7 $g.kg^{-1}$ for for freeze-dried and oven-dried grape skin powders, respectively. Addition of freeze-dried grape skin powder into processed cheese was beneficial to antioxidant capacity in terms of reducing power. Higher contents of rutin, (+)-catechin and (-)-epicatechin, as well as total phenolic content, were achieved through the incorporation of freeze-dried GS powder, as was determined using two-factor ANOVA procedure. Based on the chemical analysis, we may conclude that using freeze-dried grape skin powder for the valorisation of processed cheese spread is the better choice in comparison with oven-dried grape skin powder. The hypothesis of this research was confirmed.

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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 [265–267].

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. Currently, chocolate is considered more of a confectionery with negative effects on human health [266–269].

5.1 Typical content of cocoa and chocolate

Main steps in production of chocolates consists of cocoa beans fermentation, drying, roasting, and grinding, followed by mixing all components (cocoa liquor, sugar, aroma, emulsifiers, and milk constituents if needed), conching, and tempering. The most chemical changes occur during fermentation, drying, roasting of cocoa beans, and process of chocolate mass conching. These chemical reactions play significant role in chocolate aroma and flavour development [270].

Raw cocoa beans contain a lot of **phenolic compounds**. The most abundant are procyanidins (about 58% of total phenolics), catechins (37%; mainly (–)-epicatechin), anthocyanins (4%), quercetin and its glycoside derivatives, and phenolic acids. Total phenolic content represents approximately 10–20% of cocoa bean DM and makes direct consumption of raw cocoa bean impossible due to extremely bitter taste. During chocolate making procedure, phenolic substances can decrease from 100% to 10% depending on the different manufacturing, e.g., the cocoa fermentation can cause loss in (–)-epicatechin and other soluble polyphenols

concentration by 10–20%. Phenolic compounds are released from storage cells and oxidation follows to form insoluble high molecular compounds, mostly tannins. Also process of drying, roasting, and conching is reason for decrease in amount of thermolabile phenolics. Higher amount of phenolics is expected to be found in dark chocolate that consists of larger amount of cocoa [265, 267–269, 271, 272]. Study of Meng et al. [273] brings comparison of total phenolic content in white, milk, and dark chocolates. Values obtained for tested commercial samples were approximately 126 mg CAE per 100 g of white chocolate, 161 mg CAE per 100 g of milk chocolate, and 579 mg CAE per 100 g of dark chocolate, respectively. Noor-Soffalina and colleagues [274] concluded about the relationship between polyphenols and **sugar** content in chocolates, the higher the level of phenolic compounds, the lower the amount of sugars. The mentioned phenomenon is probably caused by the fact that a large amount of sugars is bound to the present polyphenols (e.g., tannins) via hydrogen bonds. Before fermentation, cocoa beans contain up to 4% sugars, mostly sucrose (90% of total amount), glucose, fructose, galactose, and arabinose. Fermentation of cocoa beans cause hydrolysis of sucrose into reducing sugars (glucose and fructose). Final chocolate consists of sugars approximately from 50% (mainly sucrose added into the mixture). **Organic acids**, mainly acetic and lactic acid, are produced from sugars during fermentation process [270, 274]. **Proteins** represent 10–15% of raw cocoa beans DM, most important are albumin (52% of total proteins), globulin (43%), prolamin, and glutelin. During fermentation process, proteins are hydrolysed by enzymes and there is increase in amount of free amino acids and oligopeptides. Drying and roasting of cocoa beans is important step mainly because of the Maillard reactions, where reducing sugars interact with proteins or amino acids, and melanoidins are formed [270, 272]. **Fat** content in cocoa beans varies between 50–58% with triacylglycerols (consist mainly of stearic, oleic, palmitic, and linoleic acid) being the most prominent. **Minerals** are one of the most important components of cocoa beans. For example, 100 g portion of dark chocolate contains 115 mg of magnesium. Besides magnesium, cocoa and chocolates are rich also in phosphorus, potassium, iron, copper, calcium, and selenium. Of the **vitamins**, niacin, riboflavin, or thiamine are found in cocoa and chocolate products [266, 272]. **Methylxanthines** (theobromine and caffeine) are typical alkaloids of cocoa beans and produced chocolate. Theobromine is produced predominantly by the shell of cocoa bean and is often bound to tannins in raw cocoa beans. Its level in defatted cocoa bean solids is about 4% and in chocolates can reach up to 1.2%, whereas caffeine is not such abundant alkaloid in chocolate (lower content than in tea and coffee, 0.2% in defatted cocoa solids). As expected, concentration of theobromine is higher in dark chocolate (0.88% by weight) than in white (not detected) and milk one (0.13% by weight) [269–273].

There are three main categories to which chocolates are classified – dark, milk, and white chocolate. Commercial dark chocolate contains from 47% (sweet dark) to 75%, or even 90% and more (highly dark) of cocoa bean solids, cocoa butter, and sugar. Its quality depends on amount of cocoa. Also, major part of all beneficial effect attributed to chocolate is associated with dark type. Milk chocolate is made up of cocoa bean solids (up to 50%, usually not less than 20–25%), cocoa butter, sugar, and milk (powdered or condensed). White chocolate does not consist of any cocoa solids, and it is formed only by cocoa butter (not less than 20%), milk, and sugar [266, 267, 273, 275].

Innovative types such as low-sugar, sugar-free, low-fat, fat-free, and vegan chocolate have also gained much popularity during past decade [276, 277].

5.2 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 (**Appendix VII.**), 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 (**Fig. 6 in Appendix VII.**). 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 Appendix VII.**), detection potential was set at +1.3 V, and optimal flow rate of 1 mL/min was chosen. Brcanović with colleagues [278] 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 [279] 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. [280] 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 [281] 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 [282]. As for GC, Oliveira et al. [283] 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 [284–286]. Variety and geographical origin of used cocoa beans can also be verified using ^{1}H NMR techniques followed by PCA of obtained dataset [287, 288].

5.3 Cocoa bean shell as a functional food ingredient

Cocoa bean shell (CBS) is by-product remaining from the cocoa industry when chocolate and other goods are produced. CBS is usually removed from cocoa bean before or after process of roasting. It comprises up to 20% of cocoa seed total weight. It is known that CBS is rich in phenolic content (especially flavan-3-ols and procyanidins) and alkaloids (caffeine and theobromine). The most abundant compounds found in this material were theobromine (9.89 mg/g of CBS) and epicatechin (3.5 mg/g of CBS). CBS can also form significant source of dietary fiber and fat with lipid profile similar to one of cocoa butter [271, 289].

Hernández-Hernández and colleagues [290] 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. [291], 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 [292] 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) [292].

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 usually used chromatographic procedures.

Appendix for Chapter 5

Appendix VII.

Arbneshi, T.; Frangu, A.; Frühbauerová, M.; Červenka, L.; Berisha, L.; Kalcher, K.; Sýs, M. Flow injection amperometric evaluation of antioxidant capacity of chocolates having different cocoa content at a boron-doped diamond electrode. *Food Technology and Biotechnology*, 2021, **59**(2), 194–200.

Appendix VII.

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Flow Injection Amperometric Evaluation of Trolox Equivalent Antioxidant Capacity of Chocolates with Different Cocoa Content at a Boron-Doped Diamond Electrode

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SUMMARY

Research background. The objective of this paper is to introduce an instrumentally simple analytical tool for determination of cocoa solid content in chocolates. This electroanalytical method is based on amperometric oxidation of all present antioxidants in chocolates at boron-doped diamond electrode (BDDE) that is integrated in a flow injection analysis (FIA) wall-jet electrode system.

Experimental approach. As part of optimisation, thirteen commonly occurring antioxidants were investigated using cyclic voltammetry at the BDDE in 0.1 mol/L phosphate buffer with different methanol (MeOH) content. Working parameters, such as MeOH volume fraction, flow rate and detection potential, were optimised. Principally, the height of the oxidation peak (current response) representing the oxidation of the sum of antioxidants (total antioxidant content; TAC) was expressed as Trolox equivalents.

Results and conclusions. For analytical purpose, a linear range from 5 to 100 mg/L described by regression equation and characterised by high correlation coefficient R^2=0.9994 was achieved. Obtained high positive correlation between the determined values of Trolox equivalent antioxidant capacity (TEAC) and cocoa mass fractions characterised by correlation coefficient of 0.9187 for eight randomly selected samples (one white, two milk, and five dark chocolates) confirmed that cocoa solids represent the main source of antioxidants (reducing agents).

Novelty and scientific contribution. The research demonstrates that TEAC values could be considered as an additional marker of cocoa content in the chocolate analysis to the commonly used theobromine (authenticity of food products). The developed FIA could therefore serve as simple analytical tool in the food quality control.

Key words: Trolox equivalent antioxidant capacity, amperometry, boron-doped diamond electrode, flow injection analysis, cocoa mass fraction in chocolate

INTRODUCTION

Chocolate is a favourite food product made from cocoa beans that is consumed as sweets or beverage and to flavour or coat various confectionery and bakery products (*1*). Generally, the chocolate is divided into three main categories, namely dark, milk and white chocolate (*1*,*2*). Dark chocolate usually contains 50–90 % cocoa solids, cocoa butter and sugar, whereas milk chocolate contains 10–50 % cocoa solids, cocoa butter, milk in some form and sugar. White chocolate does not contain any cocoa solids and is made simply of cocoa butter, sugar and milk powder (*3*). Lower quality chocolates may also contain butter fat, vegetable oil or artificial colours or flavours. According to EU legislation (2000/36/ES), the last-mentioned type must not be labelled as chocolate (*4*). U.S. Food and Drug Administration (FDA) issued an order that semisweet chocolate must contain a minimum of 35 % chocolate liquor (*5*).

In the recent past, Czech Agriculture and Food Inspection Authority revealed the sad fact that most commercially available chocolates do not have the declared content of cocoa solids in order to be classified as a regular chocolate. Moreover, the statutory minimum content of cocoa solids was missing in some chocolate drinks (*6*). These unsatisfactory

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reports demonstrate the urgency to develop a simple analytical method applicable in the chocolate analysis.

Theobromine (TBR) is the primary alkaloid contained in cocoa powder and chocolate. Since TBR ranges from 26 g/kg in cocoa to 140 mg/kg in cocoa butter, this alkaloid can be considered as a marker of cocoa content (*7*). Determination of fat-free cocoa solids is performed using a protocol ČSN 56 0578, based on the HPLC analysis (*8*).

In addition to TBR, dark chocolate is rich in minerals, such as potassium, iron, magnesium, copper, manganese and zinc. The cocoa in dark chocolate also contains antioxidants called flavonoids, which may provide several health benefits (*3,9*). Assuming that cocoa powder and cocoa butter are the only sources of antioxidants, it is possible to use the total antioxidant content (TAC) as another potential marker of cocoa content (*10*). Phenolic compounds, flavours (vanillin and ethylvanillin) and alkaloids (TBR and caffeine) present in chocolate represent reducing agents that can be electrochemically oxidised at carbon-based working electrodes (*11–14*).

Due to an insignificant passivation of the electrode surface, a boron-doped diamond electrode (BDDE) was integrated into wall-jet flow cell to find out whether a simple flow injection analysis (FIA) with amperometric detection could be used for evaluation of dark chocolates (*15*). A correlation (R or R^2), known as a statistical measure describing a relationship between two variables (*16*), represented ideal tool to clarify the dependence between the declared cocoa content and TAC values in numerous dark chocolate samples.

MATERIALS AND METHODS

Chemicals and reagents

Analytical standards of ≥99.0 % L-ascorbic acid, ≥98.0 % caffeic acid, 99.0 % caffeine, ≥99 % *trans*-cinnamic acid, ≥98 % (–)-epicatechin, 97 % (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ≥95 % chlorogenic acid, 97.5–102.5 % gallic acid, ≥97.0 % kaempferol, ≥98 % (+)-catechin hydrate, ≥95 % naringin, ≥98 % sinapic acid, ≥98.0 % theobromine and ≥97 % vanillin were purchased from Sigma-Aldrich, Merck (Prague, Czech Republic). All voltammetric measurements were performed in their 1.0 mmol/L aqueous solutions of 0.1 mol/L phosphate buffer, pH=7.0, prepared from sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate, both obtained from Lach-Ner, Ltd. (Neratovice, Czech Republic). Due to low solubility, naringin, (+)-catechin, (–)-epicatechin and kaempferol had to be dissolved in phosphate buffer containing volume fraction of 10 and 50 % methanol (MeOH). Deionized water (ρ=18.3 MΩ·cm) obtained with a Milli-Q® water purification system from Merck (Darmstadt, Germany) was used for the preparation of phosphate buffer.

Pretreatment of boron-doped diamond electrode

A commercially purchased boron-doped diamond electrode (BDDE) with boron to carbon ratio of 1:1000 and a surface diameter of 3 mm (Windsor Scientific Ltd, Slough, UK) was used for all experiments. The BDDE surface was mechanically pretreated by carefully polishing it with a wet filter paper to eliminate the passivation layers on the electrode caused by oxidation products of polyphenols.

Instrumentation

The electrochemical behaviour of the dominant thirteen substances with antioxidant effect present in chocolate and Trolox was studied using cycling voltammetry at BDDE which was simultaneously connected with a silver/silver chloride electrode, 3.0 mol/L KCl as salt bridge (reference electrode) from Metrohm Česká republika s.r.o. (Prague, Czech Republic) and platinum sheet (auxiliary electrode) from Elektrochemické detektory, s. r. o. (Turnov, Czech Republic) to the potentiostat/galvanostat Autolab PGSTAT101 operated *via* the Nova 1.11 software from the above-mentioned Metrohm company (*17*).

Flow injection analysis (FIA) configuration consisted of a multi-channel peristaltic pump MINIPULS 3 from Gilson (Middleton, WI, USA), Rheodyne automatic six-position dosing valve from IDEX Health & Science (Wertheim, Germany), and BDDE inserted into the cross-flow cell from Inventek Sp. z o.o. (Warsaw, Poland), as shown in **Fig. 1**.

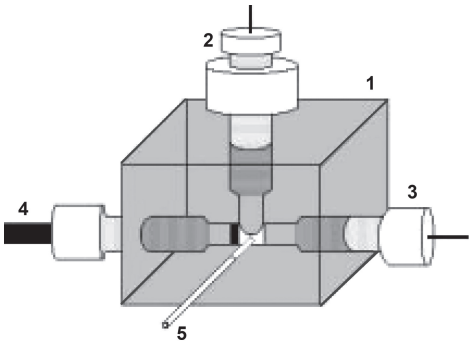

Fig. 1. Schematic diagram of the electrochemical flow cell used in the amperometric measurements in flow injection system: 1=polyurethane resin block, 2=silver chloride reference electrode, 3=auxiliary platinum electrode, 4=boron-doped diamond electrode, and 5=polyethylene tubing

Methods

Repetitive cyclic voltammetry (five cycles) was used to determine oxidation peak potentials of the investigated antioxidant substances. Potential range was set from –0.4 to +1.6 V, initial potential of 0 V, scan rate (v) 50 mV/s, and potential step (E_{step}) 2.5 mV. Flow injection analysis with amperometric detection in the wall-jet configuration was usually performed at +1.3 V *vs* a miniature silver/silver chloride reference electrode at flow rate of 1 mL/min. The 0.1 mol/L phosphate buffer (pH=7.0) containing 30 % methanol was used as

flowing carrier solution. Otherwise, all necessary changes in the working conditions are listed in the legends of the corresponding figures.

Sample preparation

Several purposefully selected chocolates of imported origin, differing in the cocoa solid content from 0 to 80 %, were purchased from common stores in Prishtina, Kosovo. The extraction of potential antioxidants from the chocolate samples of 5 g containing different amounts of cacao were carried out in a total mixture of 50 mL of water (70 %), acetone (29.8 %) and glacial acetate acid (0.2 %) using the ultrasonic bath at 30 °C for 30 min. The acetone was evaporated in ultrasonic bath at 40 °C for 20 min. After this, the solution with chocolate was adjusted with 0.1 mol/L NaOH to pH=5 and diluted in 100-mL volumetric flask using 0.1 mol/L phosphate buffer (pH=7.0) and MeOH (φ=30 %). The sample was then centrifuged five times at stirring speed of 1000 rpm for 4 min and filtered through a filter paper of pore size less than 1 μm. The filtrate obtained from the chocolate extracts was diluted fivefold to reduce the high content of extract-reducing agents. Sample volume of 100 μL was used for FIA analysis.

Statistical evaluation

Analysis of chocolate extracts was always repeated five times (N=5) and final results were calculated and presented as error bars (confidence intervals) $\bar{x}\pm st_{1-\alpha}$, where $\bar{x}$ is the arithmetic mean, s the standard deviation, and $t_{1-\alpha}$ the critical value of Student's t-distribution for five (4 degrees of freedom) determinations (2.7764) at a significance level α=0.05 (95 % probability).

RESULTS AND DISCUSSION

Electrochemical behaviour of substances present in chocolate

In this work we investigated only the reducing power of the chocolate samples, *i.e.* the potential of a substance to reduce another substance either by removal of hydrogen atom or release of electrons. We did not use conventional spectrophotometric assays, which are based on monitoring the reactions between the present antioxidants and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\cdot+}$) or di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium ($DPPH^{\cdot}$) radicals.

Since we can anodically oxidise most of the chocolate components with antioxidant activity at carbon-based working electrodes (*18–20*), we investigated the electrochemical behaviour of thirteen selected antioxidants using repetitive cyclic voltammetry (five cycles) at BDDE in 0.1 mol/L phosphate buffer (pH=7.0) from 0 to +1.6 V and back to 0.4 V. Due to low water solubility, phosphate buffer with MeOH (φ=10 %) was used for electrochemical study of (+)-catechin, (–)-epicatechin and naringin, while addition of 50 % MeOH was necessary for kaempferol due to its low solubility in water.

To set a constant working potential for the subsequent amperometric detection, it was important to determine the values of the peak potentials of individual antioxidants. All investigated antioxidants provided minimally one oxidation peak, where for the analytical purpose (determination of cocoa powder content in chocolate), peak potential values of the first peaks are shown in ascending order as follows: caffeic acid at +0.398 V, kaempferol at +0.483 V, chlorogenic acid at +0.505 V, sinapic acid at +0.620 V, gallic acid at +0.635 V, (+)-catechin at +0.640 V, L-ascorbic acid at +0.649 V, vanillin at +0.688 V, (–)-epicatechin at +0.744 V, naringin at +1.011 V, cinnamic acid at +1.133 V, caffeine at +1.384 V, and theobromine (TBR) at +1.404 V.

For demonstration, repetitive cyclic voltammograms (5 cycles) of Trolox, vanillin, and TBR are shown in Fig. 2. In all cases, a decrease in the oxidation signal was observed with each subsequent cycle, indicating a slow transport of oxidation products from the BDDE surface. This phenomenon was solved when these products were flushed from the electrode surface by amperometric detection in a flow mode. From the above-mentioned peak potential values, it is clear that if a constant potential is set for amperometric detection of +0.623 V (Trolox), antioxidants having higher oxidation peak

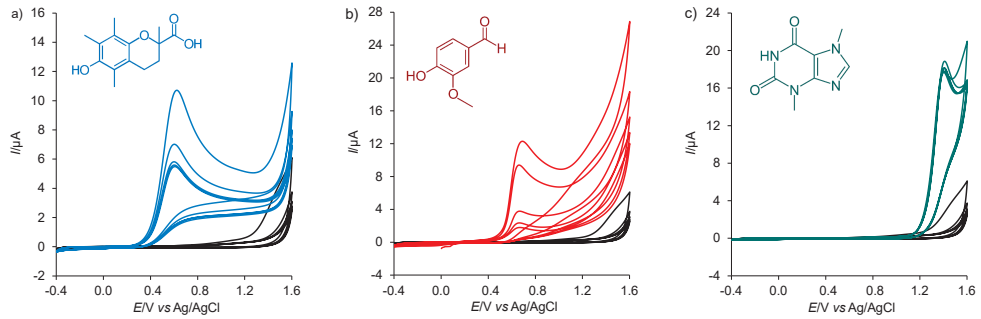

Fig. 2. Repetitive cyclic voltammograms (5 cycles) of: a) 1 mmol/L Trolox, b) vanillin, and c) theobromine recorded on boron-doped diamond electrode in 0.1 mol/L phosphate buffer (pH=7.0) at a scan rate of 50 mV/s. Black curves (blank) indicate the cyclic voltammograms obtained for phosphate buffer only

potentials will not be included in chocolate analysis. Hence, an effect of amperometric detection on total antioxidant content (TAC) values was essential for optimisation. These TAC values are usually expressed as Trolox equivalent antioxidant capacity (TEAC) (*21*). The presence of short-chain alcohols in phosphate buffer (aqueous-alcoholic mixtures) generally has no effect on peak shift. However, this assumption had to be verified for a MeOH volume fraction of 10 to 50 %.

Optimisation of flow injection analysis

The optimisation procedure included selection of optimal working parameters, such as composition of carrier solution, potential of amperometric detection, and flow rate. Due to the presence of slightly water-soluble phenolic acids, flavonoids and tannins, it was necessary to select the MeOH volume fraction in the carrier solution of 0.1 mol/L phosphate buffer (pH=7.0). The whole optimisation was carried out in the dark chocolate extract with *w*(cocoa)=80 %.

In general, polyphenolic compounds can be defined as weak organic acids for which it is known that their peak potentials are shifted to more positive potentials with decreasing pH values (*22,23*). The main reason for not performing FIA with acidic carrier solution is the necessity of amperometric detection at high positive potentials. The effect of pH in a range of pH=6–9 on current response of 1 mmol/L Trolox was investigated using cyclic voltammetry in 0.1 mol/L phosphate buffer. The obtained results indicate that the Trolox provides the maximum current response at pH=7, which was consistent with other studies that report the determination of polyphenols using FIA (*24,25*).

The optimum volume fraction of methanol in the carrier phosphate buffer solution was determined by varying *φ*(MeOH)=0–50 %. For constant detection potential of +1.3 V and flow rate of 1 mL/min, the extract of dark chocolate provided an oxidation peak whose height increased with higher volume fractions of MeOH (up to 30 %) in the phosphate buffer (**Fig. 3**) and this was taken as an optimum for further measurements.

Retaining the detection potential constant throughout the analysis is of critical significance for the application of amperometric sensing. After injection of the chocolate extract into the flowing carrier solution, an evident increase in the current response became clear for potentials greater than +0.7 V, whereas setting at higher potential values triggered only a small increase in the current response. However, a significant increase in the baseline current response was observed at detection potentials greater than +1.4 V and thus the optimal value of +1.4 V was chosen for preventive purposes.

The carrier solution flow rate was also the important FIA working parameter to be optimised as it specifies the duration of reducing agents (polyphenols) in the column where their electrochemical oxidation takes place. The flow rate of

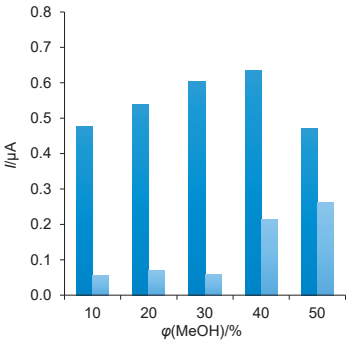

Fig. 3. Effect of MeOH volume fraction in phosphate buffer on the current response of the extract of dark chocolate with *w*(cocoa)=80 %. Results were recorded on boron-doped diamond electrode in FIA mode at a flow rate of 1 mL/min and detection potential of +1.3 V. The light blue bars indicate the baseline current responses

0.2 to 1.6 mL/min for 50 mg/L Trolox was investigated at the fixed potential of +1.3 V. A sharp rise in peak height was seen up to 1 mL/min, while a constant current response was observed above that flow rate. Therefore, a flow rate of 1 mL/min was chosen as optimum.

Analytical method validation

First, it is necessary to note that the presented contribution is not an introduction of a newly developed analytical method for TEAC determination of chocolate extracts, but an initial study to find out whether TEAC values can be used as a marker for cocoa content in chocolate samples. However, a simple validation of FIA with amperometric detection at BDDE had to take place.

Precision, defined as the level of agreement of repeated measurements, was determined as relative standard deviation (RSD) of five analyses (injections). For example, RSD values of 3.3 and 3.8 % for milk chocolate (30 % cocoa) and dark chocolate (50 % cocoa) extracts, respectively, were calculated. If significance level of 5 % (α=0.05) is taken into account, satisfactory precision can be obtained.

As shown in **Fig. 4**, the dependence of height of oxidation current on Trolox concentration was studied for calibration range from 5 to 160 mg/L. A calibration range from 5 to 100 mg/L Trolox was described by the following equation:

$$I=0.04859+0.0233c \quad R^2=0.9994 \qquad /1/$$

where 0.04859 is a slope characterising the sensitivity, 0.0233 is y-intercept, and *c* is the concentration of the standard (Trolox). This linear behaviour between Trolox concentration and peak current response can be applicable for analytical purpose. If concentrations higher than 100 to 160 mg/L Trolox are included into calculations of linear regression, the following equation will be obtained:

$$I=0.05736+0.0211c \quad R^2=0.9954 \qquad /2/$$

where 0.05736 is the slope, and 0.0211 is y-intercept. Due to the high value of the intercept, it was not possible to use the

method of standard addition, and therefore method of calibration curve was preferred. Limit of detection (LOD) and limit of quantification (LOQ) of 1.4 and 4.6 mg/L Trolox, respectively, were calculated according to the formulae:

$$LOD=3s/k \quad /3/$$

and

$$LOQ=10s/k \quad /4/$$

where 3 and 10 are statistically recommended multiples of the baseline noise, s represents the standard deviation of five repetitive measurements of 5 mg/L Trolox and k is the slope of linear regression (0.0233).

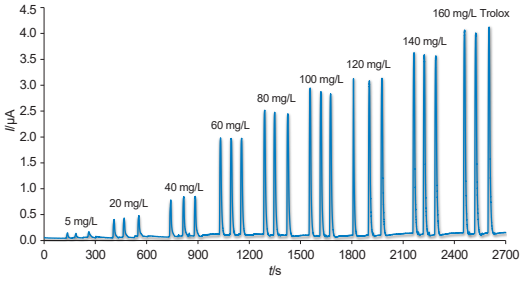

Fig. 4. Typical amperograms of flow injection analysis recorded on boron-doped diamond electrode within calibration measurements at optimum working conditions (phosphate buffer with φ(MeOH)=30 %, flow rate of 1 mL/min and detection potential of +1.3 V)

Analysis of chocolate samples

Extracts of white chocolate (0 % cocoa), two samples of milk chocolate (30 % cocoa), and three dark chocolates (50, 64 and 80 % cocoa) were analysed using FIA at BDDE. Two milk chocolates from different manufacturers with the same cocoa mass fraction were chosen to verify the accuracy of the analysis. **Fig. 5** shows that both extracts of milk chocolates provided comparable current response. In addition, a current response at the limit of detection was obtained for the extract of white chocolate which confirms that this type of chocolate cannot be considered as a rich source of antioxidants.

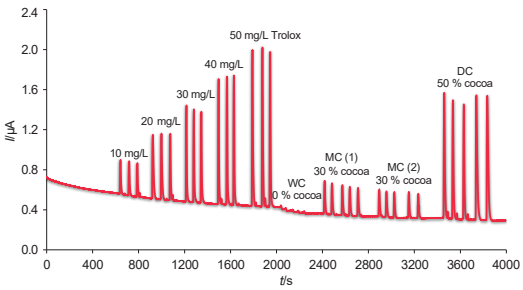

Fig. 5. Typical record obtained during flow injection analysis of white (WC), milk (MC) and dark chocolate (DC) at the boron-doped diamond electrode

Unlike this, the dark chocolate extract samples were diluted twice so that their current responses would not exceed the linear range.

Except for one sample of chocolate with 80 % cocoa (excluded from statistical evaluation), TEAC values (mg Trolox per 100 g sample) increased with higher cocoa mass fraction. The reason why the dark chocolate extract provided the current response like chocolate samples with half the cocoa content has not been further investigated. However, it can be assumed that the manufacturer probably declared false nutritional information.

Fig. 6 shows that TAC presented as TEAC could be considered as additional marker of cocoa content in the chocolate analysis to the commonly used TBR and caffeine (*7*). Moreover, a high positive correlation between the determined TEAC values and cocoa mass fractions characterised by R=0.9187 for eight randomly selected chocolate samples is proof of that. The calculated TEAC values from FIA are in close agreement with those previously reported routine spectrophotometric assays that are usually based on the reaction of antioxidants with a colour radical (*26,27*).

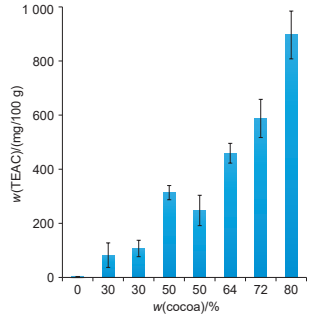

Fig. 6. Trolox equivalent antioxidant capacity (TEAC) of white (0 %), milk (30 %), and dark (50-80 % cocoa mass fraction) chocolates obtained using the flow injection analysis with integrated boron-doped diamond electrode

CONCLUSIONS

The boron-doped diamond electrode integrated in the flow injection analysis (FIA) system could represent a simple analytical tool for evaluation of chocolate quality by determining its cocoa content. This basic study represents the first step in the development of a simple analytical method for determination of cocoa content as a source of polyphenols and other potential antioxidants (reducing agents). It is expected that the analyses of more chocolate samples containing different cocoa powder mass fractions and comparisons with measured total phenolic content as Trolox equivalents will be the subjects of the upcoming investigations. The developed FIA will find application in the food quality control if the presented assumption is confirmed.

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CONFLICT OF INTEREST

All authors declare that they do not have any known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHORS' CONTRIBUTION

T. Arbneshi evaluated the measured data. A. Frangu carried out all laboratory measurements. M. Frühbauerová prepared samples of chocolates for FIA analysis. L. Červenka made the final correction of the English language. L. Berisha constructed an electrochemical flow cell. K. Kalcher designed all steps leading to the development of the present electroanalytical method. M. Sýs coordinated the work of the whole scientific team and wrote the manuscript.

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PUBLICATIONS

Publications in international journals with IF:

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