Biological activity, antioxidant capacity and volatile profile of enriched Slovak chocolates

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Summary
Different kinds and types of chocolates produced in Slovakia with various fruit and nut enrichments were analysed regarding total polyphenols, flavonoids and phenolic acids contents using spectrophotometric methods and their volatile profile was analysed using gas chromatography–mass spectrometry. The method with 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing power method (FRAP) were used to measure antioxidant activity. Plain dark chocolate without any enrichment made by a traditional process contained the highest number of total polyphenols and phenolic acids and also had the highest antioxidant activity. Positive correlation was observed between antioxidant activity and total contents of phenolic compounds, flavonoids and phenolic acids. The enrichment with different kinds of fruits did not increase the number of bioactive compounds or antioxidant capacity of chocolates but created more attractive sensory profiles due to the presence of new volatile compounds.

Keywords
antioxidant activity; enrichment; flavonoids; phenolic acids; polyphenols; pyrazines

In the ancient past, chocolate was appreciated by soldiers as an energy booster and for its mystical properties as well as medicinal effects, including being extremely helpful as an expectorant, a diuretic, an aphrodisiac and to treat the stomach weakness [1]. Nowadays, chocolate, although enjoyed by many for its flavour and textural properties, is typically considered only as a non-nutritional confectionery food that provides only energy and fats. However, certain chocolates can provide a non-negligible amount of various essential nutrients [2]. Cocoa exhibits the highest flavanol content, on weight basis, from all foodstuffs and is therefore considered a significant contributor to the total dietary flavonoid intake. For many consumers then cocoa-derived products constitute a larger proportion of the diet than foods containing bioactive compounds with similar properties, such as green tea or red wine [3]. In spite of the high content of naturally occurring bioactive substances in chocolates, a great number of various functional chocolates was developed because consumers became more interested in healthy lifestyle and nutrition, but only few attempts were made to produce chocolates enriched with naturally derived bioactive compounds [4].

The pleasant flavour is, in addition to taste, certainly the most important sensory attribute of chocolate because it determines the acceptance and leads the preference for the product [5]. The flavour profile is, however, mostly dependent on what happens to the beans during chocolate

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processing: the aroma precursors are developed during the post-harvest treatments and turned into the desirable odour notes during the manufacturing process [6]. To the best of our knowledge, up to this day, there was no such detailed study on the bioactive compounds and volatiles of Slovak chocolates. Therefore, the aim of this study was to evaluate the biological activity, including antioxidant activity and the volatile profiles of chocolates available on the Slovakian market.

MATERIALS AND METHODS

Samples

Nine different commercial plain or enriched dark, milk and white chocolates (marked as S1–S9) originating from two Slovak chocolate manufactures (Happylife, Ivanka pri Dunaji and Lyra Chocolate, Ivanka pri Nitre) were sampled directly from the market or from the producer. Each chocolate sample was sampled in triplicate from three different batches. Their main characteristics and formulation are presented in Tab. 1.

Chemicals

All chemicals were of analytical grade and were purchased from CentralChem (Bratislava, Slovakia) or Sigma Aldrich (St. Louis, Missouri, USA).

Ash, dry matter and moisture content

Ash content was determined gravimetrically after calcination of 3 g of each sample in a muffle furnace at (900 ± 50) °C for 4 h.

The dry matter content was determined by drying 5 g of the sample at (130 ± 2) °C for 60 min in a drying oven (WTB, Binder, Germany).

Moisture content was then calculated from the dry matter content.

Preparation of sample extracts

All samples were grated into small pieces with a domestic stainless steel grater. The step of lipid elimination from samples was not applied due to the possibility of loss in powders or fat as the only cocoa component during the process in samples of white chocolates. Although lipids from chocolates were removed in most of previous studies, some of them worked with whole chocolates [7]. Then, 0.25 g of a homogenized sample was extracted with 20 ml of 80% ethanol for 2 h in a laboratory shaker GFL 3005 (GFL, Burgwedel, Germany). After centrifugation at 4 600 ×*g* (Rotofix 32A, Hettich, Germany) for 10 min and subsequent filtration with Whatman Grade 3 qualitative filter paper (Merck, Darmstadt, Germany), the supernatant was used for measurements. All analyses were done in triplicate.

Total phenolic content

Total phenolic content (*TPC*) was measured by the method of Singleton and Rossi [8] using Folin-Ciocalteu reagent. A volume of 100 μl of the sample extract was mixed with 100 μl of the Folin-Ciocalteu reagent, 1 000 μl of 200 g·l^{-1} Na_2CO_3 and 8.8 ml of distilled water. Absorbance at 700 nm was measured using UV/Vis spectrophotometer Jenway 6405 (Jenway, London, United Kingdom) after 30 min. Gallic acid (25–250 mg·l^{-1}; $R^2 = 0.9978$) was used as the standard and the results were expressed as grams of gallic acid equivalents (GAE) per kilogram of chocolate.

Tab. 1. List of ingredients of the samples.

Sample	Type	Enrichment	Cocoa [%]	Composition [%]			
				Cocoa butter	Cocoa powder	Agave syrup	Enrichment
S1	Dark	Sea buckthorn	60	40	20	20	20
S2	Dark	Almond	60	37	23	20	20
S3	Dark	Mulberry	65	40	25	20	15
S4	Dark	Currant	65	40	25	20	15
S5	Dark	Cherry	65	40	25	20	15
S6	Dark	Plain	80	47	33	20	NP

Sample	Type	Enrichment	Cocoa [%]	Composition [%]					
				Cocoa butter	Cocoa mass	Cane sugar	Milk powder	Soya lecithin	Vanilla
S7	White	Plain	40	40	NP	44	15	0.5	0.5
S8	Dark	Plain	70	45	25	29	NP	0.5	0.5
S9	Milk	Plain	37	25	12	47	15	0.5	0.5

NP – not present.

Total flavonoid content

The total flavonoid content (*TFC*) was determined using the modified method of Willet [9]. Briefly, the sample extract (0.5 ml) was mixed with 0.1 ml of 100 g·l^{-1} ethanolic solution of $AlCl_3$, 0.1 ml of 1 mol·l^{-1} sodium acetate and 4.3 ml of distilled water. The absorbance at 415 nm was measured using the spectrophotometer Jenway 6405 after 30 min. Quercetin (0.01–0.50 mg·l^{-1}; R^2 = 0.9977) was used as the standard and the results were expressed as grams of quercetin equivalents (QE) per kilogram of chocolate.

Total phenolic acid content

The total phenolic acid content (*TPAC*) was determined using the method of Farmakopea Polska [10]. Sample extract (0.5 ml) was mixed with 0.5 ml of 0.5 mol·l^{-1} HCl, 0.5 ml Arnow's reagent (100 g·l^{-1} $NaNO_2$ + 100 g·l^{-1} Na_2MoO_4), 0.5 ml of 1 mol·l^{-1} NaOH and 0.5 ml of water. Absorbance at 490 nm was then measured using the spectrophotometer Jenway 6405. Caffeic acid (1–200 mg·l^{-1}, R^2 = 0.9996) was used as a standard and the results were expressed as grams of caffeic acid equivalents (CAE) per kilogram of chocolate.

Antioxidant activity

DPPH$^{\bullet}$ method

Radical-scavenging activity of samples was measured using the method of Sánchez-Moreno et al. [11] with 2,2-diphenyl-1-picrylhydrazyl (DPPH). The sample extract (0.4 ml) was mixed with 3.6 ml of DPPH solution (0.025 g DPPH in 100 ml ethanol). After 10 min of resting in a dark place, the absorbance of the sample extract was determined using the spectrophotometer Jenway 6405 at 515 nm. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 10–100 mg·l^{-1}; R^2 = 0.9881) was used as a standard and the results were expressed as grams of Trolox equivalents (TEAC) per kilogram of chocolate.

ABTS^{+} method

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS^{+}) decolourization was determined using the method of Re et al. [12] with slight modifications. Two millilitres of ABTS^{+} solution were mixed with 0.98 ml of phosphate-buffered saline (PBS, 5 mmol·l^{-1}, pH 7.4) and 0.02 ml of the sample extract. Absorbance was measured using the spectrophotometer Jenway 6405 6 min after addition of the sample extract. Trolox (10–100 mg·l^{-1}; R^2 = 0.9991) was used as a standard and the results were expressed as grams of TEAC per kilogram of chocolate.

Ferric reducing power method

Reducing power of samples was determined by the method of Oyaizu [13]. One milliliter of the sample extract was mixed with 5 ml PBS, pH 6.6 and 5 ml of 10 g·l^{-1} potassium ferricyanide. The mixture was stirred thoroughly and heated in a water bath for 20 min at 50 °C. After cooling to room temperature, 5 ml of 100 g·l^{-1} trichloroacetic acid was added. Then, 5 ml of the mixture was pipetted into the test tube and mixed with 5 ml of distilled water and 1 ml of 1 g·l^{-1} ferric chloride solution. Absorbance was measured using the spectrophotometer Jenway 6405 at 700 nm. Trolox (10–100 mg·l^{-1}; R^2 = 0.9974) was used as a standard and the results were expressed as grams of TEAC per kilogram of chocolate.

Volatile profile and composition analysis

Isolation of aromatic compounds was carried out using a headspace solid-phase microextraction procedure (HS-SPME). A manual SPME holder (Supelco, Bellefonte, Pennsylvania, USA) with divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) fibre (Supelco) was used to extract the volatile substances. Prior to use, the fibre was conditioned in a gas chromatograph injection port at 250 °C for 1 h.

Preparation and extraction of the sample were performed according to Afoakwa et al. [14] with minor modifications. For each extraction, 1 g of homogenized chocolate sample was placed in a 40-ml glass vial with a plastic screw cap and then 15 ml of distilled water were added. Sodium chloride (0.5 g) and 15 μl β-ionone were subsequently added to the sample. β-Ionone was used as an internal standard after checking that it was primarily absent in chocolate, separated well from other volatile substances, was stable at high temperatures and did not react with water. The samples prepared were subsequently heated in a water bath at 50 °C with temperature control and stirring for 70 min, the first 15 min of which were the equilibration of the components in the vial space and during the remaining time, the fibre was introduced and the adsorption of the volatile components to the fibre took place. The SPME fibre was then removed from the vial space and inserted into the gas chromatography–mass spectrometry (GC-MS) injector (230 °C), where it was exposed for 2 min. The separation and identification of volatile compounds were performed on a Shimadzu GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) with a Shimadzu GC-MS QP-5050A mass spectrometer detector. The GC-MS system was equipped with a SLB-5ms GC capillary column column (Supelco; 30 m × 0.25 mm internal dia-

meter, 0.25 μm film thickness). Helium was used as a carrier gas at a flow rate of 0.7 ml·min^{-1}, splitless mode was applied and the following temperature program: (1) initial temperature 40 °C; (2) rate of 2 °C·min^{-1} from 40 °C to 180 °C; (3) a rate of 25 °C·min^{-1} from 180 °C to 300 °C and hold for 1 min. The injector temperature was maintained at 230 °C and the detector temperature at 300 °C.

The compounds were simultaneously identified using three different analytical methods: retention indices (*RI*) with reference to *n*-alkanes (C_8–C_{24}), GC-MS retention times of authentic chemicals and mass spectra (authentic chemicals and spectral library collection NIST05; National Institute of Standards and Technology, Gaithersburg, Maryland, USA). Identification was taken as only tentative if it was based only on mass spectral data.

Quantification of the volatile compounds was performed on a gas chromatograph Shimadzu 2010 with a flame ionization detector (FID). The column and chromatographic conditions were the same as those previously reported for the GC-MS analysis. The injector temperature was 250 °C and N_2 was used as a carrier gas (1 ml·min^{-1}). Data handling was carried out by means of GCsolution 2.3 (Shimadzu).

Statistical analysis

All measurements were carried out in triplicate, if not stated differently. Experimental data were evaluated by basic statistical variability indicators using the Microsoft Excel software (Microsoft, Redmond, Washington, USA). Dependency rate between the tested traits was expressed using the linear correlation analysis. Data were also subjected to Fisher's least significant difference test to compare the means. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Ash, dry matter and moisture content

Ash, which is a part of the proximate analysis for nutritional evaluation, refers to the inorganic residues remaining after ignition or complete oxidation of organic matter in a food sample and consists mainly of the minerals. The content of ash (Tab. 2) ranged from 1.3 % in white chocolate to 2.7 % in the plain dark chocolate. In general, it can be said that chocolate samples enriched with fruits (e.g. sample S4) had a higher ash content than plain ones (e.g. sample S7). The content of dry matter was above 85 % in all tested samples. The sample with the highest moisture content, 14.6 %, was that enriched with cherry fruit (sample S5) and this addition could contribute to this increased value. The moisture content in plain chocolates, irrespective of the type, was below 2 % (samples S7, S8 and S9). Thus, in general, chocolates enriched with fruits had a higher moisture content, which might result in a shorter shelf life.

Total phenolic, flavonoid and phenolic acid contents

Dark chocolate is one of the foods with the highest content of polyphenols and is one of the major contributors to polyphenol intake in the western diet. However, white chocolate does not contain fat-free cocoa solids and, thus, lacks most of the bioactive components [15]. In Tab. 3, *TPC*, *TFC* and *TPAC* of all the evaluated samples are shown.

TPC of Slovak chocolates ranged between (4.74 ± 0.13) g·kg^{-1} and (16.25 ± 0.53) g·kg^{-1} (expressed as GAE). The lowest *TPC* values were found in samples S5 and S7, which were dark chocolate with cheery addition and white chocolate, respectively. The final *TPC* of chocolate depends on many factors related to the raw material, processing methods and storage as well, because natural compounds are easily degradable due to their sensitivity to high temperatures, presence of light and oxygen and different levels of pH [15]. These changes resulting from the differences in raw materials or processing methods can be seen when studying the experimental results reported in Tab. 3.

Sample S6, plain dark raw chocolate with 80 % cocoa solids, should have contained more bioactive substances than sample S8, plain dark chocolate with 70 % cocoa solids made by traditional process comprising steps using high temperatures according to Cerit et al. [7] who claimed that the cocoa solids content determines the amount of polyphenols in chocolates. However, our findings did not support this claim, which might be connected with the fact that several of the steps of chocolate processing significantly affected the content of biologically active compounds in final products [16]. All the samples enriched with fruits exhibited lower content of bioactive compounds in comparison to the plain dark chocolate (sample S8) or only slightly higher than plain dark raw chocolate (sample S6). Moreover, the content of all polyphenols in chocolate can vary tremendously not only between cocoa-containing final products but also depending on the source of the beans, the processing conditions and how the chocolates are manufactured [17].

TFC ranged from (0.19 ± 0.04) g·kg^{-1} to

(1.38 ± 0.00) g·kg^{-1} (expressed as QE). The highest value of this parameter, (1.38 ± 0.00) g·kg^{-1}, was found in the sample of plain milk chocolate. However, SERAFINI et al. [18] found out that milk ingredients due to the formation of secondary bonds between chocolate flavonoids and milk proteins may interfere with the absorption of antioxidants from chocolate in vivo and may, therefore, negate the potential health benefits that can be derived from eating moderate amounts of dark chocolate.

Time and temperature, as well as manufacturing processes such as alkalization, can be detrimental to the flavonoid content in chocolate but proper processing and manufacturing methods used in production can result in a cocoa product that can contain as much as 10 % flavonoids on a dry-weight basis [2]. From the groups of naturally occurring antioxidants in chocolate, especially flavonoids are getting lost during cocoa processing [19].

Also dark chocolate samples enriched with mulberry (sample S3) and sample enriched with sea buckthorn (sample S1) exhibited high values of *TFC*, possibly due to the very high flavonoid content in these two fruits, where flavonoids also belong to the main active components [20]. On the other hand, dark chocolate enriched with almonds exhibited the lowest amount of flavonoids, (0.19 ± 0.04) g·kg^{-1} (expressed as QE), despite the fact that almond polyphenols are mainly composed of tannins and flavonoids, but almonds themselves have only moderate *TPC*.

Phenolic acids are secondary metabolites of plants and fungi and are produced as a protection against UV light, insects, viruses and bacteria or to inhibit the growth of other plant competitors. The most abundant phenolic acid (69.2 %) found in cocoa liquor is protocatechuic acid followed by coumaric acid or hydroxy derivatives of cinnamic acid [21].

TPAC determined for chocolates in this study ranged from (3.21 ± 0.00) g·kg^{-1} to (15.63 ± 0.22) g·kg^{-1} (expressed as CAE). It was estimated that the sample of white chocolate (sample S7) with a lack of cocoa solids will exhibit very low content of phenolic acids compared to other samples. Although it was reported that phenolic compounds bind to a variety of proteins and this can result in the reduction in their accessibility [22], sample S7 had the second highest TPAC among the samples examined. ZHOU [23] isolated casein from a white chocolate and detected no phenolic acids there, so they concluded that these compounds in white chocolate are particularly associated with the cocoa butter or other ingredients rather than with casein. On the other hand, DIMATTIA et al. [19] claimed that the content of the minor phenolic compounds, such as phenolic acids, is usually low and its contribution to total antioxidant activity is only limited.

Tab. 2. Content of ash, dry matter and moisture in the samples.

Sample	Ash [%]	Dry matter [%]	Moisture [%]
S1	2.2^{ac}	90.8^{abd}	9.2^{a}
S2	2.3^{abc}	91.1^{abcd}	8.9^{a}
S3	2.4^{ab}	90.0^{a}	10.0^{ab}
S4	2.4^{ab}	89.3^{a}	10.7^{b}
S5	2.2^{ac}	85.4^{a}	14.6^{d}
S6	2.7^{b}	91.3^{abcd}	8.7^{a}
S7	1.3^{d}	98.4^{bcd}	1.6^{c}
S8	2.0^{c}	98.9^{cd}	1.1^{c}
S9	1.6^{d}	98.2^{d}	1.8^{c}

Values represent mean (n = 3). Values followed by the same letter in superscript within the same column were not significantly different (p < 0.05), according to Fisher's test.

Tab. 3. Total phenolic, flavonoid and phenolic acid contents in the samples.

Sample	*TPC* [g·kg^{-1}]	*TFC* [g·kg^{-1}]	*TPAC* [g·kg^{-1}]
S1	8.07 ± 0.62^{b}	1.17 ± 0.06^{ab}	6.64 ± 0.25^{cd}
S2	8.69 ± 1.69^{b}	0.19 ± 0.04^{b}	6.42 ± 0.12^{d}
S3	8.83 ± 1.29^{b}	1.30 ± 0.11^{a}	9.51 ± 0.20^{b}
S4	8.65 ± 0.84^{b}	0.27 ± 0.04^{b}	7.22 ± 0.18^{c}
S5	4.74 ± 0.13^{c}	0.37 ± 0.21^{b}	3.21 ± 0.00^{f}
S6	7.32 ± 1.02^{bc}	0.52 ± 0.25^{b}	6.68 ± 0.29^{cd}
S7	4.87 ± 0.89^{c}	1.05 ± 0.06^{a}	14.86 ± 0.68^{e}
S8	16.25 ± 0.53^{a}	0.99 ± 0.00^{a}	15.63 ± 0.22^{a}
S9	7.67 ± 0.40^{bc}	1.38 ± 0.00^{a}	6.21 ± 0.31^{d}

Values represent mean ± standard deviation (n = 3). Values followed by the same letter in superscript within the same column were not significantly different (p < 0.05), according to Fisher's test.
TPC – total phenolic content expressed as gallic acid equivalent, *TFC* – total flavonoid content expressed as quercetin equivalent, *TPAC* – total phenolic acids content expressed as caffeic acid equivalent.

Antioxidant activity

A standardized method for the determination of antioxidant activity of certain foods has not yet been established. Therefore, it is highly advisable to use at least two or more methods for comprehensive information about this property [24]. Antioxidant activity measured using DPPH$^{\bullet}$ assay (expressed as TEAC) varied from (0.95 ± 0.06) g·kg^{-1} for white chocolate (sample S7) to (6.19 ± 0.71) g·kg^{-1} for plain dark 70 % chocolate (sample S8; Tab. 4).

The same trend was observed for the antioxidant activity measured by the ABTS^{+} assay, with

the lowest and highest values being found for samples S7 and S8, respectively. The results of the FRAP assay again agreed with this trend. This may be also due to the fact that the Maillard reaction can contribute to the formation of different reducing substances whose reducing power is increasing the antioxidant effect [24]. The Maillard reaction is associated not only with formation of various compounds exhibiting antioxidant properties but also with the improvement of flavour and the sensory appeal.

Current results agreed with the finding of Medeiros et al. [25] who found the highest antioxidant activity measured by DPPH in dark chocolate followed by milk chocolate, although they did not study any sample of white chocolate. Similar results were also found by Vicente et al. [26] who used $DPPH^{\bullet}$ and $ABTS^{+}$ assays and concluded that the antioxidant activity of chocolate was mainly influenced by its cocoa solid content. According to DiMattia et al [19], the most significant loss in the antioxidant activity was, however, observed in the final steps of chocolate processing, but that could be caused by the addition of other ingredients with antagonistic effects against antioxidant activity. The obtained results may also be explained by possible interactions or formation of complexes of polyphenolic compounds with proteins, polysaccharides and/or alkaloids, as well as by low selectivity of the analytical methods or interference very often occurring in colourimetric assays.

Even if the antioxidant activity of chocolates is reduced due to a loss of bioactive compounds during the production process, it is still a better source of dietary antioxidants than tea or red wine [27]. A strong positive correlation was found between antioxidant activity and the contents of bioactive compounds: $ABTS^{+}$ vs *TPC* ($R^2 = 0.97$), $ABTS^{+}$ vs *TPA* ($R^2 = 0.93$) and FRAP vs *TPAC* ($R^2 = 0.97$). These results clearly demonstrated that specific phenolic compounds are directly related to the antioxidant activity in cocoa and its derivative products.

Tab. 4. Antioxidant activity of the samples.

Sample	$DPPH^{\bullet}$ [g·kg^{-1}]	$ABTS^{+}$ [g·kg^{-1}]	FRAP [g·kg^{-1}]
S1	4.10 ± 0.16^{bc}	18.38 ± 2.37^{b}	11.08 ± 0.23^{de}
S2	3.88 ± 0.33^{bc}	21.69 ± 9.60^{b}	11.13 ± 0.03^{e}
S3	4.39 ± 0.38^{bc}	18.11 ± 2.77^{bc}	16.79 ± 0.23^{b}
S4	4.02 ± 0.09^{bc}	19.06 ± 2.91^{bc}	14.29 ± 0.08^{c}
S5	1.80 ± 0.77^{d}	6.88 ± 1.83^{bc}	5.72 ± 0.23^{f}
S6	3.64 ± 0.42^{c}	19.80 ± 0.81^{b}	13.35 ± 0.36^{cd}
S7	0.95 ± 0.06^{d}	4.25 ± 0.54^{c}	1.18 ± 1.81^{g}
S8	6.19 ± 0.71^{a}	44.28 ± 4.73^{a}	34.06 ± 0.82^{a}
S9	5.26 ± 0.12^{ab}	12.02 ± 0.34^{bc}	10.87 ± 0.13^{e}

Values represent mean ± standard deviation ($n = 3$). Values followed by the same letter in superscript within the same column were not significantly different ($p < 0.05$), according to Fisher's test.
DPPH – 2,2-diphenyl-1-picrylhydrazyl, ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP – ferric reducing power. Results are expressed as Trolox equivalents.

Volatile compounds

Tab. 5 shows the 74 volatile compounds found in the chocolate samples. The volatile compounds isolated, identified and quantified in the volatile profile of chocolates can be grouped into 11 chemical families: alcohols (12 compounds), aldehydes (13 compounds), alkane hydrocarbons (7 compounds), aromatic hydrocarbons (2 compounds), carboxylic acids (3 compounds), disulfides (1 compound), esters (8 compounds), ethers (1 compound), pyrazines (8 compounds), ketones (7 compound) and monoterpenes (12 compounds). Up to this day, approximately 600 volatiles have been identified in cocoa flavour. Different chocolate types may exhibit various and specific flavours because the content of these compounds can vary significantly [28].

The most abundant alcohols were phenethyl alcohol and 2-heptanol, followed by 2,3-butanediol isomers. The content of phenethyl alcohol was the highest in the sample of white chocolate, 124.98 mg·kg^{-1}, and alcohols represented as much as 22.0 % of all volatile compounds identified in this sample. In all the samples, two isomers of 2,3-butanediol were identified, with the exception of sample S7 where only one of the isomers was found. This compound has a cocoa butter flavour and confers desirable flavour to chocolates. Phenethyl alcohol and 2,3-butanediol have been reported to be produced during cocoa fermentation by yeasts such as *Kloeckera apiculata* and *Saccharomyces cerevisiae* var. *chevalieri* [29]. Ascrizzi et al. [5] concluded that high contents of volatile alcohols are desirable to obtain chocolate products with candy and flowery notes.

The most abundant group was aldehydes and, within it, benzaldehyde was the most abundant with content ranging from 41.07 mg·kg^{-1} to 2099.69 mg·kg^{-1}. Benzaldehyde was found to be present in raw cocoa beans and is known to contribute to a beany almond-like odour [30]. The highest content of aldehydes, 2290 mg·kg^{-1}, was found in sample S2 containing almonds, where benzaldehyde can also be expected to be a dominant aldehyde. It is a natural compound present in almonds and, therefore, it is possible to observe

Tab. 5. Content of volatile compounds and retention indeces of chocolates.

Compound	*RI*		Content [mg·kg^{-1}]								
	EXP	LIT	S1	S2	S3	S4	S5	S6	S7	S8	S9
Alcohols											
Ethanol	490	482	1.50	4.60	1.55	0.76	0.90	0.67	0.40	1.48	0.78
2-Pentanol	698	703	t	129.26	ND	ND	ND	ND	ND	ND	ND
2,3-Butanediol (isomer 1)	775	782	22.90	31.19	12.05	23.22	12.40	36.97	2.91	119.04	9.90
1,3-Propanediol	788	793	ND	ND	ND	ND	ND	ND	ND	ND	4.46
2,3-Butanediol (isomer 2)	788	782	34.19	30.02	13.22	17.98	5.77	23.45	ND	52.67	16.39
Furfuryl alcohol	845	844	ND	ND	ND	ND	ND	ND	19.55	17.97	16.28
2-Heptanol	888	882	ND	146.26	3.74	8.53	13.93	11.40	94.30	57.60	36.91
1-Octen-5-ol	976	983	ND	ND	ND	ND	ND	ND	4.04	ND	2.73
Benzyl alcohol	1 032	1 031	ND	24.79	ND	ND	21.30	5.44	ND	28.72	60.24
2-Nonanol	1 091	1 098	ND	ND	ND	ND	ND	ND	57.39	42.91	16.52
Phenethyl alcohol	1 110	1 109	15.67	84.40	7.20	31.15	44.64	37.93	90.54	124.98	42.58
2-Butyl-1-octanol	1 290	1 277	ND	ND	ND	6.01	ND	ND	ND	ND	ND
Aldehydes											
Isobutyraldehyde	542	540	1.22	ND	1.45	ND	1.08	2.35	ND	3.70	1.18
3-Methylbutanal	628	621	11.25	31.63	24.69	ND	22.18	30.54	ND	54.00	10.57
2-Methylbutanal	640	640	6.27	13.32	15.88	14.54	14.31	26.09	ND	47.09	10.23
Hexanal	791	800	ND	ND	ND	ND	11.02	ND	5.68	ND	ND
Furfural	822	825	32.73	ND	ND	64.35	77.41	ND	ND	12.78	3.51
(*E*)-2-Hexenal	843	847	ND	ND	3.71	ND	ND	ND	ND	ND	ND
Heptanal	898	896	6.47	ND	ND	ND	ND	ND	ND	ND	ND
Methional	902	905	4.85	ND	ND	ND	ND	ND	ND	ND	ND
2-Heptenal	951	954	ND	ND	ND	ND	ND	13.03	ND	19.21	ND
Benzaldehyde	955	960	120.60	2 099.69	114.12	127.78	173.98	121.02	41.07	195.23	78.27
Hyacinthin	1 039	1 045	60.84	105.72	91.80	111.18	110.03	111.41	ND	235.27	27.04
Nonanal	1 103	1 102	56.59	39.28	28.39	21.12	40.30	26.90	ND	23.14	19.93
2-Phenyl-2-butenal	1 263	1 270	ND	ND	ND	ND	ND	ND	7.20	ND	ND
Alkane hydrocarbons											
n-Hexane	596	600	86.23	167.60	51.30	45.44	44.10	59.50	90.23	37.29	37.48
Decane	997	1 000	ND	ND	ND	ND	ND	ND	ND	ND	20.28
2-Methyldecane	1 060	1 065	ND	ND	ND	ND	12.30	ND	ND	ND	ND
Undecane	1 098	1 100	ND	ND	ND	ND	ND	ND	ND	ND	34.77
Dodecane	1 197	1 200	ND	ND	ND	ND	ND	ND	8.16	15.25	19.61
Tetradecane	1 398	1 400	ND	ND	ND	6.22	ND	ND	ND	ND	ND
Hexadecane	1 599	1 600	ND	ND	ND	4.82	ND	ND	ND	ND	ND
Aromatic hydrocarbons											
Toluene	748	759	ND	ND	3.88	ND	ND	2.96	3.89	4.69	3.34
p-Cymene	1 020	1 025	19.16	24.23	13.58	10.05	6.30	4.27	3.41	9.13	18.09
Carboxylic acids											
Acetic acid	584	594	563.30	278.59	114.33	802.92	351.28	194.04	ND	397.24	34.57
Propanoic acid	732	729	ND	ND	ND	2.88	ND	ND	ND	ND	ND
Isovaleric acid	863	857	ND	13.82	ND	ND	ND	ND	ND	ND	ND
Disulfides											
Dimethyldisulfide	739	747	3.29	ND	ND	ND	ND	ND	ND	5.46	ND
Esters											
Isoamyl acetate	865	866	ND	ND	ND	ND	ND	ND	30.37	ND	ND
2-Heptyl acetate	1 039	1 043	ND	ND	ND	ND	5.15	2.47	21.53	18.42	5.39
Isoamyl isovalerate	1 103	1 105	100.52	ND	ND	ND	ND	ND	ND	ND	ND
Benzyl acetate	1 160	1 170	ND	ND	ND	6.25	6.82	ND	13.23	9.94	7.75
Ethyl octanoate	1 190	1 196	ND	ND	ND	ND	4.29	ND	13.75	13.05	6.49
Phenylethyl acetate	1 251	1 250	8.04	ND	ND	9.18	17.35	15.02	50.24	49.85	19.55
Isoamyl benzoate	1 425	1 430	ND	ND	ND	ND	7.82	ND	10.87	6.44	5.52
Octyl hexanoate	1 581	1 570	ND	ND	ND	6.39	ND	ND	ND	ND	ND

Tab. 5. *continued*

Compound	*RI*		Content [mg·kg^{-1}]								
	EXP	LIT	S1	S2	S3	S4	S5	S6	S7	S8	S9
Ethers											
Vinyl methyl ether	514	TI	1.12	2.87	ND	ND	ND	ND	1.05	1.67	0.71
Pyrazines											
Methylpyrazine	818	819	ND	ND	ND	ND	ND	ND	ND	11.11	ND
2,3-Dimethylpyrazine	908	911	ND	ND	ND	ND	ND	ND	ND	21.63	ND
2,5-Dimethylpyrazine	916	915	ND	17.80	ND	2.99	6.52	ND	10.18	41.85	6.53
2-Ethyl-6-methylpyrazine	990	995	ND	ND	ND	ND	4.30	2.32	18.23	17.04	ND
2,3,5-Trimethyl pyrazine	1 001	1 000	38.31	93.75	26.86	32.11	43.70	37.03	79.35	120.58	36.89
2,6-Dimethyl-3-ethylpyrazine	1 075	1 080	ND	ND	ND	6.22	8.30	7.54	14.82	23.58	6.75
2,3,5,6-Tetramethylpyrazine	1 082	1 085	105.75	287.51	65.54	95.54	149.28	128.40	220.21	316.72	74.55
2,3,5-Trimethyl-6-ethylpyrazine	1 155	1 163	ND	ND	ND	7.59	8.81	7.88	8.35	29.91	ND
Ketones											
Acetoin	708	711	ND	ND	ND	ND	ND	ND	ND	13.36	ND
2-Heptanone	883	889	ND	27.37	ND	ND	ND	ND	37.14	16.74	15.10
2-Acetylfuran	902	910	7.17	ND	ND	7.40	6.97	ND	ND	13.29	ND
Acetophenone	1 045	1 042	ND	ND	ND	ND	6.32	ND	6.59	ND	ND
2-Acetylpyrrole	1 061	1 060	10.45	ND	5.26	8.43	14.01	11.05	12.36	22.44	8.02
2-Nonanone	1 089	1 091	4.95	17.36	ND	3.74	9.48	6.67	64.62	47.53	30.79
2-Decanone	1 189	1 190	ND	ND	ND	ND	ND	ND	ND	ND	3.35
Monoterpenes											
α-Thujene	919	925	ND	ND	ND	ND	ND	ND	ND	ND	3.37
α-Pinene	927	932	ND	ND	ND	ND	ND	ND	6.27	ND	7.97
β-Pinene	960	964	8.35	16.30	7.22	3.22	ND	ND	5.30	ND	12.87
Myrcene	978	984	21.28	32.51	12.70	15.22	17.01	5.70	26.04	15.14	23.62
δ-3-Carene	1 010	1 005	ND	ND	ND	ND	ND	ND	ND	ND	8.94
α-Terpinene	1 018	1 014	ND	ND	ND	ND	ND	ND	ND	5.83	ND
Limonene	1 025	1 028	329.75	445.99	249.18	164.68	94.08	65.29	60.16	102.29	298.93
(*E*)-β-Ocimene	1 035	1 037	ND	ND	ND	ND	5.44	ND	27.83	ND	ND
γ-Terpinene	1 053	1 062	21.06	30.44	15.19	11.82	8.51	6.87	11.81	17.00	17.36
(*Z*)-Linalool oxide	1 083	1 072	13.46	ND	6.79	6.61	5.76	4.71	6.53	ND	ND
Linalool	1 097	1 098	42.02	76.71	25.41	35.43	53.27	40.02	35.33	34.69	ND
Nerol	1 226	1 231	ND	ND	7.76	17.55	ND	ND	ND	ND	ND

RI – retention index, EXP – experimental, LIT – literature (database NIST05; National Institute of Standards and Technology, Gaithersburg, Maryland, USA), ND – not detected, TI – tentatively identified.

its highest content in the sample enriched with this ingredient. In this sample, benzaldehyde represented up to 49.1 % of all volatile compounds, while its content in other samples was lower than 12 %.

Two compounds belonging to the group of aromatic hydrocarbons were identified, namely, p-cymene and toluene. *p*-Cymene, found in all samples, is an important product of the chemical industry used as an additive in fragrances and musk perfumes, and as a masking odour for industrial products [31], not described in chocolates yet. Toluene was previously found in chocolate [14].

Three carboxylic acids were identified in chocolate samples. Acetic acid develops during fermentation of cocoa beans and its content decreases during roasting and conching [30], but it is commonly found in chocolates [14, 29] being often considered as an undesirable compound. It was present in all samples except for the sample of white chocolate (S7). Sample S4 enriched with currants exhibited the highest content of acetic acid (46.2 % of all present volatile compounds). Only in this sample (S4), propanoic acid was also present. Isovaleric acid (0.3 %) was also found in sample S2. It was previously found in chocolate [32] and is judged as unpleasant with a sweat smell.

From the group of disulfides, only one compound was identified, namely, dimethyl disulfide, which was present in a sample enriched with sea buckthorn and in plain dark chocolate. This com-

pound was previously found in chocolate [30] and has a cabbage odour [33].

Esters, together with aldehydes and ketones, confer a fruity aroma to the final cocoa product and are quite stable along the processing chain [5]. Roasting does not cause losses in the content of esters. However, esters were not identified in all the evaluated samples. Samples S2 and S3 lacked any of these compounds. On the other hand, the highest ester content and a more complex profile (more compounds) was found in sample 7 (white chocolate), where they represented almost 12 % of the total volatile content. The dominant ester was phenylethyl acetate, which has honey and flowery odour notes. According to Rodriguez-Campos et al. [29], it is beneficial for the aromatic quality of cocoa to contain high amounts of 2-phenylethyl acetate and ethyl phenyl acetate due to the flavour notes associated with them. A high amount of isoamyl isovalerate was found in sample S1 (101 $mg \cdot kg^{-1}$), while in other samples this compound was not present. It is one of the most abundant esters in sea buckthorn, which was an ingredient of this sample.

Pyrazines belong to main volatile compounds of the cocoa aroma [32]. The most representative compound of this group was 2,3,5,6-tetramethylpyrazine, which was the dominant pyrazine in all the studied samples. Sample S8 contained the highest amount of heterocyclic aromatic compounds of all samples examined and 2,3,5,6-tetramethylpyrazine was the dominant compound as well. This could be due to the fact that this sample was the only one containing acetoin, which is considered to be a precursor of tetramethylpyrazine [29]. Eight different heterocyclic aromatic compounds were identified, among which methyl-, 2,3-dimethyl-, 2,5-dimethyl-, 2,3,5-trimethyl-, 2,6-dimethyl-3-ethyl- and 2,3,5,6-tetramethylpyrazines were reported as having strong chocolate flavour with cocoa/roasted/nutty notes, while 2,3,5-trimethyl-6-ethylpyrazine contributed to sweet and candy notes [14]. Even if 2,3,5,6-tetramethylpyrazine and 2,3,5-trimethylpyrazine were the most significant compounds from this group, approximately 80 different pyrazines can contribute to the overall cocoa flavour [14]. In some cases, 2,3,5,6-tetramethylpyrazine can represent approximately 90 % of total pyrazines [29], because it is already present in the raw unroasted cocoa beans.

The dominant ketone was 2-nonanone, which was found in all samples except sample S3. It can be described as having a floral or fatty odour [33]. Acetophenone, which has a flower, almond or sweet odour, was found only in the sample with cherry and in the sample of white chocolate. Acetoin was only found in sample S8. This compound produces desirable notes in cocoa and acts as a precursor for tetramethylpyrazine [29]. A high content of aldehydes and ketones is favourable for cocoa quality, aldehydes being crucial for the development of a good cocoa flavour [28].

Monoterpenes were present in all the samples. The sample with the highest content (35.1 %) of all compounds was sample S3, which was enriched with mulberry. From all monoterpenes, limonene was the most abundant in all the samples. It was followed by linalool, but this was not present in the sample of milk chocolate, while myrcene was found in all the samples. Besides linalool, also (*Z*)-linalool oxide was found, and the both compounds are reported to be typical for roasted cocoa beans [28].

CONCLUSION

This is the first study evaluating the functionality of Slovak chocolates. Experimental results indicated that they are a good source of dietary polyphenols and antioxidants, which may have beneficial effects on human health, and they are rich in volatile compounds, which contribute to the chocolate sensory quality. On the basis of our findings, it can be concluded that Slovak chocolate can contribute significantly to the daily intake of dietary polyphenols, flavonoids and phenolic acids.

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