



THE COMPARISON OF BIOLOGICAL ACTIVITY OF CHOCOLATES MADE BY DIFFERENT TECHNOLOGICAL PROCEDURES

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ABSTRACT

Chocolate is one of the most consumed delicacies in the world. Nowadays, raw chocolates without vanilla or allergens are getting more attention. The aim of this study was to evaluate and compare the biological activity of different types of chocolate – cold processed chocolate and chocolate made by traditional way. Total content of fat, crude fibre, polyphenols, flavonoids, phenolic acids and methylxanthines – theobromine and caffeine was evaluated. The antioxidant activity was determined by a method using DPPH radical, reducing power method and phosphomolybdenum method. Both evaluated chocolates had similar content of fat and crude fibre, but sample of chocolate made by traditional way probably due to the higher content of cocoa mass had almost two times higher content of total polyphenols, flavonoids and phenolic acids as cold processed chocolate. Also the content of theobromine and caffeine was slightly higher in chocolate made by traditional way. This sample had the highest antioxidant activity – 93.68 mg TEAC.g⁻¹ determined by phosphomolybdenum method, while in the sample of chocolate made by cold processed way was measured value 50.82 mg TEAC.g⁻¹. Similarly, reducing power of chocolate made by traditional way was almost two times higher, but antioxidant activity determined with DPPH method was similar in both samples (3.58 and 3.62 mg TEAC.g⁻¹). The antioxidants and methylxanthines in chocolates determine its potential to be a significant source of biologically active compounds with favorable effects to human health. It can be concluded in this study, that chocolate produced by conventional production technology can have more health-promoting ingredients reserved, but more extensive researches are still needed.

Keywords: chocolate; antioxidant activity; polyphenols; flavonoids; theobromine; caffeine

INTRODUCTION

In recent decades, nutrition research has focused on the investigation of bioactive dietary flavonoids, widely found in many plant-based foods and beverages, in order to elucidate their beneficial properties to human health. Cocoa (*Theobroma cacao* L.) and chocolate products appear to be one of the most promising foods due to their high polyphenol content, which evidently highlights the link with health-promoting properties (Alañón et al., 2016).

Worldwide consumption of chocolate and cocoa-containing products increased by 10% from 2002 to 2010, which might be attributed to consumer economic enhancement and increasing knowledge of potential health benefits derived from cocoa constituents. Chocolate and cocoa-containing products are a good source of non-nutrient bioactive polyphenols with potential health benefits including reduced risk of cardiovascular disease and prebiotic activity (Hu et al., 2016).

Chocolate and cocoa products are a rich source of flavonoids. Flavonoids, naturally occurring polyphenolic compounds present in plant-based foods, represent up to 20% of the compounds present in cocoa beans. Flavanols, and in particular epicatechin, are a subgroup of flavonoids, and are the most common cocoa flavonoids. High levels of flavanols are also found in tea, red wine, and fruits such as

grapes and apples. In addition to cocoa flavanols, other psychoactive components of chocolate include the methylxanthines (caffeine and theobromine), both of which have been associated with improving alertness and cognitive function. One hundred grams of dark chocolate contain approximately 100 mg of flavanols, while 100 g of unsweetened cocoa powder without methylxanthines can contain up to 250 mg of flavanols (Crichton et al., 2016).

Chocolate represents functional properties due to its high level of flavonoid content, namely catechins and procyanidins, and beneficial impacts of chocolate consumption on human health. However, consumers are becoming more demanding in food market and they would like to have more options to choose from than ever before. Therefore, manufacturers desire to broaden their product ranges such as having organic chocolate, high-cocoa polyphenol-rich chocolate, probiotic chocolate, and prebiotic chocolate rather than ordinary chocolate. It was also showed that dark chocolate ensured a high probiotic survival rate (Gültekin-Özgüven et al., 2016).

Therefore the aim of this study was to determine the biological activity of selected types of commercially available chocolates and evaluate their antioxidant activity, amount of total polyphenols, flavonoids, phenolic acids and main methylxanthines – theobromine and caffeine.

MATERIAL AND METHODOLOGY

Biological material

The chocolates evaluated in this study were purchased from local market and signed as sample 1 and 2. Sample 1 was Italian raw chocolate named „*Ciocolatino biologico 75% cacao con zucchero da fiori di palma da cocco*“, produced in town Modica, in Sicily (Italy). It contains 75% of cocoa solids and the temperature throughout the process never exceeds 45 °C. Sample 2 was Slovakian traditionally processed chocolate named „*Bean to Bar Dark 78%*“, with 78% cocoa solids. Both samples were made only from cocoa mass and coconut flower sugar and both were organic and distributed by same company.

Chemicals

All chemicals were analytical grade and were purchased from CentralChem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

Samples were homogenized in a mortar and then defatted with petroleum ether. Defatted sample – 0.25 g of was extracted with 20 mL of 80% ethanol for 2 hours in a shaker (GFL 3005, Germany). After centrifugation at 4000 RPM (Rotofix 32A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, total polyphenols, flavonoids, phenolic acids, theobromine and caffeine – each analysis was carried out in ten replicates).

Determination of fat content

Fat content was determined by Fat extractor Ancom XT15 (ANKOM Technology, New York, USA) with the methodology recommended by the producer. The sample (1.5 g, W_1) was weighted to special filter bag (XT4, Ancom, USA) and dried for 3 hours in an oven (WTB, Binder, Germany) at 102 °C to remove moisture prior to the extraction. Samples were placed in a desiccant pouch for 15 minutes and after re-weighted (W_2) and extracted 60 minutes at 90 °C with petroleum ether. After process samples were removed and dried in an oven at 102 °C for 30 minutes, placed in desiccant pouch and re-weighted (W_3). The analysis was carried out in duplicate. Fat content was calculated by following formula:

$$\text{Fat content (\%)} = \frac{100 \times (W_2 - W_3)}{W_1}$$

Determination of crude fiber content

Dietary fiber content was determined with Ancom 200 Fiber analyzer (ANKOM Technology, New York, USA), with methodology recommended by the producer. One gram (W_2) of the sample was weighted to special filter bag (W_1 – bag tare weight; F57, Ancom, USA). Samples were defatted with petroleum ether, air-dried and placed to analyzer. 2000 mL of 0.1 M sulphuric acid was added and samples were hydrolyzed 45 minutes at 100 °C, after this process samples were washed with hot distilled water 3 times. Then 2000 mL of 0.1 M potassium hydroxide was added and samples were hydrolyzed 45 minutes at 100 °C, after this process were washed with hot distilled water 3 times. Water was gently pressed from the bags and bags were soaked in acetone for 5 minutes, removed, air-dried

and placed in the oven at 102 °C (WTB, Binder, Germany) for 2 hours. After cooled to room temperature, bags were re-weighted and ashed in pre-weighted crucibles for 2 hours at 550 °C for 2 hours. Ashed crucibles were weighted to calculate the loss of weight of organic matter (W_3). The analysis was carried out in duplicate. Crude fiber content was calculated by following formula:

$$\text{Crude fiber (\%)} = \frac{100 \times [W_3 - (W_1 \times C_1)]}{W_2}$$

C_1 – ash corrected blank bag factor (running average of loss of weight on ignition of blank bag/original blank bag)

Total polyphenol content

Total polyphenol content of chocolate extracts was measured by the method of Singleton and Rossi, (1965) using Folin-Ciocalteu reagent. A 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 minutes in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25 – 250 mg.L⁻¹; $r^2 = 0.9978$) was used as the standard and the results were expressed in mg.g⁻¹ gallic acid equivalents.

Total flavonoid content

Total flavonoids were determined using the modified method of Willett, (2002). A 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 mL of 1 M sodium acetate and 4.3 mL of distilled water. After 30 minutes in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin (0.01 – 0.5 mg.L⁻¹; $r^2 = 0.9977$) was used as the standard and the results were expressed in mg.g⁻¹ quercetin equivalents.

Total phenolic acids content

Total phenolic acids content was determined using method of Farmakopea Polska, (1999). A 0.5 mL of sample extract was mixed with 0.5 mL of 0.5 M hydrochloric acid, 0.5 mL Arnova reagent (10% NaNO₂ + 10% Na₂MoO₄), 0.5 mL of 1 M sodium hydroxide (w/v) and 0.5 mL of water. Absorbance at 490 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Caffeic acid (1-200 mg.L⁻¹; $r^2 = 0.9996$) was used as a standard and the results were expressed in mg.g⁻¹ caffeic acid equivalents.

Antioxidant activity

Radical scavenging activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sánchez-Moreno et al., 1998). The extract (0.4 mL) was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). After 10 minutes in darkness, absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 mg.L⁻¹; $r^2 = 0.9881$) was used

as the standard and the results were expressed in $\text{mg}\cdot\text{g}^{-1}$ Trolox equivalents.

Reducing power

Reducing power of samples was determined by the method of **Oyaizu, (1986)**. One milliliter of sample extract was mixed with 5 mL PBS (phosphate buffer with pH 6.6) and 5 mL of 1% potassium ferricyanide (w/v) in the test tube. Mixture was stirred thoroughly and heated in water bath for 20 minutes at 50 °C. After cooling, 5 mL of 10% trichloroacetic acid was added. 5 mL of mixture was pipetted into the test tube and mixed with 5 mL of distilled water and 1 mL of 0.1% (w/v) ferric chloride solution. Absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Reducing power was expressed in $\text{mg}\cdot\text{g}^{-1}$ Trolox equivalents, using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10 – 100 $\text{mg}\cdot\text{L}^{-1}$; $r^2 = 0.9974$) as the standard and results were expressed in $\text{mg}\cdot\text{g}^{-1}$ Trolox equivalents.

Phosphomolybdenum method

Phosphomolybdenic method was determined by a method of **Prieto et al. (1999)**. Monopotassium phosphate (2.8 mL, 0.1 M, w/v) was mixed with sulfuric acid (6 mL, 1 M), ammonium molybdate (0.4 mL, 0.1 M, w/v), distilled water (0.8 mL) and 1 mL of sample extract. Test tubes were mixed thoroughly and heated in water bath for 120 minutes at 90 °C. After cooling, absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Antioxidant activity was expressed in $\text{mg}\cdot\text{g}^{-1}$ Trolox equivalents (10 – 1000 $\text{mg}\cdot\text{L}^{-1}$; $r^2=0.9975$).

Methoxyxantines content determination by HPLC-DAD method

Theobromine and caffeine content was determined using separation gradient method RP-HPLC/UV-DAD by Agilent 1260 Infinity high performance liquid chromatograph (Agilent Technologies, Waldbronn, Germany). Separation was achieved on a Purosphere reverse phase C18 column (4 mm × 250 mm × 5 μm) (Merck, KGaA, Darmstadt, Germany). The mobile phase consisted of HPLC methanol (B) and 0.1% formic acid in HPLC water (C). The following gradient program was employed: 0 – 2 min. isocratic elution (20% B and 80% C), 2 – 15 min. linear gradient elution (40% B and 60% C), and 40% B and 60% C 15 – 20 min. The flow rate was 1 $\text{mL}\cdot\text{min}^{-1}$. Column oven temperature was set up to 25 °C and the samples were kept at 4 °C in the Peltier sample manager. The DAD signal was conducted at 210 – 400 nm with preferred wavelength 330 nm for quantitative purposes with data acquisition rate of 5 Hz.

Statistical analysis

The basic statistical analyzes were realized in SAS programming packages (THE SAS SYSTEM V 9.2.). Correlation coefficients were calculated by CORR analysis and it was also used t-test (**SAS, 2009**).

RESULTS AND DISCUSSION

Fat content

Dark chocolate can be considered as products with an important nutritional density, because of their richness in carbohydrates and fats. Cocoa butter is considered the most important cocoa by-product, due to its physical (rheology and texture) and chemical characteristics, and also organoleptic qualities. The prevalence of saturated fatty acids over unsaturated fatty acids is considered to be negative from the nutritional point of view. For many years, saturated fatty acids whose chain length is C12:0 – C16:0 have been considered to provoke atherosclerosis, and to be associated with cardiovascular disease. Thus, because of its high SFA content, chocolate is often postulated as having a hypercholesterolemic effect. However, it has been suggested in recent clinical trials that stearic acid (C18:0), a non-cholesterolemic and atherogenic type of dietary saturated fat, is neutral. These trials have shown that chocolate consumption has neutral effects on serum total cholesterol and LDL-cholesterol, as neither lowers HDL-cholesterol (**Torres-Moreno et al., 2015**).

Fat content of both evaluated samples was similar. Cold processed chocolate contained $35.24 \pm 0.01\%$ of fat, while chocolate made by traditional way contained $37.60 \pm 1.47\%$ of fat. The manufacturer of the chocolate made by traditional way maintains on the package that the chocolate contains 41 grams of fat per 100 g of chocolate, which correlates with our results. On the package of cold processed chocolate there was no data about fat content. **Rezende et al., (2015)** showed that increasing the cocoa butter content enhanced the acceptance score of chocolates. This relationship is widely accepted in chocolate production, as cocoa butter provides rheological properties that promote proper hardness, mouth feel and swallow.

Although evidence in the literature suggests that chocolate consumption may have beneficial effects on health, it must be noted that chocolate has a high total fat and sugar content; in consequence, daily consumption of large amounts of chocolate may increase weight in the long term. That is why scientific evidence suggests that chocolate consumption should be considered in the context of a healthy diet, and dark chocolate must be consumed in moderate amounts (20 – 25 g per day) (**Machálková et al., 2016; Torres-Moreno et al., 2015**).

Crude fiber content

The health benefits of dietary fiber have long been appreciated. Higher intakes of dietary fiber are linked to less cardiovascular disease and fiber plays a role in gut health. Higher intakes of fiber are linked to lower body weights (**Slavin, 2013**). The recommended dietary reference intake of total fiber is 25 g per day for young women and 38 g per day for young men; however, a usual intake of dietary fiber in the US is only about 15 g per day (**Jurasová et al., 2011; Brownawell et al., 2012**).

Content of cruder fiber was in both samples on the similar level – $0.561 \pm 0.01\%$ for cold processed chocolate and $0.535 \pm 0.02\%$ for chocolate made by traditional way. Also **Torres-Moreno et al., (2015)** reported that chocolate contains less than 2% of fibre. Thus, chocolate

Table 1 Content of biologically active compounds in chocolate samples.

| | TPC (mgGAE.g ⁻¹) | TFC (mgQE.g ⁻¹) | TPA (mgCAE.g ⁻¹) | DPPH (mgTEAC.g ⁻¹) | RP (mgTEAC.g ⁻¹) |
|-----------------|---------------------------------|--------------------------------|---------------------------------|-----------------------------------|---------------------------------|
| Sample 1 | 8.14 ±0.98 | 0.34 ±0.08 | 9.47 ±0.91 | 3.62 ±0.09 | 31.39 ±1.14 |
| Sample 2 | 16.37 ±2.07 | 0.74 ±0.02 | 16.16 ±0.63 | 3.58 ±0.03 | 55.91 ±0.95 |

TPC – total polyphenol content; TFC – total flavonoid content; TPA – total phenolic acid content; RP – reducing power; GAE – gallic acid equivalent; QE – quercetin equivalent; CAE – caffeic acid equivalent; TEAC – Trolox equivalent antioxidant capacity; ±standard deviation; sample 1 – cold processed chocolate; sample 2 – chocolated meda by traditional way.

consumption does not contribute significantly to protein and dietary fibre intake.

Erdem et al., (2014) showed that dietary fiber addition didn't show negative effects, such as off-flavor, unwanted aroma or taste, on color and organoleptic properties of chocolate samples. Among fibers, maltodextrin and lemon fiber addition had positive effects on the sensory characteristics.

Total polyphenol content

Latest studies have shown that chocolate was not only a simple blend of fat and sugar, but also a rich source of flavonoids and polyphenols which shows high antioxidant activities (**Erdem et al., 2014**). Main groups of polyphenols are the catechins (37%), procyanidins (58%) and anthocyanins (4%). The polyphenols in cocoa beans contribute to about 12 – 18% of the dry weight of the whole bean (**Bordiga et al., 2015**). Furthermore, these non-nutrient bioactive compounds have potential health benefits including reduced risk of cardiovascular disease and prebiotic activity (**Hu et al., 2016**).

Total polyphenol content (TPC) of samples is shown in Table 1. Chocolate made by traditional way had almost double polyphenol content compared to cold processed chocolated. Similar results for dark chocolates produced in Serbia reported **Todorovic et al., (2015)**, where TPC varied from 7.21 ±0.49 to 12.65 ±0.45 mg GAE.g⁻¹ for chocolates with 60 – 75% of cocoa content.

Our results are also with accordance to **Hu et al., (2015)** results, which determined TPC 27.34 ±0.20 mg GAE.g⁻¹ in defatted sample of 70% Peru origin chocolate, while our 78% Peru origin chocolate made by traditional way had similar amount – 26.23 ±2.07 mg GAE.g⁻¹ in defatted sample.

Bordiga et al., (2015) reported that both processing conditions (fermentation and drying) and the chocolate-making practices influence the polyphenolic content in the final products. **Hu et al., (2015)** explained that manufacturers use a variety of cacao cultivars as well as processing and storage parameters, and all of these impact antioxidant capacity and phenolic profiles of the final products.

Total flavonoid content

Flavonoids are an important class of plant pigments, naturally found in fruit and vegetables. This class of naturally occurring polyphenolic compounds which cannot be synthesized by humans possesses a series of biological properties, acting on biological systems as antioxidants. They act as antiviral, antiinflammatory, and antitumoral agents, affecting capillary permeability and acting as

exogenous antioxidants. Flavonoids present in the diet are directly linked to the prevention of atherosclerosis, as various studies show that the reduction of total blood cholesterol levels and the antioxidant effect lead to lower risks of atherosclerosis, teratogenicity, and coronary disease (**Calado et al., 2015**).

Total flavonoid content (TFC) of chocolate made by traditional way was again much higher than cold processed chocolate (Table 1). **Calado et al., (2015)** showed, that bitter chocolate can have higher flavonoid content compared to some kinds of cooked vegetables and advises, that people who don't like foods like broccoli or eggplant (daily dose between 20 and 26 g) could eat more chocolate (daily dose only 8 g).

The cocoa bean is one of the richest sources of flavanols, but the art is to preserve these wholesome components as much as possible in the final consumable products. The negative impact of the manufacturing process of chocolate and cocoa powder products on the flavanol content should not be underestimated (**Paoletti et al., 2012**).

Total phenolic acids content

Phenolic acids are secondary plant metabolites widely spread throughout the plant kingdom. Chemically, phenolic acids are hydroxylated derivatives of benzoic, cinnamic, phenylacetic and phenylpropanoic acids. In nature, hundreds of phenolic acids have been identified. They are most abundant in coffee, tea and especially in berries. Recent interest in phenolic acids stems from their potential protective role against oxidative stress, inflammation, diabetes and cancer in experimental studies (**Zamora-Ros et al., 2013**).

Protocatechuic acid is a hydroxybenzoic acid that can be found in many foods and it is also the most important phenolic acid (69.16%) found in cocoa liquor. It has been reported to have several physiological functions including antioxidant, antibacterial activity, antimutagenic activity, antitumour activity, and anticancer effects. Coumaric acids, hydroxy derivatives of cinnamic acid, are another important group of phenolic compounds found in cocoa (2.65%) with antioxidant and antitumorigenic properties (**Zhou et al., 2015**). Content of total phenolic acids (TPA) in chocolate samples is shown in Table 1. Chocolate made by traditional way again showed almost two times higher value with compare to cold processed chocolate. To date, limited data exist on the quantitative intake of phenolic acids (**Zamora-Ros et al., 2013**).

Antioxidant activity

Radical scavenging activity

The antioxidant activity of cocoa powder is well known, but changes occur during the lifetime of the bean, and activity often depends on its processing. The content of polyphenols can vary greatly depending on the source of beans, primary and secondary processing conditions, and packaging and processing of chocolate making. Due to these factors, the ratio and types of polyphenols found in cocoa beans, as well as their activity, are unlikely to be the same as those found in the finished products (Vertuani et al., 2014).

Cold processed chocolate has higher only radical scavenging activity determined using DPPH radical (Table 1). But if the product contains more cocoa butter, antioxidant capacity values would be lower than products with the same cacao content but more cacao solids. Because of the complexity of chocolate, many components, other than phenols are present that could influence the results in different ways (Hu et al., 2016).

High antioxidant activity also reported Vertuani et al., (2014), who showed that it is not only the amount of cocoa used that is important, but also the quality and provenience affect the product properties. In general, for organic chocolates, by increasing the percentage of cocoa, the amount of total polyphenols enhances and the antioxidant capacity of the product increases in proportion.

Reducing power

Antioxidant properties are known to be concomitant with the development of reducing power. Reductones can react directly with peroxides and can also prevent peroxide formation by reacting with certain precursors (Kim et al., 2013).

Values for reducing power (RP) expressed as trolox equivalent antioxidant capacity (TEAC) are shown in Table 1. To compare it with results of other authors, RP of samples was expressed in absorbance at 700 nm (A_{700}), too. A_{700} of cold processed chocolate (1.04 ± 0.03 nm) is almost 3 times higher than A_{700} of broccoli (0.30 ± 0.00 nm) determined by Kim et al., (2013). A_{700} of chocolate made by traditional way (1.85 ± 0.03 nm) is higher than broccoli or cold processed chocolate or even leafy green vegetable *Aralia elata* (A_{700} 1.20 ± 0.00 nm) consumed in Asia (Kim et al., 2013). Reducing power and total flavonoids in evaluated chocolates showed a high correlation ($r^2 = 0.972$, $p < 0.05$), similarly like reducing power and total phenolic acid content ($r^2 = 0.982$, $p < 0.05$), implying that the phenolic compounds were major contributors to the observed antioxidant activity.

Phosphomolybdenum method

In the presence of antioxidant compounds, Mo (VI) is reduced to Mo (V) and forms a green colored complex of phosphomolybdenum (V) that gives absorbance at 700 nm. The method is based upon the spectrophotometric quantitation of total antioxidant capacity (Prieto et al., 1999). Antioxidant capacity of cold processed chocolate was 50.82 ± 3.69 mg TEAC.g⁻¹, while chocolate made by traditional way showed almost double the reducing power – 93.68 ± 10.41 mg TEAC.g⁻¹. Ibrić and Čavar, (2014) determined total antioxidant activity using molybdenum

reduction method with catechin standard as IC₅₀, which is the concentration of extract to reduce 50% of molybdenum cation. IC₅₀ of dark chocolate was 2.54 ± 0.23 mg.mL⁻¹. There are also no previously published data concerning evaluation of antioxidant activity of cocoa products using this method.

Similarly like in reducing power, phosphomolybdenum method and total flavonoids and phenolic acids in evaluated chocolates showed a high correlation ($r^2 = 0.964$; $r^2 = 0.979$, $p < 0.05$).

Methylxanthines content

Cocoa is the major natural source of the theobromine. Compared with coffee and tea, cocoa and chocolate products have much lower content of caffeine and only traces of theophylline. The level of methylxanthines in cocoa beans depends on varietal type and is influenced by the fermentation process. Besides psycho-pharmacological effects, methylxanthines of cocoa products particularly theobromine, and to a lesser extent caffeine, may have a role in lowering plasma glucose (Todorovic et al., 2015).

As expected, theobromine was the predominant compound among methylxanthines. Its content in cold processed chocolate was 22.095 ± 0.058 mg.g⁻¹ and slightly higher in chocolate made by traditional way (27.763 ± 0.009 mg.g⁻¹). Content of caffeine was much lower, 1.326 ± 0.002 mg.g⁻¹ in cold processed chocolate and 1.758 ± 0.005 mg.g⁻¹ in chocolate made by traditional way, respectively. Theobromine/caffeine ratio was 16:1 for both samples.

Bordiga et al., (2015) and Todorovic et al., (2015) determined lower amount of these methylxanthines in dark chocolates containing 40 – 75% cocoa solids. Theobromine/caffeine ratio was lower in chocolates containing more cocoa solids. The content of methylxanthines and the theobromine/caffeine ratio vary depending on the cocoa genotype (Aprotosoie et al., 2016).

Obtained values for methylxanthines in cocoa products show that potential physiological effects of chocolate mainly come from theobromine, with only small contribution of caffeine. From literature is known that 50 g of dark chocolate can have sufficient quantity of theobromine to produce neurophysiological effects (Todorovic et al., 2015). The main pharmacological activities include: central nervous system stimulation, cardiovascular and metabolic effects, bronchodilation, diuresis, gastric secretion stimulation, and, in high doses, the stimulation of skeletal muscles. Methylxanthines, mainly caffeine, enhance physical and intellectual performance, mitigate fatigue, and cause a feeling of alertness. Cocoa products represent only a small part of the human diet and the concentration of methylxanthine is low, so these products do not normally pose a risk to human health. Furthermore, theobromine appears to be even safer for humans than caffeine (Aprotosoie et al., 2016).

CONCLUSION

On the basis of the above findings we can conclude, that high percentage dark chocolate is in general very good source of biologically active compounds, but not only

antioxidants, but also fats, crude fiber or psychologically active compounds. Our results confirmed that traditional process of chocolate making probably due to the higher content of cocoa mass can preserve more nutritionally significant components than processes with low temperatures. Even if high quality, organic chocolates have interesting biological characteristic, much higher caloric value of this delicacies should not be forgotten. Since there is lack of data for this issue, especially raw chocolates, it is necessary to expand the array of samples and performed analyzes to confirm or refute our conclusions. Results in this work can be an important tool for next scientific works and for food producers.

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