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Original Research Article

Polyphenols, methylxanthines, and antioxidant capacity of chocolates produced in Serbia

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ABSTRACT

Different kinds of chocolates produced in Serbia were analyzed regarding total polyphenol, flavonoid and proanthocyanidin content using spectrophotometric methods. Flavan-3ols and methylxanthines in all samples were determined with RP-HPLC. DPPH, FRAP, ABTS and ORAC assays were applied for measuring antioxidant capacity. The average of all four antioxidant tests for each cocoa product was used for calculating antioxidant potency composite index (ACI). Obtained results for all four assays have shown that antioxidant capacity of analyzed chocolate/cocoa extracts followed cocoa, polyphenol, flavonoid, and proanthocyanidin contents. Although the addition of raspberries to dark chocolates had no significant influence on their total polyphenol, flavonoid and proanthocyanidin contents, statistical analysis showed that there was significant increase in the antioxidant capacity of dark chocolates with raspberry compared to plain dark chocolates ($p = 0.007$). Overall range for theobromine content varied from 5.5 to 22.3 mg/g depending on the product type, while the content of caffeine was 13–30 times lower in all analyzed cocoa products. In addition, correlation between antioxidant potency composite index and declared percentage of cocoa was high ($R^2 = 0.798$, $p < 0.05$) and indicated that declared cocoa content was a reliable indication for antioxidant capacity of chocolates produced in Serbia.

1. Introduction

Chocolate has never been just a sweet. Cocoa and chocolate products have been consumed for thousands of years. To Mayan people, cocoa bean was a symbol of fertility and life. According to the legend, chocolate was the food of Gods. The Aztecs used cocoa bean as a medicine for at least 150 diseases. In the 17th and 18th centuries, chocolate was regularly prescribed as a drug in Europe for treating various kinds of diseases, from colds and coughs to digestive problems, problems with fertility, mental illnesses and as an antidepressant (Dillinger et al., 2000; Jalil and Ismail, 2008).

The main nutritional ingredients of cocoa beans are fat, carbohydrates and proteins. Besides these ingredients, cocoa bean contains a range of different biologically active compounds of diverse activities in the human body: various enzymes, vitamins soluble in water and oils, sterols, phospholipids, dietary fibers, minerals (K, Mg, Cu, Fe, P), xanthines (caffeine and theobromine) and polyphenol compounds (phenolic acids and flavonoids) (Gray, 2001). However, there is an important difference between pure low-fat cocoa powder (natural cocoa powder) and cocoa, which is used in production of chocolate and other cocoa products. It is necessary to emphasize that production process leads to significant losses of cocoa biologically active compounds, especially the process of alkalization was shown to reduce content of both methylxanthines and flavan-3-ols (Le et al., 2012). Also, polyphenol compounds have capability to associate with proteins, thus formation of milk protein-polyphenol complexes could be the reason for reduced antioxidant capacity of cocoa products with added milk (Serafini et al., 2003). In addition whey and soy proteins, which are often found in the cocoa products, may have contribution to the same reduction (Vertuani et al., 2014).

Cocoa is the major natural source of the xanthine 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

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process (Niemenak et al., 2006). Of the various cocoa compounds, theobromine and caffeine would appear to be the most likely to have some psycho-pharmacological activity. Two double-blind, placebo-controlled human trials which measured the effects of cocoa powder and methylxanthines found in 50 g of dark chocolate on cognitive performance and mood (Smit et al., 2004) showed the changes in mood and behavior, including feeling more energetic, increased motivation to work, and increased alertness. Besides psycho-pharmacological effects, methylxanthines of cocoa products particularly theobromine, and to a lesser extent caffeine, may have a role in lowering plasma glucose (Sarria et al., 2014).

Polyphenols are large and heterogeneous group of biologically active secondary metabolites in plants. Flavonoid polyphenol group which is more common in cocoa products can be subdivided into four main groups: isoflavones, neoflavonoids and chalcones; flavones, flavonols, flavanones and flavanonols; flavanols and proanthocyanidins; anthocyanidins. Flavanols or flavan-3-ols are often commonly called catechins. Monomeric flavanols (catechin and epicatechin) and their derivatives (*e.g.* gallocatechins) are the major flavonoids in cocoa bean (cocoa powder and chocolates). Catechin and epicatechin can form polymers, which are often referred to as proanthocyanidins because an acid-catalyzed cleavage of the polymeric chains produces anthocyanidins (Tsao, 2010). Unlike vitamins and minerals, polyphenols are not essential components in human diet. However, they are consumed on a daily basis because to their ubiquity in plant foods. Many studies have shown that polyphenols play an important role in preserving health, because of their antioxidant properties (Ackar et al., 2013). The antioxidant activity of cocoa and chocolate was shown to be correlated with their catechin, epicatechin and procyanidin contents (Wan et al., 2001).

So far there is no detailed report on the bioactive compounds in chocolates produced in Serbia and the present study was undertaken to investigate the quality of these products concerning the content of polyphenols and methyxanthines. Since physiological and health-related effects of cocoa are of importance to consumers and often are communicated through media the second aim of the study was to evaluate the correlation of declared cocoa content with the antioxidant activity of analyzed cocoa products.

2. Materials and methods

2.1. Samples

Twelve different commercial dark and milk chocolates originating from five leading Serbian chocolate manufactures were sampled directly from the market. Producers that were included in the study were: Soko-Stark d.o.o., Belgrade; Koncern Bambi a.d., Pozarevac; Pionir d.o.o., Belgrade; Food Industry "Dunja", Belgrade, and Delhaize Serbia d.o.o., Belgrade. Each commercial chocolate sample was sampled in triplicate and was originated from different lots. Analyzed two cocoa powders were raw materials intended for chocolate production and were obtained from two chocolate factories in three different time periods.

Characteristics of the samples are presented in Table 1.

Table 1
Sample characteristics.

Sample	Fat (%)	Declared cocoa content (%)
Milk chocolate 1 (MC1)	31	27
Milk chocolate 2 (MC2)	31.9	28
Milk chocolate 3 (MC3)	31	25
Milk chocolate 4 (MC4)	31	29
Milk chocolate 5 (MC5)	30	29
Dark chocolate 1 (DC1)	40	75
Dark chocolate 2 (DC2)	36.6	70
Dark chocolate 3 (DC3)	33	65
Dark chocolate 4 (DC4)	30	70
Dark chocolate 5 (DC5)	38	70
Dark chocolate with raspberry 1 (4%)[a] (DCR1)	36	60
Dark chocolate with raspberry 2 (1.6%)[a] (DCR2)	36	70
Cocoa powder 1 (CP1)	11	100
Cocoa powder 2 (CP2)	12	100

[a] Percentage in parenthesis defines the content of added plant material.

2.2. Chemicals

Acetone, n-butanol, hydrochloric acid, n-hexane, sodium carbonate, Folin-Ciocalteau reagent (FC), potassium peroxodisulfate and ferric chloride hexahydrate were obtained from Merck (Darmstadt, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), AAPH (2,2′-Azobis(2-methylpropionamidine)dihydrochloride), as well as Florescein sodium salt were supplied by Sigma–Aldrich (St. Luis, MO, USA). The analytical standards, *e.g.* gallic acid (98% purity) and caffeine (98.5%) were from Acros Organics (New Jersey, USA), but Trolox (97%), theobromine (≥98.5%), (+)-catechine (≥98%), (−)-epicatechine (≥98%) and cyanidin chloride (≥98.5%) were purchased from Sigma–Aldrich (St. Luis, MO, USA). All other solvents used in this study were HPLC (high pressure liquid chromatography) or of analytical grade.

2.3. Sample preparation

The chocolate/cocoa extracts were prepared as described by Guyot et al. (1998) and Hammerstone et al. (1999), with some modifications. Chocolate samples were stored in refrigerator. They were firstly manually grinded in Bosch coffee grinder (Michigan, USA). Exactly 2.0 g of each cocoa product was extracted three times with 10 mL of n-hexane, in order to eliminate lipids from the samples. Then defatted samples were air-dried during 24 h to evaporate residues of organic solvent (Adamson et al., 1999). The components of interest, polyphenols and methylxanthines were extracted from defatted cocoa products two times with 5 mL of solvent extraction: acetone + distilled water + acetic acid (70 + 29.8 + 0.2 v/v/v), for 30 min in FALC ultrasonic bath (Treviglio, Italy). The mixture was centrifuged (Janetzki T32C, Wallhausen, Germany) for 10 min at 3000 rpm after each extraction and the supernatant was decanted. After filtration to remove the residual particles, the supernatants were combined in a flask and filled up to obtain 10 mL of extract.

2.4. Determination of total polyphenol (TPC) and flavonoid content (TFC)

Total polyphenol content (TPC) of chocolate/cocoa extracts was determined spectrophotometrically according to the Follin-Ciocalteau method, which was adapted from Swain and Hills