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Comparative study of commercially available cocoa products in terms of their bioactive composition

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ABSTRACT

Recent trends in food marketing suggest that cocoa products, besides being favourite sweets among consumers, also present multiple-benefit foodstuffs, which are becoming objects of increased scientific research, mainly because of their interesting phytochemical composition. UV/VIS spectrophotometric and high-performance liquid chromatography (HPLC-PDA) methods were applied in order to compare the composition of polyphenols and methylxanthines in commercial cocoa products affected by different extraction solvents. Antioxidant capacity of water and methanol extracts was evaluated using DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and FRAP (ferric reducing/antioxidant power) assays. The obtained results confirmed that the content of polyphenols and methylxanthines, as well as the antioxidant capacity of cocoa products depend on the content of their cocoa solids. Among the tested cocoa products, the highest content of bioactive compounds (polyphenols and methylxanthines) was determined in extracts of cocoa products with the highest content of cocoa solids (cocoa liquor, cocoa powder and dark chocolate with 88% cocoa solids), while the lowest content was determined in milk chocolate and cocoa bar extracts. The most abundant phenolic compound in cocoa extracts was (−)-epicatechin, while the most abundant methylxanthine was theobromine. In comparison with water, 70% methanol demonstrated higher efficiency for the extraction of the studied bioactive compounds from cocoa products.

1. Introduction

In the past few years, consumers all over the world have become more cautious regarding food and its ingredients. Recently, a great interest in using natural plant-derived bioactive compounds in foods as "multi-functional food additives" has arisen, due to their additional nutritional and therapeutic effects (Prior, Wu, & Schaich, 2005; Shahidi & Naczk, 2003; Shetty & McCue, 2003). However, the lack of knowledge about the molecular composition and content of bioactive ingredients in the source material limits the use of natural antioxidants (Shahidi, Wanasundra, & Amarowich, 1994). In this respect, screening of various food products with beneficial health properties is very important.

The consumption of cocoa products and chocolate contributes to human nutrition through provision of lipids, sugars, minerals (potassium, magnesium, copper and iron) and antioxidants, principally polyphenols (Holland, Welch, Unwin, Buss, & Paul, 1991). Tea and red wine have long been known for their high content of polyphenolic substances, but it is less known that cocoa bean, as well as cocoa derived products, also present a rich source of polyphenols, which exhibit equal or even higher antioxidant capacity than some fruit or vegetables (Lee, Kim, Lee, & Lee, 2003). For the past 10 years, many human studies have been conducted utilizing different cocoa products (Borchers, Keen, Hannum, & Gershwin, 2000; Cooper, Donovan, Waterhouse, & Wiliamson, 2008; Ramljak et al., 2005; Richelle, Tavazzi, Enslem, & Offord, 1999). However, questions arise on which of these products would deliver the best polyphenol content and antioxidant effects.

Polyphenols in cocoa (*Theobroma cacao* L.) and cocoa products can be classified into three main groups: flavan-3-ols (37%), anthocyanins (4%) and proanthocyanidins (58%) (Wollgast & Anklam, 2000a). According to Kim and Keeney (1984) the unfermented cocoa bean contains about 120–180 g/kg of polyphenolic compounds, with (−)-epicatechin being quantitatively the main compound (approximately 35%) (Shahidi & Naczk, 2003). (+)-Catechin, traces of (+)-gallocatechin, (−)-epigallocatechin, (−)-epicatechin-3-gallate (Forsyth, 1955) and numerous procyanidins (Adamson et al., 1999; Hammerstone, Lazarus, & Schmitz, 2000), as well as small quantities of quercetin, quercetin glycosides, naringenin, luteolin, apigenin, clovamide and phenolic acids such as caffeic, ferulic, gallic and *p*-coumaric acid have also been found in cocoa products (Borchers et al., 2000; Sanchez-Rabaneda et al., 2003). Their inclination to form complex compounds with proteins, polysaccharides and alkaloids enhances the complexity of cocoa products (Hagerman & Butler, 1981).

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Although most of the studies indicate that the health benefits of cocoa or cocoa products are attributable to polyphenols, it should be noted that cocoa and cocoa products are not only rich in polyphenols, but are also rich in methylxanthines, which account for about 3.2% of defatted unsweetened chocolate composition (Schultz, Prinsen, & Pater, 1970). The main methylxanthines of cocoa are theobromine (3.7% on a fat-free basis) and caffeine (about 0.2%) (Timbie, Sechrist, & Kenney, 1978). The possible synergistic interactions between flavonoids and methylxanthines are also unclear and need further study, so the contribution of theobromine in cocoa products towards health benefits should be considered.

Cocoa polyphenols have been reported in many studies as bioactive compounds, with antioxidant, antiradical and anticarcinogenic properties (Abbe Maleyki & Amin, 2008; Ren, Qiao, Wang, Zhu, & Zhang, 2003). Oligomeric procyanidins isolated from cocoa have been shown to possess biological activities potentially relevant to oxidant defences and immune function (Arteel & Sies, 1999; Bearden et al., 2000). Also, it is well-known that methylxanthines have physiological effects on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory and renal systems (Nehlig, Daval, & Debry, 1992; Spiller, 1998).

In view of all mentioned positive effects of polyphenols and methylxanthines, and due to limited information on the composition of these biologically active compounds (polyphenols and methylxanthines) in different commercial cocoa products, the aim of this study is: (i) to apply an array of assays for the routine determination of polyphenol and methylxanthine composition of commercial cocoa product extracts, and (ii) to compare the antioxidant capacity of cocoa products affected by the extraction solvent used (water and 70% methanol).

2. Experimental

2.1. Samples

Thirteen different commercial cocoa products (cocoa liquor, cocoa powder, dark, milk, cooking and powdered chocolate) were supplied by the two leading Croatian chocolate manufacturers (see Table 1).

2.2. Chemicals

Folin–Ciocalteu, formic acid, potassium peroxodisulfate, sodium carbonate, formaldehyde, ferric chloride hexahydrate, ferrous sulfate heptahydrate and hydrochloric acid were of analytical grade and supplied by Kemika (Zagreb, Croatia). DPPH (2,2-diphenyl-1-picrylhydrazyl) was supplied by Fluka (Buchs, Switzerland), *n*-hexane was supplied by Carlo-Erba (Rodano, Italy) and methanol (HPLC grade) was supplied by J.T. Baker (Deventer, Netherlands). Vanillin, 4-dimethylaminocinnamaldehyde, Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)diammonium salt) as well as caffeine, theobromine, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (+)-gallocatechin (GC), (−)-gallocatechin gallate (GCG), (+)-catechin (C), procyanidin B_2, gallic acid (GA), caffeic acid (CA), ferulic acid (FA) protocatehuic acid (PCA) and *p*-coumaric acid (pCA) were obtained from Sigma–Aldrich (Steinheim, Germany).

Table 1
General description of cocoa products evaluated in this study.

Sample	Product description	% Cocoa solids
CL_1	Cocoa liquor	100
CL_2	Cocoa liquor	100
CP_1	Cocoa powder	100
CP_2	Cocoa powder	100
CH_{88}	Chocolate with 88% c.s.	88
CH_{72}	Chocolate with 72% c.s.	72
CH_{60}	Chocolate with 60% c.s.	60
CC_1	Cooking chocolate	42
CC_2	Cooking chocolate	35
CHP_1	Powdered chocolate	36
CHP_2	Powdered chocolate	36.5
MC	Milk chocolate	29
CB	Cocoa bar	16

2.3. Sample preparation

The cocoa samples were prepared as described by Guyot, Marnet, Laraba, Sanoner, and Drilleau (1998) and Hammerstone, Lazarus, Mitchell, Rucker, and Schmitz (1999), with some modifications. Cocoa liquor and chocolates were frozen and manually grated. In order to eliminate lipids from the samples, 2.0 g of each cocoa product was extracted three times with 10 mL of *n*-hexane. The defatted cocoa solids were air-dried during 24 h to remove the residual organic solvent (Adamson et al., 1999). The phenolic compounds and methylxanthines were extracted two times from 2.0 g of defatted cocoa products with 5 mL of distilled water or aqueous methanol (70%), for 30 min in an ultrasonic bath. After each extraction, the mixture was centrifuged for 10 min at 3000 rpm 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. The flask containing the extract was flushed with nitrogen prior to storage in a freezer at −18 °C.

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

Total phenol content (TPC) of cocoa extracts was determined spectrophotometrically according to a modified method of Lachman, Hosnedl, Pivec, and Orsák (1998). Briefly, to a 0.5 mL aliquot of samples, 2.5 mL Folin–Ciocalteu's reagent, 30 mL distilled water and 7.5 mL of 20% Na_2CO_3 were added and filled up to 50 mL with distilled water. After 2 h the absorbance of blue coloration was measured at 765 nm against a blank sample. To determine the content of total flavonoids, these compounds were precipitated using formaldehyde, which reacts with C-6 or C-8 on 5,7-dihydroxy flavonoids to form methyl derivates that further react with other flavonoid compounds also at positions C-6 and C-8. The condensed products of these reactions were removed by filtration and remaining non-flavonoid phenols were determined as previously described. Flavonoid content was calculated as the difference between total phenol and non-flavonoid content. Gallic acid was used as the standard and the results were expressed as mg gallic acid equivalents (GAE) per gram of defatted cocoa product (Kramling & Singleton, 1969). All measurements were performed in triplicate.

2.5. Flavan-3-ol content

2.5.1. Vanillin assay

Cocoa extracts were analyzed for their flavan-3-ols content using a method described by Di Stefano, Cravero, and Gentilini (1989). For the analysis, a working solution of 4% vanillin in methanol was prepared daily. Cocoa extract (500 μL) was added to 3 mL of the previously prepared vanillin reagent and after 5 min, 1.5 mL of concentrated HCl was added. The mixture was allowed to react for 15 min, in a cold water bath, where after absorbance readings were taken at 500 nm. The blank was prepared by replacing the 4% vanillin solution with methanol. Absorbance of the blank was subtracted from the absorbance of the corresponding vanillin-con-

taining sample (ΔE). The content of flavan-3-ols was calculated according to the formula: (+)-catechin = 290.8 × ΔE, and the results were expressed as mg (+)-catechin/g of defatted cocoa product.

2.5.2. Reaction of 4-dimethylaminocinnamaldehyde (p-DAC)

A standard procedure reported by Di Stefano et al. (1989) was used to estimate the flavan-3-ol content. Reagent was prepared by dissolving 100 mg *p*-dimethylaminocinnamaldehyde (*p*-DAC) in a mixture of concentrated HCl (25 mL) and methanol (70 mL), and the resulting solution was made up to 100 mL with methanol. For the analysis, 1 mL of cocoa extract was added to 5 mL of *p*-DAC reagent in a glass test tube and thoroughly shaken. After 10 min absorbance reading was taken at 640 nm, along with two blank samples prepared separately for each cocoa sample. The first blank consisted of 5 mL *p*-DAC reagent and 1 mL of distilled water, and the second one consisted of 5 mL of distilled water and 1 mL of cocoa extract. The content of flavan-3-ols was calculated according to the formula: (+)-catechin = 32.1 × ΔE, where ΔE is the difference of absorbance between the tested cocoa extract and appropriate blanks. The results were expressed as mg (+)-catechin/g of defatted cocoa product.

2.6. Quantitative determination of proanthocyanidins

Proanthocyanidins (i.e. condensed tannins) were analyzed by the procedure described by Porter, Hrstich, and Chan (1986), with some modifications. Briefly, butanol/HCl assay was carried out by mixing 2 mL of cocoa extract with 4 mL of a solution of *n*-BuOH-conc. HCl (95:5, v/v) and 0.2 mL of a 2% solution of $NH_4Fe(SO_4)_2 \times 12H_2O$ in 2 M HCl. The solution was capped and thoroughly mixed and heated for 45 min at 95 °C in a water bath. The sample was cooled and the visible spectrum recorded at $\lambda = 550$ nm. The blank value of the BuOH–HCl–Fe^{III} solvent was subtracted. The quantity of condensed tannins was determined from a standard curve of cyanidin chloride treated with BuOH–HCl–Fe^{III} mixture, and expressed as mg cyanidin chloride equivalents (CyE)/g of defatted cocoa product.

2.7. HPLC analysis of phenolic compounds and methylxanthines

The samples were filtered through a 0.45 μm filter (Nylon Membranes, Supelco, Bellefonte, USA) before HPLC analysis. Twenty microliter of each sample was injected for HPLC analysis using a Varian Pro Star Solvent Delivery System 230 (Varian, Walnut Creek, USA) and a Photodiode Array detector Varian Pro Star 330 (Varian, Walnut Creek, USA) by using a reversed-phase column Pinnacle II C-18 column (Restek, USA) (250 × 4.6 mm, 5 μm i.d.). The solvents consisted of 3% formic acid (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 mL min^{-1}. The elution was performed with a gradient starting at 2% B to reach 32% B at 20 min, 40% B at 30 min and 95% B at 40 min, and becoming isocratic for 5 min. Chromatograms were recorded at 278 nm. Detection was performed with a Photodiode Array Detector by scanning between 200 and 400 nm, with a resolution of 1.2 nm. Phenolic compounds were identified by comparing the retention times and spectral data with those of standards. The data acquisition and treatment were conducted using Star Chromatography Workstation Version 5 software. All analyses were repeated three times.

2.8. Determination of antioxidant capacity

2.8.1. Ferric reducing/antioxidant power (FRAP)

The ferric reducing/antioxidant power (FRAP) assay was carried out according to a standard procedure by Benzie and Strain (1996). Working FRAP reagent was prepared daily, containing 2.5 mL of 20 mM $FeCl_3 \times 6H_2O$, 2.5 mL of 10 mM TPTZ in 40 mM HCl and 25 mL of 300 mM acetate buffer. An aliquot of 100 μL cocoa extract was mixed with 1.9 mL of FRAP reagent solution in spectrophotometric cuvettes. Absorbance was measured at 593 nm after incubation at room temperature for 4 min. All measurements were performed in triplicate. Aqueous solutions of $FeSO_4 \times 7H_2O$ (100–1000 μM) were used for the calibration and the results are expressed as mmol/L Fe(II).

2.8.2. DPPH radical scavenging assay

Antioxidant capacity of the cocoa extracts was determined using the DPPH radical scavenging assay described by Brand-Williams, Cuvelier, and Berset (1995), with some modifications. Briefly, 100 μL of water or methanol cocoa extract was added to 1.9 mL of 0.094 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol up to completing 2 mL. The free radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 517 nm every 2 min until the reaction reached its "plateau" state. Antioxidant capacity was expressed as mmol/L Trolox equivalents, using the calibration curve of Trolox (0–1000 μM), a water soluble vitamin E analogue.

2.8.3. ABTS radical scavenging assay

The Trolox equivalent antioxidant capacity (TEAC) of cocoa extracts was also estimated by the ABTS radical cation decolorization assay (Re et al., 1999). Stock solutions of ABTS (7 mM) and potassium peroxodisulfate (140 mM) in water were prepared, and mixed together to a final concentration of 2.45 mM potassium peroxodisulfate. The mixture was left to react overnight (12–16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. All measurements were performed as follows: 100 μL of cocoa extract were added to 2.0 mL of the ABTS radical solution, and the absorbance readings were taken after exactly 6 min against the appropriate reagent blank of 100 μL of ethanol instead of the sample. The results, obtained from triplicate analyses, were expressed as Trolox equivalents, and derived from a calibration curve determined for this standard (100–1000 μM).

2.9. Statistical analysis

All measurements and analyses were carried out in triplicate. The results were analyzed statistically using the Statistica 7.0 program to determine the average value and standard error. Variance analysis, with a significance level of $\alpha = 0.05\%$, was performed to determine the efficiency of the solvent on the content of extracted polyphenols and methylxanthines as well as to establish the differences in the content of these compounds among the extracts of cocoa products. Correlation analysis was also run with the same statistical package.

3. Results and discussion

3.1. Total phenol (TPC) and flavonoid content (TFC) of cocoa extracts

Fig. 1 presents the total phenol (TPC) and flavonoid content (TFC) expressed as mg GAE/g of defatted cocoa product determined in methanol and water extracts of thirteen tested cocoa products. TPC of methanol cocoa extracts ranged from 3.48 mg GAE/g of defatted MC to 33.50 mg GAE/g of defatted CL_2, while TFC of the same cocoa extracts ranged from 1.25 mg GAE/g of defatted MC to 16.60 mg GAE/g of defatted CH_{88}. In comparison with the extraction efficiency of 70% methanol, a 1.5-fold decrease on average TPC and 1.7-fold decrease on average TFC was obtained when water

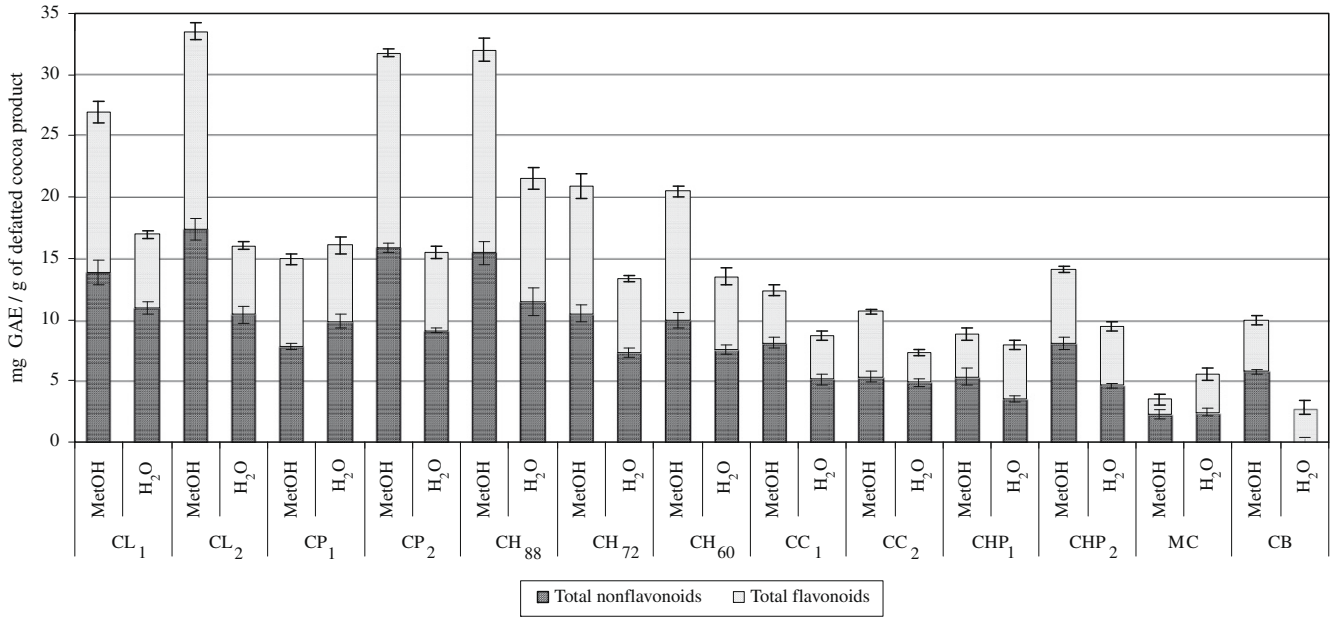

Fig. 1. Total phenol (TPC) and flavonoid contents (TFC) of methanol and water cocoa product extracts.

was applied as the extraction solvent. Generally, both TPC and TFC depend on the percentage of cocoa solids in the cocoa derived product, which is confirmed by a high correlation obtained between the cocoa solids content and the TPC and TFC for both methanol ($r_{TPC} = 0.845$ and $r_{TFC} = 0.845$) and water cocoa product extracts ($r_{TPC} = 0.910$ and $r_{TFC} = 0.751$). However, the lowest TPC and TFC were observed in MC although it contains higher cocoa solids content (29%) than CB (16%). Milk based products represent a very complex matrix where strong catechin–protein interactions are well-known to occur (Siebert, Troukhanover, & Lynn, 1996) and can directly interfere with accurate catechin determination by significantly reducing analytical recovery. These results can also be explained by the lack of selectivity of Folin–Ciocalteu reagent (Escarpa & Gonzalez, 2001), which reacts not only with phenols but also with other reducing compounds such as carotenoids, amino acids, sugars and vitamin C (Vinson, Su, Zubik, & Bose, 2001). Cocoa products also present a rich source of polysaccharides, fats and other compounds that can interfere with the determination of phenolic compounds in this assay, making their estimation inaccurate and difficult to interpret.

According to the literature data, TPC of cocoa extracts varies greatly depending on the solvent and procedure used for the extraction of polyphenols. In a study on non-alkalised cocoa powder extract, obtained by using 70% methanol as the extraction solvent, Serra Bonvehi and Ventura Coll (1997) estimated TPC of 58 mg GAE/g of cocoa powder. These results are higher than the ones obtained in our study, but it has been known that TPC varies depending on the cocoa bean variety, geographical origin, ripeness degree (harvest season) and post-harvest conditions, such as fermentation, drying, roasting, processing and storage (Wollgast & Anklam, 2000a). Waterhouse, Sirley, and Donovan (1996) analyzed cocoa powder, bitter chocolate and milk chocolate, using 95% aqueous methanol as the extraction solvent and detected 20 mg GAE/g of cocoa powder, 8.4 mg GAE/g of dark chocolate and 5 mg GAE/g of milk chocolate. The results presented in this study are in agreement with previously reported data, but they cannot be directly compared due to differences in the applied extraction solvent and presentation of the results. Additionally, some literature data mentioned above are expressed as mg of standard compound per g of cocoa product, while our research results are given as mg of standard compound per gram of defatted cocoa product, which also contributes to minor discrepancies between the results.

3.2. Proanthocyanidin (PAC) and flavan-3-ol content of cocoa extracts

In order to estimate the main classes of polyphenols present in cocoa products, three spectrophotometric methods were applied in this study. Proanthocyanidin content (PAC) of cocoa extracts was determined by the quantitative method of Porter et al. (1986), modified from the method of Bate-Smith (1954), which was originally used for the determination of cyanidins in various plant extracts. The colorimetric reaction with BuOH/HCl involves the acid-catalysed oxidative cleavage of polymeric tannins into their individual flavan-3-ol units, thus yielding a red coloured anthocyanidin moiety. The resulting colour is proportional to the number of cleavage sites between flavanol units constituting the polymers (Hagerman & Butler, 1989; Porter et al., 1986). The content of proanthocyanidins in methanol cocoa extracts varied between 0.23 mg CyE/g of defatted MC and 4.75 mg CyE/g of defatted CL_2. Among the analyzed cocoa products, cocoa liquor (CL_1 and CL_2) had the highest content of PAC, followed by cocoa powder (CP_1 and CP_2), whereas milk chocolate (MC) had the lowest content of proanthocyanidins (Fig. 2). Fig. 2 clearly displays the difference between PAC of water and methanol cocoa extracts, indicating the significantly ($p < 0.05$) lower content of proanthocyanidins in water cocoa extracts. Apparently, the presence of water in the BuOH/HCl assay increases the occurrence of competing side reactions and decreases the yield of anthocyanidins. It has long been known that proanthocyanidins readily form complexes with both proteins and carbohydrates (Davis & Hoseney, 1979; Leinmuller, Steingass, & Menke, 1991), so it is likely that these compounds also occur in the cocoa extracts. Heating proanthocyanidins in BuOH/HCl causes the acid-catalysed autooxidation, cleavage of interflavanoid bonds and the release of red anthocyanidins (Porter et al., 1986). If the BuOH/HCl does not disrupt these complexes, the proanthocyanidins they contain will escape detection. This would account for the further reduction in chromophore yield.

Vanillin assay is based on a reaction between vanillin and hydroxyl side groups at the C-6 and C-8 positions in the flavan-3-ol molecules. As with the BuOH/HCl assay, in vanillin assay the pro-

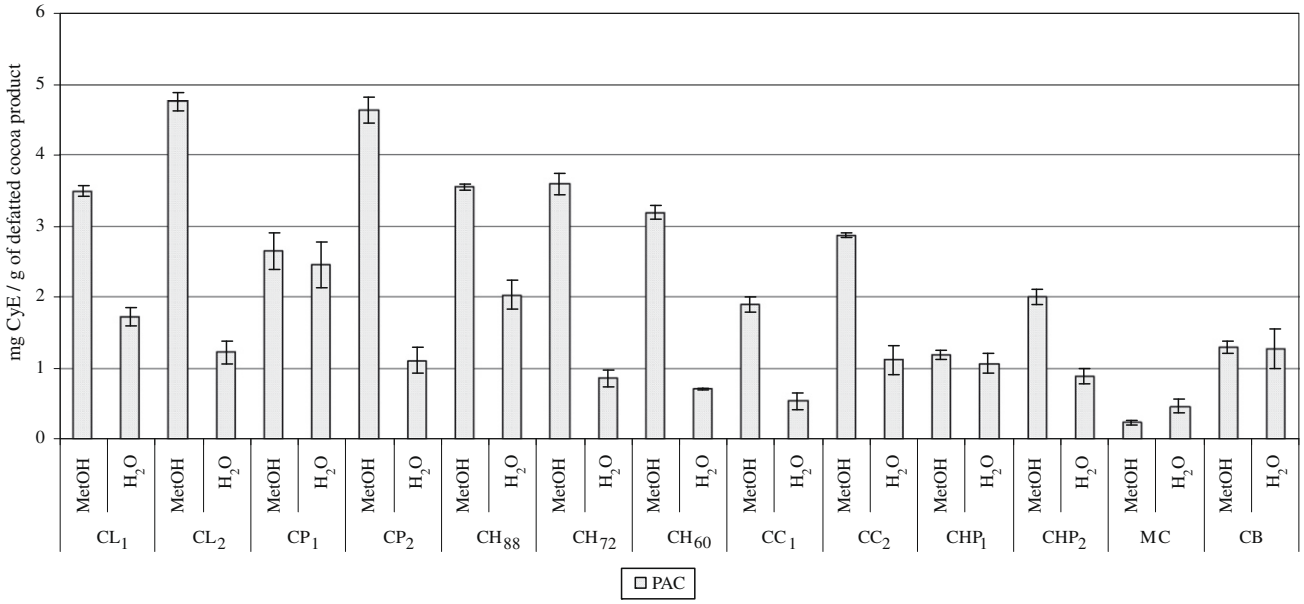

Fig. 2. Proanthocyanidin content (PAC) of methanol and water cocoa product extracts.

duction of a red coloured chromophore in the presence of HCl is consistent with the oxidative cleavage of proanthocyanidins and the release of anthocyanidins (Porter et al., 1986). In the vanillin assay only the terminal flavan-3-ol units of the polymer molecule react with vanillin, giving a red coloured product, whereas in the BuOH/HCl assay the terminal units are not taken into account (Scalbert, 1992). Fig. 3 shows the flavan-3-ol content of methanol and water extracts determined by vanillin and *p*-DAC assays, expressed as mg (+)-catechin/g of defatted cocoa product. Flavan-3-ol content determined by vanillin assay ranged from 0.63 mg (+)-catechin/g of defatted MC to 10.66 mg (+)-catechin/g of defatted CL_2 in methanol extracts, and from 0.49 mg (+)-catechin/g of defatted MC to 4.87 mg (+)-catechin/g of defatted CH_{88} in water extracts. The vanillin-HCl assay is quite specific to a narrow range of flavan-3-ols (monomers and polymers) including catechin monomers. In addition, this assay is sensitive to differences in solvent, reaction time, temperature and vanillin concentrations used (Dalzell & Kerven, 1998; Sun, da Silva, & Spranger, 1998). Therefore, the use of *p*-dimethylaminocinnamaldehyde (*p*-DAC) as an alternative to vanillin was also investigated. Vanillin assay suffers from the disadvantage of competing side reactions, which can interfere with the colour development, usually measured by the light absorbance at 500 nm. Moreover, crude plant extracts frequently contain non-flavonoid materials, which substantially contribute to the absorbance at this wavelength. *p*-DAC reagent, like vanillin, reacts with certain compounds containing *meta*-oriented di- or trihydroxy substituted benzene rings. Only flavan-3-ols with free *meta*-oriented hydroxyl groups in the A-ring and a single bond at the 2,3-position are capable of reacting with vanillin. These structural requirements also appear to be applicable for reactivity

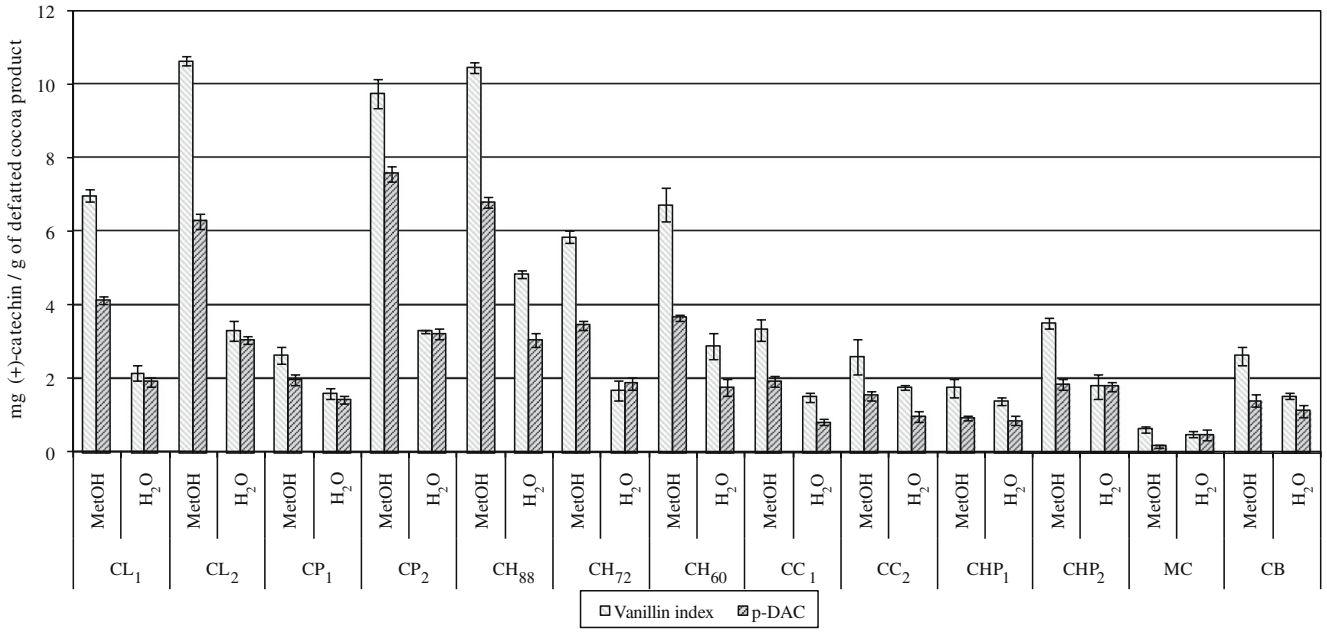

Fig. 3. Flavan-3-ol content of methanol and water cocoa product extracts evaluated by vanillin and *p*-DAC assays.

with *p*-dimethylaminocinnamaldehyde (McMorrough & McDowell, 1978). However, *p*-DAC reagent is able to react only with the hydroxyl group at the C-6 position in the benzene ring. Due to these structural differences, flavan-3-ol content of cocoa extracts determined with *p*-DAC reagent is much lower than the one determined by the vanillin assay (Fig. 3). The flavan-3-ol content of methanol cocoa extracts, determined with the *p*-DAC reagent ranged from 0.17 mg (+)-catechin/g of defatted MC to 7.58 mg (+)-catechin/g of defatted CP_2, while the flavan-3-ol content of water extracts was significantly ($p < 0.05$) lower, ranging from 0.49 mg (+)-catechin/g of defatted MC to 3.22 mg (+)-catechin/g of defatted CP_2. Just like in the case of vanillin assay, a decrease in flavan-3-ol content determined with the *p*-DAC reagent was noticed, with regard to decreasing content of cocoa solids. The average content of flavan-3-ols determined by vanillin assay is comparable to the average content of flavan-3-ols determined with the *p*-DAC reagent, but still yielding a 1.6-fold difference between the average content of methanol extracts and a 1.3-fold difference between the average content of water extracts.

3.3. High-performance liquid chromatography of cocoa extracts

High-performance liquid chromatography (HPLC) currently represents the most popular and reliable analytical technique for the characterization of polyphenolic compounds, as witnessed by the number of papers published on this subject. Regarding separation methods, reversed-phase HPLC with UV detection at 280 nm has been the primary analytical method for the quantification of procyanidins in food samples (Adamson et al., 1999). According to literature data (Liu et al., 2006; Parejo et al., 2004), the addition of acidic mobile phase allows better separation of polyphenols since it reduces the ionization of both hydroxyl and carboxyl groups. In this study, using methanol and formic acid as the solvent system, three flavan-3-ol compounds, three phenolic acids and two methylxanthines were determined.

The majority of identified compounds were methylxanthines, with theobromine being the predominant compound, in both methanol and water extracts (Fig. 4). The highest content of theobromine was detected in the water extract of CP_1 (15.50 mg/g of defatted cocoa product), while the lowest one was determined in both methanol and water extracts of MC (1.86 mg/g of defatted cocoa product). In comparison with theobromine, the content of caffeine was lower, ranging from 0.15 mg/g of defatted water extract of MC to 1.42 mg/g of defatted methanol extract of CH_{88}. The solubility of caffeine and theobromine is temperature and solvent dependent (Macrane, 1985; Pura Naik, 2001). Previous findings published on this issue claimed that good resolution of caffeine and theobromine can be achieved only when prepurification step of cocoa extracts is applied (del Rosario Brunetto et al., 2007). The results of our study are in agreement with those of Saldana, Zetzl, Mohamed, and Brunner (2002), who stated that water is an excellent extraction solvent for methylxanthines, but because of its exceptional polarity it is extremely nonselective, which results in the extraction of other compounds present in the tested sample. Thus, for the extraction of methylxanthines, dimethyl chloride or chloroform are recommended and used more often (Caudle, Gu, & Bell, 2001; Hulbert, Biswal, Walker, Meher, & Collins, 1998).

The content of polyphenolic compounds in water and methanol extracts of various cocoa products is shown in Table 2. From the class of flavan-3-ols, (−)-epicatechin (EC), (+)-catechin (C) and procyanidin B_2 were identified. Considerable variations in the quantities of these compounds in cocoa extracts were observed, depending on the extraction solvent and the content of cocoa solids in each individual cocoa product. In methanol cocoa extracts, (−)-epicatechin dominated among flavan-3-ols, with the highest content of (−)-epicatechin determined in CP_2 (1.87 mg/g of defatted product). Interestingly, the content of procyanidin B_2 was higher in water extracts than in methanol cocoa extracts, suggesting a very good water solubility of cocoa procyanidins. Also, the content of flavan-3-ols in the studied products increased with the increase in cocoa solids content from CB (only 16% of cocoa solids) to a maximum in cocoa powder (100% cocoa solids). Nevertheless, some discrepancies were noticed in the content of individual flavan-3-ols, e.g. the content of (−)-epicatechin varied greatly in cocoa liquor and cocoa powder, regarding their equal cocoa solids content. Likewise, milk chocolate containing higher cocoa solids content (29%) comprised lower content of all identified flavan-3-ols than cocoa bar, which contained only 16% of cocoa solids. These results could be the consequence of the differences in the hydrophobicity of catechins, and the synergistic or antagonistic effects of all other

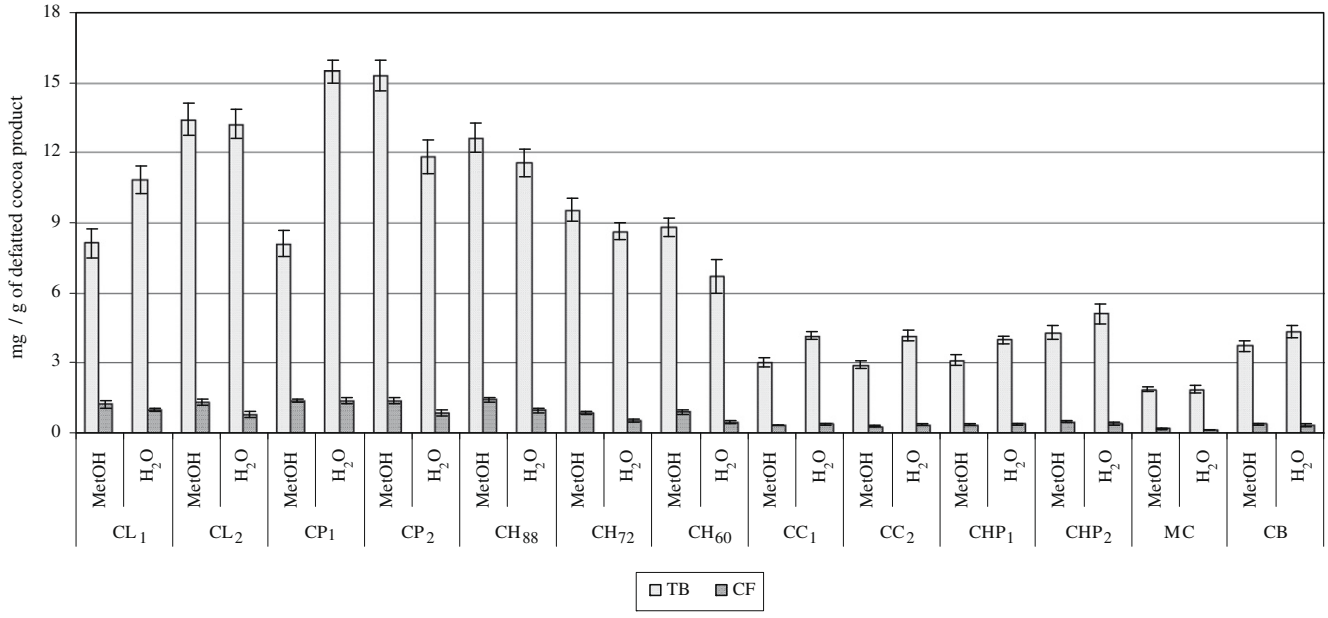

Fig. 4. Methylxanthine content of methanol and water cocoa product extracts.

Table 2
Contents of flavan-3-ols [(−)-epicatechin (EC), (+)-catechin (C) and procyanidin B_2] and phenolic acids [gallic acid (GA), caffeic acid (CA) and ferulic acid (FA)] in cocoa product extracts determined by HPLC analysis.

Sample	Extraction solvent	Flavan-3-ols			Phenolic acids		
		(−)-EC	(+)-C	Proc. B_2	GA	CA	FA
CL_1	70% Methanol	0.80 ± 0.09	0.17 ± 0.06	0.51 ± 0.09	0.47 ± 0.09	0.19 ± 0.02	0.04 ± 0.01
	Water	0.85 ± 0.12	0.59 ± 0.14	0.76 ± 0.17	0.24 ± 0.07	0.24 ± 0.05	0.01 ± 0.00
CL_2	70% Methanol	1.78 ± 0.21	0.07 ± 0.04	0.76 ± 0.26	0.23 ± 0.03	0.17 ± 0.04	n.d.[a]
	Water	0.85 ± 0.07	0.09 ± 0.02	1.14 ± 0.07	0.27 ± 0.05	0.23 ± 0.03	n.d.[a]
CP_1	70% Methanol	0.38 ± 0.03	0.04 ± 0.09	0.14 ± 0.08	0.48 ± 0.01	0.06 ± 0.01	0.02 ± 0.01
	Water	0.47 ± 0.15	n.d.[a]	0.18 ± 0.02	0.44 ± 0.10	0.18 ± 0.07	n.d.[a]
CP_2	70% Methanol	1.87 ± 0.23	0.33 ± 0.08	0.39 ± 0.03	0.28 ± 0.03	0.10 ± 0.02	0.07 ± 0.01
	Water	1.06 ± 0.16	0.38 ± 0.10	0.97 ± 0.16	0.20 ± 0.02	0.15 ± 0.04	n.d.[a]
CH_{88}	70% Methanol	1.68 ± 0.27	0.25 ± 0.17	0.79 ± 0.18	0.38 ± 0.07	0.18 ± 0.01	0.05 ± 0.02
	Water	0.75 ± 0.07	0.26 ± 0.06	0.78 ± 0.13	0.16 ± 0.06	0.21 ± 0.05	0.04 ± 0.02
CH_{72}	70% Methanol	0.75 ± 0.03	0.05 ± 0.02	0.26 ± 0.08	0.30 ± 0.06	0.06 ± 0.02	0.02 ± 0.00
	Water	0.46 ± 0.02	0.08 ± 0.03	0.47 ± 0.14	0.16 ± 0.03	0.06 ± 0.01	n.d.[a]
CH_{60}	70% Methanol	1.20 ± 0.20	0.59 ± 0.11	0.18 ± 0.06	0.25 ± 0.02	0.05 ± 0.02	n.d.[a]
	Water	0.31 ± 0.03	0.02 ± 0.01	0.65 ± 0.07	0.11 ± 0.03	0.01 ± 0.00	n.d.[a]
CC_1	70% Methanol	0.34 ± 0.05	0.11 ± 0.03	0.13 ± 0.04	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
	Water	0.38 ± 0.03	0.12 ± 0.05	0.06 ± 0.09	0.07 ± 0.01	0.05 ± 0.02	n.d.[a]
CC_2	70% Methanol	0.27 ± 0.02	0.03 ± 0.02	0.14 ± 0.03	0.01 ± 0.00	0.04 ± 0.01	n.d.[a]
	Water	0.26 ± 0.02	0.03 ± 0.01	0.34 ± 0.03	0.04 ± 0.01	0.04 ± 0.01	n.d.[a]
CHP_1	70% Methanol	0.25 ± 0.03	0.08 ± 0.03	0.17 ± 0.05	0.02 ± 0.01	0.06 ± 0.02	n.d.[a]
	Water	0.22 ± 0.02	0.05 ± 0.01	0.38 ± 0.11	0.09 ± 0.01	0.06 ± 0.02	n.d.[a]
CHP_2	70% Methanol	0.68 ± 0.09	0.51 ± 0.11	0.31 ± 0.13	0.05 ± 0.02	0.10 ± 0.02	n.d.[a]
	Water	0.45 ± 0.05	0.10 ± 0.07	0.45 ± 0.06	0.04 ± 0.01	0.06 ± 0.02	n.d.[a]
MC	70% Methanol	0.10 ± 0.01	0.02 ± 0.01	0.09 ± 0.02	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00
	Water	n.d.[a]	n.d.[a]	0.06 ± 0.01	0.10 ± 0.05	0.02 ± 0.00	n.d.[a]
CB	70% Methanol	0.41 ± 0.05	0.08 ± 0.05	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.02 ± 0.01
	Water	0.23 ± 0.02	0.04 ± 0.02	0.34 ± 0.04	0.04 ± 0.02	0.05 ± 0.03	n.d.[a]

Values are expressed as means in mg/g ± SD (n = 3) of defatted cocoa product.
[a] n.d. = not detected.

compounds present in the reaction mixture (Baptista, Tavares, & Carvalho, 1998). The obtained results confirmed the presence of phenolic compounds reported previously in cocoa and cocoa derived products (Kim & Keeney, 1984; Nelson & Sharpless, 2003; Zhu, Hammerstone, Lazarus, Schmitz, & Keen, 2003).

The comparison of flavan-3-ol content in the tested cocoa products with those obtained by other authors shows some differences. Namely, Arts, Hollman, and Kromhout (1999) determined the content of catechins by reversed-phase high-performance liquid chromatography method (RP-HPLC) using 70% methanol as the extraction solvent, and found 0.5 mg of catechin and epicatechin/g of dark chocolate and 0.16 mg of catechin and epicatechin/g of milk chocolate. Adamson et al. (1999) used 70% aqueous acetone as the extraction solvent and a modified normal phase HPLC for quantification of catechins and procyanidins in chocolates, and determined 0.7 mg of total procyanidins and 0.2 mg of catechin and epicatechin, both per g of milk chocolate, as well as 1.7 mg of total procyanidins and 0.8 mg of catechin and epicatechin per g of dark chocolate. Richelle et al. (1999) reported higher content of epicatechin in dark chocolate (2 mg/g of dark chocolate), but the used method had not been described. The content of procyanidins is not so easy to compare with the available literature data, because of the lack of authentic procyanidin standards. Thus, only procyanidin B_2 has been identified, with the highest content determined in dark chocolate with 88% cocoa solids (0.8 mg/g of defatted product).

The quantitative analysis of polyphenolic constituents by RP-HPLC analysis also displayed three different phenolic acids contributing to total phenolic content of various cocoa products (Table 2). Previously published research data on the composition of phenolic acids in cocoa products had indicated the presence of gallic, caffeic, ferulic and *p*-coumaric acids (Borchers et al., 2000; Sanchez-Rabaneda et al., 2003). The present study established the presence of gallic, caffeic and ferulic acid, while *p*-coumaric acid was not detected. Gallic acid accounted for almost half of the total phenolic acid content of various cocoa products, followed by caffeic acid, while ferulic acid was determined in minor quantities.

During fermentation of raw cocoa beans, changes in polyphenolic composition occur, which affect the chocolate flavour and metabolites typical for cocoa. Complex interactions among the polyphenols to form high-molecular weight tannins and their association with proteins play a major role in the overall quality of fermented cocoa beans for chocolate production (Forsyth, 1955). The differences in the polyphenolic profile and quantity among all tested cocoa products could also be explained by the difference in manufacturing methods, such as alkalization, removal of fats by hydraulic press at high temperature and milling to small particles, which is known to lead to chemical alteration of polyphenols (Adamson et al., 1999).

3.4. Antioxidant capacity of cocoa extracts

Recognition of many health benefits provided by polyphenolic compounds has encouraged the increased scientific interest for the determination of antioxidant capacity of various plant-derived products. However, a standardised method for the determination of antioxidant properties of certain foods and beverages has not yet been established, so using more than one method for evaluating antioxidant capacity is highly recommended. In this study three different antioxidant assays (ABTS, DPPH and FRAP) for the evaluation of antioxidant capacity of various cocoa products were applied and the obtained results are shown in Table 3. Benzie

Table 3
Antioxidant capacity of cocoa product extracts determined by DPPH, ABTS and FRAP assays.

Sample	Methanol extracts			Water extracts		
	DPPH (mmol/L Trolox)	ABTS (mmol/L Trolox)	FRAP (mmol/L Fe(II))	DPPH (mmol/L Trolox)	ABTS (mmol/L Trolox)	FRAP (mmol/L Fe(II))
CL_1	11.76 ± 0.06	20.16 ± 0.10	20.50 ± 0.39	11.40 ± 0.04	17.63 ± 0.12	15.03 ± 0.42
CL_2	11.60 ± 0.07	20.41 ± 0.04	29.23 ± 0.49	11.56 ± 0.01	18.13 ± 0.17	17.08 ± 0.81
CP_1	11.27 ± 0.04	20.19 ± 0.10	11.33 ± 0.67	9.47 ± 0.02	18.14 ± 0.06	17.05 ± 0.39
CP_2	11.68 ± 0.03	20.46 ± 0.02	26.33 ± 0.57	11.56 ± 0.05	18.20 ± 0.10	14.60 ± 0.60
CH_{88}	11.75 ± 0.09	20.40 ± 0.06	24.93 ± 0.64	11.14 ± 0.01	18.71 ± 0.29	16.95 ± 0.53
CH_{72}	11.62 ± 0.15	17.73 ± 0.19	16.40 ± 0.46	11.43 ± 0.14	14.31 ± 0.14	10.30 ± 0.60
CH_{60}	11.47 ± 0.13	18.01 ± 0.21	15.93 ± 0.49	10.33 ± 0.03	13.25 ± 0.27	11.20 ± 0.49
CC_1	11.12 ± 0.12	11.20 ± 0.31	9.68 ± 0.49	9.91 ± 0.17	11.16 ± 0.17	5.83 ± 0.35
CC_2	10.44 ± 0.05	10.78 ± 0.17	7.63 ± 0.39	10.52 ± 0.12	9.20 ± 0.29	6.53 ± 0.28
CHP_1	10.38 ± 0.11	9.50 ± 0.27	6.45 ± 0.60	11.15 ± 0.04	10.03 ± 0.27	6.48 ± 0.53
CHP_2	11.57 ± 0.10	12.94 ± 0.41	9.45 ± 0.14	11.38 ± 0.07	10.76 ± 0.48	8.05 ± 0.25
MC	5.20 ± 0.03	3.85 ± 0.14	2.33 ± 0.42	8.49 ± 0.22	4.91 ± 0.48	3.30 ± 0.57
CB	10.39 ± 0.07	7.04 ± 0.39	6.08 ± 0.21	10.01 ± 0.22	6.69 ± 0.43	6.18 ± 0.28

The results are expressed as averages of three independent measurements ± SD.

and Strain (1996) defined the FRAP assay as the «ferric reducing/antioxidant power», which measures the ability of a compound to reduce Fe^{3+} to produce Fe^{2+}. Both the Folin–Ciocalteu assay and the FRAP assay are electron transfer-based assays (Huang, Ou, & Prior, 2005), based on the assumption that a sample's reducing capacity is a direct measure of its antioxidant capacity. The decreasing order of cocoa product extracts based on the antioxidant capacity evaluated with FRAP assay, follows the TPC observed with the Folin–Ciocalteau assay: cocoa liquor > cocoa powder > chocolates with 88%, 72% and 60% cocoa solids > cooking chocolate > powdered chocolate > cocoa bar > milk chocolate. These results are also confirmed by a high correlation coefficient obtained between these two methods in both methanol and water extracts ($r = 0.994$ and $r = 0.949$, respectively). The results of both assays indicate that cocoa liquor and cocoa powder possess the highest reducing capacity.

Table 3 also shows the DPPH and ABTS radical scavenging capacities of water and methanol cocoa extracts expressed as mmol/L Trolox. It is well established that cocoa procyanidins exhibit higher antioxidant potential than most of the monomeric polyphenolic compounds found in cocoa (Adamson et al., 1999; Counet & Collin, 2003). Furthermore, the most abundant phenolic acid in the cocoa extracts determined by HPLC was gallic acid, known as the most powerful monophenolic antioxidant, which is attributed to the presence of three hydroxyl groups in its structure (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998). Comparing the results of the applied radical scavenging assays on cocoa extracts, it can be noticed that both methanol and water cocoa extracts exhibit high antioxidant potential, but the efficiency of radical scavenging differs markedly with regard to each cocoa product. Methanol extracts of cocoa liquor (CL_1) and chocolate with 88% cocoa solids (CH_{88}) were the most efficient DPPH radical scavengers (11.76 and 11.75 mmol/L Trolox, respectively). Milk chocolate (MC) exhibited the poorest DPPH radical scavenging capacity in both methanol and water extracts, 5.20 and 8.49 mmol/L Trolox, respectively. According to the results of DPPH assay, it is evident that methanol cocoa extracts exhibit significantly ($p < 0.05$) higher antioxidant capacity than water cocoa extracts, but very small differences were observed among the tested cocoa products. In the case of ABTS radical scavenging assay, the difference in methanol and water cocoa extracts is again evident, but with much higher variability. In this assay, methanol cocoa extract of CP_2 showed the best ABTS radical scavenging properties (20.46 mmol/L Trolox), slightly better than CL_2 (20.41 mmol/L Trolox) and CH_{88} (20.40 mmol/L Trolox). Just like in the case of DPPH radical scavenging, MC showed the poorest ABTS scavenging efficiency (3.85 mmol/L Trolox). The antioxidant capacity of cocoa extracts obtained by ABTS assay was higher than the one obtained by DPPH assay, except for powdered chocolate, milk chocolate and cocoa bar. Considering the fact that DPPH radical reacts only with lipophilic antioxidants, while ABTS radical reacts with both hydrophilic and lipophilic antioxidants (Prior et al., 2005), the difference between the results of these two radical scavenging assays becomes more obvious. Similar methodological differences were previously observed by Kim, Lee, Lee, and Lee (2002) and Arnao (2000), who also claim that these differences may be due to absorbance interruption at 517 nm by other compounds in the DPPH assay. Lower correlation coefficients between TPC and DPPH in both methanol ($r = 0.644$) and water ($r = 0.487$) extracts indicate that only a small content of the phenolic antioxidants in cocoa products account for the activity by scavenging free DPPH radicals. These results also indicate that high scavenging ability on DPPH and ABTS radicals could not be exclusively due to phenols in cocoa extracts. Namely, our results are in full agreement with those reported by Arlorio et al. (2005) and Othman, Ismail, Ghani, and Adenan (2007), suggesting that high scavenging ability of cocoa extract compounds on DPPH and ABTS radicals could be attributed to other methanol-soluble compounds like methylxanthines, minor flavonoids and pigments. It is well established that the DPPH radical scavenging assay determines free antioxidants in products, whereas the Folin–Ciocalteu assay reagent determines both free and bound phenolics in order to obtain the content of total phenols (Singleton, Orthofer, & Lamela-Raventós, 1999b). Therefore, the bound antioxidants in cocoa products may not contribute to radical scavenging activity in the DPPH assay.

4. Conclusion

Recognition of the health potential of chocolate is a recent development, which has finally denied the long-standing delusions about its nutritive adversity. Health benefits of various cocoa products arise from cocoa polyphenols and their antioxidant capacity, justifying the increased interest of scientists around the world on this topic. However, less attention has been dedicated to precise determination of polyphenolic composition in a vast number of cocoa products available daily to consumers all over the world. Considering the fact that a standardised method for quantification of polyphenols and antioxidant capacity of various plant-derived products has not been established yet, the minor discrepancies among the results reported in this study are not surprising, since the results depend on the sample preparation and method employed. Extracts of cocoa products containing the highest content of cocoa solids also contained the highest content of total and individual classes of polyphenols and exhibited the highest antioxidant capacity *in vitro*. Among flavan-3-ols determined in cocoa extracts, (−)-epicatechin was quantitatively the most abundant, followed by

procyanidin B_2 and (+)-catechin. Likewise, cocoa products with the highest content of cocoa solids contained the highest content of methylxanthines. Theobromine was the most abundant methylxanthine, while caffeine was present in almost 10-fold smaller content than theobromine. In comparison with water, 70% methanol is more efficient solvent to extract bioactive compounds from cocoa products. The results of this study imply that cocoa products are a major source of dietary antioxidants, which may have positive effects on human health.

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