doi: 10.17113/ftb.54.01.16.4011

Content of Total Phenolics, Flavan-3-Ols and Proanthocyanidins, Oxidative Stability and Antioxidant Capacity of Chocolate During Storage

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Received: November 26, 2014
Accepted: October 22, 2015

Summary

Antioxidant (AO) capacity of chocolates with 27, 44 and 75 % cocoa was assessed after production and during twelve months of storage by direct current (DC) polarographic assay, based on the decrease of anodic current caused by the formation of hydroxo-perhydroxyl mercury(II) complex (HPMC) in alkaline solutions of hydrogen peroxide at potentials of mercury oxidation, and two spectrophotometric assays. Relative antioxidant capacity index (RACI) was calculated by taking the average value of the AO assay (the sample mass in all assays was identical). Oxidative stability of chocolate fat was determined by differential scanning calorimetry. Measured parameters and RACI were correlated mutually and with the content of total phenols (Folin-Ciocalteu assay), flavan-3-ols (vanillin and *p*-dimethylaminocinnamaldehyde assay) and proanthocyanidins (modified Bate-Smith assay). During storage, the studied functional and health-related characteristics remained unchanged. Amongst applied AO assays, the DC polarographic one, whose validity was confirmed by two-way ANOVA and F-test, correlated most significantly with oxidative stability (oxidation onset temperature and induction time). In addition, principal component analysis was applied to characterise chocolate types.

Key words: antioxidant capacity, chocolate, differential scanning calorimetry, oxidative stability, phenols, polarography

Introduction

Chocolate represents an important source of antioxidants (AOs). This is especially significant since it contains a superior amount of AOs compared to many other foods and beverages (*1–3*), as well as taking into account its wide consumption. Thus, 40 g of dark or milk chocolate provides 951 or 394 mg of polyphenolic AOs, respectively (*4*). In the US diet, chocolate provides an estimated 100–107 mg per day of antioxidants, which represents more than 20 % of AOs originating from fruits and vegetables

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(total 488 mg per day) (*4*). Residents of the European Union consume even more chocolate per capita than the US population. In the Dutch population chocolate contributes up to 20 % of the total flavonoid intake in adults, and in children the percentage is even higher (*5,6*).

Flavan-3-ols (37 %), anthocyanins (4 %) and proanthocyanidins (58 %) are the main polyphenols in cocoa (*Theobroma cacao* L.) and cocoa products (*7*). According to *in vitro* and *in vivo* studies, such phenolics possess biological activity that is reflected in the AO capacity and regulation of the immune system. They also improve the resistance of cocoa fat towards oxidation processes (*3*). There has recently been an increasing number of experimental and clinical studies that suggest a beneficial effect of chocolate polyphenols on cardiovascular health, *i.e.* blood pressure, cholesterol level, vasodilatation, but also on insulin sensitivity, inflammation and immune function (*8–12*).

However, not all chocolates are equally rich in polyphenols. Wide variations in polyphenolic compound content depend on the origin of cocoa beans, post-harvest manipulation (*13*), the applied manufacturing process and the final chocolate composition. According to Belščak *et al.* (*14*), extracts of cocoa products containing the highest content of cocoa solids (cocoa liquor and dark chocolates) have the highest content of total and individual classes of polyphenols and exhibit the highest AO capacity *in vitro* (ferric reducing ability of plasma (FRAP), 2,2--diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3--ethylbenzothiazoline-6-sulphonic acid (ABTS) assays), as opposed to milk chocolates. These results corespond to the AO capacity determined previously by using oxygen radical absorbance capacity (ORAC) and vitamin C equivalent AO capacity, as well as total polyphenols and procyanidins determined by Miller e*t al.* (*15*). Strong linear correlation between non-fat cocoa solids in different cocoa products including chocolates and ORAC (R^2=0.9849), total polyphenols (R^2=0.9793) and procyanidins (R^2=0.46) was observed (*15*). According to the findings of Vertuani *et al.* (*16*), by increasing the percentage of cocoa, the amount of total polyphenols increases and proportionally with it the antioxidant capacity. Since several studies dealing with AO capacity of different plant products over time came to opposite results (*17–19*), it was interesting to examine possible changes in AO capacity and phenolic content of different types of chocolate during twelve months of storage.

In the present study, total content of phenolics, flavan-3-ols and proanthocyanidins, as well as AO capacity of three common types of chocolate with 27, 44 and 75 % cocoa were determined immediately after production and after six and twelve months of storage. Two widely accepted spectrophotometric assays (FRAP and ABTS) and DC polarographic assay based on the decrease of anodic current caused by hydroxo-perhydroxyl mercury(II) complex (HPMC) formation were applied. Versatility of HMPC assay developed and optimised recently (*20*) was confimed through wide application on various food samples (*21–23*). Relative antioxidant capacity index (RACI) was calculated by taking the average value of the AO assay of samples with equal mass. Relationship between AO capacity and oxidative stability of the fat extracted from chocolates assessed for the first time using differential scanning calorimetry (DSC) was of interest as well. Two parameters of the oxidative stability of cocoa butter extracted from the chocolate samples were determined: oxidation onset temperature and oxidation induction time. Until now, relationship between AO capacity determined using the assay based on HPMC anodic current decrease in the presence of AOs and oxidative stability has not been studied. Both measured and calculated parameters were correlated using regression analysis. The legitimacy of the applied DC polarographic assay has been examined by correlations to other AO assays, analysis of variance (ANOVA) and F-test. Also, the *post hoc* Tukey's honestly significant difference (HSD) test was conducted to check the differences in mean values in each AO assay (statistically significant at $p<0.05$ level). Principal component analysis (PCA) was utilized for grouping of observed samples in factor space, according to the used AO assays. Possibility to select chocolates with higher resistance to oxidation was demonstrated.

Materials and Methods

Chemicals

Folin-Ciocalteu reagent, ammonium peroxodisulphate, sodium carbonate, sodium acetate trihydrate, acetic acid, hydrochloric acid, ferric chloride hexahydrate and ferric sulphate heptahydrate were of analytical grade and supplied by Kemika (Zagreb, Croatia). Methanol (HPLC grade) was purchased from J. T. Baker (Deventer, the Netherlands). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), TPTZ (2,4,6-tripyridyl-*S*-triazine), ABTS (2.2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt) as well as gallic acid (GA) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Hydrogen peroxide was obtained from Merck (Darmstadt, Germany).

Sample preparation

In the present study three different types of chocolate were tested: milk, semisweet and dark chocolate with 27, 44 and 75 % cocoa, respectively. Chocolate composition was as follows: milk chocolate contained 53 % sugars, 31 % fat and 9 % proteins, semisweet chocolate 54 % sugars, 28 % fat and 5 % proteins, and dark chocolate 25 % sugars, 40 % fat and 10 % proteins. Chocolate production was carried out under industrial conditions in the joint-stock company Štark (Belgrade, Serbia) following standard technological operations. Determination of AO capacity of various types of chocolate was performed immediately after production, and after six and twelve months of storage at 20 °C.

The chocolate samples were prepared as described by Guyot *et al.* (*24*) and Hammerstone *et al.* (*25*) with some modifications. Chocolates were frozen and manually grated. In order to eliminate lipids from the samples, 2.0 g of each kind of chocolate was extracted three times with 10 mL of *n*-hexane. Extracted fat was used for determination of oxidative stability by DSC. The defatted cocoa was air--dried for 24 h to remove the residual organic solvent (*26*). In order to extract the phenolic compounds, twofold extraction of defatted chocolate (2.0 g) with 70 % methanol

(5 mL) was performed for 30 min in an ultrasonic bath. After centrifugation the supernatants of both extracts were combined and filled up to 20 mL. Extracts, protected with nitrogen, were kept in a freezer (–18 °C) until analysis.

Determination of total phenols

Total phenolic content (TPC) was determined spectrophotometrically according to a modified method of Lachman *et al.* (*27*) with Folin-Ciocalteu reagent. Briefly, 0.5 mL of the sample was added into a 50-mL volumetric flask containing 2.5 mL of Folin-Ciocalteu reagent, 30 mL of distilled water and 7.5 mL of 20 % Na_2CO_3, and filled up to the mark with distilled water. After two hours, the absorbance of blue colouration was measured at 765 nm against a blank sample. Gallic acid (GA) was used as the standard and the results were expressed in mg of gallic acid equivalents (GAE) per g of defatted chocolate. All measurements were performed in triplicate.

Determination of flavan-3-ol content by vanillin and p-DAC assays

The content of flavan-3-ols was determined by the vanillin assay using the daily prepared working solution of 4 % vanillin in methanol (*28*). All measurements were performed in triplicate. The content of flavan-3-ols was calculated according to the formula:

$$w(\text{flavan-3-ol})=290.8\cdot\Delta A \qquad /1/$$

where ΔA is the difference obtained by subtracting the absorbance of the blank from the absorbance of the corresponding vanillin-containing sample. The results were expressed in mg of (+)-catechin per g of defatted chocolate.

Alternatively, due to differences in the method-reaction mechanisms and selectivity of the used reagents, the content of flavan-3-ols was also estimated using the *p*-dimethylaminocinnamaldehyde (*p*-DAC) reagent, according to a procedure reported by Di Stefano *et al.* (*28*). The content of flavan-3-ols was calculated according to the formula:

$$w(\text{flavan-3-ol})=32.1\cdot\Delta A \qquad /2/$$

where ΔA is the difference of the absorbance of the tested extracts and appropriate blanks. The results were expressed in mg of (+)-catechin per g of defatted chocolate.

Determination of proanthocyanidins

Proanthocyanidins were analysed by the butanol/HCl assay, modified from the method of Bate-Smith, described by Porter *et al.* (*29*). The quantity of proanthocyanidins, determined from a standard curve of cyanidin chloride treated with butanol-HCl-Fe(III) mixture, was expressed in mg of cyanidin chloride equivalents (CyE) per g of defatted chocolate.

Determination of AO capacity using DC polarography

A dropping mercury electrode (DME) with a programmed dropping time of 1 s was used as working electrode. Saturated calomel electrode (SCE) was used as reference and a Pt-foil as auxiliary electrode. The polarographic analyser PAR model 174A (Princeton Applied Research, Artisan Technology Group®, Champaign, IL, USA), equipped with Omnigraphic® X-Y recorder 2000 (Houston Instrument, Austin, TX, USA) was used to record the current *vs.* potential curves. The initial scanning potentials were 0.10 or 0.15 V. Potential scan rate was 10 mV/s. The DME current oscillations were filtered with instrumental low pass filter positioned at 3 s. Samples were gradually added into 5 mM H_2O_2 solution obtained by adding 100 μL of 1.0 M H_2O_2 into 20 mL of Clark and Lubs buffer (pH=9.8) prepared by mixing 25 mL of 0.4 M H_3BO_3, 25 mL of 0.4 M KCl and 40.8 mL of 0.2 M NaOH. After the addition of the investigated samples decrease of the initial anodic current of 5 mM H_2O_2 was recorded. Linear slope of the decrease of the current depending on the volume of the sample, expressed in % per mL, was used to express AO capacity of chocolate extracts. The inert atmosphere during the recording of current *vs.* potential curve was kept by passing nitrogen above cell solution. A stream of pure nitrogen was passed through the cell solution for 5 min before the first recording and for 30 s after each aliquot addition. All experiments were done in triplicate at room temperature.

Determination of free radical scavenging ability by the use of $ABTS^{\bullet+}$

The Trolox equivalent antioxidant capacity (TEAC) of chocolate extracts was estimated by the $ABTS^{\bullet+}$ decolourisation assay (*30*). The results were expressed as mean value of triplicate analyses in Trolox equivalents (TE), derived from a calibration curve (c(Trolox)=100–1000 μmol/L).

FRAP assay

The ferric reducing antioxidant power (FRAP) assay was carried out according to a standard procedure by Benzie and Strain (*31*). All measurements were performed in triplicate. Aqueous solution of $FeSO_4{\cdot}7H_2O$ was used to prepare the calibration curve and the results of the assay were expressed in mmol of Fe(II) per L.

Determination of oxidative stability by DSC

Oxidation induction time and oxidation onset temperature were determined by DSC, using ASTM methods (*32,33*) on DSC Q1000 (TA Instruments, New Castle, DE, USA). Lipids obtained from the sample of 3.0–3.3 mg of chocolate extract were weighed in the open aluminium pan. For the determination of oxidation induction time, samples were heated at a specific constant test temperature in an oxygen environment (50 mL/min). For the determination of oxidation onset temperature, lipid samples were heated at a heating rate of 10.0 °C/min, in temperature interval from 25 to 250 °C, in open aluminium containers, which served as an ethalon, with an oxygen purge flow of 50 mL/min. A rapid heat increase was observed with an exothermic heat flow manifested during the beginning of the oxidation reaction. Oxidation induction time and onset temperature were determined from the resulting exotherm (Universal Analysis 2000 software v. 4.1D, TA Instruments).

Determination of RACI

The standard scores of a certain sample, calculated after the ranking of different assays, gave a single unitless value, called relative antioxidant capacity index (RACI) (*21*). This value is a particular combination of data obtained using specific measuring methods with no unit impediment and variance among them.

Statistical analysis

Descriptive statistical analyses for calculating the mean values and the standard error of the mean were performed using Microsoft Excel software (MS Office 2007, Microsoft Corporation, Redmond, VA, USA). The mean value and standard deviation of triplicate readings were recorded (N=3), and all obtained results were expressed as the mean value±standard deviation (S.D.). The evaluation of correlation matrix, two-way ANOVA and F-test, of the obtained AO assays were performed for comparison of mean values, and significant differences were determined using *post hoc* Tukey's honestly significant difference (HSD) test at the $p<0.05$ level. PCA of the obtained results was also performed using STATISTICA v. 10 software (StatSoft, Dell Software, Tulsa, OK, USA).

Results and Discussion

In the present paper, antioxidant (AO) capacity of three types of chocolate: milk, semisweet and dark chocolate with 27, 44 and 75 % cocoa, respectively, was assessed during twelve months of storage using recently developed direct current (DC) polarographic assay (*20*). Methanolic extracts of fresh, six- and twelve-month stored samples were gradually added into initial buffered solution. Significant differences were observed between polarograms of 5 mmol/L solution of H_2O_2 in Clark and Lubs buffer (pH=9.8) recorded before and after the addition of chocolate extracts (Fig. 1). The most potent effect on the anodic current originating from HPMC formation was ascribed to dark chocolate with the highest percentage of cocoa, as opposed to milk chocolate. Height of initial anodic limiting current was compared with remaining limiting current obtained after gradual addition of tested samples. After each addition, the percentage of limiting current decrease was calculated.

A dose-dependent effect of the investigated samples on HPMC anodic current was observed. Decrease of limiting current measured after each addition of chocolate extract is shown in Fig. 1. It can be seen that linear relationship was observed only for milk chocolates. Deviation from linearity was detected when 100-μL aliquots of both semisweet and dark chocolate extracts were added. Thereafter, the volume of aliquots decreased. Gradual addition of 50 μL of samples was found optimal (not shown). The slope of the linear part of dose-response curves was used to determinate the AO capacity (Table 1).

In accordance with the recommended multilateral approach to assess AO capacity (*34,35*), two common spectrophotometric AO assays were applied in parallel with DC polarographic one. Antiradical activity towards $ABTS^{\bullet+}$ and FRAP of chocolate samples were measured (Table 1). In order to compare AO activities measured using various assays, unitless RACI was used. Strong relationship between overall AO capacity and cocoa was confirmed. The highest value of RACI (0.96) was ascribed to dark chocolate with 75 % cocoa, while the lowest to milk chocolate (–1.31; Table 1). Superior AO capacity of chocolates with 75 and 44 % of cocoa in comparison with milk chocolate was confirmed using RACI (Table 1). Total phenolic, flavonoid and proanthocyanindin contents were used to characterise samples more comprehensively and also to enable better insight into the changes of health-related parameters during storage (Table 2). Time dependence of AO capacity of milk, semisweet and dark choco-

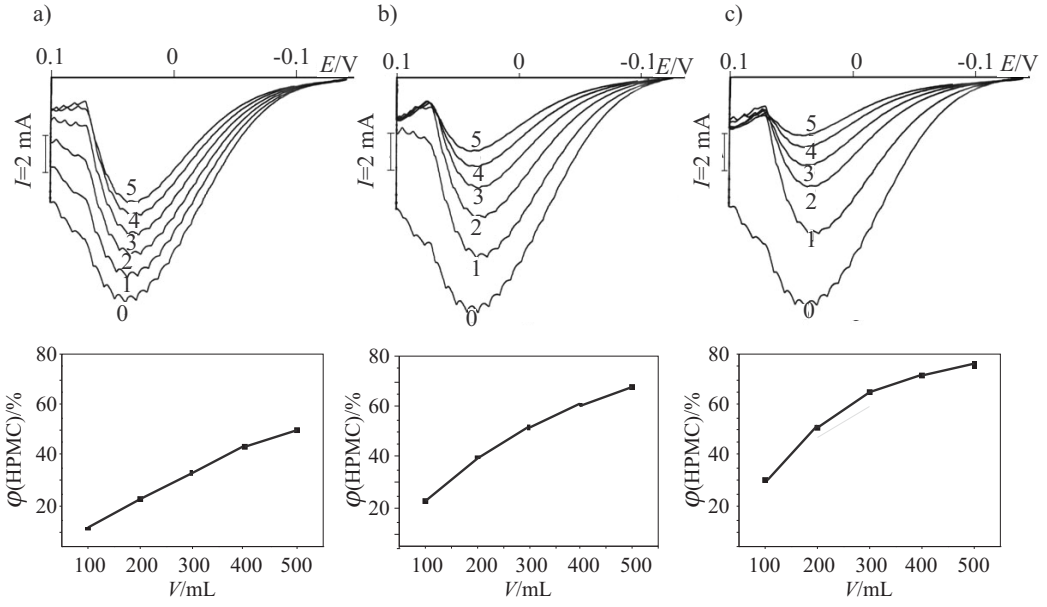

Fig. 1. Anodic polarographic curves of 5 mmol/L solution of H_2O_2 in Clark and Lubs buffer (pH=9.8) before (0) and after the addition of five equal aliquots (from 1 to 5: V=200–1000 mL) of methanolic extract of chocolate (above) and dose-dependence curves of formed HPMC in relation to volume of chocolate extracts (below) with: a) 27, b) 44 and c) 75 % cocoa

Table 1. Antioxidant capacity determined by DC polarographic (HPMC), FRAP and $ABTS^{\cdot+}$ assays, as well as calculated RACI

t(storage)/month	HPMC/(%/μL)	FRAP/(μmol of Fe(II) per g)	$ABTS^{\cdot+}$/(μmol of TE per g)	RACI
Milk chocolate (*w*(cocoa)=27 %)				
0	$(0.1\pm0.0)^{a}$	$(3.9\pm0.8)^{b}$	$(4.7\pm1.2)^{b}$	–1.31
6	$(0.1\pm0.0)^{a}$	$(4.5\pm1.0)^{b}$	$(5.0\pm0.9)^{b}$	–1.29
12	$(0.1\pm0.0)^{a}$	$(5.0\pm1.0)^{b}$	$(4.8\pm0.8)^{b}$	–1.26
Semisweet chocolate (*w*(cocoa)=44 %)				
0	$(0.23\pm0.01)^{c}$	$(33.7\pm5.8)^{a}$	$(29.7\pm5.2)^{a}$	0.26
6	$(0.27\pm0.01)^{d}$	$(35.9\pm10.2)^{a}$	$(32.9\pm5.7)^{a}$	0.50
12	$(0.24\pm0.01)^{cd}$	$(37.3\pm6.9)^{a}$	$(34.4\pm2.7)^{a}$	0.48
Dark chocolate (*w*(cocoa)=75 %)				
0	$(0.35\pm0.01)^{b}$	$(35.5\pm6.6)^{a}$	$(35.2\pm2.2)^{a}$	0.81
6	$(0.34\pm0.02)^{b}$	$(36.5\pm6.5)^{a}$	$(38.3\pm3.4)^{a}$	0.85
12	$(0.34\pm0.00)^{b}$	$(40.6\pm9.3)^{a}$	$(38.4\pm2.5)^{a}$	0.96

Values with the same letter in superscript are not statistically different at the $p<0.05$ level (Tukey's HSD test)
HPMC=hydroxo-perhydroxyl mercury(II) complex, TE=Trolox equivalent, FRAP=ferric reducing antioxidant power, $ABTS^{\cdot+}$=2,2′--azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical, RACI=relative antioxidant capacity index

Table 2. Content of total phenolics (TPC), flavan-3-ols (FLA) and proanthocyanidins (PAC)

t(storage)/month	*w*(TPC)[1]/(mg GAE/g)	*w*(FLA)[2]/(mg CAT(+)/g)	*w*(FLA)[3]/(mg CAT(+)/g)	*w*(PAC)/(mg CyE/g)
Milk chocolate (*w*(cocoa)=27 %)				
0	$(1.6\pm0.4)^{c}$	$(0.3\pm0.1)^{b}$	$(0.15\pm0.02)^{b}$	$(0.11\pm0.01)^{b}$
6	$(1.5\pm0.2)^{c}$	$(0.4\pm0.1)^{b}$	$(0.15\pm0.01)^{b}$	$(0.11\pm0.02)^{b}$
12	$(1.5\pm0.2)^{c}$	$(0.4\pm0.2)^{b}$	$(0.17\pm0.02)^{b}$	$(0.13\pm0.02)^{b}$
Semisweet chocolate (*w*(cocoa)=44 %)				
0	$(6.4\pm1.0)^{ab}$	$(2.3\pm0.2)^{a}$	$(1.0\pm0.1)^{a}$	$(0.92\pm0.18)^{a}$
6	$(6.4\pm0.7)^{a}$	$(2.4\pm0.4)^{a}$	$(0.95\pm0.06)^{a}$	$(0.90\pm0.11)^{a}$
12	$(6.6\pm1.1)^{b}$	$(2.5\pm0.4)^{a}$	$(0.93\pm0.02)^{a}$	$(1.00\pm0.15)^{a}$
Dark chocolate (*w*(cocoa)=75 %)				
0	$(8.4\pm0.5)^{b}$	$(3.1\pm0.2)^{a}$	$(1.3\pm0.4)^{a}$	$(1.1\pm0.1)^{c}$
6	$(8.2\pm0.2)^{ab}$	$(3.1\pm0.6)^{a}$	$(0.99\pm0.07)^{a}$	$(1.1\pm0.2)^{a}$
12	$(8.0\pm0.5)^{ab}$	$(3.1\pm0.6)^{a}$	$(1.02\pm0.06)^{a}$	$(1.2\pm0.1)^{a}$

Results are expressed per g of defatted chocolate
GAE=gallic acid equivalent, CAT(+)=catechine, CyE=cyanidin chloride equivalent
[1–3]Determined using Folin-Ciocalteu, vanillin and *p*-DAC assay, respectively
Values with the same letter in superscript are not statistically different at the $p<0.05$ level (Tukey's HSD test)

lates was not observed, which coincides with the results of Hurst *et al.* (*17*). In other words, AO capacity of most analysed samples measured immediately after the production was not so different from that found in the samples kept at 20 °C for 180 or 360 days.

Besides phenolic content and AO capacity, oxidative stability of chocolates during storage was measured by DSC. To the best of our knowledge, this is the first time DSC has been used to determine the parameters of the oxidative stability of cocoa butter extracted from chocolate samples, *i.e.* oxidation onset temperature and oxidation induction time. The transfer of an oxygen molecule to an unsaturated fatty acid requires energy easily determined by DSC (*33,36*). Oxidative stability is represented by oxidation induction time or onset temperature at which chocolate fat resists oxidation. While oxidation induction time was determined by heating the sample (in oxygen) to a constant temperature and measuring the developed heat in DSC experiment, oxidation onset temperature at which oxidation occurs is a relative measure of oxidative stability at the used heating rate. Previously, DSC had been mainly applied to characterise melting profiles of chocolate samples and to determine the degree of crystallisation that influences sensory and rheological properties, as well as shelf life (*37*). Lipids extracted from chocolates with higher total phenolic content and superior AO capacity are less prone to oxidation (Table 3).

In this study, two-way ANOVA was conducted to show the significant effects of the independent variables on the responses, which were significantly affected by the

Table 3. Oxidative stability of lipids extracted from milk, semisweet and dark chocolates determined by differential scanning calorimetry

t(storage)/month	Temperature (oxidation onset)/°C	*t*(oxidation induction)/min
Milk chocolate (*w*(cocoa)=27 %)		
0	(204.2±5.4)^{ab}	(37.5±14.9)^{a}
6	(196.8±10.1)^{a}	(35.7±12.2)^{a}
12	(196.5±9.9)^{a}	(36.3±11.9)^{a}
Semisweet chocolate (*w*(cocoa)=44 %)		
0	(216.4±4.3)^{ab}	(142.6±7.5)^{b}
6	(207.8±7.5)^{ab}	(136.3±3.7)^{b}
12	(203.3±17.3)^{ab}	(134.9±1.8)^{b}
Dark chocolate (*w*(cocoa)=75 %)		
0	(220.6±0.8)^{ab}	(249.6±14.6)^{c}
6	(225.8±14.8)^{ab}	(256.1±29.6)^{c}
12	(237.0±13.3)^{b}	(268.4±25.4)^{c}

Values with the same letter in superscript are not statistically different at p<0.05 level (Tukey's HSD test)

various parameter combinations. The cocoa content was the most influential variable in AO capacity calculation, statistically significant at p<0.05 level with 95 % confidence limit, while chocolate type was also very important variable in TPC, FRAP, $ABTS^{\bullet+}$ and oxidation induction time assays, statistically significant also at p<0.05. The product of these two variables was found statistically significant in TPC, flavan-3-ol, FRAP, $ABTS^{\bullet+}$ and oxidation induction time assays.

Both measured and calculated parameters were correlated using regression analysis, and the obtained DC polarographic assay was validated. Stepwise regression between RACI and the used AO assays (FRAP, $ABTS^{\bullet+}$ and HPMC) revealed that each of the three assays matched RACI, and that correlations between RACI and the used assays were highly significant (p<0.01 level). RACI strictly correlated with both FRAP and $ABTS^{\bullet+}$ assays (0.99), and HPMC (0.97) (Table 4). Strong positive correlations were established between the AO activities of dark and milk chocolates deduced from cyclic voltammograms with those determined using DPPH, $ABTS^{\bullet+}$ and FRAP assays (*38*). Reliability of HPMC assay as a measuring method with the lowest standard deviation recorded was confirmed by experimental measurements. The coefficients of variation for HPMC assay, calculated as the ratio of the standard deviation divided by the mean, are 0.01–0.05.

Correlations between RACI and total phenolic content (determined by Folin-Ciocalteu reagent), proanthocyanidin and flavan-3-ol determined using vanillin assay were high (0.99), as well as the correlation with flavan-3--ol determined using *p*-DAC assay (0.97). Correlation between RACI and oxidation onset temperature (0.79) was significantly lower than the correlation with oxidation induction time (0.93). Generally, parameters determined using both spectrophotometric and DC polarographic assays correlated well with oxidation induction time (0.86–0.98), while correlations with oxidation onset temperature were considerably lower (0.73–0.85). However, the highest correlation was found between HPMC and oxidation onset temperature (0.85) or oxidation induction time (0.98). Correlations with the results of FRAP (0.732 and 0.865) and $ABTS^{\bullet+}$ (0.764 and 0.904) assay were substantially lower. Finding that AO capacity determined by HPMC correlates well with oxidative stability might gain importance. The *post hoc* Tukey's HSD test was used for comparison between the chocolate samples within each used assay, and statistically insignificant difference was found in almost all samples during storage at p<0.05 level (Tables 1–3). HPMC and total phenolic content of semisweet chocolate varied significantly during storage. As expected, oxidation onset temperature was statistically significant for all types of chocolate.

The PCA reduced the number of variables and enabled to establish the relationship between the used AO assays and different types of chocolates that give complimentary data. The full autoscaled data matrix consisting of three different types of chocolates, after 0, 6 and 12 months of storage, and nine measured parameters, as well as the calculated RACI were submitted to PCA. For

Table 4. Correlations between antioxidant capacity determined using DC polarographic (HPMC), $ABTS^{\bullet+}$ and FRAP assays, content of total phenolics (TPC), flavan-3-ols (FLA) and proantocyanidins (PAC), oxidative stability (oxidation induction time (OIT) and onset temperature (OOT)) and reactive antioxidant capacity index (RACI)

	FLA[1]	FLA[2]	PAC	FRAP	$ABTS^{\bullet+}$	HPMC	OOT	OIT	RACI
TPC	0.998	0.979	0.996	0.973	0.988	**0.981**	0.806	0.944	0.994
FLA[1]		0.975	0.997	0.979	0.992	**0.982**	0.802	0.942	0.998
FLA[2]			0.976	0.959	0.961	**0.948**	0.728*	0.887	0.969
PAC				0.988	0.995	**0.966**	0.789*	0.924	0.997
FRAP					0.994	**0.928**	0.732*	0.865	0.987
$ABTS^{\bullet+}$						**0.956**	0.764*	0.904	0.997
HPMC							**0.853**	**0.979**	**0.974**
OOT								0.921	0.794*
OIT									0.929

[1,2]Determined using vanillin and *p*-DAC assay, respectively. All shown correlations are significant at p<0.01, *N*=12, except those marked with asterisk, which are significant at p<0.05 level

PCA modelling, the 3×3×10 data matrix was divided into a sample code and different data sets. All examples in the distinctive classifications obtained diverse scores, as anticipated by PC score plot. The variability of the results in three types of chocolate is large, so the different categories of chocolates are formed according to the content of total phenols, flavan-3-ol (vanillin and *p*-DAC assays) and proanthocyanidins, and AO capacity determined using FRAP, $ABTS^{\cdot+}$ and HPMC, as well as oxidative stability (oxidation onset temperature and oxidation induction time).

For visualising the data patterns and the discriminating efficiency, a scatter plot of the observed samples, using the first two principal components (PCs), obtained from PCA, was made (Fig. 2). As can be seen, there is a neat separation of the three sorts of chocolates. The first two principal components, accounting for 98.7 % of the total variability, are considered to be sufficient for such data, also visible from the high correlation coefficients obtained between different assays applied (Table 4).

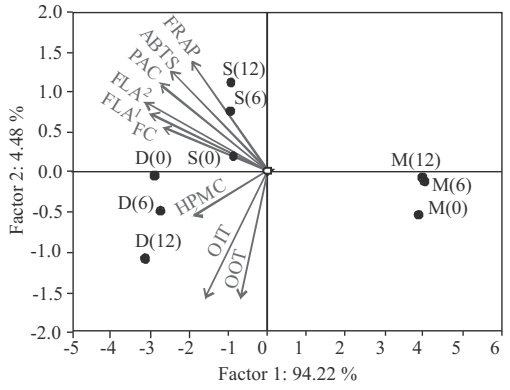

Fig. 2. PCA biplot of the results obtained for three different types of chocolates (M=milk, S=semisweet, D=dark) analysed after production, and after 6 and 12 months of storage. For abbreviations see Table 4

Conclusions

There is a strong relationship between the content of cocoa, total phenols, flavan-3-ols and proanthocyanidins on the one hand, and total antioxidant capacity (determined using two widely accepted spectrophotometric and a recently developed direct current (DC) polarographic assays) and calculated relative antioxidant index on the other. Also, strong relationship with the oxidative stability determined using differential scanning calorimetry has been confirmed unequivocally. Very good correlation between hydroxo-perhydroxyl mercury(II) complex and both oxidation induction time and oxidation onset temperature suggests that reliable, cheap and easy-to-handle DC polarographic assay, validated using regression analysis, two-way ANOVA and F-test, can be used as an alternative to spectrophotometric assays commonly applied in the analysis of chocolate and cocoa products, and as an indicator of oxidative stability of chocolates as well. Simple and fast procedure with possibility to process opalescent or turbid samples directly can be considered as additional advantage. There is a growing body of data obtained by parallel application of DC polarographic and common spectrophotometric assays on various food and beverage items and this study contributes to its extension. Multilateral approach and introduction of RACI enables more comprehensive comparison between analysed samples. Also, better insight into health-related or functional characteristics of chocolates and their changes during storage is allowed. Based on all analysed parameters, quality of chocolate remains unchanged during twelve months of storage.

Acknowledgements

This work was supported by the Ministry of Education and Science, Republic of Serbia, Grant nos. 31093, 31055, 043010 and 46010.

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