


Green and Efficient Extraction Method to Determine Polyphenols in Cocoa and Cocoa Products

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Abstract Cocoa and cocoa products have broad and well-proven health benefits, most of which are associated with the high antioxidant capacity of cocoa flavonoids. Most extraction methods for flavonoids in cocoa products use several steps including a defatting step and large amount of organic solvents. Moreover, they are labor-intensive and time-consuming. In this work, a pressurized hot water extraction (PHWE) method has been compared to conventional sonication extraction (CSE) method. The contribution of individual compounds to the total antioxidant capacity of the extracts was evaluated by developing an analytical technique consisting of high-performance liquid chromatography (HPLC) with photodiode array coupled to electrochemical and to charged aerosol detectors and HPLC-mass spectrometry for the confirmation of the identity of compounds present. Additionally, procyanidins were analyzed by fluorescence detection. PHWE turned out to be more efficient in extracting phenolics and methylxanthines, as compared to the conventional method, in addition to being more “green” in terms of using less organic solvents.

Keywords Cocoa · Pressurized hot water extraction · Flavanols · Antioxidant capacity · Electrochemical detector

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Abbreviations

CAD	Charged aerosol detector
CSE	Conventional sonication extraction
DAD	Photodiode array detector
DP	Degree of polymerization
ECD	Electrochemical detector
EE	Epicatechin equivalents
ESI	Electrospray ionization source
FLD	Fluorescence detector
G	Galloyl group
GA	Gallic acid
MS	Mass spectrometry detector
PHWE	Pressurized hot water extraction
QTOF	Quadrupole and orthogonal acceleration time-of-flight
RRFs	Relative response factors
TPC	Total phenolic compounds

Introduction

Polyphenols have become an intense focus of research interest due to their potent antioxidant properties and their perceived health-beneficial effects in the treatment and prevention of cancer, cardiovascular disease, and other pathologies (Chen and Chen 2013; Iriti and Varoni 2013; Martin et al. 2013).

Cocoa beans are one of the best-known sources of dietary polyphenols; the total polyphenol content of the dried fat free-mass of fresh cocoa beans is around 15–20% and of fermented beans approximately 5% (Wollgast and Anklam 2000). The polyphenol content in cocoa beans depends on their origin and processing, as they are subject to a combination of fermentation and drying treatments, followed by alkalisation and roasting, which affects the polyphenol content and consequently the end product quality (Hii et al. 2009). However,

the content of polyphenols in chocolate, which is the most commonly consumed cocoa product, is lower (Adamson et al. 1999). This is not only because chocolate contains processed cocoa beans but also added sugars, milk, solids, and cocoa butter. In addition, the conditions during cocoa bean processing and chocolate making may result in polyphenol degradation (Wollgast and Anklam 2000).

Cocoa polyphenols have been reported in many studies as bioactive compounds, with antioxidant, antiradical, antihypertensive, and anticarcinogenic properties (Andújar et al. 2012; Martin et al. 2013). The main polyphenols found in cocoa are flavanols, mainly catechin, epicatechin, and procyanidins (see Fig. 1).

Even though most of the studies indicate that the health benefits of cocoa products are attributed to polyphenols, it should be observed that cocoa and cocoa products also contain high amounts of methylxanthines, namely theobromine and caffeine, which represent about 2.4–3.6% of defatted dry cocoa composition (Fig. 1) (Matissek 1997). Theobromine is known to be a brain stimulant, to have diuretic action, and being a potential for blood pressure reduction (Cheng et al. 2009). Besides having their own health benefits, methylxanthines may also be involved in synergistic interactions with polyphenols, although possible health effects are unclear and further studies are needed.

Common methods to extract polyphenols from cocoa products use several steps, including defatting before the extraction of the polyphenols itself (see Fig. 2) (Adamson et al. 1999; Belščak et al. 2009; Wollgast et al. 2001). In addition, large amounts of organic solvents are used and methods are often

based on manual and are time consuming and highly dependent on the operator, i.e., not easily reproduced. The development of an efficient method to extract flavonoids from cocoa is a challenging task due to the structural diversity of phenolic compounds, their presence in a complex matrix and interaction with other compounds. Hence, an important aspect to consider is the development of fast, cost-effective, and environmentally adaptable extraction methods that are able to isolate the polyphenols of interest from cocoa and cocoa products. For instance, pressurized liquid extraction is an extraction technique that uses solvent subjected to elevated temperature (usually above the boiling point of the liquid) and pressure to keep the solvent in a liquid state. By using high temperature, the extraction process becomes more efficient due to faster diffusion rates, thereby minimizing the extraction time. It improves disruption of solute-matrix interactions, facilitating desorption of the analyte from the matrix. Additionally, by decreasing solvent viscosity and surface tension, together with higher pressures, the penetration within the sample matrix is improved and the extraction efficiency enhanced, when compared with the same solvent at ambient temperature. This leads to a significant reduction on extraction times and solvent volumes. In the present work, water was used as the solvent, i.e., the technique pressurized hot water extraction (PHWE). In PHWE, the dielectric constant of the water decreases with increasing temperature, which implies that the solvent water polarizability can be tuned by changing temperature. The dielectric constant is around 90 at 25 °C but is around 65 at 100 °C and around 35 at 250 °C. That is, the dielectric constant of water at 250 °C is the same as that for methanol at

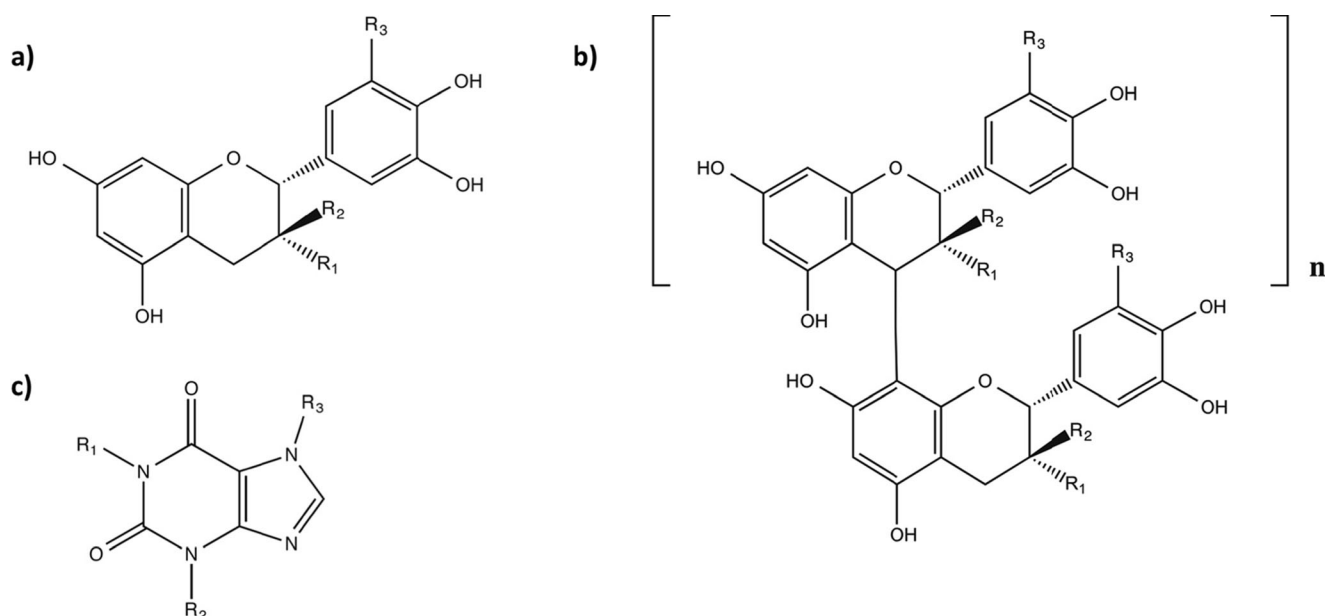


Fig. 1 Chemical structure of the main phenolic compounds, flavan-3-ol for **a** monomer units and **b** procyanidins (where catechin ($R_1 = H$, $R_2 = OH$, and $R_3 = H$) and epicatechin ($R_1 = OH$, $R_2 = H$, and

$R_3 = H$)), and **c** methylxanthines (where theobromine ($R_1 = H$, $R_2 = R_3 = CH_3$) and caffeine ($R_1 = R_2 = R_3 = CH_3$)) found in cocoa

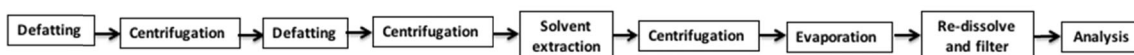
Conventional sonication extraction:**Pressurized hot water extraction:**

Fig. 2 Main steps employed to carry out conventional sonication extraction and pressurized hot water extraction

ambient conditions (Plaza and Turner 2015). Thus, water at high temperatures is an interesting alternative extraction solvent and could potentially replace many of organic solvents conventionally used in extraction.

Therefore, our hypothesis is that PHWE can be used as a green technique to extract polyphenols from cocoa and cocoa products, avoiding the defatting step and the use of organic solvents for the extraction (Fig. 2). In this work, PHWE was compared with a conventional sonication extraction (CSE) (Wollgast et al. 2001) to extract phenolic compounds and methylxanthines from cocoa and cocoa products. In addition, a full characterization of cocoa extracts and the contribution of individual compounds to the total antioxidant capacity of the extracts was evaluated for testing both extraction techniques, PHWE and CSE. The analytical techniques used were HPLC coupled to a photodiode array detector (DAD), an electrochemical detector (ECD), and a charged aerosol detector (CAD) and HPLC with mass spectrometry detector (MS). The advantage of combining all these detectors (DAD, ECD, CAD, and MS) is that information about oxidizability can be obtained from ECD, in addition to light adsorbing properties of the compounds with DAD, as well as quantitative analysis with CAD and chemical structure from the MS detector. Moreover, the different degrees of polymerization of procyanidins were analyzed by fluorescence detection (HPLC-FLD).

Materials and Methods

Chemicals and Reagents

All the chemicals were of analytical grade. Heptane, acetone, and acetonitrile were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Ammonium formate, acetic acid, theobromine, and sodium carbonate were supplied by Sigma-Aldrich (Steinheim, Germany). Formic acid, caffeine, and Folin-Ciocalteu reagent were from Merck (Darmstadt, Germany). Methanol, HPLC-MS grade, was provided by Scharlau (Barcelona, Spain). The polyphenols standards (catechin, epicatechin, and procyanidin B2) were purchased from Extrasynthese (Lyon, France). Deagglomerated alumina (α - Al_2O_3) suspensions with grain sizes 0.1 μm was provided by Struers (Ballerup, Denmark). The ultrapure water used

was obtained from a Milli-Q instrument (Millipore, Billerica, MA, USA).

Samples

All samples, cocoa powders (A and B), chocolates (B, C, and D), and nibs and nibs powder were commercially available and the chocolate breads were manufactured *in house*, following the recipe below, and using the cocoa powders A and B. The cocoa nibs are cocoa beans that have been roasted, separated from their husk, and broken in smaller pieces. Before extraction, all samples were grinded.

Bread recipe: 400 g of white flour (Kungsörnen AB, Järna, Sweden), 150 g cocoa powder, 360 g water, 4.8 g dry yeast, 40 g sugar, and 4.8 g salt. The bread was baked according to a standardized procedure in a home baking machine (Severin model n° BM 3983). The menu choice was programme 2 (white bread, 1 Kg, quick (time: 2:37)). The water content of the cocoa bread was calculated by subtracting dry weight from the total weight. The fresh cocoa bread was dried in an oven at 60 °C for 56 h. This process was carried out in triplicate. The percentage of water in the bread with cocoa A and cocoa B was 40.9 ± 1.2 and $41.5 \pm 0.9\%$, respectively.

Conventional Sonication Extraction Technique

A conventional sonication extraction technique previously described by Wollgast et al. (2001), Gu et al. (2006), and Brčanovic et al. (2013) was employed, with some modifications, to extract the polyphenols from cocoa and cocoa products. One gram of the cocoa and cocoa products was defatted twice with 10 mL heptane for 5 min using an Elmasonic S30H ultrasonic bath (Singen, Germany) with ultrasonic vibration at frequency of 37 kHz, ultrasonic power of 80 W, and a temperature of 30 °C and was subsequently centrifuged for 10 min at 3000g. Polyphenols were extracted from the pellet with 10 mL of a mixture of acetone-water-acetic acid (70:29.8:0.2, v/v/v) for 10 min at 30 °C in the ultrasonic bath at the same conditions as that in the defatting step. The sample was centrifuged for 10 min at 3000g and dried with a Reacti-Vap™ Evaporators (Thermo Fisher, Germering, Germany). Solvent evaporation was carried out using a nitrogen flow and magnet stirring at 30 °C until total sample dryness. The dry extracts were re-dissolved in water and filtered through a

0.45 μm PTFE filters (VWR International, West Chester, PA, USA) before HPLC separation. Samples were prepared in triplicate.

Pressurized Hot Water Extraction

Extractions were performed on a Dionex ASE 200 system (Thermo Fisher, Germering, Germany). The water was sonicated for 40 min for removing the dissolved oxygen. Extractions were performed at 125 °C for 3 min based on an experimental method used in a previous study (Plaza et al. 2013). Prior to each experiment, the extraction cell was heated-up for 6 min. Likewise, all extractions were performed in 11-mL extraction cells, containing 1 g of fresh sample. Samples were prepared in triplicate and stored protected from light at –20 °C until analysis. The volume of the extracts was adjusted to 25 mL with MilliQ water and filtered through a 0.45- μm PTFE filters (VWR International, West Chester, PA, USA) prior to analysis.

Total Phenolic Compounds Determination (Folin-Ciocalteu)

The Folin-Ciocalteu reducing capacity was estimated as epicatechin equivalents (EE), expressed as milligram epicatechin per gram of sample based on a previous protocol (Koşar et al. 2005). The total volume of the reaction mixture was miniaturized to 1 mL. 790 μL of water and 10 μL of sample were mixed, to which 50 μL of undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 2% (w/v) Na_2CO_3 was added. After 2 h of incubation at 25 °C, 300 μL of the mixture was transferred into a well of the microplate, the absorbance was measured at 760 nm in a microplate spectrophotometer reader (Multiskan GO, Thermo Fisher, Germering, Germany) and compared to a epicatechin calibration curve (0.025–2.000 mg/mL) prepared equally. The data was presented as the average of triplicate analyses for each extract.

Analysis of Polyphenols by HPLC with Diode Array, Electrochemical, and Charged Aerosol Detection (HPLC-DAD-ECD-CAD)

The HPLC-DAD-ECD-CAD analysis of phenolic compounds from cocoa and cocoa products extracts was performed according to Plaza et al. (2014b). An UltiMate-3000® HPLC system from Dionex (Thermo Fisher, Germering, Germany) with an online degasser, a dual-gradient pump with degasser, an autosampler with cooler, column oven, and photodiode array detector, all controlled by a Chromeleon 6.80 (Thermo Fisher) software, was used. An ECD (Bioanalytical Systems Inc., West Lafayette, IN) was attached just after the DAD. The ECD redox potential was measured at +0.6 V vs.

Ag/AgCl. A battery-powered potentiostat (Palmsens, Palm Instrument BV, Houten, The Netherlands) was connected to the flow cell and was controlled by HP iPAQ Pocket PC (HP 2200 series, Hewlett-Packard Company). PalmTime software (vs. 2.3.0.0, Palmsens) was used to collect data. A Corona CAD instrument from ESA Biosciences Inc. (a part of Thermo Fisher, Germering, Germany) was placed in series after the ECD detector. Data processing was carried out with Chromeleon 6.8 software (Thermo Fisher).

Separation was accomplished with porous-shell fused-core Ascentis Express C18 analytical column (150 \times 2.1 mm, particle size 2.7 μm) from Supelco (Bellefonte, PA, USA). The column temperature was 50 °C and the flow rate 300 $\mu\text{L}/\text{min}$. Two microliters of extract was injected from a sample tray held at 4 °C. The mobile phases consisted of (A) ammonium formate buffer (pH 3.0) 60 mM in water and (B) methanol with 0.5% of formic acid in a gradient elution analysis programmed as follows: 0 min, 5% (B); 0–5 min, 5% (B); 5–35 min, 5–40% (B); 35–40 min, 40% (B), with 10 min of post-time. To circumvent the organic solvent elicited impact on the CAD response (de Villiers et al. 2007; Plaza et al. 2014b), an inverse methanol gradient (make-up gradient) was delivered to CAD with 0.3-min delay with respect to the elution gradient (de Villiers et al. 2007). All solvents were purged with nitrogen to remove oxygen.

Phenolic compounds were quantified with the CAD detector. The calibration curve of epicatechin was selected to quantify all phenolic compounds and methylxanthines, because it is normally found in cocoa (Gu et al. 2006; Langer et al. 2011). The epicatechin standard solution was injected in triplicate at six concentrations levels (1–100 $\mu\text{g}/\text{mL}$). The calibration curve of epicatechin was obtained by plotting peak area as function of concentration (in micrograms/milliliter). Responses obtained in the examined ranges were expressed by a linear equation with high r^2 determination coefficient value (0.999).

Identification of Polyphenols by HPLC with DAD and Mass Spectrometry

The structural elucidation of phenolic compounds was carried out by a Waters Acquity UPLC chromatographic system (Waters Corp., Manchester, UK) equipped with a DAD and a quadrupole and orthogonal acceleration time-of-flight tandem mass spectrometers Xevo G2 QTOF with electrospray ionization source (ESI) (Waters MS Technologies, Manchester, UK). The HPLC instrument was equipped with a binary solvent, an auto-sampler, and a column heater compartment. The system was controlled by Waters® Empower™ Chromatography software, while MassLynx™ (V 4.1, SCN 779, Waters Corp., Manchester, UK) was used for MS data acquisition and treatment. The separation conditions were the same as the previous (see section “Analysis of polyphenols by

HPLC with diode array, electrochemical, and charged aerosol detection (HPLC-DAD-ECD-CADTM). UV-vis spectra were recorded in the range of 200–500 nm. The ESI interface was operated in both positive and negative modes, and full-scan HPLC-QTOF-MS spectra were obtained by scanning from 50 to 1000 m/z. The mass spectrometer was calibrated using a solution of sodium formate. Data were collected in continuum mode and mass was corrected during acquisition using an external reference (Lock-SprayTM) comprising a 10- μ L/min solution of leucine-enkephalin (2 ng/ μ L) via a lock-spray interface. The capillary and cone voltage were set at 3 kV and 30 V, respectively. Nitrogen was used as both cone gas (50 L/h) and desolvation gas (1000 L/h). The source and desolvation temperature were set at 120 and 400 °C, respectively. Simultaneous acquisition of exact mass at high and low collision energy, MS^E (where E represents collision energy), was used to obtain full scan accurate mass fragment, precursor ion, and neutral loss information. The collision energy in function 1 (low energy) was off while in function 2 (high energy), the collision energy ranged between 15 and 60 V.

Analysis of Procyanidins by HPLC with Fluorescence Detector

The separation of flavanol oligomers was performed as previously described (Robbins et al. 2009) using an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler, solvent degasser, quaternary pump, column heater, and DAD and fluorescence detectors. A Develosil Diol 100-Å column (250 \times 4.6 mm; 100-Å pore size; 5- μ m particle size; 35 °C) from Phenomenex (Torrance, CA, USA) was used for separation. The mobile phase consisted of (A) acetonitrile/acetic acid (98:2, v/v) and (B) methanol/water/acetic acid (95:3:2, v/v/v), and the following gradient system was used: 0 min, 7% (B), 0–3 min, 7% (B), 3–60 min, 37.6% (B), 60–63 min, 100% (B), 63–70 min, 100% (B), and 70–76 min, 7% (B), with a flow rate of 1 mL/min and the injection volume was 5 μ L. The autosampler was set to and held at 5 °C. UV data were collected at 280 nm, and fluorescence detection was conducted with an excitation wavelength of 230 nm and emission at 321 nm.

Quantification of procyanidins with different degrees of polymerization (DP) was carried out according to Robbins et al. (2012). They have developed a reliable, robust, and readily transferable method for the analysis and quantification of procyanidins with different DP in cocoa and cocoa products, allowing its broader implementation for the analysis of procyanidins in cocoa. The method was based on the relative response factors (RRFs) of epicatechin specifics for each DP to quantify DP of 2 to 10.

Monomer content was based on the external calibration standard (–)–epicatechin. The concentration of DP 2 to 10 was calculated using the various RRFs according to Robbins

et al. (Robbins et al. 2012) by the following equation:

$$\text{Concentration of flavanols in mg/g} = \left(\frac{((\text{Area}_{\text{DPn}} - b) / (m_{\text{epic}} \times \text{RRF}_{\text{DPn}}))}{(\text{Sample (g)})} \right) \times \text{DF}$$

Where DPn is the degree of polymerization indicating the oligomer, m is the slope of the epicatechin calibration curve, and DF is the appropriate dilution factor. Only a single calibration curve was needed to quantify DP 1–10 since the concentration is based on the RRF to epicatechin. The epicatechin standard solution was injected in triplicate at six concentration levels (0.1–50 μ g/mL). The calibration curve of epicatechin was obtained by plotting peak area as function of concentration (in micrograms/milliliter). Responses obtained in the examined ranges were expressed by a linear equation with a good r^2 determination coefficient value (0.999). The total procyanidin concentration was determined by summing the individual DP 1–10 concentrations.

Statistical Analysis

All the analyses were conducted in triplicate. The data generated were subjected to one-way analysis of variance (ANOVA) using R i386 version 3.3.1 (R foundation for statistical computing, Vienna, Austria). Tukey's paired comparison test was used to determine statistical significant differences ($p < 0.05$) between mean values for different samples at 95% confidence level. Results are expressed as mean value and standard deviation; differences are pointed out with superscripted letters. Differences in the tested variables were considered statistically significant, when $p \leq 0.05$, highly significant when $p \leq 0.01$, and with a trend when $p \leq 0.1$.

Results and Discussion

Two different extraction methods, CSE and PHWE, were employed and compared at an analytical scale to test our hypothesis. As far as our knowledge goes, this is the first time that PHWE is used in such samples. The extracts obtained by CSE and PHWE from cocoa and cocoa products were fully characterized by Folin-Ciocalteu assay, HPLC-DAD-CAD-ECD, HPLC-QTOF/MS, and HPLC-FLD methods.

Total Phenolic Compounds

Figure 3 shows the total phenolic compounds (TPC) measured by Folin-Ciocalteu method for all the different cocoas and cocoa products extracts, obtained by PHWE and CSE. The amounts of TPC extracted by PHWE and CSE were significantly different ($p \leq 0.05$) in cocoas (A and B) and in nibs

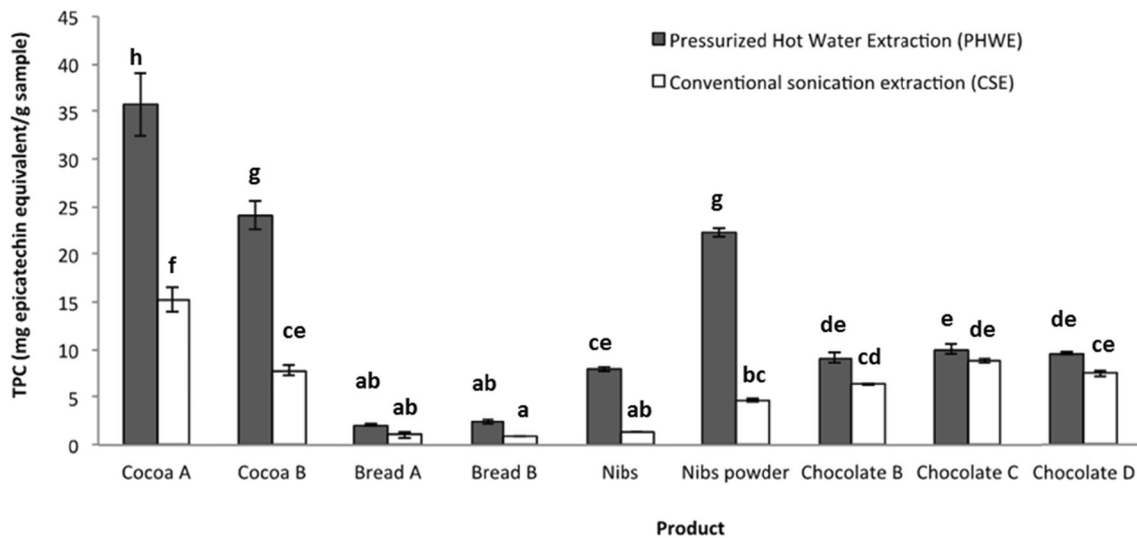


Fig. 3 Total Phenolic Compounds (TPC) (milligram of epicatechin equivalent per gram of sample) measured by the Folin-Ciocalteu method for all different cocoas and cocoa products, extracted by PHWE and CSE. Different letters represent significant differences ($p \leq 0.05$)

(nibs and nibs powder) samples being 2.3, 3.1, 5.6, and 4.7 times, respectively, higher in PHWE. This could be due to the fact that the solvation power of a liquid increases with increasing temperature. Additionally, liquid water at elevated temperature and pressure is a solvent of lower polarizability/polarity and density that enables faster mass transfer and improved wetting of the sample due to higher diffusivity and lower viscosity and surface tension (Plaza and Turner 2015). These liquid water properties at elevated temperature could explain why the TPC was significantly higher when PHWE was used to extract antioxidants from these samples.

However, the TPC extracted using PHWE and CSE were not significantly different ($p \geq 0.05$) in chocolate (B, C, and D), possibly due to the porosity of the particles. This physical characteristic can affect the extraction of TPC. In the chocolate samples, the particles lack porosity which means that the advantages of PHWE about mass transfer could be low. Also, the bread samples (A and B) did not show significant difference ($p \geq 0.05$).

It can be observed that TPC was higher for almost all PHWE extracts of cocoa products, although the highest differences could be observed among samples of different nature (Fig. 3). The highest phenolic content (mg EE/g) was found in cocoas (A and B, 35.7 ± 3.3 and 24.1 ± 1.5 , respectively) and nibs powder (22.3 ± 0.5) in PHWE extracts, while in CSE, was the cocoa A (15.3 ± 1.3) followed by chocolate C (8.8 ± 0.2), and cocoa B (7.9 ± 0.6). The lowest phenolic content in PHWE was found in breads A and B, followed by nibs and chocolates B, D, and C. For CSE extracts, the lowest values were found in breads, nibs, nibs powder, and chocolate B (Fig. 3).

The nature of the sample and its physical characteristics can also affect the capacity of the phenolic extraction. For instance, the cocoa, which is the basic ingredient of the

chocolate, depending on how the cocoa beans are cultivated and how the cocoa powder is produced, can affect the concentration of phenolic compounds present in the cocoa. Therefore, cocoa A presented higher concentration of TPC than cocoa B. Also, the TPC in chocolates depends on the cocoa origin, chocolate production, and percentage of cocoa's ingredient used. Thus, chocolate C had the highest concentration followed by chocolates D and B (Fig. 3).

The bread samples had 15% cocoa powder by wet weight; meaning that the bread should have around 15% of the TPC compared with their corresponding cocoa powders. However, bread B had only 11% and bread A had less than 6% of TPC compared to their corresponding cocoa. This means that some polyphenols or other antioxidant compounds, which react with the Folin-Ciocalteu reagent, were degraded during the bread baking.

The extraction of phenolic compounds was significantly different ($p \leq 0.05$) between nibs and nibs powder, when comparing both extraction techniques. The particle size of nibs was bigger than that of nibs powder. Thus, nibs presented lower TPC. However, when the particle size of the cocoa nibs was reduced using a grinder, the extraction of phenolic compounds increased. As expected, the particle size affected the mass transfer between the nibs and the solvent, because a larger surface area resulted in greater accessibility of the extraction solvent to the analyte and the migration rate of the analyte through the pores of the solid matrix increases with decreasing particle size. Also, the mechanical treatment used to decrease particle size can result in breakage of the cell walls and cell structure, which may enhance the diffusion of the analytes.

Anyhow, the Folin-Ciocalteu assay is not specific for phenolic compounds and it has been proposed for the measurement of total reducing capacity of samples (Magalhães et al.

2008). In this work, further advance analytical techniques were used to show the chemical composition obtained in extracts produced by PHWE and CSE specifically considering phenolic compounds from cocoa and cocoa products.

Identification of the Extracted Phenolic Compounds and Methylxanthines

Separation of phenolic acids, flavanols, flavonols, and methylxanthines of all extracts obtained by PHWE and CSE was carried out using HPLC with DAD coupled to ECD and CAD in analysis time of around 40 min. Figure 4 shows the chromatograms and amperogram of cocoa A obtained by PHWE.

A HPLC-MS^E method was set up, aiming to separate and identify the phenolic compounds present in the extracts. Table 1 shows that the profile of phenolic compounds obtained when using both extraction techniques in the different cocoa products was similar. Nibs powder extracted by PHWE was chosen as representative for being one with high content in phenols (see Fig. 3 and Table 1). The careful analysis of the separated compounds, using the information provided by the DAD detector as well as the MS detector installed in series, together with the information that could be found in the literature and from commercial standards (when available), allowed the identification of 12 phenolic compounds and 2 methylxanthines in the extracts. The identified compounds as well as their chemical characteristics are shown in

Table 2. All phenolic compounds were detected using an electrospray interface in negative, while methylxanthines were detected in positive ionization mode.

Quantification of Phenolic Compounds and Methylxanthines

Quantification of the different phenolic compounds and methylxanthines found in the extracts of cocoa and cocoa products was carried out with a CAD detector. The content of phenolics and methylxanthines of each cocoa product using PHWE and CSE is summarized in Table 1. As can be seen, from the 15 identified compounds, just the compounds with higher concentration (around 10 compounds) could be quantified. One disadvantage of CAD is its low sensitivity compared with other detectors such as DAD (Plaza et al. 2014b). Glycosylated procyanidins A and the flavonols found at lower concentrations could not be quantified (see Fig. 4).

Theobromine (peak 2) was the compound with the highest concentration in all extracts (concentrations from 152.1 to 1039.4 mg/100 g of sample), also caffeine (peak 6) was found at considerable concentrations (from 6.6 to 30.0 mg/100 g of sample) (Table 1). The total concentration of methylxanthines obtained in this study agreed with other works found in the literature (Belščak et al. 2009; Langer et al. 2011). These compounds can be of interest regarding their health benefits, as previously described (Akiba et al. 2004). The ratio of

Fig. 4 Chromatograms and amperograms corresponding to the HPLC-DAD-ECD-CAD analysis of cocoa A extract obtained by PHWE at **a** 280 nm, **b** 350 nm, **c** CAD chromatogram, and **d** amperogram. Analytical conditions are given under “Material and Methods.” For peak identification, see Table 2

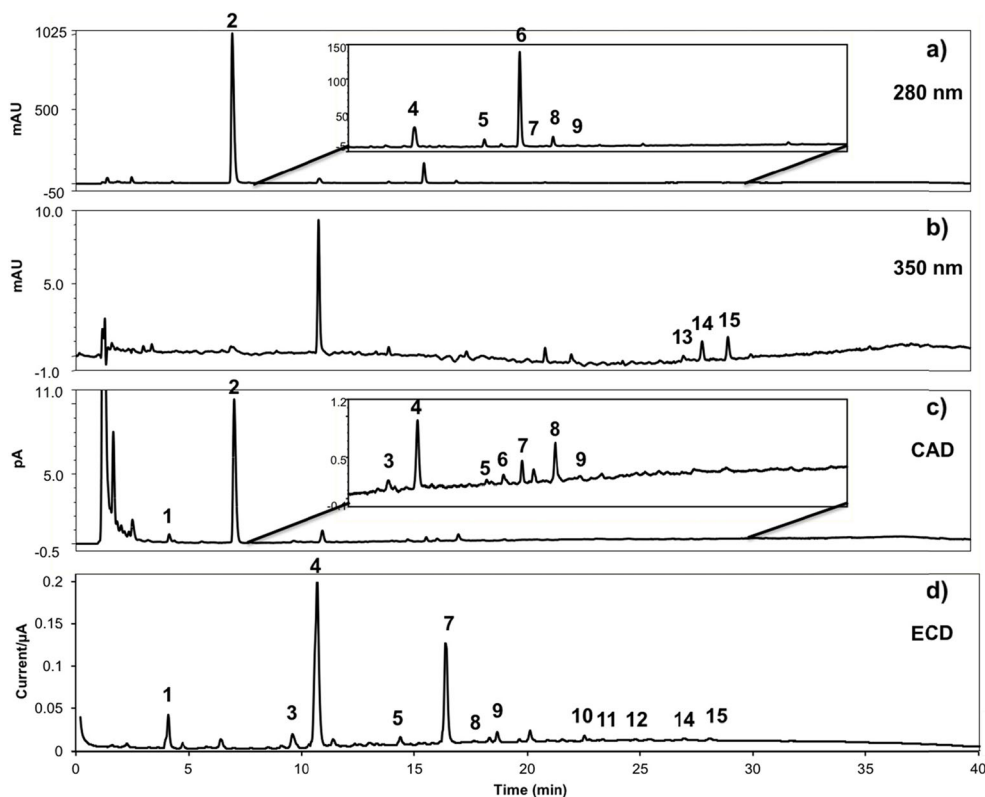


Table 2 Identification (UV-vis and MS characteristics, molecular formula (MF) and mass precision (mDa) and individual antioxidant capacity (linear coefficient (R^2) and slope of the regression line for a side by side comparison between CAD response (concentration (mg/100 g of sample) and ECD response (Area (nA*s)/100 g of sample) for each phenolic compound)) of phenolic compounds and methylxanthines in cocoa and cocoa products

ID	Rt (min)	Identification	UV-Vis maxima (nm)	[M-H] ⁻ (m/z), MF, mDa	Main fragments detected (m/z), MF, mDa	Individual antioxidant capacity	
						R^2	Slope
1	4.03	Unknown	276.8			0.761	10.36
2	6.84	Theobromine ^a	205.8, 272.8	181.0731 [M] ⁺ , C ₇ H ₉ N ₄ O ₂ , 0.5	138.0666, C ₆ H ₈ N ₃ O ₁ , -0.1	–	–
3	9.66	Procyanidin B	201.8, 279.8	577.1343, C ₃₀ H ₂₅ O ₁₂ , -0.3	451.0990, C ₂₄ H ₁₉ O ₆ , -3.9 425.0877, C ₂₂ H ₁₇ O ₆ , 0.4 407.0768, C ₂₂ H ₁₅ O ₈ , 0.1 289.0698, C ₁₅ H ₁₃ O ₆ , -1.4 137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0 109.0306, C ₆ H ₅ O ₂ , 1.6 137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0	0.835	12.99
4	10.9	Catechin ^a	203.8, 278.8	289.0698, C ₁₅ H ₁₃ O ₆ , -1.4	109.0285, C ₆ H ₅ O ₂ , -0.5 451.0990, C ₂₄ H ₁₉ O ₆ , -3.9 425.0877, C ₂₂ H ₁₇ O ₆ , 0.4 407.0768, C ₂₂ H ₁₅ O ₈ , 0.1 289.0698, C ₁₅ H ₁₃ O ₆ , -1.4 137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0	0.846	27.21
5	14.74	Procyanidin B2 ^a	205.8, 278.8	577.1343, C ₃₀ H ₂₅ O ₁₂ , -0.3	109.0285, C ₆ H ₅ O ₂ , -0.5 451.0990, C ₂₄ H ₁₉ O ₆ , -3.9 425.0877, C ₂₂ H ₁₇ O ₆ , 0.4 407.0768, C ₂₂ H ₁₅ O ₈ , 0.1 289.0698, C ₁₅ H ₁₃ O ₆ , -1.4 137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0	0.836	21.76
6	14.91	Caffeine ^a	205.8, 272.8	195.0878 [M] ⁺ , C ₈ H ₁₀ N ₄ O ₂ , -0.4	109.0285, C ₆ H ₅ O ₂ , -0.5 138.0666, C ₆ H ₈ N ₃ O ₁ , -0.1	–	–
7	15.36	Epicatechin ^a	203.8, 278.8	289.0698, C ₁₅ H ₁₃ O ₆ , -1.4	137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0 109.0285, C ₆ H ₅ O ₂ , -0.5 611.1370, C ₃₀ H ₂₇ O ₁₄ , -3.1 449.0991, C ₂₄ H ₁₇ O ₆ , 3.1 289.0698, C ₁₅ H ₁₃ O ₆ , -1.4	0.898	32.04
8	17.47	Procyanidin A glucoside	201.8, 278.8	737.1705, C ₃₆ H ₃₃ O ₁₇ , -1.3	137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0 109.0285, C ₆ H ₅ O ₂ , -0.5 611.1370, C ₃₀ H ₂₇ O ₁₄ , -3.1 449.0991, C ₂₄ H ₁₇ O ₆ , 3.1 289.0698, C ₁₅ H ₁₃ O ₆ , -1.4 137.0247, C ₇ H ₅ O ₃ , 0.8 125.0226, C ₆ H ₅ O ₃ , -1.3 109.0285, C ₆ H ₅ O ₂ , -0.5	0.796	11.30
9	18.02	Procyanidin C	209.8, 278.8	865.1942, C ₄₅ H ₃₇ O ₁₈ , -3.8	739.1582, C ₃₉ H ₃₁ O ₁₅ , -8.1 713.1498, C ₃₇ H ₂₉ O ₁₅ , -0.8 695.1346, C ₃₇ H ₂₇ O ₁₄ , -5.5 577.1287, C ₃₀ H ₂₅ O ₁₂ , -5.3 451.0947, C ₂₄ H ₁₉ O ₆ , 0.5 425.0877, C ₂₂ H ₁₇ O ₆ , 0.4 407.0768, C ₂₂ H ₁₅ O ₈ , 0.1 289.0698, C ₁₅ H ₁₃ O ₆ , -1.4 137.0247, C ₇ H ₅ O ₃ , 0.8 125.0249, C ₆ H ₅ O ₃ , 1.0 109.0285, C ₆ H ₅ O ₂ , -0.5	0.595	6.92

Table 2 (continued)

ID	Rt (min)	Identification	UV-Vis maxima (nm)	[M-H] ⁻ (m/z), MF, mDa	Main fragments detected (m/z), MF, mDa	Individual antioxidant capacity	
						R ²	Slope
10	23.30	Procyanidin A galactoside	278.8	737.1705, C ₃₆ H ₃₃ O ₁₇ , -1.3	611.1370, C ₃₀ H ₂₇ O ₁₄ , -3.1	—	—
					449.0861, C ₂₄ H ₁₇ O ₆ , -1.2	—	—
					289.0698, C ₁₅ H ₁₃ O ₆ , -1.4	—	—
					137.0247, C ₇ H ₅ O ₃ , 0.8	—	—
					125.0249, C ₆ H ₅ O ₃ , 1.0	—	—
11	23.90	Procyanidin A arabinoside	278.8	707.1645, C ₃₅ H ₃₁ O ₁₆ , -1.7	109.0285, C ₆ H ₅ O ₂ , -0.5	—	—
					581.1288, C ₂₉ H ₂₅ O ₁₃ , -0.7	—	—
					449.0861, C ₂₄ H ₁₇ O ₆ , -1.2	—	—
					289.0698, C ₁₅ H ₁₃ O ₆ , -1.4	—	—
					137.0247, C ₇ H ₅ O ₃ , 0.8	—	—
12	24.65	Procyanidin B	279.8	577.1343, C ₃₀ H ₂₅ O ₁₂ , -0.3	125.0249, C ₆ H ₅ O ₃ , 1.0	—	—
					109.0285, C ₆ H ₅ O ₂ , -0.5	—	—
					451.0990, C ₂₄ H ₁₉ O ₆ , -3.9	—	—
					425.0877, C ₂₂ H ₁₇ O ₆ , 0.4	—	—
					407.0768, C ₂₂ H ₁₅ O ₈ , 0.1	—	—
13	27.48	Quercetin glucoside	352.8	463.0815, C ₂₁ H ₁₉ O ₁₂ , 2.6	289.0698, C ₁₅ H ₁₃ O ₆ , -1.4	—	—
					137.0223, C ₇ H ₅ O ₃ , -1.6	—	—
					125.0249, C ₆ H ₅ O ₃ , 1.0	—	—
					109.0285, C ₆ H ₅ O ₂ , -0.5	—	—
					301.0337, C ₁₅ H ₉ O ₇ , -1.1	—	—
14	28.17	Quercetin galactoside	355.8	463.0858, C ₂₁ H ₁₉ O ₁₂ , -1.9	301.0337, C ₁₅ H ₉ O ₇ , -1.1	—	—
					301.0337, C ₁₅ H ₉ O ₇ , -1.1	—	—
15	29.26	Quercetin arabinoside	352.8	433.0765, C ₂₀ H ₁₇ O ₁₁ , -0.6	301.0337, C ₁₅ H ₉ O ₇ , -1.1	—	—

^a Identified and confirmed using commercial standards

theobromine to caffeine found in the cocoa products (if the concentration of theobromine is normalized as 10) was 10:0.1–0.8.

Catechin (peak 4) (9.1–88.8 mg/100 g of sample) and epicatechin (peak 7) (9.1–67.4 mg/100 g of sample) were the phenolic compounds found in higher concentration in almost all extracts of cocoa products. The concentrations detected for catechin and epicatechin agreed with the literature (Gu et al. 2006). Concentrations of procyanidin B2 (peak 5) (8.7–43.6 mg/100 g of sample), procyanidin A glucoside (peak 8) (7.5–48.3 mg/100 g of sample), and procyanidin C (peak 9) (6.7–27.7 mg/100 g of sample) in the extracts of cocoa products were significant. The unknown phenolic compound (peak 1) was one of the main compounds in some cocoa extracts (6.2–57.3 mg/100 g of sample) (Table 1).

Cocoa A was the product with the highest concentration of phenolic compounds followed by nibs powder, cocoa B, nibs, chocolate C, chocolate D, and chocolate B. The lowest concentration of phenolic compounds was found in the cocoa breads (A and B). The total phenolic compounds extracted in cocoas and nibs depended on their manufacture and variety. But the phenolic composition in chocolates and breads depends on the quality of raw cocoa material, variety of the beans, and recipe, for instance, chocolates have higher amount of cocoa powder than bread (15% of cocoa powder base on wet weight).

By comparing both extraction techniques, PHWE seemed to be a more suitable technique than CSE to extract these kinds of phenolic compounds from cocoa and cocoa products (Table 1). The differences in the extraction of the different phenolic compounds between both extraction techniques were significantly different ($p \leq 0.05$) for almost all products in most of the detected peaks, except for breads (A and B) (Table 1). Highly significant differences ($p \leq 0.01$) could be observed in cocoa A, nibs, and nibs powder for peaks 1, 4, and 8, also in chocolate C for peaks 3, 4, 5, 7, and 9. However, non-significant differences ($p \geq 0.05$) were observed for breads A and B, whereas for the different kinds of chocolates (B, C, and D), both significant and non-significant differences were found depending on the phenolic compound detected. Regarding phenolic compounds extraction, PHWE technique revealed to be way more effective than CSE. When compared, PHWE was able to extract twice the amount of phenolic compounds as compared with CSE in cocoa A (266.1 vs. 133.3 mg/100 g of sample) and nibs (153.1 vs. 72.3 mg/100 g of sample). Also, PHWE extracted 1.6, 1.5, and 1.3 times more than CSE in cocoa B (164.2 vs. 102.8 mg/100 g sample), nibs powder (218.1 vs. 146.2 mg/100 g sample), and chocolate C (140.7 vs. 103.2 mg/100 g sample), respectively. Compared with CSE, PHWE was only less efficient (although not significantly ($p \geq 0.05$)) for bread A, chocolate B, and D samples.

The results above are in agreement with the results obtained for total phenolic compounds measured by Folin-Ciocalteu method (section “Total phenolic compounds”, Fig. 3). However, the latter method did not measure just phenolic compounds, because it measures any reductive substance as well as possible antagonism and synergism effects between different compounds. For instance, methylxanthines did not show antioxidant capacity (see Table 2) but they could have synergistic interactions with polyphenols. Moreover, comparing total phenolic compounds determined by the Folin-Ciocalteu method (Fig. 3) with the total phenolic compounds obtained by HPLC-CAD analysis (Table 1), there is a decrease using the latter analysis. These differences are likely due to the nonspecificity of the Folin-Ciocalteu assay. Furthermore, some of the phenolic compounds could not be quantified with the employed HPLC-CAD method, and it did not detect all phenolic compounds. Only, the free monomers and dimers that link to small sugars were detected with the HPLC-CAD method.

Moreover, PHWE extracted also higher concentration of methylxanthines than CSE from almost all samples except for bread A and chocolate B (Table 1). Highly significant differences ($p \leq 0.01$) between PHWE and CSE were observed in all products except for breads A and B and chocolates C and D. PHWE was also able to extract 3.9, 2.5, 1.7, and 1.6, times more theobromine than CSE in nibs, cocoa B, nibs powder, and cocoa A, respectively, being less effective only in bread A and chocolate B. Regarding caffeine, again PHWE extracts revealed higher values for almost all samples, except for chocolate C and D, but only significantly ($p \leq 0.05$) for the later (Table 1).

Individual Antioxidant Capacity of Phenolic Compounds

The ECD was used to study the oxidation of phenolic compounds to indicate the antioxidant capacity of each compound. To determine the contribution of the individual phenolic compounds to the total antioxidant capacity of the cocoa extracts, the concentration of each compound as determined by CAD and the peak area obtained by ECD for each compound were correlated in scatter plots (Table 2). The antioxidant capacity was able to detect seven compounds (peaks 1, 3, 4, 5, 7, 8, and 9), because only these compounds were found in high enough concentration possible to carry out the quantification by CAD. The methylxanthines, theobromine (peak 2), and caffeine (peak 6) were found in higher concentration but no antioxidant capacity was associated with these compounds. These results are in line with data reported for these compounds found in tea (Stewart et al. 2005).

As shown in Table 2, the response between both detectors was fairly linear, with the r^2 being ≥ 0.761 . Procyanidin C (peak 9) presented a low r^2 (0.595). Using the slope, it was possible to calculate the individual antioxidant capacity of

Table 3 Quantification (mg/100 g of sample \pm standard deviation (sd)) of procyanidins in extracts obtained by PHWE and CSE from cocoa and cocoa products using HPLC-FLD

Procyanidins	Cocoa A		Cocoa B		Bread A		Bread B		Nibs	
	CSE	PHWE	CSE	PHWE	CSE	PHWE	CSE	PHWE	CSE	PHWE
DP 1	165.0 \pm 19.4 ^f	194.9 \pm 21.3 ^g	122.3 \pm 7.2 ^e	180.2 \pm 4.0 ^{fg}	37.1 \pm 0.1 ^{ab}	39.0 \pm 1.4 ^{ab}	26.9 \pm 3.7 ^a	32.8 \pm 3.3 ^a	45.0 \pm 2.6 ^{ab}	57.1 \pm 3.9 ^{ab}
DP 2	150.2 \pm 19.3 ⁱ	196.2 \pm 14.8 ^j	97.9 \pm 7.8 ^{eg}	132.4 \pm 11.8 ^{hi}	26.0 \pm 0.5 ^{ab}	15.5 \pm 2.4 ^a	16.9 \pm 0.1 ^a	25.8 \pm 1.2 ^{ab}	47.1 \pm 5.4 ^{ac}	50.2 \pm 2.7 ^{bc}
DP 3	21.5 \pm 0.4 ^{df}	55.6 \pm 8.1 ⁱ	10.2 \pm 1.5 ^{abc}	9.7 \pm 1.2 ^{abc}	3.5 \pm 0.2 ^a	4.7 \pm 0.7 ^a	3.3 \pm 0.5 ^a	3.6 \pm 0.5 ^a	8.4 \pm 1.1 ^{ab}	5.7 \pm 0.5 ^a
DP 4	10.2 \pm 0.2 ^b	24.0 \pm 1.2 ^d	1.7 \pm 0.2 ^a	1.8 \pm 0.3 ^a	ND	ND	ND	ND	0.28 \pm 0.02 ^a	0.29 \pm 0.03 ^a
DP 5	12.1 \pm 1.6 ^f	13.7 \pm 2.7 ^f	22.3 \pm 0.8 ^g	34.3 \pm 3.9 ^h	ND	ND	0.5 \pm 0.1 ^{ab}	0.77 \pm 0.04 ^{ab}	ND	0.023 \pm 0.002 ^a
DP 6	122.9 \pm 20.4 ^f	99.4 \pm 7.6 ^e	46.5 \pm 3.6 ^{cd}	86.2 \pm 6.7 ^e	11.4 \pm 0.6 ^{ab}	14.8 \pm 1.3 ^{ab}	2.5 \pm 0.1 ^a	2.5 \pm 0.3 ^a	16.0 \pm 2.3 ^{ab}	33.4 \pm 2.7 ^{bc}
DP 7	35.5 \pm 1.8 ^d	34.5 \pm 3.9 ^{cd}	ND	ND	3.4 \pm 0.4 ^a	4.2 \pm 0.1 ^a	ND	ND	7.3 \pm 0.3 ^a	27.1 \pm 2.0 ^{bd}
\geq DP 8	ND	ND	ND	ND	ND	ND	ND	ND	8.5 \pm 1.1 ^a	35.8 \pm 2.9 ^b
Total	517.4 \pm 16.6 ^j	618.4 \pm 42.8 ^k	300.9 \pm 14.7 ^{ef}	444.6 \pm 13.9 ^{hi}	81.4 \pm 0.2 ^{ab}	78.2 \pm 4.3 ^{ab}	50.1 \pm 3.1 ^a	65.5 \pm 2.9 ^a	132.6 \pm 4.9 ^{bc}	209.5 \pm 7.9 ^{de}
Procyanidins	Nibs powder		Chocolate B		Chocolate C		Chocolate D			
	CSE	PHWE	CSE	PHWE	CSE	PHWE	CSE	PHWE	CSE	PHWE
DP 1	129.1 \pm 14.2 ^e	175.7 \pm 5.5 ^{fg}	51.7 \pm 5.3 ^{ab}	62.9 \pm 1.7 ^{bc}	102.8 \pm 11.3 ^{de}	116.9 \pm 10.9 ^{de}	57.8 \pm 7.9 ^{ab}	92.0 \pm 3.9 ^{cd}	92.0 \pm 3.9 ^{cd}	92.0 \pm 3.9 ^{cd}
DP 2	153.8 \pm 15.5 ⁱ	207.9 \pm 6.8 ^j	62.7 \pm 1.6 ^{cd}	70.7 \pm 4.1 ^{ce}	110.6 \pm 9.5 ^{fgh}	124.9 \pm 8.8 ^{gi}	65.7 \pm 8.6 ^{cd}	81.8 \pm 10.0 ^{def}	81.8 \pm 10.0 ^{def}	81.8 \pm 10.0 ^{def}
DP 3	45.7 \pm 1.7 ^b	62.0 \pm 2.6 ⁱ	18.2 \pm 2.5 ^{cde}	15.4 \pm 2.5 ^{bd}	36.4 \pm 3.0 ^g	35.4 \pm 2.0 ^g	26.6 \pm 5.1 ^{ef}	30.2 \pm 1.1 ^{fg}	30.2 \pm 1.1 ^{fg}	30.2 \pm 1.1 ^{fg}
DP 4	27.9 \pm 2.8 ^d	38.0 \pm 2.0 ^e	2.9 \pm 0.3 ^a	3.7 \pm 0.6 ^a	15.3 \pm 2.7 ^c	16.4 \pm 0.9 ^c	17.0 \pm 1.8 ^c	15.0 \pm 2.6 ^c	15.0 \pm 2.6 ^c	15.0 \pm 2.6 ^c
DP 5	7.4 \pm 0.5 ^{de}	9.6 \pm 1.6 ^{ef}	1.5 \pm 0.1 ^{abc}	0.8 \pm 0.2 ^{ab}	6.7 \pm 0.8 ^{de}	4.6 \pm 0.6 ^{bd}	5.8 \pm 0.6 ^{cde}	3.8 \pm 0.7 ^{cd}	3.8 \pm 0.7 ^{cd}	3.8 \pm 0.7 ^{cd}
DP 6	12.1 \pm 1.4 ^a	92.0 \pm 10.2 ^e	34.2 \pm 2.0 ^{bc}	45.9 \pm 5.7 ^{cd}	40.9 \pm 0.5 ^{cd}	61.4 \pm 7.5 ^d	4.5 \pm 0.8 ^a	6.2 \pm 1.2 ^a	6.2 \pm 1.2 ^a	6.2 \pm 1.2 ^a
DP 7	65.6 \pm 6.1 ^e	80.8 \pm 9.3 ^f	23.1 \pm 1.5 ^{bc}	23.4 \pm 1.2 ^{bc}	28.3 \pm 1.0 ^b	28.7 \pm 0.7 ^{bd}	26.7 \pm 4.6 ^{bd}	28.2 \pm 1.5 ^{bd}	28.2 \pm 1.5 ^{bd}	28.2 \pm 1.5 ^{bd}
\geq DP 8	43.8 \pm 4.6 ^c	ND	ND	ND	1.5 \pm 0.1 ^a	ND	7.1 \pm 0.9 ^a	ND	ND	ND
Total	485.4 \pm 43.4 ^{ij}	666.1 \pm 28.3 ^k	194.2 \pm 5.3 ^{cd}	222.8 \pm 5.3 ^{de}	336.5 \pm 12.9 ^{fh}	388.0 \pm 29.7 ^{gh}	211.2 \pm 19.6 ^{de}	257.2 \pm 11.2 ^{de}	257.2 \pm 11.2 ^{de}	257.2 \pm 11.2 ^{de}

All measurements were done in triplicate. DP degrees of polymerization from monomers (DP1) to octamers (DP8), CSE conventional sonication extraction, PHWE pressurized hot water extraction, ND not detected. Mean values with different superscript letters in the same row are significantly different ($p \leq 0.05$)

each phenolic compound in cocoa and cocoa product extracts. The higher the slope, the higher the antioxidant capacity. The results revealed that the compounds with the highest antioxidant capacity were the monomers epicatechin (slope 32.04) and catechin (slope 27.21). The monomers presented higher antioxidant capacity than dimers (procyanidins B2 and B) and trimers (procyanidin C). It is not in agreement with data found in literature that showed that the antioxidant capacity of grape seed procyanidins was related to their degree of polymerization, for instance, the monomers were the compounds with less antioxidant power (Spranger et al. 2008). However, to the best of our knowledge, there is no data that shows this statement in cocoa procyanidins. Other studies suggested that the anthocyanin's monomers were better hydrogen-donating antioxidant compared to oligomeric proanthocyanidins (Kong et al. 2003). Between the dimers, the procyanidins B2 and B had higher antioxidant power than procyanidin A glucoside because the glycosylation of the latter decreased its antioxidant capacity (Plaza et al. 2014a). The unknown compound (peak 1) and procyanidin C (peak 9) possessed the lowest antioxidant capacity (see Table 2).

Determination of Procyanidins by HPLC-FLD

The HPLC-DAD-ECD-CAD-MS method allowed the identification and quantification of monomers, dimers, and some trimers of procyanidins. However, this method could not determine larger oligomers of procyanidins. These compounds with different DP belong to the group of flavanols

(see Fig. 1). They are the phenolic compounds more abundant in cocoa and have a range of positive physiological effects in humans; most notably, they reduce blood pressure and reduce the risk of cardiovascular disease and stroke (Sudano et al. 2012). Due to their structural diversity and complexity, the analytical methodology employed for the analysis of these compounds is based mainly upon DP instead of separation of individual compounds and specific isomers (Robbins et al. 2009). Therefore, a normal-phase HPLC-FLD method using a HILIC column was carried out to accomplish the analysis of procyanidins with different degrees of polymerization (Robbins et al. 2009; Robbins et al. 2013; Robbins et al. 2012). This method allowed the measurement of the large structural diversity of these compounds by oligomeric size. The procyanidins from cocoa and cocoa products have been quantified up to predefined molecular weight between DP1 and DP10. Procyanidins standards with DP2 and DP3 are commercially available; however, flavanols with DP4–10 are not. Thus, in this work, RRFs were used to achieve the quantification for these compounds due to the lack of commercial standards (see section “Analysis of Procyanidins by HPLC with fluorescence detector”). The RRFs used were specific for this analytical method according to Robbins et al. (2013, 2012). The results and chromatographic profile of cocoa procyanidins are shown in Table 3 and Fig. 5, respectively.

As can be seen in Table 3, DP1 and DP2 were the most abundant procyanidins followed by DP6 and DP7. DP7 was in higher concentration than DP6 just in chocolate D. However,

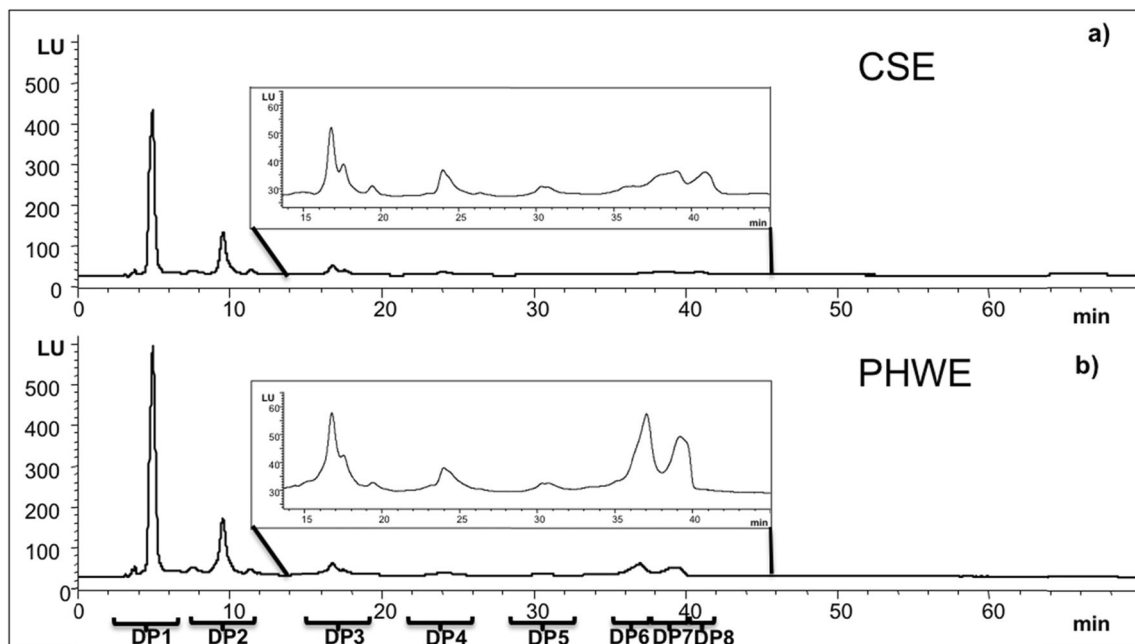


Fig. 5 HPLC-FLD profile of flavanol oligomers from nibs powder extract obtained by **a** CSE and **b** PHWE. FLD detection wavelength was 230 and 321 nm for excitation and emission, respectively. The

peaks represent different degrees of polymerization (DP), from monomers (DP1) to octomers (DP8)

\geq DP8, DP4, and DP5 were found in less concentration. Additionally, \geq DP8 was not presented in some samples as cocoas A and B, chocolate B, and the breads A and B, and DP7 was neither in cocoa B nor bread B. Mostly PHWE did not extract \geq DP8, only in nibs and nibs powder was an observed \geq DP8.

In general, PHWE was more efficient and extracted higher amounts of procyanidins from cocoa and cocoa products than CSE. Highly significant differences ($p \leq 0.01$) between both extraction methods in the extraction of total procyanidins were observed in cocoas A and B, and nibs and nibs powder, while non-significant differences ($p \geq 0.05$) were found in chocolates B, C, and D and breads A and B (Table 3). These results matched well with the results achieved for total phenolic compounds and for the phenolic compounds analyzed by reverse-phase HPLC (see Tables 1 and 3 and Fig. 3).

Cocoa A and nibs powder were the samples with more procyanidins. Nibs were a good source of polyphenols. As mentioned previously, the concentration of polyphenols in the different cocoa products depends on the percentage of cocoa, which justifies why the bread samples presented the lowest concentration of phenolic compounds. The percentage of cocoa powder in the bread was of 25% of dry weight and 15% of wet weight. And the total procyanidins on the bread represented an average of $15 \pm 2\%$ of the total procyanidins of their respective cocoas and extraction method. Hence, the procyanidin concentration in cocoa and cocoa bread correlate perfectly, also meaning that the procyanidins from the bread were not degraded during its preparation and baking.

Langer et al. (2011), Miller et al. (2006), and Todorovic et al. (2015) used a similar extraction method as CSE to measure the levels of total procyanidin oligomers in dark chocolate. Their results were 94–793, 278–410, and 187–263 mg/100 g of product, respectively. These levels were very similar to the ones found in samples B, C, and D for CSE (194–337 mg/100 g) and for PHWE (223–388 mg/100 g). However, Gu et al. (2006) and Robbins et al. (2013) presented values of total procyanidins of 852–1985 and 654–965 mg/100 g, respectively, being higher than the level detected in our samples.

Usually, higher concentrations of DP1 and DP2 flavanols were found with normal-phase HPLC-FLD method than with reverse-phase HPLC-DAD-ECD-CAD method. These differences could be due to the limited sensitivity of the CAD detector, not able to quantify compounds to low concentration. On the other hand, FLD is a sensitive detector. Also, procyanidins have a tendency to cross-link with proteins, sugars, and others compounds. HPLC-DAD-ECD-CAD method just detected the free monomers and dimers that link to small sugars. Overall, once again, PHWE technique was the most effective in extracting the target compounds.

Conclusions

The results obtained in this work have supported our hypothesis. PHWE method was more efficient in extracting phenolic compounds and methylxanthines from cocoa and cocoa products as compared to CSE method. The differences between both extraction methods were higher in the procyanidin group for all the tested samples and significantly higher for the cocoas (A and B) and nibs (nibs and nibs powder) samples. In addition, PHWE avoided many of extraction steps (i.e., defatting step) and the use of organic solvents, being a greener extraction method and more controlled. Also a full characterization of the phenolic composition of the cocoa products was possible by using advance analytical tools such as HPLC-DAD-ECD-CAD-MS and HPLC-FLD.

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Compliance with Ethical Standards

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Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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