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RESEARCH PAPER

Extraction of cocoa proanthocyanidins and their fractionation by sequential centrifugal partition chromatography and gel permeation chromatography

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Abstract Cocoa beans contain secondary metabolites ranging from simple alkaloids to complex polyphenols with most of them believed to possess significant health benefits. The increasing interest in these health effects has prompted the need to develop techniques for their extraction, fractionation, separation, and analysis. This work provides an update on analytical procedures with a focus on establishing a gentle extraction technique. Cocoa beans were finely ground to an average particle size of <100 μm, defatted at 20 °C using *n*-hexane, and extracted three times with 50 % aqueous acetone at 50 °C. Determination of the total phenolic content was done using the Folin-Ciocalteu assay, the concentration of individual polyphenols was analyzed by electrospray ionization high performance liquid chromatography-mass spectrometry (ESI-HPLC/MS). Fractions of bioactive compounds were separated by combining sequential centrifugal partition chromatography (SCPC) and gel permeation column chromatography using Sephadex LH-20. For SCPC, a two-phase solvent system consisting of ethyl acetate/*n*-butanol/water (4:1:5, *v*/*v*/*v*) was successfully applied for the separation of theobromine, caffeine, and representatives of the two main phenolic compound classes flavan-3-ols and flavonols. Gel permeation chromatography on Sephadex LH-20 using a stepwise elution sequence with aqueous acetone has been shown for effectively separating individual flavan-3-ols. Separation was obtained for (−)-epicatechin, proanthocyanidin dimer B2, trimer C1, and tetramer cinnamtannin A2. The purity of alkaloids and phenolic compounds was determined by HPLC analysis and their chemical identity was confirmed by mass spectrometry.

Keywords Cocoa proanthocyanidins · Sequential centrifugal partition chromatography · Gel permeation chromatography · Total phenolic content

Introduction

The frequently reported relationship between plant phenolic compounds, often referred to as polyphenols, and health has accelerated the research into these bioactive compounds. Promisingly, proanthocyanidins (PAs) in plant food are investigated because of their reported antioxidant, antiinflammatory, and antimicrobial activity. Especially, PAs from cocoa (*Theobroma cacao* L.) are assumed to possess health benefits [e.g., 1]. In general, cocoa beans contain compounds ranging from simple alkaloids such as theobromine and caffeine to complex polyphenols, which can be classified into the main groups of phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and the quite large group of flavonoids comprising flavan-3-ols (catechin, epicatechin), anthocyanins (cyanidins), flavonols (quercetin), flavones (luteolin), and flavanones (naringenin). Especially higher oligomeric PAs, resulting from a condensation of flavan-3-ols, are assumed to have a higher antioxidant capacity than the monomers or lower oligomeric PAs. Despite the high scientific interest in compounds like cocoa PAs, the majority of them are either not commercially available or are very expensive.

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It is generally consent that at the moment it is impossible to develop an extraction method suitable for all bioactive compounds from plant materials. Nevertheless, a technique that generates significant amounts of purified compounds is essential for analytical purposes or bioavailability and related bioactivity studies. Accordingly, one objective of this work was to evaluate the highest content of total phenols obtained with different extraction conditions. Folin-Ciocalteu method was used for the quantification of the total phenolic content. The proximate composition of the total flavonoid content and the total PA content were also determined photometrically.

In order to further fractionate complex phenolic extracts, preparative techniques in various modes are frequently used to separate bioactive compounds. Separation of PAs in cocoa according to their molecular size has already been reported for normal phase HPLC [2]. Preparative purifying processes for catechins and PAs on reversed phase HPLC of root bark have been also described [3], as well as size exclusion chromatography for PAs obtained from various plant bodies including cocoa beans [4]. In the case of phenolic compounds, solid-liquid chromatography suffers from major drawbacks. The recovery rate and the obtained amounts are much less than the other separation mechanisms. To overcome these problems, separation using liquid-liquid chromatography has attracted researches as a more efficient approach. Shibusawaa, Yanagidaa, Isozakia, Shindoa, and Ito [5] separated apple PAs with regard to degree of polymerization (DP) using *high speed countercurrent chromatography* (HSCCC). Liquid-liquid chromatographic techniques such as HSCCC or *sequential centrifugal partition chromatography* (SCPC) are covering an important niche among the chromatographic methods applied for the isolation of secondary plant metabolites. A significant advantage can be seen in the avoidance of irreversible adsorption interactions on solid stationary phases, the limited risk of sample denaturation, the possibility for a total sample recovery, a comparatively large load capacity, and the possibility to perform separations on a semi-preparative scale [6]. SCPC is a relatively new continuous cyclic liquid-liquid chromatographic separation technology [7]. In contrast to CCC, CPC chromatography enables a fast chromatographic run and an almost unlimited upscaling.

A study by Esatbeyoglu, Wray, and Winterhalter [8] showed the possibility of isolating dimeric, trimeric, tetrameric, and pentameric PAs from unroasted cocoa beans using HSCCC with *tert*-butylmethlyether/*n*-butanol/water (4.3:0.7:5, *v*/*v*/*v*) as solvent system. Further, different modes of operation do however not permit a simple transfer of a HSCCC solvent system for isolation of cocoa polyphenols using to a SCPC separation. A complete separation of the main cocoa compounds using SCPC has not been described, yet. Furthermore, as the separation efficiency of liquid-liquid chromatographic systems is typically not sufficient for achieving a complete separation of complex samples in one single chromatographic run, the combination of SCPC with other methods such as preparative column chromatography is necessary because of their complementary properties.

Combinations of HSCCC and gel permeation chromatography using Sephadex LH-20 have already been shown to be an efficient separation technique. In a study by Yang, Li, and Wan [9], the main individual tannins from black tea were purified, whereby theaflavin, theaflavin-3-gallate, theaflavin-3-gallate, and theaflavin-3,3-digallate were obtained in a separation process using a combination of HSCCC and a chromatographic separation with Sephadex LH-20. Also, Zhou, Liang, Zhang, Zhao, Guo, and Shi [10] obtained a purification of glucosidase inhibitors from *Polygonatum odoratum* by HSCCC and a following separation with Sephadex LH-20. A study done by Cheel, Minceva, Urajová, Aslam, Hrouzek, and Kopecký [11] used also preparative CPC separation followed by gel permeation chromatography to obtain on 50 mg of crude soil cyanobacteria extract a yield of 3.5 mg Aeruginosin-865, with a purity over 95 % as determined by HPLC.

Therefore, the aim of the present study was first to find an efficient extraction method for cocoa PAs. For this purpose, various extraction solvents were studied and extraction conditions such as temperature, solid-liquid ratio, and extraction time were optimized. The second objective was to establish a new method for the separation and purification of cocoa bean extracts using sequential centrifugal partition chromatography combined with gel permeation chromatography. Critical parameters such as the two-phase solvent system and the sample load for the SCPC separation were optimized. The chemical structures of the purified compounds obtained were confirmed by mass spectrometry.

Materials and methods

Materials

The phenolic reference substances (−)-epicatechin and (+)-catechin were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Proanthocyanidin B3 and B4 were purchased from TransMIT GmbH (Giessen, Germany). Further reference substances such as theobromine, caffeine, (−)-epicatechin, (+)-catechin, proanthocyanidin B1, quercetin, quercetin-3-*O*-arabinoside, and quercetin-3-*O*-glycoside were obtained from Extrasynthese (Lyon, France). Anhydrous sodium carbonate, aluminum chloride hexahydrate, *n*-butanol, Folin-Ciocalteu reagent, sodium hydroxide, and sodium nitrite were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade acetonitrile, water, ethanol, acetone, methanol, 1-propanol,

2-propanol, and formic acid were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Double-distilled water (Merck & Cie KG, Schaffhausen, Switzerland) was used throughout the whole study. Column chromatography was carried out with Sephadex LH-20 gel (GE Healthcare Bio-Sciences AB, Glattbrugg, Switzerland).

Sample preparation

Dried and fermented cocoa beans were from a genuine Trinitario variety grown and harvested in Ghana. Optimization of sample preparation was performed as described previously [12]. Primarily, frozen cocoa beans were freeze dried (Alpha 2-4 LDplus, Martin Christ, Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), manually dehulled, and crushed in a knife mill (A 11 basic Analytical Mill, IKA ®-Werke GmbH & Co. KG, Staufen, Germany) for approx. 10 s to minimize heat development. In order to remove lipids, cocoa beans were extracted five times with the fivefold volume (*v*/*w*) of *n*-hexane for 5 min at 20 °C. The hexane phase was removed by centrifugation at 2880×*g* for 1 min (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). This procedure of defatting and grinding was done four times until the *n*-hexane phase appeared colorless. After defatting, cocoa bean powder with an average particle size of <100 μm was further used for the extraction of target compounds. For the analysis of the total phenolic content, 2 g of defatted and dry cocoa powdered samples was weighed into a 50-mL centrifuge vessel and extracted three times with 6 mL of aqueous acetone mixture for 8 min at 50 °C (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany). After each extraction step, the mixture was centrifuged and the combined supernatant was considered as *liquid cocoa extract* and directly used for spectrophotometric assays, or freeze dried for SCPC analysis and named *freeze dried cocoa extract*.

Solvent and temperature optimization

A range of solvents were tested for their efficacy in extracting target compounds from ground cocoa bean extract, including methanol, ethanol, 1-propanol, 2-propanol, and acetone, and their respective mixtures with water. Thirteen extraction temperatures (20–140 °C) were evaluated for their effect on the extraction of valuable cocoa compounds.

Generally, 2 g of cocoa bean powder was extracted three times with 6 mL of solvent/water mixtures for 8 min in a 50-mL centrifuge tube at different temperatures using a benchtop shaker (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany) at 800 rpm. The liquid cocoa extract obtained was analyzed for the total phenolic content using the photometric Folin-Ciocalteu assay, and for individual components using RP-HPLC/MS. Subsequently, individual compounds of the maintained liquid cocoa extracts were measured by RP-HPLC/MS. All extraction experiments were done in triplicate.

Determination of the total phenolic content (TPC) using Folin-Ciocalteu assay

The photometric method for determining the total phenolic content is used frequently in food analysis and is applicable to polar solvents. However, there are other reducing substances present, such as sugars or proteins, which are also able to react with the Folin-Ciocalteu reagent [13]. The total phenolic content (TPC) of cocoa beans was determined according to Blois [14] with some minor modifications: For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:10, 1:100. Following, a 1-mL aliquot of the *liquid cocoa extract* was mixed with 1 mL of Folin-Ciocalteu reagent (2 N reagent diluted 1:3 with dest. H_2O); 2 mL distilled water was added and the sample incubated for 3 min at room temperature. Thereafter, 2 mL of anhydrous sodium carbonate solution (20 % Na_2CO_3, *w*/*v*) was added. The solution was kept for 2 h at room temperature for color formation, and the absorption of the blue colored sample was measured at 750 nm using an UV/Vis spectrophotometer (Genesys™ 10S, Thermo Fisher Scientific AG, Reinach, Switzerland) against a blank sample containing the same reagents and 1 mL distilled water. (−)-Epicatechin was used as a calibration standard and final results were expressed as milligrams of (−)-epicatechin equivalent per gram of non-fatty dry matter (mg ECE/g). The calibration curve was linear from 5 to 50 mg/L with a linear regression line of $y = 18.821x + 0.0357$ and $R^2 = 0.998$.

Determination of the total flavonoid content (TFC) using the aluminum chloride assay

Besides the determination of the TPC, the total flavonoid content (TFC) of the cocoa extract gives an indication of the phenolic composition of cocoa beans and the yield of the different extraction procedures. The determination of the TFC was done photometrically according to the method described by Zzaman, Bhat, and Yang [15] using the same extract as described before: for *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:10, 1:100. Further on, an aliquot (1 mL) of the *liquid cocoa extract* or the standard solution was added to a test tube containing 4 mL distilled water. Afterwards, 0.3 mL sodium nitrite solution (5 % $NaNO_2$, *w*/*v*) was added and the sample was incubated for 6 min at room temperature. Aluminum trichloride solution (0.3 mL; 10 % $AlCl_3$, *w*/*v*) were added and the sample incubated for another 6 min. Two milliliters of a 1 M sodium hydroxide solution (4 % NaOH, *w*/*v*) was added and the sample was filled up to 10 mL using distilled water. After 15 min, the absorption of the pink solution was

measured at 510 nm. A linear calibration line was obtained using (−)-epicatechin at concentration range of 25 to 225 mg/L with a linear regression line $y = 4.1072x - 0.0025$ and $R^2 = 0.998$, whereby the total flavonoid content was expressed in milligrams of (−)-epicatechin equivalent per gram non-fatty dry matter (mg ECE/g).

Determination of the total proanthocyanidin content (TPAC) using the acid butanol assay

The acid butanol assay is widely used to determine the total proanthocyanidin content (TPAC) in food. The method by Porter, Hrstich, and Chan [16] and a modified one by Pérez-Jiménez, Arranz, and Saura-Calixto [17] were used during this study. For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:5, 1:10. Further on, in a screw cap tube, 6 mL of acid butanol (5 mL 37 % HCl plus 95 mL *n*-butanol) reagent was added to 1 mL aliquot of the *liquid cocoa extract*. As a catalyst, 1 mL of a 2 % solution of ammonium iron(III)sulfate in 2 N HCl (2 % $NH_4Fe(SO_4)_2 \cdot 12H_2O$) was added. The tube was sealed and incubated at 95 °C for 50 min using a benchtop shaker, followed by an immediate cool down with cold water. Afterwards, the absorption of the pink color was measured photometrically at 550 nm against a blank. The proanthocyanidin content was calculated using cyanidin at concentration range of 10 to 70 mg/L using the following equation: $y = 13.137x - 0.009$ and $R^2 = 0.9982$. The proanthocyanidin content was expressed in milligram of cyanidin equivalent per gram non-fatty dry matter (mg CyE/g).

Sequential centrifugal partition chromatography (SCPC)

The separation was carried out on a semi-preparative SCPC liquid-liquid partition chromatography system (Armen Instruments, Saint-Avé, France) with a total volume of 250 mL. A HPLC pump (Pump Model SD-300, Rainin Instrument Co. Inc., Oakland, CA, USA) was used to deliver the mobile phase, in ascending and descending mode, respectively. The maximum rotational speed used was 3000 rpm and a maximum back pressure of 60 bar. The effluent was continuously monitored at 275 nm using an UV detector (UV1, Rainin Instrument Co. Inc. Oakland, CA, USA). A fraction collector (Foxy 200, Teledyne Isco, Lincoln, NE, USA) was connected to the detector outlet. A manual injection valve (10 mL Rheodyne loop) allowed injections ranging up to 6 g. The choice of the biphasic solvent system and the elution mode are crucial for successful liquid-liquid chromatographic separations. The biphasic solvent system ethyl acetate/butanol/water with 4:1:5 (*v*/*v*/*v*) was prepared by mixing defined portions of ethyl acetate, butanol, and water and degassing vigorously. After degassing, the solvent was equilibrated at room temperature for 30 min. In the meantime, the freeze dried cocoa extract (700 mg) was dissolved in 2 mL 40 % aqueous 2-propanol supported by the use of an ultrasonic bath for 5 min at room temperature. The sample solution was then partitioned between 4 mL each of the mobile and the stationary phase and filtered through a 5.0-μm syringe filter (Nylon 66, BGB Analytik AG, Boeckten, Switzerland). Afterwards, sample solution was injected into the SCPC column through the manual injection valve. The chromatogram was continuously monitored and fractions were collected for a period of 5 min each with the fraction collector.

For the SCPC fractionation, the column was initially filled with the stationary phase (organic phase, upper phase) in the *descending* mode at a flow rate of 1.5 mL/min and a revolution speed of 500 rpm. The mobile phase (aqueous phase, lower phase) was then pumped at a revolution speed of 1900 rpm until an equilibrium was established after 90 mL dead volume. After the separation of the first major peaks in the chromatogram was achieved, the column mode was turned to *ascending* after 220 min, in order to recover the components that were not eluted under these conditions. The preparative separation of individual substance classes was carried out in a single run. The peak fractions (I–IV) were collected automatically, correspondingly to the chromatogram obtained and subjected to further HPLC-MS analyses.

Gel permeation chromatography

As applying a single separation technique typically is not sufficient to obtain individual components covering a wide range of polarities, a combination of chromatographic methods needs to be performed. Therefore, semi-preparative SCPC was selected as first and most important separation technique for its lack of a solid stationary phase that can lead to irreversible adsorption of phenolic components (e.g., on reversed phase chromatographic material) and a complete sample recovery [18]. The second purification step applied for isolating fractions or even single compounds of high purity was done on a lipophilic Sephadex LH-20 gel. Traditional column chromatography has been already proven for being suitable of separating a number of phenolic compounds. Especially, alkylated crosslinked dextran Sephadex LH-20 is suitable for a separation of polyphenolic polymers when using aqueous acetone as eluent [19, 20].

In the present study, a glass column 10 × 120 mm with a volume of 6 mL (ECO10/120V0V, YMC Europe GmbH, Dinslaken, Germany) was packed with 1 g of Sephadex LH-20. For purification, 40 mg of 'PA fraction' was dissolved in 100 mL 40 % aqueous 2-propanol and directly subjected to Sephadex LH-20. Saturation and equilibration were done with pure water before adding the sample. The elution was performed by stepwise elution, starting with 100 mL defined solvent at a moderate flow rate of 0.6 mL/min. The effluents were collected in 100 mL volumetric flasks. Subsequently, the

elution step was performed starting with 100 mL dest. H_2O as mobile phase, continuing with 100 mL 10 % aqueous acetone, and further with 100 mL 15 % aqueous acetone etc. until 100 mL 50 % aqueous acetone. The fractions containing target compounds were freeze dried and dissolved in 40 % aqueous 2-propanol for RP-HPLC/MS analysis. Regeneration of the column was done with 40 % aqueous 2-propanol for at least twofold column volume before exposing again to sample.

Determination of individual cocoa compounds using RP-HPLC/MS analyses

Samples were analyzed with a liquid chromatograph coupled to a quadrupole mass spectrometer with electrospray ionization interface (LC/MS 6120, Agilent Technologies AG, Waldbronn, Germany). The fractions were analyzed in ESI-MS as total ion chromatogram in the positive mode *m*/*z* 100–2000. Detailed conditions of the HPLC system and gradient elution are given in Pedan, Fischer, and Rohn [12]. All data obtained was processed with LC/MSD ChemStation software version Rev. B.04.03-SP1 (Agilent Technologies AG, Waldbronn, Germany).

Results and discussion

Effect of extraction solvents on cocoa polyphenols

As already mentioned, cocoa extract is rich in monomeric and oligomeric PAs. The yield of PAs varies with the two main extraction parameters: type of solvent and extraction temperature. Initially, the efficiency of the various extraction solvents was investigated at room temperature. The results of the TPC are summarized in Fig. 1, whereby the different extraction yields of PAs are shown in relation to the type of solvent used. The yield of extracted PAs increases within the homologous series of alcohols such as methanol, ethanol, and 1-propanol and is influenced by a decreasing polarity. It can also be pointed out that extraction yield is more intense with increasing chain length of the solvent and a decrease of the amount of water in the extraction solvent. The results show that the mixture of 50 % aqueous acetone was the most efficient in extracting phenolic compounds.

Besides the determination of the TPC, also the TFC and the TPAC were measured in the different aqueous acetone mixtures. Figure 2 shows the extraction yields for all three assays. Again, the 50 % aqueous acetone mixture showed the best efficiency for extracting the different polyphenol classes in cocoa. The content of extracted total phenols of an unroasted cocoa extract was 60.4 mg ECE/g, the TFC was up to 37.2 mg ECE/g, and the TPAC was up to 25.4 mg CyE/g for 50 % aqueous acetone.

In order to substantiate the colorimetric results, RP-HPLC/MS analyses of the *liquid cocoa extracts* were carried in addition. Cocoa bean compounds were identified based on retention times and mass spectra of reference compounds. In detail, it could be shown that 100 % acetone as extraction solvent selectively extracts the two alkaloids theobromine and caffeine. With increasing water content (e.g., 10 %, 90 % aqueous acetone) (+)-catechin and (−)-epicatechin, followed by PA dimer were extracted. Extraction efficiency was improved for PA trimers and tetramers with solvent concentrations between 20 and 80 % aqueous acetone and culminates with the highest extraction yield for condensed polyphenols using 50 % aqueous acetone. The obtained cocoa extracts consist of at least oligomeric PA with DP = 4. Generally, same order of solvent efficiency as shown in Fig. 1 (50 > 40 %, 60 %, etc.) was obtained when using RP-HPLC/MS analysis for identifying the individual phenolic compounds. Extraction efficiency with lower percentage of acetone/water mixture, or even water, was more selective for extracting single or low molecular weight polyphenols. On the other side, an indication for high oligomeric polyphenols can be observed through the additional peak eluted at the end of the chromatogram.

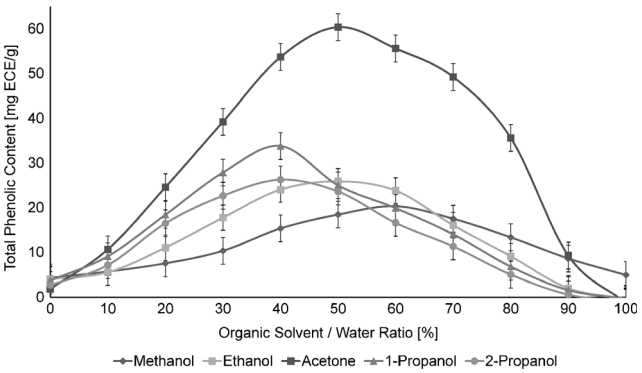

Fig. 1 The influence of extraction solvent on the total phenolic content (TPC). Values are expressed as milligram (−)-epicatechin equivalents per gram non-fat dry matter (mg ECA/g)

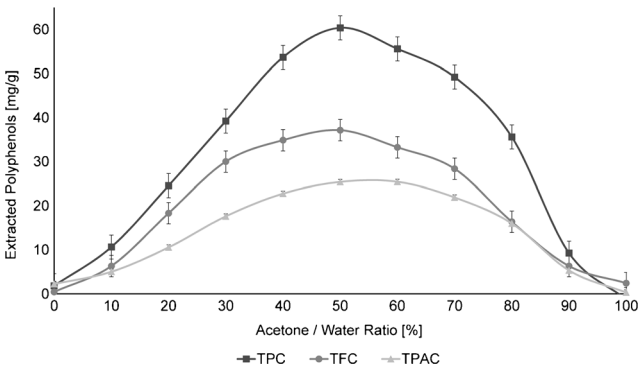

Fig. 2 The influence of 50 % aqueous acetone mixture on the total phenolic content (TPC), total flavonoid content (TFC), and total proanthocyanidin content (TPAC)

Therefore, at a combination of acetone/water (50 %), preferentially more PA oligomers are extracted (see 'PA hump').

Separation of complex mixtures typically fails when using reversed phase columns. Generally known, the efficiency of a reversed phase column for separating higher oligomeric PAs is limited due to the separation of complex polyphenols. In some cases, only peak broadening occurs, whereas in many cases, a group of higher oligomeric PAs occurs as a "hump" at the end of the chromatographic run. Tarascou et al. [21] proved that high polymerized polyphenols eluting as a hump at the end of the chromatographic profiles. Kuhnert [21] stated that a chromatographic hump is generally a consequence of a complex mixture containing a too large number of compounds to be chromatographically resolved. Similar observations in the chromatographic behavior were observed in previous studies of the so-called PA hump during the analysis of unroasted cocoa beans [12]. In case of the so-called thearubigin hump, containing condensed black tea polyphenols, major efforts have been undertaken in recent years by Kuhnert [22] to resolve this into several dozens of individual components consisting of quite similar structural subunits. It is reasonable to assume that a similar situation exists in cocoa, based on the similarity of the polyphenol (epicatechin) subunits and the proven occurrence of linearly condensed higher PAs.

In the present study, acetone-based solvents were most efficient for extracting oligomeric PAs, as compared to other extractions solvents, e.g., alcohol/water mixtures. The extraction is therefore preferably carried out with 50 % aqueous acetone. Hammerstone and Chimel [23] found acetone and ethanol being the most effective solvents in extracting higher oligomeric cocoa PAs. Especially for PAs with DP5-10, the efficiency increases significantly when comparing different water to solvent ratios, like e.g., the average percentage recovery of PA decamer increased from 0 % using 100 % acetone to more than 100 % when using 50 % aqueous acetone. Monrad, Howard, King, Srinivas, and Mauromoustakos [24] investigated red grape pomace and found 50 % aqueous ethanol to be optimal for extracting total procyanidins than other ethanol/water compositions.

Effect of temperature on the extraction of cocoa polyphenols

An extraction can be carried out at temperatures ranging from chilled solvents up to the boiling points of the solvents. Since polyphenols are heat labile components, flavon-3-ol losses occur at elevated temperatures. Further, investigations on temperature conditions for comparing changes in content and composition are mandatory. In the present study, the total phenolic content was measured by Folin-Ciocalteu extracted three times for 8 min using 50 % aqueous acetone as solvent. Hereby, the concentration of cocoa polyphenols increased significantly with increasing temperature in the range of 20–110 °C, in detail 88 mg ECE/g at 20 °C to 116 mg ECE/g at 110 °C and began to decrease again to 99 mg ECE/g at 140 °C (Fig. 3). The data obtained confirmed the significant effect of temperature on decreases or increases of TPC dependent on the applied temperature. In addition to the photometric assays, RP-HPLC/MS analyses of the *liquid cocoa extracts* from the different extraction procedures were carried out. In general, the yield of individual bioactive compounds such as (+)-catechin, (−)-epicatechin, PA dimer B2, and trimer C1 increased only slightly within the temperature range (data not shown). In contrast to another study dealing with the temperature influence [25], no degradation or loss could be observed at temperatures from 20 to 140 °C during an 8-min extraction period. However, according to the present results, the extraction temperature was kept at 50 °C to ensure a reproducible polyphenol extraction below the boiling point of acetone as part of the extraction solvent. In addition, the moderate conditions help to preserve most of the initial phenolic compounds and to obtain the genuine flavan-3-ol composition.

Studies by Kothe et al. [25] claimed for epicatechin and PA dimer B1, B2, B5 progressive changes within a temperature range from 100 to 140 °C. Temperatures above 140 °C did not have an accelerated influence on further degradation.

Hammerstone and Chimel [23] also observed a temperature effect on the recovery of oligomeric cocoa PAs and recommended an elevated extraction temperature at 50 °C with an aqueous acetone mixture. Furthermore, it was pointed out in that study that the effect of temperature is non-significant for monomeric PAs and is significantly pronounced for more complex oligomeric PAs.

As already stated above, phenolic compounds' degradation depends on roasting time and temperature. The product pattern, resulting from thermal treatment at high temperatures under non-aqueous conditions (roasting) is quite different. For some flavonol, mechanism for degradation under roasting conditions is a deglycosylation to the corresponding aglycone. The main aglycone product remains stable during further

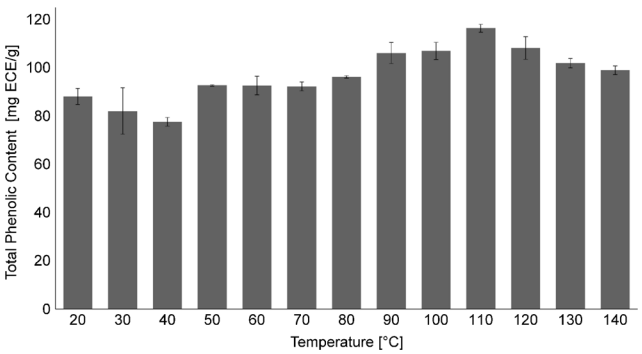

Fig. 3 Influence of temperature extraction (20–140 °C) on the total phenolic content (TPC) as determined by using 50 % aqueous acetone and 8 min of extraction

roasting. Compared to the cooking process, flavonol glycosides showed several reaction products. In this context, studies observed degradation for flavonol glycosides in aqueous solution when heating up to 100 °C, whereby glycosides showed an intensive breakdown but did not form aglycones [26, 27].

Structural elucidation of isolated PAs with RP-HPLC/MS

The chromatogram of the SCPC separation gave four fractions (Fig. 4) and the coil fraction. Separation was completed within about 2.5 h so that at least two chromatographic runs could be performed within 1 day. Fractions I and II eluted in sharp peak starting at a retention time of about 70–80 min, respectively 85–120 min. Fraction III eluted in a broad peak between 155 min followed by return to baseline at about 220 min. By switching, the elution mode fraction IV eluted within 220–250 min. Several SCPC runs were repeated under the same condition, whereby the retention times were consistent between chromatographic runs.

The following compounds were identified according to their retention time and confirmed by mass spectrometry and authentic reference compounds. PAs were detected at 280 nm. In Fig. 5, the peaks numbered with roman numerals correspond to the compounds assigned in the following text. During the separation in *descending* elution mode, polar compounds eluted earlier. The polar fraction I from the partition of crude *freeze dried cocoa extract* contained mainly a mix of hydrophilic, largely complex, mostly branched molecules. An indication might be the 'PA hump' explained earlier in the study.

For the numbered peaks, it was possible to obtain a MS signal and in some cases MS fragmentation pattern. The resulting data are shown in Table 1. Based on the experiments and knowledge, the fragmentation pattern $[M+H]^+$ of the identified compounds was summarized here as well.

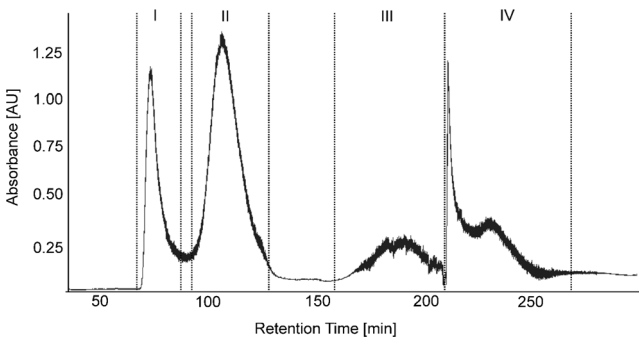

Fig. 4 SCPC chromatogram when applying 700 mg cocoa extract. SCPC conditions: solvent system, EtOAc/BuOH/H_2O 4:1:5 (*v/v/v*); stationary phase, upper phase; revolution speed, 1900 rpm; detection wavelength, 275 nm; flow rate, 1.5 mL/min. Switchover from descending to ascending after 220 min. Fr I–IV with (*I*) hydrophilic complex compounds; (*II*) theobromine; (*III*) caffeine; (*IV*) flavonols and proanthocyanidins

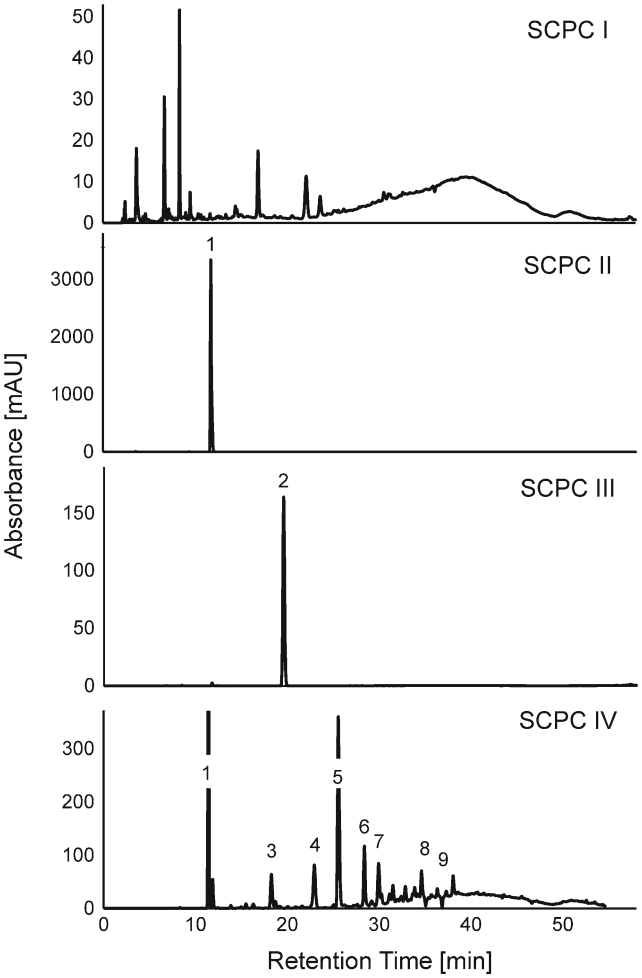

Fig. 5 HPLC chromatograms of the SCPC fractions Fr I–IV. Peaks numbered with roman numbers are in the order of increasing retention time and correspond to the compounds explained in the text. SCPC separation of fraction IV was operated three times before analyzing. (*I*) Hydrophilic complex compounds; (*II*) theobromine; (*III*) caffeine; and (*IV*) flavonols and proanthocyanidins

In detail, a major portion of polar compounds eluted preferably at the beginning of the HPLC run. Furthermore, an unresolved 'PA hump' was observed for fraction I eluting in a retention timeframe of 35–45 min. Fraction II has been identified as theobromine (1, *m/z* 181.1). Fraction III contained caffeine (2, *m/z* 195.1).

In contrast, the fraction obtained with butanol (fraction IV) contained compounds with a high structural diversity such as flavan-3-ols with up to four epicatechin subunits and flavonol aglycones, with the latter also glycosylated with arabinose, galactose, and glucose. However, the fractions were still very complex in their composition and presence of residual theobromine (1). This underlines that the partition coefficient of flavan-3-ols and flavonols like quercetin and their glycosylated products are comparatively similar, being the main reason for not finding complete separation conditions.

Table 1 RP-HPLC-ESI/MS determination of phenolic compounds in an extract of unroasted cocoa beans. Compounds with peak no. 1–17 were detected in positive ion mode

Peak no.	Compound	t_R [min]	Molecular weight [g/mol]	Molecular ion $[M+H]^+$	Major fragments $[M+H]^+$
1; 10	Theobromine	11.7	180.16	181.1	n.d.
2; 11	Caffeine	19.6	194.19	195.1	n.d.
3; 13	(+)-Catechin	18.3	290.26	291.1	139.0; 165.1
4; 14	PA B2	22.9	578.52	579.2	291.1
5; 12	(−)-Epicatechin	25.5	290.26	291.1	139.0; 165.1
6; 15	PA C1	28.4	866.77	867.2	579.2; 1155.5
7; 17	Cinnamtannin A2	30.0	1155.04	1155.3	579.2; 867.0
8	Quercetin-3-O-glucoside	34.6	464.38	465.1	303.1
9	Quercetin-3-O-arabinoside	38.1	434.35	435.1	303.0
16	Unknown PA dimer	34.8	866.77	867.2	579.2; 1155.5

n.d. not detected

In detail, fraction IV contained eight major peaks identified as target phenolic compounds (peaks 3–9). Based on the HPLC/MS analysis, they were determined as (+)-catechin (3, *m/z* 291.1), PA dimer B2 (4, *m/z* 579.1, with a dominant fragment ion *m/z* 288.9), (−)-epicatechin (5, *m/z* 291.1), PA trimer C1 (6, *m/z* 866.5), as well as PA tetramer A2 (7, *m/z* 1155.1). Further compounds were UV-active at 360 nm and therefore assigned as negative peaks in the chromatogram (peak 8, 9). They were identified as quercetin-3-*O*-glucoside (8, *m/z* 465.1, with fragment ions *m/z* 303.1) and quercetin-3-*O*-arabinoside (9, *m/z* 435.1, with fragment ions *m/z* 303.0), tentatively. However, their amount is low compared to flavan-3-ols.

Nevertheless, this method separates of about 700 mg *freeze dried cocoa extract* (I), 232 mg of hydrophilic complex compounds, (II) 88 mg theobromine, (III) 19 mg caffeine, (IV) 149 mg flavones and flavan-3-ols, and 90 mg retained in the coil.

On the basis of a high PA accumulation in fraction IV, this fraction is called 'PA fraction'. The occurrence of theobromine (1) in fraction IV is significant and due to carry-over effects in the partition cells. Nevertheless, this effect of sample carry-over is still limited compared to conventional separatory-funnel partition. Nevertheless, an additional sample clean-up is required to minimize such "contamination effects."

However, the SCPC run resulted in four fractions according to the corresponding peaks separated. A typical color pattern can be observed for the eluted fractions. Fraction I had an intensive brown color. According to Fincke and Fincke [28], the brown color typically indicates water-insoluble phlobaphene. These compounds are structurally complex (see PA hump) and are responsible for the pure brown to the violet-tinged color of the fermented cocoa bean. Moreover, it was recently shown that the color intensity increases from monomeric to polymeric PAs [12]. During cocoa fermentation, anthocyanidins are oxidized by polyphenol oxidase to quinones. The quinones can covalently react with amino acids and proteins or polymerize to form tannins. High molecular weight tannins can also interact non-covalently with proteins through hydrogen bonding, resulting as well in a brown, water-insoluble pigment that gives cocoa its characteristic brown color [29]. With increasing retention time, the color appeared more violet-like for fraction II and fraction III. Upon turning the column mode to *ascending* mode, a quick color change appeared to a bright red for fraction IV, containing the flavan-3-ols and flavonols. The occurrence of the reddish color can be explained by the presence of purple anthocyanidin pigments (e.g., cyanidin arabinoside, -galactoside) in fresh cocoa beans. Although during fermentation these pigments are mostly hydrolyzed by glycosidases, resulting in a more pale purple color [30], they still occur in the extracts of unroasted cocoa.

Isolation of PAs from the freeze dried cocoa extract obtained by gel permeation chromatographie using Sephadex LH-20

In order to compare the purification grade of the main cocoa PAs with SCPC, a further clean-up step by gel permeation chromatography on Sephadex LH-20 was employed to separate individual flavan-3-ols from the semi-purified PA extract. SCPC separation was operated three times, and the same fractions were combined to enrich compounds 3–9. After separation, each fraction was analyzed by RP-HPLC/MS. HPLC analysis of each obtained pure compound can be seen in Fig. 6. The affinity of PAs for Sephadex LH-20 was used for further fractionation. Here, the optimal separation of fraction IV was performed using aqueous acetone as eluting solvent in a gradient from 0 to 40 % in increments of 5 %. To improve the purity of the isolated sub-fractions, a stepwise elution with aqueous acetone as mobile phase was performed

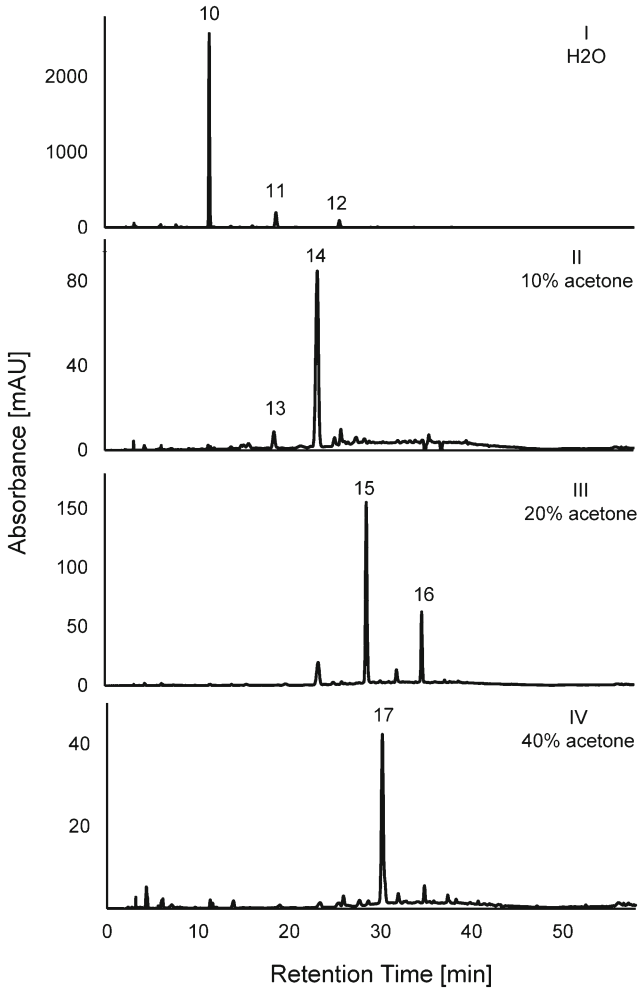

Fig. 6 Purification of the main individual PAs through gel permeation chromatography on Sephadex LH-20. Peaks numbered with roman numbers are in the order of increasing retention time and correspond to the compounds explained in the text

from 0 to 40 % in increments of 10 %. A sequential elution starting with 100 mL H_2O assigned compounds as theobromine (10, *m/z* 181.1), caffeine (11, *m/z* 195.1) and (−)-epicatechin (12, *m/z* 290.9) in fraction I. The eluate was collected and freeze dried immediately. Among the requested bioactive monomeric flavan-3-ol, also theobromine and caffeine could be detected in higher amounts. A further fractionation with 10 % aqueous acetone, the phenolic compounds (+)-catechin (13, *m/z* 290.9) and PA dimer B2 (14, *m/z* 578.7) were obtained. Anterior impurities of theobromine and caffeine could be eliminated through sufficient aqueous extraction. Further on, the elution with 20 % aqueous acetone gained PA trimer C1 (15, *m/z* 866.5) and an unknown PA dimer (16, *m/z* 578.7). The major peak of the last elution step with 40 % aqueous acetone was identified as PA tetramer A2 (17, *m/z* 1156.0). Forty milligrams of the "PA fraction" obtained a yield of 1.4 mg of fraction I, 5.6 mg of fraction II, 2 mg of fraction III, and 2 mg of fraction IV.

The intermediate elution steps with 15 % (4.5 mg), 25 % (2 mg), 30 % (3 mg), 35 % (2 mg) aqueous acetone lead to an insufficient separation of targeted PA compounds. As a consequence, those fractions were discarded. A fully desorption of the purified flavonoids could be obtained by 50 % aqueous acetone as eluent. In contrast to a direct injection of the crude cocoa extract on the Sephadex LH-20 column, the solvent is not strong enough to elute all substances, especially the group of high complex brown tannins. This complex group interacted irreversible with Sephadex LH-20.

Again, a typical color pattern could be observed, as already mentioned above. The color increased correspondingly to the elution order, beginning with a light brown by 0 % aqueous acetone and ending with bright red for 30 % aqueous acetone.

Conclusion

The present study achieved the most efficient extraction of target secondary metabolites from cocoa such as flavan-3-ols using a threefold extraction with 50 % aqueous acetone (*v*/*v*), a liquid-to-solid ratio of 1-3 (*w*/*v*), and an 8-min extraction time (data not shown). The preferred temperature was set to 50 °C for minimizing solvent evaporation. The polyphenol-rich supernatant was freeze dried and used for further semi-preparative isolation for the compounds of interest.

With regard to their chemical structure, plant polyphenols are quite diverse and complex, so that an optimized extraction method for each compound is difficult to achieve. In this study, extraction efficiencies were calculated as a function of the extraction solvent composition and temperature according to the total and individual PAs in a cocoa bean extract. It was found that 50 % aqueous acetone was an ideal solvent composition. Moreover, using a combination of liquid-liquid and gel chromatographic separation, it was possible to isolate high oligomeric PAs on a semi-preparative scale. This method therefore describes a relatively simple and fast procedure for the fractionation of monomeric, dimeric, trimeric, and tetrameric PAs by using gel permeation chromatography on Sephadex LH-20.

The results provided an efficient methodology for the separation and further identification of bioactive components. The described method has a broad applicability and is rapid, and suitable for preparing biologically active PAs from crude plant extracts. This method can be to obtain purified flavan-3-ols which can be further used for bioactive investigations.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest. The manuscript was approved for publication by all authors.

References

1. Othman A, Ismail A, Ghani NA, Adenan I. Antioxidant capacity and phenolic content of cocoa beans. Food Chem. 2007;100:1523–30.
2. Pedan V, Fischer N, & Rohn S. Fractionation of condensed cocoa polyphenols using semi-preparative NP-HPLC. 44. Deutscher Lebensmittelchemikertag (Lebensmittelchemische Gesellschaft), Karlsruhe, Germany. 2015; ISBN 978-3-936028-93-5: 119.
3. Khallouki K, Haubner R, Hull WE, Erben G, Spiegelhalde B, Bartsch H, et al. Isolation, purification and identification of ellagic acid derivatives, catechins, and procyanidins from the root bark of *Anisophyllea dichostyla* R. Br Food Chem Toxicol. 2007;45:472–85.
4. Honda S, Takahashi T, Kamimura A, Matsuoka T, Kanda T, Yanagida A, & Hieda K. Process for purification of proanthocyanidin oligomer. European Patent Application EP. 2000;1 666 476 A1 .
5. Shibusawaa Y, Yanagidaa A, Isozakia M, Shindoa H, Ito Y. Separation of apple procyanidins into different degrees of polymerization by high-speed counter-current chromatography. J Chromatogr A. 2001;915:253–7.
6. Marston A, Hostettmann K. Counter-current chromatography as a preparative tool—applications and perspectives. J Chromatogr A. 1994;658:315–41.
7. Hopmann E, Minceva M. Separation of a binary mixture by sequential centrifugal partition chromatography. J Chromatogr A. 2012;1229:140–7.
8. Esatbeyoglu T, Wray V, Winterhalter P. Isolation of dimeric, trimeric, tetrameric and pentameric procyanidins from unroasted cocoa beans (*Theobroma cacao* L.) using countercurrent chromatography. Food Chem. 2015;179:278–89.
9. Yang C, Li D, Wan X. Combination of HSCCC and Sephadex LH-20 methods. An approach to isolation and purification of the main individual theaflavins from black tea. J Chromatogr B. 2008;861:140–4.
10. Zhou X, Liang J, Zhang Y, Zhao H, Guo Y, Shi S. Separation and purification of α-glucosidase inhibitors from *Polygonatum odoratum* by stepwise high-speed counter-current chromatography combined with Sephadex LH-20 chromatography target-guided by ultrafiltration-HPLC screening. J Chromatogr B. 2015;985:149–54.
11. Cheel J, Minceva M, Urajova P, Aslam R, Hrouzek P, Kopecky J. Separation of Aeruginosin-865 from cultivated soil cyanobacterium (*Nostoc* sp.) by centrifugal partition chromatography combined with gel permeation chromatography. Nat Prod Commun. 2015;10(10):1719–22.
12. Pedan V, Fischer N, & Rohn S. An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa. Food Res Int. 2015; in press.
13. Everette JD, Bryant QM, Green AM, Abbey YA, Wangila GW, Walker RB. Thorough study of reactivity of various compound classes toward the Folin-Ciocalteu reagent. J Agric Food Chem. 2010;58:8139–44.
14. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199–200.
15. Zzaman W, Bhat R & Yang TA. Effect of superheated stream roasting on the phenolic antioxidant properties of cocoa beans. J Food Process Pres. 2013; ISSN 1745-4549.
16. Porter LJ, Hrstich LN, Chan BG. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochem. 1986;25:223–30.
17. Pérez-Jiménez J, Arranz S, Saura-Calixto F. Proanthocyanidin content in foods is largely underestimated in the literature data: an approach to quantification of the missing proanthocyanidins. Food Res Int. 2015;42:1381–8.
18. Friesen JB, McAlpine JB, Chen S-N, Pauli GF. Countercurrent separation of natural products: an update. J Nat Prod. 2015;78:1765–96.
19. Green JR, Cousineau J. Purification of phlorizin by column chromatography on Sephadex LH-20 with aqueous propan-2-ol. J Chromatogr. 1980;188:439–41.
20. Kantz K, Singleton VL. Isolation and determination of polymeric polyphenols in wines using Sephadex LH-20. Am J Enol Vitic. 1991;42(4):309–16.
21. Tarascou I, Souquet JM, Mazauric JP, Carrillo S, Coq S, Canon F, et al. The hidden face of food phenolic composition. Arch Biochem Biophys. 2010;501:16–22.
22. Kuhnert N. Unraveling the structure of the black tea thearubigins. Arch Biochem Biophys. 2010;501:37–51.
23. Hammerstone JF, & Chimel MJ. Method for extracting cocoa procyanidins. 2003; US Patent US6627232 B1.
24. Monrad JK, Howard LR, King JW, Srinivas K, Mauromoustakos A. Subcritical solvent extraction of procyanidins from dried red grape pomace. J Agric Food Chem. 2010;58:4014–21.
25. Kothe L, Zimmermann BF, Galensa R. Temperature influences epimerization and composition of flavanol monomers, dimers and trimers during cocoa bean roasting. Food Chem. 2013;141:3656–63.
26. Rohn S, Buchner N, Driemel G, Rauser M, Kroh LW. Thermal degradation of onion quercetin glucosides under roasting conditions. J Agric Food Chem. 2007;55:1568–73.
27. Buchner N, Krumbein A, Rohn S, Kroh LW. Effect of thermal processing on the flavonols rutin and quercetin. Rapid Commun Mass Spectrom. 2006;20(21):3229–35.
28. Fincke H & Fincke A. Handbuch der Kakaoerzeugnisse. Berlin, New York, Springer-Verlag. 1965; doi:10.1007/978-3-642-49937-1.
29. Aroyeun SO, Ogunbayo JO, Olaiya AO. Effect of modified packaging and storage time of cocoa pods on the commercial quality of cocoa beans. Br Food J. 2006;108:141–51.
30. Afoakwa EO, Quao J, Takrama FS, Budu AS, Saalia FK. Changes in total polyphenols, *o*-diphenols and anthocyanin concentrations during fermentation of pulp pre-conditioned cocoa (*Theobroma cacao*) beans. Int Food Res J. 2012;19(3):1071–7.