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An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa

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Abstract

Unroasted cocoa beans are rich in monomeric flavanols and particularly epicatechin-based proanthocyanidin oligomers, with the latter making up to 60% of the total polyphenol content. Although the antioxidant activity of cocoa polyphenols is well known, it is still a challenging analytical field, especially, when it comes to the determination of condensed polyphenols and the evaluation of their single contribution to the overall activity. Therefore, an online NP-HPLC-DPPH assay was developed to separate the homologous series of condensed polyphenols for assessing their antioxidant capacity in relation to their degree of polymerisation (DP), simultaneously. In this context, normal-phase chromatography allows separations of polyphenols based on their degree of polymerisation. This study showed that an unroasted cocoa extract contains condensed polyphenols with a DP of up to 10 monomer units. By means of the online post-column derivatisation with 2,2-diphenyl-1-picrylhydrazyl (DPPH), the antioxidant capacity of the separated condensed polyphenols was assessed. It could be shown that, with the exception of the dimers, the contribution to the total antioxidant activity decreased from monomers to decamers. However, from the single proanthocyanidins identified, nonameric and decameric proanthocyanidins were found to have the highest values for the antioxidant capacity. The degree of polymerisation associated with each molecular weight fraction was further confirmed using electrospray ionization mass spectrometry coupled with reverse-phase liquid chromatography. The online NP-HPLC-DPPH method can be used as qualitative and quantitative analysis of condensed proanthocyanidins and the simultaneous elucidation of the biological activity of proanthocyanidins in complex mixtures.

Keywords: condensed cocoa polyphenols; degree of polymerisation; online NP-HPLC-DPPH; antioxidant capacity

1. Introduction

Polyphenols are known to be food constituents of health-beneficial nature, often hypothesized to result from their antioxidant activity. Especially flavanol-rich diets including fruits and vegetables, tea, wine as well as cocoa are thought to have a beneficial effect on, e.g., cardiovascular health (Corti, Flammer, Hollenberg, & Lüscher, 2009). With increasing demand for polyphenol-rich extracts, there is a need to develop a rapid and efficient method to fully characterize plant extracts and to understand the physiological mechanism responsible for a corresponding mode of action of the preventive effects.

With regard to cocoa, its polyphenols are stored in the pigment cells of the cotyledons in the seeds, which are the major source for food-relevant products, such as chocolate. Three groups of polyphenols have to be considered primarily: flavan-3-ols with approx. 37%, anthocyanins with approx. 4% and proanthocyanidins with approx. 58% (Wollgast & Anklam, 2000). Among the major flavan-3-ols in cocoa and cocoa products, the monomers (-)-epicatechin and (+)-catechin are making up to 10% of the total polyphenol consumption in unroasted, fermented cocoa. These monomers can form polymers, the so-called proanthocyanidins (PA). The coupling between the monomeric flavan-3-ols occurs primarily between the positions 4 and 8, but may also involve the positions 4 and 6. Proanthocyanidin dimers (e.g., B1, B2, B3, B4, B5), trimers (e.g., C1), and tetramers (e.g., cinnamtannin A2), wherein the monomers are linked by 4->8, represent the most important cocoa PA (Prior & Gu, 2005). Further structural complexity is also given by interactions with insoluble polymeric plant material such as polysaccharides or proteins (Matthews, Mila, Scalbert, & Donnelly, 1997; Rohr, Meier & Sticher, 2000; Gu et al., 2004; Naczk & Shahidi, 2004).

The molecular weight of oligomeric PA is expressed as their degree of polymerisation (DP) and is referred to as monomers, dimers, trimers, tetramers etc. According to Gu et al. (2002), they are further defined as monomers with DP = 1, oligomers with DP = 2-10, and polymers with DP > 10. Much higher degrees of polymerisation might also exist in cocoa beans, but would be insoluble and quite challenging to analyse. However, the portion of condensed polyphenols present in cocoa is much higher than that of monomeric polyphenols (US Department of Agriculture NDL, 2004; Neveu et al., 2010).

With regard to antioxidant activity, not only low molecular polyphenols have the ability to scavenge radicals, but also oligo- and polymers of higher molecular weight are thought to possess a significant bioactivity (Saura-Calixto, 2012). Moreover, they contribute significantly to the human intake of antioxidants (Dorenkott et al., 2014; Gu et al. 2002). So far, the

majority of polyphenol studies, however, focused on the smaller, well extractable molecules, which can be easily detected by RP-HPLC (Bandoniene & Murkovic, 2002; Niederländer et al., 2008; Malherbe, de Beer, & Joubert, 2012). Consequently, most of the *in vitro* and *in vivo* studies on antioxidant activity dealt with the low molecular weight polyphenols. The question remains whether and how the antioxidant capacity varies with the degree of polymerisation. Due to the lack of reference substances, it is difficult to predict the antioxidant capacity of higher molecular polyphenols *in vitro*. None of the mentioned technique provides information about the radical scavenging activity of the separated oligomeric PA.

It was the aim of the present study to develop an online NP-HPLC-DPPH method for assessing the radical scavenging activity of high molecular weight polyphenols. This method bases on a separation with liquid chromatography coupled online with the well-known antioxidant capacity assay using DPPH as synthetic, stable radical, scavenged by the target compounds. Thus, the measurement of the antioxidant capacities and the parallel estimation of the separated compounds are permitted. From the countless assays for determining the antioxidant activity, the DPPH assay has become a quite popular method for the analysis of the antioxidant activity of all kinds of substrates. As it was the aim to only compare between the antioxidant activities of the procyanidins, the DPPH assay has been chosen, as it allows a fast reaction with most of the phenolic compounds. The optimum composition of the reagents, reaction time and temperature which affect the sensitivity range of spectrophotometric assays have been studied for the new application technique of the online NP-HPLC-DPPH assay.

2. Material and Methods

2.1. Materials and Reagents

Polyphenol standard substances such as (-)-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). 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade acetonitrile, methanol, 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.

2.2. Sample Preparation

2.2.1. Removal of lipids from the cocoa beans

Unroasted cocoa beans, from a Trinitario variety from Finca La Amistad of Costa Rica, were manually dehulled and frozen (-20°C). To minimize heat development, frozen cocoa beans were then crushed in a knife mill (A 11 basic Analytical Mill, IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 30 sec. In order to remove cocoa lipids from the beans, crushed material was extracted with n-hexane at a ratio of 1:5 (w/v) for 5 minutes at 20 °C. The hexane phase was centrifuged at 2,880 *x* g for 5 minutes (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). This procedure of defatting was repeated 5 times until the n-hexane extract appeared colourless. The defatted material was air dried for evaporating any hexane residue. After drying, the cocoa powder was sieved and particles of an average particle size of <100 µm were extracted further. The particle fraction was obtained using a sieve shaker (Vibratory Sieve Shaker AS 200 basic, measuring range < 100 µm, Retsch GmbH, Haan, Germany).

2.2.2. Extraction of the analytes

For HPLC analysis, 2 g of the sieved cocoa material were weighed into a 50 mL centrifuge vessel and extracted three times with 6 mL 50% acetone / water for 8 minutes at 50 °C. After each extraction step, the mixture was centrifuged for 5 minutes at 2,880 *x* g (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The combined polyphenol-rich supernatant (liquid cocoa extract) was used for further analysis and for semi-preparative isolation of single compounds of interest.

2.3. Online NP-HPLC-DPPH assay

The online NP-HPLC-DPPH assay can be used for a rapid assessment of antioxidant compounds in complex mixtures. As already described above, oligomeric PA are eluting in the order of their increasing degree of polymerisation, when using normal phases (Adamson et al., 1999, Hammerstone, Lazarus, & Schmitz, 1999; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Yanagida et al., 2007). A method combining the separation

of the condensed polyphenol compounds and the simultaneous determination of their antioxidant activity will be an advantage for characterizing bioactivity. However, the greatest benefit of this method is that the contribution of a single compound to the overall activity of a mixture of antioxidants can be measured. The more rapidly the absorbance decreases of the compound, the more potent is the antioxidant activity in terms of hydrogen-donating ability (Yen & Duh, 1994).

For the online NP-HPLC-DPPH assay, a solvent gradient was used on a UV/Vis-HPLC system (Knauer GmbH, Berlin, Germany), equipped with a diol-HILIC column (3.0 mm i.D. x 150 mm, 5 µm; YMC Triart Diol-HILIC, Stagroma AG, Reinach, Switzerland) and a pre-column of the same phase. The separation was carried out under the following conditions: Column temperature 30 °C; flow rate, 0.3 mL/min; sample injection volume, 1 µL. The mobile phase consisted of acetonitrile-water-formic acid (97.9:2:0.1, v:v:v) (solvent A) and methanol-water-formic acid (97.9:2:0.1, v:v:v) (solvent B), applied in the following gradient elution order: 1% B at 1-6 min, 1-20% B at 6-11 min, 20% B at 11-18 min, 20-26% B at 18-34 min, 26% B at 34-50 min, 26-95% B at 50-55 min and 95% B at 55-63 min. Between runs there was a 9 min postrun to re-equilibrate the column. The chromatogram was recorded by means of UV/Vis-detection (UVD 2.1 S, Knauer GmbH, Berlin, Germany) at a wavelength of 275 nm.

The DPPH free radical scavenging activity of the extracts was analysed using the DPPH assay, as originally described by Blois (1958) and modified by Brand-Williams, Cuvelier, & Berset (1995). Briefly, the working solution was prepared by dissolving 24 mg DPPH in 550 mL methanol, to obtain an absorbance of 1.1 ± 0.2 units at 515 nm (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006).

The DPPH reagent was filtered through a 0.45 µm membrane filter and carefully degassed before use. The scavenging reaction between DPPH and the condensed polyphenols as well as the DPPH reagent were protected from light.

Following the HPLC column, tubes were connected with a “T” piece and DPPH solution was added at a flow rate of 0.3 mL/min using a separate pump. The scavenging reaction took place in a reaction coil of 4.8 m length, i.D. of 0.38 mm and a volume of 0.550 mL, at a temperature of 60 °C. The induced bleaching of the coloured reagent was detected photometrically at 515 nm in presented in the chromatograms as negative peaks. The instrumental setup is depicted in **Figure 1**. The HPLC analysis was done in triplicate for each sample. The standard substances consists of (-)-epicatechin, proanthocyanidin dimer B2, trimer C1, and cinnamtannin A2 which are commercial available and differs in their condensation pattern.

2.4. Quantification of oligomeric PA using online NP-HPLC-DPPH assay

Quantification of individual oligomeric PA and determination of their antioxidant activity in unroasted liquid cocoa extract was done using external standard calibration. Stock solutions containing the standard substances were therefore prepared and diluted to appropriate concentrations with the same solvent. Briefly, the calibration was achieved using the standard substances (-)-epicatechin as monomer, proanthocyanidin B2 as dimer, proanthocyanidin C1 as trimer and cinnamtannin A2 as tetramer in a concentration range from c = 0.05 g/L to 1 g/L. Quantification was based on flat baseline integration as recommended by Gu et al. (2002). The calibrated curves were generated for each oligomeric class using a quadratic fit of area sum versus concentration. The method optimization was performed using standard substances and afterwards applied to liquid cocoa extracts.

The results of the PA content and their antioxidant activity are expressed for DP 1 as milligrams of (-)-epicatechin per gram non-fatty dry matter, for DP 2 as mg of procyanidin dimer B2, for DP 3 as mg trimer C1 and for DP ≥ 4 as mg cinnamtannin A2 per gram non-fatty dry matter due to the lack of standard substances.

The limits of detection and quantification were determined at a signal-to-noise ratio of about 3.0. Interday variability was chosen to validate the method and to specify the developed assay. Therefore, the mixed standard solution was analysed for three replicates within one day and analysed in duplicate weekly for a month. A linear dependence of negative peak area on concentration of the reference antioxidants was observed.

2.5. DPPH cuvette test

The different antioxidant kinetic behaviour of oligomeric PA was assayed photometrically and the results were compared with those of the online NP-HPLC-DPPH assay. It is a giving need for a new developed assay to make it compatible with an already given method of antioxidant activity measurement like the DPPH cuvette test.

Braude, Brook, & Lindstead (1954) discovered the hydrogen transfer mechanism between the stable free radical DPPH and antioxidant compounds. As it is described by Brand-Williams, Cuvelier, & Berset (1995) and some modifications introduced by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne (2006), the working solution was

prepared by dissolving 24 mg DPPH in 500 mL methanol and left to react in the dark for 24 h at room temperature to obtain an absorbance of 1.1 ± 0.2 units at 515 nm. A composite standard was prepared using commercially available (-)-epicatechin. As this is the main flavonoid compound, it was used as a representative for oligomeric PA in the preliminary tests, although its detection response factor differs from PA which should be kept in mind. Stock solutions were made at the following concentration range: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L, whereby 150 µL standard solution was allowed to react with 2,850 µL of the DPPH working solution. To determine the reaction kinetics, the DPPH radical quenching of (-)-epicatechin was measured at 25 °C and 60 °C. The absorbance was noted after an incubation time of 2 and 5 minutes at 515 nm with methanol as blank and an UV/Vis spectrophotometer (Genesys^{TM} 10S, Thermo Fisher Scientific AG, Reinach, Switzerland).

2.6. Isolation of oligomeric PA using semi-preparative NP-HPLC

As most of the PA beyond a DP of 4 are not commercially available, isolation and purification of individual oligomeric PA was required for the validation of the method.

A semi-preparative separation of the PA was achieved using a diol-HILIC column with 20.0 mm i.D. x 150 mm, 5 µm particle size (YMC-Actus Triart Diol-HILIC, Stagroma AG, Reinach, Switzerland). The separation was carried out under following conditions: The flow rate was set to 1.9 mL/min, and the column temperature was 30 °C. As described above, the mobile phase consisted of a mixture of acetonitrile-water-formic acid (97.9:2:0.1, v:v:v) (solvent A) and methanol-water-formic acid (97.9:2:0.1, v:v:v) (solvent B), applied in the following gradient elution order: 1% B at 1-8 min, 1-20% B at 8-22 min, 20% B at 22-45 min, 20-25% B at 45-55 min, 25% B at 55-80 min, 25-95% B at 80-85 min and 95% B at 85-100 min to wash remaining residues off the column. The detector wavelength was set to 275 nm. The semi-preparative NP-HPLC analysis was performed on the same HPLC system as described above for the analytical method. Sample injection was done using a 200 µL injection loop.

For semi-preparative injection, the liquid cocoa extract was freeze-dried to remove the extraction solvent entirely. The freeze-dried extract was named ‘PA cocoa extract’ (PACE). To prevent the HPLC system from precipitation of solids, 100 mg of PACE was re-dissolved in 400 µL 40% isopropanol, suspended using an ultrasonic bath, and filtered through a 0.45 µm cellulose syringe filter prior to injection.

Fractions of appropriate volumes were collected manually in 10 mL amber glass vials for a couple of minutes following the chromatographic the peak shape visually.

The content of suitable fractions (F1: from 21-22 min; F2: from 34-35 min; F3: from 39-40; F4: from 43-45; F5: from 48-50 min; F6: from 55-57 min and F7: from 62-64 min) were pooled, evaporated to dryness under nitrogen with a sample concentrator (Portmann Instruments AG, Biel-Benken, Switzerland), subsequently dissolved in 100 µL in methanol and analysed by RP-HPLC-ESI/MS.

2.7. Characterization of oligomeric PA using RP-HPLC-ESI/MS

For a satisfyingly determination of the PA, several techniques had to be combined. With an offline two dimensional diol-HILIC normal phase *x* reversed phase liquid chromatography, the analysis of PA can be effectively performed. In the first dimension, oligomeric PA can be separated according to their molecular weight and the second dimension offers a potent analytical technique of untargeted characterization of individual compounds. An advantage of this method is given by the fact that, although RP-HPLC separates flavan-3-ols monomers, dimers, and trimers without difficulties, there are obviously limitations for higher oligomers. In a crude matrix the oligomers are co-eluting up to a DP ≥ 4 as a large unresolved peak.

Identification of individual compounds up to tetramers [(+)-catechin, (-)-epicatechin, proanthocyanidin dimer B2, B3, B4, proanthocyanidin trimer C1, and proanthocyanidin tetramer A2] was confirmed by RP-HPLC-ESI/MS. This was performed on an Agilent 1200 series liquid chromatography and quadrupole mass spectrometer with electrospray ionization interface (LC/MS 6120, G6100 series, Agilent Technologies AG, Waldbronn, Germany). The liquid cocoa extract was analysed using a gradient mixture of water-formic acid (99.9:0.1, v:v) (solvent A) and acetonitrile-water-formic acid (94.9:5:0.1, v:v:v) (solvent B). A 2.1 x 150 mm Eclipse XDB-C18 (5 µm) column (Agilent Technologies AG, Waldbronn, Germany) was used. The separation was affected using a linear gradient at 30 °C with a flow of 0.7 mL/min as follows: 1% B at 0-1.5 min, 1-5% B at 1.5-5 min, 5-10% B at 5-12 min, 10% B at 12-18 min, 10-15% B at 18-25 min, 15-20% B at 25-32 min, 20-25% B at 32-37 min, 25-30% B at 37-43 min, 30% at 43-46 min, 30-40% at 46-50 min, 40-50% at 50-52 min, 50-70% B at 52-55 min, 70% B at 55-56 min and 70-10% B at 56-58 min. The re-equilibration time was 8 min.

For ESI/MS analysis, the positive capillary voltage was set at 4,000 V and the negative at 3,000 V. The drying gas temperature was 350 °C and the drying gas flow 12 mL/min. The samples were analysed using a full scan from 100-2,000 *m/z* in positive ionization mode. The comparison of retention times and characteristic fragmentation patterns was done using the aforementioned standard substances.

3. Results and Discussion

3.1. Online NP-HPLC-DPPH analysis of standard PA

So far, various research groups have used diverse protocols for the investigation of the antioxidant activity of countless plant based products. The DPPH radical is widely used for measuring the efficiency of antioxidants, because of its radical stability, the sensitivity, and the technical simplicity of the assay execution (Huang, Ou, & Prior, 2005). It has become a quite popular method for the analysis of the antioxidant activity of phenolic compounds, as it allows a fast reaction with most of the phenolic compounds. Further assays (providing different antioxidant mechanisms) have not been applied, as it was the aim to only compare between the different cocoa fractions.

The idea behind the online NP-HPLC-DPPH method was to adapt the traditional DPPH cuvette test to HPLC conditions, whereby HPLC allows a full characterisation of individual condensed PA in complex mixtures in one single run under the same conditions. For both approaches, the online NP-HPLC-DPPH derivatisation and the optimized analysis in a photometer, the reaction conditions have been adjusted according to the individual reaction kinetics. For an improved reproducibility, the radical scavenging reaction of oligomeric PA with DPPH was optimized in terms of temperature and time. The antioxidant kinetic behaviour was evaluated photometrically and the results were adapted to the online NP-HPLC-DPPH methodology. A linear relationship over a concentration range from 0.05 g/L to 1 g/L was observed at a temperature of 60 °C, when using a reaction time of 2 minutes (or a corresponding length of the reaction capillary, resp.). The HPLC-PTFE reaction coil was therefore thermostated at 60 °C and the reaction time was kept short (1 minute).

The UV and the DPPH chromatograms of the oligomeric standard PA are shown in **Figure 2.** Peaks with retention times at 4.4, 5.2, 8.7, 18.9, and 22.2 min in the UV chromatogram and the DPPH chromatogram are (-)-epicatechin, (-)-epigallocatechin, PA dimer B2, trimer C1, and cinnamtannin A2.

The reaction was optimized with regard to intensity and shape of the peak signals resulting from the DPPH radical quenching. When applying a non-standardized procedure, the DPPH radical reaction mixture can cause side reactions such as polymerisation of catechins and *ortho*-quinones to higher molecular weight oligomers, or adducts between the oxidized form of catechin and DPPH radical (Osman, 2011).

The small peak with the retention time of 5.2 min in the UV chromatogram was identified as (-)-epigallocatechin by RP-HPLC-ESI/MS. Although it has a small UV absorption, its antioxidant activity is much higher than that of (-)-epicatechin. It could be proven that the commercially available reference substance (-)-epicatechin has a slight contamination of less than 0.05% of (-)-epigallocatechin, whereby the antioxidant activity is approx. 30% higher. Previous studies of antioxidant activity supported the role of specific structural components as requisites for radical scavenging (Nanjo, Goto, Seto, Suzuki, Sakai, & Hara, 1996; Nanjo, Mori, Goto, & Hara, 1999). They also confirmed the assertion that the presence of an *ortho*-hydroxyl group in the B ring is indispensable for the radical scavenging effect. Those results showed the DPPH scavenging ability of tea catechins whereby (-)-epigallocatechin and (-)-epigallocatechin gallate were stronger than (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate. The concentration required to give a 50% decrease of the signal intensity of DPPH radical was 2.5 µM for (-)-epicatechin and 1.7 µM for (-)-epigallocatechin.

It should be kept in mind that contaminations like this or any other potentially antioxidant substances cannot be adequately considered in spectrophotometric tests.

3.2. Online NP-HPLC-DPPH analysis of a liquid cocoa extract

The method developed can be used as fast identification of bioactives in extracts without the need of a complex sample preparation. **Figure 3** shows combined UV (positive peaks) and DPPH radical-quenching (negative peaks) chromatograms. There are ten major UV- and antioxidant-active compounds in cocoa beans extract which show a significant radical-quenching capacity. From comparison with the standard substances (-)-epicatechin monomer, proanthocyanidin B2 dimer, C1 trimer, and cinnamtannin A2 tetramer, the peaks with the retention time at 4.8, 10.1, 22.9, 30.7, 34.7, 38.2, 41.2, 44.3, 46.4, and 48.9 min in the UV chromatogram, and DPPH chromatogram were identified as (-)-epicatechin monomer, dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer, and decamer, respectively. The identification is based on the retention time with standard substances, the UV spectra and MS fragmentation pattern. Obviously, there is no antioxidant capacity detected for the solvent peak (acetone) with a retention time at 3.2 or for the caffeine or theobromine peaks with a retention time at 4.1 and 5.4 min.

The content of identified PA in the unroasted liquid cocoa extract is shown in **Table 1**. Levels of PA were expressed as epicatechin equivalents per g fat free dry mass. This form of

calculation makes it easier to compare the results also with other studies. Except for dimers, the proanthocyanidin content and the antioxidant activity decrease from monomers to decamers in accordance with the descriptions of Ioannone, Di Mattia, De Gregorio, Sergi, Serafini, & Sacchetti (2015), Bordiga, Locatelli, Travaglia, Coïsson, Mazza, & Arlorio (2015), and Di Mattia et al. (2013). Although, monomers were the most abundant polyphenols (with regard to their concentration), the high antioxidant activities were also found for some other fractions (e.g., trimers to pentamers (**Table 1**).

Besides that, contents of proanthocyanidins can be expressed at hand of corresponding standard compounds. E.g., for dimers as mg proanthocyanidin B2 equivalents, for trimers as mg proanthocyanidin C1 equivalents and for fractions bigger or equal than tetramers as mg cinnamtannin A2 equivalents. The evaluation indicates that PA pentamers were the most abundant compounds in this extract with a high antioxidant activity (**Table 1**)

In general, the content and composition of PA in cocoa beans differs due to genetic, geographic origin, and environmental factors. Most of the work in literature reported a cascading decrease along monomers to decamers. Nevertheless, in this work unfermented, raw cocoa beans had a relatively high abundance of trimers and pentamers when calculating through external standards. However, data obtained in this work are not significantly different when taken the statistical variability of PA into account as well as generally natural fluctuation in cocoa beans. Further studies need to be done concerning different varieties, and regions of productions. Also Kim & Keeney (1984) observed a wide range of (-)-epicatechin from 21.89 - 43.27 mg/g non-fatty dry matter among cocoa beans of varying genetic origin.

In addition, the antioxidant capacity was determined as the ratio of the antioxidant activity per content of the single proanthocyanidin fractions (**Table 1**). This provides information on the effectiveness of PA to scavenge DPPH radicals (Vizzotto, Cisneros-Zevallos, & Byrne, 2007; Jacobo-Velázquez & Cisneros-Zevallos, 2009). A high value implies that this compound has a high relevance in the total antioxidant activity of the sample. Nevertheless, although trimeric PA show the highest antioxidant activity, it has a comparatively moderate capacity. The highest value for the capacities was found for nonamers and decamers (**Table 1**). From the chemical point of view, this seems to be kind of reasonable, as those large molecules provide more structural features for interacting with radicals.

This approach has proven that oligomeric PA are efficient DPPH radical scavengers despite their high molecular weight and potential steric hindrance. According to Arteel & Sies (1999), long chain PA are better scavengers than short chain PA, particular tetramer was found efficient against oxidation and nitration reaction. Ramljak et al. (2005) claimed for

pentameric cocoa proanthocyanidin even a growth inhibition effect of human breast cancer cells.

In an offline approach by Rösch, Bergmann, Knorr, & Kroh (2003), a quite similar order was found for the antioxidant activity of oligomeric PA from sea buckthorn (*Hippophae rhamnoides*). PA tetramers showed hereby the highest antioxidant activity. Lotito et al. (2000) reported that monomers, dimers, and trimers were the most effective antioxidants in the aqueous phase, while PA of higher molecular weight were more effective in the lipid phase. These results also underline the observation described by Lu & Yeap Foo (2000) who characterised apple polyphenols. They found trimers and tetramers to be more efficient free radical scavenger than proanthocyanidin B2 and (-)-epicatechin. But they also claimed that this trend of antioxidant activity do not extend to higher molecular PA. The present study demonstrates that to a point the increasing PA enhances the effectiveness against radicals. But with an increase of molecular weight, the in antioxidant activity leads to a slight, non-linear reduction for polyphenols with DP > 8. According to Lu & Yeap Foo (2000), the slight increase of the antioxidant activity could be explained by the increasing crowding and therefore the less availability of the hydrogen donating effect.

A further aspect to consider is the separation of PA by polymerisation degree when using normal phases, whereby different PA with the same molecular weight are often determined as one fraction. However, separation of single PA only succeeds with reversed phases, so far.

As obvious from **Figure 3**, structures larger than decamers elute as an unresolved group of peaks at the end of the chromatogram which corresponds to the majority of the material present. So far, all traditional separation methods failed to resolve this ‘proanthocyanidin (PA) hump’. This has to do with the fact that the resolution of a NP-HPLC stationary phase under HILIC conditions is not sufficient to separate such complex samples (Kalili & Villiers, 2009). There is much evidence suggesting that a chromatographic separation of larger polymers is not possible due to their complexity. Polymeric PA with a higher molecular mass cannot be determined with NP-HPLC (Lazarus, Hammerstone, Adamson, & Schmitz, 2001).

However, the cocoa ‘proanthocyanidin hump’ shows a powerful antioxidant capacity that, beyond all doubt, represents more or less the majority of the sample material. Although it is difficult to characterize the PA hump completely, it is necessary to compare the PA hump in interaction with other compounds and their antioxidant activity. With an antioxidant activity of approx. 20 mg per gram fat free dry matter (expressed as epicatechin equivalents), the PA hump is as powerful as all other PA together. However, with regard to the antioxidant

capacity of this fraction, the effectiveness to neutralize DPPH radicals can be considered as comparatively low (**Table 1**).

Unfortunately, only a few studies showed an unadorned chromatogram including the PA hump (e.g., Guyot, Marnet, & Drilleau, 2001, Gu et al., 2002, Esatbeyoglu, Wray, & Winterhalter, 2015). However, some researcher also observed analogous unresolved humps in their chromatograms when analysing different plant extracts. Kuhnert (2010) as well as Kuhnert, Dairpoosh, Yassin, Golon, & Jaiswal (2013) highlighted similar observations in the chromatographic behaviour when describing the so-called “thearubigin hump” in the analysis of black tea. Although the molecular weight of the thearubigins ranges from 1,000 to 40,000 (Yao et al. 2006), Sinha & Ghaskadbi (2013) recently presumed strong antioxidant properties of black tea thearubigins. Here again, although thearubigins are present in large amounts in black tea, the information on formation and structure of this heterogeneous polymer is very limited.

In the present study, it could be shown that the unroasted cocoa extract contains a high amount of extractable condensed polyphenols which can be separated on a normal phase column whereby their antioxidant capacity can be assessed by online post-column derivatisation.

Besides the homologous series of oligomeric PA, other UV-active flavanol moieties can be seen on a HILIC-column. The poor separation between each oligomer peak suggests that PACE is quite inhomogeneous including different structures showing a high complexity in its PA subunits and the linkages between them (Yanagida et al., 2007; Valls, Millán, Pilar Martí, Borràs, & Arola, 2009). Besides linear PA also large, branched molecules can be present. Another to date unsatisfyingly investigated phenomenon when using a HILIC column is the appearance of smaller unresolved UV-absorbing peaks. They merged into big peaks with shoulders. In the chromatogram they are marked with an asterisk (**Figure 3**). Similar effects were showing during the NP-HPLC analyses of grapes or wine, indicating the presence of different mono-galloylated, galloylated and non-galloylated PA (Nunes, Gómez-Cordovés, Bartolomé, Hong, & Mitchell, 2006). Callemien & Collin (2008) isolated dimers and trimers from lager beer extract by normal phase column and obtained, using thioacidolysis, that catechin forms the main terminal unit, whereas both catechin and gallocatechin are major constituents of the extension unit. For cocoa, it can be hypothesized that such smaller unresolved peaks are PA linked either to *C*- or *O*-glucose, -arabinose, -galactose (Hatano et al., 2002).

3.3. Quantification of oligomeric PA using the online NP-HPLC-DPPH assay

In this study, oligomeric PA were quantified using an external calibration for UV-NP-HPLC analysis with a diol-HILIC column. For each analyte, peak areas from chromatogram were plotted against the concentration of the stock solution of known concentrations. As expected, PA show a positive correlation between the concentration against UV-absorption. The calibration curves were as follows: (-)-epicatechin, y = 1618.8x + 11.922 (R^2 = 0.9994, 0.05-1.0 g/L), proanthocyanidin B2, y = 1154.5x + 3.04 (R^2 = 0.9995, 0.05-1.0 g/L), proanthocyanidin C1, y = y = 1266.7x - 16.46 (R^2 = 0.9999, 0.05-1.0 g/L) and cinnamtannin A2, y = 973.69x – 11.756 (R^2 = 0.9999, 0.05-1.0 g/L).

The DPPH radical solution is a deep violet chromogen and any quenching of the radical results in a loss of color, getting yellowish and is indicated by signal intensity decrease and represented by a negative peak in the chromatogram. The radical scavenging properties are detected at 515 nm. The DPPH scavenging activity of oligomeric PA were calculated from the working calibration curves: (-)-epicatechin, y = 1233.7x + 109.36 (R^2 = 0.9836, 0.05-1.0 g/L), proanthocyanidin B2, y = 1306.6x + 51.282 (R^2 = 0.9923, 0.05-1.0 g/L), proanthocyanidin C1, y = 1507.5x + 46.693 (R^2 = 0.9927, 0.05-1.0 g/L), and cinnamtannin A2, y = 1271.6x + 34.386 (R^2 = 0.9938, 0.05-1.0 g/L). The activities tested with the online NP-HPLC-DPPH assay decreased in following order: proanthocyanidin C1 > proanthocyanidin B2 > cinnamtannin A2 > (-)-epicatechin.

For the validation of the online NP-HPLC-DPPH assay, the limits of detection (LOD) and the limits of quantification (LOQ) of the PA compounds and their antioxidant capacity were calculated based on the standard deviation of y intercept of the regression against the slope of the calibration curve. However, the UV absorption is comparatively sensitive (LOD = 7-21 µg/mL) as the DPPH reaction (LOD = 6-27 µg/mL) (**Table 2**).

As depicted from the calibration curves the PA content is highly correlated with the antioxidant capacity (R^2 > 0.99). This suggests that PA accounts for a major portion of the antioxidant activity in unroasted cocoa beans. This could also be an indicator for potential biological activity.

The common limitation of the analysis is the weak UV absorption of higher oligomeric PA due to the wider chromatogram peaks. Furthermore, the response for UV-absorbing chromophores decreases in relation to the degree of polymerisation. This approach can be explained by the higher molecular weight and effects of steric hindrance.

It can be further discussed if measurement for condensed polyphenols might be determined at lower wavelengths such as 205 nm, where they give better response at low concentration. Lee & Ong (2000) analyzed sixteen types of tea ingredients at 205 nm and obtained a higher sensitivity with a LOD ≈ 0.05 µg/mL.

Some methodologies are also using fluorescence detectors to improve sensitivity and selectivity for the analysis of catechins (Arts & Hollman, 1998; Lazarus, Adamson, Hammerstone, & Schmitz, 1999). Typical concentrations for fluorescence measurements are in the range 5-16 µg/mL (Hemingway & Karchesy, 1989). Hellström & Mattila (2008) achieved LOD values for extractable PA varying from 1.0-15 µg/mL for a concentration level from 20-2,000 µg/mL.

3.4. Quantification of oligomeric PA using the traditional photometric assay

To evaluate whether both methods can be compared, it was important to quantify oligomeric PA using also the traditional photometric assay. To determine the reaction kinetics, the DPPH radical quenching of (-)-epicatechin was measured at 25 °C and 60 °C. The absorbance at 515 nm was noted after an incubation time of 2 and 5 minutes using a UV-visible spectrophotometer with methanol as blank. The absorption measured at 60 °C with an incubation time of 2 min showed the most linear response over the whole range of concentrations from 0.05 g/L to 1.0 g/L.

Accordingly, the four main condensed cocoa polyphenols were investigated under the optimized conditions. The working calibration curves are representing a polynomial of the second degree: (-)-epicatechin, $y = 0.5817x^2-1.3405x+0.9578$ ($R^2 = 0.9997$, 0.05-1.0 g/L), proanthocyanidin B2, $y = 0.224x^2-0.7919x+0.934$ ($R^2 = 0.9994$, 0.05-1.0 g/L), proanthocyanidin C1, $y = 0.2277x^2-0.7261+0.9289$ ($R^2 = 0.9986$, 0.05-1.0 g/L) and cinnamtannin A2, $y = 0.1855x^2-0.6405x+0.9331$ ($R^2 = 0.9996$, 0.05-1.0 g/L).

The resulting dose-response curves showed a decrease in DPPH-scavenging activity in the following order: cinnamtannin A2 > proanthocyanidin C1 > proanthocyanidin B2 > (-)-epicatechin (**Figure 4**).

The comparatively higher oligomers had a higher antioxidant activity than the monomers. Among PA, tetramers were the most potent while epicatechin was the least active. The antioxidant activity increases depending on DP. The important structural feature for DPPH scavenging is the *ortho*-hydroxyl group in the B-ring(s), the additional presence of a 3-OH

group(s), and the abundant 4->8 linkage between the subunits (Heim, Tagliaferro, & Bobilya, 2002).

3.5. Comparison of the traditional photometric assay versus the online NP-HPLC-DPPH assay

The results of the traditional photometric DPPH assay and the online NP-HPLC-DPPH assay can be expressed and calculated as (-)-epicatechin equivalents for the first four homologous polyphenols. Here again, the four main standard substances (-)-epicatechin, procyanidins B2, C1, and A2 were used for the calculation and have been indicated as representatives of DP 1, DP 2, DP 3 and DP 4 (**Figure 5**).

Proanthocyanidin B2 (DP 2), when expressed as (-)-epicatechin units/equivalents, shows a slightly higher antioxidant activity than (-)-epicatechin itself. With regard to the traditional photometric assay, this trend extends up to the tetramers: Cinnamtannin A2 has a more effective radical scavenging activity than the monomeric (-)-epicatechin.

Hence, the results of the both assays cannot be compared satisfyingly. The antioxidant activity determined with the online NP-HPLC-DPPH assay is diminished in relation to the traditional photometric assay. One explanation for that phenomenon could be the hydrogen-bond accepting ability of the HPLC solvents methanol and acetonitrile as described by Jabbari & Moallem (2015). An increase of the organic solvent influences the radical scavenging activity of flavonoids. Presumably, they undergo interactions with DPPH due to solute-solvent effects. Also Sharma & Bhat (2009) recommended for a better sensitivity and a high range of accuracy, the preparation of the DPPH solution in methanol without any further additives.

Recent studies showed that the antioxidant activity is closely related to the molecular structure of the polyphenol and the solute-solvent present (Jabarri, Mir, Kanaani, & Ajloo, 2014). Also a lower pH in the mobile phase can lead to a dramatic loss of sensitivity and to a decrease in absorbance of the DPPH radical (Malherbe, de Beer, & Joubert, 2012).

The direct comparison of the DPPH cuvette assay with the NP-HPLC-DPPH assay leads to the conclusion that each assay has its particular advantage and disadvantage. The method ultimately chosen will depend on speed of analysis, accuracy required, sensitivity required and the determination of single compounds in complex matrixes. The DPPH cuvette assay describes a simple high throughput colorimetric assay which is reliable and generates reproducible measurements, expressed as total antioxidant capacity (TAC). The

disadvantage, on the other hand, is in the analysis of complex matrixes. The online HPLC-DPPH assay gives more information about the composition of the mixture as well as a rapid assessment of the antioxidant capacity of every single compound.

However, the use of an internal standard is pretty common when using RP-HPLC. Antioxidant capacities of phenolic substances in plant extracts are usually quantified with an internal standard such as trolox (Riehle, Vollmer, & Rohn, 2013). Data are normally presented as trolox equivalent antioxidant capacity (TEAC) which is expressed as mg trolox equivalents per g sample. The resulting values are usually comparatively higher than the real amount of polyphenols. This has to do with the fact that trolox has a higher antioxidant activity than polyphenols usually have. For example, according to Arts, Haenen, Voss, & Bast, chrysin has a relatively high TEAC value while its antioxidant activity in other assays is relatively low. In the present case, the quantification using an internal standard on a diol-HILIC column proved to be rather difficult. Neither known cocoa polyphenols nor typical standards such as trolox or vitamin C could be used as internal standard due to adverse co-eluting with other known substances. For this reason, trolox was deliberately refrained as internal standard.

3.6. Isolation of oligomeric PA using semi-preparative NP-HPLC

Additional investigation of isolated oligomeric PA was needed for several reasons: Primarily to understand the contribution of each single PA to the total antioxidant activity of an isolated fraction. Furthermore, increasing the scale of the NP-HPLC chromatography is of particular interest regarding the preparation of self-made analytical standards in considerable amounts for substances that are difficult to obtain commercially.

A separation of the unroasted PA cocoa extract with a semi-preparative NP-HPLC column is illustrated in **Figure 6,** whereby PA up to hexamers can be observed in a 60 min run. Fractions were numbered F1-F6, respectively in their order of elution from the diol-HILIC column. Despite column overload, fractions were clearly separated from each other. The colour of the fractions increased from colourless for F1, upon light pink for F4 and to deep red for F7. Colour intensification results from condensation an increased number of monomeric units. Monomers such as (-)-epicatechin are known as white powder, whereas dimers such as proanthocyanidin B2 are coloured white to beige. This trend continues towards polymeric tannins known as brownish powders.

Although the analysis of the liquid cocoa extract on the analytical column gives fractions with up to decamers, it is surprising that on the semi-preparative column only monomers to hexamers could be separated although obviously material was eluting from the column (between 60 and 90 min) (**Figure 6**). A proposed rationale could be the different retention mechanism for larger diol-HILIC columns and there insufficient separation with increasing molecular weight (DP). According to the VAN DEEMTER equation the efficiency diminishes in relation to the column width and results in peak broadening.

An important element of this study was the right sample preparation and dissolution of the PACE for semi-preparative purpose. Since the knowledge of oligomeric PA is still incomplete, the conditions have to be optimised to ensure an efficient extraction and injection, so that no information is lost. On the other hand, to prevent the system from precipitation most of the researchers (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006) dissolve their extract in the HPLC mobile phase whereby some of them additionally dissolved it in ethanol before injected onto the column. Because of its limited solution power this study purposely decided not to dissolve the PACE in the HPLC solvent. To prevent the HPLC system from precipitation of solids, the 'PA cocoa extract' was re-dissolved in 40% isopropanol, suspended using an ultrasonic bath, and filtered through a 0.45 µm cellulose syringe filter prior to injection so that no resinous material was seen. Similar to Kelm, Johnson, Robbins, Hammerstone, & Schmitz (2006) and their observation on unfermented cocoa PA, in the present study only monomers to heptamers could be separated clearly. PACE could be prevented from precipitation by using the new method of complete dissolution. Various injection methods to increase the injection load for right sample preparation are currently under investigation.

A major benefit of the method described is the isolation of single compounds in high purity, whereby their identities were deduced by RP-HPLC-ESI/MS.

3.7. Identification of oligomeric PA using RP-HPLC-ESI/MS

The compounds F1-F6 of the 'PA cocoa extract' (PACE) were isolated by semi-preparative HPLC using UV-Vis detection. As this technique cannot provide compound identification or structural information the optimized RP-HPLC method was coupled to electrospray ionization to complete the characterization of PACE.

Identification was based on retention time and mass spectra summarized in **Table 3**. This table also includes the experimental *m/z*, the fragmentation of fission products and the molecular formulas.

The six successive eluted fractions were measured in positive ionization TIC mode $[M+H]^+$. For an increased sensitivity, each fraction is shown in the extraction ion mode (**Figure 7**). Briefly, the first fraction is shown by measuring at *m/z* 291, the second fraction at *m/z* 579, the third fraction at *m/z* 867, etc. A total of six oligomeric PA and the two alkaloids theobromine and caffeine were identified using RP-HPLC-ESI/MS.

As mentioned above, several PA can elute as a cluster of peaks of isomers on a normal phase column. The separation is not sensitive enough to difference PA of the same degree of polymerization. Detailed structure can be just determined by RP-HPLC.

Figure 7a shows an extracted ion chromatogram (EIC) of $[M+H]^+$ = 291 for fraction F1 which contains two peaks at t_R = 18.8 min and 26.1 min and were assigned as (+)-catechin and (-)-epicatechin after comparison with the retention time and fragmentation pattern of commercially available standards. (-)-Epicatechin has a much higher absolute intensity than (+)-catechin. The third peak with $[M+H]^+$ = 291 at t_R = 34.7 min is a pseudomolecular ion coming from procyanidin B2. All things considered, the antioxidant activity of the monomeric fraction in **Figure 7a** is contributed equally by (-)-epicatechin and (+)-catechin.

Figure 7b is an EI-chromatogram of $[M+H]^+$ = 579 for fraction F2 with proanthocyanidin B3 at t_R = 17.4 min, PA B4 at t_R = 22.1 min and PA B2 at t_R = 23.5 min. Furthermore there are characteristic signals for dimers at t_R = 25.5 min, 31.4 min and 35.1 min whereby they remain unidentified due to a lack of standard substances. So altogether, there are at least six PA dimers contributing to the antioxidant activity of the dimeric fraction.

Figure 7c is an EIC of $[M+H]^+$ = 867 with proanthocyanidin C1 at t_R = 28.9 min. In addition there are three more non-identified trimers with t_R = 23.4 min, 30.8 min, and 35.2 min. Altogether, four PA trimers contribute to the antioxidant activity of the trimeric fraction.

Figure 7d is an EIC of $[M+H]^+$ = 1,155 with cinnamtannin A2 at t_R = 30.5 min. Furthermore, there are three more non-identified tetramers with t_R = 25.7 min, 31.8 min, and 37.9 min. Due to the lack of standard substances there is no clear classification of PA besides that possible. Altogether, four PA tetramers contribute to the antioxidant activity of the tetrameric fraction.

Figure 7e is an EIC with the mass-to-charge ratio *m/z* 1,444 with clear peaks at t_R = 32.1 min, and t_R = 37.8 min. Altogether, two PA pentamers contribute to the antioxidant activity of the pentameric fraction.

Figure 7f is an EIC with the mass-to-charge ratio *m/z* 1,732 with clear peaks at t_R = 33.5 min and a smaller one at t_R = 35.6 min, providing information that a minimum of two PA hexamers contribute to the antioxidant activity of the hexameric fraction.

Behind every fraction there is a couple of isobaric, sometimes isomeric PA which altogether contributes to the overall antioxidant capacity.

However, the efficiency of the monomeric fraction and the corresponding chromatographic resolution is much higher than for the dimeric, trimeric, tetrameric fractions, etc. Motilva, Serra, & Macià (2013) observed a decrease of the absolute intensity during each fraction of cocoa flavanols and the reduction of the signal-to-noise ratio. This can be explained by the poor extraction yield of the semi-preparative NP-HPLC within each fraction. The insufficient resolution can be avoided by an efficient sample preparation after semi-preparative isolation by decreasing matrix suppression.

4. Conclusions

The method described can be used for a rapid evaluation of condensed antioxidant components in complex matrices such as plant or food extracts. For chromatographic separation of mono- and oligomeric PA, a standard procedure using a NP-HPLC system was established. To simultaneously assess the antioxidant capacities of PA, the traditional photometric assay was adapted and coupled online to a HPLC system. The well-known antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was chosen, because of its easy and rapid way to determine the antioxidant activity and its reactivity of its stable free radical (Prior, Wu, & Schaich, 2005).

The DPPH concentration was optimized and the effect of reaction time, temperature, and stability of the antioxidant compound during the assay (reaction time < 1 min) was characterized. The results suggest that this method can be used for the simultaneous determination of condensed polyphenols according to their DP as single antioxidant capacities. Several antioxidants were identified in the cocoa extract whereby the results indicate that the radical scavenging activity of the homologous series of condensed PA is increasing with increasing DP. Nonameric and decameric PA fractions showed the highest antioxidant capacity.

Besides that, it should be general questioned whether it is useful to evaluate the PA amount as epicatechin equivalents (ECE) or as PA equivalents (PAE). When calculating as ECE, the values appeared more significant. The evaluation as PAE, however, makes the

values appear more equal, but compounds used for the calculation are chemically closer related to the substance that is quantified. Despite to that, it should be also taken into account that the UV activity of oligomeric PA is decreasing with increasing DP. This can be seen in the calibration curve whereby the slope of the straight line drops from (-)-epicatechin to cinnamtannin A2.

Furthermore, it can be discussed whether the newly developed assay can be improved in terms of detection limits for higher polymeric PA with DP > 10. Also, the standard deviation for higher PA could be minimized maybe by using a fluorescence detector.

The online NP-HPLC-DPPH assay can also be considered as a promising technique for quality control in the chocolate manufacturing process whereby the question arises whether the antioxidant capacity of individual oligomeric PA changes throughout the process.

However, the most promising substances, because of their high potential antioxidant activity, still seem to be the unresolved. Further, higher condensed polyphenols occur at the end of the NP-HPLC chromatogram (‘PA hump’) providing future challenges for their analysis and characterization.

To develop a complete understanding of the biological activity of cocoa, additional structure determination is important: Research on structure elucidation can be done by isolation of oligomeric substances and their further depolymerisation reaction in presence of nucleophiles such benzyl mercaptan (thiolysis) (Matthews et al., 1997; Ramirez-Coronel, Marnet, Kumar Kolli, Roussos, Guyot, & Augur 2004; Callemien, Guyot, & Collin, 2008), phloroglucinol (phloroglucinolysis) (Lorrain, Ky, Pechamat, & Teissedre, 2013), or enzymatic treatments of the extract (Mandalari et al., 2006).

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Figure Legends

Figure 1 Instrumental setup of the online post-column NP-HPLC-DPPH methodology.

Figure 2 Correlation of standard substances of oligomeric PA from unroasted cocoa beans extracts using online screening with antioxidant capacity. The polyphenols have a concentration of 1 g/L. The asterisk indicates the contamination of the (-)-epicatechin reference sample.

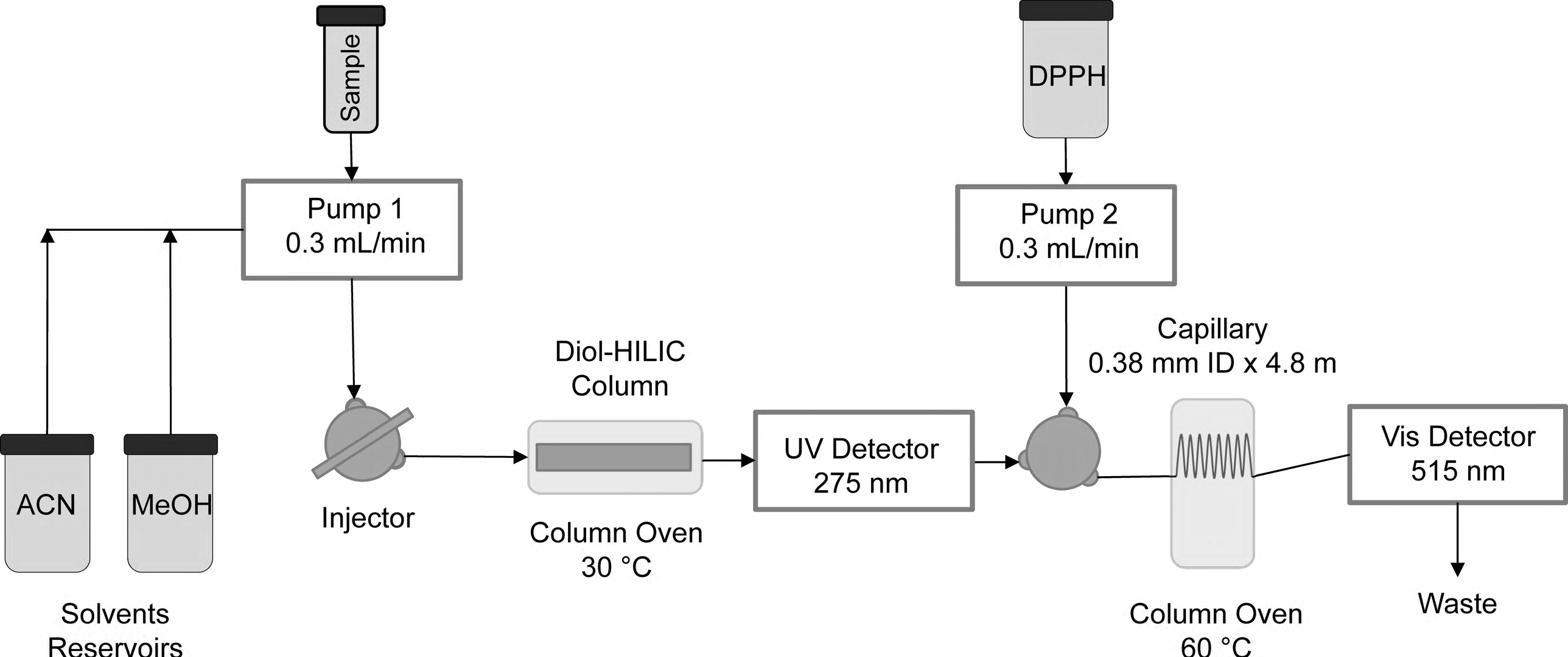
Figure 3a-b (a) Bioactive screening of condensed polyphenols from unroasted cocoa beans extract using online screening NP-HPLC-DPPH (injection volume: 1µ; UV wavelength: positive 275 nm, negative 515 nm); (**b**) Identified PA: epicatechin monomer (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6), heptamers (7), hexamers (8), nonamers (9), decamers (10), and an unresolved “proanthocyanidin (PA) hump” (11). The asterisks are indications for glycoside bound proanthocyanidin oligomers.

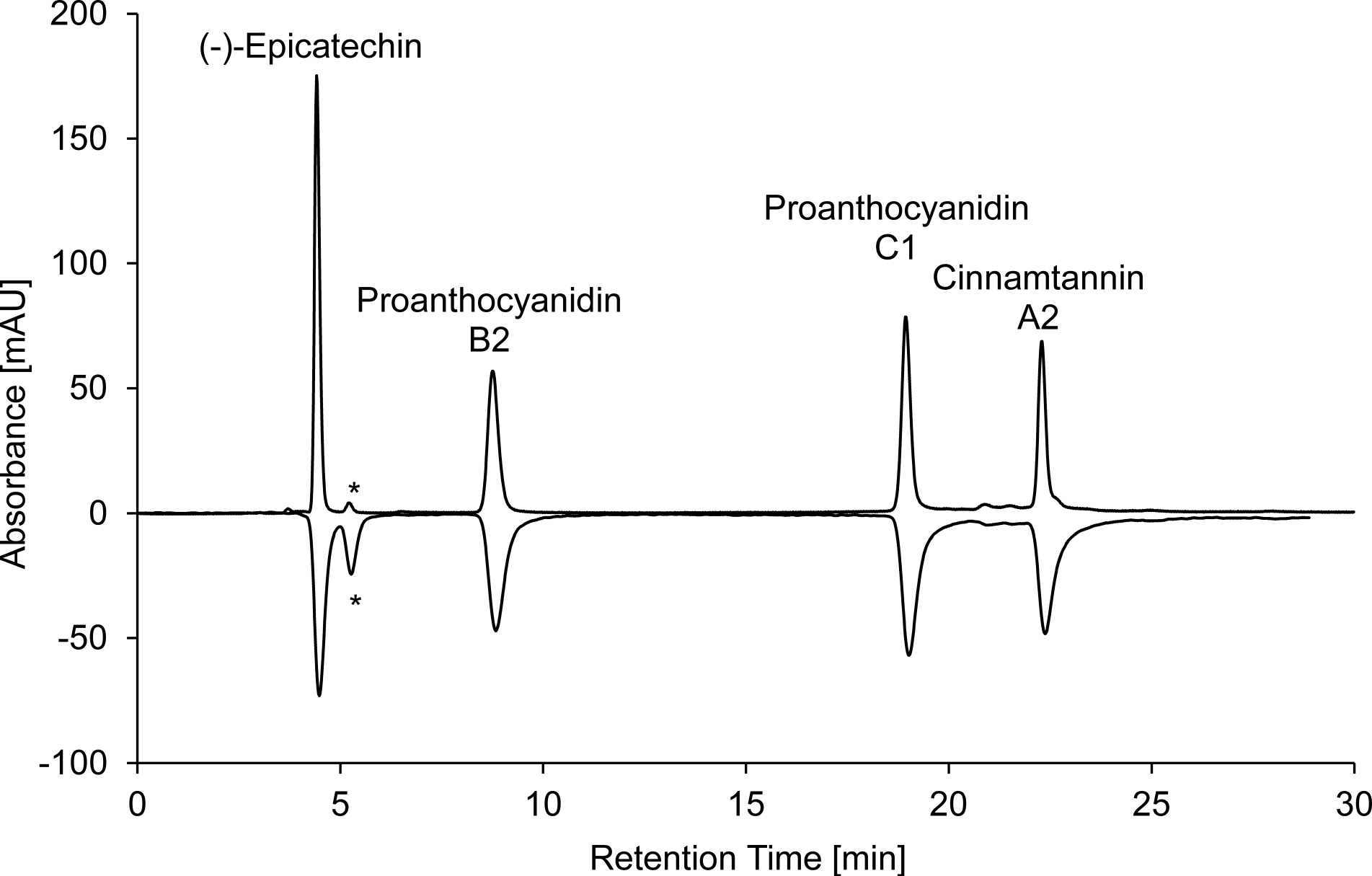
Figure 4 Dose-response curves obtained by the DPPH cuvette assay for (-)-epicatechin, proanthocyanidin B2, C1 and cinnamtannin A2 at concentrations from 0.05 g/L to 1.0 g/L measured at 515 nm and 2 minutes at 60 °C incubation. Each value represents the mean values and the standard deviations from three determinations.

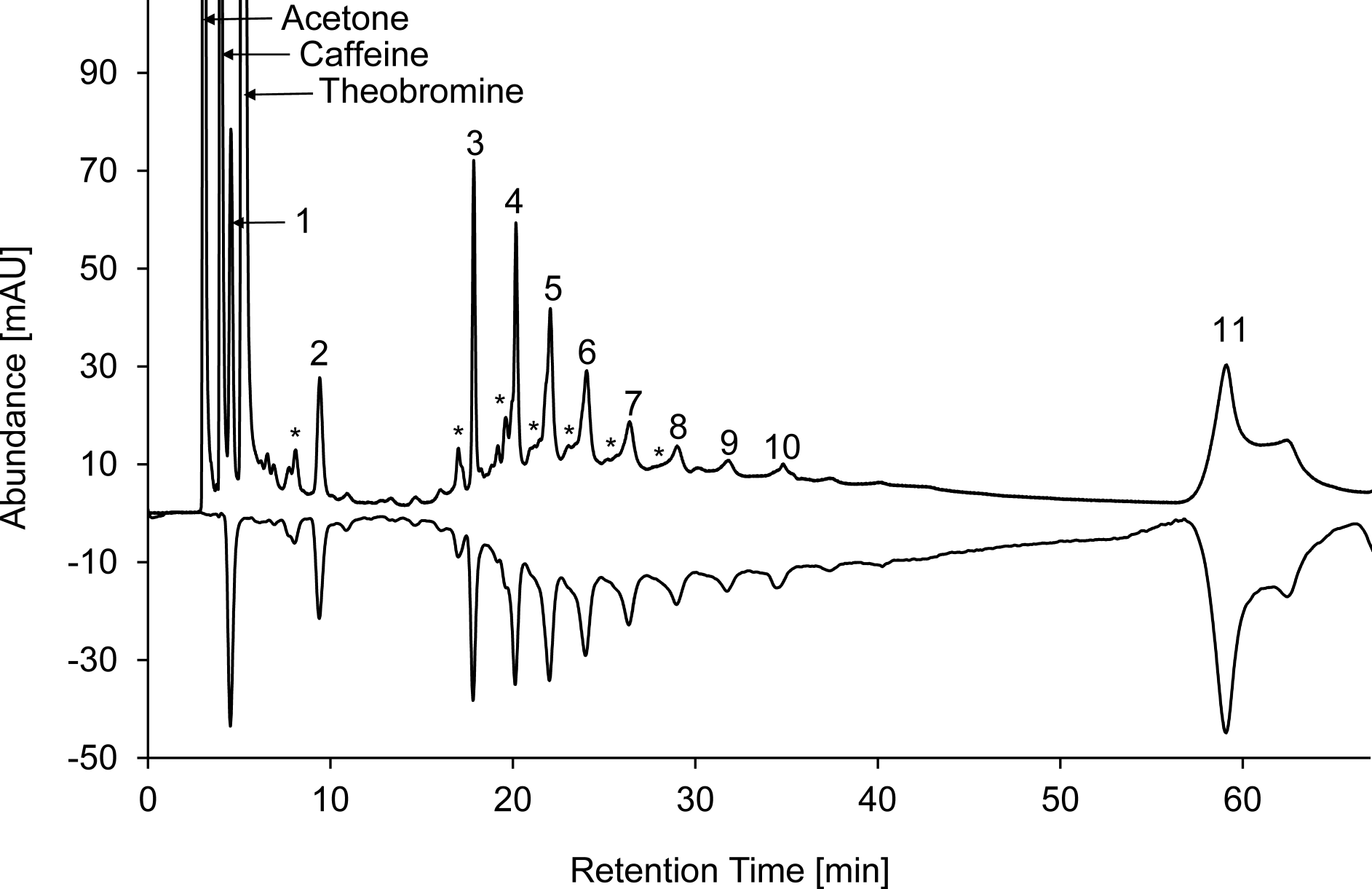
Figure 5 Comparison of DPPH scavenging activity of PA between the DPPH cuvette assay and the online NP-HPLC-DPPH assay. The results are expressed as (-)-epicatechin equivalents. The error bars are the result of a triple determination.

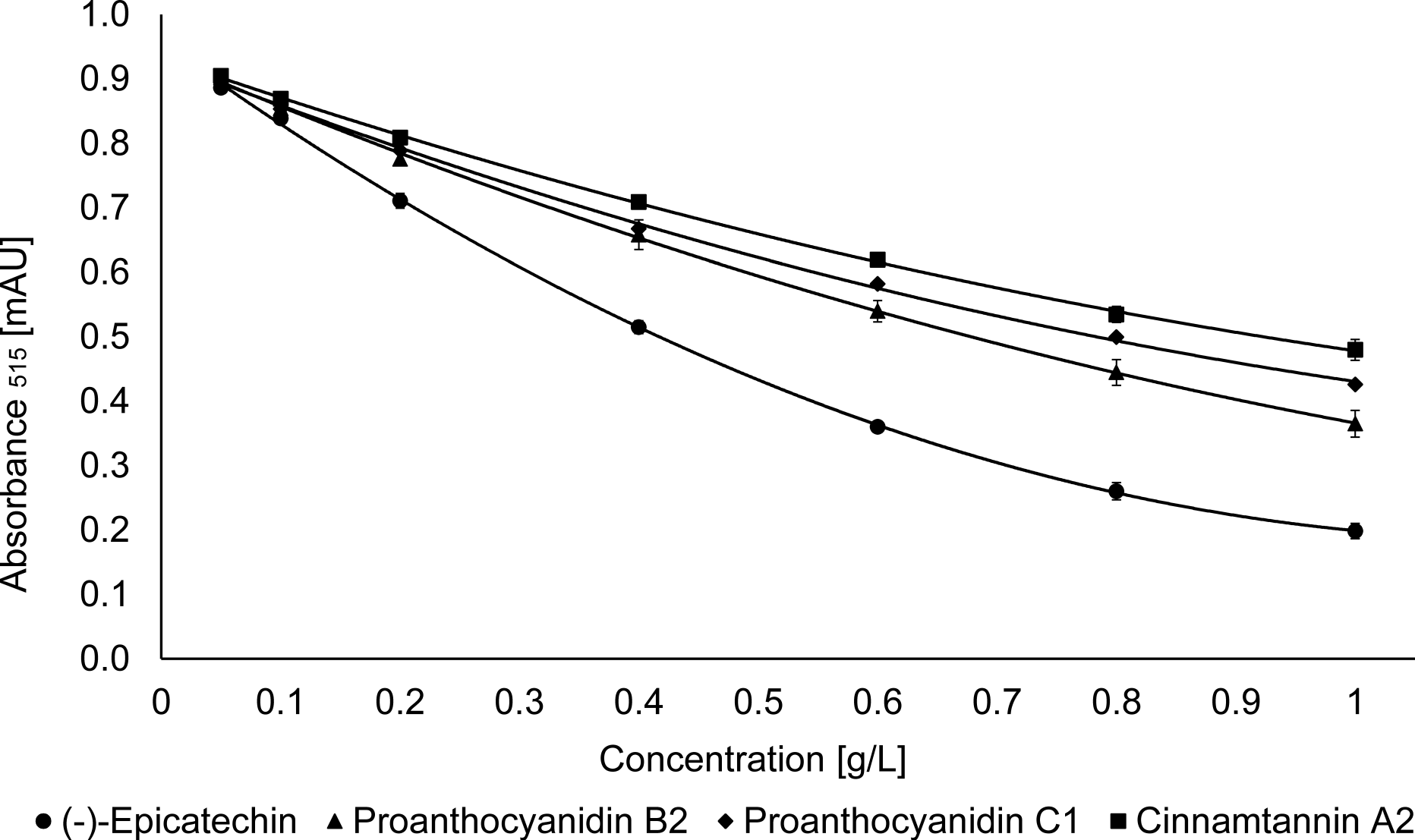
Figure 6 Preparative diol-HILIC profile of 50 mg unroasted cocoa powder extract injected onto the column (injection volume: 200 µL). The fractions are labelled F1-F7 according to their DP. The black bar in the middle of each peak reflects the manually collected fractions.

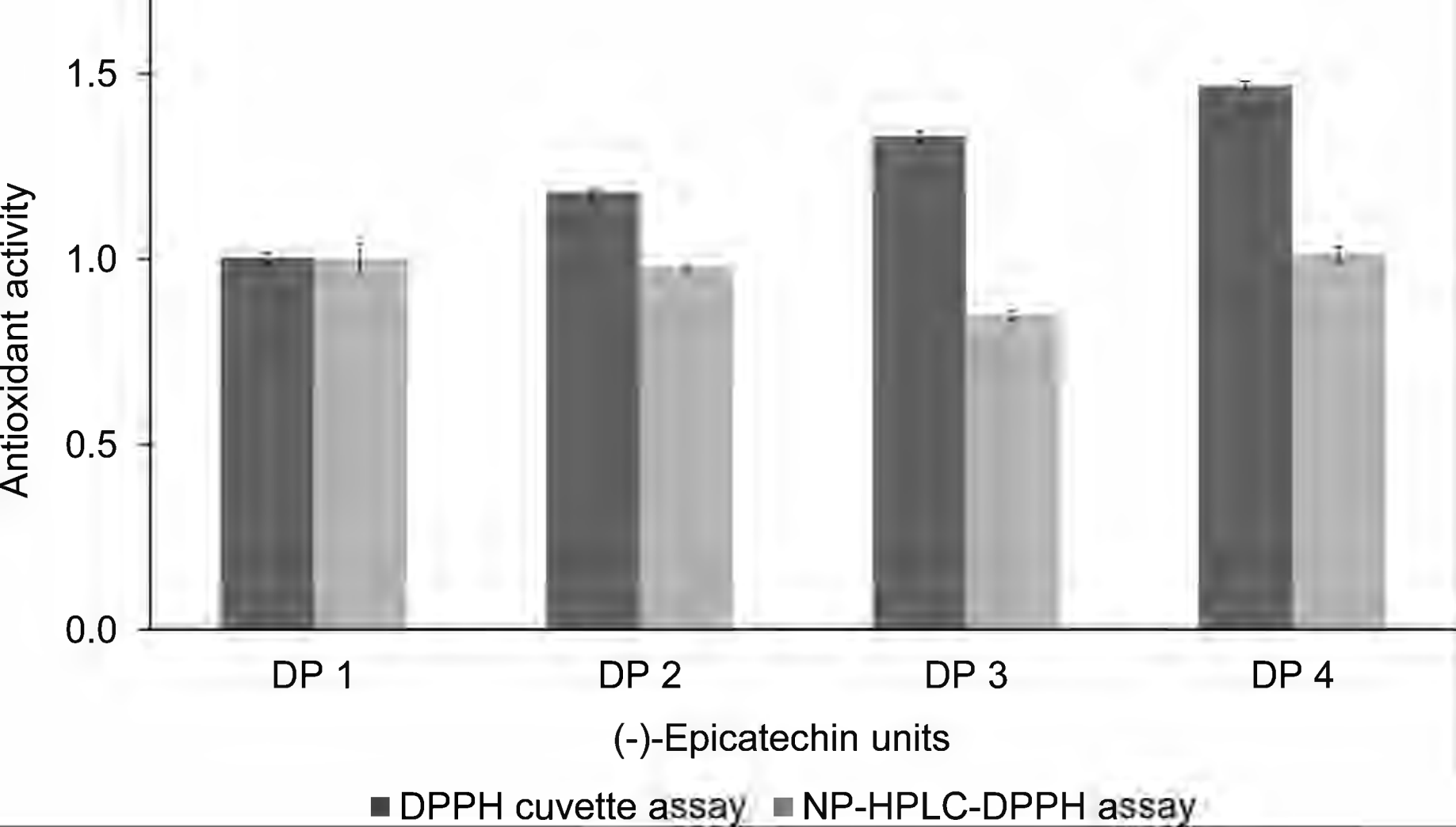
Figure 7a-f Extracted Ion Chromatograms (EIC) obtained from the analysis of the unroasted cocoa extract using RP-HPLC/MS.

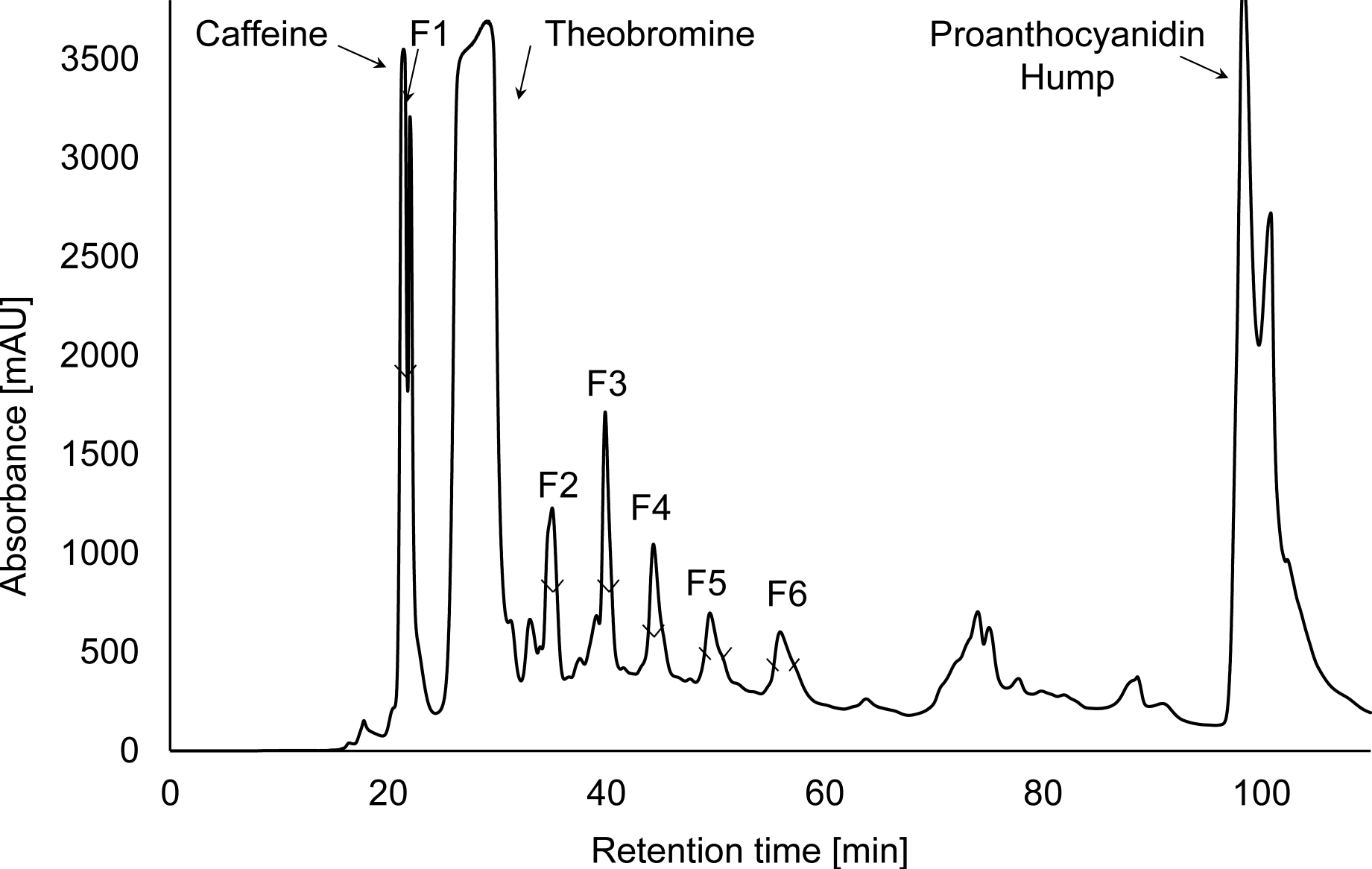

Sample
DPPH
Pump 1
0.3 mL/min
Pump 2
0.3 mL/min
Capillary
0.38 mm ID x 4.8 m
Diol-HILIC
Column
ACN
MeOH
Injector
Column Oven
30 °C
UV Detector
275 nm
Vis Detector
515 nm
Solvents
Reservoirs
Column Oven
60 °C
Waste


(-)-Epicatechin
Proanthocyanidin B2
Proanthocyanidin C1
Cinnamtannin A2
*
*
Absorbance [mAU]
Retention Time [min]
200
150
100
50
0
-50
-100
0
5
10
15
20
25
30


Acetone
Caffeine
Theobromine
1
2
3
4
5
6
7
8
9
10
11
*
Abundance [mAU]
90
70
50
30
10
-10
-30
-50
0
10
20
30
40
50
60
Retention Time [min]


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance 515 [mAU]
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Concentration [g/L]
(-)-Epicatechin
Proanthocyanidin B2
Proanthocyanidin C1
Cinnamtannin A2


1.5
1.0
0.5
0.0
Antioxidant activity
DP 1
DP 2
DP 3
DP 4
(-)-Epicatechin units
DPPH cuvette assay
NP-HPLC-DPPH assay


Caffeine
F1
Theobromine
Proanthocyanidin
Hump
F2
F3
F4
F5
F6
Absorbance [mAU]
3500
3000
2500
2000
1500
1000
500
0
0
20
40
60
80
100
Retention time [min]

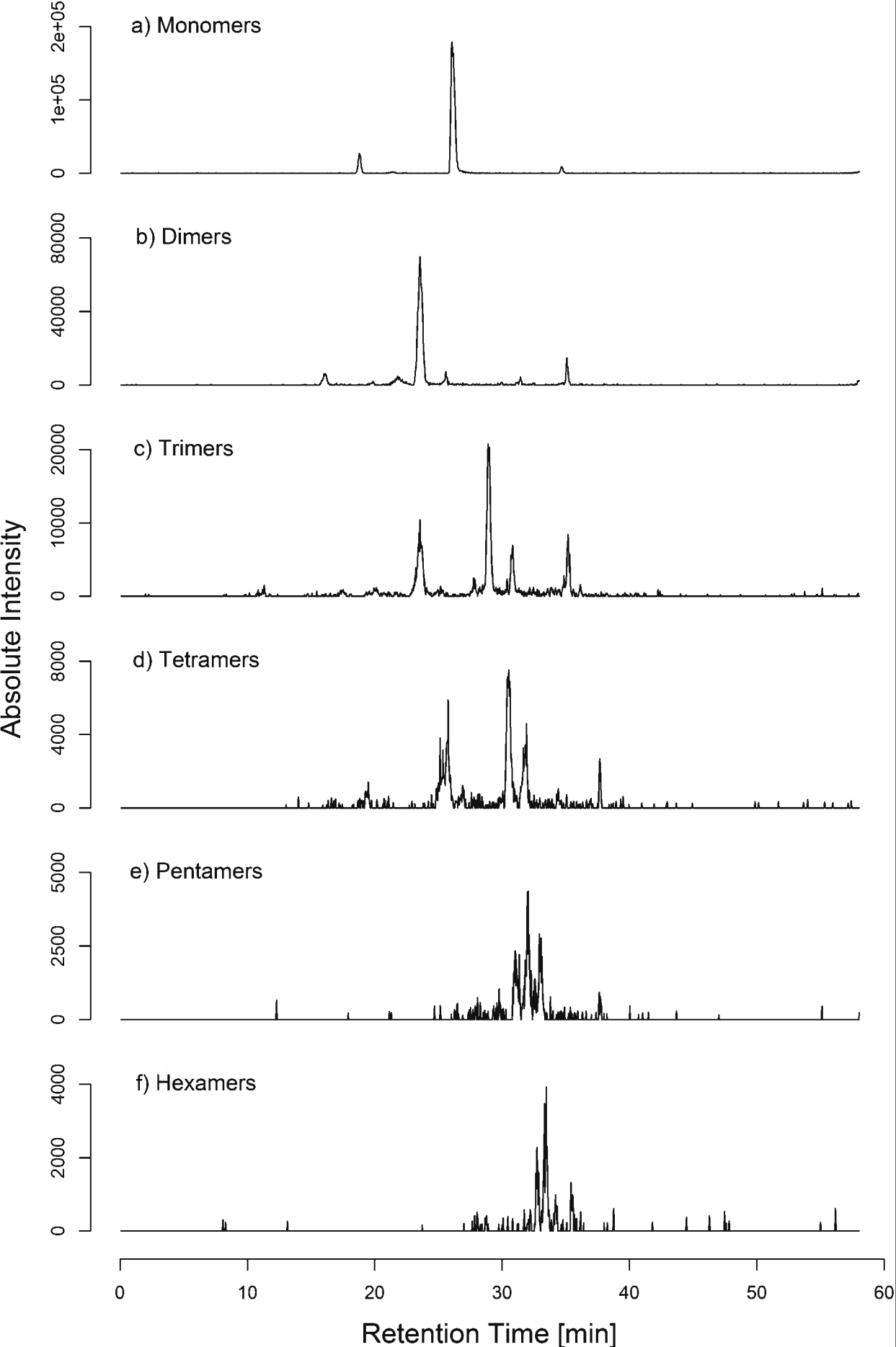

a) Monomers
b) Dimers
c) Trimers
d) Tetramers
e) Pentamers
f) Hexamers
Absolute Intensity
Retention Time [min]
0
1e+05
2e+05
40000
80000
10000
20000
4000
8000
2500
5000
2000
4000
10
20
30
40
50
60

Table 1. Quantification of oligomeric PA and their antioxidant activity in an unroasted liquid cocoa extract obtained by online NP-HPLC-DPPH. PA content and its antioxidant activity are expressed as epicatechin equivalents (using epicatechin as standard; ECE) or as proanthocyanidin equivalents (using cinnamtannin A2 as standard; PAE). Antioxidant capacity is defined in this study as the ratio of antioxidant activity per soluble PA content. Values represent value mean, with n = 3 (± standard deviation).

Peak No.	**Degree of Polymerisation**	**PA Content** [mg ECE/g]	**Antioxidant Activity** [mg ECE/g]	**Antioxidant Capacity** [for ECE]	**PA Content** [mg PAE/g]	**Antioxidant Activity** [mg PAE/g]	**Antioxidant Capacity** [for PAE]
1	DP 1	**5.02 (± 0.53)**	**3.43 (± 0.48)**	0.68 (± 0.04)	5.02 (± 0.53)	1.86 (± 0.48)	0.37 (± 0.06)
2	DP 2	2.50 (± 0.32)	2.64 (± 0.45)	1.05 (± 0.09)	3.58 (± 0.45)	1.41 (± 0.43)	0.39 (± 0.08)
3	DP 3	4.19 (± 0.54)	**3.68 (± 0.39)**	0.88 (± 0.05)	5.55 (± 0.69)	2.09 (± 0.49)	0.37 (± 0.05)
4	DP 4	3.97 (± 0.48)	**3.25 (± 0.31)**	0.81 (± 0.07)	6.81 (± 0.81) *	2.15 (± 0.49) *	0.31 (± 0.05) *
5	DP 5	4.11 (± 0.46)	**3.25 (± 0.32)**	0.79 (± 0.10)	**7.04 (± 0.77)** *	**2.16 (± 0.50)** *	0.30 (± 0.07) *
6	DP 6	3.03 (± 0.55)	2.72 (± 0.47)	0.90 (± 0.13)	5.26 (± 0.92) *	1.64 (± 0.45) *	0.31 (± 0.06) *
7	DP 7	2.00 (± 0.35)	2.24 (± 0.35)	1.13 (± 0.14)	3.54 (± 0.59) *	1.18 (± 0.34) *	0.33 (± 0.08) *
8	DP 8	1.39 (± 0.30)	1.79 (± 0.20)	1.31 (± 0.23)	2.53 (± 0.50) *	2.02 (± 0.19) *	0.29 (± 0.07) *
9	DP 9	0.55 (± 0.19)	1.61 (± 0.32)	**3.22 (± 0.21)**	1.13 (± 0.31) *	0.56 (± 0.40) *	0.56 (± 0.67) *
10	DP 10	0.56 (± 0.13)	1.27 (± 0.11)	**2.39 (± 0.21)**	1.15 (± 0.22) *	0.24 (± 0.10) *	0.23 (± 0.14) *
11	‘PA hump’	22.52 (± 3.80)	17.07 (± 2.02)	0.76 (± 0.06)	37.65 (± 6.31) *	15.56 (± 1.96) *	0.42 (± 0.03) *

* Values are expressed as cinnamtannin A2 equivalents

947 **Table 2.** LOD and LOQ of standard substances in the online NP-HPLC-DPPH assay
948 determined at a concentration range 50 µg/mL to 400 µg/mL.
949

Compound	UV-detection at 275 nm		Vis-detection at 515 nm	
	LOD [µg/mL]	**LOQ** [µg/mL]	**LOD** [µg/mL]	**LOQ** [µg/mL]
(-)-Epicatechin	7	22	27	83
Procyanidin B2	12	38	6	19
Procyanidin C1	17	54	11	33
Cinnamtannin A2	21	64	16	49

950

Table 3. RP-HPLC-ESI/MS determination of oligomeric proanthocyanidins in unroasted cocoa beans extract. Only single charged ions for F1-F7 could be detected in the positive ion mode.

Compound	**Molecular formula**	**t_R** [min]	**Peak**	**Molecular Ion** $[M+H]^+$	**Major Fragments** $[M+H]^+$
Monomer	$C_{15}H_{14}O_6$	4.3	F1	291.1	139.0; 165.1
Dimer	$C_{30}H_{26}O_{12}$	8.1	F2	579.2	291.1
Trimer	$C_{45}H_{38}O_{18}$	16.2	F3	867.2	579.2; 1155.5
Tetramer	$C_{60}H_{50}O_{24}$	18.5	F4	1155.3	n.d.
Pentamer	$C_{75}H_{62}O_{30}$	20.1	F5	1443.3	579.2; 867.0
Hexamer	$C_{90}H_{74}O_{36}$	21.8	F6	1731.4	579.9; 867.0; 1155.3

n.d. not detected