



Temperature influences epimerization and composition of flavanol monomers, dimers and trimers during cocoa bean roasting



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ABSTRACT

Cocoa consumption is suggested to promote many health benefits, since cocoa is a rich source of flavanols; but amounts and profiles of flavanols depend strongly on the bean type, origin and manufacturing process. Roasting is known as a crucial step in technical treatment of cocoa, which leads to flavanol losses and modifications, especially the epimerization of (–)-epicatechin to (–)-catechin. This study monitors the influence of cocoa bean roasting on the composition of flavanol monomers to trimers, with special focus on epimerization, which was quantified for procyanidin dimers, and also observed for trimers for the first time. Five dimeric and two trimeric potential epimerization products were detected and the extent of epimerization during cocoa roasting was shown to be a function of temperature. The data also showed remarkable variations in the change of flavanol content. The quantified flavanols decreased about 50% in Java beans and increased about 30% in Ivory Coast beans, despite being roasted under equal conditions.

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1. Introduction

Cocoa (*Theobroma cacao* L. Malvaceae) contains high concentrations of various phenolic compounds, with a total polyphenolic content between 6 to 8% by weight of the dry fermented bean (Crozier, Preston, Hurst, Payne, & Mann, 2011). Consumption of cocoa products is connected with numerous positive health benefits, often related to cardiovascular effects, due to the potential of lowering blood pressure (Buijsse, Weikert, Drogan, & Bergmann, 2010), improving endothelial function (Heiss, Keen, & Kelm, 2010), inhibiting platelet aggregation (Khawaja, Gaziano, & Djoussé, 2011) and reducing inflammatory response (Monagas et al., 2009). Polyphenolic composition of cocoa is mainly dominated by flavanols (catechins and procyanidins) and thus they are held to be responsible for these effects (Tomás-Barbéran, Borges, & Crozier, 2011). This evidence arises from data which shows a strong relationship between the intake of cocoa flavanols, their resulting plasma levels, and physiological effects (Ellinger, Reusch, Stehle, & Helfrich, 2012; Schroeter et al., 2006).

The amounts of flavanols and other phenolic substances ingested with chocolates or cocoa powders differ substantially

(Gu, House, Wu, Ou, & Prior, 2006). From the fresh harvested cocoa to its products, a number of processing stages are required to develop flavor or flavor precursors. Composition and amounts of polyphenols in cocoa products vary strongly with bean type, origin and especially with manufacturing processes. While unfermented cocoa presents high flavanol levels, fermenting, roasting and alkalizing led to a dramatic loss of flavanols (Wollgast & Anklam, 2000). Cocoa bean fermenting is the most critical step, which can reduce more than 90% of the initial flavanol concentrations (Elwers, Zambrano, Rohsius, & Lieberei, 2009; Kim & Kee-ney, 1984). Roasting of cocoa is essential for the formation of the typical chocolate aroma from the precursor compounds formed during fermentation (Arlorio et al., 2008). Time and temperature of the roasting process depends on several factors, such as cocoa material (beans, nibs or liquor roasting), final cocoa product (dark or milk chocolates) and type of cocoa (Criollo or Forastero). For cocoa beans the roasting conditions range from 15 to 45 min with temperatures from 130 to 150 °C (Krysiak, Adamski, & Żyżelewicz, 2013). Since flavanols are heat labile components, roasting results in further flavanol losses (Jolic, Redovnikovic, Markovic, Sipusic, & Delonga, 2011). Alkali treatment of cocoa powders, with the aim to reduce acidity and bitterness and to improve suspension properties, has shown to cause the lowest flavanol contents (related to nonfat cocoa solids) of all cocoa products (Gu et al., 2006).

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Structural modifications, like the epimerization of the flavanol monomers due to the roasting and alkali treatment, has been reported (Hurst et al., 2011). Unfermented cocoa seeds contain the flavan-3-ol monomer (–)-epicatechin and in much lesser concentrations (+)-catechin. The epimerization from (–)-epicatechin to (–)-catechin, and from (+)-catechin to (+)-epicatechin due to technological treatment, has often been postulated. Only a few publications confirmed this reaction by enantioseparation (Fig. 1) (Gotti, Furlanetto, Pinzauti, & Cavrini, 2006; Hurst et al., 2011; Kofink, Papagiannopoulos, & Galensa, 2007). The reaction mechanism is not fully clarified, but it is assumed that ring opening occurs on position C-2 of the oxygenated ring, and reclosing leads to the atypical enantiomers (Ellis, Yeap Foo, & Porter, 1983). High temperatures, particularly when combined with alkaline conditions, accelerate the epimerization reaction. The chiral investigation of chocolates and cocoa powders proved the presence of all four catechins (Cooper et al., 2007; Gotti et al., 2006; Kofink et al., 2007).

Human intervention studies suggest that bioavailability of the flavanol monomers in humans is influenced by stereochemical configuration (Ottaviani et al., 2011; Ritter, Zimmermann, & Galensa, 2010). In the case of catechin, the absorption of the (+)-isomer is favoured, compared to the (–)-isomer. These results were the same for catechin derived from pure reference compounds and from alkalinized cocoa powder (Ritter et al., 2010). Ottaviani et al. (2011) confirmed these findings for the catechin enantiomers and additionally found a ranking in the absorption for all four catechins in the following order: (–)-epicatechin > (+)-catechin = (+)-epicatechin > (–)-catechin.

As procyanidins are composed of catechin and epicatechin units, they also contain chiral centers where epimerization could occur. Procyanidin B2 and B5 are the main flavanol dimers in cocoa (Fig. 1). These molecules present five stereocenters, two of each monomeric unit (position C-2 and C-3) and one at the interflavan

bond (position C-4 of the upper unit). Changes in the stereochemical configuration are only postulated for the C-2 positions and no information exists about changes in configuration of the other stereocenters. Based on this, epimerization can take place at two positions in the procyanidin dimers, leading to three possible epimerization products per dimer (listed in Fig. 1). These epimerization products would be composed of one or two of the atypical (–)-catechin units.

Since not all the stereocenters are converted, epimerization products are diastereomers and analytical separation is possible by reversed phase chromatography, in the same way as for the procyanidins B1, B2, B5, and other epimeric dimers (Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003).

Procyanidin dimers are absorbed into the blood stream (Holt et al., 2002; Ritter et al., 2010; Taubert, Roesen, Lehmann, Jung, & Schömig, 2007). As postulated for the monomers, the absorption of the dimers could be influenced by stereochemical configuration.

Thus, this study was carried out to investigate if epimerization of procyanidins occurs during cocoa bean roasting. Three cocoa bean samples (two batches from Java and one from the Ivory Coast) were roasted using the same time and temperature conditions to compare changes in content and composition. One of these cocoa beans was also roasted at different temperatures to observe flavanol alterations as a function of the temperature. Data from this study may suggest how to conduct the roasting process to preserve the natural profile of the flavanols as much as possible.

The procyanidin dimers B1, B2, B5 and further unknown dimers were investigated by mass spectrometry and quantified by UHPLC–UV. Mass traces of procyanidin B-type dimers and trimers, before and after roasting, were evaluated to investigate the epimerization reaction. The monomers were chirally separated by cyclodextrin-added capillary electrophoresis to reveal the epimerization of the catechins.

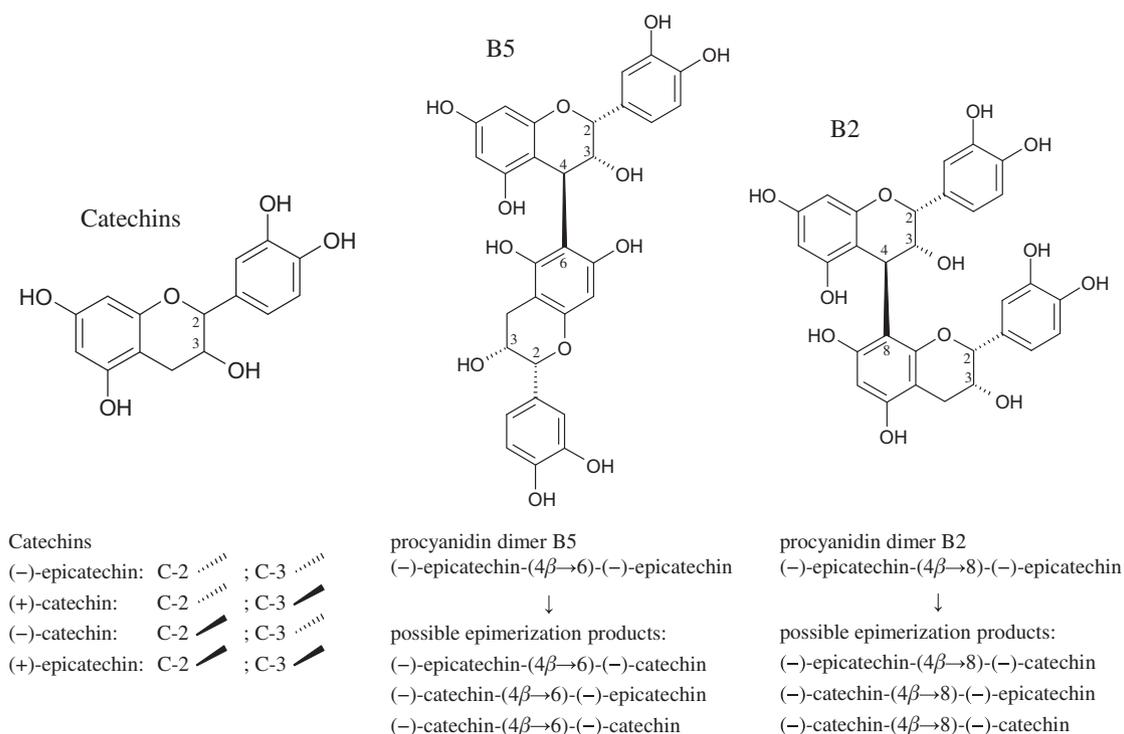


Fig. 1. Stereochemical configurations of the catechins and the main cocoa procyanidin dimers B2 and B5 with possible epimerization products listed beneath.

2. Materials and methods

2.1. Cocoa samples and roasting performance

Roasting experiments were performed using three different samples of fermented cocoa beans purchased from Quast & Consorten (Hamburg, Germany). Two of the bean samples came from Java (Java1 and Java2) and one was from the Ivory Coast. No further details except the origin were known for the cocoa beans. The beans were roasted in pilot plant scale using 5 kg of the cocoa beans with a demonstration batch roaster from F.B. Lehmann GmbH (Aalen, Germany). The three cocoa bean samples were roasted at 150 °C for 30 min and Java1 beans were roasted with five different temperatures (100, 120, 140, 150, and 160 °C) for 30 min. All roastings were performed in duplicate.

2.2. Reagents and standards

Purified water was prepared with a Millipore Direct-Q 3 system from Merck (Darmstadt, Germany). All solvents and additives used as eluents for UHPLC analysis were of LC-MS grade, while other chemicals were of analytical or LC grade. Acetonitrile and acetic acid were obtained from Fisher Scientific (Loughborough, UK). Dimethyl sulfoxide, formic acid and *n*-hexane were from Merck (Darmstadt, Germany), and water for UHPLC was from VWR International (Leuven, Belgium). Boric acid and sodium tetraborate for buffer solutions were from Merck (Darmstadt, Germany) and KMF Laborchemie (St. Augustin, Germany). Acetone and hydroxypropyl- γ -cyclodextrin were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium hydroxide and hydrochloric acid for adjusting the pH-value of the buffer solution were from Bernd Kraft GmbH (Duisburg, Germany) and Th. Geyer GmbH & Co. KG (Renningen, Germany).

(\pm)-Catechin hydrate (>98%), (+)-catechin hydrate (>98%) and (–)-epicatechin (>97%) were purchased from Sigma-Aldrich Chemie GmbH and procyanidins B1 (>80%) and B2 (>90%) from Extrasynthese (Genay, France).

2.3. Preparation of extracts

Cocoa beans were first pre-crushed in a knife mill (GM 200, Retsch, Haan, Germany) together with dry ice to prevent heat development. To obtain a fine and homogeneous powder, an additional grinding step was performed with a ball mill (MM 2000, Retsch, Haan, Germany) after cooling with liquid nitrogen.

The powdered cocoa was defatted and phenolic substances were extracted using pressurized liquid extraction (ASE 200, Dionex, Idstein, Germany), both at room temperature and with 199 MPa. Afterwards, 1 g of cocoa powder was mixed with 4 g of diatomaceous earth (Hydromatrix HM-N, Biotage, Uppsala, Sweden) and added to 11 ml extraction cells assembled with cellulose filters at the outlets. Defatting was carried out twice with *n*-hexane, for 5 min, and 100% flush volume. Analytes were then extracted with acetone/water (50/50, v/v), for 20 min and 50% flush volume. The extract was diluted with distilled water to 60 ml to decrease the organic content below 20%, in order to prevent analyte loss during solid-phase extraction.

Cocoa extract was then purified on a polyamide column (Chromabond PA cartridge, 1 g/6 ml, Macherey–Nagel, Düren, Germany) using an automated solid-phase extraction system (ASPEC XLi system, Abimed, Langenfeld, Germany). The extraction cartridges were conditioned with 6 ml dimethyl sulfoxide/formic acid (99.9/0.1, v/v) and washed with 10 ml of distilled water. Cocoa extract was loaded to the cartridge in three steps of 20 ml and each step was followed by washing with 10 ml of distilled water.

Another washing step with 1.5 ml of the conditioning solvent was done before the compounds were eluted with 4 ml dimethyl sulfoxide/formic acid (99.9/0.1, v/v). Sample extracts were stored at –80 °C until measurement.

2.4. Capillary electrophoresis

For chiral analysis Beckman P/ACE MDQ capillary electrophoretic system (Beckman Instruments, Fullerton, CA, USA) equipped with a photo diode array detector was used. The separation was obtained on a fused silica capillary with a total length of 70 cm and an inner diameter of 75 μ m (Beckman Instruments). Borate buffer (0.2 mol/l) was used as the background electrolyte adjusted to pH 8.5. For chiral separation, 0.015 mol/l hydroxypropyl- γ -cyclodextrin was solubilized in the borate buffer system. Prior to analysis buffer was filtered through a 0.45 μ m cellulose syringe filter (Chromabond, Macherey–Nagel, Düren, Germany). The capillary temperature was set to 18 °C and the applied voltage was 18 kV. Samples were injected hydrodynamically with 0.3 psi for 3 s. Capillary regeneration was done before each run with 0.25 mol/l sodium hydroxide solution for 3 min, with purified water for 2 min, and equilibrated with the running buffer for 5 min. The system was controlled by Beckman 32 Karat software version 7.0 and electropherograms were recorded at 280 nm.

2.5. UHPLC–UV–MS/MS

Analysis was carried out using an Acquity UPLC system (Waters, Milford, MA, USA) consisting of a binary pump (BSM), an autosampler (SM), a column oven (CM), a diode array detector (PDA), and a triple-quadrupole mass spectrometer (TQD) with electrospray interface, operating in negative mode. The separation column was an Acquity BEH Shield RP18 (150 mm \times 2.1 mm, 1.7 μ m, Waters) tempered at 40 °C. Solvents were water (mobile phase A) and acetonitrile (mobile phase B), both acidified with 0.1% formic acid (v/v), operating at a flow rate of 0.3 ml/min. The binary gradient was as follows: 0–28 min, 98–72% A; 28–29 min, 72–0% A; 29–31 min, 0% A; 31–33 min, 0–98% A; 33–35 min, 98% A. Injection volume was 2 μ l and sample extracts were maintained at 20 °C. The whole system was controlled by MassLynx 4.1 software (Waters). The peak identity of *m/z* 577 for the quantified procyanidin B-type dimers was confirmed by recording their MS/MS spectra in product ion scan mode (collision gas (argon) flow 0.3 ml/min; collision energy 20 and 40 V). The mass data for comparing the relative composition of procyanidin B-type dimers and trimers were recorded using selected reaction monitoring (SRM) with the transition ions at *m/z* 577 \rightarrow 289, 407, 425, 451 and *m/z* 865 \rightarrow 287, 289, 575, 577, 695, 739. Therefore, the following parameters were used: Capillary voltage of –1.5 kV, cone voltage of 50.0 V, extractor voltage of 2.0 V, RF voltage of 0.2 V, source temperature of 150 °C, desolvation temperature of 450 °C, cone gas (nitrogen) flow of 50 l/h, and desolvation gas (nitrogen) flow of 800 l/h.

2.6. Quantification and statistics

All concentrations are given as mean values and standard deviation in the fat free cocoa dry mass (ffdm) of two replicates. Fermented cocoa beans (unroasted) were extracted in duplicate, and each value of the roastings represents two independent roasted charges of cocoa beans, each extracted once. Quantification after UHPLC and CE separation was done using UV at 280 nm and external standard curves. The monomers were quantified as their corresponding reference compounds and the procyanidin dimers were quantified as epicatechin equivalents. Further investigation of the epimerization of procyanidin dimers and trimers was assessed by comparing peak area mean values ($n = 2$) of SRM chromatograms

with the transition ions at m/z 577 \rightarrow 289 for dimers and m/z 865 \rightarrow 577 for the trimers, before and after applied roasting.

3. Results and discussion

3.1. Exclusion of epimerization during sample preparation

Samples were prepared by avoiding any heat and day light exposure to ensure that no undesired reactions, in particular epimerization, of the analytes occurred. Therefore, samples were ground under cooling, brown glassware was used, and acid was added to the eluting solvent of the solid-phase extraction. Kofink et al. (2007) examined the influence of UV-light and oxygen on sample preparation under extreme conditions (exposition of UV-light and aeration for 24 h) and no epimerization of the flavanol monomers was detectable. For that reason, under the conditions applied here, we can exclude epimerization during sample preparation.

3.2. Identification of the substances

In all cocoa bean samples epicatechin, catechin, and five procyanidin dimers were quantified. These were the B-type procyanidins B1, B2 and B5, which are well known to occur in cocoa (Porter, Ma, & Chan, 1991; Tomás-Barberán et al., 2007), and two further B-type procyanidin dimers (procyanidin dimer1 and dimer2). Identification was achieved by comparison with reference compounds in the case of the monomers and the procyanidins B1 and B2. The second dominant dimer was supposed to be procyanidin B5 (Fig. 1) (Cooper et al., 2007) and further identified by its retention behavior on reversed phase column, because it elutes far later than the other procyanidin dimers due to the $4\beta \rightarrow 6$ interflavan bond (Santos-Buelga et al., 2003). All of the quantified procyanidin dimers had a $[M-H]^-$ at m/z 577 and produced the characteristic MS/MS fragmentation patterns which have been described as quinone methide (QM: m/z 289), retro-Diels–Alder (RDA: m/z 425, 407) and heterocyclic ring fission cleavages (HRF: m/z 451) (Friedrich, Eberhardt, & Galensa, 2000).

Further procyanidin dimers and trimers were assessed with MS detection by monitoring their mass traces in the SRM mode. The identity of the B-type dimers and trimers were confirmed by the characteristic fragments of the QM, RDA and the HRF cleavages of $[M-H]^-$ at m/z 577 (\rightarrow 289, 407, 425, 451) and 865 (\rightarrow 287, 289, 575, 577, 695, 739).

3.3. Flavanol contents in fermented cocoa beans

The content of the monomers and the five quantified dimers in the three samples of the fermented cocoa beans before roasting (Java1, Java2, Ivory Coast) varied, as observed in the literature. This is as flavanol concentrations (and polyphenol concentration in general), depend strongly on bean type, origin and fermentation process (Cooper et al., 2007). Java1 cocoa beans had the highest concentrations, followed by Java2. The beans from the Ivory Coast had the lowest contents of all quantified substances (Table 1). The epicatechin contents of the cocoa beans from this study ranged from 0.97 to 4.82 mg/g. The contents of catechin were 0.07–0.26 mg/g, and the concentrations of the dimers were 0.43–2.03 mg/g for procyanidin B2, 0.12–0.57 mg/g for procyanidin B5, and 0.018–0.027 mg/g for procyanidin B1. These data fall within the range of data reported in the literature (Jolic et al., 2011; Payne, Hurst, Miller, Rank, & Stuart, 2010; Wollgast & Anklam, 2000). The concentrations of procyanidin dimer1 and dimer2 were in a similar range to procyanidin B1, with 0.012–0.024 mg/g for dimer1 and 0.012–0.026 mg/g for dimer2.

Chiral separation by capillary electrophoresis showed that for epicatechin the (–)-isomer was only found. In the case of catechin the (–)-isomer was predominant (Table 1). Hurst et al. (2011) separated epicatechin and catechin enantiomers from cocoa beans with different degrees of fermentation. No (+)-epicatechin was found and the contents of (–)-catechin were higher than those of (+)-catechin in the case of heavily fermented cocoa (Hurst et al., 2011). Regarding these data, the three cocoa beans from this study were assumed to be heavily fermented.

3.4. Epimerization of flavanol monomers and dimers as a function of temperature

The Java1 cocoa bean roasting trial involved five roastings with different temperatures between 100 and 160 °C for 30 min. For all quantified substances, progressive changes caused by the different temperatures were observed (Fig. 2).

The increases and decreases of the monomers due to the epimerization reaction are illustrated in Fig. 2A for epicatechin and Fig. 2B and C for catechin. While the epicatechin content decreased substantially, the content of catechin behaved in the opposite way. Unroasted beans had 4.82 mg/g epicatechin and 0.26 mg/g catechin. The strongest roasting (160 °C/30 min) led to a loss of 68% of the initial epicatechin content (Java1, 160 °C: 1.56 mg/g, 32%), and the catechin content rose to 240% (Java1, 160 °C: 0.62 mg/g) (Table 1).

The chiral separation by capillary electrophoresis (Fig. 2A–2C, unfilled quadrates) confirmed the epimerization reaction: the (–)-epicatechin content decreased while (–)-catechin content increased, and (+)-catechin content fell in the same way as the epicatechin content. Recently, similar observations of different roastings were published for cocoa beans from the Ivory Coast. Although the data of contents, losses, and increments differed in their amounts to our findings, the trend caused by the postulated epimerization reaction was the same (Hurst et al., 2011).

The same converse reaction with temperature that was observed for the monomers was also observed for the procyanidin dimers according to the UHPLC–UV analysis (Table 1). The three procyanidin dimers B1, B2 and B5 showed progressive losses with higher roast levels (Fig. 2D and E). In contrast, the concentrations of dimer1 and dimer2 increased steadily (Fig. 2F) with higher temperatures. For procyanidin B1, B2 and B5 only 58%, 32% and 29% of the initial concentrations remained when roasted at 160 °C for 30 min, and the contents of procyanidin dimer1 and dimer2 rose to 541% and 211% in the same roasting. These two procyanidin dimers behaved in a comparable way as catechin or rather (–)-catechin did, and in the opposite way to the procyanidin dimers B1, B2, and B5. Therefore, it was assumed that these dimers were products of the epimerization reaction.

In this study, we had no detailed information on the structures of the procyanidin dimers 1 and 2, except the typical mass spectrometric fragmentation patterns for B-type dimers. Thus, nothing can be said about the stereochemical structure they might have or have had before epimerization. However, these dimers are probably not condensation products of oxidation reactions. Compared to B-type dimers, the MS/MS fragment spectra of oxidation products with m/z 577 has been shown to differ, because fragmentation would lead to other prominent fragments (Guyot, Vercauteren, & Cheynier, 1996; Sun & Miller, 2003). To prove the suggestion of epimerization, further research is needed to characterize these components stereochemically. Fig. 1 lists the potential epimerization products of the main cocoa procyanidins B2 and B5. Nine epimerization products are theoretically possible (three for each of B1, B2, and B5). Quantification by UV absorption was possible only for the two dimers 1 and 2. In Section 3.6. further dimeric epimerization products are investigated by mass spectrometric measurement,

Table 1
Concentrations of flavanol monomers and dimers in unroasted and roasted cocoa beans.^a

Temp. (°C) ^b	(-)-Epicatechin (mg/g)	(+)-Catechin (mg/g)	(-)-Catechin (mg/g)	Epicatechin (mg/g)	Catechin (mg/g)	Procyanidin B1 (mg/g)	Procyanidin B2 (mg/g)	Procyanidin B5 (mg/g)	Procyanidin dimer1 (mg/g)	Procyanidin dimer2 (mg/g)	ΣFlavanols (mg/g) (%) ^c
<i>Java1 cocoa beans</i>											
	4.77 ± 0.08	0.09 ± 0.02	0.18 ± 0.03	4.82 ± 0.16	0.26 ± 0.01	0.027 ± 0.001	2.03 ± 0.02	0.57 ± 0.02	0.018 ± 0.001	0.023 ± 0.003	7.75 (100)
100	4.25 ± 0.01	0.06 ± 0.001	0.20 ± 0.01	3.94 ± 0.57	0.24 ± 0.01	0.022 ± 0.002	1.70 ± 0.24	0.45 ± 0.03	0.018 ± 0.001	0.023 ± 0.001	6.40 (83)
120	3.35 ± 0.2	0.05 ± 0.004	0.23 ± 0.04	2.85 ± 0.16	0.24 ± 0.01	0.019 ± 0.001	1.33 ± 0.04	0.29 ± 0.01	0.021 ± 0.001	0.019 ± 0.001	4.77 (62)
140	2.08 ± 0.3	0.03 ± 0.001	0.40 ± 0.07	1.77 ± 0.30	0.33 ± 0.08	0.018 ± 0.002	0.85 ± 0.25	0.19 ± 0.04	0.051 ± 0.018	0.029 ± 0.005	3.24 (42)
150	2.08 ± 0.2	0.03 ± 0.001	0.58 ± 0.01	2.05 ± 0.15	0.48 ± 0.09	0.018 ± 0.001	0.98 ± 0.07	0.24 ± 0.01	0.081 ± 0.016	0.041 ± 0.008	3.88 (50)
160	1.68 ± 0.08	0.03 ± 0.001	0.79 ± 0.04	1.56 ± 0.11	0.62 ± 0.03	0.016 ± 0.001	0.64 ± 0.05	0.16 ± 0.01	0.099 ± 0.011	0.048 ± 0.004	3.16 (41)
<i>Java2 cocoa beans</i>											
	2.88 ± 0.2	0.05 ± 0.003	0.25 ± 0.015	2.37 ± 0.03	0.21 ± 0.01	0.020 ± 0.001	1.1 ± 0.01	0.3 ± 0.07	0.024 ± 0.001	0.026 ± 0.001	4.06 (100)
150	1.39 ± 0.1	0.03 ± 0.003	0.53 ± 0.114	1.23 ± 0.07	0.37 ± 0.02	0.015 ± 0.001	0.5 ± 0.1	0.1 ± 0.01	0.066 ± 0.002	0.033 ± 0.002	2.33 (57)
<i>Ivory Coast cocoa beans</i>											
	1.16 ± 0.03	0.02 ± 0.003	0.02 ± 0.003	0.97 ± 0.01	0.07 ± 0.01	0.018 ± 0.001	0.43 ± 0.01	0.12 ± 0.01	0.012 ± 0.001	0.012 ± 0.001	1.62 (100)
150	1.02 ± 0.02	0.04 ± 0.003	0.66 ± 0.070	0.91 ± 0.01	0.55 ± 0.06	0.019 ± 0.003	0.43 ± 0.02	0.10 ± 0.01	0.081 ± 0.014	0.033 ± 0.003	2.12 (131)

^a Concentrations are expressed in mg/g fat free cocoa dry mass and as mean ± standard deviation (n = 2). Quantification was done by UV absorption of external standard curves, in the case of the monomers with their corresponding reference compounds and as epicatechin equivalents for the dimers. (-)-Epicatechin, (+)-Catechin and (-)-Catechin, were quantified by chiral capillary electrophoresis and all other components were quantified by UHPLC-DAD.

^b Duration of all roastings was 30 min.

^c ΣFlavanols, corresponds to the sum of flavanols quantified by UHPLC. Values in brackets are the percentage amount of the Σflavanols referring to the unroasted beans, which is set to 100%.

to clarify if the reaction mechanism is limited to specific epimerized molecules.

Furthermore, the courses of the flavanol contents displayed disparities, in decreases and increases, dependent on the applied temperature (Fig. 2). The losses of epicatechin and the procyanidins B1, B2 and B5 occurred mainly at temperatures from 100 to 140 °C. At higher roasting temperatures, the decrease almost stagnated. However, at temperatures above 140 °C no further losses were recorded, but the increases of dimer 1 and 2 were accelerated. Hurst et al. (2011) roasted cocoa beans at 163 °C with varying lengths of time and made similar observations for the enantioseparated monomers. Roasting time of 25 min showed no further losses of (-)-epicatechin and (+)-catechin compared to roasting of 20 min, while (-)-catechin increased steadily due to the epimerization reaction (Hurst et al., 2011).

According to results from our trial, roasting temperature has to be held below 140 °C to get cocoa products with almost natural flavanol compositions, i.e. with limited epimerization products.

3.5. Flavanol composition of three roasted cocoa bean samples

Beside the Java1 roasting trial, two further batches of cocoa beans were roasted (150 °C/30 min) to compare epimerization reaction and alterations in the flavanol composition of different cocoa beans after equal roastings. One of these bean samples was from Java (Java2) and the other from the Ivory Coast.

As described in Section 3.3, the fermented cocoa bean samples had different initial flavanol contents. Moreover, the changes of the flavanol contents and the epimerization reaction due to roasting varied among the three cocoa bean samples (Table 1). The biggest losses after roasting were found for the Java1 cocoa beans, the sum of the quantified flavanols dropped about 50%, from 7.75 to 3.88 mg/g. The Java2 cocoa beans behaved similar, only 57% of the initial content remained after roasting (4.06 mg/g before and 2.33 mg/g after roasting). The Ivory Coast beans behaved very differently when compared to the Java beans. After roasting the quantified flavanols in total were 2.12 mg/g, this represents 131% of the initial amount (1.62 mg/g). Epicatechin and the procyanidins B1, B2 and B5 showed no or only minimal losses, whereas catechin and the dimers 1 and 2 were responsible for the increase in flavanol content of the Ivory Coast roasting trial (Table 1). The rising contents may result from cleavage products of higher polymerized procyanidins caused by roasting.

Before roasting, the three cocoa bean samples had quite similar flavanol compositions (Fig. 3), although their absolute concentrations differed (Table 1). The profiles of the roasted beans showed different portions of the epimerization products. After roasting the catechin contents rose to 183% and 174% for the cocoa beans Java1 and Java2. In contrast, the catechin content in the Ivory Coast beans increased up to 836% (0.55 mg/g) compared to the unroasted beans (0.07 mg/g). The Ivory Coast beans also had the highest increase in contents of dimer 1 and 2 (670% and 271%) (Table 1). Another study investigated unroasted and roasted cocoa beans from the Ivory Coast and from Papua New Guinea, and the catechin contents increased up to 650% and 640% due to roasting, whereas epicatechin contents dropped to 30% and 59% (Payne et al. 2010). Thus, the extent of the epimerization reaction and the flavanol reduction can vary strongly between cocoa beans, even when using equal roasting conditions. To identify criterions of cocoa beans which may cause these differences, further investigation is needed with higher sample amounts of fully characterized (e.g. origin, variety, grade of fermentation, chemical composition, pH-value, water content) cocoa beans.

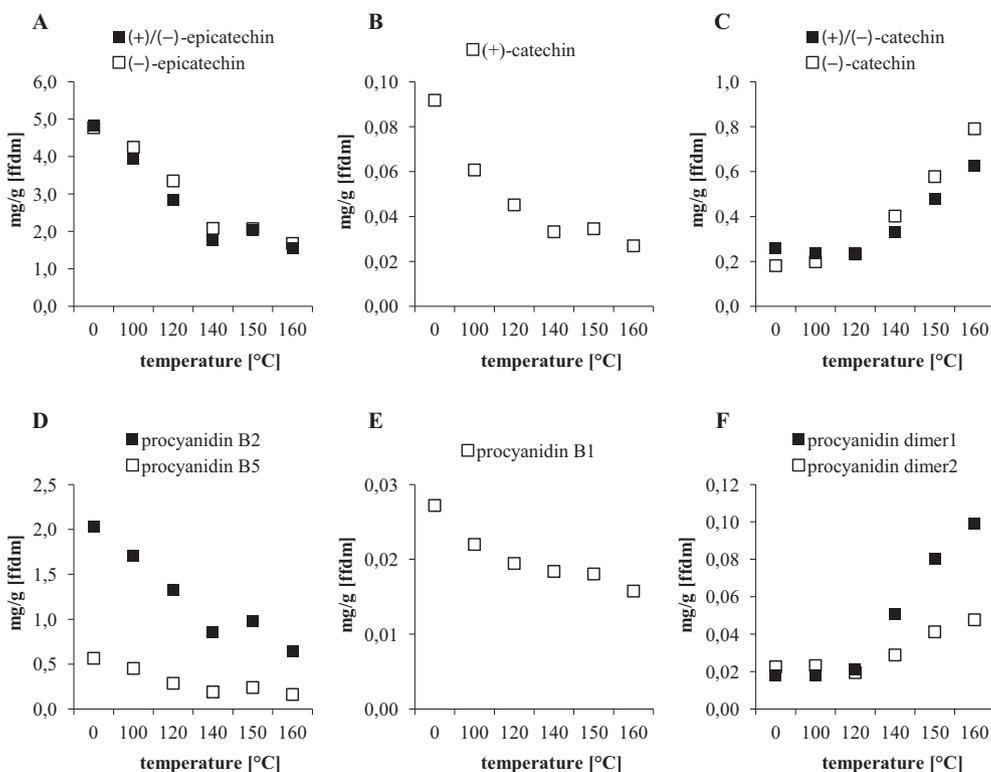


Fig. 2. Contents of flavanol monomers (A–C) and dimers (D–F) in unroasted and roasted cocoa bean samples of Java 1.

3.6. Epimerization of flavanol dimers and trimers examined by MS detection

Two dimeric epimerization products were quantifiable by UV detection in the cocoa bean samples (Sections 3.4 and 3.5), but further dimeric epimerization products are possible. Moreover, if epimerization actually takes place in flavanol monomers and dimers, it is likely that procyanidins in general undergo epimerization. But in the case of lower concentrated dimers and trimers, the quantification by UV absorption is complicated, because of many interfering procyanidins and the comparable low concentrations of the epimerization products (see dimer1 and dimer2 in Table 1). Procyanidin trimers are composed of three units where epimerization can occur. If epimerization is not limited to specific epimerized molecules, contents are divided into seven possible epimerization products from one trimer. Therefore, the more isomers that are possible, the lower the concentrations of the epimerization products will be.

The examination was carried out by comparing mass traces of dimers and trimers recorded in SRM mode. Cocoa beans from the Ivory Coast presented clear evidence that epimerization leads to further dimers and trimers (Fig. 4). This fits with the results of the procyanidin dimers quantified in Sections 3.4. and 3.5., because the Ivory Coast beans had the highest extent of the dimeric epimerization reaction (Table 1). Hence, the cocoa beans from the Ivory Coast, before and after roasting, were investigated to observe epimerization reaction of further dimers and of trimers (Table 2).

In total, nine dimeric peak areas were evaluated (Table 2, Fig. 4). The selected ion transition of m/z 577 → 289 was chosen, as it revealed the highest intensity. The transition m/z 577 → 289 could also result from in-source fragmentation of trimeric procyanidin molecules. Therefore, peaks in the m/z 577 → 289 trace with the same retention time as peaks in the m/z 865 → 577 trace were considered as artefacts and excluded from our data evaluation.

Beside the procyanidin dimers 1 and 2 (peaks 2 and 7), one dimer shows a clear rise (peak 4) after roasting and two further dimers were detectable only after roasting (peaks 3 and 8). The mass traces of the procyanidin trimers (m/z 865 → 577) also revealed different trends of decreases and increases as a consequence of the roasting. Peak areas of five procyanidin trimers were examined. These were the main cocoa procyanidin trimer C1 ((-)-epicatechin-(4 β → 8)-(-)-epicatechin-(4 β → 8)-(-)-epicatechin) and four additional B-type trimers with unknown stereochemical

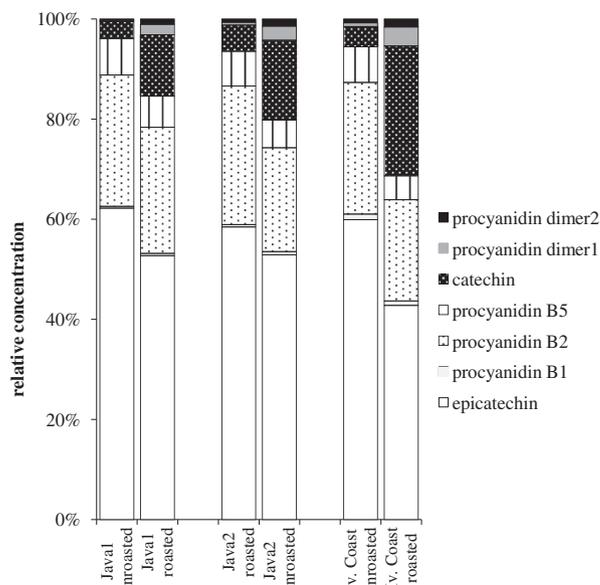


Fig. 3. Relative concentrations of the flavanol monomers and dimers in three unroasted and roasted cocoa bean samples.

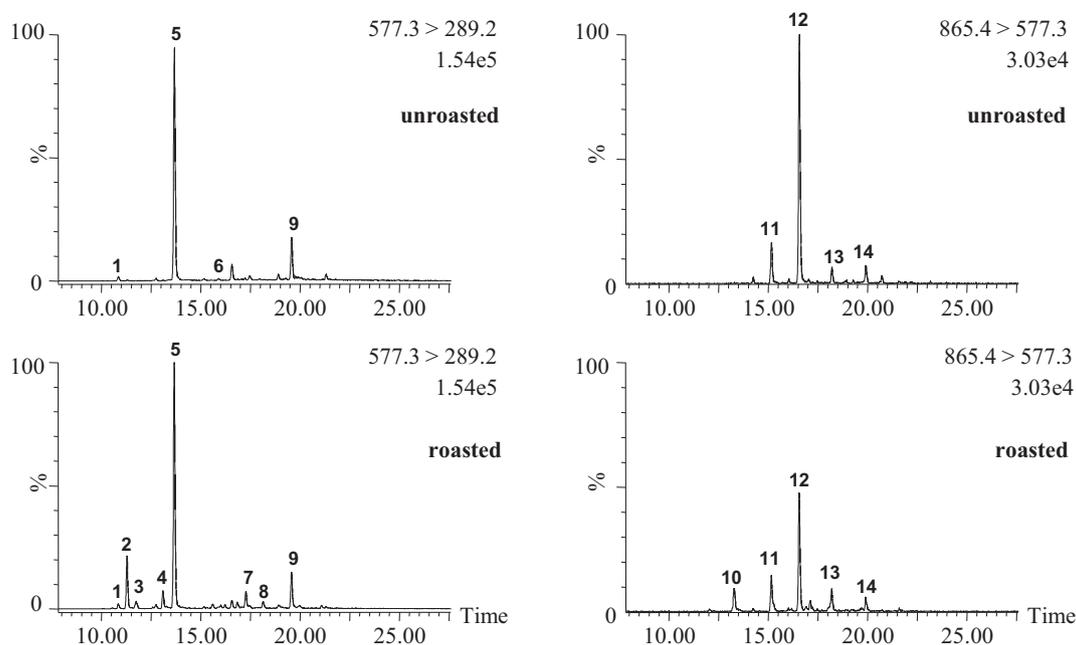


Fig. 4. SRM chromatograms at m/z 577 \rightarrow 289 for dimers and at m/z 865 \rightarrow 577 for trimers, of unroasted and roasted Ivory Coast cocoa bean extracts. Peak numbers refer to Table 2.

Table 2

Peak areas \pm standard deviation^b and percentage changes of procyanidin B-type dimers and trimers before and after roasting of Ivory Coast cocoa beans.

Peak ^a	RT (min)	Compound	Unroasted cocoa beans	Roasted cocoa beans (150 °C/min)	% Change ^c
<i>Procyanidin B-type dimers</i>					
1	10.84	procyanidin B1	265 \pm 6	315 \pm 23	119
2	11.28	procyanidin dimer 1	50 \pm 9	2536 \pm 489	5122
3	11.75	B-type dimer	n.d.	434 \pm 120	
4	13.09	B-type dimer	38 \pm 8	845 \pm 136	2224
5	13.67	procyanidin B2	14847 \pm 748	15531 \pm 628	105
6	15.89	B-type dimer	162 \pm 10	257 \pm 49	159
7	17.25	procyanidin dimer2	112 \pm 1	821 \pm 103	736
8	17.98	B-type dimer	n.d.	328 \pm 100	
9	19.59	procyanidin B5	2733 \pm 188	2180 \pm 12	80
<i>Procyanidin B-Type trimers</i>					
10	13.27	B-type trimer	n.d.	230 \pm 39	
11	15.19	B-type trimer	494 \pm 1	469 \pm 19	95
12	16.52	procyanidin C1	3110 \pm 119	1526 \pm 108	50
13	18.20	B-type trimer	199 \pm 35	341 \pm 35	191
14	19.92	B-type trimer	256 \pm 33	151 \pm 6	65

^a Peak numbers refer to Fig. 4.

^b The values are given as mean \pm standard deviation ($n = 2$) of peak areas obtained by MS measuring in SRM mode of dimers (m/z 577 \rightarrow 289) and of trimers (m/z 865 \rightarrow 577).

^c Values represent the percentage amount in the roasted beans, referring to the unroasted beans, which is set 100%. n.d.: not detectable.

structure. One trimer shows a nearly two fold higher peak area in the roasted cocoa beans compared to the unroasted (peak 13), and one trimer was detectable only in the roasted beans (peak 10).

Five dimeric and two trimeric epimerization products were detected; therefore, we claim that the epimerization reaction of the procyanidin dimers is not limited to specific molecules and that epimerization takes place in trimeric procyanidins as well. Furthermore, it can be assumed that epimerization might occur for procyanidins in general and as a consequence, roasting process augments the complexity of flavanols ingested with cocoa products.

4. Conclusion

Roasting trials with different temperatures (Java1) show that epimerization of procyanidin dimers (beside the already known epimerization reaction of the monomers) occurs. The contents of two tentatively identified B-type dimers increased in the same manner as catechin as a function of temperature. The hotter the roasting the more the epimerization accelerates and the more the flavanol profile is influenced. According to our results, temperature should be kept below 140 °C in order to preserve most of the initial flavanol concentration and to obtain almost unaffected flavanol composition.

Three different batches of roasted cocoa beans were investigated and increases of the two dimers were quantified for each bean sample. Furthermore, the extent of epimerization and flavanol losses varied strongly between the two cocoa bean samples from Java and the bean sample from the Ivory Coast, despite being roasted under equal conditions. Before roasting the three samples had different flavanol concentrations, but similar compositions. After roasting the beans presented remarkable differences in the flavanol profile, due to the extent of epimerization. Thus, we claim that the bean source may have an influence on the resulting flavanol composition, but to identify reasons for this further investigations with higher sample amounts of fully characterized cocoa beans are needed.

The comparison of mass traces of procyanidin dimers and trimers of unroasted and roasted cocoa beans from the Ivory Coast demonstrated in total, five dimeric and two trimeric potential epimerization products. These increases evidence that roasting causes epimerization in flavanol monomers, dimers and trimers and might occur in procyanidins, which may influence the bioavailability of flavanols ingested with cocoa products.

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References

- Arlorio, M., Locatelli, M., Travaglia, F., Coisson, J.-D., Del Grosso, E., Minassi, A., et al. (2008). Roasting impact on the contents of clovamide (*N*-caffeoyl-*l*-DOPA) and the antioxidant activity of cocoa beans (*Theobroma cacao* L.). *Food Chemistry*, *106*, 967–975.
- Buijsse, B., Weikert, C., Drogan, D., & Bergmann, M. (2010). Chocolate consumption in relation to blood pressure and risk of cardiovascular disease in German adults. *European Heart Journal*, *31*, 1616–1623.
- Cooper, K. A., Campos-Gimenez, E., Alvarez, D. J., Nagy, K., Donovan, J. L., & Williamson, G. (2007). Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. *Journal of Agricultural and Food Chemistry*, *55*, 2841–2847.
- Crozier, S. J., Preston, A. G., Hurst, W. J., Payne, M. J., & Mann, J. (2011). Cocoa seeds are a “Super Fruit”: A comparative analysis of various fruit powders and products. *Chemistry Central Journal*, *5*, 5.
- Ellinger, S., Reusch, A., Stehle, P., & Helfrich, H.-P. (2012). Epicatechin ingested via cocoa products reduces blood pressure in humans: A nonlinear regression model with a Bayesian approach. *American Journal of Clinical Nutrition*, *95*, 1365–1377.
- Ellis, C. J., Yeap Foo, L., & Porter, L. J. (1983). Enantiomerism: A characteristic of the proanthocyanidin chemistry of the monocotyledonae. *Phytochemistry*, *22*, 483–487.
- Elwers, S., Zambrano, A., Rohsius, C., & Lieberei, R. (2009). Differences between the content of phenolic compounds in Criollo, Forastero and Trinitario cocoa seed (*Theobroma cacao* L.). *European Food Research and Technology*, *229*, 937–948.
- Friedrich, W., Eberhardt, A., & Galensa, R. (2000). Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *European Food Research and Technology*, *211*, 56–64.
- Gotti, R., Furlanetto, S., Pinzauti, S., & Cavrini, V. (2006). Analysis of catechins in *Theobroma cacao* beans by cyclodextrin-modified micellar electrokinetic chromatography. *Journal of Chromatography A*, *1112*, 345–352.
- Gu, L., House, S. E., Wu, X., Ou, B., & Prior, R. L. (2006). Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *Journal of Agricultural and Food Chemistry*, *54*, 4057–4061.
- Guyot, S., Vercauteren, J., & Cheynier, V. (1996). Structural determination of colourless and yellow dimers resulting from (+)-catechin coupling catalysed by grape polyphenoloxidase. *Phytochemistry*, *42*, 1279–1288.
- Heiss, C., Keen, C. L., & Kelm, M. (2010). Flavanols and cardiovascular disease prevention. *European Heart Journal*, *31*, 2583–2592.
- Holt, R. R., Lazarus, S. A., Sullards, M. C., Zhu, Q. Y., Schramm, D. D., & Hammerstone, J. F. (2002). Procyanidin dimer B2 [epicatechin-(4 β -8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa. *American Journal of Clinical Nutrition*, *76*, 798–804.
- Hurst, W. J., Krake, S. H., Bergmeier, S., Payne, M., Miller, K., & Stuart, D. A. (2011). Impact of fermentation, drying, roasting and Dutch processing on flavan-3-ol stereochemistry in cacao beans and cocoa ingredients. *Chemistry Central Journal*, *5*, 53.
- Jolic, S. M., Redovnikovic, I. R., Markovic, K., Sipusic, D. I., & Delonga, K. (2011). Changes of phenolic compounds and antioxidant capacity in coco beans processing. *International Journal of Food Science & Technology*, *46*, 1793–1800.
- Khawaja, O., Gaziano, J. M., & Djoussé, L. (2011). Chocolate and coronary heart disease: A systematic review. *Current Atherosclerosis Reports*, *13*, 447–452.
- Kim, H., & Keeney, P. G. (1984). (-)-Epicatechin content in fermented and unfermented cocoa beans. *Journal of Food Science*, *49*, 1090–1092.
- Kofink, M., Papagiannopoulos, M., & Galensa, R. (2007). (-)-Catechin in cocoa and chocolate: Occurrence and analysis of an atypical flavan-3-ol enantiomer. *Molecules*, *12*, 1274–1288.
- Krysiak, W., Adamski, R., & Żyżelewicz, D. (2013). Factors affecting the colour of roasted cocoa bean. *Journal of Food Quality*, *36*, 21–31.
- Monagas, M., Khan, N., Andres-Lacueva, C., Casas, R., Urpí-Sardà, M., & Llorach, R. (2009). Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease. *American Journal of Clinical Nutrition*, *90*, 1144–1150.
- Ottaviani, J. I., Momma, T. Y., Heiss, C., Kwik-Urbe, C., Schroeter, H., & Keen, C. L. (2011). The stereochemical configuration of flavanols influences the level and metabolism of flavanols in humans and their biological activity in vivo. *Free Radical Biology and Medicine*, *50*, 237–244.
- Payne, M. J., Hurst, W. J., Miller, K. B., Rank, C., & Stuart, D. A. (2010). Impact of fermentation, drying, roasting, and Dutch processing on epicatechin and catechin content of cocoa beans and cocoa ingredients. *Journal of Agricultural and Food Chemistry*, *58*, 10518–10527.
- Porter, L. J., Ma, Z., & Chan, B. G. (1991). Cacao procyanidins: Major flavonoids and identification of some minor metabolites. *Phytochemistry*, *30*, 1657–1663.
- Ritter, C., Zimmermann, F. B., & Galensa, R. (2010). Chiral separation of (+)/(-)-catechin from sulfated and glucuronidated metabolites in human plasma after cocoa consumption. *Analytical and Bioanalytical Chemistry*, *397*, 723–730.
- Santos-Buelga, C., García-Viguera, C., & Tomás-Barberán, F. A. (2003). On-line identification of flavonoids by HPLC coupled to diode array detection. In C. Santos-Buelga & G. Williamson (Eds.), *Methods in polyphenol analysis* (pp. 92–127). Cambridge: Royal Society of Chemistry.
- Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C., & Hollenberg, N. (2006). (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences*, *103*, 1024–1029.
- Sun, W., & Miller, J. M. (2003). Tandem mass spectrometry of the B-type procyanidins in wine and B-type dehydrodiccatechins in an autoxidation mixture of (+)-catechin and (-)-epicatechin. *Journal of Mass Spectrometry*, *38*, 438–446.
- Taubert, D., Roesen, R., Lehmann, C., Jung, N., & Schömig, E. (2007). Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide. *The Journal of the American Medical Association*, *298*, 49–60.
- Tomás-Barberán, F., Borges, G., & Crozier, A. (2011). Phytochemicals in cocoa and flavan-3-ol bioavailability. In A. Crozier, H. Ashihara, & F. Tomás-Barberán (Eds.), *Teas, cocoa and coffee: Plant secondary metabolites and health* (pp. 193–217). Oxford, UK: Wiley-Blackwell.
- Tomás-Barberán, F. A., Cienfuegos-Jovellanos, E., Marin, A., Muguerza, B., Gil-Izquierdo, A., Cerdà, B., et al. (2007). A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *Journal of Agricultural and Food Chemistry*, *104*, 3926–3935.
- Wollgast, J., & Anklam, E. (2000). Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International*, *33*, 423–447.