

## Differences between the content of phenolic compounds in Criollo, Forastero and Trinitario cocoa seed (*Theobroma cacao* L.)

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**Abstract** Phenolic compounds contribute to the quality of raw cocoa, the basis of all chocolate products. Detailed research is needed about the variability of these substances in unprocessed cocoa seed and during seed processing. For the present study, seed samples of Upper Amazon Forastero, Lower Amazon Forastero, Nacional, Criollo and Trinitario from different origins were compared in order to estimate the influence of genetic and site-specific features on the phenolics. Besides ripe, fresh cocoa seed, different fermentation stages of Criollo samples were analysed. Total polyphenols were examined using Folin–Ciocalteu reagent. RP-HPLC–PDA was adapted to analyse the monomeric cocoa polyphenols. Criollo seed contains no anthocyanins, but greater amounts of caffeic acid aspartate than samples of other cocoa types. No genetically determined differences in the amounts of total polyphenols and (–)-epicatechin were found. In contrast, soil fertilisation may lead to cocoa seed with significantly smaller amounts of total polyphenols, flavan-3-ols and anthocyanins, but larger quantities of caffeic acid aspartate than those from unfertilised locations. The diminution of catechins found during fermentation and drying is stronger in the Criollo seed samples than described for other genotype groups. This may be responsible for the mild flavour of Criollo chocolates. Caffeic acid aspartate turned out to be highly

resistant to degradation, with an average of 33% of the original content remaining in fully fermented cocoa seed. The results could be used in trials to produce raw cocoa with specific contents of phenolics.

**Keywords** *Theobroma cacao* L · Phenolic compounds · Criollo · Trinitario · Forastero

### Introduction

Cocoa and chocolate are extraordinarily popular consumer goods. They are based on raw cocoa, the fermented and subsequently dried seed of the cocoa tree *Theobroma cacao* L. Originating in the Amazon basin of South America, cocoa today is cultivated throughout the entire tropical zones of the earth.

Only products from roasted raw cocoa possess the unique cocoa flavour that comprises the general impression of cocoa smell and taste [1]. The individual flavour potential of a raw cocoa determines its classification as fine or flavour cocoa with a particularly aromatic and characteristic flavour or as bulk cocoa without such a specific flavour note. Cocoa of the rare Criollo type is considered to exhibit one of the best flavour qualities [2].

Bitterness and astringency are important components of cocoa flavour. Though bitterness can be attributed in part to purines, both characteristics are mainly caused by phenolic constituents, which amount up to 18% of fat-free dry weight in unprocessed seed [3]. According to some authors, the reaction products of phenolic substances are among the most important components of cocoa flavour [4]. Clapper-ton et al. [5] correlate the flavour quality of individual cocoa samples with their content of (–)-epicatechin. Complex condensation products of phenolic cocoa compounds

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generated during fermentation and drying are responsible for the brown colour of raw cocoa and chocolate.

It is a well-documented fact that phenolic cocoa ingredients possess a strong anti-oxidative potential, which is largely based on the *o*-diphenol structure of many of these compounds. Cocoa polyphenols have been reported to have a protective effect against arteriovascular diseases, cancer and inflammatory processes in the human body [6–17]. Based on the health protective effect of cocoa seed polyphenols, various chocolate products with increased amounts of phenolic compounds were launched (e.g. CocoaVia “products from Mars Inc. and Polyphenol 3000” from Meiji Seika Kaisha).

Information is scarce about the parameters that determine the content of phenolic compounds in unfermented cocoa seed. In view of the present trends to produce authentic fine or flavour chocolates, it is of great interest to assess the individual content of polyphenols of the diverse cocoa types, varieties and origins. In addition, the documentation of the decrement of phenolic compounds during cocoa seed processing still needs further research. Earlier studies have been focusing on the decomposition of procyanidins which is only one among various groups of cocoa polyphenols. A comprehensive analysis of the genetic influence on the chemical reactions during seed processing is not available yet. Such investigations are needed to learn more about the generation of fine or flavour cocoas, like Criollo, which differs substantially from most of the other raw cocoas due to its less pronounced bitterness and astringency [18].

In the present study, total polyphenols and the main phenolic monomers in ripe and unfermented seed of Forastero, Trinitario and Criollo cocoa of six different origins were examined. The objective was to analyse the genetic and the site-specific influences on the content and composition of phenolic seed ingredients. In addition to ripe cocoa seed, samples of Criollo seed from different fermentation stages were analysed.

## Materials and methods

### Cocoa samples

Samples of ripe and unfermented cocoa seed were purchased from six different origins, as specified in Table 1.

Most of the site-specific data were obtained directly from the suppliers of the cocoa samples and listed in Table 2.

The ripe, unfermented cocoa seed samples belong to various clones, respectively, varieties (in the case of the Criollo and the Amelonado samples). The samples were grouped according to their respective, traditional cocoa

types Trinitario, Criollo and Forastero, the latter being sub-grouped in Upper Amazon Forastero, Lower Amazon Forastero and Nacional. Such a classification is justifiable as all seed samples belong either to traditionally cultivated varieties or (in the case of the Upper Amazon Forasteros) to closely related populations. Thus, the genetic variation within the same cocoa type, respectively, sub-group can be assumed to be comparatively low.

Most of the unfermented cocoa seed samples were obtained as ripe fruits (Table 1). The seeds were taken from their pods 4–7 days after harvesting, their seed shells removed and the embryos shock frozen in liquid nitrogen and subsequently freeze-dried. Seed samples from Ecuador were sent as freeze-dried nib powder. Samples from Venezuela were obtained as sun-dried seed. According to preliminary studies, sun-dried cocoa seed samples do not differ significantly from freeze-dried samples as far as their contents of polyphenols are concerned (unpublished data).

The influence of fermentation on the phenolic composition of all Criollo varieties listed in Table 1 was also studied. The samples were obtained from three fermentation batches of Criollo Mérida and one fermentation batch of Guasare and Porcelana, respectively (five Criollo fermentation batches in total). From each fermentation batch sun-dried seed was sampled after 24, 48 and 72 h of fermentation, the last stage being equal to fully fermented Criollo seed.

### Chemicals

Unless otherwise specified, all chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### Extraction

Between 1 and 2.5 g of freeze-died or sun-dried cotyledons were milled to a powder with a particle size of ca.  $1 \mu\text{m}^3$  in a Retsch MM 200 (Germany) laboratory mill with 10 mL of *n*-hexane for fat removal. The pulverised sample then was rinsed 2 times with 50 mL of 40–60 °C petroleum in a Buchner funnel to reduce the residual fat content to  $\leq 5\%$ . Subsequently, the defatted powder was dried in a vacuum oven at room temperature and 100 mbar.

### Analysis of total polyphenols

For analysis of total phenolic compounds, 0.5 g of the fat free sample was weighed in a 100 mL centrifuge vessel and put on ice. Subsequently, each sample was extracted 3 times with a mixture of acetone and water (once 80 + 20, twice 60 + 40 v/v) under constant agitating conditions. After

**Table 1** The investigated ripe, unfermented cocoa seeds

Clone/variety	Type	<i>n</i> Samples	Ecuador <sup>a</sup>	Ghana <sup>b</sup>	Malaysia <sup>c</sup>	England <sup>d</sup>	Trinidad <sup>e</sup>	Venezuela <sup>f</sup>
Amelonado	LAF	5		2			3	
BR 25	T	3			3			
CATONGO	LAF	5		2			3	
Criollo Mérida	C	3						3
EET 59	N	3				1	2	
EET 95	N	8	1	2			5	
EET 390	N	3			3			
Guasare	C	1						1
ICS 95	T	5	1	2			2	
KKM 22	T	3			3			
NA 33	UAF	8		2	3	2	1	
PBC 123	T	3			3			
Porcelana	C	1						1
SCA 6	UAF	10		2		5	3	
T 63/967	UAF	2		2				
T 79/501	UAF	2		2				
T 85/799	UAF	2		2				
UIT 1	T	3			3			

<sup>a</sup> Former plantation of Nestlé, Quevedo, Los Rios

<sup>b</sup> Genebank of the CRIG (Cocoa research Institute Ghana) near New Tafo

<sup>c</sup> Genebank of the MCB (Malaysian Cocoa Board), Quoin Hill near Tawau

<sup>d</sup> Genebank of the School of Plant Science, University of Reading near London

<sup>e</sup> Genebank of the CRU (Cocoa Research Unit), University of the West Indies

<sup>f</sup> Genbanks and plantations in Zea/Mérida, San Juan de Lagunillas/Mérida and Chama/Zúlia

C Criollo, LAF Lower Amazon Forastero; N Nacional; T Trinitario, UAF Upper Amazon Forastero

each extraction, the sample was centrifuged at room temperature and 5000 rpm for 10 min (Laborfuge GL, Heraeus Christ, Osterode, Germany). Prior to the third centrifugation, the sample was treated in an ultrasonic bath (Sonorex Super RK 510 H, Bandelin, Berlin, Germany) for 2 min.

The three supernatants were combined in a flask containing 2.5 mL of glacial acetic acid. Acetone was removed by rotary evaporation under partial vacuum at 40 °C and 80 mbar (LABO Rota SE 320, Resona Technics, Gossau, Switzerland). The aqueous residue was transferred to a volumetric flask containing 2 mL of glacial acetic acid and diluted to 100 mL with Milli-Q Plus water.

The total content of polyphenols was determined with the Folin–Ciocalteu procedure [19]. Crude extract of phenolic compounds was diluted 1/10 (v/v) with 2.5% aqueous acetic acid. This solution (1 mL) was transferred to a 10 mL volumetric flask and 0.5 mL Folin–Ciocalteu reagent and 2 mL of a 20% aqueous solution of Na<sub>2</sub>CO<sub>3</sub> was added. The mixture was then diluted to 10 mL with Milli-Q Plus water. The samples were then incubated for 10 min in a water bath at 70 °C. After cooling to room temperature, the absorbance of the samples was measured at  $\lambda = 730$  nm against the blank value by means of a

Photometer (Ultrospec 3000, Pharmacia Biotech, Freiburg, Germany). The content of total polyphenols was determined at equivalents of (–)-epicatechin.

#### RP-HPLC analysis of monomeric cocoa polyphenols

For RP-HPLC analysis, 50 mg of the defatted cocoa powder was weighed in a centrifuge tube (16 × 100 mm<sup>2</sup>), 3 mL of methanol added and the mixture stirred for 20–30 s with an ULTRA-TURRAX<sup>®</sup> T25 (Ika Labortechnik, Staufen, Germany) agitator. The agitator was then rinsed with 2 mL of methanol and the solutions combined. The centrifuge tube was cooled for 15 min at 0 °C, and then centrifuged for 10 min at 5000 rpm. The methanolic supernatant containing phenolic compounds was decanted into a 50 mL pear-shaped flask. The extraction was repeated 3 additional times, with a cooling phase of only 2 min. The methanol was removed from the combined extracts by rotary evaporation under partial vacuum at 40 °C and 100 mbar. Subsequently, the residue was dissolved in 1.5 mL methanol (Lichrosolv<sup>®</sup>). The sample was transferred into an HPLC vial through a 0.45- $\mu$ m syringe filter (PTFE Multoclear<sup>®</sup>, CS-Chromatographie Service,

**Table 2** Site-specific factors of the different origins of the cocoa samples

Growing area	Ecuador Quevedo/Nestlé farm 1°01'S; 79°28'W	Venezuela/Mérida Zea 8°22'88.3"N; 71°46'953"W	Venezuela/Zulia Chama 8°43'27"N; 71°44'33.3"W	Venezuela/ Mérida S. Juan de Lagumillas 8°31'00"N; 71°21'00"W	Ghana, New Tafo CRIG 6° 43'0"N; 1°37'0' W	Malaysia/Sabah, Tawau MCB Quoin Hill 4°16'N; 117°54'O	GB/ Reading 51°26'12"N; 0°56'33"W	Trinidad and Tobago, CRU (UWI) 10° 35'N; 61° 18'W
M above sea level	80	850	40	1050	200–300	220–230	80	15
Rainy season(s)	Dec–May	July–Dec (max Aug–Sep)	June–Nov	July–Dec (max Aug–Sep) ca. 550	1. May–June 2. Sept–Oct	Dec–March (max January)	–	June–Oct
Rainfall/a (mm)	1886–2607	1100–1335	1300–1800	ca. 550	1250–1750	2036.6	–	1484.1
AVT (°C)	24.8–25.6	21.9	27	22	26–30	27.3	22–30	26
Rel. humidity (%)	87	ca. 80	82	62	70–80	82.8	25–85	79
Plant density cocoa trees	High	Low (mixed culture)	1111 ha <sup>-1</sup>	3 × 3 m <sup>2</sup> , 1111 ha <sup>-1</sup>	2.5 × 2.5 m <sup>2</sup> (triangular) 1739 ha <sup>-1</sup>	Not exceeding 2664 ha <sup>-1</sup> (1.9 × 1.9 m <sup>2</sup> )	Pots 40–50l	1.8 × 1.8 m <sup>2</sup> ca. 3000 ha <sup>-1</sup>
Shading trees	No shading	Banana, avocado, orange trees, <i>Erythrina</i> sp., high density	<i>Erythrina</i> sp. 15 × 15 m <sup>2</sup> , 44 ha <sup>-1</sup>	<i>Erythrina</i> sp. 15 × 15 m <sup>2</sup> , 44 ha <sup>-1</sup>	Residual primary forest + <i>Glyricidia</i> sp., ca. 20 ha <sup>-1</sup>	<i>Glyricidia sepium</i> , 8 ha <sup>-1</sup> after establishment	No shading	<i>Erythrina</i> sp., ca. 200 ha <sup>-1</sup> (6 × 6 m <sup>2</sup> )
Irrigation	If necessary	Never	–	Permanent	–	–	Permanent	If necessary
Topography	Plain	Gradient of 1–2%	Plain	Gradient of 5–12%	Gradient of 5–12%	Gradient	Plain	Plain
Soil type	Sandy + loamy eutrandspts, toxic tropudalfs	Sand 55%, gravel >20%, alluvium 22.5% light, well-drained, low soil depth	Sandy, loamy and gritty tropopsammets, tropoqupts, tropofluvents and troporthents	Cambortid clay 36–44% alluvium 21–38% sand 20–39%	Fairly high moisture retention capacity, partially coarse grained	Xanthic and orthic ferralsols	Sand, gravel and vermiculit (1:2:2) (hydroponic)	Gleyic cambisols, fine, sandy clay, occasionally stagnant moisture
Nutrient content (ppm)	NH <sub>4</sub> <sup>+</sup> 21, PO <sub>4</sub> <sup>3-</sup> 24, K 0.28 meq/100 g	P 2.5, Ca 1.65, Mg 0.98 Na + K: traces	PO <sub>4</sub> <sup>3-</sup> , K, Ca Mn: rather high Mg: average Fe, SO <sub>4</sub> <sup>2-</sup> , NO <sub>3</sub> <sup>-</sup> , NH <sub>4</sub> <sup>+</sup> : low	N 26, PO <sub>4</sub> <sup>3-</sup> 75, K 81–160, S 50–100 micronutrients low	PO <sub>4</sub> <sup>3-</sup> low	High	–	Low
CEC	–	2.6 cmol kg <sup>-1</sup>	High	Average to high	–	–	High	–
pH	6	5.3	4.5–6.5	7–8	–	>5	5.6	–
Organic compounds	4.02%	ca. 1%	Average to high	Average to high	–	–	–	–
Fertilisation	200 kg N, 80 kg P <sub>2</sub> O <sub>5</sub> 120 kg K <sub>2</sub> O ha <sup>-1</sup> yr <sup>-1</sup>	>150 g of standard fertiliser per tree and year + organic material	2 applications per year, 88 kg N, P <sub>2</sub> O <sub>5</sub> and K <sub>2</sub> O ha <sup>-1</sup> yr <sup>-1</sup> , 500 g per tree	See Venezuela/ Chama	No fertilisation	4 applications per year, 100 kg N, 100 kg PO <sub>4</sub> <sup>3-</sup> , 120 kg K <sub>2</sub> O ha <sup>-1</sup> yr <sup>-1</sup>	Modified Long Ashton solution, 7 times per day, 5 min, respectively	11 samples from trees fertilised with 450 g 12/12/17/2 N/P/K/Mg, 8 samples from unfertilised trees

**Table 3** List of the phenolic standard compounds for RP-HPLC

Phenolic compound	Group of phenols	Provider
Aesculetin	Hydroxycoumarins	Aldrich
Catechin	Flavan-3-ols	Fluka
Chlorogenic acid	Hydroxycinnamic acid esters	Aldrich
Clovamid	Hydroxycinnamic acid amides	Bayer HealthCare AG
Cyanidin 3- <i>o</i> - $\alpha$ -L-arabinoside	Anthocyanins	Polyphenols AS
Cyanidin 3- <i>o</i> - $\beta$ -D-galactoside	Anthocyanins	Polyphenols AS
<i>p</i> -Hydroxycinnamic acid	Hydroxycinnamic acids	Merck
Caffeic acid	Hydroxycinnamic acids	Merck
(–)-Epicatechin	Flavan-3-ols	Sigma
(–)-Epigallocatechin	Flavan-3-ols	Sigma
Ferulic acid	Hydroxycinnamic acids	Merck
<i>p</i> -Hydroxybenzoic acid	Hydroxybenzoic acids	Merck
<i>o</i> -Hydroxy-phenyl acetic acid	Phenylacetic acids	Merck
<i>p</i> -Hydroxy-phenyl acetic acid	Phenylacetic acids	Merck
Phenylacetic acid	Phenylacetic acids	Merck
3-(4-Hydroxyphenyl) propionic acid	Similar to hydroxycinnamic acids	Merck
Phloroglucinol	Simple phenols	Aldrich
Protocatechuic acid	Hydroxybenzoic acids	Aldrich
Quercetin	Flavonols	Sigma
Quercetin 3- <i>o</i> -galactoside	Flavonol glycosides	Fluka
Quercetin 3- <i>o</i> -glucoside	Flavonol glycosides	Fluka
Syringic acid	Hydroxybenzoic acids	Merck
Vanillic acid	Hydroxybenzoic acids	Aldrich

Langerwehe, Germany). The vial was sealed hermetically and the sample stored at  $-20^{\circ}\text{C}$  until further analysis.

The RP-HPLC–PDA method was optimised to separate the reported phenolic cocoa compounds reviewed by Ziegler and Biehl [20], and, slightly modified, by Jardine [21]. Standards were purchased from different providers as stated in Table 3.

Chromatographic analyses were carried out on a HPLC system equipped with an AS-4000 (Merck, Darmstadt, Germany) automatic injector, two Knauer (Berlin, Germany) HPLC pumps 64, a Knauer HPLC programme 50 solvent controller, a Waters (Eschborn, Germany) 996 Photodiode Array Detector (PDA) and analysed by means of Millennium TM 3.2 software (Millipore Corporation, Milford, MA, USA). Separation of polyphenols was performed on a Waters Novapac C18 ( $3.9 \times 300 \text{ mm}^2$ ; end-capped) column at  $26^{\circ}\text{C}$ . The binary mobile phase (Table 4) consisted of 2% acetic acid in water (A) and a mixture of acetonitrile, water and concentrated acetic acid (400:90:10 v/v/v) (B).

Twenty microlitres of sample was injected onto the column. Phenolic compounds were detected in a spectrum from  $\lambda = 230\text{--}540 \text{ nm}$ . Quantification of the anthocyanins took place at 530 nm, the flavonols were detected at 371.6 nm and all further phenolic compounds were

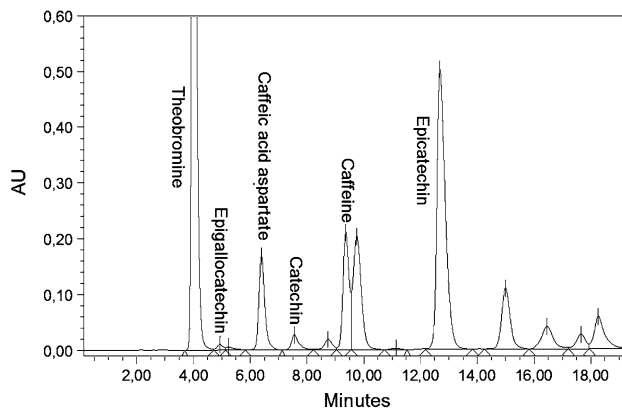
**Table 4** RP-HPLC gradient used for the separation of cocoa seed polyphenols

Time (min)	Flow rate ( $\text{mL min}^{-1}$ )	A (%)	B (%)
0	1.2	90	10
8	1.2	90	10
38	1.1	77	23
50	1	60	40
70	1	10	90
73	1	10	90
78	1.2	90	10
93	1.2	90	10

quantified at 280 nm. Each substance other than caffeic acid aspartate (see below) was identified and quantified by comparing its retention time, spectrum and peak area with an authentic standard (see Table 3).

#### Identification and quantification of (+)-*N*-(*E*)-caffeoyl-L-aspartate

The identity of (+)-*N*-(*E*)-caffeoyl-L-aspartate (caffeic acid aspartate) was established by Dr. T. Stark (Institute for Food Chemistry, University of Münster, Germany) by comparing the fractionated peak with its reference standard



**Fig. 1** Representative chromatogram of a sample of unfermented cocoa at  $\lambda = 280$  nm. AU absorption units

at the University of Münster's Institute for Food Chemistry. As this standard substance was not available in Hamburg, caffeic acid aspartate was quantified via the chlorogenic acid standard (see Table 3). As the absorption characteristics of chlorogenic acid are almost identical to those of caffeic acid aspartate, identical molar amounts of each substance produces the same peak areas (Prof. Dr. T. Hofmann, Institute for Food Chemistry, University of Münster, Germany, personal communication).

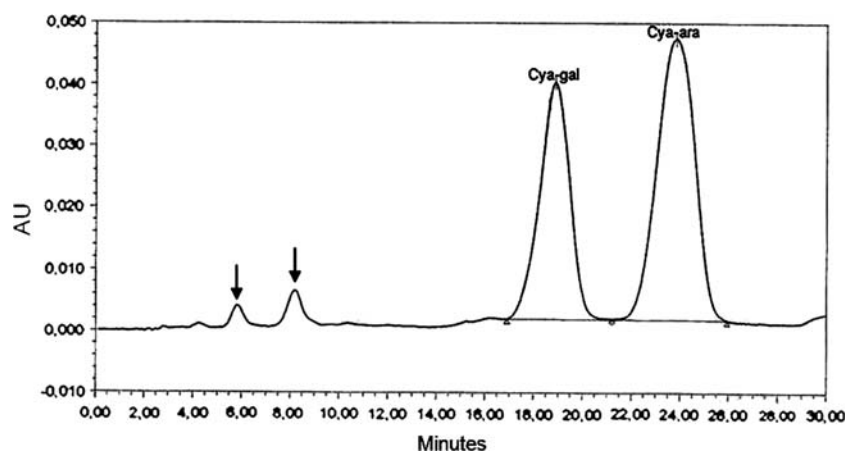
#### Statistical analysis

Statistical analysis was performed with STATISTICA (version 6, StatSoft, Inc., Tulsa, OK, USA). Data were subjected to correlation analysis and to analysis of variance (one-way ANOVA) with subsequent Scheffé post hoc test at a level of significance of  $p < 0.05$ .

## Results

Each sample of fresh and unfermented cocoa seed polyphenols exhibits 12–15 significant peaks at  $\lambda = 280$  nm

**Fig. 2** Representative chromatogram of a sample of unfermented cocoa at  $\lambda = 530$  nm. *Cya-gal* cyanidin 3-*o*- $\beta$ -D-galactoside, *Cya-ara* cyanidin 3-*o*- $\alpha$ -L-arabinoside. Arrows unidentified peaks with similar spectra, AU absorption units



(Fig. 1). Two of these peaks correspond to the two purine alkaloids caffeine and theobromine, which are commonly found in cocoa seeds. These two substances did not affect analysis of phenolics, as they did not overlap any of the standard phenolic substances.

The phenolic compounds (–)-epicatechin, caffeic acid aspartate and catechin were quantified at  $\lambda = 280$  nm. Traces of (–)-epigallocatechin also were found.

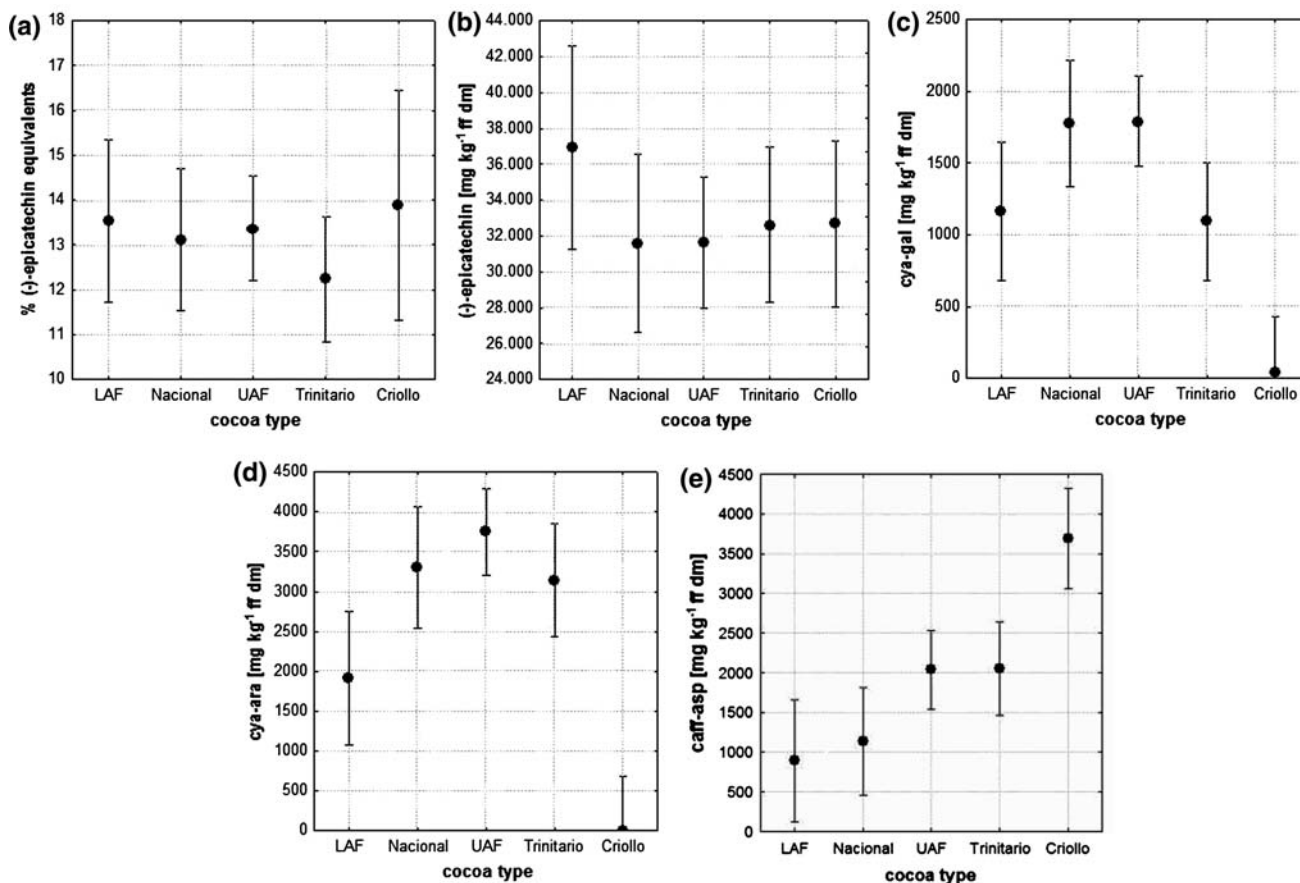
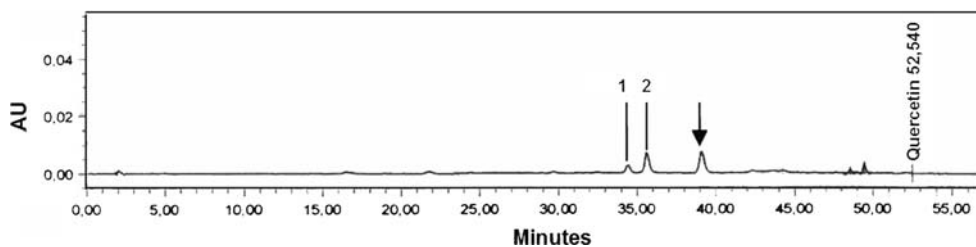
Additional peaks at  $\lambda = 280$  nm do not correspond to the phenolic standard substances listed in Table 3. As their spectra possess a maximum at 278.7 nm and are similar to that of (–)-epicatechin and (+)-catechin, these unidentified phenolic fractions presumably belong to a group of oligomeric and polymeric procyanidins that have been detected in cocoa seed by Adamson et al. [22] among others.

Four peaks are present at  $\lambda = 530$  nm in all fresh and unfermented cocoa seed samples that contained any anthocyanins (Fig. 2). The two larger peaks were identified as cyanidin 3-*o*- $\beta$ -D-galactoside and cyanidin 3-*o*- $\alpha$ -L-arabinoside. Two further fractions with similar spectra were eluted at earlier retention times (Fig. 2, arrows). These two substances probably are cyanidin rutinoside and a pentoside that have been previously described as cocoa seed compounds [23].

The flavonol content in the phenolic extracts was determined at  $\lambda = 371.6$  nm. Whereas quercetin was not found in any of the samples, each of them contained traces of quercetin-3-galactoside and quercetin-3-glucoside (Fig. 3). A third fraction that did not resemble any reference standard was detected at a retention time of ca. 39 min (Fig. 3). This substance appears to be another quercetin glycoside, like quercetin 3-*o*-arabinoside, which was previously described as a cocoa seed ingredient [24–30].

The samples of fresh and unfermented cocoa seed showed large differences in their content of total polyphenols, as well as in the content of individual phenolic compounds. The content of total polyphenols varied

**Fig. 3** Representative chromatogram of a sample of unfermented cocoa at 371.6 nm with retention time of quercetin. 1 quercetin 3-*o*-galactoside, 2 quercetin 3-*o*-glucoside. Arrow unknown substance with similar spectrum, AU absorption units



**Fig. 4** ANOVA of the type-specific content of total phenols (a), (–)-epicatechin (b), cyanidin 3-*o*-β-D-galactoside (c), cyanidin 3-*o*-α-L-arabinoside (d) and caffeic acid aspartate (e). a Actual effect:  $F = 0.60$ ,  $p = 0.67$ ; b actual effect:  $F = 0.68$ ,  $p = 0.61$ ; c actual

effect:  $F = 13.89$ ,  $p < 0.005$ ; d actual effect:  $F = 20.82$ ,  $p < 0.005$ ; e actual effect:  $F = 10.77$ ,  $p < 0.005$  type VI decomposition (effective hypothesis); vertical bars designate 95% confidence intervals

between 6.93 and 17.96% of fat-free dry mass (ff Md). As for the cyanidin 3-*o*-α-L-arabinoside, a variation between 0 and 7,737 mg kg<sup>-1</sup> ff Md was found, whereas the content of cyanidin 3-*o*-β-D-galactoside ranged from 0 to 4,234 mg kg<sup>-1</sup> ff Md. Different seed samples contained between 0 and 6546 mg kg<sup>-1</sup> ff Md caffeic acid aspartate.

The content of (+)-catechin varied broadly between 0 and 2,254 mg kg<sup>-1</sup> ff Md in the analysed cocoa samples. No significant dependencies between the content of (+)-catechin and any genetic or site-specific variables (as listed in Table 2) were found.

No significant differences in the content of total polyphenols and the dominating phenolic substance (–)-epicatechin were found between the samples of Criollo, UAF, LAF, Nacional and Trinitario cocoa (Fig. 4a, b).

Criollo seeds contain few or no anthocyanins. However, these substances occur in significantly larger amounts in most of the Forastero and Trinitario samples (Fig. 4c, d). Seeds resulting from self-pollination of the Lower Amazon Forastero clone CATONGO (Table 1) are also characterised by the absence of anthocyanins. Among the other cocoa samples, no differences in anthocyanin content were

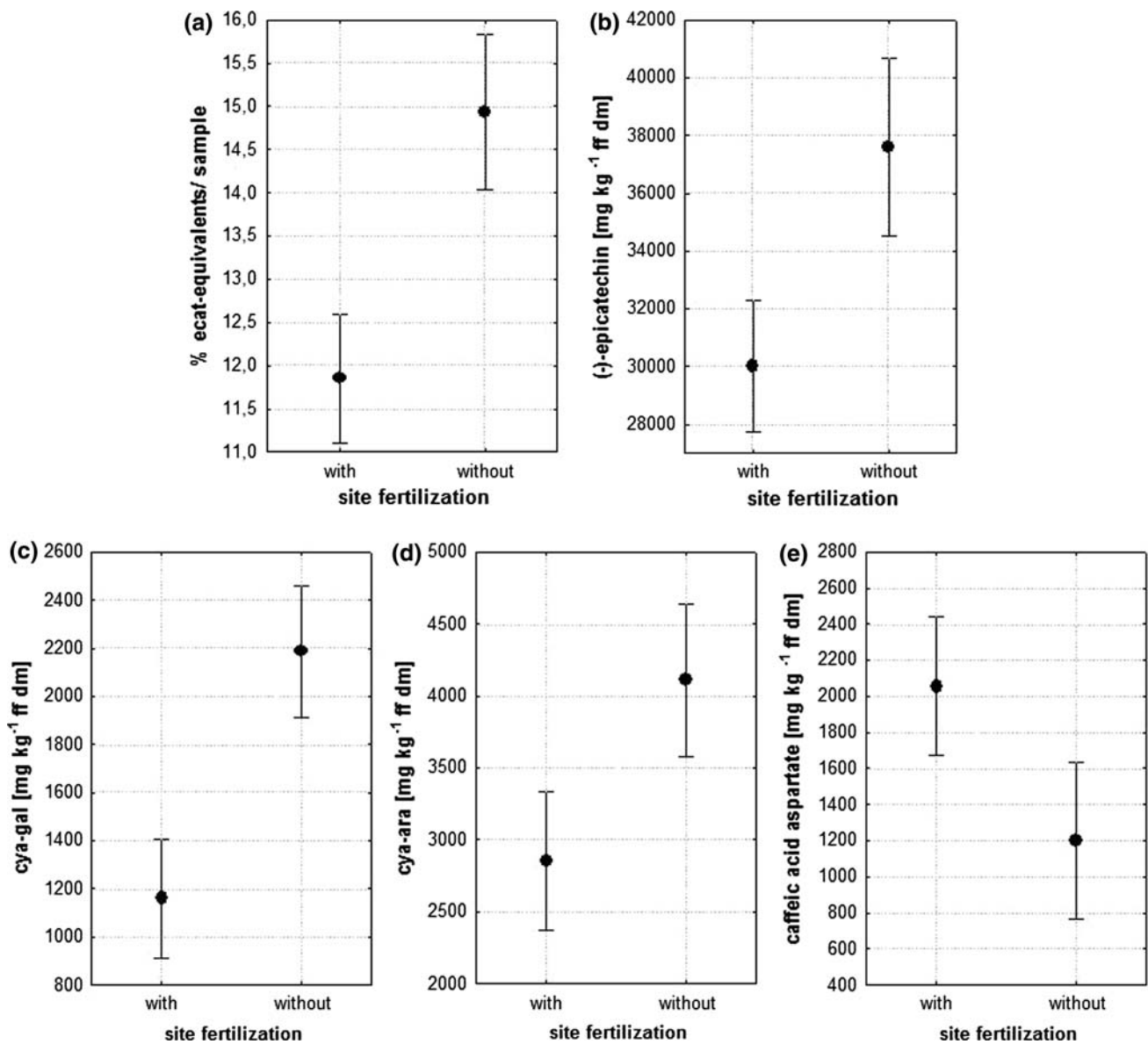
found which were specific to cocoa type, respectively, sub-group (Fig. 4c, d).

Criollo seeds contain significantly higher amounts of caffeic acid aspartate than the samples of the other cocoa types and sub-groups ( $F = 10.77$ ;  $p < 0.005$ ; Fig. 4e). Thus, in addition to their lack of anthocyanins, Criollo cocoa seeds also differ from other cocoa types and sub-groups because they have elevated amounts of caffeic acid aspartate.

Most of the site-specific variables (see Table 2) could not be linked statistically to the polyphenol contents of the

respective cocoa seed samples. However, a significant correlation was found between site fertilisation (yes/no) and the total polyphenol content as well as that of specific phenolic compounds. Significantly larger amounts of total polyphenols, flavan-3-ols and anthocyanins were found in cocoa seed from sites without fertilisation. In the same seeds the quantity of caffeic acid aspartate is significantly lower than in seeds from fertilised locations (Fig. 5a–e).

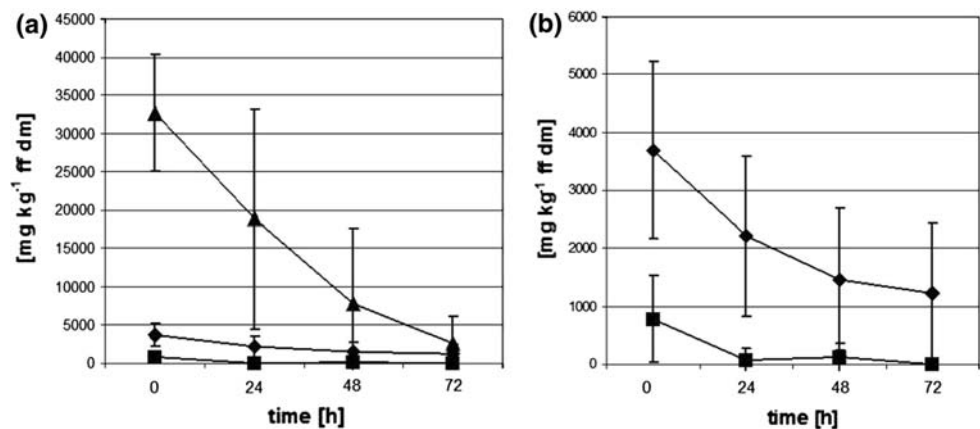
The amount of phenolic compounds in Criollo seed at different stages of processing has not previously been investigated. During fermentation the contents of all



**Fig. 5** ANOVA of the contents of total polyphenols (a), (–)epicatechin (b), cyanidin 3-*o*- $\beta$ -D-galactoside (c), cyanidin 3-*o*- $\alpha$ -L-arabinoside (d) and caffeic acid aspartate (e) depending on site fertilisation; type VI decomposition (effective hypothesis); c–e basing on all ripe samples other than Criollos. **a** Actual effect:  $F = 27.67$ ,

$p < 0.005$ ; **b** actual effect:  $F = 15.49$ ,  $p < 0.005$ ; **c** actual effect:  $F = 31.14$ ,  $p < 0.005$ ; **d** actual effect:  $F = 12.40$ ,  $p < 0.005$ ; **e** actual effect:  $F = 8.66$ ,  $p < 0.005$  type VI decomposition (effective hypothesis); vertical bars designate 95% confidence intervals

**Fig. 6** Average decrement of phenolic compounds in Criollo fermentations. **a** Loss of (–)-epicatechin (ECAT), catechin (CAT) and caffeic acid aspartate (Caff-Asp). **b** Loss of catechin (CAT) and caffeic acid aspartate (Caff-Asp) (detail from **a**). Filled Square catechin, filled triangle epicatechin, filled circle caffeic acid aspartate



phenolic Criollo seed compounds decreases (Fig. 6). Large differences were found between the individual samples. These differences did not depend on the respective Criollo variety.

During Criollo seed fermentation and subsequent drying, the dominating phenolic monomer (–)-epicatechin decreases to an average of  $2,615 \text{ mg kg}^{-1}$  ff Md which corresponds to about 8% of the content of unprocessed seed. The decrease of (+)-catechin is even greater, to a residue of  $7.2 \text{ mg kg}^{-1}$  ff Md or about 1% of the original amounts. Caffeic acid aspartate is more resistant to decrement than the two catechins during Criollo seed processing. In fully fermented Criollo seed,  $1,220 \text{ mg kg}^{-1}$  ff TM or about 33% of the original amount of this substance still remains.

Due to their different decrement rates, the relative proportion of the main phenolic Criollo seed compounds changes substantially during processing. In some of the fully fermented samples, the amount of (–)-epicatechin, which is by far the dominant phenolic substance in unprocessed Criollo seed, is even lower than that of caffeic acid aspartate.

## Discussion

Many of the polyphenols listed in Table 3 were not detected in the extracts of fresh cocoa seed by means of the methods applied in the present study, although newly adapted analytical means for these substances were applied. Most of the missing compounds were detected by means of traditional methods such as paper chromatography. Few of these substances were recovered from cocoa extracts in recent studies. This leads to the assumption that the majority of these previously described phenolic content may have been artefacts generated during post-harvest processes or during analysis of the cocoa seed material. As

for the chlorogenic acids, these compounds may have been confused with caffeic acid aspartate and other hydroxycinnamic acid amides in previous publications due to their similar spectra and the lack of precise analytical methods. Some compounds may also be present below the detection limit of the method applied. Some short annotations for particular phenolic compounds are given in Table 5.

The finding that there are no differences in the amount of total polyphenols and (–)-epicatechin of the several cocoa types and sub-groups corresponds to Griffiths [36], who describes identical polyphenol and catechin content in Forastero and Criollo type cocoa. These results are also supported by Graziani de Fariñas et al. [37] who did not find any significant differences in individual content of tannins between Forasteros, Criollos and Trinitarios.

However, the results of the present study are in contrast to those of Lange and Fincke [38], Efraim [39], cited by Efraim et al. [40] and Martini et al. [41] who state that Criollo seed contains only 30–60% of the amount of polyphenols in Forastero seed. Indeed, such differences can be found between individual Criollo and Forastero seed samples and processed Criollo seeds generally possess a mild flavour with less bitterness and astringency than Forastero seeds [18]. Therefore, differences in the amount of phenolics between individual seed samples can easily be mis-interpreted as type-specific distinctions. However, according to the results of the present study, reduced content of polyphenols in Criollo cocoa is caused by faster decrement of these substances during seed processing in comparison to the other cocoa types and sub-groups (see below).

Few or no anthocyanins were present in Criollo and CATONGO seed as reported by other authors [42].

As for the larger amounts of caffeic acid aspartate in Criollo cocoa seed, Correia et al. [43] found significant differences between the amounts of hydroxycinnamic acid amides specific to individual varieties of *Coffea* L. seed.

**Table 5** Monomeric phenolic compounds and their presence in fresh cocoa seed

Phenolic compounds	Assured presence in cocoa seeds	Possible explanation for description in previous studies
Esculetin	No	Possible degradation product of hydroxycinnamic acids [31]
(+)-Catechin	Yes	
Chlorogenic acid, neochlorogenic acid	No	Possible misidentified hydroxycinnamic acid amides [32, 34]
Cyanidin 3- <i>o</i> - $\alpha$ -L-arabinoside	Yes	
Cyanidin 3- <i>o</i> - $\beta$ -D-galactoside	Yes	
<i>p</i> -Coumaric acid, caffeic acid	No	Possible degradation product of flavonoids or hydroxycinnamic acid amides [33]
(-)-Epicatechin	Yes	
(-)-Epigallocatechin	Yes (traces)	
Ferulic acid, <i>p</i> -hydroxybenzoic acid, hydroxyphenylacetic acids, phenylacetic acid, 3-(4-hydroxyphenyl)-propionic acid, syringic acid, vanillic acid	No	Possible degradation product of flavonoids or hydroxycinnamic acid amides [34]
(+)-Gallocatechin	No	Possibly present in traces below detection limit
Hydroxycinnamic acid amides	Yes	Besides caffeic acid amide further amides are described in Stark und Hofmann [34] amongst others
Phloroglucinol, protocatechuic acid	No	Possible degradation product of catechin [35]
Quercetin	No	Possible deglycosylation product of quercetin glycosides [30]
Quercetin 3- <i>o</i> -galactoside	Yes (traces)	
Quercetin 3- <i>o</i> -glucoside	Yes (traces)	

These similar results support the assumption that the content of caffeic acid aspartate in cocoa seed is, among other things, determined genetically.

The results of the current study indicate that the nutrient supply of the mother plant affects the amounts of phenolic contents in cocoa seed. Increased phenolic compounds at sub-optimal nutrient supply have been observed in other plant species. Various ecophysiological models, such as the “Carbon/Nutrient Balance Hypothesis” [44] or the “Growth-Differentiation Balance Hypothesis” [45] provide possible explanations for this phenomenon.

Nitrogen deficit may be responsible for the lower content of caffeic acid aspartate in seed samples of cocoa trees from sites that were not treated with fertilizer. The elevated concentration of amides in plants is a typical result of fertilisation with nitrogen. These compounds were described by Pahlsson [46] from leaves of *Fagus sylvatica* L., as well as by Li [47] from vegetative parts of other plants. Tsai et al. [48] detected larger amounts of nitrogenous compounds in maize kernels after manuring.

Further experiments (e.g. formal fertilizer trials) could determine if and to what extent the content of phenolic compounds in cocoa seeds is influenced by fertilisation.

Such procedures may be suitable for directed modification of these compounds and, thus, of the cocoa seed quality.

The discovery that the amounts of (+)-catechin is not correlated with any genetic or site-specific features may be caused in part because during sample processing (-)-epicatechin epimerizes to (-)-catechin [49–52]. (-)-Catechin cannot be separated from (+)-catechin by the method applied here.

During Criollo seed fermentation and drying, a rapid decline of catechins was observed. According to previous studies, a similar loss of (-)-epicatechin from seeds of other cocoa types was found, if at all, only after a significantly longer fermentation time [3, 53, 54]. This suggests that the mild flavour of Criollo raw cocoa may be caused by the rapid decrease of phenolics during fermentation. Further research is needed to analyse the reasons for the rapid decomposition of phenolic compounds during Criollo seed processing. Characteristics of Criollo seed shell or cotyledon tissue may permit a faster invasion of pulp degradation products into the seed and to a stronger leakage of phenolic compounds during fermentation. Higher residual PPO and peroxidase activities also may facilitate a higher oxidation rate of phenolic Criollo seed compounds during drying.

The findings that caffeic acid aspartate is more resistant than catechins to decrement correspond to the results of other authors. Wollgast [30] detects less decrement of hydroxycinnamic acid amides during the processing of raw cocoa to chocolate. A high resistance of hydroxycinnamic acid amides against chemical and physical impacts was also observed by Correia et al. [43] who documented rather small losses of these substances during the roasting of coffee beans.

## Conclusion

The present study indicates that there are type-specific differences in phenolic compounds within unfermented cocoa seed. Additionally, the site-specific nutrient supply seems to influence the amounts and the composition of cocoa seed polyphenols. The individual decrement rate of these substances during seed fermentation and drying depends on cocoa type. The seed of the fine or flavour cocoa Criollo differs considerably from that of other cocoa types and sub-groups. The reason for greater amounts of caffeic acid aspartate and the faster decrement of catechins during Criollo seed fermentation should be investigated in further studies. Both aspects may contribute to the characteristic flavour potential of raw cocoa made from Criollo seed.

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## References

- Forsyth WGC, Quesnel VC (1963) The mechanism of cacao curing. *Adv Enzymol* 25:457–492
- Cuatrecasas J (1964) Cacao and its allies: a taxonomic revision of the genus *Theobroma*. *Bulletin of the United States National Museum. Proc US Natl Herb* 35:377–614
- Kim H, Keeney PG (1984) (–)-Epicatechin content in fermented and unfermented cocoa beans. *J Food Sci* 49:1090–1092
- Rohan TA, Connell M (1964) The precursors of chocolate aroma: a study of the flavonoids and phenolic acids. *J Food Sci* 29:460–463
- Clapperton JF, Lockwood G, Yow STK, Lim DHK (1994) Effects of planting materials on flavour. *Cocoa Growers' Bulletin* 48:47–57
- Engler MB, Engler MM, Chen CY, Malloy MJ, Browne A, Chiu EY, Kwak HK, Milbury P, Paul SM, Blumberg J, Mietus-Snyder ML (2004) Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *J Am Coll Nutr* 23(3):197–204
- Heiss C, Kleinbongard P, Dejam A, Perré S, Schroeter H (2005) Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *J Am Coll Cardiol* 46:1276–1283
- Jourdain C, Tenca G, Deguercy A, Troplin P, Poelman D (2006) In vitro effects of polyphenols from cocoa and [beta]-sitosterol on the growth of human prostate cancer and normal cells. *Eur J Cancer Prev* 15(4):353–361
- Karim M, McCormick K, Tissa Kappagoda C (2000) Effects of cocoa extracts on endothelium-dependent relaxation. *J Nutr* 130:2105–2108
- Kenny TP, Keen CL, Jones P, Kung H-J, Schmitz HH, Gershwin ME (2004) Pentameric procyanidins isolated from *Theobroma cacao* seeds selectively downregulate ErbB2 in human aortic endothelial cells. *Exp Biol Med* 229(2):255–263
- Mao TK, Van de Water J, Keen CL, Schmitz HH, Gershwin ME (2003) Cocoa flavonols and procyanidins promote transforming growth factor- $\beta$ 1 homeostasis in peripheral blood mononuclear cells. *Exp Biol Med* 228:93–99
- Osakabe N, Yasuda A, Natsume M, Takizawa T, Terao J, Kondo K (2002) Catechins and their oligomers linked by C4  $\rightarrow$  C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. *Exp Biol Med* 227:51–56
- Perré S (2005) Einfluss von flavanolreichem Kakao auf die Endotheldysfunktion bei Rauchern. PhD Thesis, University of Düsseldorf
- Rein D, Paglieroni TG, Wun T, Pearson DA, Schmitz HH, Gosselin R, Keen CL (2000) Cocoa inhibits platelet activation and function. *Am J Clin Nutr* 72(1):30–35
- Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Urbe C, Schmitz HH, Kelm M (2006) (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* 24(103):1024–1029
- Sies H, Schewe T, Heiss C, Kelm M (2005) Cocoa polyphenols and inflammatory mediators. *Am J Clin Nutr* 81(Suppl):304–312
- Yamagishi M, Natsume M, Nagaki A, Adachi T, Osakabe N, Takizawa T, Kumon H, Osawa T (2000) Antimutagenic activity of cacao: inhibitory effect of cacao liquor polyphenols on the mutagenic action of heterocyclic amines. *J Agric Food Chem* 48:5074–5078
- Wolters B (1999) Zur Verbreitungsgeschichte und Ethnobotanik indianischer Kulturpflanzen, insbesondere des Kakaobaums. *J Appl Bot* 73:128–137
- Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16:144–158
- Ziegleder G, Biehl B (1988) Analysis of cocoa flavour components and flavour precursors. In: Linskens HF, Jackson JF (eds) *Modern methods of plant analysis, volume 8, analysis of non-alcoholic beverages*. Springer, Berlin, Heidelberg, pp 321–393
- Jardine NJ (1999) Phytochemicals and phenolics. In: Knight I (ed) *Chocolate and cocoa: health and nutrition*. Blackwell Science, Oxford, pp 119–142

22. Adamson GE, Lazarus SA, Mitchell AE, Prior RL, Cao G, Jacobs PH, Kremers BG, Hammerstone JF, Rucker RB, Ritter KA, Schmitz HH (1999) HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J Agric Food Chem* 47(10):4184–4188
23. Cakirer MS (2003) Color as an indicator of flavanol content in the fresh seeds of *Theobroma cacao* L. Thesis, The Pennsylvania State University
24. Hammerstone JF, Lazarus SA, Mitchell AE, Rucker R, Schmitz HH (1999) Identification of procyanidins in cocoa and chocolate using high performance liquid chromatography/mass spectrometry. *J Agric Food Chem* 47:490–496
25. Leco Corporation (2006) Comparison of flavonoids in cocoa from different origins using LC-TOFMS. Leco Form No. 203-821-297, 1–7
26. Sanbongi C, Osakabe N, Natume M, Takizawa T, Gomi S, Osawa T (1998) Antioxidative polyphenols isolated from *Theobroma cacao*. *J Agric Food Chem* 46:454–457
27. Sánchez-Rabaneda F, Jáuregui O, Casals I, Andrés-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventós RM (2003) Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J Mass Spectrom* 38:35–42
28. Stark T, Bareuther S, Hofmann T (2005) Sensory-guided decomposition of roasted cocoa nibs (*Theobroma cacao*) and structure determination of taste-active polyphenols. *J Agric Food Chem* 53:5407–5418
29. Tomas-Barberán FA, Cienfuegos-Jovellanos E, Marín A, Muguerza B, Gil-Izquierdo A, Cerda B, Zafrilla P, Morillas J, Mulero J, Ibarra A, Pasamar MA, Ramón D, Espín JC (2007) A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J Agric Food Chem* 55:3926–3935
30. Wollgast J (2005) The contents and effects of polyphenols in chocolate: qualitative and quantitative analyses of polyphenols in chocolate and chocolate raw products as well as evaluation of potential implications of chocolate consumption in human health. PhD thesis, University of Giessen, Germany
31. Butler WL, Siegelmann HW (1959) Conversion of caffeic acid to esculetin during paper chromatography. *Nature* 183:1813–1814
32. Quesnel VC (1965) Chloroform-extractable aromatic acids of cacao. *J Sci Food Agric* 16:596–599
33. Rios LY, Gonthier M-P, Rémésy C, Mila I, Lapierre C, Lazarus SA, Williamson G, Scalbert A (2003) Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *Am J Clin Nutr* 77:912–918
34. Stark T, Hofmann T (2005) Isolation, structure determination, synthesis, and sensory activity of N-phenylpropenoyl-L-amino acids from cocoa (*Theobroma cacao*). *J Agric Food Chem* 53:5419–5428
35. Kenyhercz TM, Kissinger PT (1978) Determination of selected acidic, neutral, and basic natural-products in cacao beans and processed cocoa. *Liquid chromatography with electrochemical detection*. *Lloydia* 41(2):130–139
36. Griffiths LA (1960) A comparative study of the seed polyphenols of the genus *Theobroma*. *Biochem J* 74(2):362–365
37. Graziani de Fariñas L, Ortiz de Bertorelli L, Parra P (2003) Características químicas de la semilla de diferentes tipos de cacao de la localidad de cumboto, Aragua. *Agronomía Tropical* 53(2):133–144
38. Lange H, Fincke A (1970) Kakao und Schokolade. In: Acker L, Bergner K-G, Diemair W, Heimann W, Kiermeier F, Schormüller J, Souci SW (eds) *Handbuch der Lebensmittel*. Volume VI: Alkaloidhaltige Genussmittel, Gewürze, Kochsalz. Springer, Berlin, Heidelberg, pp 210–309
39. Efraim P (2004) Estudo para minimizar as perdas de flavonóides durante a fermentação de cacau para produção de chocolate. PhD Thesis, University of Campinas, Brasil
40. Efraim P, Tucci ML, Pezoa-García NH, Haddad R, Eberlin M (2006) Teores de compostos fenólicos de sementes de cacau de diferentes genótipos. *Braz J Food Technol* 9(4):229–236
41. Martini MH, Figueira A, Lenci CG, de Queiroz Tavares D (2008) Polyphenolic cells and their interrelation with cotyledon cells in seven species of *Theobroma* (*Sterculiaceae*). *Revista Brasil Bot* 31(3):425–431
42. Bartley BGD (2005) *The genetic diversity of cacao and its utilization*, 1st edn. CABI, Cambridge
43. Correia AMNG, Leitão MCA, Clifford MN (1995) Caffeoyltyrosine and Angol II as characteristic markers for Angolan *robusta* coffees. *Food Chem* 53:309–313
44. Bryant JP, Chapin FS, Klein DR (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368
45. Herms DA, Mattson WJ (1992) The dilemma of plants: to grow or defend. *Q Rev Biol* 67:283–335
46. Pahlsson AM (1992) Influence of nitrogen fertilization on minerals, carbohydrates, amino acids and phenolic compounds in beech (*Fagus sylvatica* L.) leaves. *Tree Physiol* 10(1):93–100
47. Li S (2004) Nitrate accumulation in plants and its relation to crop yields. Third international nitrogen conference, abstracts—concurrent oral sessions, 72
48. Tsai CY, Huber DM, Warren HL (1980) A proposed role of zein and glutelin as N sinks in maize. *Plant Physiol* 66:330–333
49. Cooper KA, Campos-Giménez E, Alvarez DJ, Nagy K, Donovan JL, Williamson G (2007) Rapid reversed phase ultra-performance liquid chromatography analysis of the major cocoa polyphenols and inter-relationships of their concentrations in chocolate. *J Agric Food Chem* 55(8):2841–2847
50. Gotti R, Furlanetto S, Pinzauti S, Cavrini V (2006) Analysis of catechins in *Theobroma cacao* beans by cyclodextrin-modified micellar electrokinetic chromatography. *J Chromatogr A* 1112:345–352
51. Haslam E (1998) *Practical polyphenolics: from structure to molecular recognition and physiological action*, 1st edn. Cambridge University Press, Cambridge
52. 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
53. Kealey KS, Snyder RM, Romanczyk, LJ, Geyer HM, Myers ME, Withcare EJ, Hammerstone JF, Schmitz HH (1998) Cocoa components, edible products having enhanced polyphenol content, methods of making same and medical uses. Patent Cooperation Treaty (PCT) WO 98/09533, USA, MARS INCORPORATED
54. Rohsius C (2007) Die Heterogenität der biologischen Ressource Rohkakao (*Theobroma cacao* L.). PhD Thesis, University of Hamburg