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Abstract

Cocoa products are obtained from the seeds of *Theobroma cacao* L. In this research, cocoa liquor and chocolate produced from cocoa beans from West Africa (Forastero, "bulk" cacao) and Ecuador (Nacional variety, "fine-flavor" cacao), were investigated, using a novel approach in which various analytical techniques are combined in order to obtain in-depth knowledge of the studied cocoa samples. The levels of various classes of primary metabolites were determined and a wide range of secondary metabolites, including volatile organic acids, aldehydes, esters, pyrazines, polyphenols, methylxanthines and biogenic amines, were identified and/or quantified by HS-SPME GC-MS (headspace-solid phase microextraction gas chromatography – mass spectrometry) and UPLC-HRMS (ultra-performance liquid chromatography – high resolution mass spectrometry). Odor Activity Values (OAV) were calculated to assess the contribution of individual volatiles on the final aroma.

Various volatile aroma compounds were more abundant in the West African cocoa liquor and chocolate, while the Ecuadorian samples were richer in most quantified non-volatile metabolites. Principal component analysis (PCA) confirmed that the four samples can be clearly distinguished. Alcohols, pyrazines, amino acids and biogenic amines were found to be highly influential in causing this differentiation. The proposed approach can be useful in future studies on more extensive cocoa sample collections, in order to highlight similarities and pinpoint typical differences in chemical composition among these samples.

Keywords

cocoa liquor, chocolate, proximate analysis, volatile composition, non-volatile composition, HS-SPME GC-MS, UPLC-HRMS, principal component analysis

List of abbreviations

CH	Chocolate
CL	Cocoa Liquor
$ddMS^2$	data-dependent MS fragmentation
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
EC	Ecuador
exp	experimental
HCD	Higher energy Collisional Dissociation
HESI	Heated ElectroSpray Ionization
HS-SPME	Headspace Solid-Phase Microextraction
KI	Kovats retention Index/Indices
lit	literature
OAV	Odor Activity Value
OTV	Odor Threshold Value
WA	West Africa

1. Introduction

Cocoa liquor is the product obtained from the fermented, dried, roasted and ground seeds of *Theobroma cacao* L. Together with cocoa butter (the fat pressed from cocoa liquor) and sugar, it is the major ingredient of dark chocolate. Cocoa is cultivated between 20° north and 20° south of the equator (Beckett, 2009), mainly in West Africa (representing over 70% of the world production), but also in Central and South America, and South-East Asia (ICCO, 2018). Generally, cocoa is classified into four main varieties based on geographic origin, pod morphology, bean yield, genotype, disease resistance and flavor characteristics. Forastero cocoa, the main variety cultivated in West Africa, is considered as bulk cocoa representing over 90% of the global cacao production. The Criollo, Trinitario and Nacional varieties form the fine-flavor cocoa group and represent 5 to 10% of the cocoa world production. The Nacional variety is exclusively grown in Ecuador and produces the typical Arriba beans with distinct floral and spicy flavor notes (Aprotosoaie, Luca and Miron, 2016a).

With regard to the chemical composition of cocoa samples (cocoa beans, cocoa liquors, chocolates, etc.), a rough division can be made between non-volatile and volatile components, each requiring different methods of analysis. Besides water and primary metabolites like fats, carbohydrates, proteins and dietary fibers, an important group of non-volatile components are the polyphenols, which can be further divided into three main subgroups: flavan-3-ols (mainly (+)-catechin and (-)-epicatechin), anthocyanins and proanthocyanidins (dimers, trimers and oligomers) (Wollgast and Anklam, 2000). Moreover, flavonoids like quercetin and luteolin have been reported, as were several phenolic acids, such as caffeic, ferulic, and coumaric acid (Aprotosoaie et al., 2016a). Many reports were published with regard to the beneficial health effects of polyphenols, mainly because of their antioxidant

potential and their protective effects on the cardiovascular system (Crozier and Hurst, 2014; Aprotosoaie et al., 2016b).

Another important class of non-volatile compounds in cocoa are the alkaloids, the methylxanthines being the most abundant ones. Theobromine is the major alkaloid, followed by caffeine. Moreover, theophylline is present, but in much lower amounts. Just like the polyphenols, these methylxanthines contribute to the typical bitter taste of cocoa. Compared to coffee, cocoa contains significantly lower levels of caffeine. Theobromine levels are higher though, but its stimulating effects on the central nervous system are far less pronounced than those of caffeine (Aprotosoaie et al., 2016a; Tuenter, Foubert and Pieters, 2018).

Amino acids and biogenic amines, like 2-phenylethylamine, dopamine and tyramine are also present. Especially during roasting, amino acids can be converted to biogenic amines via decarboxylation. Additional enzymatic reactions can occur, like the reaction of dopamine with acetaldehyde, which leads to the formation of salsolinol. Biogenic amines can exert important effects in the human organism. 2-phenylethylamine, for example, has been referred to as the “love drug”, as it is associated with aphrodisiac effects. Moreover, mood lifting and heightened sensitivity were also associated to the presence of phenylethylamine and N-acylethanolamine. However, 2-phenylethylamine is only present in low amounts, and it does not reach the brain after oral intake. Nevertheless, some amines, at high levels, may cause adverse effects to human health. For example, high levels of tyramine, tryptamine and 2-phenylethylamine can cause migraine (do Carmo Brito et al., 2017; Tuenter et al., 2018).

Flavor is the most important criterion for chocolate quality. It is influenced by both the volatile and non-volatile fraction. During fermentation, the formation of flavor precursors like reducing sugars and free amino acids is initiated. Mainly during drying and roasting of the cocoa beans, these compounds can

undergo further reactions, which are important for the final flavor and aroma of the cocoa liquors and chocolates. Reducing sugars can react with amino acids or oligopeptides via a Maillard reaction, leading to Amadori products. These can be further converted via multiple reactions into various compounds (aldehydes, ketones, furans, pyrazines, etc.) (Aprotosoaie et al., 2016a). Finally, conching is of utmost importance as it eliminates undesirable volatiles and reduces the moisture content, thus enhancing the flavor of the final product (Afoakwa, 2011; Aprotosoaie et al., 2016a). The specific cocoa aroma and final composition of cocoa liquors and chocolates are influenced by many factors, like the cocoa genotype, place of origin of the cocoa beans, season of harvesting, the practices of local farmers, and all subsequent processing steps (Afoakwa, Paterson, Fowler and Ryan, 2008; Caligliani et al., 2014; Rottiers et al., 2018; Hinneh et al., 2019a).

In the past decades, interest in “functional”, healthy, and high-quality food products has risen, and chocolate is one example of this. A lot of research has been carried out, mainly concerning the cardiovascular effects of (polyphenols in) chocolate. Nevertheless, it is crucial to perform these tests on well-characterized samples, and it is important to take into account also other (minor) constituents when assessing potential biological activities. However, in many studies the levels of flavan-3-ols, procyanidins, methylxanthines and the total phenolic content are determined, but a more detailed analytical characterization of the non-volatile phytochemicals in cocoa liquors and chocolates is relatively rare. Different techniques have been applied for the analysis of such samples, for example High Performance Liquid Chromatography – Ultraviolet detection (HPLC-UV), or Nuclear Magnetic Resonance (NMR) spectroscopy (Natsume et al., 2000; Caligiani et al. 2014). However, Ultra Performance Liquid Chromatography– High Resolution Mass Spectrometry (UPLC-HRMS) is the preferred technique when analyzing also minor non-volatile constituents, (like biogenic amines, as reported by Oracz and Nebesny (2014)) and is applied in this research. With regard to the volatile

compounds, different extraction procedures combined with various analytical techniques were already tested (Counet, Callemien, Ouwerx and Collin , 2002; Frauendorfer and Schieberle, 2008; Owusu, Petersen and Heimdal, 2011). Headspace Solid-Phase Micro extraction (HS-SPME) is considered the preferred extraction technique in combination with GC-MS (Gas Chromatography – MS) for the profiling of the aroma of cocoa products and was used for analysis of the aroma compounds.

In this study, a comparison is made between cocoa liquors and chocolates, produced from a batch of "blended bulk cocoa beans", harvested in West Africa, which is common practice in commercial mass production of chocolate, and cocoa beans of the Nacional variety, considered a fine-flavor cocoa, from Ecuador. The nutritional composition of the cocoa liquors was determined; the levels of various phytochemicals, volatile and non-volatile, were assessed by means of GC-MS and UPLC-HRMS. Additionally, the odor activity values (OAVs) of volatile compounds and the contribution of these compounds to overall aroma were calculated. Finally, principal component analysis (PCA) was carried out, based on the GC-MS and UPLC-HRMS data, in order to visualize whether the samples could be distinguished and if so, to look further into the exact compositional differences between the samples.

2. Materials and Methods

2.1 Raw materials

Two cocoa liquors were investigated: a commercial West African cocoa liquor (WA-CL) made from a blend of cocoa beans of different origins and varieties, supplied by Cacaolab bvba (Evergem, Belgium), and an Ecuadorian cocoa liquor (EC-CL) made from cocoa beans of the Nacional variety collected in the Sucumbíos province. The former is considered a bulk cocoa liquor, the latter a fine-flavor cocoa liquor. The cocoa liquor samples provided were obtained from well fermented and dried cocoa beans that

were roasted and ground into cocoa liquor. Pre-broken sugar, cocoa butter and soy lecithin were provided by Cacaolab bvba.

2.2 Chemicals

UPLC-grade acetonitrile, formic acid and ammonium formate were purchased from Biosolve (Valkenswaard, The Netherlands). HPLC-grade acetone was obtained from Acros Organics (Geel, Belgium), and HPLC-grade *n*-hexane came from Fisher Scientific (Loughborough, UK). Ultrapure water was generated with a Direct Pure Up system of Rephile. Analytical standards were purchased from Extrasynthese (Lyon, France), Sigma-Aldrich (Bornem, Belgium), Santa Cruz Biotechnology (Heidelberg, Germany), or Carl Roth (Karlsruhe, Germany).

2.3 Chocolate formulation and production

Dark chocolates consisting of 48.0wt% pre-broken sugar, 18.1wt% non-fat cocoa solids coming from cocoa liquor, 33.5wt% cocoa butter and 0.4wt% soy lecithin were produced on a 5 kg scale at Cacaolab. Two dark chocolates with a cocoa content of 51.6% were made: one from the West African cocoa liquor (WA-CH) and one from the Ecuadorian liquor (EC-CH). The chocolates were produced using the protocol described by Tran et al. (2016), but mixing was done at a rotational speed setting of 1. The chocolates were hand tempered on a marble plate and the temper index was measured with a Chocometer (Aasted-Mikroverk, Farum, Denmark). The chocolate was considered well-tempered when a temper index between 3.5 and 4.5 was obtained, to ensure sufficient contraction and a good gloss and snap. Finally, the tempered chocolate was molded into chocolate tablets of about 6 g (33 x 33 x 5 mm), vibrated, leveled off and cooled in a cooled cabinet at 11 °C for 45 min (Chocolate World, Antwerp,

171 Belgium). After a maturation period of 24 h at 20 °C, the chocolates were individually wrapped into
172 aluminum foil and stored until further analysis.

173

174 *2.4 Proximate analysis*

175 Moisture content of the cocoa liquors was analyzed in triplicate using Karl Fischer titration. The analysis
176 was done using a 719 Titrino (Metrohm, Herisau, Switzerland) with Hydranal solvent (Riedel de Haen,
177 34812) and Hydranal titrant 5 (Riedel de Haen, 34801). The fat, protein, total dietary fiber and ash
178 content of the cocoa liquors were analyzed in duplicate using the official AOAC methods for cocoa
179 products: methods 963.15 (Weibull method), 970.22 (Kjeldahl method using a conversion factor of
180 6.25), 985.29 and 972.1, respectively.

181

182 *2.5 HS-SPME GC-MS analysis*

183 The volatile aroma profiles were recorded using HS-SPME-GC-MS based on the method described by
184 Van Durme, Ingels and De Winne (2016), but confirmation of identified compounds was done with the
185 Wiley 275 library and by comparison of Kovats indices determined after injection of a series of *n*-alkane
186 homologues (C_5–C_{13}) (KI(exp)), with the Kovats indices from literature (KI(lit)).
187 To evaluate whether a volatile contributes to the overall flavor profile, the OAVs were calculated using
188 documented odor threshold values (OTVs) (van Gemert, 2011). The OTVs in oil media were used due
189 to the fat continuous dispersion (ca. 55% fat) of the cocoa liquors. The OAVs were calculated by dividing
190 the headspace concentrations of aroma compounds with their respective OTVs.

191

192 *2.6 UPLC-HRMS analysis*

193 Prior to extraction, samples were defatted three times with *n*-hexane (ratio 1:5 (m/V)). The samples
194 were subjected to ultrasonication for 10 min, followed by centrifugation (5 min, 1370 x G). The defatted
195 cocoa liquors and chocolates were left to dry for at least 24 h at room temperature, protected from
196 light. Extraction of 0.5 g of the samples was carried out three consecutive times with 10 mL of a mixture
197 of 70:29.8:0.2 aceton:H_2O:acetic acid. Samples were vortex mixed and submitted to ultrasonication for
198 1 h, repeating the vortex mixing after 30 min. The samples were centrifuged (5 min, 1370 x G) and the
199 supernatant was collected. All extracts were prepared in triplicate.

200 Standard stock solutions of 50 compounds (including a wide range of substances, previously reported
201 in cocoa and chocolate (Afoakwa, 2011; Aprotosoaie et al, 2016a) were prepared at a concentration of
202 1 mg/mL in methanol. Dilutions were prepared in 60:40 (V:V) methanol:40 mM ammonium formate
203 buffer. A list of the analytes can be found in the Supplementary Information, Table S1.

204 All samples were analyzed by UPLC-HRMS. For qualitative purposes, one extract per sample was
205 injected on a UPLC system and analyzed by a Q Exactive HRMS detector (Thermo Fisher Scientific,
206 Bremen, Germany), as described previously by Bijttebier et al. (2016). Quantitative UPLC-HRMS analysis
207 of the chocolates and cocoa liquors was carried out on an Acquity UPLC with XEVO G2-XS QTOF
208 (Quadrupole Time-Of-flight) MS system. Separation was achieved on an UPLC HSS T3 column (2.1 x 100
209 mm, 1.8 µm), kept at 40 °C, and H_2O (A) and CH_3CN (B) both with 0.1% formic acid, as mobile phase.
210 The following gradient was applied: 0-1 min 3% B, 7 min 15% B, 14 min 22% B, 17 min 30% B, 22-24 min
211 100% B, 26-30 min 3% B. The flow rate was 0.4 mL/min. Detection was carried out in ESI+ mode, *m/z*
212 scan range 50-1500. V_{cap} 1.0 kV, V_{cone} 40 V, source offset 80, T_{source} 120 °C, $T_{desolvation}$ 550 °C, cone gas 50
213 L/h, desolvation gas 1000 L/h. The 50 reference compounds were analyzed under the same conditions,
214 in concentrations ranging from 610 pg/mL to 5 µg/mL, and calibration curves were constructed, in order

to allow a quantitative determination of these compounds in the samples. All samples were analyzed as such, or after a ten-, hundred- or thousand-fold dilution in 50% methanol (injection volume: 5 µL).

2.7 Data-analysis

The resulting values of the UPLC-HRMS and GC-MS analysis were subjected to analysis of variance (ANOVA) at 5% significance level, using SPSS Statistics 25 (SPSS Inc., Chicago, USA) or GraphPad Prism 6 (GraphPad Software, San Diego, USA). Workflow4Metabolomics 3.0 (http://workflow4metabolomics.org/), and Matlab R2018a were used to carry out PCA.

3. Results and Discussion

3.1 Nutritional composition of cocoa liquors

Table 1 shows the nutritional composition of the cocoa liquors. Fat (>54%) was the major constituent for both origins, followed by total dietary fibers (14.1-19.8%) and proteins (14.7-15.6%). Ash and moisture content of the cocoa liquor samples were less than 3.5% and 1.5%, respectively. The nutritional composition of the cocoa liquors varied depending on their geographical origin. EC-CL showed a lower moisture content, a higher protein content and a lower total dietary fiber and ash content. No differences were found in total fat content between the two origins. Their fat content was in the range 47-60% defined by the Codex Standards (2001). The fat content of the EC-CL was slightly higher than the values reported by Luna, Crouzillat, Cirou and Buchelli (2002) for Ecuadorian Arriba cocoa liquors (52.2-53.8). The fat content of the WA-CL fell in the range 55-59%, found for most Forastero cocoa beans (Afoakwa, Quao, Takrama, Budu and Saalia, 2013). The moisture content of the cocoa liquor samples was lower than 2%, a value recommended by industrial manufacturers (Beckett,

2009). The protein, total dietary fiber and ash content of the cocoa liquors was in the same order of magnitude compared to the values reported by Torres-Moreno, Torrescasana, Salas-Salvadó and Blanch (2015) for cocoa beans from Ghana and Ecuador.

3.2 Volatile aroma composition

Table 2 shows the mean semi-quantitative volatile composition of the cocoa liquors and chocolates, and the linear KI, determined for each compound on the DB-WAX column. A total of 67 volatile components was identified, including acids, alcohols, aldehydes, esters, ketones, pyrazines, furans, furanones, lactones, pyrans, pyrroles and terpenes.

The total volatile concentration was higher in the cocoa liquors (20,394 – 63,801 ng/g cocoa liquor) than in the chocolates (7,592 – 9,673 ng/g chocolate). Both the EC-CL and EC-CH showed a lower total volatile concentration than their West African counterpart. For each compound, the OAV was calculated by dividing the values from Table 2 by their respective OTV (van Gemert, 2011) (Table 3). A volatile with OAV ≥ 1 may be considered odor-active, and only those compounds are given in Table 3. As can be seen from Table 3, not all OTVs were found in literature, and thus, OAVs could not be determined for all components. However, OAV of the most relevant volatiles could be determined. It is worth mentioning that depending on its OTV, a volatile with a relatively higher headspace concentration may not necessarily contribute to the overall flavor profile.

Table 2 and 3 show that volatiles, and particularly volatile acids, decreased during chocolate production from cocoa liquor, but that no new key odorants appeared. The reduced concentration of volatiles is mainly due to the conching step and the “dilution of the cocoa liquors” by the addition of sugar and cocoa butter. This was also observed by Counet et al. (2002). The dominating odorant identified in both

cocoa liquors and chocolates was acetic acid, which was less abundant in the EC samples than in the WA samples. The presence of acetic acid is typically characterized by sour, vinegar flavor notes (Frauendorfer and Schieberle, 2008). Off-flavor components such as butyric acid and 3-methylbutyric acid were only detected in WA-CL, but disappeared in the respective chocolate.

The second most dominant group of odorants were alcoholic compounds, of which 2,3-butanediol and 1,3-butanediol were the most prominent in all samples. However, despite the unknown odor thresholds in oil and air (van Gemert, 2011), these two alcohols are expected to have no impact on chocolate flavor and are therefore absent in Table 3. The concentration of the alcoholic volatiles, particularly 2-phenylethanol, was substantially lower in the chocolates compared to the cocoa liquors. 2-Phenylethanol is the most odor-active alcohol with equal amounts in the cocoa liquors and chocolates. It is expected to confer its typical floral note to the chocolates. Despite the low amounts of 2-heptanol in both chocolates, this volatile has a low OTV and, therefore, contributes highly to the fruity aroma. This is in accordance with the results of Rottiers et al. (2019) who studied the volatile aroma composition of fine-flavor Trinitario cocoa beans.

The aldehyde fraction contained 2-methylpropanal, and 2- and 3-methylbutanal, odorants contributing to a distinct dark chocolate flavor (Counet et al., 2002). These volatiles are formed through Strecker degradation and are derived from the amino acids valine, leucine, and isoleucine, respectively (Afoakwa et al., 2008). Although the amounts of these flavor aldehydes in EC-CL were significantly lower than in WA-CL, no significant differences were found between the chocolates. Hinneh et al. (2019b) found that high roasting intensities cause a decrease in aldehydes, due to conversion of these Strecker intermediates or their precursors into other volatiles. Benzaldehyde was the most predominant aldehyde (Table 2). With its typical roasted almond odor, it is expected to contribute to the flavor of both liquors and chocolates.

In both chocolates, a reduction of most esters was found to an undetectable level, while two important esters remained: 2-phenylethylacetate and butyl benzoate. The former is odor active and likely to confer its honey, floral, fruity character to the flavor of both liquors and chocolates. The banana-like note of 3-methylbutylacetate in the WA-CL was not retained in the chocolate.

Among the ketones detected, 2-nonanone was the most abundant and highest in WA-CL. In addition, acetophenone, 3-hydroxy-2-butanone and 1-hydroxy-2-propanone were detected in relatively high concentrations in the cocoa liquors. 2-Nonanone and 2,3-butanedione might contribute to the aroma. Interestingly, the concentration of 2,3-butanedione (buttery note (Januszewska, 2018)) did not decrease after conching in the Ecuadorian sample, but could no longer be detected in the WA-CH. Rottiers et al. (2019) proved that 2-nonanone highly contributes to the fruity aroma, while 2,3-butanedione was responsible for buttery, creamy flavor notes.

Nitrogenous heterocyclic components, such as pyrazines derived from Maillard reactions, emerged as interesting volatiles in dark chocolate. Cocoa, hazelnut, coffee, roasted, and popcorn-like attributes of those pyrazines are considered to be highly desirable for chocolate flavor (Afoakwa, 2011). Concentrations of these cyclic nitrogen-containing molecules were highest in the WA-CL. From the 12 detected pyrazines, 10 remained after conching. However, taking into consideration the odor threshold value in oil, only 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine are aroma active in all samples; 2,3-dimethyl-5-ethylpyrazine (roasted cocoa) only in CL and 3-ethyl-2,5-dimethylpyrazine (caramel) only in WA-CL. Moreover, WA-CL was extremely rich in tetramethylpyrazine; more than 87 times higher than in EC-CL. However, the contribution of the last pyrazine to the flavor is negligible due to its high OTV (OAV < 1). The processing of cocoa liquors into chocolates had a major influence on the total pyrazine level. These results correlate well with results of Hinneh et al. (2018) who also found that

304 tetramethylpyrazine was not odor-active, while trimethylpyrazine was, but the most odor-active
305 pyrazine was 2,3-dimethyl-5-ethylpyrazine.

306 The formation of furans, furanones, pyrans and pyrroles mainly occurs by caramelization of sugars. Only
307 γ-butyrolactone is odor active and imparts a sweet, caramel like flavor. The impact on the overall flavor
308 of linalool can likely also be assessed as considerable, due to its relatively low OTV (37 ng/g) (van
309 Gemert, 2011). This component contributes to the flowery, green note in chocolate aroma. No
310 significant difference in linalool content was observed between both chocolates, although the amount
311 in WA-CL was much higher.

312 PCA was carried out, based on the two datasets shown in Tables 2 and 3, to obtain more information
313 about the samples and their differences. The PCA-score plot based on the levels of all volatile
314 components (Table 2) is presented in Figure 1. The score plot of the PCA based on the OAVs alone (Table
315 3) is highly similar and can be consulted in the Supplementary Information, Figure S1. It can be
316 concluded that quantification of these 20 odor-active compounds is sufficient to discriminate the
317 samples.

318 The PCA score plot of the first two principal components shows a clear distinction between the four
319 types of samples, with PC1 accounting for 88.9% and PC2 8.7% of the total variance and 92.8% and
320 5.7%, respectively, when only data of the odor active compounds are used . Samples of the two
321 different liquors and chocolates clustered well in both score plots. EC-CL, and to a lesser extent EC-CH,
322 were related to the floral and buttery components such as 2-phenylethanol, 2-heptanol, γ-
323 butyrolactone and 2,3-butanedione, as could be deduced from the loading plot (Figure 2). WA-CL could
324 be associated with typical malty and chocolate notes such as 2- and 3-methylbutanal and all roasted

components, such as 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine. WA-CH was not characterized by any typical flavor component.

3.3 Non-volatile secondary metabolite composition

For identification purposes, the chromatographic and spectral information, without and with MS fragmentation, were analyzed. Structures were assigned to unknown peaks only when both the *m*/*z*-ratios and molecular formulae of the precursor and product ions were in agreement. Additional information for dereplication was often acquired from in-house and commercial compound databases and peer reviewed publications. The tentatively identified compounds in EC-CL and EC-CH and the diagnostic chromatographic and HRMS data used for compound identification can be found in Table S2, Supplementary Information. Unless otherwise stated (vide infra), the compounds were also present in the WA-CL and WA-CH. This table also specifies the literature consulted for confirmation of compound identity.

Cocoa is a rich source of catechins and procyanidins, which was also observed in the current study. Next to the major compounds catechin and epicatechin (identified with reference standards), several (epi)catechin hexoside and (epi)catechin sulfonic acid isomers were tentatively identified (their fragmentation was similar to that described by Patras, Milev, Vrancken and Kuhnert (2014)). Furthermore, a multitude of procyanidins ranging from dimeric to heptameric isomers was tentatively identified (Table S2, Supplementary Information). The fragmentation pattern of the detected procyanidins was similar to that of an analytical standard solution of procyanidin B2 and was in accordance with the fragmentation patterns described previously (Patras et al., 2014; Bijttebier et al., 2016). Glycosylated (pro)cyanidins (hexosides and pentosides of procyanidin A and cyanidin) were also

347 detected. However, when comparing the samples of EC with those of WA, both cyanidin glycosides
348 (cyanidin-3-O-galactoside and cyanidin-3-O-arabinoside) were present in higher levels in the former.
349 Three trimeric procyanidins were tentatively identified based on their (de)protonated molecules: one
350 containing one A-type and one B-type connection at 13.24 min, the other two containing two A-type
351 connections at 14.57 and 15.87 min. However, this could not be confirmed by MS^2 because of low signal
352 intensities, as was the case in the study of Patras et al. (2014). Interestingly, these procyanidins could
353 clearly be detected in the EC-CL an EC-CH, but the trimeric A-type procyanidins at 14.57 and 15.87 min
354 were not found in the WA samples, nor did the WA-CH contain the trimeric procyanidin eluting at 13.24
355 minutes. Furthermore, the hexameric and heptameric procyanidins were far more abundant in the EC
356 samples, compared to the WA samples.
357 Several other flavonoids such as quercetin, and glycosides thereof, luteolin and an apigenin-hexoside
358 were also tentatively identified (Table 2, Supplementary Information). In addition, some
359 hydroxycinnamic acid-amino acid conjugates such as coumaroyl-aspartate, clovamide (caffeoyl-
360 dihydroxyphenylalanine) and dideoxyclovamide (coumaroyl-tyrosine) were detected, in accordance
361 with Kothe (2013) and Arlorio et al. (2008). A wide variety of phenolic constituents was thus tentatively
362 identified in the cocoa samples in the current study.
363 Three unknown structures corresponding to molecular formulae $C_{14}H_{24}O_{10}$, $C_{17}H_{30}O_{10}$ and $C_{19}H_{34}O_{10}$
364 were detected in positive and negative ionization mode. These unknown compounds with similar
365 fragmentation patterns were previously detected by Patras et al. (2014). In negative ionization mode
366 the three compounds all fragmented to render the pseudomolecular ions $[M\text{-}H\text{-}C_4H_6O_3]^-$ (loss of 102
367 Da) and $[M\text{-}H\text{-}C_6H_8O_4]^-$ ions (loss of 144 Da), which can be indicative for the presence of a 3-hydroxy-3-
368 methylglutaryl substituent, as previously described by Mencherini et al. (2013) in citrus fruits. Another
369 product ion that was detected in this study at 161 Da (most probable molecular formula $C_6H_9O_5$)

370 corresponded to a deprotonated hexose sugar moiety with the loss of water. In positive ion mode the
371 loss of CO_2 was observed, indicative for a carboxyl group, which is in agreement with the presence of a
372 3-hydroxy-3-methylglutaryl moiety. Notwithstanding the indications for a 3-hydroxy-3-methylglutaryl
373 and a hexose moiety, further research is required to reveal the structures of these unknowns.

374 For completeness' sake, several disaccharides, amino acids and organic acids were also observed in the
375 chromatograms of the cocoa extracts. Some compounds, such as cynaroside, epicatechin-4(S)-
376 benzylthioether and (epi)gallocatechin, which have been reported before in cocoa samples were not
377 detected in the current study, probably due to natural phytochemical variations between plants and/or
378 processing conditions (Patras et al., 2014).

379 For quantitative purposes, the cocoa liquors and chocolates were analyzed by UPLC-HRMS in ESI+ mode
380 and 17 compounds could be quantified, including flavonoids, procyanidins B1 and B2, methylxanthines,
381 and some biogenic amines and amino acids (Table 4). With regard to the cocoa liquors, significantly
382 higher levels of catechin, epicatechin, procyanidin B1 and B2, caffeine, theophylline, serotonin,
383 tryptamine and salsolinol were found in the EC-CL, while luteolin, L-tyrosine and tyramine were more
384 abundant in the WA-CL. Only the levels of quercetin, theobromine and L-tryptophan did not differ
385 significantly. When comparing the levels of the different phytochemicals in the two chocolates, less
386 significant differences were found. Catechin, epicatechin and procyanidin B1 and B2 were more
387 abundant in EC-CH, and L-tyrosine was present in higher levels in WA-CH, which is in correspondence
388 to the results of the cocoa liquors. For the other compounds, however, the differences between the
389 two chocolates were not significant. *N*-oleylethanolamine could be identified in all samples by
390 comparison with the retention time and mass spectrum of the reference compound, but could not be
391 quantified, due to poor linearity of the calibration curve.

In general, the phenolic compounds were the most abundant in EC-CL. With regard to the biogenic amines and amino acids, varying results were obtained. For example, L-tyrosine is much more concentrated in the WA samples, and higher levels were also found in these samples for tyramine, but the levels of serotonin and tryptamine, were higher in EC-CL. No general conclusion can be drawn for the differences in the levels of methylxanthine compounds (caffeine, theobromine and theophylline) between the samples from EC and WA. Theobromine levels did not differ significantly, in contrast to caffeine.

Quantitative comparison of phytochemicals in cocoa bean processing products with other studies should be performed with caution, as the content is highly dependent on the origin of the beans and the conditions applied during processing (Wollgast and Anklam, 2000). Indeed, the content of polyphenols in cocoa liquor or chocolate is dependent on, among others, the time and/or temperature of heat treatment and the pH value, and depending from the applied conditions, either these compounds can be retained, or a decrease in polyphenolic compounds can occur (Toker et al., 2019; Afoakwa et al., 2008; Sulistyowati and Misnawi, 2008). In the current study, processing of cocoa liquor into chocolate consisted of mixing, refining and conching. The conching was carried out for 2 h at 60 °C, followed by 4 h at 70 °C, which can be considered common conditions in the production of dark chocolate (Toker et al., 2019; Beckett et al., 2009; Afoakwa et al., 2008). The non-volatile compounds were present in lower amounts in CH compared to CL samples. These lower values are mainly caused by a dilution factor introduced by adding other ingredients during the production of the chocolate samples. However, a certain degree of degradation or conversion of the compounds of interest can not be excluded, based on our data.

Alañón, Castle,, Siswanto, Cifuentes-Gómez and Spencer (2016) investigated the levels of epicatechin and catechin in 41 commercial milk and dark chocolates. In general, epicatechin is present in higher levels than catechin in cocoa samples, and the levels of both flavan-3-ols decrease during the different processing steps from cocoa bean to chocolate. During these steps, polymerization/condensation to procyanidins takes place, but also the epimerization of (-)-epicatechin to (-)-catechin can occur (Hurst, Krake, Bergmeier, Payne, Miller and Stuart, 2011). As mentioned before, the specific processing conditions will affect the final composition of the cocoa liquors and chocolates, and thus, it is no surprise that Alañón et al. (2016) found highly varying levels of catechin and epicatechin in the different chocolates. The ratio of epicatechin:catechin also varied greatly. Our results showed that epicatechin is present in concentrations three to four times higher than catechin, and this is true for both CL and the CH.

With regard to the methylxanthines, the level of caffeine is approximately twice as high in the samples from Ecuador, compared to the samples from West Africa, while theobromine levels did not differ significantly between the same type of samples prepared from cocoa beans of different origin. Zoumas, Kreiser and Martin (1980) reported the levels of theobromine and caffeine in 22 samples of cocoa liquor and found that caffeine levels can vary by a factor 6 to 7, while theobromine levels varied by a factor 2 at most. This is in agreement with our finding that the theobromine levels between samples show less variance than the caffeine levels. Various researchers studied the relationship between methylxanthine levels in cocoa beans and cocoa varieties (Carrillo et al., 2014; Brunetto et al., 2008; Davrieux et al., 2005). Given the fact that methylxanthines are rather stable and do not suffer much loss during further processing into cocoa liquors, a comparison of these data with ours seems justified. Typically, cocoa of the Forstero variety shows lower levels of caffeine as such, and higher theobromine/caffeine ratios, compared to Trinitario and Criollo varieties. This is in correspondence to our data, showing that the

436 level of caffeine in the WA samples is about half of the level in the fine-flavor sample. In addition, the
437 ratio of theobromine/caffeine in the cocoa liquor samples was calculated as 4.7:1 for EC-CL and 9.2:1
438 for WA-CL, so the ratio is clearly higher in the bulk cocoa sample.

439

440 Finally, PCA was carried out to obtain more information about the four types of samples and their
441 differences. Two approaches were followed: a targeted one using the results of the quantified
442 compounds, and an untargeted one using the complete UPLC-HRMS data-set. PCA based on the results
443 of the quantified compounds showed a clear distinction between the four types of samples (Figure 3).
444 PC1 explained 90.6% and PC2 8.8% of the total variance. According to the loading plot (Figure 3), the
445 cocoa liquors are characterized by higher levels of theobromine, L-tryptophan, luteolin and quercetin
446 compared to the chocolates. The samples from Ecuador typically contain procyanidin B2 and serotonin
447 in relatively high amounts, while a higher level of L-tyrosine and tyramine is characteristic for the
448 samples of West Africa.

449 The second PCA was carried out after data pre-processing of the raw UPLC-HRMS data with
450 Worklfow4Metabolomics. All data between 0 and 24 min were taken into account, but features with
451 >50% missing values were removed. This resulted in a total of >10,000 features, and thus this PCA is
452 based on a much more thorough data-comparison. In this case, PC1 could account for 64.9% of the
453 total variance, PC2 for 4.6% and PC3 for 4.5%. Interestingly, in the score plot of PC1 vs. PC2 (Figure 4),
454 chocolates and cocoa liquors can be easily distinguished, but samples of the same type and of different
455 origin are not clearly separated into different clusters. The division in four distinct clusters is clear
456 though, when PC1 is plotted against PC3. PCA of cocoa samples, based on compounds quantified by
457 GC-MS was carried out before (Cambrai, Marcic, Morville, Houer, Bindler and Marchioni, 2010; Tran et

458 al., 2015). However, this is the first report of PCA, based on the complete dataset resulting from UPLC-
459 HRMS analysis of CL and CH samples. In this case even minor compounds, and/or compounds which
460 could not be identified due to the absence of the corresponding reference compound, or due to the
461 fact that the MS^2 analysis was not conclusive, are taken into account.

462 Thus, either PCA based on the limited set of quantified compounds, or based on the complete raw
463 dataset, proved to be capable of distinguishing the four samples analyzed in this work. Future
464 research is needed though, to confirm these findings, since only a limited number of samples, which
465 differed strongly, were analyzed here. When choosing for one of the two types of PCA for assessing
466 variance amongst a set of samples, various factors need to be taken into account. For example, in
467 case of using the complete raw dataset, there is no need to analyze the reference compounds and to
468 generate calibration curves. This can save (instrument)time and money, which can be considered an
469 important advantage. However, when using the results of the quantified compounds, it is easier to
470 deduce which compounds are the most distinctive and data pre-processing and –analysis are less
471 complicated. The preferred outcome will define which method is the most appropriate.

472 **4. Conclusion**

473 In this study, cocoa liquor and chocolate, produced with either a bulk cocoa blend of *Theobroma cacao*
474 beans from West Africa or with fine-flavor cocoa beans of the Nacional variety, grown in Ecuador, were
475 analyzed for a wide range of properties. The scope of this study was broader than most researches on
476 cocoa, since a wide range of compounds and characteristics of the samples has been investigated.
477 Volatile and non-volatile constituents were analyzed, both influencing the final flavor of chocolate.

478 Most of the analyses showed clear differences between the West African and Ecuadorian cocoa
479 products. The levels of various compounds were examined by GC-MS and UPLC-HRMS, and in general

higher levels of non-volatile phytochemicals were found in the samples produced with the fine-flavor beans originating from Ecuador, compared to those produced with West African beans. In contrast, higher levels of volatile compounds were found in the West African cocoa liquor than in the Ecuadorian cocoa liquor, and the same trend was seen in the chocolates, although less pronounced.

The UPLC-HRMS methodology is of added value because of its more extended approach compared to previous analyses of this type of samples. The UPLC analysis with MS^2 detection allowed for the (tentative) identification of 40 substances. Quantification of both phenolic and non-phenolic compounds was performed. PCA was carried out, using different data-sets, obtained by UPLC-HRMS and GC-MS analysis, thus taking into account not only the major, but also the minor constituents.

In summary, in this work an approach is proposed to analyse cocoa samples in order to obtain in-depth knowledge of the composition of the samples, and to carry-out a more thorough comparison between the samples. In future research, this methodology could be applied for distinguishing, and perhaps classifying, a broader range and variety of cocoa samples.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

References

1. Afoakwa, E. O., Paterson, A., Fowler, M., Ryan, A., (2008). Flavor Formation and Character in Cocoa and Chocolate : A Critical Review. *Critical Reviews in Food science and Nutrition, 48(9),* 840-857. http://dx.doi.org/10.1080/10408390701719272.

2. Afoakwa, E.O. (2011). Chocolate Science and Technology (Google eBook). Wiley Blackwell. Retrieved from http:///book.google.com/books?id=404r7yb-618C&pgis=1

3. Afoakwa, E.O., Quao, J., Takrama, J., Budu, A.S., Saalia, F.K. (2013). Chemical composition and physical quality characteristics of Ghanaian cocoa beans as affected by pulp pre-conditioning and fermentation. *Journal of Food Science and Technology, 50(6),* 1097-1105. http://dx.doi.org/10.1007/s13197-011-0446-5.

4. Alañón, M.E., Castle, S.M., Siswanto, P.J., Cifuentes-Gómez, T., Spencer, J.P.E. (2016). Assessment of flavanol stereoisomers and caffeine and theobromine content in commercial chocolates. *Food chemistry*, *208*, 177-184. http://dx.doi.org/10.1016/j.foodchem.2016.03.116.

5. Aprotosoaie, A.C., Luca, S.V., Miron, A. (2016a). Flavor chemistry of cocoa and cocoa products – an overview. *Comprehensive Reviews in Food Science and Food Safety, 15(1),* 73-91. https://doi.org/10.1111/1541-4337.12180.

6. Aprotosoaie, A.C., Miron, A., Trifan, A., Luca, S.V., Costache, I-I. (2016b). The cardiovascular effects of cocoa polyphenols – an overview. *Diseases, 4, 39.* https://doi.org/10.3390/diseases4040039.

7. Arlorio, M., Locatelli, M., Travaglia, F., Coïsson, J.D., Del Grosso, E., Minassi, A., Appendino, G., Martelli, A. (2008). Roasting impact on the contents of clovamide (N-caffeoyl-L-DOPA) and the antioxidant activity of cocoa beans (*Theobroma cocoa* L.). *Food Chemistry, 106(3),* 967–975. https://doi.org/10.1016/j.foodchem.2007.07.009.

528 8. Beckett, S.T. (2009). *Industrial chocolate manufacture and use.* (4th ed.). West Sussex (UK):
529 Blackwell Publishing Ltd (Chapter 1, 2 & 6).
530 9. Bijttebier, S., Van der Auwera, A., Voorspoels, S., Noten, B., Hermans, N., Pieters, L., Apers, S.
531 (2016). A First step in the quest for the active constituents in *Filipendula ulmaria* (Meadowsweet):
532 comprehensive phytochemical identification by liquid chromatography coupled to quadrupole-
533 orbitrap mass spectrometry. *Planta Medica, 82(6),* 559-72. https://doi.org/10.1055/s-0042-
534 101943.
535 10. Brunetto, M.R., Gutierrez, L., Delgado, Y., Gallignani, M., Zambrano, A., Gomez, A., Ramos, G.,
536 Romero, C. (2007). Determination of theobromine, theophylline and caffeine in cocoa samples by
537 a high-performance liquid chromatographic method with on-line sample cleanup in a switching-
538 column system. *Food Chemistry, 100,* 459-467. https://doi.org/10.1016/j.foodchem.2005.10.007
539 11. Caligiani, A., Palla, L., Acquotti, D., Marseglia, A., Palla, G. (2014). Application of ^{1}H NMR for the
540 characterisation of cocoa beans of different geographical origins and fermentation levels. *Food*
541 *Chemistry, 157*, 94-99. https://doi.org/10.1016/j.foodchem.2014.01.116.
542 12. Cambrai, A., Marcic, C., Morville, A., Houer, P.S., Bindler, F., Marchioni E. (2010). Differentiation of
543 chocolates according to the cocoa's geographical origin using chemometrics. *Journal of*
544 *Agricultural and Food Chemistry, 58(3),* 1478-1483. https://doi.org/10.1021/jf903471e.
545 13. Carrillo, L.C., Londoño-Londoño, J., Gil, A. (2014). Comparison of polyphenol, methylxanthines and
546 antioxidant activity in *Theobroma cacao* beans from different cocoa-growing areas in Colombia.
547 *Food Research International, 60,* 273-280. https://doi.org/10.1016/j.foodres.2013.06.019
548 14. Codex Standards 141-1983: Standard for cocoa (cacao) mass (cocoa/chocolate liquor) and cocoa
549 cake.
550 Codex Alimentarius 1983, revision 2001.

15. Counet, C., Callemien, D., Ouwerx, C., Collin, S. (2002). Use of Gas Chromatography − Olfactometry To Identify Key Odorant Compounds in Dark Chocolate . Comparison of Samples before and after Conching. *Journal of Agricultural and Food Chemistry, 50(8),* 2385-2391. https://doi.org/10.1021/jf0114177.
16. Crozier S.J., Hurst W.J. (2014). Cocoa polyphenols and cardiovascular health. In: Ross Watson R., Preedy V.R., Zibadi S., editors. Polyphenols in human health and disease. Waltham: Academic Press. p. 1077–1082.
17. Davrieux, F., Assemat, S., Sukha, D.A., Bastianelli, D., Boulanger, R., Cros, E. (2007). Genotype characterization of cocoa into genetic groups through caffeine and theobromine content predicted by near infra red spectroscopy. In : Near infrared spectroscopy : Proceedings of the 12th International Conference, Auckland, New Zealand, 9th - 15th April 2005. Burling-Claridge G.R. (ed.), Holroyd S.E. (ed.), Sumner R.M.W. (ed.). NIRCE. Chichester : IM Publications, pp. 382-386. ISBN 978-0-473-11646-0|978-0-473-11746-7
18. do Carmo Brito, B.N., Chisté, R.C., da Silva Pena, R., Abreu Gloria, M.B., Santos Lopes, A. (2017). Bioactive amines and phenolic compounds in cocoa beans are affected by fermentation. *Food Chemistry, 228,* 484-490. https://doi.org/10.1016/j.foodchem.2017.02.004.
19. Frauendorfer, F., Schieberle, P. (2008). Changes in key aroma compounds of Criollo cocoa beans during roasting. *Journal of Agricultural and Food Chemistry*, *56(21)*, 10244-10251. https://doi.org/10.1021/jf802098f.
20. Hinneh, M., Semanhyia, E., Walle, Van De Walle, D., De Winne, A., Tzompa-Sosa, D. A., Scalone G.L.L., De Meulenaer, B. Messens, K., Van Durme, J. Afoakwa, E.O., De Cooman, L., Dewettinck,K. (2018). Assessing the influence of pod storage on sugar and free amino acid profiles and the

implications on some Maillard reaction related flavor volatiles in Forastero cocoa beans. *Food Research International*, *111*, 607–620. https://doi.org/10.1016/j.foodres.2018.05.064.

21. Hinneh, M., Van de Walle, D., Tzompa-Sosa, D.A., De Winne, A., Termote, S., Messens, K., Van Durme, J., Afoakwa, E.O., De Cooman, L., Dewettinck, K. (2019a). Tuning the aroma profiles of *FORASTERO* cocoa liquors by varying pod storage and bean roasting temperature. *Food Research International, 125,* 108550. https://doi.org/10.1016/j.foodres.2019.108550.
22. Hinneh, M., Van de Walle, D., Tsomp-Sosa, D.A., Haeck J., Abotsi, E.E., De Winne, A. Messens, K. Van Durme, J. Afoakwa, E.O., De Cooman, L., Dewettinck, K. (2019b). Comparing flavor profiles of dark chocolates refined with melanger and conched with Stephan mixer in various alternative chocolate production techniques. *European Food Research and Technology*, *245*(4), 837-852. https://doi.org/10.1007/s00217-018-3221-2.
23. Hurst, W.J., Krake, S.H., Bergmeier, S.C., Payne, M.J., Miller, K.B., 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.* https://doi.org/10.1021/jf102391q.
24. ICCO (2018). ICCO Quarterly Bulletin of Cocoa Statistics, Vol. XLIV, No. 3, Cocoa year 2017/18. Published: 31-08-2018.
25. Januszewska, R. (2018) Hidden persuaders in cocoa and chocolate, a flavor lexicon for cocoa and chocolate sensory professionals. Woodhead publishing, an imprint of Elsevier.
26. Kadow, D., Bohlmann, J., Philips, W., Lieberei, R. (2013). Identification of main fine flavor components in two genotype of the cocoa tree (*Theobroma cacao L.*). *Journal of Applied Botany and Food Quality, 86(1)*, 90-98. https://doi.org/10.5073/JABFQ.2013.086.013.

27. Kothe, L. (2013). Analyse technologisch bedingter Veränderungen phenolischer Verbindungen in Kakao und Kakaoprodukten mit LC-MS und Kapillarelektrophorese. PhD thesis, Rheinischen Friedrich-Wilhelms-Universität Bonn.
28. Luna, F., Crouzillat, D., Cirou, L., Buchelli, P. (2002). Chemical composition and flavour of Ecuadorian cocoa liquor. *Journal of Agricultural and Food Chemistry, 50(12),* 3527-3532. https://doi.org/10.1021/jf0116597.
29. Mencherini, T., Campone, L., Piccinelli, A.L., García, Mesa M., Dulce Armenteros, D.M., Aquino, R.P., Rastrelli, L. (2013). HPLC-PDA-MS and NMR characterization of a hydroalcoholic extract of *Citrus aurantium* L. var. *amara* peel with antiedematogenic activity. *Journal of Agricultural and Food Chemistry, 61 (8),* 1686–1693. https://doi.org/10.1021/jf302815t.
30. Natsume, M., Osakabe, N., Yamagishi, M., Takizawa, T., Nakamura, T., Miyatake, H., Hatano, T., Yoshide, T. (2000). Analyses of polyphenols in cacao liquor, cocoa and chocolate by normal-phase and reversed-phase HPLC. *Bioscience, Biotechnology and Biochemistry, 64 (12)*, 2581-2587. https://doi.org/10.1271/bbb.64.2581.
31. Oracz, J., Nebesny, E. (2014) Influence of roasting conditions on the biogenic amine content in cocoa beans of different *Theobroma cacao* cultivars*. Food Research International, 55,* 1-10. https://doi.org/10.1016/j.foodres.2013.10.032.
32. Owusu, M., Petersen, M. A., & Heimdal, H. (2011). Effect of fermentation method , roasting and conching conditions on the aroma volatiles of dark chocolate. *Journal of food processing and preservation, 36(5),* 446–456. https://doi.org/10.1111/j.1745-4549.2011.00602.x.
33. Patras, M.A., Milev, B.P., Vrancken, G., Kuhnert, N. (2014). Identification of novel cocoa flavonoids from raw fermented cocoa beans by HPLC–MS^n. *Food Research International, 63,* 353–359. https://doi.org/10.1016/j.foodres.2014.05.031.

34. Rottiers, H., Tzompa Sosa, D.A., Van de Vyver, L., Hinneh, M., Everaert, H., De Wever, J., Messens, K., Dewettinck, K. (2018). Discrimination of cocoa liquors based on their odor fingerprint: a fast GC electronic nose suitability study. *Food Analytical Methods, 12(2),* 475-488. https://doi.org/10.1007/s12161-018-1379-7.

35. Rottiers, H., Tzompa Sosa, D.A., De Winne, A., Ruales, J., De Clippeleer, J., De Leersnyder, I., De Wever, J., Everaert, H., Messens, K., Dewettinck, K. (2019). Dynamics of volatile compounds and flavor precursors during spontaneous fermentation of fine flavor Trinitario cocoa beans. *European Food Research and Technology*, https://doi.org/10.1007/s00217-019-03307-y.

36. Sulistyowati, M., Misnawi, J. (2008). Effects of alkali concentration and conching temperature on antioxidant activity and physical properties of chocolate. *International Food Research Journal, 15(3),* 297-304.

37. Toker, O.S., Palabiyik, I., Konar, N. (2019). Chocolate quality and conching. *Trends in Food Science & Technology, 91,* 446-453. https://doi.org/10.1016/j.tifs.2019.07.047.

38. Torres-Moreno, M., Torrescasana, E., Salas-Salvadó, J., Blanch, C. (2015). Nutritional composition and fatty acids profile in cocoa beans and chocolates with different geographical origin and processing conditions. *Food Chemistry, 166,* 125-132. https://doi.org/10.1016/j.foodchem.2014.05.141.

39. Tran, P.D., Van de Walle, D., De Clercq, N., De Winne, A., Kadow, D., Lieberei, R., Messens, K., Tran, D.N., Dewettinck, K., Van Durme, J. (2015). Assessing cocoa aroma quality by multiple analytical approaches. *Food Research International, 77,* 657-669. https://doi.org/10.1016/j.foodres.2015.09.019.

40. Tran, P. D., Van Durme, J., Van De Walle, D., De Winne, A., Delbaere, C., De Clercq, N., Phan, T.T.Q., Nguyen, C.P., Tran, D.N., Dewettinck, K. (2016). Quality attributes of dark chocolate

produced from Vietnamese cocoa liquors. *Journal of Food Quality, 39 (4),* 311–322. https://doi.org/10.1111/jfq.12200.

41. Tuenter, E., Foubert, K., Pieters, L. (2018). Mood components in cocoa and chocolate: the mood pyramid. *Planta Medica, 84(12/13)*, 839-844. https://doi.org/10.1055/a-0588-5534.
42. Van Durme, J., Ingels, I., De Winne A. (2016). Inline roasting hyphenated with gas chromatography-mass spectrometry as an innovative approach for assessment of cocoa fermentation quality and aroma formation potential. *Food chemistry, 205,* 66-72. https://doi.org/10.1016/j.foodchem.2016.03.004.
43. van Gemert, L.J. (2011). Odour thresholds: Compilations of odour threshold values in air, water and other media. Den Haag: Oliemans Punter & Partners BV, Nederland.
44. 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. https://doi.org/10.1016/S0963-9969(00)00068-5.
45. Zoumas ,B.L., Kreiser, R., Martin, R.A. (1980). Theobromine and caffeine content of chocolate products. *Journal of Food Science*, *45*, 314-316. https://doi.org/10.1111/j.1365-2621.1980.tb02603.x.

Figure captions

Figure 1. PCA-score plot of volatile components in cocoa liquors (CL) and chocolates (CH) of Ecuador (EC) and West Africa (WA).

Figure 2. PCA-loading plot of odor-active volatile components in cocoa liquors (CL) and chocolates (CH) of Ecuadro (EC) and West Africa (WA).

Figure 3. PCA-score plot (left) and loading plot (right) of quantified non-volatile components in cocoa liquor (CL) and chocolates (CH) of Ecuador (EC) and West Africa (WA).

Figure 4. PCA-score plots of non-volatile components in cocoa liquor (CL) and chocolates (CH) of Ecuador (EC) and West Africa (WA).

672 **Table 1.** Nutritional composition of the cocoa liquors (CL) from Ecuador (EC) and West Africa (WA).

Proximates (g/100 g)	EC-CL	WA-CL
Moisture	1.13 (1.09, 1.19)	1.35 (1.28, 1.45)
Fat	54.8 (54.7, 54.8)	55.0 (54.9, 55.2)
Protein	15.6 (15.4, 15.8)	14.7 (14.6, 14.8)
Total dietary fiber	14.1 (13.7, 14.4)	19.8 (19.3, 20.3)
Ash	2.8 (2.8, 2.9)	3.3 (3.2, 3.3)

673

Table 2. Semi-quantitative concentrations measured by HS-SPME GC-MS (expressed as ng/g undecane equivalents) of aroma volatiles identified in cocoa liquors (CL) and chocolates (CH) of Ecuador (EC) and West Africa (WA). Data are mean ± SD (*n* = 3). KI = Kovats retention index, exp = experimental, lit = literature.

	EC-CL	WA-CL	EC-CH	WA-CH	KI (exp)	KI (lit)*
Acids						
Acetic acid	$13.06 \cdot 10^{3\,b} \pm 0.98 \cdot 10^3$	$33.7 \cdot 10^{3\,a} \pm 2.2 \cdot 10^3$	$5.07 \cdot 10^{3\,c} \pm 0.96 \cdot 10^3$	$6.5 \cdot 10^{3\,c} \pm 1.1 \cdot 10^3$	1418	1404 - 1477
Butyric acid	n.d.	$89^a \pm 11$	n.d.	n.d.	1604	1607 - 1663
3-Methylbutyric acid	n.d.	$1{,}02 \cdot 10^{3\,a} \pm 0.20 \cdot 10^3$	n.d.	n.d.	1634	1624 - 1665
Alcohols						
Ethanol	$350^a \pm 50$	$53.3^b \pm 7.9$	$70.4^b \pm 8.8$	$14.8^b \pm 3.2$	949	929 - 959
Isobutanol	$22.7^a \pm 4.1$	n.d.	$6.1^b \pm 0.9$	n.d.	1087	1054 - 1125
2-Pentanol	$95^b \pm 14$	$210^a \pm 13$	n.d.	n.d.	1121	1118
2-Methyl-1-butanol	$19.4^b \pm 2.3$	$28.8^a \pm 4.4$	$6.4^c \pm 1.2$	n.d.	1208	
3-Methyl-1-butanol	$83.2^a \pm 8.3$	$85.1^a \pm 7.9$	$24.8^b \pm 3.6$	n.d.	1219	1205 - 1247
2-Heptanol	$249^b \pm 40$	$361^a \pm 31$	$22.4^c \pm 2.9$	$6.4^c \pm 0.6$	1330	1273 - 1332
2,3-Butanediol	$1.30 \cdot 10^{3\,b} \pm 0.13 \cdot 10^3$	$4.33 \cdot 10^{3\,a} \pm 0.44 \cdot 10^3$	$553^c \pm 92$	$0.99 \cdot 10^{3\,b,c} \pm 0.15 \cdot 10^3$	1516	1492 - 1582
1,3-Butanediol	$1.09 \cdot 10^{3\,b} \pm 0.10 \cdot 10^3$	$2.30 \cdot 10^{3\,a} \pm 0.14 \cdot 10^3$	$488^c \pm 88$	$470^c \pm 83$	1546	1566 - 1600
1-Phenylethanol	$28.3^b \pm 3.6$	$95.4^a \pm 5.0$	$5.7^c \pm 0.4$	$6.5^c \pm 0.7$	1723	
2-Phenylethanol	$1.39 \cdot 10^{3\,a} \pm 0.23 \cdot 10^3$	$1.75 \cdot 10^{3\,a} \pm 0.11 \cdot 10^3$	$323^b \pm 28$	$184^b \pm 20$	1810	1837 - 1927
Benzyl alcohol	$59.5^b \pm 5.5$	$124^a \pm 18$	$71.7^b \pm 5.7$	$79.8^b \pm 7.2$	1840	1837 - 1864
Aldehydes						
2-Methylpropanal	$31.6^b \pm 2.3$	$93.4^a \pm 7.6$	$13.2^c \pm 1.2$	$14.3^c \pm 2.5$	814	611 - 834
2-Methylbutanal	$27.3^b \pm 5.7$	$115.5^a \pm 0.8$	$7.9^c \pm 1.6$	$10.2^c \pm 1.8$	935	864 - 936
3-Methylbutanal	$132^b \pm 12$	$593^a \pm 25$	$64^c \pm 11$	$88^{b,c} \pm 16$	937	912 - 936
Hexanal	$34.9^b \pm 4.4$	$63.1^a \pm 6.5$	$26.4^b \pm 1.5$	$28.4^b \pm 2.7$	1068	1059 - 1089
5-Methyl-2-isopropyl-2-hexenal	n.d.	$217^a \pm 16$	n.d.	n.d.	1374	1388
Benzaldehyde	$215^b \pm 18$	$1.58 \cdot 10^{3\,a} \pm 0.15 \cdot 10^3$	$99^b \pm 13$	$275^b \pm 29$	1499	1470 - 1568
2-Phenyl-2-butenal	$17.2^b \pm 3.1$	$244^a \pm 18$	$10.1^b \pm 1.6$	$19.4^b \pm 1.9$	1862	1872 - 1965
5-Methyl-2-phenyl-2-hexenal	$12.2^b \pm 2.3$	$118.0^a \pm 9.4$	$4.1^b \pm 0.5$	$8.9^b \pm 1.3$	2038	2024 - 2083
Esters						
Methyl acetate	$21.6^b \pm 1.5$	$88^a \pm 11$	$7.1^b \pm 2.6$	$7.2^b \pm 2.0$	888	828 - 864
Pentyl acetate	n.d.	$247^a \pm 16$	n.d.	n.d.	1052	1050 - 1080
2-Methylbutyl acetate	n.d.	$130.6^a \pm 2.2$	n.d.	n.d.	1097	1055 - 1147
3-Methylbutyl acetate	n.d.	$7.1 \cdot 10^{2\,a} \pm 1.2 \cdot 10^2$	n.d.	n.d.	1109	1100 - 1117
Heptyl acetate	$32.7^b \pm 3.2$	$112.7^a \pm 9.9$	n.d.	n.d.	1278	1250 - 1266

	EC-CL	WA-CL	EC-CH	WA-CH	KI (exp)	KI (lit)*
Ethyl isobutanoate	n.d.	$108.1^{a} \pm 4.1$	n.d.	n.d.	1588	
4-Ethylphenyl acetate	$26.1^{b} \pm 2.6$	$95.3^{a} \pm 7.7$	n.d.	n.d.	1700	1724
2-Phenylethyl acetate	$74.7^{b} \pm 8.4$	$533^{a} \pm 50$	$28.6^{b} \pm 2.1$	$53.3^{b} \pm 4.4$	1757	1771 - 1821
Butyl benzoate	$71^{b} \pm 11$	$160.2^{a} \pm 9.3$	$22.5^{c} \pm 2.2$	$22.3^{c} \pm 2.4$	1808	1839
Ketones						
2-Butanone	n.d.	$87.5^{a} \pm 2.3$	n.d.	n.d.	930	917 - 950
2,3-Butanedione	$30.5^{b} \pm 4.3$	$68.0^{a} \pm 8.5$	$29.6^{b} \pm 3.8$	n.d.	975	970 - 984
2-Heptanone	$72^{b} \pm 14$	$156.8^{a} \pm 5.3$	$13.4^{c} \pm 1.9$	$6.0^{c} \pm 0.7$	1170	1160 - 1181
1-Hydroxy-2-propanone	$72.1^{b} \pm 5.6$	$173^{a} \pm 14$	$25.9^{c} \pm 4.3$	$29.5^{c} \pm 3.1$	1296	1300 - 1323
3-Hydroxy-2-butanone	$190^{a} \pm 19$	$260^{a} \pm 53$	n.d.	n.d.	1274	1250 - 1295
2-Nonanone	$124^{b} \pm 11$	$434^{a} \pm 41$	$27.8^{c} \pm 2.7$	$28.8^{c} \pm 1.0$	1388	1347 - 1420
Acetophenone	$71.2^{b} \pm 6.7$	$3.6 \cdot 10^{2\ a} \pm 1.5 \cdot 10^{2}$	$16.0^{b} \pm 1.0$	$15.7^{b} \pm 4.6$	1623	1600 - 1655
Pyrazines						
Methylpyrazine	$38.3^{b} \pm 3.4$	$236^{a} \pm 17$	$31.0^{b} \pm 2.2$	$37.1^{b} \pm 5.7$	1266	1235 - 1312
2,5-Dimethylpyrazine	$28.6^{b} \pm 2.9$	$532^{a} \pm 43$	$11.1^{b} \pm 0.4$	$30.9^{b} \pm 5.2$	1323	1290 - 1358
2,6-Dimethylpyrazine	$21.7^{b} \pm 2.1$	$493^{a} \pm 49$	$11.6^{b} \pm 3.5$	$22.2^{b} \pm 3.0$	1328	1300 - 1370
2,3-Dimethylpyrazine	$27.2^{b} \pm 7.5$	$301^{a} \pm 19$	$15.8^{b} \pm 2.3$	$20.5^{b} \pm 0.7$	1346	1315 - 1344
2-Ethyl-6-methylpyrazine	$10.2^{b} \pm 0.6$	$217^{a} \pm 19$	n.d.	$9.1^{b} \pm 0.4$	1376	1381 - 1415
2-Ethyl-5-methylpyrazine	$12.3^{b} \pm 1.4$	$233^{a} \pm 20$	n.d.	$13.2^{b} \pm 1.2$	1384	1386 - 1453
2-Ethyl-3-methylpyrazine	n.d.	$88^{a} \pm 16$	n.d.	$7.4^{b} \pm 1.2$	1392	1397 - 1414
2,3,5-Trimethylpyrazine	$57.8^{b} \pm 3.9$	$1.59 \cdot 10^{3\ a} \pm 0.18 \cdot 10^{3}$	$18.2^{b} \pm 1.6$	$41.0^{b} \pm 5.4$	1404	1381 - 1413
3-Ethyl-2,5-dimethylpyrazine	n.d.	$3.6 \cdot 10^{2\ a} \pm 1.1 \cdot 10^{2}$	n.d.	n.d.	1465	
2,3-Dimethyl-5-ethylpyrazine	$20.9^{b} \pm 1.6$	$4.1 \cdot 10^{2\ a} \pm 1.4 \cdot 10^{2}$	n.d.	n.d.	1468	1445 - 1493
Tetramethylpyrazine	$53.3^{b} \pm 4.8$	$4.67 \cdot 10^{3\ a} \pm 0.50 \cdot 10^{3}$	$67.3^{b} \pm 5.6$	$156^{b} \pm 18$	1472	1438 - 1474
2,3,5-Trimethyl-6-ethylpyrazine	n.d.	$229.6^{a} \pm 30.9$	n.d.	$12.9^{b} \pm 2.6$	1498	
Furans, furanones, lactones, pyrans, pyrroles						
2-Pentylfuran	$24.6^{b} \pm 2.3$	$148.5^{a} \pm 9.4$	$23.8^{b} \pm 2.1$	$24.4^{b} \pm 1.9$	1228	1193 - 1265
2-Methyl-tetrahydro-3-furanone	$10.2^{b} \pm 1.8$	$66.4^{a} \pm 6.3$	$3.9^{b} \pm 0.3$	$7.0^{b} \pm 0.4$	1257	
γ-Butyrolactone	$357^{a} \pm 29$	$408^{a} \pm 50$	$152^{b} \pm 20$	$92^{b} \pm 13$	1564	1595 - 1673
2-Furanmethanol	$103.4^{b} \pm 7.8$	$388^{a} \pm 42$	$37.3^{c} \pm 4.6$	$72.7^{b,c} \pm 8.9$	1633	1614 - 1669
2-Acetylpyrrole	$54.4^{b} \pm 7.6$	$379^{a} \pm 32$	$19.4^{b} \pm 1.9$	$60.8^{b} \pm 7.0$	1951	1930 - 2020
2-Formylpyrrole	$24.9^{b} \pm 6.5$	$100^{a} \pm 23$	$12.1^{b} \pm 0.5$	$27.7^{b} \pm 4.7$	1984	2028 - 2048
2-Pyrrolidinone	$56^{b} \pm 15$	$130.5^{a} \pm 6.0$	$17.4^{c} \pm 1.0$	$26.9^{c} \pm 6.9$	2004	1988 - 2019
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$97^{b} \pm 35$	$381^{a} \pm 39$	$27.3^{b} \pm 2.3$	$54^{b} \pm 18$	2166	2211
Terpenes						
α-Pinene	$53.7^{a} \pm 7.8$	$35.5^{b} \pm 0.6$	n.d.	n.d.	993	
δ-3-Carene	$70^{a} \pm 13$	$33.6^{b} \pm 4.9$	$11.5^{c} \pm 0.8$	n.d.	1136	

	EC-CL	WA-CL	EC-CH	WA-CH	KI (exp)	KI (lit)*
β-Myrcene	18.1^{b} ± 0.4	272^{a} ± 11	n.d.	n.d.	1159	1145 - 1180
Limonene	72.9^{a} ± 7.4	73.4^{a} ± 7.3	70.6^{a} ± 2.5	73.6^{a} ± 9.0	1189	
trans-β-Ocimene	34.3^{b} ± 5.3	284^{a} ± 21	n.d.	n.d.	1241	1225 - 1252
Linalool	146^{b} ± 16	473^{a} ± 50	20.2^{c} ± 2.3	23.8^{c} ± 2.8	1518	1500 - 1537
cis-Linalool oxide	45.1^{b} ± 3.3	275^{a} ± 78	n.d.	n.d.	1431	1423 - 1468
trans-Linalool oxide	15.3^{b} ± 0.9	84^{a} ± 20	n.d.	n.d.	1456	1451 - 1453
Others						
Dimethyltrisulfide	n.d.	47.5^{a} ± 1.3	n.d.	n.d.	1357	1377
Grand total	***$20,394^{b}$ ± 1,610***	***$63,801^{a}$ ± 4,542***	***$7,592^{c}$ ± 1,281***	***$9,673^{c}$ ± 1,552***		

** KI(lit) sourced from webbook.nist.gov or pherobase.com. n.d. = not detected. Different superscripts in a row point out significant differences ($p < 0.05$).*

Table 3. Odor Actvity Values (OAVs) of aroma components in cocoa liquor (CL) and chocolates (CH) of Ecuador (EC) and West Africa (WA). Data are mean ± SD (*n* = 3).

Chemical group	Chemical component	OTV* (ng/g)	Odor Activity Value				Odor description**
			EC-CL	WA-CL	EC-CH	WA-CH	
Acids	Acetic acid	124	105.3^{b} ± 7.9	271.6^{a} ± 17.8	40.9^{c} ± 7.7	52.4^{c} ± 9.1	sour, vinegar
	3-Methylbutyric acid	22	n.d.	46.3^{a} ± 9.2	n.d.	n.d.	acidic, overripe fruit
Alcohols	2-Heptanol	10	24.9^{b} ± 4.0	36.1^{a} ± 3.1	2.2^{c} ± 0.3	0.6^{c} ± 0.1	fruity, citrus, herbal
	2-Phenylethanol	211	6.6^{a} ± 1.1	8.3^{a} ± 0.5	1.5^{b} ± 0.1	0.9^{b} ± 0.1	honey, rummy, floral
Aldehydes	2-Methylpropanal	3.4	9.3^{b} ± 0.7	27.5^{a} ± 2.2	3.9^{c} ± 0.4	4.2^{c} ± 0.7	(un)roasted cocoa,malty
	2-Methylbutanal	2.2	12.4^{b} ± 2.6	52.5^{a} ± 0.4	3.6^{c} ± 0.7	4.7^{c} ± 0.8	(un)roasted cocoa,malty
	3-Methylbutanal	5.4	24.4^{b} ± 2.2	109.8^{a} ± 4.7	11.9^{c} ± 2.0	$16.2^{b,c}$ ± 2.9	(un)roasted cocoa,malty
	Benzaldehyde	60	3.6^{b} ± 0.3	26.3^{a} ± 2.5	1.7^{b} ± 0.2	4.6^{b} ± 0.5	roasted almonds
Esters	Pentyl acetate	13	n.d.	19.0^{a} ± 1.2	n.d.	n.d.	fruity, orange, tropical
	3-Methylbutyl acetate	9.6	n.d.	73.8^{a} ± 12.3	n.d.	n.d.	banana
	2-Phenylethyl acetate	137	0.5^{b} ± 0.1	3.9^{a} ± 0.4	0.2^{b} ± 0.0	0.4^{b} ± 0.0	rose, floral, fruity, honey
Ketones	2,3-Butanedione	4.5	6.8^{b} ± 1.0	15.1^{a} ± 1.9	6.6^{b} ± 0.8	n.d.	buttery
	2-Nonanone	100	1.2^{b} ± 0.1	4.3^{a} ± 0.4	0.3^{c} ± 0.0	0.3^{c} ± 0.0	milky
Pyrazines	2,3-Dimethylpyrazine	123	0.2^{b} ± 0.1	2.4^{a} ± 0.2	0.1^{b} ± 0.0	0.2^{b} ± 0.0	caramel, cocoa
	2,3,5-Trimethylpyrazine	290	0.2^{b} ± 0.0	5.5^{a} ± 0.6	0.1^{b} ± 0.0	0.1^{b} ± 0.0	cocoa, roasted nuts
	3-Ethyl-2,5-dimethylpyrazine	24	n.d.	14.9^{a} ± 4.6	n.d.	n.d.	caramel, cocoa
	2,3-Dimethyl-5-ethylpyrazine	60	0.3^{b} ± 0.0	6.8^{a} ± 2.4	n.d.	n.d.	roasted cocoa
Lactone	γ-Butyrolactone	35	10.2^{a} ± 0.8	11.7^{a} ± 1.4	4.4^{b} ± 0.6	2.6^{b} ± 0.4	sweet, caramel flavor
Terpenes	β-Myrcene	9.18	2.0^{b} ± 0.0	29.6^{a} ± 1.2	n.d.	n.d.	pistachio
	Linalool	37	3.9^{b} ± 0.4	12.8^{a} ± 1.4	0.5^{c} ± 0.1	0.6^{c} ± 0.1	floral

** OTV, odor threshold value according to van Gemert, 2011; n.d. not detected; **Odor description, according to Januszeska (2018), Kadow, Bohlmann, Philips and Lieberei (2013). Different superscripts in a row point out significant differences (p < 0.05).*

Table 4. Levels of various compounds in cocoa liquor (CL) and chocolate (CH) from Ecuador (EC) and West Africa (WA). Values are reported in μg/gram defatted sample. Data are mean ± SD (*n* = 3).

Compound	EC-CL	WA-CL	EC-CH	WA-CH
Flavan-3-ols				
Catechin	$3.2 \cdot 10^3 \pm 0.2 \cdot 10^{3\,a}$	989 ± 20^{b}	$6.610^2 \pm 1.210^{2\,c}$	164 ± 15^{d}
Epicatechin	$10.5 \cdot 10^3 \pm 0.4 \cdot 10^{3\,a}$	$3.5 \cdot 10^3 \pm 0.2 \cdot 10^{3\,b}$	$1.9 \cdot 10^3 \pm 0.2 \cdot 10^{3\,c}$	542.3 ± 8.6^{d}
Proanthocyanidins				
Procyanidin B1	639 ± 39^{a}	224.6 ± 7.3^{b}	180 ± 16^{b}	53.0 ± 2.2^{c}
Procyanidin B2	$6.0 \cdot 10^3 \pm 0.2 \cdot 10^{3\,a}$	$1.12 \cdot 10^3 \pm 0.03 \cdot 10^{3\,b}$	$1.09 \cdot 10^3 \pm 0.08 \cdot 10^{3\,b}$	371 ± 13^{c}
Flavonoids				
Apigenin	0.54 ± 0.09^{a}	0.41 ± 0.03^{a}	0.12 ± 0.02^{b}	0.11 ± 0.02^{b}
Naringenin	1.0 ± 0.1^{a}	0.62 ± 0.04^{b}	0.29 ± 0.09^{c}	0.167 ± 0.008^{c}
Luteolin	3.4 ± 0.2^{b}	4.4 ± 0.1^{a}	$<LOQ^{\alpha}$	$<LOQ^{\alpha}$
Quercetin	88.4 ± 7.7^{a}	78.1 ± 3.8^{a}	21.8 ± 3.9^{b}	17.1 ± 0.9^{b}
Methylxanthines				
Caffeine	$33.8 \cdot 10^3 \pm 3.9 \cdot 10^{3\,a}$	$17.6 \cdot 10^3 \pm 2.5 \cdot 10^{3\,b}$	$9.8 \cdot 10^3 \pm 0.6 \cdot 10^{3\,c}$	$4.3 \cdot 10^3 \pm 0.8 \cdot 10^{3\,c}$
Theobromine	$159 \cdot 10^3 \pm 17 \cdot 10^{3\,a}$	$163 \cdot 10^3 \pm 20 \cdot 10^{3\,a}$	$54.5 \cdot 10^3 \pm 2.7 \cdot 10^{3\,b}$	$48.2 \cdot 10^3 \pm 7.6 \cdot 10^{3\,b}$
Theophylline	13.1 ± 1.8^{a}	7.4 ± 0.2^{b}	2.4 ± 0.1^{c}	$<LOQ^{\beta}$
Amino acids				
L-Tryptophan	76.6 ± 8.3^{a}	78.1 ± 4.5^{a}	14.6 ± 2.4^{b}	16.0 ± 0.8^{b}
L-Tyrosine	454 ± 15^{b}	$1.15 \cdot 10^3 \pm 0.01 \cdot 10^{3\,a}$	134 ± 24^{d}	228 ± 13^{c}
Biogenic amines and related compounds				
Serotonin	68.9 ± 9.1^{a}	12.7 ± 0.9^{b}	11.8 ± 1.6^{b}	2.0 ± 0.1^{b}
Tyramine	9.0 ± 0.3^{b}	12.9 ± 0.4^{a}	2.8 ± 0.8^{c}	3.6 ± 0.5^{c}
Tryptamine	6.6 ± 1.2^{a}	3.1 ± 0.3^{b}	0.77 ± 0.13^{c}	0.38 ± 0.03^{c}
Salsolinol	66.3 ± 7.5^{a}	36.6 ± 2.1^{b}	14.3 ± 1.6^{c}	7.8 ± 0.1^{c}

$^{\alpha}$LOQ = 1.2 μg/g, $^{\beta}$ LOQ = 1.8 μg/g. *Different superscripts in a row point out significant differences ($p < 0.05$).*