Influence of Aerobic and Anaerobic Moist Incubation on Selected Non-Volatile Constituents – Comparison to Traditionally Fermented Cocoa Beans

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Abstract

Recently, moist incubation has been proposed as an alternative postharvest processing method for cocoa beans. During this treatment, unfermented and dried cocoa nibs are rehydrated with a lactic acid solution containing ethanol and subsequently incubated for 72 h at 45 °C before drying. Previous studies focused on the aroma formation during this treatment and the further processing to chocolate. The current study focused on the influence of aerobic and anaerobic moist incubation on selected non-volatile components in comparison with the unfermented raw material and traditionally fermented cocoa. Total phenolic content (TPC) and total flavan-3-ol content (TFC), contents of (+)-catechin, (-)-epicatechin, procyanidins B2 and C1, cinnamtannin A2, methylxanthines (theobromine and caffeine), contents of sugars (sucrose, D-glucose, D-fructose) and free amino acids (17 proteinogenic amino acids) were determined. Fermentation index (FI) was also evaluated. The aerobic incubated and fermented cocoa showed low levels of phenolic compounds in comparison to the unfermented cocoa and the anaerobic incubated cocoa. The level of methylxanthines was unaffected by all treatments. Contents of reducing sugars were more than two-fold higher after both incubation treatments compared to fermentation. The level of free amino acids liberated was highest after anaerobic incubation followed by fermentation and aerobic incubation. The aerobic incubated cocoa showed the highest fermentation index, while the anaerobic incubated cocoa may be considered under fermented (fermentation index < 1.0). Statistical analysis (ANOVA) showed significant differences between all treatments, which was verified by principal component analysis (PCA).

Keywords

Cocoa postharvest treatment; cocoa incubation; polyphenols; aroma precursors

Introduction

Cocoa with its distinct flavor properties serves as the basis for a broad variety of chocolate and confectionary products consumed and highly appreciated worldwide. During traditional postharvest processing, ripe and fresh cocoa beans are subjected to microbial fermentation of the fruit pulp and subsequent drying before further processing to consumable cocoa-based products. After harvest, beans and the surrounding fruit pulp are removed manually from the cocoa pods and put in wooden boxes or on heaps before covering with banana leaves or jute bags.[1] During the early anaerobic stage of fermentation, sugars and citric acid contained in the fruit pulp are metabolized by yeasts and lactic acid bacteria under anaerobic conditions yielding mainly ethanol, CO_2,, and lactic acid.[2] Due to drainage of the fruit pulp, which is supported by pectinolytic activity of certain yeast strains, oxygen availability within the pile of biomass increases.[3] With increasing aerobic conditions usually supported by periodic turning and mixing of the beans, acetic acid bacteria proliferate metabolizing ethanol into acetic acid. This causes the temperature to rise to approximately 45 °C to 50 °C within the fermentation mass.[1,2] Acidification of the beans by diffusion of acidic and lactic acid to reach a pH-value within the beans between 4.5 – 5.5, combined with the rise in temperature, leads to the death of the embryo. This initiates breakdown of the cell structure within the bean, so endogenous enzymes and beans` major constituents, i.e., soluble proteins, carbohydrates, and polyphenols, get into contact by diffusion to form important aroma precursors and facilitate transformation of phenolic compounds.[4,5] Sucrose is degraded by invertase to the monomeric reducing sugars D-glucose and D-fructose.[6] Proteins are hydrolyzed by proteolytic enzymes to a variety of peptides and free amino acids.[5,7] Especially hydrophobic amino acids such as phenylalanine, valine, leucine, isoleucine and hydrophilic peptides have been identified as specific cocoa aroma precursors. They are released by the sequenced activity of an aspartic endoprotease and a carboxypeptidase preferably at a pH-value of 5.0-5.5.[4,5] During the later stages of fermentation when oxygen availability in the fermenting mass increases, flavan-3-ols such as (–)-epicatechin and (+)-

48 catechin and short chained procyanidins such as procyanidin B1 (dimer with one unit (–)-epicatechin and
49 one unit (+)-catechin), procyanidin B2 (dimer with two units (–)-epicatechin), procyanidin C1 (trimer
50 with three units (–)-epicatechin), and cinnamtannin A2 (tetramer with four units (–)-epicatechin) are
51 oxidized to form polymeric tannins.[8] These polymers can form complexes with other constituents like
52 proteins, lowering the astringency and bitterness of the raw material.[1,4] The chemical and enzymatic
53 catalyzed oxidation, polymerization and complexation reactions of the polyphenols are responsible for
54 browning of the cocoa beans. The browning continues during drying which is typically initiated after
55 approximately 5-8 days after the start of fermentation by spreading the beans to sun-dry or using artificial
56 dryers.[1]
57 Many recent studies used incubations of fresh cocoa seeds to study the transformations within the beans
58 without the influence of microorganisms. Fresh seeds were incubated in pH-adjusted solutions under
59 controlled temperature and oxygen regimes, which were adjusted to simulate the conditions during
60 fermentation.[9–12] It was shown that the desired transformations of major components in the beans such
61 as sugars, proteins, and polyphenols could be achieved as well.[11,12] Thus, a possible implementation of
62 this process on cocoa farms is discussed controversially. However, upscaling this process may not be
63 feasible, because of the expensive infrastructure(s) required.
64 Recent studies proposed “moist incubation” of unfermented and dried cocoa nibs as a possible time- and
65 location independent postharvest treatment.[13,14] During this treatment, the beans are sun-dried
66 immediately after harvest to a moisture content of approximately 6 - 8% to be stable for transportation
67 or storing. During drying the embryo is inactivated, while the beans` endogenous enzymes stay active
68 but separated from their substrates.[15–17] After deshelling of the beans, the nibs are rehydrated with a lactic
69 acid solution containing ethanol to reach a pH-value ~ 5.0. This presumably facilitates contact between
70 enzymes and substrates in the same manner as during fermentation. The nibs are then incubated at 45 °C
71 for approximately 72 h under aerobic conditions before drying. It was shown that this method can be
72 used to produce chocolate with pleasant flavor properties.[13,14] Fruity esters and malty Strecker aldehydes

were found in higher quantities in the moist incubated samples in comparison to fermented cocoa. On the other hand, volatile acids with unpleasant odor-qualities like acetic acid, and 2- and 3-methylbutanoic acid were found in higher quantities in the fermented samples. These previous studies focused on the identification and quantification of some key aroma compounds, but no measurements of non-volatile components were made. Furthermore, the moist incubations done in the previous studies were conducted under strict aerobic conditions. It is well-known that the availability of oxygen during postharvest processing plays an important role supporting the oxidation of phenolic substances and browning process.[1] After the analysis of the sensory impact and the influence on aroma generation which were conducted in the previous investigations, the impact of the treatment on the non-volatile constituents should be examined. Consequently, the aim of the present study was to investigate the effect of moist incubation with and without the addition of oxygen on the evolution of important non-volatile components, such as flavan-3-ols, caffeine, theobromine, sugars, and free amino acids in comparison to traditional fermentation.

Material and Methods

Raw Materials and Experiments

The raw materials were obtained from the same source and were processed in the same manner as described before.[13,14] Cocoa of the cultivar Trinitario was harvested on a farm in Bijagua, Costa Rica during the harvest of 2021 and the batch was separated to obtain fermented and unfermented cocoa beans: approximately 80 kg of the batch was directly spread as a single bean layer on drying trays with a meshed bottom (mesh size ~0.75 cm) to allow for excessive pulp to drain off and support sufficient aeration. The beans were turned, mixed and kneaded manually at least two times per day to support homogenous drying and avoid the formation of bean clusters. Drying was finished when a final moisture content of ~ 6-8 % was reached in the beans. To produce the reference, the traditionally fermented cocoa beans, about 80 kg

of the fresh beans were filled in a wooden fermentation box and covered with banana leaves to start the fermentation. Mixing and aeration was performed manually after 48 h and was repeated every 24 h until a total fermentation time of approximately 120 h was reached. The beans were then dried as described above to stop the fermentation. About 20 kg of these samples were packed in a plastic bag and closed with a cable-tie to prevent the beans from possible re-humidification, transported by air-cargo to Wädenswil, Switzerland and stored at 12 °C until further processing. The beans were broken and deshelled using a lab-scale breaker (Limprimita cocoa breaker, Capco/Castlebroom Engineering, Ltd., Ipswich, U.K.) and winnower (cocoa winnower large, Capco/Castlebroom Engineering, Ltd., Ipswich, U.K.) to obtain unfermented and fermented cocoa nibs. The moist incubations were performed as described before[13,14] with the difference that they were conducted under aerobic as well as anaerobic conditions to investigate the influence of forced and suppressed aeration on the yield of the different analytes.

For the aerobic moist incubation three portions of 20 g (± 0.1 g) of unfermented nibs were rehydrated under vacuum in a sealed bag for 12 h at 4 °C with 10.6 g (± 0.01 g) of aqueous solution containing lactic acid (0.1 mol/L) and ethanol (5 % v/v) to reach a pH value in the cocoa solids of 5.2 and a final moisture content of approximately 35 %. The bags were then opened, fumigated with oxygen, sealed, and then incubated at 45 °C for 72 h in a laboratory incubator under occasional mixing by turning and kneading the bags every 12 h. After incubation the samples were dried on trays at 40 °C for 24 h in a laboratory oven with air circulation under occasional turning until a final moisture content < 6 % was reached. For the anaerobic incubated material, the same protocol was followed with the difference that the vacuum bags were kept sealed until the end of incubation time, so the material was only subjected to oxygen during drying.

From the fermented and unfermented cocoa only one batch was available. For their analysis, three random samples of 15 g deshelled nibs were taken and defatted. The results are expressed as mean values with the calculated standard deviations (n=3).

The incubation experiments were performed in triplicates. For analysis, three random samples of each batch were taken, defatted and analyzed individually, leading to 9 replicates for each incubation treatment (n=9). The results are expressed as mean values with the calculated standard deviations.

The materials are hereafter referred to as “unfermented cocoa”, “aerobic incubated cocoa”, “anaerobic incubated cocoa”, and “fermented cocoa”.

Chemicals and reagents

All solvents and chemicals that were used were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) unless differing supplier is given in parenthesis.

Chemicals used for sample preparation, extraction and analysis:

Acetic acid, dimethylsulfoxide, ethanol absolute (VWR International GmbH, Dietikon), Folin & Ciocalteu reagent (2 N), n-hexane (VWR International GmbH, Dietikon), hydrochloric acid (37 %), L-(+)-lactic acid, OPA reagent, potassium hexacyanoferrate (II) trihydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), sodium acetate (Carl Roth GmbH & Co. KG, Germany), sodium carbonate, trifluoroacetic acid, zinc acetate dihydrate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)

Solvents and chemicals used for HPLC-MS/MS, HPLC-UV/Vis, HPLC-FLD analysis (MS grade)

Acetone, acetonitrile, ammonium acetate, Borax, formic acid, methanol, sulfuric acid, water (Carl Roth GmbH & Co. KG, Karlsruhe, Germany)

Standards used for identification and quantitation

For the preparation of standards for identification and quantitation following substances were used: L-alanine, L-arginine, L-asparagine, L-aspartic acid, caffeine, L-glutamine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-serine, theobromine, TraceCERT® (amino acid standard mix), L-tryptophane, L-tyrosin, L-valine. (+)-catechin, cinnamtannin A2, (-)-epicatechin, procyanidin B2 and procyanidin C1 were purchased from PhytoLab GmbH und Co. KG (Vestenbergsreuth, Germany). For enzymatic sugar measurements Enzytec™ sugar standard (E8445) (R-Biopharm AG, Darmstadt, Germany) was used for calibration.

Methods

Sample Preparation

Preparation of cocoa material was performed in the same manner as described by Pedan et al. (2016) with slight modifications.[18] The dried cocoa nibs were frozen with liquid nitrogen and ground for 30 s to a fine powder using a laboratory mill (A 11 basic analytical mill, IKA Werke GmbH und Co. KG, Staufen, Germany). The ground samples were defatted with n-hexane (Carl Roth GmbH und Co. KG, Karlsruhe, Germany) eight times using the following protocol: sample material and hexane were extracted in a 50 mL tube with a sample to solvent ratio of 1:3 (w/v) for 10 min using an overhead shaker (Reax 2, Heidolph Instruments GmbH und Co. KG, Schwabach, Germany) before centrifugation at 2880 × g (Type 5810, Vaudaux-Eppendorf, Schönenbuch, Switzerland) for additional 10 min. The solvent was then decanted before the next defatting cycle was started using fresh solvent. After the final defatting cycle, the sample material was spread in a Petri dish and dried under the fume hood overnight to evaporate excess hexane. The material was then kept at - 20°C in sealed bags until further analysis.

Extraction of samples

For the determination of different analytes three different extracts were prepared.

Acetone/water-extracts for analysis of phenolic compounds and methylxanthines

Extracts were prepared as described by Pedan et al (2016) with slight modifications.[18] Samples of 0.5 g (± 0.01 g) were extracted three times with 1.5 mL of 50 % acetone in water (v/v) at 50 °C for 8 minutes using a heated laboratory shaker (Hettich AG, Tuttlingen, Germany) and subsequently centrifuged at 2880 × g for 5 minutes (Type 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). After centrifugation supernatants were combined and subsequently filtered through a syringe filter (RC membrane, 0.2 µm, Phenomenex Helvetia GmbH, Basel, Switzerland) into glass vials which were then sealed and kept at - 20 °C until further analysis.

Trifluoroacetic acid extracts for analysis of free amino acids

Extractions for the determination of free amino acids were performed in the same manner as described for the acetone/water-extracts, except 0.30 g (± 0.01 g) of sample was extracted three times with 1 mL of a modified solution to extract free amino acids from the study by Murthy et al (1997), containing 0.11 mol/L trifluoro acetic acid, 0.22 mol/L sodium acetate and 0.33 mol/L acetic acid.[19]

Water extracts for analysis of sugars

Extracts for the determination of sugars were obtained using the same protocol, except 0.50 g (± 0.01 g) of defatted sample were extracted with water. After complete extraction Carrez-clarification was performed by adding 0.25 mL of potassium hexacyanoferrate (II) trihydrate (150 g/L) and 0.25 mL of zinc acetate dihydrate (230 g/L) to the combined supernatants to precipitate proteins. After centrifugation the clear supernatant was filtered through a syringe filter (RC membrane, 0.2 µm, Phenomenex Helvetia GmbH, Basel, Switzerland) and kept in sealed glass vials at -20°C until further analysis.

Analysis

Total phenolic content (TPC)

TPC was determined based on the method described by Blois (1958).[20] First, the acetone/water-extracts of the samples were diluted with water 1:400. Then, 20 µL of diluted sample extract, 20 µL Folin reagent (25 % solution (v/v) prepared with 2 N Folin reagent reagent in water) and 80 µL sodium carbonate/water solution (100g/L) were mixed in a 96-well plate and incubated at room temperature in the dark for 2 h. The absorption of the samples was then measured using an automated UV/Vis-spectrophotometer (BioTek Instruments Epoch 2, Agilent Technologies AG, Switzerland) at 750 nm. For the calibration, standard solutions in the range of 0.01 mg/mL to 0.06 mg/mL with (-)-epicatechin were prepared in the same manner and water was used as a blank.

Every sample was measured in duplicates and the means were used for further calculations. TPC was calculated using linear regression. Calibration equations are shown in Table S1. Results are expressed as mg (-)-epicatechin equivalents per g fat free dry matter of sample (mg EE/g ffdm).

Total content of flavan-3-ols (TFC)

196 TFC was determined using the method described by Payne et al (2010) with minor modifications.[21] To
197 prepare the 4-(dimethylamino)cinnamaldehyde (DMAC) solution 0.0300 g (± 0.001 g) of DMAC was
198 added to 30 mL of a 10 % hydrochloric acid/ethanol solution (v/v). This solution was stored at 4°C for
199 at least 15 min before use. For the measurements 50 µL of acetone/water-extract of sample was pipetted
200 in a 96-well plate and mixed with 250 µL of DMAC solution. The absorption at 640 nm was read
201 immediately with an automated plate reader as described for the TPC measurements. For a five-point
202 calibration, procyanidin B2 in concentrations of 0.001 mg/mL to 0.1 mg/mL in 50 % methanol in water
203 (v/v) were prepared. Ethanol was used as a blank. The unfermented samples were diluted 1:2 using
204 ethanol to meet a concentration within calibration range. Every sample was measured in duplicates and
205 the means were used for further calculations. TFC was calculated using linear regression. Calibration
206 equations are shown in Table S1. Results are expressed as mg procyanidin B2 equivalents per g fat free
207 dry matter of sample (mg PE/g ffdm).
208 *Quantitation of selected flavan-3-ols, procyanidins and methylxanthines (HPLC-MS/MS)*
209 As determined, the acetone/water-extracts were diluted prior the analysis with a solution containing 50
210 % (v/v) acetonitrile in water: 1:100 for the unfermented beans, 1:4 for the fermented beans and 1:2 for
211 the incubated beans.
212 HPLC-MS/MS analysis was conducted on a system consisting of an Agilent 1290 Infinity II chromato-
213 graphic system coupled to an Agilent 6530 Q-TOF mass spectrophotometer. Separation of analytes was
214 performed using an Agilent Poroshell 120 EC-C18 (2.1 x 150 mm, 2.7 µm) column preceded by a guard
215 column (Agilent Poroshell 120 EC-18, 2.1 × 5 mm, 2.7 µm). The flow rate was set to 0.7 mL/min, and
216 the column temperature set at 35 °C. The two elution mobile phases were made up of water + 0.1 %
217 formic acid (FA) (mobile phase A) and acetonitrile + 0.1 % FA. HPLC gradient: 0-3 min., 5 % B; 5 min,
218 9 % B; 10 min, 9 % B; 12 min, 20 % B; 14 min, 24 % B; 19 min, 25 % B; 21 min, 30% B, 22-30 min,
219 100 % B; 30.10-37 min, 5 % B. Injection volume was 2 µL. UV spectra were recorded at 275, 320, and
220 360 nm.

The MS analyses were performed using Agilent 6530 Q-TOF instrument in negative ionization mode (ESI -), in the spectral range of 100-3200 Da. Nitrogen served as the nebulizer and collision gas. The MS parameters were as follows: gas temperature, 350 °C; drying gas, 10 L/min; nebulizer, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; capillary voltage, 4,000 V.

For the analysis of the methylxanthines, the acetone/water extracts were diluted 1:100 with 50 % (v/v) acetonitrile in water. The HPLC parameters were identical, and the MS analyses were performed using Agilent 6530 Q-TOF instrument in positive ionization mode (ESI +), in the spectral range of 100-3,200 Da. Nitrogen served as the nebulizer and collision gas. The MS parameters were as follows: gas temperature, 350 °C; drying gas, 10 L/min; nebulizer, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; capillary voltage, 4,000 V. Pure substances were used for determination of retention times and for the preparation of calibration lines (see table S1). The contents of individual substances were calculated using linear regression. Results are expressed as mg/g ffdm.

Quantitation of free amino acids (HPLC- FLD)

The free amino acids were quantitated using an Agilent 1260 Infinity HPLC (Agilent Technologies (Schweiz) AG, Basel, Switzerland) with a fluorescent detector (FLD). TFA-extracts were derivatized prior to injection by programming the autosampler of the HPLC: 1 µL TFA-extract was mixed with 2 µL Borax solution (25 mmol/L in water), then 3 µL OPA reagent was added and mixed, and finally 1 µL 10 % (v/v) acetic acid in water solution was added and mixed. The derivatized sample solution (7 µL) was injected into the column (Agilent Poroshell 120 EC-C18 2.1 × 50 mm, 2.7 µm) tempered at 20 °C. As mobile phase A ammonium acetate buffer (50 mmol/L in water) was used. As mobile phase B a solution made of 40 % acetonitrile, 40 % methanol, and 20 % water (v/v/v) was used. HPLC gradient: 0 min: 15 % B; 3 min: 30 % B; 10 min: 45 % B; 12 min: 53 % B; 20 min: 100 % B. Fluorescent spectra were recorded at 340 nm (Ex) and 450 nm (Em). For identification of the individual amino acids and the determination of the corresponding retention times an amino acid standard mix and solutions of each individual substances were used. Calibration lines were prepared by diluting the standard mix.

Calibration ranges and equations are shown in Table S1. The fermented, aerobic incubated and anaerobic incubated sample were diluted 1:2 to meet the calibration range. The contents of individual substances were calculated using linear regression. Results are expressed as mg/g ffdm. For evaluation, interpretation, and illustration of the results amino acids were split into two groups and the amounts of the individual compounds were summed up: hydrophobic (L-alanine, L-tyrosine, L-valine, L-methionine, L-tryptophan, L-phenylalanine, L-isoleucine, and L-leucine) and other amino acids (L-aspartic acid, L-glutamic acid, L-asparagine, L-serine, L-histidine, L-glutamine, L-glycine, L-arginine, L-lysine).

Quantitation of sugars (enzyme assays)

Sucrose, D-glucose, and D-fructose were determined using enzymatic assays Enzytec™ Liquid D-glucose (E8140), Sucrose/D-Glucose (E8180), D-Glucose/D-Fructose (E8160) (r-biopharm AG, Darmstadt, Germany) with an automated biochemistry analyzer (Type: Chemwell 2910, Awareness Technology Inc., Palm City, USA). The system was equipped with 96-well plates and the temperature set 37 °C for incubation after each pipetting step according to the specific assay. Absorbance readings were made at 340 nm. For calibration Enzytec™ sugar standard (E8445) (r-biopharm AG, Darmstadt, Germany) was used. Calibration lines were prepared by diluting the standard mix. Calibration ranges and equations are shown in Table S1. The contents of individual substances were calculated using linear regression. The measuring principle of all assays is based on the detection of NADH with D-glucose as an intermediate, which is achieved by inversion of sucrose and subsequent isomerization of D-fructose to D-glucose (E8180) or direct isomerization of D-fructose (E8160). To calculate the specific contents of sucrose and D-fructose the amount of D-glucose (E8140) was subtracted from the results of the other assays. Results are expressed as mg/g ffdm.

Determination of fermentation index (FI)

The fermentation indexes of the samples were determined with the method described by Gourieva and Tserrevitinov (1979) with minor modifications.[22] 0.1 g (± 0.01 g) were extracted with 10 mL hydrochloric

acid/water solution (3:97 v/v) at 4 °C for approximately 15 h. After centrifugation the absorption of the supernatants were measured at 460 nm and 530 nm using an automated UV/Vis-spectrophotometer (BioTek Instruments Epoch 2, Agilent Technologies AG, Switzerland). The fermentation index is defined as the ratio of the absorption measured at 460 nm and 530 nm (FI = A460/A530). Values ≥ 1.0 are considered well fermented and values ≤ 1.0 are considered under fermented.

Statistical analysis

The complete data set was evaluated by analysis of variance (ANOVA) using XLSTAT (version 2022.2.1, Addinsoft Inc. USA) between the calculated means of the samples. Significant differences were tested using Tukey`s honestly significant difference test (HSD) with a confidence interval of 95% ($p < 0.05$). The results are shown in Table 1. Different letters within one row indicate significant differences between the means of samples. Principal component analysis (PCA) was performed with the complete data set to visualize differences and similarities of the samples by reducing the dimensions.[23] The loading and score plots of the PCA are displayed in Figure 5 and Figure 6 respectively.

Results and Discussion

Determination of phenolic compounds and methylxanthines

Total phenolic content (TPC) and Total flavan-3-ol content (TFC)

The results of TPC and TFC analysis are shown in Figure 1 and Table 1. For the unfermented cocoa sample, a TPC of 239 mg EE/g ffdm was measured. Contents in unfermented Trinitario, Forastero and Criollo samples range from 120-140 mg EE/g ffdm according to literature.[24] The results of the present study showed a high TPC in comparison. This may be caused by differences in the work-up procedure used and variations between raw materials. After fermentation and anaerobic incubation, a significant decrease of approximately 25 % and 35 % to a final content of 181 mg EE/g ffdm and 157 mg EE/g ffdm was measured. For fermented cocoa beans the values found in literature range from 40-140 mg EE/g ffdm.[25] Considering the high initial TPC content measured in the unfermented cocoa sample, the

comparably high values obtained after fermentation and anaerobic incubation are plausible. After aerobic incubation a significantly lower TPC of 66.5 mg EE/g was measured, which corresponds to a total decrease of approximately 70 %. The activity of polyphenol oxidases is known to be reduced to below 5 % of the initial activity during fermentation and drying.[16,26] However, browning continues throughout postharvest processing, despite low activity levels. It is assumed that the remaining low polyphenol oxidase activity in combination with chemical oxidation is sufficient for further oxidation and browning of phenolic compounds.[8,16,26] The steep decrease of the TPC induced by the aerobic incubation, may therefore be caused by the excessive availability of oxygen. Higher polymerization products, such as condensed tannins may have been formed, which can further react with proteins, peptides and amino acids to form insoluble complexes.[27,28] The results of the TFC measurements show a similar trend. In the unfermented sample, an initial concentration of 217 mg PE/g ffdm was measured. The fermented and anaerobic sample showed a significant lower concentration of 107 mg PE/g ffdm and 147 mg PE/g ffdm respectively. On the other hand, a significantly lower content of 32.4 mg PE/g ffdm was measured in the aerobic sample. TFC determination using DMAC specifically reacts with the monomeric flavan-3-ols (–)-epicatechin, (+)-catechin, epigallocatechin, gallocatechin, and their respective gallates, oligomeric procyanidins of cocoa up to n = 4, and A-type procyanidins.[21] A higher loss of these compounds was induced by the aerobic treatment (- 85 %) compared to the fermentation (- 51 %) and anaerobic incubation (- 32 %). The comparably moderate reduction of the TFC in the anaerobic sample is most likely due to the limited availability of oxygen during the incubation process. The low TFC measured in the aerobic sample on the other hand, suggests the treatment supports oxidation and polymerization of these compounds to higher condensed tannins, which cannot be detected with the used method.[21]

Selected flavan-3-ols and procyanidins

The results of the HPLC-MS/MS measurements of the most abundant flavan-3-ols are displayed in Figure 2 and Table 1.

Among the monomeric flavan-3-ols (–)-epicatechin is known to be the most abundant compound in unfermented cocoa beans reaching concentrations between 30 - 50 mg/g ffdm,[8,24–26,29–31] which is well in accordance with our result of 38.9 mg/g ffdm measured in the unfermented sample. (+)-Catechin is present in lower amounts in the unfermented sample with 1.67 mg/g ffdm which is also in the range of 0.5 – 8.0 mg/g ffdm found in literature.[24–26,31,32]

The significant decrease of (–)-epicatechin measured after aerobic incubation and fermentation is comparable, with only 0.33 mg/g ffdm left in the aerobic incubated sample and 2.72 mg/g ffdm in the fermented sample. On the other hand, the anaerobic incubation induced a significant, but much lower reduction of (–)-epicatechin with a measured concentration after the treatment of 29.9 mg/g ffdm. During fermentation a steep decrease of (–)-epicatechin has been observed in several studies within the first 72 h, which is assumed not only to be caused by oxidation and polymerization reactions, but also by exudations of soluble phenols out of the beans during fermentation.[24,29,30]

The results obtained for procyanidin B2, procyanidin C1 and cinnamtannin A2 (dimer, trimer and tetramer of (–)-epicatechin) show the same trend. While initial contents of 22.0 mg/g ffdm, 11.6 mg/g ffdm and 13.3 mg/g ffdm were measured in the unfermented sample, only traces of these compounds were measured after aerobic incubation. The fermented sample also showed a significant decrease of ≥ 90 % with 0.16 mg/g ffdm procyanidin B2, 1.35 mg/g ffdm procyanidin C1, and 1.16 mg/g ffdm, and cinnamtannin A2 respectively. On the other hand, values obtained in the anaerobic incubated samples also showed a significant but lower reduction of approximately 20 - 30 % of the initial content, reaching a final concentration of 17.5 mg/g ffdm procyanidin B2, 8.41 mg/g ffdm procyanidin C1, and 9.21 mg/g ffdm cinnamtannin A2. This suggests oxidation and polymerization of the monomer (–)-epicatechin and the measured (–)-epicatechin based proanthocyanidines procyanidin B2, procyanidin C1 and cinnamtannin A2 is promoted during the aerobic incubation and fermentation in equal matters, while the anaerobic incubation left a higher proportion of these compounds in the final raw material.

Methylxanthines

The results obtained for the quantitation of caffeine and theobromine can be found in Table 1. According to literature, among the methylxanthines theobromine and caffeine can be found in concentrations ranging from approximately from 10.0 – 30.0 mg/g ffdm and 1.00 – 6.00 mg/g ffdm respectively in unfermented cocoa.[33,34] During fermentation a loss of theobromine and caffeine of approximately 30 % in the first 72h has been reported, most likely due to diffusion out of the beans.[33] The theobromine and caffeine contents of 29.5 mg/g ffdm and 8.64 mg/g ffdm analyzed in the unfermented materials are well in accordance with the values given in literature. Present results showed that none of the applied postharvest treatments of the current study caused a significant decrease of these compounds, suggesting the fermentation as well as the incubation do not affect the level of methylxanthines.

Determination of Sugars and Amino Acids

Sugars

The results of sugar analysis are shown in Figure 3 and Table 1. In the unfermented cocoa an initial sucrose, D-glucose, D-fructose content of 36.3 mg/ffdm, 2.53 mg/g ffdm and 2.63 mg/g ffdm was determined, which is in accordance with contents reported in literature.[35,36] One major goal of postharvest processing is the release of D-glucose and D-fructose from sucrose caused by invertase activity to act as aroma precursors during further processing.[16] Both incubation treatments as well as the fermentation caused a significant reduction of sucrose and a significant increase in the reducing sugars. After incubation treatments, there was no sucrose detectable in the incubated samples, while only traces were found in the fermented sample. However, D-glucose and D-fructose were measured with a much higher content after both incubation treatments in comparison to the fermentation. The highest contents of 20.4 mg/g ffdm were measured for both D-glucose and D-fructose after aerobic incubation, while the contents measured in the aerobic incubated sample were comparable (18.5 mg/g ffdm and 17.9 mg/g ffdm). On the other hand, a significant lower content of 4.95 mg/g ffdm of D-glucose and 11.7 mg/g ffdm of D-fructose was measured in the fermented sample. In theory, the contents of D-glucose and D-fructose should approximately sum up to the initial sucrose content before postharvest processing, which is

roughly the case for the aerobic and anaerobic incubated cocoa. The lower contents measured in the fermented sample, however, only add up to approximately 45 % of the initial sucrose content. This difference has been reported by several authors, who concluded that parts of the monomeric sugars are lost by exudations out of the beans, and drain away with fermentation sweatings.[1,4,35,36] On the contrary, the incubation treatments were performed in a closed system, where no exudations, and therefore only minimal losses can occur. In conclusion, both incubation treatments yield more reducing sugars in comparison to fermentation, thus, more of these aroma precursors are available for aroma formation during further processing like roasting.

Amino acids

Amino acids play a major role in the aroma formation during processing of cocoa beans. Especially hydrophobic amino acids and hydrophilic peptides released during fermentation have been identified as key components for cocoa flavor.[5,37,38]

The measured amounts of free hydrophobic and other amino acids are illustrated in Figure 4. The results for individual amino acids are shown in Table 1. A significant increase in the amount of total free amino acids was detected for all applied postharvest treatments. While 5.33 mg/g ffdm total free amino acids were measured in the unfermented sample, the highest increase was measured in the anerobic incubated sample with 13.77 mg/g ffdm, which was significantly higher in comparison to the fermented sample where 12.31 mg/g ffdm was measured. The measured contents of total free amino acids were well in line with values given in literature, where an initial content of unfermented cocoa ranging from approximately 5.00 – 8.00 mg/g ffdm, and after fermentation contents of up 24.0 mg/g have been reported.[25,36,39,40] However, in the aerobic incubated sample a significantly lower concentration of total free amino acids of 9.40 mg/g ffdm was determined. This lower content in comparison to the fermented and anaerobic incubated cocoa, may be caused by interactions with flavan-3-ols. Oxidation of flavan-3-ols leads to the corresponding *o*-quinone form, which can react with the nucleophilic groups of proteins, peptides and amino acids to form insoluble complexes.[27] Furthermore, it is known from different processes, such as

394 tea-, tobacco- and wine-making, that the reaction of *o*-quinones may induce Strecker degradation of
395 amino acids resulting in the corresponding Strecker aldehydes.[27] A connection between aroma formation
396 and the oxidation of phenolic compounds during cocoa fermentation has been suggested by several
397 authors before.[1,41,42] However, the possible importance of interaction of phenolic- and amino compounds
398 for aroma formation during postharvest processing of cocoa has not been a subject of attention in more
399 recent research works. The low levels of flavan-3-ols and lower levels of free amino acids measured in
400 the aerobic incubated cocoa of the present study may explain the results obtained in one of our previous
401 studies, where higher contents of Strecker aldehydes were measured before and after roasting in the
402 aerobic incubated material in comparison to the fermented cocoa.[13,14] Even though lower measured
403 contents of free amino acids in the aerobic incubated cocoa suggested that the aroma formation potential
404 during further processing such as roasting is limited in comparison to the anaerobic incubation and
405 fermentation. The results of both studies indicate that the availability of oxygen during postharvest
406 processing may play a major role in the formation of aroma compounds. Increasing the availability of
407 oxygen during fermentation could also be achieved by increasing the frequency of mass turning and
408 mixing, but it is connected to a higher acidification of the beans by promoting acetic acid and lactic acid
409 bacteria growth.[43] Although acetic acid and its formation was shown to be of major importance during
410 fermentation by inducing bean death, supporting enzyme substrate reactions by lowering the pH-value
411 and diffusing throughout the bean, excess acidification is detrimental to flavor.[9] Our results suggest that
412 the desired transformation can also be achieved using lactic acid with the proposed moist incubation of
413 unfermented and dried cocoa cotyledons. In comparison to traditional fermentation however, moist
414 incubation allows direct control of key postharvest processing parameters like the degree of acidification
415 and oxygen availability.

416 **Determination of the fermentation index (FI)**

417 The FI was measured to evaluate the degree of fermentation of the samples. Values ≥ 1.0 indicate a
418 higher level of brownness and are considered well-fermented. FI values ≤ 1.0 indicate a higher proportion

of red color and are considered underfermented.[22] The unfermented cocoa and the anaerobic incubated cocoa both reached an FI < 1.0 with 0.36 and 0.65 respectively. The aerobic incubated cocoa and the fermented cocoa on the other hand, showed an FI of 1.72 and 1.19. The limited availability of oxygen during anaerobic incubation therefore inhibited browning, which also correlated with the high values obtained for the measurements of the phenolic compounds. The aerobic incubated cocoa and the fermented cocoa were considered well-fermented.

Principal Component Analysis

The two first principal components (PC1 and PC2) explained a total of 81.7 % of variance in the data with 43.1 % (PC1) and 38.7 % (PC2) respectively. The loading plot and score plot are shown in Figure 5 and Figure 6 respectively. The loading plot shows that PC1 was highly influenced by different amino acids, especially L-glycine, L-methionine, L-tyrosin, L-arginine, L-asparagine, L-phenylalanine on the positive side of the PC1 axis (correlation between variable and factor > 0.9). L-valine, D-glucose and D-fructose on the other hand, had the highest influence on the positive side of the PC2 axis. The phenolic compounds (TPC, TFA, and flavan-3-ols) also strongly influenced the positive side of the PC1 axis, while being on the lower right quadrant on the negative side of the PC2 axis. On the score plot in Figure 6, all treatments form distinct clusters underlining the significant differences between the samples.

The fermented samples cluster is located around the cross section of PC1 and PC2. The fermented samples are characterized by low amounts of reducing sugars and phenolic compounds, high amounts of amino acids and a high fermentation index. The cluster corresponding to the anaerobic incubated cocoa is located on the positive side of the PC1 axis and is characterized by higher amounts of free amino acids and phenolic compounds than the one of the fermented samples. The cluster formed by the aerobic incubated samples is located in the upper left quadrant of the PCA. This cluster is characterized by low amounts of phenolic compounds a high fermentation index as well as high contents of reducing sugars. The cluster corresponding to the unfermented cocoa samples is located in the lower left quadrant and is

443 characterized by high contents of sucrose and phenolic compounds, while low amounts of amino acids,
444 reducing sugars and a low fermentation index were determined.
445 Overall, it can be summarized that there are significant differences among the aerobic incubated cocoa,
446 the anaerobic incubated cocoa, the fermented and unfermented cocoa, regarding their composition of
447 selected cocoa non-volatiles. Aerobic incubation and fermentation lead to a strong reduction of phenolic
448 compounds, while the anaerobic incubation reduced these compounds to a lesser extent. The availability
449 of oxygen may therefore be adapted during incubation to control the final concentration of phenolic
450 substances in the resulting material. This provides the opportunity to influence the content of bioactive
451 compounds (low polymerized flavanols), which are also responsible for bitterness and astringency in
452 cocoa. Furthermore, it was shown that both moist incubation treatments lead to a comparable release of
453 free amino acids and two-fold higher amounts of reducing sugars as during fermentation. The obtained
454 results underline the findings of our previous investigations that moist incubation can serve as a
455 controllable alternative postharvest treatment, which results in cocoa raw material with high flavor
456 potential.[13,14]
457

458 ## Abbreviations Used

459 ANOVA-analysis of variance, DMAC- 4-(dimethylamino)cinnamaldehyde, EE-(-)-epicatechin
460 equivalents, Em-emission, Ex- excitation, FI-fermentation index, FLD-fluorescence detector, HPLC-
461 high performance liquid chromatography, MS/MS-tandem mass spectrometry, PE-procyanidin B2
462 equivalents, PCA-principal component analysis, PC1-principal component 1, PC2-principal component
463 2, SD standard deviation, TFC-total flavan-3-ol content, TPC-total phenolic content

464 ## Conflict of Interest

465 The authors declare no competing financial interest.

Supporting Information Description

Table S1. Information on Calibration and Detection Parameters Used for the Analysis of the Different Quantitated Compounds

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Figure Captions

Figure 1: **Total Phenolic Content (TPC) and Total Flavan-3-ol Content (TFC) Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa , and Fermented Cocoa. Standard Deviations (SD) are Shown as Bars**

Figure 2: **Contents of Selected Flavan-3-ols Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa. Standard Deviations (SD) are Shown as Bars**

Figure 3: **Amount of Sucrose, D-Glucose, D-Fructose Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa. Standard Deviations (SD) are Shown as Bars**

Figure 4: **Amount of Total Hydrophobic and Other Amino Acids Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa. Standard Deviations (SD) are Shown as Bars**

Figure 5: **Loading Plot of Principal Component Analysis**

Figure 6: **Score Plot of Principal Component Analysis**

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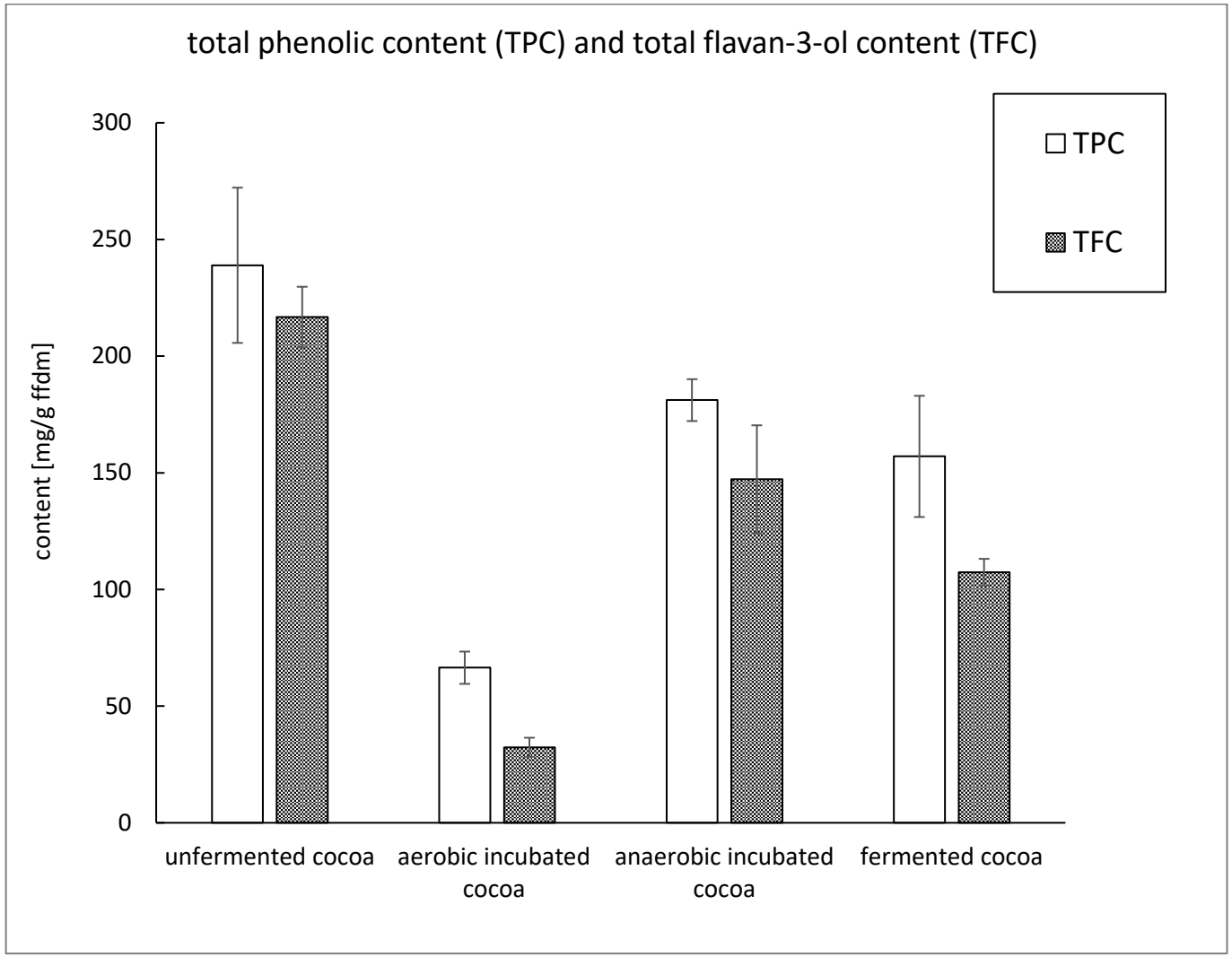

592
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594 **Figure 1: Total Phenolic Content (TPC) and Total Flavan-3-ol Content (TFC) Determined in the**
595 **Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa.**
596 **Standard Deviations are Shown as Bars**

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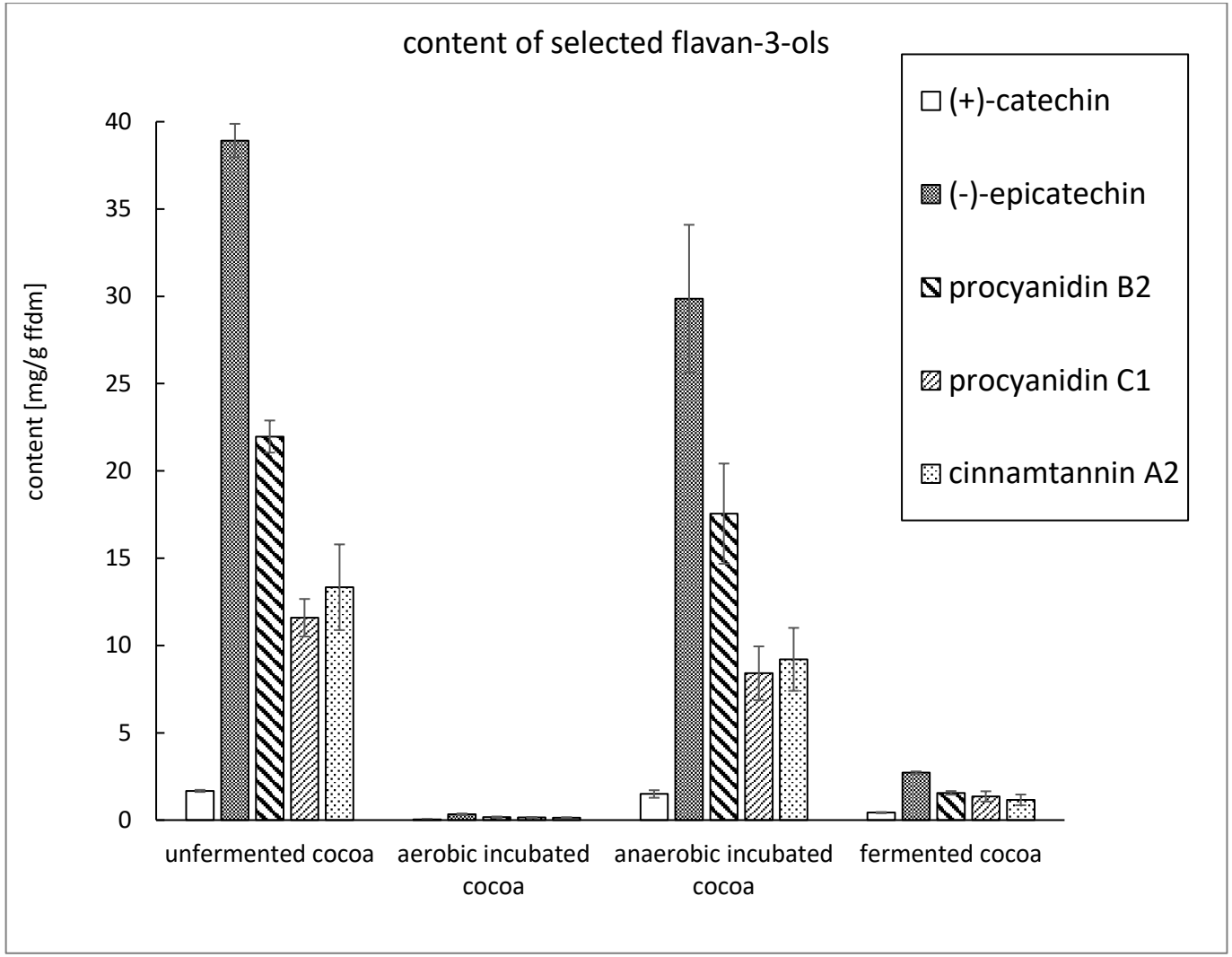

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Figure 2: Contents of Selected Flavan-3-ols Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa. Standard Deviations are Shown as Bars

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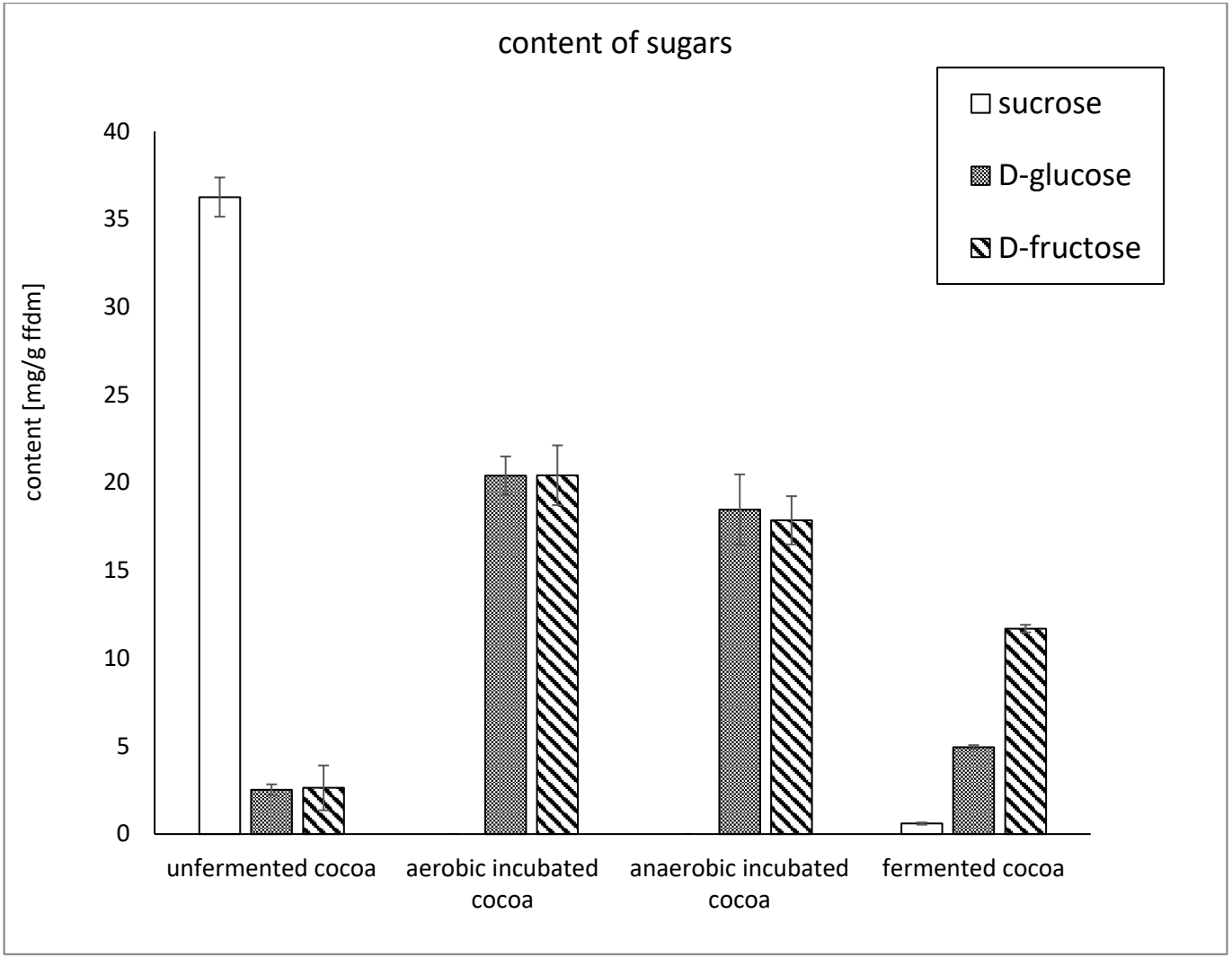

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Figure 3: Amount of Sucrose, D-Glucose, D-Fructose Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa. Standard Deviations are Shown as Bars

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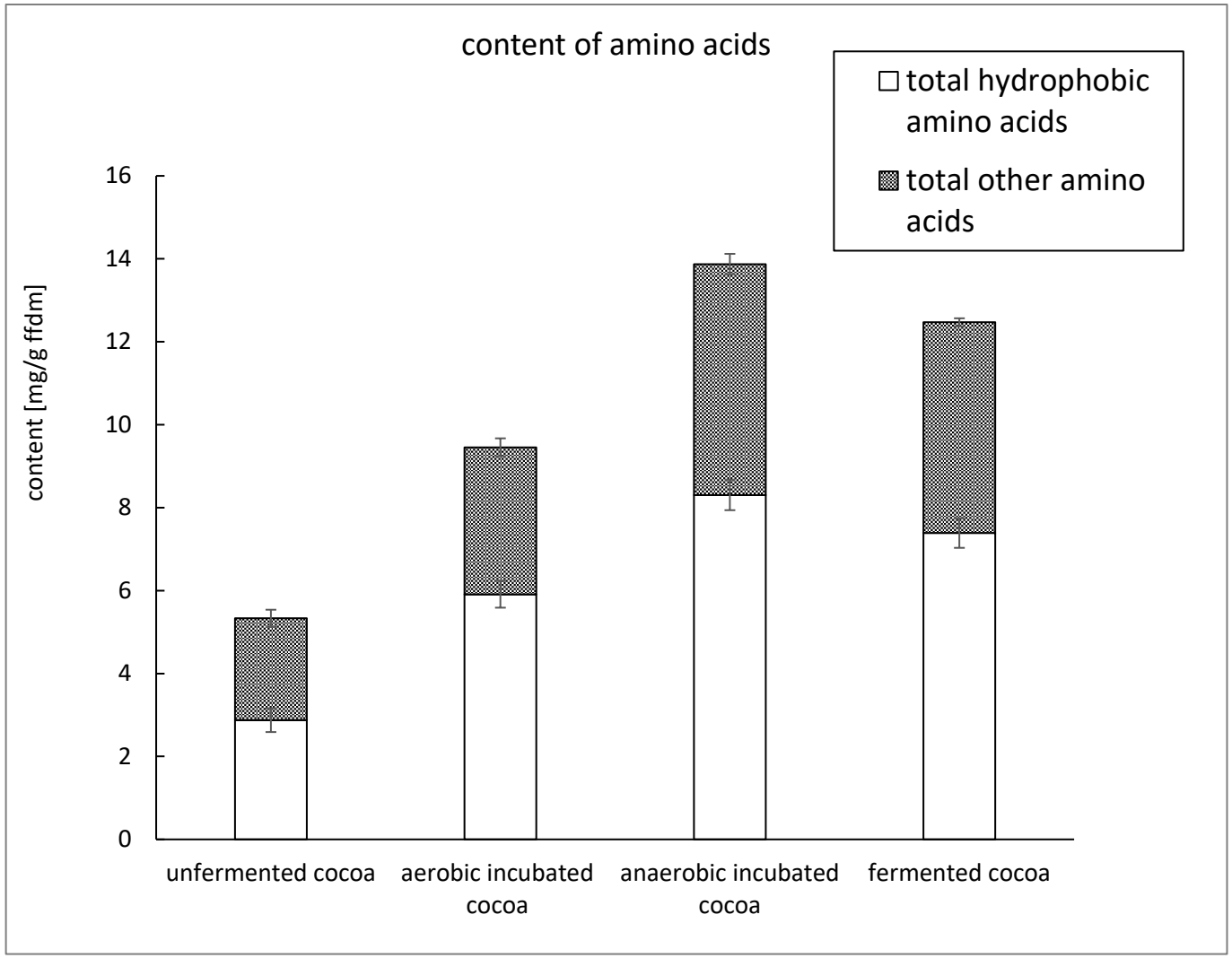

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Figure 4: Amount of Total Hydrophobic and Other Amino Acids Determined in the Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa, and Fermented Cocoa. Standard Deviations are Shown as Bars

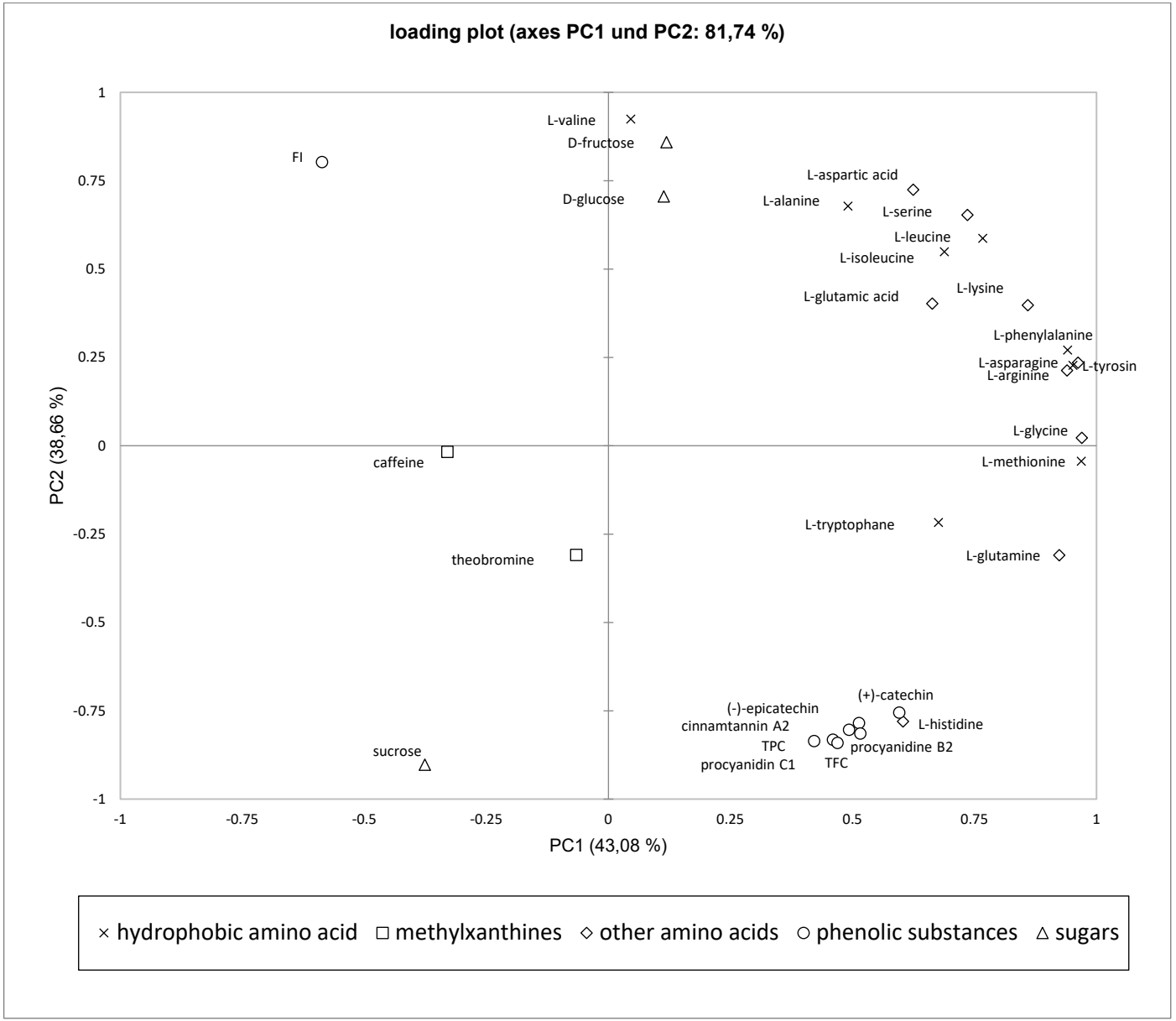

Figure 5: Loading Plot of Principal Component Analysis

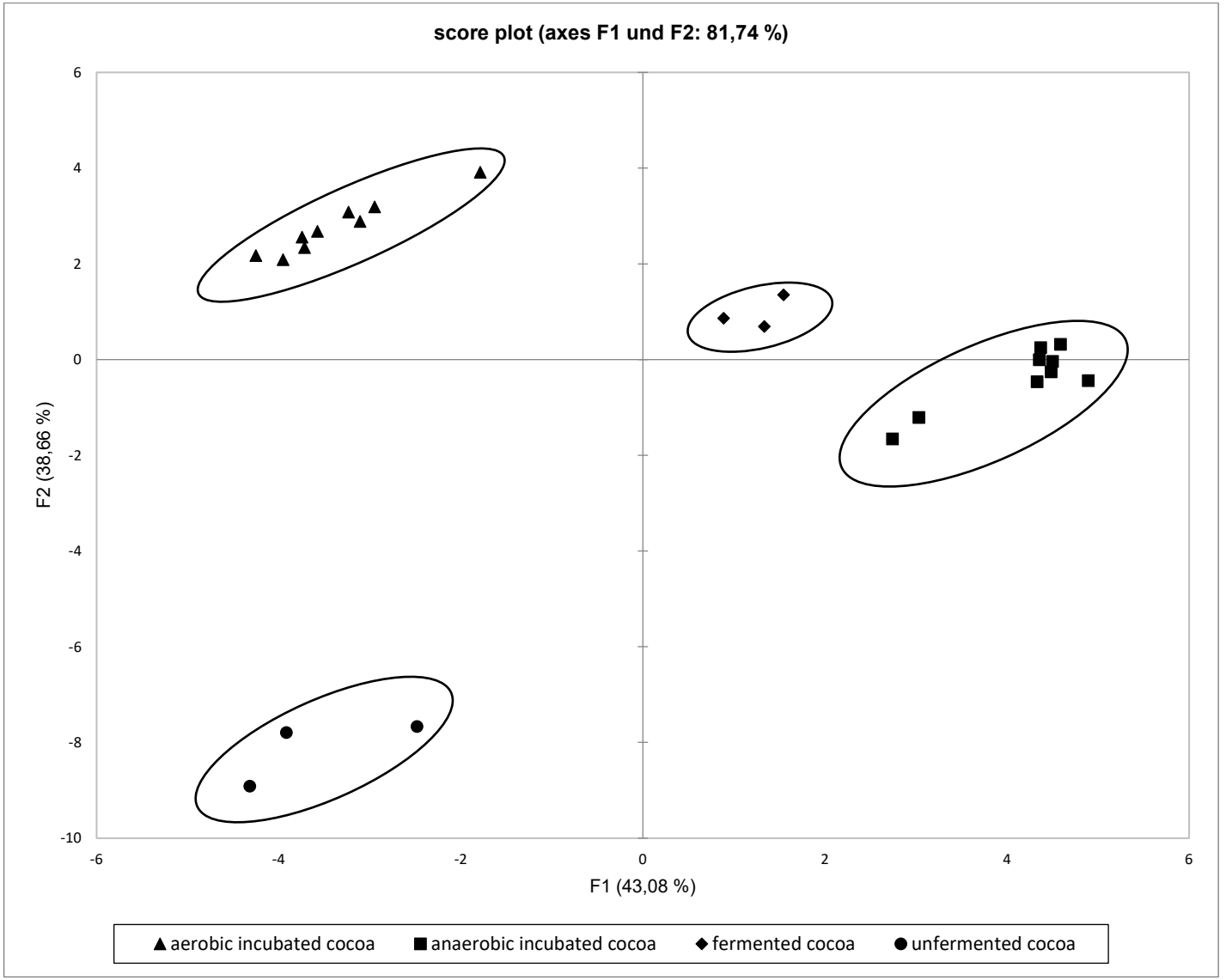

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Figure 6: Score Plot of Principal Component Analysis

611

612 **Table 1: Results of the Determination of Phenolic Compounds, Methylxanthines, Sugars, Amino Acids, and Fermentation Index in**
613 **Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa and Fermented Cocoa**

sample	unfermented cocoa		aerobic incubated cocoa		anaerobic incubated cocoa		fermented cocoa	
	content [mg/g ffdm]							
	mean	SD[1)]	mean	SD[2)]	mean	SD[2)]	mean	SD[1)]
phenolic compounds								
TPC[3)]	239_{c}	33.3	66.5_{a}	6.91	181_{b}	8.98	157_{b}	26.0
TFC[4)]	217_{d}	13.0	32.4_{a}	4.09	147_{c}	23.1	107_{b}	5.79
(+)-catechin	1.67_{c}	0.06	0.04_{a}	0.00	1.5_{c}	0.22	0.43_{b}	0.03
(-)-epicatechin	38.9_{c}	0.96	0.33_{a}	0.05	29.9_{b}	4.23	2.72_{a}	0.07
procyanidin B2	22.0_{c}	0.92	0.17_{a}	0.04	17.5_{b}	2.87	1.56_{a}	0.10
procyanidin C1	11.6_{c}	1.07	0.15_{a}	0.03	8.41_{b}	1.54	1.35_{a}	0.30
cinnamtannin A2	13.3_{c}	2.45	0.13_{a}	0.03	9.21_{b}	1.80	1.16_{a}	0.31
methylxanthines								
theobromine	29.5_{a}	4.58	27.9_{a}	1.45	28.3_{a}	1.71	28.0_{a}	1.26
caffeine	8.64_{a}	0.65	8.63_{a}	0.52	8.28_{a}	0.51	8.38_{a}	0.22
sugars								
sucrose	36.3_{b}	1.12	n.d.	-	n.d.	-	0.60_{a}	0.06
D-glucose	2.52_{a}	0.32	20.4_{b}	1.10	18.5_{b}	2.00	4.95_{a}	0.11
D-fructose	2.63_{a}	1.28	20.4_{d}	1.70	17.9_{c}	1.37	11.7_{b}	0.22
total reducing sugars[5)]	5.15_{a}	0.73	40.8_{d}	1.85	36.3_{c}	1.28	16.6_{b}	0.31

(table continues)

614

Table 1 Results of the Determination of Phenolic Compounds, Methylxanthines, Sugars, Amino Acids, and Fermentation Index in Unfermented Cocoa, Aerobic Incubated Cocoa, Anaerobic Incubated Cocoa and Fermented Cocoa (continued)

sample	unfermented cocoa		aerobic incubated cocoa		anaerobic incubated cocoa		fermented cocoa	
	content [mg/g ffdm]							
	mean	SD[1)]	mean	SD[2)]	mean	SD[2)]	mean	SD[1)]
amino acids								
L-aspartic acid	0.15_{a}	0.01	0.28_{b}	0.03	0.31_{c}	0.02	0.33_{c}	0.01
L-glutamic acid	0.97_{a}	0.13	1.09_{ab}	0.10	1.2_{b}	0.07	1.27_{b}	0.00
L-asparagine	0.32_{a}	0.03	0.38_{b}	0.03	0.51_{c}	0.03	0.42_{b}	0.00
L-serine	0.13_{a}	0.01	0.36_{b}	0.03	0.47_{c}	0.03	0.39_{b}	0.01
L-histidine	0.26_{d}	0.02	0.02_{a}	0.00	0.21_{c}	0.02	0.14_{b}	0.00
L-glutamine	0.19_{b}	0.01	0.15_{a}	0.01	0.25_{c}	0.01	0.18_{b}	0.01
L-glycine	0.08_{a}	0.01	0.09_{a}	0.00	0.23_{b}	0.01	0.21_{b}	0.01
L-arginine	0.23_{a}	0.01	0.56_{b}	0.04	1.27_{d}	0.06	1.00_{c}	0.00
L-alanine	0.70_{a}	0.07	1.29_{c}	0.07	1.41_{d}	0.09	1.02_{b}	0.02
L-lysine	0.13_{a}	0.01	0.61_{b}	0.03	1.13_{c}	0.06	1.15_{c}	0.11
L-tyrosine	0.39_{a}	0.07	0.56_{b}	0.04	0.98_{c}	0.08	0.92_{c}	0.05
L-valine	0.38_{a}	0.03	0.80_{c}	0.06	0.69_{b}	0.07	0.64_{b}	0.02
L-methionine	n.d.	-	n.d.	-	0.30_{a}	0.04	0.13_{b}	0.01
L-tryptophan	0.07_{b}	0.02	0.04_{a}	0.00	0.10_{b}	0.02	0.16_{c}	0.01
L-phenylalanine	0.60_{a}	0.06	1.02_{b}	0.07	1.81_{c}	0.09	1.79_{c}	0.05
L-isoleucine	0.29_{a}	0.03	0.37_{b}	0.03	0.41_{bc}	0.03	0.44_{c}	0.01
L-leucine	0.44_{a}	0.05	1.83_{b}	0.09	2.59_{c}	0.17	2.29_{c}	0.23
total hydrophobic amino acids[6)]	2.88_{a}	0.29	5.91_{b}	0.32	8.31_{d}	0.37	7.39_{c}	0.36
total other amino acids	2.46_{a}	0.21	3.54_{b}	0.22	5.57_{d}	0.25	5.08_{c}	0.10
total amino acids	5.33_{a}	0.49	9.46_{b}	0.52	13.9_{d}	0.56	12.5_{c}	0.27
other								
FI	0.36_{a}	0.00	1.72_{d}	0.02	0.65_{b}	0.01	1.19_{c}	0.01

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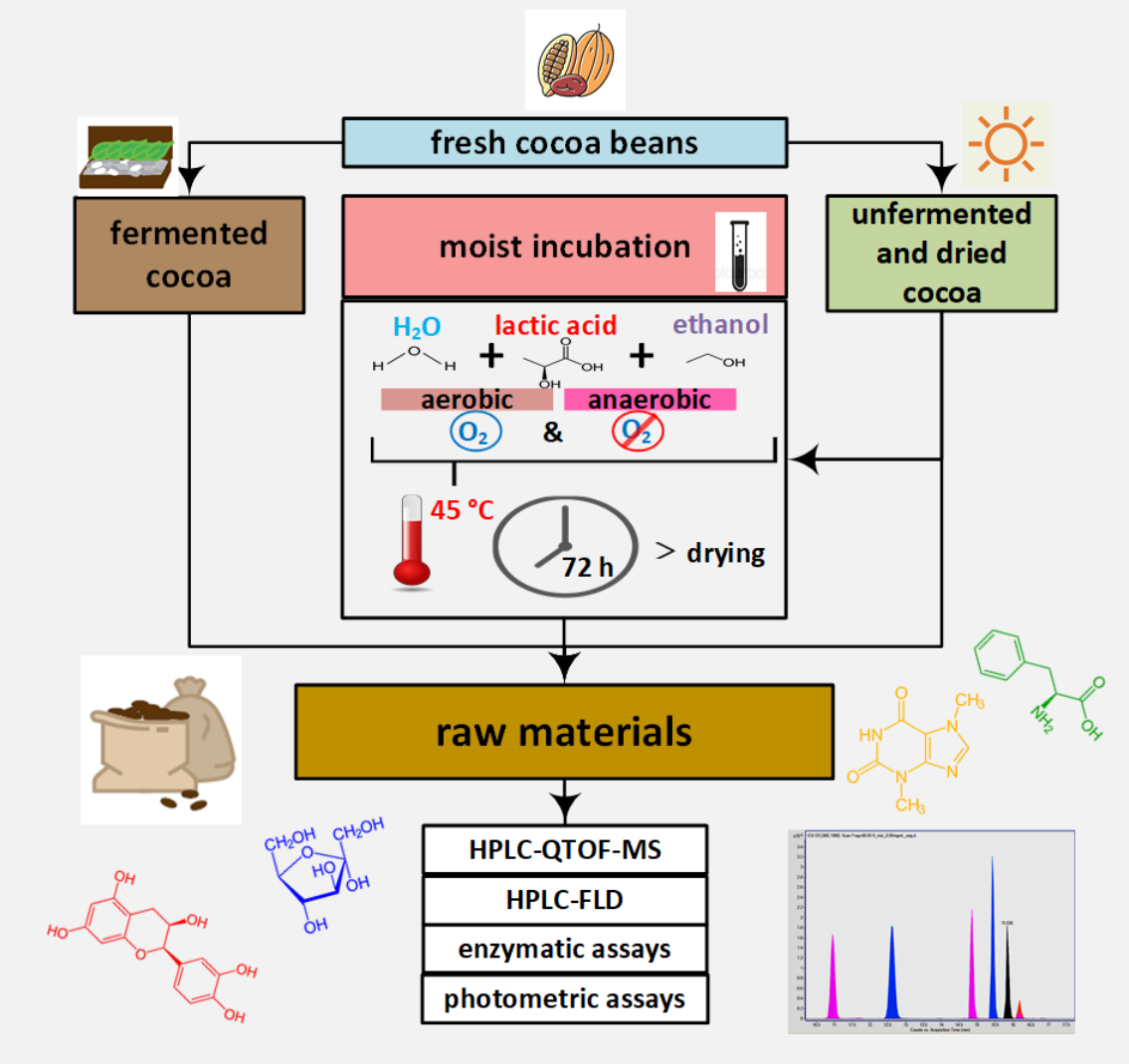
1) mean and standard deviation (SD) was calculated from extractions of three random samples from the same batch of raw material (n=3); 2) mean and standard deviation (SD) was calculated from data obtained from three experimental replicates with three random samples of each experiment (n=9); 3) total polyphenol content expressed as mg (-)-epicatechin equivalents per g fat free dry matter (mg EE/g ffdm); 4) total flavan-3-ol content expressed as mg procyanidin B2 equivalents per g fat free dry matter (mg PE/g ffdm); 5) sum of D-glucose and D-fructose; 6) sum of L-alanine, L-tyrosine, L-valine, L-methionine, L-tryptophan, L-phenylalanine, L-isoleucine, L-leucine. Values with different letters within one row show significant difference between treatments according to Tukey (HSD) test with a significancy level of 95 % ($p<0.05$)

TOC: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fermented cocoa
moist incubation
unfermented and dried cocoa
H2O
lactic acid
ethanol
aerobic
anaerobic
O2
&
45 °C
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> drying
raw materials
HPLC-QTOF-MS
HPLC-FLD
enzymatic assays
photometric assays