The impact of fermentation on the distribution of cadmium in cacao beans

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ABSTRACT

A large fraction of the South-American cacao production is affected by new cadmium (Cd) regulations in cacao. This work was set up to characterize the distribution and speciation of Cd within the cacao fruit and to monitor potential Cd redistribution during cacao fermentation. In cacao fruits from four locations, Cd concentrations decreased with testa > nib ~placenta ~pod husk > mucilage. The distribution of Cd within cacao beans was successfully visualized using laser ablation inductively coupled plasma spectrometry (LA-ICP-MS) and confirmed higher Cd concentrations in the testa than in the nib. Speciation analysis by X-ray absorption spectroscopy (XANES) of unfermented cacao beans revealed that Cd was bound to O/N-ligands in both nib and testa. Fermentation induced an outward Cd migration from the nibs to the testa, i.e. against the total concentration gradient. This migration occurred only if the fermentation was sufficiently extensive to decrease the pH in the nib to <5.0, likely as a result of increased Cd mobility due to organic acid penetration into the nibs. The change in dry weight based nib Cd concentrations during fermentation was, on average, a factor 1.3 decrease. We propose that nib Cd can be reduced if the nib pH is sufficiently acidified during fermentation. However, a balance must be found between flavor development and Cd removal since extreme acidity is detrimental for cacao flavor.

1. INTRODUCTION

Although cacao-derived products are generally consumed in small quantities compared to staple foods, they can be an important source of dietary Cd because of potentially high Cd concentrations. The European Food Safety Authority estimated that cacao-derived products account for 4.3% of the total dietary Cd exposure in the European population (EFSA, 2012). Therefore, the European Commission approved threshold limits for Cd in cacao-derived products, which were enforced in January 2019 (European Commission, 2014) Similar limits were adopted by the Codex Alimentarius (Codex Alimentarius Commission, 2018). This new regulation on Cd in cacao will impact South-American cacao farmers, as Cd concentrations in South-American cacao are generally higher than those in cacao from other origins. Bertoldi et al. (2016) reported Cd concentrations in South-American cacao beans more than tenfold larger than those in West-African cacao. These findings have prompted researchers to explore potential techniques to lower Cd in the final product, focusing mostly on the relation between cacao bean Cd and soil Cd. For example, Argüello et al (2019) has mapped the concentrations of Cd in cacao beans and corresponding soils in Ecuador and was able to identify soil parameters to predict cacao bean Cd. Ramtahal et al. (2019) investigated potential soil amendments and showed that both biochar and lime application may reduce Cd accumulation in cacao.

A recent survey by Vanderschueren et al. (2019) showed that the Cd concentration in cacao-derived products is correlated to the cacao content of the product, indicating that Cd originates from the raw material (cacao nibs) rather than other ingredients or contamination during processing. Similar results have also been reported by Abt et al. (2018), Villa et al. (2014) and Yanus et al. (2014). The cacao fruit (*Theobroma cacao* L.) consists of an outer pod husk containing 20–50 cacao beans (seeds), surrounded by a sugary mucilage and attached to a central tissue (cacao

placenta). Each cacao bean comprises of a seed coat or testa and two cotyledons known as the nib (Beckett, 2008). Chocolate production requires extensive post-harvest processing, starting with fermentation. The pod husk and placenta are discarded, and the cacao beans with surrounding mucilage are fermented for two to ten days depending on the cultivar and local practices. Fermentation is anaerobic during the first one to two days and the microbial population mostly consists of yeasts. Pectinolytic enzymes produced by the yeasts liquefy the mucilage and cause fermentation sweatings, which are drained through holes at the bottom of the fermentation boxes. This results in an increase in oxygen levels, allowing growth of lactic acid and acetic acid bacteria. The concentrations of lactic acid and acetic acid in the mucilage start to increase after two days of fermentation (De Vuyst & Weckx, 2016). The fermentation process results in a temperature increase from ambient temperature to 45–50 °C; an increase in mucilage pH from 3–4 to 4.5–5 due to conversion of citric acid to ethanol; and a decrease in nib pH from 6.5–7 to 4.5–5 due to penetration of lactic and acetic acid (De Vuyst & Weckx, 2016; Thompson et al., 2007). After fermentation, cacao beans are dried to reach a moisture content of maximum 6–8% (Afoakwa, 2010). During the second more industrial stage of the chocolate manufacturing process, the cacao beans are roasted and the testa is removed. The roasted nibs are then ground to cacao liquor and further processed to obtain consumer products (i.e. chocolate and cacao powder).

Post-harvest strategies that lower the Cd concentration in the cacao nib may offer viable options to lower the Cd concentration in the final product because the nib is the only part of the cacao fruit retained during processing. Developing such strategies requires better understanding regarding the distribution and speciation of Cd in the different cacao tissues, as well as the influence of conventional post-harvest processes on this distribution. Most previous work reports on the cacao nib, testa and pod husk with little attention to the mucilage and placenta. The Cd concentration in

the testa is generally reported higher than in the nibs. For example; Lewis et al. (2018) found more than twofold higher Cd concentrations in the testa compared to the nib, and Ramtahal et al. (2016) reported higher Cd concentrations in the testa compared to the nib, in cacao from Trinidad and Tobago. The reverse has also been observed. Chavez et al. (2015) measured the Cd concentrations in cacao from 19 Ecuadorian farms and found generally higher Cd concentrations in the nib compared to the testa. Sample treatment often differs, cacao samples were provided by chocolate manufacturers with little information regarding sample processing (Lee & Low, 1985), samples were either or not washed with water which can affect Cd concentrations in the outer tissues (Gramlich et al., 2018; Lewis et al., 2018; Ramtahal et al., 2016), or samples were washed with chelating agent solutions (Chavez et al., 2015). To the best of our knowledge, the influence of fermentation on the Cd distribution in cacao has not been reported to date. Thyssen et al. (2018) mapped the 2D distribution of Cd in sections of fermented cacao beans using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and found elevated signals for Cd, Cu, K, Mg, Na, Pb and Zn in the testa compared to the nib, however they did not investigate unfermented samples. Fermentation has been reported as a possible technique for reducing Cd in rice (Zhai et al., 2019; Zhang et al., 2017). Zhai et al. (2019) reported Cd removal efficiencies >90% for rice fermented with lactic acid bacteria and related this to a combination of the Cd binding potential of the bacteria, and the effects of the organic acid production mobilizing Cd. This phenomenon may also occur during cacao fermentation due to the lactic and acetic acid production, but has not been studied to date.

The objectives of this study were (i) to determine the distribution and speciation of Cd between and within the different cacao fruit tissues, i.e. pod husk, placenta, mucilage, testa and nibs; and (ii) to investigate the influence of fermentation on the distribution of Cd between these different

cacao bean tissues. Better understanding of the distribution and speciation of Cd in cacao and the influence of post-harvest processing may shed light on opportunities to lower Cd concentrations in the final product.

2. MATERIALS AND METHODS

2.1. Cacao material

Ripe cacao fruits were collected at four fields in the provinces El Oro (batch A, CCN-51 cultivar), Guayas (batches B and C, Nacional cultivar) and Sucumbíos (batch D, Nacional cultivar) in Ecuador (Table 1). Unfermented cacao beans for XANES spectroscopy were collected at different fields in the provinces Esmeraldas (Nacional cultivar), Guayas (CCN-51 cultivar) and Sucumbíos (Nacional cultivar).

2.2. Sampling and sample preparation

A minimum of three intact cacao fruits was collected for each batch, and each fruit was considered as an independent replicate. The intact fruits were manually separated to obtain pod husk, placenta, mucilage and cacao beans. Residual mucilage was removed from the cacao beans using paper towels and all cacao tissues were oven dried for 72 hours at 65 °C. After drying, subsamples of intact cacao beans of batches C and D were collected for LA-ICP-MS imaging. The remainder of the cacao beans was manually separated in nibs and testa, and all dried fractions (pod husk, placenta, mucilage, testa and nib) were ground using a coffee grinder before chemical analyses.

2.3. Fermentation

Fermentation experiments were conducted to assess the effect of fermentation on the distribution of Cd within cacao tissues, and comprised two cultivars (CCN-51 in batch A and Nacional in batches B, C and C_{bis}) and three common fermentation methods (cascade fermentation in batches A and B, single box fermentation in batch C and single box fermentation with pre-drying in batch

C_{bis}). No fermentation experiments were set up for the cacao of batch D. Batch C was split in two fermentation batches, C and C_{bis}, which comprised of the same cacao (same variety and plantation) but were subjected to different fermentation conditions (Table 1). Fermentation experiments were conducted in Ecuador following local practices using wooden boxes with perforated floors to allow drainage of the fermentation sweatings. The boxes were covered with jute bags to retain heat. For each batch, the total mass of cacao needed to fill two fermentation boxes (about 580–1180 kg, Table 1) was thoroughly mixed and divided in duplicate fermentation boxes. Different subsamples of 1 kg cacao mass were taken and placed in mesh bags (Figure S1) to facilitate bean sampling during fermentation. All mesh bag subsamples (3–7 per box, depending on the batch) were placed in the center of the fermentation boxes at the start of fermentation and relocated in the same position after mixing. Daily sampling was performed by taking out one of the mesh bag subsamples from each fermentation box. Each mesh bag subsample was considered an independent replicate for that fermentation day and the two fermentation boxes were considered as the duplicates for the batches or fermentation experiments. Fermentations A and B were performed in cascades of three wooden boxes measuring 60×60×60 cm (width×depth×height) and the fermenting masses were mixed every two days by depositing them in the next box of the cascade (Figure S2). Batches C and C_{bis} were fermented in single 100×100×60 cm wooden boxes. This cacao was mixed manually after one day (C_{bis}) or two days (C) and remained in the initial box throughout the fermentation period. Cacao beans of batch C_{bis} were pre-dried over night before fermentation, mimicking a common practice in some fermentation facilities. In this pre-drying method, fresh cacao was spread out on a concrete floor and left to dry overnight. This method, also referred to as bean spreading, is a common practice to prevent excessive acidity of cacao beans during fermentation (Biehl et al., 1990; Meyer et al., 1989; Schwan & Wheals, 2004). The total fermentation time for each batch

was determined by local practices (Table 1). The endpoint of each fermentation was based on quality assessment by local farmers. Beans were sampled daily by removing one mesh bag subsample (1 kg cacao mass) from the center of each box. The cacao beans in the mesh bags were then manually separated in mucilage and beans and oven dried at 65 °C for 72 hours, with beans split in nibs and testa and further ground as described above.

2.4. Temperature and pH

The mucilage pH was measured immediately after sampling or after opening of the cacao fruits. Cacao beans with mucilage attached were vigorously shaken for 2 min in a 1:10 solid to deionized water ratio and the pH of the suspension was measured. To determine nib and testa pH, dried and ground material was treated likewise in a 5:10 solid sample to deionized water ratio and filtered (F2040 filter paper, retention 7–9 µm, CHMLAB GROUP, Barcelona, Spain) to obtain a clear supernatant for pH measurement. The temperature in the fermentation experiments was measured daily in the center of the fermentation boxes using a digital thermometer (VWR International, Darmstadt, Germany).

2.5. Determination of the elemental composition of cacao beans

Duplicates of 100 mg dry material were digested in 3 mL concentrated Suprapur® nitric acid (HNO_3, 65% w/w; Merck, Darmstadt, Germany) in an open digestion block for 8 hours at a maximum temperature of 130 °C. Digests were diluted five times with Milli-Q water (18.2 MΩ cm^{-1}) and the Cd concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, USA). The ICP-MS analysis was performed in helium (He) collision cell mode monitoring the ^{111}Cd isotope, using ^{103}Rh as an on-line internal standard. The limit of quantification (LOQ) for the ICP-MS analysis was 0.02 µg Cd L^{-1} which corresponded to a solid sample LOQ of 0.006 mg Cd kg^{-1} dry matter. Blank samples (in

quadruplicate) and certified reference material NIST 2384 baking chocolate (in triplicate) were included in all digestions and treated the same way as the cacao tissue samples. Recoveries of the certified reference material ranged 98–108% and the coefficient of variation (CV) for the duplicate digestions ranged 0.1–23% (average CV 5%). The concentrations of several other elements were also determined, i.e. ^{27}Al, ^{75}As, ^{44}Ca, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{39}K, ^{24}Mg, ^{55}Mn, ^{95}Mo, ^{60}Ni, ^{31}P, ^{208}Pb and ^{66}Zn.

2.6. Visualization of the elemental distribution by LA-ICP-MS in unfermented cacao beans

Unfermented cacao beans for LA-ICP-MS imaging were sampled from batches C and D. For each LA-ICP-MS sample (which was a single bean), the Cd concentration was determined from other cacao beans obtained from the same fruit using the digest method and ICP-MS analysis as described previously. Transverse cacao bean cross-sections with a thickness of 65 µm were made following the method described by Lombi et al. (2009) for rice grains. The cacao beans were sliced with a vibrating microtome (Microm HM 650 Vibratome, Thermo Scientific, Walldorf, Germany) using diamond blades (GFD Gesellschaft für Diamantprodukte, Ulm, Germany). The cross-sections were made at approximately 80% of the length of the cacao bean, measured from the cacao radicle side. Once a flat surface was obtained, the surface of the cacao bean was defatted using hexane (HiPerSolv Chromanorm 97%, VWR, Leuven, Belgium) to enable sticking of the tape on the cacao surface. Then, a piece of Kapton polyimide tape was pressed on the surface and the diamond blade cut underneath, leaving a cacao bean cross-section glued on the Kapton tape. For elemental detection, a quadrupole 7700cs ICP-MS (Agilent Technologies, Santa Clara, USA) was used, mounted with platinum (Pt) cones. The sensitivity and operational conditions (stability, background and mass calibration) were checked using a 1 µg L^{-1} Y, Tl, Li, Ba and Ce tuning solution. The ICP-MS was then coupled to a 213-nm laser ablation system equipped with a TV2

cell (NWR213, ESI, Freemont, CA) and coupling was optimized using a NIST 612 glass by monitoring ^{238}U and ^{232}Th for maximum sensitivity and a U to Th ratio as close as possible to the unit. Imaging of cacao cross-sections was performed by subsequent line scans with a 20 Hz laser shot repetition rate, a fluency maintained between 5–5.3 J cm^{-2}, a laser beam of 20 μm^2 and 50 μm^2, a scan speed of 23 and 50 $\mu m\ s^{-1}$, and a distance between each line of 40 and 100 µm for high and low resolution images, respectively. The ablated cacao particles were transported with 800 mL min^{-1} He and mixed with Ar gas from ICP-MS before the ICP torch inlet. The ICP-MS was used in He mode, allowing monitoring of ^{111}Cd (0.2 s), ^{114}Cd (0.2 s), ^{31}P (0.1 s), ^{39}K (0.005 s), ^{44}Ca (0.15 s), ^{60}Ni (0.1 s) and ^{64}Zn (0.1 s as integration time). The acquisition time was set according to the ablation time needed for one line. One data file recording the intensity of each element versus time was acquired for each line, and a homemade program under Python was used to generate 2D images of element intensities per pixel with a colour code.

2.7. X-ray absorption near-edge structure spectroscopy

The speciation of Cd in unfermented cacao was investigated using X-ray absorption near-edge structure spectroscopy (XANES). The samples comprised of cacao beans from CCN-51 and Nacional cultivars. The cacao testa and nib were separated manually and both materials were dried, ground and pressed into pellets for XANES analysis. The XANES spectra were collected at the X-ray absorption spectroscopy beamline (Australian synchrotron, ANSTO). The Cd K-edge (26711 eV) was scanned rather than the Cd L_{III}-edge (3538 eV) to avoid interference with the K K-edge (3608 eV), as K is expected to be abundant in cacao tissues. Sample spectra were measured in fluorescence mode with a 100-elements solid-state Ge detector, at 10 °K to prevent beam damage. One spectrum represents the average of 2–26 scans, depending on the concentration of Cd. Each scan was measured on a different spot on the pellet to limit beam damage. Reference X-ray spectra

for Cd metal foil were collected simultaneously with the sample spectra and were used for both energy calibration and spectral alignment. Several Cd reference compounds were also measured: Cd-chloride, Cd-phosphate, Cd-sulfate, Cd-oxide, Cd-acetate, Cd-lactate, Cd-citrate, Cd-histidine, Cd-phytate, Cd-malate, Cd-cysteine and Cd-glutatione (details about reference compound preparation are given in the supplementary information). Data extraction was performed using Sakura (https://sakura.readthedocs.org) while background subtraction, normalization and linear combination fitting (LCF) were done using Athena software (Ravel & Newville, 2005). For each sample spectrum, LCF was performed by fitting regions between -25 and 70 eV using the library of Cd reference compounds. Satisfactory fits were obtained with a combination of two reference compounds. Three compound LCFs were not retained as the residual factor (R-factor) used to assess the goodness of fit was not significantly smaller compared to the R-factor of the two compound LCFs. Linear combination results were normalized to 100% to compare the relative speciation between samples.

2.8. Statistical analysis

All statistical analysis was executed using JMP® Pro version 14.0.0 (SAS Institute 2018). The differences in Cd concentration between the different cacao tissues were tested using Tukey's Honestly Significant Difference test (P-value≤0.05) using the mean data of sampling replicates (e.g. the two fermentation boxes), as the independent replicates. The effect of fermentation time on the elemental composition of the different tissues was tested using Pearson's correlation test (P-value≤0.05).

3. RESULTS AND DISCUSSION

3.1. Distribution of Cd in unfermented cacao beans

Cacao beans from batch D showed the highest Cd concentrations in nib and testa, followed by batch C, and finally batches A and B (Table 2). The coefficients of variation (CV) of nib Cd between cacao fruits of the same batch ranged between 20 and 37%, indicating variation in cacao bean Cd between fruits from the same plantation. In a large survey of 159 Ecuadorian fields, Argüello et al. (2019) observed that the average CV in bean Cd concentration among fruits of different trees within the same field was 39%. They related this variation in bean Cd to the large spatial variation in soil Cd. The Cd concentrations were overall highest in the testa, followed by the nibs, placenta and pod husk (all similar in Cd content) and finally the mucilage. No information was found in literature regarding the Cd concentration in the placenta or the mucilage. Gramlich et al. (2018) measured the Cd concentration in cacao from 55 farms in Honduras and did not find a significant difference between the Cd concentration in the pod husks (1.1 ± 0.2 mg kg^{-1}) and that in the nibs (1.1 ± 0.1 mg kg^{-1}). Conversely, Ramtahal et al. (2016) reported higher Cd concentrations in the pod husks (0.53–4.49 mg Cd kg^{-1}) compared to the nibs (0.35–3.82 mg Cd kg^{-1}) for cacao from 45 farms in Trinidad and Tobago.

Testa Cd concentrations were higher than nib Cd concentrations in all batches (ratio testa Cd to nib Cd 1.8 for A, 1.7 for B, 1.5 for C and 1.7 for D). Considering the average weight fractions for nib (0.93) and testa (0.07), 91% of the total bean Cd was located in the nib and 9% in the testa in unfermented cacao beans. In accordance to the present work, Ramtahal et al. (2016) reported significantly higher Cd concentrations in testa (0.44–4.41 mg Cd kg^{-1}) compared to nibs (0.35–3.82 mg Cd kg^{-1}) for unfermented cacao beans from Trinidad and Tobago. Lewis et al. (2018) reported more than two-fold higher Cd concentrations in the testa (average 1.83 mg kg^{-1}) compared to those in the nibs (average 0.88 mg kg^{-1}) for unfermented cacao beans from the same genetic group and grown in a common garden. Similarly, Lee & Low (1985) determined the Cd

concentrations in raw cacao beans from two different sources and reported higher Cd concentrations in the testa (1.32 ± 0.06 and 2.05 ± 0.01 mg Cd kg^{-1}) compared to Cd concentrations in the nibs (0.76 ± 0.02 and 1.01 ± 0.01 mg Cd kg^{-1}) for the two sources, respectively. Conversely, Chavez et al. (2015) analyzed the Cd concentration in unfermented cacao nibs and testa from 19 different small-scale farms in the south of Ecuador and consistently found higher Cd concentrations in the cacao nibs compared to the testa. However, the cacao beans in that study were washed with a hypochlorite solution before peeling which may have removed some of the Cd in the outer testa.

3.2. Imaging of elemental distribution in unfermented cacao beans with LA-ICP-MS

The elemental imaging maps obtained by LA-ICP-MS for ^{111}Cd and ^{114}Cd isotopes showed close agreement, which indicates that there were no interferences affecting the Cd measurement (Figure 1, and Figures S3 and S4). There were no zones found with consistently elevated intensities for the measured isotopes (^{44}Ca, ^{111}Cd, ^{114}Cd, ^{39}K, ^{60}Ni, ^{31}P and ^{64}Zn, Figures S3 and S4) in the obtained elemental maps, indicating that the samples were sufficiently planar for reliable image interpretation and comparison. Regions with consistently lower signal intensity corresponded to inherent cracks in the cacao bean samples as visible on the sample pictures (Figure 1). The overall Cd concentrations in the nib and testa from batch D were nearly seven times higher than the Cd concentrations in the tissues of batch C. When LA-ICP-MS imaging is performed at a higher resolution, a smaller surface area of the sample is ablated and thus less sample material is transported to the ICP-MS detector. Therefore, higher resolution imaging requires samples with larger elemental concentrations (or very long measurement times). Because of this, the batch D sample could be scanned at higher resolution (20 µm² laser beam size) than the cross-section of batch C (laser beam size 50 µm²). The ICP-MS integration time was equal in both scans to limit

the measurement duration. Therefore, the signal intensity (expressed as counts per second, cps) of the batch D sample was lower than that of the batch C sample even though the overall Cd concentrations in D were larger than those in C.

The testa layer was clearly distinguishable from the cacao nib and showed elevated Cd intensities for both samples (Figure 1), which is in line with the measured Cd concentrations in these tissues (Table 2). Apart from Cd, only Ca displayed clearly elevated intensities in the testa (Figures S3 and S4). The distribution of K was approximately uniform between nib and testa; and P, Ni and Zn were more abundant in the nib compared to the testa (Figures S3 and S4). Cadmium and zinc were not co-located within the cacao bean tissues even though they are considered similar in chemical properties and are often transported in plants through similar mechanisms (Smolders & Mertens, 2013). Dissimilar Cd and Zn distribution patterns have also been visualized in rice (Meharg et al., 2008). This may indicate a difference in transport mechanisms for Cd and Zn into the cacao seed, possibly related to a defense mechanism of the plant as Zn is an important micronutrient while Cd has no known function in plant growth. The distribution of Cd within the cacao nib was not homogeneous throughout the cross-section. Further identification of nib zones with higher Cd intensities may shed light on the way Cd is translocated into the cacao bean during plant growth. Thyssen et al. (2018) created elemental maps of longitudinal cross-sections of fermented cacao beans using LA-ICP-MS and observed elevated signal intensities for Cd in the testa. However, they reported accumulation of P, K and Zn in the testa, which was not observed in this study. These differences compared to the present work may be explained by the influence of cacao fermentation or by possible differences in cultivars and overall different elemental concentrations between these samples.

3.3. Speciation of Cd in unfermented cacao beans

The LCF procedure identified two reference compounds to describe the speciation of Cd in all samples (Figure 2 and Figure S5). Optimal fits were obtained with a combination of Cd-histidine, where Cd is bound to amino and carboxyl groups, and Cd-citrate, where Cd is bound to alcohol and carboxyl groups. The proportions of both ligands were similar in most samples (39–58% Cd-histidine and 42–61% Cd-citrate), except for nib 3 (84% Cd-histidine and 16% Cd-citrate) and testa 1 (22% Cd-histidine and 78% Cd-citrate). These results indicate that Cd in the cacao nib and the testa is bound to O/N-ligands. In hyperaccumulator plants, Cd was found with both S-ligands and O-ligands, and the association with O-ligands was reported as a detoxification strategy in contrast to non-hyperaccumulating plants where S-ligands were predominant (Huguet et al. 2012, Isaure et al. 2006 & 2015, Vogel-Mikuš et al. 2007). In the present work, no evidence was found for complexation with S-ligands (thiols) in the cacao nib or testa.

3.4. Changes in pH and temperature during fermentation

The nib pH in batches A and B decreased with fermentation from 6.2 to about 4.5, the mucilage pH increased from about 3.7 to 4.5, and the testa pH increased from 4.3 to 5.0 (Figure 3). Changes in pH were less pronounced in batches C and C_{bis} (final nib pH 5.2 and 6.0, mucilage pH 3.8 and 4.0, and testa pH 4.8 and 4.4 for the two batches, respectively). The fermentation times for C and C_{bis} were shorter than in A and B (3–4 versus 5–7 days, Table 1), suggesting a lower extent of fermentation. The temperature profile was similar in all batches and increased from the start of fermentation reaching 45 °C after three to four days of fermentation. The pH and temperature values are in line with values reported in literature, which state that the temperature of the fermenting cacao bean mass increases from ambient temperature to about 45–50 °C and nib pH decreases from 6.3–7.0 to 4.0–5.5 during fermentation (Belitz et al., 2009; De Vuyst & Weckx, 2016; Papalexandratou et al., 2011; Schwan & Wheals, 2004; Thompson et al., 2007).

3.5. The influence of fermentation on the distribution of Cd in cacao beans

One replicate sample of mucilage (fermentation day 3, batch B) showed an extreme Cd concentration (6.6 mg Cd kg^{-1}) and was excluded from analysis. The concentration of Cd within the different cacao bean tissues before the start of fermentation (day zero, Figure 4) was in line with the values observed in intact fruits (testa > nib > mucilage, Table 2). The nib Cd concentration in batches A and B decreased with fermentation time by a factor 1.3 (Figure 4). The final nib Cd concentration in B was lower than 0.60 mg kg^{-1}, which is commonly considered the maximum allowed Cd concentration in cacao beans destined for export to the EU. The mucilage and testa Cd concentrations in A increased with fermentation time (factor 2.1 in testa and 7.8 in mucilage) reaching a similar plateau concentration after four days of fermentation. The same was true for the mucilage Cd in B (increased by factor 2.5) but no significant trend in testa Cd was observed. To correct for changes in nib and testa weight fractions throughout fermentation, the Cd concentrations in the different tissues were multiplied with the weight fraction of that tissue. These weight fraction corrected results still showed a decrease in nib Cd content (expressed in mg nib Cd kg^{-1} total cacao bean) and an increase in testa Cd content (mg testa Cd kg^{-1} total cacao bean) for A and B (Figure S6). This suggests that Cd migrates from the nib to the testa and the mucilage during fermentation, resulting in lower Cd concentrations in the final cacao based product because the outer tissues (testa and mucilage) are removed at later stages of the post-harvest process. At the end of fermentations A and B, approximately 80% of the total cacao bean Cd was located in the nibs whereas 20 % was found in the testa.

As stated previously, batches C and C_{bis} were fermented less extensively than batches A and B. This may explain why no change in nib Cd was observed by the end of fermentation in these batches. The mucilage Cd concentration in C increased significantly with fermentation time (factor

355 6.2) in a similar pattern as observed for batches A and B. But in contrast to A and B, the testa Cd
356 concentration in C decreased with time. Fermentation of batch C_{bis} had no significant influence on
357 the Cd concentrations in the testa. The mucilage Cd concentration did increase by factor 1.7 but
358 this change was not of the same magnitude as observed in the other batches. The overall Cd
359 concentrations in C and C_{bis} were higher than those in A and B. However, results from a pilot scale
360 fermentation (5 kg) using the same high Cd cacao, showed that Cd concentrations in the nibs of
361 this cacao did decrease with fermentation time if the cacao was fermented more extensively, i.e.
362 four days with a decrease in nib pH from 6.1 to 4.8 (data not shown). This demonstrates that the
363 pH, rather than the total Cd concentration, explains Cd migration. Mass balance calculations of
364 bean Cd showed that the total bean Cd concentrations reduced with 15% by the end of fermentation
365 in batches A, B and C (Figure S7). This may be related to the loss of mucilage through fermentation
366 sweatings. No Cd loss was observed over the course of fermentation for batch C_{bis} which had been
367 air-dried prior to fermentation. Farmers estimate that the cacao loses approximately 25% of its
368 fresh weight during pre-drying as the mucilage liquid runs off and evaporates. As a result,
369 sweatings during the fermentation may be much smaller in such fermentation practices and the
370 total Cd mass may remain constant over the course of fermentation.
371 The nib Cd concentration was strongly correlated to nib pH throughout fermentation in batches A
372 and B, but not in C and C_{bis} (Figure 5). The nib pH in C and C_{bis} decreased by over one unit during
373 fermentation but remained >5, while the pH in batches A and B dropped to 4.5. This may indicate
374 the importance of fermenting long enough to reach nib pH values <5, in order to generate Cd
375 migration from the nib outwards. XANES speciation analysis showed that Cd was mostly bound
376 to O/N-ligands such as histidine and citrate. The pKa value for the dissociation of the second
377 carboxylic group in citrate is 4.77, which can explain the increase in Cd mobility when the tissue

pH drops below that value. Conversely, the pKa values for histidine are 1.82 (carboxylic group), 6.00 (N in the imidazole ring) and 9.17 (amine group). Zhai et al. (2019) stated that rice fermentation may decrease Cd concentrations and that this Cd removal capacity is related to the acid producing abilities of lactic acid bacteria present during fermentation. They reported a pH decrease from 6 (initial pH) to <4.5 by the end of fermentation, depending on the strain of lactic acid bacteria used. Testa Cd concentrations were approximately a factor two larger than nib Cd concentrations in unfermented cacao (Table 2). The migration of Cd during fermentation thus occurred against the total Cd concentration gradient. However, the cacao testa has been identified as a heavy metal adsorbent with potential applications in the treatment of industrial effluents (Meunier et al., 2003). Because of the Cd sorption capacity of the testa and the differences in pH between the nib and the testa, the concentration gradient of mobile Cd in fermented cacao beans may be the inverse of the total concentration gradient, a key speculation that requires further validation.

3.6. The influence of fermentation on other elements in cacao

Apart from Cd, several other elements were analyzed by ICP-MS (Al, As, Ca, Co, Cr, Cu, K, Mg, Mn, Mo, Ni, P, Pb and Zn) and the concentrations of most of these elements in each tissue (nib, testa, mucilage) were correlated with fermentation time (Table S1 and Figures S8 I–X). Concentrations of Al, As, Cr and Pb were <LOQ in relevant fractions of the nib samples (Al 98%, As 80%, Cr 72% and Pb 93%), the testa samples (Pb 22%) or the mucilage samples (As 37%) and were not further discussed. Fermentation had no significant effect on Mo in any of the cacao tissues. Elemental concentrations in the nibs generally decreased while testa and mucilage concentrations increased. The nib concentrations decreased in batches A and B for Cu (factor A 1.4, B 1.2), K (A 1.6, B 1.4), Mg (A 1.4, B 1.3), Mn (A and B 1.1), Ni (A 1.7, B 1.6) and P (A

1.04, B 1.4). No significant changes were observed in the nib elemental composition for batches C and C_{bis} with the exception of a significant increase in nib Ni concentration in C_{bis} (factor 1.1). The testa concentrations increased with fermentation in batches A and B for Cu (A 3.2, B 1.8), K (A 2.8, B 2.6), Mg (A 4.4, B 2.9), Mn (A 3.1, B 3.0), Ni (A 3.9, B 3.4) and P (A 9.8, B). Calcium was the only element that displayed a reverse change in concentration, nib Ca increased while testa Ca decreased with fermentation time. The elemental concentrations in the mucilage generally increased, which might be caused by microbial deterioration of the outer layers of the testa. If present, this deterioration was not strong enough to cause a significant decrease in the testa weight fraction with fermentation time. The testa weight fraction remained in the range 0.05–0.10 throughout fermentation in all batches. However, regardless of the minimal change in testa weight fraction with fermentation, changes in the morphology of nib and testa may still be possible. To confirm migration of the elements rather than changes in the morphology of the tissues during the fermentation process, the elemental concentrations in each tissue (nib and testa) were multiplied by the weight fraction of that tissue. The weight fraction corrected concentrations corroborated the migration of aforementioned elements (Cu, K, Mg, Mn, Ni and P) from the nib to the testa in batches A and B. The observed migration pattern of Ni might be of importance in the future because the European Commission mentioned cacao based products among important food sources of Ni in the European population (EFSA, 2015). Based on the similar behavior of Ni and Cd observed in this work, post-harvest strategies to lower Cd concentrations in cacao during fermentation will likely also be effective for Ni.

4. CONCLUSION

In unfermented cacao fruits, Cd concentrations are highest in the testa, followed by nibs, placenta and pod husks which all contain similar Cd concentrations, and finally the mucilage. This study is

probably first to report the fate of Cd and its distribution in cacao tissues during fermentation. Migration of Cd from the nibs to the testa was only observed if the nib pH dropped below 5. This acidic pH resulted from longer fermentation times. More extensive fermentation can thus result in lower Cd concentrations in the final product as the testa and mucilage are removed later in the post-harvest process. After fermentation, nib Cd concentrations decreased by a factor 1.3, indicating that fermentation may be useful to comply to the new Cd requirements (0.60 mg Cd kg^{-1}) in cacao beans with initial unfermented nib Cd concentrations up to 0.78 mg Cd kg^{-1}. Further work is required to assess the full potential of Cd migration from the nib to the testa during fermentation. Nevertheless, it is often recommended to avoid very low nib pH as this can cause an unpleasant acidic taste in the final product (De Vuyst & Weckx, 2015; Schwan & Wheals, 2004). A balance must thus be found between flavor quality and Cd concentration. This acidic flavor is the main reason for pre-drying practices and results confirmed that the nib pH in pre-dried cacao decreased less extensively compared to the other fermentation experiments. Our results indicate that pre-drying and short fermentation times may reduce the extent of outward Cd migration.

SUPPLEMENTARY INFORMATION

The supplementary information includes a description of the preparation of XANES Cd reference compounds, pictures of the mesh sample bags (Figure S1) and the cascade set-up (Figure S2) used in the fermentation experiments, LA-ICP-MS imaging of both cacao bean cross-sections for ^{44}Ca, ^{114}Cd, ^{39}K, ^{31}P, ^{60}Ni and ^{64}Zn (Figures S3 and S4), XANES spectra for cacao tissue samples and Cd reference compounds (Figure S5), weight fraction corrected Cd concentrations in nib and testa throughout fermentation for all fermentation batches (Figure S6), Cd mass balances of the fermentation experiments (Figure S7), Pearson correlation coefficients indicating the effect of fermentation time on the elemental composition (Ca, Cd, Co, Cu, K, Mg, Mn, Mo, Ni, P and Zn) of each cacao tissue (nib, testa and mucilage) (Table S1), and the effect of fermentation on the elemental composition (Ca, Co, Cu, K, Mg, Mn, Mo, Ni, P and Zn) (Figures S8 I–X).

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