Cadmium speciation in cacao beans changes during a fermentation-like incubation

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ABSTRACT

Cadmium (Cd) concentrations in cacao often exceed food limits. Recently, it was shown that cacao bean fermentation enhances Cd solubility, opening potential for Cd mitigation in cacao products. This study was set-up to identify changes in Cd speciation during fermentation. X-Ray absorption spectroscopy (XAS) complemented with speciation calculations, were used on samples collected from high and low Cd farms, that were subjected to a fermentation-like incubation that reached high temperatures (>45°C) and acidic pH (<5). Incubation decreased nib Cd concentration up to a factor 1.5 and changed Cd complexation in high Cd beans from sulphur to oxygen ligands, likely due to pH changes. In beans with lower Cd concentrations, Cd was complexed before and after incubation with oxygen-ligands. A combination of pH changes and/or phytate breakdown may explain the migration of Cd outward from the nib. XAS and speciation calculations proved complimentary techniques and indicated similar speciation changes during fermentation.

KEYWORDS

Cd speciation, Fermentation-like incubation, Chelation, XANES, Metal detoxification, Phytate

1. INTRODUCTION

The European Commission and the Codex Alimentarius enforced a new regulation which limits the allowed concentration of cadmium (Cd) in cacao-derived consumer products, such as chocolate (European Commission 2014; Codex Alimentarius Commission 2018). These limits range between 0.10 and 0.80 mg Cd kg\(^{-1}\) depending on the type of product and the percentage of cocoa solids in the final product. To comply with those limits, the cacao processing industry has introduced unofficial Cd requirements that apply to the cacao beans they purchase. Those unofficial industry limits vary between 0.10 and 1.10 mg Cd kg\(^{-1}\) bean dry weight (CBI Ministry of Foreign Affairs n.d.; Meter et al. 2019; Vanderschueren et al. 2021). To facilitate the discussion below, 0.60 mg Cd kg\(^{-1}\) bean dry weight is further used as a threshold. These Cd-cacao thresholds can strongly impact the cacao industry, especially in Central and South America, where bean Cd concentrations are higher compared to those in other cacao producing regions (Knezevic 1979; Bertoldi et al. 2016; Abt et al. 2018; Vanderschueren et al. 2019; reviewed in Vanderschueren et al. 2021). Studies on mitigation strategies for Cd in cacao have mostly focussed on agronomic
practices, e.g., use of soil amendments or cultivar selection. While results of that research are promising, the practical implementation of such mitigation strategies is not yet relevant, probably because it takes a longer time before effects are detected (Vanderschueren et al. 2021).

Postharvest mitigation strategies focusing on fermentation may offer a valid short-term alternative to address the Cd contamination in cocoa products. Previous work has indicated that conventional fermentation practices can reduce the Cd concentration in the cacao nib (the inner part of the cacao beans that is used in chocolate production) by up to a factor 1.3 (Vanderschueren et al. 2020). This reduction of nib Cd concentration has been attributed to a combination of high temperature (>45°C) and acidic pH (<5). The study showed that Cd mobilized from the nib to the testa (seed envelope that is discarded for chocolate production). However, the biochemical mechanisms behind this mobilization remain unclear.

Cadmium complexation with ligands i.e., Cd speciation, has been studied in non-fermented cacao beans. The few existing speciation data indicates that Cd in nibs is bound to a combination of carboxylate, phosphate and thiol (sulphide) ligands (Vanderschueren 2021; Blommaert et al. 2022). An abundant phosphate ligand in seeds is phytate. This molecule is the typical storage for P in seeds and serves as storage site for K and Mg, and sometimes also Ca, Fe and Zn (Hawkesford et al. 2012; Silva et al. 2021). The chemical similarity between Cd and Zn suggests that Cd may also be bound to phytate (Schlemmer et al. 2009). Data on Cd speciation in other plant seeds is currently limited, and mostly restricted to plants that cope with high exposure levels of Cd due to the current detection limits of synchrotron X-Ray Absorption Spectroscopy (XAS) (within 1 to 10 mg Cd kg⁻¹ (Gu et al. 2020; Yan et al. 2020; Wiggenhauser et al. 2021)). For cereals, speciation data indicated primarily a binding of Cd to S-ligands in rice (Gu et al. 2020; Wiggenhauser et al. 2021), and a mixture of O and S ligands in durum wheat (Yan et al 2020) despite the high phytate content in these cereal grains. Sulphur is a constituent of the amino acid cysteine and acts as a functional group (R-SH, thiol group) which has very high affinity for Cd. Plant cells respond to metal exposure by synthesis of the tripeptide glutathione, and/or of more complex thiol-containing polypeptides including phytochelatins and metallothioneins (Hawkesford et al. 2012; Clemens 2019).

Taken together that i) phytate is a plausible Cd ligand in the cacao nibs (Blommaert et al. 2022) and ii) that fermentation conditions may activate phytase in the nibs, which may hydrolyse phytate and thereby mobilize chelated metals, it was suggested that Cd in the cacao nibs was reduced after fermentation by a release of Cd from the phytate complex due to acidification and/or phytate breakdown (Vanderschueren et al. 2023). Cadmium speciation after fermentation may also have implications for the food chain risk of cacao-derived Cd because phytate can inhibit the gastrointestinal absorption of Cd in humans similarly as it does for Zn (Schlemmer et al. 2009). To our knowledge, there is no Cd speciation data in fermented nibs. This data may help to decipher the biochemical mechanisms behind the Cd mobilization outward from the nib.

The main objective of this study was to reveal the changes in chemical properties and Cd speciation in cacao nibs and testa before and after fermentation-like incubation. We used a controlled incubation setup to mimic fermentation (see Vanderschueren et al. 2023 for original reference).
2. MATERIALS AND METHODS

2.1. Harvest of cacao pods and experimental setup

Eight ripe cacao pods of the Nacional cultivar were collected in Sucumbios province (Ecuador). The samples were sourced from a Cd contaminated location previously identified as a hotspot for Cd accumulation (Argüello et al. (2019)) and are further mentioned as the high Cd beans (H1-H4). The pods were cut open using a kitchen knife and the beans from each pod were divided in two subsamples of equal size: one subsample that was not incubated (untreated) and the other that was incubated (treated). The subsamples consisted of 6 to 25 beans depending on the pod. The subsamples for incubation weighed 40 ± 22 g (mean ± standard deviation, wet weight without removing mucilage). The beans were incubated in 300 mL deionized water or acetic acid solution (20 g L⁻¹) for 72 hours at 25°C, 45°C or 65°C. The experimental setup was a reduced setup derived from the incubation experiment of 44 runs previously described by Vanderschueren et al. (2023), where the effect of temperature (T), ethanol, and acetic acid on the Cd mobilisation in the nibs was assessed. The reduced set-up in this study included four treatments that presented the lower and upper range of effects on Cd mobilisation observed in Vanderschueren et al. (2023). Ethanol was not included in this experiment since it did not affect significantly Cd content in beans (Vanderschueren et al., 2023). Lactic acid, another organic acid present in a real fermentation processes, was not included either because although it has a similar affinity for Cd²⁺, it is less acidic (Smith & Martell, 1987), and it is present at lower concentration than acetic acid in real fermentation conditions (Leal Junior et al 2023). The treatments consisted of: deionized water at 45°C (H1); 20 g L⁻¹ acetic acid at 25°C (H2); 20 g L⁻¹ acetic acid at 45°C (H3); and 20 g L⁻¹ acetic acid at 65°C (H4) (Table 1). Each treatment was performed on two different pods (i.e. biological replicate, n=2). From the same pod the incubation was performed in duplicate to determine the coefficient of variation (CV) for the Cd concentration measurements in the testa and nibs (i.e. process replicate, n=2). To minimise the effect of sample variation, beans from different pods were never mixed.

Two additional samples from the setup of Vanderschueren et al. 2023 were included in this study. These pods were sourced from soils with natural concentrations of Cd in Guayas (Argüello et al. 2019). These beans are further mentioned as the low Cd beans (L1 and L2). The low Cd beans were incubated in duplicate (process replicate, n=2) in 20 g L⁻¹ acetic acid and 45 °C (L1) or 40 g L⁻¹ acetic acid and 65 °C (L2) (Table 1).

2.2. Sample processing and chemical analyses

2.2.1. Incubation solutions

The pH of the incubation solutions was measured before and after incubation for each biological replicate. A 10 mL subsample was taken from each biological replicate before and after incubation. Subsamples were diluted 10 (samples before incubation) or 100 times (samples after incubation), acidified to 1.0 % (v/v) HNO₃ (NORMATOM ® 67 – 69 % w/w, VWR International, Radnor, PA, USA) and analysed by inductively coupled plasma mass spectrometry to measure the elemental content (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA).

2.2.2. Nib pH

Three cacao beans were randomly selected from each pod (untreated and treated). The mucilage on the beans was removed and the beans were oven-dried for 72 h at 70 °C. After drying, the testa of the beans was removed manually, and peeled nibs were ground in an electric mill (IKA, Staufen, Germany). The ground nibs were sieved to obtain a more homogeneous particle size (800 µm test
sieve, VWR International) and incubated in an end-over-end shaker at a 1:20 (g:mL) solid:deionized water ratio for 24 h at 20°C. Samples were then centrifuged for 10 minutes at 2000 g (Heraeus Multifuge X3R, Thermo Scientific, Waltham, MA, USA) and the pH of the supernatants was measured to determine the nib pH.

2.2.3. Nib and testa elemental composition
Two beans were selected randomly from each biological and process replicate (untreated and treated) and dried and peeled as described above. Individual nibs were ground in the electric mill and 300 (± 1) mg subsamples were acid digested in 8 mL HNO₃ (NORMATOM ® 67 – 69 % w/w, VWR International) using a microwave digestion system (MARS 6, CEM, Matthews, NC, USA). Nib digests were then diluted to 200 mL with Milli-Q water (18.2 MΩ cm⁻¹) prior to ICP-MS analysis. Intact testa samples (154 ± 96 mg, not ground) were acid digested in 3.0 mL HNO₃ (NORMATOM ® 67 – 69 % w/w, VWR International) for 8 h in an open digestion block at a maximum temperature of 135 °C. Testa digests were then brought to a volume of 10.0 mL and diluted ten times with Ultrapure Type 1-water prior to ICP-MS analysis. Blank samples (triplicate for the microwave digestion and quadruplicate for the open block) and certified reference material NIST 2384 baking chocolate (Sigma Aldrich) (in triplicate) were included in each digestion batch (certified concentration 0.0734 ± 0.0077 mg Cd kg⁻¹). The limit of quantification was 0.01 mg Cd kg⁻¹ dry weight, and the Cd recovery of the certified reference material was 107 ± 18 % (n = 6). The average coefficient of variance between the process replicates (CV) was 7% for both nib and testa Cd analyses.

2.2.4. Phytate analysis
The phytate content was determined in oven-dried (60°C) nibs (treated and untreated) after extraction with 0.66 M HCl (incubation overnight in an end-over-end shaker at 20°C), with the Megazyme K-Phyt essay (Megazyme, Bray, Ireland). Extracts were treated or not with phytase and alkaline phosphatase to convert polyphosphates to monophosphate and the phosphate content was then colorimetrically measured using the molybdenum blue method (Murphy and Riley 1962). The colorimetric measurements were performed at a wavelength of 655 nm using a UV/VIS spectrophotometer (Lambda 25, PerkinElmer, Waltham, MA, USA). The P present as phytate (phytate-P) content in the nibs was then calculated from the difference in phosphate between extracts with and without enzyme treatment, thereby assuming that all P released from enzymatic hydrolysis originated from phytate. An internal reference wheat flour sample was included in duplicate in the phytate analysis and had a recovery of 95% (based on the median measured phytate concentration after 36 individual analyses of that material).

2.3. Speciation analysis: synchrotron X-ray absorption spectroscopy (XAS)
Speciation analysis was performed on the untreated and treated nib and testa samples from H3, H4, L1 and L2. For H3 and H4, two biological replicates were measured for each treatment (n=2). The mucilage was manually removed from the cacao beans (minimum four beans), and the beans were peeled to separate nib and testa without drying. Those fresh nib and testa samples were flash-frozen in liquid N₂ and stored at -80°C until further processing. Frozen hydrated samples were milled in liquid N₂ using a cryo-grinder (Pulverisette23, Fritsch) and then pressed into pellets in cryo conditions (diameter 6 mm, thickness 3-5 mm). In addition to frozen hydrated samples, subsamples of H4 (biological replicate 1), H3 (biological replicate 2), L1, and L2 were oven-dried (60°C) for 48 h, ground, and pressed into pellets of the same size.
Cadmium K-edge XAS measurements were conducted at SAMBA beamline (SOLEIL synchrotron, Saint Aubin, France) and BM30 beamline (ESRF synchrotron, Grenoble, France). The X-ray absorption near edge structure (XANES) part, was analysed. The low Cd concentrations in the samples did not allow to retrieve valuable spectra from the extended-X-ray absorption fine structure (EXAFS) part. The monochromator was a Si220 double crystal, and acquisition was done in fly scan (SAMBA) and in step by step (BM30) mode. The acquisition time for one scan at SAMBA was 4.5 minutes, and on average 50 scans were necessary for one sample. At BM30, one scan took 17 minutes, and around 5 scans were needed for the high Cd beans, and 20 for the low Cd beans. All samples were recorded at 20 K using a He cryostat, in fluorescence mode with a 35-element germanium detector (SAMBA) or a 13-element germanium detector (BM30). A Cd foil was recorded simultaneously in transmission mode for the energy calibration of XANES spectra.

The raw XANES spectra were averaged, calibrated in energy and normalized, and treated by linear combination fits (LCFs) using the XAS software Fastosh (Landrot 2018). Linear combination fitting was performed by fitting the normalized XANES spectra in regions between −20 and 80 eV with the use of a database of Cd reference compounds. A previously recorded database of Cd reference spectra recorded at 15 to 20K was exploited that contained Cd-cell wall, Cd-glutathionine, Cd-phytochelatin, Cd-hydrated, Cd-malate, Cd-histidine, Cd-phosphate, Cd-phytate, Cd-metallothionein. The preparation and measurements of this dataset is described in (Huguet et al. 2012; Huguet et al. 2015; Blommaert et al. 2022). The R-factor (\( \frac{\sum[\mu_{\text{exp}} - \mu_{\text{fit}}]^2}{\sum[\mu_{\text{exp}}]^2} \)) assessed the goodness of fit. A combination of two reference compounds was used. For some spectra, particularly the noisier ones, there was not a unique solution since the LCF procedure provided fits of equivalent quality (R-factor increased <10% compared to the best fit). For these spectra, several fits are shown.

### 2.4. Modelling Cd speciation in nibs before and after incubation

Cadmium speciation in the nib was modelled with Visual MINTEQ software (version 3.1, available online at https://vminteq.lwr.kth.se/). Three main ligand groups were defined; cysteine (Cd-S; i.e. sulphide ligands), phytate (Cd-O (P)), and organic acids (citric acid, acetic acid, oxalic acid, and lactic acid (Cd-O (C); i.e. carboxylate ligands). Metal-phytate and metal-cysteine complexation data were added into Visual MINTEQ after consulting literature data that was corrected to infinite dilution (see section 1.1 SI).

Cadmium speciation was modelled for untreated and treated nibs (H3-H4 and L1-L2). The input for the model can be found in Table S1. The nib pH and phytate content measured before and after incubation were used as input parameters. Dry weight element concentrations measured were converted to liquid concentrations based on the solid:liquid ratio measured in the cacao nibs of 1:0.09 g mL\(^{-1}\). The model considers the nib thus as a single compartment, the cell plasma, containing all elements. The ionic strength was fixed at 0.1 M. The possible precipitation of Ca-oxalate, Mg-oxalate, magnetite and ferrisildite was allowed in the model to avoid an oversaturated solution. An estimation of the amount of organic acids in cacao beans was derived from Watson et al. 2013. From Calvo et al. 2021, we derived that the acetic acid concentration in the nib increased with 60% after incubation, and that the concentration of lactic acid increased from 0 to 1 g/kg nib. The concentrations of cysteine and glutathione in nibs are reported to be respectively 4.3 mg kg\(^{-1}\) dry weight and 11.67 mg kg\(^{-1}\) dry weight (Minyaka et al. 2007). These values were used as input concentrations for the nibs of the low Cd beans. As only cysteine was added into the software to represent all S-ligands, the sum of molar liquid concentrations of glutathione and
cysteine (0.82 mM) was used as input for cysteine in the model. For the high Cd beans, it was estimated that the higher Cd concentrations induced the synthesis of thiol ligands with a factor 10, and the cysteine input was thus estimated at 8.2 mM. The Cd complexation was very sensitive to the occupation of Mg on the phytate complex (Fig. S1). Therefore, two different scenarios were modelled at two different Mg input concentrations, details are given in the result section (Mg = 1.8 M (scenario A) and Mg = 1.0 M (scenario B), Table S1).

2.5. Calculations and statistics
The mean and SD (standard deviation) reported throughout the study were calculated with the biological replicates of each treatment. The coefficient of variation (CV %) was calculated with the process replicates for Cd nib and testa concentrations. Concentration ratios for different elements were calculated in the nibs and testa (exemplified for Cd: \( \frac{\text{Cd untreated sample}}{\text{Cd treated sample}} \) (mg kg\(^{-1}\) DW/mg kg\(^{-1}\) DW)). The SD of these ratios was calculated by using the formula for error propagation for multiplication. Statistical analysis was conducted with JMP® Pro version 18.0.0 (SAS Institute, 2022).

3. RESULTS
3.1. Characterization of untreated cacao beans
For the high Cd beans (H1-H4), the average Cd concentration before the incubation treatment was 9.1 ± 2.6 mg Cd kg\(^{-1}\) in the nibs and 8.2 ± 2.0 mg Cd kg\(^{-1}\) in the testa (± standard deviation, \(n=8\)). For the low Cd treatments (L1 and L2), the average Cd concentrations in the nib were 2.5 ± 0.4 mg Cd kg\(^{-1}\) and 2.8 ± 0.6 mg Cd kg\(^{-1}\) in the testa (\(n=2\)). Testa concentrations were generally lower than the corresponding nib Cd concentration in H1-H4 (except for H2), and higher in treatments L1-L2, but this finding should be confirmed with more replicates (Table 1). The mean nib pH was 6.7 ± 0.1 (H1-H4, \(n=4\)) and 7.0 ± 0.0 (L1-L2, \(n=2\)). The mean nib phytate content was 5.2 ± 0.5 g phytate-P kg\(^{-1}\) (H1-H4, \(n=4\)) and was not measured in L1-L2 (Table 1).

3.2. Effect of incubation on cacao bean composition
Incubation decreased nib pH. The pH decrease was more pronounced with increasing temperature and acetic acid concentrations (Table 1). Nib Cd concentrations decreased mostly in treatments with highest temperature and with acetic acid addition, i.e. H4 and L2. In these treatments, nib Cd concentration was about factor 1.5 lower than the corresponding original, non-treated beans (Table 1). The testa concentrations increased after incubation, and the increase was most pronounced for treatment H1 (Table 1). The Cd mass distribution between nibs, testa, and incubation solution after incubation confirmed that there was a gradient in Cd migration from nib into testa and incubation solutions with increasing temperature and decreasing pH (Figure 1).

For other elements, the nib elemental concentrations decreased mostly for treatments H4 and L2 after incubation (Table S2). The decrease of nib elemental concentrations after incubation was especially pronounced for K. For Ca and Sr, incubation did not affect the concentration ratio. The testa was overall enriched in the element after incubation (except for Ca, Sr, and Mo) (Table S3). The increase in testa elemental content due to incubation was variable and could be less related to incubation conditions. Testa Ca and K were not affected during incubation, and in general for the other elements the largest increase in testa elemental composition was in treatment L1 (Table S3). Incubation at higher temperatures and higher acetic acid concentrations (H3 and H4) lowered the
phytate concentrations in the nibs with a factor 1.4-2.4 (Table 1), which was similar to the experiment with the low Cd beans (Vanderschueren et al. 2023).

<table>
<thead>
<tr>
<th>AA $\text{g L}^{-1}$</th>
<th>T $^\circ\text{C}$</th>
<th>Fraction of total Cd</th>
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<tr>
<td>H1</td>
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<td>45</td>
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<td>H2</td>
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<td>L2</td>
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Figure 1: Cadmium distribution between the nib, testa, and incubation solution after incubation. Higher temperature/acetic acid conditions promote mobilization of Cd from the nib to the incubation solution. AA= dose acetic acid and T= incubation temperature. The error bars indicate the standard deviation of the replicate measurements ($n=2$).

3.3. Cadmium speciation analyses in cacao beans

We identified two major groups of ligands that complex Cd in cacao beans; (i) O -ligands represented by the references of Cd-hydrated, Cd-cellulose, Cd-cell wall, Cd-Ca-oxalate, Cd-malate and Cd bound to phosphate ligands (i.e. Cd-phosphate and Cd-phytate), and (ii) S-ligands represented by Cd-phytochelatin, Cd-cysteine, Cd-glutathione and Cd-metallothionein. Group (i) represents O donors (Cd-O) and group (ii) S donors (Cd-S) in the first shell. For Cd-S, the amplitude of the white line (the intense absorption peak after the edge; Figure 2) is reduced compared to Cd-O. The references with phosphate (Cd-O (P)) and carboxylate (Cd-O (C)) ligands display spectral differences (the first oscillation maximum of O (P)-ligands is slightly shifted towards higher energy), yet the quality or signal to noise ratio of the spectra obtained in this study was not sufficient enough to differentiate between these two O-ligands.

3.3.1. Effect of sample preparation on Cd speciation

Cadmium speciation was compared between samples prepared in frozen hydrated and oven-dried state between treatments H4 (replicate 1) and H3 (replicate 2). The absorption spectra recorded for the frozen hydrated and dried samples were alike, although the spectra of dried samples showed less noise (Figure S2). Linear combination fitting confirmed minimal differences in speciation. However, the noisier spectra in frozen-hydrated state of nibs (Figure S2) and testa (Figure S3) gave equivalent fits with slight changes in speciation (see below), whereas with the spectra of dried samples the low R-factor allowed to fit a unique solution.
3.3.2. Effect of Cd concentration on Cd speciation in untreated beans
The spectra of the untreated nibs of the high Cd treatments (H3-H4), resembled mostly the Cd-S reference spectra (Figure 2). The linear combination fits also confirmed that Cd was mostly coordinated with sulphur donors ((75-100%), Figure S2 and Figure 3A). The spectra of the lower Cd beans were noisier than the high Cd beans (lower R-factor) due to the lower abundance of Cd in the sample. The linear combination fits in the nibs indicated that for L1-L2 Cd was mostly bound with O-ligands (60-100%), and that S-ligands could occur as minor species (0-40 %).

3.3.3. Effect of incubation on Cd speciation in cacao beans
The XANES spectra revealed clear differences in speciation between the untreated and treated cacao nibs (Figure 2) in treatments H1-H4. Cadmium was mainly bound to S-ligands before incubation (varying from 70-100%, Figure 3). After incubation, most Cd was bound to O-ligands (60-100%) with Cd-S as possible minor ligand. In the low Cd beans, Cd speciation seemed less impacted by incubation as the Cd coordination remained mainly with O-ligands (Figure 3A). Similarly, in the testa no systematic shift in speciation was observed (Figure 3B). Before
incubation, Cd was mostly bound to O ligands, with possibly a fraction of Cd-S. After incubation, Cd-O was the only species found.

Figure 3: The fraction of Cd species in the cacao nibs (A) and testa (B) determined by XANES for treatments H3-H4, L1-L2. The grey zone represents the variation in the replicate measurements or the quality of the linear combination fit. For more detailed fits of each run, see Figure S3 and S4. FH=frozen hydrated.

3.4. Modelling Cd speciation in nibs before and after incubation
The modelled Cd speciation was sensitive to the assumed Mg concentrations (Fig. S1). The Mg concentrations in the mean dry bean Mg concentration corrected to the water content was equivalent to 1.8 M. At that concentration, Mg binding to phytate was outcompeting Cd (Figure S1). At a lower Mg concentration, here arbitrarily reduced to 1.0 M, complexation of Cd with free phytate became relevant. Therefore, two scenarios with different Mg concentrations were modelled (Mg = 1.8 M (scenario A) and Mg = 1.0 M (scenario B), Table S1).

In scenario A, the low Cd beans prior to incubation (L1-L2) were mainly bound to carboxylate ligands (54% Cd-O (C)) and sulphide-ligands (42% Cd-S) (Figure 4A). After incubation, 85% of Cd was associated with the organic carboxylate anions, 10% was free, 3% was on the phytate complex, and 2% was associated with cysteine. With lower Mg competition (Scenario B, Figure 4B), most Cd was coordinated with phytate (78%), and the remainder was complexed with sulphide (15%) and carboxylate ligands (7%). After incubation, most Cd was still complexed with phytate (78%), but the remainder was complexed with carboxylate ligands (21%) and free Cd$^{2+}$ (1%).
In the high Cd beans (H3-H4), the predictions showed that most Cd was coordinated with S-ligands before incubation, at both scenarios A (84% Cd-S) and B (58% Cd-S) (Figure 4). In scenario A, the remainder was complexed with carboxylate (15%), and in scenario B with phytate (40%). After incubation, the model predicted that the proportion of Cd bound to S-ligands decreased to less than 2% in both scenarios. The remaining ligands complexing Cd were O-ligands, and more specifically carboxylate ligands in scenario A (Figure 4A), and phytate ligands in scenario B (Figure 4B).

Figure 4: Simulated Cd speciation in the nibs before and after incubation. Simulations used Visual Minteq in which the complexation database was complemented with stability constants for phytate and cysteine ligand (see SI, 1.1). Cadmium is either free or complexed with carboxylate ligands (Cd-O(C)), phytate (Cd-O(P)) and sulphide ligands (Cd-S). The predicted Cd speciation was sensitive to the assumed Mg concentrations (Fig. S1), therefore two scenarios are shown here. A: available Mg in the nibs estimated from total nib Mg concentration and B available Mg in the nibs estimated factor 1.8 lower than in A to illustrate how partial complexation of Mg$^{2+}$ to unaccounted other ligands may lower Mg-phytate complexation and enhance Cd-phytate complexation. In the SI, the input for the different treatments and conditions can be found.

4. DISCUSSION

4.1 Cadmium speciation and chemical composition in the untreated nibs

The Cd concentrations in both treatments H1-H4 and L1-L2 (Table 1) exceed the threshold commonly used for processing of cacao beans of 0.60 mg Cd kg$^{-1}$ and the median bean Cd concentration of 0.63 Cd mg kg$^{-1}$ reported in a meta-analysis in Latin America ($n=785$, Vanderschueren et al. 2021)). These samples with relatively high Cd content were selected for
this study as the detection limit for Cd XANES currently ranges between one and a few mg kg\(^{-1}\) (depending on matrix) at the beamlines used in this study.

The nib phytate content (5.2 ± 0.5 g phytate-P kg\(^{-1}\)) prior to incubation was in a similar range as in studies with lower bean Cd concentrations (4.4 g phytate-P kg\(^{-1}\) ± 1.00 (Blommaert et al. 2022), 4.5 ± 0.3 g phytate-P kg\(^{-1}\) (Vanderschueren et al. 2023)). The phytate content seems thus not to be affected by higher Cd concentrations in the nib. The Cd/phytate molar ratio is only 5.1% on average and maximally 9.1% in the H1-H4 beans, illustrating that even in contaminated beans, phytate is in large excess for Cd.

Cadmium speciation in the nibs was clearly impacted by the concentration of Cd in the beans (Figure 2 and 3). In the high Cd beans (H3-H4), Cd was mainly bound to S-ligands. In plant cells it is well documented that to cope with elevated Cd concentrations, a common mechanism to maintain low concentrations of Cd\(^{2+}\) in the cytoplasm is metal complexation or chelation by low-molecular-weight compounds like the thiol-containing ligands cysteine, glutathione (GSH), and phytochelatins (PCs) (Seth et al. 2012; Clemens 2019). The Minteq model indeed clearly confirmed that thiol-containing compounds have high affinity for Cd, as the majority of Cd was bound to cysteine in the high Cd beans. In rice grains, the current speciation data indicates mainly a binding of Cd to S-ligands, although these data represent a variety of Cd concentrations (0.98 mg kg\(^{-1}\) (Wiggenhauser et al. 2021), 3.51-11.4 mg kg\(^{-1}\) (Gu et al. 2020)). Rice thus stores Cd differently in the seeds and/or detoxification by thiol-containing compounds is already triggered at lower Cd concentrations in rice than in cacao nibs. Previous studies in cacao with similar nib Cd concentrations as the low-Cd conditions (2-3 mg kg\(^{-1}\)) confirmed binding of Cd with O-ligands (Vanderschueren 2021; Blommaert et al. 2022), and in these studies it was hypothesized that Cd was mostly bound with phytate. In our study, the quality of the spectra did not allow differentiation between phosphate and carboxylate ligands. It is thus also possible that carboxylate ligands like the anions of organic acids (Watson et al. 2013), and/or cell wall components like pectines (Huguet et al. 2012) may be complexing Cd in the cacao nibs.

Our study revealed that in the testa of H3 and H4, Cd was mostly coordinated with O-ligands in unfermented state (Figure 3). Hence, speciation in the testa differed vastly from their corresponding nibs. This may indicate that detoxification mechanisms (by chelation with S-ligands) are more activated in the nibs than in the testa and/or that in the testa other mechanisms are at stake, like the binding of Cd with a cell wall component as has been observed in other plant tissues (Huguet et al. 2012; Tian et al. 2017).

To further disentangle the speciation of the low Cd nibs, the Minteq model was used, as the model could differentiate between Cd-O (P) and Cd-O (C) complexation. With relatively higher Mg concentrations in the system (1.8 M, Figure 4A), the modelled Cd speciation predicted a predominant binding to carboxylate ligands (mostly acetate, not shown) for L1-L2. This was mostly due to the occupation of the phytate complex by Mg. Phytate is known to have high affinity for Mg (Hawkesford et al. 2012). The relative concentration of available Mg in the plasma was probably largely overestimated in this model. When Mg concentrations were lowered from 1.8 M to 1.0 M, most of Cd was complexed with phytate (about 78%, Figure 4B). Briefly, the model indicated that the modelled metal-phytate and metal-organic acid interaction depended strongly on the concentrations of other competing elements. With higher Mg competition, less Cd was associated with phytate, and more with organic acids. It has been reported that proportions of K,
Mg and Ca associated with phytate vary considerably among plant species and between tissues of a seed (Hawkesford et al. 2012).

4.2 Cadmium mobilization and changes in Cd speciation during incubation

After incubation, the concentration of elements increased in the testa and decreased in the nibs, except for Ca (Table S2 and S3), as observed in Vanderschueren et al. 2020. In the conditions with low pH and high T, around 30% of Cd was mobilized into the incubation solution (Figure 1). The measured Cd speciation in the left-over Cd fraction in the nibs indicated for all treatments that Cd was bound to O-ligands (Figure 3) and this was confirmed by the calculated speciation when there was lower Mg competition (Figure 4B).

Our model indicated that for samples H3-H4 the mobilization of nib Cd can be mostly explained by pH dependent interactions between Cd and thiol groups (i.e. cysteine or glutathione) in the nibs. The pKa of thiol groups is estimated around 9.6 (Tummanapelli and Vasudevan 2015), and of carboxyl groups around 4 (Visual Minteq database). Decreasing the pH increased Cd\(^{2+}\):H\(^+\) competition on the functional binding groups of thiol groups, by which the binding sites on the cysteine complex become unavailable whereas the carboxylate and phosphate groups are well accessible (unless in the very acid pH range). Further, as the complexation with the organic acids is less strong, part of the Cd may be mobilized towards the testa and diffuse into the incubation solution.

For the low Cd beans (L1-L2) in scenario B, where the phytate complex is less occupied by Mg, Cd is complexed with phytate before complexation and remains bound to phytate after incubation (Figure 4B). This model thus suggests that Cd releases from the phytate complex due to phytate breakdown by phytase activation. This is corroborated by the fact that up to 58% of phytate broke down in the nib during incubation (Table 1). Furthermore, we observed that the breakdown of phytate has a large impact on the migration of Mg and K towards the nib and outer solution. Before and after incubation, Mg and K in the nibs are mostly complexed with phytate due to their high affinity for phytate (Hawkesford et al. 2012), and these elements are largely mobilized into the incubation solution (Figure S5). When the competition with Mg is higher (scenario A, Figure 4A), the model indicated that about 54% of Cd was bound to carboxylate before and the remainder with thiol groups. After incubation, almost all Cd was bound to carboxylate. For the Cd bound to thiols, a similar analogy can be drawn as in the case of high Cd beans of scenario A; i.e. the release of Cd is related to the decrease in pH. For the Cd bound to carboxylate, a possible ligand could be the polysaccharides of the cell wall (Cd-O (C)-ligand) (Isaure et al. 2015; Tian et al. 2017), and fermentation could cause a disruption of these cells by which Cd releases and binds with organic acids that are available. This mechanism could lead to a partial mobilization of Cd into the incubation solution.

4.3 Implications, limitations and outlook

This study provided new insights on the chemical form of Cd in cacao beans and on the mechanisms responsible for the decrease of nib Cd concentrations during fermentation. However, some limitations affected this study. The conditions H3-H4-L1-L2, had pH ranging between 3.8 and 4.6 after incubation. It has been reported that pH between 4.8 and 5.2 indicates a good fermentation process (Calvo et al. 2021). The incubation conditions in this study were thus more acidic than an ideal fermentation. Consequently, the processes observed may be more pronounced than in real conditions. In addition, a real fermentation includes three phases, with production of
ethanol, acetic acid and then lactic acid. Although the acetic acid phase is the most likely to mobilize Cd (see the experimental setup section), this incubation experiment is a simplified version of the real process. Moreover, the Cd concentration in the low Cd beans in this study was still relatively high compared to the average Cd concentration reported in cacao beans. In general, samples for studies using XANES have higher metal content due to the detection limit. It is very plausible that other speciation may be detected at lower concentrations, since we observed that speciation depends on the metal concentration.

This study has its importance as postharvest mitigation strategies focussing on fermentation may offer a valid short-term alternative to manage the Cd-cacao issue. Our study indicates that Cd speciation in unfermented beans may differ, according to different bean Cd concentrations. However, in all treatments, the final Cd speciation after incubation was with O-ligands. This information can be used to decipher biochemical mechanisms behind the reduction of Cd during fermentation to optimize these processes. Lastly, the complexation of Cd after fermentation may have important implications for human nutrition studies.
CREDIT AUTHORSHIP CONTRIBUTION STATEMENT
Hester Blommaert: Conceptualization, Investigation, Data curation, Writing – original draft. Géraldine Sarret: Conceptualization, Investigation, Supervision, Writing - review & editing. Eduardo Chavez: Resources, Writing - review & editing. Erik Smolders: Resources, Supervision, Writing - review & editing. Ruth Vanderschueren: Conceptualization, Investigation, Supervision, Writing – review & editing.

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Table 1: Bean compositions for the different treatments before (untreated) and after (treated) incubation.

<table>
<thead>
<tr>
<th></th>
<th>H1 0 g L(^{-1}) AA</th>
<th>H2 20 g L(^{-1}) AA</th>
<th>H3 20 g L(^{-1}) AA</th>
<th>H4 20 g L(^{-1}) AA</th>
<th>L1 ((^{1})) 20 g L(^{-1}) AA</th>
<th>L2 ((^{1})) 40 g L(^{-1}) AA</th>
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<tr>
<td></td>
<td>45 °C</td>
<td>25 °C</td>
<td>45 °C</td>
<td>65 °C</td>
<td>45 °C</td>
<td>65 °C</td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nib Cd (mg kg(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>2 10.1 4.76</td>
<td>2 8.58 1.18</td>
<td>2 8.86 1.49</td>
<td>2 9.03 2.41</td>
<td>1 2.19 /</td>
<td>1 2.83 /</td>
</tr>
<tr>
<td>Treated</td>
<td>2 9.68 5.17</td>
<td>2 9.07 1.54</td>
<td>2 7.47 1.69</td>
<td>2 6.09 2.51</td>
<td>1 1.96 /</td>
<td>1 1.8 /</td>
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<tr>
<td>Ratio</td>
<td>1.04 0.74</td>
<td>0.95 0.21</td>
<td>1.19 0.33</td>
<td>1.48 0.73</td>
<td>1.12 /</td>
<td>1.57 /</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>2 8.00 3.11</td>
<td>2 9.02 2.51</td>
<td>2 8.09 1.26</td>
<td>2 7.78 0.45</td>
<td>1 2.32 /</td>
<td>1 3.32 /</td>
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<tr>
<td>Treated</td>
<td>2 23.73 8.01</td>
<td>2 12.54 2.28</td>
<td>2 19.49 2.62</td>
<td>2 19.57 9.7</td>
<td>1 6.35 /</td>
<td>1 6.13 /</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.34 0.17</td>
<td>0.72 0.24</td>
<td>0.42 0.09</td>
<td>0.40 0.20</td>
<td>0.37 /</td>
<td>0.54 /</td>
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<td>Testa/nib Cd ratio</td>
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<td>Untreated</td>
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<td>2 1.04 0.32</td>
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<td>1 1.08 /</td>
<td>1 1.18 /</td>
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<tr>
<td>Treated</td>
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<td>2 1.38 0.34</td>
<td>2 2.66 0.70</td>
<td>2 3.13 2.02</td>
<td>1 3.24 /</td>
<td>1 3.41 /</td>
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<tr>
<td>Nib phytate-P (g P kg(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>2 5.19 0.97</td>
<td>2 5.12 0.29</td>
<td>2 5.34 0.39</td>
<td>1 5.18</td>
<td>0 /</td>
<td>0 /</td>
</tr>
<tr>
<td>Treated</td>
<td>2 5.04 0.51</td>
<td>2 5.33 0.00</td>
<td>2 3.78 0.23</td>
<td>1 2.19</td>
<td>0 /</td>
<td>0 /</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.03 0.22</td>
<td>0.96 0.06</td>
<td>1.41 0.13</td>
<td>2.37</td>
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<td></td>
<td></td>
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<tr>
<td>Untreated</td>
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<td>2 6.76 0.07</td>
<td>2 6.64 0.06</td>
<td>1 6.74</td>
<td>1 7.00</td>
<td>1 7.00</td>
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<tr>
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<td>2 5.19 0.10</td>
<td>2 4.59 0.12</td>
<td>1 4.10</td>
<td>1 4.40</td>
<td>1 3.80</td>
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The mean and SD (standard deviation) were calculated from the biological replicates of each treatment. The SD of the ratios was calculated by using the formula of error propagation for multiplication.

(1) Dried nib samples from a previous experiment (Vanderschueren et al. 2023). Treatments L1 and L2 each refer to a single experimental run in that previous study, and thus to a single cacao pod. Phytate content was only analysed in a subset of samples in that study, not including the experimental runs that were analysed here.

(2) Pod 7 contained only 13 beans. Hence, insufficient beans were available for replicate measurements of pH and phytate measurements.
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