MANAGING CADMIUM IN CACAO PRODUCTS FROM FARM TO FORK

Ruth VANDERSCHUEREN

Supervisors:
Prof. E. Smolders
Prof. J. A. Delcour

Members of the
Examination Committee:
Prof. J. Lammertyn (Chair)
Prof. C. Courtin
Dr. J. Steensels
Prof. G. Sarret
Dr. N. Waegeneers

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Uitgegeven in eigen beheer, Ruth Vanderschueren, Overwinningstraat 62, 3010 Leuven

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Throughout these past years, I have made multiple references to the words of the wise Mr. Forrest Gump: *"Life is like a box of chocolates; you never know what you're gonna get!"*. While I have used this statement mostly to describe surprising research results, an analogy can also be made to these past years as a PhD researcher. When I started this adventure, I had no idea that I would learn so much, both scientifically and personally, that I would gain so many incredible experiences, have the opportunity to work with outstanding colleagues in Leuven and far beyond, and that I would meet so many wonderful people. A PhD may seem like a lonely job, but this could not be more untrue. Throughout these past years, I have received help and support from multiple people. In the text below I have attempted to thank all those wonderful people

The chocolate production process logically starts with the plant. Cacao trees only start to produce fruits after multiple years and prefer to grow up in the shade of other, larger crops that protect them from the bright tropical sun until they are, themselves, strong enough to withstand the weather.

Lieve mama, het is misschien wat atypisch om jou eerst te bedanken maar voor mij is dit niet meer dan logisch. Jij bent er altijd voor mij (en voor Robin) geweest doorheen onze studies, inclusief op momenten waarop we deze hulp misschien maar moeilijk wouden aanvaarden. Tijdens mijn doctoraat was dat niet anders. Ik kon altijd bij jou terecht, met vragen rond wetenschappelijke grammatica, hulp om beursaanvragen te schrijven, of gewoon (en vooral) wanneer ik een luisterend oor nodig had omdat het allemaal eventjes wat te veel was. Dus mama, dikke dikke merci. Ik kan me geen beter rolmodel inbeelden.

After a few years, the cacao tree starts to produce its first fruits. A flower, smaller than a 1 eurocent coin, grows out to a fruit the size of a rugby ball.

Similarly, this work grew from an idea to the manuscript you are reading today. For this, I would like to thank my promotors Erik and Jan, and the members or the examination committee. Dear Chairman and members of the jury, thank you for your invaluable feedback and for examining this manuscript with a fine-tooth comb. Thank you also to the FWO for funding this research.

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Once the cacao fruits are ripe, they are harvested manually, and the cacao beans are collected for fermentation. Fermentation is a vital step in the chocolate production process. During this phase, flavour precursors are formed inside the cacao beans, which are essential for the quality of the final product. Cacao fruits can only be stored for up to seven days between harvest and fermentation, which is why this step is performed in the country of origin.

La gran ventaja de trabajar con un cultivo tropical como cacao, es que también se puede trabajar afuera de Bélgica. Aunque aún no he tenido la oportunidad de ver mucho de Ecuador, ya sé que es un país magnifico y que la gente es muy simpática.

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After fermentation and drying, the cacao beans are transported to the industrial processing facility. There, any extraneous material is removed, and the beans are roasted to develop the typical chocolate flavour.

A PhD is far from a one-woman job. Throughout these past years, I had the opportunity to collaborate with, and learn from, several inspiring researchers. Sandra Mounicou and Marie-Pierre Isaure, thank you for the great collaboration on the laser ablation work and for the coffee breaks in Pau. Jakob Santner, thank you for you great idea with the Chelex gels and for the opportunity to go to BOKU to try it all out.

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After roasting, the shell is removed through a process called breaking and winnowing. This is important because the shell can add off-flavours to the final product, and it can damage the processing equipment. While this industrial process is highly optimised, deshelling of small batches (e.g. in experimental work) is done by hand and this is a tedious and time-consuming job.

Doorheen de jaren hebben verschillende thesisstudenten hun steentje bijgedragen aan dit werk. Vincent, Jasmien en Florence, superhard bedankt voor al jullie harde werk en voor de gezellige dagen in het labo. Wanneer je de materiaal en methode paragrafen in dit manuscript leest lijkt het soms allemaal niet zo veel werk, maar jullie weten natuurlijk beter. Boontjes kuisen, boontjes pellen, boontjes malen, boontjes digesteren, het zijn stuk voor stuk enorm tijdrovende en ééntonige taken en zonder jullie hulp zou al dit werk nooit zijn afgeraakt. Daarnaast hebben jullie ook alle drie veel input gegeven en zo elk je stempel gedrukt op een stuk van dit werk, dikke merci daarvoor. Hester, Stan, Vincent, Jesse en Jasmien, bedankt voor de superfijne maanden in Ecuador, de gezellige avonden en de uitstapjes.

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The roasted cacao beans are then ground, the mixture is refined and mixed with additional ingredients, such as milk powder and sugar, or nuts, seeds, fruits and other inclusions. The chocolate bar is moulded and is now ready to enjoy.

Zoals de stukjes karamel en zeezout in een Tony's Chocolonely reep, maken vrienden, collega's en familie alles toch net iets beter, of eerder heel veel beter.

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Summary

A new EU regulation was enforced in 2019 which sets the maximum allowed cadmium (Cd) concentration in chocolates and cacao powders sold to the European consumers. The cacao market in Central and South America is affected by this regulation because Cd concentrations are markedly higher in cacao beans from that region compared to beans from Africa, the main global cacao producing region. Lowering the cacao nib Cd concentrations by a factor 1.3 or more could largely improve the sustainability of the cacao sector in Central and South America. Cadmium in cacao beans originates from the soil and several agronomic mitigation strategies are being explored to lower the soil-plant transfer of Cd. However, none of these have had large success to date. Mitigation strategies can also be implemented during the extensive postharvest process of cacao because the new EU Cd regulations only apply to the final product, but such strategies had not been explored thus far.

The objective of this dissertation was to study the effect of conventional postharvest processing on the Cd concentrations in the different cacao bean tissues, with specific focus on fermentation, and to reveal potential postharvest mitigation strategies to lower the Cd concentration in the final product.

As a first step, a study was set up to confirm the suspected effect of the cacao origin on the Cd content of chocolate products. The relation between the elemental composition of chocolates and the origin of the cacao used was studied in 139 single origin chocolates. The EU Cd regulations were exceeded in 10 % of the samples, which were all produced with cacao from Central or South America. Increasing cacao content of the product was associated with increased concentrations for most elements, indicating cacao as the main source of minerals and trace elements (incl. Cd) in chocolate. Classification and Regression Tree (CART) analysis resulted in a decision tree that could effectively classify chocolate samples by cacao origin based on the concentrations of five elements (Ba, Cd, Mo, Sr and Zn). Samples of South America were differentiated from the other samples based on their Cd concentration, indicating the geogenic origin of Cd.

The second step was devoted to the Cd distribution in the cacao fruit and its changes during fermentation. Chocolate products are made of the *cacao nibs*, the central part of the fermented cacao bean. Cadmium concentrations in the different tissues of unfermented cacao fruits decrease in the order testa > nib ~ placenta ~ pod husk > mucilage. Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) was used to visualise the Cd distribution within cacao beans and confirmed higher Cd concentrations in the testa than in the nib. The impact of fermentation on the distribution of Cd in cacao was studied using a top-down approach, starting with a full scale fermentation setup, followed by lab scale and micro-fermentations, and finally controlled incubations to mimic the fermentation conditions.

Fermentation in full scale commercial setups (> 200 kg) induced an outward Cd migration from the nib to the testa, i.e. from a lower Cd concentration tissue to a higher Cd concentration tissue, which decreased nib Cd concentrations by maximally a factor 1.3. This migration occurred only if fermentation was sufficiently extensive to acidify the nibs to pH < 5.0, which was the first indication that pH-driven nib Cd mobilisation occurs during fermentation. Next, a micro-fermentation experiment was performed to study the fermentation effect with higher precision than in the commercial setups. Single pod derived cacao beans in mesh bags were embedded in a full scale commercial fermentation box, and four days of fermentation reduced the nib Cd concentration by a factor 1.25. The mobility of Cd within the cacao beans was mapped with imprints of transversal cuts exposed to a metal binding gel, followed by LA-ICP-MS analysis. That showed that fermentation enhances the Cd mobility in cacao nibs and that it establishes a mobile concentration gradient from inside the nibs to the outer testae and mucilage. In subsequent lab scale fermentation experiments (5 kg), lactic and acetic acid were applied during or after fermentation in an attempt to enhance the nib Cd mobilisation. Total nib Cd concentrations did not decrease due to organic acid treatments, which was likely related to the lower temperatures reached in these small fermentation vessels in comparison to full scale commercial setups. It was hypothesised that loss of structural integrity in the cacao nibs due to fermentation heat, acetic acid and/or ethanol is required for acidification-driven nib Cd mobilisation. That hypothesis was tested in an incubation experiment using combinations of different incubation temperatures, and acetic acid and ethanol concentrations in the media. Mobilisation of Cd in the nibs was most pronounced when acetic acid addition was associated with higher temperatures, whereas ethanol did not have statistical effects. The incubation of cacao beans in typical fermentation conditions (45 °C and 20 g L^{-1} acetic acid) reduced the nib Cd concentration by a factor 1.3, and this reduction factor increased to 1.6 in more extreme conditions. If such incubation was followed by an overnight water extraction of ground nibs, nib Cd concentrations were even reduced by factors > 2. Fine-tuning of fermentation parameters for optimal production of heat and acetic acid thus holds promise for lowering cacao nib Cd concentrations.

In a final step, water and chelating agent extractions of cacao nibs were evaluated as mitigation measures. Water extractions of ground cacao nibs had initially been used in this work as a diagnostic technique to compare Cd mobility among samples. These data, however, indicated large water extractable Cd fractions in fermented cacao nibs, but not in unfermented nibs. Water and chelating agent (EDTA) extractions with short contact times (1 or 2 hours) and small liquid:solid ratios (1 – 6 mL g^{-1}) were performed on broken cacao nibs derived from a commercial cacao fermentation. Incubation for 1 hour in 10 mM EDTA at liquid:solid ratios of 2 mL g^{-1} or higher removed 50 – 60 % of nib Cd. Water and/or chelating agent washing prior to nib roasting likely influences the flavour quality of the final product but implementation of such technique is not implausible because similar practices are already adopted in selected cacao processing technologies (i.e. alkalinisation or Dutch processing).

In conclusion, this study revealed that cacao nib Cd concentrations can be decreased by fermentation due to pH-driven mobilisation and that this effect only occurs with adequate fermentation heat, likely because it requires loss of structural integrity in the nib tissue related to *bean death*, i.e. loss of germination potential due to heat and acetic acid. Conventional fermentation practices can reduce nib Cd concentrations by a factor 1.3, while fine-tuning of fermentation parameters can potentially increase this reduction factor to 1.6. Follow-up experiments are required to reveal if such fermentation conditions are practically feasible. It is also suggested to identify to what extent the breakdown of phytate during cacao fermentation is a driving factor for the enhanced Cd mobilisation. Washing of fermented cacao nib fragments with a solution containing low concentrations of a chelating agent, e.g. EDTA, can reduce nib Cd concentrations by a factor > 2. Both fine-tuning of fermentation parameters and nib washing can be readily and widely implemented and are, therefore, highly promising as mitigation strategies to address the Cd issue in Central and South America.

Samenvatting

In 2019 ging een nieuwe Europese regelgeving van kracht die de maximale toegelaten cadmiumconcentratie (Cd) bepaalt in chocolades en cacaopoeders die verkocht worden op de Europese markt. Deze nieuwe limieten hebben een grote impact op de cacaomarkt in Centraal- en Zuid-Amerika, aangezien cadmiumconcentraties in cacaobonen van die gebieden merkbaar hoger zijn dan in bonen van andere regio's zoals Afrika, de voornaamste cacaoproducerende regio wereldwijd. De duurzaamheid van de cacaosector in Centraal- en Zuid-Amerika kan sterk verbeterd worden door de cadmiumconcentratie in cacaonibs te verlagen met een factor 1.3 of meer. Het Cd in de cacaobonen is afkomstig van de bodem en verschillende agronomische mitigatiestrategieën worden onderzocht om de bodem-plant overdracht van Cd te beperken. Echter, tot dusver behaalde dat onderzoek nog geen groot succes. Aangezien de nieuwe EU-regelgeving enkel van toepassing is op het eindproduct, kunnen mitigatiestrategieën ook toegepast worden tijdens het uitgebreide naoogst proces van cacao, maar zulke technieken waren tot dusver nog niet onderzocht.

Het doel van dit onderzoek was om de effecten van conventionele naoogst behandelingen op de cadmiumconcentraties in de verschillende weefsels van de cacaovrucht te bestuderen, met specifieke focus op fermentatie, en om potentiële naoogst-mitigatiestrategieën te identificeren die de cadmiumconcentratie in het eindproduct kunnen verlagen.

Als eerste stap werd een studie opgezet om het vermoedelijke effect van de cacao-origine op de cadmiumconcentratie in chocoladeproducten te bevestigen. Het verband tussen de elementaire samenstelling van chocolades en de oorsprong van de gebruikte cacao werd bestudeerd in 139 originechocolades. De nieuwe cadmiumlimieten werden overschreden in 10 % van de stalen, allen geproduceerd met cacao uit Centraal- en Zuid-Amerika. Voor de meeste elementen kon een verband gevonden worden tussen toenemende cacaogehaltes en toenemende elementconcentraties, wat erop wijst dat de cacaobonen de voornaamste bron zijn van deze mineralen en spoorelementen (incl. Cd) in chocolade. *Classification and Regression Tree* (CART) analyse resulteerde in een keuzeboom die cacaostalen efficiënt kon indelen naargelang de oorsprong van de cacao op basis van vijf elementen (Ba, Cd, Mo, Sr en Zn). Zuid-Amerikaanse chocolades werden onderscheiden van de andere stalen op basis van hun cadmiumconcentratie, wat aangeeft dat Cd in cacao waarschijnlijk van geogene oorsprong is.

Vervolgens werd de cadmiumverdeling in de cacaovrucht en de verandering van deze verdeling tijdens fermentatie bestudeerd. Chocoladeproducten worden gemaakt van de cacaonibs, het binnenste deel van de gefermenteerde cacaobonen. In de verschillende weefsels van ongefermenteerde cacaovruchten daalt de cadmiumconcentratie in de volgorde testa > nib ~ placenta ~ pod husk > mucilage. Laser ablatie inductief gekoppeld plasma massaspectrometrie (LA-ICP-MS) werd gebruikt om de verdeling van Cd in cacaobonen in beeld te brengen. Deze analyse bevestigde dat cadmiumconcentraties hoger zijn in de testa dan in de nib.

Het effect van fermentatie op de verdeling van Cd in de verschillende cacaoweefsels werd bestudeerd met behulp van een *top-down* benadering, startend met een opstelling op commerciële schaal, gevolgd door laboschaal en micro-fermentaties, en tenslotte gecontroleerde incubaties die de fermentatiecondities nabootsten. Fermentatie in commerciële fermentatiebakken (> 200 kg) veroorzaakte een buitenwaartse migratie van Cd van de nib naar de testa, i.e. van een weefsel met lagere cadmiumconcentratie naar een weefsel met hogere cadmiumconcentratie, en hierdoor daalde het cadmiumgehalte in de nibs met een factor 1.3. Deze migratie vond echter enkel plaats wanneer fermentatie voldoende extensief was zodat de pH in de nib daalde tot < 5.0. Dit was de eerste aanwijzing dat fermentatie een pH-gedreven cadmiummobilisatie veroorzaakt in cacaonibs. Vervolgens werd een microfermentatie-experiment opgesteld om het effect van fermentatie te bestuderen met hogere nauwkeurigheid dan in de commerciële opstellingen. Netzakjes met de cacaobonen van individuele vruchten werden in een commerciële fermentatiebak geplaatst en gefermenteerd gedurende vier dagen. Dit resulteerde in een verlaging van de cadmiumconcentratie in de nib met een factor 1.25. De mobiele cadmiumgradiënt in de cacaobonen werd in beeld gebracht doormiddel van afdrukken van transversaal doorgesneden cacaobonen op een metaalbindende gel, gevolgd door LA-ICP-MS analyse. Dit toonde aan dat fermentatie de mobiliteit van Cd in cacaonibs verhoogt en dat zo een mobiele concentratiegradiënt tot stand komt van de binnenkant van de nibs naar de testae en het mucilage. In de daaropvolgende laboschaal fermentatie-experimenten (5 kg) werden melkzuur en azijnzuur toegevoegd tijdens of na de fermentatie, om zo te proberen de cadmiummobilisatie te versterken. De organische zuurbehandelingen veroorzaakten geen daling in de totale cadmiumconcentratie in de nibs, wat waarschijnlijk verklaard kan worden door de lagere temperaturen die bereikt werden in deze kleine fermentatiebakken in vergelijking met commerciële opstellingen. Verzuring-gedreven cadmiummobilisatie in cacaonibs komt mogelijks enkel voor indien de structurele integriteit van de nibs verstoord wordt door de toenemende fermentatiehitte, en azijnzuur- en ethanolconcentraties. Deze hypothese werd onderzocht in een incubatie-experiment, met verschillende combinaties van incubatietemperaturen, en azijnzuur- en ethanolconcentraties in de incubatiemedia. De mobilisatie van Cd in de nibs was het meest uitgesproken bij incubaties met toevoeging van azijnzuur en bij verhoogde temperaturen, terwijl ethanol geen statistisch significant effect veroorzaakte. Incubatie van cacaobonen bij typische fermentatiecondities (45 °C en 20 g L^{-1} azijnzuur) verlaagde de cadmiumconcentratie in de nib met een factor 1.3, en deze reductiefactor steeg tot 1.6 in meer extreme condities. Indien zulke incubatie werd gevolgd door een waterextractie van de vermalen cacaonibs, werd de cadmiumconcentratie in de nib zelfs gereduceerd met een factor > 2. Aanpassen van de fermentatieparameters voor een optimale productie van hitte en azijnzuur is bijgevolg een veelbelovende strategie om de cadmiumconcentratie in cacaonibs te doen dalen.

Tot slot werden extracties van cacaonibs met water en chelerende agentia geëvalueerd als mitigatiestrategie. Waterextracties van vermalen cacaonibs werden in dit werk initieel gebruikt als een diagnostische techniek om de cadmiummobiliteit te vergelijken tussen stalen. De data toonden echter aan dat een groot aandeel van het Cd in gefermenteerde cacaonibs waterextraheerbaar is, maar dat dit niet het geval is in ongefermenteerde nibs. Extracties met water en chelerende agentia (EDTA), korte contacttijden (1 tot 2 uur) en kleine vloeistof:vast verhoudingen (1 – 6 mL g^{-1}) werden uitgevoerd op gebroken cacaonibs afkomstig van een commerciële cacaofermentatie. Incubatie van 1 uur in 10 mM EDTA bij een vloeistof:vast verhouding 2 mL g^{-1} of hoger verwijderde 50 – 60 % van het nib Cd. Cacaonibs wassen met water en/of chelerende agentia voordat de nibs geroosterd worden, heeft waarschijnlijk een invloed op de smaak van het eindproduct. Echter, implementatie van zulke technieken is niet onrealistisch aangezien gelijkaardige technieken reeds worden toegepast in specifieke procestechnologieën voor de verwerking van cacao (i.e. alkalinisatie of *Dutch processing*).

Ter conclusie, deze studie bracht aan het licht dat cadmiumconcentraties in cacaonibs gereduceerd kunnen worden doormiddel van fermentatie omwille van pH-gedreven cadmiummobilisatie in de nibs. Dit effect doet zich enkel voor indien voldoende fermentatiehitte geproduceerd wordt, waarschijnlijk omdat mobilisatie een verstoring van de structurele integriteit in de nibweefsels vereist. Conventionele fermentatie kan de cadmiumconcentratie in de nib doen dalen met een factor 1.3, en door middel van optimalisatie van de fermentatieparameters kan deze reductiefactor stijgen tot 1.6. Vervolgexperimenten zijn vereist om na te gaan of zulke extreme fermentatiecondities praktisch haalbaar zijn. Daarnaast wordt ook voorgesteld te onderzoeken wat de invloed is van eventuele fytaatafbraak tijdens cacaofermentatie op de mobilisatie van Cd. Gefermenteerde cacaonibs wassen met oplossingen met lage concentraties van een chelerend agens zoals EDTA kan de cadmiumconcentratie in de nib meer dan een factor twee verlagen. Zowel optimalisatie van fermentatieparameters als het wassen van cacaonibs, kunnen eenvoudig en grootschalig worden geïmplementeerd en beide technieken zijn daarom veelbelovende mitigatiestrategieën om het cadmiumprobleem aan te pakken in Centraal- en Zuid-Amerika.

List of abbreviations

AAB Acetic Acid Bacteria
AAF Air Accumulation Factor
AAS Atomic Absorption Spectroscopy
ANOVA Analysis of Variance
bw body weight
CART Classification and Regression Tree
CCN 51 Colección Castro Naranjal 51
CEC Cation Exchange Capacity
CI Confidence Interval
cps counts per second
CRM Certified Reference Material
CV Coefficient of Variation
DA Discriminant Analysis
DGT Diffusive Gradient Thin film
dw dry weight
EDTA Ethylenediaminetetraacetic Acid
EFSA European Food Safety Authority
FAO Food and Agriculture Organisation
HMA Heavy Metal transporting ATPase
HSD Honestly Significant Difference (Tukey test)
ICCO International Cacao Organisation
ICP-MS Inductively Coupled Plasma Mass Spectrometry
ICP-OES Inductively Coupled Plasma Optical Emission Spectrometry
ITF Internal Translocation Factor
JECFA Joint FAO/WHO Expert Committee on Food Additives
LAB Lactic Acid Bacteria
LA-ICP-MS Laser Ablation Inductively Coupled Plasma Mass Spectrometry
LCF Linear Combination Fitting
LOD Limit of Detection
LOQ Limit of Quantification
NRAMP Natural Resistance Associated Macrophage Protein
PCA Principal Component Analysis
RF Reduction Factor
SOC Soil Organic Carbon
stdev standard deviation
TF Transfer Factor
US FDA United States Food and Drug Agency
WHO World Health Organisation
XANES X-ray Absorption Near-Edge Structure
ZIP Zinc-Iron Permease

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Context and aims

In January 2019, the European commission enforced a new regulation which limits the allowed cadmium (Cd) concentration in cacao-derived products such as chocolate sold to the European consumer. These limits range between 0.10 and 0.80 mg Cd kg^{-1} depending on the product type. The objective of the regulation is to protect European consumers from potential adverse effects related to Cd exposure through chocolate consumption. Whether this concern is justified or not, is outside of the scope of this dissertation. Similar thresholds for Cd in cacao-derived products were approved by the Codex Alimentarius in 2018 and are, or likely will be, in force in different parts of the world. However, these regulations are expected to negatively impact the cacao market, especially in Central and South America. Cacao from Central and South America generally contains larger Cd concentrations compared to cacao from Africa or Asia, and this is likely related to geogenic rather than anthropogenic sources. The cacao industry is confronted with increasing demands but also with increasing concern about child labour and social issues in the main cacao producing countries of West Africa. The industry is, therefore, willing to intensify production in Central and South America but is confronted with the Cd issue.

The large potential impact of the new EU regulations on the cacao market in Central and South America has sparked researchers to investigate mitigation strategies to lower Cd concentrations in cacao. On the short term, mixing cacao from different geographic origins is likely the most suitable mitigation strategy, especially since mixing is already a common practice in the industry. However, mixing is not an option for much of the Central and South American market as they produce fine flavour cacao and sell much of their crop for the production of single origin chocolates, a luxury product with increasing demand. A growing number of technical strategies to mitigate Cd accumulation in cacao is being tested. The use of soil amendments to reduce Cd availability to the plant by immobilising Cd in the soil has shown some, but limited effects. Mitigation through soil amendments requires long term applications by the farmers and treatments needs to be finetuned to each location. For example, application of lime to increase soil pH and thereby immobilise Cd in the soil is only beneficial in acidic soils, not in alkaline or pH neutral soils. Selection of low Cd accumulating cultivars has been proven useful for dealing with elevated Cd concentrations in several food crops, but research on cacao cultivar selection for low Cd accumulation is still in an early stage and will likely take years to complete as cacao is a large perennial tree and, therefore, long term field trials are required to study the cultivar effect.

Postharvest mitigation should also be considered as a potential strategy to deal with the Cd-cacao issue, because regulations apply only to the final product and not to the raw material or to intermediate products. Cacao undergoes an extensive postharvest process and it is unclear how the different processing steps affect the Cd concentration in the final product. In contrast to agricultural management techniques, postharvest mitigation is likely more universally applicable, and it offers more control to the cacao processing industry. Against this background, the objectives of this work are to reveal the effect of current postharvest practices on the Cd distribution in the different cacao tissues, and to identify potential methods for postharvest mitigation to reduce the Cd concentration in the final cacao-derived product. The outline of this dissertation is visualised in Figure 0.1.

The first chapter offers a comprehensive overview of the postharvest process of cacao, with specific focus on fermentation as this step was hypothesised to potentially cause Cd redistribution and, hence, affect Cd concentrations in the different cacao bean tissues. Chapter 2 entails a detailed review of the journey of Cd from soil to chocolate bar. The existing knowledge on the various factors influencing Cd in cacao at all stages of the production process is summarised and potential mitigation strategies at these different stages of the cacao value chain are discussed. Chapter 3 reports a study which was the starting point of the present work, it studies the relation between the mineral elements in single origin chocolate, and both its cacao content and the origin of the cacao used. Chapters 4, 5 and 6 discuss the effect of fermentation on the distribution of Cd within the different tissues of cacao beans. First (Chapter 4), the Cd distribution within unfermented cacao fruits is studied and the effect of conventional full scale fermentation on the Cd concentrations in the different tissues is investigated. The observations from the full scale conventional fermentation are used to develop a lab scale fermentation experiment with manipulation of the organic acid concentrations in Chapter 5. Fermentation conditions are mimicked in an incubation experiment to study which factors truly determine the effect of fermentation on Cd in cacao (Chapter 6). Finally, the potential of water and/or chelating agent washing of cacao nibs for lowering the Cd concentrations in the final product is explored in Chapter 7.

To identify the effect of postharvest processing on the Cd concentration in cacao tissues and to reveal potential postharvest mitigation strategies to lower the Cd concentration in the final product.

Objective

From bean to bar: the postharvest process of cacao
Chapter 1

The transfer of cadmium from soil to chocolate bar
Chapter 2

Introduction

The issue of Cd in the final product

The elemental composition of chocolates is related to cacao content and origin: a multi-element fingerprinting analysis of single origin chocolates
Chapter 3

The impact of fermentation on the Cd distribution in cacao bean tissues

Full scale
The impact of fermentation on the distribution of cadmium in cacao beans
Chapter 4

Lab scale
Cadmium migration from nib to testa during cacao fermentation is driven by nib acidification
Chapter 5

Controlled system
Outward migration of cadmium from cacao nibs during cacao fermentation is related to the combination of enhanced acidity and temperature: mimicking fermentation through incubation
Chapter 6

The potential of water and/or chelating agent washing as a mitigation strategy

Cacao nib washing prior to roasting effectively lowers nib cadmium concentrations: the first step towards nib washing as a mitigation strategy
Chapter 7

Experimental work

General conclusions, relevance of the present findings and perspectives for future work

Figure 0.1: Dissertation outline.

Part 1
Introduction

CHAPTER 1

From bean to bar: the postharvest process of cacao

CHAPTER 2

The transfer of cadmium from soil to chocolate bar

Chapter 1
From bean to bar: the postharvest process of cacao

Summary
Cacao undergoes an extensive postharvest process before it ends up on the shelf in the form of a chocolate bar. The journey from plant to final product takes several months. Cacao beans are the seeds of the cacao tree (*Theobroma cacao* L.), a perennial tree with a cauliflorous flowering pattern, i.e. the fruits grow on the trunk and thicker branches. Each cacao fruit consists of a thick outer pod husk filled with 20–50 cacao beans incapsulated in a white sugary mucilage. Each bean is made up of an outer shell or testa and an inner part called the nib, which is the only part of the fruit that is retained in the final product. Upon harvest, cacao beans with mucilage are collected and fermented for 2–10 days depending on the cultivar and local practices. Even though fermentation is considered vital for the flavour quality of the final product, it is mostly performed using uncontrolled and non-standardised methods. After fermentation, the beans are dried and transported to cacao processing facilities. There, the beans are roasted to develop typical chocolate flavours and the testa is removed to avoid contamination of the product and to protect processing equipment. The roasted nibs are then ground at elevated temperature to obtain cacao liquor, which is further processed depending on the intended final product.

1.1. History and economics of chocolate production

The cacao tree is native to Central and South America. It was introduced in Asia in the 16th and 17th century and in Africa in the 19th century (Fowler and Coutel 2017). Most of modern day cacao production is located in West Africa (e.g. 73.1 % of the total production in the cacao year 2015/16[1] originated from West Africa, of which more than half was produced in Ivory Coast), followed by Central and South America (16.9 %, mostly Brazil and Ecuador), and Asia and Oceania (9.9 %, with Indonesia as the main producer) (ICCO 2018). The use of cacao dates back to the time of the Aztec and Inca cultures, who used cacao beans as a currency and to make a drink called chocolatl, roasted and ground cacao mixed with water and spices or honey. Although the Spanish introduced chocolatl in Europe in the 16th century, it was too expensive and only consumed by the highest classes. This changed in the beginning of the 19th century, when Van Houten invented the cacao press and started producing low fat cacao powders that were more easily mixed with water. The first solid chocolate bars were produced in the mid-19th century (Afoakwa 2010). Today, chocolate has become a staple snack and/or desert for people all over the world, especially in Europe and North America. In 2012, Switzerland had the highest per capita chocolate consumption (11.9 kg yr^{-1}), followed by Ireland (9.9 kg yr^{-1}), UK (9.5 kg yr^{-1}), Austria (8.8 kg yr^{-1}) and Belgium (8.3 kg yr^{-1}); while consumption in North America was 6.4 kg yr^{-1} in Canada and 5.5 kg yr^{-1} in the USA (Thomas 2017). The specialty dark chocolate market is considered the strongest growing market segment, while most of the chocolate market worldwide is made up of bulk chocolate, i.e. milk chocolate, confectionary products, biscuits, etc. (Santander Muñoz et al. 2019). The importance of the specialty chocolate market is also indicated by the establishment of the ICCO Ad Hoc Panel on Fine or Flavour Cocoa, who recognised 23 countries as (partly) fine flavour producers: Belize, Bolivia, Colombia, Costa Rica, Dominican Republic, Ecuador, Grenada, Guatemala, Honduras, Indonesia, Jamaica, Madagascar, Mexico, Nicaragua, Panama, Papua New Guinea, Peru, Saint Lucia, São Tome and Principe, Trinidad and Tobago, Venezuela and Vietnam (ICCO 2015). Although the premium single origin chocolate market is growing rapidly, sales are focused mostly on North America and Europe, who accounted for 98 % of the global premium chocolate market value in 2007 (Afoakwa 2010). As a result, chocolate related regulations in the EU and/or North America can be expected to have a strong impact on the premium single origin chocolate market.

1.2. The cacao plant

1.2.1. Occurrence and cultivation

The cacao tree (*Theobroma cacao* L., part of the family Sterculiaceae) is a perennial plant which is cultivated within 20° latitude from the equator at low elevation, below 600–1000 m. Cacao prefers humid conditions with a rainfall of 1500 – 2500 mm spread evenly throughout the year and a humidity of 70 – 80 % during the day (Belitz et al. 2009; Afoakwa 2010; Fowler and Coutel 2017). Originally, cacao trees grew to heights of 10 m and more at maturity, but modern breeding techniques have reduced the height to approximately 3 m, which allows for easier harvesting (Afoakwa 2010).

[1] Cacao years run from October until September

Cacao cultivars are generally divided in three groups: the two original subspecies Criollo (*Theobroma cacao* L. ssp. *cacao* Cuat.) and Forastero (*Theobroma cacao* L. ssp. *shaerocarpum* Cuat.), and a hybrid of those subspecies called Trinitario. Most of the cacao produced today is Forastero, which is commonly considered as bulk cacao and is used for the production of a wide array of products, including milk chocolate and cacao butter for both consumption and other markets such as cosmetics (Fowler and Coutel 2017). Criollo cacao has a more specific aroma and is referred to as fine flavour cacao, but because Criollo cultivars tend to be more susceptible to diseases and have lower yield compared to Forastero, they are now rare and mostly found on older plantations in Central America, Venezuela, Madagascar, Sri Lanka and Samoa (Fowler and Coutel 2017). Trinitario originated in Trinidad at the end of the 18th century, when farmers introduced Forastero cacao from Venezuela to recover the cacao farms on the island after an event that was reported as a 'blast', likely either a weather phenomenon or a disease. This cacao formed hybrids with the native Criollo, yielding Trinitario (Wood and Lass 1985; Loor et al. 2009). Trinitario is described as a more disease resistant fine flavour cacao and makes up approximately 5 % of the world cacao production (Afoakwa 2010). Besides these three main cultivar groups, Nacional cacao is at times considered as a fourth fine flavour cacao type and is almost exclusively cultivated in the Pacific coast area of Ecuador (Loor et al. 2009). The exact genetic origin of Nacional, and whether it should be considered a Forastero or Criollo cultivar or something separate, is unknown. Nacional cacao is internationally renowned for its fine flavour profile, often referred to as *Arriba* (Fowler and Coutel 2017). However, true Nacional cacao has become rare due to its high sensitivity to the fungal disease witches' broom, which prompted breeding programs in the 20th century to introduce hybrid materials (Loor et al. 2009). As a result, *Arriba* flavour cacao found in Ecuador nowadays is often a hybrid between Nacional and Trinitario (Fowler and Coutel 2017). These classifications are mostly based on geographical origin and on morphological differences such as colour and shape of the pod, and colour of the nib, rather than on genetic data. In more recent years, advanced breeding techniques have led to the development of specific cacao cultivars with increased yield and/or disease resistance. An example is the CCN 51 cultivar (Colección Castro Naranjal 51) which is taking over parts of the cacao market in Ecuador due to its high yield and better resistance for diseases compared to the native Nacional cultivar.

1.2.2. Flowering and fruit development

The cacao tree is a cauliflorous or truncate plant, the flowers and fruits develop on the trunk and thicker branches of the tree (Wood and Lass 1985). Healthy cacao trees produce 20000 – 100000 flowers per year, divided over several blooming periods depending on the cultivar and local climate conditions, but only 1 – 5 % of these flowers are pollinated and eventually develop into fruits (Afoakwa 2010). Once pollinated, the fruit or cacao pod will start to grow. The young pods, up to half of the total development time, are called cherelles (Figure 1.1). Even though only a limited number of flowers is pollinated, cacao trees generally produce too many cherelles for the plant to carry. Therefore, part of the cherelles will stop growing and decay while still on the plant. This is called cherelle wilt (Greathouse et al. 1971; Wood and Lass 1985; Fowler and Coutel 2017). After a total development time of 5–6 months, the remaining pods reach maturity

and can be harvested. This maturation can be identified visually as the pod husk will change colour from green or purple to yellow, red or orange depending on the cultivar.

Cacao yield is highly variable and depends on factors such as cultivar, tree density, shade, tree age, climate, soil parameters, and prevalence of pests and diseases. Dry cacao bean yields vary widely, and yield potential is often not reached. For example, the national average yield in Ghana is approximately 400 kg ha^{-1} (Aneani and Ofori-Frimpong 2013), while simulations have indicated that yields of > 5000 kg ha^{-1} can be achieved through more intense management (Zuidema and Leffelaar 2002). Although yield varies throughout the year, with peaks in productivity in certain periods determined by the local climate, there is no specific harvest season and plants produce fruit all year round.

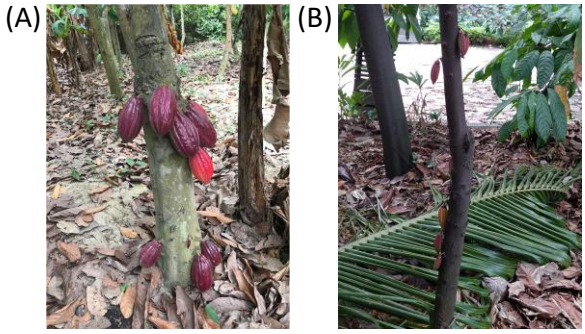


Figure 1.1 Cacao fruits maturing on the trunks of CCN 51 cacao trees in Ecuador, the pod colour will change from purple to orange/yellow upon maturation. (A) Cacao pods matured past the cherelle stage. (B) Immature cherelle pods. (Pictures made by the author)

1.2.3. Composition of the cacao pod and cacao bean

The mature cacao fruit or pod has a teardrop shape measuring 10 – 35 cm in length and the wet weight of a single pod varies from 200 g to > 1 kg (Fowler and Coutel 2017). The pod is made up of an outer woody husk with a thickness of 10 – 15 mm that varies in colour depending on the cultivar and ripeness of the fruit (Belitz et al. 2009). This husk is filled with 20 – 50 cacao beans (the seeds) that are attached to the plant through a central tissue (the placenta) and embedded in a white mucilaginous pulp (the mucilage) (Figure 1.2).

The cacao mucilage comprises approximately 40 % of the cacao bean fresh weight and is the only tissue that is consumed without additional processing (Schwan and Wheals 2004). The mucilage consists of endocarp cells with large intercellular spaces. It has a high moisture content (82 – 87 %), contains 10 – 15 % sugars, and has an acid pH of 3.0 – 3.5 due to its citric acid concentration (1 – 3 %) (Roelofsen 1958; Schwan and Wheals 2004; Lima et al. 2011). Because of its composition, the mucilage forms an ideal growth medium for microbial activity during fermentation.

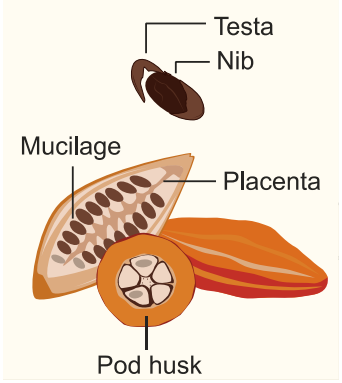


Figure 1.2 The different tissues of the ripe cacao pod.

Each cacao bean is made up of two cotyledons (the cacao nibs) and a rootlet, which is also referred to as germ or radicle, encapsulated in an outer shell or testa (Figure 1.2). The nibs are the only part of the cacao pod that is retained in the final product, all other tissues are removed during postharvest processing. Within the cotyledons, two main cell types can be differentiated: storage cells and pigment cells. The storage cells contain mostly fat (45 – 65 % of the bean dry weight, the cacao butter) and protein (10 – 15 % of the bean dry weight), comprised in small vacuoles packed inside the cytoplasm together with starch granules (Wood and Lass 1985; Belitz et al. 2009; Afoakwa 2010). The pigment cells or polyphenolic cells make up 14 – 20 % of the bean dry weight and contain mostly polyphenolic compounds, theobromine and caffeine in a single large vacuole (Afoakwa 2010; Lima et al. 2011). The high polyphenol content in unfermented cacao beans (> 7 % of the total dry weight) yields the distinct colour of the cacao nibs, i.e. purple in Nacional, Forastero and Trinitario (Figure 1.3), and white in Criollo due to the lower anthocyanin content. Unfermented and unroasted cacao beans are generally considered to have an unpleasant bitter and astringent taste due to the high polyphenol content. During fermentation, the polyphenol content decreases due to several (bio)chemical reactions described below, and the astringent flavour thus also decreases. A polyphenol content above 5 % is often considered a sign of poor fermentation practices (Afoakwa 2010). This reduction in polyphenol content and related astringency is one of the reasons why Forastero cacao generally requires longer fermentation times compared to Criollo, as Forastero has approximately a 1.5 times higher polyphenol content. The most important polyphenolic compounds in cacao are catechins, anthocyanins and proanthocyanidins (Afoakwa 2010).

Figure 1.3 From left to right, Nacional cultivar: (1) Cacao pod growing on the trunk. (2) Ripe cacao pods. (3) Fresh cacao beans with mucilage. (4) Longitudinal cross-sections of unfermented cacao beans revealing the purple colour of the nibs. (Picture 1 made by the author; pictures 2, 3 and 4 by Jasmien Doevenspeck)

The testa or seed coat makes up 10 – 14 % of the total dry weight of the cacao bean, it is made up of two layers: an outer testa and an inner tegmen and is covered in a layer of closely adhering mucilage (Afoakwa 2010). Vascular bundles can be visually identified on the testa, after removal of the mucilage (Figure 1.4). The surface of the testa is highly permeable for aqueous solutions (Andersson et al. 2006). During fermentation, several compounds enter the nib via the testa barrier, e.g. ethanol and acetic acid. Because the testa acts as the protective barrier during fermentation and drying, it is exposed to several potential sources of contamination. Cacao bean drying is often performed in open air which exposes the testa to dust and may result in higher levels of metals such as lead. Mycotoxins, such as aflatoxins and ochratoxin A, have also been found in the testa (Copetti et al. 2010 and 2012). The testa is a highly fibrous material which can damage processing equipment. Therefore, it is removed either before or after bean roasting and it is thus considered a by-product of the cacao industry. It is mainly used as fuel, animal feed or in fertiliser preparation (Okiyama et al. 2017).

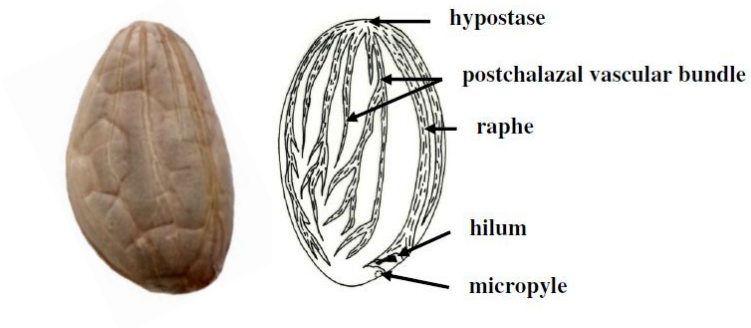


Figure 1.4 Left, picture of the testa of a dried unfermented Nacional cultivar cacao bean, mucilage removed (picture made by the author). Right, schematic visualisation of the vascular bundles on the cacao testa (Andersson et al. 2006).

1.3. Postharvest processing

Production of chocolate from cacao beans involves an extensive postharvest process. The pods are harvested when ripe and the husk is cut open to remove the cacao beans with attached mucilage. Both the pod husk and the placenta are discarded. Then, cacao beans and mucilage are fermented for 2 – 10 days depending on the cultivar and local practices. This fermentation is vital for chocolate production because it initiates several (bio)chemical reactions inside the cacao nibs that produce flavour precursors which, in turn, yield characteristic chocolate flavours during roasting. Most of the mucilage liquefies during fermentation and drains away as mucilage sweatings. After fermentation, the fermented beans are dried to stop the fermentation process and to enable safe storage. These first steps are performed in the country of origin, either at the farm or in cooperatives who collect cacao from farmers in the area. These cooperatives are especially important for small scale farmers because their yield can be too low to allow adequate fermentation. The fermented and dried beans are then sold to cacao processing companies in the USA or Europe, or to smaller local or foreign processing facilities. This more industrial phase of the process starts with the removal of stones and other extraneous materials, after which the beans are roasted and the testa is removed. The roasted nibs are then ground at elevated temperature to obtain cacao liquor and this intermediate product is further processed depending on the intended final product, as explained in more detail below.

1.3.1. Fermentation

Despite the importance of fermentation for product quality, fermentation practices are mostly non-standardised and depend on local practices, cultivar and weather conditions, which yields variable product quality.

1.3.1.1. Fermentation setups

The two most common fermentation setups are heap and box fermentation. In heap fermentation, cacao beans are piled on top of a layer of banana leaves and these large heaps or piles (up to > 1000 kg) are then covered with more banana leaves to retain heat. At consistent intervals of one or two days, the heaps are turned manually to allow air access into the fermenting mass. Heap fermentations are most common in Africa. In Central and South America, cacao is fermented in large wooden boxes (200 to > 2000 kg). The floor of the boxes is perforated to allow air access and leaching of the fermentation sweatings. Fermentation boxes are often set up in cascades to allow for easy mixing (Figure 1.5).

1.3.1.2. The three phases of the fermentation process

Fermentation begins immediately after pod opening, when the mucilage is spontaneously inoculated by microorganisms present in the environment, i.e. in the air, dust or soil, the machetes used to open the cacao pods, or microorganisms left behind in dried mucilage on the fermentation box walls. Despite the large variation in fermentation practices in the field, three general phases can be discerned: (1) the anaerobic yeast phase, (2) the microaerobic lactic acid bacteria phase, and (3) the aerobic acetic acid bacteria phase. While it has been reported that these phases cannot always be fully discerned as they may

overlap (Camu et al. 2007), the division of the process in these three steps helps to explain the sequence of microbial activity and (bio)chemical reactions.

Phase 1: anaerobic yeast phase (0 to 24 – 36 hours)

At the onset of fermentation, the tight packing of the cacao mass in the fermentation vessels creates an anaerobic environment and conditions are acid, as the unfermented cacao mucilage has a pH of 3.0 – 3.5 due to the presence of citric acid (Ardhana and Fleet 2003). These conditions promote growth of yeasts that tolerate the acid environment and lack of oxygen. During this first phase, several yeast species have been identified but *Saccharomyces cerevisiae* is generally considered to be the most abundant. The main metabolic activity of the yeasts is the conversion of fermentable sugars in the mucilage to ethanol and CO_2. As a result, ethanol concentrations increase and reach peak concentrations that range between 2 and 20 mg g^{-1} mucilage, after 24 to 54 hours of fermentation (Lima et al. 2011). Apart from ethanol production, three other important changes in the (bio)chemical environment can be discerned. First, some yeast species (e.g. *Candida* spp. and *Pichia* spp.) break down citric acid and increase the mucilage pH (Schwan and Wheals 2004). Some yeast species present in cacao fermentation convert citric acid to other organic acids such as acetic, malic and oxalic acids. This also increases the mucilage pH because of the higher pK_a values of these organic acids compared to citric acid (De Vuyst and Leroy 2020). Second, yeasts produce pectinolytic enzymes, e.g. polygalacturonase or pectin methylesterease, that break down the pectin in the mucilage parenchyma cell walls (Schwan and Wheals 2004). This causes liquefaction of the mucilage, which drains out in the form of mucilage sweatings. As a result, air access in the fermenting mass increases and conditions become more aerobic. The loss of mucilage sweatings generally ceases after 24 – 36 hours of fermentation and by that time the wet weight of the fermenting cacao mass is decreased by 12 – 15 % (Wood and Lass 1985). Third, yeasts produce aromatic compounds that contribute to the flavour profile of the final product, e.g. fusel alcohols, fatty acids and fatty acid esters (Schwan and Wheals 2004). At the end of the first fermentation phase, the increasing mucilage pH and increased ingress of air in the fermenting mass creates favourable growing conditions for lactic acid and acetic acid bacteria. In the following fermentation phases, bacteria oxidise the ethanol in the mucilage and this exothermal reaction heats up the fermenting mass. The yeast population decreases due to that increasing temperature, the increasing mucilage pH, and the depletion of fermentable sugars in the mucilage.

Figure 1.5 (A) Fermenting cacao in a wooden fermentation box with removable front panels in Guayas province, Ecuador. (B) and (C) Cascade fermentation boxes at a cooperative in Manabí province, Ecuador. (Pictures made by the author)

Phase 2: microaerobic lactic acid bacteria phase (24 – 36 to 48 – 72 hours)
Lactic acid bacteria (LAB) tolerate acid environments and are, therefore, present in the cacao mass from the onset of fermentation. However, they only become dominant when conditions become more favourable at the end of phase 1; and they are quickly defeated by acetic acid bacteria once conditions become more aerobic on the third fermentation day. Two types of LAB can be discerned: homofermenters converting sugars (e.g. glucose) to lactic acid using the Embden-Meyerhof pathway (lactic acid yield 85%) and heterofermenters converting sugars to lactic acid while also producing acetic acid, mannitol, glycerol, ethanol and CO_2 using the hexose monophosphate pathway (lactic acid yield 50%) (Schwan and Wheals 2004). The effect of the second fermentation phase on the mucilage pH is ambiguous. On the one hand, LAB produce lactic and acetic acid and thus decrease the mucilage pH. On the other hand, LAB can metabolise the citric and malic acid remaining in the mucilage after phase 1, thus counteracting the decrease in pH (Schwan and Wheals 2004; Lima et al. 2011). Lactic acid peak concentrations in the cacao mucilage have been reported to range from < 1 to 10 mg g^{-1} mucilage (Lima et al. 2011). It is generally assumed that most of the produced lactic acid is broken down in the third fermentation phase, but some may remain depending on the overall course of fermentation.

Phase 3: aerobic acetic acid bacteria phase (48 – 72 hours to end)
Similar to lactic acid bacteria, acetic acid bacteria (AAB) are generally present from the onset of fermentation but only take the upper hand towards the end, when conditions become more aerobic. Acetic acid bacteria metabolise ethanol to acetic acid, which reaches peak concentrations between 5 and 20 mg g^{-1} mucilage after three to four days of fermentation (Lima et al. 2011). However, acetic acid concentrations then rapidly decrease because it is either metabolised by AAB of the *Acetobacter* and *Gluconoacetobacter* genera to water and CO_2; it is volatised due to the high temperature; or it penetrates the testa causing *bean death* and disrupting cellular structures in the nibs, as explained in more detail below. Penetration of acetic acid into the cacao nibs acidifies the nibs from an initial nib pH of 6.5 – 7.0 (prior to fermentation) to 4.0 – 5.5 at the end of fermentation (Schwan and Wheals 2004; Belitz et al. 2009; Papalexandratou et al. 2011; De Vuyst and Weckx 2016). The acetic acid production also buffers the alkaline pH drift in the mucilage. The overall net effect of fermentation is an increase of the mucilage pH

from 3.0 – 3.5 to 4.5 – 5.0 (Ardhana and Fleet 2003; Thompson et al. 2007; Lima et al. 2011; De Vuyst and Weckx 2016). The AAB metabolism is strongly exothermic, and while the fermentation temperature increases from the onset of fermentation, the temperature only increases to > 45 °C during the third fermentation phase. The maximum temperature within the fermenting cacao mass can even exceed 50 °C (Lima et al. 2011). Because of this high temperature, metabolic activity ceases and the temperature thus decreases again. This temperature peak is often used as an indication that fermentation is complete.

Over fermentation: outgrowth of Bacilli.

At the end of fermentation, fermentable sugars in the mucilage have been depleted and the most important remaining carbon sources are mannitol and organic acids such as lactic and acetic acid. In combination with the aerobic conditions and increased temperature and mucilage pH at this stage, conditions become favourable for the growth of aerobic spore-forming *Bacillus*. Their outgrowth has been related to several off-flavours in cacao fermentation, including undesired excessive acidity.

1.3.1.3. Overall changes in cacao bean composition and chemistry due to fermentation

Bean death and the production of flavour precursors

Although microbial activity during cacao fermentation takes place in the mucilage, the cacao nibs are affected by the heat and the metabolites that can penetrate the testa. These metabolites (mostly acetic acid and ethanol) allow the formation of flavour precursor molecules, which give rise to the typical cacao flavour during subsequent roasting (Schwan and Wheals 2004). The high temperatures within the fermenting bean mass and the penetration of fermentation metabolites into the nibs result in *bean death*, which has been defined either as the loss of viability or germination potential of the beans, or as the diffusion of polyphenol pigments throughout the cacao nib tissue (Quesnel 1965).

Bean death breaks down the cell walls and membranes within the nibs and causes substantial changes to the subcellular structure. Biehl et al. (1982) reported that incubation of cacao beans in acetic acid at high temperatures resulted in destruction of subcellular compartmentation and fusion of lipid bodies inside the storage cells of the nibs. The fused lipids accumulate in the centre of the cells, pushing water and hydrophilic substances to the periphery. This change in subcellular organisation favours exudation of water-soluble substances from the cells. After bean death, the beans absorb water and swell (Forsyth and Quesnel 1963; Roelofsen 1985). The compounds that exudated from the storage cells after bean death accumulate in this liquid phase in between the nib and the testa (Forsyth 1952) and can subsequently migrate out of the beans through the permeable testa barrier. The substantial changes in the subcellular structure of the nibs during bean death bring several enzymes in contact with their substrates. These interactions between the cacao enzymes and various substrates produce the cacao flavour precursors (Fowler and Coutel 2017). There is no evidence for penetration of extraneous enzymes from the microorganisms in the mucilage into the cacao beans. It has even been suggested that incubation of cacao beans in acetic acid solutions at elevated temperature, in the absence of microorganisms, can yield the required flavour precursors for chocolate production (Biehl et al. 1985; Kadow et al. 2015).

Three main factors are reported to induce bean death: fermentation temperature, acetic acid and ethanol. However, some discussion remains regarding their relative importance. Roelofsen (1958) and Schwan and Wheals (2004) attributed bean death mostly to acetic acid, while Fowler and Coutel (2017) stated that bean death is caused by penetration of both acetic acid and ethanol into the nibs and that temperature is relatively unimportant. In contrast, Quesnel (1965) performed artificial fermentation incubations and found that fermentation heat becomes the dominating factor causing bean death above a certain temperature (in the range 43 – 53 °C). Their results also indicated that acetic acid is not the only organic acid able to induce bean death, as incubations in formic acid generated similar results compared to acetic acid, but not all organic acids have this capability. Citric acid, which is naturally present in the cacao mucilage, does not induce bean death, likely due to its larger size prohibiting penetration into the nibs. Lactic acid can also penetrate into the cacao beans and is considered an off-flavour (Holm et al. 1993). It is, however, not generally considered to contribute to bean death.

The flavour chemistry of cacao is complex and not yet fully understood. In their review discussing the effect of fermentation on chocolate quality, Schwan and Wheals (2004) state that the production of cacao flavour precursors can essentially be attributed to the interaction between vicilin (7S)-class globulin storage proteins and two protease enzymes with highly pH-dependant activity: aspartic endopeptidase (optimal pH 3.8) and serine carboxy-(exo)peptidase (optimal pH 5.8). Because of the pH-dependent activity of these enzymes, excessive nib acidification early on in the fermentation process can reduce the production of flavour precursors and thus will generate a final product with poor flavour quality (Schwan and Wheals 2004, and references therein). Proteolysis of the globulin storage proteins generates peptides and amino acids that further react with reducing sugars during roasting, thereby generating typical cacao flavour compounds. The most prevalent sugar in unfermented cacao beans is sucrose, which is not a reducing sugar and thus does not interact in Maillard reactions. During fermentation, sucrose is converted to the reducing sugars glucose and fructose due to invertase activity inside the cacao beans (Schwan and Fleet 2014). The methylxanthines theobromine and caffeine are stored in the polyphenol storage cells in unfermented cacao beans (see above) and contribute to the bitter taste of unfermented cacao (Belitz et al. 2009). During fermentation, disruption of cell structures releases the methylxanthines from the storage cells. Theobromine concentrations in the cacao nibs have been reported to decrease by up to 25% during fermentation due to outward migration or exudation to the testa and/or mucilage, together with other soluble compounds such as polyphenols (see below) (Roelofsen 1958; Timbie et al. 1978; Nazaruddin et al. 2006; Brunetto et al. 2007; Camu et al. 2008a). This decrease in nib theobromine content explains why fermentation reduces the bitterness of cacao. Caffeine concentrations, which typically range between 0.1 and 0.5 % (m/m) in unfermented cacao nibs, have been reported to either decrease with fermentation (Brunetto et al. 2007) or to remain unaffected (Camu et al. 2008a).

The effect of fermentation on polyphenols

Moderate cacao consumption has been related to improved cardiovascular health due to its high polyphenol content (Lee et al. 2003; Corti et al. 2009; Hooper et al. 2012). However, the polyphenolic content of cacao beans is strongly affected by fermentation and other postharvest processing steps. Polyphenolic compounds give unfermented cacao beans their typical dark purple colour (mostly the anthocyanin fraction) and contribute to the astringent flavour. The polyphenol content in cacao beans is reduced during fermentation and this removes most of the astringent flavour (Fowler and Coutel 2017). For example, Camu et al. (2008a) reported a 10 to 50 % decrease in polyphenol content in cacao beans with fermentation. This decrease in the nib polyphenol content is assumed to be related to both diffusion of the polyphenolic compounds to the surrounding mucilage (exudation), and to oxidation of the polyphenols to insoluble tannins, which can occur both non-enzymatically or through the activity of the polyphenol oxidase enzyme (Nazaruddin et al. 2006). In fresh cacao beans, the polyphenolic compounds and polyphenol oxidising enzymes are separated within the plant cells. Cell damage during fermentation (bean death) brings the enzymes together with their polyphenol substrate and rapidly yields hydrolysation and, together with increased oxygen ingress, oxidation of polyphenolic compounds (Camu et al. 2008a; Lima et al. 2011). Glycosidase enzymes release saccharides from anthocyanins, thereby bleaching the purple colour of the cacao nibs (Forsyth and Quesnel 1957). In addition, polyphenols migrate outward from the nib to the testa and the mucilage and are lost via exudation (Forsyth 1952; Kim and Keeney 1984). Later in the fermentation process and during subsequent drying, when conditions become more oxygenated, polyphenol oxidase hydroxylates monophenols to o-diphenols and oxidises o-diphenols to o-quinones which causes enzymatic browning and yields the typical chocolate brown colour of cacao (Forsyth and Quesnel 1957; Camu et al. 2008a).

1.3.2. Drying

After fermentation, cacao beans are dried to stop the fermentation process and thus prevent production of off-flavours, and to lower their moisture content to levels adequate for storage and transportation, i.e. < 7 – 8 % (Wood and Lass 1985; Fowler and Coutel 2017). Drying is the last step of the postharvest process that is performed at the cacao farm or cooperative. After drying, the beans are sold to processing companies all over the world. During drying, polyphenol concentrations in the nib further decrease due to oxidation (Camu et al. 2008a). The acetic acid concentration in the nibs is also reduced due to volatilisation. Similar to fermentation, several drying methods exist, and practices vary among countries and farmers. The most common method is to expose the cacao beans in shallow layers to the sun for several days until they reach the desired moisture content. Sun drying can be performed on trays or mats or on a concrete surface, and drying is sometimes performed under transparent plastic roofs to protect the cacao beans from rain (Figure 1.6). Throughout the drying process, the beans are mixed with large wooden rakes to ensure even drying. Artificial dryers are also used but they are generally considered to produce a product of lower quality (Afoakwa 2010). In artificial dryers, the temperature is often too high which hardens the testa. As a result, the volatile acetic acid is trapped inside the beans and the final product retains more acid flavours compared to

sundried beans (Wood and Lass 1985; Fowler and Coutel 2017). Wood and Lass (1985) reported that cacao fermentation in Ecuador was conventionally performed in combination with drying. The cacao beans were piled in heaps on concrete floors during the night and spread out to dry during the day. Similar practices are still performed in some Ecuadorian farms for the fermentation of Nacional cacao, as an adjusted drying step after conventional box fermentations (Figure 1.6).

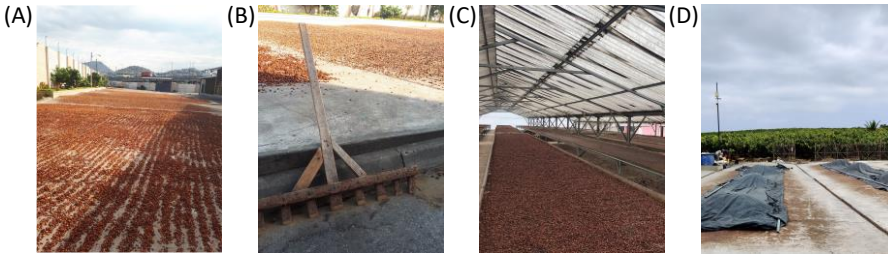


Figure 1.6 (A) Fermented cacao beans drying on concrete flooring at a cooperative in Guayas province, Ecuador. (B) Rake used to mix the drying cacao beans several times per day to ensure even drying of the product. (C) Fermented cacao beans drying on elevated tray tables under a transparent roof at a cooperative in Manabí province, Ecuador. (D) Nacional cultivar cacao beans are heaped in between drying phases as a form of extended fermentation at a cacao plantation in Guayas province, Ecuador. (Pictures A and B by Vincent De Mesmaeker, picture C made by the author, picture D by Jasmien Doevenspeck)

1.3.3. Breaking and winnowing (deshelling) and roasting

Upon arrival in the processing facility, the cacao beans are cleaned and extraneous material such as rocks is removed. The beans are then roasted and the testae are removed. Roasting and deshelling can be performed either as nib roasting or whole bean roasting. In whole bean roasting, the intact cacao beans are roasted prior to testa removal. The main advantage of this method is that roasting makes the testa more brittle and loosens it from the nibs, which allows for easier removal. Similar to what happens in artificial dryers operated at high temperature (see above), the testa hardens and prevents loss of volatile compounds during roasting (Kamphuis and Fowler 2017). This can be both disadvantageous because acetic acid is retained in the nibs, or advantageous because volatile flavour compounds are protected. In nib roasting, the testa is removed prior to roasting which allows volatilisation of acetic acid and reduces the acid flavour in the final product. Nib roasting also allows alkalinisation prior to roasting, which is a common practice in the cacao powder production (also referred to as Dutch processing, see below).

Testa removal

Optimal removal of the testa has several benefits. Up to this point in the postharvest process, the shell or testa has acted as a protective barrier for the cacao nibs and has thus been exposed to potential contaminants including mycotoxins. The testa does not contribute to chocolate flavour and it is a hard fibrous material that can damage processing equipment. To remove the testa, cacao beans are broken through impact using centrifugal force and nibs and testae are separated in a winnower based on the difference in their specific weight (Kamphuis and Fowler 2017). Cacao liquor, the intermediate product obtained after roasting and grinding (see below) should not contain more than 5

% (m/m) shell or germ (radicle) material on a fat free dry basis (Codex Alimentarius 2014).

Roasting

During roasting, the reducing sugars, amino acids and peptides that were formed during fermentation are transformed through Maillard and Strecker degradation reactions to cacao flavour compounds such as pyrazines or Strecker aldehydes. The moisture content is further reduced from 7 – 8 % in dried beans to < 2 % in roasted beans (De Vuyst and Weckx 2015). Roasting times and temperatures vary between 5 minutes and 2 hours, and between 100 and 150 °C depending on the intended final product and on the roasting setup (i.e. whole beans vs. nibs) (Wood and Lass 1985; Wollgast and Anklam 2000; Kamphuis and Fowler 2017). Roasting is the only high temperature step in cacao powder production, whereas cacao intended for chocolate will undergo several other heat steps later in the production process (e.g. grinding and conching, see below). Therefore, cacao intended for the production of chocolate is generally roasted at lower temperatures compared to cacao intended for the production of cacao powder (Wood and Lass 1985).

1.3.4. Alkalinisation or Dutch processing

Alkalinisation is the practice of adding alkaline compounds (e.g. sodium hydroxide, calcium carbonate or potassium carbonate) to introduce specific flavours and colours in the final product (Winkler 2014). This practice was first introduced by the Dutch cacao processing company Van Houten in the 1800s and is, therefore, also referred to as *Dutch processing* (Minifie 1989; Ziegleder 2017). Alkalinisation is optional and mostly used in the production of cacao powder, cacao-derived drinks, or cacao intended as an ingredient in products such as cakes or cookies (Minifie 1989). Alkalinisation decreases the fat content of cacao due to hydrolysis and saponification of triglycerides (Valverde García et al. 2020). It also generates specific colours during subsequent roasting. The pH of the nibs is increased to 6.8 – 7.5 and, while there is some controversy regarding the effect on flavour, alkalinised cacao is generally considered to have a reduced, more mild flavour compared to non-alkalinised cacao (Minifie 1989; Wollgast and Anklam 2000; Belitz et al. 2009; Ziegleder 2017). Bitterness is also reduced as alkalinisation decreases the theobromine content in the cacao beans (Li et al. 2012). In practice, the nibs are soaked in an alkaline solution at elevated temperature, the solution is removed through drying and the alkalinised cacao nibs are roasted. This can be done either as separate steps or in the form of a continuous process inside a roasting drum (Kamphuis and Fowler 2017). Alkalinisation can also be performed by mixing alkaline solutions with the cacao liquor or by adding dry alkaline products to the cacao cake, but this does not influence the colour of the product as the alkaline products are added after roasting.

1.3.5. Further processing

After roasting, testa removal and alkalinisation (optional), the nibs are ground at a temperature > 35 °C (the melting point of cacao butter) to produce cacao mass or *cacao liquor*, which is essentially pure unprocessed chocolate. Further processing depends on the intended final product. The first option is to separate the cacao butter from the non-fat cacao solids (the *cacao cake*) using a hydraulic press. The resulting cacao cake typically has a fat content of 10 – 24 %, depending on the applied pressure and the pressing time

(Afoakwa 2010). The cake is then ground to obtain cacao powder, which is classified either as regular (≥ 20 % cacao butter) or fat-reduced cacao powder (< 20 % cacao butter) (EU Directive 2000/36/EC 2000). The cacao butter can be used as an ingredient in food or non-food products (e.g. cosmetics). Chocolate can be produced by mixing cacao powder and butter with additional ingredients such as sugar or milk powder. The second option is to use the cacao liquor directly. Additional ingredients such as sugar or milk powder are mixed with the cacao liquor, and this mixture is subjected to a refining step to reduce the particle size to < 30 µm (Afoakwa 2010). Additional cacao butter or other vegetable fats can also be added, but the product can only be labelled as chocolate if the non-cacao vegetable fats amount to less than 5 % (m/m) (EU Directive 2000/36/EC 2000). The final step in the production of bulk chocolate is conching, i.e. agitation of the chocolate mass for several hours at temperatures > 50 °C. The objectives of conching are to remove some of the volatile off-flavours that are still present and to improve viscosity and flow parameters of the product, potentially through addition of fat or emulsifiers such as lecithin (Afoakwa 2010; Beckett et al. 2017). Conching coats the solid particles, mostly non-fat cacao solids and sugar, with fat so that the particles can flow easily past one another and this largely improves the product texture. Conching is followed by tempering and moulding to obtain the desired final product.

Chapter 2
The transfer of cadmium from soil to chocolate bar

This chapter is based on the following reference:
Vanderschueren R, Argüello D, Blommaert H, Montalvo D, Barraza F, Schreck E, Gramlich A, Schulin R, Maurice L, Lewis C, Vazquez JL, Umaharan P, Chavez E, Sarret G, & Smolders E. 2021. Mitigating the level of cadmium in cacao products: reviewing the transfer of cadmium from soil to chocolate bar. *Science of the Total Environment*. 781:146997. doi:10.1016/j.scitotenv.2021.146779

Summary
The new EU regulation on cadmium (Cd) in cacao-derived products affects the cacao market worldwide. This chapter reviews the journey of Cd from soil to chocolate bar and collates current data on the topic, giving due attention to data quality. Cacao bean Cd concentrations are typically about a factor two larger compared to the soil on which the cacao trees grow, this is high but not unusual and, therefore, the cacao plant is not classified as a Cd hyperaccumulator. Average Cd concentrations in cacao beans range between 0.02 and 12 mg kg^{-1}, and are markedly higher in Central and South America, where more than half of cacao bean samples exceed the commonly applied industry threshold for export to the EU (0.60 mg kg^{-1}). This regional enrichment is related to relatively high soil Cd concentrations in the young soils of Central and South America. The source of Cd is, in general, likely geogenic rather than derived from phosphate fertilisers or contamination. A meta-analysis of 780 soil-plant paired data shows that soil Cd, soil pH and soil organic carbon largely explain bean Cd concentrations. Detection of effects of cultivars, soil amendments or agronomic practices are strongly hampered by the spatial variability in phytoavailable soil Cd concentrations. Application of lime or biochar has the potential to lower bean Cd in acid soils. In the long term, breeding low Cd cultivars likely provides high potential for mitigation but genetics and breeding research is currently limited by the lack of understanding of how Cd is loaded into the developing fruit of the cauliflorous cacao tree. In the short term, mixing of cacao from different origins may be the most feasible strategy to meet the EU limits.

2.1. Introduction

Cadmium (Cd) is a potentially toxic trace metal that has no known biological function in humans. Cadmium accumulates in the human body and has a biological half-life of 10 – 35 years. Chronic Cd exposure has been related to several adverse health effects, including renal tubular dysfunction and osteomalacia (World Health Organization 2010). The human diet is the main source of body burden Cd in the non-smoking population and staple foods such as rice, wheat grain products or potatoes contribute largely to dietary Cd exposure because of their high consumption. Certain luxury foods such as chocolate, although ingested in smaller quantities, can also contribute to dietary Cd exposure due to their elevated Cd concentrations. To protect consumers, the European Commission approved a new regulation in 2014 (in force from 2019), which sets the maximum allowed Cd concentration in cacao powder at 0.60 mg Cd kg^{-1}, while the thresholds for chocolates range between 0.10 and 0.80 mg Cd kg^{-1} depending on the cacao solids content of the product (European Commission 2014). Similar regulations have been, and are expected to be, implemented worldwide, e.g. in Australia and New Zealand (Australia New Zealand Food Standards Code 2017), Russia (Ministry of Health of the Russian Federation 2011), and the countries within the Southern Common Market (Mercosur 2011). In California (USA), products with elevated Cd concentrations must have a warning on the packaging (Meter et al. 2019). The Cd-cacao regulations have also been adopted by the Codex Alimentarius (Codex Alimentarius Commission 2018). These regulations have fuelled research worldwide to monitor and mitigate Cd accumulation in cacao. The number of research papers on Cd in cacao was only 29 up to 2014 but rose almost exponentially to > 100 by 2020.

Cadmium in cacao products originates from the cacao beans, rather than from contamination during processing. This is demonstrated by the strong correlations between the Cd concentration in the final chocolate product and its cacao content, and between the Cd concentration in chocolate and the geographical origin of the cacao (Villa et al. 2014; Yanus et al. 2014; Abt et al. 2018; Lo Dico et al. 2018). While all plants take up Cd to some extent, the cacao tree (*Theobroma cacao* L.) is rather effective in doing so. The oldest study reporting Cd concentrations in cacao beans and chocolates dates back to 1979 and that study already highlighted that elevated Cd concentrations are typically found in cacao and chocolates from Central and South American origin, as well as in specialty origin chocolates (Knezevic 1979). The recently enforced EU limits and the Codex Alimentarius recommendations apply to the final product sold to consumers, not to the cacao beans. Therefore, the processing industry has translated the EU limits to requirements regarding the maximum Cd concentrations in the beans purchased from their suppliers. Those unofficial industry limits vary among companies and have been reported to range between 0.50 and 1.10 mg Cd kg^{-1} (Meter et al. 2019; CBI Ministry of Foreign Affairs n.d.), but some cacao farmers are confronted with purchaser thresholds as low as 0.10 – 0.30 mg Cd kg^{-1} (personal communication). To facilitate the discussion, 0.60 mg Cd kg^{-1} was used as a threshold throughout this dissertation.

Mitigation of Cd accumulation has been intensively studied for several food crops including durum wheat, spinach, potatoes and rice (McLaughlin et al. 2021), but for cacao this research is in its infancy. This chapter summarises the existing knowledge on the various factors influencing Cd concentrations in cacao-derived products at all stages of the production process. That includes soil Cd phytoavailability, Cd uptake and translocation within the plant, postharvest processing and the effect of dietary intake through chocolate consumption on the Cd body burden. A meta-analysis of the currently available Cd-cacao studies is presented with due attention given to the quality of the analytical data, thereby only selecting data for which the quality of chemical analyses met predefined quality criteria (more details below and in Appendix I). Research gaps are identified and potential mitigation strategies at the different stages of the cacao value chain are discussed.

2.2. Cadmium in soils and its relation to cacao plants

2.2.1. Origin of Cd in cacao beans: soil, air, fertiliser and irrigation water

Generally, most of the Cd in plant tissues originates from the soil through root uptake, while only a small fraction is derived from air through foliar uptake. This is likely also true for cacao, first because of the positive associations reported between soil Cd and bean and leaf Cd concentrations (see below) and, second because of the large Cd concentrations found in cacao leaves and beans, which are well above what can be reasonably expected from foliar uptake. The contribution of airborne Cd to crops can be measured using isotopes that indicate the Cd provenance, and can be quantified using Air Accumulation Factors (AAFs, $m^3\ g^{-1}$), calculated as the ratio of the Cd concentration in the plant to that in the air. For trace metals in general, the AAF is estimated at 20 $m^3\ g^{-1}$ for plant leaves, with a range of 2 – 100 $m^3\ g^{-1}$ depending on the type of plant and trace metal (Mclaughlin et al. 2011). Reported air Cd concentrations in the rural areas where cacao is grown range from 0.07 to 0.57 ng Cd m^{-3} [cacao production areas near oil activities (Barraza et al. 2017)]. Leaf Cd concentrations derived from air in these cacao growing areas will thus range between 0.001 and 0.011 mg Cd kg^{-1} (considering an AAF of 20 $m^3\ g^{-1}$), with a maximum of 0.06 mg Cd kg^{-1} (AAF of 100 $m^3\ g^{-1}$), which is only 6 % of the typical leaf Cd concentrations measured in cacao plants in Central and South America (> 1 mg Cd kg^{-1}, see below).

Cadmium occurs naturally in the environment, with background soil concentrations ranging from 0.1 to 1.0 mg Cd kg^{-1} and estimated global averages of 0.1 to 0.3 mg Cd kg^{-1} (Smolders and Mertens 2013). In contrast, polluted soils (e.g. near smelting sites) can contain Cd concentrations up to three orders of magnitude higher than the background (He et al. 2015). The soil Cd concentrations in cacao producing areas differ worldwide and tend to be higher in the geologically young soils of Central and South America, with a range of reported averages between 0.22 and 10.8 mg Cd kg^{-1} (Chavez et al. 2015; Ramtahal et al. 2016; Arévalo-Gardini et al. 2017; Barraza et al. 2017; Gramlich et al. 2017; Barraza et al. 2018; Gramlich et al. 2018; Lewis et al. 2018; Argüello et al. 2019; Engbersen et al. 2019; Rodríguez Albarrcín et al. 2019; Scaccabarozzi et al. 2020) and 0.12 to 0.85 mg kg^{-1} in Asia (Fauziah et al. 2001; Zarcinas et al. 2004). Unfortunately, reliable information about Cd concentrations in soils of cacao producing areas in Africa remains scarce. The majority of global cacao is produced in West Africa ,where soils are highly weathered and

are expected to have low Cd concentrations. Indeed, a study in different crops of Ghana reported an average total soil Cd concentration of 0.026 mg Cd kg^{-1} (Bortey-Sam et al. 2015). It is important to note that, while soil Cd concentrations are thus generally reported to be higher in Central and South America compared to other continents, soil Cd concentrations in Central and South American cacao producing areas are mostly < 1 mg Cd kg^{-1} (Figure 2.2), and thus not categorised as contaminated. Multiple researchers have indicated large geographical differences in cacao bean Cd, with elevated Cd concentrations found in cacao from Central and South American origin compared to origins, e.g. Africa (Bertoldi et al. 2016; Abt et al. 2018). The strong effect of geographical origin on bean Cd concentrations is outlined below and suggests that Cd in cacao generally does not originate from anthropogenic activity, as anthropogenic activities occur worldwide. Indeed, three independent studies by Gramlich et al. (2018), Argüello et al. (2019) and Scaccabarozzi et al. (2020) found that high soil Cd concentrations in cacao farms were associated with alluvial soils from sedimentary materials, which can be explained by the higher Cd concentrations usually found in sedimentary rocks compared to igneous rocks (Thornton 1981; Birke et al. 2017). However, the substrate or alluvium source is likely also of importance, as alluvial soils in Africa show different Cd concentrations compared to their Central and South American counterparts. Nevertheless, there are specific cacao producing areas in Central and South America where soil Cd concentrations are exceptionally high (Rodríguez Albarrcín et al. 2019), potentially due to historical enrichment related to mining activities.

The use of mineral P-fertilisers has been suggested to be at the origin of high Cd in cacao beans (Zug et al. 2019). However, it is highly unlikely that the use of P-fertilisers can explain elevated cacao bean Cd concentrations on a large scale. Gramlich et al. (2017) measured the Cd concentration in P-fertilisers in a long term system comparison trial on an experimental cacao farm in Bolivia and found an average Cd concentration of 102 mg Cd kg^{-1} P_2O_5, which is high compared to fertilisers available in Europe [average 28 mg Cd kg^{-1} P_2O_5, < 10 % of samples contained 60 mg Cd kg^{-1} P_2O_5 (Verbeeck et al. 2020)]. At a sustained annual dose of 20 kg P_2O_5 ha^{-1} $year^{-1}$ with that local fertiliser, fertilisation would add about 2 g Cd ha^{-1} $year^{-1}$, equivalent to a net addition to soil of 0.001 mg Cd kg^{-1} soil $year^{-1}$ at 15 cm incorporation depth or an accumulation of only 0.1 mg Cd kg^{-1} soil after 100 years. This mineral fertiliser dose is unrealistically high, as most of the cacao production worldwide is in the hands of low-income smallholders who do not commonly use fertilisers (Snoeck et al. 2016; Vaast et al. 2016). This worst-case scenario calculation hence indicates that mineral P-fertilisers are not the main source of Cd in cacao plantations, at least not at a large scale.

Irrigation water contains Cd and moderate Cd contamination in the water may be a significant source of Cd in soils and plants. An annual irrigation application of 500 mm with irrigation water containing 1 µg Cd L^{-1} is equivalent to 5 g Cd ha^{-1} $year^{-1}$ and leads to an accumulation of 0.25 mg Cd kg^{-1} soil, at a sustained irrigation over 100 years and 15 cm incorporation depth. Such accumulation is not unlikely in areas affected by mining activities. For example, in the Puyango-Tumbes river basin at the border between Ecuador and Peru, dissolved Cd concentrations range between 1 and 10 µg L^{-1} due to upstream gold mining (Tarras-Wahlberg et al. 2001; Carling et al. 2013; Marshall et al.

2018). The elevated Cd concentrations in these rivers have been mentioned as a potential source of Cd to nearby cacao fields (Chavez et al. 2015).

Several soil profile studies have reported that soil Cd concentrations in cacao fields decline with increasing soil depth. The Cd concentration in the top 0 – 15 cm of the soil is about a factor 1.5 larger than the Cd concentration at depths 15 – 60 cm (Chavez et al. 2015; Arévalo-Gardini et al. 2016; Barraza et al. 2017; Gramlich et al. 2018). Although this might be interpreted as evidence for anthropogenic sources, it is more plausible that this concentration profile reflects the cycling effect caused by decomposition of litter from the aerial biomass (Reimann et al. 2019). The dry leaf biomass production of cacao is about 3.9 Mg ha^{-1} y^{-1} (Heuveldop et al. 1988) with an average leaf Cd concentration of 2.6 mg kg^{-1} dry weight (dw) (n = 762, derived from the meta-analysis described below). Hence, the annual Cd flux returned to the soil by leaf litter decomposition is about 10 g Cd ha^{-1} y^{-1}, which is much larger than the worst-case inputs calculated from application of contaminated P-fertilisers or irrigation water as indicated above. Enriching the topsoil from a background soil concentration of 0.89 mg Cd kg^{-1} [estimated by the subsoil Cd concentrations reported by Gramlich et al. (2017)] to 1.09 mg Cd kg^{-1} [topsoil Cd concentrations reported by Gramlich et al. (2017)] would thus require approximately 50 years of leaf litter decomposition, which is not an unrealistic age for the many cacao orchards in the hands of small-scale farmers. Indeed, an economic survey conducted in Ecuador (Vazquez et al., in preparation) reported that the age of cacao orchards ranged from 1 to 100 years, with an average of 13.8 years (n = 1534), and 11 % of the orchards surpassed the age of 30. Barraza et al. (2019) determined the stable isotope ratio ($\delta^{114/110}Cd$) in cacao leaves, litter and topsoil from cacao plantations. While the isotopic ratios in topsoil differed among the different sites, there was a consistent fractionation between leaves and soil ($\Delta_{leaf\text{-}soil}$), indicating that the topsoils were likely enriched with heavier Cd isotopes originating from the leaf litter. Considering all the above, the main Cd source in cacao producing soils is likely geogenic rather than anthropogenic, and local enrichments may be related to point sources such as mining activities, input of sedimentary materials or the use of contaminated irrigation water. The annual Cd cycling due to plant uptake and leaf litter decomposition can explain the larger Cd concentrations in the surface soil compared to the subsoil.

2.2.2. Bean Cd concentrations and their relationship with soil properties: a meta-analysis

Cacao bean Cd concentration data reported in different studies have been collated here to identify general trends (Table 2.1). Studies included in this table were selected based on the quality criteria elaborated in Appendix I, i.e. quality assurance of the chemical analysis combined with a minimal number ($n \geq 4$) of different samples to characterise the region, as well as information on sample treatment (drying, peeling and digestion procedures). Studies that used Flame Atomic Absorption (AAS) or older Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) instruments were excluded from this compilation because of their poor detection limits for Cd in plant tissues (0.1 – 0.5 mg Cd kg^{-1} dw). Average or median bean Cd concentrations clearly vary by geographical origin, with cacao beans originating from Central and South America showing Cd concentrations four to six times higher compared to beans from Asia or Africa (Table 2.1, data from

Central and South America marked in grey). While most included studies have reported the data as bean Cd concentrations, sample treatments before analyses differ strongly among studies. Processing steps such as fermentation, peeling or roasting may influence the Cd concentration but require further research as explained in section 2.4.3.

Cadmium concentrations in crops depend on the availability of soil Cd, which increases with increasing total soil Cd, increasing soil acidity, decreasing soil organic matter, and, in some cases, Zn deficiency and Cl salinity (Smolders and Mertens 2013). These relationships have also been corroborated for cacao (Gramlich et al. 2018; Argüello et al. 2019). To better illustrate these general trends, the raw data of different soil-plant studies were collated. Over 400 studies on soil-plant Cd relationships in cacao (including all plant tissues) were found. However, only seven passed all quality criteria elaborated in Appendix I (Table I.1). A total of 785 paired soil-plant data points were gathered from these seven datasets, which were all based on studies in Central and South America (six published studies and one unpublished dataset Appendix I, Table I.2). The selected studies reported total soil Cd, soil pH, soil organic carbon (SOC) and bean Cd, among other variables. In studies I and VII, bean Cd referred to the Cd concentration in the peeled bean or nib; while in studies II, III, IV, V and VI, samples were not peeled and bean Cd thus referred to the total bean Cd concentration. The frequency distribution of these data shows that bean Cd concentrations are 1.08 mg kg^{-1} (arithmetic mean) and 0.63 mg kg^{-1} (median; n = 779) with distinct differences among studies (Figure 2.1). A total of 403 samples (52 % of the compiled data) showed bean Cd concentrations exceeding 0.60 mg kg^{-1}, the threshold commonly used for export to the EU (Figure 2.1). Per study, the number of observations exceeding this threshold ranged between 7 and 100 %. This clearly emphasises the magnitude of the impact of the new EU Cd regulations on cacao producers.

Table 2.1 Average cacao bean Cd concentrations (mg kg^{-1}) from several studies across the world clearly illustrate geographical differences, with the highest bean Cd concentrations reported for cacao grown in Central and South American countries (rows marked in grey). Incomplete information on analytical data quality is indicated by superscripts. The variation is indicated either by the standard deviation or the range min-max; P = peeled; UP = unpeeled; F = fermented; UF = unfermented; R = roasted.

Sample origin	Bean Cd [mg kg^{-1}]			Processing	Study
	Average	Variation	n		
Ghana [a,c]	0.02	0.003	3	P, F	Vītola and Ciproviča (2016)
Nigeria [a,c]	0.02	0.003	3	P, F	Vītola and Ciproviča (2016)
Ghana [a,c]	0.05	0.045–0.058	30	UP, F	Nnuro et al. (2020)
Cameroon [a,c]	0.05	0.01	3	P, F	Vītola and Ciproviča (2016)
Ghana [a,c]	0.05	0.005–0.095	20	UP, F	Amankwaah et al. (2015)
Ivory Coast [a,c]	0.05	0.04	9	P, F	Yapo et al. (2014)
West Africa	0.09	0.04	21	UP, F	Bertoldi et al. (2016)
Brazil [a]	0.10	/	1	P, F	Knezevic (1979)
Ivory Coast [a]	0.12	0.09–0.14	3	P, F	Knezevic (1979)
Sao Thomas and Principe [a]	0.12	0.09–0.15	4	P, F	Knezevic (1979)
Dominican Republic	0.13	0.031	-	UP, F	Kruszewski et al. (2018)
Tanzania [a]	0.13	/	1	P, F	Knezevic (1979)
Ghana [a]	0.14	0.09–0.18	8	P, F	Knezevic (1979)
Brazil (Bahía) [a]	0.19	0.09–0.29	8	P, F	Knezevic (1979)
Ecuador [a,c]	0.20	0.04	3	UP, F	Vītola and Ciproviča (2016)
Bolivia [a]	0.21	0.02	64	P, F	Gramlich et al. (2017)
New Guinea [a]	0.22	0.14–0.29	8	P, F	Knezevic (1979)
Samoa [a]	0.22	/	1	P, F	Knezevic (1979)
Malaysia [a]	0.25	0.01–1.27	86	/	Mohamed et al. (2020)
Sri Lanka [a]	0.26	0.24–0.27	2	P, F	Knezevic (1979)
Ghana [a,c]	0.3	0.248–0.336	67	P, F	Takrama et al. (2015)
Mexico [a]	0.28	/	1	P, F	Knezevic (1979)
Asia	0.33	0.18	8	UP, F	Bertoldi et al. (2016)
Ecuador	0.35	0.24	50	UP, UF	Acosta and Pozo (2013)
East Africa	0.51	0.59	8	UP, F	Bertoldi et al. (2016)
Indonesia (Sumatra) [a]	0.52	/	1	P, F	Knezevic (1979)
Central America	0.54	0.30	10	UP, F	Bertoldi et al. (2016)
Malaysia [a]	0.55	0.26	10	/	Fauziah et al. (2001)
Brazil	0.55	0.10–1.50	36	P, F	De Araujo et al. (2017)
Caribbean [a]	0.57	/	1	P, F	Knezevic (1979)
Central and South America	0.62	0.38	7	P, F, R	Abt et al. (2018)
Ecuador	0.63	0.067	-	UP, F	Kruszewski et al. (2018)
Malaysia [a]	0.67	0.20–1.68	5	/	Zarcinas et al. (2004)

Table 2.1 continued

Sample origin	Bean Cd [mg kg^{-1}]			Processing	Study
	Average	Variation	n		
Trinidad & Tobago [a]	0.68	/	1	P, F	Knezevic (1979)
Ecuador	0.75	0.27–1.72	81	UP, F	Romero-Estévez et al. (2019)
Indonesia (Java) [a]	0.76	/	1	P, F	Knezevic (1979)
Grenada [a]	0.77	/	1	P, F	Knezevic (1979)
Ecuador	0.78	0.12–1.52	4	UP, UF	Barraza et al. (2018)
Ecuador	0.90	0.09–3.51	31	UP, UF	Barraza et al. (2017)
Ecuador	0.90	0.03–10.4	560	P, UF	Argüello et al. (2019)
Malaysia (Sabah) [a]	0.94	0.59–1.29	2	P, F	Knezevic (1979)
Ecuador [c]	0.94	0.02–3.00	19	P, UF	Chavez et al. (2015)
Jamaica [a]	0.95	/	1	P, F	Knezevic (1979)
Peru [a]	0.96	0.34	72	UP, UF	Rosales-Huamani et al. (2020)
Trinidad & Tobago	0.98	0.50–2.34	45	P, F	Ramtahal et al. (2015)
Trinidad & Tobago [b]	1.00	0.17–2.31	100	UP, UF	Lewis et al. (2018)
Costa Rica [a]	1.02	/	1	P, F	Knezevic (1979)
Honduras	1.10	0.10	110	P, UF	Gramlich et al. (2018)
Malaysia [a]	1.14	0.45–1.83	9	P, F	Knezevic (1979)
Peru [a, d]	1.13	0.11–6.30	70	UP, UF	Arévalo-Gardini et al. (2017)
Peru [a]	1.31	1.26–1.36	3	P, F	Knezevic (1979)
South America [a]	1.39	1.09	14	UP, F	Bertoldi et al. (2016)
Venezuela [a]	1.96	1.75–2.17	3	P, F	Knezevic (1979)
Costa Rica [a,c]	2.20	0.56–8.7	24	UP, UF	Furcal-Beriguete and Torres-Morales (2020)
Trinidad & Tobago	2.27	1.78	402	P, UF	Ramtahal et al. (2016)
Ecuador (Arriba) [a]	2.45	0.55–4.34	6	P, F	Knezevic (1979)
Honduras	2.56	0.81–10.6	60	UP, UF	Engbersen et al. (2019)
Ecuador [d]	2.68	1.26–3.92	5	UP, UF	Barraza et al. (2019)
Colombia [a]	12.0	6.94	57	UP, F	Rodríguez Albarrcín et al. (2019)

[a] No information was found on whether certified reference materials (CRM) were included in the analysis or not.

[b] CRM included with certified Cd concentrations above the reasonable range of cacao Cd concentrations.

[c] Reported sample Cd concentrations lower than the limit of detection of the equipment used for Cd analysis.

[d] Mean and variation were calculated from the raw data which was kindly provided by the authors of these studies.

The average total topsoil Cd concentrations of each study (Appendix I, Table I.2), as well as the overall average (± standard deviation, stdev) of the compiled dataset (0.43 ± 0.41 mg Cd kg^{-1}, n = 776) are within the range for non-polluted soils. Only 50 soil samples across all studies exceeded 1.0 mg Cd kg^{-1}, such "hotspots" thus represent only 6 % of the compiled data. Multivariate regression analysis was first applied to each individual dataset (details on methods can be found in Appendix I). The results of this multivariate analysis (Table 2.2 and Appendix I, Table I.3) show a generally emerging trend: bean Cd increases with increasing total soil Cd, with decreasing pH (except in study II) and with decreasing SOC (except in study VI). The effect of organic carbon can be explained by the high affinity of Cd for sorption sites in organic matter (Christensen 1989). The effect of pH on Cd availability is due to the competition between Cd^{2+} and protons for soil sorption sites, as Cd^{2+} binds to carboxylic or phenolic groups present in organic matter and oxyhydroxides (Smolders and Mertens 2013).

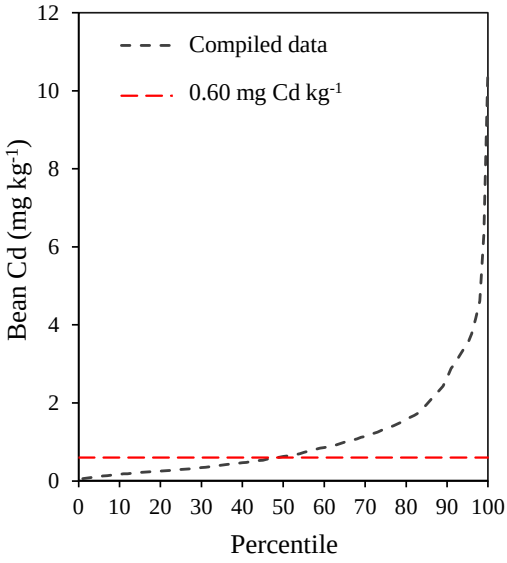


Figure 2.1 Percentile distribution of cacao bean Cd concentrations of the compiled meta-analysis data (n = 780, overall mean 1.08 mg Cd kg^{-1}), with indication of the unofficial industry threshold of 0.60 mg Cd kg^{-1}. Cacao beans were either peeled or not depending on the study. A detailed description of the studies included in the meta-analysis can be found in Appendix I.

Total soil Cd alone might not be the best predictor for crop Cd concentrations as the majority of Cd is sorbed to different soil particles and is not directly available for plant uptake. Therefore, plant Cd concentrations are better predicted if soil pH is included in the prediction equation, as it is the key soil property controlling the availability of soil Cd. Available soil Cd has been proposed as a proxy to predict crop Cd concentrations. Available soil Cd often refers to the soluble fraction in the soil and is operationally defined by the extraction method used to measure that soluble fraction (McLaughlin et al. 2000). Study VII reported available Cd in the topsoil (measured either by simple extraction with ammonium-acetate-EDTA or using Diffusive Gradients in Thin film, DGT), and available soil Cd was identified as a significant predictor for bean Cd for that study (Table 2.2 and

Appendix I, Table I.3). Soil pH, which affects the soluble fraction of Cd in the soil, is logically not a significant variable when available soil Cd rather than total soil Cd is used. Multivariate regression was also applied to the compiled dataset including data from all seven studies, with correction for the disproportionate weight of their sample sizes (n = 334, methods are described in Appendix I). Total soil Cd, pH and SOC explained > 41 % of the variance in bean Cd concentrations within the compiled dataset.

The equation of the model reads as follows:

$$log_{10}[Bean\ Cd] = 1.34 + 0.86 * log_{10}[Total\ Soil\ Cd] - 0.18 * pH - 0.25 * log_{10}[SOC]$$

where bean Cd and total soil Cd are expressed in mg kg^{-1}, pH is standardised as pH_{CaCl2} using the equation proposed by Kissel et al. (2009), and SOC is expressed as a percentage. This significant multivariate model suggests (i) that bean Cd increases almost proportionally to total soil Cd; (ii) that bean Cd increases by a factor of 1.5 per unit decrease in soil pH (Figure 2.2); and (iii) that doubling SOC reduces bean Cd by a factor of 1.8. The Cd-cacao issue is thus clearly not related to a single factor such as total soil Cd, but rather to an interaction of several factors, i.e. total soil Cd, soil pH and SOC, which control the solubility of Cd and, therefore, also its availability to the cacao plant.

Table 2.2 Predictors for bean Cd concentrations for each of the included studies, determined by multivariate regression analysis. All variables were log transformed before analysis except for pH. The sign in brackets corresponds to the sign of the coefficient of that predictor. Top = Topsoil (0 – 20 cm), Sub = Subsoil (20 – 40 cm), Av = Available, SOC = Soil Organic Carbon, DGT = Diffusive Gradient Thin film.

Study	n ¥	Significant predictors	R^2
I	559	Total Cd Top (+); pH Top(-); SOC Top(-)	0.57
II a §	28	SOC Sub (-); Total Zn Sub (+); Total Mn Top (+)	0.64
II b §	28	Total Cd Top (+); SOC Sub(-)	0.49
VI	60	Total Cd Top (+); pH Top (-)	0.23
VII a §	107	Total Cd Top (+); pH Top(-); SOC Top(-)	0.36
VII b §	106	Av Cd Top (+); SOC Top (-); Av Zn Sub (+); Av Mn Top (-)	0.41
VII c §	105	DGT Cd Top (+); SOC Top (-)	0.43

§ There are three regression models for study VII as the variables Total Cd Top, Av Cd Top and DGT Cd Top were highly correlated thus running only one regression with all variables included would have violated the assumption of no multicollinearity. For study II, two models are included because Total Cd Top was significantly correlated to Total Zn Sub and Total Mn Top.

¥ The number of observations included in each regression can differ from the number of observations mentioned in Appendix I (Table I.2) due to missing data for some of the predictors.

Because Zn and Cd are considered analogues, low available soil Zn has been suggested to trigger plant Cd uptake (Oliver et al. 1994; Chaney et al. 2006; Chaney 2010). In a recent greenhouse study, Souza dos Santos et al. (2020) reported that increasing soil Zn inhibited the uptake and translocation of Cd by CCN 51 cacao plants. However, this effect was only found in soils spiked to Cd concentrations well above environmentally relevant soil Cd concentrations. Argüello et al. (2020) found that Cd uptake in cacao from deeper soil layers was enhanced by surface liming and the authors related this to increased activity

of deeper roots to cope with micronutrient deficiency in the topsoil (i.e. Zn) caused by surface liming. The multivariate regressions presented here did not identify total soil Zn as a significant predictor for bean Cd concentrations (with exception of study II, Table 2.2), even when using leaf Zn concentrations as regressors (details not shown). The overall average leaf Zn concentration in the meta-analysis was 97 ± 68 mg kg^{-1} (n = 700, data from studies I, II, IV and VII) which falls within the range reported as optimal (> 20 mg Zn kg^{-1} dw) for plant development (Fageria et al. 2002). Only a small fraction of samples (< 4 %) presented suboptimal leaf Zn concentrations, which may explain why no significant effect of soil Zn was identified in the regression analysis. The lack of Zn deficiency within the included datasets may also indicate that Zn deficiency is not a widespread issue in Central and South American cacao production areas, where Cd accumulation is considerable. However, study II showed a significant effect of total subsoil Zn and total topsoil Mn on bean Cd (regression II a, Table 2.2). Similarly, study VII showed a significant effect of available subsoil Zn and available topsoil Mn on bean Cd, when available soil Cd was used as a predictor instead of total soil Cd (regression VII b, Table 2.2). Additional research regarding the effect of available Zn and Mn is required to unravel their potential interaction with Cd and their effect on Cd accumulation in cacao.

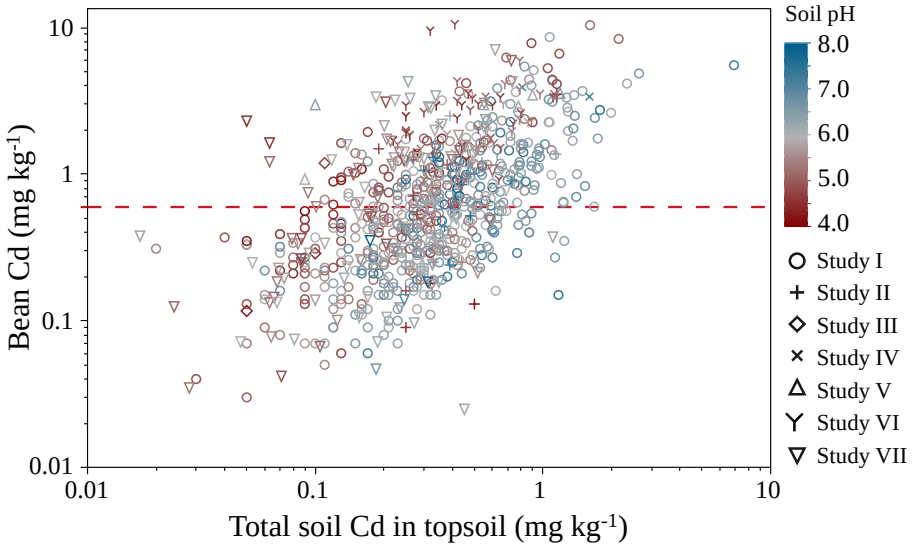


Figure 2.2 Cacao bean Cd concentrations as influenced by total soil Cd and soil pH for the selected studies in the meta-analysis. Soil pH values for all studies are standardised to pH_{CaCl2} (Kissel et al. 2009). The dashed line indicates the industry threshold of 0.60 mg Cd kg^{-1} for export of cacao beans to the EU. Details of the studies can be found in Appendix I.

Transfer factors (TF) indicate to what extent a crop accumulates Cd in its edible parts and are calculated as the ratio of the Cd concentration in the plant leaf or the edible part over the total Cd concentration of the soil. Average TFs for cacao (i.e. bean Cd over soil Cd) in the different studies range from 0.26 to 19 and are significantly affected by soil pH (Figure 2.3). Transfer factors for plants grown in acid soils are higher compared to TF for plants grown in more neutral or alkaline conditions, confirming the importance of soil pH for

plant Cd availability. The TF expression shows that average bean Cd will remain below the 0.60 mg Cd kg^{-1} industry threshold if soil Cd is below 0.32 mg kg^{-1} (soil pH 7.0 – 8.0), below 0.29 mg kg^{-1} (pH 6.0 – 7.0), below 0.19 mg kg^{-1} (pH 5.0 – 6.0) or below 0.10 mg kg^{-1} for the most acid soils (pH < 5.0). Transfer factors are also influenced by plant genetics and allow comparison of Cd accumulation among crops (see below).

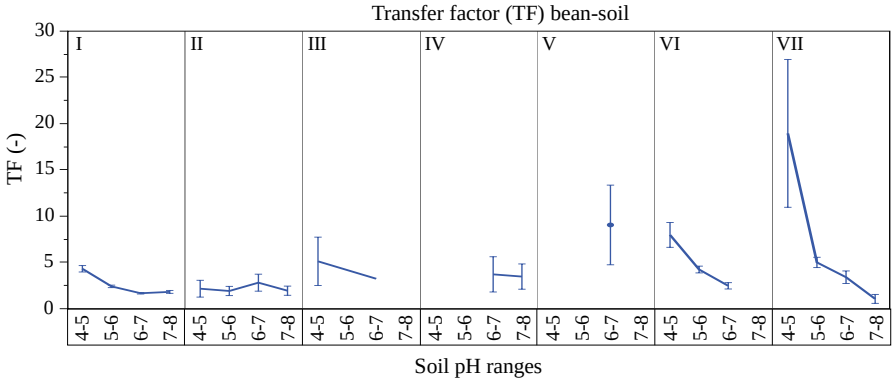


Figure 2.3 Average transfer factors (dry weight bean Cd concentrations divided by dry weight-based soil Cd concentrations, dimensionless) vs soil pH range per study (I – VII). Bars represent the standard error of the average. For study III, no error bars are given in pH range 6 – 7 as only one observation was available. Details of the studies can be found in Appendix I.

2.2.3. Agronomic factors affecting Cd uptake in cacao

Some researchers have argued that Cd uptake is affected by cacao management, e.g. monoculture vs agroforestry or organic vs conventional mineral fertilisation (Maddela et al. 2020). However, there is not enough information available to date to assess the effect of those agronomic practices on Cd in cacao, and the scarce existing information is not standardised among studies. Two studies investigated the effect of cropping systems (monoculture and agroforestry) on Cd uptake in cacao. Argüello et al. (2019) found no significant effect of cropping systems on bean Cd concentrations in their large national study in Ecuador. Gramlich et al. (2017) also found no such effect on bean Cd concentrations in a field trial but did find higher leaf Cd concentrations when cacao was grown as a monoculture compared to agroforestry. Gramlich et al. (2017) suggested that the higher plant density in agroforestry systems may be the reason for the lower leaf Cd concentration in cacao plants in this cropping system. Higher plant density could be translated to higher competition for nutrients, water and light, which may slow down the growth rate of cacao. The authors proposed that plants with a lower growth rate take up less Cd, because lower growth rate infers lower nutrient uptake and Cd is usually taken up as a hitchhiker element along with essential nutrients such as Zn.

The effect of fertiliser application, i.e. organic vs conventional fertilisers, on Cd uptake in cacao has not been widely studied. Three studies presented the effect of fertiliser application on Cd uptake with inconsistent results. On one hand, Gramlich et al. (2017) found no significant differences in soil and bean Cd concentrations between soils treated with either organic or conventional fertilisation, but this could be related to the low number of observations. On the other hand, Argüello et al. (2019) reported higher Cd

concentrations in cacao beans from trees that received organic compared conventional fertilisers, whereas Zug et al. (2019) found higher Cd concentrations in cacao beans from trees that received conventional N-fertilisation but no effect of P-fertilisation. Even though the results are contradictory, both situations could be plausible. First, compost is the most common source of nutrients in organic farming. As compost is primarily made with vegetal residues, the quality of the compost product, i.e. amount of trace metals, is directly related to the composition of the raw materials. The quality of compost and the high rates of application could result in higher Cd concentration in organically managed cacao as reported by Argüello et al. (2019). Second, increased N application has been mentioned to enhance Cd uptake and accumulation in plants (Yang et al. 2020). As N-fertilisation increases aboveground vegetative growth of plants, it stimulates active nutrient uptake and thus uptake of hitchhiker elements such as Cd will also increase. Additionally, N-fertilisation can lead to soil acidification and reduced soil pH can mobilise Cd, as explained above.

2.3. Uptake, translocation and partitioning of cadmium in the cacao tree

2.3.1. Soil-plant transfer of Cd in cacao compared to other plant species

Soil-plant transfer factors (TFs) allow identification of Cd accumulating characteristics, although they are influenced by genotype and external conditions such as soil Cd availability, as mentioned in section 2.2.2. Soil-plant TFs reported in cacao based on both leaves and beans are higher compared to TFs reported for most other crops (Table 2.3). The soil-leaf TFs for Cd in cacao are close to those reported for other woody species such as willow (Van Slycken et al. 2013) and poplar (Laureysens et al. 2004). The soil-bean TFs for cacao range between 2 and 6 depending on the study and are distinctly larger than TF reported for wheat and rice, but of similar magnitude as TFs reported for sunflower (Table 2.3). The Cd accumulation potential of the cacao plant is thus high, but not unusual. Cacao has been described as a Cd accumulator because of its high soil-plant TF, but this term lacks a clear definition. Several criteria indicate whether a plant can be classified as a Cd hyperaccumulator: (i) in natural conditions the plant should be able to accumulate ≥ 100 mg Cd kg^{-1}; (ii) both the TF and the internal shoot-to-root translocation factor ($ITF_{shoot-root} = Cd_{shoot}/Cd_{root}$) should be larger than one; and (iii) extreme metal tolerance should be achieved through efficient biochemical detoxification (van der Ent et al. 2013). While cacao does not meet the first criterion, TF > 1 have been reported (Table 2.3). The average Cd concentrations in the vegetative tissues of the cacao plant reported in different field studies are collated in Table 2.4, and these data suggest a relatively homogeneous Cd distribution between roots, scions and leaves. Engbersen et al. (2019) reported a similar $ITF_{stem-root}$ for cacao (0.99) compared to $ITF_{stem-root}$ reported for known moderately Cd accumulating woody species, i.e. $ITF_{stem-root} \approx 1$ in poplar and cotton (Vollenweider et al. 2011; Chen et al. 2015). To decide on the third criterion and to allow classification of cacao, additional research on the speciation of Cd in cacao tissues is crucial to elucidate the molecular detoxification mechanisms. Nonetheless, the Cd uptake potential of the cacao tree can be conceived as rather high compared to other agricultural crops, and more similar to woody species used for phytoextraction such as willow and poplar. Therefore, it is proposed to term cacao a moderate Cd accumulator.

It is unlikely that the accumulation of Cd in cacao results in Cd toxicity in the field. Different critical Cd toxicity levels have been proposed for various crops in mature leaves, e.g. 6 – 10 mg Cd kg^{-1} (Krämer 2010) or 5 – 30 mg Cd kg^{-1} (Kabata-Pendias 2010). Leaf Cd concentrations in cacao exceed 10 mg Cd kg^{-1} in only 3 % of the data included in the meta-analysis, suggesting that Cd toxicity to cacao plants is unlikely to occur at a large scale. There is only one study available to date discussing Cd toxicity in cacao seedlings (de Araújo et al. 2017). However, the soils in that study were spiked to high Cd concentrations (50 and 100 mg Cd kg^{-1} soil) and the reported leaf Cd concentrations (230 – 390 mg kg^{-1}) were not in the range of leaf Cd concentrations found in most cacao fields.

Table 2.3 Average cadmium transfer factors (TF) (min-max) for cacao and other selected agricultural crops, i.e. Cd concentration ratios between leaf or seed (e.g. cacao bean) and soil. The TFs predicted from soil-plant regression models used median values of the soil properties from the studies that yielded the corresponding models. Additional information regarding specific study conditions (e.g. study location and soil Cd concentrations) can be found in Appendix I (Table I.4).

TF calculated as concentration ratio						
Plant	**TF (leaf – soil)**		**TF (seed – soil)**		**n**	**Reference**
Cacao	4.5	(0.34–42.8)	1.6	(0.13–12.5)	560	Argüello et al. (2019)
	3.4	(0.56–20.1)	2.0	(0.26–7.8)	28	Barraza et al. (2017)
	4.9	(1.9–29.2)	5.6	(1.4–29.8)	60	Engbersen et al. (2019)
	7.1	(1.9–21.1)	2.7	(0.5–16.3)	108	Gramlich et al. (2018)
Willow		(1.6–10.3)			8	Van Slycken et al. (2013)
Oak	0.26[$]				2	Sevel et al. (2009)
Poplar		(5.0–40.0)			13	Laureysens et al. (2004)
Leafy vegetables	0.19	(0.001–4.5)			170	Zhang et al. (2014)
Sunflower			2.2	(1.8–3.4)	200	Li et al. (1995)
Cotton			0.46[$]		6	Chen et al. (2015)
Wheat	0.27	(0.06–0.64)	0.15	(0.03–0.34)	40	Puschenreiter and Horak (2000)
Rye	0.11	(0.03–0.29)	0.04	(0.01–0.16)	40	Puschenreiter and Horak (2000)
Pistachio			0.03	(0.01–0.04)	220	Shirani et al. (2018)
TF calculated from predicted crop Cd concentration model based on soil properties						
Plant			**TF (seed – soil)**		**n**	**Reference**
Cacao			1.76		334	This chapter
Indica rice			0.76		1043	Römkens et al. (2009)
Japonica rice			0.30		2155	Römkens et al. (2009)
Wheat			0.27		246	Adams et al. (2004)

[$]No range available, only average is given

2.3.2. Uptake and translocation of Cd within the cacao plant: trends and mechanisms

Cadmium is known as a hitchhiker element, using transporters for essential elements such as Zn, Fe, Mn and Ca at all steps of these nutrient pathways, from root uptake to grain or seed loading (Clemens and Ma 2016). Important transporter gene families for the uptake and translocation of Cd in plants are Zinc-Iron Permease (ZIP), Natural Resistance Associated Macrophage Proteins (NRAMPs) and Heavy Metal transporting ATPases (HMAs). These proteins transport divalent transition metals such as Fe(II), Zn, Mn and Cd. The limited information available to date regarding the role of these transporter genes for Cd uptake in cacao is discussed below. For a more profound synthesis about the genetic and molecular mechanisms of Cd uptake in plants in general, the reader is referred to specialised reviews by Clemens and Ma (2016) and Shahid et al. (2016). The identification of key genes involved in Cd uptake and translocation in cacao is of great importance as it can be used to develop mitigation strategies by using these genes as targets for genetic engineering, or through marker-assisted breeding programs.

2.3.2.1. Uptake of Cd in the cacao root system

Ullah et al. (2018) identified five genes from the NRAMP family in cacao and demonstrated that TcNRAMP5 can encode for a protein which transports Cd, Mn(II) and Fe(II). It has been reported that NRAMP5 plays a role in the entry of Cd in the root cells for rice (Ishikawa et al. 2012; Sasaki et al. 2012), barley (Wu et al. 2016), Polish wheat (Peng et al. 2018) and tobacco (Tang et al. 2017). More specifically, Ullah et al. (2018) showed that the TcNRAMP5 transcript was expressed in the roots of cacao seedlings. Usually, the expression of NRAMP transporter genes is up-regulated under divalent metal deficiency. In cacao seedlings, expression of the TcNRAMP5 transporter genes in the roots was down-regulated in the presence of Cd and up-regulated under Fe deficiency, while Zn or Mn deficiency did not affect gene expression. To link the expression pattern to its function, Ullah et al. (2018) cloned five TcNRAMP genes and expressed them in yeast strains. The yeast cells expressing TcNRAMP5 accumulated up to three times more Cd compared to the empty vector control cells. Considering the above, TcNRAMP5 likely plays a major role in the regulation of Cd uptake in cacao plants (Figure 2.4) and may be targeted for the development of mitigation strategies through induction of loss-of-function mutations. Yet, extrapolation of these findings to relevant cacao soil-plant systems is limited. First, activities used in the hydroponic experiment are about 100 times higher than natural soil Cd concentrations. The observed expression pattern may thus differ from natural field conditions. Second, uptake in yeast may not be suitable to predict uptake in cacao plants.

The isotopic signature of Cd in plants is increasingly used to infer biogeochemical processes but only few studies have reported Cd isotope fractionation data for cacao. Several processes can generate stable isotope fractionation during the transfer of Cd from soil to plant tissues, such as membrane transport or chemical binding. Moore et al. (2020) compared the isotopic shift of TcNRAMP5 transporter expressed yeast relative to the empty vector control cells ($\Delta^{114/110}Cd_{trans\text{-}ev} \approx -0.8$ ‰), with the isotopic shift in the cacao seedlings relative to the hydroponic solution ($\Delta^{114/110}Cd_{tot\text{-}sol} = -0.22 \pm 0.08$ ‰). Because both systems showed a shift toward lighter isotopes, and because the expressed TcNRAMP5 proteins were localised in the external plasma membrane of the yeast cells, the authors confirmed that TcNRAMP5 transporters can be a major pathway for Cd uptake

in cacao. However, these observations cannot exclude the possibility that other transporters can also be involved in Cd uptake. Compared to other species, the total isotopic fractionation due to absorption of Cd from hydroponic solutions is less pronounced in cacao seedlings, but it is similar to the isotopic shift reported for Cd tolerant and accumulator species (Wei et al. 2016). The reported fractionation towards lighter isotopes is similar to the fractionation from soil solution to plants in cereals and rice, although there is some variation in the rate of fractionation (Table 2.5). It is noteworthy that the cacao plants used in the hydroponic study of Moore et al. (2020) had leaf Cd concentrations of about 200 mg Cd kg^{-1} which far exceeds the Cd toxicity threshold of 10 mg Cd kg^{-1} and the relevant concentrations in the field (1 – 10 mg Cd kg^{-1}). This may have influenced the Cd uptake and translocation system due to activation of internal systems to cope with Cd toxicity.

Experiments with identification or induction of loss-of-function mutations in the TcNRAMP5 gene are indispensable to confirm the role of TcNRAMP5 for Cd uptake in cacao. Moreover, other proteins that were previously identified as major Cd transporters in other crops [e.g. AtIRT1 in *A. Thaliana*, and OsIRT1 and OsIRT2 in rice (McLaughlin et al. 2021)] should be studied, since information regarding the role of these transporters in cacao is not available to date. Knowledge on such transporters is important to predict potential effects of soil Zn concentrations on Cd uptake. Recent work with cacao seedlings showed that leaf Cd concentrations can be reduced by soil Zn addition (Souza dos Santos et al. 2020), but these effects were only observed in soils spiked to Cd concentrations well above the environmentally relevant range. Follow-up experiments with more realistic soil Cd concentrations are required.

2.3.2.2. Partitioning and translocation of Cd inside the cacao plant

From plant roots, Cd is generally transported radially across the root cells and loaded into the xylem, followed by long-distance transport via xylem and phloem and transfer into the plant organs (Clemens and Ma 2016). The rate of translocation of Cd from roots to above-ground tissues depends on vacuolar sequestration, xylem loading, and intervascular and xylem-to-phloem transfer (Figure 2.4). Cadmium can be sequestered in vacuoles during the radial transport through the root cells, as a protective measure to reduce Cd translocation to other tissues. A key transporter in this vacuolar sequestration is the Heavy Metal transporting ATPase 3 (HMA3) influx transporter, which is located at the membrane of vacuoles and which has been proven to control the sequestration of Cd in the roots of rice (Wang et al. 2019), soybean (Wang et al. 2012), and Chinese cabbage and pak choi (L. Zhang et al. 2019). For a discussion of the further translocation of Cd, loading in the xylem, root-to-shoot translocation and intervascular transfer in plants in general, the reader is referred to more specialised reviews by Clemens and Ma (2016) and McLaughlin et al. (2021). The role of the HMA family in Cd sequestration in cacao has not yet been determined to date. Moore et al. (2020) transformed yeast with HMA family transporters to compare the isotope fractionation pattern due to Cd sequestration in cacao seedlings. The data were, however, not conclusive on the specific role of HMA transporters in controlling Cd transport in cacao seedlings. In both rice and durum wheat, root-to-shoot Cd translocation via the xylem has been identified as a major determinant for shoot Cd accumulation (Harris and Taylor 2004; Uraguchi et al. 2009). These

observations highlight the need for studies focused on unravelling the mechanisms for Cd translocation from roots to aboveground tissues.

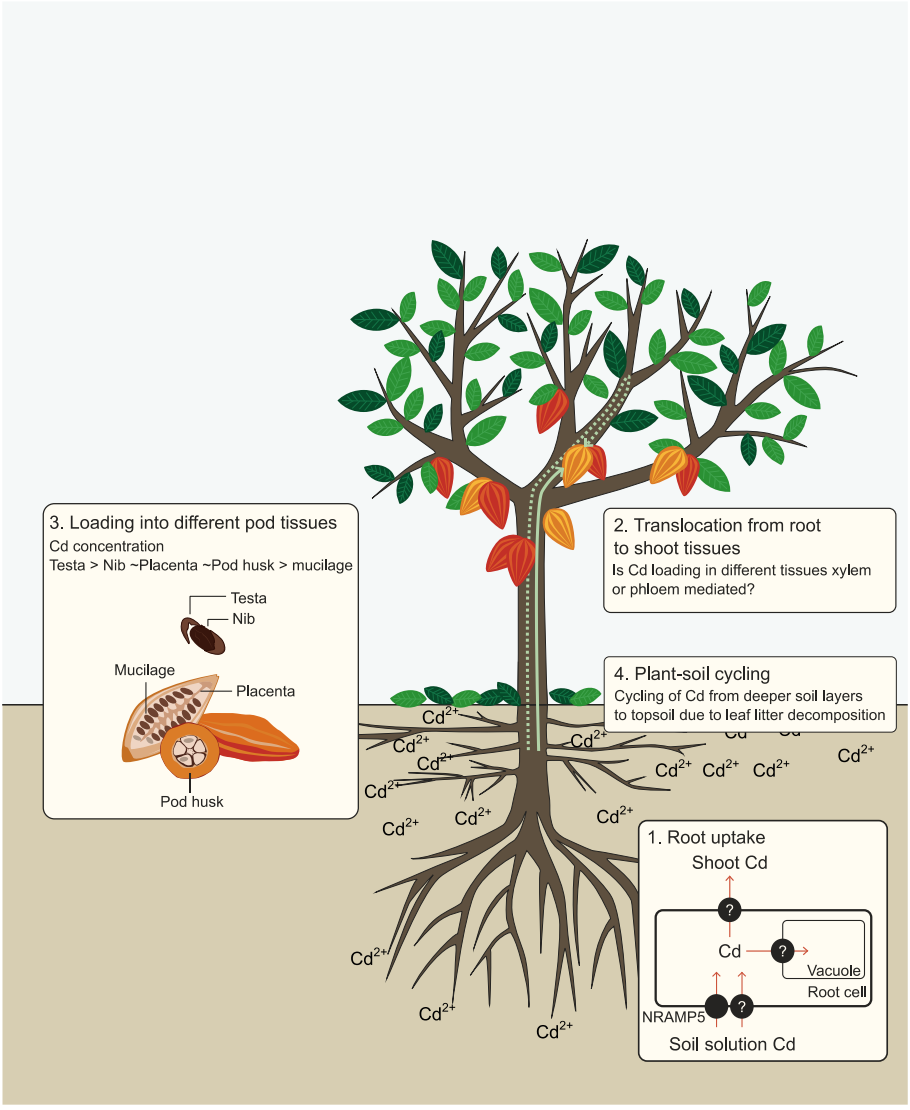


Figure 2.4 Uptake and translocation of Cd within the cacao plant-soil system. (1) Cadmium uptake in a simplified cacao root cell. TcNRAMP5 has been identified as an important transporter for Cd uptake from soil solution, but it is likely not the only transporter. Potential further pathways include sequestration in vacuoles and root-to-shoot translocation. (2) Translocation of Cd from root to cacao pod may occur through direct uptake from the xylem or through remobilisation of Cd from the leaves and transportation through the phloem. (3) Loading of Cd in the different pod tissues. (4) Cycling of Cd from deeper soil layers to the topsoil through leaf litter decomposition.

Table 2.4 Average (min-max) dry weight-based Cd concentrations (mg Cd kg^{-1}) for different cacao plant tissues, only including unfermented cacao beans. Dashes indicate that the parameter was not reported for this specific study.

	Tissue Cd (mg kg^{-1})					
Reference	**Root**	**Scion** [§]	**Leaf**	**Nib**	**Testa**	**Pod husk**
Argüello et al. (2019)	/	/	2.62 (0.13–55.5) n = 560	0.90 (0.03–10.4) n = 560	/	/
Barraza et al. (2017)	/	/	1.99 (0.19–7.9) n = 28	0.90 (0.09–3.5) n = 28	/	0.98 (0.08–4.4) n = 31
Engbersen et al. (2019)	2.44 (0.68–9.70) n = 60	2.25 (0.41–13.0) n = 60	2.31 (0.64–9.3) n = 60	2.56 (0.81–10.6) n = 60	/	/
Gramlich et al. (2018)	/	/	2.64 (0.06–28.0) n = 110	1.06 (0.03–7.1) n = 109	/	1.12 (0.04–10.2) n = 108
Lewis et al. (2018)	/	/	2.18 (0.48–5.2) n = 198	0.99 (0.17–2.3) n = 137	/	/
Ramtahal et al. (2016)	/	/	3.56 (0.45–17.4) n = 482	2.27 (0.48–9.3) n = 402	2.55 (0.33–15.4) n = 441	2.55 (0.46–8.0) n = 241

[§] Analysis of scion wood cores.

Cacao is a cauliflorous tree and mechanisms and pathways for Cd loading into the developing cacao beans thus may be different from other plant species. The relative importance of either xylem or phloem for Cd loading in cacao beans has not been revealed to date. Most studies report leaf Cd concentrations to be higher than corresponding bean Cd concentrations (Table 2.4; Figure 2.5). The data from the meta-analysis (section 2.2.2) shows a markedly positive correlation between bean and leaf Cd concentrations, with average ITF $_{\text{leaf-bean}}$ ranging from 1.1 to 4.2 among studies (Figure 2.5). This clear correlation may justify the use of leaf Cd concentration measurements as a proxy for bean Cd because leaf sampling is convenient as it does not depend on fruit availability. However, the use of leaf Cd measurements as a proxy for bean Cd may only be justified when considering a genetically homogeneous sample set, as both Engbersen et al. (2019) and Lewis et al. (2018) reported only moderate correlations between leaf and bean Cd concentrations in their genetically diverse studies.

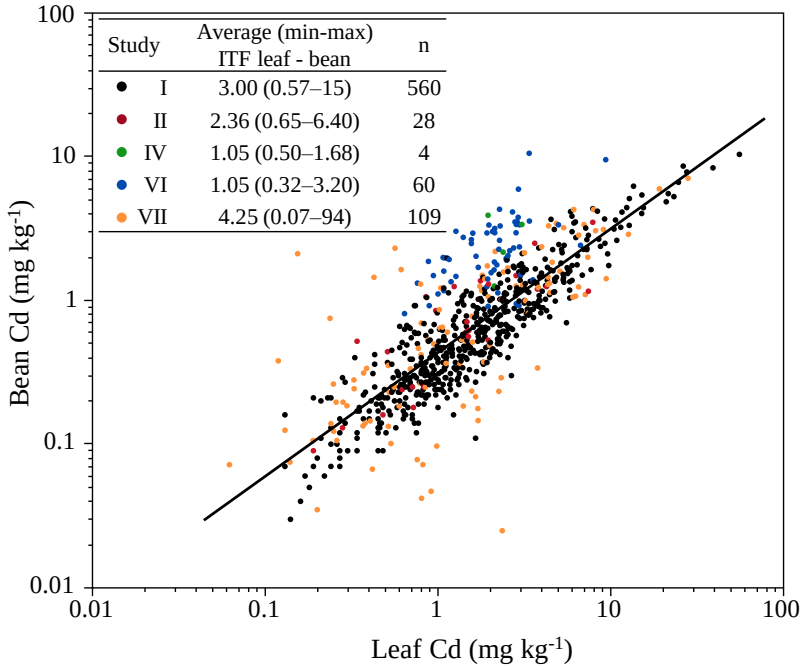


Figure 2.5 Cadmium concentrations in cacao leaves and beans are significantly correlated (Pearson correlation coefficient r = 0.82) and Cd concentrations are generally higher in leaves compared to beans. Studies III and V are not included as they did not include leaf Cd measurements. Details of the studies can be found in Appendix I. ITF =internal transfer factor calculated as the ratio of leaf Cd over bean Cd concentrations.

Another approach to study the translocation of Cd within plants, is to quantify isotope discrimination among different tissues (Table 2.5). In the hydroponic system of Moore et al. (2020), cacao leaves were strongly enriched in heavy Cd isotopes compared to the roots ($\Delta^{114/110}Cd_{\text{leaf-root}}$ = 0.26 ‰). Similar hydroponic studies with *Ricinus communis* and *Solanum nigrum* (Wei et al. 2016) showed a less systematic fractionation than reported in the cacao study of Moore et al. (2020). This may indicate that cacao seedlings exert mechanisms to cope with Cd that differ from the coping mechanisms found in Cd accumulator plants. A larger fractionation between roots and aboveground tissues may indicate that additional Cd retention mechanisms are invoked to inhibit the translocation of Cd to aerial plant parts, as discussed before. In most plants, Cd becomes enriched in heavy isotopes in the order roots < stem < leaf < seed (Table 2.5). The only study available on Cd isotope discrimination in mature cacao trees suggests that the transfer of Cd from stem to leaves and seeds in cacao is different compared to isotope discrimination studies in other species available thus far (Barraza et al. 2019). This pilot field study in Ecuador reported an average trend towards lighter isotopes in the cacao beans compared to the leaves ($\Delta^{114/110}Cd_{\text{bean-leaf}}$ = - 0.27 ‰). That different isotopic signature may be related to the cauliflorous character of cacao, and hints to the hypothesis that Cd is directly transferred from xylem to phloem in developing cacao beans, without first passing through the leaves.

2.3.2.3. Distribution of Cd within the cacao fruit

Inside the cacao fruit, reported average Cd concentrations are 0.90 – 2.56 mg kg^{-1} in the nibs, 0.99 – 2.55 mg kg^{-1} in the testae and 0.98 – 2.55 mg kg^{-1} in the pod husks (Table 2.4). There is no data available thus far regarding the Cd content of the mucilage. Lewis et al. (2018) studied the partitioning of Cd between testa and nib, and found that testa Cd concentrations were, on average, twice as large as nib Cd concentrations. However, the authors reported that the concentration ratio (testa over nib Cd) varied depending on the genotype, ranging from 1.2 to >7. The testa accounts for only a small part of the total bean weight and is thus likely also responsible for only a small fraction of total bean Cd, despite its elevated Cd concentration.

The outer pod husk has been reported to be a storage organ providing nutrients to the developing cacao beans through the mucilage (Toxopeus 1985), but its role in Cd transport to the cacao beans is not known thus far. Several studies have compared Cd concentrations between nibs and pod husks, and all reported similar Cd concentrations in both tissues, i.e. $ITF_{\text{husk-nib}}$ close to one (Ramtahal et al. 2016; Barraza et al. 2017; Gramlich et al. 2018). It has been reported that different nutrient distribution pathways exist between cacao pod husks and beans (De Araujo et al. 2020). Engbersen et al. (2019) stated that Cd partitioning within the cacao fruit may change during maturation of the fruit on the tree. Their results showed decreasing Cd concentrations in the pod husk and increasing Cd concentrations in the nibs with maturation, which indicates that there may be remobilisation of Cd occurring from the husk to the bean. However, additional research is required to corroborate this hypothesis, as the observed differences may have been related to the variation in Cd concentration among fruits on one tree, rather than to an effect of maturation.

Table 2.5 Average (min/max) or average ± stdev apparent Cd isotope fractionation ($\Delta^{114/110}Cd_{A-B} = \delta^{114/110}Cd_A - \delta^{114/110}Cd_B$) (‰) for different plants. Positive values indicate that tissue A is enriched in heavy isotopes compared to tissue B.

Plant	$\Delta^{114/110}Cd_{A-B}$(‰)							
	Plant-substrate	**Plant-soil**	**Shoot-root**	**Leaf-stem**	**Leaf-root**	**Grain-stem**	**Grain-leaf**	**Reference**
Hydroponics								
Ricinus communis	-0.38 [a] (-0.46/-0.32)		0.03 (-0.08/0.19) [$]	-0.11 (-0.18/-0.04)	-0.05 (-0.26/0.15)			Wei et al. (2016)
Solanum nigrum	-0.41 [a]		0.02[$]	0.09	0.11			Wei et al. (2016)
Cacao	-0.22 [a] (-0.34/0.01)				0.33 (0.13/0.9)			Moore et al. (2020)
Pot experiments								
Wheat	-0.07 [b] (-0.21/0.03)	0.23 (0.13/0.39)	0.29 (0.21-0.41)			0.30 (0.1/0.5) [$]		Wiggenhauser et al. (2016)
Wheat	(-0.36/-0.20) [c]	(0.31/0.46)	0.26 (0.19/0.35)			0.32 (0.16/0.46) [$]		Imseng et al. (2019)
Barley	(-0.1/-0.06) [c]	(0.51/0.55)	0.34 (0.27/0.45)			0.59 (0.44/0.82) [$]		Imseng et al. (2019)
Rice	-0.30 ± 0.01 [c]		0.16 ± 0.03					Wiggenhauser et al. (2020)
Rice	-0.43 ± 0.01 [c]		-0.02 ± 0.05					Wiggenhauser et al. (2020)
Rice			0.28	0.19[$$]	0.44[$$]		0.51	Wiggenhauser et al. (2021)
Field experiments								
Cacao	0.34 (0.22/0.41)						-0.27 (-0.40/-0.08)	Barraza et al. (2019)
Rice	0.45							Zhang et al. (2021)

[$]Shoot = stem; [$$]Leaf = flag-leaves; [a] Source: Hydroponic solution; [b] Source: $Ca(NO_3)_2$ soil extract; [c] Source: Soil solution

2.3.2.4. Cultivar-related differences in Cd uptake and partitioning

The cultivar effect on Cd uptake in cacao was first reported in 2018, with a factor 13 variation in bean Cd and a factor 7 variation in leaf Cd concentrations observed among cultivars grown on the field (Lewis et al. 2018). The data suggest a differential partitioning of Cd between vegetative (leaf) and reproductive (bean) tissues, favouring the hypothesis that there are cultivar-specific differences in xylem-to-phloem transfer of Cd to those different tissues. In their genetically diverse database, Engbersen et al. (2019) reported that available soil Cd was closely related to Cd in vegetative parts (R^2 = 0.50) but to a minor extent to bean Cd (R^2 = 0.26), which was attributed to a cultivar effect in the loading of Cd into the beans. The authors used this as indirect evidence suggesting that cultivar-related differences in bean Cd concentrations are mainly related to cultivar-specific differences in xylem-to-phloem transfer. While Cd transfer to the leaves is probably mostly governed by the xylem, the contributions of either the xylem or the phloem pathway for bean Cd loading are yet to be revealed and may differ among cultivars. However, the cacao trees in the study of Engbersen et al. (2019) were grafted onto rootstocks of unknown genetic identity, which may have affected the uptake of metals and the further translocation from the root, obscuring the cultivar effect. The design of that study also did not allow to account for potential effects of local spatial variability, which may have influenced Cd uptake and partitioning. Barraza et al. (2019) reported a genotype effect in the isotopic fractionation between cacao leaves and beans (Nacional: $\Delta^{114/110}Cd_{\text{bean-leaf}}$ = - 0.34 to - 0.40 ‰, CCN 51 hybrid: $\Delta^{114/110}Cd_{\text{bean-leaf}}$ = - 0.08 ‰). Similarly, distinctly different isotope fractionation patterns have also been reported between shoots and roots of an excluder ($\Delta^{114/110}Cd_{\text{shoot-root}}$ = 0.19 ‰) and a non-excluder type of rice ($\Delta^{114/110}Cd_{\text{shoot-root}}$ = -0.02 ‰) (Wiggenhauser et al. 2020). The consistent isotope fractionation between cacao leaves and soil reported by Barraza et al. (2019) is intriguing and indicates clear differences in the soil-bean fractionation among cultivars. This confirms the previous hypothesis that the translocation and sequestration of Cd in different cacao tissues varies among cultivars. This pilot study has some limitations due to the limited number of observations but confirms that the isotope approach can provide insights on Cd pathways in the cacao plant and pave the road for further studies in the field.

2.4. Mitigation strategies to lower the cadmium concentration in the final product

The current Cd-cacao regulations apply to the final product, not the cacao beans. Therefore, mitigation strategies can be applied at all stages of the production process, from tree to chocolate bar. Mitigation practices at different production steps will likely have to be combined to achieve a final product that complies with the new EU regulations. Mitigation practices can be based on soil amendments to reduce Cd uptake into the plant; plant-based strategies, i.e. selection of cultivars with reduced translocation of Cd to the cacao beans; or postharvest processing. Agronomic practices have also been suggested to influence Cd concentrations in cacao but additional research on their potential effects is required (see section 2.2.3).

2.4.1. Mitigating Cd uptake in cacao using soil amendments

While several soil amendment techniques exist, lime, biochar, gypsum and Zn supplementation are the only amendments discussed here, as these are the only amendments that have been or are being investigated for the mitigation of Cd in cacao. A reduction factor (RF), defined as the ratio between the crop Cd concentration in the control (no treatment) and the Cd concentration in the treatment was calculated to quantify the effect of each amendment. A RF > 1 indicates that crop Cd was reduced by the treatment.

2.4.1.1. Lime

The application of liming materials [i.e. $CaCO_3$, CaO, $Ca(OH)_2$ or $CaMg(CO_3)_2$] has often been recommended as an agronomic practice to reduce the uptake and accumulation of Cd in edible parts of crops. Liming decreases the solubility of soil Cd and thus decreases available soil Cd, by increasing the soil pH and through competition between Ca^{2+} and Cd^{2+} at root uptake sites (Christensen 1984; Bolan et al. 2003a). However, increasing the solution concentration of Ca^{2+} can also result in competitive desorption of surface bound Cd^{2+} (Christensen 1984). The latter is one of the plausible reasons why liming does not consistently decrease Cd concentrations in plant tissues (Bolan et al. 2003b). Significant reductions in crop Cd by the application of lime have been reported in field experiments with paddy rice in China: the application of 7.5 Mg ha^{-1} $CaCO_3$ led to RF 3 – 4, and this was sufficient to also have an effect on rice grain Cd grown in the 2nd year crop (H. Chen et al. 2018); while application of 1.5 Mg ha^{-1} burned lime (75 % CaO) resulted in a RF of 1.5 (Zhu et al. 2016). However, it is unclear if such high RFs can also be reached with lime application for cacao.

A recent study by Ramtahal et al. (2019) evaluated the effect of $Ca(OH)_2$ application at different rates (0 – 6 Mg ha^{-1}) on Cd uptake in cacao plants grown in pots (6 month old cuttings) and in the field (30 year old trees). Results from the pot trial showed effectively reduced leaf Cd concentrations 6 months after lime application, with a RF of 3. In the field trial, however, leaf Cd concentrations initially decreased by a factor 2 (measured 4 months after lime application), after which they started to increase in both control and limed treatments so that for the last sampling at 9 months after lime application, leaf Cd in the limed treatment was only reduced by a factor 1.1. Interestingly, soil pH remained at the target pH of 7.0 up until the 9th month after application. Similar contrasts in liming effects between field trials and pot experiments have been reported previously. Earlier work conducted in potatoes (Maier et al. 1996; Sparrow and Salardini 1997), sunflower kernels (Li et al. 1996) and peanuts (McLaughlin et al. 1997) suggested the difficulty in fully incorporating lime to deeper soil layers as an explanation for the lack of effect and/or limited effectiveness of liming in reducing crop Cd concentrations in the field. The lack of effect of liming in the cacao field trial may thus be related to Cd being taken up by deeper rooted soil horizons that were not affected by the lime treatment. In established plantations, lime incorporation to deeper soil horizons is not possible without damaging the root system, which may compromise the efficacy of the amendment. Argüello et al. (2020) showed in a pot experiment with cacao seedlings that liming the topsoil compartment only, as would be the case in field liming, enhanced Cd uptake from the non-limed bottom compartment.

2.4.1.2. Gypsum

The effectiveness of lime in reducing soil Cd availability to cacao is limited by its shallow penetration into the soil. Amendments such as gypsum may be able to overcome such limitations because gypsum can reach deeper soil layers. The mechanism by which gypsum reduces soil Cd availability is yet to be revealed; it is not a liming material and thus does not increase the soil pH. Nevertheless, studies have shown that adding gypsum as a soil amendment reduces Cd uptake in different crops: RF 1.9 with application of 3 % (w/w) in a pot trail with *Angelica gigas* (Kim et al. 2018); RF 2.5 with application of 0.15 g S kg^{-1} soil in a pool experiment with rice (D. Zhang et al. 2019); and RF 2 – 3 with application of 0.8 % (w/w) in a field trial with wheat and rice (Rehman et al. 2015). Based on the data available in other crops, gypsum might be a plausible amendment to ameliorate Cd accumulation in cacao beans. However, research is needed to reveal the potential of gypsum as a soil amendment for Cd mitigation in cacao.

2.4.1.3. Biochar

Biochar has received a lot of attention as an effective material for soil immobilisation of metals such as Cd. The sorption properties of this carbon rich material are related to its high specific surface area, high cation exchange capacity (CEC), its alkaline pH and the presence of carboxylic, hydroxyl or phenolic surface functional groups (Li et al. 2017). Because of the complex composition of biochar, the exact mechanism of metal sorption is not yet completely understood. However, results from laboratory experiments indicate that, at least in the short term, metal immobilisation is controlled by an increase in soil pH due to alkalinisation processes and by intra-particle diffusion of the elements within the biochar pores (Rees et al. 2014). The effectiveness of biochar application also depends on soil properties, the type of crop and the type of biochar used. According to the meta-analysis of D. Chen et al. (2018), positive effects in reducing plant Cd are expected when biochar is applied in soils with acid pH, coarse texture and intermediate organic carbon content.

In cacao, Ramtahal et al. (2019) evaluated the effect of a commercially available biochar (charcoal green®) on the phytoavailability of soil Cd in pot and field experiments. Biochar was applied at rates of 0, 326, 489, 652 kg ha^{-1} and the materials were incorporated at 20 cm depth. In the greenhouse study, biochar significantly increased the soil pH by about one pH unit and reduced the Cd concentration in the leaves of cacao seedlings, and this effect was dose dependent. Results from the field experiment with 30-year-old cacao trees showed that biochar did not increase the soil pH in contrast to the pot trial. Biochar applied at the highest dose in the field trials reduced leaf Cd by a factor 1.9 compared to the control, and the effect was more consistent over time than the effect of lime (last sampling done 6 months after biochar application). Results of studies with other crops are promising but the effectiveness of the treatment varies: RF 2 with application of 20 – 40 Mg ha^{-1} in a field study with rice and the effect of biochar application on grain Cd could still be observed three years after application to the soil (Bian et al. 2013 and 2014); and RF 1.2 determined one year after biochar application with doses up to 40 Mg ha^{-1} in a field study with wheat on alkaline soil, but this effect was no longer present or highly variable in the second and third year after application (Sui et al. 2018). Such high application rates of soil amendments would likely not be cost effective in cacao farming.

2.4.1.4. Fertiliser management: Zn supplementation

Although no studies in cacao to date have reported the effect of Zn fertilisation in reducing Cd uptake in the field, this topic is briefly described here because, based on information collected in other crops, the application of soil or foliar Zn can effectively reduce Cd concentrations in crops. The effect of Zn fertilisation on crop Cd depends on the status of Zn in the soil, with largest effects reported in Zn deficient soils: RF 2 with application of up to 5 kg Zn ha^{-1} in wheat grain (Oliver et al. 1994); RF 2 with application of 13 kg Zn ha^{-1} in rice (Fahad et al. 2015); and a linear decrease in Cd concentration in grain and shoots of yellow lupin with increasing Zn doses up to 6.4 kg Zn ha^{-1} (Brennan and Bolland 2014). It has been suggested that the effect of Zn application on plant Cd can be explained by competition between Zn and Cd at the root uptake sites, or that the application of Zn reduces the Zn deficiency of the plant, thereby preventing loss of integrity of the root cells which can facilitate non-selective Cd uptake through mass flow (Grant et al. 1999). In studies conducted in soils where soil Zn levels were not deficient, Zn fertilisation did not significantly affect the Cd concentration in wheat grain (Grant and Bailey 1998; Rojas-Cifuentes et al. 2012; Forster et al. 2018). An exception is the study on potatoes by McLaughlin et al. (1995), which showed that the addition of very large doses of Zn at planting (up to 100 kg Zn ha^{-1}) in non-deficient soils could significantly decrease Cd in the tuber (RF 2) in one of the studied sites.

2.4.1.5. Factors complicating the use of soil amendments to remediate Cd in cacao

Theobroma cacao L. is a large perennial tree and it is thus essential to test the efficacy of soil amendments in field conditions. The root system of the cacao tree is most dense in the near-surface soil (Nygren et al. 2013; Neither et al. 2019), which complicates effective incorporation of soil amendments. The poorly understood complexity of rhizosphere processes in the roots of cacao may also explain the lack of observed effect for some soil amendments and additional research is needed to better understand how cacao roots shape the local biochemical environment in the rhizosphere. In addition, high spatial variability is a major obstacle to detect effects of soil treatments on bean Cd concentrations in the field. A power analysis was performed based on the variation in bean Cd within fields reported by Argüello et al. (2019) (Appendix I). Beans from only 5 trees per treatment are required to identify a statistically significant effect of treatment if a reduction factor (RF) of 2 is expected and if the field has an average spatial variability. For fields with a realistic worst-case variability, that number increases to 15 trees per treatment. In contrast, an unfeasibly large number of 198 trees are required to identify significant effects if the treatments yield only a RF of 1.2 and if the variability is a realistic worst case. A RF of 1.5 can have large consequences on the economy of the cacao sector but it is clear that detection of such moderate but realistic RFs is challenged by soil and bean Cd variability within fields.

2.4.2. Genetics as a potential mitigation strategy for Cd in cacao

The research on the potential of genetic mitigation strategies, i.e. traditional genetic selection, plant breeding techniques, marker-assisted molecular breeding and/or genetic engineering, is still in an early phase as the genetic and molecular mechanisms for Cd uptake and partitioning in cacao are yet to be revealed. However, there are indications that genetics-based techniques could offer effective and sustainable strategies for Cd

mitigation in cacao. As discussed above, the work by Ullah et al. (2018) and Moore et al. (2020) identified TcNRAMP5 as a potentially important gene for Cd uptake in cacao, and thus as a potential target for genetic selection or modification. A recent greenhouse experiment with 53 wild and domesticated cacao genotypes proposed 11 cacao clones as low Cd accumulators which can be potentially exploited for future work (Arévalo-Hernández et al. 2020). However, soils in that study were spiked to extreme Cd concentrations (25 mg Cd kg^{-1}). A follow-up experiment with more environmentally relevant soil Cd concentrations is needed to truly confirm those cultivars as low Cd accumulators. Lewis et al. (2018) found significant differences in cacao bean and leaf Cd concentrations among cultivars grown in the field (up to a factor 13) and authors claimed that soil properties were homogeneous throughout the field, despite its large size (34 ha). Conversely, Engbersen et al. (2019) observed only a factor 2 – 3 difference in bean Cd among cultivars. The differences in bean Cd concentrations among cultivars observed in these studies might be related to other factors such as soil variability and other site conditions, e.g. specific growth dilution or differences in flushing and pod development cycles, rather than cultivar differences in Cd uptake and translocation. Future studies of the cultivar effect on Cd in cacao should evaluate multiple sites and consider large genotype-environment interactions.

The use of low Cd accumulating rootstocks for grafting, which is a common technique in cacao cultivation, may be promising to reduce Cd uptake in cacao but has not been studied to date. Grafting has been used to mitigate plant stress due to adverse soil chemical conditions in the root environment, e.g. heavy metal uptake and accumulation (Savvas et al. 2010). The role of the rootstock versus the scion in Cd accumulation in cacao beans is not yet known and this is a key knowledge gap for breeding. Rootstock effects on above-ground Cd concentrations appear to be species dependent. For example, Arao et al. (2008) demonstrated that grafting eggplants onto *Solanum torvum* rootstock reduced Cd concentrations in the eggplants by 63 − 74 %, compared to grafting onto *Solanum melongena* or *Solanum integrifolium*. In addition, the Cd concentration measured in the xylem sap was significantly lower in *S. torvum* compared to *S. melongena*. This suggests that *S. torvum* can limit translocation of Cd from the root to the shoot, by preventing Cd loading into the xylem. In contrast, studies conducted on potato showed that the rootstock regulates the total Cd uptake by the plant but that the scion regulates the distribution of Cd between the shoots and the tubers (Mengist et al. 2018). The use of cacao rootstocks with low Cd uptake/translocation potential could be the basis to renovate cultivars and eventually reduce Cd concentrations in cacao while maintaining the flavour of specific cultivars, which is likely under control of the scion.

2.4.3. Postharvest processing

A detailed overview of the different steps in the postharvest process of cacao is given in Chapter 1. There are four main approaches within the postharvest process that may affect the Cd concentration in the final cacao-derived product: testa removal, choice of product recipe, bean mixing and fermentation (Figure 2.6). First, optimal removal of the testa may reduce the Cd concentration in the final product, as the testa Cd concentration is approximately twice that of the nib [Table 2.4 and (Lee and Low 1985; Yanus et al. 2014; Ramtahal et al. 2016; Lewis et al. 2018)]. The testa is removed after roasting in a process

called *breaking and winnowing*, after which the nibs are ground to obtain cacao liquor (Chapter 1). However, testa removal is not complete and the intermediate product cacao liquor is allowed to contain up to 5 % (m/m) residual testa and/or germ (also referred to as radicle) on a fat-free dry weight basis or approximately 2.5 % on a total dry weight basis (Codex Alimentarius 2014). Considering that the testa comprises approximately 10 % of the dry weight of a cacao bean, complete testa removal can reduce the Cd concentration by a factor 1.16 from intact cacao beans to cacao liquor. This also indicates that cacao beans may be unnecessarily rejected by the industry because of elevated Cd concentrations if this decision is based on analysis of total bean Cd (nib and testa) instead of nib Cd.

Second, Cd is mostly partitioned in the non-fat cacao solids (Mounicou et al. 2003; Yanus et al. 2014; Kruszewski et al. 2018). Therefore, products made from the same cacao beans can have different Cd concentrations depending on the product type, i.e. Cd concentrations in white chocolate are negligibly small while fat-reduced cacao powder contains much higher Cd concentrations. Indeed, Cd concentrations are reported to be higher in cacao powders compared to cacao liquor and/or beans (Mounicou et al. 2003; Kruszewski et al. 2018). Considering this, it can be relevant for processing companies to take the intended final product into consideration when setting Cd requirements for their suppliers (Figure 2.6). In addition, the new EU Cd thresholds differ depending on the cacao solids content of the final product. To produce a typical milk chocolate with 35 % cacao solids (EU limit 0.30 mg Cd kg^{-1}), cacao beans can be used with nib Cd concentrations up to 0.86 mg Cd kg^{-1}, not considering effects of fermentation and/or testa removal. For dark chocolate with 70 % cacao solids (EU limit 0.80 mg Cd kg^{-1}), beans with nib Cd concentrations up to 1.14 mg Cd kg^{-1} could be used, which is nearly double the unofficial industry requirement of 0.60 mg Cd kg^{-1}. A webtool was recently created which allows farmers to easily calculate the acceptable bean Cd concentrations based on the cacao solids content of the intended product (https://www.chocosafe.org).

Third, it is common practice to mix cacao from different geographical sources during the production process. Considering the large geographical differences in cacao Cd concentrations (Table 2.1), beans from different origins can be mixed to ensure acceptable Cd concentrations in the final product, e.g. mixing cacao from West Africa which is generally reported to contain low Cd concentrations, with cacao from Central or South America where Cd concentrations are higher. This strategy can also offer viable solutions on a national scale, which is especially relevant for countries specialising in fine flavour origin chocolates. For example, based on the bean Cd concentrations measured by Argüello et al. (2019) in their nationwide study, and on the cacao production per province reported by the Ecuadorian government between 2014 and 2019 (Instituto Nacional de Estadística y Censos - Gobierno de La República del Ecuador 2019), the average bean Cd concentration per province exceeds the recommended value of 0.60 mg Cd kg^{-1} in 10 of the 23 Ecuadorian provinces. This corresponds to 32 000 metric tonnes or 15.5 % of the national production. The extensive database of Argüello et al. (2019) indicates an overall average bean Cd concentration of 0.90 mg Cd kg^{-1}, with 48 % of fields exceeding the guideline of 0.60 mg Cd kg^{-1}. Mixing all production at a national scale would result in an average bean Cd concentration of 0.59 mg kg^{-1} and the product would thus comply with

the Cd requirements (Table 2.6). Although mixing on a national scale is likely not practically feasible, there is potential for mixing strategies on smaller scales. The cacao market in many Central and South American countries is already set up in a way that would allow structural mixing of cacao from different sources, as larger cooperatives purchase the cacao from small scale farmers and then sell the (mixed) product to their clients.

Table 2.6 The effect of cacao mixing as a strategy to comply with the 0.60 mg Cd kg^{-1} threshold, using Ecuador as an example. The distribution of Cd concentrations without mixing is based on the national study of Argüello et al. (2019), which included 159 fields. The national and provincial estimates were calculated with spatial explicit data from that study and weighed by provincial production, details on the calculations can be found in Appendix I.

Produce is not mixed	Average cacao bean Cd concentration (mg kg^{-1})	Fields with average bean Cd > 0.60 mg kg^{-1} (%)
All included data (Argüello et al. 2019)	0.90	48
1 % of fields with largest average bean Cd concentration excluded	0.81	47
5 % of fields with largest average bean Cd concentration excluded	0.71	43
10 % of fields with largest average bean Cd concentration excluded	0.64	38
13 % of fields with largest average bean Cd concentration excluded	0.59	34
Produce is mixed at national level [£]	Average cacao bean Cd concentration (mg kg^{-1})	Production lost (%)
All provinces included	0.59	0
Excluding provinces with average > 1 mg Cd kg^{-1}	0.53	8
Excluding 10 provinces with highest bean Cd concentrations	0.51	15

[£] Average bean Cd concentrations per province estimated by combining data from the national agricultural land use map (Sistema de Informatión Pública Agropecuaria 2016) with the map of spatial distribution of Cd in cacao beans from Argüello et al. (2019). Average total annual production calculated based on the survey of continuous agricultural production and surface area (Instituto Nacional de Estadística y Censos - Gobierno de La República del Ecuador 2019). More detailed information on this calculation can be found in Appendix I.

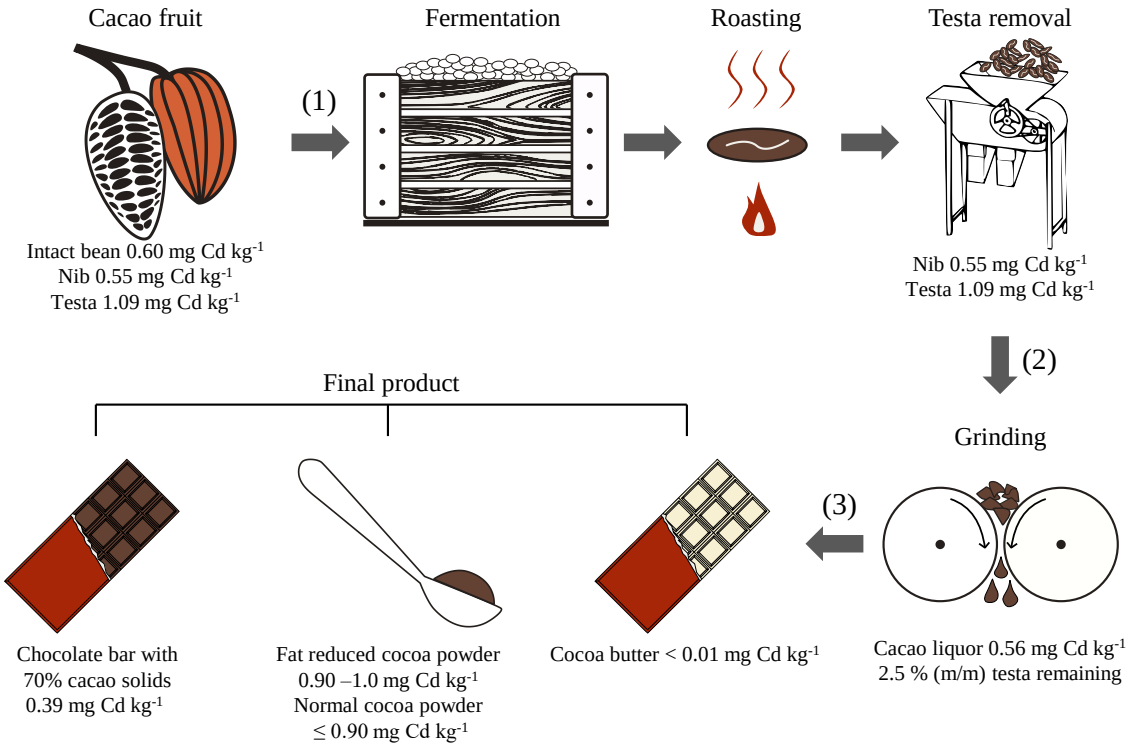


Figure 2.6 Effect of postharvest processing steps on Cd concentrations in (intermediate) cacao-derived products, considering an initial total bean Cd concentration of 0.60 mg Cd kg^{-1} (the unofficial industry threshold). Only the processing steps which can potentially reduce the Cd concentration in the final product are given: (1) the effect of fermentation is not yet revealed; (2) current regulations allow 2.5 % (m/m) testa material to remain in cacao liquor, more efficient testa removal may lower the Cd concentrations due to high testa Cd (i.e. in this case complete testa removal would result in cacao liquor with 0.55 mg Cd kg^{-1}); (3) Cd is contained in the non-fat cacao solids and thus Cd concentrations in the final product depend on product type.

Fourth, the potential impact of fermentation on Cd in cacao and the need for research on this topic has been indicated (Meter et al. 2019), but published research remains scarce to date. Some authors have reported Cd concentrations measured in unfermented beans and intermediate products after fermentation. For example, Barraza et al. (2017) measured Cd in unfermented unpeeled cacao beans (1.02 – 1.37 mg kg^{-1}) and in cacao liquor (1.47 – 3.88 mg kg^{-1}), i.e. suggesting some Cd enrichment after fermentation and processing. However, those samples did not originate from the same location and the observed difference in Cd concentrations was thus likely related to sample variation rather than a processing effect. Yanus et al. (2014) reported Cd concentrations in unfermented nibs (0.072 ± 0.001 mg kg^{-1}) and testae (0.085 ± 0.001 mg kg^{-1}), and in cacao powder (0.125 ± 0.011 mg kg^{-1}), again suggesting enrichment which could be related to fat removal during cacao powder production. However, it is again unclear to what extent this trend is related to variability of samples or to processing. During fermentation, the cacao beans are exposed to high temperatures and an acid environment (Chapter 1). Such conditions may mobilise Cd within the different cacao tissues, i.e. nib, testa or mucilage. During fermentation, the microbial community in the mucilage produces metabolites such as ethanol and acetic acid, which are known to penetrate the testa and thus migrate inward from the mucilage to the nib. While some information is available regarding the distribution of Cd between nib and testae, mucilage Cd concentrations have not been reported thus far. If the mucilage contains relevant Cd concentrations, some of this Cd may migrate inward with the metabolites and thus increase the nib Cd concentration. In contrast, polyphenols and methylxanthines have been reported to migrate outward from the nib to the testa and the mucilage, in the form of exudates. It is thus also possible that Cd migrates outward from the nib during fermentation, thereby reducing the nib Cd concentration. Targeted fermentation studies are required to test those hypotheses.

2.5. From chocolate bar to body burden

While different regulatory bodies are implementing limitations regarding the maximum allowed Cd concentration in chocolate and other cacao-derived products, controversy remains regarding tolerable intake levels, and regarding the relation between dietary intake and body burden. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) set a provisional tolerable monthly intake of 25 µg Cd kg^{-1} body weight (bw), which corresponds to a tolerable daily intake of 0.83 µg Cd kg^{-1} bw (FAO/WHO 2010). Conversely, the European Food Safety Authority (EFSA) set a stricter tolerable weekly intake of 2.5 µg Cd kg^{-1} bw or a daily tolerable intake of 0.36 µg Cd kg^{-1} bw (EFSA 2011). Children and infants have been identified as a high risk group within the population, due to differences in their diet compared to adults, and because they have a higher food consumption per unit body weight. However, as adverse effects due to dietary Cd intake are mostly related to accumulation in the human body over a lifetime of exposure, focussing on these young age groups may not be relevant. Although research has indicated that only a small part of dietary Cd is absorbed in the human body (European Chemicals Bureau 2007), regulations and health based guidelines are based on dietary Cd intake rather than on the Cd body burden. In contrast to the assumption in the risk assessment models, most dietary Cd studies show that the Cd body burden increases less than proportional with increasing dietary intake, and this is related to lower Cd bioavailability

and/or differences in micronutrient status with diet when relying on high Cd diets (Vahter et al. 1996; Reeves et al. 2001). Only limited information is currently available regarding the gastro-intestinal absorption rate of Cd from cacao-derived products and studies thus far have focussed only on *in vitro* experiments. Mounicou et al. (2002 and 2003) studied the *in vitro* bioaccessibility of Cd in cacao liquor and cacao powder and found accessible fractions ranging between 10 and 50 %. Conversely, Barraza et al. (2017) determined the gastric bioaccessibility of Cd using the unified method from the Bioaccessibility Research Group of Europe and reported a gastric bioaccessibility of > 90 % in cacao liquor from Ecuador. These studies have not highlighted if such fractions are lower than for other staple foods such as cereals. There is currently no chemical reason to expect lower Cd bioavailability and/or bioaccessibility in cacao compared to cereals. However, considering the strong influence of dietary status (e.g. dietary Zn, Fe and Ca) on the gastro-intestinal absorption of Cd (Reeves and Chaney 2008), it remains to be demonstrated if long term high chocolate consumption truly increases the Cd body burden to levels of concern. *In vivo* duplicate diet studies with increased dietary Cd through chocolate consumption would offer vital insights to assess the assumptions behind the existing and future food regulations.

2.6. Conclusions

Recent (and upcoming) regulations on the maximum allowed Cd concentrations in cacao-derived products are threatening cacao producers worldwide and are especially causing concern in Central and South America. Elevated soil Cd concentrations found in cacao production areas are likely related to geogenic rather than anthropogenic origin. Bean Cd concentrations are generally larger in cacao plants grown on acid soils with low soil organic carbon, when considering equal total soil Cd. Several mitigation strategies are being explored to deal with elevated Cd concentrations in cacao. However, mitigation strategies are not a cure-all and their use comes at a cost, either due to the financial cost of implementing the strategy or due to adverse effects on the final product quality. First, soil amendments can limit Cd plant uptake by reducing the phytoavailable soil concentration (e.g. biochar, lime or gypsum) or by saturating uptake mechanisms through which Cd can otherwise enter the plant (e.g. Zn fertilisation). Application of lime and biochar in cacao field trials has been reported to yield reduction factors (RF) up to 2, but as both treatments are based on a liming effect (i.e. increasing soil pH), these amendments are mostly effective in acid soils. While soil amendments could offer a readily applicable medium term solution, their incorporation is limited due to the dense rooting system of the perennial cacao tree. Second, root uptake and root-to-shoot translocation of Cd within the plant may be targeted for selection of low Cd accumulating cultivars. While the research on Cd uptake and translocation mechanisms in cacao is only in its early stages, genetics-based mitigation strategies can be highly valuable in the future. The available work on cacao indicates potential RF ranging from 2 to 13. However, to avoid current uncertainties related to the different soils under the cultivars, experiments on the same soil (i.e. same location, also termed common garden experiments) should be performed to identify low Cd accumulating cultivars and their potential RF. The use of specific rootstocks in grafting should also be explored as this may offer the benefit of genetics-based mitigation (selection of a low Cd uptake rootstock) while maintaining the flavour

profile of the original cultivar in the scion. There is a need to better characterise the pathways of Cd loading into cacao beans and to reveal the role of the xylem and phloem pathways. Third, postharvest mitigation strategies may offer the benefit of control for industrial scale cacao processers but have not yet been explored, and the effect of such strategies on the flavour quality of the final product should always be taken into consideration. On the short term, a realistic and easily implementable strategy is mixing of cacao from different origins (and thus with different bean Cd concentrations), both on larger and smaller scales. The intended final product should be kept in mind when setting Cd requirements, as current industry thresholds may be overly strict. For example, to produce dark chocolate with 70 % cacao solids, cacao beans with nib Cd concentrations up to 1.14 mg kg^{-1} could be used, which is almost double the currently common requirement of 0.60 mg Cd kg^{-1}. Finally, considering the indications that cacao is a moderate Cd accumulator, farmers may be recommended to change to a lower Cd accumulating crop if mitigation for cacao is not feasible.

Part 2
Experimental work

CHAPTER 3

The elemental composition of chocolates is related to cacao content and origin: a multi-element fingerprinting analysis of single origin chocolates

CHAPTER 4

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

CHAPTER 5

Cadmium migration from nib to testa during cacao fermentation is driven by nib acidification

CHAPTER 6

Outward migration of cadmium from cacao nibs during fermentation is related to the combination of enhanced acidity and temperature: mimicking fermentation through incubation

CHAPTER 7

Cacao nib washing prior to roasting effectively lowers nib cadmium concentrations: the first step towards nib washing as a mitigation strategy

Chapter 3
The elemental composition of chocolates is related to cacao content and origin: a multi-element fingerprinting analysis of single origin chocolates

This chapter is based on the following reference:
Vanderschueren R, Montalvo D, De Ketelaere B, Delcour J A, Smolders E. 2019. The elemental composition of chocolates is related to cacao content and origin: A multi-element fingerprinting analysis of single origin chocolates. *Journal of Food Composition and Analysis*. 83:103277. doi:10.1016/j.jfca.2019.103277

Summary
Commercially available single origin chocolates (n = 139) were analysed by ICP-MS to identify the potential of elemental fingerprinting for tracing cacao origin in chocolate, and to compare the chocolate composition relative to trace metal limits. Cadmium (Cd) concentrations exceeded the EU limit of 0.80 mg Cd kg^{-1} in 16 samples, all produced with cacao from South or Central America. Six samples contained lead (Pb) concentrations > 0.10 mg kg^{-1}, the limit of the Codex Alimentarius for edible fats. Increasing cacao content was associated with increased element concentrations for most elements, indicating cacao as the main source of minerals and trace elements. Significant differences in elemental composition between origins (P-value < 0.05) were found for Ba, Cd, Mo and Sr. Classification and Regression Tree (CART) analysis resulted in a decision tree that could effectively classify chocolate samples by cacao origin (overall misclassification rate 23 %) based on the concentrations of five elements (Ba, Cd, Mo, Sr and Zn). Samples of South America were classified based on their Cd concentration, indicating the geogenic origin of Cd.

3.1. Introduction

Chocolate is made from the seeds of the cacao tree (*Theobroma cacao* L.) through an extensive postharvest process that includes fermentation, drying and roasting. Cacao is a perennial crop grown at low altitude in tropical climates (within 20° latitude from the equator). The plant originates from Central and South America and was exported to other suitable geographical areas such as Africa in the 19th century (Chapter 1). Several health benefits have been attributed to moderate cacao consumption mainly due to its high polyphenol content, which has been related to cardiovascular health (Lee et al. 2003; Corti et al. 2009; Hooper et al. 2012). However, cacao-derived products may contain potentially toxic trace elements, such as cadmium (Cd), nickel (Ni) and lead (Pb).

In 2014, the EU Commission approved Cd limits in chocolate that have been in force since January 2019. These limits range from 0.10 mg Cd kg^{-1} in chocolates with < 30 % cacao solids, to 0.80 mg Cd kg^{-1} in chocolates with ≥ 50 % cacao solids (European Commission 2014). Recently, the Codex Alimentarius adopted similar limits for Cd in chocolate, ranging from 0.80 mg Cd kg^{-1} for chocolates that contain between 50 and 70% cacao solids, to 0.90 mg Cd kg^{-1} for chocolates with ≥ 70 % cacao solids (Codex Alimentarius Commission 2018). The European Food Safety Authority (EFSA) reviewed the exposure of the European population to Ni in food. This study concluded that chronic dietary exposure to Ni is of concern for the general European population and proposed a tolerable daily intake level of 2.8 µg Ni kg^{-1} bw (EFSA CONTAM Panel 2015). In addition, that review highlighted that cacao-derived products are among the main high Ni food sources, e.g. some types of chocolate (average 3.8 mg Ni kg^{-1}), and cacao beans and derived products (average 9.5 mg Ni kg^{-1}). This information prompted the European Commission to recommend that the member states monitor the Ni content of foods from 2016 until 2018 (European Commission 2016), which may result in limits for Ni in chocolate in the future. The EU regulation currently does not include explicit limits for Pb in chocolate, however the European Commission Regulation on maximum levels for contaminants in foodstuffs states that fats and oils, including cacao butter which is one of the main ingredients of chocolate, should not contain more than 0.10 mg Pb kg^{-1} (European Commission 2006). This limit of 0.10 mg Pb kg^{-1} is endorsed by the Codex Alimentarius for edible fats (Codex Alimentarius Commission 2017), and by the US Food and Drug Agency (US FDA) for candy destined for young children (US FDA 2006).

The presence of trace metals in chocolate can be influenced by several factors. The concentrations of different minerals in chocolate increase with increasing cacao content (Villa et al. 2014; Yanus et al. 2014; Abt et al. 2018; Lo Dico et al. 2018). This indicates that these elements mainly originate from the cacao beans, rather than from other ingredients or contamination during processing. The mineral content of cacao is affected by its geographical origin. Notable geographical differences in the elemental composition of cacao beans have been reported (Table 2.1). Chocolate is often made using a mix of cacao beans from different origins. However, single origin chocolates are becoming increasingly popular and are often marketed as luxury products, which raises interest in the influence of cacao origin on the quality and food safety of the final product. The composition of single origin chocolates allows to trace back the origin of the cacao, provided that the geogenic signal in the composition is strong and conserved. This has already been proven

for cacao beans: multi-element fingerprinting followed by principal component analysis (PCA) and discriminant analysis (DA) have been used to identify the geographical origin of cacao beans (Cambrai et al. 2010; Caligiani et al. 2014 and 2016; Diomande et al. 2015; Acierno et al. 2016 and 2017; Bertoldi et al. 2016; D'Souza et al. 2017; Kumari et al. 2018). Much less information is available for the similar analysis of the final product, i.e. chocolate. Junior et al. (2018) used DA based on elemental composition to differentiate between organic and conventional chocolates, while Bertoldi et al. (2016) used 13 chocolates as a validation set to test the efficiency of their DA model for the classification of cacao beans by geographical origin.

The present study was set up to assess the elemental composition of single origin chocolates and to identify the potential for tracing cacao origin by element fingerprinting of chocolates. This information may shed light on the impact of trace metal regulations on the cacao market as well as on the role of geographical origin. Classification and Regression Tree (CART) analysis is used to identify elements that most strongly characterise the cacao origin.

3.2. Materials and methods

3.2.1. Sample collection and preparation

Between February and May 2018, 139 single origin chocolates (not including white chocolates or chocolates with inclusions or noticeable food colouring) were purchased online and in commercial retail points from 47 different manufacturers in several countries (Belgium, n = 86; Colombia, n = 3; Ecuador, n = 25; France, n = 10; Italy, n = 3; Madagascar, n = 6; Mexico, n = 2; New Zealand, n = 2; Peru, n = 1 and Switzerland, n = 1) (Appendix II, Figure II.1). The samples were divided into four regions based on the cacao origin mentioned on the packaging: Africa (n = 34), Asia Pacific (n = 14), Central America (n = 22) and South America (n = 69) (Table 3.1). The chocolates were grated using a manual plastic grater and 100 mg of each sample was digested in 3.0 mL Suprapur® nitric acid (HNO_3, 65 % w/w; Merck, Kenilworth, NJ, USA) in an open digestion block for 11 hours at 130 °C. The obtained digests were brought to a volume of 10 mL and diluted five times with Milli-Q water (18.2 MΩ cm^{-1}), and the total elemental composition of macro (Ca, K, Mg, Na, P, S) and trace elements (Al, As, B, Ba, Be, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, Li, Mn, Mo, Ni, Sb, Se, Sn, Sr, Pb, Ti, Tl, U, V, Zn, Zr) was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA). Blank samples were included in quadruplicate in each digestion batch and treated in the same way as the chocolate samples.

3.2.2. Instrument calibration and sample analysis

Multi-element analysis was performed using an ICP-MS equipped with a collision cell, in the No Gas mode for ^{27}Al, ^{11}B, ^{137}Ba, ^{9}Be, ^{133}Cs, ^{115}In, ^{7}Li, ^{208}Pb, ^{121}Sb, ^{118}Sn, ^{205}Tl, ^{238}U and ^{90}Zr; in the Helium mode (5 mL He min^{-1}) for ^{75}As, ^{44}Ca, ^{111}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{56}Fe, ^{69}Ga, ^{39}K, ^{24}Mg, ^{55}Mn, ^{95}Mo, ^{23}Na, ^{60}Ni, ^{78}Se, ^{88}Sr, ^{47}Ti, ^{51}V and ^{66}Zn; and in the High Energy Helium mode (10 mL He min^{-1}) for ^{31}P and ^{34}S. An internal standard containing ^{72}Ge, ^{103}Rh and ^{193}Ir was added online to correct for measurement drift. Calibration for all elements was performed using Certipur® multi-element certified standard solutions (Merck). Measurements were performed in triplicate and instrument calibration was validated

using three certified reference solutions (Reference Material for Measurement of Elements in Surface Waters, Spectapure Standards, Oslo Norway; SRM 1643f Trace Elements in Water, National Institute of Standards and Technology, Maryland USA; and TM-28.4 Trace Elements in Water, Environment and Climate Change, Canada). All solutions for ICP-MS analyses were acidified to 1 % HNO_3 (v/v, Suprapur®, Merck). Measurements below the limit of quantification (LOQ) were reported as < LOQ. The LOQ is defined here as 10 times the standard deviation of the four procedural blanks, reconverted to a chocolate weight based concentration using equal sample weight to digest volume.

Table 3.1 Geographical origin and cacao content (%) of single origin chocolates (n = 139). The range of cacao contents of collected samples did not differ among the regions.

Region	n	Cacao content [a] (%) min-max (median)	Countries of origin (n)
Africa	34	32 – 100 (72)	Cameroon (1), D.R. Congo (4), Ivory Coast (1), Madagascar (19), Tanzania [b] (5), Uganda (4)
Asia Pacific	14	42 – 73 (68)	India (4), Indonesia (3), Papua New Guinea (1), Samoa (1), Vanuatu (2), Vietnam (3)
Central America	22	35 – 100 (71)	Belize (1), Costa Rica (5), Cuba (2), Dominican Republic (5), Grenada (2), Haiti (1), Mexico (2), Nicaragua (2), Trinidad (2)
South America	69	35 – 100 (70)	Bolivia (1), Brazil (3), Colombia (12), Ecuador (34), Peru (16), Venezuela (3)

[a] From package information

[b] One sample from Tanzania (70% cacao solids) was considered an outlier and excluded from statistical analysis due to its extremely high Cr concentration (24 mg Cr kg^{-1}).

3.2.3. Quality assurance

Two chocolate certified reference materials were included in triplicate in each digestion batch as quality assurance; NIST 2384 baking chocolate certified for Ca, Cd, Cu, Fe, K, Mg, Mn, P, Pb and Zn; and ERM®-BD512 dark chocolate certified for Cd, Cu, Mn and Ni. Iron was excluded from further analysis due to poor recovery of the reference materials. For all other elements, recoveries ranged between 79 % and 102 % (Appendix II, Table II.1). To verify reproducibility, duplicate samples were included at intervals of 15 samples. The coefficient of variation (CV) was calculated for these duplicates (13 duplicate sets) and ranged between 1 and 23 % depending on the element (overall average CV = 6 %). Gallium was excluded as a strong correlation was found with Ba (R^2 = 0.99), likely caused by isobaric interference with the Ba^{2+} ion which is also detected at m/z = 69.

3.2.4. Statistical analysis

All statistical analysis was executed using JMP® Pro version 14.0.0 (SAS Institute 2018). Elements for which more than 15 % of the data points were below LOQ (Al 19 %, As 100 %, Be 96 %, In 90 %, Li 81 %, Na 84 %, Pb 60 %, Sb 86 %, Se 41 %, Sn 98 %, Ti 38 %, Tl 59 %, U 81 % and Zr 91 %) were excluded from statistical analysis, however the raw data are given in Appendix II (Table II.2). Statistical analyses were thus performed using 18 elements (B, Ba, Ca, Cd, Co, Cr, Cs, Cu, K, Mg, Mn, Mo, Ni, P, S, Sr, V and Zn). Measurements below the LOQ were replaced by LOQ/2 and concentrations were log

transformed prior to statistical analysis to ensure normal distribution of the data, except for Classification and Regression Tree (CART) analysis as this technique does not require normal distribution of the data.

A one factor Analysis of Variance (ANOVA) was used to investigate the effect of different origins on the 18 elements considered (Kutner et al. 2005). After a positive omnibus F-test, a post-hoc Tukey's Honestly Significant Difference (HSD) test was used to identify significant differences between the average elemental concentrations in chocolates from the different origins (P-value < 0.05). This analysis only allows to consider each of the 18 elements separately and does not give insight in the overall differences in elemental composition. In order to overcome this limitation, CART analysis was performed to provide a combined analysis that allows for an appealing visualisation of the differences amongst the regions (Breiman et al. 1984). The CART method is a non-parametric technique that identifies variables with the power to classify samples (in this case according to the cacao origin) and uses these variables to build a decision tree. Each node in the decision tree corresponds to a conditional IF-statement in the model and splits the dataset in two subgroups based on the cut-off value of a single variable. Samples that are higher than the cut-off value for this variable belong in one subgroup and samples with lower values belong in the other. Splits and cut-off values are chosen so that the difference in the average response (in this case the origin) between the subgroups is maximised, consecutive splits thus yield subgroups that are more homogeneous with regard to cacao origin. At each node and leaf of the decision tree, the model calculates probabilities for each category. Although this was not the primary goal of this work, these probabilities can then be used to predict the classification of unknown samples. The cacao content differed strongly between chocolate samples (Table 3.1) and as stated in the introduction, the elemental composition of chocolates is strongly influenced by their cacao content. Therefore, CART analysis was applied to the residuals of a linear regression of the considered element concentrations against the cacao content of the samples. This correction mimics the real-life situation in which the origin of a chocolate sample is to be determined given its known cacao content. Fivefold cross-validation was used to prevent over fitting of the CART model. In fivefold cross-validation, the dataset is randomly split in five subsets, also referred to as folds, and each fold is consecutively used as a validation dataset for the remainder of the data (the other four folds combined). Splitting of the CART tree is stopped when the addition of another split no longer improves the cross-validation R^2, i.e. the average R^2 of all five folds.

3.3. Results and discussion

3.3.1. The elemental composition of chocolates and its relation to origin and cacao content

The 139 samples originated from 27 countries and were split in four regions (Table 3.1). The fractions of collected samples for each region were 24 % (Africa), 10 % (Asia Pacific), 16 % (Central America) and 50 % (South America). Globally, cacao production is highest in Africa (73 %), followed by Central and South America (together 17 %) and Asia Pacific (10 %) [based on cacao production between October 2015 and September 2016 (ICCO 2018)]. This indicates a sample bias towards South America. However, that bias was likely related to the single origin market as South American cacao is renowned and respected

for its high quality. For example, the Nacional cultivar native to Ecuador is known for its aromatic flavour (Afoakwa 2010). The average cacao content (%) in the collected samples was unaffected by origin (P-value > 0.05).

Median, minimum and maximum concentrations of all measured elements can be found in Appendix II (Table II.2). One sample (Tanzania, 70 % cacao solids) was considered an outlier and excluded from analysis due to its extremely high Cr concentration (24 mg Cr kg^{-1}). Of all elements, the macro elements Ca (median 720 mg kg^{-1}), K (median 6437 mg kg^{-1}), Mg (median 1887 mg kg^{-1}), P (median 2748 mg kg^{-1}) and S (median 925 mg kg^{-1}) were present in chocolate in the largest concentrations, with the exception of Na which was below LOQ in 84 % of the samples. The most prevalent micro element was Zn (median 27 mg kg^{-1}), followed by Mn (median 14 mg kg^{-1}) and Cu (median 13 mg kg^{-1}). Concentrations of Al, As, Be, In, Li, Na, Pb, Sb, Se, Sn, Ti, Tl, U and Zr were below the LOQ in more than 15 % of the samples and these elements were excluded from statistical analysis.

Single correlation analyses between element concentrations and cacao content of the chocolates were significant for all analysed elements (Pearson's correlation coefficient, P-value < 0.05), except for Cr and V (Table 3.2), suggesting that cacao is the main origin of these minerals in chocolate. Calcium was the only element that negatively correlated to cacao content, which can be explained by the presence of Ca in milk powder used in the preparation of milk chocolates. Since most minerals originate from the cacao, it might be argued to normalise all data for cacao content (%) to identify the geogenic signature of, for example, 100 % cacao solids. However, single correlations between normalised element concentrations (to 100 % cacao solids) and cacao content were lower, but still significant (P-value < 0.05) for B (r = 0.54), Mg (r = 0.60), Mn (r = 0.49), Co (r = 0.17), Ni (r = 0.31), Cu (r = 0.45), Sr (r = 0.30) and Cd (r = 0.24). In the sections below, we first discuss the elemental concentration without normalisation since metal limits are expressed on the final product and since the cacao contents in the samples were similar, i.e. the average cacao content (%) was unaffected by origin. For CART analysis however, data were corrected for cacao content as explained in the materials and methods section. The average elemental concentrations for each region are reported in Table 3.3. The average concentrations of Ba, Cd, Mo and Sr differed significantly between regions (P-value < 0.05). No significant differences were observed in the macro element (Ca, K, Mg, P and S) concentrations between the different regions.

3.3.1. Presence of potentially toxic trace elements in chocolate and their relation to cacao content and origin

Arsenic concentrations were below the LOQ (0.08 mg As kg^{-1}) in all samples, which is in accordance with the results reported previously by Lo Dico et al. (2018). No regulations exist so far regarding the As concentrations in chocolates, but measured values were below the lowest threshold value for As in rice, i.e. 0.10 mg As kg^{-1} for rice for the production of food for infants or young children (European Commission 2015).

Table 3.2 Pearson's correlation coefficients (r) between log transformed element concentrations and cacao content (%) in single origin chocolates.

	Correlation coefficients				
	All regions	Africa (n = 33)	Asia Pacific (n = 14)	Central America (n = 22)	South America (n = 69)
B	0.84*	0.87*	0.85*	0.92*	0.81*
Ba	0.68*	0.87*	0.60*	0.46*	0.65*
Ca	-0.34*	-0.30	-0.84*	-0.43*	-0.21
Cd	0.45*	0.75*	0.53	0.76*	0.31*
Co	0.54*	0.64*	0.65*	0.33	0.57*
Cr	0.11	0.04	0.06	0.20	0.13
Cs	0.24*	0.26	0.36	0.17	0.30*
Cu	0.79*	0.78*	0.91*	0.75*	0.95*
K	0.79*	0.87*	0.76*	0.72*	0.78*
Ni	0.55*	0.76*	0.56*	0.74*	0.36*
Mg	0.90*	0.92*	0.93*	0.93*	0.87*
Mn	0.77*	0.87*	0.80*	0.72*	0.74*
Mo	0.25*	0.56*	-0.30	0.18	0.25*
P	0.73*	0.81*	0.51	0.76*	0.74*
S	0.76*	0.82*	0.58*	0.86*	0.78*
Sr	0.71*	0.91*	0.53	0.48*	0.67*
V	0.12	0.06	0.22	0.50*	0.08
Zn	0.85*	0.93*	0.79*	0.84*	0.86*

* significant correlation (P-value < 0.05)

Nickel concentrations ranged between 0.30 and 12.5 mg Ni kg^{-1}, which is in accordance to values reported in literature by Bertoldi et al. (2016) (3.3 to 4.7 mg Ni kg^{-1} depending on the origin of the chocolates), Junior et al. (2018) (on average 2.6 and 2.8 mg Ni kg^{-1} in organic and conventional chocolates respectively), Mrmošanin et al. (2018) (between 1.90 to 5.90 mg Ni kg^{-1} in ten chocolates purchased in Serbia) and Dahiya et al. (2005) (on average 2.76 mg Ni kg^{-1} in cacao based candy). Nickel concentrations were strongly correlated with cacao content (r = 0.55), indicating that at least some part originates from the raw material (Table 3.2). This observation was previously corroborated by the European Food Safety Authority which reported on average higher Ni concentrations in cacao beans and derived products (9.5 mg Ni kg^{-1}) compared to the final chocolate products (3.8 mg Ni kg^{-1}) (EFSA CONTAM Panel 2015).

The Pb limit of 0.10 mg kg^{-1} as referred to in the introduction, was exceeded in six chocolate samples originating from India (0.11 mg kg^{-1} purchased in Belgium), Trinidad (0.12 mg kg^{-1} purchased in Belgium), Peru (0.68 mg kg^{-1} purchased in Belgium) and Ecuador (0.17 and 0.36 mg kg^{-1} purchased in Ecuador and 0.10 mg kg^{-1} purchased in Belgium). In practice, however, regulatory agencies take the measurement error in consideration when assessing whether samples exceed the limit. The average CV for

duplicate Pb measurements was 23 % (n = 6 as all other duplicate measurements were < LOQ). Considering an error equal to twice the CV, samples exceed the limit only if their measured Pb concentration surpasses 0.15 mg Pb kg^{-1}, which was true for only three samples. Values for Pb were below LOQ (0.02 mg Pb kg^{-1}) in 60 % of the samples and the median concentrations per region were also < LOQ (Appendix II, Table II.2). To test whether there was a relationship between the Pb concentrations in the chocolates and their cacao content and/or origin, a categorical variable with two values (> LOQ and < LOQ) was used. No significant relationship (X^2 statistic, $\alpha = 0.05$) was found between the proportions of detectable Pb concentrations and either cacao content or origin. This indicates that Pb in chocolates likely originates from the postharvest process rather than the raw material, i.e. the cacao beans. For example, open air drying of fermented cacao beans in industrial areas may contribute to the Pb concentration in the final product. The measured Pb concentrations were lower than those mentioned by Mrmošanin et al. (2018) (0.25 to 0.86 mg Pb kg^{-1}) but higher than the values reported by Bertoldi et al. (2016) (0.008 to 0.024 mg Pb kg^{-1}). Abt et al. (2018) found significant differences in the average Pb concentrations measured in milk chocolate (0.01 mg Pb kg^{-1}) compared to those in dark chocolate (0.03 mg Pb kg^{-1}) and cacao powder (0.11 mg Pb kg^{-1}) purchased on the US market. However, their results showed much lower Pb concentrations in the raw product (0.003 mg Pb kg^{-1} in cacao nibs), which again suggests that Pb in chocolate originates from the production process rather than the raw material. In contrast, Villa et al. (2014) did find a significant correlation between cacao content and Pb concentration in 30 chocolates purchased in Brazil.

From the total number of samples collected for this study, 16 chocolates (all > 50 % cacao solids) exceeded the new EU regulation limit for Cd, i.e. 0.80 mg Cd kg^{-1} for chocolate with ≥ 50 % cocoa solids (Figure 3.1). A correlation was found between cacao content and Cd concentration (r = 0.45, Table 3.2). This observation is in line with reports in literature (Villa et al. 2014; Abt et al. 2018; Lo Dico et al. 2018). Abt et al. (2018) found on average higher Cd concentrations in cacao nibs (0.62 mg Cd kg^{-1}) and cacao powder (0.70 mg Cd kg^{-1}) compared to dark chocolate (0.27 mg Cd kg^{-1}) and milk chocolate (0.06 mg Cd kg^{-1}). Likewise, Lo Dico et al. (2018) reported median Cd concentrations increasing from chocolate with < 30 % cacao solids (< LOQ) to chocolate with 30 to 50 % cacao solids (0.019 mg Cd kg^{-1}), chocolate with > 50 % cacao solids (0.116 mg Cd kg^{-1}) and finally cacao powder (0.159 mg Cd kg^{-1}); and Villa et al. (2014) found a linear relationship between the cacao content and the Cd concentration measured in chocolates from Brazil.

Table 3.3 Average concentrations (± stdev) of 18 elements measured in chocolate bars from different origins. Superscript letters indicate significant differences in the elemental concentrations between regions, levels which are not connected by the same letter are significantly different (Tukey HSD test, P-value < 0.05, ns = no significant effect). Other elements were below LOQ in over 15 % of the samples and are not shown.

	Average concentration ± stdev (mg kg^{-1})				
	LOQ	Africa (n = 33)	Asia Pacific (n = 14)	Central America (n = 22)	South America (n = 69)
B	0.45	8.0 ± 3.0 ^{ns}	7.1 ± 2.6	8.7 ± 2.4	7.9 ± 2.2
Ba	2.0	8.5 ± 5.2 ^{A}	3.9 ± 2.7 ^{B}	3.7 ± 2.1 ^{B}	4.9 ± 3.3 ^{B}
Ca	26	1030 ± 560 ^{ns}	1130 ± 640	810 ± 400	830 ± 460
Cd	0.01	0.20 ± 0.11 ^{BC}	0.12 ± 0.07 ^{C}	0.33 ± 0.30 ^{AB}	0.58 ± 0.52 ^{A}
Co	0.01	0.32 ± 0.14 ^{ns}	0.39 ± 0.32	0.31 ± 0.13	0.35 ± 0.18
Cr	0.04	0.65 ± 0.75 ^{ns}	0.44 ± 0.38	0.52 ± 0.36	0.58 ± 1.1
Cs	0.001	0.016 ± 0.013 ^{ns}	0.022 ± 0.017	0.020 ± 0.013	0.021 ± 0.031
Cu	0.07	11.8 ± 4.0 ^{ns}	11.4 ± 4.2	13.2 ± 3.8	13.0 ± 4.2
K	8.9	7100 ± 1990 ^{ns}	6360 ± 1170	7030 ± 1950	6330 ± 1550
Mg	1.6	1960 ± 650 ^{ns}	1640 ± 475	1840 ± 499	1870 ± 511
Mn	0.03	17.4 ± 7.6 ^{ns}	16.3 ± 11.9	14.1 ± 4.4	13.6 ± 5.9
Mo	0.01	0.17 ± 0.07 ^{B}	0.10 ± 0.05 ^{C}	0.10 ± 0.04 ^{C}	0.24 ± 0.10 ^{A}
Ni	0.16	3.7 ± 1.7 ^{ns}	3.3 ± 2.2	4.6 ± 2.8	3.2 ± 1.8
P	0.76	2920 ± 595 ^{ns}	2670 ± 296	2600 ± 435	2870 ± 654
S	58	960 ± 170 ^{ns}	920 ± 90	930 ± 140	900 ± 190
Sr	0.04	7.5 ± 2.9 ^{A}	5.0 ± 2.1 ^{B}	5.6 ± 2.2 ^{AB}	6.3 ± 3.5 ^{AB}
V	0.007	0.064 ± 0.066 ^{ns}	0.052 ± 0.051	0.056 ± 0.034	0.041 ± 0.036
Zn	1.2	27 ± 7 ^{ns}	23 ± 6	26 ± 6	29 ± 8

From the chocolates collected for this study, samples originating from South America contained significantly higher Cd concentrations compared to chocolates from Africa and Asia Pacific (Table 3.3) and the chocolates that exceeded the new EU limits were all from Central and South America (Figure 3.1): Haiti (1.44 mg Cd kg^{-1}, purchased in France); Peru (1.07 and 1.34 mg Cd kg^{-1} purchased in Belgium and 0.81 mg Cd kg^{-1} purchased in Peru); Colombia (1.68 mg Cd kg^{-1} purchased in Italy; 0.94 and 0.97 mg Cd kg^{-1} purchased in Colombia; 1.22, 2.30 and 2.61 mg Cd kg^{-1} purchased in Belgium) and Ecuador (0.81, 0.91, 0.93, 1.66 and 2.00 mg Cd kg^{-1} purchased in Ecuador and 0.85 mg Cd kg^{-1} purchased in Belgium). However, considering the measurement error (i.e. two times the average CV for duplicate Cd analyses which was 2 %, n = 13), regulatory agencies will only reject samples if their measured Cd concentration exceeds 0.83 mg Cd kg^{-1}, which was the case for 14 samples. The concentration range for Cd was much larger in the South American sample set (from 0.02 up to 2.61 mg Cd kg^{-1}) compared to that from other origins (Figure 3.1 and Appendix II, Table II.2). This supports the hypothesis of Cd hotspot areas rather than a homogeneous geographical presence, where elevated Cd uptake by the cacao plant and

consecutive accumulation in the cacao beans can yield high Cd chocolates. In a recent study conducted in the cacao growing areas in Ecuador, Argüello et al. (2019) reported spatial variability on Cd concentrations in cacao beans (39 %) and cacao cultivating soils (32 %) within a large number of sampled fields, and identified clear cacao bean Cd hotspot areas (of geogenic origin) based on prediction maps. Both the measured concentrations and geographical differences observed in the present study are in accordance to findings reported in literature for unprocessed and processed cacao. For example, Bertoldi et al. (2016) reported Cd concentrations ranging from 0.09 to 1.39 mg Cd kg^{-1} in cacao beans and 0.14 to 0.62 mg Cd kg^{-1} in chocolates from different origins. They found that Cd concentrations in cacao beans from South America were on average four times higher than those in cacao beans from Asia, and more than ten times higher than cacao beans from West Africa. Mounicou et al. (2003) also reported higher average Cd concentrations in cacao powders from Venezuela and different provinces in Ecuador (0.533 to 1.833 mg Cd kg^{-1}) compared to Ivory Coast (0.094 mg Cd kg^{-1}) and Ghana (0.133 mg Cd kg^{-1}).

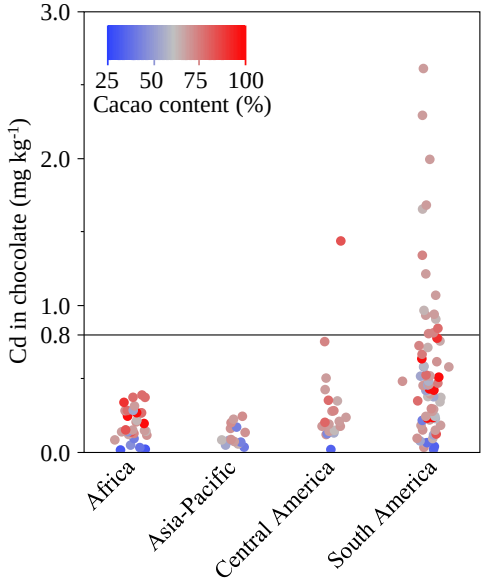


Figure 3.1 Cadmium concentrations in chocolate classified by region of origin and cacao content (%). The horizontal line represents the EU limit for Cd in chocolates with > 50 % cacao content (0.80 mg Cd kg^{-1}) (European Commission 2014). Sixteen samples from South America (n = 15) and Central America (n = 1) exceed this limit.

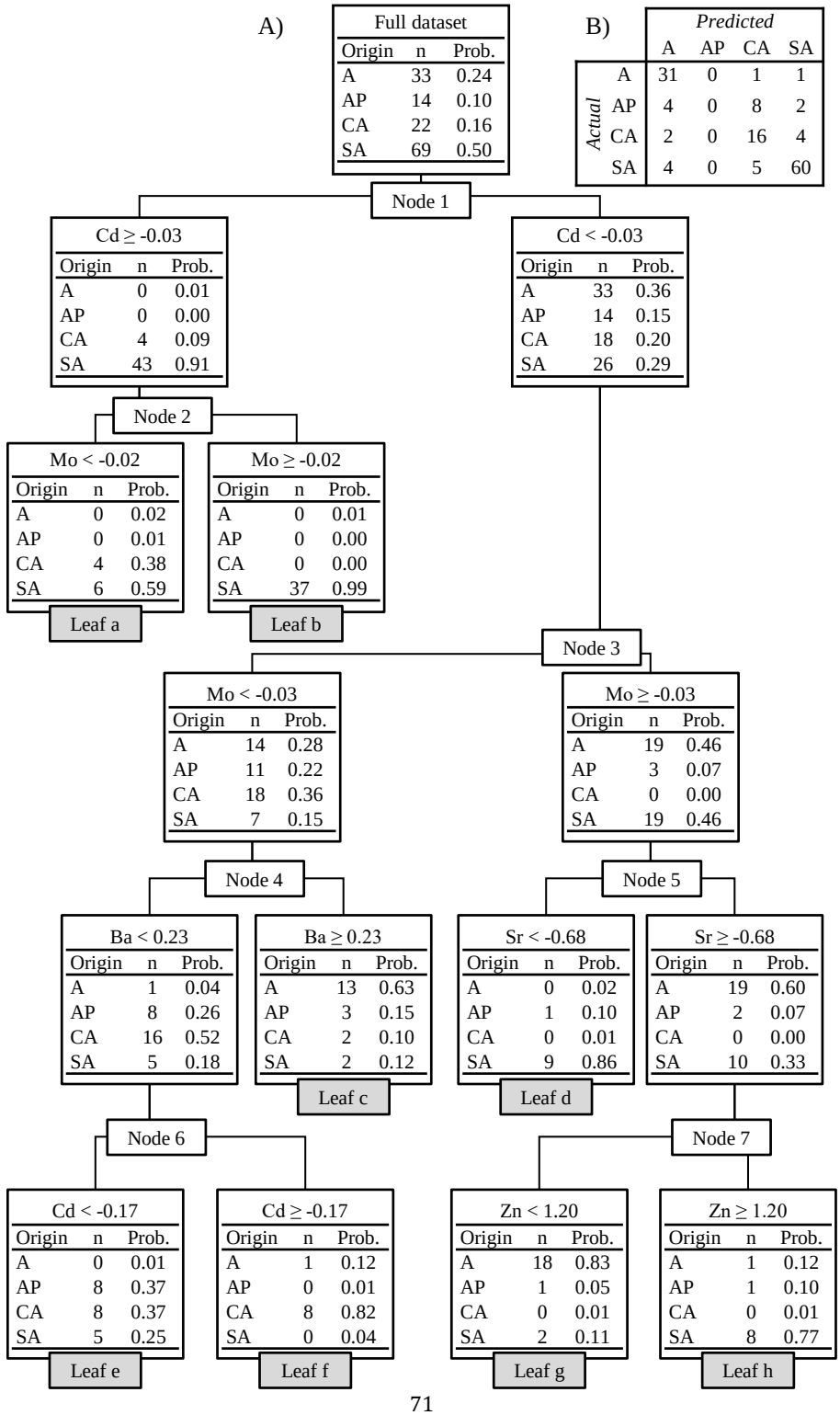
To obtain a desirable product (with regard to flavour and cost), cacao from different geographical origins is often mixed during the chocolate production process. The large differences in Cd concentration among cacao from different origins indicates that this mixing strategy may be a suitable technique to ensure that the final product complies with the new Cd regulations. For this study, single origin chocolates were selected specifically since they are likely most affected by the new EU Cd regulation, as they can only be produced using cacao beans from the indicated country. The large variation within the regions also indicates that blending strategies within countries may be suitable to ensure that single origin chocolates comply with the EU regulation.

3.3.2. Classification and Regression Tree (CART) analysis

Due to the large number of unknowns and complexity of the recipes, fingerprinting of the final chocolate product requires robust methods. Classification and Regression Tree (CART) analysis is a non-parametric technique that has been used to verify the origin of food products based on their elemental composition, e.g. to verify the origin of wine (Capron et al. 2007; Gonzálvez et al. 2009). Here, fivefold cross-validated CART analysis was used to identify elements that most strongly characterise chocolates from different origins, and to generate a decision tree for the classification of chocolates by origin, based on their elemental composition. The CART method was chosen over other chemometric techniques that are often used to generate elemental fingerprints in food, such as principal component analysis and discriminant analysis, because it does not rely on the normality assumption and is more robust to the influences of the unknown variables in the chocolate recipe. Each datapoint has a weight of 1 among the n datapoints in the analysis. As such, the CART method has a robustness similar to that of a median, and the effect of outliers or mislabelled datapoints on the final result is limited (Breiman et al. 1984). In this specific case for example, chocolate samples with deviating element concentrations due to an additional unknown ingredient will not strongly affect the final decision tree. As mentioned previously, the elemental composition of chocolate bars is significantly correlated to their cacao content. To correct for this relation, CART analysis was applied to the residuals of the linear regression fit of each element to the cacao content of the sample. The regression parameter estimates can be found in Appendix II (Table II.3).

Five elements (Ba, Cd, Mo, Sr and Zn) capable of separating the dataset using seven splitting nodes (Cd and Mo were both repeated in two nodes) were identified by CART analysis. The first node of the decision tree was determined by Cd and divided the dataset in two subgroups (Figure 3.2). The subgroup with high Cd residuals, i.e. the subgroup with higher Cd concentrations than what would be expected based on the cacao content of the samples, was dominated by South America, whereas its counterpart was made up of a mixture of origins. As such, the analysis identified Cd concentration as the first variable capable of discriminating South American chocolates from other origins, which is in agreement with the statistically higher average Cd concentration in South American chocolates (Table 3.3).

Figure 3.2 A) Decision tree for the classification of chocolates by cacao origin. Classification is based on fivefold cross-validated CART analysis applied to the residuals of the individual regressions of the elemental concentrations of chocolate to their cacao content (parameter estimates can be found in the Appendix, Table II.3). Nodes are indicated by numbers and leaves (end points) are indicated by lowercase letters. For each node and leaf, the cut-off value of the residuals, the composition of the subgroup and the probabilities for each origin are given. B) Confusion matrix for the CART model showing that 60 of the 69 South American samples were correctly classified and that the overall misclassification rate was 23 %. The model was not able to correctly classify Asia Pacific samples. A = Africa, AP = Asia Pacific, CA = Central America, SA = South America.


A)
Full dataset
Origin n Prob.
A 33 0.24
AP 14 0.10
CA 22 0.16
SA 69 0.50
Node 1
Cd ≥ -0.03
Origin n Prob.
A 0 0.01
AP 0 0.00
CA 4 0.09
SA 43 0.91
Node 2
Mo < -0.02
Origin n Prob.
A 0 0.02
AP 0 0.01
CA 4 0.38
SA 6 0.59
Leaf a
Mo ≥ -0.02
Origin n Prob.
A 0 0.01
AP 0 0.00
CA 0 0.00
SA 37 0.99
Leaf b
Cd < -0.03
Origin n Prob.
A 33 0.36
AP 14 0.15
CA 18 0.20
SA 26 0.29
Node 3
Mo < -0.03
Origin n Prob.
A 14 0.28
AP 11 0.22
CA 18 0.36
SA 7 0.15
Node 4
Ba < 0.23
Origin n Prob.
A 1 0.04
AP 8 0.26
CA 16 0.52
SA 5 0.18
Node 6
Cd < -0.17
Origin n Prob.
A 0 0.01
AP 8 0.37
CA 8 0.37
SA 5 0.25
Leaf e
Cd ≥ -0.17
Origin n Prob.
A 1 0.12
AP 0 0.01
CA 8 0.82
SA 0 0.04
Leaf f
Ba ≥ 0.23
Origin n Prob.
A 13 0.63
AP 3 0.15
CA 2 0.10
SA 2 0.12
Leaf c
Mo ≥ -0.03
Origin n Prob.
A 19 0.46
AP 3 0.07
CA 0 0.00
SA 19 0.46
Node 5
Sr < -0.68
Origin n Prob.
A 0 0.02
AP 1 0.10
CA 0 0.01
SA 9 0.86
Leaf d
Sr ≥ -0.68
Origin n Prob.
A 19 0.60
AP 2 0.07
CA 0 0.00
SA 10 0.33
Node 7
Zn < 1.20
Origin n Prob.
A 18 0.83
AP 1 0.05
CA 0 0.01
SA 2 0.11
Leaf g
Zn ≥ 1.20
Origin n Prob.
A 1 0.12
AP 1 0.10
CA 0 0.01
SA 8 0.77
Leaf h
B)
Predicted
A AP CA SA
Actual
A 31 0 1 1
AP 4 0 8 2
CA 2 0 16 4
SA 4 0 5 60

The left branch was then split by Mo (node 2), further separating South America from Central America. Similarly, node 3 also separated South American samples from Central American and Asia Pacific samples based on Mo. This was in accordance with the statistically higher Mo concentrations measured in South American chocolates (average 0.24 mg Mo kg^{-1}) compared to samples from Central America (average 0.10 mg Mo kg^{-1}) and Asia Pacific (average 0.10 mg Mo kg^{-1}) (Table 3.3). The fourth node was determined by Ba and separated African samples from a mixture of origins. This was again in accordance with the results of the Tukey test (Table 3.3), which showed that the average Ba concentration in African samples was statistically higher compared to chocolates from the other regions. In node 5, the dataset was split based on Sr, which separated African chocolates but divided the group of South American chocolates evenly, and the final node (node 6) was determined by Zn although overall no significant difference between the average Zn concentrations per origin was identified (Table 3.3). Bertoldi et al. (2016) showed similar differences in the average concentrations of Cd and Ba in cacao beans from different origins, i.e. Cd concentrations were significantly higher in Central and South American compared to Asian and West African cacao beans, and Ba was significantly higher in beans from East Africa compared to all other origins. However, they reported higher Mo concentrations in East Africa compared to Asia, Central America and West Africa, which was not observed in the present study. The elements identified by the CART model (Cd, Ba, Mo, Sr and Zn) were also reported by Bertoldi et al. (2016) as part of their discriminant model obtained through stepwise discriminant analysis for the separation of cacao beans by geographical origin.

The CART model was evaluated through several aspects. First, the fivefold cross-validated misclassification rate was 23 %. This represents the fraction of the data for which the predicted category (or origin) was not equal to the observed category, i.e. the origin mentioned on the sample packaging. The confusion matrix displays more details regarding these misclassified samples (Figure 3.2 B). Second, the probabilities of each origin in the leaves (= endpoints) of the decision tree provide an additional indication of how well the model was able to separate the origins from each other. High probabilities in the leaves were obtained for South American (0.99 in leaf b) and African samples (0.83 in leaf g). The efficiency of the model for the identification of South American and African samples was corroborated by the low misclassification rates for these regions, only 10 % of African samples and 13 % of South American samples were misclassified. Separation of the Asia Pacific samples from the rest of the dataset was unsuccessful, all Asia Pacific samples were misclassified. This may be explained by the large geographical heterogeneity and corresponding differences in soil composition and climate within the Asia Pacific sample group, as it comprised a combination of islands and mainland countries (Appendix II, Figure II.1). This likely resulted in a larger variety of elemental compositions complicating the separation of these samples from the bulk data set. Also, the small sample size of this group may have caused it to be underrepresented in the dataset. As the CART method strives to minimise the overall error rate, underrepresentation of a sample group may cause it to be poorly predicted by the model. Improved misclassification rates may be reached using other parameters instead of total

element concentrations, e.g. stable isotope ratios (Diomande et al. 2015; Perini et al. 2016).

3.4. Conclusions

This study revealed that 10 % of the 139 collected single origin chocolates (13 samples from South America and one from Central America) would be rejected based on the EU Cd regulation. A significant and strongly positive correlation was found between the Cd concentration measured in chocolates and their cacao content, suggesting that the trace element most likely originates from the raw material (the cacao beans) rather than from other ingredients or processing. The average Cd concentration was significantly higher in South American chocolates compared to chocolates made with cacao from Africa and Asia Pacific, indicating the geogenic origin of the element as argued in several cacao and chocolate studies. This observation was validated by the CART model, which identified Cd as the first node, splitting the dataset in a group that consisted of mostly South American samples and a group with samples from all four regions. The recommended maximum Pb concentration of 0.10 mg Pb kg^{-1} was exceeded in three samples originating from Ecuador and Peru (taking the measurement error in consideration), but the data suggests that Pb concentrations in chocolate were unrelated to the raw cacao material and were likely the result of postharvest processing. This is not unexpected given the ubiquity of Pb in the environment. Classification and regression tree analysis proved to be a suitable technique to trace the origin of chocolates based on their elemental composition. It provided both a decision tree to classify unknown samples and information regarding the elements which characterise chocolates from each origin. The total misclassification rate was 23 % and was mostly related to samples from Asia Pacific which were not separated from the rest of the data set.

Chapter 4
The impact of fermentation on the distribution of cadmium in cacao beans

This chapter is based on the following reference:
Vanderschueren R, De Mesmaeker V, Mounicou S, Isaure M-P, Doelsch E, Montalvo D, Delcour J A, Chavez E, Smolders E. 2020. The impact of fermentation on the distribution of cadmium in cacao beans. *Food Research International*. 127:108743. doi:10.1016/j.foodres.2019.108743

Summary
A large fraction of the South American cacao production is affected by new EU cadmium (Cd) regulations in chocolate. This work was set up to characterise the distribution and speciation of Cd within the cacao pod and to monitor potential Cd redistribution during fermentation. In cacao pods from four locations, Cd concentrations decreased with testa > nib ~ placenta ~ pod husk > mucilage. The distribution of Cd within cacao beans was successfully visualised using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) and confirmed higher Cd concentrations in the testa than in the nib. Speciation analysis by X-ray Absorption Near-Edge Structure (XANES) spectroscopy of unfermented cacao nibs revealed that Cd was bound to a combination of oxygen, sulphur and phosphate ligands. Fermentation induced an outward Cd migration from the nibs to the testae, i.e. from a lower to a higher Cd concentration tissue. This migration occurred only if the fermentation was sufficiently extensive to decrease the nib pH 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. It is proposed that nib Cd can be reduced if the nib pH is sufficiently acidified during fermentation. However, a balance must be found between flavour development and Cd removal since extreme acidity is detrimental for cacao flavour.

4.1. 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 their potentially high Cd concentrations. The European Food Safety Authority (EFSA) 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 also adopted by the Codex Alimentarius (Codex Alimentarius Commission 2018). These new regulations will impact South American cacao farmers, as Cd concentrations in South American cacao are generally higher than those in cacao from other origins (Table 2.1). For example, Bertoldi et al. (2016) reported Cd concentrations in South American cacao beans more than tenfold larger than those in West African beans. These findings have prompted researchers to explore mitigation techniques to lower Cd concentrations in the final product. Such efforts have focused mostly on the relation between bean and soil Cd concentrations. For example, Argüello et al. (2019) mapped the concentrations of Cd in cacao beans and corresponding soils in Ecuador and was able to identify soil parameters predicting bean Cd concentrations. Ramtahal et al. (2019) studied potential soil amendments and showed that both biochar and lime application may reduce Cd accumulation in cacao in the field.

Chapter 3 concluded that the Cd concentration in chocolates is related to their cacao content, indicating that Cd originates from the raw material (the cacao beans) rather than from 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). Postharvest strategies that lower the Cd concentration in the cacao nib may offer viable solutions to reduce the Cd concentration in the final product because the nib is the only part of the cacao pod retained during processing. A detailed overview of the postharvest process of cacao is given in Chapter 1. Developing postharvest mitigation strategies requires better understanding of the distribution and speciation of Cd in the different cacao tissues, as well as the influence of conventional postharvest processes on this distribution. Most previous work reported on the cacao nib, testa and pod husk, with little attention to the mucilage or the placenta. The Cd concentration is generally reported to be higher in the testae 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 generally found higher Cd concentrations in the nib compared to the testa. Sample treatment often differs in the literature available thus far, cacao samples were provided by chocolate manufacturers with little information regarding sample processing (Lee and Low 1985), and samples were either or not washed with water (Ramtahal et al. 2016; Gramlich et al. 2018; Lewis et al. 2018) or hypochlorite solutions (Chavez et al. 2015), which can affect Cd concentrations in the outer tissues.

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 study unfermented samples. Fermentation has been reported as a possible technique for reducing Cd in rice (Zhang et al. 2017; Zhai et al. 2019). 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 organic acid production on Cd mobilisation. 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, testae and nibs; and (ii) to investigate the effect of fermentation on the distribution of Cd among these different cacao bean tissues. Better understanding of the distribution and speciation of Cd in cacao and the effect of postharvest processing, may shed light on mitigation opportunities to lower Cd concentrations in the final product.

4.2. Materials and methods

4.2.1. Cacao material

Ripe cacao pods 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 4.1). Unfermented cacao beans for X-ray Absorption Near-Edge Structure (XANES) spectroscopy were collected at different fields in the provinces Esmeraldas (Nacional cultivar), Guayas (CCN 51 cultivar) and Sucumbíos (Nacional cultivar).

4.2.2. Sampling and sample preparation for unfermented cacao pod tissues

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

4.2.3. Fermentation

Fermentation experiments were conducted to assess the effect of fermentation on the Cd distribution in 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 consisted of the same cacao (same cultivar and field) but were subjected to different fermentation conditions (Table 4.1).

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 4.1) was thoroughly mixed and divided over duplicate fermentation boxes. Different subsamples of 1 kg cacao mass were taken and placed in mesh bags (Appendix III, Figure III.1) to facilitate bean sampling during fermentation. All mesh bag subsamples (3 – 7 per box, depending on the batch) were placed in the centre 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 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 (Appendix III, Figure III.2). 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 overnight before fermentation, mimicking a common practice in some fermentation facilities. In this pre-drying method, fresh cacao was spread out on a concrete floor in open air and left to dry overnight. This method, also referred to as bean spreading, is a common practice to prevent excessive acidity in the fermented beans (Meyer et al. 1989; Biehl et al. 1990; Schwan and Wheals 2004). The total fermentation time for each batch was determined by local practices (Table 4.1). The endpoint of each fermentation was based on a quality assessment by local farmers. Beans were sampled daily by removing one mesh bag subsample (1 kg cacao mass) from the centre of each box. The 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 testae and further ground as described above.

4.2.4. Temperature and pH

The mucilage pH was measured immediately after sampling or after opening of the cacao pods. Cacao beans with mucilage attached were vigorously shaken for 2 min in a 1:10 solid to deionised water ratio and the pH of the suspension was measured to determine the mucilage pH. To determine nib and testa pH, dried and ground material was treated likewise in a 5:10 solid sample to deionised 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 boxes was measured daily in the centre of the boxes using a digital thermometer (VWR International, Radnor, PA, USA).

Table 4.1 Origin of the different cacao batches and setup of the fermentation experiments. For batch D, no fermentation experiment was performed.

	Origin	Cultivar	Box dimensions (w × d × h, cm)	Cacao per box (kg)	Box setup	Times mixed in fermentation	Fermentation time (days)	Immediate fermentation
A	El Oro	CCN 51	60 × 60 × 60	290	Cascade	2	7	Yes
B	Guayas field 1	Nacional	60 × 60 × 60	290	Cascade	1	5	Yes
C	Guayas field 2	Nacional	100 × 100 × 60	590	Single	1	4	Yes
C_{bis}	Guayas field 2	Nacional	100 × 100 × 60	590	Single	1	3	Pre-dried [a]
D	Sucumbíos	Nacional	/	/	/	/	/	

[a] Pre-dried overnight at ambient temperature before fermentation by spreading the cacao beans on a concrete floor in open air.

4.2.5. 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, Kenilworth, NJ, USA) in an open digestion block for 8 hours at a maximum temperature of 130 °C. Digests were brought to a volume of 10 mL, 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, CA, USA). The ICP-MS analysis was performed in the helium (He) collision cell mode monitoring the ^{111}Cd isotope, using ^{103}Rh as an online 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 between 98 and 108 % and the coefficient of variation (CV) for the duplicate digestions ranged between 0.1 and 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.

4.2.6. Visualisation of the elemental distribution in unfermented beans by LA-ICP-MS

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 beans obtained from the same pod using the digestion method and ICP-MS analysis described above. Transverse bean cross-sections with a thickness of 65 µm were made following the method described by Lombi et al. (2009) for rice grains. The beans were sliced with a vibrating microtome (Microm HM 650 Vibratome, Thermo Scientific, Waltham, MA, USA) using diamond blades (GFD Gesellschaft für Diamantprodukte, Ulm, Germany). The cross-sections were made at approximately 80 % of the length of the bean, measured from the radicle side. Once a flat surface was obtained, the surface of the cacao bean was defatted using hexane (HiPerSolv Chromanorm 97 %, VWR International, Radnor, PA, USA) 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) was used, mounted with platinum 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, Fremont, CA, USA) and coupling was optimised 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 and 5.3 J cm^{-2}, a laser beam of 20 μm^2 or 50 μm^2, a scan speed of 23 or 50 µm s^{-1}, and a distance between each line of 40 or 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 the 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.

4.2.7. X-ray absorption near-edge structure spectroscopy (XANES)

The speciation of Cd in unfermented cacao nibs was studied using XANES spectroscopy. The samples consisted of unfermented cacao beans from CCN 51 and Nacional cultivars. The testae and nibs were separated manually, and the nibs were dried, ground and pressed into pellets for XANES analysis. The XANES spectra of the samples 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 Cd concentration of the sample. Each scan was measured on a different spot on the pellet to limit beam damage. Normalisation and linear combination fitting (LCF) were done with Athena software (Ravel and Newville 2005), using the databases of Cd reference spectra recorded at 15 – 20 °K from Isaure et al. (2006) and Huguet et al. (2012 and 2015), with the addition of Cd phytate, both as a precipitate and in solution (phytate/Cd molar ratio = 5). For each sample spectrum, LCF was performed by fitting regions between -20 and 100 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.

4.2.8. Statistical analysis

All statistical analysis was executed using JMP® Pro version 14.0.0 (SAS Institute 2018). The differences in Cd concentration among the different cacao tissues were tested using Tukey's Honestly Significant Difference (HSD) test (P-value < 0.05) using the average 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).

4.3. Results and discussion

4.3.1. Distribution of Cd within the different cacao pod tissues

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 4.2). The coefficients of variation (CV) of nib Cd between cacao pods of the same batch ranged between 20 and 37 %, indicating variation in bean Cd concentrations between pods from the same field. Similarly, Argüello et al. (2019) observed an average CV of 39 % in bean Cd concentration among fruits of different trees within the same field, in a large study of 159 Ecuadorian fields. They related this to the large spatial variation in available soil Cd.

Table 4.2 Distribution of Cd among the different tissues of unfermented cacao pods. Placenta and pod husk were only collected for batches A and B. For batch D, no mucilage material was collected. Concentrations are averages (± stdev of sampling replicates), different letters denote significant differences within each row (Tukey's HSD test, P-value < 0.05).

Batch	n	Cd concentration (average ± stdev, mg kg^{-1} dry weight)				
		Nib	Testa	Mucilage	Placenta	Pod husk
A	3	0.52 ± 0.11 ^{BC}	0.94 ± 0.22 ^{A}	0.08 ± 0.01 ^{D}	0.18 ± 0.008 ^{CD}	0.59 ± 0.21 ^{AB}
B	3	0.39 ± 0.07 ^{AB}	0.66 ± 0.26 ^{A}	0.09 ± 0.03 ^{B}	0.31 ± 0.12 ^{AB}	0.28 ± 0.01 ^{B}
C	6	2.4 ± 0.88 ^{A}	3.7 ± 1.6 ^{A}	0.48 ± 0.24 ^{B}	/	/
D	6	9.6 ± 2.3 ^{B}	16 ± 4.6 ^{A}	/	/	/

The Cd concentrations were overall highest in the testae, followed by the nibs, placenta and pod husk (all similar), and finally the mucilage (Table 4.2). No information was found in literature regarding the Cd concentration in the placenta or the mucilage. Gramlich et al. (2018) analysed 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 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 (average ratio testa over nib Cd was 1.8 for batch A, 1.7 for batch B, 1.5 for batch C and 1.7 for batch D). Considering the average weight fractions for nib (0.93) and testa (0.07) in these samples, 91 % of the total bean Cd was located in the nibs and 9 % in the testae in unfermented cacao beans. In accordance to the present work, Ramtahal et al. (2016) reported significantly higher Cd concentrations in the testa (0.44 – 4.41 mg Cd kg^{-1}) compared to the nibs (0.35 – 3.82 mg Cd kg^{-1}) for unfermented cacao from Trinidad and Tobago. Lewis et al. (2018) reported more than twofold 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. Similarly, Lee and Low (1985) determined the Cd concentrations in 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 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) analysed the Cd concentration in unfermented cacao nibs and testae from 19 small scale farms in Ecuador and consistently found higher Cd concentrations in the nibs compared to the testae. However, the beans in that study were washed with a hypochlorite solution which may have removed some of the Cd from the testa.

4.3.2. Visualising the elemental distribution in unfermented beans with LA-ICP-MS

The elemental imaging maps obtained by LA-ICP-MS for the ^{111}Cd and ^{114}Cd isotopes showed close agreement, which indicates that there were no interferences affecting the Cd measurement (Figure 4.1 and Appendix III, Figures III.3 and III.4). 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), indicating that the samples were sufficiently planar for reliable image interpretation and comparison. Regions with consistently lower signal intensities corresponded to inherent cracks in the bean samples as visible on the sample pictures (Figure 4.1).

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. Therefore, higher resolution imaging requires samples with higher elemental concentrations or very long measurement times. Because of this, the batch D sample could be scanned at a higher resolution (20 μm^2 laser beam size) than the cross-section of batch C (50 μm^2). The ICP-MS integration time was equal in both scans to limit measurement time. 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 batch D were larger than those in batch C.

The testa was clearly distinguishable from the nib and showed elevated Cd intensities for both samples (Figure 4.1), which is in line with the measured Cd concentrations in those tissues (Table 4.2). Apart from Cd, only Ca displayed clearly elevated signal intensities in the testa (Appendix III, Figures III.3 and III.4). The distribution of K was approximately uniform between nib and testa; while P, Ni and Zn were most abundant in the nib. 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 and Mertens 2013). Dissimilar Cd and Zn distribution patterns have also been visualised 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 defence mechanism of the plant as Zn is an important micronutrient while Cd has no known function in plant growth.

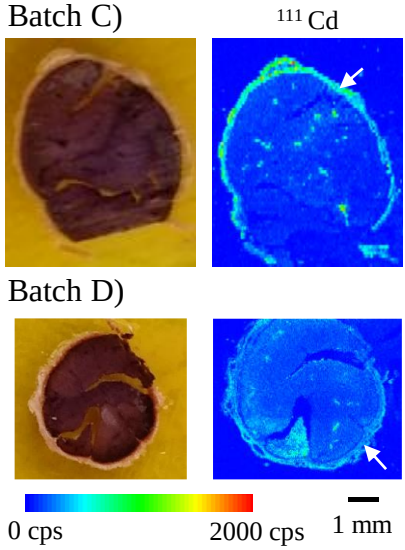


Figure 4.1 Relative distribution of ^{111}Cd in two unfermented cacao bean transversal cross-sections (batch C and batch D) visualised using LA-ICP-MS, and photography images of the samples before LA-ICP-MS analysis. White arrows indicate the testa. The average Cd concentrations measured in beans from the same pod (as determined by ICP-MS) were 1.7 mg Cd kg^{-1} in the nib and 3.1 mg Cd kg^{-1} in the testa (batch C); and 11 mg Cd kg^{-1} in the nib and 22 mg Cd kg^{-1} in the testa (batch D). The cross-section from batch C was analysed with a laser beam size of 50 µm^2 and a scan speed of 50 µm s^{-1}, whereas the cross-section from batch D was analysed with a laser beam size of 20 µm^2 and a scan speed of 23 µm s^{-1}. cps = counts per second.

The distribution of Cd within the nib was not homogeneous throughout the cross-sections. Further identification of nib zones with higher Cd intensities may shed light on the way Cd is loaded into the cacao beans during plant growth. Thyssen et al. (2018) created elemental maps of longitudinal cross-sections of fermented cacao beans using LA-ICP-MS and also 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 fermentation, or by possible differences in cultivars and overall different elemental concentrations between these samples.

4.3.3. Speciation of Cd in unfermented cacao beans

The results of the LCFs indicated that Cd in cacao nibs is bound to a combination of O-ligands (cellulose, cell wall material and organic acids mix), P-ligands (phosphate and phytate) and S-ligands (phytochelatin and metallothionein) (Figure 4.2 and Appendix III, Table III.1 and Figure III.5). In general, 40 – 80 % of nib Cd was bound to O-ligands, while the remainder was associated to (poly)phosphates or S-ligands. In hyperaccumulator plants, Cd was found to be associated 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 (Isaure et al. 2006; Vogel-Mikuš et al. 2007; Huguet et al. 2012). Similarly, Yan et al. (2020) reported that Cd in the crease of durum grains was associated half-half to O-ligands and S-ligands, and the authors stated that their results could not unambiguously exclude P-ligands.

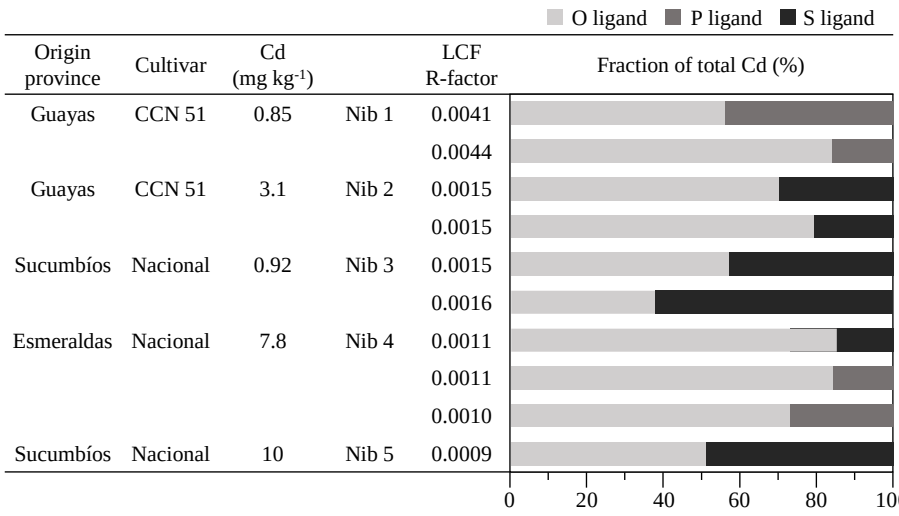


Figure 4.2 Fractions of Cd species in unfermented cacao nibs determined by XANES followed by LCF analysis, and corresponding R-factors indicating the goodness of fit. Different fits for the same sample could not be reasonably differentiated as the difference in R-factor between the fits was < 10 %. Total Cd concentrations in each tissue were determined by acid digestion and ICP-MS analysis. The different ligands were malate, cellulose or cell wall (O-ligands); phosphate or phytate (P-ligands); and metallothionein or phytochelatin (S-ligands). Detailed XANES spectra and LCF fits for the samples are given in Appendix III (Table III.1 and Figure III.5).

4.3.4. 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 3.7 to 4.5, and the testa pH increased from 4.3 to 5.0 (Figure 4.3). Changes in pH were less pronounced in batches C and C_{bis}, with 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 batches C and C_{bis} were shorter than for A and B (3 – 4 versus 5 – 7 days, Table 4.1), 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. The pH and temperature values are in line with values reported in literature, which state that the temperature of the fermenting 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 (Schwan and Wheals 2004; Thompson et al. 2007; Belitz et al. 2009; Papalexandratou et al. 2011; De Vuyst and Weckx 2016).

4.3.5. 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 tissues at the onset of fermentation (day 0, Figure 4.4) was in line with the values observed in intact fruits (testa > nib > mucilage, Table 4.2). The nib Cd concentrations in batches A and B decreased with fermentation time by a factor 1.3 (Figure 4.4). The final nib Cd concentration in B was lower than 0.60 mg kg^{-1}, the unofficial industry threshold for export to the EU.

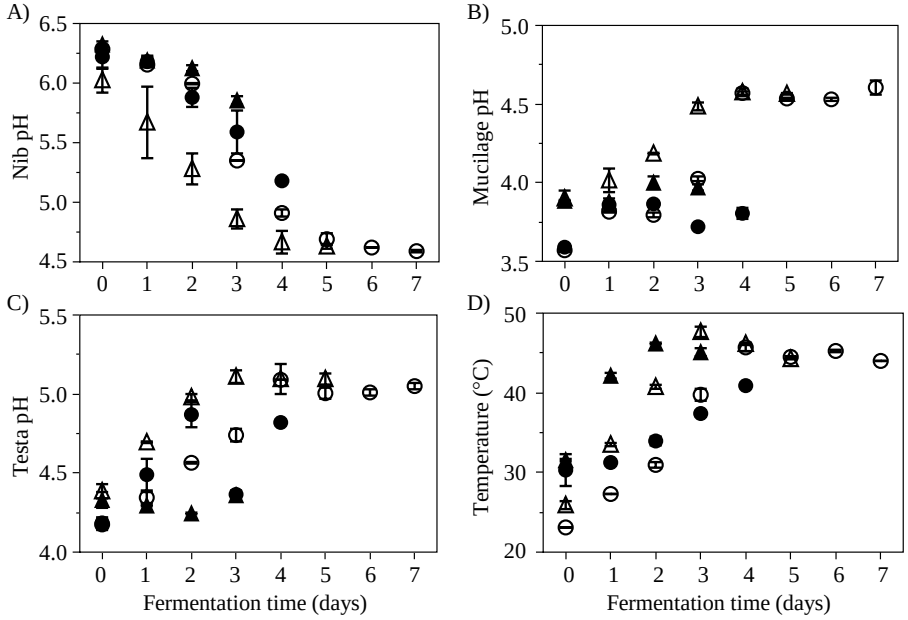
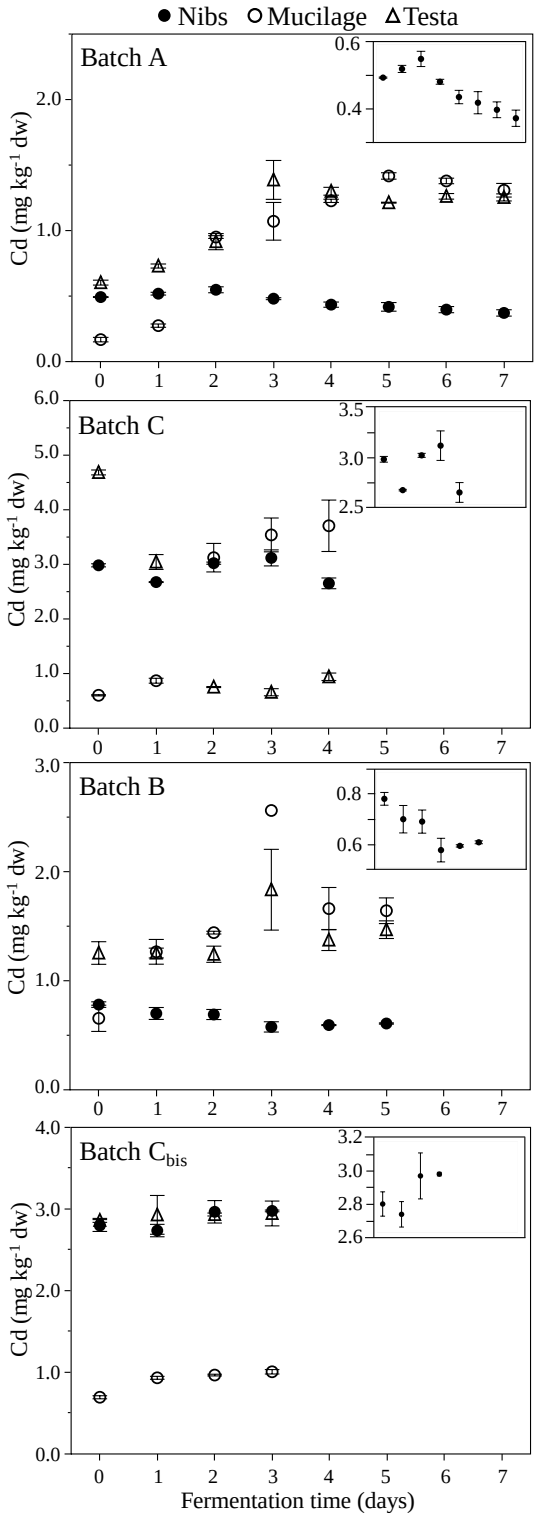


Figure 4.3 Changes in nib pH (A), mucilage pH (B), testa pH (C) and temperature in the centre of the fermenting mass (D), for three experimental setups: batch A (○), batch B (△), batch C (●) and batch C_{bis} (▲). Each point represents the average of duplicate samples and the error bars are standard errors.

The mucilage and testa Cd concentrations in batch A increased with fermentation time (factor 2.1 in the testa and 7.8 in the mucilage) reaching a similar plateau concentration after four days of fermentation. The same was true for the mucilage Cd in batch B (increased by a factor 2.5) but no significant trend in testa Cd was observed. The decreasing Cd concentrations in the nibs during fermentation are unlikely related to a change in the ability to remove the high Cd testa from the low Cd nib during peeling. Budget analysis using concentrations and weight fractions of the tissues showed that the nib Cd content (expressed in mg nib Cd kg^{-1} total cacao bean) decreased and testa Cd content (mg testa Cd kg^{-1} total cacao bean) increased for batches A and B (Appendix III, Figure III.6). This suggests that Cd migrates from the nib to the testa and the mucilage during fermentation, which reduces the Cd concentrations in the final product because the outer tissues (testa and mucilage) are removed at later stages of the postharvest process. At the end of fermentation in batches A and B, approximately 80 % of the total 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 batch C increased significantly with fermentation (factor 6.2) in a similar pattern as observed for batches A and B. But in contrast to batches A and B, the testa Cd concentration in batch C decreased with time. Fermentation of batch C_{bis} had no significant effect on the Cd concentration in the testa. The mucilage Cd concentration did increase by a factor 1.7 but this change was not of the same magnitude as observed in the other batches. The overall Cd concentrations in batches C and C_{bis} were higher than those in batches A and B. However, results from a lab scale fermentation (5 kg) using similar high Cd cacao, showed that Cd concentrations in the nibs did decrease with nib pH if the cacao was fermented more extensively, i.e. four days with a decrease in nib pH from 6.5 to 4.6 (see Chapter 5, lab fermentation A). This demonstrates that the pH, rather than the total Cd concentration, explains Cd migration. Mass balance calculations showed that the total bean Cd concentrations were reduced by 15 % by the end of fermentation in batches A, B and C (Appendix III, Figure III.7). This may be related to the loss of mucilage through fermentation sweatings. No Cd loss was observed over the course of fermentation for batch C_{bis} which had been air-dried prior to fermentation. The farmers estimated that the cacao loses approximately 25 % of its fresh weight during pre-drying as the mucilage liquid runs off and evaporates. As a result, sweatings during fermentation may be much smaller in such fermentation practices and the total Cd mass may remain constant over the course of fermentation.

Figure 4.4 Concentrations of Cd in the different cacao bean tissues [nib (●), testa (Δ) and mucilage (O)] measured at different days of fermentation. Each point represents the average of duplicate fermentation boxes and error bars denote the standard error. Inset figures further zoom in on the changes in Cd concentration in the nibs to facilitate visualisation.


Nibs
Mucilage
Testa
Batch A
Batch C
Batch B
Batch C$_{bis}$
Cd (mg kg^{-1} dw)
Fermentation time (days)

Nib Cd concentrations were strongly correlated to the nib pH throughout fermentation in batches A and B, but not in C and C_{bis} (Figure 4.5). The nib pH in batches C and C_{bis} decreased by over one unit during fermentation but remained > 5, while the pH in batches A and B dropped to 4.5. This may indicate the importance of extensive fermentation to reach nib pH values < 5, in order to generate Cd migration from the nib outwards. Zhai et al. (2019) stated that fermentation can lower Cd concentrations in rice 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 4.2). The migration of Cd during fermentation thus occurred against the total Cd concentration difference. 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.

4.3.6. The influence of fermentation on other elements in cacao

Apart from Cd, several other elements were analysed 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 and mucilage) were correlated with fermentation time (Appendix III, Table III.2 and Figures III.8 – III.17). 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 1.1 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} except for a significant increase in the 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 9.4). 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 related to microbial deterioration of the outer layers of the testa. If present, this deterioration was not strong enough to significantly decrease 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 (Appendix III, Figure III.6). The observed migration pattern of Ni might be of importance in the future because the European Commission mentioned cacao-derived products among important food sources of Ni in the European population (EFSA CONTAM Panel 2015). Based on the similar behaviour of Ni and Cd observed in this work, postharvest strategies to lower Cd concentrations in cacao during fermentation will likely also be effective for Ni.

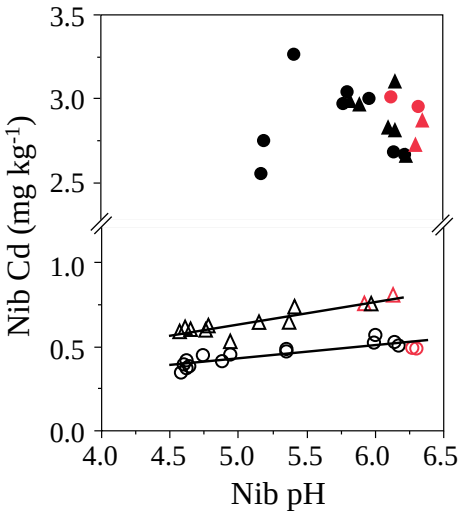


Figure 4.5 Nib Cd concentrations are significantly (P-value < 0.05) correlated to nib pH during fermentation of batch A (○, Pearson correlation r = 0.87) and batch B (△, r = 0.90), but this correlation was not significant in the fermentation of batches C (●) and C_{bis} (▲). The red color indicates the starting point of each independent fermentation replicate (day 0).

4.4. Conclusions

In unfermented cacao pods, Cd concentrations are highest in the testae, followed by the nibs, the placenta and the pod husk 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 testae was only observed if the nib pH dropped below 5. This acidic pH resulted from longer fermentation times. More extensive fermentation can thus lower the Cd concentrations in the final product as the testa and mucilage are removed later in the postharvest process. After fermentation, nib Cd concentrations decreased by up to a factor 1.3, indicating that fermentation may be useful to comply to the new Cd requirements (0.60 mg Cd kg^{-1}) in 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 low nib pH as this can yield an unpleasant acidic taste in the final product. A balance must thus be found between the flavour quality and the Cd concentration. This acidic flavour 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. Pre-drying and short fermentation times may reduce the extent of outward Cd migration.

Chapter 5
Cadmium migration from nib to testa during cacao fermentation is driven by nib acidification

Summary

The Central and South American cacao industry is threatened by recent cadmium (Cd) regulations in cacao. Previous work has shown that cacao nib Cd concentrations can slightly decrease during fermentation, but this reduction in nib Cd concentrations was only noticeable with strong nib acidification. First, lab scale fermentation experiments (5 kg units) were set up with lactic and acetic acid amendments during or after fermentation, both were ineffective in reducing the total nib Cd concentration. In contrast, the water extractable Cd fraction in cacao nibs clearly increased with decreasing nib pH, but the same was also true for unfermented cacao when artificially lowering the pH from 6.5 to 4.5 in the equilibrium solution. Second, single pod derived cacao beans were inserted in mesh bags and embedded in a full scale fermentation box, which allowed monitoring of the fermentation effect with high precision. In this setup, the nib Cd concentration significantly decreased by a factor 1.25 (P-value < 0.05) after four days of fermentation. The gradient in mobile Cd within the cacao beans was mapped with Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS), using imprints of transversal cuts exposed to a metal binding gel. That analysis showed that fermentation enhances the Cd mobility and establishes a mobile concentration gradient from inside the nibs to the outer testa and mucilage. The large fractions of Cd that could be extracted from fermented cacao nibs suggest that washing nibs with water and/or chelating agents prior to roasting holds promise as a mitigation strategy for Cd in cacao.

5.1. Introduction

In 2019, the European Commission enforced a regulation which sets the maximum allowed cadmium (Cd) concentrations in cacao-derived products sold to the consumer (European Commission 2014). Similar limits have also been approved by the Codex Alimentarius (Codex Alimentarius Commission 2018). These new regulations have a large impact on the cacao industry, especially in Central and South America as their soils naturally contain elevated Cd concentrations. Larger Cd concentrations have been reported in cacao beans and chocolates from Central and South America, compared to other geographical origins such as Africa (Chapter 3). The impact of the new Cd regulation on cacao farmers in Central and South America was indicated by an extensive meta-analysis (Chapter 2). More than 50 % of compiled bean Cd data in that meta-analysis exceeded the unofficial industry threshold for export to the EU (0.60 mg Cd kg^{-1}). The EU regulation has prompted researchers to study mitigation strategies to lower the Cd concentrations in cacao-derived products. While research thus far has focused mostly on reducing Cd uptake in the cacao plant through soil amendments (Ramtahal et al. 2019) or through selection of specific cacao cultivars for reduced Cd uptake and translocation (Lewis et al. 2018; Engbersen et al. 2019), the potential of postharvest mitigation during processes such as fermentation has also been pointed out (Meter et al. 2019).

To date, only limited information is available regarding the effect of postharvest processing on cacao bean Cd concentrations. A detailed overview of the postharvest process of cacao, including fermentation, can be found in Chapter 1. The potential effect of fermentation on bean Cd concentrations was demonstrated in Chapter 4, the results indicated that Cd migrates from the nib to the testa and the mucilage during fermentation. This migration decreased the nib Cd concentration by up to a factor 1.3, but this effect was only present when fermentation resulted in sufficient nib acidification, i.e. nib pH < 5. This migration is counterintuitive since testa Cd concentrations are typically higher than nib Cd concentrations, even before fermentation. It is speculated that the concentration gradient of mobile Cd in cacao beans is opposite to the total concentration gradient, especially in more acid, fermented beans. This might be related to a higher content of Cd^{2+} binding agents in the testa compared to the nib.

The objective of this study is to better understand the causes of Cd migration during cacao fermentation. More specifically, this study was set up (i) to reveal whether nib acidification is the driving force behind Cd migration during cacao fermentation; and (ii) to identify whether artificial acidification during the fermentation process using organic acids typically present during fermentation (acetic and lactic acid) can be used as a strategy to lower the Cd concentration in the final product. The work consisted of two lab scale fermentation experiments with different organic acid treatments, and one micro-fermentation experiment established in a full scale commercial fermentation unit. The idea behind the lab scale fermentation experiments is that the effects of acidity on mobile and total nib Cd concentrations can be disentangled from the other effects in the fermentation process, such as heat induced cell breakdown or the effects of ethanol penetration into the nibs. A novel method was adopted to map local Cd mobility at mm resolution. An imprinting technique was used followed by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysis of the imprint. This

visualisation method allows to test the hypothesis that the mobile Cd concentration gradient in cacao beans is opposite to the total Cd concentration gradient.

5.2. Materials and methods

5.2.1. Lab scale fermentations with organic acid treatments

5.2.1.1. Cacao material and lab scale fermentation with organic acid treatments

Two separate lab scale fermentation experiments were executed in a greenhouse in Guayaquil (Ecuador). These experiments consisted of 5 kg cacao fermentations with administration of lactic and acetic acid before, during, or at the end of fermentation (Table 5.1). Lab scale fermentations were executed in plastic planter pots with a flat cone shape (small diameter 17.5 cm, large diameter 23.5 cm, height 22.5 cm). Three to five holes were pierced in the bottom of each planter pot to ensure drainage of fermentation sweatings. Cacao beans (Nacional cultivar) were provided by local farmers in Manabí and Guayas provinces (Ecuador) and transported to the lab within 24 hours after harvest. Prior to setting up each fermentation experiment, 60 kg fresh cacao beans were deposited on a plastic sheet and homogenised manually before dividing the beans among the planter pots (5 kg each). The planter pots were placed inside Styrofoam boxes to retain fermentation heat. The fermentation lasted 5 (lab scale fermentation A) or 6 days (lab scale fermentation B). The cacao mass was mixed manually on day 2 in experiment A, and on days 2 and 5 in experiment B. Treatment doses, treatment time and total fermentation time for both experiments are shown in Table 5.1. To apply treatments, the cacao was spread out in plastic trays and treatment solutions were applied using plastic spray bottles. The application of acids before fermentation and on the second fermentation day was logically expected to disturb the fermentation process and associated temperature increase. Therefore, control treatments (water only) were included on days 0 and 2. The treated cacao beans were mixed manually to ensure an even distribution of the treatment components and were redeposited in the plastic planter pots.

5.2.1.2. Cacao sampling and determining the elemental composition

The cacao bean samples comprised 500 g cacao beans with attached mucilage, and were taken after mixing, at the start of fermentation, at the end of fermentation, and before treatment. Samples of the first experiment (A) thus included day 0, day 2, day 4 for the cacao treated that day, and day 5; samples of the second experiment (B) included day 0, day 5 and day 6. The mucilage was removed manually using paper towels and cacao beans were oven dried for 72 hours at 65 °C. After drying, beans were peeled to separate nib and testa, and both fractions were ground using an electric coffee grinder. Subsamples of 100 mg dry material were acid digested in an open digestion block for 8 h in 3.0 mL Normatom® nitric acid (HNO_3 67 – 69% w/w, VWR International, Radnor, PA, USA) and reached a maximum temperature of 130 °C. Digests were brought to a volume of 10 mL and diluted ten times with Milli-Q water (18.2 MΩ cm^{-1}) prior to elemental analysis with Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA). Cadmium concentrations were measured by monitoring the ^{111}Cd isotope in helium (He) collision cell mode, using ^{103}Rh as an online internal standard. The limit of quantification (LOQ) for the analysis was 0.001 mg Cd kg^{-1} dw. Duplicate samples were included in the digestions at intervals of ten samples and the

coefficient of variation (CV) of these duplicates for Cd analysis ranged from 0.4 to 13 % (average CV 3.5 %). Blank samples (in quadruplicate) and certified reference material NIST 2384 baking chocolate (in triplicate, certified concentration 0.073 ± 0.008 mg Cd kg^{-1}) were included in all digestions and treated the same way as the cacao tissue samples. Recoveries of the certified reference material ranged between 87 and 100 %.

5.2.1.3. Temperature and pH

The temperature (T) in the centre of the fermentation boxes was measured daily using a digital thermometer (VWR International). Because analysis of nib and mucilage pH required bean sampling, pH was determined only at sampling times to avoid excessive disturbance of the fermentation process. The mucilage pH was determined in suspension after shaking 25 g beans with mucilage attached in 100 mL deionised water on an orbital shaker (KS 130 orbital shaker, IKA laboratory equipment, Staufen, Germany) for 5 minutes at 320 rpm. The nib pH was determined by shaking 5.0 g dried ground material in 20 mL deionised water on an orbital shaker for 5 min and measuring the pH in the supernatant after centrifugation for 25 minutes at 1800 g (MTL-5MS Tabletop Low Speed Centrifuge, Micronlab, Shandong, China).

5.2.1.4. Water extractable Cd

The Cd mobility was assessed by measuring Cd concentrations in deionised water extracts of the nibs. Ground nib samples were sieved to obtain a more homogenous particle size distribution (800 µm test sieve, VWR), after which 0.5 g ground nib was incubated with 4.0 mL deionised water in an end-over-end shaker at 20 °C for 5 days. After incubation, samples were centrifuged for 15 minutes at 2000 g (Heraeus Multifuge X3R, Thermo Scientific, Waltham, MA, USA), and the supernatant was filtered through a 0.45 µm syringe filter. Filtered samples were diluted 200 times prior to ICP-MS analysis.

Additionally, the effect of artificial acidification on Cd mobility was determined using water extractable Cd as a proxy. To this end, ground nib and testa material from the blank treatments of experiment A (unfermented samples at day 0 and fermented samples at day 5) were used. Duplicate ground materials (0.5 g for nibs and 0.25 g for testae) were incubated in 12.0 mL deionised water, either acidified to pH 4.5 with hydrochloric acid (HCl, 37 % w/w) or not. The pH of the contact solutions was adjusted prior to incubation and remained unchanged after incubation (< 0.1 pH unit difference). Samples were incubated in an end-over-end shaker for 5 days at 20 °C. After incubation, the samples were centrifuged for 15 minutes at 2000 g, and the supernatant was filtered using a 0.45 µm syringe filter. Filtered samples were diluted 200 times (nibs) or 20 times (testae) prior to ICP-MS analysis.

Table 5.1 Organic acid treatments in the different lab scale fermentation experiments. Control samples were treated with deionised water.

Lab scale fermentation experiment A						
Treatment	Blank	Control (water)	Control (water)	Acetic acid	Acetic acid	Acetic acid
Treatment dose (g kg^{-1} cacao)	/	/	/	13	13	15
Treatment day	/	0	2	0	2	4 §
Treatment volume (L)	/	1	1	1	1	1
Fermentation time (days)	5					
Replicates per treatment	2					
Lab scale fermentation experiment B						
Treatment	Blank	Control (water)		Acetic acid	Lactic acid	
Treatment dose (g kg^{-1} cacao)	/	/		35	53	
Treatment day	/	5		5	5	
Treatment volume (L)	/	0.5		0.5	0.5	
Fermentation time (days)	6					
Replicates per treatment	3					

§ Because of the treatment, this cacao was mixed a second time on day 4.

5.2.2. Micro-fermentation

5.2.2.1. Cacao material and micro-fermentation setup

The micro-fermentation experiment was carried out at a plantation in Guayas province (Ecuador), using Nacional cultivar cacao. Fifteen ripe pods were harvested and opened using a machete. Half of the beans from each pod were immediately sampled and processed without fermentation. The other half of the beans from each pod were collected in individual mesh bags with drawstring closing and placed in the centre of a wooden fermentation box (82 x 65 x 65 cm) filled with Nacional cacao from the same field (225 kg). Fermentation lasted four days and the cacao mass was mixed manually on the second day. The mesh sample bags were kept in the centre of the fermentation box throughout the process and were sampled when fermentation was complete (the fermented samples). A picture of the experimental setup can be found in Appendix IV, Figure IV.1.

Unfermented and fermented beans were cleaned with paper towels to remove the mucilage, oven dried for 72h at 65 °C, and peeled to separate nib and testa. Due to the small sample size (half of the beans from a single pod), testae and nibs of only three individual beans per sample were digested separately and their elemental composition was measured with ICP-MS, as described above. The temperature in the centre of the fermentation box was measured daily using a digital thermometer (Custom, CT-422WR, Hitchcock, TX, USA). Nib and mucilage pH were determined before and after fermentation in the bulk cacao surrounding the micro-fermentation samples, using the methods described above.

5.2.2.2. Visualisation of Cd mobility on a metal binding gel using LA-ICP-MS

A novel visualisation method was developed to map mobile Cd in cacao beans at mm resolution, thereby using the principles for mapping mobile elements in 2D in soils with the Diffusive Gradient in Thin film technique (DGT) developed by Santner and co-workers (Santner et al. 2015). In brief, a sample (here a cacao bean cut transversally) is exposed to a gel that contains a metal ion selective binding agent. The mobile metal ions migrate from the source (the cacao bean) to the gel. After a given deployment time, the gel is removed, dried, and its surface composition is scanned with LA-ICP-MS.

Metal binding gels containing micro-milled Chelex-100 resin were prepared as described by Zhou et al. (2018), with a resin-to-gel ratio of 4.5 % (w/w). Due to the small size of the resin beads (< 10 µm), this gel can be used for chemical analysis with high spatial resolution. Visualisation of mobile Cd was performed on 5 of the 15 micro-fermentation pods and included two unfermented and two fermented beans from each pod. Immediately after sample collection (either before or after fermentation, not dried), beans were transversely cut in half using a clean razor blade and placed on the micro Chelex gel overnight. A Nuclepore® membrane (0.2 µm pore size, Whatman Maidstone, UK) was placed in between the beans and the gel as a barrier to avoid particulate transfer from the beans to the gel. The micro Chelex gel acts as a zero sink for cationic trace metals. It binds mobile Cd ions present in the contact surface with the cacao bean and thereby creates a print of the mobile Cd in the beans. Imprinted Chelex gels were stored in plastic Ziplock bags with several drops of ultrapure water to keep the gels hydrated until further analysis. Gels were dried on 0.45 µm Millipore membrane with Whatman chromatography paper as a support (3 mm CHR), in a slab gel dryer (DrygelSr., Hoefer Inc., Holliston, MA, USA) for at least 48 hours. During drying, gels were covered with a sheet of clean polyethylene plastic to protect the reactive layer. Dried gels were then fixed on glass microscopy slides using double sided tape and submitted to LA-ICP-MS analysis.

For elemental analysis, a quadrupole 7700cs ICP-MS (Agilent Technologies) mounted with platinum cones was used. The sensitivity and operational conditions of the ICP-MS (stability, background and mass calibration) were initially checked using a 1.0 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, Fremont, CA, USA) and coupling was optimised using a NIST 612 glass by monitoring ^{238}U and ^{232}Th for maximal sensitivity, and a U to Th ratio as close as possible to the unit. Visualisation of Ca, Cd, K, Ni and Zn fixed on micro Chelex gel was performed by running several ablation line scans with a 20 Hz laser shot repetition rate, fluency maintained between 4.1 and 4.5 J cm^{-2}, a laser beam of 50 μm^2 and a scan speed of 50 µm s^{-1}. Ablated material was transported with 800 mL min^{-1} He and mixed with Ar gas 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), ^{39}K (0.005 s), ^{44}Ca (0.15 s), ^{60}Ni (0.1 s), and ^{64}Zn (0.1 s as integration time).

Several sets of two or three ablation lines (distance between lines within one set was 100 µm) were run on each sample as can be seen in Appendix IV (Figure IV.7). Signal intensities were averaged for each set of two to three ablation lines. To account for the differences in elemental composition between samples and between elements, signal intensities measured for each element (counts per second, cps) were divided by the elemental concentration determined in each sample by acid digestion and ICP-MS analysis as described above. Several gels were lost prior to LA-ICP-MS imaging as they were either compromised during storage and transportation or broken during gel drying. In addition, gels that displayed disproportionally low K intensities, i.e. factor 10 lower than the other samples, were excluded from the discussion below. Even though the affinity of the Chelex resin is lower for K compared to the other elements evaluated here (Cd, Ca, Ni and Zn), K concentrations in the nibs are high and K is readily water extractable (see results below). Therefore, low K intensities were considered as an indication for poor contact between the gel and the cacao bean, or as an indication that the gel was erratically used upside down, as these gels are typically asymmetric because Chelex resin beads settle to the bottom during gel casting.

5.3. Results and discussion

5.3.1. Lab scale fermentations with organic acid treatments

5.3.1.1. Effect of treatments on fermentation parameters

Both water and organic acid treatments had a clear effect on fermentation temperature. It dropped to ambient temperature within 24 hours after treatment (Figure 5.1). Pre-fermentation treatment (i.e. treatment on day 0) delayed heat production in the fermenting mass. In fermentation A, this effect was stronger for the acid treatment compared to the corresponding water treatment. The temperature profiles of lab scale fermentation A suggest that the effect of treatment on temperature was smallest when applying the acids after fermentation, as was performed in fermentation B.

The mucilage pH was strongly affected by organic acid treatments as this tissue was in direct contact with the treatment solutions. The final mucilage pH was significantly lower in acid treated cacao compared to blank and water treatments in both fermentation experiments (Table 5.2). Water treatment on day 0 largely increased the mucilage pH from 4.0 to 6.9 at the end of fermentation (day 5), potentially indicating suboptimal fermentation. Water treatment prior to fermentation likely delayed and/or impeded the outgrowth of yeasts in the first fermentation phase, which reduced the ethanol production. Ethanol is the main substrate for the acetic acid bacteria (AAB) in the third and final fermentation phase. During this phase, the mucilage pH decreases as AAB convert ethanol to acetic acid, and the fermentation temperature increases to > 45 °C because this process is strongly exothermic. Thus, reduced production of ethanol during the yeast phase may explain the higher final mucilage pH and lower maximum temperature observed in the pre-fermentation water treatment.

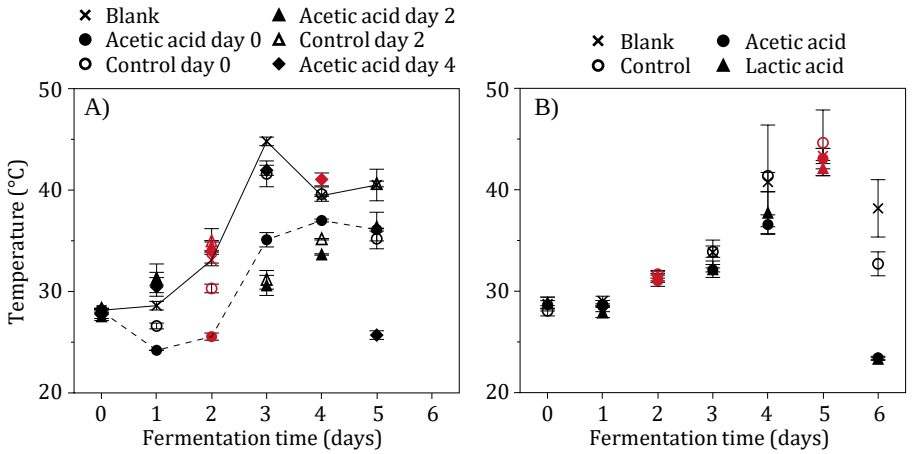


Figure 5.1 Treatment of fermenting cacao with water (control) and organic acids influences the temperature of the fermenting mass. Symbols represent the average temperature of duplicate (experiment A) or triplicate (experiment B) treatments, error bars are standard deviations. Red symbols correspond to mixing and/or treatment application. The temperature courses of blank (solid line) and day 0 acetic acid treatment (dashed line) are indicated for fermentation A. Graphs displaying the changes in nib and mucilage pH with fermentation time for the different treatments can be found in Appendix IV, Figure IV.2.

At the end of experiment A, the nib pH was significantly lower in the blank, and acetic acid day 2 and day 4 treatments compared to acetic acid day 0 and water treatments (Table 5.2). The acetic acid treatments in experiment A thus did not lower the nib pH below that of the blank, and results of the water treatments indicated that liquid application hindered nib acidification with fermentation. This again suggests reduced availability of ethanol substrate for the AAB, as nib acidification mostly results from penetration of acetic acid into the nibs. Acetic acid treatment before fermentation (day 0) immediately decreased the nib pH, but this effect disappeared with fermentation time (significant decrease from pH 6.5 to 5.9 within one hour after treatment, results not shown). Despite this immediate penetration of the acetic acid into the nibs, pre-fermentation treatments yielded higher final nib pH values compared to blank treatments, which was likely related to the effects of pre-fermentation liquid application on the overall fermentation (i.e. less acetic acid production from fermentation activity). Much larger acid doses were applied in fermentation experiment B compared to experiment A (35 and 53 g kg^{-1} cacao compared to 13 and 15 g kg^{-1} cacao, Table 5.1). These treatments were applied only after fermentation and resulted in significantly lower final nib pH values (on average 4.4 for lactic acid and 4.5 for acetic acid treatment) compared to blank or water treatment (average nib pH 5.2). A more detailed overview of the changes in nib pH with fermentation time can be found in Appendix IV (Figure IV.2). The effect on nib pH was again already present shortly after acid application. The average nib pH decreased from 5.3 to 4.6 with lactic acid application and from 5.3 to 4.7 with acetic acid application, within the first hour after application (P-value < 0.05). Penetration of both lactic and acetic acid into the nib thus occurs quickly and does not require fermentation, as equally fast acid penetration was also observed in the pre-fermentation treatment of experiment A. It should be noted

that, while both lactic and acetic acid treatments in experiment B resulted in a lower nib pH compared to the blank and water treatments, final nib pH values were still within the range for fermented cacao beans reported in literature, i.e. pH 4.0 – 5.5 (Schwan and Wheals 2004; Belitz et al. 2009; Papalexandratou et al. 2011; De Vuyst and Weckx 2016). The differences in final nib pH between acid treated and untreated cacao (blank or water treatments) in experiment B thus may have been related to less extensive fermentation in these small planter pots with untreated cacao, compared to a full scale commercial fermentation.

5.3.1.2. Effect of treatments on tissue Cd concentrations

A detailed overview of nib and testa Cd concentrations with fermentation time can be found in Appendix IV (Figures IV.3 and IV.4), a summary is given in Table 5.2. Nib Cd concentrations were not significantly affected by fermentation for any of the applied treatments, except for the replicates treated with acetic acid on day 2 in experiment A, where nib Cd decreased from 2.3 ± 0.3 mg Cd kg^{-1} before fermentation to 2.0 ± 0.1 mg kg^{-1} after fermentation (day 5) (Appendix IV, Figures IV.3 and IV.4). In both fermentation experiments, no significant differences could be observed in final nib Cd concentrations among the different treatments (Table 5.2). However, final testa Cd concentrations did differ significantly among treatments in both experiments. Because the testa represents a much smaller weight fraction compared to the nib, the total Cd stock in the testa is also smaller. This explains why outward Cd migration from nib to testa can result in a significant increase in the testa Cd concentration but not in a detectable decrease in the nib Cd concentration. However, patterns were not consistent as testa Cd concentrations increased in some treatments but decreased in others (Appendix IV, Figures IV.3 and IV.4).

While both lactic and acetic acid treatments in experiment B resulted in a similar final nib pH of 4.5, testa Cd increased more extensively with acetic acid compared to lactic acid treatment. Both lactic and acetic acid thus penetrated and acidified the cacao nib, but testa Cd results suggested that only acetic acid treatment resulted in relevant Cd mobilisation. However, this hypothesis was not confirmed by the results of the water extractions, as the water extractable Cd fractions in the nibs of both lactic and acetic acid treated cacao were similar at the end of fermentation and did not differ from blank or water treatments (P-value > 0.05). In experiment A, the total nib and testa Cd concentrations decreased and increased significantly with decreasing nib pH (Figure 5.2). In experiment B, no significant correlations could be found. Still, a trend of increasing testa Cd concentrations with decreasing nib pH could be discerned, with the acetic acid treated samples standing out in the low pH/high testa Cd area of the graph (Figure 5.2.B, top left). This relation between tissue Cd and nib pH suggests a pH dependent mobilisation of Cd in the nib tissue. Indeed, the water extractable nib Cd fraction increased significantly with decreasing nib pH in both experiments. The effects of different fermentation treatments on tissue elemental concentrations and water extractable nib fractions of Ca, Cu, K, Mn, Ni, P and Zn are reported in Appendix IV (Table IV.1 and Figure IV.5).

Table 5.2 Nib pH, mucilage pH and tissue Cd [average ± standard deviation of duplicates (experiment A) or triplicates (experiment B)] at the end of fermentation (day 5 for experiment A, day 6 for experiment B) depending on the cacao treatment. Letters denote significant differences within rows (Tukey's HSD test, P-value < 0.05). ns = not significant.

Fermentation experiment A, n = 2						
Treatment	Blank	Control (water)	Control (water)	Acetic acid	Acetic acid	Acetic acid
Day of treatment	/	Day 0	Day 2	Day 0	Day 2	Day 4
Nib pH	4.58 ± 0.04 ^{B}	4.88 ± 0.01 ^{A}	5.02 ± 0.02 ^{A}	4.88 ± 0.15 ^{A}	4.54 ± 0.01 ^{B}	4.34 ± 0.04 ^{B}
Mucilage pH	4.79 ± 0.30 ^{B}	6.91 ± 0.25 ^{A}	5.03 ± 0.02 ^{B}	3.23 ± 0.04 ^{C}	3.82 ± 0.01 ^{C}	3.68 ± 0.03 ^{C}
Nib Cd (mg kg^{-1})	1.98 ± 0.12^{ns}	2.10 ± 0.10	2.09 ± 0.004	2.23 ± 0.12	2.00 ± 0.02	2.06 ± 0.20
Testa Cd (mg kg^{-1})	3.75 ± 0.72^{AB}	2.85 ± 0.36^{AB}	2.50 ± 0.03 ^{B}	2.85 ± 0.35 ^{AB}	4.19 ± 0.17 ^{A}	4.25 ± 0.21 ^{A}
Fermentation experiment B, n = 3						
Treatment	Blank		Control (water)	Acetic acid		Lactic acid
Day of treatment	/		Day 5	Day 5		Day 5
Nib pH	5.23 ± 0.04 ^{A}		5.24 ± 0.07 ^{A}	4.51 ± 0.03 ^{B}		4.44 ± 0.03 ^{B}
Mucilage pH	6.63 ± 0.30 ^{A}		5.53 ± 0.80 ^{A}	3.54 ± 0.02 ^{B}		2.73 ± 0.01 ^{B}
Nib Cd (mg kg^{-1})	0.51 ± 0.02 ^{ns}		0.44 ± 0.04	0.51 ± 0.01		0.51 ± 0.04
Testa Cd (mg kg^{-1})	0.55 ± 0.06 ^{BC}		0.40 ± 0.04 ^{C}	1.07 ± 0.11 ^{A}		0.65 ± 0.05 ^{B}

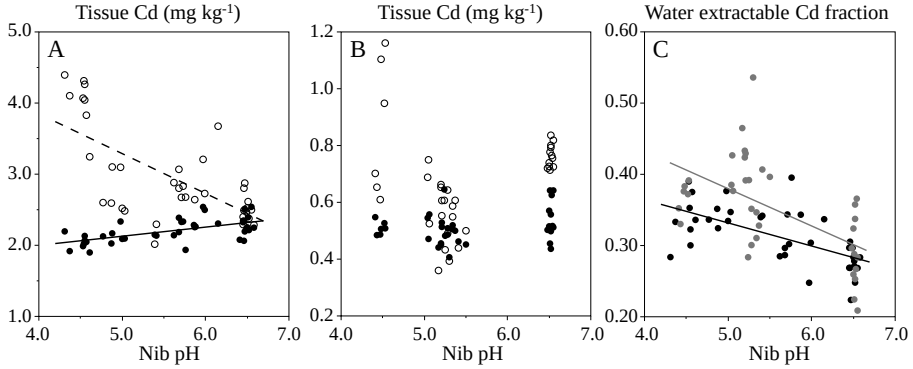


Figure 5.2 (A) Both nib Cd (●, solid line) and testa Cd (○, dashed line) are significantly correlated to nib pH in the combined dataset of experiment A; Pearson correlation coefficients: r = 0.65 (nib) and r = -0.67 (testa). (B) Nib Cd (●) and testa Cd (○) are not significantly correlated to nib pH in experiment B, testa Cd concentrations in the acetic acid treated samples (top left in graph) are significantly higher compared to blank, water and lactic acid treated samples (Table 5.2). (C) Water extractable nib Cd (fraction of total Cd) is significantly correlated to nib pH in both experiments; r = -0.63 (experiment A, black) and r = -0.57 (experiment B, grey). Water extractions were performed in liquid:solid ratio 8:1 (mL g^{-1}) with a total extraction time of 5 days.

The water extraction of unfermented ground bean tissues artificially acidified to pH 4.5 indicated an increase of water extractable nib Cd at lower pH (Figure 5.3), again suggesting the importance of nib acidification for Cd migration during fermentation. Similar effects of acidification were also observed for e.g. Mn and Zn (Appendix IV, Figure IV.6). Water extractable Cd fractions were much lower in the testa compared to the nib. Although testa Cd mobility increased with fermentation, it was not significantly affected by artificial acidification. The increased water extractability of Cd in the fermented testa may represent the Cd that migrated from nib to testa during fermentation, while the Cd present in this tissue prior to fermentation was less water extractable (Figure 5.3). Interestingly, Cd in fermented nibs was largely extractable with water, the extracted fraction in fermented samples ranged between 28 and 54 %, depending on the fermentation treatment. Although the experimental setup used here is not practical for industrial treatments (contact time of five days, high liquid:solid ratio and finely ground nibs, which are not an intermediate product in industrial chocolate production), these results do indicate that extraction of Cd from cacao nibs through washing with water or even chelating agent solutions has potential as a postharvest mitigation strategy. If effective, water and/or chelating agent washing can be applied to the broken nib fragments obtained after testa removal.

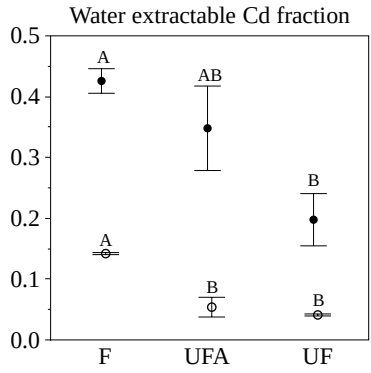


Figure 5.3 The water extractable Cd fraction (used as a proxy for Cd mobility) is larger in the nib (●) compared to the testa (○), and is significantly higher in fermented (F, nib pH 5.0 and testa pH 5.2) than in unfermented (UF, nib pH 6.5 and testa pH 4.4) tissues. Water extraction of unfermented beans in an acid medium (UFA, pH 4.5 to mimic pH of fermented nibs) had intermediate effects. The cacao used here originated from the blank treatments of lab fermentation experiment A, day 0 and day 5. Symbols represent average water extractable fractions (n = 2) and error bars are standard deviations. Letters indicate significant differences for each material (nib and testa), based on Tukey's Honestly Significant Difference test (P-value < 0.05). Water extractions were performed in liquid:solid ratio 24:1 for nibs and 48:1 for testa (mL g^{-1}) with a total extraction time of 5 days.

5.3.2. Micro-fermentation

5.3.2.1. Effect of fermentation on tissue Cd concentrations

The temperature in the centre of the fermentation box increased from ambient temperature before fermentation (26 °C) to a maximum of 50 °C on the third day, after which it decreased to 46 °C by day 4. These changes were more pronounced than those in the lab scale fermentations described above. The mucilage pH increased from 3.9 (day 0) to 4.4 (day 4), while the nib pH decreased from 6.3 (day 0) to 4.8 (day 4). Even though all cacao pods were collected from a single field, both nib and testa Cd concentrations varied by more than a factor 3 among the different pods (CV 37 % for nibs and 31 % for testae), while variations in tissue Cd among bean replicates within a pod were relatively small (average CV 12 % for nibs and 10 % for testae) (Figure 5.4). Similar large variations in bean Cd within a single field have been reported previously by Argüello et al. (2019), who reported an average within-field CV of 39 % in a nationwide study in Ecuador. Pairwise analysis of single pod micro-fermentations revealed an effect of fermentation on both nib and testa Cd concentrations, which could have been masked by field variation in an experiment using composite samples. A pairwise t-test indicated a significant decrease in the nib Cd concentration with fermentation by - 0.30 mg Cd kg^{-1}, and a significant increase in the testa Cd concentration by + 0.75 mg Cd kg^{-1} (P-value < 0.05). The nib Cd concentration decreased on average by a factor 1.25, which is similar to the factor 1.3 decrease in nib Cd concentrations with fermentation reported in Chapter 4. Significant decreases in nib elemental concentrations were also observed for Cu (factor 1.3), K (factor 1.3), Ni (factor 1.3) and P (factor 1.1), while testa elemental concentrations increased for Cu (factor 2.9), K (factor 2.5), Mn (factor 2.6), Ni (factor 2.2), P (factor 5.5) and Zn (factor 2.1) (Appendix IV, Table IV.2).

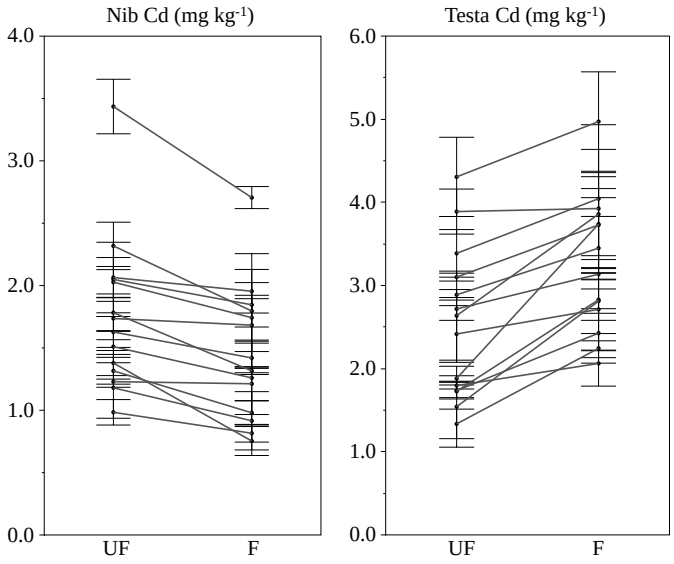


Figure 5.4 Fermentation decreased the nib Cd concentrations (left) and increased the testa Cd concentrations (right) in the micro-fermentation experiment. Solid lines connect data from the same pod (same mesh bag subsample), points are averages of the three sampling replicates of each pod and error bars are standard deviations. UF = unfermented, F = fermented.

Considering the weight fractions of nib and testa from which mucilage had been removed (average weight fractions nib 0.93 and testa 0.07, Chapter 4), the average mass of Cd lost from the nibs (0.28 ± 0.20 mg Cd kg^{-1} $_{bean}$) was significantly larger than the mass of Cd gained in the testae (0.05 ± 0.03 mg Cd kg^{-1} $_{bean}$). Similar discrepancies could be found in the mass balances of Cu, K, Ni and P, i.e. the decrease in mass of these elements in the nib was significantly larger than the mass gained in the testa. This discrepancy in mass balances is likely related to leaching of elements into the cacao mucilage (the liquid phase surrounding the cacao beans). Indeed, Cd, Cu, K, Ni and P concentrations in the mucilage (among other elements) have been reported to increase with fermentation by a factor 2 – 8 depending on the element and on the fermentation conditions (Chapter 4). Results in Chapter 4 also indicated that fermentation reduced the total cacao bean Cd level by 15 %, likely due to leaching of Cd in the mucilage sweatings.

5.3.2.2. Visualisation of elemental mobility in unfermented and fermented cacao beans

While duplicate mobile elemental prints were originally generated before and after fermentation for five micro-fermentation pods, only eight samples are discussed here as several samples had to be excluded, as explained in the materials and methods section. The contact area between the bean and the Chelex resin gel could be clearly discerned on dried gels, as the samples stained the gel and left a dark imprint (Figure 5.5 and Appendix IV, Figure IV.7). This imprint allowed targeting the contact area during LA-ICP-MS analysis. However, the edges of the imprints were blurry and thus the exact location of contact between the gel and the testa and/or mucilage could not be discerned from the nib imprint. Visualisation of the elemental imprints on the Chelex resin gels by LA-ICP-MS indicated that mobility of Cd, Ni and Zn increased with fermentation, with largest mobile

element signals located in the centre of the nib imprints (Figure 5.5). Mobile K also increased while mobile Ca decreased with fermentation (Appendix IV, Figure IV.8). For Cd and Zn, the observations are in accordance with the increased water extractability of these elements in fermented nibs (Figure 5.3 and Appendix IV, Figure IV.5). The water extractability of Ni in nibs was not significantly affected by fermentation, more than 70 % of nib Ni was water extractable regardless of the nib pH (Appendix IV, Figure IV.5). However, signal intensities for Ni on the Chelex gels were clearly larger for fermented than for unfermented samples, and nib Ni concentrations were positively correlated to nib pH in both fermentation experiments (Appendix IV, Table IV.2 and Figure IV.5). The maps of the mobile element prints obtained here (signal larger for nib than for testa) were markedly different from the LA-ICP-MS images of total element composition in cacao beans (signal larger for testa than for nib) (Figure 4.1). The lack of element signal peaks at the edges of the Chelex imprints was in accordance with the lower water extractable Cd fraction found in the testa compared to the nib.

5.3.3. Impact of the experimental setup on effects of fermentation on cacao nib Cd

The results of the micro-fermentation experiment revealed a factor 1.25 decrease in nib Cd with fermentation. No such effect was found in the lab scale fermentation experiments (5 kg cacao fermented in plastic planter pots). The lack of effect observed in the lab scale experiments was likely unrelated to a lack of sufficient acidification. Indeed, the final nib pH for the blank treatments in lab scale fermentation A (pH 4.6) was even lower than that reached in the micro-fermentation experiment (pH 4.8). The difference in observed effects between both setups may be related to differences in fermentation temperatures. While small lab scale fermentations of 5 kg cacao have several practical advantages over full scale or micro-fermentations for experimental work, the small fermentation volume offers less heat retention compared to that in a full scale fermentation box of > 200 kg cacao. The temperature in the centre of the lab scale fermentation vessels reached 45 °C in the blanks of both experiments (A and B), which was on the lower end of maximum temperatures typically reported in literature (Lima et al. 2011) and lower than the temperature reached in the full scale fermentation described above (50 °C). The lab scale temperature profiles showed a delay in initial temperature rise, especially in experiment B where the fermentation temperature only started to increase after two days (Figure 5.1). Jespersen et al. (2005) reported that yeast outgrowth in the outer layers of the fermenting cacao mass is delayed in comparison to that in the centre of the cacao bean mass. They found that the maximum yeast cell count was reached after 24 hours in the centre of the fermenting mass, while it was only reached after 72 h in the outer layers. The delay in fermentation activity observed in the lab scale fermentations, indicated by the lag phase in the temperature profile, may be related to the small size and large surface to volume ratio of the lab scale fermentation vessels. Fermentation temperature may thus be the missing piece of the puzzle explaining the lack of decrease in nib Cd concentrations in the lab scale experiments. Sufficient heat may be required to allow acidification-driven mobilisation of Cd in cacao nibs during fermentation. Additional research under controlled temperature conditions is required to verify or reject this hypothesis.

The differences in detected fermentation effects between both setups may also be related to the large variation in bean Cd concentrations. Results of the micro-fermentation

experiment demonstrated how large inter-pod variability (up to a factor 3, all pods collected from the same field) can mask the effect of fermentation on bean Cd concentrations. The coefficient of variation of the nib Cd concentration between all nib samples prior to treatment in lab scale fermentation B was large (CV 13 %). A power test was performed to assess the number of replicates in the lab scale experiments, using the online tool developed by Rollin Brant [university of California (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html)]. To account for differences in the initial bean Cd concentrations, the CV was used in the calculation instead of the standard deviation. Based on that analysis, at least four replicates are required to detect a factor 1.3 effect with statistical significance (CV = 13 %, α = 5 % and power = 80 %). However, the power test also indicated that the two replicates per treatment in lab scale fermentation A were sufficient to detect that effect (CV between all nib samples prior to treatment in experiment A was only 6 %).

5.3.4. Organic acid fermentation treatments as a mitigation strategy for Cd in cacao

The micro-fermentation experiment indicated that nib acidification is the driver for Cd mobilisation in cacao nibs, and thus also for reducing the final nib Cd concentration. However, organic acid application in lab scale fermentation setups did not reduce the nib Cd concentration, probably because of the lower temperatures compared to full scale fermentations, a factor that will be further explored in the subsequent Chapter 6. Organic acid treatments reduced the nib pH, but only when applying large acid doses, and the final nib pH was still within the range of nib pH values typically reported for full scale commercial setups (pH 4 – 5). Thus, nib Cd does decrease with decreasing nib pH but organic acid treatments during fermentation may not be the best option to obtain the required nib acidification. Optimising fermentation conditions (e.g. duration, aeration frequency or size of fermentation boxes) for optimal nib acidification can likely yield similar final nib pH values compared to organic acid treatments. For example, acetic acid concentrations in cacao mucilage have been reported to be higher when turning is performed (Camu et al. 2008b). Finetuning of fermentation parameters for increased acetic acid production is likely also easier to implement. It is cheaper and it entails less interference in the fermentation process. Still, the impact of such fermentation practices on the flavour should be taken into account, as excessive acetic acid has been related to less pronounced cacao flavour in the final product (Camu et al. 2008a).

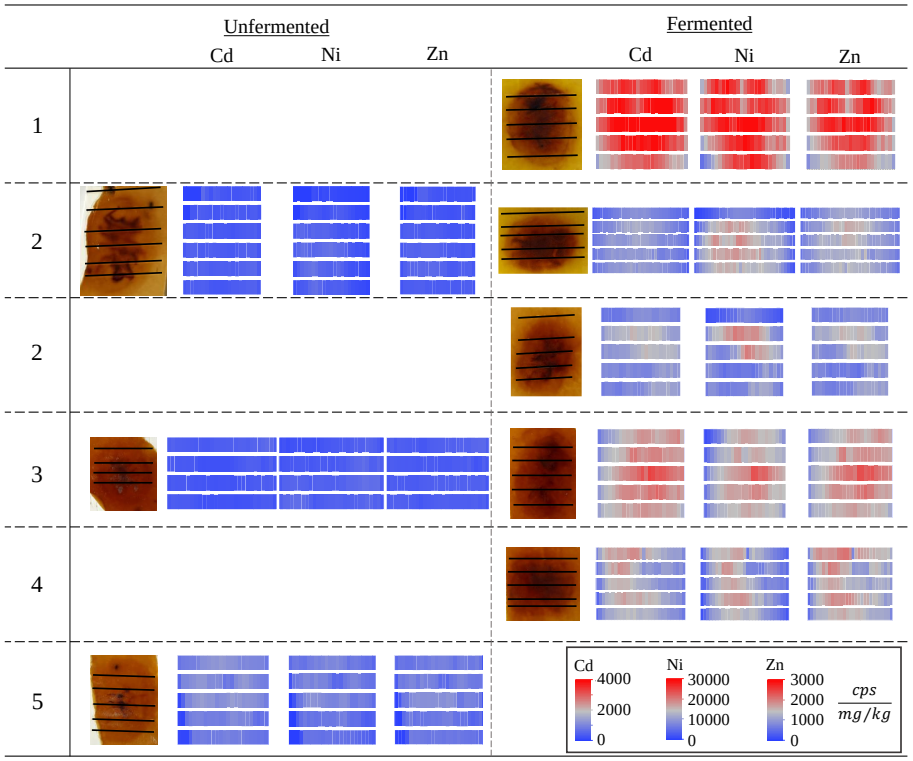


Figure 5.5 Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) visualisation of Cd, Ni and Zn diffused from the bean toward ground Chelex 100 gel ("mobile elements"). These maps indicate higher element mobility in fermented compared to unfermented cacao beans. Cacao bean samples originate from different single pod micro-fermentation samples (1–5). Measured signals for each element (counts per second, cps) are normalised to the elemental composition determined for that sample by acid digestion and ICP-MS analysis. Each horizontal line represents the average signal of two or three ablation lines run in close parallel, indicated by black lines on the corresponding pictures. The width of each pixel corresponds to a distance of 0.5 mm on the ablation line. Larger scale pictures of gel imprints with ablation lines can be found in Appendix IV (Figure IV.7).

5.4. Conclusions

The mobility of Cd in cacao nibs increases during fermentation, as indicated by enhanced water extractions and by enhanced diffusible Cd identified by LA-ICP-MS imaging. Water extractable nib Cd was negatively correlated to nib pH and increased through artificial acidification, indicating nib pH as the driving force behind nib Cd mobilisation. Migration of Cd from the cacao nibs to the testae during fermentation is thus likely related to increased Cd mobility in the nibs due to nib acidification. Organic acid amendments before or during fermentation were not effective in reducing the nib pH due to their negative impact on fermentation (lower fermentation temperatures and excessively high mucilage pH). The application of acetic or lactic acid as a post-fermentation treatment lowered the nib pH values compared to blank and water treatments, but only when applying large acid doses. Although no significant reductions in nib Cd concentrations could be observed with organic acid treatment, the reduced nib pH did increase testa Cd concentrations and nib Cd mobility. Micro-fermentations with single cacao pods inside a full scale fermentation box indicated a factor 1.25 reduction in nib Cd with fermentation. Optimisation of fermentation parameters for acetic acid production can likely offer similar nib acidification compared to organic acid treatments, without the added cost. However, a trade-off needs to be made between nib Cd reduction and flavour quality of the final product as excessive nib acidity is considered an off-flavour. Considering the large fraction of Cd that could be extracted with water from fermented cacao nibs (up to 50 %), washing of cacao nibs with water and/or chelating agents after fermentation and prior to roasting holds promise as a mitigation strategy for Cd in cacao.

Chapter 6
Outward migration of cadmium from cacao nibs during fermentation is related to the combination of enhanced acidity and temperature: mimicking fermentation through incubation

Summary

Previous chapters showed that cacao fermentation may reduce cadmium (Cd) concentrations in the cacao nibs, the central part of the cacao bean used to produce chocolate. Nib acidification initiates migration of Cd from nib to testa during fermentation, but experimental addition of acids did not mimic the trends, suggesting that other factors are involved. This study was set up to reveal the potential effect of fermentation factors on Cd migration out of the nib. Fermentation conditions were mimicked through incubation of cacao beans with combinations of different incubation temperatures, and acetic acid and ethanol concentrations in the incubation media, using a D-optimal experimental design with 44 runs. Mobilisation of Cd in the nibs was most pronounced when acetic acid addition was associated with higher temperatures, whereas ethanol did not have statistical effects. Incubation of cacao beans in typical fermentation conditions (45 °C and 20 g L^{-1} acetic acid) reduced the nib Cd concentration by a factor 1.3, and this reduction factor increased to 1.6 in more extreme conditions, i.e. 65 °C with 40 g L^{-1} acetic acid. If such incubation was followed by an overnight water extraction of ground nibs, nib Cd concentrations were even reduced by factors > 2. Fine-tuning of fermentation parameters for optimal production of heat and acetic acid thus holds promise for reducing cacao nib Cd, especially when followed by nib washing prior to roasting.

6.1. Introduction

In 2019, threshold limits were implemented to limit the cadmium (Cd) concentration in cacao-derived products such as chocolate (European Commission 2014; Codex Alimentarius Commission 2018). Strong correlations have been reported between the Cd concentration in chocolate and the cacao solids content of the product; and between the chocolate Cd concentration and the origin of the cacao beans used in production (Chapter 3). These correlations demonstrate that Cd in chocolate originates mostly from the cacao beans, rather than from other ingredients or contamination during processing. As a result, the cacao processing industry has set requirements for the cacao beans that they purchase for their suppliers and these unofficial industry thresholds vary among companies (Chapter 2). To facilitate the discussion below, a cacao bean threshold value of 0.60 mg Cd kg^{-1} dw in is used. Those Cd-cacao thresholds are especially causing concern in Central and South America, as cacao beans from those geographical areas have been reported to contain larger Cd concentrations compared to cacao from other origins such as Africa (Table 2.1). Research for mitigation strategies to lower Cd concentrations in cacao has focussed mostly on the use of soil amendments, agronomic practices and selection of low Cd accumulating cultivars. Results for these techniques are promising but reduction factors obtained thus far are limited, the use of soil amendments is restricted by the soil properties of the field, and research on genetics-based mitigation strategies is still in an early phase and requires long term studies (Chapter 2). However, considering that the EU regulations only apply to the final product, mitigation strategies may also be implemented during postharvest processing. A detailed overview of the postharvest process of cacao is given in Chapter 1.

Previous results have indicated that cacao fermentation can decrease nib Cd concentrations by up to a factor 1.3 (Chapters 4 and 5). Despite the recurring observation that nib Cd mobilisation during fermentation is related to nib acidification, addition of organic acids during the fermentation process in lab scale fermentations was ineffective for reducing nib Cd concentrations (Chapter 5). Part of that may be related to high bean Cd variability but it was also speculated that temperature effects may be involved because lab scale fermentations presented lower temperature peaks compared to full scale fermentations. Cacao bean death during fermentation, i.e. loss of germination potential and disruption of internal cellular structures, has been attributed to fermentation heat and to penetration of ethanol and acetic acid from the mucilage into the cacao nibs (Quesnel 1965; Schwan and Wheals 2004; Thompson et al. 2007; Fowler and Coutel 2017). Considering that impact of temperature and ethanol on the structural integrity of the cacao nibs, Cd mobilisation in cacao during fermentation may require cacao bean death and the related loss of structural integrity. Elevated fermentation temperatures and/or ethanol concentrations in the mucilage may be required for nib Cd mobilisation, in addition to nib acidification due to acetic acid penetration. The present study was set up to better understand the impact of fermentation on Cd in cacao, and to reveal if fermentation temperature and/or ethanol concentrations in the mucilage determine acidification-driven mobilisation of Cd in cacao nibs. To this end, cacao bean incubations were performed to identify the effects of temperature, acetic acid and ethanol on Cd mobilisation in cacao nibs. The experimental setup was adapted from the incubation

experiments described previously by Biehl and Passern (1982), Kadow et al. (2015), Eyamo Evina et al. (2016) and John et al. (2020), among others. These incubation experiments are models for the fermentation under controlled conditions, the use of such experimental setup dates back to the beginning of the 20th century (Roelofsen 1958, and references therein). Better understanding of the effect of the fermentation parameters temperature, ethanol and acetic acid on Cd concentrations in cacao, as well as their potential interactions, can allow development of fermentation guidelines for optimal reduction of nib Cd concentrations during fermentation.

6.2. Materials and methods

6.2.1. Experimental design

A D-optimal experimental design was used to assess the effect of temperature (T), ethanol and acetic acid on the mobilisation of Cd in cacao nibs. Three levels were included for each factor: a baseline level [T = 25 °C; $C_{ethanol}$ = 0 g L^{-1}; $C_{acetic\ acid}$ = 0 g L^{-1}], a central level corresponding to typical fermentation conditions [T = 45 °C; $C_{ethanol}$ = 35 g L^{-1}; $C_{acetic\ acid}$ = 20 g L^{-1}], and an upper extreme level [T = 65 °C; $C_{ethanol}$ = 70 g L^{-1}; $C_{acetic\ acid}$ = 40 g L^{-1}]. A D-optimal experimental design with 40 runs was generated in JMP® Pro version 15.1.0 (SAS Institute 2019) and four additional runs were added to the central level of the design to ensure that typical fermentation conditions were represented in the experiment. A run is a treatment or a replicate of that treatment. The D-optimality criterion was selected for the design of this experiment because it optimises the precision of effect estimates and is, therefore, most suitable to test for significance of different factors. The final design comprised 44 runs and a detailed description of the design can be found in Appendix V (Table V.1). To minimise the effect of sample variation, beans of different pods were never mixed and each of the 44 runs used beans derived from one pod; the pairwise comparison of compositions between the beans of a run and the untreated beans of a corresponding pod allowed to derive the fermentation effect (see below: data analysis).

6.2.2. Cacao material and experimental setup

Ripe cacao pods (Nacional cultivar) were collected by local technicians in Guayas province (Ecuador) and shipped to Belgium within 48 hours after harvest. Pods were opened using a kitchen knife and the beans from each fruit were split in two equal subsamples: one subsample to determine the bean composition prior to incubation (*untreated*), and one subsample for incubation treatment (*treated*). The cacao pods contained 16 to 51 beans and subsamples for incubation consisted of 8 – 26 beans with an average (± stdev) subsample weight of 49 ± 9 g (wet weight, mucilage not removed). These subsamples for incubation were placed in individual sealed plastic containers with 300 mL of the contact solution, i.e. deionised water with 0 – 40 g L^{-1} acetic acid and 0 – 70 g L^{-1} ethanol. Samples were incubated for 72 hours at 25 °C (incubation room), 45 °C or 65 °C (oven).

6.2.3. Cacao bean sample preparation and chemical analyses

6.2.3.1. Sampling the contact solutions

The contact solution pH was measured before and after treatment. After treatment, a 10 mL subsample was taken from the contact solution of each run. Samples were diluted 20 (25 °C runs), 100 (45 °C runs) or 200 times (65 °C runs) with deionised water and filtered

with a 0.45 µm syringe filter prior to Inductively Coupled Plasma Mass Spectrometry analysis (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, USA).

6.2.3.2. Determining the water extractable element fractions and nib pH

The adhering mucilage was removed from the beans using paper towels and the beans were oven dried for 72 hours at 70 °C. For each subsample (both untreated and treated), four dried cacao beans were randomly selected. Beans were peeled to remove the testae, peeled nibs were ground in an electrical coffee grinder and the ground nibs were sieved to obtain a more homogeneous particle size (800 µm test sieve, VWR). Ground nibs were then incubated in an end-over-end shaker in a 1:20 (g:mL) solid:deionised water ratio for 24 hours. After incubation, samples were centrifuged for 25 min at 2000 g (Heraeus Multifuge X3R, Thermo Scientific, Waltham, MA, USA), the pH of the supernatants was measured to determine the nib pH, and subsamples of the supernatants were diluted 80 times and acidified to 1 % nitric acid (HNO_3, NORMATOM® 67 – 69 % w/w, VWR International, Radnor, PA, USA) prior to ICP-MS analysis. Duplicates were included at intervals of seven samples and the average coefficient of variation (CV) for duplicate Cd analyses was 10 %.

6.2.3.3. Determining the elemental composition of nib and testa

For each sample, two dried cacao beans were randomly selected and peeled to separate nibs and testae. Nib and testa from each individual bean were analysed separately. Cacao nibs (sample weight 1.0 ± 0.2 g) were acid digested in 20 mL HNO_3 using a microwave digestion system (MARS 6, CEM, Matthews, NC, USA). Microwave assisted digestion was executed in two steps (max. temperature 100 °C and 180 °C) and the pressure was released from the digestion vessels in between steps to prevent excessive pressure build-up. Nib digests were brought to a volume of 50 mL and diluted 100 times with Milli-Q water (18.2 MΩ cm^{-1}) prior to ICP-MS analysis. Testa samples (sample weight 0.07 ± 0.02 g) were acid digested in 3 mL HNO_3 for 8 hours in an open digestion block at a max. temperature of 135 °C. Samples were digested until near-dry, brought to a volume of 10 mL and diluted ten times with Milli-Q water (18.2 MΩ cm^{-1}) prior to ICP-MS analysis. The method limit of quantification (LOQ) was 0.004 mg Cd kg^{-1} dw. In each digestion, blank samples (triplicate for microwave digestion, quadruplicate for open block digestion) were included and treated equal to the unknown samples. Certified reference material NIST 2384 baking chocolate was included in triplicate in each batch (certified concentration 0.0734 ± 0.0077 mg Cd kg^{-1}) at a sample weight of 1 g (microwave digestion) or 0.09 g (open block digestion). Recovery of the certified reference material ranged between 87 and 111 %. The distributions of elements between the different tissues at the end of fermentation (contact solution, nib and testa) were calculated based on the weight of duplicate nibs and testae for each run. To obtain total nib and testa dry weight in each incubation container, the average nib and testa weight from duplicate beans was multiplied by the number of beans in each incubation run. The average CV for duplicate beans was 7 % for the nib Cd measurements and 8 % for testa Cd measurements.

6.2.4. Data analysis

All statistical analysis was performed in JMP® Pro version 15.1.0 (SAS Institute 2019). To account for the large inter-pod (or inter-run) variation in elemental composition prior to treatment, concentration ratios ($\frac{Element\ conc.\ treated\ samples}{Element\ conc.\ untreated\ samples}$) were used as responses for all statistical analyses. Multivariate linear regression analysis including second and third order interactions was performed to assess the effect of the different factors (temperature, acetic acid and ethanol) on these elemental concentration ratios in nib or testa. Significance of the model effects was determined using the F statistic effect test (P-value < 0.01).

6.3. Results

6.3.1. Elemental composition of untreated cacao beans

Although all cacao pods were sourced from the same field, average nib Cd concentrations per pod before treatment ranged from 1.1 to 5.0 mg Cd kg^{-1}, with a coefficient of variation (CV) of 33 %. Similar high CVs were observed for Ni (28 %) and Cu (41 %), while CVs for essential elements and macro nutrients were < 10 % (K, Mg, P and Zn) (Appendix V, Table V.2). Elemental concentrations measured in the testa were generally lower than, or equal to, the concentrations measured in the nib (Cu, K, Mg, Mn, Ni, P and Zn), except for Ca and Cd. Testa Cd concentrations were on average 20 % higher than nib Cd concentrations measured in the same pod [95 % confidence interval (CI) for ratio testa Cd over nib Cd was 1.16 – 1.27, Appendix V Table V.2].

6.3.2. Effect of incubation treatments on the elemental composition of cacao beans

Incubations modified the colour of the contact solutions, which ranged from clear (samples incubated at 25 °C) to dark brown (samples incubated at 65 °C) (Appendix V, Figure V.1). Increasing concentrations of acetic acid generated pink hues in the contact solutions after incubation at 45 °C, likely due to polyphenol exudation from the nibs. Pink hues were also observed in the 65 °C treatments after 24 hours of incubation but became less noticeable as the colour of these samples darkened with incubation time.

Analysis of the contact solutions in which the cacao beans were incubated revealed that most of the Cd lost from the cacao nibs was transferred to the contact solution, rather than to the testa (Figure 6.1). This transfer of Cd to the solution was small (< 10 %) at low temperature and in the absence of acetic acid but was much larger at the highest temperature and in the presence of acetic acid. More than 40 % of total Cd was located in the contact solution after incubation at 65 °C with 20 or 40 g L^{-1} acetic acid. Similar migrations from nib to contact solution were observed for the other evaluated elements (Appendix V, Figure V.2). Increased incubation temperature resulted in a large migration from nib to contact solution for K and Ni, regardless of acetic acid concentrations (Appendix V, Figure V.2).

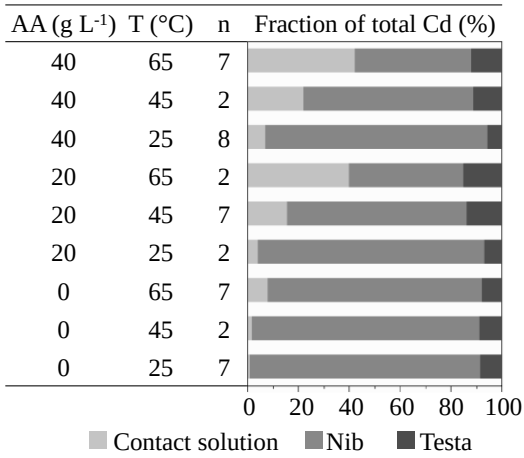


Figure 6.1 The distribution of Cd between the different tissues after treatment (nib, testa and contact solution) indicates migration of Cd from nib to contact solution and testa, especially in high temperature / high acetic acid conditions. AA = Acetic Acid, T = Temperature.

The concentrations of mineral elements in the testa increased with incubation (95 % CI of mean concentration ratio > 1 across all runs), for all elements except K. Testa Cd concentrations increased with incubation, on average, by a factor 1.7 (95 % CI of mean 1.5 – 1.9 across all runs). In contrast, elemental concentrations in the nib generally decreased with incubation (95 % CI < 1), for all elements except Ca and Cu. The nib Cd concentrations after incubation were, on average, a factor 0.89 of the initial concentration (95 % CI of mean 0.84 – 0.95 across all runs). Because Ca and Cu concentrations in the nibs were not significantly affected by the incubation treatments, both elements are not further discussed.

Ethanol had no significant effect on either nib or testa elemental compositions (Appendix V, Figure V.5). Increasing incubation temperature significantly reduced the nib concentrations for all elements (Table 6.1). Both acetic acid and the second order interaction between temperature and acetic acid had a significant negative effect on nib concentration ratios of Cd, whereas testa Cd concentrations were positively affected by temperature only, not by acetic acid. Similar trends were found for nib Mg, Mn, P and Zn. Conversely, changes in the testa elemental composition with incubation were only poorly explained by incubation temperature and/or acetic acid concentration (Table 6.1). Although testa Ni and P increased with incubation, this change in testa concentrations was not explained by incubation temperature and/or acetic acid concentration.

The nib pH decreased with increasing temperature and increasing acetic acid concentrations (Figure 6.2). Surprisingly, incubation at elevated temperature in the absence of acetic acid also resulted in slight nib acidification. The element concentrations in the nib, i.e. the treated over untreated concentration ratios, were positively correlated to nib pH for all elements and nib acidification largely explained the effect of incubation on nib Cd, Mg, Mn, P and Zn (R^2 values for correlations between nib element concentrations and nib pH were close to R^2 values of multivariate regression models, Figure 6.3).

Table 6.1 Incubation temperature and acetic acid concentrations significantly affect nib and testa elemental compositions, as determined by multivariate regression analysis (P-value < 0.01). Concentration ratios for each tissue were used as the response ($\frac{Element\ conc.\ treated\ samples}{Element\ conc.\ untreated\ samples}$) instead of absolute concentrations to minimise the effect of variability in initial concentrations among beans from different pods. The sign in brackets corresponds to the sign of the coefficient of that predictor.

	Nib		Testa	
	Significant factors	R^2	Significant factors	R^2
Cd	Temperature (-), Acetic acid (-), Temperature * Acetic acid (-)	0.69	Temperature (+)	0.27
K	Temperature (-)	0.89	/	/
Mg	Temperature (-), Acetic acid (-), Temperature * Acetic acid (-)	0.89	Temperature (+)	0.32
Mn	Temperature (-), Acetic acid (-), Temperature * Acetic acid (-)	0.83	Temperature (+), Acetic acid (+), Temperature * Acetic acid (+)	0.47
Ni	Temperature (-)	0.83	/	/
P	Temperature (-), Acetic acid (-), Temperature * Acetic acid (-)	0.80	/	/
Zn	Temperature (-), Acetic acid (-), Temperature * Acetic acid (-)	0.86	Acetic acid (+)	0.40

6.3.3. Effect of incubations on the extraction of Cd and other elements from cacao nibs

The water extractable element fractions in dried and ground nibs were lower in samples that were incubated at the highest temperature and acetic acid doses (results not shown). This is in contrast with the earlier findings (Figure 5.3) that fermentation enhances the water extractable fractions. However, the incubations performed here had a high solution to bean ratio, which explains the apparent contrast: a large fraction of the water extractable elements had already migrated towards to the contact solutions during incubation (Figure 6.1 and Appendix V, Figure V.2). To account for the effect of extraction during sample incubation, total water extractable fractions were calculated by summing the fractions extracted into the incubation solutions with the corresponding extracted fractions from the water extraction analysis performed on ground, dried material after incubation. The total extractable fractions increased with decreasing nib pH for Cd, Mg, Mn and Zn; while nib K, P and Ni were highly extractable regardless of nib pH (Figure 6.4). Water extractions performed on dried nibs before incubation indicated that large fractions of K (80 %), Ni (85 %) and P (50 %) were already water extractable in untreated cacao beans, while this share was much smaller for Cd (10 %), Mg (40 %), Mn (20 %) and Zn (10 %) (Appendix V, Figure V.3).

6.4. Discussion

6.4.1. Variation in bean Cd concentrations among pods

The large variation in nib Cd concentrations observed among pods prior to incubation (CV 33 %) was in accordance with previous reports. In an extensive nationwide study in Ecuador, Argüello et al. (2019) reported that cacao bean Cd concentrations vary on average 39 % between pods harvested from different trees in a single field. However, the variation in nib Cd concentration between beans from a single pod was small (average CV 7 %), demonstrating the benefit of using single cacao pods as replicates instead of larger composite samples. The difference in Cd concentrations between nib and testa observed here (testa Cd was a factor 1.2 higher than nib Cd) was smaller than the ratios generally reported in literature, i.e. factor > 1.5 larger Cd concentrations in the testa compared to the nib (Chapter 4).

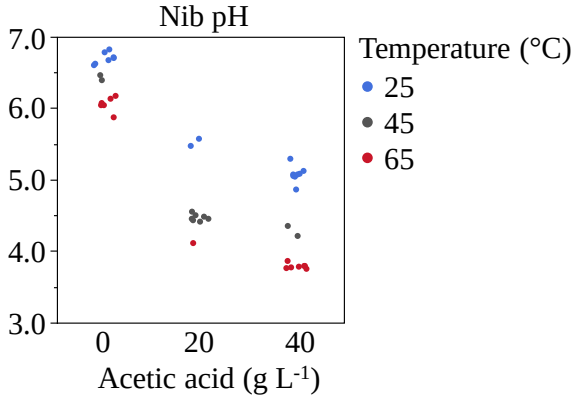


Figure 6.2 Effect of incubation temperatures and acetic acid concentrations on the acidification of cacao nibs during incubation.

6.4.2. Incubation of cacao beans at higher temperatures acidifies the nibs

Incubation of cacao beans in the absence of acetic acid resulted in a decrease of the nib pH from 6.9 ± 0.1 before incubation to 6.7 ± 0.1 after incubation at 25 °C, and 6.1 ± 0.1 after incubation at 65 °C (Figure 6.2). Increased incubation temperature thus resulted in nib acidification. Cacao mucilage typically contains 1 – 3 % citric acid and has a pH of 3.5 (Schwan and Wheals 2004). As a result, contact solutions for incubation were acid even in treatments where no acetic acid was added (average solution pH 4). However, it is unclear if citric acid is able to cross the testa barrier due to its large size. Quesnel (1965) reported that both formic and acetic acid can induce cacao bean death in artificial incubation experiments but that cacao beans remained viable after incubation with citric acid.

6.4.3. Mobilisation of Cd in cacao nibs is driven by nib acidification but only occurs at higher temperature

The three factors included here (heat, ethanol and acetic acid) were selected as they are generally assumed responsible for loss of seed viability during cacao fermentation, i.e. *bean death*. Increasing fermentation temperature and penetration of acetic acid and/or ethanol into the nibs induce structural changes within the nibs during fermentation and thus may also impact mobilisation of Cd and other elements in the nibs (Quesnel 1965;

Biehl et al. 1977; Biehl et al. 1982; Thompson et al. 2007). Ethanol did not affect the nib pH or the elemental composition of nib, testa or contact solution, and is not further discussed.

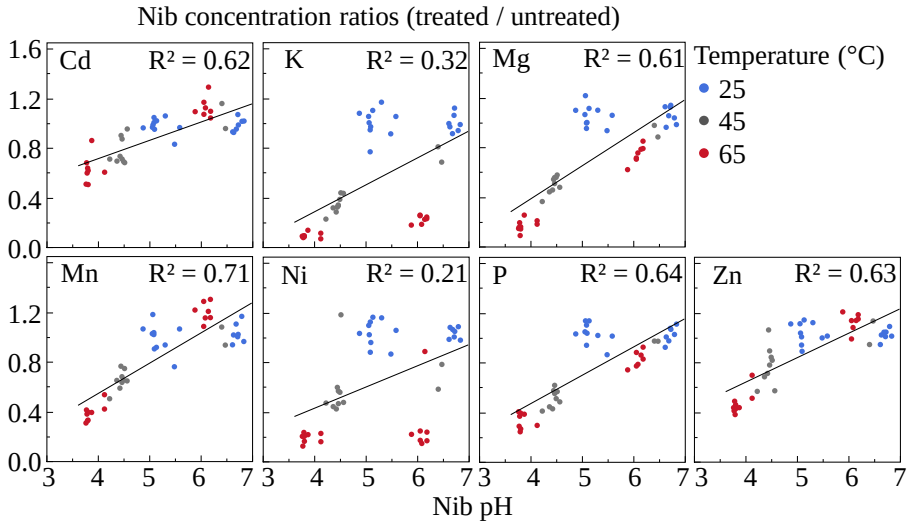


Figure 6.3 Nib acidification is the driving factor behind decreasing elemental concentrations in cacao nibs during incubation in different temperatures and acetic acid concentrations. Nib concentration ratios ($\frac{Element\ conc.\ treated\ samples}{Element\ conc.\ untreated\ samples}$) are significantly correlated to nib pH for all evaluated elements (P-value < 0.01).

Based on the results of the multivariate regression analysis and based on the correlations between nib concentration ratios and nib pH (Figure 6.3), the evaluated elements could be divided in three groups. The first group are the elements Ca and Cu, which were not significantly affected by incubation treatment. The second group of elements are Cd, Mg, Mn, P and Zn. The concentrations of these elements in the nibs decreased with incubation and their water extractability from cacao nibs was enhanced by increasing incubation temperature and increasing acetic acid concentrations. The interaction between temperature and acetic acid was identified as a significant negative predictor for nib Cd, Mg, Mn, P and Zn concentrations (Table 6.1), which indicates that the effect of acetic acid on nib elemental concentrations was enhanced at higher incubation temperatures, and vice versa. However, nib acidification explained most of the variation in nib concentration ratios for these elements. The R^2 values for the correlation between nib pH and nib concentration ratios (Figure 6.3) were similar to the R^2 values for the multivariate regression models (Table 6.1). Hence, the interaction effect of temperature and acetic acid is explained by the effect on nib pH, which was clearly lower when acid addition and high temperature were combined rather than when only a single factor was changed. The reduction of the nib concentrations of these elements (Cd, Mg, Mn, P and Zn) is, hence, related to pH dependent mobilisation in the nibs. However, based on the findings in Chapter 5, a minimum temperature is required to allow that pH dependent mobilisation to take place, as nib pH in the lab scale experiments in Chapter 5 dropped as low as 4.3 but no effect on nib Cd was observed (Table 5.2). This combined effect of acetic acid and

temperature on the elemental composition of the cacao nibs may be related to structural changes inside the nib tissues. Biehl et al. (1982) reported more extensive disruption of cellular structures when peeled cacao beans were incubated in acetic acid solutions, compared to incubations in the absence of acetic acid at higher temperature (50 °C). They stated that higher concentrations of acetic acid during fermentation separate the lipid bodies from the hydrophilic substances within the storage cells, and that this favours exchange and exudation of water soluble compounds from the cells. However, Biehl et al. (1982) reported that the effect of acetic acid on cellular disruption was less pronounced at higher temperatures (50 °C vs. 40 °C). In contrast, the results of Quesnel (1965) indicated that, while loss of germination can be obtained after incubation of cacao beans in acetic acid at relatively low temperature (< 39 °C), pigment (i.e. polyphenol) diffusion only occurred at temperatures > 43 °C.

The third group of elements contains K and Ni, the concentrations of these elements in the nibs decreased significantly with increasing incubation temperature but were not significantly affected by the acetic acid concentration. Nib pH did explain some of the variation in nib K and Ni concentrations, but R^2 values for the correlation to nib pH were much lower compared to R^2 values for Cd, Mg, Mn, P and Zn. Both K and Ni were readily extractable with water, even from nib samples prior to incubation (Appendix V, Figure V.3). Potassium is generally mobile in plant tissues and complexation is mostly weak and readily exchangeable (Hawkesford et al. 2012), which may explain why it is highly mobilised from the cacao nibs. Nickel was more readily extracted from cacao beans compared to other divalent cations (Cd, Mg, Mn and Zn) even before incubation (Appendix V, Figure V.3) and Ni mobilisation in cacao nibs was related mostly to increasing incubation temperature rather than nib acidification (Figure 6.3). These results suggest that, despite their chemical similarities, Ni complexation inside cacao nibs differs from complexation of Cd, Mg, Mn or Zn.

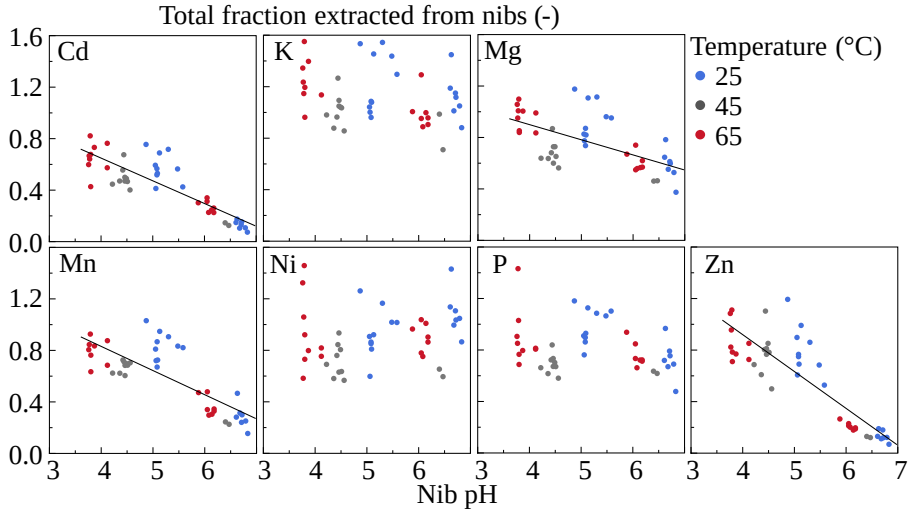


Figure 6.4 Total fractions of elements in cacao nibs extracted after incubation and water extraction, in relation to nib pH and incubation temperature. Lines of fit are given only for elements where the extracted fraction was significantly correlated to the nib pH (P-value < 0.01).

As Cd concentrations are generally reported to be higher in the testa compared to the nib, outward migration of Cd during fermentation occurs against the absolute concentration difference. However, water extractability of Cd, and thus Cd mobility, has been reported to be much lower in the testa compared to the nib (Chapter 5). In Chapter 4, it was hypothesised that Cd migrates from nib to testa during fermentation due to this difference in mobile Cd concentrations between nib and testa, and that the testa acts as a sink due to its high sorption capacity. However, the distribution of elements among the different tissues after incubation (i.e. contact solution, nib and testa) indicated that a large part of the elements mobilised in the cacao nibs was directly extracted to the contact solution, with only partial retention in the testa. Changes in testa element concentrations with incubation were only poorly explained by incubation conditions (Table 6.1). However, testa element concentrations did increase with incubation for all evaluated elements, except K (95 % CI > 1, Appendix V, Figure V.4). Thus, once elements are mobilised in the nibs due to increasing temperature and/or nib acidification, they are only partly fixed in the testa while most can migrate through this barrier to the contact solution in an incubation setup with high liquid to bean ratios. Andersson et al. (2006) reported that the cacao testa is highly permeable for aqueous solutions, and they stated that this permeability explains why acetic acid is able to penetrate the testa during fermentation. In addition, the testa is also permeable to alkaloids and polyphenols, as they have been reported to migrate out of the cacao beans during fermentation, resulting in lower concentrations of these compounds in fermented compared to unfermented nibs (Forsyth 1952; Roelofsen 1958; Timbie et al. 1978; Kim and Keeney 1984; Nazaruddin et al. 2006). Previous results from full scale fermentations confirm these trends. Fermentation generally decreased the elemental concentrations in the nibs and increased those in both the testa and the mucilage (sweatings), i.e. the liquid phase (Chapters 4 and 5). The loss of Cd from the beans to the mucilage sweatings in full scale operations is likely lower than those in the model incubations here, due to the high liquid to bean ratios.

6.4.4. Fine-tuning fermentation conditions as a mitigation strategy

Incubation of cacao beans at typical fermentation conditions (45 °C and 20 g L^{-1} acetic acid) lowered the average nib Cd concentration to a factor 0.79 ± 0.11 of the initial (untreated) concentration, i.e. an average reduction by a factor 1.27. Incubation of cacao beans at higher temperatures (65 °C) and acetic acid concentrations (40 g L^{-1}) further enhanced that factor to 1.6. Despite the large volume of liquid applied in these incubations in comparison to normal cacao fermentation practices, the observed factor reduction for the incubations in typical fermentation conditions was similar to the factor 1.3 observed previously in full scale fermentations studied using composite samples (Chapter 4), and the factor 1.25 reduction observed in a micro-fermentation experiment inside a full scale cacao fermentation box (Chapter 5). This indicates that a similar factor 1.6 reduction in nib Cd concentration may also be obtainable in commercial fermentation processes, if the fermentation temperature and the acetic acid concentration in the mucilage are increased. Previous results indicated that treatment with organic acids during fermentation was ineffective for the reduction of nib Cd concentrations (Chapter 5). The results of the incubations confirm the hypothesis stated in Chapter 5, i.e. that nib acidification is the driving force behind Cd mobilisation in cacao nibs during fermentation, but that this only

occurs if adequate fermentation temperatures are reached. Temperature was thus the missing piece of the puzzle in the small fermentations with 5 kg cacao used in Chapter 5. Indeed, incubation at 25 °C did not decrease the nib Cd concentration, regardless of the acetic acid concentration (95 % CI 0.95 – 1.01 across all 25 °C runs).

Generation of both fermentation heat and acetic acid are mostly attributed to the metabolic activity of acetic acid bacteria (AAB). Thus, optimisation of fermentation conditions to favour AAB growth may reduce nib Cd concentrations. For example, Camu et al. (2008b) reported increased activity of AAB and increased acetic acid concentrations in the mucilage of turned compared to non-turned fermentations heaps in Ghana, because aeration promotes the growth of the aerobic AAB. However, it is important to note that changes in fermentation practices will likely affect the final product quality. Acid flavours in chocolate have been related to high acetic acid concentrations in the cacao beans after fermentation (Holm et al. 1993) and, as mentioned in Chapter 1, a low nib pH early on in the fermentation process (pH < 4.5) hinders the production of flavour precursors which results less pronounced cacao flavour in the final product (Schwan and Wheals 2004; Camu et al. 2008a). This impact of excessive bean acidity on the final product quality may be limited through practices at later stages of the postharvest process. For example, nib roasting (deshelling prior to roasting) can reduce the amount of acetic acid in the final product due to volatilisation during roasting. Nib roasting also allows the option of alkalinisation or Dutch processing, i.e. treating the cacao nibs with alkaline solutions prior to roasting.

6.4.5. Potential of cacao nib washing as a mitigation strategy further down the postharvest processing chain

Incubation of cacao beans at high temperature (65 °C) with addition of acetic acid (20 or 40 g L^{-1}) followed by water extraction of ground nibs (solid:liquid ratio 1:20, incubation for 24 hours) extracted on average 66 % of the total Cd from the nibs (Figure 6.4). Such treatment thus removes more than half of the Cd originally present in the unfermented cacao nibs. Washing of cacao nibs with water after fermentation but prior to roasting is highly promising as a mitigation strategy and should be further explored using more realistic extraction conditions, i.e. higher solid:liquid ratios and using broken nib fragments instead of ground nibs, as ground nibs are not an intermediate product in the postharvest process of cacao. This concept is further explored in Chapter 7.

6.5. Conclusions

Incubation of cacao beans at typical fermentation conditions resulted in a factor 1.27 decrease of the nib Cd concentration and increasing the incubation temperature and acetic acid concentrations in the incubation media further increased this factor to 1.6. Fine-tuning of fermentation conditions for optimal production of heat and acetic acid thus has potential for reducing Cd concentrations in cacao nibs and should be further explored in full scale fermentation setups. However, high acetic acid concentrations in cacao nibs are generally considered an off-flavour and strong acidification of the nibs early on in the fermentation process has been related to poor product quality. While specific processing strategies may be able to limit the impact of excessive acidification on the final product quality (e.g. nib roasting and/or alkalinisation), a trade-off will likely have to be made between optimal Cd removal and flavour of the final product.

Chapter 7
Cacao nib washing prior to roasting effectively lowers nib cadmium concentrations: the first step towards nib washing as a mitigation strategy

Summary

Water extractions have been performed previously on ground cacao nibs as an analytical technique to compare Cd mobility between samples, e.g. unfermented and fermented beans. Results revealed that unexpectedly large fractions of nib Cd could be extracted by water from fermented, but not from unfermented cacao nibs. Here, water and chelating agent (EDTA) extractions with short contact times (1 or 2 hours) and small liquid:solid ratios (1 – 6 mL g^{-1}) were performed on broken cacao nibs derived from a commercial cacao fermentation. Incubation for 1 hour in 10 mM EDTA was able to remove 50 – 60 % of the nib Cd, at a liquid:solid ratio of 2 mL g^{-1} or higher. Incubation in water removed up to 40 % of nib Cd but required an incubation time of at least 2 hours. While this chapter offers only the first step in revealing the potential of cacao nib washing as a mitigation strategy for Cd in cacao, results are promising and should be further explored.

7.1. Introduction

In 2019, the European Commission enforced a new regulation limiting the maximum allowed cadmium (Cd) concentration in cacao-derived products such as chocolate (European Commission 2014), and similar thresholds have since been accepted by the Codex Alimentarius Commission (Codex Alimentarius Commission 2018). Cadmium found in chocolates mostly originates from the raw material, i.e. the cacao beans, rather than from contamination during processing or other ingredients (Chapter 3). As a result, the cacao processing industry has set requirements for their cacao suppliers and these unofficial thresholds, which apply to the cacao beans, generally range between 0.30 and 0.60 mg Cd kg^{-1}. Conventional full scale fermentation can reduce the Cd concentration in cacao nibs by a factor 1.3 (Chapters 4 and 5) and fine-tuning of fermentation parameters may be able to increase this reduction factor to 1.6 (Chapter 6). Water extractions were performed in previous chapters as an analytical method to compare the Cd mobility between unfermented and fermented samples and indicated markedly large water extractable Cd fractions in fermented compared to unfermented nibs. For example, incubation of cacao beans at high temperatures (65 °C) and acetic acid concentrations (20 or 40 g L^{-1}), followed by a 24 hour water extraction of the ground cacao nibs in a liquid:solid ratio of 20 mL g^{-1} removed 66 % of the nib Cd, i.e. a reduction factor of 2.3 (Chapter 6). Considering the high water extractability of Cd in fermented nibs, implementation of water and/or chelating agent washing steps during postharvest processing may be an effective strategy to reduce the Cd concentration in the final product. Washing steps are likely more effective when applied to fermented cacao because the water extractable Cd fraction increases with fermentation (Chapters 5 and 6). Washing prior to fermentation can even result in higher nib Cd concentrations after processing. As indicated by the results of the blank treatments in Chapter 5, addition of water to the cacao mass prior to fermentation slows down the fermentation process and reduces the production of heat and acetic acid in the mucilage. Considering that heat and acetic acid are the driving factors behind Cd mobilisation in the nibs (Chapter 6), water treatment before fermentation limits the potential reduction of the nib Cd concentration with fermentation and thus results in a final product with higher Cd concentrations.

Although water and/or chelating agent washing steps prior to nib roasting will likely influence the flavour quality of the final product, implementation of such techniques is not implausible. Liquid incubations of cacao prior to roasting are already commonly performed by the industry during alkalinisation. Alkalinisation or Dutch processing is a common practice to introduce specific colours or flavours during the production of cacao-derived products and to increase the solubility of cacao powder in drinks. The cacao nibs are incubated in an alkaline solution (1 – 6 % of NaOH, $CaCO_3$, Na_2CO_3, K_2CO_3 or other alkaline compounds or mixtures) at high temperature and pressure, prior to roasting (Kamphuis and Fowler 2017; Valverde García et al. 2020). This treatment increases the nib pH from < 5 after fermentation to pH values ranging from 5 to > 7.6 after alkalinisation (Miller et al. 2008). Alkalinisation also reduces the acidity, bitterness and astringency of the final product.

Details regarding alkalinisation conditions are often proprietary but the processes described in literature mention liquid:solid ratios ranging from 10 – 50 % [m/m (Valverde García et al. 2020)] to values closer to 100 % (Minifie 1989; Li et al. 2012). The alkalinisation treatment time ranges between 5 and 180 min (Valverde García et al. 2020). Thus, while nib washing will likely have an effect on the flavour of the final product, it is expected to yield an industrially relevant product, as indicated by the similar treatments already applied in the industry. The effects of current alkalinisation practices on nib Cd concentrations have not yet been revealed. The only change in the elemental composition of the cacao powder related to alkalinisation reported thus far, is an increase in the concentrations of minerals introduced by the alkalinisation salt (i.e. Na, Ca or K) (Valverde García et al. 2020). The alkaline solutions are often removed through drying rather than draining. However, it is not unlikely that some of the nib Cd is extracted into the solution during alkalinisation and, in that case, draining of the alkaline solutions instead of drying may reduce the Cd concentration in the final product.

Extraction experiments in the previous chapters have been performed using large liquid:solid ratios and long contact times and have focussed on ground cacao nibs. Ground nibs are not an intermediate product in the postharvest process of cacao, as grinding is typically performed at higher temperature yielding a homogeneous cacao mass, the cacao liquor. In the present study, a first step is made towards the development of water and/or chelating agent washing treatments as a mitigation strategy for Cd in cacao. Water and chelating agent incubations are performed on fermented broken cacao nib fragments, the intermediate product obtained after deshelling, and different contact times and liquid:solid ratios are compared to reveal the potential of such treatments for implementation in the industrial phase of the postharvest process.

7.2. Materials and methods

7.2.1. Cacao nibs

All cacao beans were of Nacional cultivar and were fermented on-site at a cacao plantation in Guayas province, Ecuador. Fermentation was performed in a large wooden box containing 225 kg of fresh cacao beans, for a total of four days without intermediate mixing. The temperature in the centre of the fermentation box reached its maximum on the third fermentation day (50 °C) and fermentation resulted in an increase of the mucilage pH from 3.9 to 4.4 and a decrease in the nib pH from 6.3 to 4.8. At the end of fermentation, a large composite sample (> 1 kg) was collected from the centre of the cacao bean mass and oven dried at 65 °C for 72 hours before transportation to Belgium. Cacao beans were peeled manually to remove the testae, and nibs were broken into nib fragments (several mm in size, Figure 7.1).

Figure 7.1 Fermented cacao beans, peeled and broken into nib fragments to represent the intermediate product obtained after breaking and winnowing in the industrial postharvest process.

7.2.2. Incubation treatments

Cacao nibs were incubated for 1 or 2 hours in an end-over-end shaker in different liquid:solid ratios (Table 7.1). Contact solutions were either deionised water, 10 mM EDTA (ethylenedinitrilotetraacetic acid disodium salt dihydrate, Titriplex III®, Merck, Kenilworth, NJ, USA), 10 mM tri-sodium citrate (VWR chemicals, Radnor, PA, USA) or 10 mM sodium acetate (Acros Organics, Geel, Belgium). All incubations were performed in duplicate. After incubation, a subsample of each extract was filtered using a 0.45 µm syringe filter (Chromafil® Xtra PET). Filtered solutions were diluted 20 times, acidified to 1 % (v/v) nitric acid (HNO_3, NORMATOM® 67 – 69 % w/w, VWR Chemicals) and their elemental composition was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent 7700x, Agilent Technologies, Santa Clara, CA, USA). Cadmium analysis was performed in the He mode by monitoring the ^{111}Cd isotope and the analysis was corrected online with ^{103}Rh as an internal standard. The limit of quantification (LOQ) for the ICP-MS analysis was 0.001 µg Cd L^{-1}.

Table 7.1 Different cacao nib incubation treatments.

Contact time (hours)	Contact solution	Liquid:solid ratio (mL g^{-1})
1	Deionised water	1
1	Deionised water	2
1	Deionised water	4
1	Deionised water	6
1	10 mM EDTA	1
1	10 mM EDTA	2
2	Deionised water	4
2	10 mM EDTA	4
2	10 mM citrate	4
2	10 mM acetate	4

7.3. Results and discussion

The Cd concentration in the cacao nibs prior to the washing treatments was 1.2 mg Cd kg^{-1}, double the generally accepted industry threshold of 0.60 mg Cd kg^{-1}. Water incubations with a contact time of 1 hour extracted on average 15 % of the Cd present in the nibs regardless of the liquid:solid ratio, whereas 1 hour incubations in 10 mM EDTA removed on average 41 % of nib Cd. Incubations in 10 mM acetate or citrate did not extract more Cd from the nibs compared to deionised water and are not further discussed (Figure 7.2). The liquid:solid ratio did not affect the water-extractable Cd fraction (Figure 7.3). However, this may have been related to the short contact time, as increasing the contact time from 1 to 2 hours significantly increased the water-extractable Cd fraction from 15 to 39 %. In contrast, EDTA incubations were affected by the liquid:solid ratio, with significantly larger Cd fractions extracted during EDTA incubations at 2 mL g^{-1} compared to 1 mL g^{-1} (incubation time 1 hour). However, further increasing the liquid:solid ratio to 4 mL g^{-1} with a contact time of 2 hours did not further increase the extracted Cd fraction (Figure 7.3). Extraction of Cd from cacao nibs through incubation thus depends on contact time and liquid:solid ratio but is limited to a maximum of 50 – 60 %, and this maximum extraction capacity is reached more readily in the presence of a chelating agent. This maximum is similar to the 66 % extractable nib Cd reported in Chapter 6 after incubation of intact cacao beans at higher temperature (65 °C) and acetic acid concentration (20 or 40 g L^{-1}), followed by a 24 hour water extraction of the ground nibs. Considering the results reported in Chapter 6, the extractable Cd fraction in cacao nibs increases with nib acidification during fermentation. However, if cacao beans are fermented in conditions promoting the production of heat and acetic acid, extractable nib Cd fractions in the fermented cacao nibs may be smaller compared to cacao beans fermented in less extreme conditions, as part of the extractable Cd was already extracted into the mucilage during fermentation (Chapter 6).

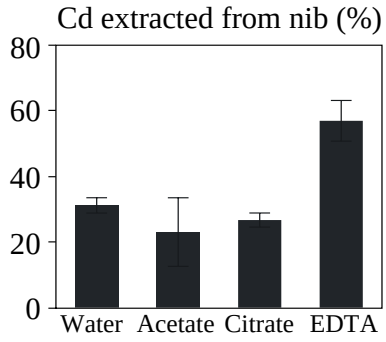


Figure 7.2 Extraction of Cd from cacao nib fragments (2 hour extraction at a liquid:solid ratio of 4 g mL^{-1}) is significantly larger for extractions in 10 mM EDTA compared to deionised water, but not for extractions in 10 mM acetate or citrate.

The fraction of Cd extracted through these relatively mild extraction conditions, i.e. short contact times and incubation in either water or solutions with low concentrations of chelating agents, is highly relevant for the Cd-cacao issue. The current EU limits range between 0.10 and 0.80 mg Cd kg^{-1} depending on the cacao content of the product (European Commission 2014). As a result, the cacao processing industry typically accepts fermented cacao beans with up to 0.60 mg Cd kg^{-1} (unofficial industry threshold). Considering that water extraction treatments prior to roasting can remove up to 60 % of the total Cd content from the cacao nibs, implementation of such mitigation strategies could allow acceptance of cacao beans with up to 2 mg Cd kg^{-1} for the production of dark chocolate, more than triple the current threshold (EU limit 0.80 mg Cd kg^{-1} for chocolate with ≥ 50 % total dry cacao solids). Such a change in the unofficial industry threshold would strongly limit the negative impact of the EU regulation on cacao farmers in Central and South America. For example, the average bean Cd concentration exceeded 0.60 mg Cd kg^{-1} in 48 % of the Ecuadorian fields sampled in the extensive nationwide study of Argüello et al. (2019), whereas only 9 % of fields had average bean Cd concentrations > 2 mg Cd kg^{-1}.

This chapter offers only the first step towards the development of water and/or chelating agent washing mitigation strategies. Follow-up studies can focus on the following experiments: (i) incubation of cacao nibs in alkalinisation conditions to study the effect of current practices on Cd in cacao; (ii) incubation of cacao nibs in water at different liquid:solid ratios with extended incubation time (> 1 hour) to reveal the effect of the liquid:solid ratio on water extractions and to identify if prolonged water incubations can reach a similar extraction potential compared to chelating agent solutions; (iii) incubation of cacao nibs in acid conditions and at higher temperatures to investigate whether such conditions can increase nib Cd extraction as suggested by the results in Chapter 6; and (iv) continuous flow nib washing experiments to mimic an industrial implementation. Adoption into practice will logically also require extended taste panel tests.

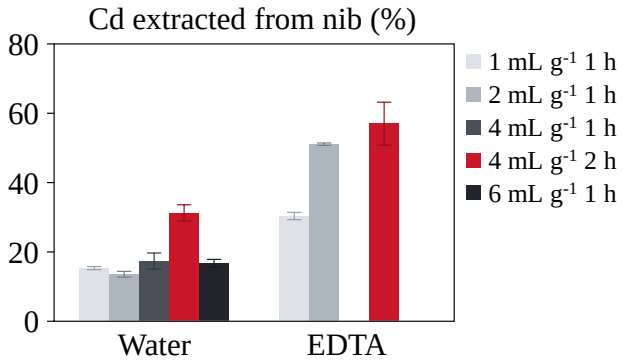


Figure 7.3 Cadmium extracted from broken cacao nib fragments after incubation in deionised water or 10 mM EDTA, for 1 hour (grey bars) or 2 hours (red bars) in different liquid:solid ratios.

7.4. Conclusion

More than half of the Cd present in fermented cacao nibs can be readily extracted with water and/or low concentrations of chelating agents such as EDTA. Considering the short contact time (1 – 2 hours) and small liquid:solid ratios (1 – 6 mL g^{-1}) required for Cd extraction, the implementation of cacao nib washing after fermentation but prior to roasting is highly promising as a postharvest mitigation strategy, it yields nib Cd reduction factors that exceed those of the agronomic countermeasures tested so far. Similar incubation treatments are already commonly applied by the industry during alkalinisation, which increases the likelihood that the proposed washing treatments are practically implementable. It is expected that treatments will influence the flavour of the final product. However, while treated cacao may no longer be suited for the production of specialty single origin chocolates, it can be reasonably assumed that the obtained product will still be of value to produce other cacao-derived products. This chapter offers only the first step towards the development of a postharvest mitigation strategy for Cd in cacao based on nib washing, follow-up experiments are required to reveal the true potential of this promising technique.

Part 3
Conclusions and perspective for future work

The general objectives of this work were to identify the effect of current postharvest practices on the distribution of Cd within and among the different tissues of the cacao fruit, and to use this knowledge for the development of postharvest mitigation techniques to lower Cd concentrations in cacao-derived products. The development of such techniques can offer universally implementable strategies for dealing with the Cd-cacao issue, with added control for the cacao processing industry. This chapter summarises the effect of the different postharvest processing steps on the Cd distribution in cacao, interprets the detected effects, and highlights best practices to reduce the Cd concentration in the final product.

Fermentation as a mitigation strategy for Cd in cacao

In ripe cacao pods, the Cd concentrations decrease in the order testa > nib ~ placenta ~ pod husk > mucilage (Chapter 4). During fermentation, the mucilage and the cacao beans are exposed to high temperatures, acid pH and a variety of metabolites, which are produced by the microorganisms in the mucilage (e.g. ethanol and acetic acid). Cellular integrity is disrupted both in the mucilage, which is liquefied and drained as mucilage sweatings, and in the nibs, where loss of membrane integrity brings enzymes in contact with their substrates to produce flavour precursors. Considering the above, fermentation was expected to have an impact on the distribution of Cd among the different cacao bean tissues. The impact of fermentation on the distribution of Cd in cacao was studied using a top-down approach, starting with a full scale fermentation setup (Chapter 4), followed by lab scale and micro-fermentations (Chapter 5) and finally incubations to mimic the fermentation conditions in a controlled system (Chapter 6). During fermentation, Cd is mobilised in the nibs and moves outward to the testae and the mucilage. This mobilisation is caused by nib acidification due to acetic acid penetration into the nibs and it only occurs if fermentation temperatures are sufficiently high. During conventional fermentation, this mobilisation can decrease the nib Cd concentration by up to a factor 1.3. The incubation experiment performed in Chapter 6 suggests that this reduction factor (RF) can be increased to 1.6 in more extreme fermentation conditions, i.e. higher temperatures and increased acetic acid concentrations.

Considering the above, fermentation practices that promote increased temperature and acetic acid production are recommended, while other fermentation practices may be discouraged when dealing with elevated bean Cd concentrations. Cacao bean spreading, for example, is a common practice that entails pre-drying of fresh beans prior to fermentation to reduce the acidity of the fermented cacao (Meyer et al. 1989; Biehl et al. 1990; Schwan and Wheals 2004). However, reduced nib acidification also implies reduced Cd mobilisation in the nibs, and, in addition, pre-drying removes part of the mucilage, i.e. the liquid phase to which Cd otherwise migrates after mobilisation. Indeed, pre-dried fermentations in Chapter 4 did not reduce nib Cd concentrations.

Trade-offs need to be made between optimal Cd removal and the acid flavours in the fermented cacao beans. It is, however, important to note that there are several techniques available to remove part of the acid flavour generated in fermentation, during subsequent processing. For example, deshelling the cacao beans prior to roasting (nib roasting) allows volatilisation of acetic acid during roasting and thus removes some of the acid off-flavours. Nib roasting can also be combined with alkalinisation or Dutch processing to further reduce the acidity of the final product.

The driving force behind Cd mobilisation in the nibs during fermentation

The XANES analysis (Chapter 4) revealed that more than half of the Cd in unfermented cacao nibs was bound to O-ligands, while the remainder was associated to thiol groups and polyphosphate ligands. More recent synchrotron data has demonstrated that Cd in unfermented nibs is mostly bound to phytate (60 – 70 %) and in a smaller extent to S-ligands such as phytochelatins or metallothioneins (30 – 40 %) (Blommaert et al., in preparation). During fermentation, up to 60 – 70 % of the Cd in the nibs is mobilised and part of that mobilised Cd migrates outward from the nibs to the testae and the mucilage. This mobilisation is driven by nib acidification and only occurs if fermentation temperatures are sufficiently high. Here, it is speculated that the fraction of Cd that can be mobilised during fermentation corresponds to the fraction of Cd that is associated to phytate in the unfermented nibs. More specifically, it is hypothesised that nib Cd mobilisation is related to a combination of (i) the effect of pH on the interactions between Cd and the phytate ligand; and (ii) the effect of fermentation on the phytate content itself. Both arguments are elaborated below.

First, the effect of fermentation on nib Cd concentrations can be partially explained by pH dependent interactions between Cd and the polyphosphate ligands (i.e. phytate) in the nibs. The interpretation of that pH dependent Cd mobilisation is logically the increased Cd^{2+}:H^{+} competition on the functional binding groups. To approximate this interaction, mobile Cd fractions were calculated for the different runs of the incubation experiment described in Chapter 6, using a geochemical model, i.e. Visual MINTEQ software (version 3.1, available online at https://vminteq.lwr.kth.se/). Visual MINTEQ is a chemical equilibrium software developed for calculating the speciation, solubility and sorption of metals in natural waters. As far as known, metal-phytate complexation data have not yet been added to the open source speciation codes of Visual MINTEQ. Therefore, original literature data were consulted to include the stability constants in the speciation code and to perform speciation calculations (Crea et al. 2006; Cigala et al. 2010; De Stefano et al. 2010; Bretti et al. 2012 and 2015). A more detailed description of that phytate database can be found in Appendix VI. The average elemental composition (Cd, Cu, Fe, K, Mn, Ni and Zn) of the cacao nibs across all runs from the incubation experiment (Chapter 6) was first used to determine the capacity of the ligand, i.e. the phytate concentration required to obtain that 7.7 % of Cd present in solution at a pH of 6.9, which corresponds to the conditions prior to incubation in Chapter 6. That phytate capacity was equal to 0.0011 g phytate g^{-1} nib, which corresponds to only 6 % of the total nib P concentration. The phytate concentration was the single adjustable parameter in the model. An overview of the input data can be found in Appendix VI, Table VI.1. The input Cd concentration was adjusted to 60 % of the total measured Cd concentration, so that only the Cd fraction

bound to P-ligands was considered. Magnesium and calcium were not added to the system because their free ion activity is typically low in plant cells (Hawkesford et al. 2012), suggesting only minor fractions of phytate occupied by Mg and Ca *in vivo*. Then, the measured pH was adjusted to mimic the conditions at the end of incubation for each individual run. The calculated mobile Cd fractions increased with decreasing pH (Figure A). However, the chemical speciation model predicted only 25 % mobile Cd at the lowest pH values, while total extractable Cd fractions in the experimental data amounted to up to 80 %. Thus, the increased Cd^{2+}:H^{+} competition at low pH explained only part of the observed nib Cd mobilisation with fermentation.

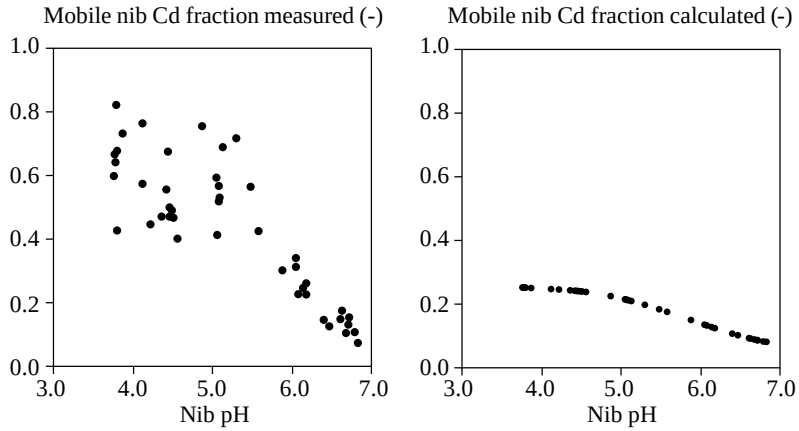


Figure A Mobile (total water extractable) nib Cd fractions in the different runs of the incubation experiment described in Chapter 6 (left) compared to mobile nib Cd fractions calculated based on Cd^{2+} complexation with phytate, determined with Visual MINTEQ (right).

Second, it is not unlikely that fermentation affects the phytate content in cacao nibs. During fermentation, subcellular structures within the nibs are disrupted (i.e. *bean death*), which brings several enzymes in contact with their substrates. It is not unlikely that fermentation conditions activate phytase in the nibs, which hydrolyses phytate and thereby can mobilise the minerals bound on these polyphosphate ligands. Hydrothermal processing in conditions similar to cacao fermentations (pH 4.0 – 5.0 and 50 – 60 °C) has been reported to break down > 80 % of phytate in wheat, rye, barley and oats (Fredlund et al. 1997; Lemmens et al. 2018). Although phytate is water soluble, it is unclear if this large compound is able to migrate through the testa barrier. Strikingly, more than 50 % of the total nib P content was readily extracted with water from dried unfermented nibs (Figure B.II), while incubation of intact beans at low temperatures and in neutral pH conditions only extracted < 5 % (Figure B.I). Phosphorous was thus not able to cross the testa barrier before fermentation. However, after incubation at high temperatures and in the presence of acetic acid, up to 70 % of the nib P had migrated through the testa to the contact solution (Figure B.I). Such observations support the hypothesis that phytate in the nibs is broken down during fermentation, thereby releasing associated minerals and metals.

It is, however, unclear if those observations are related only to phytate or to other P-compounds. Both the phytate content and phytase enzyme activity should be measured before, during and after fermentation to assess that hypothesis. Reported information on phytate in cacao nibs is scarce. Valiente et al (1996) reported that defatted cacao powder has a high affinity for Cd and the authors proposed that this could be related to phytate, but they did not report on the phytate concentration in the powders.

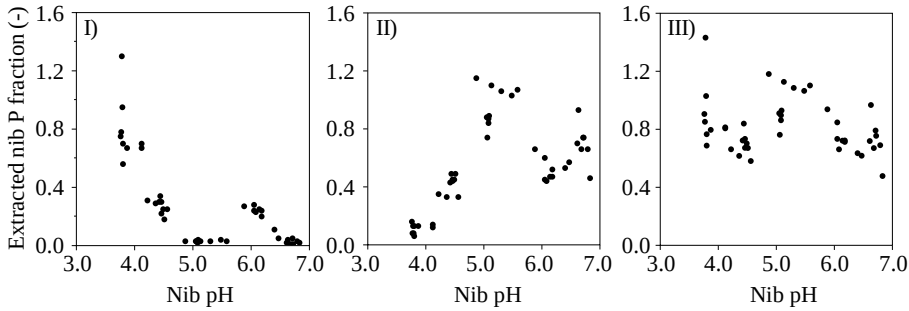


Figure B Effect of incubations on water extractable P fraction of cacao nibs in function of nib pH (Chapter 6). I) Nib P fraction extracted through the testa to the contact solutions during incubation. II) Nib P fraction extracted with deionised water from dried ground nibs after incubation. III) Total extracted nib P fraction, i.e. sum of A and B.

Cadmium mitigation during fermentation: can the use of starter cultures affect nib Cd concentrations?

While cacao fermentations are still mostly uncontrolled and based on spontaneous inoculation, researchers have studied the potential of starter cultures for decades, with early research dating back to the 1980s (Sánchez et al. 1985; De Vuyst and Weckx 2016; Pereira et al. 2016; and references therein). More recent work has indicated that the use of starter cultures can impact the sensory quality of the final product (Crafack et al. 2013; Meersman et al. 2015 and 2016). Most work thus far has reported similar temperatures and acetic acid concentrations in both the mucilage and the nibs during starter culture mediated fermentations compared to spontaneous fermentations (Lefeber et al. 2012; Ramos et al. 2014; Meersman et al. 2015 and 2016). However, the objective of starter culture studies thus far was to closely mimic spontaneous fermentation conditions, albeit with increased consistency. It is unclear if the use of starter cultures can increase heat and/or acetic acid production during fermentation, and thereby increase the outward Cd migration from the nibs. Increased consistency in fermentation parameters with the use of starter cultures may already offer substantial advantages for the Cd-cacao issue. Fermentation conditions, i.e. temperature and acidification, are affected by farmer practices (e.g. fermentation setup and frequency of mixing), cultivar, equipment (e.g. presence of inoculum in walls of fermentation boxes) and weather, among other factors. The pH and temperature conditions required for Cd mobilisation in the nibs are likely not always met, even in different fermentation batches from the same farm or cooperative. The use of starter cultures, as well as clear guidelines for fermentation practices, may offer more consistent reductions of nib Cd concentrations with fermentation.

In addition to the effect of starter cultures on the fermentation conditions, specific lactic acid bacteria have been reported to take up more Cd than other strains. For example, Zhai et al. (2019) reported > 90 % reduction in the Cd concentration of rice after fermentation with *Lactobacillus plantarum*, and the authors related this partly to the Cd uptake capacity of the bacteria. However, the specifics of the cacao fermentation process need to be considered when assessing the potential effect of using Cd-accumulating strains as starter cultures on nib Cd concentrations. The microbial community in cacao fermentations is confined to the mucilage. There is no microbial activity inside the nibs and bacteria can thus only take up Cd present in the mucilage. Thus, even if specific Cd-accumulating strains can withstand the competition of the native microbial community present during fermentation, it is unlikely that their presence can substantially affect nib Cd concentrations.

Mitigation during the industrial postharvest phase: nib washing

Water extractions were initially used in Chapters 5 and 6 as an analytical technique to compare mobile Cd fractions between unfermented and fermented cacao bean tissues. Water extractable nib Cd fractions were markedly high in fermented samples, suggesting the potential of cacao nib washing as a mitigation strategy applied after fermentation. A first step towards the development of such technique was presented in Chapter 7. Cacao nib washing with a small volume of a low concentration EDTA solution can reduce the nib Cd concentration by a factor > 2. The implementation of such a technique is not implausible, considering that liquid treatments prior to roasting are already performed in the industry during alkalinisation or Dutch processing. Nib washing clearly has high potential as a mitigation strategy for Cd in cacao and should be further explored.

Relevance for the cacao industry

The postharvest process of cacao can typically be divided in two phases. The first phase takes place on the cacao farm or in local cooperatives that collect the cacao from small scale farmers and consists of harvest, fermentation and drying. After drying, the cacao beans are stable for long term storage and transportation and are typically sold to large cacao processing companies in Europe or the USA. The second, more industrial processing phase takes place at the processing companies and entails deshelling, roasting, alkalinisation (optional), grinding and refining to obtain the cacao liquor. The cacao liquor is then further processed depending on the final product, e.g. pressing to separate cacao butter and cacao cake, or conching and mixing with additional ingredients for chocolate production.

The EU Cd regulations apply only to the final product sold to the consumer, not to the raw material or intermediate products. Therefore, the cacao processing industry has translated the EU regulations to unofficial industry thresholds which apply to the cacao beans they purchase from their suppliers. Such thresholds are, however, only justified if Cd in chocolates originates from the beans rather than from other ingredients or contamination during processing. The extensive study of single origin chocolates in Chapter 3 revealed a strong correlation between the Cd concentration in chocolates and their cacao solids content. The CART analysis identified a geographical signature in the Cd content of chocolates, with higher Cd concentrations measured in chocolates made from

Central and South American cacao, compared to other origins. Both observations form evidence that Cd in chocolate originates from the cacao beans and that it is logical that the industry uses bean Cd concentration thresholds to judge on the quality of their raw materials. However, the industry should take care when setting their threshold limits, as excessively low thresholds can have an unnecessary negative impact on the cacao industry, especially in Central and South America.

The bean Cd concentration thresholds imposed by the industry typically range between 0.30 and 0.60 mg Cd kg^{-1} (based on personal communications). However, cacao beans with much higher Cd concentrations can be used depending on the intended final product. Considering the EU limit for a dark chocolate with ≥ 50 % cacao solids (0.80 mg Cd kg^{-1}, Table A), cacao beans with up to 0.80 mg Cd kg^{-1} can be used to produce chocolates with 100 % cacao solids, while beans with up to 1.14 mg Cd kg^{-1} can be used in the production of typical dark chocolates with 70 % cacao solids (1.14 = 0.8/07). Excessively strict industry thresholds can have a large impact on the cacao producing regions. The effect of such regulations on local farmers can be illustrated using the extensive nationwide dataset of Argüello et al. (2019) for Ecuador, which included 159 fields. Considering the calculations above, 25 % of the Ecuadorian fields would be rejected for the production of a dark chocolate with 70 % cacao solids (bean Cd < 1.10 mg Cd kg^{-1}). However, 48 % of fields are rejected based on the unofficial industry threshold of 0.60 mg Cd kg^{-1}, and this number increases to 77 % when considering the stricter threshold of 0.30 mg Cd kg^{-1}. This discrepancy between the EU limits and the industry thresholds should be addressed as it can have a large and unnecessary impact on cacao farmers in Central and South America.

Farmers should also consider the effect of fermentation on nib Cd concentrations as it implies that analysis of fermented, rather than unfermented, cacao beans should be used to assess their export options. Fermentation can decrease nib Cd concentrations by a factor 1.3 but, to obtain such effect, fermentation practices should favour heat and acetic acid production. The results in Chapter 6 suggest that this reduction factor (RF) may even be increased to a factor 1.6 in more extreme conditions, follow-up experiments are required to reveal if such conditions are practically feasible. Considering again the example of a dark chocolate with 70 % cacao solids, a RF of 1.3 with fermentation implies that cacao beans can be accepted with initial nib Cd concentrations prior to fermentation up to 1.40 mg Cd kg^{-1}, this concentration was exceeded in only 16 % of fields in the dataset of Argüello et al. (2019). More extreme fermentation conditions with a RF of 1.6 even allow use of cacao beans with up to 1.8 mg Cd kg^{-1}, or rejection of only 9 % the Ecuadorian fields.

Cacao nib washing with chelating agent solutions reduced the nib Cd concentration by up to a factor 2.3 (Chapter 7). Such treatment is likely most relevant for the production of cacao powders, as liquid treatments are already common in cacao powder production during alkalinisation. Cacao beans with nib Cd concentrations between 0.40 and 0.60 mg Cd kg^{-1} can be used to produce regular cacao powders under the EU regulations, depending on the cacao butter content of the product and without any additional treatment. Cacao nib washing can increase these thresholds to 0.90 – 1.40 mg Cd kg^{-1} in the cacao nibs, depending on the cacao butter content. For a cacao powder with 20 %

cacao butter, this is the difference between rejecting 65 % of the Ecuadorian cacao fields without additional treatment or rejecting only 30 % of the fields if nib washing is implemented.

Table A Maximum allowed Cd concentrations in cacao-derived foodstuffs, determined by the European Commission and in force since January 2019 (European Commission 2014).

Cacao-derived product	Cd (mg kg^{-1} wet weight)
Milk chocolate with < 30 % total dry cocoa solids	0.10
Chocolate with < 50 % total dry cocoa solids; milk chocolate with ≥ 30 % total dry cocoa solids	0.30
Chocolate with ≥ 50 % total dry cocoa solids	0.80
Cocoa powder sold to the final consumer or as an ingredient in sweetened cocoa powder sold to the final consumer (drinking chocolate):	
Regular cocoa powder, ≥ 20 % to 50 % cacao butter	0.60
Fat reduced cocoa powder < 20 % cacao butter	0.60

The examples above focus on dark chocolates and cacao powder only because the cacao from the areas most affected by the Cd-cacao issue, i.e. Central and South America, is often used to produce dark specialty chocolates. The effects of fermentation and nib washing described above are likely not additive, as both are based on the mobilisation and extraction of Cd from the nibs. Stronger migration of Cd from the nib to the testa and the mucilage during fermentation likely leaves less to be extracted during nib washing later in the process. However, it is not unlikely that total RFs > 2 can be obtained through a combination of these treatments and, as demonstrated by the examples above, the impact of such high reduction factors on the cacao industry in Central and South America is large. Considering the above, postharvest mitigation is a highly promising short to medium term strategy for the Cd-cacao issue.

Apart from postharvest processing, three main other mitigation strategies can be discerned. First, mixing of cacao beans from different origins can dilute the Cd concentration in the final product because of the large geographical differences in cacao bean Cd concentrations (Chapter 3). Mixing can be readily implemented by the industry and is considered highly effective as a short term solution for the Cd-cacao issue, but it is not a viable option for the single origin specialty chocolate industry in Central and South America.

Second, soil amendments can be applied to reduce the phytoavailability of Cd in the soil. Results for lime and biochar application are promising (biochar RF 2) but available data for field trials is limited to date. The potential of soil amendments for the mitigation of Cd in cacao is restricted by the root system of the perennial cacao tree, the wide surface roots complicate incorporation of amendments into the soil and the deeper part of the root system is likely unaffected by the treatments. In addition, both lime and biochar application immobilise Cd in soils by increasing the soil pH and are thus only effective in acid soils, not in neutral or alkaline soils.

Third, genetics based mitigation, e.g. cultivar selection, seems promising but research on those strategies is still in an early phase. Studies on Cd uptake in different cacao cultivars are complicated by the large within-field variation, i.e. it is difficult to discern the cultivar effect from the soil effect. Besides, several knowledge gaps remain to allow development of genetics based mitigation strategies. It is not yet clear how Cd is taken up and translocated from root to shoot, Cd loading into cacao beans may occur (partly) via direct transport from the xylem as fruits grow directly on the trunk of the tree. The relative importance of rootstock cultivar versus scion cultivar for Cd uptake and translocation in grafted cacao plants also needs to be revealed, as selection of low Cd accumulating rootstocks may offer reduced bean Cd concentrations while maintaining the flavour quality of the scion cultivar.

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Appendices

APPENDIX I

The transfer of cadmium from soil to chocolate bar

APPENDIX II

The elemental composition of chocolates is related to cacao content and origin: a multi-element fingerprinting analysis of single origin chocolates

APPENDIX III

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

APPENDIX IV

Cadmium migration from nib to testa during cacao fermentation is driven by nib acidification

APPENDIX V

Outward migration of cadmium from cacao nibs during fermentation is related to the combination of enhanced acidity and temperature: mimicking fermentation through incubation

APPENDIX VI

Chemical equilibrium calculations

Appendix I
The transfer of cadmium from soil to chocolate bar

I.1. Meta-analysis: methods and description of included studies

I.1.1. Selection of individual studies

First, a search in three databases (Scopus, Web of Science and PubMed) was performed using the following search words: cacao, cocoa, chocolate, cadmium and heavy metals. All the results from the databases using all different search word combinations were merged into a general database which was revised by removing duplicate results. After this revision, a total of 429 publications remained. The selection of studies was based on the following criteria:

(i) *Bean Cd concentrations with indication of origin*: the publication must report Cd concentrations of cacao bean samples with information about the sample origin and the pre-processing before chemical analysis (i.e. fermentation, roasting, peeling, etc.).
(ii) *Reported quality assurance and quality control protocols*: the article must report the use of suitable certified reference materials (CRM), as well as the percentage of recovery. The CRM should have certified Cd concentrations within reasonable range of the Cd concentrations expected in the samples.
(iii) *Acceptable limit of quantification (LOQ) for relevant environmental Cd concentrations*: the acceptable LOQ were defined according to the instrument used for measurement, i.e. atomic absorption spectrometer (AAS) LOQ ≥ 1.0 mg Cd kg^{-1}; inductively coupled plasma optical emission spectrometer (ICP-OES) LOQ ≥ 0.1 mg Cd kg^{-1}; atomic absorption spectrometer with graphite furnace (AAS-GF) LOQ ≥ 0.05 mg Cd kg^{-1}; and inductively coupled plasma mass spectrometer (ICP-MS) LOQ ≥ 0.02 mg Cd kg^{-1}.
(iv) *Availability of paired soil and bean Cd information*: paired data were defined as bean and soil Cd measurements from the same tree.

Bean cadmium data

From a total of 439 publications gathered using the search words, 393 were rejected as their topic did not fit the objective of this review, i.e. experiments with cacao seedlings, chocolate quality, analytical techniques to measure Cd, etc. Only 46 articles passed the first selection criterium. From these 46 publications, 15 publications were rejected as they reported only soil Cd data (5), no bean Cd data (3), no data available online (1), reported Cd concentrations were below the limit of detection (4), or no Cd data was reported but other heavy metals were analysed (2). Thus, 31 publications were used to summarise Cd concentrations in cacao beans across the world (Table 2.1).

Paired soil and bean cadmium data

To analyse the effect of soil properties and agronomic factors on the uptake of Cd in the cacao tree, it was necessary to have paired bean-soil Cd data, i.e. soil and bean samples from the same cacao tree. Starting with 31 publications, 25 of them were rejected as no paired data were reported (16), no CRM was reported (6), reported CRM was not suitable for the expected Cd concentrations (2), or pre-processing of bean samples was unconventional (1).

The selection process resulted in a total of six studies that fulfilled the listed criteria (Table I.1). To avoid data losses, corresponding authors from the selected studies were contacted to gather the original databases. Only for study VI, no contact was made with the authors as most of the information was already published. During the communication to gather the data, authors from study II provided an additional unpublished dataset that fulfilled the selection criteria and was added to this meta-analysis.

I.1.2. Description of included studies

Study I (Argüello et al. 2019) is a nationwide study conducted in cacao producing areas of Ecuador where 560 paired bean-soil samples were collected. Study II (Barraza et al. 2017) is a localised study conducted in cacao producing areas in the Northern part of the Amazonian region of Ecuador where 31 paired bean-soil samples were collected. Study III (Barraza et al. 2018) is also a localised study conducted in the Northern part of the Amazonian region of Ecuador, focused on areas influenced by oil extraction activities. A total of 15 sites were sampled within study III but only four sites reported paired bean-soil samples, therefore, only these four observations were included in the meta-analysis. Study IV (Barraza et al. 2019) is a pilot field study focused on determining the isotope fractionation in the soil-cacao system. A total of five samples were collected from three farms in the Northern province of Sucumbíos in Ecuador. Study V is a localised study conducted in 15 small scale cacao farms, along the Pacific coast in the Northern part of Ecuador (Esmeraldas province). This study was realised in collaboration with the French cooperative Ethiquable. All cacao trees grew in an agroforestry system without any addition of fertilisers or other treatment. The monitoring of Cd concentrations in topsoils and cacao beans lasted 2 years. The results from study V have not been published. Study VI (Engbersen et al. 2019) is a localised study within the cacao plantation of the research station CEDEC-JAS in Northern Honduras where a total of 60 paired bean-soil samples were collected. Finally, study VII (Gramlich et al. 2018) is a nationwide study conducted in the cacao producing areas of Honduras, where a total of 110 paired bean-soil samples were gathered. Main differences in the methods to determine pH, soil organic carbon, total soil Cd and bean Cd, as well as the equipment used to measure Cd concentrations, are presented in Table I.1.

I.1.3. Standardisation of selected variables

All variables were standardised to the same units before analysis. Some studies collected soil samples at different depths which complicated the analysis and resulted in data losses during the different regressions. To simplify the analysis, two new variables were created, i.e. [Cd Top] and [Cd Sub]. The variable [Cd Top] was defined as the soil Cd concentration at a depth of 0 – 20 cm, whereas [Cd Sub] was defined as the soil Cd concentration at a depth of 20 – 40 cm. For the studies that gathered information in several soil depths (i.e. 0 – 5, 5 – 10, 10 – 20, etc.) a weighted average was used to calculate [Cd Top]. Likewise, to account for differences in applied methods between the studies, pH_{water} was converted to pH_{CaCl2} using the equation described by Kissel et al. (2009). Based on a comparison of pH in water with pH in 0.01 M calcium chloride ($CaCl_2$) for 1186 soil samples, the median difference in pH was 0.67. The pH measured in water was thus slightly higher than the pH measured in 0.01 M $CaCl_2$.

The equation used to convert pH_{water} to pH_{CaCl2} proposed by Kissel et al. (2009) reads as follows:

$$pH_{H2O} = 0.9228 * pH_{CaCl2} + 1.1008$$

I.1.4. Multivariate regression analysis on individual studies

For individual study regression analysis, studies III through V were excluded because of their small sample size. As the selected studies reported different variables, the analysis was based on the soil characteristics considered to be most important for Cd uptake, i.e. pH, SOC, % clay, total soil Cd, Zn, and Mn concentrations, and available soil Cd, Zn and Mn concentrations. Statistical data analysis was performed using JMP® Pro Version 15.1.0 (SAS Institute 2019). All variables were log transformed before analysis, except pH. Statistical significance was established throughout all analysis at P-value < 0.05. Not all the variables were present in all studies and, therefore, the predictors included in each regression varied among studies (Table I.3). For study VII, three different regressions were made because the variables Total Cd Top, Cd Av Top and DGT Cd Top were highly correlated and thus running a single regression including all those variables would violate the assumption of no multicollinearity. For the same reason, two different regressions were made for study II because the variables Total Cd Top, Total Zn Sub and Total Mn Top were significantly correlated. Stepwise regression analysis (forward and backward) was conducted to select soil variables predicting Cd concentration in beans. The variables were selected based on the maximisation of the adjusted R-square and the minimisation of the Bayesian Information Criterion (BIC). Multivariate regression analysis using ordinary Least Square Model (LSM) was performed using the selected variables for different models. To fulfil the assumption of multivariate regression, the Variation Inflation Factor (VIF) was evaluated for all significant variables in each model.

I.1.5. Multivariate regression applied to the compiled dataset

A new model was constructed using the compiled data (n = 334), focusing on the most important soil variables identified in the multivariate regression analysis of the individual studies (pH, SOC and total soil Cd). The high number of observations in study I created a bias and any new model would thus be dominated by the results of this study. To overcome this issue, an unbiased subset of study I (n = 127) was selected using the algorithm described by Ortell et al. (2019), which retains representative averages and standard deviations from the original dataset for the selected variables. As such, an equal number of observations (167) was randomly selected from the data of the two countries available in the dataset (Ecuador: study I, n = 127, study II, n = 28, study III, n = 4, and study V, n=8; Honduras: study VI, n = 60 and study VII, n = 107).

Table I.1 Analytical methods used in the selected studies for soil and plant analysis.

Study	Reference	pH	SOC	Soil*	Leaves*	Beans*	Equipment
I	Argüello et al. (2019)	1 mM $CaCl_2$ 1:5 w/v	combustion	Aqua regia	HNO_3	HNO_3	ICP-MS
II	Barraza et al. (2017)	water 1:5 w/v	combustion	Aqua regia + HF	HNO_3 + H_2O_2	HNO_3 + H_2O_2	ICP-MS
III	Barraza et al. (2018)	water 1:5 w/v	combustion	Aqua regia + HF	HNO_3 + H_2O_2 + HF	HNO_3 + H_2O_2 + HF	ICP-MS
IV	Barraza et al. (2019)	water 1:5 w/v	combustion	Aqua regia + HF	HNO_3 + H_2O_2 + HF	HNO_3 + H_2O_2 + HF	ICP-MS & MC-ICP-MS
V	Maurice & Schreck (unpublished)	water 1:5 w/v	combustion	Aqua regia + HF	HNO_3 + H_2O_2	HNO_3 + H_2O_2	ICP-MS
VI	Engbersen et al. (2019)	Water 1:2 w/v	Walkley & Black	Aqua regia	HNO3	HNO3	ICP-MS
VII	Gramlich et al. (2018)	water 1:2 w/v	Walkley & Black	HNO_3 + H_2O_2	HNO_3 +H_2O_2	HNO_3 +H_2O_2	AAS & AAS-GF

* Method used for digestion to determine total elemental concentrations: HNO_3 = Nitric Acid, H_2O_2 = oxygen peroxide; HF = hydrofluoric acid.

SOC = Soil Organic Carbon; AAS = Atomic Absorption Spectrometry; GF = Graphite Furnace; ICP-OES = Inductively Coupled Plasma Optical Emission Spectrometry; ICP-MS = Inductively Coupled Plasma Mass Spectrometry; MC-ICP-MS = Multi Collector Inductively Coupled Plasma Mass Spectrometry

Table I.2 Average values (min-max) of selected soil characteristics from the topsoil (0 – 20 cm) and subsoil (20 – 40 cm) for each study. A detailed description of the analytical methods used in each study can be found in Table I.1. SOC = Soil Organic Carbon.

Study	Reference	Country	n	pH		SOC (%)		Total Cd (mg kg^{-1})	
				Topsoil	Subsoil §	Topsoil	Subsoil §	Topsoil	Subsoil §
I	Argüello et al. (2019)	Ecuador	560	6.0 (4.1–7.9)	/	2.59 (0.19–13.1)	/	0.44 (0.02–6.90)	/
II	Barraza et al. (2017)	Ecuador	31	5.9 (4.2–7.7)	6.0 (4.4–7.9)	0.86 (0.17–2.41)	0.69 (0.11–4.82)	0.46 (0.19–1.15)	0.40 (0.16–1.02)
III	Barraza et al. (2018)	Ecuador	4	4.9 (4.3–6.0)	5.5 (4.9–6.5)	0.96 (0.49–1.85)	0.32 (0.16–0.58)	0.18 (0.05–0.47)	0.15 (0.08–0.30)
IV	Barraza et al. (2019)	Ecuador	5	6.8 (6.3–7.1)	6.9 (6.9–7.0)	/	/	0.68 (0.26–1.60)	0.33 (0.25–0.40)
V	Maurice & Schreck (unpublished)	Ecuador	15	6.6 (6.1–7.1)	/	1.95 (0.61–3.09)	/	0.55 (0.09–1.19)	/
VI	Engbersen et al. (2019)	Honduras	60	5.2 (4.3–6.5)	/	2.04 (1.03–3.34)	/	0.53 (0.22–1.15)	/
VII	Gramlich et al. (2018)	Honduras	110	5.8 (4.6–7.8)	5.7 (4.6–7.8)	2.30 (0.97–4.56)	1.44 (0.16–2.74)	0.25 (0.02–1.11)	0.16 (0.01–0.82)

§ Dashes indicate that the parameter was not reported for this specific study.

Table I.3 Included variables and resulting prediction equation for the regression analysis for each separate study.

Study	Predictors included in the multivariate regression analysis [§]	Prediction equation for bean Cd
I	pH Top, SOC Top, Total Cd Top, Total Zn Top, Total Mn Top	1.7 + 0.94 * $\log_{10}$[Total Cd Top] -0.21 * [pH Top] - 0.94 * log[SOC Top]
II a	pH Top, pH Sub, SOC Top, SOC Sub, Total Cd Top, Total Cd Sub, Total Zn Top, Total Zn Sub, Total Mn Top, Total Mn Top	-3.4 - 0.49 * log[SOC Sub] + 1.0 * log[Total Zn Sub] + 0.41 * log[Total Mn Top]
II b	pH Top, pH Sub, SOC Top, SOC Sub, Total Cd Top, Total Cd Sub	-0.056 + 0.93 * log[Total Cd Top] - 0.74 * log[SOC Sub]
VI	pH Top, SOC Top, Av Cd Top, Clay Top, Av Zn Top	1.6 + 0.49 * log [Total Cd Top] - 0.21 * [pH Top]
VII a	pH Top, pH Sub, SOC Top, SOC Sub, Total Cd Top, Total Cd Sub, Clay Top, Clay Sub, Total Zn Top, Total Zn Sub	2.2 + 0.84 * log[Total Cd Top] - 0.25 * [pH Top] - 1.2 * log[SOC Top]
VII b	pH Top, pH Sub, SOC Top, SOC Sub, Av Cd Top, Av Cd Sub, Clay Top, Clay Sub, Av Zn Top, Av Zn Sub, Av Mn Top, Av Mn Sub	1.2 + 0.55 * log[Av Cd Top] + 0.49 * log[Av Zn Sub] - 0.25* log[Av Mn Top]
VII c	pH Top, pH Sub, SOC Top, SOC Sub, DGT Cd Top, DGT Cd Sub, Clay Top, Clay Sub, DGT Zn Top, DGT Zn Sub	0.47 + 0.82 * log[DGT Cd Top] - 0.73 * log[SOC Top]

[§] Top = topsoil (0 – 20 cm), Sub = subsoil (20 – 40 cm), Av = available, SOC = soil organic carbon, DGT = diffusive gradient thin film.

Table I.4 Soil-plant transfer factors for different crops, including cacao. $TF_{leaf\text{-}soil}$ = leaf Cd / soil Cd; $TF_{seed\text{-}soil}$ = seed Cd / soil Cd. Additional information to support Table 2.3. Table continued on the following page.

Transfer factor calculated as concentration ratio					
Plant	**TF (leaf – soil)**	**TF (seed – soil)**	**n**	**Reference**	**Remarks**
Cacao	0.34–42.8 (4.46)	0.13–12.5 (1.60)	560	Argüello et al. (2019)	Ecuador Soil Cd: 0.025 – 6.90 (0.44 average) mg kg^{-1}, pH: 6.02
	0.56–20.1 (3.39)	0.26–7.84 (1.96)	28	Barraza et al. (2017)	Ecuador Soil Cd: 0.05 – 1.60 (0.46 average) mg kg^{-1}, pH: 6.01
	1.88–29.2 (4.93)	1.44–29.8 (5.62)	60	Engbersen et al. (2019)	Honduras Soil Cd: 0.22 – 1.15 (0.53 average) mg kg^{-1}, pH: 5.15
	1.9–21.1 (7.1)	0.5–16.3 (2.7)	108	Gramlich et al. (2018)	Honduras Soil Cd: 0.017 – 1.11 (0.25 average) mg kg^{-1}, pH: 5.77
Willow	1.6–10.3		8	Van Slycken et al. (2013)	France Soil Cd 6.5 ± 0.8 mg kg^{-1}; soil Zn 377 ±69 mg kg^{-1}; pH 6.6 ± 0.2
Oak	0.26 [$]		2	Sevel et al. (2009)	Two oaks in tree forest ecosystem with soil Cd A horizon (0 – 35 cm) 0.15 mg kg^{-1}, pH 5.2.
Poplar	5.0–40.0		13	Laureysens et al. (2004)	Short rotation coppice culture on a former waste disposal site. Soil Cd 0.05 – 1.60 mg kg^{-1}.
Leaf vegetables	0.001–4.5 (0.189)		170	Zhang et al. (2014)	South China. Soil Cd: 0.012 – 1.18 mg kg^{-1}
Wheat	0.06–0.64 (0.27)	0.03–0.034 (0.015)	40	Puschenreiter and Horak (2000)	Austria. Soil Cd: 0.13 – 0.44 (0.25 mean) mg kg^{-1}
Rye	0.03–0.29 (0.11)	0.01–0.16 (0.04)	40	Puschenreiter and Horak (2000)	Austria. Soil Cd: 0.13 – 0.44 (0.25 mean) mg kg^{-1}
Sunflower		1.76–3.4 (2.19)	200	Li et al. (1995)	USA Soil Cd 0.39 mg kg^{-1}.

[$] No range available, only average is given

Table I.4 continued

Transfer factor calculated as concentration ratio					
Plant	**TF (leaf – soil)**	**TF (seed – soil)**	**n**	**Reference**	**Remarks**
Cotton		0.46 [$]	6	Chen et al. (2015)	Soil Cd 0.26 ± 0.04 mg kg^{-1}; TF calculated based on average seed Cd and average soil Cd.
Pistachio		0.01–0.04 (0.03)	220	Shirani et al. (2018)	Iran Soil Cd: 0.61 – 2.9 (1.6 average) mg kg^{-1}, pH: 7.9

Transfer factor calculated from predicted crop Cd concentration model based on soil properties [£]					
Plant	**TF (seed – soil)**	**Prediction model**	**n**	**Reference**	**Remarks**
Cacao	1.76	log[Bean Cd] = 1.34 + 0.86 log[Total Soil Cd] - 0.18 [soil pH] - 0.25 log[SOC]	334	Chapter 2 [§]	Ecuador and Honduras
Indica rice	0.76	log[Grain Cd] = 1.20 + 0.76 log[Cd Soil] - 0.17 [Soil pH] - 0.32 log[CEC]	1043	Römkens et al. (2009)	Taiwan. Soil Cd ranges from 0.1 mg kg^{-1} to 30 mg kg^{-1} (i.e. from background to heavily polluted soils)
Japonica rice	0.30	log[Grain Cd] = 0.97 + 0.74 log[Cd Soil] - 0.18 [Soil pH] - 0.43 log[CEC]	1043	Römkens et al. (2009)	Taiwan. Soil Cd ranges from 0.1 mg kg^{-1} to 30 mg kg^{-1} (i.e. from background to heavily polluted soils)
Wheat	0.27	log[Grain Cd] = 0.12 + 0.43 log[Cd Soil] - 0.16 [Soil pH]	246	Adams et al. (2004)	USA

[$] No range available, only average is given

[§] See paragraph 2.2.2.

[£] Transfer factors were calculated with median values from studies I-VII (see above). pH 5.99, CEC 17.84 $cmol_c$ kg^{-1}, soil Cd 0.33 mg kg^{-1}.

I.2. Power analysis to test the effect of mitigation strategies

It is essential to test mitigation strategies in field conditions because cacao is a perennial tree. However, there is scarce experience testing the effect of soil amendments on the reduction of Cd uptake in perennial trees. A statistical power test can be used to estimate the minimum sample size needed to identify a significant treatment effect on bean Cd. In order to calculate such power analysis, it is necessary to know the standard deviation in the population of interest, as well as the plausible effect size of the applied treatments. Argüello et al. (2019) reported an average coefficient of variation of 39 % for bean Cd within 157 different cacao fields in Ecuador, with a 90th percentile of 70 %. Bean Cd data in that study were log-normally distributed and a power analysis for mitigation strategies can be readily calculated using the standard deviation of log-transformed bean Cd values. The standard deviation of log-transformed bean Cd values varied between 0.0027 and 0.53, with an average of 0.18 and a 90th percentile of 0.32. Assuming that the effect of any treatment would be a certain reduction factor (RF), defined as the ratio of the bean Cd concentration in the treatment over the bean Cd concentration in the control, a power analysis can be calculated. Table I.5 shows the results of a power analysis for a scenario with average bean Cd variability and a scenario with high bean Cd variability, as well as differences in the size of replicates, i.e. replicates corresponding to individual trees or replicates corresponding to plots of five or ten trees. All analyses were performed using the online tool developed by Rollin Brant [university of California (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html)]. All data were rounded to two significant digits to maintain consistency in the calculations. To estimate the reduction in standard deviation due to increment of the number of trees per replicate, the following equation was used:

$$Stdev_{plot} = \frac{Stdev_{population}}{\sqrt{n}}$$

Table I.5 Statistical power test to calculate the minimum number of replicates required to identify a significant effect of treatment in cacao field trials (with 80 % power and 5 % type I error, one side comparison of two averages and equal sample size in both) as affected by the variance and expected treatment effect on bean Cd concentration expressed as a reduction factor (RF).

	Number of replicates needed according to the expected factor reduction					
RF	>2.0*	>1.5*	>1.2*	>2.0**	>1.5**	>1.2**
Replicate = 1 tree	5	13	63	15	40	198
Replicate = 5 trees	1	3	13	3	8	38
Replicate = 10 trees	1	2	7	2	4	20

* Assumed Stdev= 0.18 [average Stdev log(bean Cd) within fields in Ecuador]

** Assumed Stdev = 0.32 [90th percentile of Stdev log(bean Cd) within fields in Ecuador]

I.3. Calculations on cacao mixing in Ecuador

Data from the national agricultural land use map for Ecuador (Sistema de Informatión Pública Agropecuaria 2016) was combined with the map of spatial distribution of bean Cd in Ecuador reported by Argüello et al. (2019) to obtain an estimate for the average cacao bean Cd concentration in each province adjusted for province yield. Specifically, all the geographic sectors identified as cacao plantations from satellite data were singled out and the Cd distribution map reported by Argüello et al. (2019) was used to obtain expected bean Cd concentrations for each of their centroid coordinates. Assuming each sector had the same bean Cd concentration as its centroid, these values were then used to calculate an average for each province, weighted by plantation area. To calculate the national average bean Cd in a mixing scenario, these provincial averages were combined with the 5-year (2014 – 2019) average annual cacao production per province as reported in the national survey of continuous agricultural production and surface area (Instituto Nacional de Estadística y Censos - Gobierno de La República del Ecuador 2019). Provinces without reported cacao production during this five-year period, or for which no average bean Cd concentration was available in the study of Argüello et al. (2019), were excluded. This resulted in a total of 20 provinces with 5-year average annual production ranging 69 – 54566 metric tonnes and provincial average bean Cd concentrations ranging 0.26 – 1.41 mg Cd kg^{-1}. While this short calculation can provide a first approximation for the average bean Cd in each province, it ignores any potential variability due to different cacao cultivars and harvesting seasons. Furthermore, this estimate assumes that any later expansion in cacao plantation areas is uniformly distributed within each province, so that the Cd average remains unchanged. More precise estimates can be obtained by accounting for these variables in future calculations.

Appendix II
The elemental composition of chocolates is related to cacao content and origin: a multi-element fingerprinting analysis of single origin chocolates

Table II.1 Quality assurance of the chemical analyses: recoveries (Rec.) of the certified reference materials (CRM) included in triplicate in each digestion: NIST baking chocolate 2384 and dark chocolate ERM® – BD512, data are averages and, for measured concentrations, the standard deviation.

	NIST Baking chocolate			ERM® dark chocolate		
	Certified (mg kg^{-1})	Measured (mg kg^{-1})	Rec. (%)	Certified (mg kg^{-1})	Measured (mg kg^{-1})	Rec. (%)
Ca	840 ± 74	845 ± 8.3	101	/	/	/
Cd	0.0734 ± 0.0077	0.072 ± 0.004	98	0.302 ± 0.013	0.31 ± 0.02	102
Cu	23.9 ± 1.0	23 ± 0.70	96	14.3 ± 0.7	14 ± 0.37	99
Fe	132 ± 11	35 ± 17	27	/	/	/
K	8650 ± 400	8579 ± 383	99	/	/	/
Mg	2610 ± 120	2559 ± 37	98	/	/	/
Mn	20.8 ± 1.3	20 ± 0.47	94	15.7 ± 0.6	15.0 ± 0.28	95
Ni	/	/	/	3.01 ± 0.23	2.8 ± 0.06	93
P	3330 ± 210	3054 ± 39	92	/	/	/
Pb	0.036 ± 0.005	0.029 ± 0.008	79	/	/	/
Zn	37.6 ± 1.9	36 ± 1.0	95	/	/	/

Table II.2 Median element concentration (min - max) measured in chocolates from different geographical origins. LOQ = limit of quantification. Table continued on following page.

	Element concentration (mg kg^{-1})							
	LOQ	Africa (n = 33)		Asia Pacific (n = 14)		Central America (n = 22)		South America (n = 69)	
Al	2.9	8	(<LOQ – 53)	7	(4 – 35)	10	(<LOQ – 32)	7	(<LOQ – 30)
As	0.08	<LOQ [a]		<LOQ [a]		<LOQ [a]		<LOQ [a]	
B	0.45	8.0	(1.6 – 12.7)	8.0	(2.6 – 10.0)	9.1	(2.2 – 14.1)	8.0	(2.0 – 13.2)
Ba	2.0	8	(<LOQ – 19)	4	(<LOQ – 9)	3	(<LOQ – 8)	5	(<LOQ – 24)
Be	0.002	<LOQ	(<LOQ – 0.003)	<LOQ [a]		<LOQ [a]		<LOQ	(<LOQ – 0.003)
Ca	26	820	(490 – 2550)	770	(490 – 2290)	690	(430 – 2120)	720	(470 – 2460)
Cd	0.01	0.20	(0.02 – 0.39)	0.09	(0.04 – 0.25)	0.23	(0.02 – 1.44)	0.46	(0.02 – 2.61)
Co	0.01	0.30	(0.05 – 0.67)	0.26	(0.09 – 0.99)	0.33	(0.07 – 0.52)	0.33	(0.06 – 1.30)
Cr	0.04	0.24	(0.05 – 2.53)	0.29	(0.06 – 1.25)	0.42	(0.09 – 1.48)	0.22	(<LOQ – 8.18)
Cs	0.001	0.013	(0.004 – 0.072)	0.013	(0.003 – 0.059)	0.018	(0.002 – 0.048)	0.012	(0.002 – 0.237)
Cu	0.07	12.7	(1.6 – 17.1)	12.2	(4.2 – 17.5)	14.7	(2.9 – 18.5)	12.9	(3.0 – 26.0)
Ga	0.47	1.6	(<LOQ – 4.0)	0.8	(<LOQ – 1.7)	0.7	(<LOQ – 1.6)	0.9	(<LOQ – 4.9)
In	0.002	<LOQ	(<LOQ – 0.010)	<LOQ	(<LOQ – 0.016)	<LOQ	(<LOQ – 0.011)	<LOQ	(<LOQ – 0.024)
K	8.9	6776	(3956 – 10971)	6297	(4353 – 9015)	6611	(3562 – 11023)	6119	(3715 – 10780)
Li	0.009	<LOQ	(<LOQ – 0.019)	<LOQ	(<LOQ – 0.012)	<LOQ	(<LOQ – 0.017)	<LOQ	(<LOQ – 0.064)
Mg	1.6	2004	(451 – 3088)	1835	(697 – 2172)	1867	(494 – 3017)	1876	(580 – 3183)

[a] All datapoints were below LOQ and, therefore, no range is given.

Table II.2 continued

	Element concentration (mg kg^{-1})							
	LOQ	Africa (n = 33)		Asia Pacific (n = 14)		Central America (n = 22)		South America (n = 69)	
Mn	0.03	17.7	(1.9 – 33.9)	11.8	(3.4 – 39.0)	14.0	(2.7 – 21.7)	13.5	(2.4 – 46.1)
Mo	0.01	0.15	(0.07 – 0.36)	0.09	(0.04 – 0.23)	0.09	(0.05 – 0.17)	0.22	(0.04 – 0.85)
Na	89	<LOQ	(<LOQ – 800)	<LOQ	(<LOQ – 580)	<LOQ	(<LOQ – 600)	<LOQ	(<LOQ – 780)
Ni	0.16	3.8	(0.4 – 7.2)	3.3	(0.4 – 7.0)	4.3	(0.4 – 12.5)	3.0	(0.3 – 8.0)
P	0.76	2859	(1722 – 4113)	2728	(2121 – 3051)	2594	(1730 – 4004)	2792	(1840 – 4822)
Pb	0.02	<LOQ	(<LOQ – 0.08)	<LOQ	(<LOQ – 0.11)	0.02	(<LOQ – 0.12)	<LOQ	(<LOQ – 0.68)
S	58	960	(660 – 1340)	930	(700 – 1040)	920	(660 – 1340)	880	(580 – 1470)
Se	0.06	0.06	(<LOQ – 0.22)	0.06	(<LOQ – 0.11)	<LOQ	(<LOQ – 1.09)	0.09	(<LOQ – 0.78)
Sn	0.02	<LOQ [a]		<LOQ [a]		<LOQ	(<LOQ – 0.04)	<LOQ	(<LOQ – 0.03)
Sr	0.04	7.7	(1.6 – 12.6)	4.7	(2.2 – 8.5)	5.7	(1.8 – 9.9)	5.7	(1.5 – 25.0)
Ti	0.1	0.2	(<LOQ – 0.9)	0.1	(<LOQ – 0.6)	0.1	(<LOQ – 1.6)	0.1	(<LOQ – 0.6)
Tl	0.004	0.007	(<LOQ – 0.032)	<LOQ	(<LOQ – 0.010)	<LOQ	(<LOQ – 0.031)	<LOQ	(<LOQ – 0.026)
U	0.001	<LOQ	(<LOQ – 0.004)	<LOQ	(<LOQ – 0.003)	<LOQ	(<LOQ – 0.002)	<LOQ	(<LOQ – 0.007)
V	0.007	0.024	(0.009 – 0.214)	0.028	(0.015 – 0.200)	0.055	(<LOQ – 0.156)	0.027	(<LOQ – 0.175)
Zn	1.2	28	(11 – 38)	23	(12 – 33)	26	(12 – 35)	28	(12 – 55)
Zr	0.02	<LOQ	(<LOQ – 0.21)	<LOQ	(<LOQ – 0.03)	<LOQ	(<LOQ – 0.04)	<LOQ [a]	

[a] All datapoints were below LOQ and, therefore, no range is given.

Table II.3 Parameter estimates for the linear regression of the elemental concentrations of chocolates compared to their cacao content. The residuals of these fits were used as input for the CART decision tree analysis.

Element	Intercept (mg kg^{-1})	Slope of the elemental concentration per cacao percentage (mg kg^{-1} / %)
B	-2.0	0.14
Ba	-6.7	0.17
Ca	1982	-16
Cd	0.03	0.005
Co	-0.03	0.005
Cr	0.34	0.003
Cu	-2.8	0.22
Cs	-0.008	0.0004
K	412	90
Mg	-513	34
Mn	-7.9	0.33
Mo	0.08	0.002
Ni	-1.0	0.07
P	806	29
S	301	8.9
Sr	-3.1	0.14
V	0.01	0.0005
Zn	-2.1	0.42

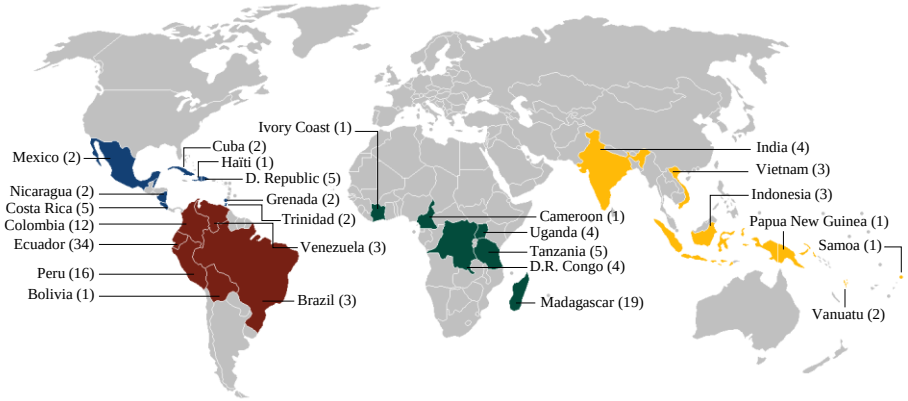

Figure II.1 World map indicating the different cacao origins in the sampled chocolates, as well as the number of samples for each origin. One sample from Tanzania (70 % cacao) was considered an outlier and was not considered in statistical analysis.

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

Figure III.1 Pictures of the mesh bags used for the 1 kg subsamples in the fermentation experiments. Top left, empty mesh bag. Bottom left, mesh bag Velcro closing. Right, mesh bag with 1 kg subsample, sampled after one day of fermentation.

Figure III.2 Picture of a cascade fermentation setup, similar to the setup used in the fermentation experiments for batches A and B.

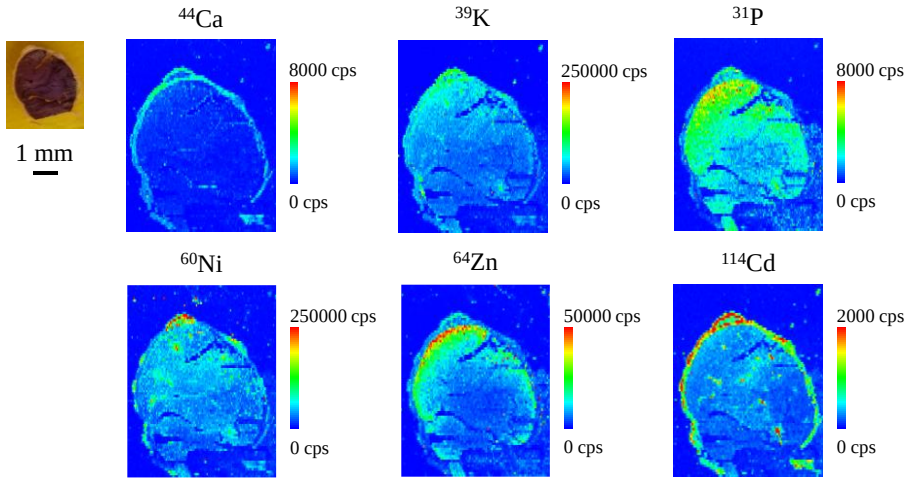

Figure III.3 Distribution images of ^{44}Ca, ^{114}Cd, ^{39}K, ^{31}P, ^{60}Ni and ^{64}Zn obtained by LA-ICP-MS imaging of the cacao bean cross-section from batch C. The total elemental concentrations of the tissues determined in beans from the same pod are 824 mg Ca kg^{-1}, 11097 mg K kg^{-1}, 5893 mg P kg^{-1}, 5.5 mg Ni kg^{-1}, 58 mg Zn kg^{-1} and 1.7 mg kg^{-1} Cd in the nibs; and 2341 mg Ca kg^{-1}, 9271mg K kg^{-1}, 844 mg P kg^{-1}, 6.9 mg Ni kg^{-1}, 37 mg Zn kg^{-1} and 3.1 mg kg^{-1} Cd in the testa. cps = counts per second.

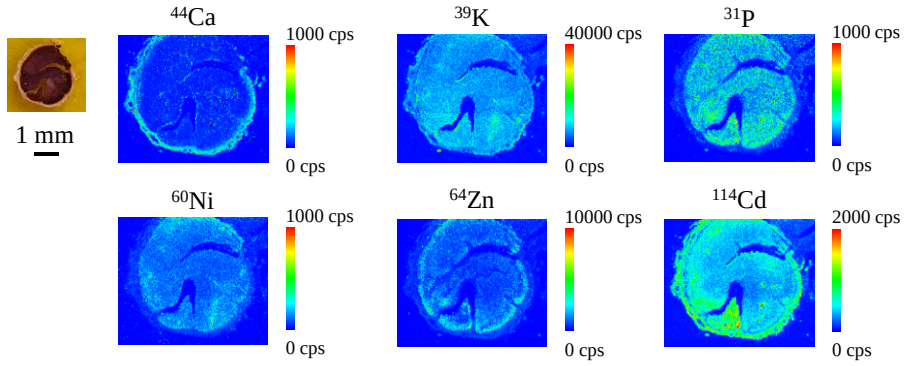

Figure III.4 Distribution images of ^{44}Ca, ^{114}Cd, ^{39}K, ^{31}P, ^{60}Ni, ^{64}Zn obtained by LA-ICP-MS imaging of the cacao bean cross-section from batch D. The total elemental concentrations of the tissues determined in beans from the same pod are: 1118 mg Ca kg^{-1}, 111035 mg K kg^{-1}, , 6137 mg P kg^{-1}, 4.4 mg Ni kg^{-1}, 44 mg Zn kg^{-1} and 11 mg kg^{-1} Cd in the nibs; and 6418 mg Ca kg^{-1}, 11810 mg K kg^{-1}, 686 mg P kg^{-1} 2.5 mg Ni kg^{-1}, 32 mg Zn kg^{-1} and 22 mg kg^{-1} Cd in the testa. cps = counts per second.

Table III.1 Detailed results of the linear combination fits (LCF) for XANES spectra of unfermented cacao nibs.

			O-ligands			P-ligands		S-ligands	
Sample	Cd (mg kg^{-1})	LCF R-factor	Cell wall	O-ligand mix [$]	Cellulose	Phosphate	Phytate	Phytochelatin	Metallothionein
Nib 1	0.85	0.0041			56	44			
		0.0044	84			32			
Nib 2	3.1	0.0015	70					30	
		0.0015	79					22	
Nib 3	0.92	0.0015	57						43
		0.0016	38					63	
Nib 4	7.8	0.0010		73		27			
		0.0011	84				16		
		0.0011		85				15	
Nib 5	10	0.0009					49	51	

[$] Mix of organic acid ligands citrate, malate and succinate.

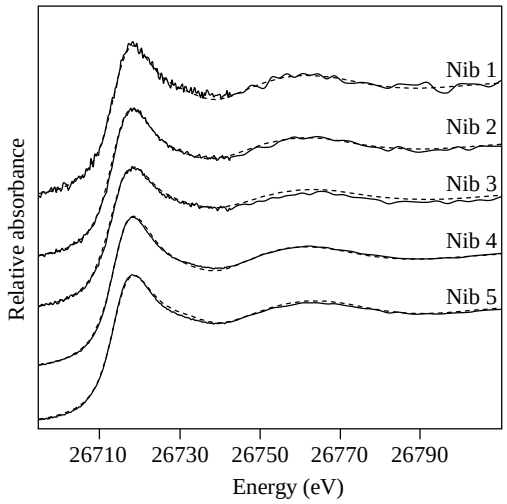

Figure III.5 XANES spectra (solid lines) and linear combination fits (dashed lines) of the Cd K-edge for unfermented nibs with Cd concentrations as determined by ICP-MS analysis: Nib 1 CCN 51 0.85 mg Cd kg^{-1}, Nib 2 CCN 51 3.1 mg Cd kg^{-1}, Nib 3 Nacional 0.92 mg Cd kg^{-1}, Nib 4 Nacional 7.8 mg Cd kg^{-1}, Nib 5 Nacional 10 mg Cd kg^{-1}.

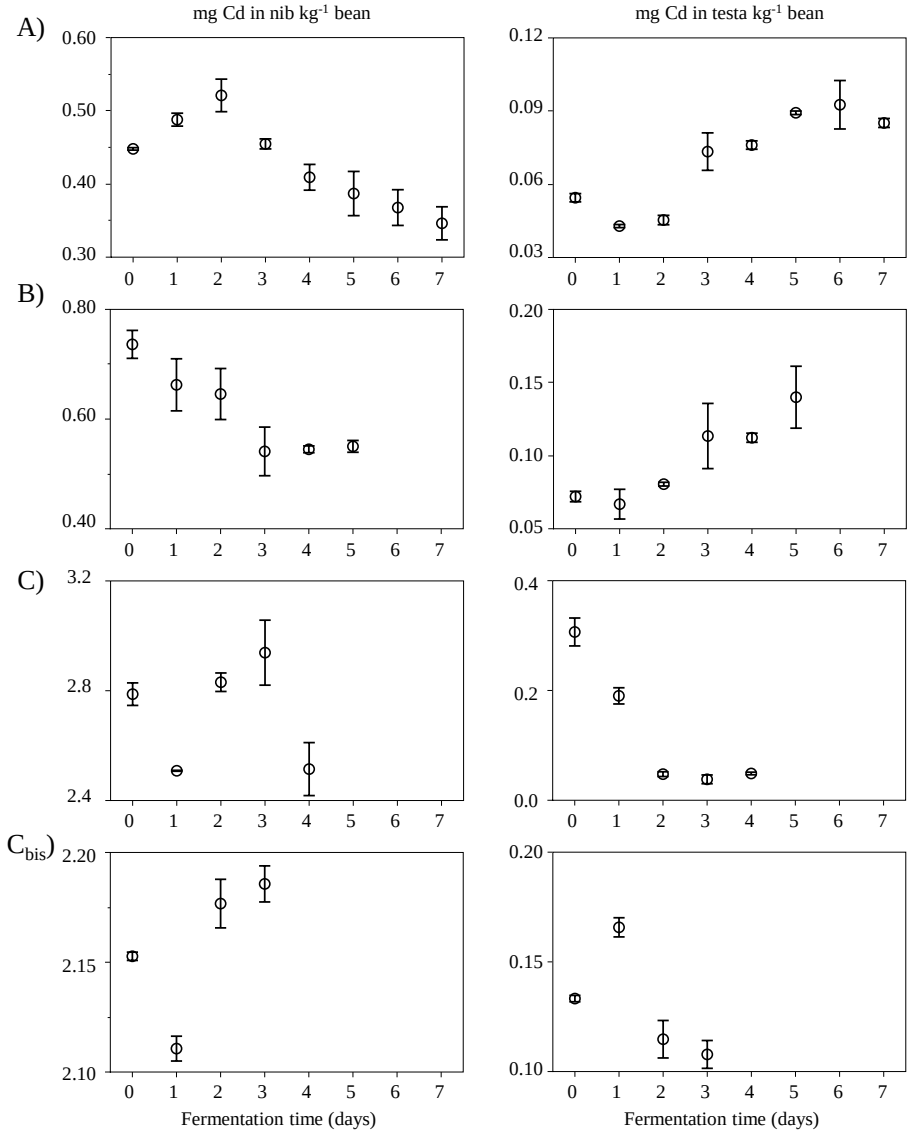

Figure III.6 Influence of cacao fermentation on weight fraction corrected Cd concentrations in nib (left) and testa (right) for the different full scale fermentation batches.

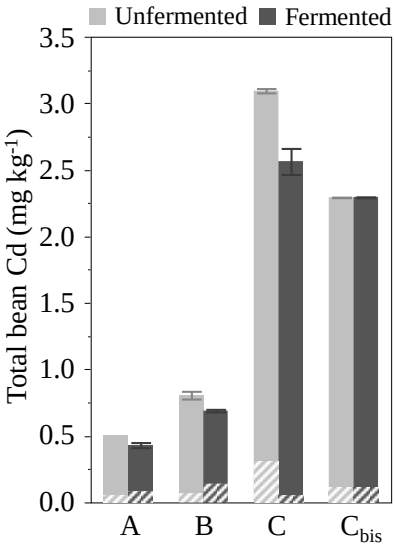

Figure III.7 Cadmium mass balances in fermented and unfermented cacao beans. The weight fractions of nib and testa were determined for three cacao beans per sample and Cd concentrations in each fraction were converted to weighed average concentrations in the oven dried cacao beans. Error bars represent the standard deviation of total bean Cd for three replicates and shaded pattern indicates the fraction of Cd that is located in the testa. Total bean weighed average Cd concentrations decreased (Students t-test) after fermentation in batches A (on average 14 % loss of Cd, P-value 0.08), B (15 %, P-value 0.06) and C (17 %, P-value 0.03) but not in batch C_{bis} (P-value 0.61).

Table III.2 Correlations of the concentrations of the different elements with fermentation time (days). Significant correlations (P-value < 0.05) are indicated with an asterisk. As stated in the main text, one mucilage sample (batch B, day 3) was considered an outlier and excluded from analysis.

	Batch	Nib	Testa	Mucilage
Ca	A	0.66*	-0.42	0.55*
	B	0.65*	-0.96*	0.08
	C	-0.43	0.25	0.75*
	C_{bis}	0.62	0.76*	-0.24
Cd	A	-0.83*	0.78*	0.89*
	B	-0.78*	0.37	0.67*
	C	-0.15	-0.87*	0.91*
	C_{bis}	0.63	0.20	0.88*
Co	A	0.17	0.96*	0.96*
	B	-0.91*	0.90*	0.90*
	C	0.19	0.66*	0.71*
	C_{bis}	-0.11	-0.39	0.21
Cu	A	-0.91*	0.93*	0.94*
	B	-0.75*	0.92*	0.93*
	C	0.25	0.31	0.03
	C_{bis}	0.13	0.23	0.08
K	A	-0.94*	0.91*	0.93*
	B	-0.91*	0.83*	0.90*
	C	-0.55	0.85*	0.90*
	C_{bis}	0.48	-0.08	0.96*
Mg	A	-0.95*	0.98*	0.98*
	B	-0.91*	0.96*	0.94*
	C	-0.15	0.63*	0.86*
	C_{bis}	0.69	-0.003	0.40
Mn	A	0.42	0.90*	0.59*
	B	-0.63*	0.88*	-0.34
	C	-0.37	-0.82*	0.91*
	C_{bis}	0.02	0.62	-0.16
Mo	A	0.32	0.31	0.05
	B	0.26	-0.30	-0.58
	C	-0.11	-0.54	0.22
	C_{bis}	0.13	0.60	<LOQ
Ni	A	-0.95*	0.94*	0.95*
	B	-0.92*	0.95*	0.83*
	C	-0.17	-0.45	0.66*
	C_{bis}	0.85*	-0.89*	0.81*
P	A	0.06	0.96*	0.99*
	B	-0.89*	0.93*	0.90*
	C	-0.55	0.37	0.90*
	C_{bis}	0.64	-0.96*	0.85*
Zn	A	-0.35	0.60*	0.69*
	B	-0.16	0.57	-0.66*
	C	0.13	0.43	0.48
	C_{bis}	0.24	-0.79	-0.65

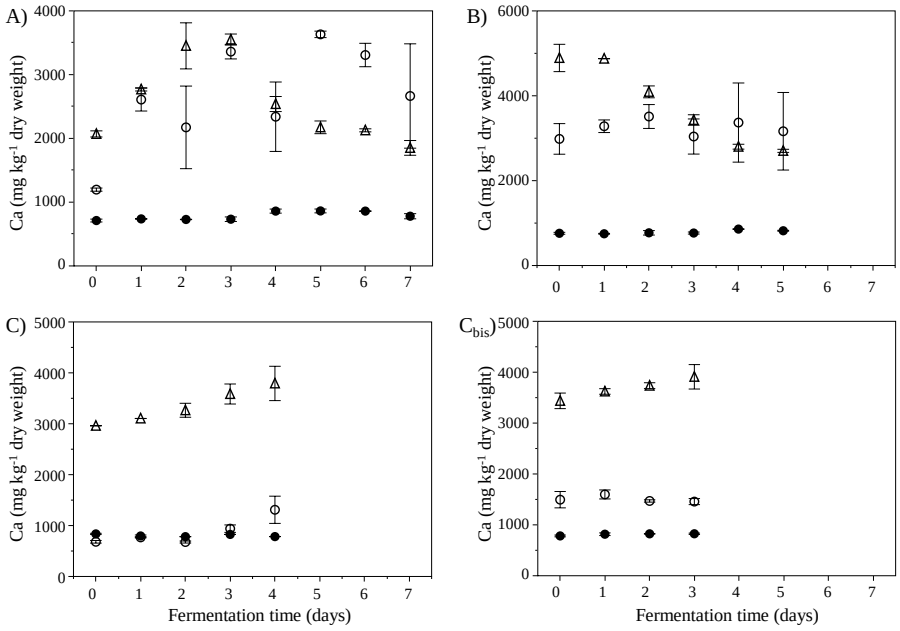

Figure III.8 Concentrations of Ca in the different cacao bean tissues [nib (●), testa (Δ) and mucilage (O)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

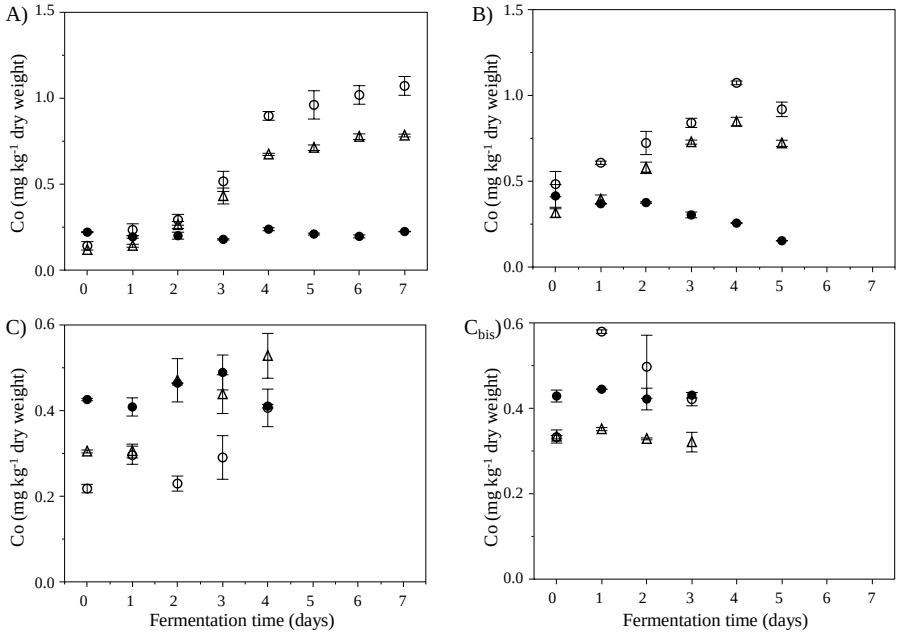

Figure III.9 Concentrations of Co in the different cacao bean tissues [nib (●), testa (Δ) and mucilage (O)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

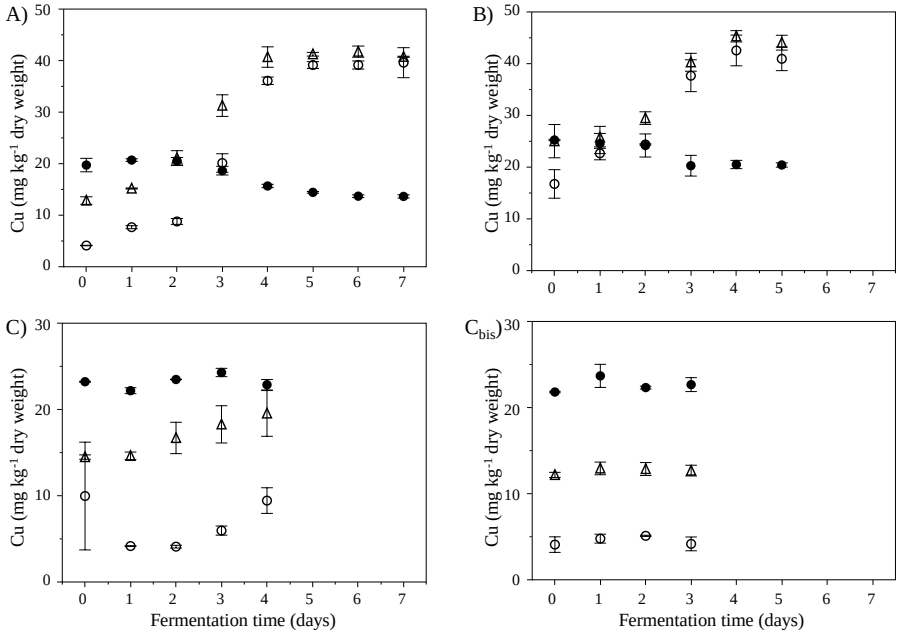

Figure III.10 Concentrations of Cu in the different cacao bean tissues [nib (●), testa (△) and mucilage (○)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

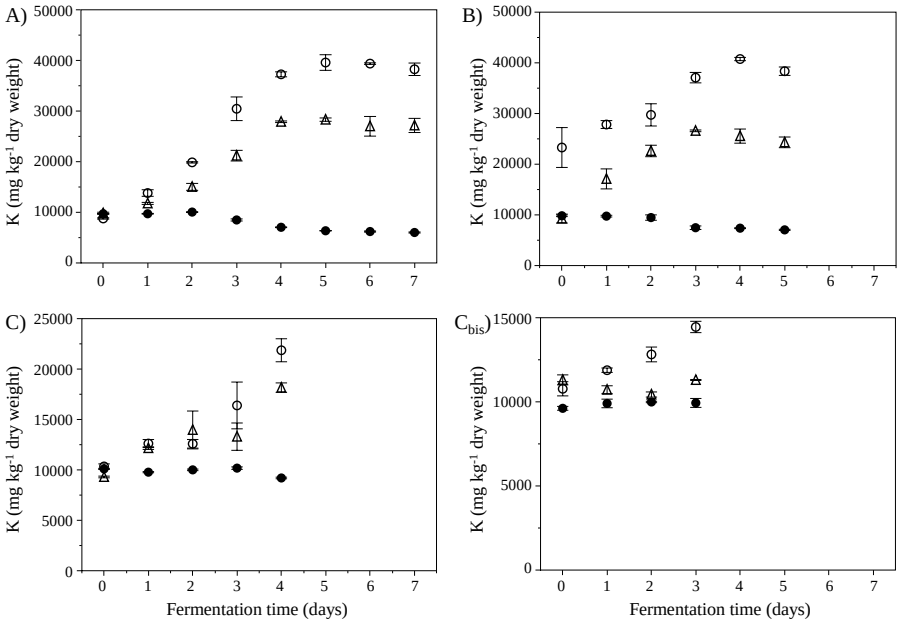

Figure III.11 Concentrations of K in the different cacao bean tissues [nib (●), testa (△) and mucilage (○)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

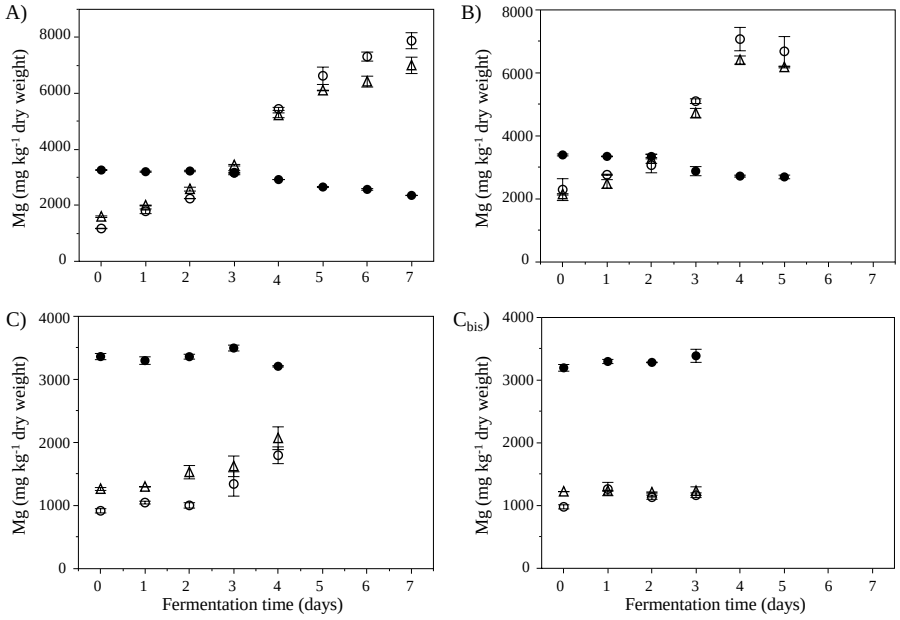

Figure III.12 Concentrations of Mg in the different cacao bean tissues [nib (●), testa (Δ) and mucilage (O)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

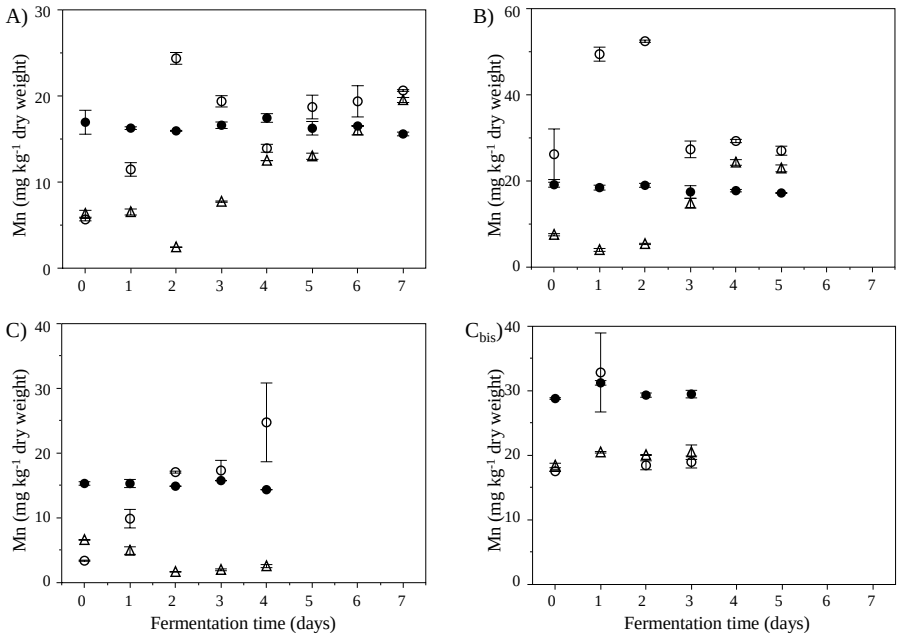

Figure III.13 Concentrations of Mn in the different cacao bean tissues [nib (●), testa (△) and mucilage (○)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

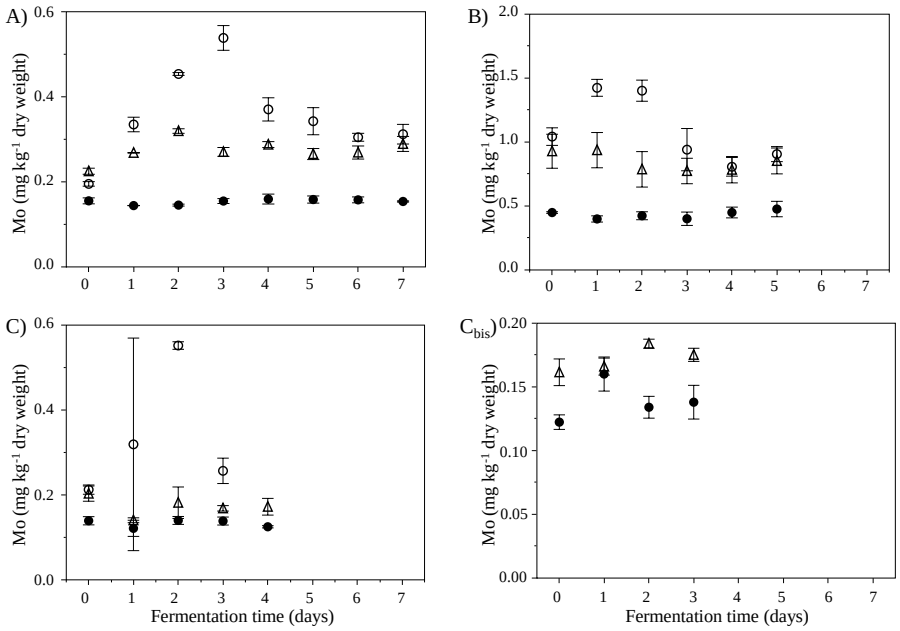

Figure III.14 Concentrations of Mo in the different cacao bean tissues [nib (●), testa (△) and mucilage (○)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

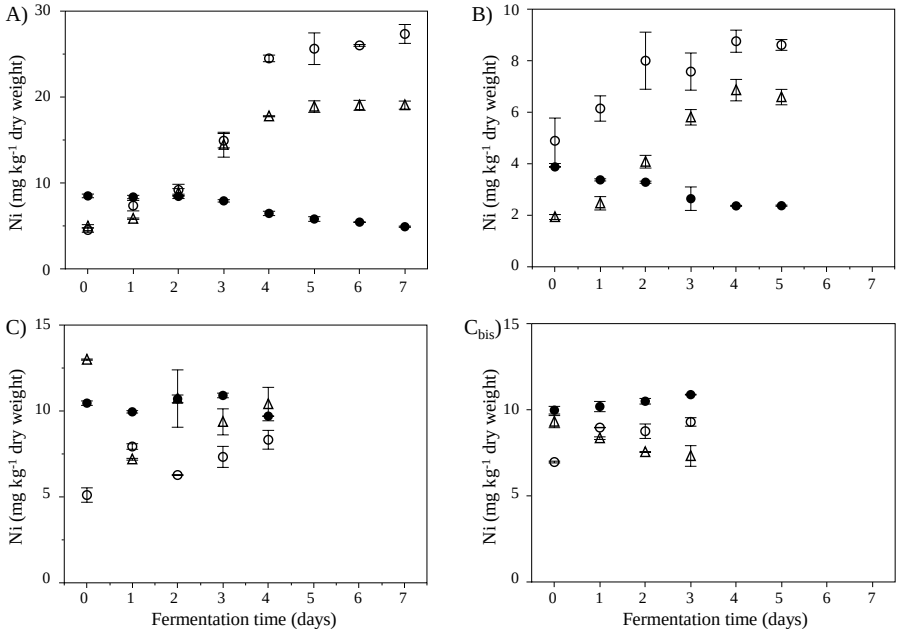

Figure III.15 Concentrations of Ni in the different cacao bean tissues [nib (●), testa (Δ) and mucilage (O)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

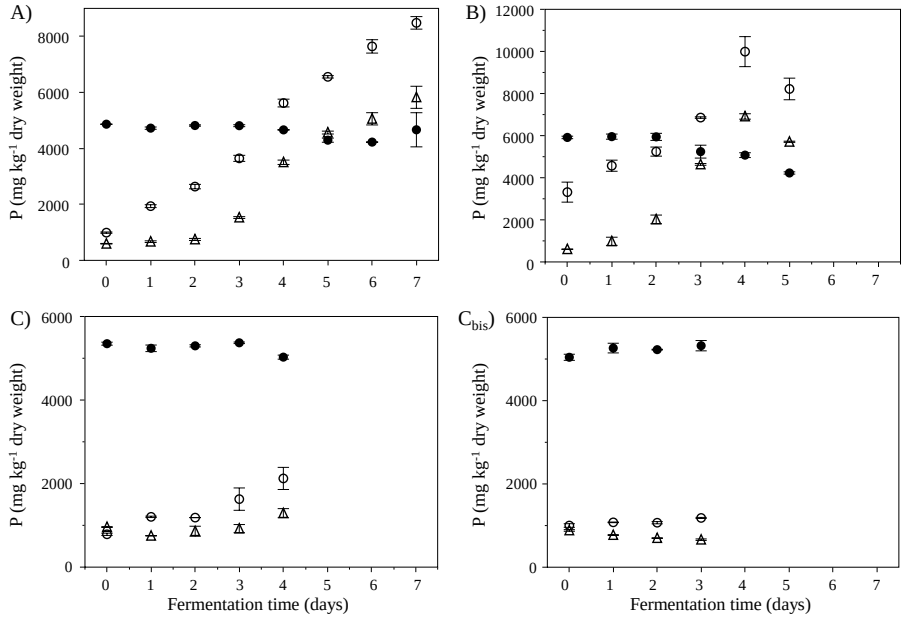

Figure III.16 Concentrations of P in the different cacao bean tissues [nib (●), testa (Δ) and mucilage (Ο)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

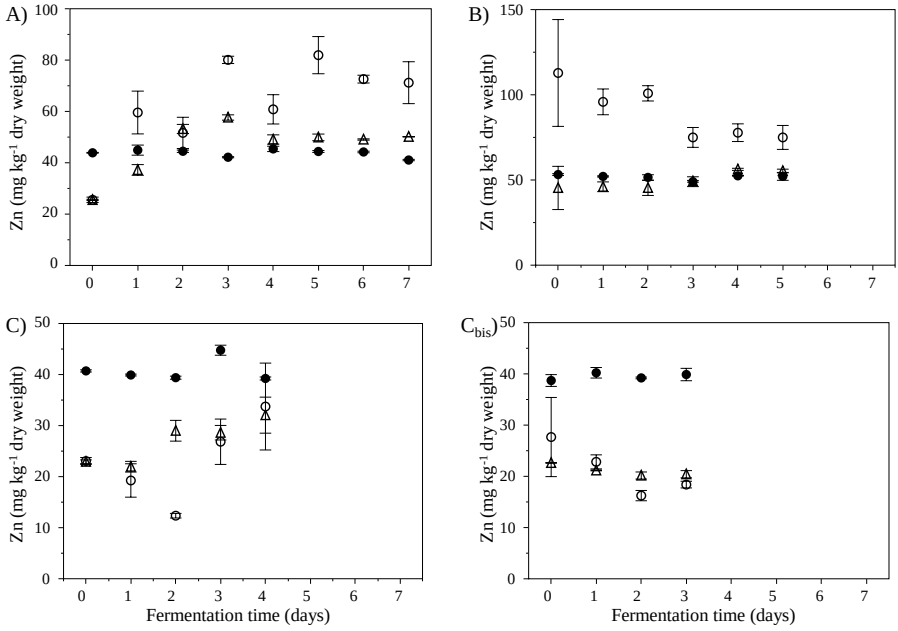

Figure III.17 Concentrations of Zn in the different cacao bean tissues [nib (●), testa (△) and mucilage (○)] measured at different days of fermentation. Each point represents the average of duplicate samples and error bars denote the standard error.

Appendix IV
Cadmium migration from nib to testa during cacao fermentation is driven by nib acidification

Figure IV.1 Mesh sample bags in the micro-fermentation setup at the start of the experiment. The cacao fermentation box is filled halfway at the time of the picture.

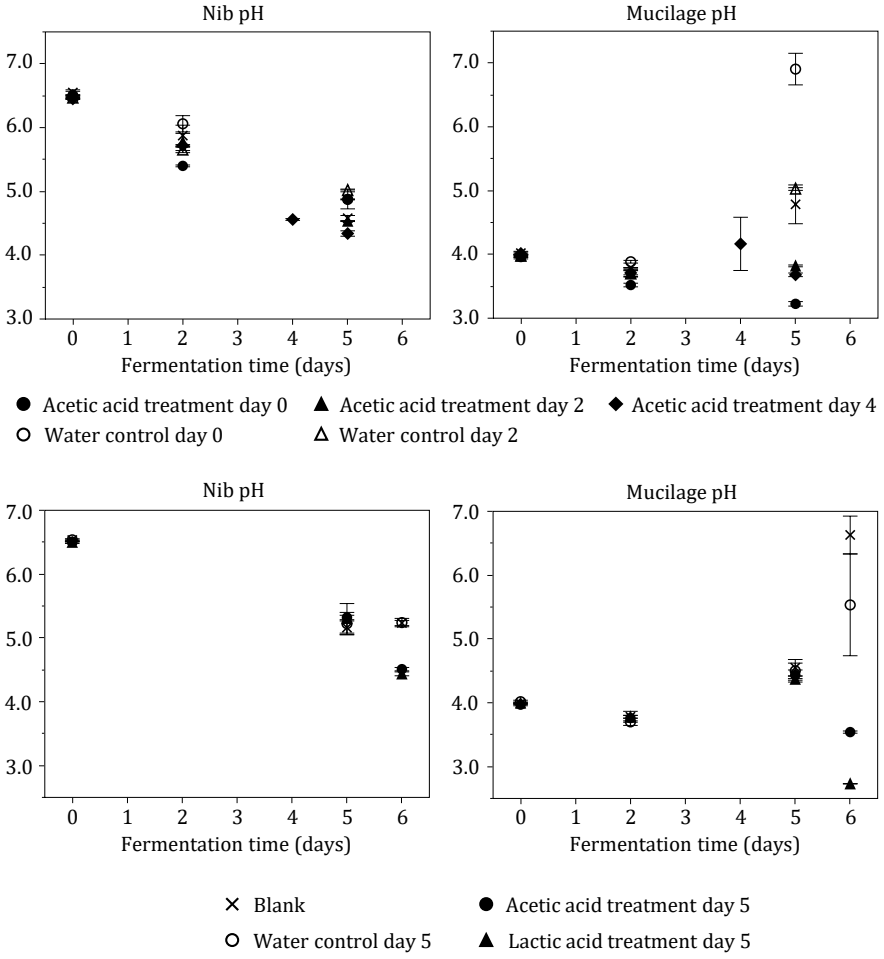

Figure IV.2 Changes in nib and mucilage pH during fermentation in lab scale fermentation A (top) and B (bottom).

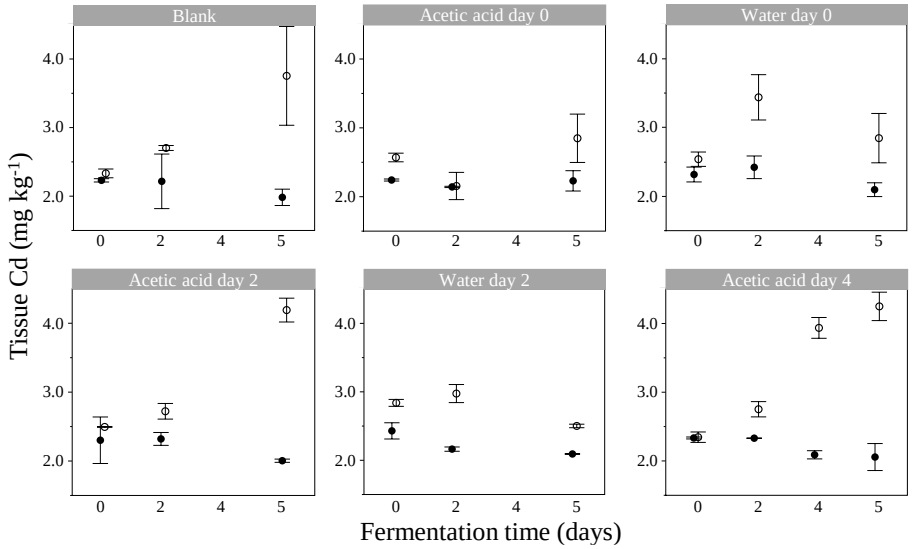

Figure IV.3 Cadmium concentrations in nib (●) and testa (○) with fermentation time, for different treatments in fermentation experiment A.

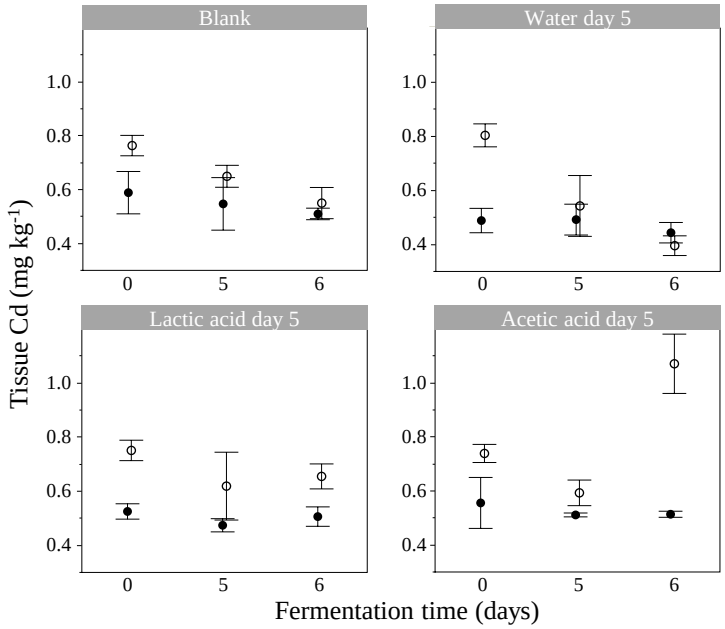

Figure IV.4 Cadmium concentrations in nib (●) and testa (○) with fermentation time, for different treatments in fermentation experiment B.

Table IV.1 Tissue elemental concentrations [average ± stdev of duplicates (experiment A) or triplicates (experiment B)] at the end of fermentation (day 5 for experiment A and day 6 for experiment B) depending on the cacao treatment. Letters denote significant differences within rows (Tukey's HSD test, P-value < 0.05). Table continued on following page.

Fermentation trial A, n = 2						
Treatment	Blank	Control Day 0	Control Day 2	Acetic acid day 0	Acetic acid day 2	Acetic acid day 4
Nib Ca (mg kg^{-1})	1070 ± 50 ^{ns}	1020 ± 50	1000 ± 40	1020 ± 40	1000 ± 10	970 ± 10
Testa Ca (mg kg^{-1})	5370 ± 250 ^{ns}	5490 ± 70	5450 ± 350	5790 ± 80	5760 ± 190	5350 ± 70
Nib Cd (mg kg^{-1})	1.98 ± 0.12 ^{ns}	2.10 ± 0.10	2.09 ± 0.004	2.23 ± 0.12	2.00 ± 0.02	2.06 ± 0.20
Testa Cd (mg kg^{-1})	3.75± 0.72^{AB}	2.85± 0.36^{AB}	2.50 ± 0.03 ^{B}	2.85±0.35 ^{AB}	4.19 ± 0.17 ^{A}	4.25 ± 0.21 ^{A}
Nib Cu (mg kg^{-1})	12.3 ± 0.2 ^{ns}	12.0 ± 0.5	12.1 ± 0.4	12.7 ± 0.5	12.5 ± 1.1	13.0 ± 0.9
Testa Cu (mg kg^{-1})	17.7 ± 1.4 ^{AB}	17.9 ± 1.1 ^{A}	18.4 ± 0.7 ^{A}	14.2 ± 0.1 ^{B}	16.3 ± 0.9 ^{AB}	16.7 ± 0.7 ^{AB}
Nib K (mg kg^{-1})	7870 ± 180 ^{ns}	7650 ± 260	8280 ± 160	8700 ± 480	8530 ± 250	7970 ± 130
Testa K (mg kg^{-1})	21100 ± 800 ^{A}	21500 ± 1100 ^{A}	19700 ± 1200 ^{A}	13800 ± 200 ^{B}	17900 ± 1600 ^{A}	18700 ± 500 ^{A}
Nib Mn (mg kg^{-1})	12.3 ± 0.09 ^{ns}	12.3 ± 0.84	13.0 ± 0.05	13.6 ± 0.01	12.5 ± 0.71	12.2 ± 0.06
Testa Mn (mg kg^{-1})	18 ± 2 ^{A}	13 ± 2 ^{AB}	9 ± 1 ^{B}	12 ± 2 ^{AB}	18 ± 2 ^{A}	19 ± 1 ^{A}
Nib Ni (mg kg^{-1})	9.2 ± 0.1 ^{B}	8.8 ± 0.6 ^{B}	9.4 ± 0.1 ^{AB}	10.4 ± 0.2 ^{A}	9.5 ± 0.2 ^{AB}	9.7 ± 0.2 ^{AB}
Testa Ni (mg kg^{-1})	18 ± 0.5 ^{AB}	19 ± 0.8 ^{A}	18 ± 0.9 ^{AB}	11 ± 0.8 ^{C}	15 ± 0.8 ^{B}	17 ± 0.5 ^{AB}
Nib P (mg kg^{-1})	4400 ± 160 ^{B}	4410 ± 50 ^{B}	4660 ± 40 ^{AB}	4940 ± 150 ^{A}	4780 ± 10 ^{AB}	4410 ± 80 ^{B}
Testa P (mg kg^{-1})	5350 ± 830^{A}	5640 ± 420 ^{A}	4540 ± 400 ^{AB}	2880 ± 690 ^{B}	4490 ± 620 ^{AB}	4640 ± 350^{AB}
Nib Zn (mg kg^{-1})	36.4 ± 0.1 ^{ns}	37.2 ± 0.4	38.9 ± 0.2	38.8 ± 1.6	38.3 ± 0.9	37.9 ± 1.2
Testa Zn (mg kg^{-1})	53 ± 8 ^{BC}	44 ± 2 ^{C}	64 ± 16 ^{ABC}	44 ± 8 ^{C}	91 ± 5 ^{A}	86 ± 1 ^{AB}

ns = not significant

Table IV.1 continued.

Fermentation trial B, n = 3				
Treatment	Blank	Control	Acetic acid day 5	Lactic acid day 5
Nib Ca (mg kg^{-1})	889 ± 20 ^{ns}	878 ± 41	853 ± 6	867 ± 17
Testa Ca (mg kg^{-1})	4660 ± 350 ^{ns}	4630 ± 440	5080 ± 60	4430 ± 170
Nib Cd (mg kg^{-1})	0.51 ± 0.02 ^{ns}	0.44 ± 0.04	0.51 ± 0.01	0.51 ± 0.04
Testa Cd (mg kg^{-1})	0.55 ± 0.06 ^{BC}	0.40 ± 0.04 ^{C}	1.07 ± 0.11 ^{A}	0.65 ± 0.05 ^{B}
Nib Cu (mg kg^{-1})	25 ± 2 ^{ns}	24 ± 1	25 ± 1	26 ± 2
Testa Cu (mg kg^{-1})	46 ± 1 ^{A}	50 ± 3 ^{A}	35 ± 3 ^{B}	28 ± 3 ^{C}
Nib K (mg kg^{-1})	9310 ± 150 ^{ns}	8910 ± 130	9180 ± 340	9180 ± 160
Testa K (mg kg^{-1})	26000 ± 500 ^{A}	25100 ± 600 ^{A}	19900 ± 300 ^{B}	15300 ± 800 ^{C}
Nib Mn (mg kg^{-1})	18.9 ± 0.7 ^{ns}	18.4 ± 0.3	17.7 ± 0.3	18.6 ± 0.7
Testa Mn (mg kg^{-1})	10.1 ± 0.5 ^{B}	9.2 ± 0.7 ^{B}	18.9 ± 0.9 ^{A}	9.4 ± 0.4 ^{B}
Nib Ni (mg kg^{-1})	8.6 ± 0.3 ^{AB}	8.2 ± 0.1 ^{B}	8.5 ± 0.2 ^{AB}	9.0 ± 0.4 ^{A}
Testa Ni (mg kg^{-1})	23.1 ± 0.7 ^{A}	23.6 ± 2.3 ^{A}	22.0 ± 0.3 ^{A}	11.5 ± 0.3 ^{B}
Nib P (mg kg^{-1})	5420 ± 200 ^{ns}	5240 ± 80	5330 ± 120	5450 ± 50
Testa P (mg kg^{-1})	4910 ± 140 ^{A}	4880 ± 490 ^{A}	3230 ± 240 ^{B}	1470 ± 100 ^{C}
Nib Zn (mg kg^{-1})	44 ± 1 ^{ns}	43 ± 1	42 ± 2	41 ± 1
Testa Zn (mg kg^{-1})	36 ± 4 ^{B}	45 ± 8 ^{B}	103 ± 26 ^{A}	34 ± 2 ^{B}

ns = not significant

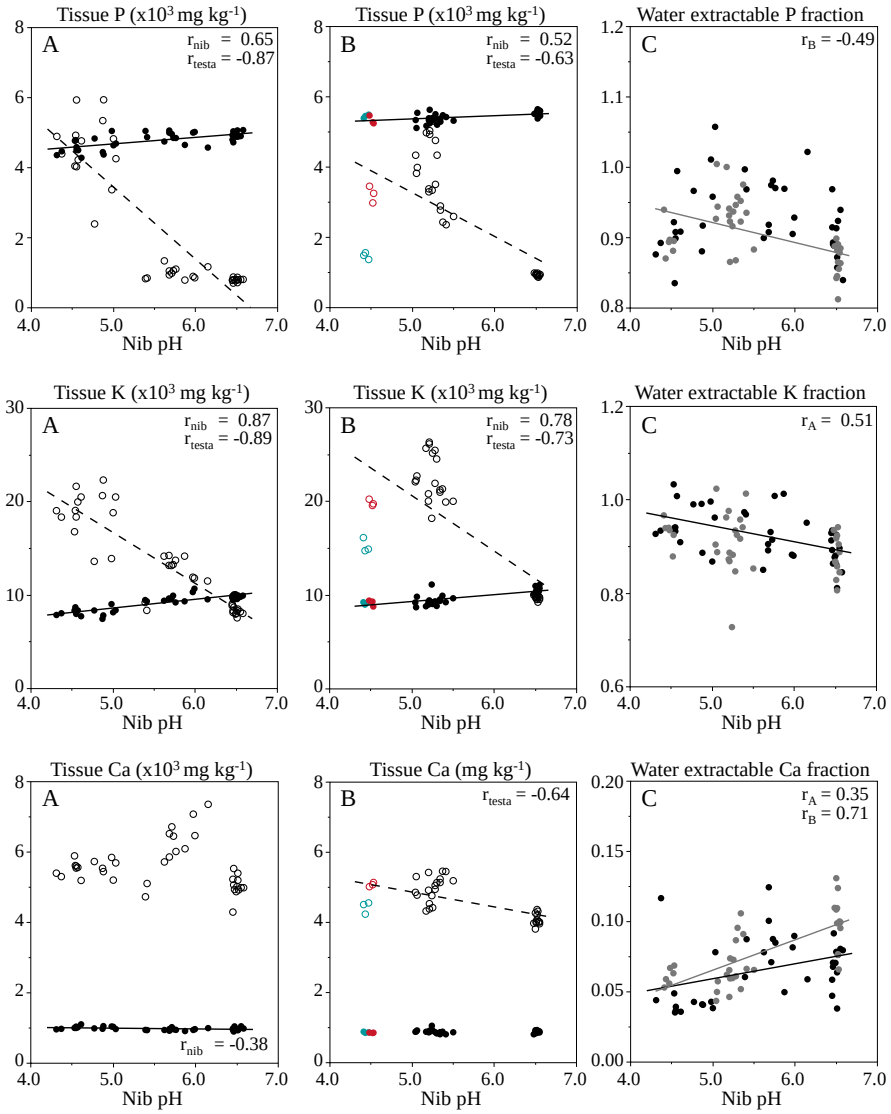

Figure IV.5 (A) Correlations between nib pH and nib (●, solid line) and testa (○, dashed line) elemental concentrations for fermentation trial A. (B) Correlations between nib pH and nib (●, solid line) and testa (○, dashed line) elemental concentrations for fermentation trial B, red symbols indicate acetic acid treated samples at the end of fermentation (day 6) and blue symbols indicate lactic acid treated samples at the end of fermentation (day 6). (C) Correlations between nib water extractable element fractions and nib pH for both fermentation trials (black symbols for experiment A, grey symbols for experiment B). Pearson correlation coefficients (r) are indicated when significant (P-value < 0.05). Figure continued on following pages.

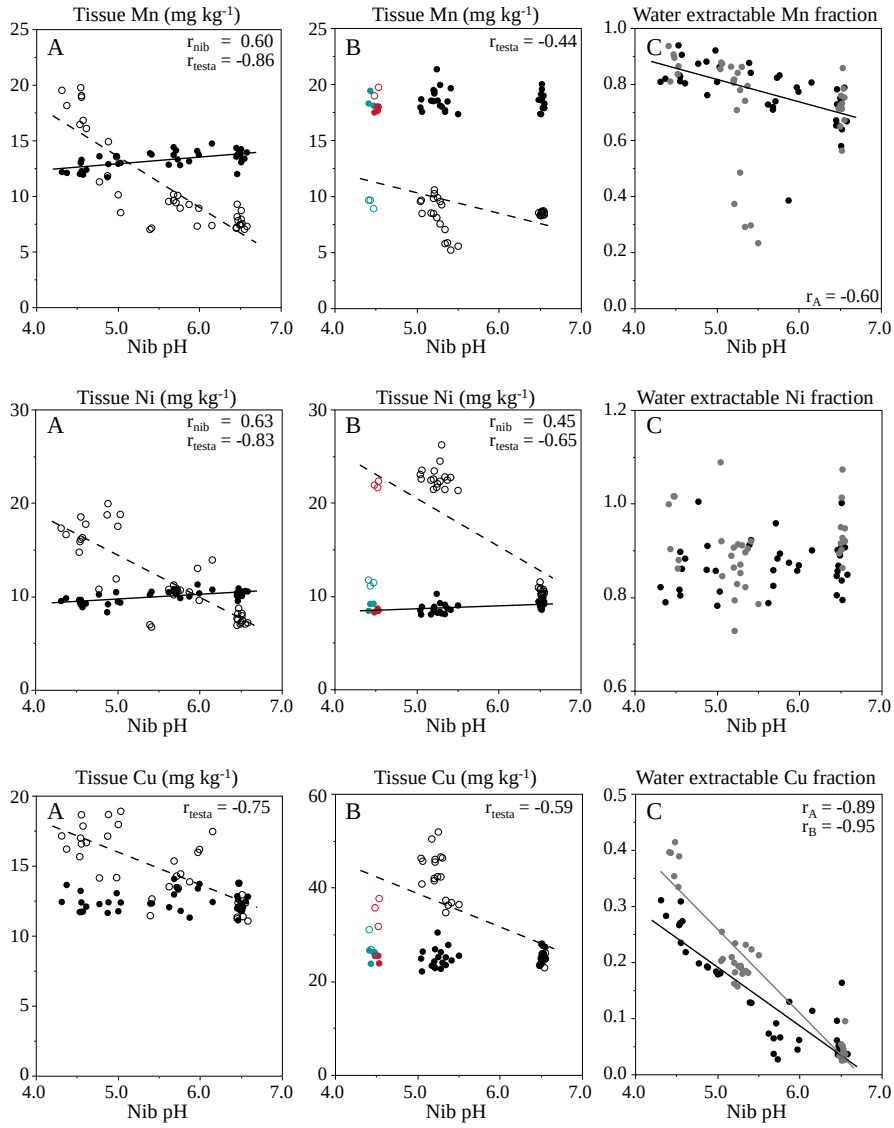

Figure IV.5 continued.

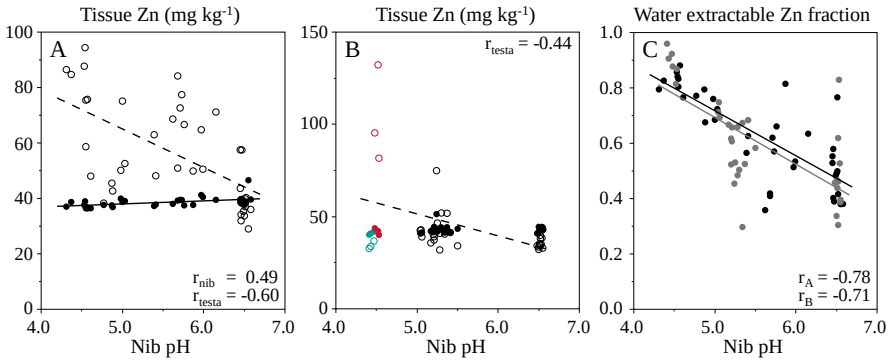

Figure IV.5 continued.

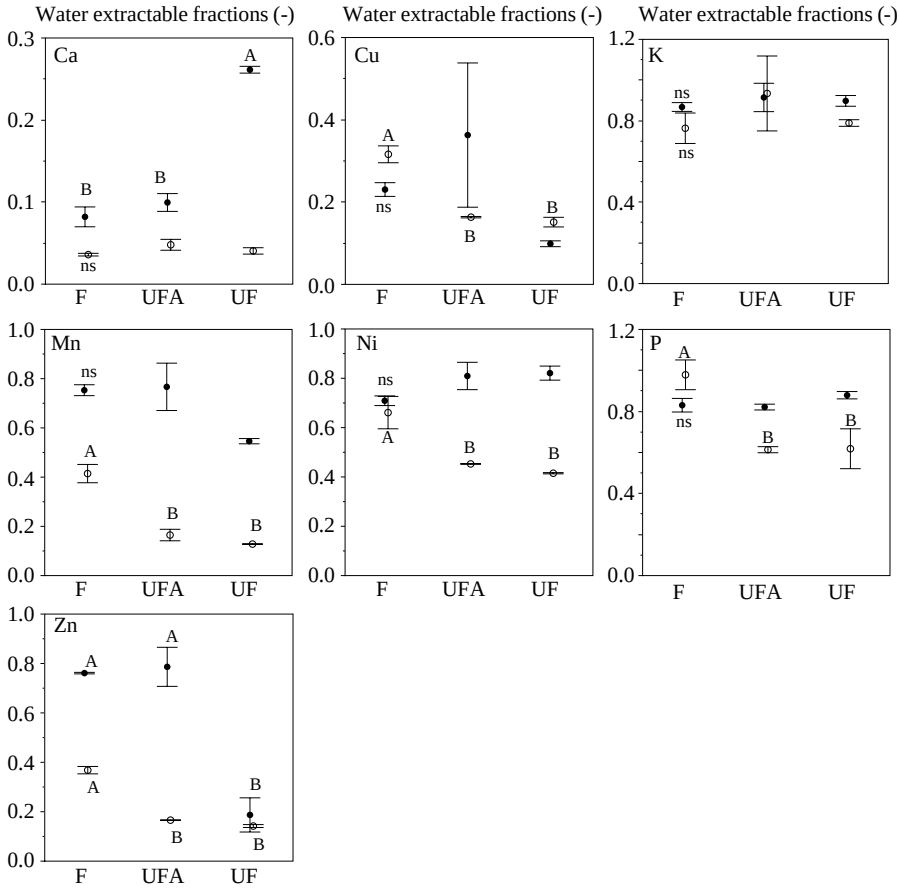

Figure IV.6 Water extractable fractions of elemental concentrations in nib (●) and testa (○) in unfermented samples (UF), samples artificially acidified to typical pH values for the end of fermentation (pH 4.5, UFA), and fermented samples (F). Letters indicate significant differences for each material (nib and testa), based on Tukey's HSD test (P-value < 0.05). ns = not significant.

Table IV.2 Elemental concentrations (average ± stdev, mg kg^{-1}) in nib and testa as affected by micro-fermentation. The average concentration ratios (elemental concentration in the fermented sample over that in the unfermented sample) indicate that concentrations generally decrease in the nib and increase in the testa, with fermentation. CI = confidence interval.

	Nib concentration (mg kg^{-1})			Testa concentration (mg kg^{-1})		
	Unfermented	Fermented	Average concentration ratio [95 % CI]	Unfermented	Fermented	Average concentration ratio [95 % CI]
Ca	833 ± 156	836 ± 178	1.01 [0.97–1.04]	2550 ± 890	2560 ± 730	1.04 [0.91–1.17]
Cd	1.76 ± 0.62	1.46 ± 0.54	0.83[£] [0.76–0.89]	2.53 ± 0.92*	3.28 ± 0.82*	1.37[£] [1.19–1.54]
Cu	25 ± 6 *	20 ± 5 *	0.80[£] [0.73–0.86]	19 ± 5 *	53 ± 15 *	2.90[£] [2.51–3.28]
K	9770 ± 790 *	7590 ± 840 *	0.78[£] [0.73–0.83]	11500 ± 2900 *	27500 ± 2100 *	2.50[£] [2.24–2.77]
Mn	16 ± 3	15 ± 3	0.99 [0.94–1.04]	8 ± 4 *	18 ± 5 *	2.63[£] [2.18–3.07]
Ni	9 ± 2 *	7 ± 2 *	0.80[£] [0.75–0.85]	13 ± 8 *	20 ± 4 *	2.18[£] [1.54–2.83]
P	5340 ± 420 *	4750 ± 430 *	0.89[£] [0.85–0.93]	980 ± 210 *	5260 ± 1140 *	5.49[£] [4.82–6.16]
Zn	46 ± 5	45 ± 4	0.98 [0.96–1.01]	25 ± 3 *	52 ± 21 *	2.10[£] [1.77–2.43]

* Tissue elemental concentration is significantly affected by fermentation (Students t-test, P-value <0.05)

[£] Tissue elemental concentration is significantly affected by fermentation, as identified through pairwise testing (Pairwise t-test; P-value <0.05).

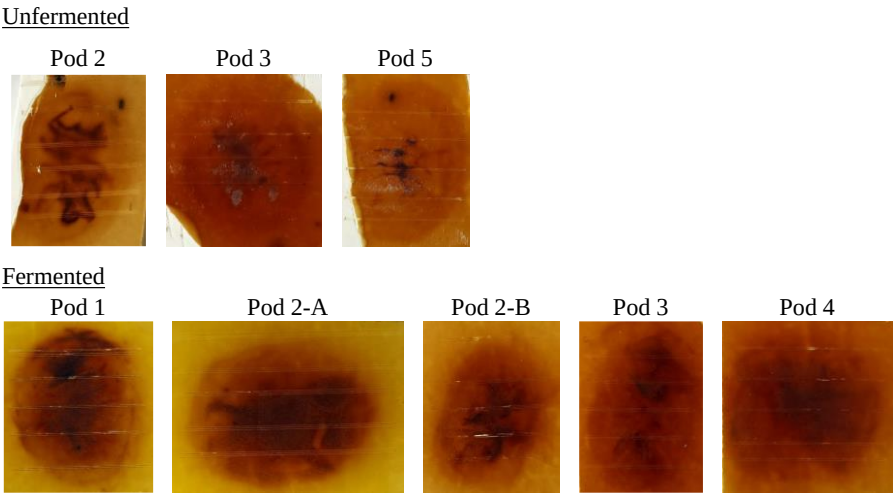

Figure IV.7 Pictures of the Chelex 100 gel imprints, after LA-ICP-MS analysis showing the location of the different ablation lines.

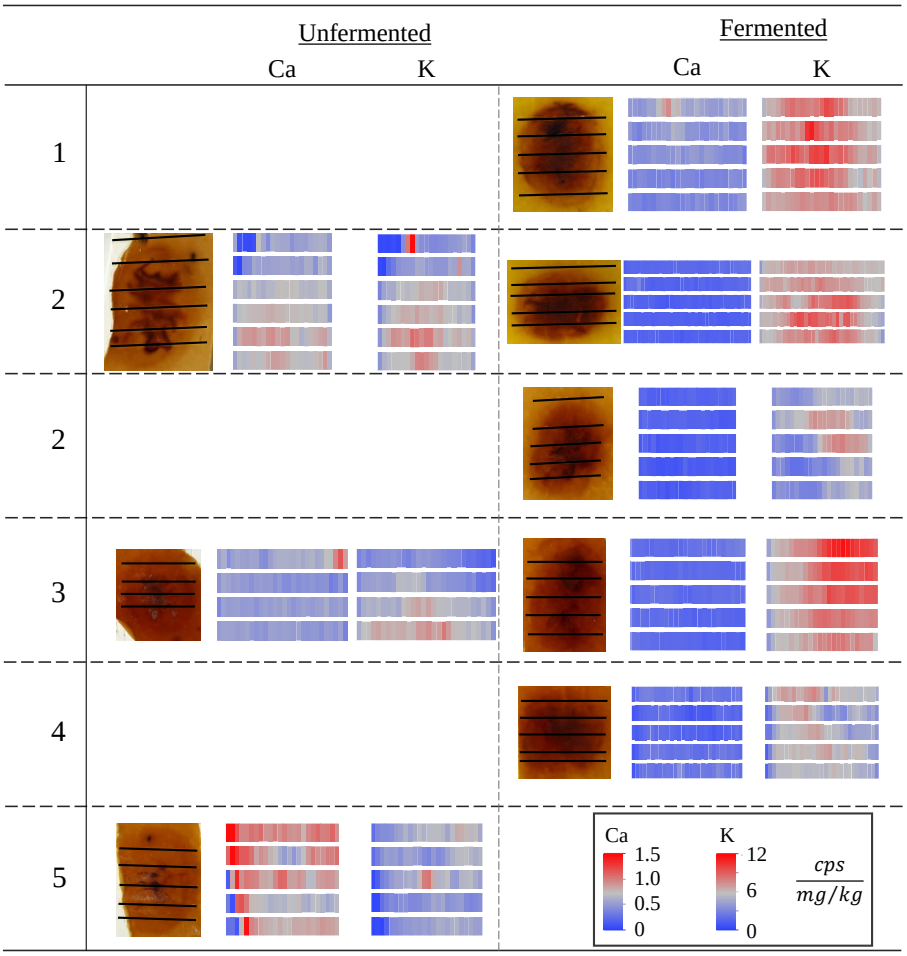

Figure IV.8 Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) visualisation of Ca and K diffused from the bean toward ground Chelex 100 gel ("mobile elements"). Cacao bean samples originate from different single pod micro-fermentation samples (1 – 5). Measured signals of each element (counts per second, cps) are normalised to the elemental composition determined for that sample by acid digestion and ICP-MS analysis. Each horizontal line represents the average signal of two or three ablation lines run in close parallel, indicated by black lines on pictures. The width of each pixel corresponds to a distance of 0.5 mm on the ablation line. Larger scale pictures of gel imprints with ablation lines can be found in Figure IV.7.

Appendix V
Outward migration of cadmium from cacao nibs during fermentation is related to the combination of enhanced acidity and temperature: mimicking fermentation through incubation

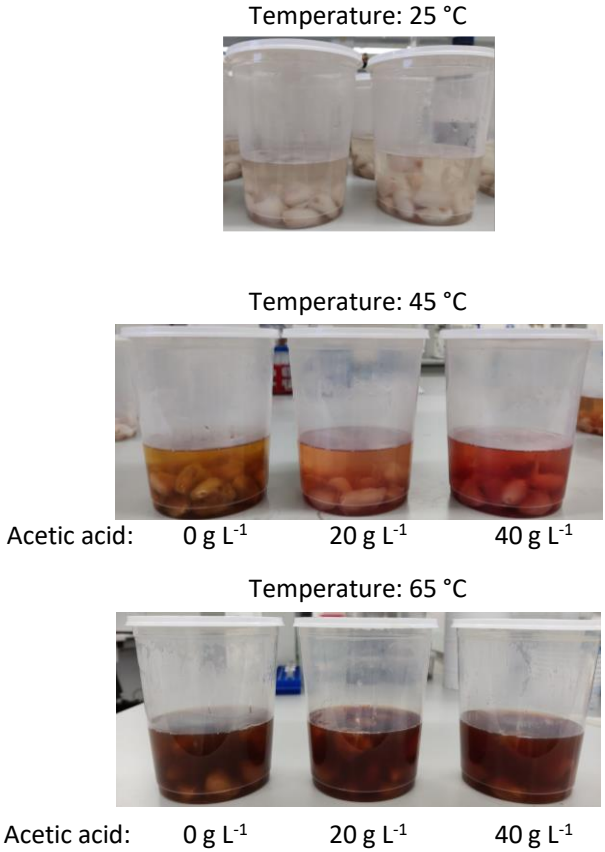

Figure V.1 Aseptic artificial fermentation samples at the end of 72 hours incubation. Samples incubated at 25 °C did not change visually, while samples incubated at higher temperature resulted in a dark brown or pink colour in the contact solution. The intensity of the pink hues increased with increasing acetic acid concentration.

Table V.1 D-optimal design of 40 runs, with four additional runs added to the centre level to represent the conditions in a typical cacao fermentation (indicated with an asterisk).

Run no.	Temperature (°C)	Acetic acid (g L^{-1})	Ethanol (g L^{-1})
1	25	0	0
2	25	0	0
3	25	0	0
4	25	0	70
5	25	0	70
6	25	0	70
7	25	0	70
8	25	20	35
9	25	20	35
10	25	40	0
11	25	40	0
12	25	40	0
13	25	40	0
14	25	40	70
15	25	40	70
16	25	40	70
17	25	40	70
18	45	0	0
19	45	0	35
20	45	20	0
21*	45	20	35
22*	45	20	35
23*	45	20	35
24*	45	20	35
25	45	20	70
26	45	20	70
27	45	40	35
28	45	40	35
29	65	0	0
30	65	0	0
31	65	0	0
32	65	0	35
33	65	0	70
34	65	0	70
35	65	0	70
36	65	20	0
37	65	20	35
38	65	40	0
39	65	40	0
40	65	40	0
41	65	40	70
42	65	40	70
43	65	40	70
44	65	40	70

Table V.2 Average (± standard deviation, n = 44), range (min-max) and coefficient of variation (CV) of elemental concentrations measured in nib and testa, prior to treatment. CI = confidence interval.

	Material	Average ± stdev (mg kg^{-1})	Range min-max (mg kg^{-1})	CV (%)	Ratio testa / nib 95% CI
Ca	Nib	1000 ± 170	740 – 1500	17	2.86 – 3.43
	Testa	3100 ± 1100	1500 – 5300	34	
Cd	Nib	2.1 ± 0.7	1.1 – 5.0	33	1.16 – 1.27
	Testa	2.5 ± 0.9	1.3 – 5.8	34	
Cu	Nib	18 ± 7	7 – 43	41	0.75 – 0.81
	Testa	14 ± 5	6 – 37	40	
K	Nib	10664 ± 752	9113 – 11964	7	0.83 – 0.91
	Testa	9200 ± 1200	6100 – 12000	13	
Mg	Nib	3534 ± 244	3050 – 3916	7	0.32 – 0.35
	Testa	1200 ± 160	740 – 1600	13	
Mn	Nib	15 ± 2	11 – 20	17	0.28 – 0.34
	Testa	5 ± 2	2 – 12	38	
Ni	Nib	6 ± 2	3 – 14	28	0.79 – 1.11
	Testa	6 ± 3	2 – 19	57	
P	Nib	5337 ± 389	4381 – 6102	7	0.16 – 0.18
	Testa	910 ± 180	530 – 1300	20	
Zn	Nib	47 ± 4	38 – 58	9	0.42 – 0.46
	Testa	21 ± 3	16 – 30	16	

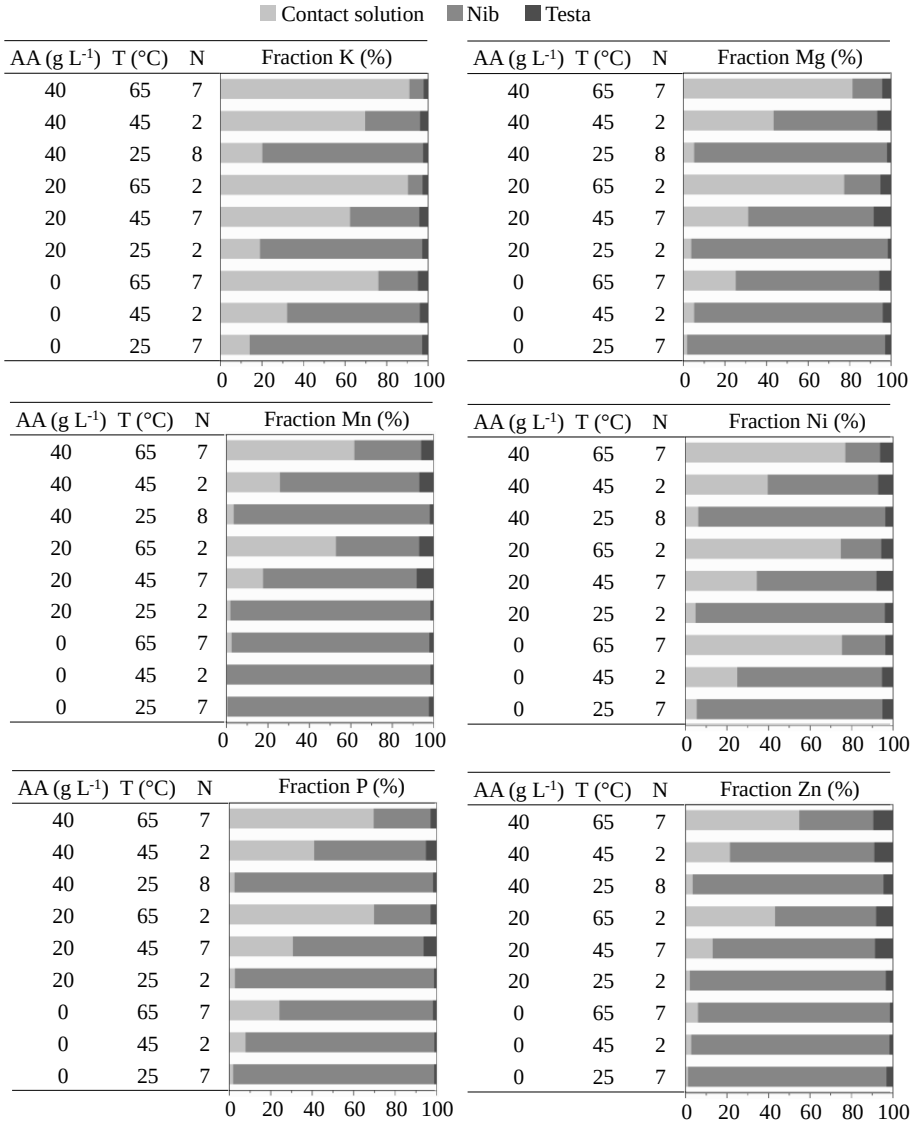

Figure V.2 The distribution of elements between the different tissues after treatment (nib, testa and contact solution) for different incubation conditions. AA = Acetic Acid, T = Temperature.

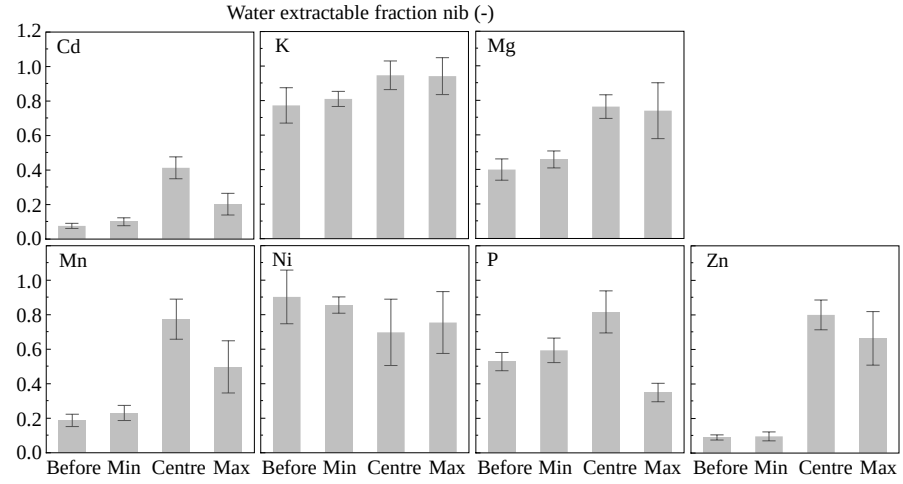

Figure V.3 Water extractable element fractions in nibs, determined by overnight water extractions with ground nib material. Lower water extractable fractions of Cd, Mn and P in max treatments are due to relevant extraction that took place during sample incubations in the different treatments (see Figure 6.1 and Figure V.2). Before = untreated samples, n = 44; Min = 25 °C, no ethanol or acetic acid added, n = 7; Centre = 45 °C, 20 g L^{-1} acetic acid and 35 g L^{-1} ethanol, n = 7; Max = 65 °C, 40 g L^{-1} acetic acid and 70 g L^{-1} ethanol, n = 7.

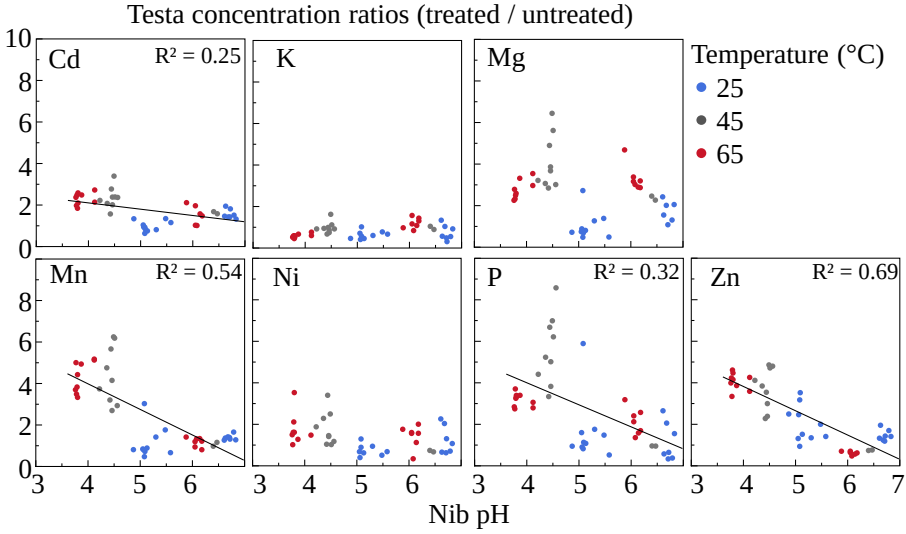

Figure V.4 Testa concentration ratios (concentration measured in testa after treatment divided by concentration measured in the testa prior to treatment) are significantly correlated to nib pH for Cd, Mn, P and Zn (P-value < 0.01).

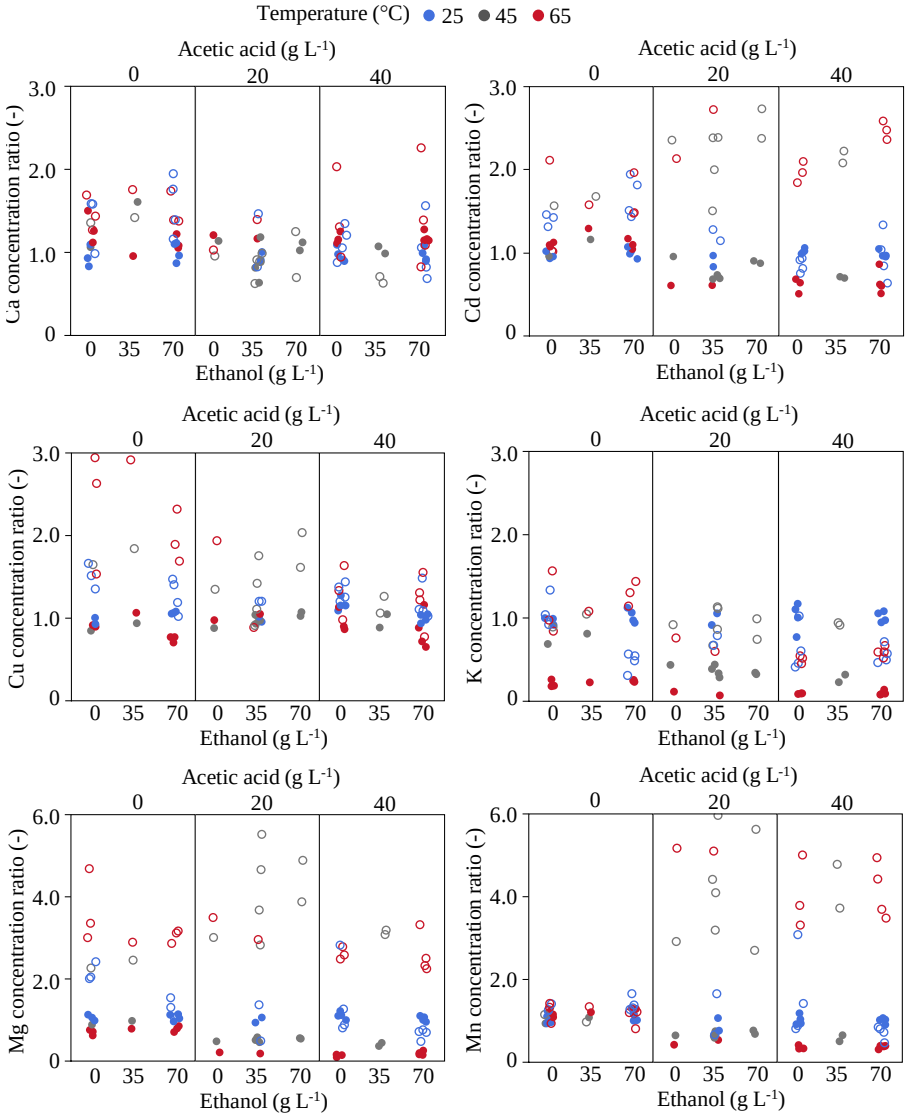

Figure V.5 Ethanol addition in the incubation solutions did not affect the element concentrations in either nib (●) or testa (○). Figure continued on the next page.

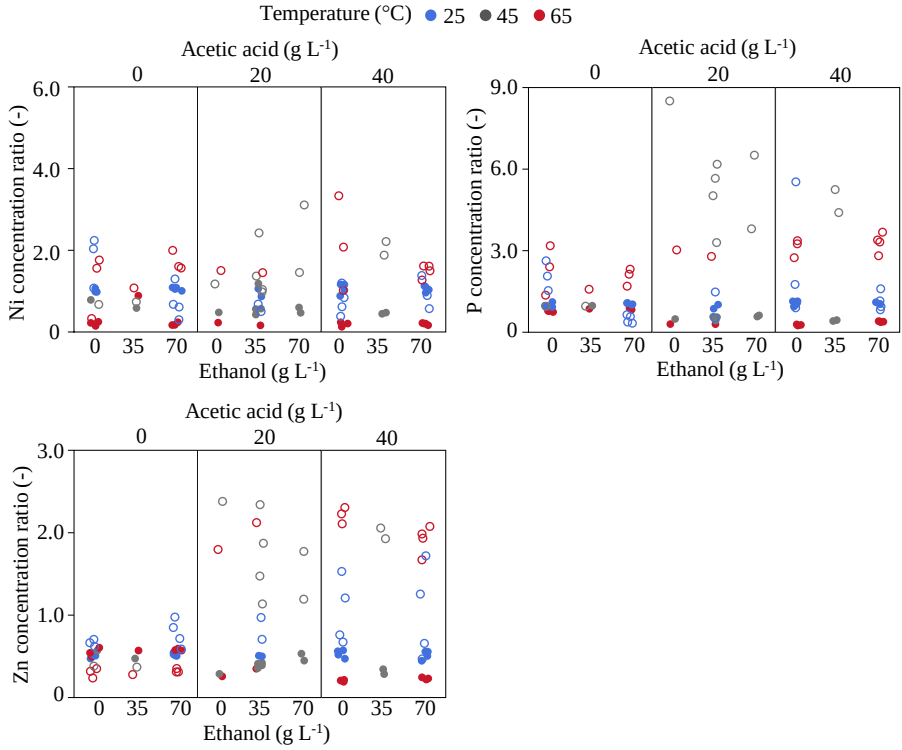

Figure V.5 continued.

Appendix VI
Chemical equilibrium calculations

VI.1. Addition of phytate complexation data to the Visual MINTEQ database

As far as known, metal-phytate complexation data have not yet been added to the open source speciation codes of Visual MINTEQ. The phytate complexation data for H^+ was derived from Bretti et al. (2015), for Ca^{2+} and Mg^{2+} from Crea et al. (2006), for Cu^{2+}, Zn^{2+} and Ni^{2+} from Cigala et al. (2010), for Cd^{2+} from De Stefano et al. (2010) and for Fe^{2+} and Mn^{2+} from Bretti et al. (2012). For all these ions, the various complexes (different stoichiometry's) from the original publications were entered in the speciation model VMinteq 3.1 (Visual MINTEQ software version 3.1, available online at https://vminteq.lwr.kth.se/). Metal and proton complexations with phytate were modelled by attributing the maximal negative charge of the anion as -7 in line with most recent modelling (Bretti et al. 2015). Phytate has a maximal net charge of -12 (2 charges per PO_4 unit) but this highly charged anion does not occur and is associated with the cations such as Na^+, K^+ or NH_4^+ in the media in which the speciation is measured. Most of the metal complexation data are modelled assuming that the completely deprotonated form is Na_5-Phytate^{7-}, which only occurs in alkaline conditions. The protonation data of phytate are very similar in media of NaCl, $NaNO_3$, KNO_3, KCl or NH_4Cl, hence this approach is likely valid for the K^+ dominated conditions in plant cells (Bretti et al. 2015).

The ionic strength corrections of the complexation constants for these polyvalent anions have been subject of intensive modelling. The metal cation – phytate complexation constants logically decrease with increasing ionic strength as the complexes have a reduced charge compared to the reagents. Here, the raw phytate complexation data, typically measured at I = 0.1 – 0.25 M, or recommended data at I = 0.1 M, were corrected to infinite dilution with the Davies equations and its parameters at 298 °K:

$$-\log f_i = 0.5108\, {z_i}^2 \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3286\, I \right)$$

with f_i the mean molal activity coefficient of an ion with charge z_i, at the ionic strength I.

All calculations for the extractions were equally calculated with the Davies equation. That Davies model is relatively consistent with the most recent model (Bretti et al. 2012) for ionic strength dependent complexation of divalent trace metals (Co^{2+}, Fe^{2+} and Ni^{2+}) with phytate. Between infinite dilution and I = 0.1 M, both models agree on the change in log K of the relevant complexes within 0.1 log units; such is acceptable given that most complexation data have a statistical uncertainty of 0.1.

Table VI.1 Input concentrations used for the chemical equilibrium calculations in Visual MINTEQ, calculated based on the average elemental composition of cacao nibs prior to incubation across all runs from the incubation experiment (Chapter 6). Dry weight element concentrations in cacao nibs (mg kg^{-1}) were converted to liquid concentrations (mmol L^{-1}) based on the solid:liquid ratio used in the water extractions in Chapter 6, i.e. 1:20 g mL^{-1}. For Cd, only Cd bound to P-ligands was considered, i.e. 60 % of the total nib Cd concentration. Magnesium and calcium were not included because their free ion activity is typically low in plant cells (Hawkesford et al. 2012) and Mg and Ca do not largely complex phytate at such concentrations. The total Fe concentration was divided in equal parts Fe (II) and Fe (III) based on the results for wheat from De Brier et al. (2016). To determine the ligand capacity, the phytate concentration in the model was adjusted to obtain 7.7 % of Cd in solution at 25 °C and pH 6.9, i.e. respectively the average water extracted Cd fraction and nib pH prior to incubation across all runs in Chapter 6.

Element	Concentration (mmol L^{-1})
Cd	0.000566
Cu	0.0137
Fe (II)	0.0102
Fe (III)	0.0102
K	13.7
Mn	0.0137
Ni	0.00525
Zn	0.036

List of publications

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