Comprehensive Analysis of Cocoa Lipidomics – unraveling the unknown chemistry of cocoa butter

by

Diana Sirbu

a Thesis submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy in Chemistry

Approved Dissertation Committee

Prof. Dr. Nikolai Kuhnert, Jacobs University Bremen, Germany

Prof. Dr. Matthias Ullrich, Jacobs University Bremen, Germany

Dr. Frédéric Depypere, Barry Callebaut Lebbeke-Wieze, Belgium

Date of Defense: June 1st, 2018

Department of Life Sciences and Chemistry

Statutory Declaration

Family Name, Given/First Name	Sirbu Diana
Matriculationnumber	20331305
What kind of thesis are you submitting: Bachelor-, Master- or PhD-Thesis	Ph. D. Thesis

English: Declaration of Authorship

I hereby declare that the thesis submitted was created and written solely by myself without any external support. Any sources, direct or indirect, are marked as such. I am aware of the fact that the contents of the thesis in digital form may be revised with regard to usage of unauthorized aid as well as whether the whole or parts of it may be identified as plagiarism. I do agree my work to be entered into a database for it to be compared with existing sources, where it will remain in order to enable further comparisons with future theses. This does not grant any rights of reproduction and usage, however.

The Thesis has been written independently and has not been submitted at any other university for the conferral of a PhD degree; neither has the thesis been previously published in full.

German: Erklärung der Autorenschaft (Urheberschaft)

Ich erkläre hiermit, dass die vorliegende Arbeit ohne fremde Hilfe ausschließlich von mir erstellt und geschrieben worden ist. Jedwede verwendeten Quellen, direkter oder indirekter Art, sind als solche kenntlich gemacht worden. Mir ist die Tatsache bewusst, dass der Inhalt der Thesis in digitaler Form geprüft werden kann im Hinblick darauf, ob es sich ganz oder in Teilen um ein Plagiat handelt. Ich bin damit einverstanden, dass meine Arbeit in einer Datenbank eingegeben werden kann, um mit bereits bestehenden Quellen verglichen zu werden und dort auch verbleibt, um mit zukünftigen Arbeiten verglichen werden zu können. Dies berechtigt jedoch nicht zur Verwendung oder Vervielfältigung.

Diese Arbeit wurde in der vorliegenden Form weder einer anderen Prüfungsbehörde vorgelegt noch wurde das Gesamtdokument bisher veröffentlicht.

07.09.2018 Sirbu Diana

Date, Signature

I dedicated this thesis to my family

for their encouragement, lifelong support and inconditional love.

Table of contents

Preface

"It's not enough to do your best, you must know what to do and then do your best."

W. Edwards Deming

The years as a Ph.D. student at Jacobs University have been full of memorable experiences, challenges, and opportunities. Coming to the end of this learning path, I would like to express my gratitude to everyone who contributed to it accomplishment.

I would like to acknowledge and thank my supervisor Prof. Dr. Nikolai Kuhnert for his guidance, encouragements, and for allowing me the opportunity to do research in my way while always pushing me indirectly to overcome my limits. I have learned a lot from him, from chemistry to human social interactions. Appreciations also go to Prof. Dr. Matthias Ullrich for his continuous engagement and advisement, as well for his 'keep going, you are doing well!' smile whenever we met in the university corridors. I would like to thank Dr. Frédéric Depypere for joining my dissertation committee and reviewing this thesis. I am grateful for the support and help from my former and current colleagues, group and lab members. I am especially grateful to Dr. Maria Alexandra Patras for always being there for me for whatever issues. I am thankful to Anja Mueller for her technical support and to Britta Behrens for helping me to sort out the enormous number of cocoa lipid samples. Having great colleagues made my tough days better. I would like to thank CoMeta team for their support, commitment, and valuable advises.

Moreover, I would like to thank Anja Muller, Britta Behrens, Nina Boettcher and Dr. Hussein Ali El Damrany Hussein for their technical support, for helping out with sample preparation and in sample logistics. Many thanks to Dr. Maria Alexandra Patras, Dr. Abhinandan Shrestha, Dr. Sergio Grimbs, Dr. Anne Grimbs, Dr. Roy D'Souza and Dr. Foad Tehrani Najafian for their valuable guidance on performing the data analysis, excel platforms and advises. Dr. Gino Vrancken for support and many productive discussions. Enis Bardhi for helping with the nuts lipid extractions and TLC. Audrey's Bergounhou for helping to perform analytical measurments on fatty acids amide.

My immeasurable gratitude is to my mother, Ioana Sirbu, whose support; love and patience have no equal. This achievement I have to share with her. Further, I would like to thank the rest part of my family for always being proud of me and having the patience for a forever student in the family. Besides, I want to show my love and appreciation for my partner and best friend who found the best way to support me during the most stressful period of my Ph. D. Another thank you must go to my physiotherapist Ariane von der Kaus for helping me to relax my back after long hours in front of computer.

Additionally, I would like to thank all my friends outside Kuhnert's group for their support, friendship, and encouragement during the ups and downs of the last few years. I would like to express

my gratitude to all of my co-authors for their openness, collaboration, and patience. I wish to acknowledge all my students whom I have got to supervise during my Ph. D. studies. Many thanks to the people of other research groups on Campus. It felt encouraging when I could meet so many people ready to help. I am thankful to Jacobs' community for teaching me to be more patient and to understand and accept diversity.

Finally, I would like to thank Barry Callebaut Company for financial support and people for the constant exchanges.

Without the support of all these people, it would not have been possible to complete this thesis. Thank you

Abstract

Theobroma cacao is a tree that produces pods containing high valuable seeds, referred to as cocoa beans. About 50 million people around the world depend on cocoa as a source of income. From tree to bar, cocoa beans undergo an intricate processing chain leading to various chemical changes within the seed. Lipids constitute half of the dry weight of a cocoa bean and the most significant part of the entire cocoa market value taking the name of cocoa butter. Cocoa butter is composed of about 95% triacylglycerols (TAGs) and 5% of other minor components. The physical and chemical characteristics of this fat have been at the forefront of many research studies in the past decade. However, there are several knowledge gaps regarding the chemical composition and modifications that cocoa butter undergoes during the production process. This research aimed at investigating the chemistry of lipids in cocoa samples from different production steps and geographical regions, thus enriching the present knowledge of cocoa chemistry.

In the first place, analytical methods for the extraction, separation, and detection of cocoa lipids were developed and optimized. The overall lipid profile of unfermented, dried and roasted cocoa beans was investigated, and a solid phase extraction method, separating cocoa lipid extract in five fractions was established to aid the evaluation of the all cocoa lipid constituents.

A non-aqueous reversed-phase liquid chromatographic method using ethanol as the mobile phase, coupled to electrospray ionization (ESI) tandem mass spectrometry was developed for TAGs analysis, which allowed the characterization of 83 different TAGs in unfermented cocoa beans, the largest number of TAGs identified in cocoa so far. From these, 58 TAGs were not previously reported in cocoa and 31 TAGs were characterized by the presence of one to three hydroxyl groups on the unsaturated fatty acid chain. Applying the same analytical approach, relative quantification of the TAGs in lipid extracts of unfermented raw and fermented dried beans from different origins was performed. Results revealed that the presence of hydroxy-allyl fatty acid derived TAGs (proposed name cacaoic acid) is unique to unfermented beans and they may serve as biomarkers for the fermentation grade of the beans. A simple analytical test, based on thin layer chromatography, testing for the presence of these biomarkers has been introduced.

Multivariate statistical analysis was carried out to compare triacylglycerol profiles across the different origins. Only small variations in individual compound concentrations across the different origins were observed. Nonetheless, TAGs profile of each geographical origin

reflected the influence of the climate conditions. Therefore, a new softness/hardness indicator was proposed, which takes into account only four di-unsaturated TAGs relative quantities. In addition, the fatty acids profile showed the impact of both fermentation status and origin of the beans. 18 fatty acids were successfully identified and many others detected. The primary outcome was the significantly increased quantity of palmitic and linoleic acid in dried fermented beans when compared to unfermented wet beans in all geographical locations. Moreover, the analysis of variance showed significant differences in the amount of the short chain fatty acids for South American cocoa beans, whereas Malaysian samples contained a significantly higher amount of long chain fatty acids.

Concerning the chemical changes occurring during the fermentation process, 72 and 96 hours (h) of fermentation showed to be crucial time points for both triacylglycerol and fatty acid content. Hydroxy-allyl fatty acids containing TAGs decreased almost entirely after 96 h of fermentation. The lipid extracts obtained from different time points have shown a distinct change of the color from pale yellow to brown yellow. Absorbance at 400 nm revealed a maximum between 96 and 120 h. Moreover, the lipid profile of minor components pointed out towards the influence of the microorganisms involved in the fermentation process. These results suggest that lipids could also be involved in defense and stress response and in the cellular metabolism of cocoa.

Furthermore, the evolution of cocoa lipids during the factory production process was studied. While the total lipid amount decreased from bean to liquor, considerably random amount dissimilarities were observed in the entire TAGs profile, which suggests that other chemical reactions of an unknown nature might take place during the roasting and grinding processes. Moreover, the impact of the alkalization treatment was explored. HPLC-MS data revealed the presence of novel compounds, identified as fatty acids amides, namely palmitamide, oleamide, and stearamide, which were proposed as markers of the alkalization process.

Separation of cocoa lipid extract in 5 fractions allowed structure elucidation of 62 minor lipid components which are part of the six different lipid classes including sterol esters, diacylglycerols, waxes, free fatty acids, sterol esters glucosides and phospholipids.

The lipidomics approach using the HPLC-MS method developed for cocoa was applied for the analysis of the lipid extracts of 2 algae species, *Scenedesmus sp.* (*Chlorophyceae*) and *Cylindrotheca closterium* (*Bacillariophyceae*). Nine different lipid classes were identified and monitored in response to nitrogen stress during 96 h. Although the two algae exhibited different

lipid metabolic responses with respect to nitrogen starvation, both of them showed a significant increase of triacylglycerols, which is valuable for biofuel production.

A large part of the research was carried out aiming at product valorization and exploration of new possible industrial applications performing multiple analysis on different samples provided by the company. The outcomes give insights into the potential of cocoa butter and open new horizons for future studies and industrial applications.

List of abbreviations

A	arachidic acid
ANOVA	one-way analysis of variance
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
CB	cocoa butter
$CHCl_3$	chloroform
CN	carbon number
DAG	diacylglycerol
DBs	double bonds
DCM	dichloromethane
DSC	differential scanning calorimetry
ECN	equivalent carbon number
EFSA	European Food Safety Authority
EIC	extracted ion chromatogram
ELSD	evaporative light scattering detector
ESI	electrospray ionization
F	fermented
FA	fatty acid
FAA	fatty acid amide
FAME	fatty acid methyl esters
FID	flame-ionization detector
FTICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
GLC	gas liquid chromatography
HCA	hierarchical cluster analysis
HPLC	high performance liquid chromatography
L	linoleic acid
LC	liquid chromatography
Ln	linolenic acid
LOQ	limit of quantification
LOX	Lipoxygenase
M	miristic acid
Ma	margaric acid
MANOVA	multivariate analysis of variance
MeOH	methanol
MOSH	mineral oil saturated hydrocarbons

MOAH	mineral oil aromatic hydrocarbons
MS	mass spectrometry
NARP	non-aqueous reversed-phase
NMR	nuclear magnetic resonance spectroscopy
O	oleic acid
OC19:0	1-oleoyl-2-nonadecanoyl-*sn*-glycerol
OO	1,2-dioleoyl-*sn*-glycerol
P	palmitic acid
PCA	principal component analyses
PLP	1,3-dipalmitoyl-2-linoleoyl-*sn*-glycerol
PLS	1-palmitoyl-2-linoleoyl-3-stearoyl-*sn*-glycerol
POA	1-palmitoyl-2-oleoyl-3-arachidoyl-*sn*-glycerol
POO	1-palmitoyl-2,3-dioleoyl-*sn*-glycerol
POP	1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol
POS	1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol
R_f	retention factor
R_t	retention time
S	stearic acid
SFC	solid fat content
SLR	1-stearoyl-2-linoleoyl-3-ricinoleoyl-*sn*-glycerol
SLS	1,3-distearoyl-2-linoleoyl-*sn*-glycerol
sn	stereospecific number
SOA	1-stearoyl-2-oleoyl-3-arachidoyl-*sn*-glycerol
SOO	1-stearoyl-2,3-dioleoyl-*sn*-glycerol
SOS	1,3-distearoyl-2-oleoyl-*sn*-glycerol
sp.	species
SP	1-stearoyl-2-palmytoyl-glycerol/2-palmytoyl-3-stearoyl-*sn*-glycerol
SPE	solid phase extraction
SPS	1-stearoyl-2-palmytoyl-3-stearoyl-*sn*-glycerol
SS	1,3-distearoyl-*sn*-glycerol
S'U'S'	1,3-disaturated-2-unsaturated-*sn*-glycerol
S'U'U'/U'U'S'	1(3),2-diunsaturated-3(1)-saturated-*sn*-glycerol
TAG	triacylglycerol
TLC	thin layer chromatography
TOF	time of flight
2D map	two dimensional map
2D NMR	two dimensional nuclear magnetic resonance spectroscopy
U	unfermented

List of figures

List of tables

Chapter 1:

1 Introduction

1.1 Lipidomics

1.1.1 General concept of Lipidomics

Lipidomics is an emerging field of metabolomics centered on the analysis of all lipids present in a biological system known as a lipidome. A lipidome is the comprehensive and quantitative description of a set of lipid species present in an organism (Navas-Iglesias, Carrasco-Pancorbo, & Cuadros-Rodríguez, 2009; Wenk, 2005). Lipidomics covers not only the study of all lipids, identifying the structures of cellular lipid species including the number of atoms, the number and location of double bonds, the core structures and head groups, individual fatty acyl chains, and the regiochemistry of each isomer, but also the molecules with which they interact, and their function within the cell. A more comprehensive definition describes lipidomics as: the full characterization of lipid molecular species and their biological functions, with respect to expression of proteins involved in lipid metabolism and function, including gene regulation (Spener, Lagarde, Géloên, & Record, 2003). This branch of metabolomics uses multiple techniques to quantify hundreds of chemically different lipids and define pathways of the molecular mechanisms through which they enable cellular function (Meer, 2005; Wenk, 2010).

1.1.2 Lipid classification and functions

Although there is not a unified definition of lipids, most of the authors accept the definition provided by Christie, which restricts the use of 'lipids' to "fatty acids, their naturally-occurring derivatives (esters or amides), and substances related biosynthetically or functionally to these compounds" (Christie & Han, 2010). Lipids are various compounds that are insoluble in water, soluble in organic solvents and chemically and structurally diverse. The number of distinct lipid chemical species in a living organism is estimated to be between 10^4–10^5. Recently, The LipidMaps consortium has developed the first internationally accepted lipid classification

system (Brügger, 2014; LIPID MAPS Lipidomics Gateway), as well as nomenclature and structural representation system. This database contains over 40,000 unique lipid structures of biological relevant lipids from human, mouse, plants, bacteria, fungi, algae and marine organisms, and other computationally generated structures. There are eight lipid categories, each complete with its classification and sub-classification hierarchy (Fig. 1.1). Each lipid is chemically different and thus set with different functional properties.

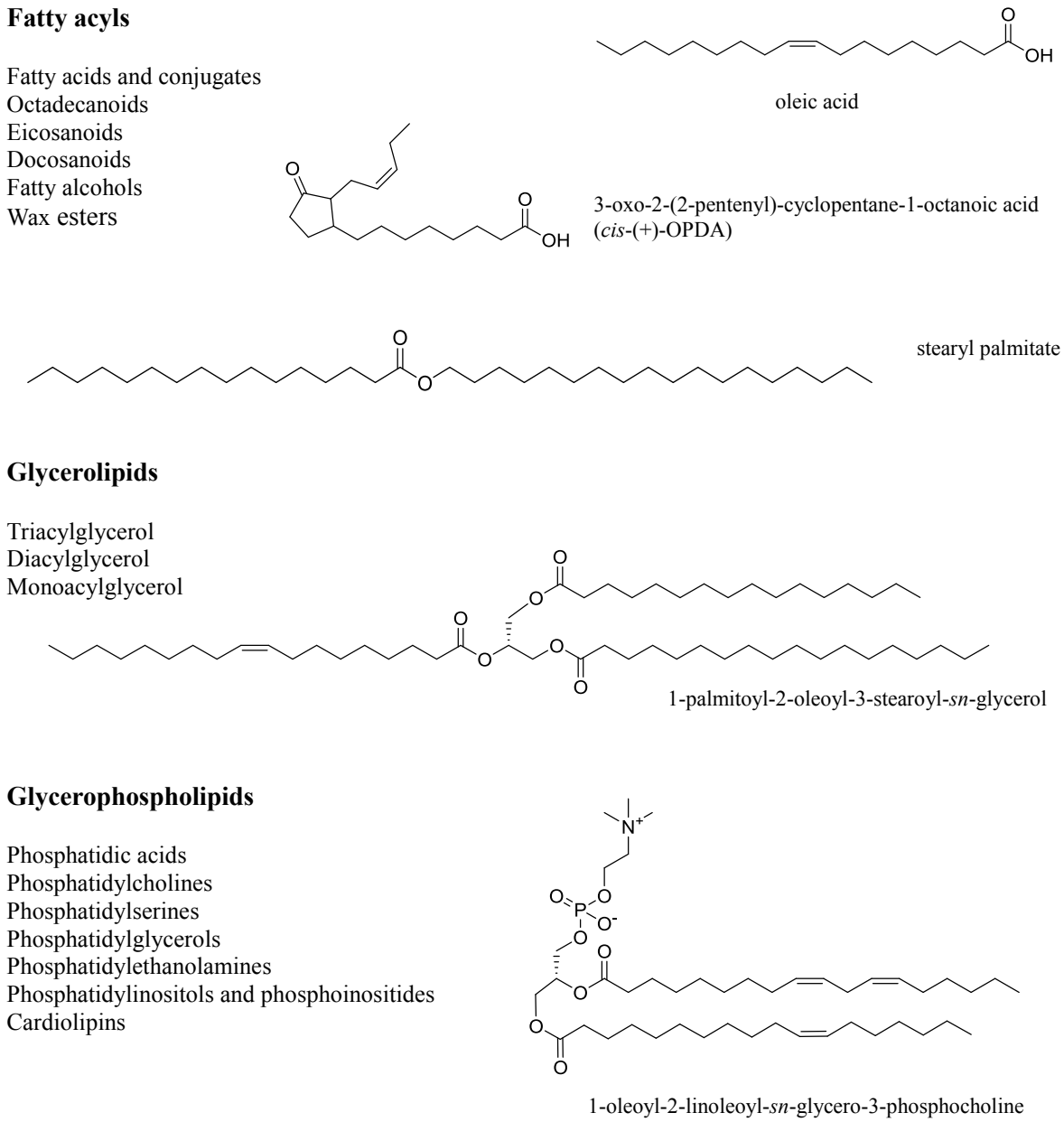
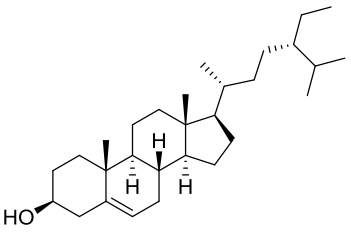


Figure 1.1 Lipid classes and prominent examples (continues on next page).

Sterol lipids

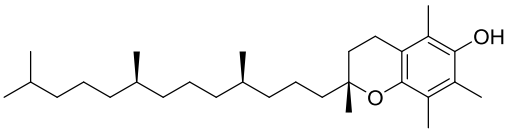
Sterols and derivatives



sitosterol

Prenol lipids

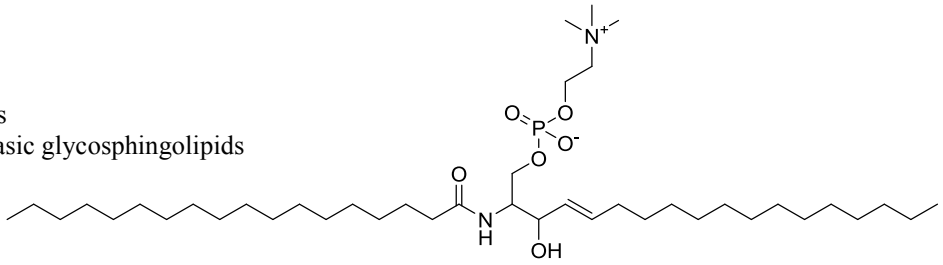
Isoprenoids



α tocopherol – vitamin E

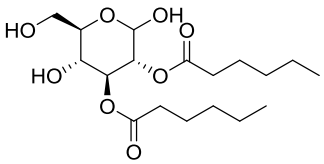
Sphingolipids

Sphingoid bases
Ceramides
Phosphosphingolipids
Neutral, acidic and basic glycosphingolipids



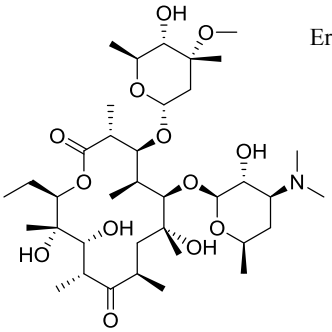
N-(octadecanoyl)-sphing-4-enine-1-phosphocholine

Saccharolipids



2,3-di-o-hexanoyl-glucopyranose

Polyketides



Erytromycin

Figure 1.1 (continued) Lipid classes and prominent examples.

Fatty acids are considered the simplest lipid compounds. The common fatty acid chain contains an even number of carbon atoms in a straight chain, which can vary from 2 to 36

carbon atoms in animal and plant tissues. Fatty acids from plant tissues can contain a wide variety of functional groups including double bonds, hydroxyl, keto and other groups. Fatty acids are the building blocks of more complex lipids such as acylglycerols, sterol-esters and sphingolipids, and precursors of biologically active lipids such as the eicosanoids, prostaglandins, and leukotrienes. Combinations of different fatty acids, typically linked by a backbone, and functional head groups lead to a massive structural diversity in lipids (Christie & Han, 2010).

A typical complex structure formed by one molecule of glycerol esterified by three fatty acid molecules is a triacylglycerol (TAG). The three fatty acids may be identical, completely different, or two of them the same. Because they are biosynthesized from acetyl-CoA, fatty acids in plants and animals are in general composed of only even-numbered chain lengths. Bacteria, on the other hand, can synthesize acids with odd chain lengths and branched chains. Therefore, ruminant fat also contains acids with an odd number of carbon atoms, for example, 15 or 17, because of the bacteria in the rumen.

A special nomenclature is used for the stereochemistry of TAGs instead of the usual D/L or R/S nomenclature, where the primary hydroxyl groups are often termed α- and α' and the secondary β, or the "stereospecific numbering" (*sn*) system was recommended by the IUPAC-IUB commission.

The number of theoretically possible molecular species increases with the growing number of fatty acids forming a mixture of TAGs, see Table 1.1. However, many of these theoretically possible species do not exist in nature or exist in very low abundance.

Table 1.1 The number of possible TAGs.

Description	Number of Possible TAGs	Number of Possible TAGs for y = 10
without isomers	$x = (y^3 + 3y^2 + 2y)/6$	220
without enantiomers	$x = (y^2 + y^3)/2$	550
all isomers	$x = y^3$	1000

(where x is the number of TAGs and y is the number of FAs in TAGs)

Lipid compounds have diverse physical and chemical properties and occur in vastly different concentration ranges. Therefore, lipids exhibit a wide variety of functions within cells. Their primary function is to form the matrix of our cell membranes, including cellular and sub-cellular partitioning, where they support a variety of tasks essential for life. Moreover, lipids also store energy; they serve as hormones and signaling molecules; participate in the

maintenance of electrochemical gradients; and a recent described function as cofactors in modulating protein activity (Coskun, Grzybek, Drechsel, & Simons, 2011).

1.1.3 Experimental approaches in lipidomics research

The workflow of lipidomics uses a set of tools defined as novel analytical approaches mainly based on liquid chromatography (LC) and mass spectrometry (MS) (Han, 2016b; Wenk, 2010). A typical analytical procedure for the characterization of individual lipid compounds includes four steps: sample preparation and lipid extraction; chromatographic separation; identification and quantification; and data analysis and interpretation. Despite this, due to the vast chemical diversity of lipid species, it is nearly impossible to analyze all lipid classes with a standard method for extraction, chromatography, and detection. Therefore, numerous methods are available for lipid analysis. It is the task of the analyst to choose the most appropriate tool for each application. Many lipid analysis techniques are using advances in electronics and computer science (especially the areas of data acquisition, data analysis, and interpretation), but some "low tech" analytical methods, such as solid phase extraction (SPE) and thin layer chromatography (TLC), can also provide valuable information at modest costs (Christie & Han, 2010).

Lately, the global analysis of lipids, namely shotgun lipidomics has acquired interest. The workflow of shotgun lipidomics is based on direct infusion of the sample into the mass spectrometer. Because there is no chromatographical separation involved, several MS scan can be performed for both identification and quantitation (Ryan & Reid, 2016). The disadvantage of the shotgun lipidomics workflow is that many isobaric species may overlap. Therefore, high mass resolution and/or multiple stages of MS^n are required to separate and distinguish the many isobaric and isomeric species found within biological lipid extracts. Additionally, ion suppression and enhancement in a direct infusion hamper quantification. Nevertheless, MS is the most preferred method of detection, enabling structure elucidation and quantification studies at a previously unattainable level of complexity.

Lipid research has experienced overgrown concerning both developments and applications since the development of numerous different LC systems and sensitive mass spectrometry technologies for the quantitative characterization of the lipidome on the market (Holčapek, Jirásko, & Lísa, 2012). Separation of lipid classes is generally achieved by adsorption chromatography. Due to the complexity of the lipid extract, quite often, it is not possible to analyze different lipid classes with a single analytical procedure. Therefore, it is required to

isolate distinct lipid fractions prior to analysis. Typically, systems employing silica gel columns, TLC, and solid phase extraction cartridges are the most applied. However, the separation of individual lipid molecules entails the use of HPLC, mostly centered on reversed-phase columns. Many other different types of column packing materials (bonded phases) such as diol, nitrile, cyanopropyl, or polyvinyl alcohols are also used. Reversed-phase HPLC separates the lipid molecules according to the combined chain-lengths of the fatty acid and the double bonds in the fatty acids, which reduces the retention time of the lipid molecule by approximately two carbon atoms. Silver ion chromatography is an auxiliary technique, which separates the lipid molecules according to their degree of unsaturation. Simplifying the lipid molecules by synthesis and extraction procedures of some of their building blocks, fatty acids methyl esters, individual fatty acids can be determined. Fatty acids are usually analyzed by gas chromatography (GC), following derivatization, which allows the separation of volatile molecules. Fused silica gel column with a polyester phase, with different polarity, enable high resolution and accurate quantitative analysis. With the non-polar phases, unsaturated fatty acids are eluted before the saturated compounds of the same chain-length, while the opposite is true for the polar phases (Christie & Han, 2010). Current lipidomics encompasses the combination of different methods, technics and columns. However, the utmost effort is in the separation and analysis of individual molecular species in its native form, to better assess its function and physical properties in a living organism or material. This task is mostly achievable by HPLC.

The coupling of an HPLC with the high vacuum required for ion analysis in a mass spectrometer obliges rapid evaporation of high amounts of liquid mobile phase to release the molecules into the gas phase, along with ionization of those molecules so that the detector can detect them. Nitrogen gas is usually the gas employed to spray the effluent stream, in the process called nebulization. Nebulization and ionization regularly occur at atmospheric pressure, and thus the formation of ions under these conditions is called “atmospheric pressure ionization” (API). The two most commonly used API techniques are atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). In APCI the nebulized solvent/analyte mixture is sprayed down a heated vaporizer tube to aid in desolvation, and the vaporized mist passes by either a corona discharge needle held at high voltage (~2-5 kV). Therefore, the nature of the solvents used in the LC mobile phase has a substantial effect on the ionization of the analyte molecules (William C. Byrdwell, 2017).

Electrospray ionization uses a concentric nebulization gas to assist desolvation by charging of the liquid to accomplish nebulization. It also includes the capability to provide heat to aid in

desolvation, which allows higher flow rates. Evaporation of the solvent caused a decrease in droplet size and simultaneously caused an increase in surface-charge density, finally leading to desorption of molecular ions into the gas phase (Coulomb explosion) at atmospheric pressure. ESI almost always produces an intact pseudomolecular ion, $[M+H]^+$ (not to be confused with a 'molecular ion', which is an odd-electron species formed by loss or addition of an electron only), or adduct ion, $[M+X]^+$ (X = Li, Na, NH_4, etc.), and just a small abundances of fragments, unless tandem MS is applied. While phospholipids are naturally amenable to ESI, triacylglycerols frequently require the addition of an electrolyte, such as ammonium formate or ammonium acetate, to produce adduct ions, $[M+NH_4]^+$ (i.e. ammonium ions in this example) with small amount of Li and Na adduct in addition (William Craig Byrdwell, 2003).

APCI produces diacylglycerol-like fragment ions ($[DAG]^+$), in case of TAGs, and their the ratios are indicative of the composition of regioisomers. However, with this technique, polyunsaturated TAGs produce a $[M+H]^+$ base peak, while saturated TAGs often yield little or no $[M+H]^+$ ion, whereas with ESI-MS intact molecule adduct ions are obtained, but the ratios of the regioisomers are less clear. Consequently, for these reasons, acquisition of both ESI-MS and APCI-MS is desirable(William C. Byrdwell, 2017).

Electrospray ionization is the most popular ionization method as it provides minimal in-source fragmentation and an outstanding sensitivity (femtomole range). This ionization technique can comprehensively analyze thousands of lipid molecular species in an extract in a reasonably short time (Christie & Han, 2010).

Although high-resolution mass spectrometry approaches were shown to be successful in lipid profiling, and some structural elucidation, other methods such as chemical derivatization or 2D NMR may be needed for the unequivocal assignment of structures such as double bond configurations (Iijima et al., 2008).

1.1.4 Plant lipidomics

Both direct infusion-based shotgun lipidomics and LC-MS based approaches are applicable in plant lipidomics (Han, 2016a). Due to the remarkable increase of the vegetable oil market, both edible oils and sustainable substitutes for petrochemicals, plant lipid biochemistry is facing massive growth. Moreover, there is a significant interest in widening the array of applications and considering new techniques of oil-formation into non-traditional tissues. To achieve this scope, it is necessary to fully understand the complex pathways at the core of plant lipid synthesis, at both genetic and biochemical level. At this point in time, the overall "picture"

of plant lipid metabolism can be grasped, yet there is still much left to learn. Lipidomics offers the possibilities to couple functional genomics and systems biology with cutting-edge lipid analytical techniques, bridging a vital gap between gene function and in vivo physiological outcomes (Li-Beisson et al., 2013).

Plants accumulate lipids mostly in their seeds. The lipid content comprises mainly of triacylglycerols and other various kinds of lipidic compounds including fatty acids, tocopherols, phospholipids, sphingolipids, and sterols. The variation in the composition of these compounds in plant seeds is very high and partially characteristic of individual plant families. Databases containing a vast collection of information about the fatty acid composition of wild plant seeds, of particular interest to food scientists, biochemists, chemists and botanists, managers of food and oleochemical industry, is nowadays available online (Matthäus, 2012).

1.1.5 Lipidomics and food science

Oils and fats are a primary source of energy; provide essential fatty acids; and other minor components as fat-soluble vitamins. In foods, lipids contribute to the texture and the flavor. Human's brain integrates the texture, taste, and flavor in the orbitofrontal cortex to give the overall perception of a particular food. Sensorial perception of foods is affected by the microstructural composition often determined by the emulsion properties associated with the continuous phase. This fact denotes the complexity of the effect of lipids on the overall flavor of a product. Nevertheless, the presence of lipid sensors in taste is still under discussion.

A significant source of oils are oilseeds and tropical fruits whereas fats are usually of animal origin. TAGs account for over 95% of the components of a fat or oil. The remaining minor constituents comprise phospholipids, glycolipids, tocopherols, sterols and other compounds referred to as non-TAG components. During the processing, both TAG and minor constituents undergo chemical and physical changes, which play a strong impact on the quality of the oils, and hence the importance of post-extraction active processing, as, deodorization, degumming or refining (Akoh, 2017; Shahidi & Weenan, 2006).

Lipidomics can be efficiently used to understand the effect of the processing on edible oils and fats. Consequently, it can be used to streamline food handling and to enhance the physicochemical properties of a desirable product. Nonetheless, it aids understanding the dietary value, and provides indicators for the evaluation of food-related health effects and toxicity, through monitoring the lipids changes (Sebedio & Brennan, 2014).

1.2 *Theobroma cacao* – from bean to bar

Theobroma cacao is a small evergreen perennial crop tree in the family *Sterculiaceae*. The scientific name was given for the first time, in 1753, by the Swedish botanist Carl Linnaeus in his book Species Plantarum. *Theobroma* means "food of the gods" and cacao is derived from the Nahuatl (Aztec language) word xocolatl, from xococ (bitter) and atl (water) (Coe & Coe, 2007).

1.2.1 History, origin, and cocoa varieties

The story of cocoa begins around 4,000 years ago when the first cocoa plantations were established by Olmecs in the south of Yucatan (Mexico) and Central America (Coe & Coe, 2007). The Maya and the Aztecs of Mexico after them, as well as the Incas from Perù were also growing cocoa trees. Thus, cocoa beans have been at the center of Mesoamerican mythology for thousands of years (Coe & Coe, 2007; Dreiss & Greenhill, 2008). Centuries before, a sophisticated process was necessary to transform the bitter seeds into what is known today as chocolate, it was consumed as unsweetened liquid form and used as currency by the Maya and the Aztecs. The chocolate was introduced to Europe in the 1520s, after the Spanish conquest of Central America. At first, it was consumed as a drink by the kings and aristocrats of Spain, France, United Kingdom, and Italy and then it was spread in coffeehouses. Later, in the nineteenth and twentieth centuries, industrialization made chocolate available for all, and now, it has turned out to be once again a more than just a bar – a luxury product (Beckett, 2008; Coe & Coe, 2007).

There are four different species of cocoa tree: Criollo – produces pods with a very thin peel and beans with white cotyledons. This variety produces small harvests, and it is very fragile, but its cocoa has a unique, refined aroma; Forastero – is a more vigorous type of tree and it is easier to cultivate and produces larger harvests. The fruit has a thicker peel and a stronger aroma. Cocoa from the Forastero beans is often called bulk cocoa because it gives chocolate a typical chocolate aroma; Trinitario – is a cross between Criollo and Forastero and is not found in the wild. Its characteristics are a strong but relatively refined aroma moreover and it is easier to cultivate, and National – the fourth type of cocoa tree is grown only in Ecuador and probably originates from the Amazonian area of Ecuador. Cocoa from National beans has a full cocoa flavor with additional floral and spicy flavors (Beckett, 2008; Coe & Coe, 2007a; Young, 2007).

International Cocoa Germplasm Database ("ICGD Homepage") stores records on about 30,000 reported cocoa clone names but only slightly more than 1,000 clones could be grouped into ten cocoa varieties (genetic groups). For example, the clone CCN51 is a hybrid of tree varieties called Amelonado, Iquitos and Criollo. Motamayor et al. proposed a new classification based on the analyses of 1241 genotyped individuals of wild and cultivated cacao covering a large geographical area with 106 microsatellite markers that resulted in 10 genetic clusters named according to the geographical location: Amelonado, Contamana, Criollo, Curaray, Guiana, Iquitos, Maranon, Nacional, Nanay, and Purus. In this way, Forastero definition disappears because it includes various genetic clusters, while Criollo definition remains (Motamayor et al., 2008).

1.2.2 Agriculture

Cocoa trees naturally grow within 20° latitude of the equator because they need about 2000 mm of rainfall a year and temperatures in the range of 21 to 32 °C. The humidity should be very close to 100% during the day and between 70-80% during the night. Very often cocoa crops are cultivated in mixed crops among tall standing plants like bananas or palms, which offer shadow to cocoa plants protecting them from the sun. The soil needs to be permeable and nutrients rich to a depth of around 1.5 m, this will allow the roots to grow. The pH of the soil should be between 5 and 7.5 as lower, or higher pH would be harmful to the plant. The sowing density affects the yield; thus, most of the farmers use a density between 1000 and 1200 trees per hectare (Young, 2007). The tree starts bearing fruits in the 5-6th year of its life when peak production levels occur, and they can continue at this level for ten years. The average life of a cocoa tree is 25 years, and after this time, the trees must be replaced to provide the same yield from year to year. The fruit is called cacao pod and it is ovoid, 15-30 cm long, and 8-10 cm wide, colored yellow to red-orange and weighs about 500 g when ripe. The cocoa tree flowers in 2 cycles of 6 months the year around and about 40 flowers develop into cocoa pods. The pod contains up to 60 seeds, usually called "beans", implanted in a white pulp (Cuatrecasas, 1964; Young, 2007). Around 80% to 90% of cocoa comes from small, family-run farms, with approximately five to six million cocoa farmers worldwide. In Africa and Asia, the typical farm covers two to four hectares. Each hectare produces 300 to 400 kilograms of cocoa beans in Africa and about 500 kilograms in Asia. Cocoa farms in the Americas tend to be a little larger and produce 500 to 600 kilograms of cocoa beans per hectare. More than 75% of the global production in the last year originated from African countries, while the leading cocoa bean

processing countries are European countries grinding 38.7% of the 4.6 million tons of cocoa harvested in 2016. Indonesia is third in world exports with around 290 thousand tons produced last year ("The International Cocoa Organization (ICCO) | Cocoa Producing and Cocoa Consuming Countries,"). Other counties as Ecuador, Cameroon, Brazil, Madagascar, Nigeria, Sri Lanka, and Venezuela export significant amounts of beans. Cocoa is also cultivated for export in Columbia, Tanzania, Congo/Zaire, Costa Rica, Cuba, Dominican Republic, Fiji, Gabon, Grenada, Haiti, Jamaica, Malaysia, South Central Mexico, Panama, Papua New Guinea, Peru, Philippines, Sao Tome, Sierra Leone, Togo, Trinidad and Western Samoa (Update, 2014).

Cacao trees produce pods throughout the year, but large harvests occur twice annually. The main crop is harvested from October to March and is usually of a higher quality than midcrop, harvested from May to August. There are no machines for harvesting cacao so the process may take weeks to accomplish. Cacao trees produce flowers and fruit year-round in this way machines could harm the clusters of flowers or pods that grow from the trunk. Workers must harvest the pods by hand, using short, hooked blades mounted on long poles to reach the highest fruit. The pods are collected into baskets and then carried to the edge of the field.

1.2.3 Post-harvesting treatments

Collected pods are opened one by one; the pulp-covered cacao seeds are scooped out and quickly placed into boxes, or heaped into piles, and covered with banana leaves. For the next three to seven days, the intense bitter seeds ferment, allowing natural yeasts and bacteria to grow and produce physicochemical transformations into the beans. Fermentation is the first essential step that creates the familiar chocolate flavor (Afoakwa, Paterson, Fowler, & Ryan, 2008). This process is divided into 2 phases: a first anaerobic and a second aerobic phase. The anaerobic phase takes place in the first 24-48 h in which the beans are exposed to numerous various microorganisms. The major species include *Hanseniaspora* sp., *Pichia* sp., *Saccharomyces cerevisiae* for the yeasts and *Acetobacter* sp., *Lactobacillus* sp. and *Leuconostoc* sp. for the bacteria (Ho, Zhao, & Fleet, 2014). During this stage, the initial pH is bellow 4 and yeasts produce ethanol from the sugar present in the pulp. Moreover, some yeasts degrade the pectin of the pulp cell walls, which favor aeration (Aprotosoaie, Luca, & Miron, 2016). After 48 to 96, the aerobic phase takes place in which the yeasts are inhibited by aeration, alcohol concentration and increased pH, giving space to the growth of lactic acid bacteria. At the end of this phase, lactic acid bacteria are replaced by acetic acid bacteria which are responsible for the oxidation of ethanol to acetic acid in an exothermic reaction which leads

to a temperature increase up to 45-52 °C. During the fermentation process ethanol, lactic acid, acetic acid, carbon dioxide are produces and the pH values increases. These compounds and pH conditions play a key role in the formation of flavor precursor. Acetic acid diffuses into the bean causing the death of the embryo and the pH values of 3.8 and 5.8 offer the optimal conditions for the activity of numerous enzymes as, for instance, proteases. Hence, complex biochemical reactions occur generating flavor precursor as reducing sugars, peptides and amino acids, oxidized polyphenols and other compounds. Besides, the color of the beans changes developing from purple or light color to brown color (Afoakwa et al., 2008; Aprotosoaie et al., 2016; Giacometti, Jolić, & Josić, 2015). If the seeds are over-fermented, unwanted molds may grow which produce the hammy off-flavor compounds. Moreover, the pH value continues to increase causing a consequent darkening of the beans (Biehl & Ziegleder, 2003; Schwan & Wheals, 2004). Most of the beans around the world are fermented under spontaneous conditions. However, controlled conditions using specific culture strains are implemented as well (De Vuyst L. & Weckx S., 2016).

After fermentation, the beans have to be dried. Drying process lasts between 6 to 14 days and consists of spreading out the beans in the sun or under solar dryers. The beans are regularly turned so that they retain just a fraction of their moisture content (less than 8%). Drying is crucial, both for stopping the fermentation process and for storage; hence, to avoid microorganisms growth. Sometimes farmers are compelled to use equipment like hot pipes or air dryers to speed the drying process and this can generate changes in the cocoa flavor.

Once the cacao seeds are dry, the cocoa farmers pack them into jute sacks immediately. They inspect the crops to make sure that they have properly fermented. After weighing and packing them into bales of 50-60 kg jute sacks or in bulk or containers, cocoa beans are shipped to their final destinations – chocolate and cocoa-making companies (Beckett, 2008).

1.2.4 Factory production process

Once the shipments of seeds have reached the factories, they quickly begin the process that transforms the beans into chocolate or other cocoa products. The conversion is a complex and time-consuming process. An individual chocolate bar production can take anywhere from two to four days, or more. Initially, the seeds are sorted according to type and country of origin. They are passed through a cleaning machine that removes bits of remaining pulp and debris. Each manufacturer uses special formulations to blend the seeds to create different types of chocolates. African beans, for instance, are known for the basic aroma that they give to

chocolate. American and Asian beans give the chocolate a more refined aroma. Then large, rotating ovens at temperatures of 120 °C or more are used to roast the seeds in order to release their rich aromas and delicious taste. Roasting is the second most important step in the cocoa processing. This process takes from 15 minutes to two hours and it depends upon the variety of the seeds. The beans lose much of their moisture during the roasting. Eventually, they turn into a deep brown color, similar to coffee beans. A sterilization step is normally applied in order to destroy the heat-resistant bacteria and spores even if most of the microorganisms are destroyed in the early stages of roasting under moist conditions. The procedure consists of an injection, over a period of 20 sec, of a fine water spray into the roasting drum at the end of the roasting period. This procedure guarantees acceptable values in terms of bacteria and spores for a good quality cocoa. The roasting processes makes the shells of the cacao rather brittle and after the beans are cooled, the giant winnowing machine open and remove the thin shells. Giant fans then blow away these empty crusts. The remaining broken seed bits called nibs are then passed through a series of sieves, which strain and sort the nibs according to their size, a process called “winnowing”. Sometimes this process along with the roasting occurs together.

In the next step, the nibs are milled – crushed by heavy steel discs, hammer mills, ball mills, three-roll refiners or a combination of the four. During this process, sufficient friction and heat are generated which liquefies the nibs into a thick paste, called chocolate liquor. The heat emanated by this process ensures that the cocoa butter present in the liquor melts, making the cocoa liquor into liquid. The cocoa liquor is used as an ingredient of chocolate, or it can be further processed into cocoa powder and cocoa butter. The nibs themselves are made up of 53-55% cocoa butter and 45-47% pure cocoa solids. The separation of these two substances is a laborious process. Huge hydraulic presses are used to squeeze out the cocoa butter from the chocolate liquor. Once cocoa butter is obtained, the remaining solid cocoa is pulverized into cocoa powder – both are essential for several different applications as beverages, cooking, chocolate, baking, and in cosmetics.

About 50% of the cocoa liquor that is used to produce cocoa powder is alkalized. Solutions of alkali, typically potassium, ammonium or sodium carbonate are commonly employed. Usually, the solutions are added to the cocoa nibs before roasting, even though the cocoa liquor or the cocoa powder can also be treated. The initial reason for doing this procedure was to make the powder less predisposed to agglomerate or sink to the bottom, when mixed with milk or water-based drink. Now the alkalization is mainly used to produce changes in the color and the flavor.

For chocolate production, manufacturers mix cocoa liquor with milk powder, sugar, and extra cocoa butter. The extra cocoa butter keeps the chocolate solid at room temperature and that is why it melts in the warmth of the mouth. The raw mixture of milk powder, liquor, sugar, and cocoa butter is churned until it becomes a coarse, brown powder called “crumb”. The ingredients are first weighed according to the recipe for the particular type of chocolate, mixed, and then blended into homogeneous chocolate mass. The chocolate crumb mixture is then pressed between rollers to form a fine powder. The rollers break down the tiny particles of milk powder, cocoa, and sugar within the crumb and give a smooth texture and a homogenous flavor to the finished chocolate. The average size of the particles in this chocolate powder should be smaller than the distance between the taste buds on our tongues – so that we can never physically feel them, this guarantees a smooth chocolate. There is a right proportion in the mixing of this mixture – not enough crushing will give a coarse and grainy chocolate and too much blending will give a pasty and gummy chocolate. In general, Swiss and German chocolates are refined for a longer period. This makes them smoother and finer than American or English candy, which is a matter of national taste preferences. Subsequently, the refined chocolate paste is transferred into a vat in which a large heavy roller kneads, blends, and grinds it for hours until the aromas have fully developed. These machines are called conches and they are large tanks with a powerful stirring apparatus inside that slowly kneads the mixture. Due to the friction caused by the stirring, heat develops. This heat melts the powder into a homogeneous paste and makes the unpleasant, acid aromas evaporate. Moreover, agitating and aerating this paste smoothes out the sugar grains and give a silky texture to the chocolate, which creates a mellower, more well-rounded flavor. This process, called “conching” can take up to six days to complete.

The refined chocolate is cooled and warmed repeatedly in a process called “tempering”. This gives to the chocolate its glossy sheen, and ensures that it will melt properly. Tempering ensures the formation of the right cocoa butter crystals so that the chocolate will harden into shiny, hard and solid shapes. Finally, only after it has been tempered, the chocolate can be poured into molds or processed further into blocks, drops or other solid shapes. In a classic assembly-line fashion, the machines squirt tempered chocolate into several hundred molds per minute. Some devices dispense chocolate over flavored centers, in a process called “enrobing”. Others create chocolate shapes that will be filled with liquid before their bottoms are sealed. Eventually, wrapping-and-packaging machines box the chocolates at speeds unattainable by human hands (Beckett, 2008; Minifie, 1989).

1.2.5 Economic impact and health benefits

It has been discovered that cocoa was already playing an important economic and social role in the communities living in the Ulúa valley in Honduras about some thousand years ago. Nowadays, people consume more than 4.5 million tons of cocoa beans annually in a multitude of different forms. Each country has unique preferences and distinctive assortments of candy and desserts (Young, 2007). More than half of the top 20 consuming countries are European with people in the Northern nations in average consuming more than people in the South. Cocoa is a cash crop and a critical export commodity for producing countries and is also a key import for consuming countries, which typically do not have suitable climates for cocoa production. When traveling a global supply chain, cocoa beans go through a complicated production process that includes farmers, buyers, shipping organizations, processors, manufacturers, chocolatiers, and distributors. Many people are involved in cocoa and chocolate industry all around the world, and they are crucial users of other agricultural commodities such as sugar, dairy, nuts, and fruits. According to the World Cocoa Foundation, some 50 million people around the world depend on cocoa as a source of livelihood. Progressively increasing demand from worldwide consumers encourages some global efforts and funds committed to support and improve cocoa farm sustainability ("World Cocoa Foundation,"). The world leader in business news, CNBC, reports that the overall chocolate market between 2010 and 2015 rose to hit 101 billion US $, according to a market research firm. Cocoa prices had increased over the last four months reaching the price of € 2,259.33 per metric ton on April 26th, 2018 ("ICCO Daily Prices of Cocoa Beans,").

Evidence for the use of cacao or chocolate for medicinal purposes can be traced to ancient Aztecs sources. Moreover initially, Spanish and Italian empire entered cocoa in their pharmacopoeia (Coe & Coe, 2007). Cocoa is particularly rich in polyphenols. The total polyphenol content of the bean arises 6% to 8% by weight of the dry bean (Rusconi & Conti, 2010; Zumbé, 1998) with monomeric, oligomeric, and polymeric procyanidins being the main constituents. They are well-known antioxidant agents, which reduce the risk of cardiovascular diseases. The European Food Safety Authority (EFSA) has recently recognized that cocoa is a precious source of compounds with specific antioxidant properties (flavanols). Daily consumption of cocoa powder (2.5 g) or dark chocolate (10 g) can help protect the walls of the arteries, stimulate blood circulation, and regulate blood pressure; thus, safeguarding the heart (EFSA Panel on Dietetic Products, 2014). A long-term feeding study to examine the relative

activities of cocoa constituents on diet-induced obesity and insulin resistance was accomplished by Dorenkott, M. R. et al. (2014). The oligomer-rich fraction showed to be most effective in preventing weight gain, fat mass, impaired glucose tolerance, and insulin resistance in mice model (Dorenkott et al., 2014). Other authors' results claim that stearic acid is a unique saturated FA in cocoa beans, having unique effects on plasma total cholesterol levels (Kris-Etherton et al., 1993). Although the mechanism of its hypocholesterolemic action is still not clear, a short-term cocoa consumption significantly reduced blood cholesterol, but the changes were dependent on the dose of cocoa consumption and the health status of participants (Jia et al., 2010). Cocoa butter has a high digestibility similar to that of corn oil (Shahkhalili, Duruz, & Acheson, 2000), and its absorption can be reduced by calcium supplementation (Shahkhalili et al., 2000).

Cocoa butter is used on a tiny scale in the pharmaceutical industry for the preparation of suppositories and in the cosmetics industry for the manufacturing of lipstick and body lotions. Some decennia ago cocoa butter was used intensively for the production of cosmetics, but now it has mostly been replaced by synthetic products (Pontillon, 1998), which are preferred because of their price despite their high content of mineral oil hydrocarbons (MOSH and MOAH). Niederer reported that in 38 paraffin oils contained in lip-gloss products, MOAH range is between 0.05-4.5% (Niederer, 2014), whereas in cocoa beans, MOSH and MOAH represent contaminants absorbed during the transportation and storage, and their concentration is much lower. EFSA has expressed concern and issued regulation since some mineral oil aromatic hydrocarbons (MOAH) may be mutagenic and carcinogenic ("Scientific Opinion on Mineral Oil Hydrocarbons in Food," 2012). Nevertheless, the most important use of cocoa butter is, of course, the preparation of chocolate.

References

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

Akoh, C. C. (2017). *Food Lipids: Chemistry, Nutrition, and Biotechnology, Fourth Edition*. CRC Press.

Aprotosoaie, A. C., Luca, S. V., & Miron, A. (2016). Flavor Chemistry of Cocoa and Cocoa Products—An Overview. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 73–91. https://doi.org/10.1111/1541-4337.12180

Beckett, S. T. (2008). *The Science of Chocolate*. Royal Society of Chemistry.

Biehl, B., & Ziegleder, G. (2003). COCOA | Chemistry of Processing. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 1436–1448). Oxford: Academic Press. https://doi.org/10.1016/B0-12-227055-X/00261-3

Brügger, B. (2014). Lipidomics: Analysis of the Lipid Composition of Cells and Subcellular Organelles by Electrospray Ionization Mass Spectrometry. *Annual Review of Biochemistry*, *83*(1), 79–98. https://doi.org/10.1146/annurev-biochem-060713-035324

Byrdwell, William C. (2017). Chapter 10 - Multiple Parallel Mass Spectrometry for Liquid Chromatography. In *Handbook of Advanced Chromatography/Mass Spectrometry Techniques* (pp. 365–405). AOCS Press. https://doi.org/10.1016/B978-0-12-811732-3.00010-8

Byrdwell, William Craig. (2003). Dual Parallel Liquid Chromatography/Dual Mass Spectrometry (LC2/MS2) of Bovine Brain Total Lipid Extract. *Journal of Liquid Chromatography & Related Technologies*, *26*(19), 3147–3181. https://doi.org/10.1081/JLC-120025516

Christie, W. W., & Han, X. (2010). *Lipid Analysis, Fourth Edition: Isolation, Separation, Identification and Lipidomic Analysis* (4 edition). Bridgwater, England: Oily Press.

Coe, S. D., & Coe, M. D. (2007). *The True History of Chocolate* (Second Edition edition). New York: Thames & Hudson.

Coskun, Ü., Grzybek, M., Drechsel, D., & Simons, K. (2011). Regulation of human EGF receptor by lipids. *Proceedings of the National Academy of Sciences*, *108*(22), 9044–9048. https://doi.org/10.1073/pnas.1105666108

Cuatrecasas, J. (1964). *Cacao and its allies a taxonomic revision of the genus Theobroma*. Washington: Smithsonian Institution.

De Vuyst L., & Weckx S. (2016). The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *Journal of Applied Microbiology*, *121*(1), 5–17. https://doi.org/10.1111/jam.13045

Dorenkott, M. R., Griffin, L. E., Goodrich, K. M., Thompson-Witrick, K. A., Fundaro, G., Ye, L., … Neilson, A. P. (2014). Oligomeric Cocoa Procyanidins Possess Enhanced Bioactivity Compared to Monomeric and Polymeric Cocoa Procyanidins for Preventing the Development of Obesity, Insulin Resistance, and Impaired Glucose Tolerance during High-Fat Feeding. *Journal of Agricultural and Food Chemistry*, *62*(10), 2216–2227. https://doi.org/10.1021/jf500333y

Dreiss, M. L., & Greenhill, S. E. (2008). *Chocolate: Pathway to the Gods* (Har/DVD edition). Tucson: University of Arizona Press.

EFSA Panel on Dietetic Products, N. and A. (NDA). (2014). Scientific Opinion on the modification of the authorisation of a health claim related to cocoa flavanols and maintenance of normal endothelium-dependent vasodilation pursuant to Article 13 (5) of Regulation (EC) No 1924/2006 following a request in accordance with Article 19 of Regulation (EC) No 1924/2006. *EFSA Journal*, *12*(5), 3654.

Giacometti, J., Jolić, S. M., & Josić, D. (2015). Chapter 73 - Cocoa Processing and Impact on Composition. In V. Preedy (Ed.), *Processing and Impact on Active Components in Food* (pp. 605–612). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-404699-3.00073-1

Han, X. (2016a). *Lipidomics: Comprehensive Mass Spectrometry of Lipids*. John Wiley & Sons.

Han, X. (2016b). Lipids and Lipidomics. In *Lipidomics* (pp. 1–20). John Wiley & Sons, Inc. https://doi.org/10.1002/9781119085263.ch1

Ho, V. T. T., Zhao, J., & Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation. *International Journal of Food Microbiology*, *174*, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014

Holčapek, M., Jirásko, R., & Lísa, M. (2012). Recent developments in liquid chromatography-mass spectrometry and related techniques. *Journal of Chromatography A*, *1259*, 3–15. https://doi.org/10.1016/j.chroma.2012.08.072

ICCO Daily Prices of Cocoa Beans. (n.d.). Retrieved April 27, 2018, from https://www.icco.org/statistics/cocoa-prices/daily-prices.html

ICGD Homepage. (n.d.). Retrieved April 26, 2018, from http://www.icgd.reading.ac.uk/

Iijima, Y., Nakamura, Y., Ogata, Y., Tanaka, K., Sakurai, N., Suda, K., … Shibata, D. (2008). Metabolite annotations based on the integration of mass spectral information. *The Plant Journal*, *54*(5), 949–962. https://doi.org/10.1111/j.1365-313X.2008.03434.x

Jia, L., Liu, X., Bai, Y. Y., Li, S. H., Sun, K., He, C., & Hui, R. (2010). Short-term effect of cocoa product consumption on lipid profile: a meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition*, *92*(1), 218–225. https://doi.org/10.3945/ajcn.2009.28202

Kris-Etherton, P. M., Derr, J., Mitchell, D. C., Mustad, V. A., Russell, M. E., McDonnell, E. T., … Pearson, T. A. (1993). The role of fatty acid saturation on plasma lipids, lipoproteins, and apolipoproteins: I. Effects of whole food diets high in cocoa butter, olive oil, soybean oil, dairy butter, and milk chocolate on the plasma lipids of young men. *Metabolism: Clinical and Experimental*, *42*(1), 121–129.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., … Ohlrogge, J. (2013). Acyl-Lipid Metabolism. *The Arabidopsis Book*, e0161. https://doi.org/10.1199/tab.0161

LIPID MAPS Lipidomics Gateway : Home. (n.d.). Retrieved January 22, 2018, from http://www.lipidmaps.org/

Matthäus, B. (2012). The new database Seed Oil Fatty Acids (SOFA). *Lipid Technology*, *24*(10), 230–234. https://doi.org/10.1002/lite.201200227

Meer, G. van. (2005). Cellular lipidomics. *The EMBO Journal*, *24*(18), 3159–3165. https://doi.org/10.1038/sj.emboj.7600798

Minifie, B. W. (1989). *Chocolate, Cocoa and Confectionery: Science and Technology*. Springer Science & Business Media.

Motamayor, J. C., Lachenaud, P., Mota, J. W. da S. e, Loor, R., Kuhn, D. N., Brown, J. S., & Schnell, R. J. (2008). Geographic and Genetic Population Differentiation of the Amazonian Chocolate Tree (Theobroma cacao L). *PLOS ONE*, *3*(10), e3311. https://doi.org/10.1371/journal.pone.0003311

Navas-Iglesias, N., Carrasco-Pancorbo, A., & Cuadros-Rodríguez, L. (2009). From lipids analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part II: Analytical lipidomics. *TrAC Trends in Analytical Chemistry*, *28*(4), 393–403. https://doi.org/10.1016/j.trac.2008.12.004

Niederer, M. (2014). 1.1. 1 Lippenpflegeprodukte/Mineralparaffine (MOSH/MOAH) und allergene Duftstoffe. *Food and Chemical Toxicology*, *72*, 312–321.

Pontillon, J. (1998). Cocoa and chocolate: production, utilization, characteristics. *Cocoa and chocolate: production, utilization, characteristics.* Retrieved from https://www.cabdirect.org/cabdirect/abstract/20026788577

Published Articles | Blommer Chocolate Company. (n.d.). Retrieved March 23, 2018, from http://www.blommer.com/articles.php

Rusconi, M., & Conti, A. (2010). Theobroma cacao L., the Food of the Gods: A scientific approach beyond myths and claims. *Pharmacological Research*, *61*(1), 5–13. https://doi.org/10.1016/j.phrs.2009.08.008

Schwan, R. F., & Wheals, A. E. (2004). The Microbiology of Cocoa Fermentation and its Role in Chocolate Quality. *Critical Reviews in Food Science and Nutrition*, *44*(4), 205–221. https://doi.org/10.1080/10408690490464104

Scientific Opinion on Mineral Oil Hydrocarbons in Food. (2012). *EFSA Journal*, *10*(6). https://doi.org/10.2903/j.efsa.2012.2704

Sebedio, J.-L., & Brennan, L. (2014). *Metabolomics as a Tool in Nutrition Research*. Elsevier.

Shahidi, F., & Weenan, H. (Eds.). (2006). *Food Lipids: Chemistry, Flavor, and Texture* (1 edition). Washington, DC : New York: American Chemical Society.

Spener, F., Lagarde, M., Géloên, A., & Record, M. (2003). Editorial: What is lipidomics? *European Journal of Lipid Science and Technology*, *105*(9), 481–482. https://doi.org/10.1002/ejlt.200390101

The International Cocoa Organization (ICCO) | Cocoa Producing and Cocoa Consuming Countries. Retrieved April 26, 2018, from https://www.icco.org/

Update, C. M. (2014). World Cocoa Foundation. *Retrieved March*.

Wenk, M. R. (2005). The emerging field of lipidomics. *Nature Reviews Drug Discovery*, *4*(7), 594. https://doi.org/10.1038/nrd1776

Wenk, M. R. (2010). Lipidomics: New Tools and Applications. *Cell*, *143*(6), 888–895. https://doi.org/10.1016/j.cell.2010.11.033

World Cocoa Foundation. Retrieved February 26, 2018, from http://www.worldcocoafoundation.org/

Young, A. M. (2007). *The Chocolate Tree: A Natural History of Cacao* (Enlarged/Expanded edition). Gainesville: University Press of Florida.

Zumbé, A. (1998). Polyphenols in cocoa: are there health benefits? *Nutrition Bulletin*, *23*(1), 94–102. https://doi.org/10.1111/j.1467-3010.1998.tb01088.x

Chapter 2:

2 Review on cocoa lipidomics – state of knowledge and future needs

Diana Sirbu[a] and Nikolai Kuhnert[a]*

[a]*Department of Life Sciences and Chemistry, Jacobs University, Campus Ring 1, D-28759 Bremen, Germany*

*Correspondence to:
Prof. Dr. Nikolai Kuhnert,
Analytical Chemistry Laboratory,
Jacobs University, Campus Ring 1,
D-28759 Bremen, Germany.
Phone: +49 421 200 3120,
Fax: +49 421 200 3102.
E-mail: n.kuhnert@jacobs-university.de

Abstract

Cocoa butter is an essential ingredient in many commercial products. The chemical composition of cocoa butter can vary depending on the growing conditions and on the production process of the cocoa on the one hand, and on the refining treatments of cocoa butter on the other hand. These slight differences in the chemical composition also lead to small differences in the physical properties, such as the crystallization kinetics.

This review provides a state of the art information on chemical compositions of cocoa butter, taking into account the paramount interest on the aspects essential for a good crystallization performance. Besides some links are drawn helping the understanding of physical properties of cocoa butter based on its chemistry. Understanding better the chemical composition and consequently, its correlation with the physical properties of the cocoa butter will be of excellent use for setting adequate, cost-effective parameters for the tempering of chocolate and the development of products with tailored melting characteristics.

Keywords: Cacao butter, chemistry, fatty acids, triacylglycerols, minor components, physical properties, polymorphism.

2.1 Introduction

Cocoa butter (CB) is an ivory colored fat extracted from cocoa beans and constitutes the primary part of the final industrial product of cocoa. The physical and chemical characteristics of CB have made this fat of high demand in food, cosmetic and pharmaceutical industries (Liendo, Padilla, & Quintana, 1997). Identified as the primary transport and suspension medium (continuous phase) for the cocoa powder, chocolate liquor, sugar and several other ingredients used in chocolate production, CB gives chocolate its substance, consistency, plasticity, natural diffusion of taste and flavor, and the typical melt-in-the-mouth characteristics (Chaiseri & Dimick, 1989; Lipp & Anklam, 1998; Ribeiro et al., 2013). Since cocoa butter has a cocoa flavor and aroma, it is used for making white chocolate as the only cocoa ingredient (Beckett, 2011).

Cocoa Butter is considered to be a precious commodity because not only cocoa trees are difficult to cultivate, and the extraction of this fat is a very complex and costly process, but also because the income derived from the sale of CB constitutes the most significant part of the entire cocoa market value (Badrie et al., 2015). Global cocoa production is increasing every year and in 2016/2017 cocoa year (Oct-Sep) has reached 4,748 thousand tons with more than 90% produced by the smallholder farmers. Cocoa butter prices have seen fluctuations over the years. The year of 2014 has seen cocoa butter prices increases by 28%, to as high as $ 8,200 a ton, because the global grinders cut processing to reduce inventory (“As cocoa price soars, chocolate makers devour substitutes,” *Reuters*, 2014). However, it has declined progressively over the last years. Since the start of the 2017/2018 crop year, cocoa butter prices followed a near upward trend and stood at over $ 5,000 per ton, reflecting the growing demand for cocoa registered in recent months, as reported by the leading regional cocoa associations for the fourth quarter of 2017. Worldwide the annual trade of cocoa butter exceeds 800,000 tons (“The International Cocoa Organization (ICCO)| Cocoa Producing and Cocoa Consuming Countries,”).

The quality of commercial cocoa butter is defined by a sum of properties including appearance, snap, hardness, elasticity, and smoothness, which regulates its use in confectionery, cosmetic, and pharmaceutical applications. Nevertheless, there is still a significant knowledge gap on how the production process affects the character of the final product. Despite many investigators have directed their attention towards the physical

properties of cocoa butter and its significance in the confectionary industry, striving to connect them to the chemical properties of this fat has so far not been possible.

While extensive work has addressed the understanding of the chemical changes occurring during cocoa bean processing within constituent proteins, carbohydrates and polyphenols, considered as the prime reagents of the Maillard reaction and flavor precursors, little research has been done so far on the chemical changes occurring within cocoa lipids, which account for 50% or more of the dry mass of cocoa beans. Recently, Garti & Widlak wrote a book covering mostly the progress on the physical characteristics of the past ten years' studies on cocoa butter (Garti & Widlak, 2015).

Therefore, the motivation behind writing this review is primarily to give researchers and industry a valuable concise overview of the state of art of cocoa butter research covering the progress of many years of studies on cocoa butter chemistry. Our principal focus is to describe the cocoa butter chemical composition in detail and to emphasize the importance of understanding how this chemical composition may reflect the physical properties of the final product (like crystallization behavior, etc.). Moreover, the data obtained by various authors were compared to individualize the possible missing pieces of the puzzle thus, to relate it to the lipid composition and hardness/softness characteristics of cocoa butter. Additionally, the review attempts to correlate numerous studies conducted worldwide to investigate the crystallization properties of cocoa butter concerning the various constituents of this fat.

2.2 From cocoa tree to cocoa butter

Cocoa beans are the seeds of the *Theobroma cacao* tree growing on small farms in tropical environments. Three main species rule over the global cocoa production, namely: Forastero, Criollo, and Trinitario. About 95% of the world's cacao is now derived from Forastero trees, and in the trade, this cacao is termed bulk cacao. Criollo has white cotyledons and is the originally cultivated type. The origin of the Trinitarios is usually asserted as the result of hybridization between Forastero and Criollo trees. The Trinitario and Criollo varieties produce mainly the fine flavor cocoa. Plant characteristics, genetic diversity and agronomy approaches have been reviewed and acknowledged by Lima et al. and recently by Badrie et al. (Badrie, Bekele, Sikora, & Sikora, 2015; Lima, Almeida, Nout, & Zwietering, 2011).

The cocoa fruits called pods, sprout directly from the trunk and the thicker branches of the cocoa tree. Being a delicate and sensitive crop, cocoa trees must be protected from the wind

and sun, as well as from pests and disease attacks and soil fertilization is required. With proper care, most cocoa trees begin to yield pods at peak production levels by the fifth year of life, which can

continue for another ten years. Cocoa pods grow throughout the year starting from thousands of small flowers, but normally there are two harvests periods per year, namely main crop, harvested from October to March, and mid-crop, harvested from May to August. Usually, during the main-crop trees produce higher quantity and quality of pods than the mid-crop. Each pod contains 30 to 40 seeds implanted in a mucilaginous pulp, which accounts for approximately 40% of the fresh beans mass (Schwan & Wheals, 2004). The pulp is characterized mainly by high water content, 80-86% (w/w), and around 9 to 13% (w/w) sugar. A seed consists of two cotyledons (the nib) and a small embryo plant, all enclosed in a skin (the shell). In the cotyledons fat is the storage substance, which constitutes the food for the developing plant and represents half of the weight of the dried beans (Fowler M. S., 2009; Hanneman, 2000; Lima et al., 2011). Fat is stored in vesicles called lipid droplets with a diameter of 12 nm, homogeneously distributed in the cotyledons (K. D. Chapman, Dyer, & Mullen, 2012). The quality and the amount of each constituent in the bean depends on agricultural factors such as cocoa variety, origin, climate conditions, soil conditions, ripening, time of harvesting. A schematic representation of the main cocoa process practices is illustrated in Figure 2.1.

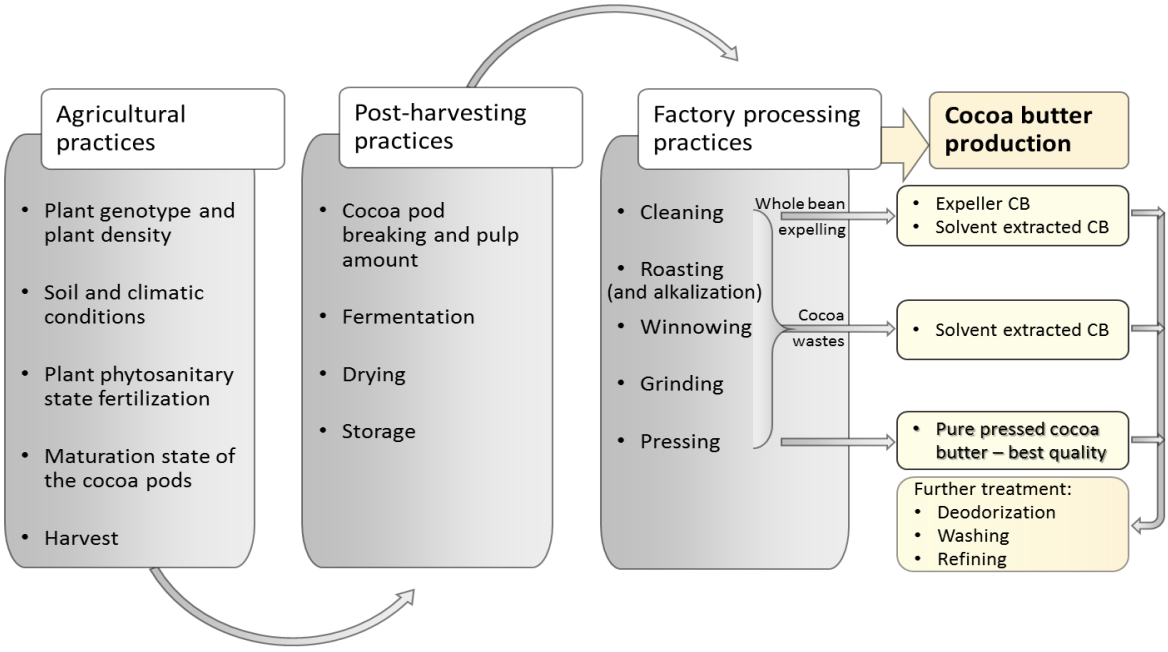


Figure 2.1 Representation of the main cocoa process practices and the steps in cocoa butter production.

Once collected, the seeds undergo fermentation – a process causing many chemical changes in both the pulp surrounding the seeds and within the beans themselves. These changes lead to the development of chocolate flavor precursors and produce color change in the seeds. The process of the fermentation has been studied extensively. Afoakwa et al., Lima et al., Schwan & Wheals, and Aprotosoaie et al. cover most of the scientific work achieved on understanding the chemical transformation occurring during the fermentation process (Afoakwa, Paterson, Fowler, & Ryan, 2008; Aprotosoaie, Luca, & Miron, 2016; Lima et al., 2011; Schwan & Wheals, 2004). The method for cocoa beans fermentation starts with piling a certain amount of beans or transferring it to heaps, boxes, or baskets allowing naturally present microorganisms to reproduce in the pulp (Thompson, Miller, & Lopez, 2007). The process of fermentation takes between 2 to 3 days for the beans of Criollo species and from 5 to 7 days for the beans of Forastero species. Moreover, other factors as such as the country of origin and the farmers' practice influence the duration of the fermentation process (Afoakwa et al., 2008; Fowler M. S., 2009; Lima et al., 2011). In the first stage (24-36 h), part of the pulp drains off and the anaerobic conditions favors yeasts to convert the sugars in the remaining pulp to ethanol. Then, bacteria start to oxidize the ethanol to acetic acid and further on to carbon dioxide and water, producing heat and therefore raising the temperature to 40-55 °C. In the second stage of the fermentation (48-96 h), namely the aerobic phase, the beans are mixed and the bacterial activity continues as the rest of the pulp drains away allowing air to diffuse between the beans. At this stage, ethanol and acetic acid inhibit germination causing bean death and enzymes and substrates lose compartmentalization moving and being active at the level of the cotyledons. As the amount of bacteria further increases, lactic acid bacteria become dominant and they convert sugar and certain organic acids into lactic acid. The formation of too much lactic acid is not desirable since it enters the cotyledons increasing the acidity of the beans, but it is important in later reactions. After approximatively 72 h, lactic acid is produced and the acetic acid bacteria more actively oxidize ethanol to acetic acid, which penetrates into the beans leading pH drop to 4.5 and causing biochemical transformations. Good fermentation process is characterized by the dominancy of the acetic acid bacteria (Afoakwa et al., 2008; Fowler M. S., 2009; Schwan & Wheals, 2004; Thompson et al., 2007). Due to membrane break down, various beans' constituents interact with each other(de Brito Edy S et al., 2000). As results, during the fermentation, precursors of cocoa taste and flavor are produced from the protein degradation into peptide and free aminoacids and reducing sugars from the enzymatic degradation of sucrose (Afoakwa, Quao, Takrama, Budu, & Saalia, 2013; Kumari et al., 2016).

Another reaction of particular importance is the breakdown and oxidation of the polyphenols, which results in reduced bitterness and astringency of the fermented beans. The colored polyphenols, the anthocyanins, are hydrolysed in cyanidins and sugars, causing the lightning of the purple color of Forastero cocoa beans. Other oxidative and browning reactions take place leading to darkening of the beans tissue (D'Souza et al., 2017; Kadow, Bohlmann, Phillips, & Lieberei, 2013; Lima et al., 2011).

After the fermentation, the seeds are dried to reduce their still high moisture content. The drying procedure is conducted either by sun drying, when the weather is sunny and dry, or it can be done artificially using mechanical dryers. It takes between 7 to 15 days to dry the beans to the desired <8% moisture content, necessary to prevent mold infestation during the storage and transport (Wood, 2008). Drying rate must not be too high as it can result in acidic beans as the volatile organic acids tend to be trapped by the hard shells and this is detrimental for the flavor. Moreover, artificial drying might make use of a source of fire, thus producing an unpleasant smoky taste which cannot be removed by further processing. On the other hand slow or insufficient drying would results in beans having low acidity, poorer color, major presence of molds, and high moisture contents in the bean (Kongor et al., 2016). This can lead to degradation of the triacylglycerols, causing high free fatty acid and diacylglycerol contents degrading both flavor and physical characteristics of cocoa butter (Guehi et al., 2008; Hancock & Fowler, 1994). Chemical changes continue taking place in the beans during the drying process involving polyphenol oxidation, inducing the formation the brown color (Biehl & Ziegleder, 2003). This results in bitterness, astringency, and sourness reduction (Giacometti, Jolić, & Josić, 2015; Kyi Tin Mar et al., 2005).

After the drying process is complete, the beans are shipped to the factories and industrial processing of cocoa begins with cleaning the beans from foreign objects. Thermal treatment is applied to facilitate the shell removal and the beans are broken into nibs (Kleinert-Zollinger, 1994). Then the nibs proceed to the roasting. The roasting process is accomplished at temperatures between 120 and 160 °C during a period of 5 to 120 min (Afoakwa et al., 2008; Aprotosoaie et al., 2016). Most of the roasting recipes use 100-130 °C for 50 to 70 min. Roasting is a crucial step of the bean processing for the cocoa aroma enhancement and for further development of flavor compounds from the precursors formed during the fermentation and drying, as well as for sterilization. During roasting the main chemical reactions are associated with non-enzymatic Maillard reactions producing carbonyl derivatives, which react with free aminoacids during Strecker degradation (Afoakwa et al., 2008). Compounds such as

pyrazines, esters, aldehydes, ketones, furans, imines, ethers, and alcohols are formed during the roasting process (Counet, Ouwerx, Rosoux, & Collin, 2004; Frauendorfer & Schieberle, 2008; Kongor et al., 2016). Subsequently, the nibs are ground into a liquid mass called cocoa mass or cocoa liquor. After or during roasting, an alkalizing process, consisting of treating the nibs with an alkaline solution, using K_2CO_3 or $(NH_4)_2CO_3$, can take place to alter flavor and color, and to improve the dispensability of cocoa powder. This determines the color and taste of the cocoa liquor, which, as an intermediate or semi-finished product, is supplied to the chocolate industry or is further processed into cocoa powder and cocoa butter (Beckett, 2008; Giacometti et al., 2015; Meursing, 1994).

The Dutchman Casparus J.Van Houten extracted cocoa butter for the first time in 1828, who patented the first cocoa press. Nowadays, the best quality cocoa butter is extracted from the cocoa liquor made from good quality beans through industrial hydraulic presses and gains the name of pure pressed cocoa butter. These machines operate at pressures of 100 MPa and temperatures between 90 and 110 °C. The butter is afterwards filtered, to remove the particles of cocoa solids. Continuous expeller presses obtain cocoa butter of inferior quality from the undeshelled beans, nibs, mass, and pressed cake. Expeller cake and other cocoa waste materials are then defatted by solvent extraction. The use of organic solvents poses a potential health threat, thus supercritical fluid extraction using carbon dioxide and ethane have been proposed (Asep et al., 2008; Mohamed, Saldaña, Mazzafera, Zetzl, & Brunner, 2002; Rossi, Arnoldi, Salvioni, & Schiraldi, 1989; Saldaña, Mohamed, & Mazzafera, 2002). Press, expeller and solvent extracted cocoa butter undergo refining by treating the fat with an alkali solution and silica adsorbents (Kamphuis, 2009). Silica pretreatment has been shown to decrease phosphorus and iron content by 85%, theobromine by 50%, and removing small amount of tocopherols (De Clercq et al., 2012). Due to the industry demand for different quality CB, other refining processing as filtering, steam refining or deodorization, bleaching, and degumming are the most applied. Deodorization is the most often used refining process after the pressing. The role of the deodorization is to minimize the variability of the flavor components, since the chocolate industry's demand is usually for cocoa butter with a neutral taste. This process consists in removing part of flavor volatiles, free fatty acids, phospholipids, diacylglycerols and tocopherols means of high temperatures, high vacuum and steam injection. Thus, obtaining a cocoa butter with a "smooth" flavor (De Clercq et al., 2012; Hanneman, 2000). Another specific refining step of CB is the removal of the alkaloids: theobromine and caffeine mostly for technological reasons. Bleaching is used for white chocolate production. Cocoa butter

obtained from alkalized cocoa liquor can have a higher soap content, which is removed by washing (Beckett, Fowler, & Ziegler, 2017).

2.3 Quality criteria of cocoa butter

European low clearly defines the quality criteria of the different types of butter. Pure press cocoa butter must not contain more than 1.75% free fatty acids and not more than 0.35% unsaponifiable matter (Table 2.1) measured by standardized AOAC official methods of analysis ("AOAC Official Methods of Analysis,"; "Directive 2000/36/EC of the European Parliament and of the Council relating to cocoa and chocolate products intended for human consumption.,"). In expeller and refined cocoa butter, the value for free fatty acids is the same as for the pure pressed cocoa butter but the value of unsaponifiable matter is increased to 0.50%, due to the higher amount of the expeller butter obtained from the shell (Rossi et al., 1989).

Table 2.1 Definitions and characteristics of cocoa butter products according to EU Directive 2000/36/EC and Codex Standards.

Pressed cocoa butter	
Free fatty acids (expressed as oleic acid)	≤ 1.75%
Unsaponifiable matter (determined using petroleum ether)	≤ 0.35%
Expeller and refined cocoa butter	
Free fatty acids (expressed as oleic acid)	≤ 1.75%
Unsaponifiable matter (determined using petroleum ether)	≤ 0.5%

The process may affect drastically the quality of cocoa butter. Thus, high quality beans are needed to produce the best quality cocoa butter.

Pressed cocoa butter is most commonly used in the chocolate industry, which is only filtered and often deodorized. The shelf life in solid form is up to one year when stored under suitable conditions. For refined cocoa butter, the shelf life is reduced dramatically to a maximum of 6 months. The shelf life of liquid cocoa butter is up to one month. Thus, for longer periods storage, the use of nitrogen to prevent oxidation by air is recommended (Beckett, 2011). The major factor contributing to the lipid oxidation are: light, temperature, the presence of oxygen, and metal ions. Low amount of tocopherols were shown to inhibit considerably the oxidation of the refined CB (Hashim, Hudiyono, & Chaveron, 1997).

2.4 Chemical composition background and significance

2.4.1 Cocoa fat content

Similar to all vegetable oils and fats, CB is mainly composed of a mixture of TAGs with a small quantity of other minor components. The quality of CB and its properties such as melting point and hardness depend on the variety of cocoa, age of the plant, geographical origin, environmental growing circumstances, and nonetheless the processing conditions.

Fat content or yield is an important quality parameter for cocoa grinders during purchasing of dried fermented cocoa beans. Usually, the fat content of cocoa beans is measured by extraction using petroleum ether or hexane solvent with subsequent gravimetric assessment, AOAC methods ("AOAC Official Methods of Analysis,"). Fat accumulates in the cocoa seeds until the ripened pods are harvested, when in fact, values near to that of matured seed are reached after 150 days post-pollination (Lehrian & Keeney, 1980). Brazilian cocoa beans had been considered to have lower fat content in comparison to West African or Malaysian ones (Wood & Lass, 2001). Thus, seeds from Amazonian hybrids present a range between 58 to 60% fat in fermented beans and Criollo is reported to contain 53%. In West Africa, fermented and dried cocoa beans have shown a fat content range between 56 and 58% and most Forastero hybrids revealed a range of 55 and 59% (Afoakwa et al., 2013; Rohan, 1963). Luis et al. reported that fat content from beans from genotypes collected in the Upper Amazonian region showed higher fat levels, while the Trinitario-Criollo and Bahian genotypes tended to have lower fat content (Pires, Cascardo, Lambert, & Figueira, 1998). Moreover, authors report that there is a significant negative correlation between dry seed yield per plant and the fat content. Despite the trend, authors have identified 40 genotypes showing high-fat content and high dry seed yield. Variations in the bean sizes could also explain the difference in the lipid amount. A positive correlation between seed weight and fat content was observed in cocoa beans from West Africa (Toxopeus & Wessel, 1970). Authors described the relationship between the fat content and the environmental conditions; thus, smaller seeds originated from pods developed during the dry season have shown low-fat content. Afoakwa et al. reported that the lipid content decreases with the increasing pod storage time. A fat reduction by 3% after 21 days of pod storage in unfermented and fermented beans was observed (Afoakwa et al., 2013).

2.4.2 Fatty acids composition

In terms of its fatty acid composition, authors have labelled cocoa butter as a relatively simple fat because palmitic, stearic, and oleic acid account for over 95% of cocoa fatty acids. Table 2.2 illustrates the fatty acid composition of cocoa butter according to several authors. Thus, cocoa butter accounts for about 25% palmitic acid, 34% oleic acid, 35% stearic acid, as main fatty acids and other minor fatty acids where linoleic acid and arachidic acid are in a proportion of 3 and 1% respectively. Figure 2.2 illustrates the chemical structure of these fatty acids. Only Marangoni et al. reported values above the average for linoleic and arachidic acid in all the origins, despite the total amount of around 98% for the five most abundant fatty acids (Marangoni & McGauley, 2003). Fatty acid chemical analysis is performed regularly by methylation of the fatty acids after alkaline hydrolysis of the triacylglycerols and other lipid components containing fatty acids according to AOAC methods, followed by GC-FID separation and detection (Foubert, Vanrolleghem, Thas, & Dewettinck, 2004; Torres-Moreno, Torrescasana, Salas-Salvadó, & Blanch, 2015).

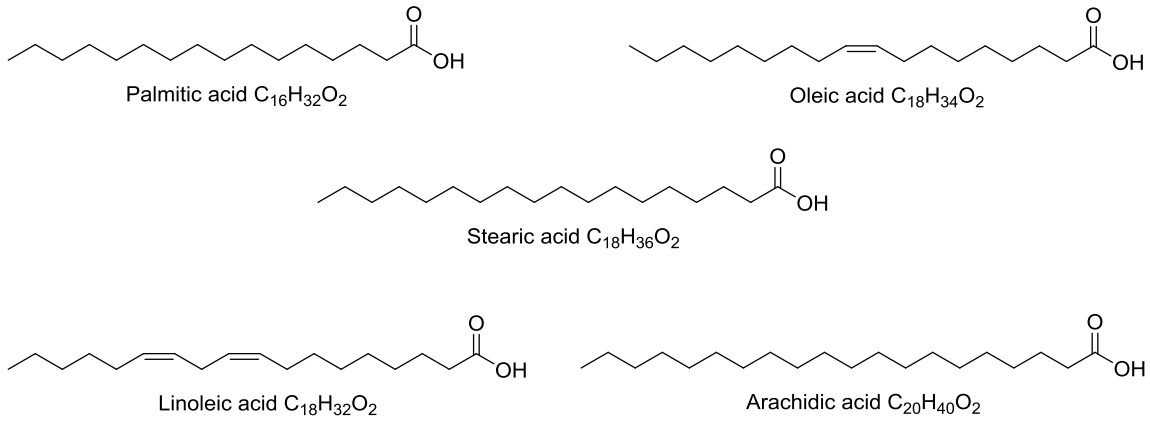


Figure 2.2 Chemical structure of the most predominant fatty acids in cocoa.

The content of fatty acids, which are the building blocks of energy storage molecules, rise rapidly during pod maturation. Studies report that stearic acid showed an increase during the last 75-80 days of pod maturation, 100-180 days post-pollination, whereas palmitic acid and linoleic acid decreased. Oleic acid has shown a notable enhancement after the ovules solidification, 110-130 days post pollination; then, it showed a decrease by 3 to 4% in the following 10 days, but remaining subsequently relatively constant until pod harvesting

(Lehrian & Keeney, 1980). Thus, pod ripeness degree applies to a role in the final fatty acids composition.

Authors suggest that Brazilian cocoa contains a higher amount of unsaturated fatty acids, compared to other countries of origin, with the most representative being oleic and linoleic acid ranging from 36.4-37.8% and 3.5-4.1% respectively (Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015). In Brazil, cocoa trees are cultivated between 5 °N and 24 °S latitude, but most of the commercial Brazilian cocoa beans are coming from Bahia's region. Vieira et al. and Esteves et al. and have shown high variability among Brazilian cocoa beans (Esteves, Barreraarellano, Nunes, Galvão, & Antoniassi, 1994; Vieira, Efraim, Walle, Clercq, & Dewettinck, 2015). Only the fatty acids values of beans from Bahia region were in the range described by most the other authors. Vieira et al. and Esteves et al. report values for cultivars grown in warmers regions of Brazil near to those found in Malaysian cocoa butters (A.h.g & Zainuddin, 1986), thus showing the prominent influence of the environmental temperature, observed by other authors (Lehrian, Keeney, & Butler, 1980). The same variability was observed in Criollo cultivars from Venezuela (Liendo et al., 1997). Notwithstanding, other authors found no difference among the beans from 3 different regions of Brazil (A. P. B. Ribeiro et al., 2012) although they ascertained significant variations among the triacylglycerols. In consequence, data suggest that geographical origin and climate are the primary factors influencing the fatty acid content in cocoa beans.

Herein, a graphical representation of normalized values (to 100) for the 5 major fatty acids is presented for better visualization of the differences across the origins (Fig. 2.3). Only data from dried cocoa beans and cocoa butter samples are available in literature. What emerges from the graph, and so from the table, is the very distinctive fatty acid profile of the South and Central American sample, where the ratio between oleic/stearic acid contents is close to 1 and in some Brazilian and Peruvian cocoa beans, it is even higher. This possibly indicates the hybrid effect on the fatty acid profile.

From the graph and the values collected in the table, we could observe a good negative correlation between stearic and oleic acid and the same for stearic and linoleic acid. By contrast, oleic and linoleic acid show a very good positive correlation. This indicates that these fatty acids are interchangeable, however in in-depth studies are needed to elucidate this hypothesis. A high correlation value was obtained when fewer data from the literature were taken into account. However, it is quite difficult to compare the data collected from different authors since the relative quantification was provided for a variable number of fatty acids as it

Table 2.2 Fatty acid content (%) in cocoa beans from different origins.

Country of origin / *Fatty acid*	Ecuador	Brazil	Ghana	Ivory Coast	Malaysia	Java	India	Nigeria	Indonesia	Peru	Dominican Republic	References
Myristic acid (C14:0)	0.06-0.09	0.08	0.09-0.12	0.05	0.12	-	-	0.16	-	0.07	0.09	(Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Palmitic acid (C16:0)	25.6-27.6	23.75-25.1	25.1-25.46	25.37-26.4	-	24.1	25.6	25.22	24.2-26.1	27.86	25.91-26.9	(Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Palmitoileic acid (C16:1)	0.3-0.32	0.25	0.2-0.23	0.27	0.24	-	-	0.26	0.27	0.29	0.26	(Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Margaric acid (C17:0)	0.22-0.27	0.21	0.27-0.32	0.26	0.24	-	-	0.2	0.24	0.24	0.26	(Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Stearic acid (C18:0)	33.89-36	32.79-34.3	36.34-37.8	35.4-36.9	37.01-37.6	37.3	36.6	35.92-37.1	36.88-37.4	31.66	35.93-34.4	(Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Oleic acid (C18:1)	33.7-36	36.4-37.8	32.7-34.31	32.84-34.5	32.98-33.7	34.3	33.9	33.1-33.6	33.06-34.4	35.14	33.2-34.8	(Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Linoleic acid (C18:2)	2.43-3.29	3.5-4.1	2.02-2.8	2.7-3.4	2.41-2.6	2.7	2.8	2.25-3.2	2.4-2.8	3.25	2.67-2.93	(Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Linolenic acid (18:3)	0.1-0.22	0.2-0.21	0.13-0.2	0.17-0.2	0.19-0.2	0.2	-	0.19	0.17	0.17	0.19	(Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Arachidic acid (C20:0)	1-1.09	0.91-1.2	1.1-1.24	0.99-1.2	1.08-1.2	1.2	1.1	1.03-1.1	0.99-1.2	0.96	1-1.1	(Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)
Gadoleic acid (C20:1)	0.05	-	0.04	-	-	-	-	-	-	-	-	(Torres-Moreno et al., 2015)
Behenic acid (C22:0)	0.1-0.2	0.2-0.22	0.14-0.2	0.20	0.2	0.2	-	0.18	0.22	0.2	0.18	(Klagge & K. Sen Gupta, 1990; Spangenberg & Dionisi, 2001; Torres-Moreno et al., 2015)

can be seen from the table. A more accurate quantification would allow a better understanding of the correlation between these three fatty acids. In view of that, absolute quantification is needed for equal comparison, since nowadays standards are available for all the major fatty acids identified in cocoa butter.

As regarding the production process, no data are describing the fatty acids profile during the fermentation process, while no significant effect was observed for the factory processing conditions in Ecuadorian and Ghanaian fatty acids (Torres-Moreno et al., 2015). Consequently, efforts towards determining other factors influencing the final fatty acid profile of cocoa are necessary.

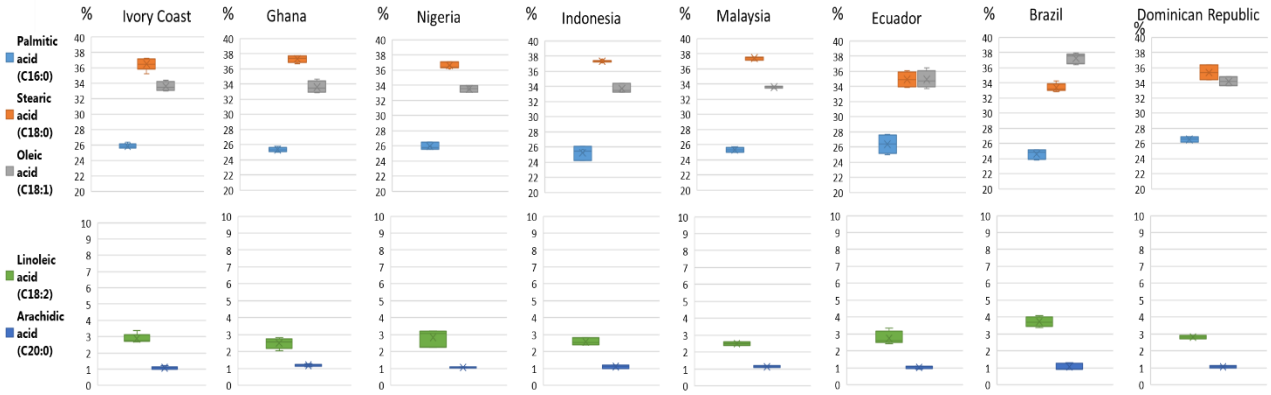


Figure 2.3 Box plots* illustrating the distribution of major fatty acids** in different origin countries.

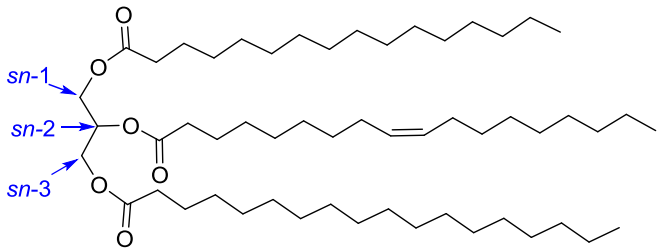
*Normalization to 100 was performed to reduce the variability due to analytical discrepancies by different authors.

**Fatty acids data from the table were used to build the box plots.

2.4.3 Triacylglycerol composition

The distribution of the fatty acids (FAs) between the different positions on the glycerol backbone creating triacylglycerols affects the physical and nutritional properties of CB. Like in most vegetable oils, in cocoa butter unsaturated FAs usually occupy the *sn*-2 position, whereas saturated FAs are mostly located in the *sn*-1 and *sn*-3 positions (Fig. 2.4). There are two main classes of triglycerides in cocoa; the major compounds are of the type 1,3-disaturated-2-unsaturated-*sn*-glycerol (S'U'S'), and the minor ones are of the type 1(3),2-diunsaturated-3(1)-saturated-*sn*-glycerol (S'U'U' and U'U'S'). The S'U'S' group is principally composed of 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol (POP, 100% regioisomeric purity), 1,3-distearoyl-2-oleoyl-*sn*-glycerol (SOS, 100% regioisomeric purity), rac-palmitoyl-stearoyl-2-

oleoyl-*sn*-glycerol (rac-POS, >90% regioisomeric purity) and, to a lesser extent, 1,3-dipalmitoyl-2-linoleoyl-*sn*-glycerol (PLP, 100% regioisomeric purity). The other triacylglycerol class is minor (rac-S'U'U') and it is composed of 1(3),2-dioleoyl-3(1)-stearoyl-*sn*-glycerol (SOO, 90% regioisomeric purity) and 1(3),2-dioleoyl-3(1)-palmitoyl-*sn*-glycerol (POO,100% regioisomeric purity). The observed fatty acid distribution confirmed that saturated acyl groups occur almost exclusively in the 1- and 3-positions of glycerol (Fauconnot et al., 2004). Glycerol 3-phosphate acyltransferases pathway catalyses cocoa triacylglycerol biosynthesis in the cytoplasm of cocoa cotyledon cells (Fritz, Kauffman, Robertson, & Wilson, 1986).



1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (POS)

Figure 2.4 Triacylglycerol structure of a 1,3-disaturated-2-unsaturated-sn-glycerol represented by POS.

Theobroma cacao genome contains 84 orthologues genes potentially involved in lipid biosynthesis (Argout et al., 2011). Griffiths & Harwood have previously appraised the distribution of the saturated fatty acids in the 1- and 3-positions of glycerol (Griffiths and Harwood, 1991). Authors have observed that diacylglycerol and phosphatidate, the two product-steps of triacylglycerols synthesis in plants as in animals arise in the early stages of maturation, were being relatively rich in both palmitate (33% and 36%, respectively) and oleate (54% and 46%, respectively). Whereas the proportion of stearate was significantly lower with values of 13% and 18%, respectively. In these lipids, the saturated fatty acids were almost exclusively confined to position *sn*-1 and the oleate to the *sn*-2 position. By contrast, the triacylglycerols were relatively rich in stearate (28%). Their results affirm that at position *sn*-1, palmitate is predominant over stearate and oleate is largely excluded, whereas position *sn*-2 is acylated exclusively with oleate. By contrast, position *sn*-3 is almost equimolar concerning both of the saturated fatty acids. Later, Tagaki and Ando (Takagi and Ando, 1995) confirmed the fatty acids distribution by chiral-phase HPLC, but only in one sample of cocoa butter obtained from unroasted beans. Initially, the three main TAGs were separated into three

fractions using reversed-phase HPLC. Then, the authors analyzed each fraction by chiral-phase HPLC concluding that the regioisomeric purity of the 3 main peaks, observed in reversed-phase HPLC, was as follows: POP (100%), POS (51%), SOP (49%), SOS (96.2%), and POA (3.8). Thus, these results and the results obtained by Fauconnot et al. that were described above, indicate that cocoa lipid extract showed a regioisomeric mixture. However, no attempts at studying the racemic content in different hybrids and cultivars from different origins were reported. Moreover, it is worth noting that the correct assignment of the TAGs was recorded only after Podlaha et al. (Podlaha, Toregard, & Puschl, 1984).

Studies on the lipid biosynthesis during maturation of cocoa pods have shown changes in the quantitative composition of TAGs during ripening where oleic fatty acid and linoleic fatty acid decreased as maturation proceeds and the typical high levels of stearate rise in the storage fat (Lehrian & Keeney, 1980). Similar behaviour was observed during the ripening of Sea Buckthorn seeds (Tsydendambaev & Vereshchagin, 2003). Moreover, a linear increase rate of TAGs between 120 and 170 days post-pollination was observed over 2 cropping seasons, though the fat accumulation started earlier in pods developed in the May-June period (Lehrian & Keeney, 1980). Same authors have studied the influence of the temperature during the latter stages of growth and ripening finding that fruits matured under elevated temperatures showed an increase in monounsaturated TAGs (S'U'S') with a concomitant decrease of diunsaturated TAGs (S'U'U') (Lehrian et al., 1980).

In literature, the analysis of TAGs in cocoa butter was performed by capillary GC (Lipp et al., 2001; A. P. B. Ribeiro et al., 2012; Rossi et al., 1989; Vieira et al., 2015) or reversed-phase HPLC coupled with evaporative light scattering detector (ELSD) (De Clercq et al., 2012b; Foubert et al., 2004), and rarely by reversed-phase HPLC coupled with mass spectrometry (Lísa & Holcapek, 2008; Segall, Artz, Raslan, Ferraz, & Takahashi, 2005). Table 2.3 (a) and 2.3 (b) illustrates the relative amount of the TAGs found in cocoa by several authors. It appears that amount of the main TAGs in cocoa butter is as follows: POP is between 12.6% and 22.6%; POS is between 34.6% and 46.3%; and SOS is between 21.7% and 31%. There was a wide range of variation among the samples from the same country, which could be caused by temperature differences throughout the whole year and the peculiarities of cocoa trees (Chaiseri & Dimick, 1989). Moreover, Chaiseri et al. found that factory process affects the quantity of the major TAGs, by decreasing significantly the amount of POS and SOS (Chaiseri & Dimick, 1989). Not much is known about the impact of the fermentation on CB chemistry. Only our recent paper gave some insides on CB chemistry of row unfermented beans illustrating the

presence of some TAGs ever described before (Sirbu, Corno, Ullrich, & Kuhnert, 2018). This indicates that the chemical composition of the bean changes through the fermentation process; nevertheless, the type of beans, the quality of the fermentation and drying and the subsequent bean processing will all affect the composition and properties of the final product. It is recognised that these slight differences in the chemical composition also lead to small differences in the physical properties, such as the crystallization kinetics (Campos, Ollivon, & Marangoni, 2010; Chaiseri & Dimick, 1989, 1995; Lehrian et al., 1980; A. P. B. Ribeiro et al., 2012; Vieira et al., 2015). Furthermore, we were able to describe the presence of a larger number of TAGs than previously reported, confirming and adding on top of the so far most comprehensive list given by Lisa et al. (Lísa & Holcapek, 2008).

Concerning origins, the assumption that the farther from the Equator is the origin, the more polyunsaturated TAGs in the composition of CB, associated with the climate conditions has been accepted by several authors (Chaiseri & Dimick, 1989; Foubert et al., 2004; Klagge & K. Sen Gupta, 1990; Lehrian et al., 1980; Liendo et al., 1997; Marty & Marangoni, 2009; Shukla, 1995). Box plots graphical representation of normalized values (to 100) for the 7 major TAGs gives an overview of the triacylglycerol variation across the countries (Fig. 2.5). It can be observed that although Dominican Republic is farther from the Equator than Bahia, it has a higher average temperature, thus supporting the temperature influence considerations. The contribution of Vieira et al. and Esteves et al. gave the further jutting piece showing that there is big variability within different Brazilian cocoa samples with respect to the TAGs composition (Esteves et al., 1994; Vieira et al., 2015).

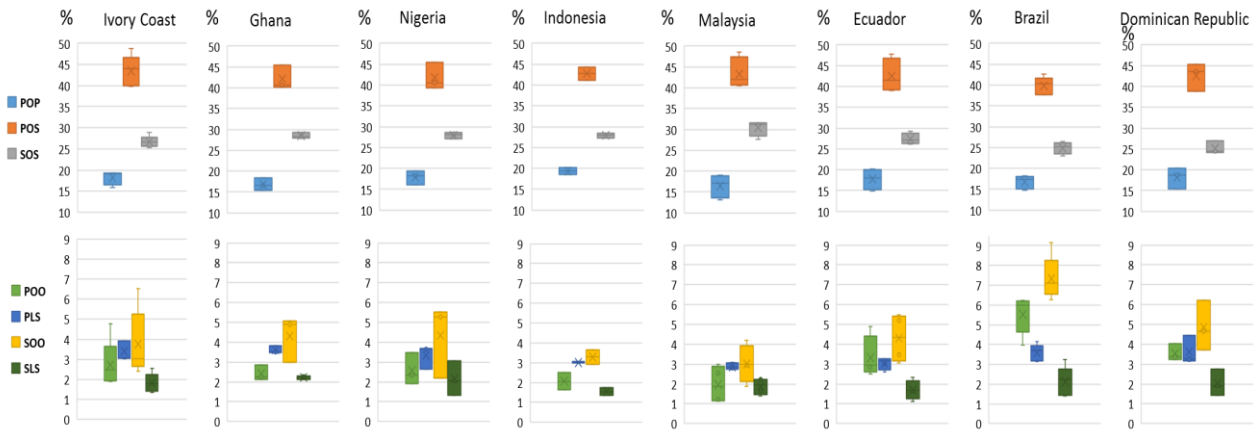


Figure 2.5 Box plots* illustrating the distribution of major TAGs** in different origin countries.

*Normalization to 100 was performed to reduce the variability due to analytical discrepancies by different authors.

**Triacylglycerol data from the table were used to build the box plots.

Table 2.3 (a) Triacylglycerol content (%) in cocoa beans from different origins.

TAG \ Country of origin	Ivory Coast	Nigeria	Cameroon	Ghana	Gabon	Indonesia	Malaysia	Samoa	Solomon Islands	References
PPP	0.38-0.39	0.51	-	-	-	0.33	0.22	-	-	(Foubert et al., 2004)
MOO/MMP	0.2	0.2	-	0.2	-		0.5	0.3	-	(Podlaha et al., 1984)
MOP	0.25	0.26	-		-	0.23	0.2		-	(Foubert et al., 2004)
PPS	0.4-0.73	0.7-0.84	-	0.8	-	0.72	0.6-0.84	0.4	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POP	15-19	14.8-18.3	17.9	14.5-17.8	17.5	17.5-19.9	12.6-18.5	16.4	19.3	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
PLP	1-1.9	1.36-1.9	-	1.8-1.9	-	1.1-1.79	0.7-1.84	1.6	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004; Podlaha et al., 1984)
PSS	0.33-1	0.4-1	1	0.6-1.3	0.9	0.49-1	0.4-1.03	0.7	1	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POS	36.6-46.3	37.4-43	38.3	37.3-42.8	37.1	40.6-41.8	36.6-46.9	38.3	40.7	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POO	1.8-4.4	1.8-3.2	3	2-2.6	3.7	1.6-2.37	1.1-2.7	2.2	0.9	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
PLS	2.88-3.7	2.51-3.6	3.4	3.2-3.6	3.5	2.81-3	2.6-2.8	2.8	3	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POL	0.36-0.7	0.32-0.8	-	0.6-1	-	0.47	0.4-0.6	0.8	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004; Podlaha et al., 1984)
SSS	0.22-0.4	0.28	-	0.4	-	0.29	0.2-0.46	-	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004)
SOS	23.8-28.5	25.7-27.8	27.7	26.3-27.5	27.5	25.8-28.1	25.9-31.1	26.8	29.5	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
SOO	2.3-3.9; 6*	2.09-5.2	5.8	2.8-4.9	7.3	2.75-3.6	1.8-2.9; 3.8*	3.7	3.8	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
SLS	1.28-2.5	1.27-1.9	2.5	2-2.2	3	1.28-1.7	1.29-2.2	2.1	2	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
OOO	0.4-0.8	0.4	-	0.5-0.8	-	-	0.1-0.8	0.2	-	(Chaiseri & Dimick, 1995; Podlaha et al., 1984)
SOL	0.9	0.8	-	0.4	-	-	0.7	0.5	-	(Podlaha et al., 1984)
SOA	0.6-1.6	0.5-1.9	0.5	0.4-2.2	0.4	0.5-1.31	0.8-2.5	2.2	0.7	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
AOO	1	1.2	-	1.4	-	-	1.6	1.6	-	(Podlaha et al., 1984)

*Podlaha et al., 1984 considered SOO+PPP as one value.

Table 2.3 (b) Triacylglycerol content (%) in cocoa beans from different origins.

TAG \ Country of origin	Brazil	Ecuador	Dominican Republic	Bolivia	Peru	Colombia	Venezuela	Costa Rica	Guatemala	Mexico	Panama	References
PPP	0.26-0.31	0.38	0.36	-	-	-	-	-	-	-	-	(Foubert et al., 2004)
MOO/MMP	0.2	0.3		-	-	-	-	-	-	-	-	(Podlaha et al., 1984)
MOP	0.18-0.21	0.3	0.23	-	-	-	-	-	-	-	-	(Foubert et al., 2004)
PPS	0.3-0.58	0.3-0.95	0.58-0.7	-	-	-	-	-	-	-	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POP	13.9-17.9	14.1-19.2	14.6-19.4	22.6	18.3	20.4	20.4	17.8	19.3	19.1	19.1	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
PLP	0.9-2.18	1.2-1.9	1.1-1.91	1.1	-	-	-	-	-	-	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004; Podlaha et al., 1984)
PSS	0.19-0.9	0.4-1.1	0.26-1.1	1.1	1.5	1.1	0.9	1	1	1.1	1	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POS	34.6-40.2	36.3-45.5	38.2-42.8	40.4	35.8	39.4	40.4	38.7	39	38.8	41.4	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POO	3.9-5.96	2.36-4.5	3.08-3.8	3.3	4.3	3.3	1	2.6	2.3	2.4	1.5	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
PLS	2.97-3.9	2.46-3.2	3-4.2	3.5	3.9	3.6	3.1	3.5	3.4	3.5	3	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
POL	0.32-1.1	0.38-0.7	0.44-0.7	-	-	-	-	-	-	-	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004; Podlaha et al., 1984)
SSS	0.21-0.6	0.3-0.62	0.18-0.4	-	-	-	-	-	-	-	-	(Chaiseri & Dimick, 1995; Foubert et al., 2004)
SOS	21.7-26	24.8-26.9	22.8-26.5	22.8	24.6	25	28.8	27.4	27.5	27.8	27.3	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
SOO	5.96-6.7; 8.4*	2.86-5.4	3.54-6.1*	4	7.4	4.4	2.8	5.5	4.9	4.1	3.1	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
SLS	1.33-3.2	1.04-2.3	1.35-2.7	2.1	3.7	2.3	1.9	3	2.2	3	2.7	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
OOO	0.7-0.9	0.7-0.8	0.6	-	-	-	-	-	-	-	-	(Chaiseri & Dimick, 1995; Podlaha et al., 1984)
SOL	1	0.8										(Podlaha et al., 1984)
SOA	0.04-1.6	0.4-2.1	0.6-1.13	0.5	0.4	0.6	0.8	0.4	0.4	0.6	0.8	(Chaiseri & Dimick, 1989, 1995; Foubert et al., 2004; Podlaha et al., 1984)
AOO	1.5	1.2	-	-	-	-	-	-	-	-	-	(Podlaha et al., 1984)

*Podlaha et al., 1984 considered SOO+PPP as one value

Only Bahian cocoa followed the global trend of categorizing Brazilian cocoa butter as a butter rich in polyunsaturated TAGs. The average temperature in West Africa are rather constant with a minimum temperature of 20 °C and a maximum 28 °C. Asian islands face the maximum average temperature of slightly above 30 °C. In contrast, in South American countries, in certain period of the year, the average temperature goes below 20 °C. Thus, Peru, Colombia and Bolivia showed a higher amount of diunsaturated TAGs. In regards to major TAGs, not significant differences could be found, due to high internal variability.

2.4.4 Minor components

As triacylglycerols account for 97-98% of cocoa butter, the remaining part is constituted by minor components. These compounds include free fatty acids, mono- and diacylglycerols, phospholipids, glycolipids and unsaponifiable matter. The role of these compounds is distributed in part on the synthesis triacylglycerols and in part as structural elements of the cell and compartmentalization of signalling proteins (Griffiths & Harwood, 1991; Lehrian & Keeney, 1980). There are two potential sources of the minor components in cocoa: these compounds may be intrinsic to cocoa beans as part of the original cocoa constituents, or they can be produced during the production process upon degradation of major cocoa butter constituents. Lehrian et al. observed that the amount of monoacylglycerols, sterols, free fatty acids, and glycolipids increase during the growth and ripening of cocoa pods. Moreover, phospholipids and diacylglycerols were present in a higher amount than those above mentioned, though showing a significant decrease during the last 20 days of pod development. Thus, a value of a little more than 0.4% of free fatty acids is the intrinsic values of the beans (Lehrian & Keeney, 1980). Chaiseri et al. reported values of 0.79, 1.25, 1.27, 1.44% for Central American, South American, African, and Asian cocoa butter respectively (Chaiseri & Dimick, 1989). Foubert et al. have shown a range between 1.16 and 2.77% in 20 sample of different origin (Foubert et al., 2004). Thus, 8 of these butters presented values above the EU legal requirements. Authors did not observe any correlation between fatty acids and the origin; therefore, high amounts of fatty acids were attributed to the inadequate post harvesting practices such as prolonged storage of fresh beans, extended fermentation or insufficient drying (Chaiseri & Dimick, 1989; Pontillon, 1998).

Diacylglycerols, as the free fatty acids, have shown no correlation between the amount and the origin. Studies showed values between 0.59 and 2.8% (Foubert et al., 2004; Pontillon, 1998; Shukla, 1995). Whereas monoacylglycerols were present only in traces (Pontillon, 1998).

Regarding phospholipids the amount varies and depends on the sources cited: 0.28 and 0.45% in raw cocoa beans (Parsons, Keeney, & Patton, 1969); 0.37% (T. R. Davis & Dimick, 1989), 0.34% (Arruda & Dimick, 1991), 0.1-0.2% (Chaiseri & Dimick, 1995), 0.8-0.9% (Savage & Dimick, 1995), 0.72-0.94% (Shukla, 1995), and 0.1-0.2% (Foubert et al., 2004) in cocoa butter. Reported values for glycolipids were of 0.3% (Pontillon, 1998) and 0.3-0.8% (Chaiseri & Dimick, 1995). Figure 2.6 illustrates a TLC plate illustrating the separation of phospholipids and glycolipids from seed crystals of cocoa butter (adapted from Arruda & Dimick, 1991).

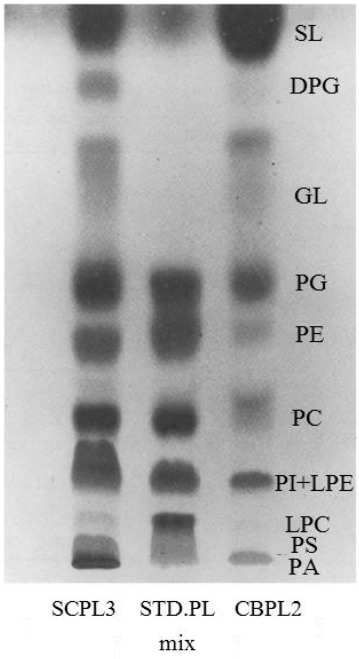


Figure 2.6 Thin-layer chromatographic separation of phospholipids from seed crystals (SCPL 3), phospholipid standard mix (STDPL mix) and Ivory Coast cocoa butter sample (CBPL2). SL–simple lipids, DPG–diphosphatidylglycerol, GL–glycolipid, PG–phosphatidylglycerol, PA–phosphatidylethanolamine, PC–phosphatidylcholine, PI+LPE–phosphatidylinositol and lysophosphatidylethanolamine, PS–phosphatidylserine, PA–phosphatidic acid (adapted from Arruda & Dimick, 1991).

The amount of unsaponifiable matter ranges from 0.3 to 1.5% (Itoh, Tamura, & Matsumoto, 1973; Schlichter-Aronhime & Garti, 1988). Sterol lipids in cocoa butter are present as mainly free sterols, with the major being sitosterol (123.3 mg/100 g fat) and stigmasterol (60.1 mg/100

g fat). Besides, esterified sterols amount were found to be 11.5% of the total sterols and glucosidic sterols 16% (Lipp & Anklam, 1998; Staphylakis & Gegiou, 1985). Other authors had reported similar sterol composition, 56.2 and 29.3% for sitosterol and stigmasterol respectively (Homberg & Bielefeld, 1982). El-Saied et al. reported values of 51.48% sitosterol, 39.45% stigmasterol, 5.77% campersterol and 3.29% cholesterol (El-Saied, Morsi, & Amer, 1981). However, no recent studies are available regarding the detailed composition of minor components in cocoa from beans of different geographical location and coming from various production stages.

Row cocoa beans contain between 200 and 250 mg/kg fat of tocopherols, and there are four types of tocopherols in cocoa: α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol, with γ-tocopherol and αα-tocopherol constituting the majority: approximately 85% and 10% respectively (De Clercq, 2011). The amount of tocopherols drops dramatically during the roasting, the parameters of which greatly influence the tocopherol content in cocoa nibs (Żyżelewicz, Krysiak, Budryn, Oracz, & Nebesny, 2014).

Expeller cocoa butter is obtained mostly from the cocoa bean shells. The lipid content of the shells was found to vary between 2 and 20% (El-Saied et al., 1981; Okiyama, Navarro, & Rodrigues, 2017; Rossi et al., 1989). Shell lipid extract showed a higher amount of linoleic acid and palmitic acid (El-Saied et al., 1981; Ruiz, Antequera, Andres, Petron, & Muriel, 2004), and different sterol composition as compared with cocoa butter (El-Saied et al., 1981).

2.5 Correlation of chemical composition with the physical properties of cocoa butter

The degree of unsaturation of the length of the hydrocarbon chain determine the physical properties of a fatty acid. In the solid state, fatty acids exist in a very regularly ordered structures called crystals. There are only a few basic crystalline structures that are stable under a given set of thermodynamic conditions. Some molecules have the ability to group together in multiple ways giving rise to several distinct crystal structures. This phenomenon is called polymorphism. The word polymorphism derives from Greek words *poly* and *morphos* meaning multiple forms. The polymorphism of CB triglycerides is the most important advancement in the understanding of the intricate structure of fat crystal networks, and it has been known since 1820 (Narine & Marangoni, 1999). Fat crystallization affects several properties of cocoa butter

such as the consistency, the mouthfeel, flavor release, and the emulsion stability (Hanneman, 2000; Sato, 2001). Thus, the physical properties of CB affect the status of the final product, chocolate. Consequently, controlling the crystallization behavior of this fat is of crucial importance (Chaiseri & Dimick, 1995; Ziegleder G., 2006). This topic was intensively studied, and it will not be addressed with much of details here, but it will give necessary information to understand how the chemistry of cocoa butter influences its physical properties in the solid state. For detailed information, we refer readers to (De Clercq, 2011; Foubert, 2003; Himawan, Starov, & Stapley, 2006; Langevelde, 2000; Sato, 2001; Stewart, 2017).

2.5.1 Cocoa butter polymorphism

TAGs are "three-legged" molecules that can pack together in two different structures, a double-chain packing and a triple chain packing. A double-chain structure is usually formed when the chemical properties of the three fatty acids moieties are the same or very similar. In contrast, when the chemical properties of one or two of the three fatty acid chain moieties are different from the other, a triple chain length structure is formed because of chain sorting and steric hindrance (Sato, 2001) (Fig. 2.7 (a)). The angle of tilt at which the double or the triple chain pack together determines the stability of the polymorph, and it defines the crystal plan. The differences between polymorphs are the most apparent from a top view of the planes, which showed the subcell structure identified by XRD patterns, giving the information regarding the spacing between the crystal plans and the inter-chain distance. Thus, three polymorphic forms can be defined: α, β', and β, in this order of increasing stability and melting point (Fig. 2.7 (b)). In α polymorph, the chains are arranged in hexagonal structure (H) with no angle. β' polymorph is characterized by the orthorhombic and perpendicular chain packing (O⊥) with an angle of tilt between 50° and 70°. Whereas in β polymorph, the chain pack in a triclinic chain packing (T//), in which adjacent chains are in step (parallel), and thus pack tightly together having an angle of tilt between 50° and 70° (Himawan et al., 2006).

Due to its chemical properties, cocoa butter is an extremely polymorphous fat. The polymorphic form is determined by the melting temperature of the individual TAGs in CB (Sato, 2001). The number and the type of polymorphs have been subject for debate for many years (Table 2.4), but nowadays it is accepted by many scientists that CB can crystallize into 6 different polymorphic forms. Considering a variety of nomenclatures that has been used for the different polymorphs, herein, a mixed nomenclature recurrently used in the literature based on 6 roman numerals and 5 Greek letters was adopted.

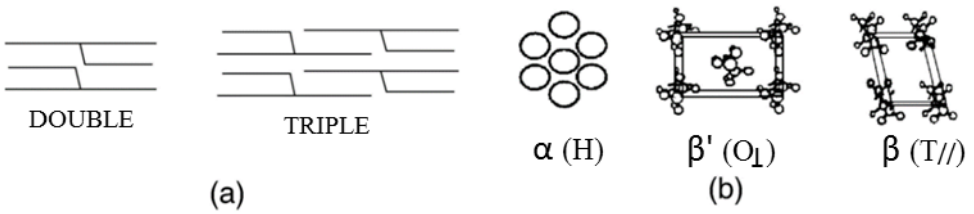


Figure 2.7 (a) Chain-length packing structures and **(b)** the subcell structures of the 3 most common polymorphs in TAGs (Himawan et al., 2006; Sato, 1999, 2001).

Table 2.4 Cocoa butter polymorphs and their melting point (°C) according to selected authors.

(Vaeck, 1960)		(Duck, 1964)		(Wille & Lutton, 1966)		(Chapman, Akehurst, & Wright, 1971)		(Lovegren, Gray, & Feuge, 1976)		(Davis & Dimick, 1986)		(Van Malssen, *et al.* 1999)	
γ	17	γ	18	I	17.3	I	melting	VI	13	I	13.1	γ	-5 to +5
α	21-24	α	23.5	II	23.3	II	point not	V	20	II	17.7	α	17-22
				III	25.5	III	provided	IV	23	III	22.4	β2'	
		β''	28										22-27
β'	28	β'	33	IV	27.5	IV	25.6	III	25	IV	26.4	β1'	
				V	33.8	V	30.8	II	30	V	30.7	β2	
β	34-35	β	34-35										29-34
				VI	36.3	VI	32.2	I	33.6	VI	33.8	β1	

The possible phase transitions in cocoa butter, γ, α and β' polymorphic phases can crystallize directly from the melt (Fig. 2.8 (a)). The transformation from α to β' is much quicker than if directly from the melt. The polymorphs β_V and β_{VI} are only obtained via a phase transformation from the β' phase (Van Malssen et al., 1999). Figure 2.8 (b) shows an example of phase transition in a saturated-O-saturated system.

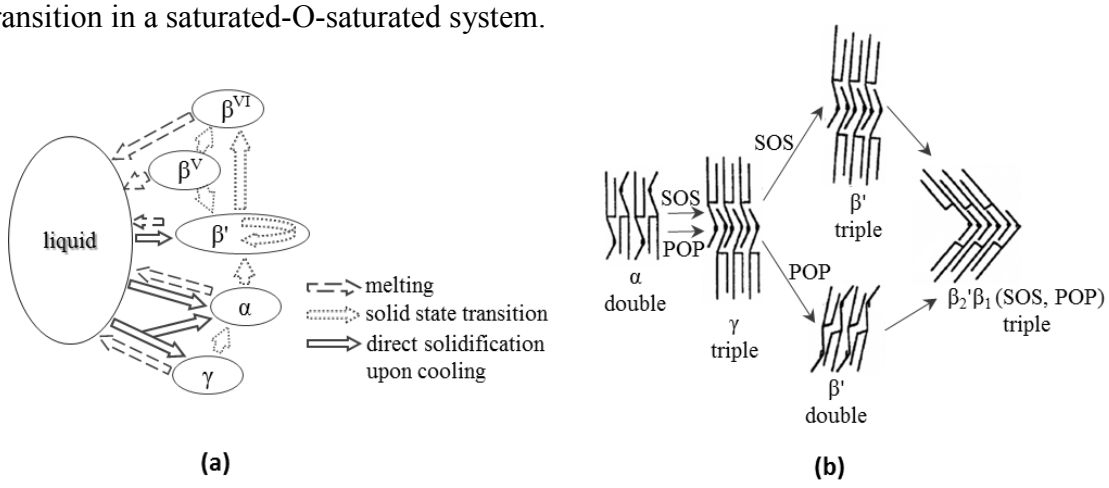


Figure 2.8 (a) Phase transition scheme of cocoa butter as proposed by Van Malssen et al., (1999) **(b)** polymorphic behavior of POP and SOS as shown by Yano et al., (1993).

2.5.2 Crystallization and melting behavior

The crystallization behavior of lipid molecules has essential repercussions in the industrial processing of food products whose physical characteristics depend mainly on fat crystallization, thus playing a critical role in determining the sensorial properties and stability of many fat containing food products (Domingues et al., 2015). The primary crystallization starts with nucleation and crystal growth after the system reached the supersaturation zone, then it is followed by aggregation into clusters and network formation (Chaiseri & Dimick, 1995; Himawan et al., 2006; Rousset, 2002; Sato, 2001). The crystallization of fat is often described under isothermal conditions (van Malssen et al., 1999; Marangoni & McGauley, 2003). A representative time-temperature state diagram for the polymorphism of statically crystallized cocoa butter based on DSC and XRD data is illustrated in Figure 2.9. Different polymorphs exhibit various morphologies: the α-form produces an amorphous mass of very tiny crystals, the β'-form is usually a bulky shape or spherulitic, while the β-form presents a needle shape (Sato, 2001).

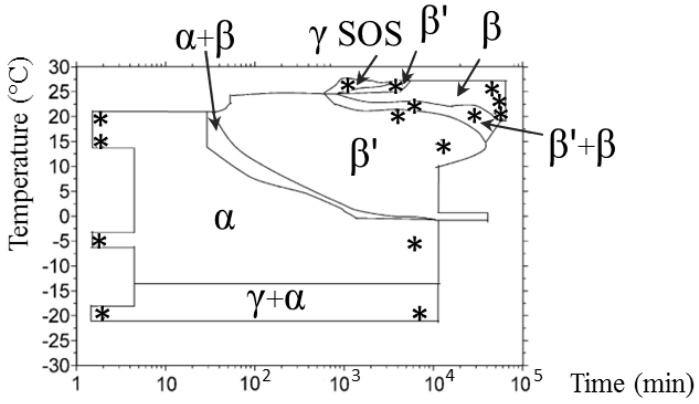


Figure 2.9 Time-temperature state diagram for the polymorphism of statically crystallized cocoa butter. Symbol (*) represents the polymorphic forms that have been determined by XRD (adapted from Marangoni & McGauley, 2003).

Tempering is a precrystallization technique used to control cocoa butter crystallization. This technique consists in subjecting the chocolate to carefully controlled shear-temperature-time parameter combinations to induce crystallisation of CB in a thermodynamically stable β_V polymorphic form. The tempering process produces seed crystals of the form β_V of CB crystal, which enables this polymorph to be the dominant form, the most desirable as it gives to chocolate a glossy appearance, good snap, contraction, and enhanced shelf life characteristics

(Beckett, 2008; Ziegleder & Schwingshandl, 1998). Poor tempering and storage conditions and other still unknown factors lead to fat bloom, a physical condition characterized by migration of high melting TAGs which recrystallize at the surface giving to the chocolate whitish colour (Beckett, 2008). Studies aiming at overcoming this problem showed that the low-temperature storage retards TAG migration (Depypere, De Clercq, Segers, Lewille, & Dewettinck, 2009), whereas diacyglycerols did not have any influence on fat blooming (De Clercq et al., 2014). Moreover, studies on molecular composition and physical dynamics showed that small amounts of trisaturated and triunsaturated TAGs influence the crystallization structure, times and temperature (Campos et al., 2010). Other authors have shown the impact of other additives on the chocolate crystallization such as sorbitan esters and lecithin (Sonwai, Podchong, & Rousseau, 2017; Svanberg, Ahrné, Lorén, & Windhab, 2011).

The melting point of triacylglycerol species depends on the fatty acid composition and the distribution of the fatty acids between the different stereospecific positions of the glycerol backbone. β' polymorphs of POP melts at 33.5 °C, PPO has a melting point of 35.2 °C, whereas POP/PPO (50/50) has a melting point of 28 °C (Sato, 2001). The melting points of many TAG have been measured (Cebula & Smith, 1991), yet a large number of the natural molecular species remains uncharacterized. Maruzeni et al. have developed a mathematical model for correlating the value of the melting point of a TAG with the values of the melting points of the three fatty acids (Maruzeni, 2009). Approximately 20% of the triacylglycerols are liquid at room temperature (Shukla, 1995).

Determined by its typical chemical composition, cocoa butter has a unique melting temperature range from 15 to 26 °C, depending on the polymorphic forms present. To describe the physical properties of CB authors mostly use the solid fat content (SFC) as a function of temperature (Fig 2.10). The solid fat content below 25 °C is an indication of the hardness of the butter. Besides, at 25 °C, the solid content should be at least 75%, this contributes to the acceptable chocolate snap when it is broken. The behavior between 25 and 30 °C designates the melting resistance of the chocolate. Values lower than 48%, will suggest that the chocolate will quickly melt in summer. The steepness of the curve (between 25 and 35 °C) shows a rapid melting of CB, thus releasing intense flavors and a pleasant mouthfeel. Above 37 °C, the absence of solid content means that cocoa butter is completely melted at body temperature and there is there is no waxy sensation after eating (Hanneman, 2000; Klagge & K. Sen Gupta, 1990).

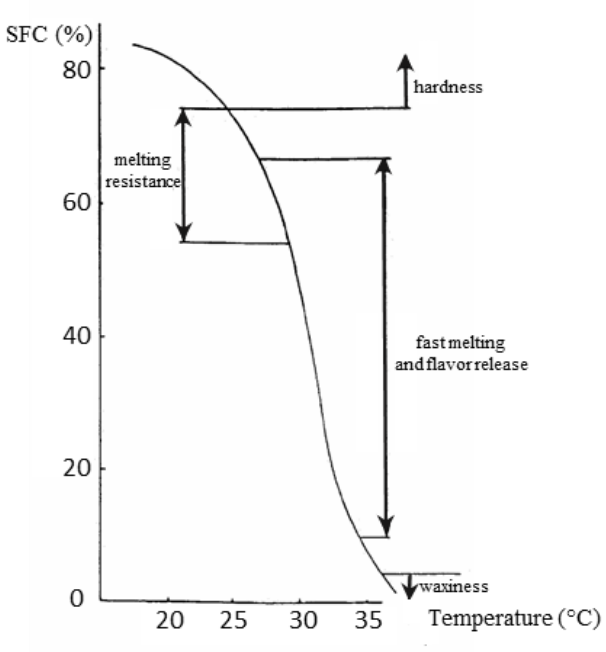


Figure 2.10 Ideal solid fat content curve of chocolate as function of time (adapted from Klagge & K. Sen Gupta, 1990).

2.5.3 Influence of the origin – softness assessment

The physical properties of cocoa butter vary according to the chemical composition. The softness and the hardness of cocoa butter are tightly correlated with the triacylglycerol composition. Thus, authors classify cocoa butters from Asian countries as hard butters, whereas butter from South American countries, especially from Bahia (Brazil) as soft butters, African butters being in the middle (Chaiseri & Dimick, 1989; Ana Paula Badan Ribeiro et al., 2013; Schlichter, Sarig, & Garti, 1985; Shukla, 1995; Vieira et al., 2015). The softness of butters was correlated to some parameters such as high iodine value and low solid fat content explained by increased POO and SOO content, which lead to undesirable crystallization characteristics (Chaiseri & Dimick, 1995; A. P. B. Ribeiro et al., 2012). Chaiseri & Dimick revealed that under the same crystallization conditions cocoa butters from Malaysia and Ivory Coast showed high nucleation rates, whereas cocoa butters from the Dominican Republic and Bahia (Brazil) had uniquely lower nucleation rates whereas Ghanaian and Ecuadorian samples have demonstrated average nucleation rates. Moreover, variation in the triacylglycerol composition was best correlated with the different crystallization induction times of cocoa butter. Same authors further showed that cocoa butters with high nucleation rates generally have high crystal growth rates and vice versa. Only the crystallization characteristics of the Ecuadorian sample deviate from this general rule in having a slightly high nucleation rate but a low growth rate

(Chaiseri & Dimick, 1995). Besides, Foubert et al. have shown the influence of other minor components on the crystallization of cocoa butter (Foubert et al., 2004).

2.6 Cocoa butter alternatives

Chocolate typically contains about 20-30% cocoa butter. As cocoa butter is an expensive product, confectioners in Asia and Europe are looking to save money and replace about a quarter of the cocoa butter with something substitutes designed to be used as alternatives. Such non-cocoa fats in chocolate products are usually referred to as compounds. The United States law requires in chocolate production that 100% cocoa butter must be used. The European Directive 2000/36/EC allows to mix cocoa butter with not more than 5% by weight of other vegetable fats in the end product, and it must be clearly indicated on the label. According to this law, six vegetable fats can be used: palm oil, illipe fat, sal fat, shea butter, coconut butter, and mango kernel. If this percentage is above 5%, the product should not be called chocolate. Much of work has been addressed towards studying the chemical composition and physical properties of cocoa alternatives (Jahurul et al., 2013; Lipp et al., 2001; Lipp & Anklam, 1998; Spangenberg & Dionisi, 2001; Ulberth & Buchgraber, 2003). Besides, Buchgraber et al. described a validated method for detection and quantification of cocoa butter equivalents in chocolate (Buchgraber, Ulberth, & Anklam, 2004).

2.7 Conclusions

The quality of cocoa butter relies on the combination of multiple factors along the entire production chain. Therefore, it is essential to understand the critical reactions taking place, which can lead to significant transformation into the lipid content of cocoa. Due to the complexity of this fat, a lot of research has been conducted, and there is still much more to be investigated. During the years, authors have taken into consideration a higher number of TAGs. SOA, for example, although it seems to be present in high amount in cocoa butter, very few authors report this TAG, and no authors account it for the crystallization behavior despite studies have shown that minor components influence the crystallization behavior. Therefore, following questions arises: ‘what about the influence of the remaining TAGs not yet taken into account by the majority of the authors?’; ‘how does the pull of the entire TAG changes during the production process?’

On the other hand, driven by flavor peculiarities, nowadays cocoa manufactures tend to make the classification according to the origins, which is particularly appealing to the consumers. Data showed that CB could as well be classified according to the source. However, chocolate producers must consider the hardness and the softness of cocoa butter for the future of such applications.

Due to rising demand for cocoa butter substituents, a consequent adulteration issue of cocoa butter might advance. If food industry takes into account only the TAGs described in the literature, very soon we might come across fats having the same TAG composition but of an artificial source.

We believe that is time to appraise as much as it is possible on the chemical composition of cocoa butter and we would like to emphasize our still limited knowledge of the variability of lipids in cocoa. Furthermore, still many questions require determination of lipid analyte absolute quantification.

2.8 Aim of the thesis

The rise in cocoa butter demand has determined the industry to invest more in the research in the past decade. Due to its intricate physical properties, cocoa butter requires deeper insights into the chemistry of this fat. The current literature does not provide a clear link between the chemical profile and the physical properties, but rather it offers assumptions often taking into account only a small number of lipid constituents. Nevertheless, there is very little evidence regarding the chemical modifications that cocoa butter undergoes during the production process. To advance the research on understanding the physical behavior of cocoa fat, several important research gaps need to be addressed.

The work of this thesis is a part of the project between Jacobs University and Barry Callebaut cooperation named CoMeta – cocoa metabolomics. The main objectives of this project are to recognize and map the precursor molecules during the production process and link them with the flavor and color of the final product. Within this framework, variations in the lipidomics of cocoa from tree to bar have been evaluated. To achieve the general purpose a series of sub-goals have been intended:

✦ Appraisal of the current state of the art in lipid chemical analysis and cocoa lipidomics research (literature review);

- Analysis of proposed methodologies for the full analysis of plant lipids with particular attention to oil seeds (literature review);
- Optimization of the extraction method to obtain a fast and reliable methodology suitable for the analysis of a large number of samples;
- Optimization of the separation of different lipid classes and characterization of each lipid classes by chromatographic techniques, along with the development of a fast and reliable methodology suitable for the analysis of a large number of samples;
- Characterization and identification of the largest possible number of lipid molecules present in cocoa by mass spectrometry techniques;
- Qualitative and quantitative assessment of the lipid profile of cocoa concerning different origins and qualities of cocoa beans;
- Study of the influence of selected process parameters in the cocoa and chocolate manufacturing procedure on the profile and content of the lipids present to identify critical process conditions responsible for lipid degradation and modification.
- Identification, selection, and characterization of biomarkers in each process step and development of a suitable tool for their assessment.

While the research focuses on cocoa butter chemistry from bean to bar important for the science of cocoa and chocolate, the overarching aim of this thesis is to derive associations and practical industrial applications for relevant eventual findings and understandings.

References

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

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

A.h.g, C., & Zainuddin, N. (1986). Characteristics of Malaysian cocoa butter. Presented at the International Conference on Cocoa and Coconuts: Progress and Outlook. Kuala Lumpur (Malaysia). 15-17 Oct 1984. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=MY19870059372

AOAC Official Methods of Analysis. (n.d.). Retrieved April 27, 2018, from http://www.aoac.org/aoac_prod_imis/AOAC/Publications/Official_Methods_of_Analysis/AOAC_Member/Pubs/OMA/AOAC_Official_Methods_of_Analysis.aspx

Aprotosoaie, A. C., Luca, S. V., & Miron, A. (2016). Flavor Chemistry of Cocoa and Cocoa Products—An Overview. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 73–91. https://doi.org/10.1111/1541-4337.12180

Argout, X., Salse, J., Aury, J.-M., Guiltinan, M. J., Droc, G., Gouzy, J., … Lanaud, C. (2011). The genome of *Theobroma cacao*. *Nature Genetics*, *43*(2), 101–108. https://doi.org/10.1038/ng.736

Arruda, D. H., & Dimick, P. S. (1991). Phospholipid composition of lipid seed crystal isolates from ivory coast cocoa butter. *Journal of the American Oil Chemists' Society*, *68*(6), 385–390. https://doi.org/10.1007/BF02663754

As cocoa price soars, chocolate makers devour substitutes. (2014, September 5). *Reuters*. Retrieved from https://www.reuters.com/article/asia-cocoa-substitute/as-cocoa-price-soars-chocolate-makers-devour-substitutes-idUSL4N0QR2XV20140905

Asep, E. K., Jinap, S., Tan, T. J., Russly, A. R., Harcharan, S., & Nazimah, S. A. H. (2008). The effects of particle size, fermentation and roasting of cocoa nibs on supercritical fluid extraction of cocoa butter. *Journal of Food Engineering*, *85*(3), 450–458. https://doi.org/10.1016/j.jfoodeng.2007.08.008

Badrie, N., Bekele, F., Sikora, E., & Sikora, M. (2015). Cocoa Agronomy, Quality, Nutritional, and Health Aspects. *Critical Reviews in Food Science and Nutrition*, *55*(5), 620–659. https://doi.org/10.1080/10408398.2012.669428

Beckett, S. T. (2008). *The Science of Chocolate*. Royal Society of Chemistry.

Beckett, Steve T. (2011). *Industrial Chocolate Manufacture and Use*. John Wiley & Sons.

Beckett, Steve T., Fowler, M. S., & Ziegler, G. R. (2017). *Beckett's Industrial Chocolate Manufacture and Use*. John Wiley & Sons.

Biehl, B., & Ziegleder, G. (2003). COCOA | Chemistry of Processing. In B. Caballero (Ed.), *Encyclopedia of Food Sciences and Nutrition (Second Edition)* (pp. 1436–1448). Oxford: Academic Press. https://doi.org/10.1016/B0-12-227055-X/00261-3

Buchgraber, M., Ulberth, F., & Anklam, E. (2004). Method validation for detection and quantification of cocoa butter equivalents in cocoa butter and plain chocolate. *Journal of AOAC International*, *87*(5), 1164–1172.

Campos, R., Ollivon, M., & Marangoni, A. G. (2010). Molecular Composition Dynamics and Structure of Cocoa Butter. *Crystal Growth & Design*, *10*(1), 205–217. https://doi.org/10.1021/cg900853e

Cebula, D. J., & Smith, K. W. (1991). Differential scanning calorimetry of confectionery fats. Pure triglycerides: Effects of cooling and heating rate variation. *Journal of the American Oil Chemists Society*, *68*(8), 591–595. https://doi.org/10.1007/BF02660159

Chaiseri, S., & Dimick, P. S. (1989). Lipid and hardness characteristics of cocoa butters from different geographic regions. *Journal of the American Oil Chemists' Society*, *66*(12), 1771–1776. https://doi.org/10.1007/BF02660745

Chaiseri, S., & Dimick, P. S. (1995). Dynamic crystallization of cocoa butter. II. Morphological, thermal, and chemical characteristics during crystal growth. *Journal of the American Oil Chemists' Society*, *72*(12), 1497–1504. https://doi.org/10.1007/BF02577843

Chapman, G. M., Akehurst, E. E., & Wright, W. B. (1971). Cocoa butter and confectionery fats. Studies using programmed temperature X-ray diffraction and differential scanning calorimetry. *Journal of the American Oil Chemists Society*, *48*(12), 824–830. https://doi.org/10.1007/BF02609292

Chapman, K. D., Dyer, J. M., & Mullen, R. T. (2012). Biogenesis and functions of lipid droplets in plants Thematic Review Series: Lipid Droplet Synthesis and Metabolism: from Yeast to Man. *Journal of Lipid Research*, *53*(2), 215–226. https://doi.org/10.1194/jlr.R021436

Clercq, N. D., Depypere, F., Delbaere, C., Nopens, I., Bernaert, H., & Dewettinck, K. (2014). Influence of cocoa butter diacylglycerols on migration induced fat bloom in filled chocolates. *European Journal of Lipid Science and Technology*, n/a-n/a. https://doi.org/10.1002/ejlt.201300476

Counet, C., Ouwerx, C., Rosoux, D., & Collin, S. (2004). Relationship between Procyanidin and Flavor Contents of Cocoa Liquors from Different Origins. *Journal of Agricultural and Food Chemistry*, *52*(20), 6243–6249. https://doi.org/10.1021/jf040105b

Davis, T., & Dimick, P. (1986). Solidification of cocoa butter (Vol. 40, pp. 104–108). Presented at the Proc. PMCA Prod. Conf.

Davis, T. R., & Dimick, P. S. (1989). Lipid composition of high-melting seed crystals formed during cocoa butter solidification. *Journal of the American Oil Chemists Society*, *66*(10), 1494–1498. https://doi.org/10.1007/BF02661979

de Brito Edy S, García Nelson H Pezoa, Gallão MI, Cortelazzo Angelo L, Fevereiro Pedro S, & Braga Márcia R. (2000). Structural and chemical changes in cocoa (Theobroma cacao L) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture*, *81*(2), 281–288. https://doi.org/10.1002/1097-0010(20010115)81:2<281::AID-JSFA808>3.0.CO;2-B

De Clercq, N. (2011). *Changing the functionality of cocoa butter* (dissertation). Ghent University. Retrieved from http://hdl.handle.net/1854/LU-1909786

De Clercq, N., Moens, K., Depypere, F., Vila Ayala, J., Calliauw, G., De Greyt, W., & Dewettinck, K. (2012). Influence of cocoa butter refining on the quality of milk chocolate. *Journal of Food Engineering*, *111*(2), 412–419. https://doi.org/10.1016/j.jfoodeng.2012.01.033

Depypere, F., De Clercq, N., Segers, M., Lewille, B., & Dewettinck, K. (2009). Triacylglycerol migration and bloom in filled chocolates: Effects of low-temperature storage. *European Journal of Lipid Science and Technology*, *111*(3), 280–289.

Directive 2000/36/EC of the European Parliament and of the Council relating to cocoa and chocolate products intended for human consumption. (n.d.). Retrieved April 27, 2018, from https://www.ecolex.org/details/legislation/directive-200036ec-of-the-european-parliament-and-of-the-council-relating-to-cocoa-and-chocolate-products-intended-for-human-consumption-lex-faoc034904/

Domingues, M. A. F., Ribeiro, A. P. B., Kieckbusch, T. G., Gioielli, L. A., Grimaldi, R., Cardoso, L. P., & Gonçalves, L. A. G. (2015). Advances in Lipids Crystallization Technology. https://doi.org/10.5772/59767

D'Souza, R., Grimbs, S., Behrends, B., Bernaert, H., Ullrich, M., & Kuhnert, N. (2017). Origin-based polyphenolic fingerprinting of Theobroma cacao in unfermented and fermented beans. *Food Research International*. https://doi.org/10.1016/j.foodres.2017.06.007

Duck, W. (1964). The measurement of unstable fat in finished chocolate. *Manufacturing Confectioner*, *35*(6), 67–72.

El-Saied, H. M., Morsi, M. K., & Amer, M. M. A. (1981). Composition of cocoa shell fat as related to cocoa butter. *Zeitschrift Für Ernährungswissenschaft*, *20*(2), 145–151. https://doi.org/10.1007/BF02021260

Esteves, W., Barreraarellano, D., Nunes, M., Galvão, M., & Antoniassi, R. (1994). Composição de ácidos graxos e triglicerídeos de quatro cultivares de cacau. *Cienc. Tecnol. Aliment*, *14*(2), 247–252.

Foubert, I. (2003). Modelling isothermal cocoa butter crystallization : influence of temperature and chemical composition /.

Foubert, I., Vanrolleghem, P., Thas, O., & Dewettinck, K. (2004). Influence of chemical composition on the isothermal cocoa butter crystallization. *JOURNAL OF FOOD SCIENCE*, *69*(9), E478–E487. Retrieved from http://hdl.handle.net/1854/LU-307446

Fowler M. S. (2009). Cocoa Beans: From Tree to Factory. *Industrial Chocolate Manufacture and Use*. https://doi.org/10.1002/9781444301588.ch2

Frauendorfer, F., & Schieberle, P. (2008). Changes in Key Aroma Compounds of Criollo Cocoa Beans During Roasting. *Journal of Agricultural and Food Chemistry*, *56*(21), 10244–10251. https://doi.org/10.1021/jf802098f

Fritz, P. J., Kauffman, J. M., Robertson, C. A., & Wilson, M. R. (1986). Cocoa butter biosynthesis. Purification and characterization of a soluble sn-glycerol-3-phosphate acyltransferase from cocoa seeds. *The Journal of Biological Chemistry*, *261*(1), 194–199.

Garti, N., & Widlak, N. R. (2015). *Cocoa Butter and Related Compounds*. Elsevier.

Giacometti, J., Jolić, S. M., & Josić, D. (2015). Chapter 73 - Cocoa Processing and Impact on Composition. In V. Preedy (Ed.), *Processing and Impact on Active Components in Food* (pp. 605–612). San Diego: Academic Press. https://doi.org/10.1016/B978-0-12-404699-3.00073-1

Griffiths, G., & Harwood, J. L. (1991). The regulation of triacylglycerol biosynthesis in cocoa *Theobroma cacao* L. *Planta*, *184*(2), 279–284. https://doi.org/10.1007/BF01102429

Guehi, T. S., Dingkuhn, M., Cros, E., Fourny, G., Ratomahenina, R., Moulin, G., & Clement, A. (2008). Impact of cocoa processing technologies in free fatty acids formation in stored raw cocoa beans. *African Journal of Agricultural Research*, *3*(3), 174–179. Retrieved from http://publications.cirad.fr/une_notice.php?dk=543904

Hancock, B. L., & Fowler, M. S. (1994). Cocoa bean production and transport. In *Industrial Chocolate Manufacture and Use* (pp. 8–24). Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-2111-2_2

Hanneman, E. (2000). The Complex World of Cocoa. *MANUFACTURING CONFECTIONER*, *80*, 107–112.

Hashim, L., Hudiyono, S., & Chaveron, H. (1997). Volatile compounds of oxidized cocoa butter. *Food Research International*, *30*(3), 163–169. https://doi.org/10.1016/S0963-9969(97)00039-2

Himawan, C., Starov, V. M., & Stapley, A. G. F. (2006). Thermodynamic and kinetic aspects of fat crystallization. *Advances in Colloid and Interface Science*, *122*(1), 3–33. https://doi.org/10.1016/j.cis.2006.06.016

Homberg, E., & Bielefeld, B. (1982). Sterine und Methylsterine in Kakaobutter und Kakaobutter-Ersatzfetten. *Dtsch. Lebensm. Rundsch*, *78*, 73.

Itoh, T., Tamura, T., & Matsumoto, T. (1973). Sterol composition of 19 vegetable oils. *Journal of the American Oil Chemists Society*, *50*(4), 122–125. https://doi.org/10.1007/BF02633564

Jahurul, M. H. A., Zaidul, I. S. M., Norulaini, N. A. N., Sahena, F., Jinap, S., Azmir, J., Omar, A. K. M. (2013). Cocoa butter fats and possibilities of substitution in food products concerning cocoa varieties, alternative sources, extraction methods, composition, and characteristics. *Journal of Food Engineering*, *117*(4), 467–476. https://doi.org/10.1016/j.jfoodeng.2012.09.024

Jia, L., Liu, X., Bai, Y. Y., Li, S. H., Sun, K., He, C., & Hui, R. (2010). Short-term effect of cocoa product consumption on lipid profile: a meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition*, *92*(1), 218–225. https://doi.org/10.3945/ajcn.2009.28202

Kadow, D., Bohlmann, J., Phillips, W., & Lieberei, R. (2013). Identification of main fine or flavour components in two genotypes of the cocoa tree (Theobroma cacao L.). *Journal of Applied Botany and Food Quality*, *86*(1), 90–98. https://doi.org/10.5073/JABFQ.2013.086.013

Kamphuis, H. J. (2009). Production and Quality Standards of Cocoa Mass, Cocoa Butter and Cocoa Powder. In *Industrial Chocolate Manufacture and Use* (pp. 121–141). Wiley-Blackwell. https://doi.org/10.1002/9781444301588.ch6

Klagge, P., & K. Sen Gupta, A. (1990). *Kakaobutter und ihre Alternativen fir Schokoladenprodukte* (Vol. 92). https://doi.org/10.1002/lipi.19900921208

Kleinert-Zollinger, J. (Lindt und S. (1994). (Cocoa butter provenance and attributes of process technics - Decisive for product quality). *Zucker- und Suesswarenwirtschaft (Germany)*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=DE94Y0220

Kongor, J. E., Hinneh, M., de Walle, D. V., Afoakwa, E. O., Boeckx, P., & Dewettinck, K. (2016). Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour profile — A review. *Food Research International*, *82*, 44–52. https://doi.org/10.1016/j.foodres.2016.01.012

Kris-Etherton, P. M., Derr, J., Mitchell, D. C., Mustad, V. A., Russell, M. E., McDonnell, E. T., … Pearson, T. A. (1993). The role of fatty acid saturation on plasma lipids, lipoproteins, and apolipoproteins: I. Effects of whole food diets high in cocoa butter, olive oil, soybean oil, dairy butter, and milk chocolate on the plasma lipids of young men. *Metabolism: Clinical and Experimental*, *42*(1), 121–129.

Kumari, N., Kofi, K. J., Grimbs, S., D'Souza, R. N., Kuhnert, N., Vrancken, G., & Ullrich, M. S. (2016). Biochemical fate of vicilin storage protein during fermentation and drying of cocoa beans. *Food Research International (Ottawa, Ont.)*, *90*, 53–65. https://doi.org/10.1016/j.foodres.2016.10.033

Kyi Tin Mar, Daud Wan Ramli Wan, Mohammad Abu Bakar, Wahid Samsudin Mohd., Kadhum Abdul Amir Hassan, & Talib Meor Zainal Meor. (2005). The kinetics of polyphenol degradation during the drying of Malaysian cocoa beans. *International Journal of Food Science & Technology*, *40*(3), 323–331. https://doi.org/10.1111/j.1365-2621.2005.00959.x

Langevelde, A. J. van. (2000). *Triacylglycerol Structures and Cocoa-butter Crystallization*. Universiteit van Amsterdam [Host].

Lehrian, D. W., & Keeney, P. G. (1980). Changes in lipid components of seeds during growth and ripening of cacao fruit. *Journal of the American Oil Chemists' Society*, *57*(2), 61–65. https://doi.org/10.1007/BF02674361

Lehrian, D. W., Keeney, P. G., & Butler, D. R. (1980). Triglyceride characteristics of cocoa butter from cacao fruit matured in a microclimate of elevated temperature1. *Journal of the American Oil Chemists' Society*, *57*(2), 66–69. https://doi.org/10.1007/BF02674362

Liendo, R., Padilla, F. C., & Quintana, A. (1997). Characterization of cocoa butter extracted from Criollo cultivars of Theobroma cacao L. *Food Research International*, *30*(9), 727–731. https://doi.org/10.1016/S0963-9969(98)00025-8

Lima, L. J. R., Almeida, M. H., Nout, M. J. R., & Zwietering, M. H. (2011). Theobroma cacao L., "The Food of the Gods": Quality Determinants of Commercial Cocoa Beans, with Particular Reference to the Impact of Fermentation. *Critical Reviews in Food Science and Nutrition*, *51*(8), 731–761. https://doi.org/10.1080/10408391003799913

Lipp, M., & Anklam, E. (1998). Review of cocoa butter and alternative fats for use in chocolate—Part A. Compositional data. *Food Chemistry*, *62*(1), 73–97. https://doi.org/10.1016/S0308-8146(97)00160-X

Lipp, M., Simoneau, C., Ulberth, F., Anklam, E., Crews, C., Brereton, P., … Wiedmaier, C. (2001). Composition of Genuine Cocoa Butter and Cocoa Butter Equivalents. *Journal of Food Composition and Analysis*, *14*(4), 399–408. https://doi.org/10.1006/jfca.2000.0984

Lísa, M., & Holcapek, M. (2008). Triacylglycerols profiling in plant oils important in food industry, dietetics and cosmetics using high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography. A*, *1198–1199*, 115–130. https://doi.org/10.1016/j.chroma.2008.05.037

Lovegren, N. V., Gray, M. S., & Feuge, R. O. (1976). Effect of liquid fat on melting point and polymorphic behavior of cocoa butter and a cocoa butter fraction. *Journal of the American Oil Chemists Society*, *53*(3), 108–112. https://doi.org/10.1007/BF02635960

Marangoni, A. G., & McGauley, S. E. (2003). Relationship between Crystallization Behavior and Structure in Cocoa Butter. *Crystal Growth & Design*, *3*(1), 95–108. https://doi.org/10.1021/cg025580l

Marty, S., & Marangoni, A. G. (2009). Effects of Cocoa Butter Origin, Tempering Procedure, and Structure on Oil Migration Kinetics. *Crystal Growth & Design*, *9*(10), 4415–4423. https://doi.org/10.1021/cg9004505

Maruzeni, S. (2009). A mathematical technique for estimating the melting points of triacylglycerols from the component fatty acid melting points. *European Journal of Lipid Science and Technology*, *111*(12), 1240–1248.

Meursing, E. H. (1994). Cocoa mass, cocoa butter, cocoa powder. In *Industrial Chocolate Manufacture and Use* (pp. 70–82). Springer, Boston, MA. https://doi.org/10.1007/978-1-4615-2111-2_6

Mohamed, R. S., Saldaña, M. D. A., Mazzafera, P., Zetzl, C., & Brunner, G. (2002). Extraction of Caffeine, Theobromine, and Cocoa Butter from Brazilian Cocoa Beans Using Supercritical CO2 and Ethane. *Industrial & Engineering Chemistry Research*, *41*(26), 6751–6758. https://doi.org/10.1021/ie0203936

Narine, S. S., & Marangoni, A. G. (1999). Relating structure of fat crystal networks to mechanical properties: a review. *Food Research International*, *32*(4), 227–248. https://doi.org/10.1016/S0963-9969(99)00078-2

Okiyama, D. C. G., Navarro, S. L. B., & Rodrigues, C. E. C. (2017). Cocoa shell and its compounds: Applications in the food industry. *Trends in Food Science & Technology*, *63*, 103–112. https://doi.org/10.1016/j.tifs.2017.03.007

Parsons, J. G., Keeney, P. G., & Patton, S. (1969). Identification and Quantitative Analysis of Phospholipids in Cocoa Beans. *Journal of Food Science*, *34*(6), 497–499. https://doi.org/10.1111/j.1365-2621.1969.tb12069.x

Pires, J. L., Cascardo, J. C. M., Lambert, S. V., & Figueira, A. (1998). Increasing cocoa butter yield through genetic improvement of Theobroma cacao L.: Seed fat content variability, inheritance, and association with seed yield. *Euphytica*, *103*(1), 115–121. https://doi.org/10.1023/A:1018327411530

Podlaha, O., Toregard, B., & Puschl, B. (1984). TG-type composition of 28 cocoa butters and correlation between some TG-type components. *Lebensmittel - Wissenschaft + Technologie = Food Science + Technology*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201302561682

Pontillon, J. (1998). Cocoa and chocolate: production, utilization, characteristics. *Cocoa and chocolate: production, utilization, characteristics.* Retrieved from https://www.cabdirect.org/cabdirect/abstract/20026788577

Ribeiro, A. P. B., Silva, R. C. da, Gioielli, L. A., Gonçalves, M. I. de A., Grimaldi, R., Gonçalves, L. a. G., & Kieckbusch, T. G. (2012). Physico-chemical properties of Brazilian cocoa butter and industrial blends. Part I Chemical composition, solid fat content and consistency. *Grasas y Aceites*, *63*(1), 79–88. https://doi.org/10.3989/gya.069011

Ribeiro, Ana Paula Badan, Basso, R. C., dos Santos, A. O., Andrade, G. C., Cardoso, L. P., & Kieckbusch, T. G. (2013). Hardfats as crystallization modifiers of cocoa butter. *European Journal of Lipid Science and Technology*, *115*(12), 1462–1473. https://doi.org/10.1002/ejlt.201300052

Rohan, T. A. (1963). *Processing of raw cocoa for the market*. Rome : FAO. Retrieved from https://trove.nla.gov.au/version/22982824

Rossi, M., Arnoldi, C., Salvioni, G., & Schiraldi, A. (1989). Characterization of cocoa extracts obtained with supercritical carbon dioxide. *ITALIAN JOURNAL OF FOOD SCIENCE*, *1*(3), 41–50. Retrieved from https://air.unimi.it/handle/2434/176331#.WsvEki5uapo

Rousset, P. (2002). Physical properties of lipids. *Physical Properties of Lipids. Marcell Dekker Inc., New York*, 1–36.

Ruiz, J., Antequera, T., Andres, A. I., Petron, M. J., & Muriel, E. (2004). Improvement of a solid phase extraction method for analysis of lipid fractions in muscle foods. *Analytica Chimica Acta*, *520*(1–2), 201–205. https://doi.org/10.1016/j.aca.2004.04.059

Saldaña, M. D. A., Mohamed, R. S., & Mazzafera, P. (2002). Extraction of cocoa butter from Brazilian cocoa beans using supercritical CO2 and ethane. *Fluid Phase Equilibria*, *194–197*, 885–894. https://doi.org/10.1016/S0378-3812(01)00719-1

Sato, K. (1999). Solidification and phase transformation behaviour of food fats—a review. *Lipid/Fett*, *101*(12), 467–474.

Sato, K. (2001). Crystallization behaviour of fats and lipids — a review. *Chemical Engineering Science*, *56*(7), 2255–2265. https://doi.org/10.1016/S0009-2509(00)00458-9

Savage, C. M., & Dimick, P. S. (1995). Influence of phospholipids during crystallization of hard and soft cocoa butters. *The Manufacturing Confectioner*, *75*(5), 127–132.

Schlichter, J., Sarig, S., & Garti, N. (1985). Polymorphic transformations of cocoa butter in the presence of emulsifier, studied by the DSC. *Thermochimica Acta*, *85*, 517–520. https://doi.org/10.1016/0040-6031(85)85634-3

Schlichter-Aronhime, J., & Garti, N. (1988). Solidification and polymorphism in cocoa butter and the blooming problems. *Crystallization and Polymorphism of Fats and Fatty Acids*, *31*, 363–393.

Schwan, R. F., & Wheals, A. E. (2004). The Microbiology of Cocoa Fermentation and its Role in Chocolate Quality. *Critical Reviews in Food Science and Nutrition*, *44*(4), 205–221. https://doi.org/10.1080/10408690490464104

Shahkhalili, Y., Duruz, E., & Acheson, K. (2000). Digestibility of cocoa butter from chocolate in humans: a comparison with corn-oil. *European Journal of Clinical Nutrition*, *54*(2), 120–125. https://doi.org/10.1038/sj.ejcn.1600905

Shukla, V. K. S. (International F. S. C. A. (1995). Cocoa butter properties and quality. *Lipid Technology (United Kingdom)*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=GB9609755

Sirbu, D., Corno, M., Ullrich, M. S., & Kuhnert, N. (2018). Characterization of triacylglycerols in unfermented cocoa beans by HPLC-ESI mass spectrometry. *Food Chemistry*, *254*, 232–240. https://doi.org/10.1016/j.foodchem.2018.01.194

Sonwai, S., Podchong, P., & Rousseau, D. (2017). Crystallization kinetics of cocoa butter in the presence of sorbitan esters. *Food Chemistry*, *214*, 497–506. https://doi.org/10.1016/j.foodchem.2016.07.092

Spangenberg, J. E., & Dionisi, F. (2001). Characterization of Cocoa Butter and Cocoa Butter Equivalents by Bulk and Molecular Carbon Isotope Analyses: Implications for Vegetable Fat Quantification in Chocolate. *Journal of Agricultural and Food Chemistry*, *49*(9), 4271–4277. https://doi.org/10.1021/jf001509g

Staphylakis, K., & Gegiou, D. (1985). Free, esterified and glucosidic sterols in cocoa butter. *Lipids*, *20*(11), 723–728. https://doi.org/10.1007/BF02534394

Stewart, D. I. (2017). *The effect of low melting oils on the crystallisation of confectionery fats* (Thesis). © David Stewart. Retrieved from https://dspace.lboro.ac.uk/dspace-jspui/handle/2134/24420

Svanberg, L., Ahrné, L., Lorén, N., & Windhab, E. (2011). Effect of sugar, cocoa particles and lecithin on cocoa butter crystallisation in seeded and non-seeded chocolate model systems. *Journal of Food Engineering*, *104*(1), 70–80. https://doi.org/10.1016/j.jfoodeng.2010.09.023

The International Cocoa Organization (ICCO) | Cocoa Producing and Cocoa Consuming Countries. (n.d.). Retrieved March 13, 2018, from https://www.icco.org/

Thompson, S. S., Miller, K. B., & Lopez, A. S. (2007). Cocoa and Coffee. *Food Microbiology: Fundamentals and Frontiers, Third Edition*, 837–850. https://doi.org/10.1128/9781555815912.ch39

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

Toxopeus, H., & Wessel, M. (1970). Studies on pod and bean values of Theobroma cacao L. in Nigeria. I. Environmental effects on West African Amelonado with particular attention to annual rainfall distribution. *Neth J Agr Sci*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201301201159

Tsydendambaev, V. D., & Vereshchagin, A. G. (2003). Changes in Triacylglycerol Composition during Ripening of Sea Buckthorn (Hippophaë rhamnoides L.) Seeds. *Journal of Agricultural and Food Chemistry*, *51*(5), 1278–1283. https://doi.org/10.1021/jf020593s

Ulberth, F., & Buchgraber, M. (2003). Analytical platforms to assess the authenticity of cocoa butter. *European Journal of Lipid Science and Technology*, *105*(1), 32–42. https://doi.org/10.1002/ejlt.200390003

Vaeck, S. (1960). Cocoa butter and fat bloom. *Manuf Confect*, 35–75.

Van Malssen, K. van, Langevelde, A. van, Peschar, R., & Schenk, H. (1999). Phase behavior and extended phase scheme of static cocoa butter investigated with real-time X-ray powder diffraction. *Journal of the American Oil Chemists' Society*, *76*(6), 669–676. https://doi.org/10.1007/s11746-999-0158-4

Vieira, L. R., Efraim, P., Walle, D. V. de, Clercq, N. D., & Dewettinck, K. (2015). Influence of Brazilian Geographic Region and Organic Agriculture on the Composition and Crystallization Properties of Cocoa Butter. *Journal of the American Oil Chemists' Society*, *92*(11–12), 1579–1592. https://doi.org/10.1007/s11746-015-2728-y

Wille, R., & Lutton, E. (1966). Polymorphism of cocoa butter. *Journal of the American Oil Chemists Society*, *43*(8), 491–496.

Wood, G. a. R. (2008). From Harvest to Store. In *Cocoa* (pp. 444–504). Wiley-Blackwell. https://doi.org/10.1002/9780470698983.ch13

Wood, G. A. R., & Lass, R. A. (2001). *Cocoa* (4th ed). Oxford : Blackwell Science. Retrieved from https://trove.nla.gov.au/version/46558938

Yano, J., Ueno, S., Sato, K., Arishima, T., Sagi, N., Kaneko, F., & Kobayashi, M. (1993). FT-IR study of polymorphic transformations in SOS, POP, and POS. *The Journal of Physical Chemistry*, *97*(49), 12967–12973.

Ziegleder G. (2006). DSC-Thermoanalyse und Kinetik der Kristallisation von Kakaobutter. *Lipid / Fett*, *92*(12), 481–485. https://doi.org/10.1002/lipi.19900921207

Ziegleder, G., & Schwingshandl, I. (1998). Kinetics of fat migration within chocolate products. Part III: fat bloom. *Lipid/Fett*, *100*(9), 411–415.

Żyżelewicz, D., Krysiak, W., Budryn, G., Oracz, J., & Nebesny, E. (2014). Tocopherols in cocoa butter obtained from cocoa bean roasted in different forms and under various process parameters. *Food Research International*, *63*(Part C), 390–399. https://doi.org/10.1016/j.foodres.2014.03.027

Chapter 3:

3 Exploring cocoa lipid classes

Abstract

Cocoa beans store their energy in the form of lipids, which constitute up to 55% of the seed. It is known that neutral lipids account for more than 95% of the cocoa lipid extract and it mainly consists of triacylglycerols (TAGs). In order evaluate the remaining part, which includes more polar lipids (as glycolipids and phospholipids) and other components like sterols, tri-terpenes, and tocopherols, it is essential to develop efficient extraction and separation methods. Method development includes all those steps involved in the selection of an adequate solvent system for chromatographic separation using strength and selectivity as key features. Herein, a simple procedure for the extraction of lipids from seed material has been developed. Various extraction conditions, including the solvent, time of extraction, different type of seeds, have been investigated to obtain the maximum yield of the extract.

Moreover, this chapter examines the overall chemical composition of the cocoa lipids by fractionating the lipid extract. Five fractions of cocoa lipids were obtained by employing solid phase extraction (SPE) cartridges, and all the fractions were analysed by thin layer chromatography (TLC) and by mass spectrometry with electrospray ionization (ESI) on direct injection analysis and LC-MS. Main TAGs and diacylglycerol (DAG) profiles have not showed substantial differences during the production process. However, significant differences were observed in the polar fractions of cocoa lipids, where a variety of more polar lipid compounds were transformed or degraded during the fermentation process.

Keywords: extraction; dichlomethane (DCM); Soxhlet; cocoa; seeds; solid phase extraction (SPE); thin layer chromatography (TLC); fraction.

Supplementary material is available on request. E-mail: n.kuhnert@jacobs-university.de

3.1 Introduction

Comprehensive lipidomics requires a complete extraction of the lipid from any type of sample. There are many factors responsible for the solubility of lipids in a particular solvent, and it occurs very frequently that a particular lipid from a specified source is soluble in one solvent but poorly soluble in another. Suitable extraction solvents have to have the capacity to overcome the strong forces of association between the lipids and cellular constituents, such as proteins and polysaccharides. To release all lipids from their association with cell constituents, the solvent (or solvent mixture) must have a suitable polarity. However, the given polarity must not permit any chemical reaction with the lipids nor that triacylglycerols and other non-polar simple lipids do not dissolve and are left adhering to the tissues. Besides, the optimal extracting solvent may have a function in preventing any enzymatic hydrolysis or other side reactions. Nevertheless, it is difficult to predict the behavior of a certain solvent as a lipid extractant (Christie & Han, 2010).

The two main structural features of lipids controlling their solubility in organic solvents are the hydrophobic hydrocarbon chains of the fatty acid or other aliphatic moieties and any polar functional groups, such as phosphate or sugar residues, which are markedly hydrophilic. Simple lipids including fatty acids, triacylglycerols, sterols, sterol and wax ester, are often a large part of storage tissues, from which they are relatively easy to extract. Complex lipids in contrast, including glycerophospholipids, glyceroglycolipids, and sphingolipids, are usually constituents of membranes, being in a close association with compounds like proteins and polysaccharides, with which they interact; thus, the extraction of these lipids is more laborious. Lipids with functional groups of low polarity only, such as triacylglycerols or cholesterol esters, are very soluble in hydrocarbon solvents like hexane, cyclohexane or toluene, and in solvents of somewhat higher polarity, such as chloroform or ethers. They tend to be rather insoluble in polar solvents such as alcohols, and methanol especially; solubility in polar solvents arises as the chain-length of the fatty acid moieties in these lipids decrease or as the chain-length of the solvent alcohol increases. The polar complex lipids tend to be only sparingly soluble in hydrocarbon solvents, though the presence of other lipids can aid dissolution, they do dissolve readily in more polar solvents such as chloroform, methanol, and ethanol (Christie & Han, 2010; Romanik, Gilgenast, Przyjazny, & Kamiński, 2007; Zahler & Niggli, 1977).

There are a large number of methods for lipid extraction, which vary in their lipid extraction efficiency (Christie, 1997; Romanik et al., 2007; Self, 2007). A peculiar method presents its advantages and disadvantages as, for example, the technique developed by Bligh and Dyer it is known to be suitable for substrates having less than 10% of lipid content; otherwise the error will be more prominent (Bligh & Dyer, 1959). As for Soxhlet extraction, it has the advantages of always applying fresh solvent into contact with the solid matrix and relatively simple and inexpensive equipment that is easy to operate but requires long extraction time (Luque de Castro & García-Ayuso, 1998). Some of the most common methods for lipid extraction (Asep et al., 2008; Folch, Lees, & Sloane Stanley, 1957; Hara & Radin, 1978; Matyash, Liebisch, Kurzchalia, Shevchenko, & Schwudke, 2008) are summarized in Table 3.1.

Table 3.1 Common methods for lipid extraction in literature and their characteristics.

Methods	***Extraction Solvent***	***Types of Lipid (Extracted)***	***Observations***
Soxhlet-related methods	Hexane or petroleum ether	Non-polar	**crude extract, extremely time consuming**
SFE (supercritical fluid extraction)	CO_2	Non-polar	**polar lipids are not extracted**
Folch	Chloroform, methanol, H_2O	Non-polar and polar	**good extraction method**
Bligh and Dyer	Chloroform, methanol, H_2O	Non-polar and polar	**samples with a high % of endogeneus H_2O**
Hara and Radin	Hexane, Isopropanol,	Non-polar and polar	**glycolipids are not extracted**
	Butanol	Non-polar and polar	**elimination (SPE)**
Matyash	MTBE	Non-polar and polar	**similar recoveries as those of chloroform but longer incubation**

Regarding the separation of the lipid compounds, solid phase extraction (SPE) is known to be an efficient approach for sample preparation in the chromatographic analysis of edible fats. This technique requires a small amount of the sample, low volumes of organic solvents, and it is accomplished in a relatively shorter time if compared to other procedures (Panagiotopoulou & Tsimidou, 2002; Ruiz-Gutiérrez & Pérez-Camino, 2000). Aminopropyl modified silica phase it is a moderately polar phase used in alternative to silica offering better recoveries of the neutral and acidic phospholipids than silica (Alvarez & Touchstone, 1992; Pernet, Pelletier, & Milley, 2006). Moreover, aminopropyl columns are commercially available in the pre-

packed form and different sizes, constituting a convenient and reproducible tool for lipid separation.

Considering the absence of a standard extraction method and that about 98% of cocoa lipid extract are neutral lipids, mainly constituted by triacylglycerols, the main propose of this work was to achieve the complete extraction of the lipid constituents in cocoa and to analyse the potential impact of different lipid extraction methods on cocoa lipidomics. Moreover, separation of the other lipid classes from the TAG lipid class on cocoa beans will allow the analysis of minor lipid components. For this purpose, selected well-established analytical methods for lipid extraction and separation have been applied in comparison for a comprehensive, focused lipidomics in seed plant material. Therefore, this chapter emphasizes the importance of separation of the lipids in different classes for the effective identification and further quantification of lipid compounds.

3.2 Material and Methods

3.2.1 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany), isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade), chloroform (Rotisolv® HPLC grade) and Tetra-dodecylammonium bromide, petroleum ether, boiling range 35–60 °C, heptane, methanol, diethyl ether, ethyl acetate, was purchased from Carl Roth (Karlsruhe, Germany), dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany), ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry), ferric chloride, perchloric acid, ninhydrin reagent, dinitrophenylhydrazine, Dragendorff reagent (ready-to-use), 4-methoxybenzaldehyde, and acetic acid were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethanol was subjected to distillation prior use.

3.2.2 Preparation of the seed sample

Cocoa bean samples, received in several sets from Barry Callebaut and four types of nuts (almonds, hazelnuts, peanuts, walnuts), coffee beans, one type of oily seed (sunflower seeds), and a sample of tea (white tea leaves), purchased from the local supermarket (all, except the tea, are produced by the same company) were deshelled and ground using a grinder Retsch

Grindomix GM200 knife mill (Haan, Germany) to make a fine an homogenous powder. Henceforth, several extraction methods (Folch et al., 1957; Hara & Radin, 1978; Hirsch & Ahrens, 1958; Li et al., 2014) have been used to perform the final extraction. Finally, an overnight Soxhlet (Buchi Extraction System B811 instrument, Flawil, Switzerland) using dichloromethane as extraction solvent, 150 mL for 5 g of powdered seed material, was used. All the samples were subjected to each extraction method at least 3 times. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis. Moisture content was determined by heating around 2.5 g of cocoa powder in the oven at 105 °C for 24, cooled in the dry atmosphere of a desiccator, and then reweighed. Total lipid content was normalized according to: $= \frac{\text{Lipid extract (g)} \times 100}{\text{Cocoa Powder (g)} - \text{Weight Loss (g)}}$.

3.2.3 Thin layer chromatography (TLC)

The lipid extract (100 mg) was dissolved in 1 mL dichloromethane/methanol (2/1, v/v) and 8 µL were spotted on the TLC Silica gel 60 F_{254} HPTLC Aluminium plate 20 cm x 20 cm (Macherey-Nagel, München, Germany). The dried lipid spots were developed with hexane/diethyl ether/glacial acetic acid (80:20:1, by vol.). Plates were allowed to dry and run another time, for half of the plate, with chloroform/methanol/glacial acetic acid (97:3:1, by vol.). After first drying time spots having chromophores were visualized under UV light at 254 nm. Then, after a second complete drying time, the plate was sprayed with a general purpose stain, Cerium Molybdate stain (Hanessian's stain), to visualize all the separated spots of the lipid extract. Others stain solutions were used to support compounds identification: iodine vapor (high affinity for both unsaturated and aromatic compounds), ferric chloride (ideal for phenols), ninhydrin (excellent for amino acids), dinitrophenylhydrazine (suitable for aldehydes and ketones), p-anisaldehyde (multipurpose stain, especially for functional groups which are strongly and weakly nucleophilic), and Dragendorff's reagent (optimal for choline and to detect alkaloids).

3.2.4 Solid phase extraction (SPE)

Chromabond aminopropyl modified silica gel NH_2 (Chromabond® LV-NH_2, Macherey-Nagel GmbH & Co. KG, Düren, Germany) column cartridges (3 mL/500 mg) were used for

the SPE fractionation. The procedure was developed by reviewing the available SPE separation methods, starting with the original method Kaluzny et al. (Kaluzny, Duncan, Merritt, & Epps, 1985) and observing the modifications applied by other authors (Alvarez & Touchstone, 1992; Laffargue, de Kochko, & Dussert, 2007; Pernet et al., 2006). After few trials and modifications, the following method has been achieved. The cartridges were first activated with 20 mL heptane/ethylacetate (80/20, v/v), then, 20 ml heptane. Then, 10 mg of the lipid extract dissolved in 1 mL of heptane were applied, after which the fractions were eluted with 5 different solvent mixtures: 1) 20 mL heptane, first fraction collected ; 2) 20 mL heptane/ethyl acetate (97/3, v/v), for the second fraction; 3) 20 mL heptane/ethyl acetate (85/15, v/v), third fraction collected; 4) 20 mL heptane/ethyl acetate (85/15, v/v) plus 1% acetic acid, for the forth fraction; and 5) 20 mL methanol. Extracted lipids were evaporated to dryness in a rotavapor and stored at -20 °C until further analysis.

3.2.5 Silica gel column chromatography

A glass column packed with silica gel 60 (230 mesh) was prepared in petroleum ether and used. 1 g of raw cocoa lipid extract was passed through the column (15 cm × 3 cm i.d.), using a solvent gradient from 100% heptane (0% ethylacetate) until 50% ethylacetate in heptane. 15 fractions were collected, evaporated to dryness in a rotavapor, and stored at -20 °C until further analysis.

3.2.6 HPLC chromatographic conditions

TAGs molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). The column used in this study was a Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 5 mmol/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. The gradient elution program consisted of holding solvent steady A/B (40/60) for 3 min; followed by a linear gradient to solvent B (100) for 33 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 40/60 solvent A/B for 5 min before reuse.

3.2.7 Mass spectrometry detection

The samples (10 μg/mL lipid) were mixed with the corresponding HCCA (α-Cyano-4-

hydroxycinnamic acid) matrix solution (1:1, v/v) and 1 µL of this mixture was transferred onto the MALDI target. The samples were allowed to crystallize at room temperature under ambient conditions. All mass spectra were acquired on a Bruker Autoflex MS device (Bruker Daltonics, Bremen, Germany). One thousand single laser shots were averaged for each mass spectrum.

High-resolution masses were acquired using a time of flight MicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of m/z 200–1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode.

LC-tandem MS was carried out using an Ion-Trap detector in positive ion mode equipped with an ESI source (Bruker Daltonics UHT Ultra, Bremen, Germany). The full scan mass spectra were recorded in the range m/z 200-1200 operating in positive ion mode. Capillary temperature was set to 350 °C, drying gas flow rate of 10 L/min and nebulizer pressure of 10 psi. Tandem mass spectra were acquired in Auto MSn (smart fragmentation) using a ramping of the collision energy.

3.3 Results and discussion

3.3.1 Extraction optimization

To analyze the entire content of cocoa, first, it is necessary to optimize the extraction method. Much of work has been conducted on observing the efficiency of different extraction procedures for extracting lipids from plant matrices. Among different kinds of extraction procedures, Soxhlet extraction proved to be the most exhaustive method for the samples. Moreover, it offers better reproducibility compared to the other methods. Table 3.2 displays the list of methods used for the lipid extraction in cocoa beans. The extracted total lipid percentage is highly depending on the extraction solvent used (Table 3.2). It is known that a more polar solvent will extract a greater amount of lipids due to its releasing properties of the lipids from their protein-lipid complexes (Christie & Han, 2010; Ryckebosch, Muylaert, & Foubert, 2012). Nevertheless, our results showed that other non-lipid compounds are extracted each time

mixtures containing methanol were applied (Fig. 3.1). These compounds change their color passing through red-pink color in acidic environment to a purple then yellow color in basic environment (Fig. 3.1 (b) and (c)). These compounds are anthocyanins (Pina, 2012) well known compounds present in cocoa (D'Souza et al., 2017; Patras, Milev, Vrancken, & Kuhnert, 2014). By treating the TLC plate with different stain solutions, we can obtain information about the nature of the compounds analyzed. Figure 3.1 (d), illustrates the TLC stained with ferric chloride, which indicates that the extracting mixture DCM/MeOH or $CHCl_3$/MeOH extracts also some phenolic compounds. From the amount of the lipid extracted (Table 3.1) and the lipid profile image (Fig. 3.1 (a)) offered by the TLC, it could be asserted that DCM was the most suitable solvent to extract the highest amount of lipids from qualitative and quantitative perspective. Cequier-Sanchez et al. have studied the efficiency of DCM with respect to the well-known Folch method (Cequier-Sánchez, Rodríguez, Ravelo, & Zárate, 2008). Their results indicate that, for lipid extraction and fatty acid assessment, dichloromethane/methanol can successfully replace the commonly employed chloroform/methanol, giving the same or even better yields. DCM is a less hazardous and less toxic solvent and some times more efficient than $CHCl_3$. Optimization of the extraction (Fig 3.2) has shown that already after 6 h extraction time, more than 55% of lipid is extracted. DCM has proven to be a good extraction solvent not only for cocoa but also for other seeds including a tea sample (Fig. 3.3).

Table 3.2 Amount of cocoa lipids extracted from 3 g of dried beans by different extraction methods and different solvents.

METHODS	***SOLVENTS (150 ML)***	***LIPID EXTRACT (GRAMS)***	***WEIGHT LOSS IN THE EXTRACTION THIMBLE (GRAMS)***
Soxhlet (continuous mode)	Heptane	1.64 (±0.13)	**1.51 (±0.03)**
	Petroleum ether	1.41 (±0.07)	**1.44 (±0.03)**
	Dichloromethane (DCM)	1.61 (±0.06)	**1.63 (±0.03)**
	Methyl tert-butyl ether (MTBE):Methanol (MEOH) (10:3)	1.65 (±0.1)	**1.46 (±0.03)**
	Chloroform ($CHCl_3$):MeOH (2:1)	1.65 (±0.01)	**1.87 (±0.04)**
	DCM:MeOH (2:1)	1.6 (±0.01)	**1.8 (±0.13)**
Ultrasonic Bath	Petroleum ether	1.23	**1.4**
	Heptane	1.16	**1.58**
	$CHCl_3$:MeOH (2:1)	1.31	**1.34**
Homogenization	DCM	1.58	***NOT PRESENT***
	DCM:MeOH (2:1)	1.69	***NOT PRESENT***
	$CHCl_3$:MeOH (2:1)	1.66	***NOT PRESENT***
	Petroleum ether	1.27	***NOT PRESENT***

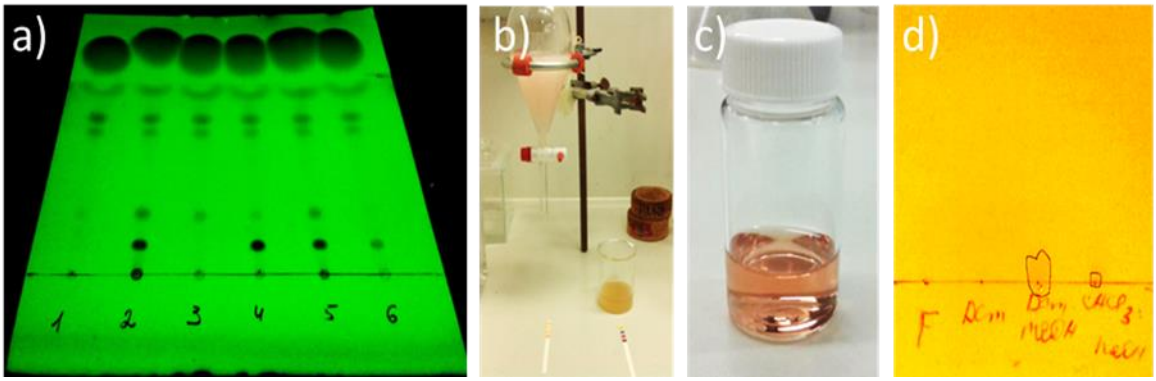


Figure 3.1 (a) TLC plate image showing the lipid profile of fermented dried beans extracted with Soxhlet apparatus: 1 – petroleum ether; 2 – CHCl3:MeOH (1:1); 3 – heptane; 4 –DCM; 5 – Folch extraction CHCl3:MeOH:0.05 N potassium chloride (8:4:3); and 6 – diethyl ether. Iodine staining and UV light at 254 nm visualized the spots. **(b)** and **(c)** show non-lipid compounds extracted with CHCl3:MeOH (2:1, v/v), and **(d)** TLC plate treated with ferric chloride for the phenols presence (F – Folch; DCM, DCM:MeOH, CHCl3:MeOH).

Only in coffee sample, DCM has shown to extract less lipid amount than heptane. It might be because coffee seeds contain less than 10% lipid amount compared to the other oils seed under study. Moreover, this indicates that the optimization of an extraction has to take into account the lipid amount in a certain sample. The reasons of choosing a tea leafs sample were two: tea is a beverages, next to coffee and cocoa (in some applications), and we wanted to test the extraction power of the three most used organic solvents (petroleum ether, heptane, and DCM) in extracting pigments. For the last point, DCM revealed good extraction efficiency.

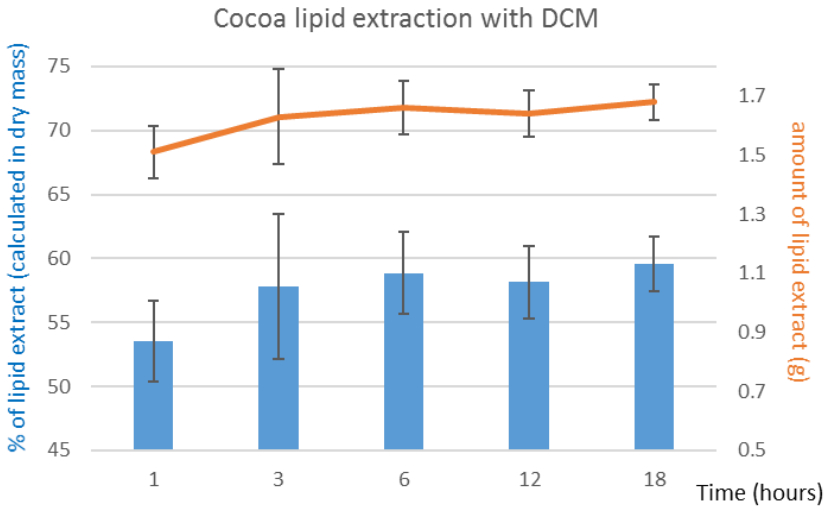


Figure 3.2 Optimization of lipid extraction in cocoa by Soxhlet method using DCM as extraction solvent. 3 g of fermented dried powdered cocoa beans and 150 mL solvent.

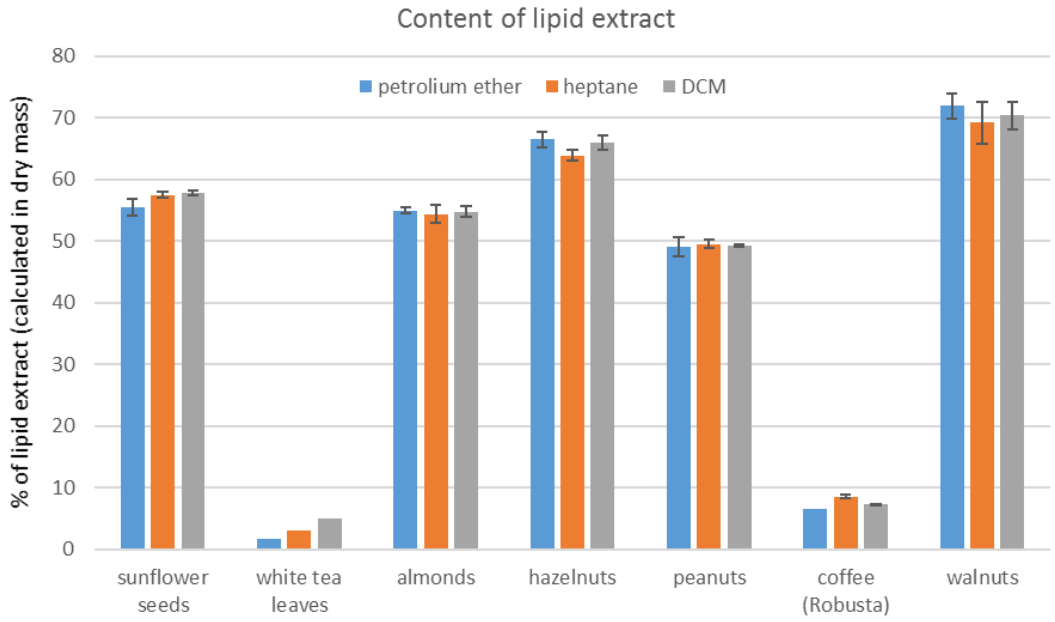


Figure 3.3 Percentage of lipid extracted form 6 seeds and 1 dried leaves samples by Soxhlet method using three different extraction solvents. 3 g of powdered plant material and 150 mL solvent.

3.3.2 TLC lipid profile

TLC is an established technique of separation of lipids. Although, TLC analyses on cocoa lipids have been used for the separation of cocoa phospholipids in the past (Levanon, Rossetini, Raskin, & Mesquita, 1967; Parsons, Keeney, & Patton, 1969), it has proved to be reliable tool for current lipidomics. In this study, TLC analyses were performed with the aim to assess the cocoa lipid content in terms of quality and quantity of extracted cocoa lipids. As observed in Figure 3.4 (a) and (b) TLC separation could provide an important piece of information. In this example, a continuous extraction employing first petroleum ether for 3 h, then heptane for another 3 h on the same sample. And ending with another 3 h extraction time with DCM on the same sample. As result, we could assert that DCM is a more exhaustive solvent compared to heptane which in turn is more efficient than petroleum ether. Cocoa bean processing implies many treatments, with the main being: fermentation, drying, and roasting. TLC image (Fig. 3.4) show that processing modifies the lipid profile of the beans as it occurs in proteins (Amin I, Jinap S, & Jamilah B, 1999; Kumari et al., 2016) and flavor related compounds (Hoskin & Dimick, 1994). Moreover, Figure 3.4 (a) illustrates the possible presence of aromatic compounds or heterocyclic compounds which have electron donating or electron withdrawing functional groups that quench fluorescence and can be seen under UV light. Asep et al. have

shown that the particle size and the extraction time have in impact on the yield but not on the lipid profile of the fat (Asep et al., 2008). This analysis approach gives relevant evidence on how the production process affects the lipid extract of cocoa. Figure 3.5 and Figure 3.6 illustrate the lipids profile of the coffee sample, tea leafs, sunflower seeds, hazelnuts and almonds in comparison to cocoa lipid profile when extracted with different organic solvents. Images show the efficiency of different solvent and how diverse are the lipid profile of the samples under study. Although solvent extraction has shown no significant differences in terms of lipid amount extracted (Fig. 3.3), DCM extracted the spot at the bases (Fig. 3.5 and Fig 3.6, spot at the origin), known as polar lipids, in higher quantity. As illustrated in the Figures, TLC image offers a comparison element for assessing lipid profile similarities in different seed samples. Thus, we can perceive how cocoa lipid profile differs, concerning lipids classes, from other seed plant material. Along with qualitative results, TLC can also provide a chromatographic measurement known as an R_f value, which is the retention or the ratio-to-front factor. This value is determined as the distance traveled by the compound divided by the distance traveled by the solvent, expressed as a decimal fraction. Compounds with the higher R_f are less polar as they interact less with the polar adsorbent on the TLC plate.

Accordingly, the polarity of the lipid compounds could be easily inferred. Thus, cocoa lipid extract showed four prominent spots on the TLC plate with R_f values of 0.95, 0.64, 0.18, and 0 in order of increasing polarity. The spot at R_f of 0.64 are compounds present in the highest amount in the lipid extract – triacylglycerols.

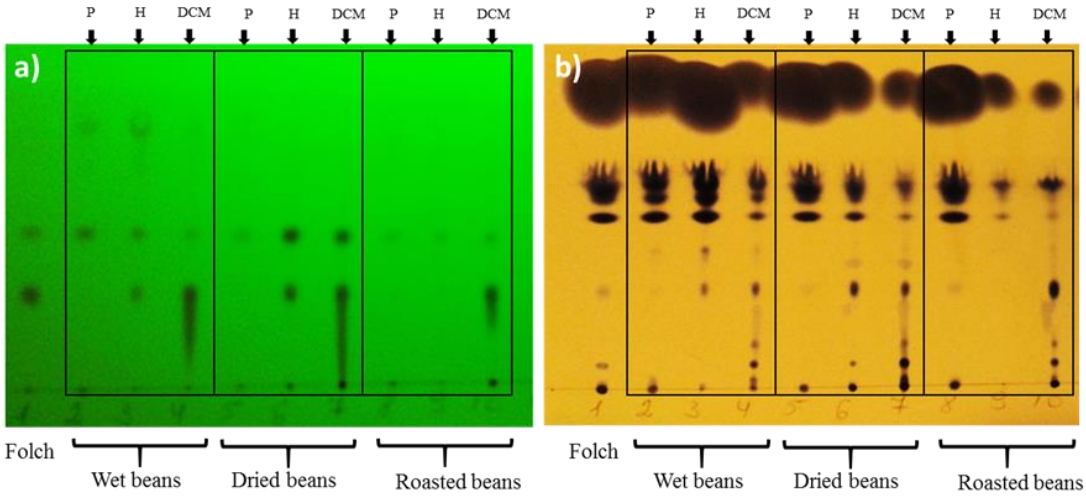


Figure 3.4 (a) Visualization under UV light at 254 nm and **(b)** Hanessian's stained TLC plate image showing the lipid profile of different treated beans extracted with Soxhlet apparatus using P – petrolium ether (2, 5, 8), H – heptane (3, 6, 9) and DCM – dichloromethane (4, 7, 10) by subsequent extraction compared with Folch extraction of the dried cocoa beans.

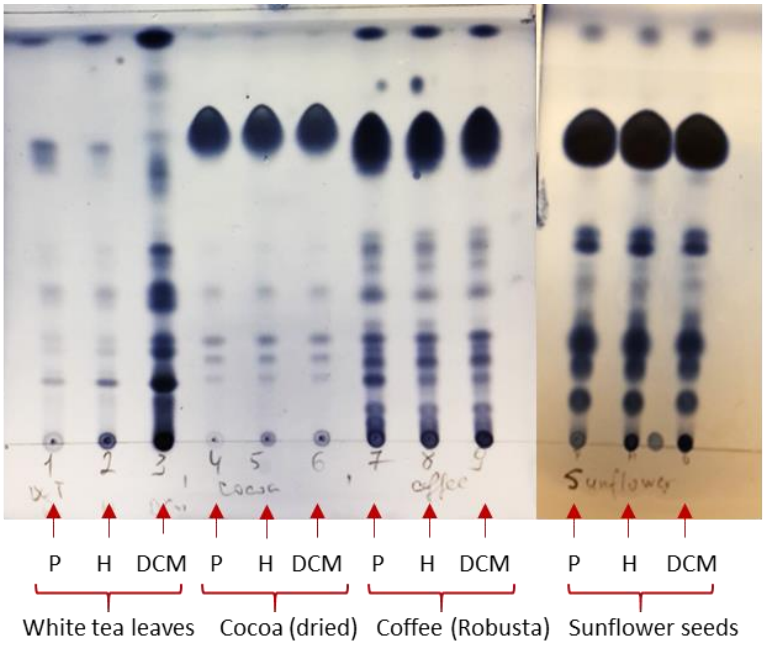


Figure 3.5 TLC plate* image showing the lipid profile of white tea leaves, cocoa beans, coffee beans and sunflower seeds extracted with Soxhlet apparatus using P – petrolium; H – heptane; and DCM – dichloromethane (3 g sample and 150 mL solvent). *Hanessian's stain was used.

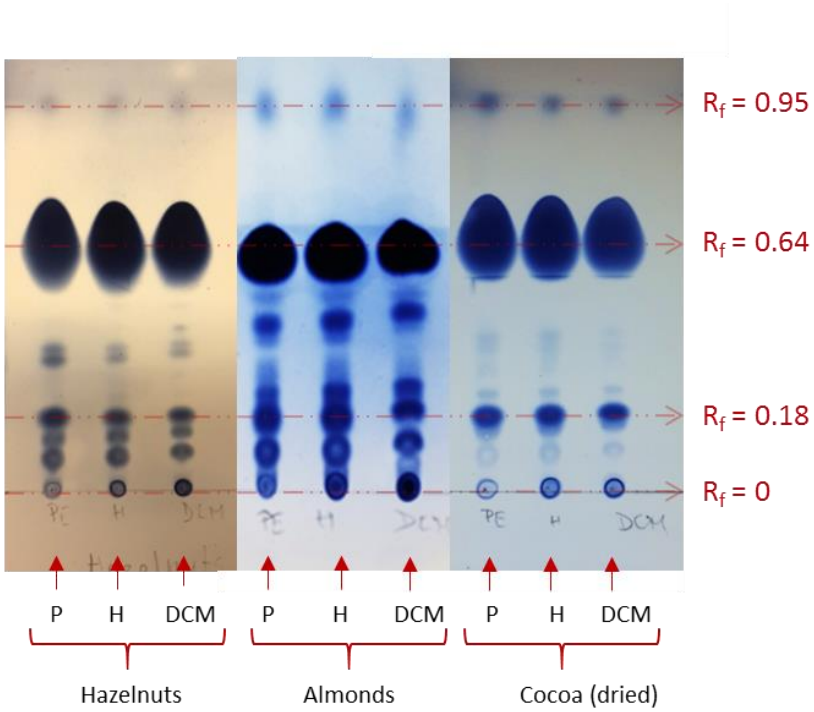


Figure 3.6 TLC plate* image showing the lipid profile of white hazelnuts and almonds in comparison to cocoa beans extracted with Soxhlet apparatus using P – petrolium; H – heptane; and DCM – dichloromethane (3 g sample and 150 mL solvent). *Hanessian's stain was used.

3.3.3 Separation of lipid classes

Due to the high amount of triacylglycerols in cocoa lipid extract, the SPE methods described in the literature for the separation of lipids (Alvarez & Touchstone, 1992; Kaluzny et al., 1985; Laffargue et al., 2007; Pernet et al., 2006) seemed not very efficient. Nonetheless, these results served as a starting point for the optimization of a SPE method for cocoa lipids. Thus, the optimized method, using heptane/ethyl acetate gradient solvent system for the first four fractions and methanol for the last elution, can efficiently separate the lipid compounds of cocoa in five fractions, corresponding to at least one class of lipid compounds. The method has a relatively rapid speed, simple process, and low cost, providing a practicable analytical method for basic research.

In oilseeds, the lipid matrix is generally composed by 80 to 90% triacylglycerols, 1 to 5% diacyl- and monoacylgycerols, 1 to 2% sterols and sterol esters, 0.1-1% polar lipids and <1% free fatty acids (Laffargue et al., 2007). TLC staining with different staining reagents (Fig. A3) and compound assignment based on mass spectrometry data have allowed the identification and assignment of the main classes in cocoa (Fig. 3.7), namely sterol esters, triacylglycerols, diacylglycerols, free fatty acids, and polar lipids. Fraction 5 contains, besides sterols esters glucosides, identified by tandem MS fragmentation, other polar lipids containing amine and amide groups as this fraction reacts positively to the ninhydrin and Dragendorff's reagent (Jork, 1990). Most probably, these compounds belong to phospholipids class.

The other separation method was a simple separation of cocoa lipids using a preparative silica gel column (Fig. 3.8). The results have been shown to be satisfactory for the separation of the components on a large scale. With minor changes, this method can be improved to achieve the separation of each spot seen on the TLC plate. Notwithstanding, an SPE method would be more appropriate and certainly the information provided by the two methods should be integrated. The fractions obtained by silica gel column separation were further analyzed by HPLC-MS. The main purpose of this separation was to illustrate the overall profile of cocoa fat. In addition, this analysis confirmed that there are more than one class of lipid compounds when considering separation by SPE. The two dimensional (2D map) maps of each fraction illustrates the complexity of cocoa lipid extract (Fig. 3.8 (b)). The 2D map of the fraction 3 showed all triacylglycerol known in cocoa, while the rest of the fractions (1, 4-16) represent other hundreds of compounds not yet fully described in literature.

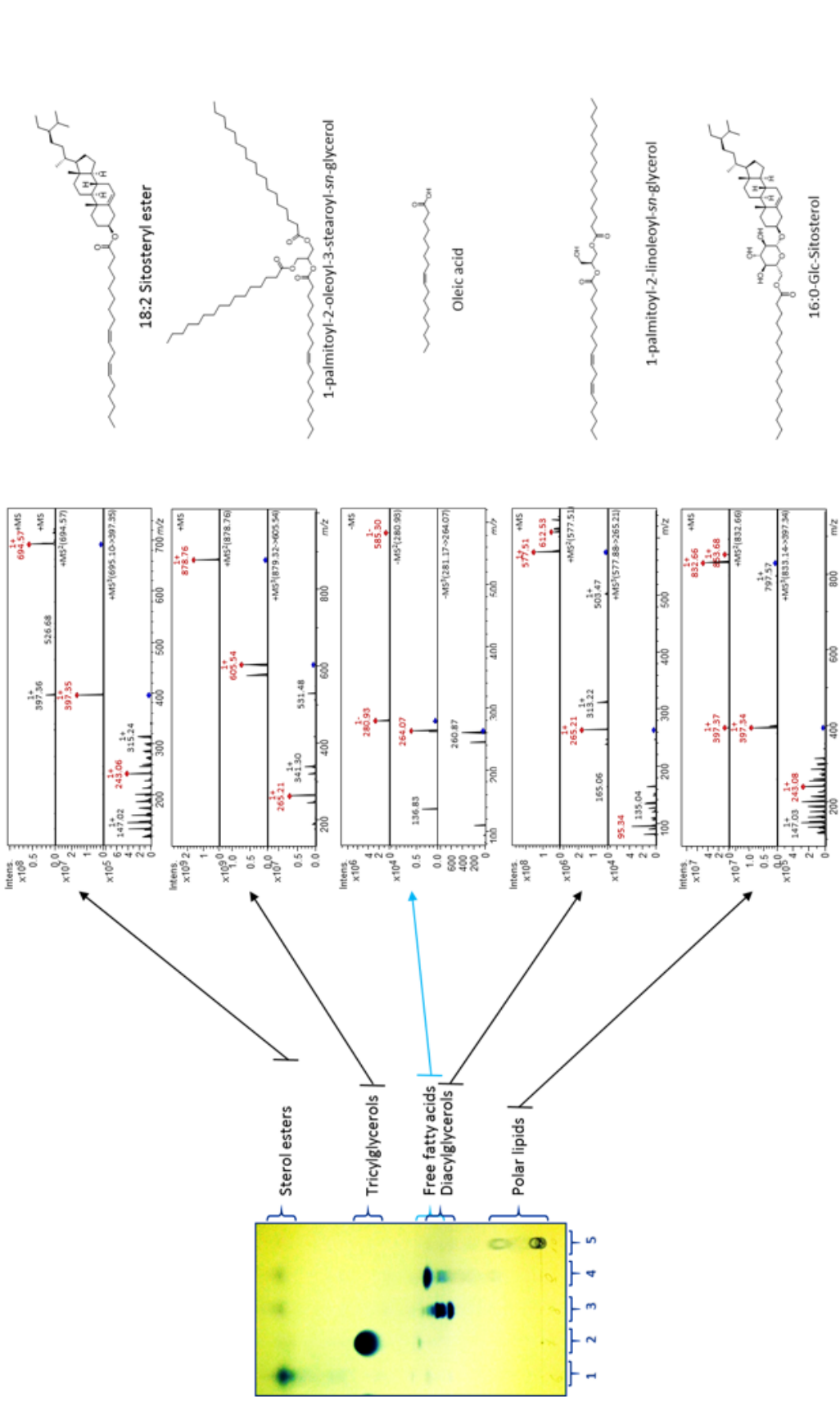


Figure 3.7 TLC plate* image showing the separation of 5 lipid fractions corresponding to: cholesterol esters; tryacylglicerols, diacylglycerols, free fatty acids and polar lipids classes asserted by MS fragmentation. On the right side, the proposed structures of identified compounds in cocoa lipid extract. *Hanessian's stain was used.

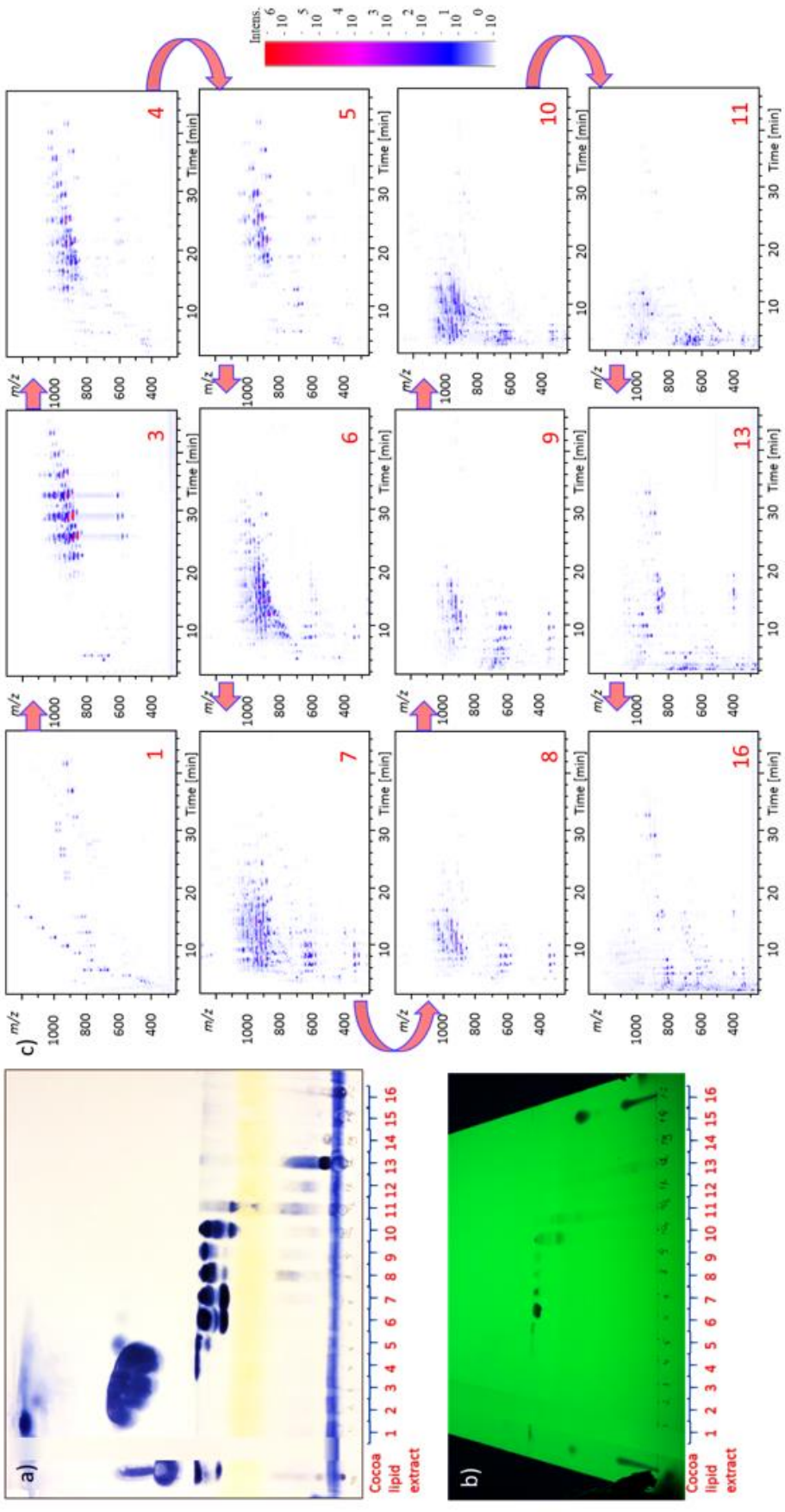


Figure 3.8 (a) TLC plate image showing the separation of a silica column fractions of lipidic compounds stained with Hannessian stain and **(b)** under UV light. **(c)** 2D maps build by HPLC-MS data of the main fractions illustrating the complexity of cocoa lipid. Fraction 3 contains the well known TAGs in cocoa.

3.4 Conclusions

Although cocoa butter is known to be a relatively simple fat, in which the predominant compounds are the triacylglycerols, with an appropriate extraction and separation method the complexity of this fat can be thorough fully appreciated. There is no doubt that this approach in combination with proper identification and quantification assessment can give more insight into the chemistry of cocoa butter and thus help to explain the intricate nature of its physical properties. Relative changes in lipid amounts with varying conditions may point out to novel targets.

References

Alvarez, J. G., & Touchstone, J. C. (1992). Separation of acidic and neutral lipids by aminopropyl-bonded silica gel column chromatography. *Journal of Chromatography B: Biomedical Sciences and Applications*, *577*(1), 142–145. https://doi.org/10.1016/0378-4347(92)80609-T

Asep, E. K., Jinap, S., Tan, T. J., Russly, A. R., Harcharan, S., & Nazimah, S. A. H. (2008). The effects of particle size, fermentation and roasting of cocoa nibs on supercritical fluid extraction of cocoa butter. *Journal of Food Engineering*, *85*(3), 450–458. https://doi.org/10.1016/j.jfoodeng.2007.08.008

Bligh, E. G., & Dyer, W. J. (1959). A Rapid Method of Total Lipid Extraction and Purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Cequier-Sánchez, E., Rodríguez, C., Ravelo, Á. G., & Zárate, R. (2008). Dichloromethane as a Solvent for Lipid Extraction and Assessment of Lipid Classes and Fatty Acids from Samples of Different Natures. *Journal of Agricultural and Food Chemistry*, *56*(12), 4297–4303. https://doi.org/10.1021/jf073471e

Christie, W. W. (1997). *Advances in Lipid Methodology*. Elsevier.

Christie, W. W., & Han, X. (2010). *Lipid Analysis, Fourth Edition: Isolation, Separation, Identification and Lipidomic Analysis* (4 edition). Bridgwater, England: Oily Press.

D'Souza, R., Grimbs, S., Behrends, B., Bernaert, H., Ullrich, M., & Kuhnert, N. (2017). Origin-based polyphenolic fingerprinting of Theobroma cacao in unfermented and fermented beans. *Food Research International*. https://doi.org/10.1016/j.foodres.2017.06.007

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry*, *226*(1), 497–509.

Hara, A., & Radin, N. S. (1978). Lipid extraction of tissues with a low-toxicity solvent. *Analytical Biochemistry*, *90*(1), 420–426.

Hirsch, J., & Ahrens, E. H. (1958). The Separation of Complex Lipide Mixtures by the Use of Silicic Acid Chromatography. *Journal of Biological Chemistry*, *233*(2), 311–320. Retrieved from http://www.jbc.org/content/233/2/311

Jork, H. (1990). *Thin-layer chromatography: reagents and detection methods*. VCH.

Kaluzny, M. A., Duncan, L. A., Merritt, M. V., & Epps, D. E. (1985). Rapid separation of lipid classes in high yield and purity using bonded phase columns. *Journal of Lipid Research*, *26*(1), 135–140.

Laffargue, A., de Kochko, A., & Dussert, S. (2007). Development of solid-phase extraction and methylation procedures to analyse free fatty acids in lipid-rich seeds. *Plant Physiology and Biochemistry*, *45*(3–4), 250–257. https://doi.org/10.1016/j.plaphy.2007.01.012

Levanon, Y., Rossetini, S. M. O., Raskin, M., & Mesquita, M. T. P. (1967). Thin-Layer Chromatographic Study on the Lipid Components of Cocoa Beans and Cocoa Butter. *Journal of Food Science*, *32*(6), 609–610. https://doi.org/10.1111/j.1365-2621.1967.tb00845.x

Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T. C., Thurecht, K. J., Ghafor, W. A., … Schenk, P. M. (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. *Microbial Cell Factories*, *13*, 14. https://doi.org/10.1186/1475-2859-13-14

Luque de Castro, M. D., & García-Ayuso, L. E. (1998). Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. *Analytica Chimica Acta*, *369*(1–2), 1–10. https://doi.org/10.1016/S0003-2670(98)00233-5

Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A., & Schwudke, D. (2008). Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *Journal of Lipid Research*, *49*(5), 1137–1146. https://doi.org/10.1194/jlr.D700041-JLR200

Panagiotopoulou, P. M., & Tsimidou, M. (2002). Solid phase extraction: Applications to the chromatographic analysis of vegetable oils and fats. *Grasas y Aceites*, *53*(1). https://doi.org/10.3989/gya.2002.v53.i1.292

Parsons, J. G., Keeney, P. G., & Patton, S. (1969). Identification and Quantitative Analysis of Phospholipids in Cocoa Beans. *Journal of Food Science*, *34*(6), 497–499. https://doi.org/10.1111/j.1365-2621.1969.tb12069.x

Patras, M. A., Milev, B. P., Vrancken, G., & Kuhnert, N. (2014). Identification of novel cocoa flavonoids from raw fermented cocoa beans by HPLC–MSn. *Food Research International*, *63*(Part C), 353–359. https://doi.org/10.1016/j.foodres.2014.05.031

Pernet, F., Pelletier, C. J., & Milley, J. (2006). Comparison of three solid-phase extraction methods for fatty acid analysis of lipid fractions in tissues of marine bivalves. *Journal of Chromatography A*, *1137*(2), 127–137. https://doi.org/10.1016/j.chroma.2006.10.059

Pina, F. (2012). Chemistry and applications of flavylium compounds: a handful of colours. *Chemical Society Reviews*, *41*, 869–908. https://doi.org/10.1039/c1cs15126f

Romanik, G., Gilgenast, E., Przyjazny, A., & Kamiński, M. (2007). Techniques of preparing plant material for chromatographic separation and analysis. *Journal of Biochemical and Biophysical Methods*, *70*(2), 253–261. https://doi.org/10.1016/j.jbbm.2006.09.012

Ruiz-Gutiérrez, V., & Pérez-Camino, M. C. (2000). Update on solid-phase extraction for the analysis of lipid classes and related compounds. *Journal of Chromatography A*, *885*(1–2), 321–341. https://doi.org/10.1016/S0021-9673(00)00181-3

Ryckebosch, E., Muylaert, K., & Foubert, I. (2012). Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae. *Journal of the American Oil Chemists' Society*, *89*(2), 189–198. https://doi.org/10.1007/s11746-011-1903-z

Self, R. (2007). *Extraction of Organic Analytes from Foods: A Manual of Methods*. Royal Society of Chemistry.

Zahler, P., & Niggli, V. (1977). The Use of Organic Solvents in Membrane Research. In *Methods in Membrane Biology* (pp. 1–50). Springer, Boston, MA. https://doi.org/10.1007/978-1-4684-2910-7_1

Chapter 4:

4 Characterization of triacylglycerols in unfermented cocoa beans by HPLC-ESI mass spectrometry

Diana Sirbu[a], Marcello Corno[b], Matthias S. Ullrich [a], and Nikolai Kuhnert[a]*

[a]*Department of Life Sciences and Chemistry, Jacobs University, Campus Ring 1, D-28759 Bremen, Germany*

[b]*Barry Callebaut, Aalstersestraat 122, B-9280 Lebbeke-Wieze, Belgium*

*Correspondence to:
Prof. Dr. Nikolai Kuhnert,
Analytical Chemistry Laboratory,
Jacobs University, Campus Ring 1,
D-28759 Bremen, Germany.
Phone: +49 421 200 3120,
Fax: +49 421 200 3102.
E-mail: n.kuhnert@jacobs-university.de

Published in Food Chemistry: doi.org/10.1016/j.foodchem.2018.01.194

Supplementary material available with the published article.

Abstract

The nutritional value of cocoa butter is mainly determined by the composition of triacylglycerols (TAGs). In this paper we have developed a non-aqueous reversed-phase liquid chromatographic method, using ethanol as the mobile phase, coupled to electrospray ionization (ESI) tandem mass spectrometry to identify TAGs in raw cocoa beans from six different origins. Tandem mass spectrometry was adopted to facilitate the identification of TAGs using unique diacylglycerol product ions and neutral losses. Additionally, two-dimensional m/z retention time maps aided the identification of entire homologous series of TAGs. A total of 83 different TAGs were identified in unfermented cocoa beans, 58 of which were not previously reported in cocoa. 31 of these compounds represent a new class of TAGs characterized by the presence of one to three hydroxyl groups on the unsaturated fatty acid chain. To date, this represents the largest number of TAGs identified in cocoa.

Keywords: triacylglycerol; high-performance liquid chromatography; tandem mass spectrometry; raw cocoa beans; homologous series; 2D map; neutral loss.

4.1 Introduction

Theobroma cacao is a small evergreen tree in the family *Sterculiaceae*, native to Central and South America. Cacao trees need rich, well-drained soils; they naturally grow within 20° latitude of the equator as they need about 2000 mm of rainfall a year, and temperatures in the range of 21 to 32 °C (Young, 2007). The seeds of *Theobroma cacao*, cocoa beans contain next to proteins, polyphenols, carbohydrates, alkaloids and minerals, lipids as their main chemical constituents accounting for 45-60% of their dry mass (Visioli et al., 2009). About 98% of cocoa lipid mass is represented by triacylglycerols (TAGs), which are hydrophobic molecular species formed by esterification of three fatty acids (FAs) with a glycerol backbone. Their FAs composition varies depending on the geographical origins, varieties of *Theobroma cacao*, growing seasons, and cultivation method (Lipp and Anklam, 1998a). Two saturated FAs with oleic acid in the middle characterize a typical pattern of distribution of FAs on the TAG body. The main TAGs are 1,3-distearoyl-2-oleoyl-*sn*-glycerol (SOS), 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (POS) and 1,3-dipalmitoyl-2-oleoyl-*sn*-glycerol (POP) that are known to be major components in cocoa butter (CB) and discriminatory marker compounds allowing identification of adulterations. Furthermore, this distribution of the FAs within the glycerol backbone has an essential nutritional and functional value highly appreciated in food, cosmetic and pharmaceutical industries (Ulberth and Buchgraber, 2003). TAGs are relative simple lipid substances, but they play an indispensable role in energy production and storage in a living organism (Carrasco-Pancorbo et al., 2009). TAGs also have an important value in nutrition and health being the main suppliers of essential fatty acids involved in maintaining life activities (Visioli et al., 2009). In seed plants, carbohydrates are accumulated as storage energy, and then transformed in TAGs during the growth stages. TAGs are then reconverted back into carbohydrates during germination with the incorporation of a high amount of water. One gram of oil is converted into approximately 2 g of carbohydrates (Murphy, 1994).

The analytical procedure for the characterization of individual TAG compounds involves three elementary steps: extraction, chromatographic separation, and identification. TAG profiling and analysis is performed by gas liquid chromatography (GLC) (Ulberth and Buchgraber, 2003) and high performance liquid chromatography (HPLC) using normal phase HPLC and non-aqueous reversed-phase (NARP) HPLC (Christie and Han, 2010; Jakab et al., 2002; Lísa and Holcapek, 2008). NARP-HPLC has been widely applied for the separation of

the intricate acylglycerol class because it offers, under optimized conditions, better resolution of the peaks and separation of TAGs according to the acyl chain lengths and the number of double bonds (DBs). The retention is ruled by equivalent carbon number (ECN) defined as ECN=CN-2DB, where CN is the number of carbon atoms (Holčapek et al., 2003; Lísa and Holcapek, 2008). Due to lack of chromophores characterization of TAGs is carried out mainly using mass spectrometry (MS) using either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) techniques. TAGs show a unique fragmentation pattern in tandem MS, since the most abundant fragment ions are formed by loss of one acyl moiety with consequent formation of diaclyglycerols product ions. In spite of this fact, they provide a challenge for mass spectrometric analysis because of the intricacy of naturally occurring TAGs, formation of multiple adduct ions and co-elution in chromatographic separation. Combinations of different fatty acyl substituents in TAGs, different stereochemical positions *sn*-1, 2 or 3 on the glycerol backbone (regioisomers) or *R/S* optical configuration of TAGs esterified in *sn*-1 and *sn*-3 positions by two different FAs (optical isomers) results in a large number of molecular species having the identical elemental composition. Ammonium adducts of TAGs, $[M+NH_4]^+$, generated by electrospray ionization, enable the molecular weight of each TAG molecular species to be determined. This allowed for tandem mass spectroscopy to be used for the elucidation of TAG (Cvacka et al., 2006; Holčapek et al., 2003; Hsu and Turk, 2010; McAnoy et al., 2005; Murphy et al., 2007). Kuhnert et al. have recently shown that fermented cocoa beans constitute an extremely complex food matrix containing more than 20 000 chemical entities by using Fourier-transform ion cyclotron resonance (FTICR) mass spectrometry technique (Kuhnert et al. 2013; Milev et al., 2014). It is, therefore, a challenge to detect and differentiate different types of TAGs in cocoa beans and various tools have to be applied.

Despite the many studies of the chemical composition of TAGs in cocoa butter previously published, there is no full characterization of the TAGs in unfermented cocoa beans which are also known as raw or wet cocoa beans (Lipp and Anklam, 1998b; Lipp et al., 2001; Segall et al., 2005; Simoneau et al., 1999; Ulberth and Buchgraber, 2003). This contribution presents a new approach to the analysis of TAGs in different origin raw cocoa beans using a NARP HPLC with ethanol as mobile phase, ESI tandem mass spectrometry and two dimensional (2D) map analysis revealing the true complexity of cocoa butter and thus allowing structure elucidation the of the most abundant lipid molecular species in cocoa.

4.2 Material and methods

4.2.1 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany), isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade) and chloroform (Rotisolv® HPLC grade) was purchased from Carl Roth (Karlsruhe, Germany), dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany), ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethanol was subjected to distillation prior use. Standard TAG samples used in this study were: TAG (16:0/18:1/16:0), TAG (16:0/16:0/16:0), TAG (18:0/18:0/18:0), and TAG (18:1/18:1/18:1) all at ≥ 99% were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

4.2.2 Sample preparation

Frozen cocoa bean samples from different origins were received in several sets from Barry Callebaut (Lebbeke-Wieze, Belgium). A total of 12 samples coming from Ivory Coast, Indonesia, Ecuador, Tanzania, Malaysia, and Brazil were analyzed. Firstly, the seed material was crushed using a grinder Retsch Grindomix GM200 knife mill (Haan, Germany) with the purpose of making homogenous powder. Henceforth, an overnight Soxhlet (Buchi Extraction System B811 instrument) method using dichloromethane as an extraction solvent, 150 mL of 5 g of crushed beans, was used. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis. For HPLC analysis, a concentration of 0.045 mg/mL in chloroform/ethanol (50/50) of cocoa lipid extract was prepared.

4.2.3 HPLC chromatographic conditions

TAGs molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). The column used in this study was a Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample

were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 5 mmol/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. The gradient elution program consisted of holding solvent steady A/B (40/60) for 3 min; followed by a linear gradient to solvent B (100) for 33 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 40/60 solvent A/B for 5 min before reuse.

4.2.4 High-resolution Mass spectrometry conditions

High-resolution masses were acquired using a time of flight (TOF) MicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of *m/z* 200–1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode. Molecular formulae suggestions were accepted if the mass error was below 5 ppm.

4.2.5 Tandem Mass spectrometry conditions

LC-tandem MS was carried out using an Ion-Trap detector in positive ion mode equipped with an ESI source (Bruker Daltonics UHT Ultra, Bremen, Germany). The full scan mass spectra were recorded in the range *m/z* 200-1200 operating in positive ion mode. Capillary temperature was set to 350 °C, drying gas flow rate of 10 L/min and nebulizer pressure of 10 psi. Tandem mass spectra were acquired in Auto MS^n (smart fragmentation) using a ramping of the collision energy.

4.2.6 Enrichment of polar TAG fraction

1 g of raw cocoa lipid extract was passed through a preparative silica gel column (15 cm × 3 cm i.d.), using a solvent gradient from 0% ethylacetate in heptane until 50% ethylacetate in heptane, in order to prepare an enriched fraction of the TAGs eluting at lower retention times

(from 4 to 25 min) in HPLC-MS. This fraction shown a more polar character and was used for further analysis by nuclear magnetic resonance spectroscopy (NMR).

4.2.7 Derivatization of hydroxylated fatty acids

Derivatization of the hydroxyl group was carried out on 100 mg of the enriched fraction obtained from the preparative silica gel column. One 0.5 mL benzoyl chloride with 200 µL of 0.1M DMAP (4-(N,N-dimethylamino)pyridine) in pyridine. The reaction was performed in dichloromethane and stirred at room temperature for 1 h. The benzoate obtained were diluted in chloroform/ethanol (50/50) and 3 µL were injected in HPLC-MS.

4.2.8 NMR spectroscopy

^{1}H NMR and ^{13}C NMR spectra were acquired on a JEOL ECX-400 spectrometer (Eching, Germany) operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR at room temperature in $CDCl_3$, using a 5 mm probe. The chemical shifts (δ) are reported in parts per million and were referenced to the residual solvent peak. Enriched cocoa lipid extract fraction was prepared by dissolving 0.1g of sample in 2.0mL of deuterated chloroform. The ^{1}H-NMR and ^{13}C NMR shifts were determined from a single analysis of the enriched fraction. To verify the ^{1}H-NMR and ^{13}C NMR assignments, $^{1}H^{1}H$ correlation spectroscopy (COSY) and $^{1}H^{13}C$ correlation spectroscopy (HMBC and HMQC) experiments were conducted.

4.3 Results and discussion

Triacylglycerols of cocoa butter (CB) have been analyzed on several occasions (Podlaha et al., 1984; Řezanka and Mareš, 1991) and reviewed by Lipp and Anklam (Lipp and Anklam, 1998a, 1998b; Lipp et al., 2001). At this stage, a total of 23 different TAGs were reported in cocoa butter obtained from fermented cocoa beans. However, no attention has been paid to the correct assignment of the fatty acid regiochemistry concerning the glycerol backbone. The first correct assignment was accomplished by a chiral phase HPLC method by Takagi and Ando (Takagi and Ando, 1995). Fauconnot et al. shown that TAG regioisomers elute at the same retention time on a C18 column. They have described the major regioisomers in cocoa: 1,3-

dipalmitoyl-2-oleoyl-*sn*-glycerol (POP, 100% regioisomeric purity), 1,3-distearolyl-2-oleoyl-*sn*-glycerol (SOS, 100% regioisomeric purity), rac-palmitoylstearoyl-2-oleoyl-*sn*-glycerol (rac-POS, >90% regioisomeric purity), 1,3-dipalmitoyl-2-linoleoyl-*sn*-glycerol (PLP, 100% regioisomeric purity), 1(3),2-dioleoyl-3(1)-stearoyl-*sn*-glycerol (SOO, 90% regioisomeric purity), and 1(3),2-dioleoyl-3(1)-palmitoyl-*sn*-glycerol (POO, 100% regioisomeric purity) identified on the basis of diaclyglycerol fragment abundance in comparison with authentic standards (Fauconnot et al., 2004). Other analyses of CB were carried out via GLC by Ulberth and Buchgraber, and Segall et al. using HPLC coupled to APCI-MS (Ulberth and Buchgraber, 2003; Segall et al., 2005). There ten already known TAGs were described in cocoa butter. Lísa and Holcapek extended the list of the TAGs by identifying TAGs which possess longer chain fatty acid in their structure, such as lignoceric and behenic acid to a total of 25 compounds (Lísa and Holcapek, 2008). However, no reports have been published to date on TAGs in unfermented cocoa beans.

We selected raw cocoa beans from six different origins, representative of beans of different botanical origin, including beans from Ivory Coast, Tanzania, Ecuador, Brazil, Malaysia and Indonesia. The total lipids were extracted using an optimized Soxhlet extraction with dichloromethane as a solvent. It should be noted that most cocoa lipid extraction methods use hexane or similar resulting in an incomplete extraction, omitting polar lipids. Gravimetric data of total lipids for each sample are given in Table 4.1. For TAG analysis, we developed an optimized reversed-phase HPLC method using ESI-mass spectrometry for detection. Ethanol proved to be an efficient eluent for the good separation of lipids from a reversed C18 stationary phase. TAG ionization was facilitated by the addition of ammonium formate. A gradient comprising ethanol and acetonitrile was shown to elute TAGs at earlier retention times if compared to methanol (at 100%) having a positive effect on the separation efficiency, which has been described by other authors (Ribeiro et al., 2004). A representative chromatogram is shown in Figure 4.1 (a). TAGs were eluting at retention times from 4.5 min to 48 min. Further minor polar lipid species were observed at earlier retention times, which were neglected in this contribution. For TAG nomenclature we use the characteristic shorthand defining the three fatty acid substituents by a single letter/abbreviation of three capital letters code (for example POS for 1-palmytoyl-2-oleoyl-3-stearoyl-glycerol) for the most common fatty acids or a universal fatty acid code for the less abundant fatty acids in nature (Table 4.2) (Lísa & Holcapek, 2008).

Table 4.1 Origin data and total lipid amount of raw cocoa samples used in this study.

No.	*Hybrid name*	*Geographical origin*	*Total lipid amount[a]* (%)*
1	C308XC5	Ivory Coast	41.09
2	C308XC5[b]*	Ivory Coast	40.03
3	G3UTA409XC1	Ivory Coast	37.34
4	C38XC5	Ivory Coast	37.26
5	UPA405XC412	Ivory Coast	37.35
6	ICS60	Indonesia	31.58
7	ICCRI 04	Indonesia	25.61
8	BL2936	Indonesia	30.68
9	PA191	Indonesia	35.15
10	PBS123	Indonesia	30.54
11	ICCRI 03	Indonesia	26.38
12	ICCRI 06H	Indonesia	30.79
13	EET544	Ecuador	32.62
14	EET103	Ecuador	35.47
15	CCN51	Ecuador	33.27
16	CCN51[b]*	Ecuador	36.13
17	PBC140	Malaysia	34.10
18	PBC123	Malaysia	28.53
19	PBC159	Malaysia	36.43
20	Comun	Brazil	33.55
21	*unknown*	Tanzania	38.51
22	*unknown*	Tanzania	37.10
23	*unknown*	Tanzania	35.10

Hybrid name was assigned according to the terminology used by local suppliers.

[a]*calculated as % of mass ± 1% from raw wet cocoa beans. The moisture content of these samples diverges between 30 and 43%.

[b]* collected from a different location.

4.3.1 High resolution LC-ESI-MS and Molecular Formulae assignment

Compound identification was carried out using a high-resolution ESI-TOF mass spectrometer operating in the positive ion mode leading to assignment of molecular formulae. Molecular formulae were accepted if the mass error was found to be below 5 ppm. Using our LC-ESI-MS method for TAG identification we observed that for most TAGs two or three pseudomolecular ions were present, including $[M+H]^+$, $[M+NH_4]^+$, and $[M+Na]^+$ with $[M+NH_4]^+$, as the most common base peak. However, not all of TAGs show $[M+NH_4]^+$ as main adduct ion. TAGs at lower retention time have shown to have an intense $[M+Na]^+$ adduct and

the pseudomolecular $[M+H]^+$ in a higher abundance if compared to $[M+NH_4]+$. Byrdawell et al. (Byrdwell and Neff, 2002) have described this phenomenon with both ESI and APCI ionization. The relative ratio of these adduct ions varied depending on the structure and the abundance of the TAGs.

Table 4.2 Abbreviations of fatty acids with the characteristic fragment ion acyls and neutral losses.

Common name	*Mass*	*Symbol*	*CN:DB*	*[RCO]+*	*$[RCOONH_4]+$*
Myristic acid	228.2089	M	C14:0	211.2067	245.2338
Pentadecylic acid	242.2246	C15:0	C15:0	225.2224	259.2495
Palmitic acid	256.2402	P	C16:0	239.2380	273.2651
Palmitoleic acid	254.2246	Po	C16:1	237.2224	271.2495
Margaric acid	270.2559	Ma	C17:0	253.2537	287.2808
Stearic acid	284.2715	S	C18:0	267.2693	301.2964
Oleic acid	282.2559	O	C18:1	265.2537	299.2808
Linoleic acid	280.2402	L	C18:2	263.2380	297.2651
Linolenic acid	278.2246	Ln	C18:3	261.2224	295.2495
Unusual 1 fatty acid	298.2351	C18:1-OH	C18:1	281.2481	315.2773
Unusual 2 fatty acid	296.2351	C18:2-OH	C18:2	279.2324	313.2616
Unusual 3 fatty acid	312.2301	$C18:2-(OH)_2$	C18:2	295.2274	329.2566
Unusual 4 fatty acid	310.2144	$C18:3-(OH)_2$	C18:3	293.2117	327.2410
Unusual 5 fatty acid	328.2250	$C18:3-(OH)_3$	C18:2	311.2223	345.2516
Nonadecylic acid	298.2872	C19:0	C19:0	281.2845	315.3138
Arachidic acid	312.3028	A	C20:0	295.3006	329.3277
Heneicosanoic acid	309.3163	C21:0	C21:0	325.3112	343.3434
Behenic acid	340.3341	B	C22:0	323.3319	357.3590
Tricosanoic acid	337.3476	C23:0	C23:0	353.3425	371.3747
Lignoceric acid	368.3654	Lg	C24:0	351.3632	385.3903

Therefore, within the mass spectra corresponding to the individual chromatographic peaks molecular formulae for two to three ions needed to be evaluated complicating the overall analysis of the TAG profile. Additionally, database searches using the lipidmaps database (Sud et al., 2012) were used to compare experimental *m/z* values with database entries for further confirmation.

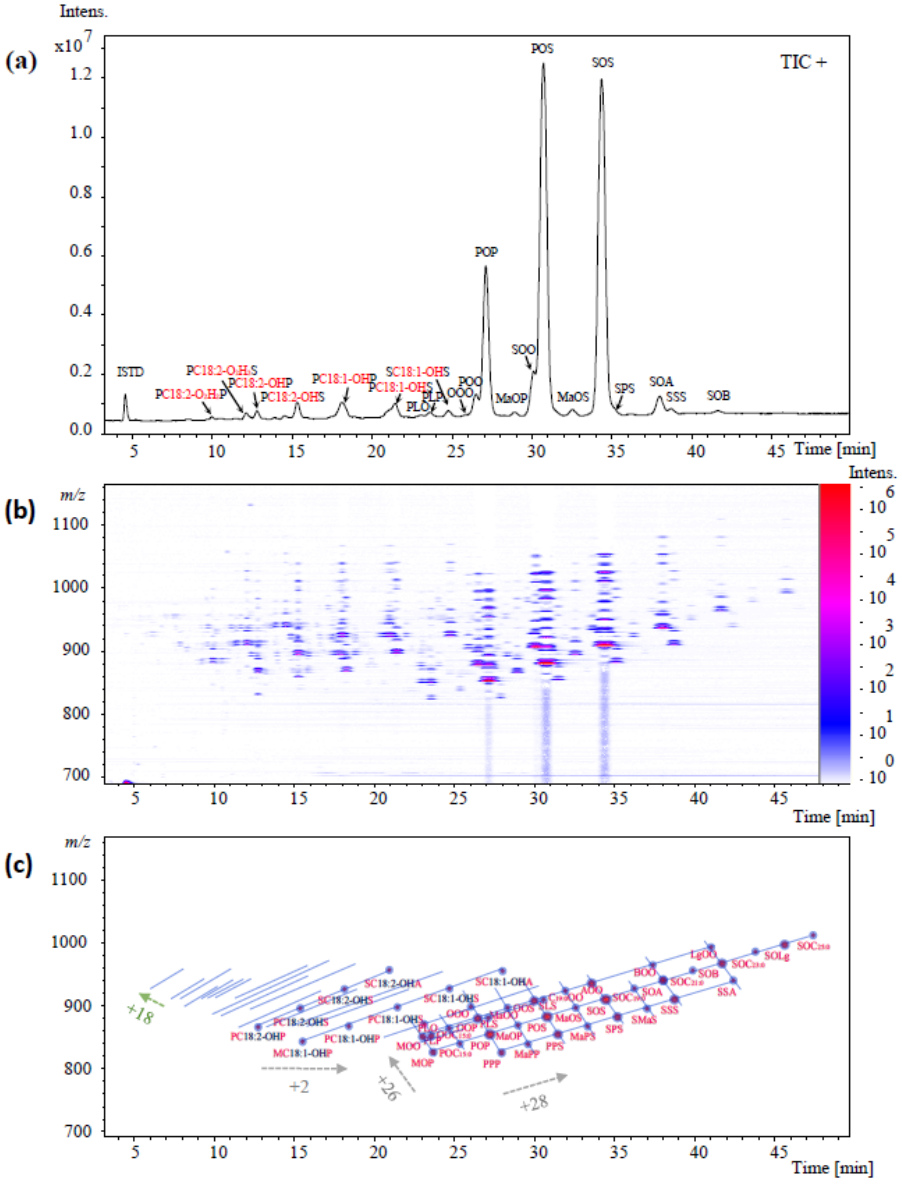


Figure 4.1 (a) HPLC-MS profile of Ivory Coast raw unfermented cocoa TAGs in positive ionization mode; **(b)** Two-dimensional (2D) map of TAGs from raw cocoa beans, Ivory Coast origin, by reversed-phase high resolution HPLC/ESI-MicroOTOF MS analysis. Colour rapresents the intensity of the peaks with red being the highest and blue the lowest: **(c)** 2D HPLC-MS feature map displaying TAGs building blocks of homologous series.

LC-MS data for CB containing signals originating from TAGs can be displayed as a 2D map with retention times on the x-axis and *m/z* values on the y-axis (Fig. 4.1(b)). This representation provides an improved overview revealing all molecular species detected in a complex sample, in particular showing minor components co-eluting with major components. These plots have been previously used for phospholipid assignment (Taguchi et al., 2000; Yamada et al., 2013). Here we used it the first time for TAG identification in plant material. On the plot each TAG species appear as two or three peaks (peak size in the third dimension corresponds to peak intensity) with mass differences of 18 Da, corresponding to $[M+H]^+$ and $[M+NH_4]^+$ and 23 Da corresponding to $[M+Na]^+$ at identical retention times. Series of TAGs lie within the plot on straight lines with a positive slope within an almost equidistant period as indicated in Figure 4.1(c). These series generate TAG homologues series. Homologous series are series of compounds, which differ in a defined mass increment corresponding to addition or subtraction of a fixed moiety and hence *m/z* increment, eg. $-H_2$ for unsaturation of FAs or $+C_2H_4$ for chain elongation of fatty acids. Therefore the information obtained allows identification of homologous series of fatty acids within the data set similar to the Kendrick formalism frequently used in petroleum analysis or processed food analysis (Hughey et al., 2001).

The 2D map allows for the assignment of two types of homologous series (Fig. 4.1(c)), the most common increments of 28 Da corresponding to $+C_2H_4$ and a second increment of 26 Da corresponding $+C_2H_2$. The retention time of TAGs is governed by ECN. Retention time typically increases with ECN number, whereas an increase in unsaturation (measured by double bond equivalents DB) leads to decrease in retention time. This trend can be clearly seen in the two dimensional plot. The separation of most TAGs within one ECN group was feasible and readily predicted by the elution regularity also described by Holčapek et al. (Holčapek et al., 2003). The molecular formula for a total of 83 TAGs were assigned, 58 of which were not previously reported in the literature. Table 4.3 shows high resolution MS data and molecular formulae assignment of all cocoa TAGs identified in Ivory Coast raw unfermented cocoa beans, the sample showing the highest diversity.

4.3.2 Tandem mass spectrometry

Assignment of individual TAG structures was based on ESI-tandem MS (MS^n) measurements. For tandem mass spectrometry an Ion-Trap mass spectrometer was used,

Table 4.3 TAG species in raw cocoa beans lipid extract subdivided into homologous series.

No.	*R. time (min)*	*Experimental mass $[M+NH_4]^+$*	*Theoretical mass $[M+NH_4]^+$*	*Error ([ppm])*	*Molecular formulae*	*Common Name*	*Short Name*
1	27.8	824.7705	824.7702	0.4	$C_{51}H_{102}NO_6$	TAG(16:0/16:0/16:0)	PPP
2	29.7	838.7854	838.7858	0.5	$C_{52}H_{104}NO_6$	TAG(17:0/16:0/16:0)	PMaP
3	31.4	852.8013	852.8015	0.1	$C_{53}H_{106}NO_6$	TAG(16:0/16:0/18:0)	PSP
4	33.2	866.8212	866.8284	4.8	$C_{54}H_{108}NO_6$	TAG(17:0/16:0/18:0)	PMaS
5	35.1	880.8313	880.8328	1.7	$C_{55}H_{110}NO_6$	TAG(18:0/16:0/18:0)	SPS
6	36.9	894.8513	894.8484	3.2	$C_{56}H_{112}NO_6$	TG(18:0/18:0/18:0)	SSS
7	38.6	908.8616	908.8641	2.7	$C_{57}H_{114}NO_6$	TG(18:0/18:0/20:0)	SAS
8	42.3	936.8982	936.8954	2.7	$C_{59}H_{118}NO_6$	TG(18:0/18:0/22:0)	SBS
9	46.4	964.9274	964.9267	0.7	$C_{61}H_{122}NO_6$	TG(18:0/18:0/24:0)	SLgS
10	23.6	822.7535	822.7445	1.2	$C_{51}H_{100}NO_6$	TG(16:0/18:1/14:0)	MOP
11	25.3	836.7707	836.7702	0.6	$C_{52}H_{102}NO_6$	TG(15:0/18:1/14:0)	MOC15:0
12	27.1	850.7851	850.7858	0.8	$C_{53}H_{104}NO_6$	TG(16:0/18:1/16:0)	POP
13	28.9	864.7994	864.8015	2.4	$C_{54}H_{106}NO_6$	TG(17:0/18:1/16:0)	POMa
14	30.6	878.8171	878.8171	0.0	$C_{55}H_{108}NO_6$	TG(16:0/18:1/18:0)	POS
15	32.5	892.8322	892.8328	0.7	$C_{56}H_{110}NO_6$	TG(17:0/18:1/18:0)	SOMa
16	34.2	906.8484	906.8484	0.0	$C_{57}H_{112}NO_6$	TG(18:0/18:1/18:0)	SOS
17	36.2	920.8646	920.8641	0.6	$C_{58}H_{114}NO_6$	TG(18:0/18:1/19:0)	SOC19:0
18	37.9	934.8800	934.8797	0.3	$C_{59}H_{116}NO_6$	TG(18:0/18:1/20:0)	SOA
19	39.7	948.8962	948.8954	0.9	$C_{60}H_{118}NO_6$	TG(18:0/18:1/21:0)	SOC21:0
20	41.6	962.9101	962.9110	0.9	$C_{61}H_{120}NO_6$	TG(18:0/18:1/22:0)	SOB
21	41.6	962.9101	962.9110	0.9	$C_{61}H_{120}NO_6$	TG(16:0/18:1/24:0)	POLg
22	43.6	976.9242	976.9267	2.6	$C_{62}H_{122}NO_6$	TG(18:0/18:1/23:0)	SOC23:0
23	45.7	990.9446	990.9423	2.3	$C_{63}H_{124}NO_6$	TG(18:0/18:1/24:0)	SOLg
24	47.8	1004.9575	1004.9580	0.4	$C_{64}H_{126}NO_6$	TG(18:0/18:1/25:0)	SOC25:0
25	49.5	1018.9577	1018.9736	1.3	$C_{65}H_{128}NO_6$	TG(18:0/18:1/26:0)	SOC26:0
26	18.8	820.7362	820.7389	3.2	$C_{51}H_{98}NO_6$	TG(14:0/18:2/16:0)	MLP
27	23.6	848.7704	848.7702	0.7	$C_{53}H_{102}NO_6$	TG(16:0/18:2/16:0)	PLP
28	27.1	876.7981	876.8015	3.8	$C_{55}H_{106}NO_6$	TG(16:0/18:2/18:0)	PLS

29	31.1	904.8318	904.8328	1.1	$C_{57}H_{110}NO_6$	TG(18:0/18:2/18:0)	SLS
30	23	848.7718	848.7702	2.0	$C_{53}H_{102}NO_6$	TG(14:0/18:1/18:1)	MOO
31	24.8	862.7868	862.7858	1.1	$C_{54}H_{104}NO_6$	TG(15:0/18:1/18:1)	C15:0OO
32	26.5	876.8007	876.8015	0.9	$C_{55}H_{106}NO_6$	TG(16:0/18:1/18:1)	POO
33	28.3	890.8169	890.8171	0.3	$C_{56}H_{108}NO_6$	TG(17:0/18:1/18:1)	MaOO
34	30.0	904.8320	904.8328	0.9	$C_{57}H_{110}NO_6$	TG(18:0/18:1/18:1)	SOO
35	31.9	918.8528	918.8484	1.2	$C_{58}H_{112}NO_6$	TG(19:0/18:1/18:1)	C19:0OO
36	33.5	932.8612	932.8641	3.1	$C_{59}H_{114}NO_6$	TG(20:0/18:1/18:1)	AOO
37	37.3	960.8965	960.8954	1.2	$C_{61}H_{118}NO_6$	TG(22:0/18:1/18:1)	BOO
38	40.9	988.9267	988.9267	0.1	$C_{63}H_{122}NO_6$	TG(24:0/18:1/18:1)	LgOO
39	19.7	846.7546	846.7545	0.1	$C_{53}H_{100}NO_6$	TG(14:0/18:2/18:1)	PLPo
40	23.0	874.7879	874.7858	2.4	$C_{55}H_{104}NO_6$	TG(16:0/18:2/18:1)	PLO
41	26.5	902.8152	902.8171	2.1	$C_{57}H_{108}NO_6$	TG(18:0/18:2/18:1)	SOL
42	29.2	930.8484	930.8466	1.9	$C_{59}H_{110}NO_6$	TG(20:0/18:2/18:1)	ALO
43	25.9	902.8200	902.8171	3.2	$C_{57}H_{108}NO_6$	TG(18:1/18:1/18:1)	OOO
44	20.6	846.7562	846.7545	2.0	$C_{53}H_{100}NO_6$	TG(16:0/18:3/16:0)	PLnP
45	24.0	874.7879	874.7858	2.6	$C_{55}H_{104}NO_6$	TG(16:0/18:3/18:0)	PLnS
46	27.6	902.8208	902.8171	4.1	$C_{57}H_{108}NO_6$	TG(18:0/18:3/18:0)	SLnS
47	15.4	838.7480	838.7494	1.7	$C_{51}H_{100}NO_7$	TG(14:0/18:1-OH/16:0)	MC18:1(-OH)P*
48	16.3	852.7632	852.7651	2.2	$C_{52}H_{102}NO_7$	TG(15:0/18:1-OH/16:0)	C15:0C18:1(-OH)P*
49	18.1	866.7796	866.7807	1.3	$C_{53}H_{104}NO_7$	TG(16:0/18:1-OH/16:0)	PC18:1(-OH)P*
50	19.9	880.7982	880.7964	2.1	$C_{54}H_{106}NO_7$	TG(16:0/18:1-OH/17:0)	PC18:1(-OH)Ma*
51	21.4	894.8121	894.8120	0.0	$C_{55}H_{108}NO_7$	TG(16:0/18:1-OH/18:0)	PC18:1(-OH)S*
52	23.1	908.8015	908.8277	3.2	$C_{56}H_{110}NO_7$	TG(17:0/18:1-OH/18:0)	C17:0C18:1(-OH)S*
53	24.8	922.8411	922.8433	2.4	$C_{57}H_{112}NO_7$	TG(18:0/18:1-OH/18:0)	SC18:1(-OH)S*
54	28.1	950.8789	950.8746	4.5	$C_{59}H_{116}NO_7$	TG(18:0/18:1-OH/20:0)	SC18:1(-OH)A*
55	15.9	864.7657	864.7651	0.7	$C_{53}H_{102}NO_7$	*not assigned*	
56	18.9	892.7664	892.7664	0.0	$C_{55}H_{106}NO_7$	*not assigned*	
57	22.1	920.8275	920.8277	0.2	$C_{57}H_{110}NO_7$	*not assigned*	
58	14.9	864.7627	864.7651	2.7	$C_{53}H_{102}NO_7$	TG(14:0/18:1-OH/18:1)	MC18:1(-OH)O*
59	17.8	892.7952	892.7964	1.3	$C_{55}H_{106}NO_7$	TG(16:0/18:1-OH/18:1)	PC18:1(-OH)O*
60	21	920.8272	920.8277	0.5	$C_{57}H_{110}NO_7$	TG(18:0/18:1-OH/18:1)	SC18:1(-OH)O*

61	24.2	948.8600	948.8590	1.1	$C_{59}H_{114}NO_7$	TG(20:0/18:1-OH/18:1)	AC18:1(-OH)O*
62	13.3	862.7468	890.7494	3.1	$C_{53}H_{100}NO_7$	TG(14:0/18:1-OH/18:2)	MC18:1(-OH)L*
63	15.9	890.7842	890.7807	3.9	$C_{55}H_{104}NO_7$	TG(16:0/18:1-OH/18:2)	PC18:1(-OH)L*
64	18.8	918.8089	918.8120	3.4	$C_{57}H_{108}NO_7$	TG(18:0/18:1-OH/18:2)	SC18:1(-OH)L*
65	12.8	864.7648	864.7651	0.3	$C_{53}H_{102}NO_7$	TG(16:0/18:2-OH/16:0)	PC18:2(-OH)P*
66	15.3	892.7963	892.7964	0.1	$C_{55}H_{106}NO_7$	TG(16:0/18:2-OH/18:0)	PC18:2(-OH)S*
67	16.6	906.8120	906.8147	2.9	$C_{56}H_{108}NO_7$	TG(17:0/18:2-OH/18:0)	MaC18:2(-OH)S*
68	18.0	920.8274	920.8277	0.4	$C_{57}H_{110}NO_7$	TG(18:0/18:2-OH/18:0)	SC18:2(-OH)S*
69	21.0	948.8624	948.8590	3.6	$C_{59}H_{114}NO_7$	TG(18:0/18:2-OH/20:0)	SC18:2(-OH)A*
70	12.3	890.7840	890.7807	3.6	$C_{55}H_{104}NO_7$	TG(16:0/18:2-OH/18:1)	PC18:2(-OH)O*
71	14.7	918.8158	918.8120	4.1	$C_{57}H_{108}NO_7$	TG(18:0/18:2-OH/18:1)	SC18:2(-OH)O*
72	17.5	946.8431	946.8433	0.2	$C_{59}H_{110}NO_7$	TG(20:0/18:2-OH/18:1)	AC18:2(-OH)O*
73	10.0	880.7574	880.7600	2.9	$C_{53}H_{102}NO_8$	TG(16:0/18:2-$(OH)_2$/16:0)	PC18:2(-$OH)_2$P*
74	12.1	908.7914	908.7913	0.1	$C_{55}H_{106}NO_8$	TG(16:0/18:2-$(OH)_2$/18:0)	PC18:2(-$OH)_2$S*
75	14.6	936.8203	936.8266	2.4	$C_{57}H_{110}NO_8$	TG(18:0/18:2-$(OH)_2$/18:0)	SC18:2(-$OH)_2$S*
76	17.2	964.8585	964.8539	4.7	$C_{59}H_{114}NO_8$	TG(18:0/18:2-$(OH)_2$/20:0)	SC18:2(-$OH)_2$A*
77	9.3	880.7616	880.7600	1.8	$C_{53}H_{102}NO_8$	*not assigned*	
78	11.5	908.7929	908.7913	1.8	$C_{55}H_{106}NO_8$	*not assigned*	
79	13.9	936.8207	936.8226	2.0	$C_{57}H_{110}NO_8$	*not assigned*	
80	7.5	906.7799	906.7756	4.7	$C_{55}H_{104}NO_8$	TG(16:0/18:3-$(OH)_2$/18:0)	PC18:3(-$OH)_2$S*
71	9.3	934.8081	934.8069	1.2	$C_{57}H_{108}NO_8$	TG(18:0/18:3-$(OH)_2$/18:0)	SC18:3(-$OH)_2$S*
82	5.9	922.7742	922.06	4.0	$C_{55}H_{104}NO_9$	TG(16:0/18:3-$(OH)_3$/18:0)	PC18:2(-$OH)_3$S*
83	7.0	950.8042	950.8019	2.4	$C_{57}H_{108}NO_9$	TG(18:0/18:3-$(OH)_3$/18:0)	SC18:2(-$OH)_3$S*

*tentatively assigned

operating in the positive ion mode using an auto MS^n routine. The mechanism of TAG fragmentation of ammonia adducts of TAGs was first suggested by McAnoy et al. (McAnoy et al., 2005). Collision induced dissociation of $[M+NH_4]^+$ precursor ions resulted in the neutral loss of NH_3 and an acyl side-chain (lost as a carboxylic acid) to generate a diacyl product ion (Scheme B.1 Appendix). Loss of the first carboxylic acid moiety can occur from the *sn*-1/3 position or the *sn*-2 position of the glycerol backbone. The mechanism of fragmentation would involve a 1,2 acyl migration to produce a five membered oxonium ion (Hsu and Turk, 2010). In tandem MS the first acyl substituent is believed to be lost from the *sn*-2 position, therefore allowing unambiguous assignment of TAG regiochemistry. This assignment requires in our view a note of caution. It is applied throughout the literature, however based on intuition rather than on a sufficient set of authentic standards. Therefore, we like to add a further argument. Migration from the *sn*-2 to the *sn*-1/3 position would in terms of organic chemistry be defined as a "down-hill migration" from a more substituted carbon to a less substituted carbon, whereas loss of any of the *sn*-2 acyl group would result in "up-hill migration" towards the *sn*-1/3 carbon atoms (Eames et al., 1999).

MS/MS data were used to identify each acyl group present for a given $[M+NH_4]^+$ precursor ion, and this information could be combined with high resolution MS data to identify possible TAG molecular species present in cocoa bean extracts. Subsequent MS^3 experiments on the resultant diacyl product ions, which gave rise to acylium (RCO^+) and related ions, enabled unambiguous TAG molecular assignments. MS^3 experiments using the diacyl product ion as precursor ion allowed further assignment of the two remaining TAG fatty acid substituents.

Assignment of TAG structures with tandem MS data is given in the supplementary information. In this section, two representative examples are discussed in more detail. A compound with a pseudomolecular ion of *m/z* 881 ($C_{55}H_{110}NO_6$, $[M+NH_4]^+$) as precursor ion yielded fragment ions at *m/z* 607.6 ($C_{39}H_{74}O_6$, $[M–P–NH_4]^+$), 41.4% relative intensity, and 579.6 ($C_{37}H_{70}O_6$, $[M–S–NH_4]^+$), 100% relative intensity, corresponding to 1,3-distearoyl-*sn*-glycerol (SS) and 1-stearoyl-2-palmytoyl-glycerol/2-palmytoyl-3-stearoyl-*sn*-glycerol (SP) diacyl product ions. SP *m/z* 579.6 was further fragmented and acylium ions of stearic $[C_{17}H_{35}CO]^+$ *m/z* 276.2 and palmitic acid $[C_{15}H_{31}CO]^+$ *m/z* 239.1 could be observed. Hence, on the basis of MS^2 and MS^3 fragments and their abundance, the overall structure was assigned as 1-stearoyl-2-palmytoyl-3-stearoyl-glycerol SPS, TG(18:0/16:0/18:0). A second example comprises the pseudomolecular ion at *m/z* 919 ($C_{58}H_{112}NO_6$, $[M+NH_4]^+$) as precursor ion, which yielded two diacyl product fragment ions at *m/z* ions at *m/z* 619.6 ($C_{40}H_{74}O_6$, [M–O–

$NH_4]^+$), 100% relative intensity, and 605.7 ($C_{39}H_{70}O_6$, $[M–C_{19:0}–NH_4]^+$), 41.7% relative intensity, corresponding to 1-oleoyl-2-nonadecanoyl-*sn*-glycerol (OC19:0) and 1,2-dioleoyl-*sn*-glycerol (OO) diacyl product ions. Further fragmentation led to MS^3 acylium ions of oleic $[C_{17}H_{33}CO]^+$ *m/z* 265.1 and nonadecylic acid $[C_{19}H_{39}CO]^+$. Therefore, the structure was assigned as $C_{19:0}OO$, TAG (19:0/18:1/18:1). Figure 4.2 (a, b, c, d) illustrates the typical tandem mass spectra of SPS and of 3 other different TAGs of increasing unsaturation fatty acid chain in position 2 of the glycerol backbone. Interestingly we observed TAGs with fatty acid substituents carrying an odd number of carbon atoms from pentadecylic acid $C_{15}H_{30}O_2$ to pentacosylic acid $C_{25}H_{50}O_2$. Such derivatives have been reported in other plants (Řezanka and Sigler, 2009), however, they are relatively rare.

As an additional tool for facilitating compound assignment, we used neutral loss analysis. The first fragmentation step in TAG fragmentation involves loss of the *sn*-1/3 acyl group followed by loss of the *sn*-2 acyl group. Hence, neutral loss chromatograms with defined neutral losses corresponding to fatty acids could be created. Such neutral loss chromatograms show all compound displaying loss of a defined acyl group. Figure 4.2 (e, f, g, h, i) shows exemplary neutral loss chromatograms with neutral loss of 273 Da ($-C_{16}H_{37}O_2N$), 301 Da ($-C_{18}H_{41}O_2N$), 299 Da ($-C_{18}H_{39}O_2N$), 297 Da ($-C_{18}H_{37}O_2N$) and 295 Da ($-C_{18}H_{35}O_2N$) corresponding to ammonia adducts of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid (Table 4.1) (Murphy et al., 2007). Because one of TAG was efficiently identified, another TAG is more easily recognized from the molecular related mass values with the help of the assembled information from the retention time point, MS^2 fragments, and neutral loss scanning of the corresponding fatty acid moiety. Combined LC-tandem MS and neutral loss scanning have been applied to the assignment of other lipid classes so far (Byrdwell and Neff, 2002; Taguchi et al., 2005). Furthermore, the fragmentation pattern of some available standards was cautiously studied and compared with cocoa TAGs. Their spectra are displayed in supplementary material. We consider that the above-described approach for the identification of TAGs will be a useful tool for the identification of TAG in any plant material, especially seeds lipids.

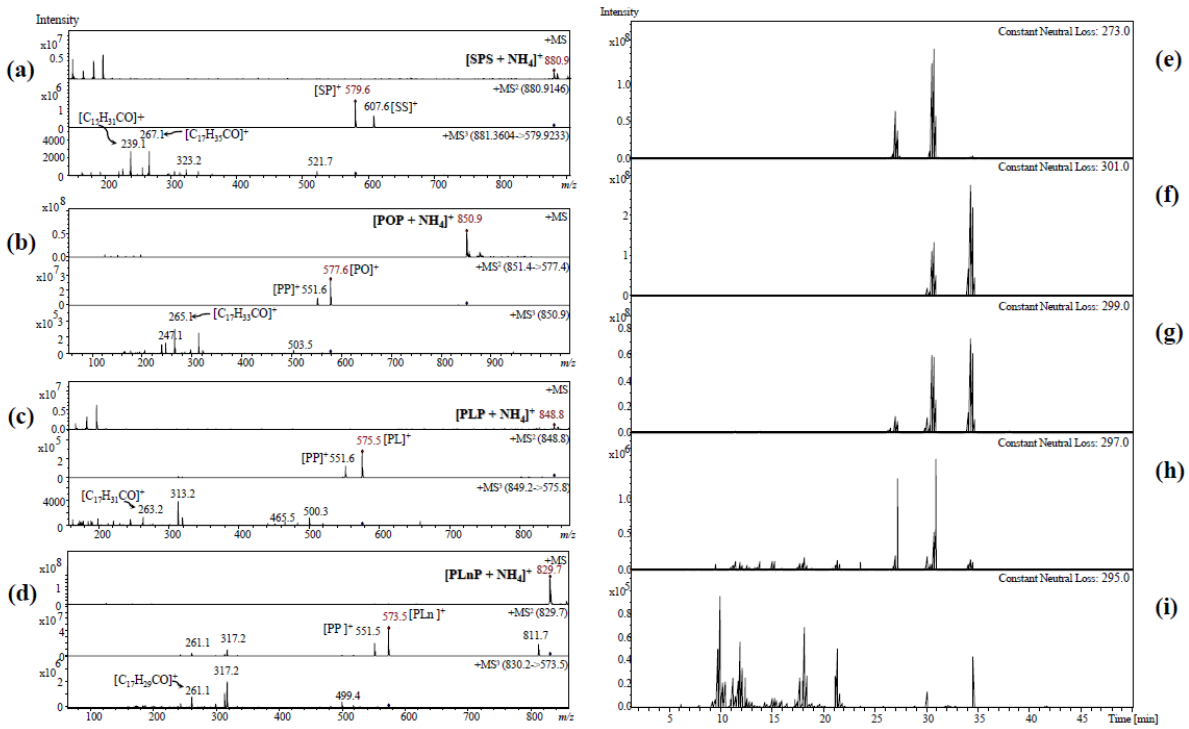


Figure 4.2 Positive ion mode tandem mass spectrum showing the fragmentation of triacylglycerols **(a)** SPS (m/z 881.9), **(b)** POP (m/z 851.9), **(c)** PLP (m/z 848.8) and **(d)** PLnP (m/z 829.7) ammonium adduct ions. P, palmitic; O, oleic; S, stearic; L, linoleic; Ln, linolenic and neutral loss chromatograms of **(e)** 273 Da, **(f)** 301 Da, **(g)** 299 Da, **(h)** 297 Da and **(i)** 295 Da (from up to down) corresponding to ammonia adducts of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid in Ivory Coast cocoa lipid extract.

4.3.3 Characterization of TAGs with hydroxylated fatty acids substituents

Next, to the TAGs containing saturated and unsaturated fatty acids, a second class of unusual TAGs was detected in unfermented beans. These compounds eluted at earlier retention times (from 4 to 25 min) indicating a more polar character. Assignment of molecular formulae from high resolution mass data indicated the presence of one, two or three additional oxygen atoms in the TAGs: $C_{55}H_{112}NO_7$, $C_{55}H_{110}NO_8$ and $C_{57}H_{108}NO_8$. The compounds appeared as three sets of homologues series, each with three to four members, with mass increments of C_2H_4 corresponding to +28 Da. Taking into account the TAG abundancy, three main subclasses of these unusual TAGs could be observed (Fig. 4.3(a)-(f)).

The observed molecular formulae suggest compounds with either an epoxide functionality, as reported as oxidation products of oleic acid, or an unsaturated fatty acid carrying an additional alcoholic moiety. The tandem MS data of these polar lipids revealed in MS^2 a loss

of 18 Da and 36 Da corresponding to the loss of respectively one or two molecule of H_2O arguing in favour of OH-derivatives. In a second step both in MS^2 and MS^3 a neutral loss -276 Da, -278 Da and -280 Da was observed corresponding to a C_{18} fatty acid with respectively four, three and two double bonds such as linolenic acid $C_{18:2}$ ($-C_{18}H_{32}O_2$). This finding suggests that hydroxylation occurs at an unsaturated fatty acid producing a more polyunsaturated fatty acid following fragmentation. For example, the compound assigned as $PC_{18:1\text{-}OH}PS$, pseudomolecular ion of *m/z* 895 ($C_{55}H_{108}NO_7$, $[M+NH_4]^+$) as precursor ion, in which neutral losses of -273 Da, -301 Da and -280 Da corresponding to stearic acid, palmitic acid, and linoleic acid were observed defining all three fatty side chains of this polar TAG. The MS^2 spectra of $[M+NH_4]^+$ and $[M+Na]^+$ of one representative compound of each class is presented in Figure 4.3(a)-(f). $[M+Na]^+$ were found to be extremely useful for a better understanding of the hydroxyl fatty acids moieties for the reason that during MS^2 fragmentation these fatty acid do not lose their hydroxyl group and the value of the neutral loss matches to the absolute *m/z* of the corresponding fatty acid. In case of Figure 4.3(b), (d) and (f), it corresponds to palmitic acid, *m/z* of 256.

The elemental composition suggests the presence of oxygen within one of the fatty acid side chains. To answer the question of the nature of oxygen functionality an extract enriched in this TAG fraction was prepared using preparative column chromatography on silica gel, and NMR spectra were recorded as well as a derivatization reaction was carried out using benzoyl chloride.

The ^{1}H-NMR spectrum shows four olefinic signals, two of them at 5.33 and 5.25 ppm corresponding to a *cis*-olefinic moiety, probably in (N-9) oleic acid like environment. Two further signals at 6.46 and 5.63 ppm show cross peaks in the $^1H^1H$-COSY spectrum and an additional cross peak to a triplet at 5.96 ppm revealing the presence of an allylic alcohol functionality. They must be assigned to olefinic protons with a *trans* double bond geometry due to a large ^{3}J coupling of 16.0 Hz. In the $^1H^{13}C$-HSQC spectrum, the allylic alcohol C<u>H</u>OH carbon can be assigned to a signal at 73.1 ppm. The $^1H^{13}C$-HMBC spectrum reveals from the C<u>H</u>OH hydrogen signal a $^2J_{HCC}$ interaction to the olefinic carbon at 129.1 ppm and two further interaction to a CH_2 carbon at 30.3 ppm as well as a $^3J_{HCCC}$ interaction to the sn-2 carbonyl carbon at 173.2 ppm. These interactions clearly place the OH substituent in the *β*-position of the carbonyl. A characteristic pair of highfield-shifted carbons as expected for an epoxide are clearly absent (Xia et al., 2016). Taking into account the tandem Ms data placing the hydroxy-

fatty acid substituent into the *sn*-2 position we propose the structures shown in Figure 4.3. All NMR data and key 2D NMR correlations are given in the supplementary material.

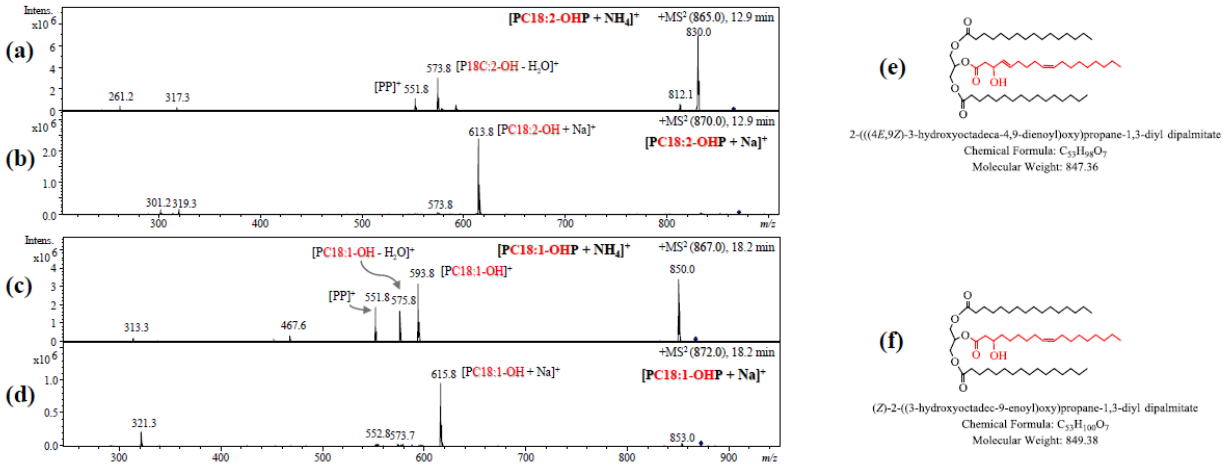


Figure 4.3 Positive ion mode tandem mass spectrum showing the fragmentation of triacylglycerols **(a)** PC18:2-OHP (*m/z* 865.0), **(c)** PC18:1-OHP (*m/z* 867.0) ammonium adduct ions and **(b)** PC18:2-OHP (*m/z* 870.0) and **(d)** PC18:1-OHP (*m/z* 872.0) sodium adduct ions. **(e)** and **(f)** proposed possible structures of the unusual TAGs in raw cocoa beans.

Following derivatization with benzoyl chloride the intensity of LC-MS signals of all polar TAGs were significantly reduced, indicating high yielding derivatization. Additional peaks were observed. In particular, a compound with MS data reminiscent of PL_nP was observed with a fatty acid with three double bonds in the *sn*-2 position, probably obtained by facile elimination from the allylic alcohol functionality by *β*-elimination. EICs signals corresponding to compounds with *m/z* values of 971.7, 999.7 or 1027.8 could be observed. Tandem MS data reveal neutral losses corresponding to loss of 139 Da ($-C_7H_6O_2 + NH_4^+$) from the precursor ions. These findings indicate the presence of benzoyl derivatives, but further tandem MS data are inconclusive. Chromatographic, MS data and 2D map for the derivatives are provided in the supplementary material.

In nature hydroxylated fatty acids occur prominently in castor oil with the TAG containing ricinoleic and dihydroxystearic acid (Lin et al., 2003). We have used castor oil as surrogate standard (Clifford and Madala, 2017) in a direct comparison with raw cocoa bean lipid extract. LC-MS data showed that none of the compounds in castor oil were identified in cocoa. However, contrary to Lin et al. (Lin et al., 2003) assignment as SLR (1-stearoyl-2-linoleoyl-3-ricinoleoyl-glycerol) TAG, we could suggest from tandem MS data (fragmentation of $[M+Na^+]$

spectra shown in supplementary material) of molecular species with *m/z* 924 a *sn*-2 position for the ricinoleic acid therefore revising the initially suggested structures. We could affirm that castor oil contain SRL and not SLR TAG moiety, eluting at 0.6 min earlier than $SC_{18:1(-OH)}O$ ($C_{57}H_{110}NO_7$) TAG moiety, same *m/z* ratio, in cocoa but having a different fragmentation pattern.

Oxygenated FAs, containing hydroxyl, epoxy, or oxo groups are an uncommon component of seed oils, but have widespread distribution among higher plant families (Smith et al., 2014). Studies confirm that hydroxy fatty acids are plant self-defense substances (Hou and III, 2000). In addition, hydroxylated fatty acids have been suggested as precursors in jasmonic acid biosynthesis, a plant hormone involved in defence reaction that has been observed in fermented cocoa beans in high abundance (Patras et al., 2014).

When comparing different origins of beans, the large majority of the TAGs was found in all samples. Indeed, unusual TAGs were found in all origin samples analyzed. However, there were subtle differences in relative concentrations when comparing samples within the raw unfermented beans. A detailed analysis of such differences is outside the scope of this contribution, and accurate quantification will be reported elsewhere.

4.4 Conclusions

A new method for TAG analysis using reversed-phase HPLC coupled to ESI mass spectrometry was developed, which allowed for the identification not only of the most abundant TAGs but also of the low abundant TAGs showing weak signals buried into the noise. Lipid extracts from raw cocoa beans from six different origins were profiled for the very first time for their TAG content. A total of 83 TAGs could be characterized in unfermented cocoa beans, 77 of which were positively identified. 58 of these compounds are reported for the first time in cocoa. Among the new identified compounds, TAGs carrying a hydroxyl side chain were characterized. The reason why these compounds were not reported before may lay on the fact that these compounds possibly undergo transformation or degradation during cocoa production process. These unusual constituents may offer the promise to blend cocoa butter with unfermented cocoa lipids to produce CB with special physical and chemical properties in new dietary or cosmetic products. However, further research studies needed to be carried out to validate this possibility and to elucidate their function.

References

Byrdwell, W.C., and Neff, W.E. (2002). Dual parallel electrospray ionization and atmospheric pressure chemical ionization mass spectrometry (MS), MS/MS and MS/MS/MS for the analysis of triacylglycerols and triacylglycerol oxidation products. Rapid Commun. Mass Spectrom. RCM 16, 300–319.

Carrasco-Pancorbo, A., Navas-Iglesias, N., and Cuadros-Rodríguez, L. (2009). From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part I: Modern lipid analysis. TrAC Trends Anal. Chem. 28, 263–278.

Christie, W.W., and Han, X. (2010). Lipid Analysis, Fourth Edition: Isolation, Separation, Identification and Lipidomic Analysis (Bridgwater, England: Oily Press).

Clifford, M.N., and Madala, N.E. (2017). Surrogate Standards: A Cost-Effective Strategy for Identification of Phytochemicals. J. Agric. Food Chem. 65, 3589–3590.

Cvacka, J., Krafková, E., Jiros, P., and Valterová, I. (2006). Computer-assisted interpretation of atmospheric pressure chemical ionization mass spectra of triacylglycerols. Rapid Commun. Mass Spectrom. RCM 20, 3586–3594.

Eames, J., Kuhnert, N., Sansbury, F.H., and Warren, S. (1999). Kinetic and Thermodynamic Control in the Synthesis of Tetrahydro-Pyrans and -Furans from 1,4-Diols by Stereospecific Phenylsulfanyl (PhS) Migration: Competition Between exo and endo Transition States and between [1,2] and [1,4]Sulfanyl Participation. Synlett *1999*, 1211–1214.

Fauconnot, L., Hau, J., Aeschlimann, J.-M., Fay, L.-B., and Dionisi, F. (2004). Quantitative analysis of triacylglycerol regioisomers in fats and oils using reversed-phase high-performance liquid chromatography and atmospheric pressure chemical ionization mass spectrometry. Rapid Commun. Mass Spectrom. RCM 18, 218–224.

Holčapek, M., Jandera, P., Zderadička, P., and Hrubá, L. (2003). Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 1010, 195–215.

Hou, C.T., and III, R.J.F. (2000). Growth inhibition of plant pathogenic fungi by hydroxy fatty acids. J. Ind. Microbiol. Biotechnol. 24, 275–276.

Hsu, F.-F., and Turk, J. (2010). Electrospray ionization multiple-stage linear ion-trap mass spectrometry for structural elucidation of triacylglycerols: assignment of fatty acyl groups on the glycerol backbone and location of double bonds. J. Am. Soc. Mass Spectrom. 21, 657–669.

Hughey, C.A., Hendrickson, C.L., Rodgers, R.P., Marshall, A.G., and Qian, K. (2001). Kendrick Mass Defect Spectrum: A Compact Visual Analysis for Ultrahigh-Resolution Broadband Mass Spectra. Anal. Chem. 73, 4676–4681.

Jakab, A., Héberger, K., and Forgács, E. (2002). Comparative analysis of different plant oils by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 976, 255–263.

Kuhnert, N., Dairpoosh, F., Yassin, G., Golon, A., and Jaiswal, R. (2013). What is under the hump? Mass spectrometry based analysis of complex mixtures in processed food--lessons from the characterisation of black tea thearubigins, coffee melanoidines and caramel. Food Funct. 4, 1130–1147.

Lin, J.-T., Turner, C., Liao, L.P., and McKeon, T.A. (2003). Identification and Quantification of the Molecular Species of Acylglycerols in Castor Oil by HPLC Using ELSD. J. Liq. Chromatogr. Relat. Technol. 26, 773–780.

Lipp, M., and Anklam, E. (1998a). Review of cocoa butter and alternative fats for use in chocolate—Part A. Compositional data. Food Chem. 62, 73–97.

Lipp, M., and Anklam, E. (1998b). Review of cocoa butter and alternative fats for use in chocolate—Part B. Analytical approaches for identification and determination. Food Chem. 62, 99–108.

Lipp, M., Simoneau, C., Ulberth, F., Anklam, E., Crews, C., Brereton, P., de Greyt, W., Schwack, W., and Wiedmaier, C. (2001). Composition of Genuine Cocoa Butter and Cocoa Butter Equivalents. J. Food Compos. Anal. 14, 399–408.

Lísa, M., and Holcapek, M. (2008). Triacylglycerols profiling in plant oils important in food industry, dietetics and cosmetics using high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A 1198–1199, 115–130.

McAnoy, A.M., Wu, C.C., and Murphy, R.C. (2005). Direct qualitative analysis of triacylglycerols by electrospray mass spectrometry using a linear ion trap. J. Am. Soc. Mass Spectrom. 16, 1498–1509.

Milev, B.P., Patras, M.A., Dittmar, T., Vrancken, G., and Kuhnert, N. (2014). Fourier transform ion cyclotron resonance mass spectrometrical analysis of raw fermented cocoa beans of Cameroon and Ivory Coast origin. Food Res. Int. 64, 958–961.

Murphy, D.J. (1994). Biogenesis, function and biotechnology of plant storage lipids. Prog. Lipid Res. 33, 71–85.

Murphy, R.C., James, P.F., McAnoy, A.M., Krank, J., Duchoslav, E., and Barkley, R.M. (2007). Detection of the abundance of diacylglycerol and triacylglycerol molecular species in cells using neutral loss mass spectrometry. Anal. Biochem. 366, 59–70.

Patras, M.A., Milev, B.P., Vrancken, G., and Kuhnert, N. (2014). Identification of novel cocoa flavonoids from raw fermented cocoa beans by HPLC–MSn. Food Res. Int. 63, 353–359.

Podlaha, O., Toregard, B., and Puschl, B. (1984). TG-type composition of 28 cocoa butters and correlation between some TG-type components. Lebensm. - Wiss. Technol. Food Sci. Technol.

Řezanka, T., and Mareš, P. (1991). Determination of plant triacylglycerols using capillary gas chromatography, high-performance liquid chromatography and mass spectrometry. J. Chromatogr. A 542, 145–159.

Řezanka, T., and Sigler, K. (2009). Odd-numbered very-long-chain fatty acids from the microbial, animal and plant kingdoms. Prog. Lipid Res. 48, 206–238.

Ribeiro, R.L.V., Bottoli, C.B.G., Collins, K.E., and Collins, C.H. (2004). Reevaluation of ethanol as organic modifier for use in HPLS-RP mobile phases. J. Braz. Chem. Soc. 15, 300–306.

Segall, S.D., Artz, W.E., Raslan, D.S., Ferraz, V.P., and Takahashi, J.A. (2005). Analysis of triacylglycerol isomers in Malaysian cocoa butter using HPLC–mass spectrometry. Food Res. Int. 38, 167–174.

Simoneau, C., Hannaert, P., and Anklam, E. (1999). Detection and quantification of cocoa butter equivalents in chocolate model systems: analysis of triglyceride profiles by high resolution GC. Food Chem. 65, 111–116.

Smith, M.A., Zhang, H., and Purves, R.W. (2014). Identification and Distribution of Oxygenated Fatty Acids in Plantago Seed Lipids. J. Am. Oil Chem. Soc. 91, 1313–1322.

Sud, M., Fahy, E., and Subramaniam, S. (2012). Template-based combinatorial enumeration of virtual compound libraries for lipids. J. Cheminformatics 4, 23.

Taguchi, R., Hayakawa, J., Takeuchi, Y., and Ishida, M. (2000). Two-dimensional analysis of phospholipids by capillary liquid chromatography/electrospray ionization mass spectrometry. J. Mass Spectrom. 35, 953–966.

Taguchi, R., Houjou, T., Nakanishi, H., Yamazaki, T., Ishida, M., Imagawa, M., and Shimizu, T. (2005). Focused lipidomics by tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci. 823, 26–36.

Takagi, T., and Ando, Y. (1995). Stereospecific analysis of monounsaturated triacylglycerols in cocoa butter. J. Am. Oil Chem. Soc. 72, 1203–1206.

Ulberth, F., and Buchgraber, M. (2003). Analytical platforms to assess the authenticity of cocoa butter. Eur. J. Lipid Sci. Technol. 105, 32–42.

Velíšek, J., and Cejpek, K. (2006). Biosynthesis of food constituents: Lipids. 1. Fatty acids and derived compounds - A review. Czech J. Food Sci. 24, 193–216.

Visioli, F., Bernaert, H., Corti, R., Ferri, C., Heptinstall, S., Molinari, E., Poli, A., Serafini, M., Smit, H.J., Vinson, J.A., et al. (2009). Chocolate, Lifestyle, and Health. Crit. Rev. Food Sci. Nutr. 49, 299–312.

Xia, W., Budge, S.M., and Lumsden, M.D. (2016). 1H-NMR Characterization of Epoxides Derived from Polyunsaturated Fatty Acids. J. Am. Oil Chem. Soc. 93, 467–478.

Yamada, T., Uchikata, T., Sakamoto, S., Yokoi, Y., Fukusaki, E., and Bamba, T. (2013). Development of a lipid profiling system using reversed-phase liquid chromatography coupled to high-resolution mass spectrometry with rapid polarity switching and an automated lipid identification software. J. Chromatogr. A 1292, 211–218.

Young, A.M. (2007). The Chocolate Tree: A Natural History of Cacao (Gainesville: University Press of Florida).

Chapter 5:

5 Variation of triacylglycerol profiles in unfermented and dried fermented beans

Diana Sirbu[a], Anne Grimbs[a], Marcello Corno[b], Matthias S. Ullrich [a], and Nikolai Kuhnert[a]*

[a]*Department of Life Sciences and Chemistry, Jacobs University, Campus Ring 1, D-28759 Bremen, Germany*

[b]*Barry Callebaut, Aalstersestraat 122, B-9280 Lebbeke-Wieze, Belgium*

*Correspondence to:
Prof. Dr. Nikolai Kuhnert,
Analytical Chemistry Laboratory,
Jacobs University, Campus Ring 1,
D-28759 Bremen, Germany.
Phone: +49 421 200 3120,
Fax: +49 421 200 3102.
E-mail: n.kuhnert@jacobs-university.de

Submitted to Food Research International Journal. Status: accepted.

Supplementary material available with the published article.

Abstract

Fermentation and drying are the two crucial processing steps required to produce cocoa beans with desired properties, especially taste and flavor. To understand their impact on the lipid profile of cocoa, the lipid composition of unfermented raw and fermented dried beans from six different origins was investigated using high-performance liquid chromatography-mass spectrometry methods. While the comparison of triacylglycerol profiles across the different origins showed only small variations in individual compound concentrations, the comparison along the fermentation status showed major differences regarding the occurrence of polar lipids. These compounds may serve as biomarkers for the fermentation status of the beans and a simple analytical method suitable for field trials is proposed. Finally, a hypothesis identifying key unsaturated triacylglycerols contributing to the hardness and softness of cocoa butter is presented.

Keywords: fermentation; drying; cocoa; origin; triacylglycerol; soft butter; lipid; profile.

5.1 Introduction

The production and marketing of origin chocolate constitute one of the latest trends in the cocoa industry, applying winemakers' concept of terroir to chocolate. Therefore chocolate manufactures spend significant effort on sourcing raw material with specific characteristics form a particular country. Even that might be the trend, price, trade balances, politics, and the volume of cocoa beans available determine the choice of bean origin for lower value cocoa products. The main cocoa bean producing countries are African countries supplying 73.3% of the global production in the last year, and the leading cocoa bean processing countries are European countries grinding 38.7% of the 4.6 million tons of cocoa harvested in 2016 ("The International Cocoa Organization (ICCO) | Cocoa Producing and Cocoa Consuming Countries,") The origin can have an impact for several reasons: first, different countries cultivate botanically different subspecies of *Theobroma cacao*. There are four main cultivars of cocoa: Criollo, Forastero, Trinitario, and Nacional (Lima, Almeida, Rob, & Zwietering, 2011; Rusconi & Conti, 2010). Secondly, the practice of producing dried beans, the first stage of the complex production process of chocolate, cocoa powder and butter, can occur in different ways. Cocoa beans are submitted to an extensive production chain, starting from harvesting the fruits on farms and plantations followed by pod opening and bean removal from the pod. Subsequently, beans are fermented and then dried to less than 8% (Badrie, Bekele, Sikora, & Sikora, 2015; Beckett, Harding, & Freedman, 2008). Drying is essential, both for stopping the fermentation process and reducing moisture thus minimizing microbial growth during transport and storage. Climate conditions at the geographical areas of cultivation have been shown to significantly influence bean chemical composition and quality (Chaiseri & Dimick, 1989; Lehrian, Keeney, & Butler, 1980). Finally, different storage and shipping methods have their contribution to the quality of the dried beans (Beckett, Fowler, & Ziegler, 2017).

Lipids represent approximately half of cocoa beans' chemical constituents. As in many other plant seed oils, triacylglycerols (TAGs) are the predominant species, defining the physical characteristic of cocoa butter (CB). Despite the extensive work made on studying the formation of lipid-related flavor precursors during the fermentation process (Afoakwa, Paterson, Fowler, & Ryan, 2008; Kadow, Bohlmann, Phillips, & Lieberei, 2013) and ample progress on understanding the chemistry of CB, there are few attempts on describing the TAGs in unfermented (please note that the terms wet beans and raw beans are used as well) cocoa beans

and changes of lipids occurring during fermentation (Liendo, Padilla, & Quintana, 1997; Lima et al., 2011). Studies on CB classify cocoa butter as soft and hard butter on origin basis with each butter used in dedicated product applications. It was previously suggested that soft CBs are characterized by higher 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1-stearoyl-2,3-dioleoyl-glycerol (SOO) content (Chaiseri & Dimick, 1989; Lipp & Anklam, 1998) whereas hard CBs are characterized by increased saturated fatty acid content (Chin, 1989). Therefore, Brazilian and Malaysian CBs are generally considered as extremes since Brazilian cocoa butter is rich in di- and tri-unsaturated TAGs (di- and tri- are referred to the number of double bonds in the fatty acid chain), while Malaysian cocoa butter was reported to contain a lower concentration of polyunsaturated TAGs and a higher concentration of saturated TAGs (Figueira et al., 1997; Marty & Marangoni, 2009).

For the description of chemical differences between cocoa beans from varying origin, several different analytical techniques have been employed to profile the typical metabolite classes in cocoa beans including polyphenol profiling, protein profiling, and lipid profiling (Caligiani et al., 2014; Kumari et al., 2016; Marseglia et al., 2016; D'Souza et al., 2017). HPLC-MS provides most chemical information (Hernandez, Castellote, & Permanyer, 1991; Lísa, Holčapek, & Boháč, 2009) to profile the chemical composition of CB including minor lipids and species co-eluting with each other.

Previously we presented data concerning the TAGs diversity in wet unfermented cocoa beans, identifying 83 TAG species in these beans. To achieve this, we have developed a suitable analytical procedure, which employs HPLC coupled to mass spectrometry (MS) (Sirbu, Corno, Ullrich, & Kuhnert, 2018). We now apply this method to assess the differences in the lipid profile specific to geographic origin and the process status of the samples. In this manuscript, we profile TAGs in unfermented wet beans and fermented dried beans from six different origins including Brazil, Ecuador, Ivory Coast, Tanzania, Malaysia, and Indonesia. These countries were chosen mostly based on their global cocoa production, as well as sourcing opportunities and geographical diversity. Multivariate statistical analysis has been employed to confirm the key compositional difference in TAG profiles when comparing unfermented and fermented beans, as well as to visualize the chemical differences according to origins of the seeds identifying in the process biomarkers allowing a clear distinction between sample groups. This is the first study examining the TAG profile in unfermented raw and fermented dried cocoa beans.

5.2 Material and methods

5.2.1 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany), isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade), chloroform (Rotisolv® HPLC grade) and Tetra-dodecylammonium bromide was purchased from Carl Roth (Karlsruhe, Germany), dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany), ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethanol was subjected to distillation prior use.

5.2.2 Sample preparation

Frozen fresh unfermented cocoa beans samples and dried fermented cocoa beans samples from 6 different origins were received in several sets from Barry Callebaut. A total of 24 pairs of samples coming from Ivory Coast, Indonesia, Ecuador, Tanzania, Malaysia, and Brazil were analyzed. Firstly, the seed material was deshelled and ground using a grinder with the purpose of making homogenous powder. Henceforth, the samples were extracted employing an overnight Soxhlet (Buchi Extraction System B811 instrument, Flawil, Switzerland) method using dichloromethane as an extraction solvent, 150 mL and 5 g of powdered beans. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis. For HPLC analysis, a concentration of 0.045 mg/mL in chloroform/ethanol (50/50) of cocoa lipid extract was prepared.

5.2.3 Moisture analysis

Moisture content was determined by heating around 2.5 g of cocoa powder in the oven at 105 °C for 24 hours, cooled in the dry atmosphere of a desiccator, and then reweighed. Total lipid content was normalized according to: $= \frac{\text{Lipid extract (g)} \times 100}{\text{Cocoa Powder (g)} - \text{Weight Loss (g)}}$

5.2.4 TLC separation

Total lipid extracts were dissolved in chloroform/ethanol (50/50) at the concentration of 10 mg/mL and applied to the origin of a Silica Gel 60 HPTLC plate 20 cm x 20 cm (Macherey-Nagel, München, Germany). The dried lipid spots were developed with hexane/diethyl ether/glacial acetic acid (80:20:1, by vol.). Plates were allowed to dry and run another time with chloroform/methanol/glacial acetic acid (97:3:1, by vol.). After a second complete drying time, the plate was sprayed with Cerium Molybdate Stain (Hanessian's Stain) in order to visualize the separated spots of the lipid extract (Christie & Han, 2010).

5.2.5 HPLC chromatographic conditions

TAGs molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). The column used in this study was a Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 10 mM/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. The gradient elution program consisted of holding solvent steady A/B (40/60) for 3 min; followed by a linear gradient to solvent B (100) for 33 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 40/60 solvent A/B for 5 min before reuse. Tetra-dodecyl ammonium bromide at the concentration of 0.001 mg/mL, mass-to-charge (*m/z*) 690.7850, and retention time (R_t) 4.5 min was used as internal standard.

5.2.6 High-resolution Mass spectrometry conditions

High-resolution masses were acquired using a time of flight MicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of *m/z* 200–1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-

port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode.

5.2.7 Statistical analysis

The individual TAG lists of the high-resolution masses were combined for peak matching and retention time alignment using a *m/z* tolerance of $\Delta m/z = 0.0001$ and a R_t tolerance of 0.5 min for elution time <20 min and 1.0 min for an elution time ≥20 min. Extensive manual curation resulted in a set of 126 peaks whereas for 74 peaks a TAG could be uniquely assigned with *m/z* and R_t tolerances of $\Delta m/z = 0.01$ and $\Delta R_t = 0.8$ min. For further analyses, the peak areas were normalized to the peak area of the internal standard (Tetra-dodecyl ammonium bromide). Reproducibility data on the internal standard, are given in the supplementary material (Appendix, Table C.1). For TAG nomenclature we use the common three-letter shorthand, for example, POO, SOO, POL or POS to designate the three acyl substituent on the glycerol moiety (P – palmitic acid, O - oleic acid, S - stearic acid, L linoleic acid). TAG identification was based on high-resolution mass spectrometry establishing molecular formulae followed by tandem mass spectrometry using a quadrupole ion trap mass spectrometer. Fragment spectra allowed the definition of the three TAG acyl moieties as well as their regiochemistry (Sirbu, Corno, Ullrich, & Kuhnert, 2018). To allow relative quantification an internal standard was added to each sample and peak areas normalized with respect to the internal standard.

Each one-way analysis of variance (ANOVA) was conducted for the normalized peak areas of the 12 major TAGs providing the country of origin classification with a significance level of $p < 0.05$. In case of significant effects for multiple comparisons, the ANOVAs are followed by post-hoc pairwise T-tests with multiple comparisons using Benjamini-Hochberg procedure ($\alpha = 0.1$) (Benjamini & Hochberg, 1995, 2000).

The hierarchical clustering of the scaled normalized peak areas excluding the 12 TAGs was calculated using averaged linkage method and the Hamming distance metric. The peak profiles in the attached heat map were sorted column-wise in ascending retention time order. The herein performed principal component analyses (PCA) were applying mean centering and Pareto-scaling on the respective normalized data (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006). For highlighting the data separation, colored two-standard deviational ellipses were added for each group based on the mean standard deviation, and the covariance of the

corresponding data. Additionally, symbol coloring and symbol shape facilitate the group assignment concerning country of origin and fermentation status, respectively.

For the softness assessment, the relative normalized peak areas were used as cumulative values and ratio, respectively. More precisely, the sum of particular TAGs was considered and the ratio of TAGs with specific properties, i.e., poly-unsaturated, mono-unsaturated and saturated ones.

5.3 Results and discussion

For this study, a sample collection was chosen, comprising 24 pairs of unfermented and fermented dry cocoa beans from six different countries of origin.

5.3.1 Total lipid content

Soxhlet extraction enabled us to obtain an extraction yield ranging from 49% to 63% of total lipid content in cocoa beans. Results showed that fermentation and drying lead to an increase of the lipid amount in the beans. Considerable variations in the total lipid content were observed for several origins, while there were no significant differences when comparing countries (Fig. 5.1). However, African samples appear to have a slightly higher total lipid content in comparison to South American and South Asian samples. These differences can be mostly attributed to biological variabilities. Lehrian and Keeney estimate an increase between 4 and 20% of the fat during the last ten days of cocoa fruit ripening (Lehrian & Keeney, 1980).

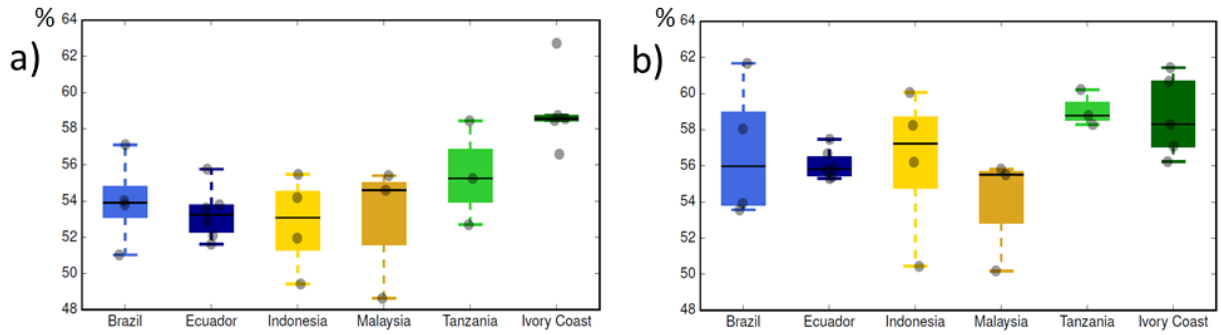


Figure 5.1 Box plots of total lipid content determined gravimetrically following Soxhlet extraction for **(a)** unfermented raw and **(b)** fermented dried cocoa beans from different countries of origin.

Other factors such as the variety of cocoa, environmental conditions, and the harvesting and post-harvesting conditions account for the differences observed (Beckett, Fowler, & Ziegler, 2017). Using a much larger sample set compared to these authors our study estimates the increase of lipid content during fermentation to 3-6%.

5.3.2 Triacylglycerol profiles

Total lipid extracts were firstly investigated by thin layer chromatography (TLC). The TLC showed a separation of cocoa lipids into five distinct fractions. Direct comparison of fermented and unfermented beans revealed the absence of a spot at retention factor (R_f) of 5.5 in fermented dry beans (Fig. 5.2 (a)). Subsequent use of ESI-HPLC (Fig. 5.2 (b)) coupled to a TOF mass spectrometer has confirmed those differences with unfermented beans displaying a variety of peaks between 10 and 22 min retention time, which are absent in dried fermented beans. To appreciate the full complexity of the lipid profile we present 2D chromatograms displaying retention time on the x-axis and *m/z* values on the y-axis (Fig. 5.2 (c) and (d)). 117 TAG compounds were detected in unfermented beans and as inferable from the 2D map and around 70 TAGs in fermented samples. A full list of detected TAGs is provided along with their MS relative quantification data in the supplementary information (Appendix, Tables C.2-C.4). Further chromatograms and 2D graphs of samples for the other five origins to allow direct comparison are presented in the supplementary material (Appendix, Fig. C1-C5). The relative composition as percentage of total lipids measured as the sum over all LC-MS peak intensities of the 25 most abundant TAGs in cocoa lipid extract from different geographical sources is presented in Table 5.1. The relative quantification was carried out from extracted ion chromatograms (EICs) by normalization with respect to the internal standard, assuming that the detector response for all TAG-classes was identical. Our findings revealed that the amounts for the three main TAGs were overestimated whereas values of minor TAGs were underestimated if compared to literature data (Chaiseri & Dimick, 1995; Podlaha, Toregard, & Puschl, 1984; Shukla, 2005). This fact is probably due to the limitations of previously employed TAG detection by refractive index detectors, not accounting for co-eluting minor TAG species.

As it can be deducted from data presented in Table 5.1, cocoa TAG profiles are fairly variable across geographical origins. Figure 5.3 displays variations of key TAGs. Key TAGs denote those TAGs that were considered to show crucial variations by other authors (Chaiseri

& Dimick, 1989; Lipp et al., 2001; Podlaha et al., 1984; Shukla, 2005; Simoneau, Lipp, Ulberth, & Anklam, 2000; Vieira, Efraim, Van, De, & Dewettinck, 2015). The relative quantities of the key TAG species show variations of up to ± 20% if unfermented and dried fermented bean pairs. For instance, 9.5% POP was observed in unfermented Malaysian beans compared to 10.8% in dried fermented beans or 27.3% SOS in Ecuadorian unfermented beans compared to 31.3% in the corresponding dried fermented beans. These difference seemed to result from process variability, in terms of fermentation and drying, but no clear trend was observed. Considerably higher variations of individual TAGs were observed comparing samples from different origins. These variations extend up to 2.5 fold difference for certain TAGs, for example, POP showed a maximum 16.1% and a minimum of 6.9%, whereas SOS showed a maximum of 42.8% and a minimum of 22.7%. Besides the aspects of variability discussed above, these large differences might cover a significant biological variability concerning cocoa varieties and ripeness degree of cocoa pods. Despite what stated above, TAG lipid profile of cocoa beans from some countries has shown significant differences.

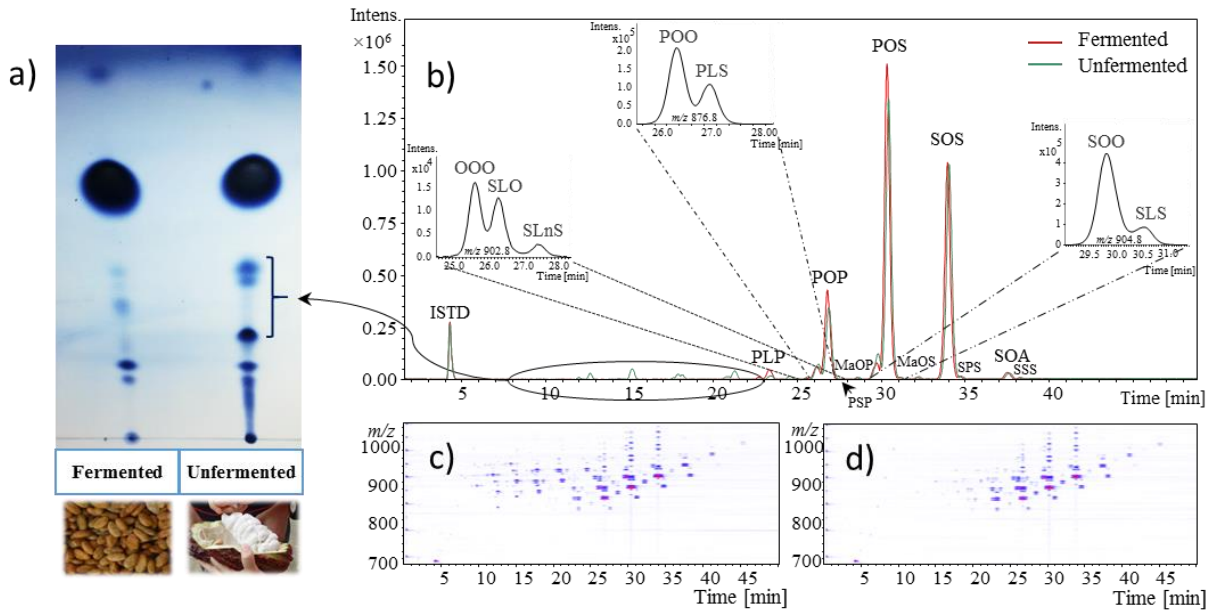


Figure 5.2 Representative chromatography results for fermented dried and unfermented raw cocoa lipid extract of Ivory Coast origin. **(a)** TLC plate image and the parallel corresponding **(b)** HPLC-MS chromatograms in positive ion mode (P – palmitic acid, O - oleic acid, S – stearic acid, L – linoleic acid, Ln – linolenic acid, Ma – margaric acid, A – arachidic acid). **(c)** Two-dimensional (2D) map of reversed-phase high-resolution MS of unfermented raw and **(d)** fermented dried cocoa bean samples from Ivory Coast. The colormap of the 2D map represents the peak intensity with red being the highest and blue the lowest.

Table 5.1 Triacylglycerol composition of cocoa lipid extract of different origins*

	Brazil		Ecuador		Tanzania		Ivory coast		Indonesia		Malaysia	
TAG (%)	U	F	U	F	U	F	U	F	U	F	U	F
POS	44.21(±1.81)	43.97(±1.65)	44.36(±2.59)	44.2(±2.98)	41.87(±1.55)	43.44(±0.71)	41.94(±2.15)	41.72(±1.34)	45.47(±0.33)	43.97(±1.47)	45.27(±2.99)	45.43(±2.22)
SOS	25.54(±2.89)	24.38(±3.92)	28.2(±3.37)	29.27(±3.99)	28.21(±3.98)	29.26(±4.56)	37.83(±3.06)	36.43(±2.27)	27.86(±2.37)	28.004(±0.97)	33.97(±6.5)	31.96(±2.91)
POP	14.85(±2.23)	15.89(±3.21)	13.23(±2.29)	12.83(±2.51)	13.86(±2.47)	12.88(±1.85)	9.8(±2.12)	10.27(±1.84)	14.1(±0.81)	14.32(±0.64)	11.59(±4.06)	12.18(±2.25)
PLS	2.69(±0.17)	3.43(±0.44)	1.86(±0.57)	2.42(±0.6)	2.67(±0.02)	2.75(±0.22)	1.45(±0.72)	2.33(±0.24)	1.87(±0.32)	2.05(±0.28)	1.32(±0.11)	1.87(±0.46)
SOO	3.01(±1.13)	2.78(±1.25)	2.67(±1.55)	2.75(±1.58)	3.29(±0.09)	2.6(±0.52)	1.45(±0.83)	1.61(±0.7)	2.05(±0.62)	2.24(±0.66)	1.04(±0.35)	1.62(±0.8)
SLS	2.24(±0.29)	2.67(±0.17)	1.64(±0.36)	1.82(±0.84)	2.14(±0.42)	2.32(±0.36)	1.6(±0.5)	2.34(±0.32)	1.6(±0.23)	1.65(±0.14)	1.5(±0.3)	1.8(±0.48)
POO	2.06(±0.39)	2.02(±0.43)	1.91(±1.02)	1.87(±0.95)	2.15(±0.37)	1.79(±0.69)	0.73(±0.27)	0.9(±0.25)	1.53(±0.48)	1.64(±0.4)	0.69(±0.18)	1.06(±0.35)
PLP	0.75(±0.07)	1.04(±0.24)	0.58(±0.22)	0.79(±0.38)	1.06(±0.34)	1.02(±0.41)	0.33(±0.18)	0.59(±0.05)	0.76(±0.19)	0.88(±0.14)	0.33(±0.03)	0.53(±0.13)
SOA	0.63(±0.01)	0.59(±0.037)	0.97(±0.16)	0.9(±0.17)	0.91(±0.27)	0.8(±0.18)	1.05(±0.12)	0.99(±0.11)	1.16(±0.13)	1.26(±0.2)	1.07(±0.1)	0.9(±0.15)
SOL	0.38(±0.09)	0.46(±0.08)	0.18(±0.05)	0.27(±0.09)	0.36(±0.03)	0.38(±0.11)	0.1(±0.07)	0.21(±0.04)	0.2(±0.06)	0.24(±0.06)	0.09(±0.03)	0.2(±0.12)
POL	0.24(±0.03)	0.33(±0.06)	0.13(±0.05)	0.19(±0.07)	0.27(±0.04)	0.29(±0.13)	0.06(±0.03)	0.13(±0.01)	0.16(±0.05)	0.2(±0.06)	0.06(±0.02)	0.13(±0.07)
SPS	0.25(±0.06)	0.26(±0.05)	0.41(±0.09)	0.34(±0.11)	0.35(±0.1)	0.34(±0.07)	0.44(±0.13)	0.45(±0.14)	0.44(±0.07)	0.54(±0.19)	0.42(±0.06)	0.36(±0.07)
OOO	0.20(±0.08)	0.22(±0.08)	0.19(±0.15)	0.19(±0.11)	0.19(±0.04)	0.18(±0.06)	0.05(±0.03)	0.08(±0.04)	0.13(±0.06)	0.18(±0.07)	0.07(±0.03)	0.11(±0.04)
PSP	0.19(±0.06)	0.19(±0.05)	0.28(±0.08)	0.24(±0.1)	0.26(±0.08)	0.22(±0.04)	0.27(±0.1)	0.27(±0.08)	0.33(±0.04)	0.36(±0.1)	0.27(±0.04)	0.25(±0.09)
SOMa	0.17(±0.04)	0.19(±0.02)	0.25(±0.06)	0.22(±0.05)	0.22(±0.05)	0.18(±0.03)	0.22(±0.05)	0.23(±0.05)	0.23(±0.008)	0.3(±0.04)	0.21(±0.06)	0.17(±0.04)
POMa	0.17(±0.05)	0.18(±0.03)	0.22(±0.07)	0.19(±0.06)	0.18(±0.06)	0.16(±0.05)	0.15(±0.03)	0.16(±0.03)	0.19(±0.02)	0.25(±0.04)	0.16(±0.03)	0.15(±0.03)
MOO	0.09(±0.01)	0.09(±0.01)	0.11(±0.04)	0.12(±0.03)	0.12(±0.04)	0.1(±0.05)	0.04(±0.009)	0.06(±0.01)	0.11(±0.03)	0.11(±0.02)	0.04(±0.006)	0.06(±0.02)
AOO	0.12(±0.03)	0.11(±0.03)	0.14(±0.06)	0.13(±0.05)	0.14(±0.04)	0.12(±0.02)	0.08(±0.03)	0.09(±0.02)	0.15(±0.03)	0.15(±0.02)	0.08(±0.02)	0.09(±0.01)
SAS	0.12(±0.03)	0.12(±0.02)	0.24(±0.05)	0.19(±0.05)	0.2(±0.08)	0.19(±0.03)	0.29(±0.04)	0.3(±0.06)	0.27(±0.04)	0.31(±0.11)	0.26(±0.04)	0.2(±0.04)
SOB	0.13(±0.01)	0.11(±0.01)	0.16(±0.04)	0.16(±0.03)	0.16(±0.03)	0.14(±0.03)	0.16(±0.03)	0.16(±0.02)	0.22(±0.03)	0.23(±0.06)	0.18(±0.03)	0.17(±0.04)
PLnS	0.06(±0.004)	0.08(±0.01)	0.06(±0.02)	0.08(±0.02)	0.08(±0.02)	0.08(±0.02)	0.03(±0.02)	0.06(±0.006)	0.08(±0.02)	0.1(±0.01)	0.05(±0.002)	0.06(±0.01)
MOP	0.06(±0.003)	0.07(±0.01)	0.09(±0.02)	0.08(±0.02)	0.09(±0.04)	0.08(±0.03)	0.06(±0.01)	0.06(±0.01)	0.11(±0.03)	0.12(±0.01)	0.06(±0.03)	0.07(±0.02)
PPP	0.06(±0.02)	0.07(±0.02)	0.08(±0.03)	0.07(±0.04)	0.07(±0.03)	0.06(±0.01)	0.06(±0.03)	0.05(±0.02)	0.09(±0.02)	0.11(±0.03)	0.07(±0.03)	0.07(±0.03)
SLnS	0.04(±0.01)	0.05(±0.003)	0.04(±0.009)	0.06(±0.01)	0.06(±0.01)	0.06(±0.007)	0.03(±0.01)	0.05(±0.004)	0.06(±0.01)	0.07(±0.02)	0.04(±0.009)	0.05(±0.01)
PLnP	0.03(±0.004)	0.04(±0.008)	0.02(±0.008)	0.03(±0.01)	0.04(±0.01)	0.04(±0.01)	0.01(±0.006)	0.02(±0.004)	0.03(±0.006)	0.05(±0.003)	0.02(±0.004)	0.025(±0.007)

U - unfermented lipid extract; **F** – fermented lipid extract. All data were expressed as means ± SD. *only the most abundant TAGs are presented in the table, other relative quantities are showed in the supplementary material. P – palmitic acid, O - oleic acid, S – stearic acid, L – linoleic acid, Ln – linolenic acid, Ma – margaric acid, A – arachidic acid, M – miristic acid.

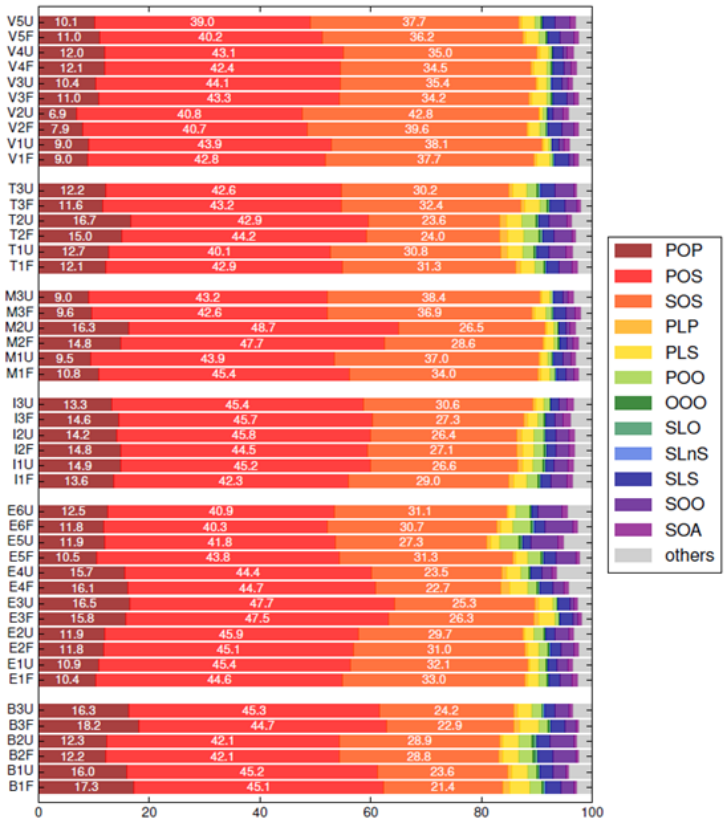


Figure 5.3 Relative abundance of the key 12 TAGs for unfermented and fermented cocoa beans from different countries of origin. The sample labels denote the countries of origin, namely, B – Brazil, E – Ecuador, I – Indonesia, M – Malaysia, T – Tanzania, and V – Ivory Coast, as well as the fermentation status, F – fermented and U – unfermented. The gray painted bars illustrate the remaining detected TAGs.

5.3.3 Comparison of Triacylglycerol profiles of different origins

To obtain a global overview of significant variations of TAGs within the sample set Principal Component Analysis (PCA) of the LC-MS data was used as an unsupervised machine learning method. Both score and loading plot based on 12 most relevant TAGs: 10 most abundant TAGs plus SLnS and OOO having the same *m/z* ratio of SLO are shown in Figure 5.4.

No overall separation according to countries of origin can be observed in the score plots (Fig. 5.4 (a)); however, pairs of countries on a given continent can be distinguished, for example, Brazil *versus* Ecuador, Indonesia *versus* Malaysia, and Tanzania *versus* Ivory Coast. Moreover, sample pairs of unfermented and dried fermented beans are located side by side, indicating that total variance of the key 12 TAGs is not attributed to fermentation status but

rather to the origin. Major contributors to that separation are the high-abundant TAGs POP, POS, and SOS while the remaining TAGs seemed to have the same influence (Fig. 5.4 (b)). Interestingly, the total variation of the entire peak profile is very similar to the 12 key TAGs (Fig. 5.4, Fig. C.6 and Fig. C.7). These observations demonstrated that these key 12 TAGs alone are sufficiently characterize the overall variations among samples.

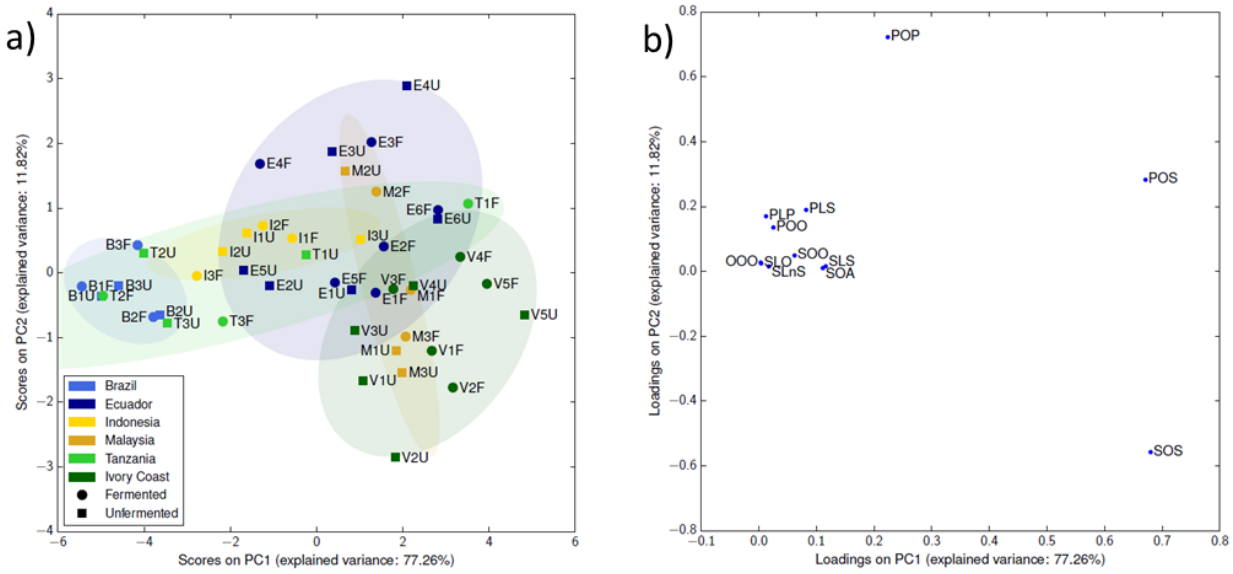


Figure 5.4 PCA of the 12 most relevant TAGs for unfermented and fermented cocoa beans from different countries of origin. The sample labeling and coloring in **(a)** the scores plot as well as the coloring of the two-standard deviational ellipses illustrate the different countries of origin, namely, B – Brazil, E – Ecuador, I – Indonesia, M – Malaysia, T – Tanzania, and V – Ivory Coast. In addition, the sample label as well as the shape indicate the fermentation status, F – fermented and U – unfermented. The labelling in **(b)** the loadings plot denotes the major TAGs.

Therefore, the differences of these 12 key TAGs for unfermented and fermented cocoa beans were further examined across the different countries of origin and visualized using box plots (see Fig. 5.5). Significant differences across the countries of origin for POP, POS, SOS, POO, SLS, and SOA. Using ANOVA, p-values were determined for these 12 key TAGs comparing pairwise samples from two origin countries. The results are visualized in Figure 5.5, lower-right panel. The shading in the pairwise comparison indicates the difference of a given country pair for the 12 TAGs, the brighter the more significant differences were found. Full data are given in the supplementary information (Appendix, Tables C.5-C.10). Thus, dissimilarities as for Ecuador and Brazil become clear as well as country couples which seemed to be more similar. For instance, statistically significant variations of POP, POS SOS, and SLS are

observed if Brazilian and Ivory Coast lipids are compared (bottom left corner) or statistically significant variations of POP, POS SOS, and SLS for the pair Ecuador and Brazil. TAG profiles from Tanzania and Indonesia, however, showed no statistically significant difference in any of the TAG constituents, indicating resemblance between these two countries.

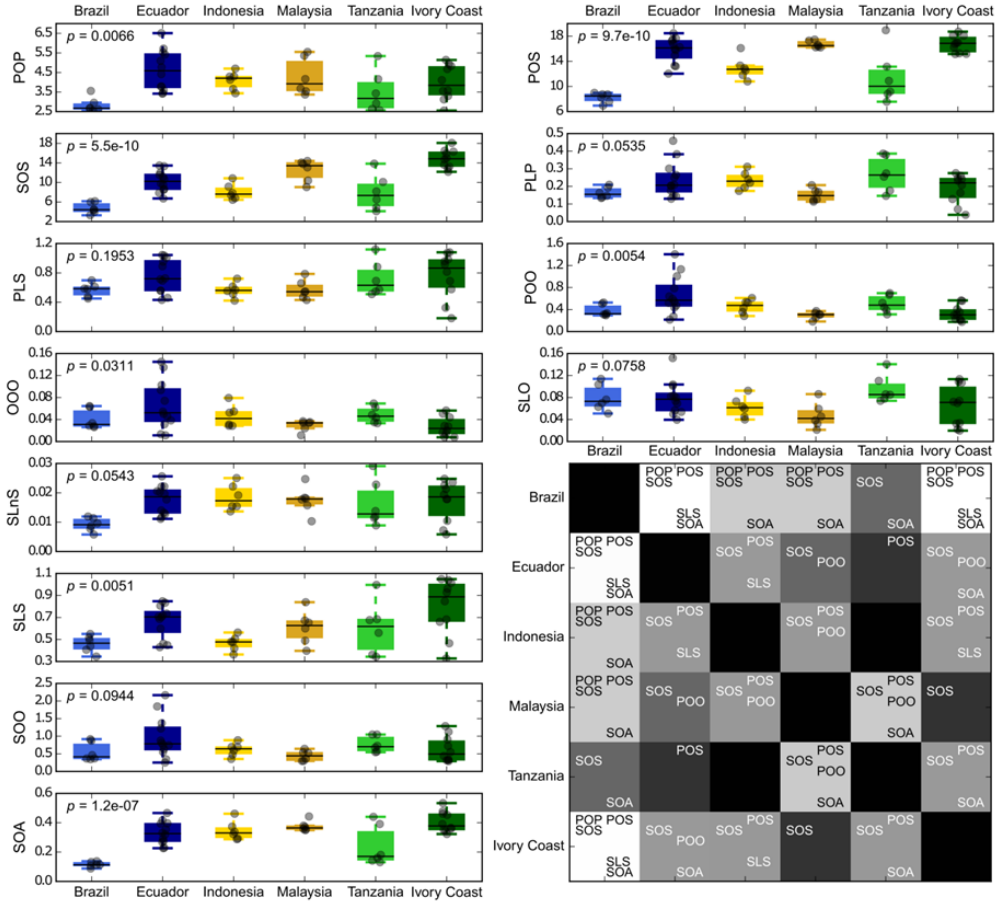


Figure 5.5 Box plots of the 12 TAGs for unfermented and fermented cocoa beans from different countries of origin. The subplot label denotes the significance value (p) of the respective ANOVA. The heat map on the lower right illustrates the pairwise T-tests with a name for each TAG, which is significantly different for the respective country pair. The brightness of the heat map additionally indicates the number of significantly different TAGs per country couple.

5.3.4 Correlation of triacylglycerol profiles with cocoa butter hardness

It is generally accepted that the softness of CB is affected by the ratio between the sum of di- and polyunsaturated TAGs and the sum of monounsaturated and saturated TAGs (Beckett et al., 2008; Chaiseri & Dimick, 1989, 1995; Ribeiro, Correa Basso, et al., 2012; Vieira, Efraim, Van, De, & Dewettinck, 2015). The softness of butters was correlated to some parameters such

as high iodine value and low solid fat content as well as increased POO and SOO content (Lipp & Anklam, 1998; Marty & Marangoni, 2009) leading to unsatisfactory crystallization characteristics (Chaiseri & Dimick, 1995; Ribeiro, Silva, et al., 2012). The main TAGs, namely POP, POS, SOS are solid at room temperature whereas POO and SOO are more liquid at room temperature. POO and SOO together with PLS and SLS are the second most abundant species in cocoa lipid extract, with the last ones not always taken into account by other authors until now (Table 5.1). Using our statistical analysis alternative biomarkers defining softness and hardness of cocoa butter can be identified. Such statistical analysis comparing soft and hard butters with each other reveal according to p-values variations between the ratios of POO/PLS and SOO/SLS after the fermentation and drying of the beans as key markers. EICs illustrating these variations are shown in Figure 5.6 (a) and (c). In Ivory Coast, for example, wet and dried beans showed a different profile of these four TAGs. This phenomenon is also observed in other countries but to a lower extent. In all the samples, the major change noted was the increase in TAGs containing linoleic acid in particular PLS and SLS. However, across all samples the ratios of POO/PLS and SOO/SLS exposed a strong correlation (Pearson correlation coefficient of 0.967). Following this line of argumentation, it can be determined that the first stage of production process affects all the members of the two homologs compounds in combination (Fig. 5.6 (b)).

Figure 5.7 (a) illustrates the assessment of the softness according to the literature. The box plots suggest a tendency of the distribution of the TAGs according to the distance to the equator, hard butters are produced closer to the equator, in contrast, soft butters further away from the equator as highlighted by other authors (Ribeiro et al., 2012; Tucci et al., 1996; Vieira et al., 2015). The same authors describe that the softness of the butter increase according to the average ambient temperature of the cultivation location. Only Ecuador does not follow this trend showing a much larger variability. This variation can be rationalized by the presence of two cocoa varieties Nacional and Forastero (manly CCN51 hybrid) and the diverse climate in this region. Geographic coordinates (GPS data) and hybrid name for all samples are provided in supplementary material (Appendix, Table C.11).

Taking into account the previous reports and the strong correlation between the ratios of POO/PLS and SOO/SLS, we have summed up their contents. Bar plots representation of the sum of the 4 TAGs (Fig. 5.7 (b)) shown to be an identical projection to the bar plots representation of the inferred established way of softness estimation. Therefore, we suggest using the sum of the quantities of these selected TAGs to predict trends of the softness of CB

from any other source. Thus, a descriptive model of a reduced complexity is proposed. This new indicator of softness relies only on the abundance/intensity of four specific TAGs and its determination, accordingly, requires less expenditure. Nevertheless, a standard reference of the four TAGs would be expected as means of comparison. Hence, a prediction regarding the relative softness of any given samples could be done in a faster way routinely.

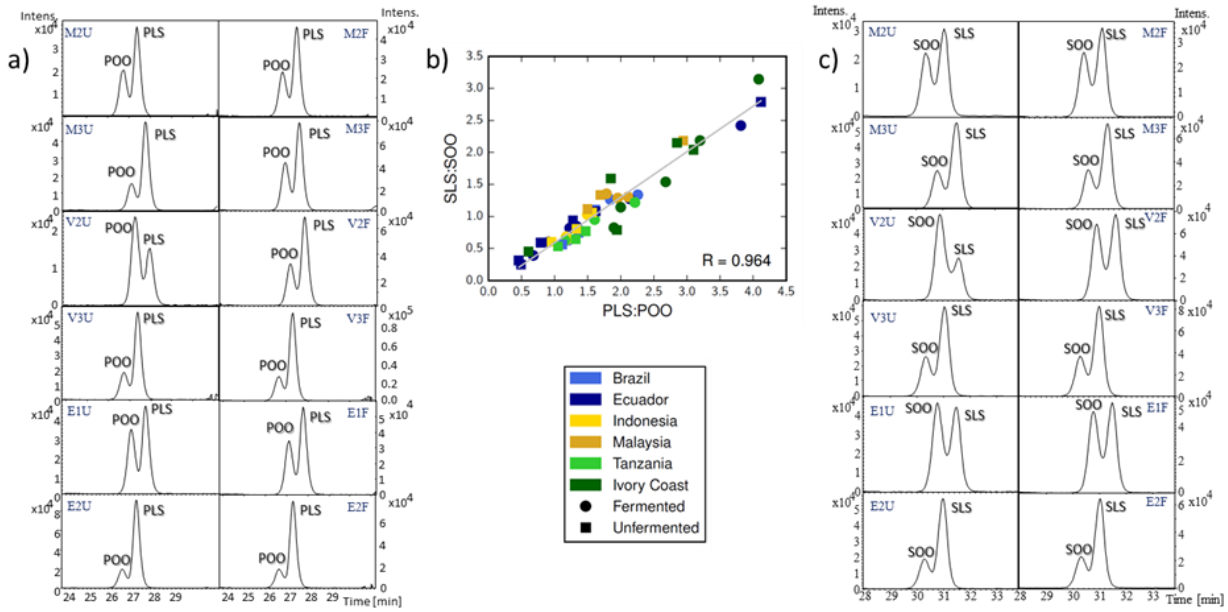


Figure 5.6 (a) Extract ion chromatogram of *m/z* 876.8015 corresponding to POO (left peak) and PLS (right peak); **(b)** Scatter plot on the SLS:SOO ratio and the PLS:POO (of all the samples) and **(c)** Extract ion chromatogram of *m/z* 904.8328 corresponding to SOO (left peak) and SLS (right peak) of 2 Malaysian samples, 2 Ivorian and 2 Ecuadorian samples.

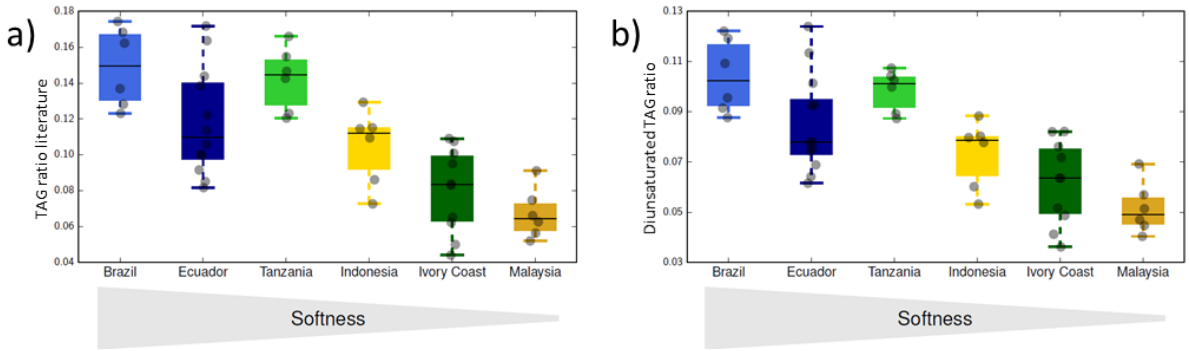


Figure 5.7 (a) Box plots of softness indicators for unfermented and fermented cocoa beans from different countries of origin. As softness indicator serve the established **(a)** ratio of polyunsaturated and monounsaturated plus saturated TAGs and **(b)** the newly conceived cumulative values of POO, PLS, SOO and SLS.

5.3.5 Comparison of triacylglycerol format in unfermented and fermented dried beans

Above, we demonstrated that TLC analysis and LC-MS analysis showed marked differences of TAG profiles when comparing unfermented and dried fermented beans are directly compared. However, this distinction was not apparent in the PCA using the entire TAG peak profile (see Fig. C6), illustrating the failure of PCA to identify key minor biomarkers in the absence of biased normalization or scaling. Since the major 12 TAGs dominate the total variance of the entire peak profile, we decided to perform a modified PCA on the peak profile excluding the data for the 12 key TAGs and secondly carry out a complementary multivariate analysis, in this case, a hierarchical clustering analysis using the LC-MS data. The restricted PCA showed a clear separation of unfermented and dried fermented bean samples in the score plot (Fig. 5.8). While the fermented samples are mainly separated along the second principal component, the unfermented samples showed higher variance across the first two principal components. These first 2 principal components explain together more than 69% of the total variance. Moreover, a hierarchical cluster analysis was performed corroborating the findings of the restricted PCA. In detail, the hierarchical cluster analysis showed a net separation of unfermented from dried fermented beans (Fig. 5.9). Aligning the underlying TAG profiles to the clustering illustrated the major differences of the unfermented and fermented samples. Polar TAGs, here, eluting at retention time from 10 to 20 min, were present in unfermented beans and absent in dried fermented beans.

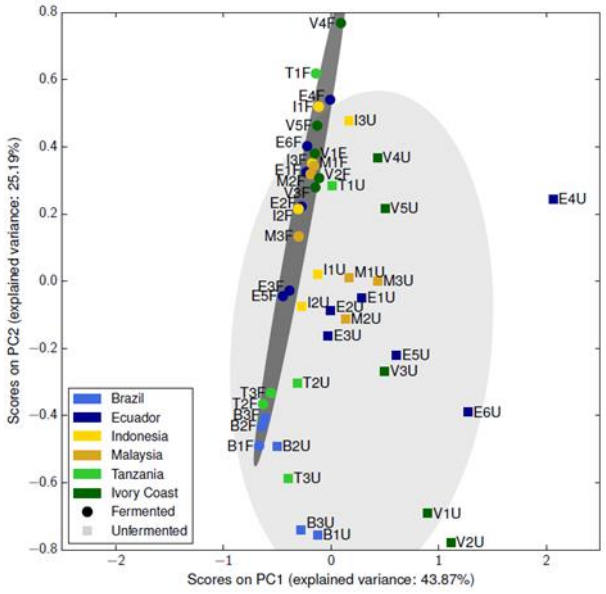


Figure 5.8 PCA of the TAGs profiles from LC-MS data, excluding the major 12 TAGs for unfermented and fermented cocoa beans from different countries of origin. The sample labeling and coloring illustrate the different countries of origin, namely, B – Brazil, E – Ecuador, I – Indonesia, M – Malaysia, T – Tanzania, and V – Ivory Coast. In addition, the sample label, as well as the shape and the coloring of the two-standard deviational ellipses indicate the fermentation status, F – fermented and U – unfermented.

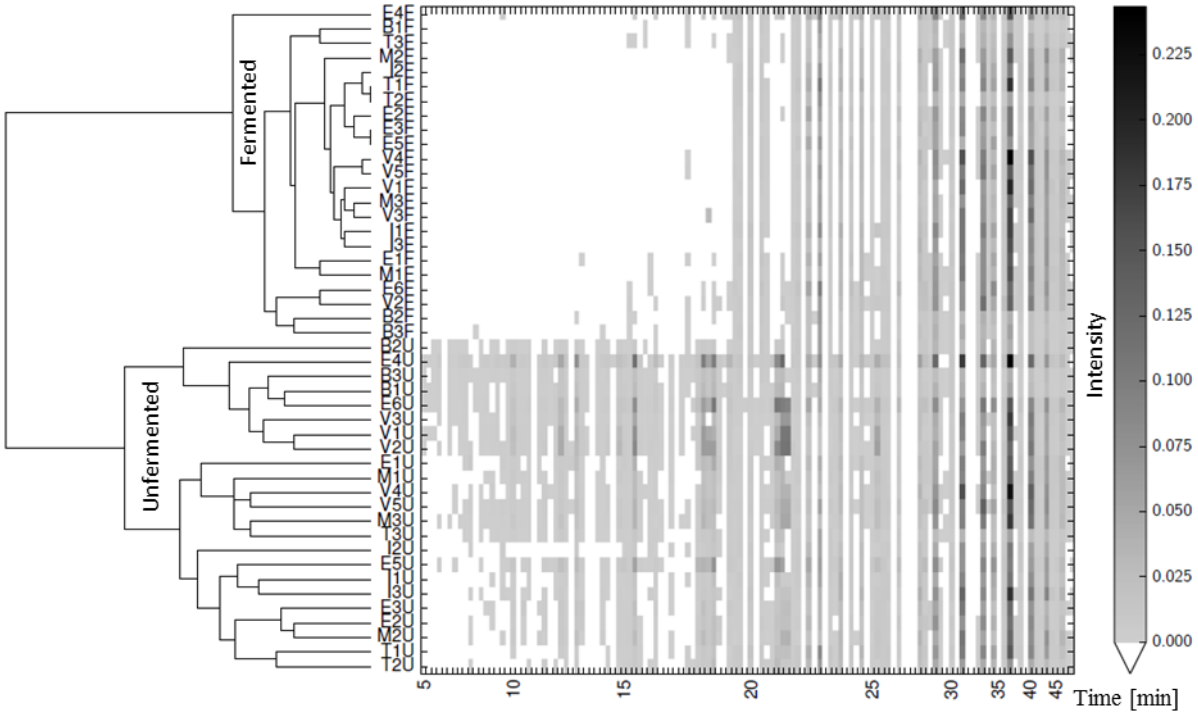
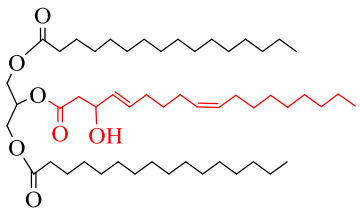


Figure 5.9 Heat map of hierarchical clustering of the peak profiles from LC-MS data, excluding the major 12 TAGs for unfermented (U) and fermented (F) cocoa beans from different countries of origin. The detected peaks are given in ascending retention time order.

Our previous work allowed structural identification of these polar TAGs by a combination of 2D-NMR and mass spectrometry as TAGs with a hydroxyl-allyl fatty acid substituent (Sirbu, Corno, Ullrich, & Kuhnert, 2018). These compounds are apparently powerful biomarkers of cocoa fermentation. At this stage, we propose to name these fatty acid moieties as *cacaoic acid*. A structure of one of the biomarkers is shown in Fig. 5.10.



2-*cacaoyl*-1,3-diyl dipalmitate

Figure 5.10 Proposed structure of a triacylglycerol containing a hydroxyl-allyl fatty acid substituent, namely cacaoic acid.

The identification of such suitable biomarkers for fermentation status constitutes a challenge in cocoa quality control. As described above, TLC analysis using Hanessian's stain provides a simple tool for detecting the presence/absence of TAGs at R_f 5.5 containing cacaoic acid suitable to aid sourcing in origin countries lacking sophisticated scientific infrastructure. Hence we propose to use this TLC method in the assessment of cocoa fermentation status.

5.4 Conclusions

In this manuscript, we extended the understanding of TAG profiles in cocoa of different origins. We have shown, to which degree variations of concentrations of TAGs occur in cocoa butters from different origins. We have, based on multivariate statistical analysis, and proposed a new softness indicator by taking into account only four di-unsaturated TAGs relative quantities. We have revealed that the presence of hydroxyl-allyl fatty acid derived TAGs (now cacaoic acid) is unique to unfermented beans and allows chemical differentiation between unfermented and fermented cocoa beans. A simple analytical test suitable for field work and sourcing, testing for the presence of these biomarkers has been introduced.

References

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

Badrie, N., Bekele, F., Sikora, E., & Sikora, M. (2015). Cocoa Agronomy, Quality, Nutritional, and Health Aspects. *Critical Reviews in Food Science and Nutrition*, *55*(5), 620–659. https://doi.org/10.1080/10408398.2012.669428

Beckett, Stephen T., Harding, J., & Freedman, B. (2008). *The Science of Chocolate* (2nd edition). Cambridge, UK: Royal Society of Chemistry.

Beckett, Steve T., Fowler, M. S., & Ziegler, G. R. (2017). *Beckett's Industrial Chocolate Manufacture and Use*. John Wiley & Sons.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*(1), 289–300. Retrieved from http://www.jstor.org/stable/2346101

Benjamini, Y., & Hochberg, Y. (2000). On the adaptive control of the false discovery rate in multiple testing with independent statistics. *Journal of Educational and Behavioral Statistics*, *25*(1), 60–83.

Caligiani, A., Palla, L., Acquotti, D., Marseglia, A., & Palla, G. (2014). Application of 1H NMR for the characterisation of cocoa beans of different geographical origins and fermentation levels. *Food Chemistry*, *157* (Supplement C), 94–99. https://doi.org/10.1016/j.foodchem.2014.01.116

Chaiseri, S., & Dimick, P. S. (1989). Lipid and hardness characteristics of cocoa butters from different geographic regions. *Journal of the American Oil Chemists' Society*, *66*(12), 1771–1776. https://doi.org/10.1007/BF02660745

Chaiseri, S., & Dimick, P. S. (1995). Dynamic crystallization of cocoa butter. I. characterization of simple lipids in rapid- and slow-nucleating cocoa butters and their seed crystals. *Journal of the American Oil Chemists' Society*, *72*(12), 1491–1496. https://doi.org/10.1007/BF02577842

Chin, A. H. G. (mardi. (1989). Cocoa butter characteristics of Malaysian clonal cocoa [*Theobroma cacao*]. Retrieved from http://agris.upm.edu.my:8080/dspace/handle/0/4247

Christie, W. W., & Han, X. (2010). *Lipid Analysis, Fourth Edition: Isolation, Separation, Identification and Lipidomic Analysis* (4 edition). Bridgwater, England: Oily Press.

Dsouza, R., Grimbs, S., Behrends, B., Bernaert, H., Ullrich, M., & Kuhnert, N. (2017). Origin-based polyphenolic fingerprinting of Theobroma cacao in unfermented and fermented beans. *Food Research International*. https://doi.org/10.1016/j.foodres.2017.06.007

Figueira, A., Lambert, S., Carpenter, D., Pires, J., Cascardo, J. C. M., & Romanczyk, L. (1997). *The similarity of cocoa flavour of fermented seeds from fingerprinted genotypes of Theobroma cacao L. from Brazil and Malaysia* (Vol. 74).

Kadow, D., Bohlmann, J., Phillips, W., & Lieberei, R. (2013). Identification of main fine or flavour components in two genotypes of the cocoa tree (Theobroma cacao L.). *Journal of Applied Botany and Food Quality*, *86*(1), 90–98. https://doi.org/10.5073/JABFQ.2013.086.013

Kumari, N., Kofi, K. J., Grimbs, S., D'Souza, R. N., Kuhnert, N., Vrancken, G., & Ullrich, M. S. (2016). Biochemical fate of vicilin storage protein during fermentation and drying of cocoa beans. *Food Research International (Ottawa, Ont.)*, *90*, 53–65. https://doi.org/10.1016/j.foodres.2016.10.033

Lehrian, D. W., & Keeney, P. G. (1980). Changes in lipid components of seeds during growth and ripening of cacao fruit. *Journal of the American Oil Chemists' Society*, *57*(2), 61–65. https://doi.org/10.1007/BF02674361

Liendo, R., Padilla, F. C., & Quintana, A. (1997). Characterization of cocoa butter extracted from Criollo cultivars of Theobroma cacao L. *Food Research International*, *30*(9), 727–731. https://doi.org/10.1016/S0963-9969(98)00025-8

Lima, L. J. R., Almeida, M. H., Rob, N., & Zwietering, M. H. (2011). Theobroma cacao L., "the food of the gods": Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. *Critical Reviews in Food Science and Nutrition*, *51*(8), 731–761. https://doi.org/10.1080/10408391003799913

Lipp, M., & Anklam, E. (1998). Review of cocoa butter and alternative fats for use in chocolate—Part A. Compositional data. *Food Chemistry*, *62*(1), 73–97. https://doi.org/10.1016/S0308-8146(97)00160-X

Lipp, M., Simoneau, C., Ulberth, F., Anklam, E., Crews, C., Brereton, P., … Wiedmaier, C. (2001). Composition of Genuine Cocoa Butter and Cocoa Butter Equivalents. *Journal of Food Composition and Analysis*, *14*(4), 399–408. https://doi.org/10.1006/jfca.2000.0984

Lísa, M., Holčapek, M., & Boháč, M. (2009). Statistical Evaluation of Triacylglycerol Composition in Plant Oils Based on High-Performance Liquid Chromatography−Atmospheric Pressure Chemical Ionization Mass Spectrometry Data. *Journal of Agricultural and Food Chemistry*, *57*(15), 6888–6898. https://doi.org/10.1021/jf901189u

Marseglia, A., Acquotti, D., Consonni, R., Cagliani, L. R., Palla, G., & Caligiani, A. (2016). HR MAS 1H NMR and chemometrics as useful tool to assess the geographical origin of cocoa beans – Comparison with HR 1H NMR. *Food Research International*, *85*(Supplement C), 273–281. https://doi.org/10.1016/j.foodres.2016.05.001

Marty, S., & Marangoni, A. G. (2009). Effects of Cocoa Butter Origin, Tempering Procedure, and Structure on Oil Migration Kinetics. *Crystal Growth & Design*, *9*(10), 4415–4423. https://doi.org/10.1021/cg9004505

Podlaha, O., Toregard, B., & Puschl, B. (1984). TG-type composition of 28 cocoa butters and correlation between some TG-type components. *Lebensmittel - Wissenschaft + Technologie = Food Science + Technology*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201302561682

Ribeiro, A. P. B., Correa Basso, R., Goncalves, L. a. G., Gioielli, L. A., Oliveira dos Santos, A., Pavie Cardoso, L., & Guenter Kieckbusch, T. (2012). Physico-chemical properties of Brazilian cocoa butter and industrial blends. Part II - Microstructure, polymorphic behavior and crystallization characteristics. *GRASAS Y ACEITES*, *63*(1), 89–99. https://doi.org/10.3989/gya.069111

Rusconi, M., & Conti, A. (2010). Theobroma cacao L., the Food of the Gods: A scientific approach beyond myths and claims. *Pharmacological Research*, *61*(1), 5–13. https://doi.org/10.1016/j.phrs.2009.08.008

Shukla, V. (2005). *Cocoa Butter, Cocoa Butter Equivalents, and Cocoa Butter Substitutes*. https://doi.org/10.1201/9781420039184.ch12

Simoneau, C., Lipp, M., Ulberth, F., & Anklam, E. (2000). Quantification of cocoa butter equivalents in mixtures with cocoa butter by chromatographic methods and multivariate data evaluation. *European Food Research and Technology*, *211*(2), 147–152. https://doi.org/10.1007/s002179900145

Sirbu, D., Corno, M., Ullrich, M. S., & Kuhnert, N. (n.d.). Characterization of triacylglycerols in unfermented cocoa beans by HPLC-ESI mass spectrometry. *Food Chemistry*. https://doi.org/10.1016/j.foodchem.2018.01.194

The International Cocoa Organization (ICCO) | Cocoa Producing and Cocoa Consuming Countries. (n.d.). Retrieved February 1, 2018, from https://www.icco.org/

Tucci, M. L. S., Abreu, M. F. de, Coral, F. J., Futino, A. M., Alfonsi, L. R. R., & Saes, L. A. (1996). Fat and fatty acids content of cocoa clones under the conditions of Vale do Ribeira, State of São Paulo, Brazil. *Bragantia*, *55*(2), 207–213. https://doi.org/10.1590/S0006-87051996000200001

van den Berg, R. A., Hoefsloot, H. C., Westerhuis, J. A., Smilde, A. K., & van der Werf, M. J. (2006). Centering, scaling, and transformations: improving the biological information content of metabolomics data. *BMC Genomics*, *7*, 142. https://doi.org/10.1186/1471-2164-7-142

Vieira, L. R., Efraim, P., Van, D. W., De, C., & Dewettinck, K. (2015). Influence of Brazilian Geographic Region and Organic Agriculture on the Composition and Crystallization Properties of Cocoa Butter. *JAOCS, Journal of the American Oil Chemists' Society*, *92*(11–12), 1579–1592. https://doi.org/10.1007/s11746-015-2728-y

Chapter 6:

6 Fatty acids profiles in unfermented and fermented dried cocoa beans of different origins and correlation with the triacylglycerol profile

Abstract

Fatty acid profile, obtained as FAME data, of unfermented wet cocoa beans and fermented dried cocoa beans from six different origins were analyzed and statistical analysis was applied to assess the significance of the differences observed. Results showed that the origin and the fermentation status have an impact on the fatty acid profile. Samples from Ivory Coast showed a significance value of $p<0.0001$ for pentadecylic acid, margaroleic acid, and margaric acid. Palmitoleic acid and linoleic acid increases after the fermentation whereas gadoleic acid decreases significantly. Besides, correlation studied between the most abundant fatty acids and most abundant triacylglycerols were investigated. A high correlation between palmitic acid and the main triacylglycerols were observed. The relations between fatty acids and triacylglycerols diminished within the fermentation.

Keywords: fatty acids; GC-FID; unfermented cocoa beans; fermented cocoa beans; p value; correlations; variance.

Supplementary material is available on request. E-mail: n.kuhnert@jacobs-university.de

6.1 Introduction

The Aztec emperor Montezuma II (1502-1520) has already recognized the nutritional value of cocoa beans: “The divine drink, which builds up resistance and fights fatigue. A cup of this precious drink permits a man to walk for a whole day without food” (Coe & Coe, 2007). The high-energy values of the cocoa are almost entirely derived from the fat content. One gram of lipid provides around 9 kcal/g, as compared to protein and carbohydrate each yielding around 4.5 kcal/g. The fat content of cocoa consists mostly of palmitic acid, oleic acid, and stearic acid forming a selection of triacylglycerols. The geographical origin influences the composition content of the fatty acids. Thus, palmitic acid content ranges in between 24.1-27.9%, stearic acid between 33.3-37.6%, and oleic acid between 32.7-36.5% (Lipp & Anklam, 1998; Lísa & Holcapek, 2008; Podlaha, Toregard, & Puschl, 1984). Vieira et al. showed a big variability within the Brazilian samples, in which, samples from trees growing in warmer regions shown higher values of saturated fatty acids in contrast to the samples from trees growing in areas of lower temperatures which shown higher values of unsaturated fatty acids (Luana Reis Vieira, Efraim, Walle, Clercq, & Dewettinck, 2015). Torres-Moreno et al. have determined the fatty acid profile in unroasted cocoa beans and chocolates of two different origins, where they have shown that only the origin had an impact on the fatty acid profile. To date, there are no studies showing the influence of the fermentation on the fatty acids profile.

Fatty acids constitute the nutritional components of cocoa butter. Although the chocolate contains large amount of saturated, the health benefits of chocolate are controversial due to presence of the polyphenols having strong antioxidant activity (Lee, Kim, Lee, & Lee, 2003). Epidemiological data have shown that unsaturated fatty acids have a hypocholesterolemic effect, while saturated fatty acids have an opposite effect (Hu et al., 1999). Stearic acid seems to exert a neutral effect on plasma LDL-cholesterol and shown have fewer implications for the progression of coronary heart disease (Kris-Etherton et al., 1993). Although the mechanism of its hypocholesterolemic effect is still not clear, the reason may lay in the limited absorption of this fatty acid from the intestinal tract in contrast to most other lipids (Weisburger, 2001). A short-term cocoa consumption significantly reduced blood cholesterol, but the changes were dependent on the dose of cocoa consumption and the health status of participants (Jia et al., 2010).

The analysis of fatty acids is performed by converting the triacylglycerols to the corresponding fatty acids methyl esters (FAME) (Petrović, Kezić, & Bolanča, 2010). The aim of this study was to evaluate the fatty acid profile of cocoa in unfermented wet beans, and fermented dried beans of different origins. Moreover, a correlation between the fatty acid and triacylglycerol profile was attempted to expand the understanding of the chemistry of cocoa butter.

6.2 Material and methods

6.2.1 Chemicals and reagents

Dichloromethane 99,8% stabilized with amylene for synthesis, potassium hydroxide, sulphuric acid, chloroform, heptane, methanol, ethanol, molecular sieves. All chemicals were purchased from Panreac AppliChem (Darmstadt, Germany), Sigma-Aldrich Chemie (Steinheim, Germany), Carl Roth (Karlsruhe, Germany).

6.2.2 Sample preparation

Frozen fresh unfermented cocoa beans samples and dried fermented cocoa beans samples from 6 different origins (Ivory Coast, Tanzania, Indonesia, Ecuador, Malaysia, and Brazil) were received in several sets from Barry Callebaut. Firstly, the seed material was deshelled and a standardized grinding method were used to grind wet unfermented frozen beans and dried fermented beans, employing the Grindomix Retsch GM 200 equipment with the purpose to make a homogenous powder. Henceforth, an overnight Soxhlet (Buchi Extraction System B811 instrument, Flawil, Switzerland) method using dichloromethane as an extraction solvent, 150 mL of 5 g of powdered beans, was used. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis.

6.2.3 FAME synthesis

Optimization of the synthesis of FAME and the limits of detection and quantification was

achieved after by choosing carefully few suitable methods from the literature. The results of the procedure are presented in the bachelor thesis of Megi Mustafai (May, 2015) under author's supervision. The final optimized method was based on the method developed by O'Fallon et al. with modifications (O'Fallon, Busboom, Nelson, & Gaskins, 2007). The maximum limit of quantification (LOQ) was found at 300 mg and the minimum at 5 mg. Thus, 100 mg initial lipid extract mass was applied for each FAME synthesis method. Method 1 was chosen to extract the bulk samples. More specifically, 0.7 mL 10 N KOH, 5.3 mL methanol and 100 mg initial lipid extract were stirred continuously at 50 ^{0}C for one hour. 500 μL chloroform was added together with potassium hydroxide and methanol, to allow good dissolvement of lipid extracts. The samples were cooled down at room temperature and 0.58 mL 24 N H_2SO_4 was added. All samples were stirred continuously at 50 ^{0}C for one additional hour. FAME were extracted with 2 mL heptane, vortexed for 5 minutes and centrifuged at 3.500 g for 3 minutes. Molecular sieves were added for 10 minutes in the final FAME solution to further neutralize the mixture. Then solution was diluted 1 to 10 and 1 to 20 and 2 vials and nitrogen was blown on every sample to avoid oxidation and then the samples were directly analysed by gas chromatography.

6.2.4 GC-FID method

A capillary column VF-5ms, 30 m x 0.25 mm was used, having an injector temperature of 265 °C and detector temperature 300 °C. Helium was used as a carrier gas, at a flow rate of 16.2 mL/min. The split ratio was 1:10 and the sample injection volume 1.0 μL. GC analysis started at 60 °C for 1 minute, followed by a second step of 10 °C /min temperature rate until 200 °C, with a hold time of 10 minutes, then 7 °C /min rate until 300 °C, at a hold time of 3 minutes and lastly having a temperature rate of 10 °C /min at 300 °C and a final holding time of 10 minutes. Fatty acids identification was performed comparing with a Supelco 37 comp. FAME MIX standard and GC-MS determined according to Elvert at al. by using the same instrument (Elvert, Boetius, Knittel, & Jørgensen, 2003).

6.2.5 Statistical analysis

Microsoft Office Excel 2016 was used to order and process the data. Known compounds were quantified based on the calibration curve for FAME MIX standard using serial dilutions,

for which at least 8 points were acquired (Appendix, Table D.1 and Table D.2). Unknown compounds were relatively quantified using the calibration curve of the nearest lower retention time fatty acid methyl ester standard reference. A one-factor and a two-factors ANOVA were used to study the effect of origin and the effect of fermentation status on fatty acid profile. The analysis were carried out with XLSTAT Evaluation 2018.2 software version 2018 (Addinsoft, France).

6.3 Results and discussion

6.3.1 Fatty acids profile

GC-FID chromatograms shown a high diversity of peaks in FAME cocoa extract (Fig. 6.1). 18 compounds have been positively identified by comparison of retention times to FAME standard mix. Additionally, GC-MS data helped to confirm the identification for the most abundant peaks. Palmitic acid, oleic acid, and stearic acid are the most abundant fatty acids, followed by palmitoleic acid, linoleic acid, and arachidic acid as second-level most abundant fatty acid (Fig. 6.1). The complete fatty acid composition is given in Table D.2 Appendix D, along with the calibration data for the quantification of fatty acids displayed in Table D.1.

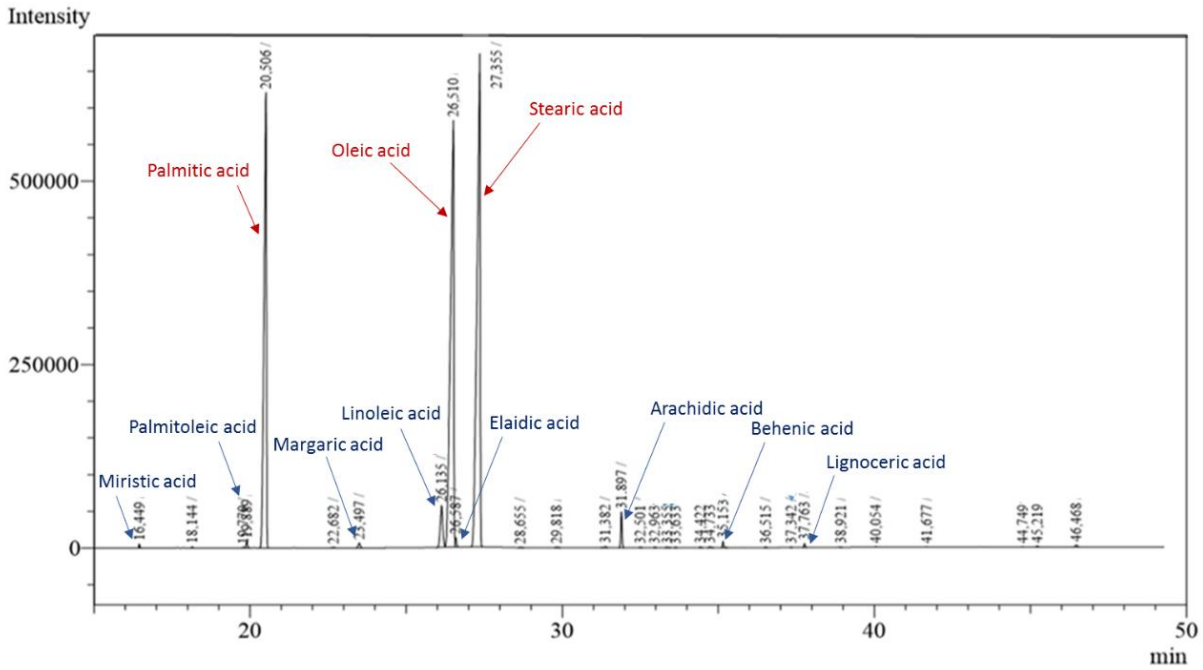


Figure 6.1 Representative GC-FID chromatogram of fatty acids methyl esters of cocoa lipid extract, Ecuador unfermented beans.

As it can be seen from these Tables, palmitic acids varies between 18 and 25% of the total fatty acids, oleic acid between 24 and 34% of the total fatty acids, and stearic acid between 25 and 36% of the total fatty acids. These values showed a significantly larger spread than what is described in the literature to date. A plausible explanation could be a smaller sample number investigated. Authors described a biased normalisation procedure taking only identified fatty acids into consideration. Therefore, we would like to emphasize the importance of a correct evaluation of the fatty acid amount.

Analysis of samples of different origins and fermentation status showed significant differences in the fatty acids profile (Fig. 6.2). The FAME extract of unfermented wet beans demonstrated the presence of a higher number of peaks if compared to the FAME extract of fermented dried beans. Furthermore, already from a first glance, the fermentation process seems to have in impact on the major peaks as well, since differences in the intensity of the peaks could be observed. Representative chromatograms of unfermented wet and fermented dried FAME extract from Ecuador and Indonesia are shown in Figure 6.2.

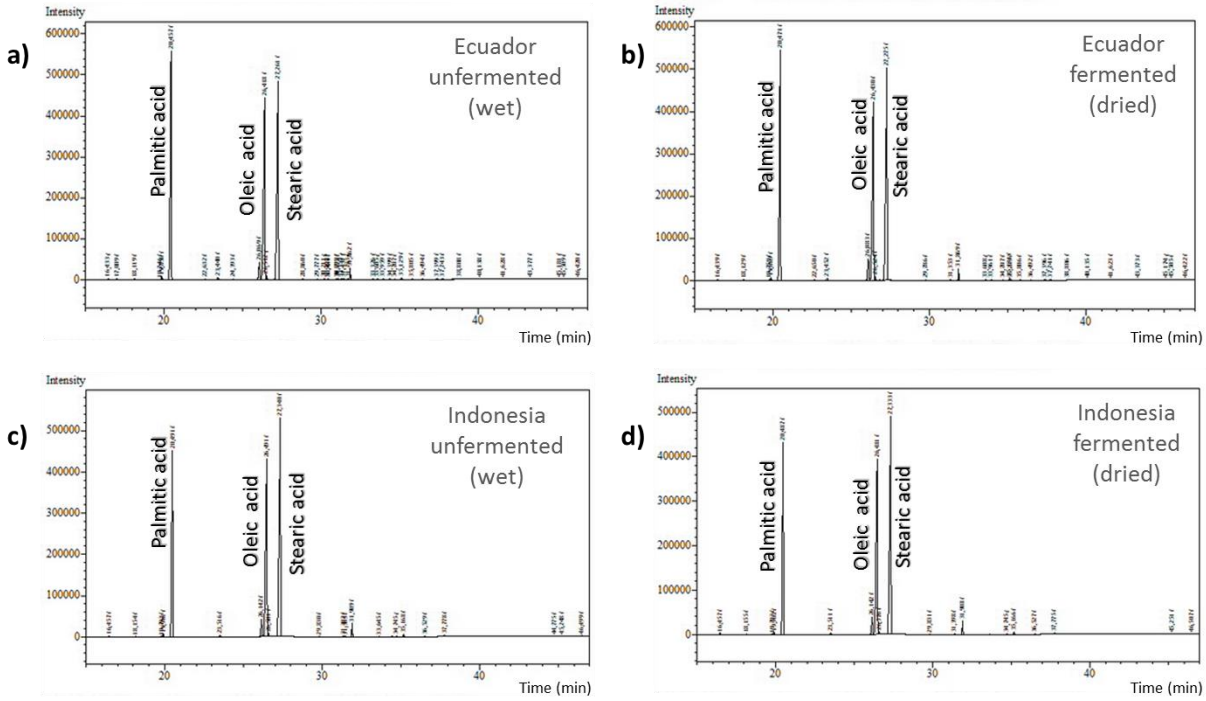


Figure 6.2 Representative GC-FID chromatogram of FAME extract of (a) and (c) unfermented wet beans and (b) and (d) fermented dried beans from Ecuador and Indonesia respectively.

6.3.2 Fatty acids distribution

Figure 6.3 displays box plots indicating the distribution of the most abundant fatty acids if origins are directly compared. From the Figure, it can be deduced that there is an unexpected variability across origins. Nevertheless, few remarks can be made: the difference between the amount of palmitic acid and stearic acid showed to be less in Ecuadorian samples, whereas Brazilian and Ivorian samples indicate a substantial distance between the quantities of these fatty acids. Brazilian and Ecuadorian samples showed higher values of palmitic acid if compared to the other 5 countries. Lower values of palmitic acids are present in Ivorian sample whereas stearic is more abundant in this country with respect to the others. A higher amount of oleic acid is present in Brazilian samples, while other countries maintain almost the same profile. Interestingly, Brazilian samples showed a higher amount of stearic acid and oleic acid and lower amount of palmitic acid if compared to the Ecuadorian samples. Another observation is that linoleic acid profile follows almost the same pattern of oleic acid.

Bar plots of the most abundant fatty acids in unfermented wet and fermented dried illustrated in Figure 6.4 (a) and (b) allow some insights on how the fermentation process is affecting the fatty acids profile. Although all the fatty acids variations are random, linoleic fatty acid in particular, displays a clear increasing after the fermentation with more than 50% increment in some samples.

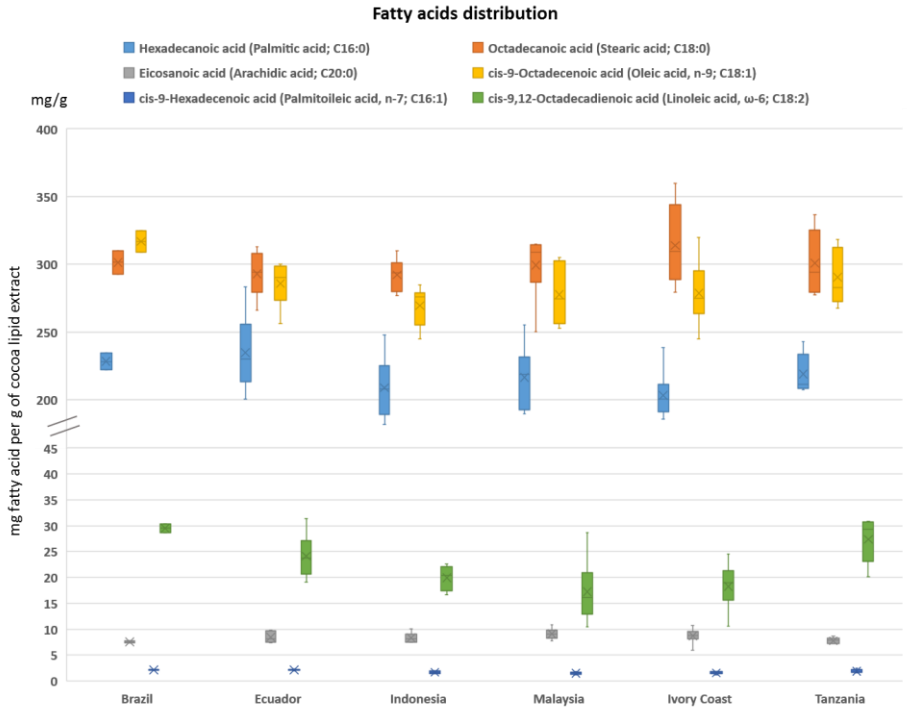


Figure 6.3 Most abundant fatty acid distribution according to the origin.

Figure 6.5 shows the total fatty acids amount the ratio between total unsaturated and total saturated fatty. Large fluctuations in the total fatty acids amount were observed across all origin pairs, defined as unfermented wet beans and fermented dried beans, in which no trend could be reasoned. An increase of the ratio between total unsaturated and total saturated fatty during the fermentation would be desirable for cocoa manufacturers however, it was only observed in some samples from Ecuador, Malaysia, Ivory Coast, and Tanzania.

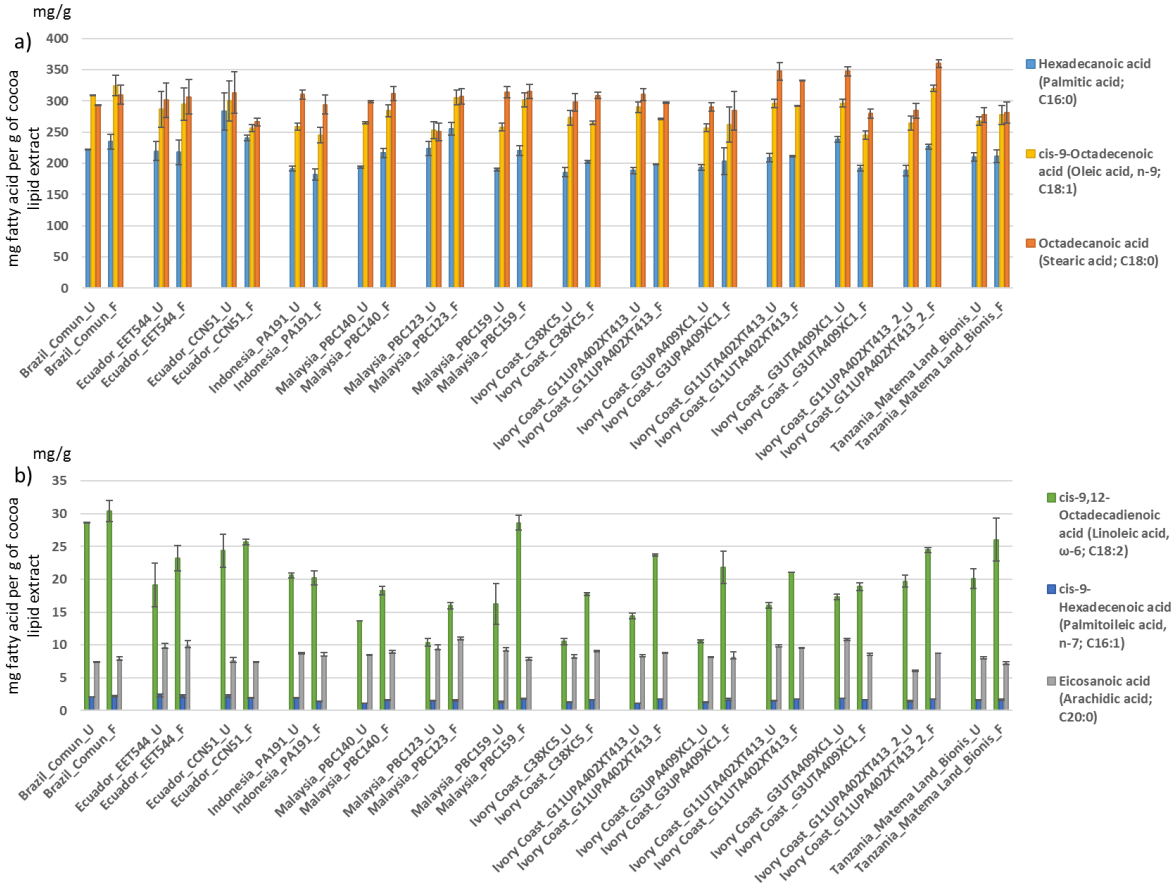


Figure 6.4 (a) Bar plot of the most abundant 3 fatty acids and **(b)** of the other 3 fatty acids in unfermented wet and fermented dried cocoa beans of different origins.

Besides the identified peaks, GC-FID analysis of the fatty acids methyl esters derived from unfermented cocoa beans revealed the occurrence of additional peaks if compared to fermented dried beans, as it can be inferred from the Figure 6.2. From all unknown peaks, 13 of them have shown a high difference between the unfermented wet beans and fermented dried beans (Fig. 6.6). These compounds eluting at retention times between stearic and gadoleic acid were tentatively assigned as hydroxyl fatty acids, well described in Chapter 4.

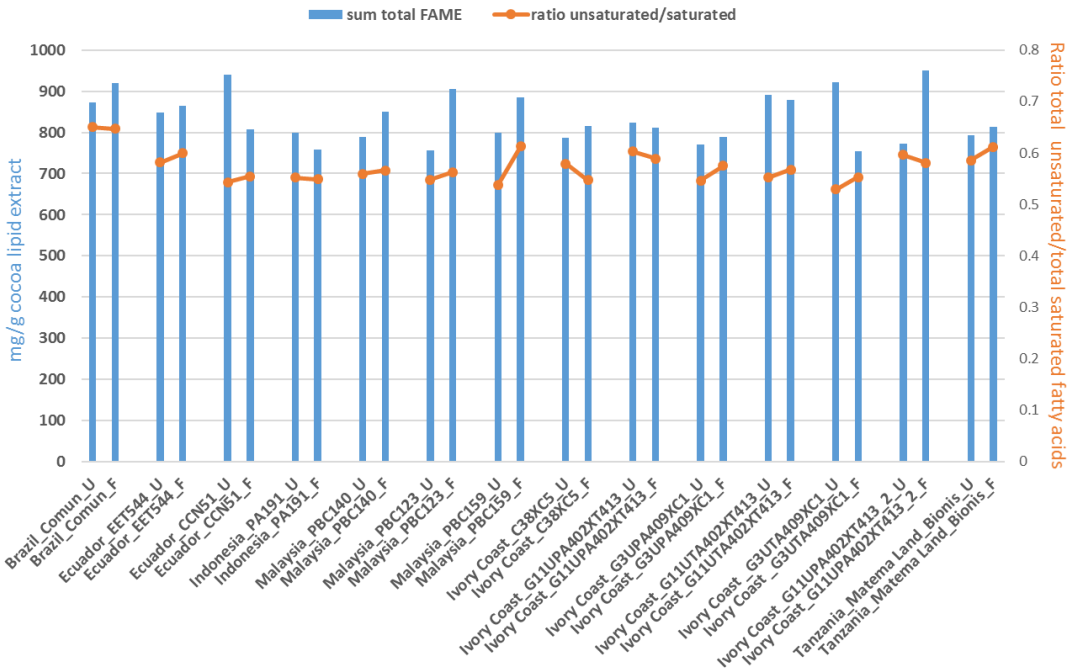


Figure 6.5 Bar plot illustrating the total fatty acids* content in unfermented wet and fermented dried cocoa beans of different origins and their corresponding ratio unsaturated/saturated fatty acids*.

*Only the data of the positively identified peaks were taken into account.

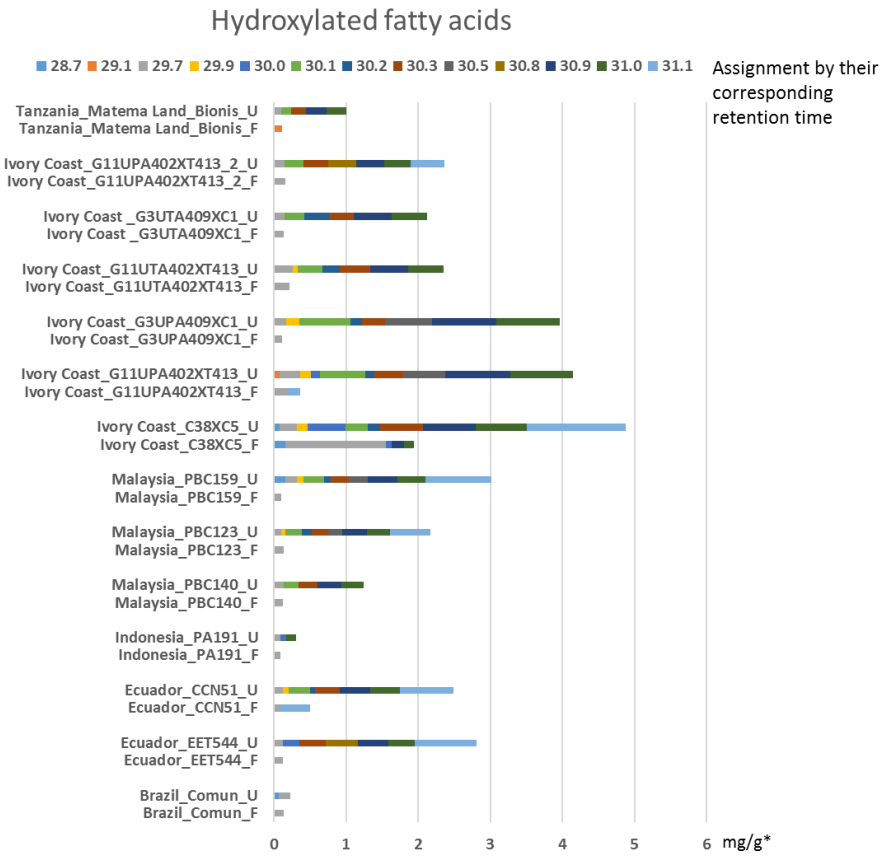


Figure 6.6 Bar plot illustrating the hydroxyl fatty acids content in unfermented wet and fermented dried cocoa beans of different origins. *the amount was calculated as mg of stearic acid/g of cocoa lipid extract.

Unfortunately, the exact assignment could not be achieved as the main fatty acid dictated the concentration level, at which these fatty acids were much diluted and therefore complicated to perform a GC-MS analysis. In all the samples, significant differences between unfermented wet beans and fermented dried beans could be estimated (Fig. 6.6). The total amount of hydroxyl fatty acids, determined as stearic acid equivalents, varied between 0.5 to almost 5 mg/g of cocoa lipid extract in unfermented wet beans, dropping down to less than 0.5 mg/g of cocoa lipid extract in fermented dried beans. These type of fatty acids are described for the first time in cocoa lipid extract, and these results are in complete accordance with what has been found in Chapter 5.

6.3.3 Statistical analysis

One-factor and two-factor ANOVA were used to study the effect of the geographical origin and the fermentation status on the fatty acids profile. For this purpose quantification date were employed. Results showed a significant effect on the following fatty acids: pentadecylic acid, margaroleic acid and margaric acid, p-value <0.0001, concerning origins. Indeed, Ivory Coast has shown higher amounts of these fatty acids. With the same confidence interval at 95% statistical significant difference for myristic acid, palmitoleic acid, linoleic acid, oleic acid, elaidic acid, gadoleic acid, eicosanoic acid, henecosylic acid, and lignoceric acid could be observed. Their p values are given in Table 6.1.

From the analysis of variance, it could be inferred that certainly, the fatty acid profile changes with the geographical origin. Brazilian and Ecuadorian samples showed significant differences in the amount of the short chain fatty acid, whereas Malaysian samples contain a higher amount of long chain fatty acids, such as eicosanoic acid (C20:0), heneicosylic acid (C21:0), and lignoceric acid (C24:0). These results are in line with the data for triacylglycerol (TAGs) profiling, Chapter 5, where Malaysian samples show a higher amount of saturated TAGs and Brazilian and Ecuadorian samples showed a higher amount of unsaturated TAGs. Vieira et al. have observed significant variance for margaric, linoleic and arachidic acid within Brazilian samples from different areas in Brazil (L.R. Vieira, Efraim, Van, De, & Dewettinck, 2015); and Torres-Moreno have observed significant variance for myristic, margaric, margaroleic, palmitic, and stearic acid when comparing samples from Ecuador and Ghana. Very few authors describe the presence of palmitoleic acid and margaric acid in cocoa butter (Rossi, Arnoldi, Salvioni, & Schiraldi, 1989; Torres-Moreno, Torrescasana, Salas-Salvadó, &

Blanch, 2015). Concerning the fermentation status, palmitoleic acid, linoleic acid, and gadoleic acid have shown a significant difference between fermented and unfermented beans. The first two fatty acids increase after the fermentation, whereas gadoleic acid decreases.

Table 6.1 Significance value (p) of the respective ANOVA of the five samples compared to Q1-Tanzania origin as reference point for the origins and Q2-U (unfermented) as reference point for unfermented wet and fermented dried comparison.

Source	Myristic acid	Pentadecylic acid	Palmitoileic acid	Margaroleic acid	Margaric acid	Linoleic acid	Oleic acid	Elaidic acid	Gadoleic acid	Eicosanoic acid	Heneicosylic acid	Lignoceric acid
Q1-Brazil	**0.028**	0.347	0.060	**0.046**	0.470	0.138	**0.038**	0.437	0.875	0.849	0.637	0.762
Q1-Ecuador	**0.020**	**0.040**	**0.020**	**0.009**	0.063	0.342	0.977	0.275	0.734	0.073	0.542	0.206
Q1-Indonesia	0.456	0.559	0.421	0.525	0.904	**0.016**	0.172	0.067	0.597	0.105	0.816	0.393
Q1-Ivory Coast	**0.013**	**< 0.0001**	0.063	**< 0.0001**	**< 0.0001**	**0.0002**	0.789	**0.009**	0.065	**0.003**	0.268	0.133
Q1-Malaysia	0.197	0.152	**0.039**	0.409	0.249	**0.0005**	0.528	**0.003**	0.722	**0.005**	**0.002**	**0.023**
Q1-Tanzania												
Q2-F	0.262	0.566	**0.031**	0.987	0.728	**< 0.0001**	0.108	0.138	**0.002**	0.467	0.122	0.551
Q2-U												

6.3.4 Correlation of fatty acids and triacylglycerols

Correlation studies between the most abundant fatty acids and the most abundant TAGs were evaluated to give more insights whether the fatty acid profile is hybrid-related or it mostly biosynthetically controlled during the fermentation. Figure 6.7 shows a scatter plot of the distribution of oleic acid and the most abundant TAGs in unfermented wet cocoa beans and fermented dried cocoa beans.

R^2 value of 0.69 and 0.77 between oleic acid and POO, and between oleic acid and SOO respectively were obtained. Thus, a correlation between the oleic acid amount and the amount of the TAGs containing two oleic acid moieties was found.

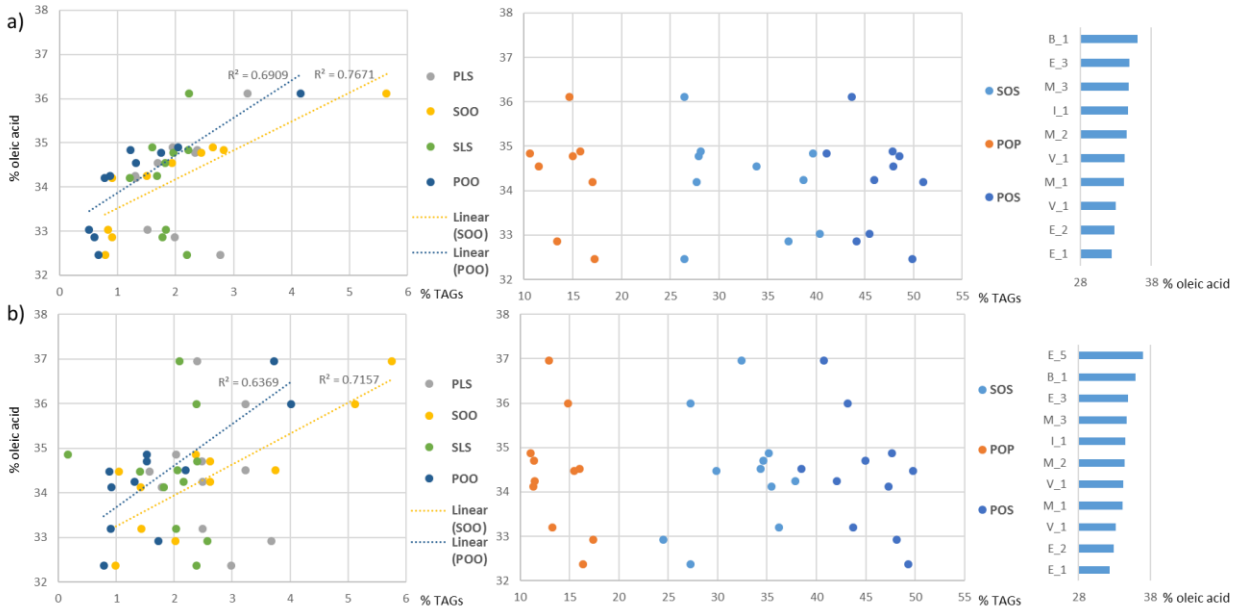


Figure 6.7 (a) Scatter plots illustrating the variation of oleic acid and main TAGs in unfermented wet cocoa beans and **(b)** in fermented dried cocoa beans and of different origins E – Ecuador, M – Malaysia, I– Indonesia, B – Brazil, V – Ivory Coast.

Usually, in statistics, three types of correlations are measured: Pearson correlation, Kendall rank correlation, and Spearman correlation to evaluate the strength of the association between two variables and the direction of the relationship where a value of ± 1 indicates a perfect degree of association between the two set of variables. Thus, to confirm what observed correlation coefficient of Pearson, Kendall, and Spearman were calculated. Results are shown in Table 6.2. The correlation coefficients showed a high negative correlation between palmitic and stearic acid. Palmitic acid showed a high positive correlation between POP and POS and a negative relationship with SOS. On the other hand, stearic acid showed a strong association with SOS and a negative correlation with POP. Linoleic acid showed a positive correlation with all 4 diunsaturated TAGs, namely POO, PLS, SOO, and SLS. From these results, it could be inferred that the profile of the most abundant fatty acids is reflected by the occurrences of the most abundant TAGs. Moreover, the values may give an indication until which degree such reflection is covered. The best correlation was observed between stearic and SOS showing a Pearson correlation coefficient value of 0.945, whereas palmitic acid showed a Pearson

correlation coefficient value of 0.889. This indicates that the contribution of SOS to the total amount of stearic fatty acid is higher than the contribution of POP to the total amount of palmitic acid.

Table 6.2 Correlation matrixes of fatty acid and TAGs in unfermented wet and dried fermented cocoa beans.

Correlation matrix_**Pearson**: UNFERMENTED WET BEANS

Variables	Palmitic acid	Linoleic acid	Oleic acid	Elaidic acid	Stearic acid	POO	POP	PLS	SOO	POS	SLS	SOS
Palmitic acid	**1**	-0.168	-0.271	0.353	**-0.780**	-0.139	**0.889**	0.109	-0.295	**0.792**	-0.287	**-0.747**
Linoleic acid	-0.168	**1**	0.443	0.435	-0.316	**0.718**	0.100	**0.913**	**0.751**	-0.352	**0.881**	-0.337
Oleic acid	-0.271	0.443	**1**	0.174	-0.349	**0.831**	0.008	0.295	**0.876**	-0.255	0.118	-0.286
Elaidic acid	0.353	0.435	0.174	**1**	-0.549	0.294	0.494	0.398	0.201	0.406	0.213	-0.615
Stearic acid	**-0.780**	-0.316	-0.349	-0.549	**1**	-0.461	**-0.882**	-0.483	-0.342	-0.546	-0.008	**0.945**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix_**Pearson**: FERMENTED DRIED BEANS

Variables	Palmitic acid	Linoleic acid	Oleic acid	Elaidic acid	Stearic acid	POO	POP	PLS	SOO	POS	SLS	SOS
Palmitic acid	**1**	0.188	**-0.637**	0.137	**-0.740**	-0.395	**0.707**	0.185	-0.577	**0.771**	0.137	**-0.763**
Linoleic acid	0.188	**1**	-0.172	0.601	-0.431	0.329	0.353	**0.859**	0.250	-0.055	0.498	-0.520
Oleic acid	**-0.637**	-0.172	**1**	0.044	0.023	**0.798**	-0.366	-0.215	**0.846**	-0.510	-0.180	0.171
Elaidic acid	0.137	0.601	0.044	**1**	-0.419	0.319	0.161	0.455	0.318	-0.064	0.262	-0.337
Stearic acid	**-0.740**	-0.431	0.023	-0.419	**1**	-0.274	**-0.662**	-0.343	-0.073	-0.485	-0.191	**0.938**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix_**Kendall**: UNFERMENTED WET BEANS

Variables	Palmitic acid	Linoleic acid	Oleic acid	Elaidic acid	Stearic acid	POO	POP	PLS	SOO	POS	SLS	SOS
Palmitic acid	**1**	0.022	-0.111	0.422	**-0.689**	0.111	**0.822**	0.067	-0.156	**0.644**	-0.200	**-0.689**
Linoleic acid	0.022	**1**	0.333	0.422	-0.244	0.378	0.111	**0.689**	0.378	-0.067	**0.689**	-0.333
Oleic acid	-0.111	0.333	**1**	0.200	-0.200	**0.778**	-0.022	0.200	**0.867**	-0.200	0.200	-0.111
Elaidic acid	0.422	0.422	0.200	**1**	-0.289	0.422	0.422	0.378	0.244	0.156	0.111	-0.378
Stearic acid	**-0.689**	-0.244	-0.200	-0.289	**1**	-0.422	**-0.778**	-0.289	-0.156	-0.422	-0.022	**0.733**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix_**Kendall**: FERMENTED DRIED BEANS

Variables	Palmitic acid	Linoleic acid	Oleic acid	Elaidic acid	Stearic acid	POO	POP	PLS	SOO	POS	SLS	SOS
Palmitic acid	**1**	0.091	**-0.491**	-0.018	-0.418	-0.455	0.382	0.018	**-0.600**	**0.600**	0.018	-0.309
Linoleic acid	0.091	**1**	0.055	0.455	-0.382	0.455	0.273	**0.636**	0.309	-0.091	**0.636**	-0.418
Oleic acid	**-0.491**	0.055	**1**	0.018	-0.018	**0.527**	-0.309	-0.164	**0.600**	-0.382	-0.164	0.018
Elaidic acid	-0.018	0.455	0.018	**1**	-0.345	0.273	0.164	0.236	0.200	0.018	0.382	-0.309
Stearic acid	-0.418	-0.382	-0.018	-0.345	**1**	-0.127	-0.455	-0.164	0.091	-0.382	-0.309	**0.891**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix_**Spearman**: UNFERMENTED WET BEANS

Variables	Palmitic acid	Linoleic acid	Oleic acid	Elaidic acid	Stearic acid	POO	POP	PLS	SOO	POS	SLS	SOS
Palmitic acid	**1**	0.055	-0.188	0.564	**-0.818**	0.139	**0.939**	0.127	-0.261	**0.818**	-0.309	**-0.733**
Linoleic acid	0.055	**1**	0.455	0.636	-0.285	0.564	0.212	**0.867**	0.491	-0.176	**0.855**	-0.442
Oleic acid	-0.188	0.455	**1**	0.345	-0.152	**0.915**	-0.055	0.273	**0.952**	-0.370	0.236	-0.164
Elaidic acid	0.564	0.636	0.345	**1**	-0.442	0.588	0.552	0.515	0.345	0.297	0.188	-0.503
Stearic acid	**-0.818**	-0.285	-0.152	-0.442	**1**	-0.382	**-0.915**	-0.370	-0.055	-0.564	-0.006	**0.903**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix_**Spearman**: FERMENTED DRIED BEANS

Variables	Palmitic acid	Linoleic acid	Oleic acid	Elaidic acid	Stearic acid	POO	POP	PLS	SOO	POS	SLS	SOS
Palmitic acid	**1**	0.091	**-0.673**	0.045	-0.582	-0.536	0.527	0.091	**-0.782**	**0.818**	0.100	-0.545
Linoleic acid	0.091	**1**	0.064	0.591	-0.573	0.555	0.382	**0.782**	0.373	-0.055	**0.782**	-0.591
Oleic acid	**-0.673**	0.064	**1**	0.027	0.055	**0.691**	-0.445	-0.227	**0.800**	-0.500	-0.218	0.073
Elaidic acid	0.045	0.591	0.027	**1**	-0.436	0.300	0.209	0.345	0.255	-0.045	0.527	-0.400
Stearic acid	-0.582	-0.573	0.055	-0.436	**1**	-0.100	-0.609	-0.309	0.127	-0.509	-0.473	**0.964**

Values in bold are different from 0 with a significance level alpha=0.05

Remarkably, a decrease in the value of the correlation coefficient in fermented dried beans if compared to unfermented wet beans was observed. The greatest difference was observed for linoleic acid grieved, which showed no correlation with POO and SOO in fermented dried cocoa beans. The reduction in the value of the correlation coefficient after the fermentation and drying of the beans suggest that the amount of the total fatty acids was subjected to alterations from an external source by adding or subtracting fatty acids. Another hypothesis is that the fermentation process implies chemical modification. Consequently, the fatty acids undergo other chemical transformation pathways. Besides, during the fermentation, as well as during the transportation, the temperature rises, which can entail melting of the low melting point TAGs that consequently could leak outside the beans, therefore, changing the total profile of fatty acids.

6.4 Conclusions

Although fermentation has shown to have a significant impact on the solids of cocoa, evaluation of the changed of the fatty acids profile before and after fermentation was not yet completed. This study shows clear differences supported by statistical analysis between geographical origins and fermentation status of the beans. From the results, key features can be selected to help the quality assessment of cocoa beans. Herein, the quantity of palmitoleic acid, linoleic acid, and unusual fatty acids could be considered as markers of the fermentation status, whereas the quantity of specific saturated fatty acids could indicate the geographical origin. The distinct fatty acids fingerprint may be used in the future to qualify the beans according to their lipid profile. Moreover, it provides insight into how to optimize and tailor fatty acid profile with respect to innovative applications and health products.

References

Coe, S. D., & Coe, M. D. (2007). *The True History of Chocolate* (Second Edition edition). New York: Thames & Hudson.

Elvert, M., Boetius, A., Knittel, K., & Jørgensen, B. B. (2003). Characterization of Specific Membrane Fatty Acids as Chemotaxonomic Markers for Sulfate-Reducing Bacteria Involved in Anaerobic Oxidation of Methane. *Geomicrobiology Journal*, *20*(4), 403–419. https://doi.org/10.1080/01490450303894

Hu, F. B., Stampfer, M. J., Manson, J. E., Ascherio, A., Colditz, G. A., Speizer, F. E., … Willett, W. C. (1999). Dietary saturated fats and their food sources in relation to the risk of coronary heart disease in women. *The American Journal of Clinical Nutrition*, *70*(6), 1001–1008. https://doi.org/10.1093/ajcn/70.6.1001

Jia, L., Liu, X., Bai, Y. Y., Li, S. H., Sun, K., He, C., & Hui, R. (2010). Short-term effect of cocoa product consumption on lipid profile: a meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition*, *92*(1), 218–225. https://doi.org/10.3945/ajcn.2009.28202

Kris-Etherton, P. M., Derr, J., Mitchell, D. C., Mustad, V. A., Russell, M. E., McDonnell, E. T., … Pearson, T. A. (1993). The role of fatty acid saturation on plasma lipids, lipoproteins, and apolipoproteins: I. Effects of whole food diets high in cocoa butter, olive oil, soybean oil, dairy butter, and milk chocolate on the plasma lipids of young men. *Metabolism: Clinical and Experimental*, *42*(1), 121–129.

Lee, K. W., Kim, Y. J., Lee, H. J., & Lee, C. Y. (2003). Cocoa Has More Phenolic Phytochemicals and a Higher Antioxidant Capacity than Teas and Red Wine. *Journal of Agricultural and Food Chemistry*, *51*(25), 7292–7295. https://doi.org/10.1021/jf0344385

Lipp, M., & Anklam, E. (1998). Review of cocoa butter and alternative fats for use in chocolate—Part A. Compositional data. *Food Chemistry*, *62*(1), 73–97. https://doi.org/10.1016/S0308-8146(97)00160-X

Lísa, M., & Holcapek, M. (2008). Triacylglycerols profiling in plant oils important in food industry, dietetics and cosmetics using high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography. A*, *1198–1199*, 115–130. https://doi.org/10.1016/j.chroma.2008.05.037

O'Fallon, J. V., Busboom, J. R., Nelson, M. L., & Gaskins, C. T. (2007). A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *Journal of Animal Science*, *85*(6), 1511–1521. https://doi.org/10.2527/jas.2006-491

Petrović, M., Kezić, N., & Bolanča, V. (2010). Optimization of the GC method for routine analysis of the fatty acid profile in several food samples. *Food Chemistry*, *122*(1), 285–291. https://doi.org/10.1016/j.foodchem.2010.02.018

Podlaha, O., Toregard, B., & Puschl, B. (1984). TG-type composition of 28 cocoa butters and correlation between some TG-type components. *Lebensmittel - Wissenschaft + Technologie = Food Science + Technology*. Retrieved from http://agris.fao.org/agris-search/search.do?recordID=US201302561682

Vieira, L.R., Efraim, P., Van, D. W., De, C., & Dewettinck, K. (2015). Influence of Brazilian Geographic Region and Organic Agriculture on the Composition and Crystallization Properties of Cocoa Butter. *JAOCS, Journal of the American Oil Chemists' Society*, *92*(11–12), 1579–1592. https://doi.org/10.1007/s11746-015-2728-y

Vieira, Luana Reis, Efraim, P., Walle, D. V. de, Clercq, N. D., & Dewettinck, K. (2015). Influence of Brazilian Geographic Region and Organic Agriculture on the Composition and Crystallization Properties of Cocoa Butter. *Journal of the American Oil Chemists' Society*, *92*(11–12), 1579–1592. https://doi.org/10.1007/s11746-015-2728-y

Weisburger, J. H. (2001). Chemopreventive Effects of Cocoa Polyphenols on Chronic Diseases. *Experimental Biology and Medicine*, *226*(10), 891–897. https://doi.org/10.1177/153537020122601003

Chapter 7:

7 Monitoring lipids of *Theobroma cacao* during the fermentation

Abstract

Within the frame of understanding the impact of the fermentation on the lipid profile in cocoa, 6 fermentation trials from 3 different origins were analyzed. Triacylglycerols and fatty acids content showed significant variations between 72 and 96 h. Hydroxyl derivatives of the triacylglycerols decrease entirely after 96 h of fermentation whereas linoleic acid showed a considerable increment throughout the process. The lipid profile of minor components indicated that the microorganisms involved in the fermentation process give their contribution in determining the profile of the less abundant lipid compounds in cocoa.

Keywords: fermentation; triacylglycerols; fatty acids; bacteria; p-value; linoleic acid; 2D map.

Supplementary material is available on request. E-mail: n.kuhnert@jacobs-university.de

7.1 Introduction

The fermentation process is the most important contributing factor to the quality of flavour and final composition of cocoa. The beans of cocoa are surrounded by a mucilaginous pulp rich in sugars and citric acid, which provide an excellent medium for the growth of the yeast and bacteria (Badrie, Bekele, Sikora, & Sikora, 2015; Ho, Zhao, & Fleet, 2014). The fermentation process consists of two phases: an anaerobic phase in the first 24 to 36 h after harvesting and opening the pods; and an aerobic phase after 48 to 96 h, when the beans are turned to allow aeration. In the first phase, yeasts exhibit maximum development after 24 h, after which increased alcohol concentration and pH activates the growth of lactic acid bacteria. During this phase lactic acid, acetic acid, ethanol, and carbon dioxide are created from the pulp sugars. The second phase is characterized by a dynamic development of acetic acid bacteria responsible for the oxidation of ethanol to acetic acid, and the exothermic process leading to a temperature increase in the cocoa mass up to 45-52 °C (Aprotosoaie, Luca, & Miron, 2016; Lima, Almeida, Rob, & Zwietering, 2011). This phase is considered essential for flavour development because of the diffusion of metabolites in the cotyledons causing the death of the embryo and activation of endogenous enzymes (Afoakwa, Paterson, Fowler, & Ryan, 2008; Ho et al., 2014; Schwan & Wheals, 2004). The duration of the fermentation varied between 96 and 168 h in a range of different methods depending on the country traditional common practices (Lima et al., 2011). During the fermentation, major biochemical changes occur (Kadow, Bohlmann, Phillips, & Lieberei, 2013; Kongor et al., 2016). Thus, the physicochemical transformation is the result of the following concatenation of micro-effects: acidification, subcellular decompartimentalization, enzymatic degradation, and components leaching (Lima et al., 2011; Schwan & Wheals, 2004; Ziegleder Gottfried, 2017). Most of the cocoa bean fermentations in the world are performed in a spontaneous process, which shows vast variations due to the inconstant environmental microorganisms. Hitherto, the fermentation can occur under controlled condition using specific culture strains (De Vuyst L. & Weckx S., 2016).

Cocoa pods are harvested at maturation, the time point when the germination of seed plant commences. Therefore, mobilization of the storage lipids, primary carbon source in oil seeds, takes place to offer energy for the germinating seedling (Feussner, Kühn, & Wasternack, 2001). This process is catalysed by Lipoxygenases (LOXs, linoleate:oxygen oxidoreductase, EC

1.13.11.12), a non-heme iron containing dioxygenases that catalyse the formation of hydroperoxy derivatives of polyunsaturated fatty acids (Rosahl, 1996). Moreover, these enzymes monitor the production of multiple other growth regulators, antimicrobial compounds, flavours and odours as well as signal molecules. One of the compounds regulated by LOX is jasmonic acid, a phytohormone, known to be involved growth processes and responses to abiotic and biotic stress (Ghasemi Pirbalouti, Sajjadi, & Parang, 2014; Turner, Ellis, & Devoto, 2002). Patras et al. have identified the presence of jasmonic acid derivative, 12-hydroxyjasmonic acid-O-sulfate (Patras, Milev, Vrancken, & Kuhnert, 2014).

Microbial attack during the fermentation constitutes an enormous stressed induced process that triggers stress response mechanisms in cocoa seeds. A recent study revealed an increase of the antioxidant capacity in fermented *versus* unfermented beans, extract of which contained besides the polyphenols 12-hydroxyjasmonic acid-O-sulfate (Dsouza et al., 2017). Much of work has been done in understanding how the post harvesting procedures affect the formation of flavour precursors, sugars, and proteins, and antioxidant capacity of the polyphenols, extensively reviewed by several authors (Afoakwa et al., 2008; Aprotosoaie et al., 2016). However, not much is known about the fate of the lipids during the fermentation. Above and beyond, authors have drawn attention towards the need of elucidation of the fermentation impact on the triacylglycerol composition (Lima et al., 2011).

Herein, the present study aimed at covering the knowledge gap by observing the changes occurring in the triacylglycerol profile, fatty acids, and other minor components during the fermentation. Within this aim, a lipidomics approach via a recently established method based on reversed-phase high-performance liquid chromatography (HPLC) coupled with high resolution mass spectrometry was used to analyze the lipid composition in cocoa. Besides, fractionation of the lipid extract, described in Chapter 3, was applied for better visualization of the minor lipid components.

7.2 Material and methods

7.2.1 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany), isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade), chloroform

(Rotisolv® HPLC grade) and Tetra-dodecylammonium bromide, heptane, methanol, ethyl acetate, was purchased from Carl Roth (Karlsruhe, Germany), dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany), ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry), and acetic acid were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethanol was subjected to distillation prior use.

7.2.2 Sample preparation

Cocoa beans samples from 6 spontaneous fermentation trials and 1 controlled fermentation trial from 3 different origins, including Ecuador, Malaysia, and Ivory Coast, were received in several sets from Barry Callebaut. A fermentation trial represents a series of samples collected at specific time points during the fermentation process of a batch of cocoa beans. Usually, the time length of a fermentation process is between 5 and 7 days. Firstly, the seed material was deshelled and ground using a grinder with the purpose of making homogenous powder. Henceforth, an overnight Soxhlet (Buchi Extraction System B811 instrument, Flawil, Switzerland) method using dichloromethane as an extraction solvent, 150 mL of 5 g of powdered beans, was used. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis. For HPLC analysis, a concentration of 0.045 mg/mL in chloroform/ethanol (50/50) of cocoa lipid extract was prepared.

7.2.3 Moisture analysis

Moisture content was determined by heating around 2.5 g of cocoa powder in the oven at 105 °C for 24, cooled in the dry atmosphere of a desiccator, and then reweighed. Total lipid content was normalized according to: $= \frac{\text{Lipid extract (g)} \times 100}{\text{Cocoa Powder (g)} - \text{Weight Loss (g)}}$.

7.2.4 Solid phase extraction (SPE)

Chromabond aminopropyl modified silica gel NH_2 (Chromabond® LV-NH_2, Macherey-Nagel GmbH & Co. KG, Düren, Germany) column cartridges (3 mL/500 mg) were used for

the SPE fractionation. The cartridges were first activated with 20 mL heptane/ethylacetate (80/20, v/v), then, 20 ml heptane. Then, 10 mg of the lipid extract dissolved in 1 mL of heptane were applied, after which the fractions were eluted with 5 different solvent mixtures: 1) 20 mL heptane, first fraction collected ; 2) 20 mL heptane/ethyl acetate (97/3, v/v), for the second fraction; 3) 20 mL heptane/ethyl acetate (85/15, v/v), third fraction collected; 4) 20 mL heptane/ethyl acetate (85/15, v/v) plus 1% acetic acid, for the forth fraction; and 5) 20 mL methanol. Extracted lipids were evaporated to dryness in a rotavapor and stored at -20 °C until further analysis.

7.2.5 HPLC chromatographic conditions

TAGs molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). The column used in this study was a Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 10 mM/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. Two different elution gradients were used. One for the total lipid separation and another to separate minor lipid components in cocoa. The first gradient elution program consisted of holding solvent steady A/B (40/60) for 3 min; followed by a linear gradient to solvent B (100) for 33 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 40/60 solvent A/B for 5 min before reuse. The second gradient elution program consisted of holding solvent steady A (100) for 5 min; followed by a linear gradient to solvent A/B (70/30) for another 5; then by a linear gradient to solvent B (100) for 90 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 100 solvent A for 5 min before reuse.

The individual TAG lists of the high-resolution masses were combined for peak matching and retention time alignment using a mass-to-charge (m/z) tolerance of $\Delta m/z = 0.0001$ and a retention time (R_t) tolerance of 0.5 min for elution time <20 min and 1.0 min for an elution time ≥20 min. Extensive manual curation of the TAGs peaks was performed to be uniquely assigned with m/z and retention time (R_t) tolerances of $\Delta m/z = 0.01$ and $\Delta R_t = 0.8$ min respectively. For further analyses, the peak areas were normalized to the internal standard (Tetra-dodecylammonium bromide) at R_t 4.4 min.

7.2.6 High-resolution Mass spectrometry conditions

High-resolution masses were acquired using a time of flight MicrOTOF Focus mass spectrometer (Bruker Daltonics UHT Ultra, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of m/z 200–1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode.

7.2.7 FAME synthesis

The method was based on the method developed by O'Fallon et al. with modifications (O'Fallon, Busboom, Nelson, & Gaskins, 2007). Thus, 100 mg initial lipid extract mass was applied for each FAME synthesis method. Method 1 was chosen to extract the bulk samples. More specifically, 0.7 mL 10 N KOH, 5.3 mL methanol and 100 mg initial lipid extract were stirred continuously at 50 ^{0}C for one hour. 500 μL chloroform was added together with potassium hydroxide and methanol, to allow good dissolvement of lipid extracts. The samples were cooled down at room temperature and 0.58 mL 24 N H_2SO_4 was added. All samples were stirred continuously at 50 ^{0}C for one additional hour. FAME were extracted with 2 mL heptane, vortexed for 5 minutes and centrifuged at 3.500 g for 3 minutes. Molecular sieves were added for 10 minutes in the final FAME solution to further neutralize the mixture. Nitrogen was blown on every sample to avoid oxidation and then the samples were directly analysed by gas chromatography.

7.2.8 GC-FID method

A capillary column VF-5ms, 30 m x 0.25 mm was used, having an injector temperature of 265 ^{0}C and detector temperature 300 ^{0}C. Helium was used as a carrier gas, at a flow rate of 16.2 mL/min. The split ratio was 1:10 and the sample injection volume 1.0 μL. GC analysis started at 60 ^{0}C for 1 minute, followed by a second step of 10 ^{0}C/min temperature rate until 200^{0}C, with a hold time of 10 minutes, then 7 ^{0}C/min rate until 300 ^{0}C, at a hold time of 3

minutes and lastly having a temperature rate of 10 ^{0}C/min at 300 ^{0}C and a final holding time of 10 minutes. Fatty acids identification was performed comparing with a Supelco 37 comp. FAME MIX standard and GC-MS determined according to Elvert at al. by using the same instrument (Elvert, Boetius, Knittel, & Jørgensen, 2003).

7.2.9 Spectrophotometric analysis

Color analysis of cocoa lipid extract was measured by absorption. Spectra were recorded on a Cary 4000 UV-Vis spectrophotometer (Varian, now Agilent, Waldbronn, Germany). For this purpose, 50 mg of cocoa lipid extract were dissolved in 3 ml chloroform/methanol (50/50).

7.2.10 Lipid extraction from the pulp

Three mL of pulp were extracted with 6 mL chloroform ethanol (50/50, v/v) in a liquid/liquid extraction. The mixture was placed in the ultrasound bath for 15 min and then vigorously shacked with using a vortex. After that, the mixture was centrifuged at 3000 rpm for 5 min and the organic phase was collected and evaporated using Turbovap. The lipid content was redissolved in 1 ml chloroform ethanol (50/50, v/v) and after adding the internal standard it was analyzed by LC-MS.

7.2.11 Statistical analysis

Microsoft Office Excel 2016 was used to order and process the data. A one-factor, two-factors ANOVA and MANOVA were used to study the effect of origin and the effect of fermentation status on the TAG profile. The analysis were carried out with XLSTAT Evaluation 2018.2 software version 2018 (Addinsoft, France).

7.3 Results

7.3.1 Lipid amount

Fluctuations in the lipid amount were observed in all fermentation trials (Fig. 7.1), defined as series of samples collected at specific time points. Although no definite pattern was found, from the graph it seems that the profile shows turning point at 72 hours (h), 96 h and 120 h. Only Malaysian samples, in both trials, showed a continuous increase during the fermentation process.

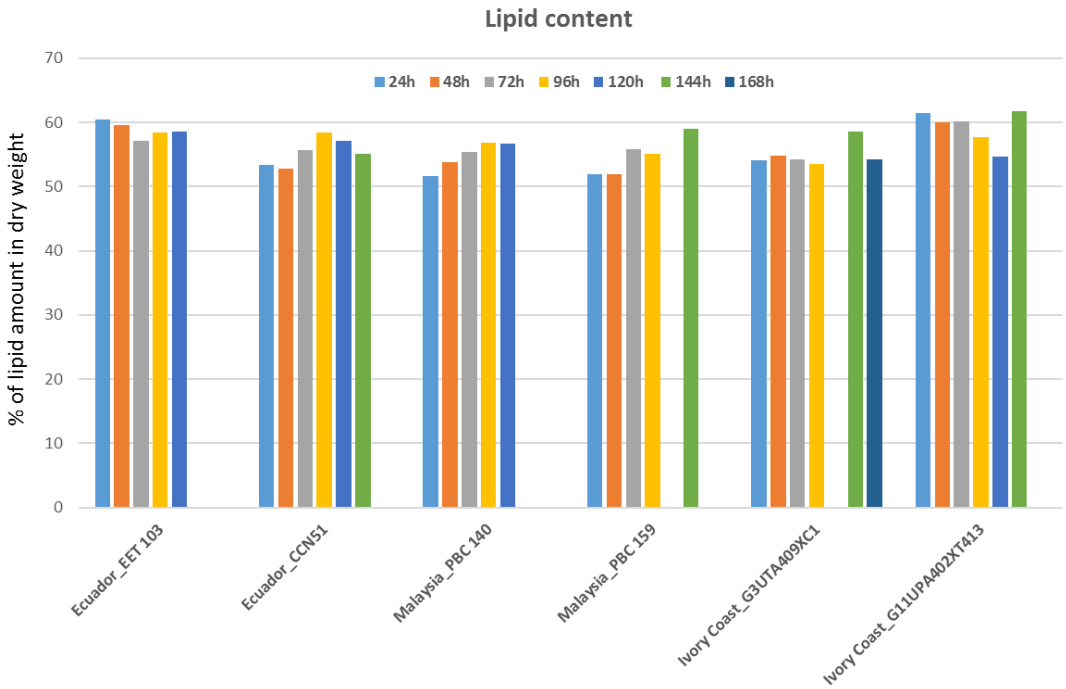


Figure 7.1 Lipid amount as the percentage in dry mass of six fermentation trials from 6 different origins.

7.3.2 TAGs profile during the fermentation

Individual TAGs have been analysed and compared in all fermentation trials. Figure 7.2 illustrates the relative amount of the main TAGs in cocoa beans, namely POP, POS, and SOS, and the overall monounsaturated TAGs in the lipid extract. For all the trials it appears that 72 h (in Ecuadorian and Malaysian samples) and 96 h (in Ivorian beans) are crucial time points for the monounsaturated TAGs during the fermentation. Ecuadorian sample showed fluctuation but not an overall change in the total amount of monounsaturated TAGs, whereas the other two origins exhibit a decrease during the fermentation.

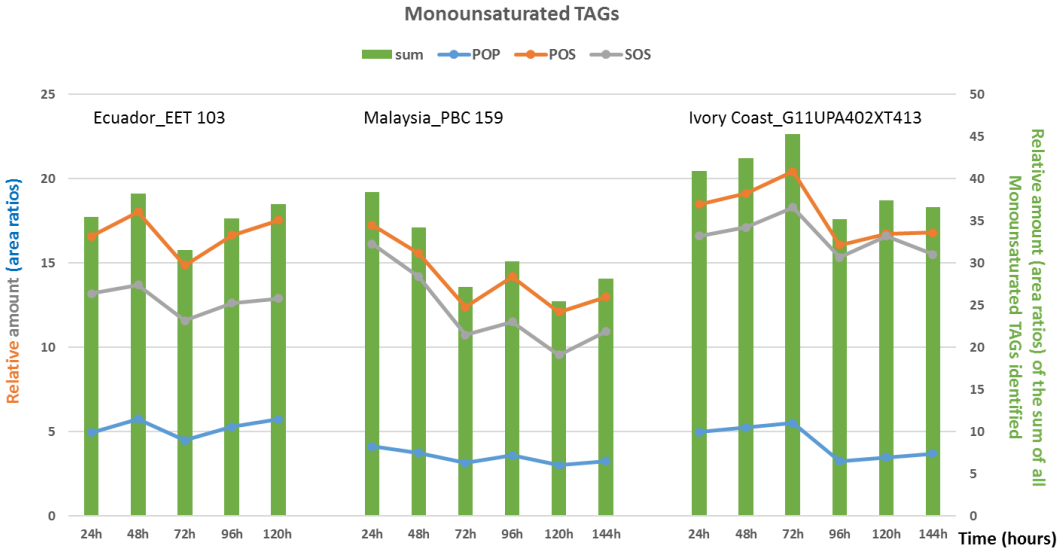


Figure 7.2 Monounsaturated TAGs relative amount in 3 fermentation trials from 3 different origins.

A second observation could be made over the variation content of the three main triacylglycerols during the second phase of the fermentation process. The variation pattern of POP, POS, and SOS have shown differences when comparing the fermentation trials in the three countries. In Ecuadorian samples, POS and SOS have shown the same kinetics whereas in Malaysian beans the ratio between SOS and POS is lower during the second half of the fermentation process. This ratio in Ivorian samples is close to one.

Surprisingly, POO and SOO have shown an increase at 72 h in all fermentation series (Fig. 7.3), which then drops at 96 h showing an opposite trend concerning the main TAGs described above. Ecuador and Ivory Coast origins exposed a second increase at 120 h. In contrast, PLS and SLS have shown a completely different behavior. In Ecuadorian and Ivorian samples the amount of these 2 TAGs increase at 48 h and decrease at 72 h, followed by a second increase; whereas in Malaysian samples, PLS and SLS increase until 96 h and then lower.

During the fermentation, Malaysian samples double their amount of saturated TAGs (Fig. 7.4), characteristic for this origin as already known from our previous work, Chapter 5. No significant variations were observed in Ecuadorian samples. The lipid extract of the beans from Ivory Coast showed a decrease of saturated TAGs during the fermentation.

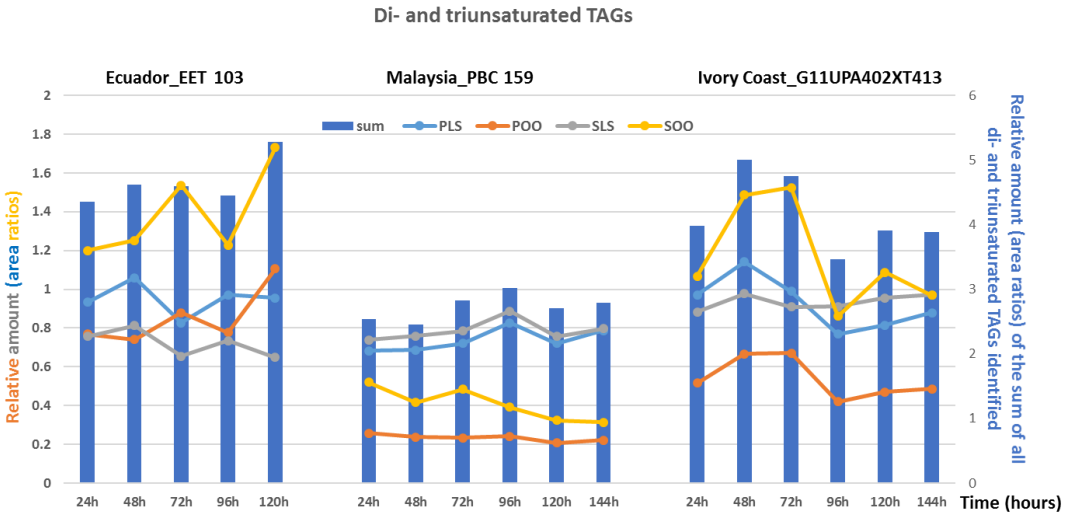


Figure 7.3 Di- und triunsaturated TAGs relative amount in 3 fermentation trials from 3 different origins.

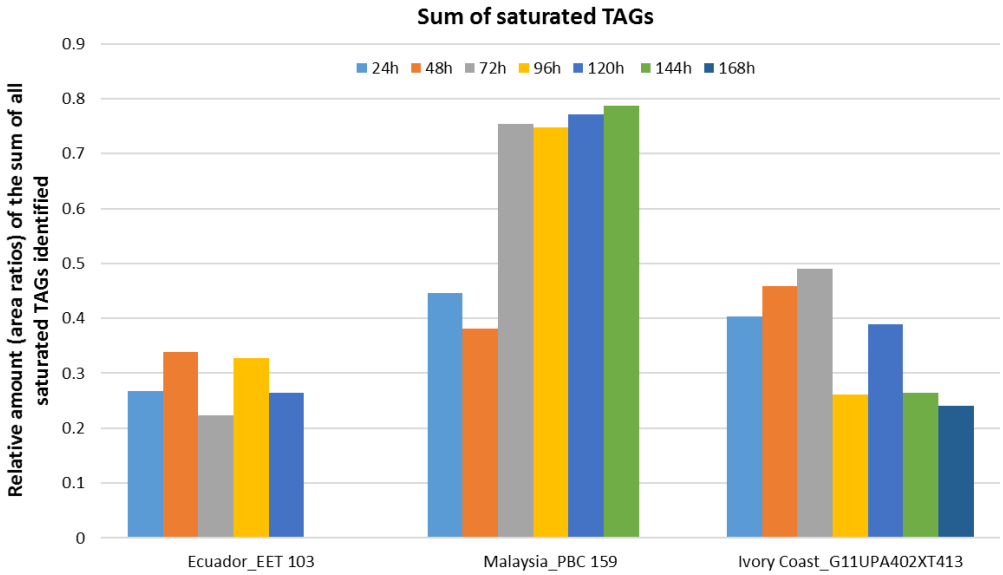


Figure 7.4 Saturated TAGs relative amount in 3 fermentation trials from 3 different origins.

Figure 7.5 illustrates the evolution of the monounsaturated, di- and triunsaturated, and saturated TAGs in the Ecuadorian hybrid CCN51. All three series of TAGs showed a trend disturbance at 72 h, where an increase was observed.

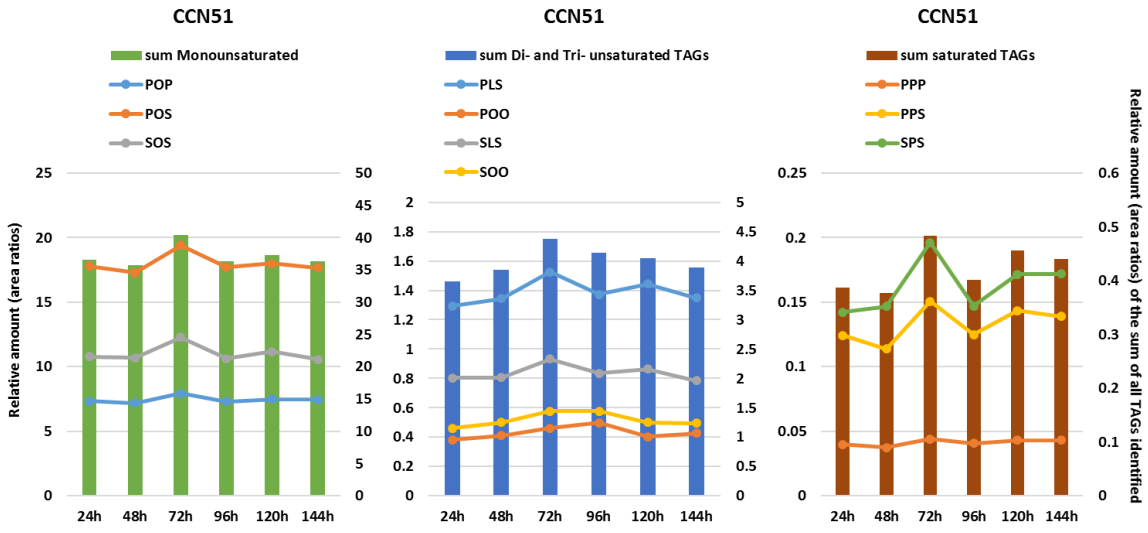


Figure 7.5 Evolution of TAGs profiles in CCN51 hybrid during the fermentation.

In Chapter 4 we have described the presence of the unusual TAGs presenting hydroxyl fatty acids esterified to the glycerol backbone, and in Chapter 5 the disappearance of this unusual TAGs suggesting to consider these compounds as markers for unfermented cocoa beans. These compounds decrease rapidly throughout the first 3 days of fermentation (Fig. 7.6). Two batched of the same hybrid were compared and showed large variability (Fig. 7.7 (a)), yet with the same decreasing development. Identical results were observed when comparing two different hybrids (Fig. 7.7 (b)).

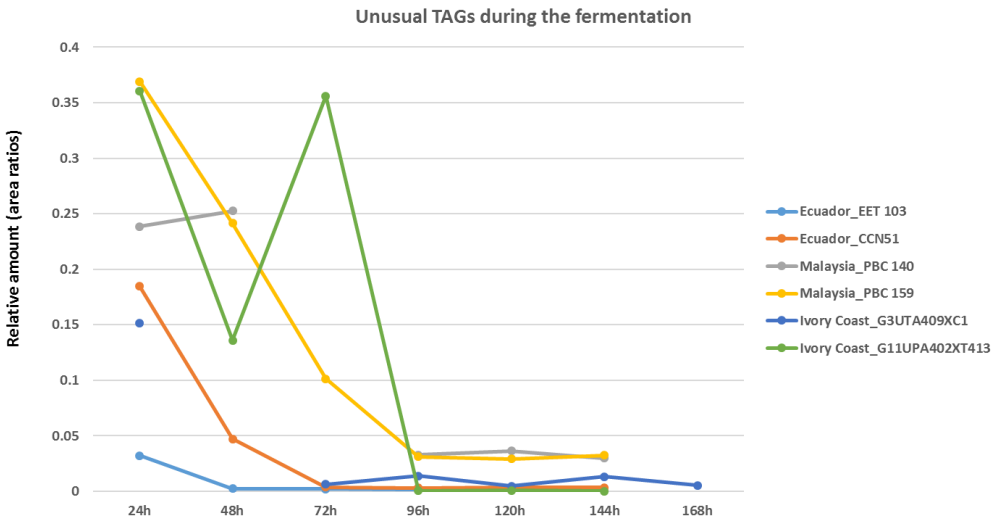


Figure 7.6 Unusual TAGs during the fermentation process.

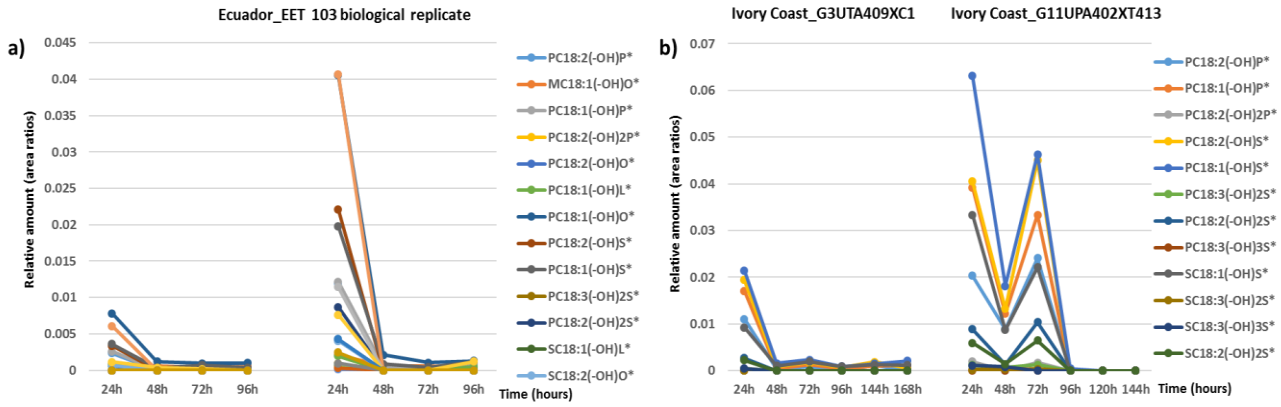


Figure 7.7 Unusual TAGs during the fermentation in **(a)** 2 batched of the same hybrid and **(b)** in 2 batched of two hybrids of the same origin.

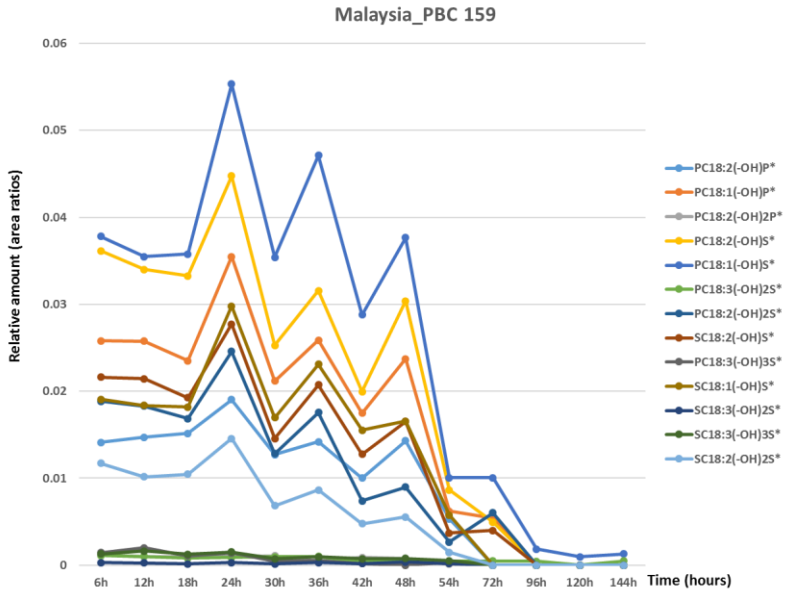


Figure 7.8 Unusual TAGs during the fermentation in samples from Malaysia.

Ivory Coast hybrids showed a quite singular zigzag decrease of the unusual TAGs. Same it was observed in the samples from Malaysia but more thickly than in Ivorian samples. It appears that during the first 18 h the profile stays constant and after a series of increasing and decreasing values follows reaching an almost complete decrease at 96 h.

7.3.3 Statistical analysis on TAGs

Analysis of variance has shown that there are statistical differences in the number of different TAGs during the fermentation. Figure 7.9 illustrates the mean values of 12 TAGs subdivided as being part of a homologues series. A homologous series is a series of compounds that differ in a defined mass increment corresponding to addition or subtraction of a fixed moiety, in this case or $+C_2H_4$. As it can be observed, all the members of a homologues series follow the same trend.

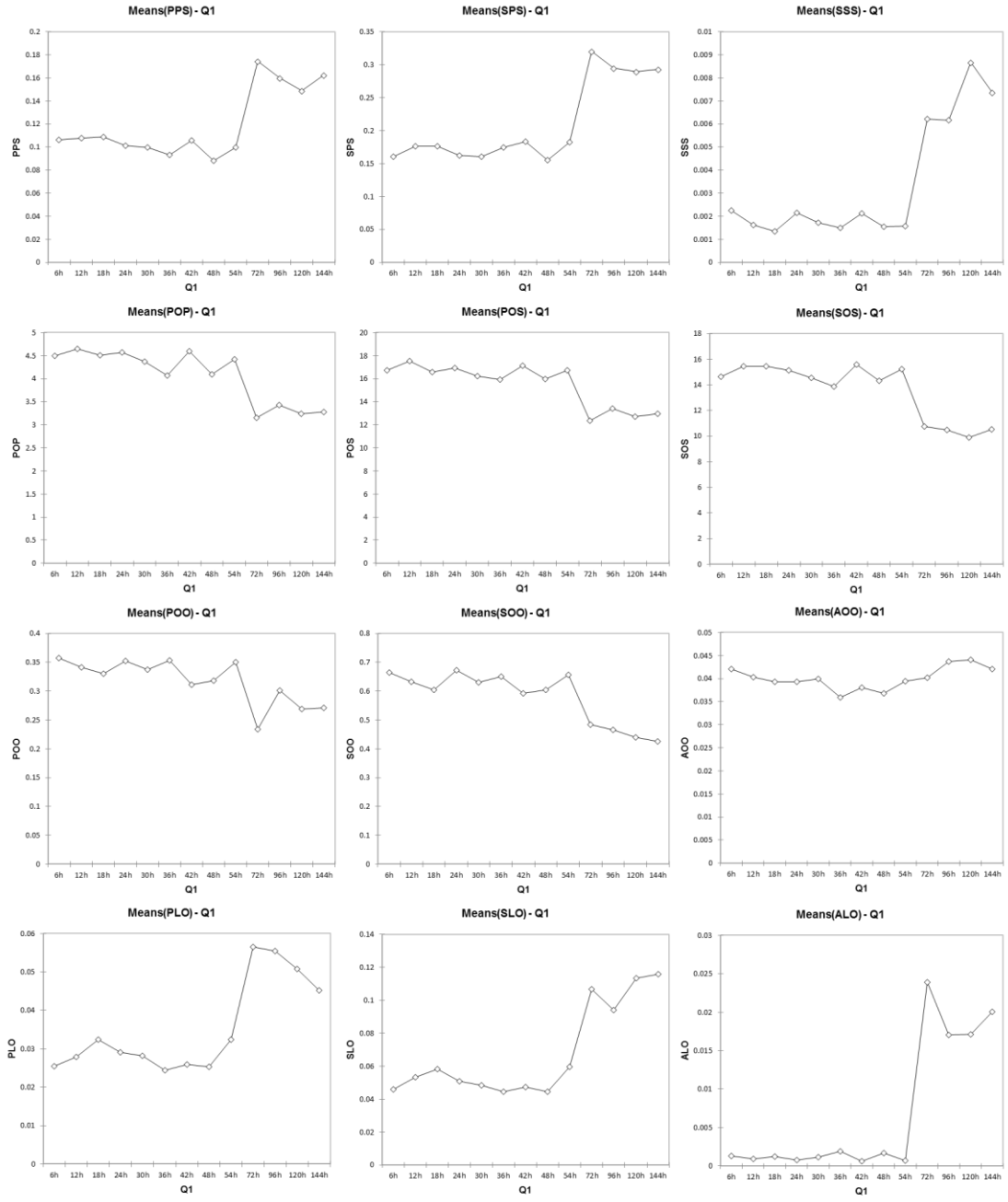


Figure 7.9 Means of the distribution of 4 homologues series TAGs in Malaysian samples.

Only AOO did not show the same trend. As for POS, the analysis of variance provide <0.05 p values, presented in Table 7.1, indicating statistical difference between the first days of fermentation and the last days. With the aim of encompassing all usual TAGs, a multivariate analysis of variance (MANOVA) was conducted to assess if mean differences exist. Multivariate F value (Wilks' λ) gave 1.643, p-value 0.005 for monounsaturated TAGs; F 1.49 and p-value 0.0001 for saturated TAGs; F 1.49 and p-value <0.0001 for diunsaturated POO type; F 1.784 and p-value 0.08 for diunsaturated PLS type; F 1.508 and p-value <0.0001 for triunsaturated TAGs; and F 1.946 and p-value <0.0001 for tetraunsaturated TAGs.

Table 7.1 Analysis of variance p-value for POS in two Malaysian fermentation trials.

POS	Q1-144h	Q1-120h	Q1-96h	Q1-72h	Q1-54h	Q1-48h	Q1-42h	Q1-36h	Q1-30h	Q1-24h	Q1-18h	Q1-12h	Q1-6h
Q1-6h	**0.002**	**0.002**	**0.008**	**0.006**	0.973	0.502	0.703	0.471	0.686	0.861	0.926	0.474	
Q1-12h	**0.000**	**0.000**	**0.002**	**0.002**	0.468	0.176	0.745	0.164	0.303	0.298	0.453		
Q1-18h	**0.002**	**0.002**	**0.010**	**0.007**	0.892	0.565	0.635	0.533	0.752	0.958			
Q1-24h	**0.001**	**0.001**	**0.006**	**0.005**	0.845	0.559	0.574	0.516	0.761				
Q1-30h	**0.005**	**0.005**	**0.020**	**0.012**	0.621	0.817	0.425	0.782					
Q1-36h	**0.006**	**0.005**	**0.022**	**0.013**	0.400	0.963	0.257						
Q1-42h	**0.001**	**0.001**	**0.004**	**0.003**	0.704	0.295							
Q1-48h	**0.009**	**0.007**	**0.031**	**0.017**	0.466								
Q1-54h	**0.002**	**0.002**	**0.008**	**0.006**									
Q1-72h	0.650	0.810	0.441										
Q1-96h	0.654	0.503											
Q1-120h	0.802												
Q1-144h													

7.3.4 Fatty acid profile during the fermentation

FAME analysis has shown an overall increase of the fatty acids during the fermentation (Fig. 7.11). Herein, the turning points are also present at 72 and 96 h, as observed for TAGs. Figure 7.12 illustrates this occurrence in another hybrid from Ivory Coast which was fermented in spontaneous and controlled fermentation.

Interestingly, the same batch of sample fermented in 2 different conditions showed a considerably different profile. The spontaneous fermentation of the hybrid G11UTA402XT413 showed an overall slight increase of the total fatty acids at the end of the fermentation with a significant increase of all fatty acids 96 h that started decreasing at 120 h, reaching the initial values at 144 h. This fact is almost not observed in the batch fermented under controlled conditions. Moreover, spontaneous fermentation showed a significant increase of linoleic acid, started at 48 h, and of palmitoleic acid, started at 72 h. The content of these 2 fatty acid return at the average level at 144 h.

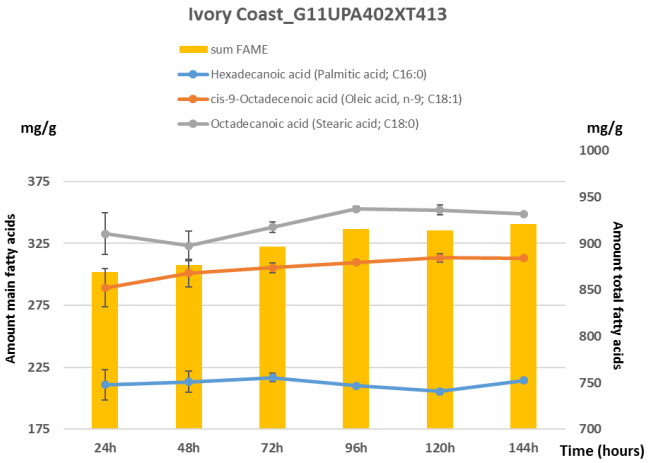


Figure 7.11 Total fatty acids and main fatty acids profile of a fermentation series from Ivory Coast.

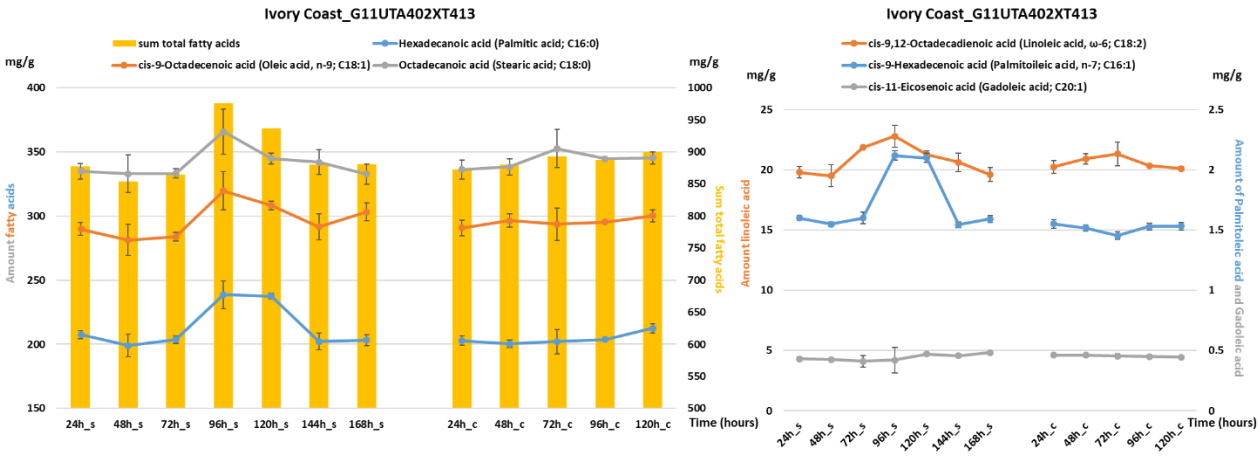


Figure 7.12 Total fatty acids and main fatty acids profile of a spontaneous and controlled fermentation series from Ivory Coast.

7.3.5 Colour of the lipid extract during the fermentation

Upon extraction, the lipid obtained from different time points have shown a varying range of colors going through pale yellow to a brownish color. Therefore, the lipid extract was analyzed by spectrophotometry. Results showed that the absorbance at 400 nm increased within the fermentation reaching the maximum point at 96 and 120 h (Fig. 7.13). Similar events were observed in other trials.

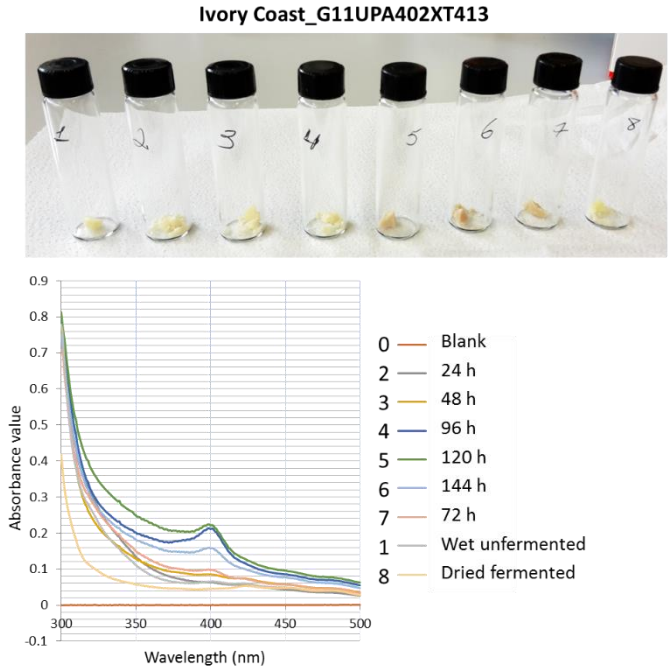


Figure 7.13 Evolution of the color during the fermentation of cocoa beans.

7.3.6 Minor lipid classes during the fermentation

Solid phase extraction was performed with the aim of separating the TAGs fraction from the remaining part of the lipid extract. Minor components were further analysed by HPLC-MS. Figure 7.14 and 7.15 illustrates the evolution of the minor components during the fermentation as two-dimensional (2D) map of the chromatograms with retention time on the x axes and *m/z* ratios on the y axes. The interrupted blue circle at 24 h indicated the presence of the unusual TAGs, which decrease in intensity and number at 48 h and then increase at 72 h. These results are following what observed when analyzing the total lipid profile (Fig. 7.1).

At 96 h these compound disappear almost entirely and another class of compounds appears. This class was identified as phospholipids, mainly phosphatidylcholines and phosphatidylethanolamines. This class of compound persists until the last day of the fermentation. In Ecuadorian samples, the loss of the unusual TAGs and the joint appearance of the phospholipidic class can be detected at 72h.

Lipid extract from the pulp at 24 and 48 hours illustrate a different profile than the lipid extract from cocoa (Fig. 7.16). This statement indicates that the pulp lipid reflects the lipid profile of bacteria present in the pulp during the fermentation. The green circle shows the TAGs class whereas the red circle indicates the phospholipid class. It can be noticed that the intensity

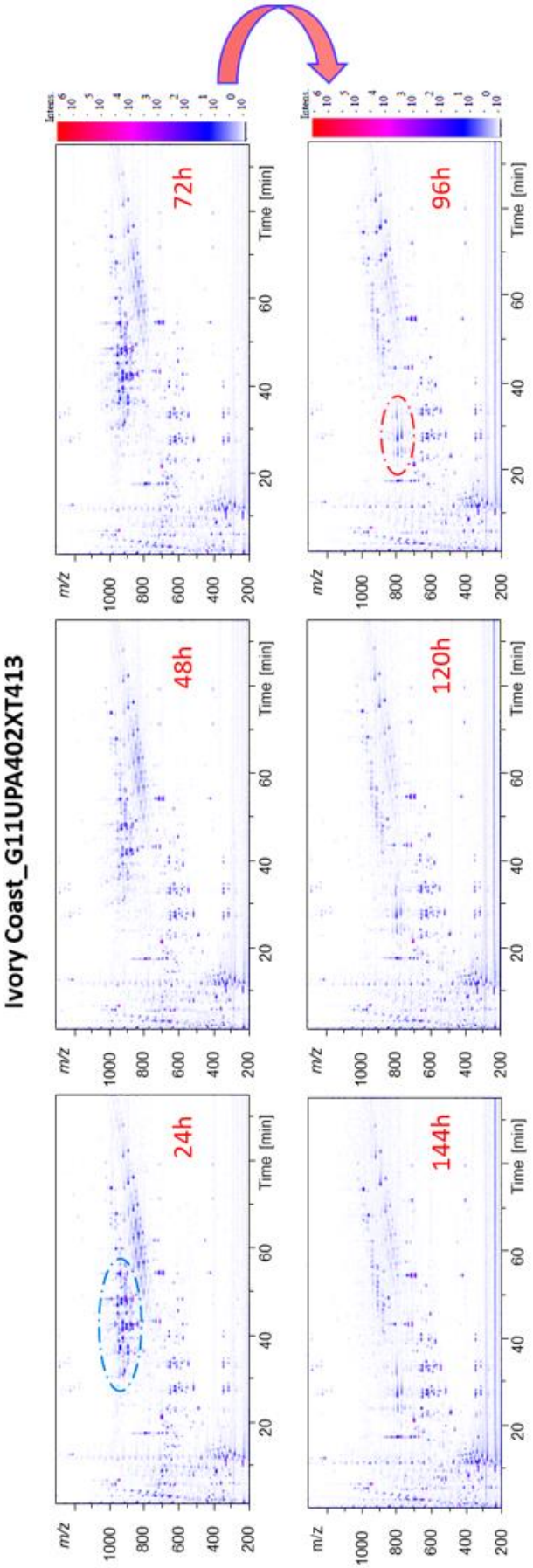


Figure 7.14 2D map of the LC-MS chromatogram in positive ion mode of minor components of the lipid extract of a fermentation trial from Ivory Coast. Blue circle indicates the unusual hydroxyl TAGs and red circle the phospholipid class occurrence.

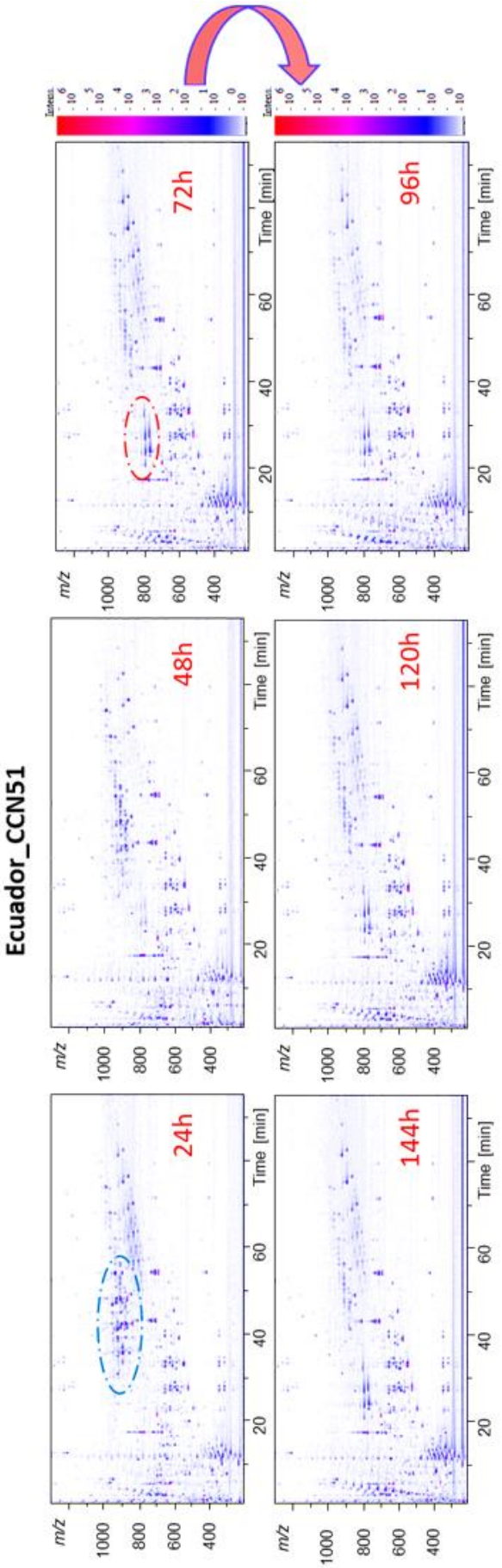


Figure 7.15 2D map of the LC-MS chromatogram in positive ion mode of minor components of the lipid extract of a fermentation trial from Ecuador. Blue circle indicates the unusual hydroxyl TAGs and red circle the phospholipid class occurrence.

of the spots is increasing passing from 24 h to 48 h. It can be assumed that due to the earlier retention time, the TAGs in the pulp extract are represented by more unsaturated species. A second observation can be made on the overall lipid profile of the pulp of different origins. The pulp from the German Forastero showed a different profile from the pulp of PBC 140 and PBC 159.

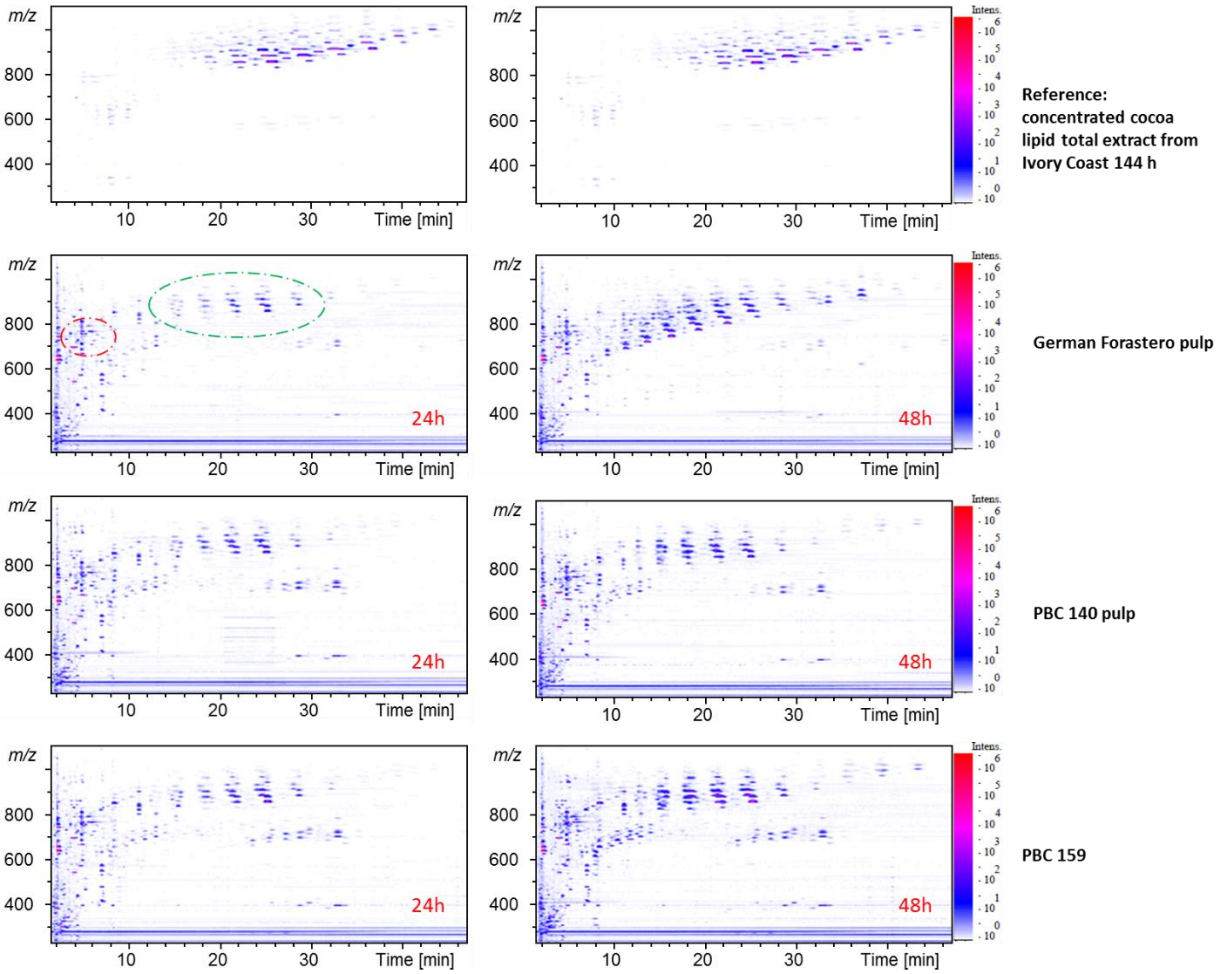


Figure 7.16 2D map of the LC-MS chromatogram in positive ion mode of the lipid extract from pulp at 24 and 48h from 3 different hybrids from 2 origins: Ivory Coast and Malaysia. At the upper part, same 2D map* left and right side of a reference chromatogram from Ivory Coast. *these 2D maps are inserted for comparison purposes.

7.4 Discussion

Oscillations of the lipid amount during the fermentation were observed in all the fermentation trials, and this phenomenon was not reported previously. As presented in Chapter

5, the lipid amount showed an increase in fermented dried beans compared to unfermented wet cocoa. Although that appeared as a trend, considerable variability among the samples was present. However, in this study, such a conclusion can be drawn only for Malaysian fermentation trials. Contradictory to our findings, Afoakwa et al. revealed that the fat content of the samples decreased significantly with fermentation and pod storage in samples from Ghana and Aremu et al. also found a decrease from 62.9% to 55.7% by the sixth day of fermentation in Nigerian samples (Afoakwa, Quao, Takrama, Budu, & Saalia, 2013; Aremu, Agiang, & Ayatse, 1995).

During the fermentation, the beans were turned at 48 h and 96 h in trials from Malaysia and Ivory Coast and the beans from Ecuador were turned every day. The decrease of all monounsaturated TAGs at 72 h in almost all fermentation trials, could suggest a consequence of the aerobic phase. Only the trials from Ivory Coast showed the decrease happening at 96 h, indicating perhaps a one-day delay in the fermentation process. At this point of fermentation, the acetic acid bacteria started to convert ethanol to acetic acid and to penetrate into bean, causing biochemical reactions. De Britto et al. observed maximum alterations of the cell wall and reduction in the cytoplasmic content of phenolic compounds and protein bodies at 72 h (de Brito Edy S et al., 2000). The decrease of the most abundant TAG profile could happen as a response of the beans against the exponential increase of the bacterial reproduction by using intensively the fat resources to produce secondary metabolite aimed at defense. Due to the increased amount of ethanol, lactic acid, acetic acid, and temperature increment, it could be speculated that the main TAGs undergo hydrolysis, however this will not explain the increase of diunsaturated TAGs containing oleic acid observed in all fermentation trials. Therefore, it could be deduced that the bacteria involved in the fermentation use the lipid from cocoa as substrate for building their own lipids. On the other hand, microorganisms and germinating oil seeds convert lipid to carbohydrate via gluconeogenesis, employing the glyoxylate cycle (Itamar Luís Gonçalves, Albanin Aparecida Mielniczki-Pereira, Ana Claudia Piovezan Borges, & Alice Teresa Valduga, 2016). At 96 h, a second turning of the beans occurs which could explain the variation in the TAG profile observed in the second part of the fermentation. It appears that the turning of the beans perturbs the trend of rising or declining of the di- and triunsaturated TAGs. CCN 51 Ecuadorian hybrid has shown an increase of the mono, di, tri, and saturated fatty acids only at 72 h, with POO and SOO continuing at this level for another 24 h. This hybrid become one of the most planted cultivars in Ecuador, mainly because of its high productivity and disease resistance (Boza et al., 2014).

A rapid decrease of unusual TAGs during the first 4 days of fermentation was observed. Hydroxyl-containing TAGs are known to be produced during the germination (Weichert, Kolbe, Kraus, Wasternack, & Feussner, 2002) catalyzed by lipoxygenases. Moreover, this compounds are involved in the synthesis of cutin derived mostly from oleic fatty acid (Blée & Schuber, 1993). Since the fermentation process inhibits many of the endogenous enzymes (Hansen Carl E, del Olmo Margarita, & Burri Christine, 1999) it could inhibit LOX; thus, no production of this enzyme occurs after the second phase of the fermentation. Besides, Sadik et al. showed that flavonoids of cocoa inhibited mammalian LOX (Sadik, Sies, & Schewe, 2003). Unusual TAGs were consumed which might indicate that they rapidly undergo degradation or these compounds are redirected into Jasmonic acid synthesis pathway to the increase the defense response (Ghasemi Pirbalouti Abdollah, Sajjadi Seyed Ebrahim, & Parang Keykavous, 2014; Mosblech, Feussner, & Heilmann, 2009). Two batched of the same hybrid from Ecuador showed different amount of the hydroxyl TAGs but the same decreasing trend during the fermentation. A control experiment, applying a thermal treatment of the beans at 45 °C for 48 h in aerobic condition, has shown no decrease in the amount of these TAGs. Therefore, a higher amount in the usual TAGs might indicate the germination status of the beans. The zigzag shaped reduction of the unusual TAGs might be explained by the exposure of the beans at different types of bacteria during the first phase of the fermentation or sampling inhomogeneity. Statistical analysis supported the trend of all the TAGs.

FAME analysis has shown an overall increase of the fatty acids during the fermentation. A remarkable rise in linoleic acid starting at 48 h in spontaneous fermentation and at 24 h in controlled fermentation followed by a decrease until reaching the initial levels in the last day of the fermentation in Ivorian samples. In other studies, fermentation of castor oil showed that the fermented seeds contained more unsaturated fatty acids than the unfermented seeds (Anosike & Egwuatu, 1980). Whereas during the fermentation of must of *Vitis vinifera* fatty acids were consumed, by the time that 50% of the sugar was used up, with the high consumption of large-chain saturated and unsaturated fatty acids (Ancín, Ayestarán, García, & Garrido, 1998).

The 2D map visualization of the chromatographic data, a tool embedded in the analytical software, offered a direct visual display of the changes occurring in the lipid profile of the cocoa and pulp extracts. 2D map of minor components showed correlations with the quantification data. Key differences in the minor lipid profile were observed at the same time when significant changes in the main TAGs occurred. The evolution of minor components is

described by the loss of hydroxyl derivative of TAGs and the appearance at 72 h, and 96 h of phospholipidic class, which is most probably of a different provenience. 2D map of spontaneous fermentation illustrated the presence of the phospholipid class already at 24 h (Appendix Fig. E.1). Additional analysis of the pulp showed a very different lipid profile if compared to cocoa bean lipid profile. The major lipid class in the pulp is represented by more unsaturated and shorter chain fatty acids TAGs, characteristic of yeast and bacterial origin (Botha & Kock, 1993; O'Leary, 1962). From the 2D map of the pulp lipid profile, it can be noted a prominent variation in the bacterial strains from two different origins. Badrie et al. reviewed the microbiological diversity related to the origin of the beans (Badrie et al., 2015). The profile of TAGs going up during the first 48 h and then decreasing, follows the profile of Yeasts and Lactobacillus species which, increase reaching the max amount at around 36 h (Schwan & Wheals, 2004). Results indicate that the major transformation in the lipid profile of cocoa samples occurred when acetic acid bacteria and Bacillus species became the dominant microorganisms (Ardhana & Fleet, 2003; Lima et al., 2011). They remained at high population until the fermentations were considered complete. Studies showed that acetic acid bacteria promote lipid accumulation in yeast which is mainly constituted by palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). Lipids produced in media containing a high concentration of acetic acid had 19% C16:0, 16% C18:0, 44% C18:1, and 10% C18:2 (Huang et al., 2016). Moreover, a special class of lipids found in acetic acid bacteria, dihydroceramides, showed to improve cognitive function in dementia model rats (Fukami, Tachimoto, Kishi, Kaga, & Tanaka, 2010). Other studies showed that during storage the amount of linoleic and α-linolenic acids increased in the probiotic cream fermented with B. lactis compared to the control cream (Yilmaz-Ersan, 2013). Moreover, many studies reported the capacity of the yeasts to synthetize cocoa lipid-like compound (Papanikolaou, Muniglia, Chevalot, Aggelis, & Marc, 2003; Wei, Gossing, Bergenholm, Siewers, & Nielsen, 2017). The fatty acids composition of the lipids in yeast changes with temperature: the lower the temperature, the more unsaturated the membrane fatty-acyl composition; while the higher the temperature, the more medium-chain fatty acids (C8:0-C12:0) are produced influencing the volatile compounds produced during the fermentation (Torija et al., 2003). Giving this fact, no correlation between the temperature during the fermentation and the amount of the polyunsaturated TAGs could be perceived. Although, substantial variations occurring in the lipid profile of all fermentation trials have happened at 72 and 96 h time points when the batch temperature reaches its maximum (see Appendix, Fig. E.5).

Nevertheless, further investigations are needed to elucidate the lipid profile of the pulp at different stages of the fermentation process and to correlate the values with the occurring fermentative microbiota. An important research question is to consider and evaluate up to which rate the bacteria involved in the fermentation causes the remodeling of the fatty acid profile and up to which degree environmental factor and hybrid type. The results showed that the ecological conditions highly dictate the lipid profile. Nonetheless, understanding the factors contributing to variations in the chemical composition and physical qualities of cocoa beans during the fermentation processes would have significant commercial implications.

7.5 Conclusions

This study showed the impact of the fermentation on the lipid profile of cocoa. The results of this chapter could put the basis for further understanding of the effects of the microbial activity in cocoa during fermentation since as it could be seen, already from the first hours of fermentation changes of the lipid profile occurred. Although not substantial alterations happen in the main TAGs profile at the end of the fermentation, the microorganisms involved in the fermentation shaped the profile of the less abundant lipid compounds. This fact helps to explain the findings of Chapter 5 regarding the POO, PLS, SOO, and SLS irregular trend. Therefore, a part of the temperature at the origin during fruit ripening a second microbiological factor must be considered.

References

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

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

Anosike, E. O., & Egwuatu, C. K. (1980). Biochemical changes during the fermentation of castor oil (Ricinus communis) seeds for use as a seasoning agent. *Plant Foods for Human Nutrition*, *30*(3–4), 181–185. https://doi.org/10.1007/BF01094021

Aprotosoaie, A. C., Luca, S. V., & Miron, A. (2016). Flavor Chemistry of Cocoa and Cocoa Products—An Overview. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 73–91. https://doi.org/10.1111/1541-4337.12180

Ardhana, M. M., & Fleet, G. H. (2003). The microbial ecology of cocoa bean fermentations in Indonesia. *International Journal of Food Microbiology*, *86*(1–2), 87–99. https://doi.org/10.1016/S0168-1605(03)00081-3

Aremu, C. Y., Agiang, M. A., & Ayatse, J. O. I. (1995). Nutrient and antinutrient profiles of raw and fermented cocoa beans. *Plant Foods for Human Nutrition*, *48*(3), 217–223. https://doi.org/10.1007/BF01088443

Badrie, N., Bekele, F., Sikora, E., & Sikora, M. (2015). Cocoa Agronomy, Quality, Nutritional, and Health Aspects. *Critical Reviews in Food Science and Nutrition*, *55*(5), 620–659. https://doi.org/10.1080/10408398.2012.669428

Blée, E., & Schuber, F. (1993). Biosynthesis of cutin monomers: Involvement of a lipoxygenase/peroxygenase pathway. *Plant Journal*, *4*(1), 113–123.

Botha, A., & Kock, J. L. F. (1993). Application of fatty acid profiles in the identification of yeasts. *International Journal of Food Microbiology*, *19*(1), 39–51. https://doi.org/10.1016/0168-1605(93)90122-W

de Brito Edy S, García Nelson H Pezoa, Gallão MI, Cortelazzo Angelo L, Fevereiro Pedro S, & Braga Márcia R. (2000). Structural and chemical changes in cocoa (Theobroma cacao L) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture*, *81*(2), 281–288. https://doi.org/10.1002/1097-0010(20010115)81:2<281::AID-JSFA808>3.0.CO;2-B

De Vuyst L., & Weckx S. (2016). The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *Journal of Applied Microbiology*, *121*(1), 5–17. https://doi.org/10.1111/jam.13045

Dsouza, R., Grimbs, S., Behrends, B., Bernaert, H., Ullrich, M., & Kuhnert, N. (2017). Origin-based polyphenolic fingerprinting of Theobroma cacao in unfermented and fermented beans. *Food Research International*. https://doi.org/10.1016/j.foodres.2017.06.007

Elvert, M., Boetius, A., Knittel, K., & Jørgensen, B. B. (2003). Characterization of Specific Membrane Fatty Acids as Chemotaxonomic Markers for Sulfate-Reducing Bacteria Involved in Anaerobic Oxidation of Methane. *Geomicrobiology Journal*, *20*(4), 403–419. https://doi.org/10.1080/01490450303894

Feussner, I., Kühn, H., & Wasternack, C. (2001). Lipoxygenase-dependent degradation of storage lipids. *Trends in Plant Science*, *6*(6), 268–273. https://doi.org/10.1016/S1360-1385(01)01950-1

Fukami, H., Tachimoto, H., Kishi, M., Kaga, T., & Tanaka, Y. (2010). Acetic Acid Bacterial Lipids Improve Cognitive Function in Dementia Model Rats. *Journal of Agricultural and Food Chemistry*, *58*(7), 4084–4089. https://doi.org/10.1021/jf9045842

Ghasemi Pirbalouti, A., Sajjadi, S. E., & Parang, K. (2014). A Review (Research and Patents) on Jasmonic Acid and Its Derivatives. *Archiv Der Pharmazie*, *347*(4), 229–239. https://doi.org/10.1002/ardp.201300287

Ghasemi Pirbalouti Abdollah, Sajjadi Seyed Ebrahim, & Parang Keykavous. (2014). A Review (Research and Patents) on Jasmonic Acid and Its Derivatives. *Archiv Der Pharmazie*, *347*(4), 229–239. https://doi.org/10.1002/ardp.201300287

Hansen Carl E, del Olmo Margarita, & Burri Christine. (1999). Enzyme activities in cocoa beans during fermentation. *Journal of the Science of Food and Agriculture*, *77*(2), 273–281. https://doi.org/10.1002/(SICI)1097-0010(199806)77:2<273::AID-JSFA40>3.0.CO;2-M

Ho, V. T. T., Zhao, J., & Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation. *International Journal of Food Microbiology*, *174*, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014

Huang, X.-F., Liu, J.-N., Lu, L.-J., Peng, K.-M., Yang, G.-X., & Liu, J. (2016). Culture strategies for lipid production using acetic acid as sole carbon source by Rhodosporidium toruloides. *Bioresource Technology*, *206*, 141–149. https://doi.org/10.1016/j.biortech.2016.01.073

Itamar Luís Gonçalves, Albanin Aparecida Mielniczki-Pereira, Ana Claudia Piovezan Borges, & Alice Teresa Valduga. (2016). &60;b&62;Metabolic modeling and comparative biochemistry in glyoxylate cycle. *Acta Scientiarum &58; Biological Sciences*, *38*(1), 1–6. https://doi.org/10.4025/actascibiolsci.v38i1.24597

Kadow, D., Bohlmann, J., Phillips, W., & Lieberei, R. (2013). Identification of main fine or flavour components in two genotypes of the cocoa tree (Theobroma cacao L.). *Journal of Applied Botany and Food Quality*, *86*(1), 90–98. https://doi.org/10.5073/JABFQ.2013.086.013

Kongor, J. E., Hinneh, M., de Walle, D. V., Afoakwa, E. O., Boeckx, P., & Dewettinck, K. (2016). Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour profile — A review. *Food Research International*, *82*(Supplement C), 44–52. https://doi.org/10.1016/j.foodres.2016.01.012

Lima, L. J. R., Almeida, M. H., Rob, N., & Zwietering, M. H. (2011). Theobroma cacao L., "the food of the gods": Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. *Critical Reviews in Food Science and Nutrition*, *51*(8), 731–761. https://doi.org/10.1080/10408391003799913

Mosblech, A., Feussner, I., & Heilmann, I. (2009). Oxylipins: Structurally diverse metabolites from fatty acid oxidation. *Plant Physiology and Biochemistry*, *47*(6), 511–517. https://doi.org/10.1016/j.plaphy.2008.12.011

O'Fallon, J. V., Busboom, J. R., Nelson, M. L., & Gaskins, C. T. (2007). A direct method for fatty acid methyl ester synthesis: application to wet meat tissues, oils, and feedstuffs. *Journal of Animal Science*, *85*(6), 1511–1521. https://doi.org/10.2527/jas.2006-491

O'Leary, W. M. (1962). THE FATTY ACIDS OF BACTERIA. *Bacteriological Reviews*, *26*(4), 421–447. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC441163/

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., & Marc, I. (2003). Accumulation of a Cocoa-Butter-Like Lipid by <Emphasis Type="Italic">Yarrowia lipolytica</Emphasis> Cultivated on Agro-Industrial Residues. *Current Microbiology*, *46*(2), 0124–0130. https://doi.org/10.1007/s00284-002-3833-3

Patras, M. A., Milev, B. P., Vrancken, G., & Kuhnert, N. (2014). Identification of novel cocoa flavonoids from raw fermented cocoa beans by HPLC–MSn. *Food Research International*, *63*(Part C), 353–359. https://doi.org/10.1016/j.foodres.2014.05.031

Rosahl, S. (2014). Lipoxygenases in Plants -Their Role in Development and Stress Response. *Zeitschrift Für Naturforschung C*, *51*(3–4), 123–138. https://doi.org/10.1515/znc-1996-3-401

Sadik, C. D., Sies, H., & Schewe, T. (2003). Inhibition of 15-lipoxygenases by flavonoids: structure–activity relations and mode of action. *Biochemical Pharmacology*, *65*(5), 773–781. https://doi.org/10.1016/S0006-2952(02)01621-0

Schwan, R. F., & Wheals, A. E. (2004). The Microbiology of Cocoa Fermentation and its Role in Chocolate Quality. *Critical Reviews in Food Science and Nutrition*, *44*(4), 205–221. https://doi.org/10.1080/10408690490464104

Torija, M. J., Beltran, G., Novo, M., Poblet, M., Guillamón, J. M., Mas, A., & Rozès, N. (2003). Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. *International Journal of Food Microbiology*, *85*(1), 127–136. https://doi.org/10.1016/S0168-1605(02)00506-8

Turner, J. G., Ellis, C., & Devoto, A. (2002). The Jasmonate Signal Pathway. *The Plant Cell*, *14*(Suppl), s153–s164. https://doi.org/10.1105/tpc.000679

Wei, Y., Gossing, M., Bergenholm, D., Siewers, V., & Nielsen, J. (2017). Increasing cocoa butter-like lipid production of Saccharomyces cerevisiae by expression of selected cocoa genes. *AMB Express*, *7*(1), 34. https://doi.org/10.1186/s13568-017-0333-1

Weichert, H., Kolbe, A., Kraus, A., Wasternack, C., & Feussner, I. (2002). Metabolic profiling of oxylipins in germinating cucumber seedlings – lipoxygenase-dependent degradation of triacylglycerols and biosynthesis of volatile aldehydes. *Planta*, *215*(4), 612–619. https://doi.org/10.1007/s00425-002-0779-4

Yilmaz-Ersan, L. (2013). Sastav masnih kiselina vrhnja fermentiranog probiotičkim bakterijama. *Mljekarstvo : Časopis Za Unaprjeđenje Proizvodnje i Prerade Mlijeka*, *63*(3), 132–139. Retrieved from https://hrcak.srce.hr/106561

Ziegleder Gottfried. (2017). Flavour development in cocoa and chocolate. *Beckett's Industrial Chocolate Manufacture and Use*. https://doi.org/10.1002/9781118923597.ch8

Chapter 8:

8 Evolution of cocoa lipids across factory production process

8.1 Introduction

After the fermentation and drying, the manufacture of cocoa liquor involves roasting, another important stage of the cocoa processing. During roasting, evaporation of volatile acids occurs, and the moisture content reduces (Afoakwa, Paterson, Fowler, & Ryan, 2008; Aprotosoaie, Luca, & Miron, 2016; Beckett, Harding, & Freedman, 2008). The degree of roasting is a time/temperature dependent process, and it ranges between 5 to 120 min (usually 10 to 35) and 110 to 150 °C (Afoakwa et al., 2008; Aprotosoaie et al., 2016; Kongor et al., 2016). Roasting process provide ideal conditions for the Maillard reactions, where small molecules as amino acids, short chain peptides, and reducing sugars, produced during the fermentation and drying, undergo important chemical reactions having an impact on the final flavour of cocoa (Counet, Ouwerx, Rosoux, & Collin, 2004; Frauendorfer & Schieberle, 2008). Roasting can be achieved by 3 methods: whole bean roasting, nib roasting, and liquor roasting (Afoakwa et al., 2008; Aprotosoaie et al., 2016).

In 1828 Coenraad Van Houten combined alkaline salts with cocoa powder (Fig. 8.1), which resulted in reduction of acidity (natural cocoa is typically 5.0 –6.0 pH) reducing the bitterness and sourness of the chocolate, making the drink more palatable. It improved the dispersibility of cocoa powder in liquid and enhancement of the, giving it the appearance of a more intense flavor and darker color. Coenraad Van Houten patented his cocoa alkalizing discovery in Netherlands in 1828, hence the phrases dutched cocoa or alkalized cocoa are now used synonymously in today's chocolate industry (Beckett, 2011; Coe & Coe, 2007). Nowadays, alkalization is carried out primarily to change the colour and to improve the flavour of cocoa powder. It reduces astringency by polymerization of flavonoids during alkali treatment (Afoakwa et al., 2008). Alkalization occur at 3 different process stages: nib alkalization, cocoa

cake alkalization and cocoa liquor alkalization. Each process has distinct advantages and disadvantages. Nib and cocoa liquor alkalization will have an effect on cocoa butter.

Alkalizing Ingredients Used in Alkalizing Cacao

CFR-Code of Federal Regulations Sec. 163.110-114

	Potassium	Sodium	Ammonium	Magnesium
Carbonate	K_2CO_3	Na_2CO_3	$(NH_4)_2CO_3$	$MgCO_3$
Bicarbonate	$KHCO_3$	$NaHCO_3$	NH_4CO_3	
Hydroxide	KOH	NaOH	NH_4OH	
Oxide				MgO

Figure 8.1 Alkalized ingredients used in alkalization of cocoa adapted from ("Published Articles | Blommer Chocolate Company,").

In the first part of this chapter, the triacylglycerol profile of dried beans and their correspondent cocoa liquor were analyzed. The aim of this study was to determine the impact of the alkalization on the lipid profile. The second part of this chapter describes structure elucidation of markers of alkalization process. Quantification data of these compounds can be found in Audrey's Bergounhou master thesis, achieved under author's supervision. Correlation studies between the markers of dutching process and physical properties, and samples specifications are described in Appendix I.

8.2 Material and methods

8.2.1 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany), isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade), chloroform (Rotisolv® HPLC grade) and Tetra-dodecylammonium bromide was purchased from Carl Roth (Karlsruhe, Germany), dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany), ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethanol was subjected to distillation prior use.

8.2.2 Sample preparation

Dried cocoa beans samples and cocoa liquor sample from 5 different origins were received in several sets from Barry Callebaut. A total of 7 pairs of samples coming from Cuba, San Thome, Madagascar, Tanzania, and Venezuela were analyzed. Firstly, the seed material was deshelled and ground using a grinder with the purpose of making homogenous powder. Henceforth, an overnight Soxhlet (Buchi Extraction System B811 instrument, Flawil, Switzerland) method using dichloromethane as an extraction solvent, 150 mL of 5 g of powdered beans, was used. Cocoa liquors were treated in the same way. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis. For HPLC analysis, a concentration of 0.045 mg/mL in chloroform/ethanol (50/50) of cocoa lipid extract was prepared.

8.2.3 HPLC chromatographic conditions

TAGs molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). The column used in this study was a Pursuit XRs C18 (150 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 10 mM/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. The gradient elution program consisted of holding solvent steady A/B (40/60) for 3 min; followed by a linear gradient to solvent B (100) for 33 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 40/60 solvent A/B for 5 min before reuse.

8.2.4 High-resolution Mass spectrometry conditions

High-resolution masses were acquired using a time of flight MicrOTOF Focus mass spectrometer (Bruker Daltonics UHT Ultra, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of m/z 200-1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-

port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode.

8.2.5 DSC analysis

Thermal measurements were made using a Mettler Toledo DSC 822 instrument (Barcelona, Spain). A sealed aluminum pan containing n-eicosane was used as reference, and temperature and enthalpy were calibrated using cyclohexane and indium. The lipid samples (10 mg) were sealed in aluminum pans. Measurements were performed as follows: initially, the temperature was increased from 20 °C to 60 °C at the rate of 20 K/min to erase any thermal memory; then holding at 60 °C for 3 min; after this period, crystallization was carried out by cooling from 60 °C to -10 °C at a rate of -10 K/min; then, after holding for 3 min at -10 °C melting thermograms were obtained by heating the samples to 60 °C at 10 K/min. Results were analysed using Mettler Toledo Star software.

8.2.6 Statistical analysis

The individual TAG lists of the high-resolution masses were combined for peak matching and retention time alignment using a mass-to-charge (*m/z*) tolerance of $\Delta m/z = 0.0001$ and a retention time (R_t) tolerance of 0.5 min for elution time <20 min and 1.0 min for an elution time ≥20 min. Extensive manual curation of the peaks could allow the assignment with *m/z* and R_t tolerances of $\Delta m/z = 0.01$ and $\Delta R_t = 0.8$ min respectively. For further analyses, the peak areas were normalized to the internal standard (Tetra-dodecylammonium bromide) at R_t 4.4 min and consequently all the ratio were normalized to 100. Thus, data are presented as percentage.

Microsoft Office Excel 2016 was used to order and process the data. A one-factor and a two-factors ANOVA were used to study the effect of origin and the effect of fermentation status on fatty acid profile. The analysis were carried out with XLSTAT Evaluation 2018.2 software version 2018 (Addinsoft, France).

8.3 Results and discussion

8.3.1 Lipid content in dried beans and cocoa liquor

The roasting process have shown to affect the total lipid amount (Fig. 8.2). Only the samples from San Thome did not show variation in the lipid content after roasting. In all other samples, the total lipid amount decreases from bean to liquor. Two explanation can be drawn, first, the roasting affect the extractability of the lipid amount. Studies showed that the roasting process increase the extractability of the butter (Asep et al., 2008). Secondly, a loss of lipid content could occur due to some chemical reactions of an unknown nature involving the lipids that might take place during the roasting and grinding processes.

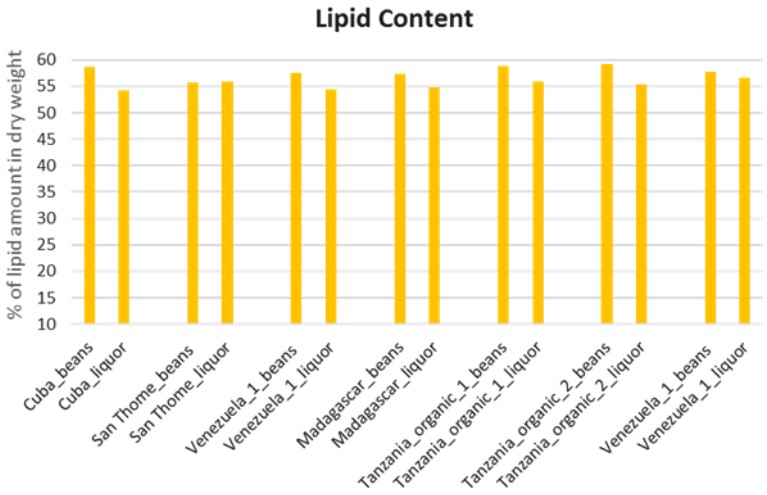


Figure 8.2 Lipid amount in cocoa beans and cocoa liquors from different origin.

8.3.2 TAGs profile of dried beans *versus* roasted beans in form of cocoa liquor

Monounsaturated TAGs showed an overall decrease with the roasting of the beans (Fig. 8.3, Table F.3 Appendix). However, not all samples exhibit the same behavior. The most abundant TAG present a variable behavior. No trend could be delineated, 5 of 7 samples displayed a decrease of POS.

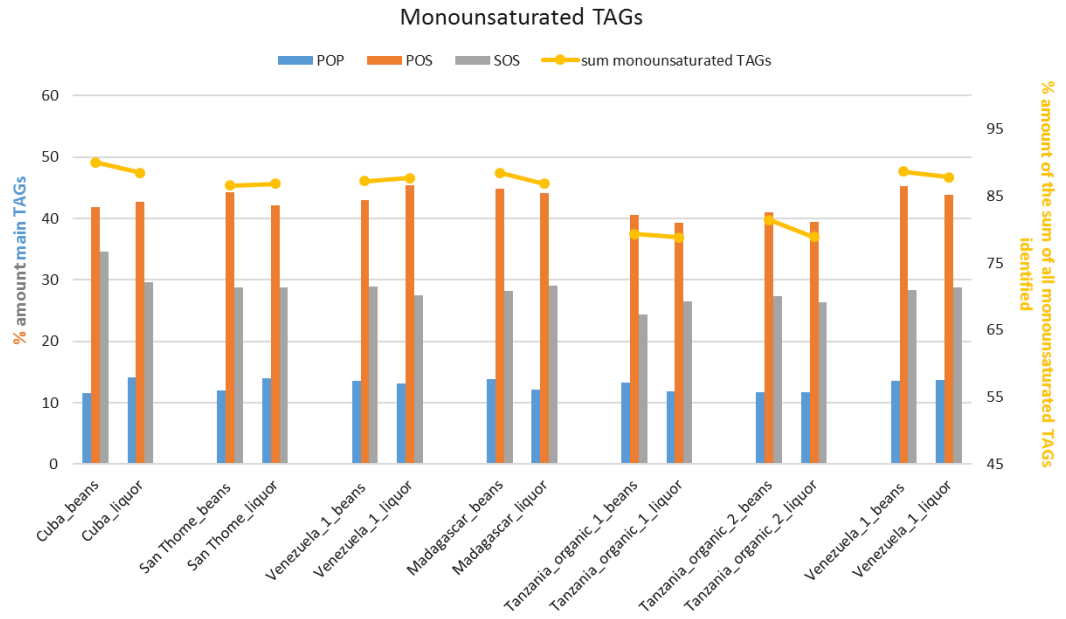


Figure 8.3 Profile amount of monounsaturated TAGs in cocoa beans and cocoa liquors from different origin.

Di- and triunsaturated TAGs total amount revealed an increasing trend after roasting. Notwithstanding no distribution pattern of the individual unsaturated TAGs could be observed (Fig. 8.4, Table F.4 Appendix). The ratio between di- and triunsaturated/monounsaturated TAGs seems to increase after roasting, whereas the ratio saturated/monounsaturated TAGs has a random comportment (Fig. 8.5). It is worth remarking that form the graph we can grasp the similarities and differences between the samples. Thus, samples from Cuba have higher amount of saturated TAGs, while Tanzanian samples own higher amount of di- and triunsaturated TAGs.

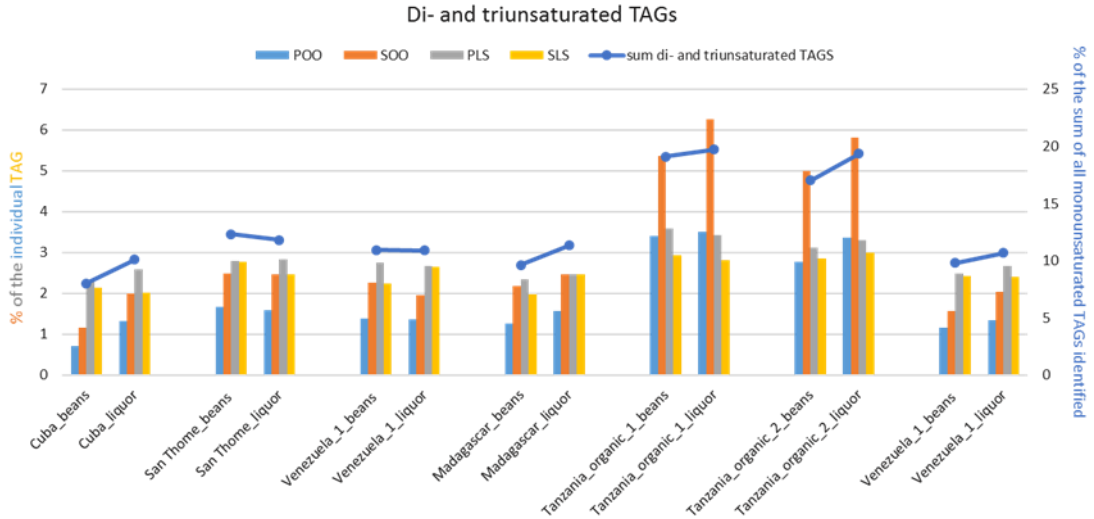


Figure 8.4 Profile amount of di- and triunsaturated TAGs in cocoa beans and cocoa liquors from different origin.

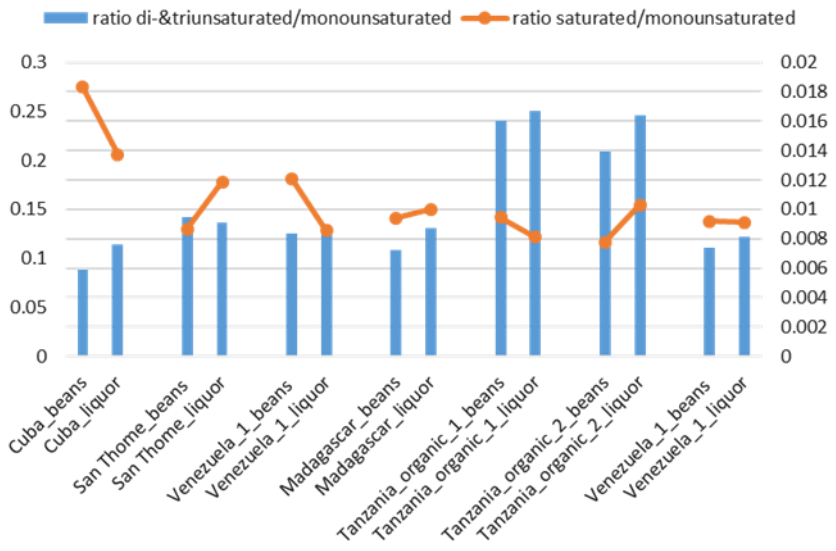


Figure 8.5 Ratios between di- and triunsaturated TAGs and ratios between saturated and monounsaturated TAGs in cocoa beans and cocoa liquors from different origin.

Hydroxyl-TAGs were found to be markers of the fermentation status of the beans (Chapter 7). Consequently, the presence of these compounds in the cocoa samples might indicate that incomplete fermentation of the beans (Fig. 8.6, Table F.5 Appendix). Only the samples from Cuba and San Thome have shown lower amount of hydroxyl-TAGs. Figure 8.7 illustrate a complete random nature of saturated TAGs (Table F.1 Appendix).

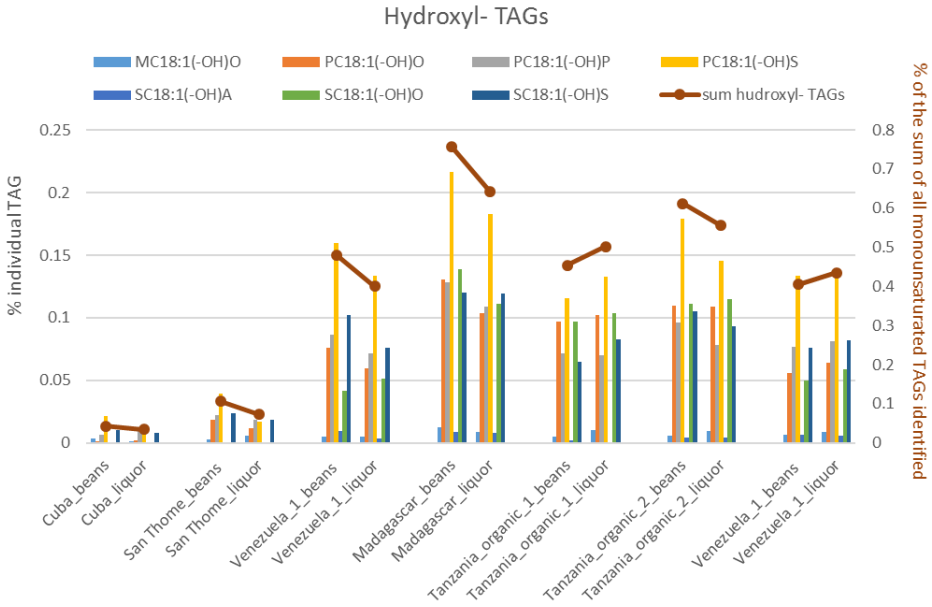


Figure 8.6 Profile amount of hydroxyl-TAGs in cocoa beans and cocoa liquors from different origin.

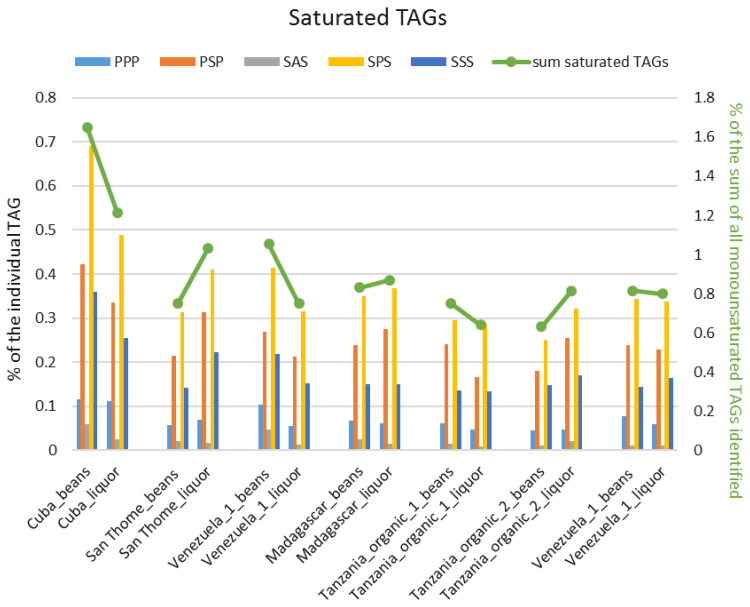


Figure 8.7 Profile amount of saturated TAGs in cocoa beans and cocoa liquors from different origin.

8.3.3 DAGs profile of dried beans *versus* roasted beans in form of cocoa liquor

In the same order, DAGs did not show a particular demeanor. In 4 origins out of 7 the amount decreases with the bean roasting, whereas in 2 samples DAG increases with roasting (Fig. 8.8, Table F.2 Appendix).

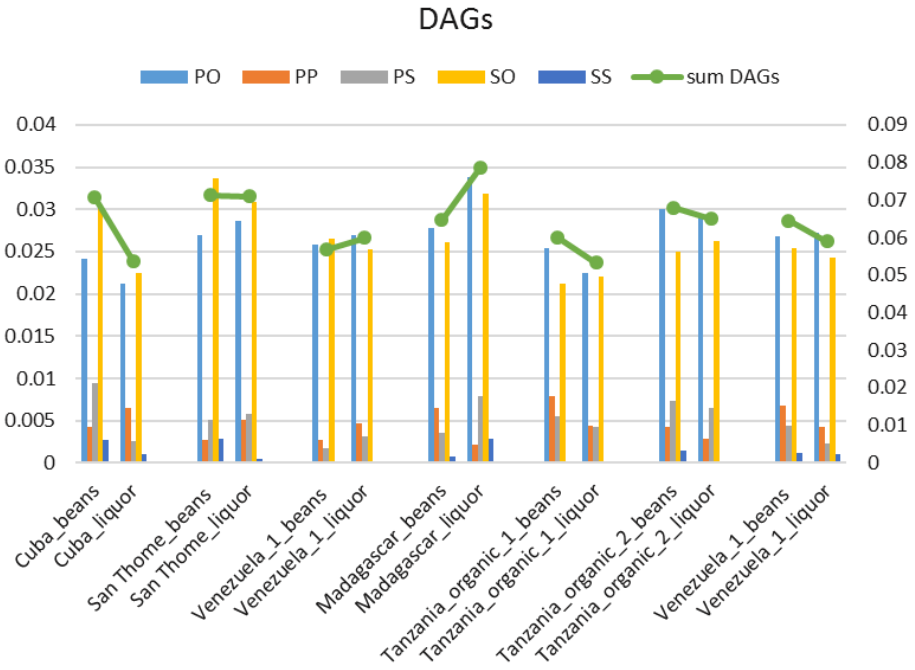


Figure 8.8 Profile amount of DAGs in cocoa beans and cocoa liquors from different origin.

8.3.4 Correlations studies on TAG and DAG profiles

Correlation studies have shown that there is a significant negative correlation between the amount of monounsaturated TAGs and the amount of di and tri-unsaturated TAGs, which indicated that there is an inverse proportion of these 2 subclasses of TAGs. Saturated TAGs have shown a positive correlation with monounsaturated TAGs and a negative correlation with hydroxyl- TAGs and di- and triunsaturated TAGs.

Table 8.1 Correlation values of the TAG lipid classes, DAG, and total lipid amount.

Correlation matrix (Pearson):

Variables	DAGs	lipid amount	unknown compounds	di- and triunsaturated TAGS	monounsaturated TAGs	saturated TAGs	Hydroxyl- TAGs
DAGs	**1**	0.066	-0.110	-0.216	0.210	0.129	-0.016
lipid amount	0.066	**1**	0.376	0.088	-0.109	0.092	0.174
unknown compounds	-0.110	0.376	**1**	**0.695**	**-0.727**	-0.467	**0.784**
di- and triunsaturated TAGS	-0.216	0.088	**0.695**	**1**	**-0.998**	**-0.580**	0.354
monounsaturated TAGs	0.210	-0.109	**-0.727**	**-0.998**	**1**	**0.560**	-0.384
saturated TAGs	0.129	0.092	-0.467	**-0.580**	**0.560**	**1**	**-0.617**
Hydroxyl- TAGs	-0.016	0.174	**0.784**	0.354	-0.384	**-0.617**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix (Spearman):

Variables	DAGs	lipid amount	Hydroxyl- TAGs	unknown compounds	monounsaturated TAGs	di- and triunsaturated TAGS	saturated TAGs
DAGs	**1**	0.081	0.077	-0.046	-0.015	0.011	0.147
lipid amount	0.081	**1**	0.182	0.336	0.077	-0.068	-0.143
Hydroxyl- TAGs	0.077	0.182	**1**	**0.842**	-0.363	0.327	-0.345
unknown compounds	-0.046	0.336	**0.842**	**1**	**-0.547**	0.534	-0.499
monounsaturated TAGs	-0.015	0.077	-0.363	**-0.547**	**1**	**-0.996**	**0.565**
di- and triunsaturated TAGS	0.011	-0.068	0.327	0.534	**-0.996**	**1**	**-0.574**
saturated TAGs	0.147	-0.143	-0.345	-0.499	**0.565**	**-0.574**	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Correlation matrix (Kendall):

Variables	DAGs	saturated TAGs	di- and triunsaturated TAGS	monounsaturated TAGs	unknown compounds	Hydroxyl- TAGs	lipid amount
DAGs	**1**	0.121	-0.055	0.033	-0.011	0.055	0.033
saturated TAGs	0.121	**1**	**-0.451**	**0.429**	-0.363	-0.253	-0.099
di- and triunsaturated TAGS	-0.055	**-0.451**	**1**	**-0.978**	0.385	0.231	-0.055
monounsaturated TAGs	0.033	**0.429**	**-0.978**	**1**	**-0.407**	-0.253	0.077
unknown compounds	-0.011	-0.363	0.385	**-0.407**	**1**	**0.670**	0.253
Hydroxyl- TAGs	0.055	-0.253	0.231	-0.253	**0.670**	**1**	0.099
lipid amount	0.033	-0.099	-0.055	0.077	0.253	0.099	**1**

Values in bold are different from 0 with a significance level alpha=0.05

Assuming that the sampling error is very low, the outcome of the results can be rationalized as following: a random acyl-rearrangement, or interesterification, on the glycerol molecule occurred under the high temperature conditions of the roasting process. Interesterification has long been used to modify modify the physico-chemical and nutritional characteristics of oils and fats (Willis, Lencki, & Marangoni, 1998). Two types of interesterification are recognised: chemical and enzymatic (Sreenivasan, 1978). Many studies were conducted to produce cocoa butter-like fats through interesterification (Abigor et al., 2003; Chang, Abraham, & John, 1990).

8.3.5 Elucidation of the fatty acids amides as markers of alkalization

Separation of lipid compounds from normal and alkalized cocoa butter (CB) by HPLC-MS (Fig. 8.9) revealed the presence of significant differences in the lipid profile of the less abundant components. To be noted that the butter underwent SPE fractionation prior analysis.

These compounds were identified as the amide form of the most abundant fatty acids present in cocoa, namely palmitamide, oleamide, and stearamide (Fig. 8.10). Figure 8.11 illustrates the tandem MS spectra of oleamide as example.

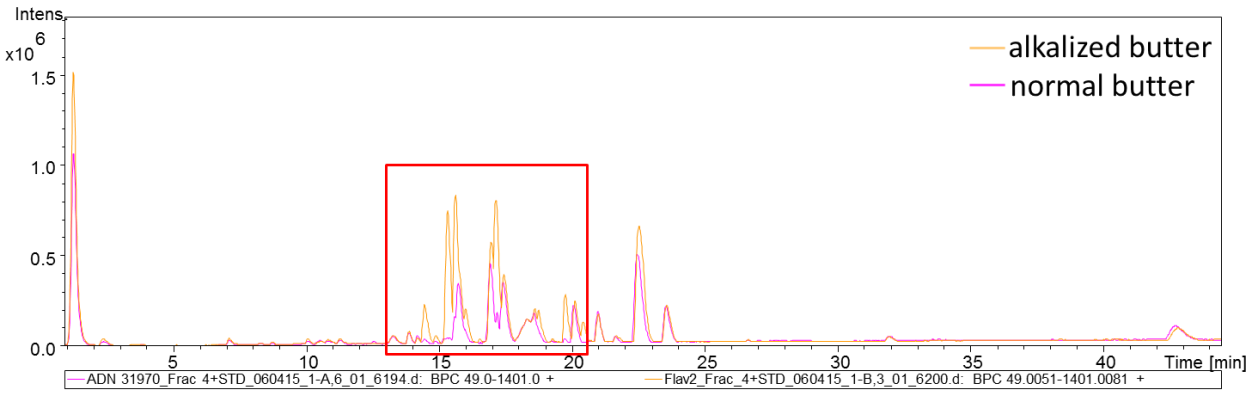


Figure 8.9 Positive ion mode HPLC-MS chromatogram of normal and alkalized lipid profile.

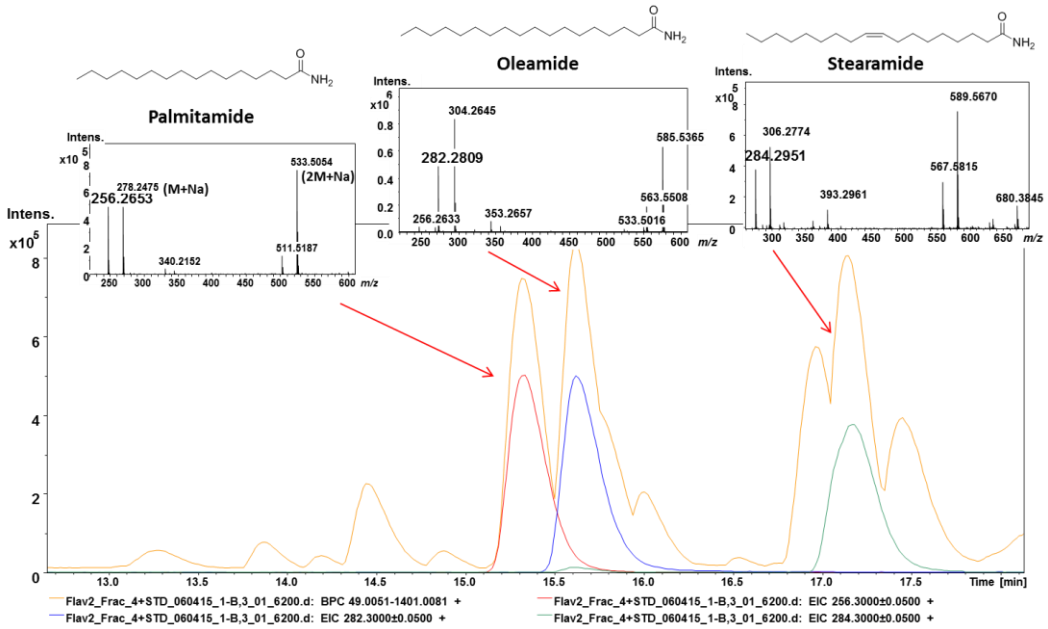


Figure 8.10 Positive ion mode HPLC-MS chromatogram (zoomed) of alkalized butter and mass spectra of the new identified compounds.

Fragmentation of oleamide showed the loss of 17 Da corresponding to ammonia and subsequent water loss. MS^3 revealed the typical fatty acid fragmentation, each 14 Da corresponds to a CH_2.

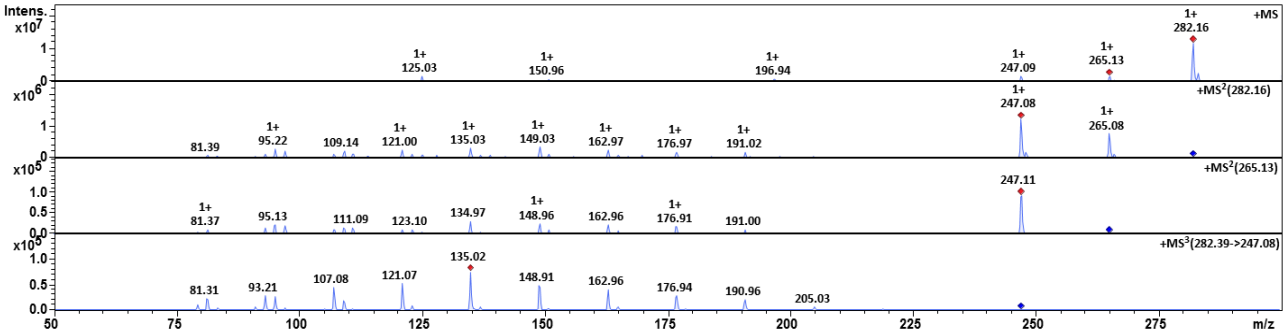


Figure 8.11 Tandem MS spectra of oleamide.

8.3.6 Influence of the fatty acid amides on the crystallization behavior of cocoa butter

To gain more insight into the impact of fatty acids amides (FAA) on the crystallization of cocoa butter thermal measurements using DSC were performed. Figure 8.12 and 8.13 displays the thermograms acquired for natural cocoa butter (CB), natural CB mixed with 0.1% fatty acid amides mix, natural CB plus 1% FAA mix, and natural CB plus 2% FAA mix. The shape of the DSC curves (Fig. 8.12) indicates a two-step crystallization process when natural butter was mixed with 1 and 2% of FAA. The complete crystallization of cocoa butter in the presence of FAA occurred at a lower temperature suggesting that FAA affects crystallization to lower polymorphic forms such as γ and α. Concerning the melting peaks (Fig. 8.13), the absolute values were lower for cocoa butter containing the highest amount of FAA. The solid fat content (SFC), defined as a parameter that expresses the solid/liquid mass relation of a fat at different temperatures, have shown that an amount between 0.1 and 1% FAA can be related to an increase of approximately 3% in SFC value at 25 °C (Fig. 8.14). In conclusion, DSC analysis revealed that FAA has a measurable effect on the crystallization of cocoa butter.

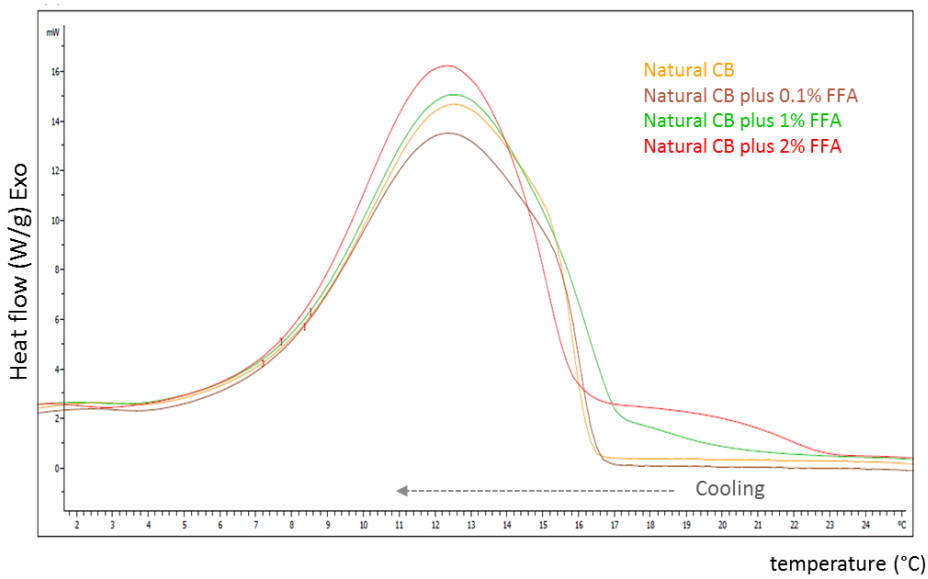


Figure 8.12 DSC crystallization thermograms recorded on cooling of natural CB, natural cocoa butter (CB), natural CB plus 0.1% fatty acid amides (FAA) mixture, natural CB plus 1% FAA, and natural CB plus 2% FAA mixture.

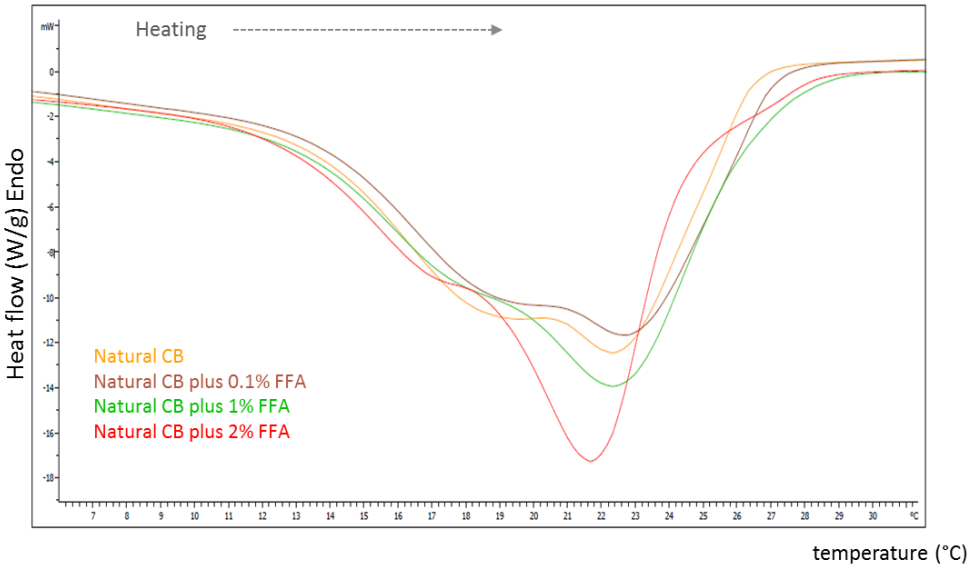


Figure 8.13 DSC crystallization melting curves of natural CB, of natural cocoa butter (CB), natural CB plus 0.1% fatty acid amides (FAA) mixture, natural CB plus 1% FAA, and natural CB plus 2% FAA mixture.

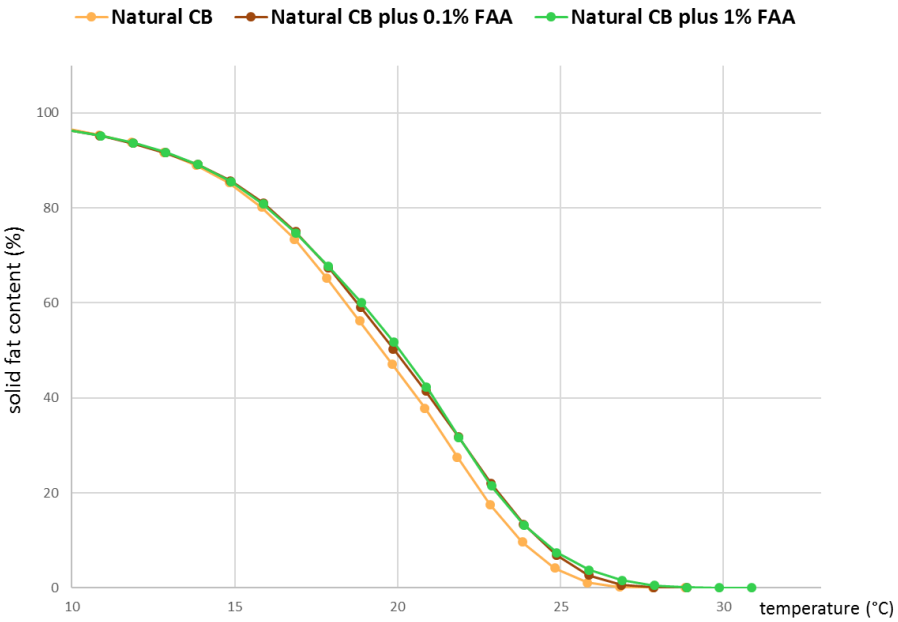


Figure 8.14 Solid fat content (in %) as a function of temperature (°C) of natural cocoa butter (CB), natural CB plus 0.1% fatty acid amides (FAA) mixture, and natural CB plus 1% FAA mixture.

8.4 Conclusions

Analysis of dried beans and their respective cocoa liquor did not show any pattern on TAGs and DAGs distribution. However, saturated TAGs have shown a positive correlation with monounsaturated TAGs. Therefore, a conclusion could be that an interesterification of the fat or other chemical reactions might occur and this process happens completely random. Successful elucidation of the compounds responsible for the differences observed between normal and alkalized cocoa butter, namely fatty acids amides, was achieved. These compounds were consequently designated as markers of the alkalization process. FAA affect the crystallization of cocoa butter by promoting the crystallization of lower polymorphic forms from the melt. Further work is necessary to complete the picture on the physical properties of cocoa butter containing FAA such as crystallization under isothermal conditions.

Supplementary material is available on request. E-mail: n.kuhnert@jacobs-university.de

References

Abigor, R. D., Marmer, W. N., Foglia, T. A., Jones, K. C., DiCiccio, R. J., Ashby, R., & Uadia, P. O. (2003). Production of cocoa butter-like fats by the lipase-catalyzed interesterification of palm oil and hydrogenated soybean oil. *Journal of the American Oil Chemists' Society*, *80*(12), 1193–1196. https://doi.org/10.1007/s11746-003-0841-7

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

Aprotosoaie, A. C., Luca, S. V., & Miron, A. (2016). Flavor Chemistry of Cocoa and Cocoa Products—An Overview. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 73–91. https://doi.org/10.1111/1541-4337.12180

Asep, E. K., Jinap, S., Tan, T. J., Russly, A. R., Harcharan, S., & Nazimah, S. A. H. (2008). The effects of particle size, fermentation and roasting of cocoa nibs on supercritical fluid extraction of cocoa butter. *Journal of Food Engineering*, *85*(3), 450–458. https://doi.org/10.1016/j.jfoodeng.2007.08.008

Beckett, Stephen T., Harding, J., & Freedman, B. (2008). *The Science of Chocolate* (2nd edition). Cambridge, UK: Royal Society of Chemistry.

Beckett, Steve T. (2011). *Industrial Chocolate Manufacture and Use*. John Wiley & Sons.

Chang, M.-K., Abraham, G., & John, V. T. (1990). Production of cocoa butter-like fat from interesterification of vegetable oils. *Journal of the American Oil Chemists' Society*, *67*(11), 832–834. https://doi.org/10.1007/BF02540501

Coe, S. D., & Coe, M. D. (2007). *The True History of Chocolate* (Second Edition edition). New York: Thames & Hudson.

Counet, C., Ouwerx, C., Rosoux, D., & Collin, S. (2004). Relationship between Procyanidin and Flavor Contents of Cocoa Liquors from Different Origins. *Journal of Agricultural and Food Chemistry*, *52*(20), 6243–6249. https://doi.org/10.1021/jf040105b

Frauendorfer, F., & Schieberle, P. (2008). Changes in Key Aroma Compounds of Criollo Cocoa Beans During Roasting. *Journal of Agricultural and Food Chemistry*, *56*(21), 10244–10251. https://doi.org/10.1021/jf802098f

Kongor, J. E., Hinneh, M., de Walle, D. V., Afoakwa, E. O., Boeckx, P., & Dewettinck, K. (2016). Factors influencing quality variation in cocoa (Theobroma cacao) bean flavour profile — A review. *Food Research International*, *82*(Supplement C), 44–52. https://doi.org/10.1016/j.foodres.2016.01.012

Published Articles | Blommer Chocolate Company. (n.d.). Retrieved March 23, 2018, from http://www.blommer.com/articles.php

Sreenivasan, B. (1978). Interesterification of fats. *Journal of the American Oil Chemists' Society*, *55*(11), 796–805. https://doi.org/10.1007/BF02682651

Willis, W. M., Lencki, R. W., & Marangoni, A. G. (1998). Lipid Modification Strategies in the Production of Nutritionally Functional Fats and Oils. *Critical Reviews in Food Science and Nutrition*, *38*(8), 639–674. https://doi.org/10.1080/10408699891274336

Chapter 9:

9 Minor lipid compounds in cocoa

9.1 Introduction

Cocoa butter is mainly constituted by triacylglycerols (TAGs) (95-98%) and a complex mixture of minor components (2-5%) of a diverse chemical nature. These compounds show a wide qualitative and quantitative composition, depending on the species, climatic conditions, pre- and the post-fermentation process of the beans (Cert, Moreda, & Pérez-Camino, 2000; Lipp et al., 2001; Lipp & Anklam, 1998).

The main groups of minor constituents present in a vegetable oil and animal fat are: long-chain aliphatic and triterpenic alcohols, methyl sterols, sterols, waxes, sterol esters, tocopherols, tocotrienols, pigments, diacylglycerols (DAGs), monoacylglycerols (MAGs), glycolipids, phospholipids and triterpenic acids. This group encloses numerous species with a broad range of polarities, concentration and chemical structures and functions; therefore, characterization of these compounds is quite tricky. The required methods imply isolation and analysis of minor constituents from a lipid extract through several steps of separation, identification, and quantification (Christie & Han, 2010).

Until this moment, the research studies of this thesis were mostly focused on the analysis of samples coming from the post-harvesting process, wet unfermented and dried fermented beans with a major attention on triacylglycerols identification. Identification of the minor lipid component is still at the early stage and the results shown here must be considered as preliminary and not yet comprehensive.

To analyses the minor components, a preliminary qualitative and quantitative isolation step from the TAGs matrix was required. Solid phase extraction (SPE) was applied, as it is a quicker technique and saves solvent volumes (Kim & Salem, 1990). SPE allowed the separation of 5 fractions (see Chapter 3).

Identification and quantification of individual molecular species required further chromatography separation and since lipids lack chromophores mass spectrometry (microTOF

MS, Bruker) is used to obtain molecular mass data, structural information, and identification of compounds. Separation of the individual components that belong to one constituent class by reversed-phase HPLC using Ethanol as a polar solvent was achieved. Each lipid class has a unique fragmentation pattern, which aids unambiguous identification of the species eluted from a column through product-ion MS analysis (IonTrap MS, Bruker).

9.2 Material and methods

9.2.1 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany), isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade) and chloroform (Rotisolv® HPLC grade) was purchased from Carl Roth (Karlsruhe, Germany), dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany), ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ethanol was subjected to distillation prior use. Standard DAG 1-palmitoyl-2-oleoyl-*sn*-glycerol and cholesterol all at ≥ 99% were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

9.2.2 Sample preparation

Frozen unfermented wet cocoa bean samples and fermented dried cocoa beans from different origins were received in several sets from Barry Callebaut (Lebbeke-Wieze, Belgium). Firstly, the seed material was crushed using a grinder Retsch Grindomix GM200 knife mill (Haan, Germany) with the purpose of making homogenous powder. Henceforth, an overnight Soxhlet (Buchi Extraction System B811 instrument) method using dichloromethane as an extraction solvent, 150 mL of 5 g of crushed beans, was used. Extracted lipids were quantified gravimetrically after evaporation to dryness in a rotavapor. The dry residue was then stored at -20 °C until further analysis. For HPLC analysis, a concentration of 0.045 mg/mL in chloroform/ethanol (50/50) of cocoa lipid extract was prepared.

9.2.3 Solid phase extraction (SPE)

Chromabond aminopropyl modified silica gel NH_2 (Chromabond® LV-NH_2, Macherey-Nagel GmbH & Co. KG, Düren, Germany) column cartridges (3 mL/500 mg) were used for the SPE fractionation. The cartridges were first activated with 20 mL heptane/ethylacetate (80/20, v/v), then, 20 ml heptane. Then, 10 mg of the lipid extract dissolved in 1 mL of heptane were applied, after which the fractions were eluted with 5 different solvent mixtures: 1) 20 mL heptane, first fraction collected ; 2) 20 mL heptane/ethyl acetate (97/3, v/v), for the second fraction; 3) 20 mL heptane/ethyl acetate (85/15, v/v), third fraction collected; 4) 20 mL heptane/ethyl acetate (85/15, v/v) plus 1% acetic acid, for the forth fraction; and 5) 20 mL methanol. Extracted lipids were evaporated to dryness in a rotavapor and stored at -20 °C until further analysis.

9.2.4 HPLC chromatographic conditions

TAGs molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). The column used in this study was a Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 10 mM/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. Two different elution gradients were used. One for the total lipid separation and another to separate minor lipid components in cocoa. The first gradient elution program consisted of holding solvent steady A/B (40/60) for 3 min; followed by a linear gradient to solvent B (100) for 33 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 40/60 solvent A/B for 5 min before reuse. The second gradient elution program consisted of holding solvent steady A (100) for 5 min; followed by a linear gradient to solvent A/B (70/30) for another 5; then by a linear gradient to solvent B (100) for 90 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 100 solvent A for 5 min before reuse.

9.2.5 High-resolution Mass spectrometry conditions

High-resolution masses were acquired using a time of flightMicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of *m/z* 200-1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode. Molecular formulae suggestions were accepted if the mass error was below 5 ppm.

9.2.6 Tandem Mass spectrometry conditions

LC-tandem MS was carried out using an Ion-Trap detector in positive ion mode equipped with an ESI source (Bruker Daltonics UHT Ultra, Bremen, Germany). The full scan mass spectra were recorded in the range *m/z* 200-1200 operating in positive ion mode. Capillary temperature was set to 350 °C, drying gas flow rate of 10 L/min and nebulizer pressure of 10 psi. Tandem mass spectra were acquired in Auto MS^n (smart fragmentation) using a ramping of the collision energy.

9.3 Results and discussion

Minor components have been separated from the major components in 4 fractions: fraction 1, fraction 3, fraction 4 and fraction 5. Fraction 2 represents the TAGs (see Chapter 3, Fig. 3.6), and they constitute major components, the usual TAGs found in cocoa butter and most abundant ones. These TAGs constitute the macro components of cocoa butter, and they have been extensively characterized in Chapter 4.

9.3.1 Molecular species fraction 1

Highly lipophilic compounds characterize fraction 1 (Fig. 9.1). So far, one class of compounds could be identified in this fraction, which are sterol esters. Assignment of individual sterol ester structures was based on ESI-tandem MS (MS^n) measurements.

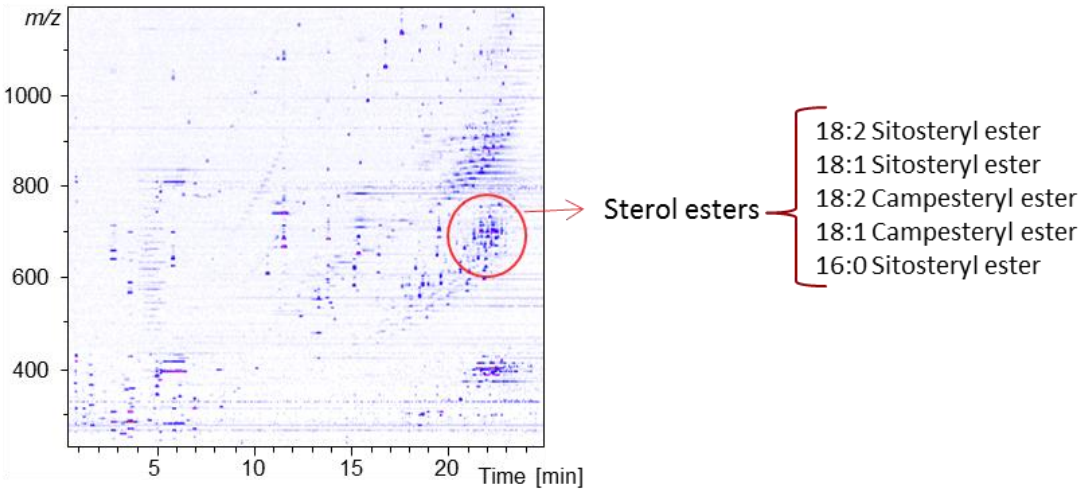


Figure 9.1 2D map of the LC-MS chromatogram in positive ion mode of fraction 1 minor components of the SPE lipid extract from fermented dried beans. Red circle indicates the sterol esters class.

Collision induced dissociation of $[M+NH_4]^+$ precursor ions resulted in the neutral loss of 297.3 Da, acyl group and ammonium adduct, generating a sterol fragment ion at *m/z* 383.4 and *m/z* 397.4 for the 2 sterol esters respectively (Fig. 9.2 (a) and (b)). In the absence of sterol ester standard, standard cholesterol was used as surrogate standard for comparison (Fig. 9.2 (c)). MS^3 of the two illustrated sterol esters, 18:2 Campesteryl ester and 18:2 Sitosteryl ester, showed very similar fragmentation pattern if compared to MS^2 of the standard. Herein, 5 compounds were identified.

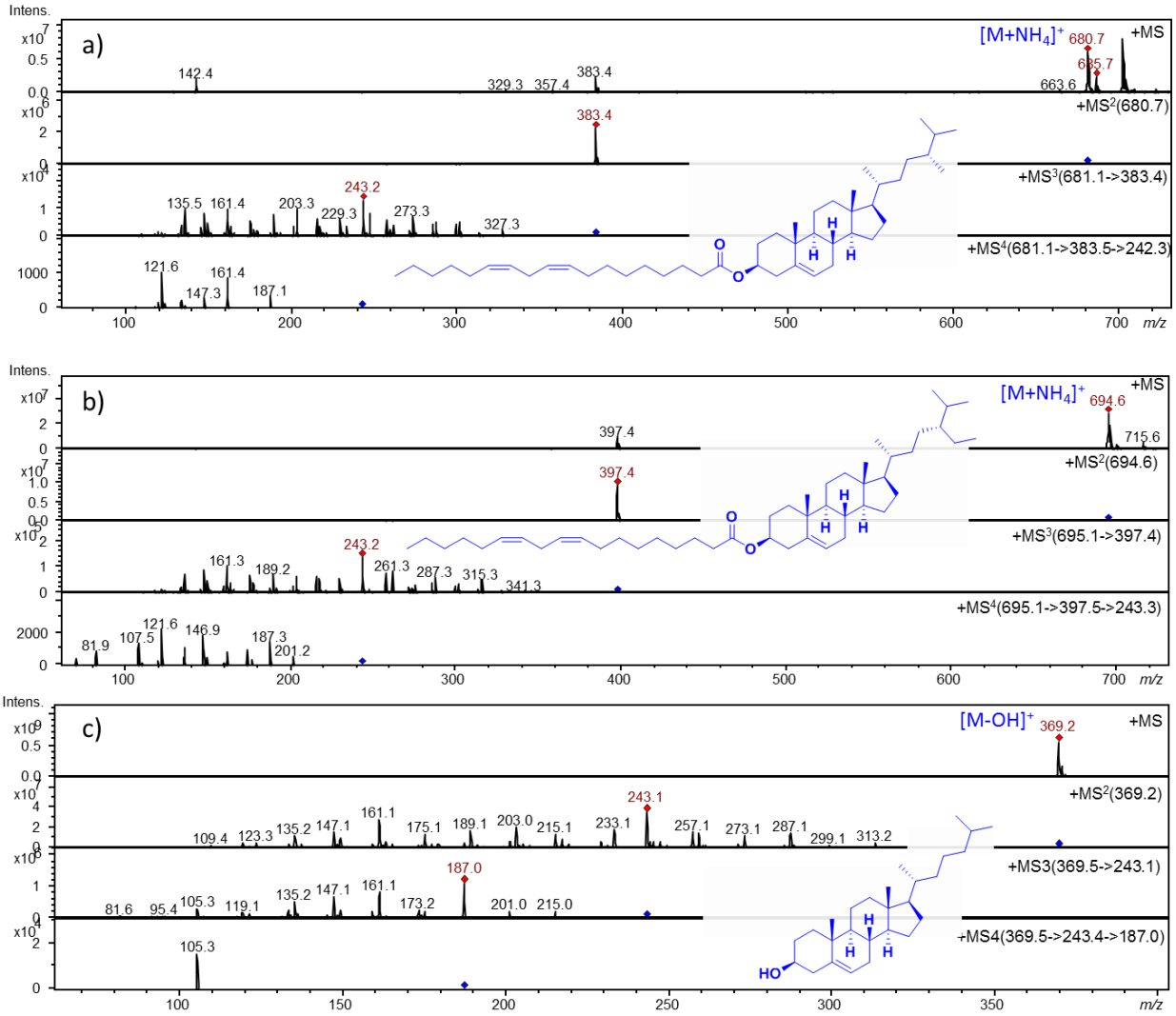


Figure 9.2: Positive ion mode tandem mass spectrum of sterol esters **(a)** 18:2 Campesteryl ester (*m/z* 680.7) ammonia adducts, **(b)** 18:2 Sitosteryl ester (*m/z* 694.6) ammonia adducts, **(c)** cholesterol standard (*m/z* 369.2) corresponding to $[M\text{-}OH]^+$ molecular ion.

9.3.2 Molecular species fraction 3

A different profile was observed when comparing the chromatograms of the fraction 3 from unfermented wet cocoa beans and fermented dried cocoa beans (Fig. 9.3). The difference is attributable to the unusual TAGs in unfermented wet beans, which have been described as hydroxyl TAG derivatives and thus, labeled as markers of the fermentation status of the beans (see Chapter 4 and 5). Other classes were identified in fraction 3 and show no substantial variations between the two types of cocoa beans, which are diacylglycerols (DAGs) and waxes.

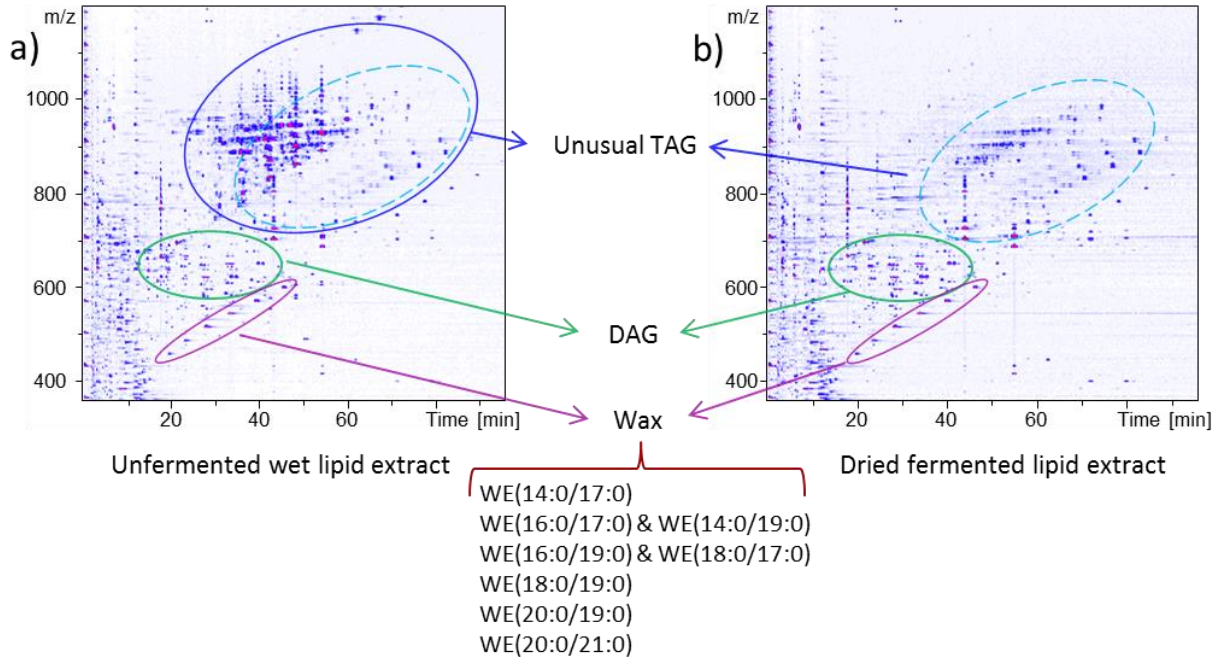


Figure 9.3 2D map of the LC-MS chromatogram in positive ion mode of fraction 3 minor components of the SPE lipid extract from **(a)** unfermented wet cocoa beans and **(b)** fermented dried cocoa beans. Blue circle indicates the hydroxyl-TAGs, green circle indicates the diacylglycerol class, and magenta circle indicates waxes class.

Concerning DAGs, two peaks at different retention time were observed (Fig. 9.4 (a)) thus, indicating the occurrence of two regioisomers 1,2-acyl-*sn*-glycerol and 1,3-acyl-*sn*-glycerol. In order to determine in which sequence the two regioisomers elute on the reversed-phase column, 1-palmitoyl-2-oleoyl-*sn*-glycerol standard was injected and tandem MS of the standard and the DAGs containing palmitic and oleic acid esterified to the glycerol backbone, *m/z* 577.5, were studied (Fig. 9.4 (b)). The *m/z* 577.5 corresponds to $[M\text{-}H_2O\text{-}NH_4]^+$ molecular ion and showed higher intensity than the precursor ion. By taking into account the retention time of the standard, it could be deduced that the first peak eluting at 27.6 min corresponds to 1-palmitoyl-2-oleoyl-*sn*-glycerol. However, such conclusion could not be inferred from MS^2 fragments since the two regioisomers displayed same fragmentation pattern. Notably, adduct ions of 1-palmitoyl-2-oleoyl-*sn*-glycerol and 1-palmitoyl-3-oleoyl-*sn*-glycerol have shown different stability at given MS conditions (Fig. 9.5). From the MS spectrum, it can be supposed that 1-palmitoyl-3-oleoyl-*sn*-glycerol is more stable because the molecular ion $[M+NH_4]^+$ is present in higher intensity but entirely absent for 1-palmitoyl-2-oleoyl-*sn*-glycerol. Sodium adduct is present for both regioisomers.

Identification of DAGs was carried out using a high-resolution ESI-TOF mass spectrometer operating in the positive ion mode leading to assignment of molecular formulae. Molecular formulae were accepted if only the mass error was found to be below 5 ppm. LC-ESI-TOF MS method gave two pseudomolecular ions, including $[M\text{-}H_2O\text{-}NH_4]^+$, and $[M+Na]^+$, as the most common base peak with $[M+Na]^+$ being the most abundant. Figure 9.6 and Table 9.1 illustrates the HPLC-MS extract ion chromatogram in positive ion mode of all diacylglycerols identified in cocoa lipid extract. Moreover, lipidmaps database was used to compare experimental *m/z* values with database entries to confirm the masses assigned.

Besides DAGs, wax lipid compounds were identified in fraction 3 by tandem MS. As an example, heptadecyl-palmitate, *m/z* 512.5 $[M+NH_4]^+$, produced MS^2 base peak at *m/z* 283.07 and *m/z* 256.06, showing a neutral loss of 230 and 256 Da respectively (Fig. 9.7). 256 Da corresponds to the loss of palmitic acid.

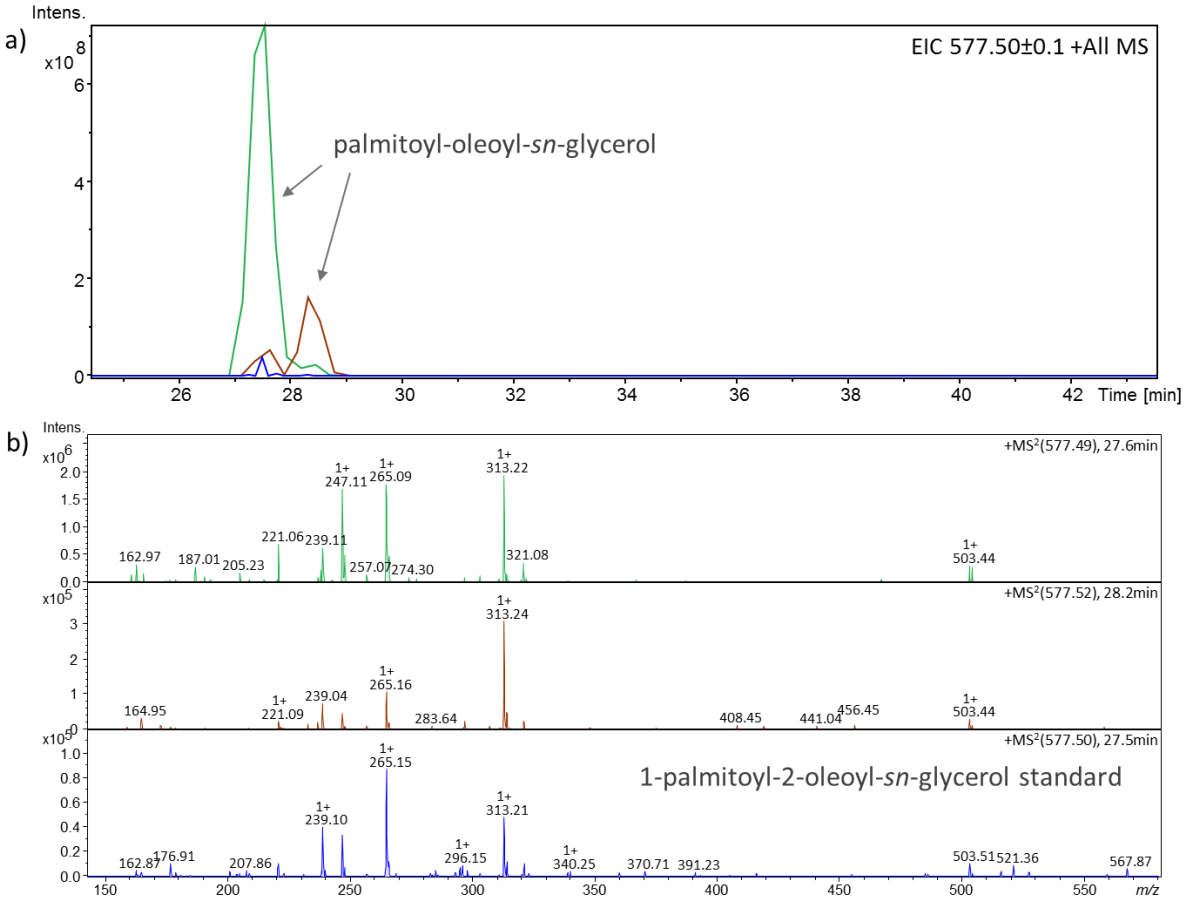


Figure 9.4 (a) HPLC-MS extract ion chromatogram of *m/z* 577.5 in positive ion mode corresponding to palmitoyl-oleoyl-*sn*-glycerol and **(b)** tandem mass spectrum displaying the fragmentation of 2 regioisomers and tandem MS spectrum of the standard 1-palmitoyl-2-oleoyl-*sn*-glycerol.

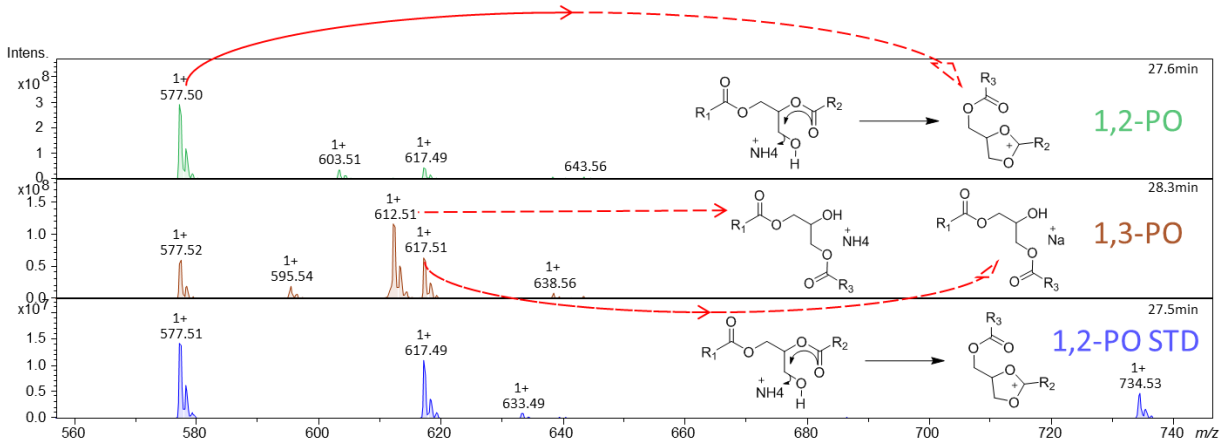


Figure 9.5 MS spectrum illustrating the adduct ions of 1-palmitoyl-2-oleoyl-*sn*-glycerol, 1-palmitoyl-3-oleoyl-*sn*-glycerol from cocoa lipid extract, and of 1-palmitoyl-2-oleoyl-*sn*-glycerol standard compound.

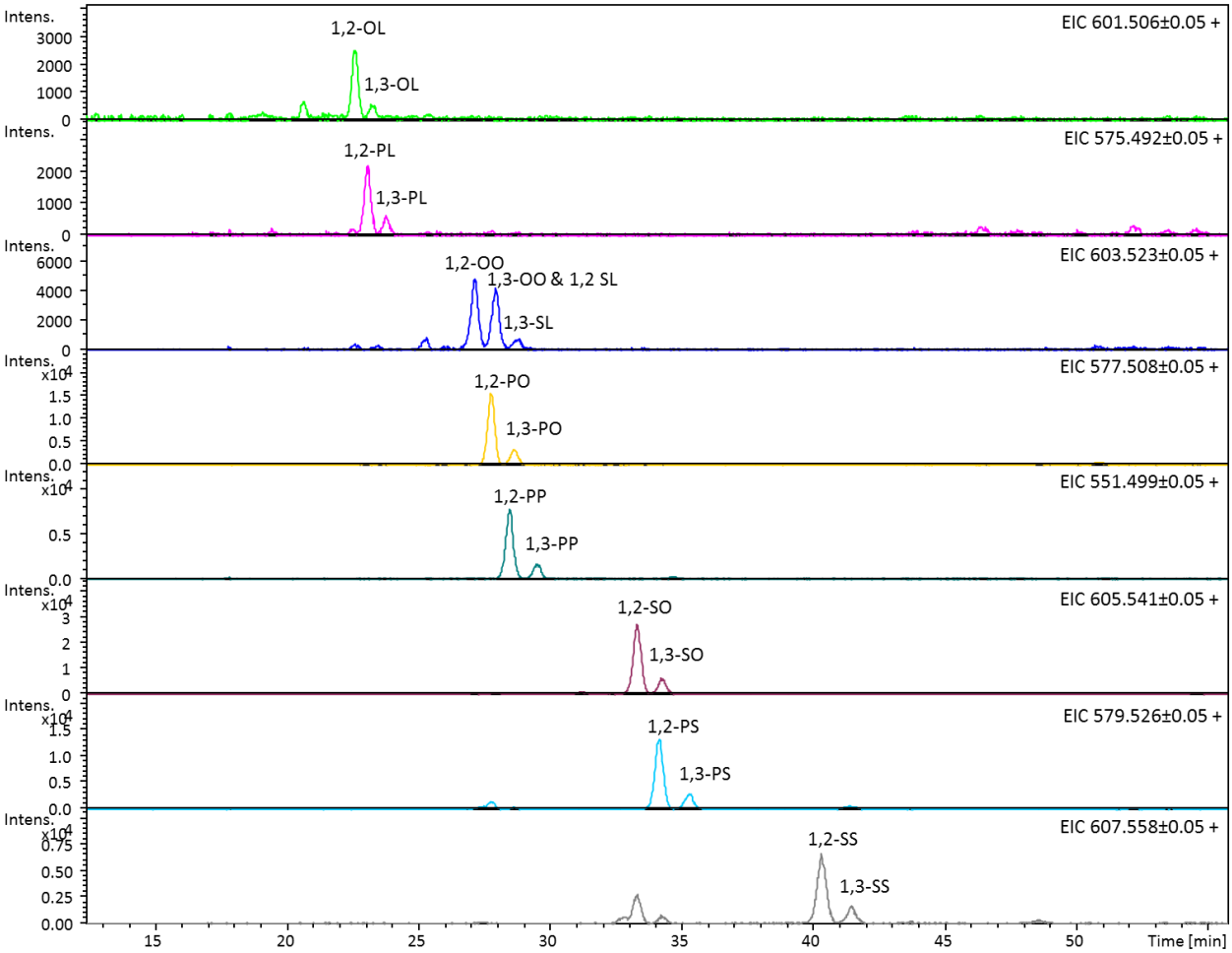


Figure 9.6 HPLC-MS extract ion chromatogram in positive mode of diacylglycerols identified in cocoa lipid extract.

Table 9.1 Diacylglycerol molecular species in cocoa beans lipid extract.

No.	*R. time (min)*	*Experimental mass $[M+Na]^+$*	*Theoretical mass $[M+Na]^+$*	*Error ([ppm])*	*Molecular formulae*	*Common Name*	*Short Name*
1	22.5	641.5124	641.5115	1.4	$C_{39}H_{70}NaO_5$	DAG(18:1/18:2/0:0)	1,2-OL
2	23.1	641.513	641.5115	2.3	$C_{39}H_{70}NaO_5$	DAG(18:1/0:0/18:2)	1,3-OL
3	23	615.4973	615.4959	2.2	$C_{37}H_{68}NaO_5$	DAG(16:0/18:2/0:0)	1,2-PL
4	23.7	615.4969	615.4959	1.6	$C_{37}H_{68}NaO_5$	DAG(16:0/0:0/18:2)	1,3-PL
5	27.1	643.5293	643.5272	3.2	$C_{39}H_{72}NaO_5$	DAG(18:1/18:1/0:0)	1,2-OO
6	27.9	643.528	643.5272	1.3	$C_{39}H_{72}NaO_5$	DAG(18:1/0:0/18:1)	1,3-OO
7	27.7	617.5131	617.5115	2.5	$C_{37}H_{70}NaO_5$	DAG(16:0/18:1/0:0)	1,2-PO
8	28.6	617.5133	617.5115	2.8	$C_{37}H_{70}NaO_5$	DAG(16:0/0:0/18:1)	1,3-PO
9	27.9	643.5267	643.5272	0.8	$C_{39}H_{72}NaO_5$	DAG(18:0/18:2/0:0)	1,2-SL
10	28.8	643.5274	643.5272	0.4	$C_{39}H_{72}NaO_5$	DAG(18:0/0:0/18:2)	1,3-SL
11	28.4	591.4976	591.4959	3	$C_{35}H_{68}NaO_5$	DAG(16:0/16:0/0:0)	1,2-PP
12	29.4	591.4976	591.4959	2.8	$C_{35}H_{68}NaO_5$	DAG(16:0/0:0/16:0)	1,3-PP
13	33.3	645.5449	645.5428	3.2	$C_{39}H_{74}NaO_5$	DAG(18:0/18:1/0:0)	1,2-SO
14	34.2	645.5442	645.5428	2.1	$C_{39}H_{74}NaO_5$	DAG(18:0/0:0/18:1)	1,3-SO
15	34.1	619.5285	619.5272	2.2	$C_{37}H_{72}NaO_5$	DAG(18:0/16:0/0:0)	1,2-SP
16	35.3	619.5276	619.5272	0.6	$C_{37}H_{72}NaO_5$	DAG(18:0/0:0/16:0)	1,3-SP
17	40.3	647.5579	647.5585	1	$C_{39}H_{76}NaO_5$	DAG(18:0/18:0/0:0)	1,2-SS
18	41.4	647.5584	647.5585	0.2	$C_{39}H_{76}NaO_5$	DAG(18:0/0:0/18:0)	1,3-SS

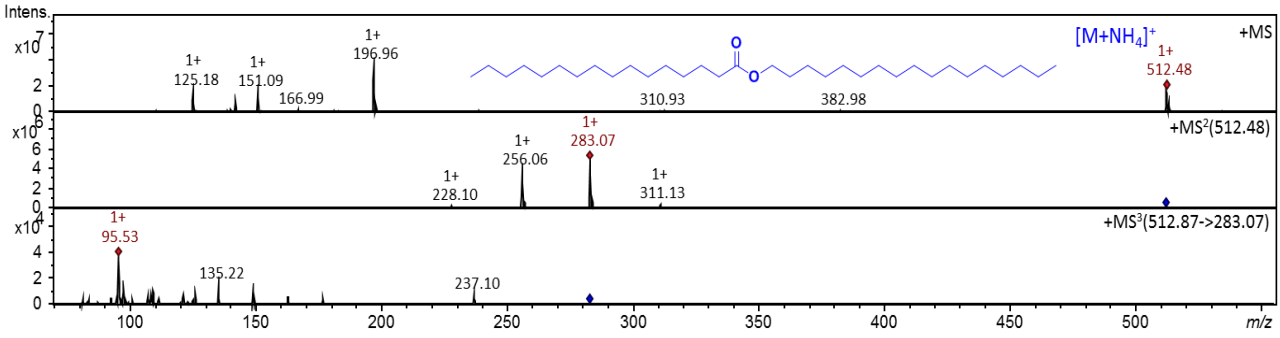


Figure 9.7 Tandem mass spectrum heptadecyl-palmitate, *m/z* 512.5, as representative of wax class.

9.3.3 Molecular species fraction 4

HPLC-MS analysis of fraction 4 in negative ion mode displays the occurrence of free fatty acids (Fig. 9.8) and other unknown compounds present with very low intensity. On the other hand, positive ion mode shows the presence of other classes of compounds, so far unidentified,

one of which is present in unfermented wet cocoa beans but subsequently absent in dried fermented cocoa bean (Fig. 9.9).

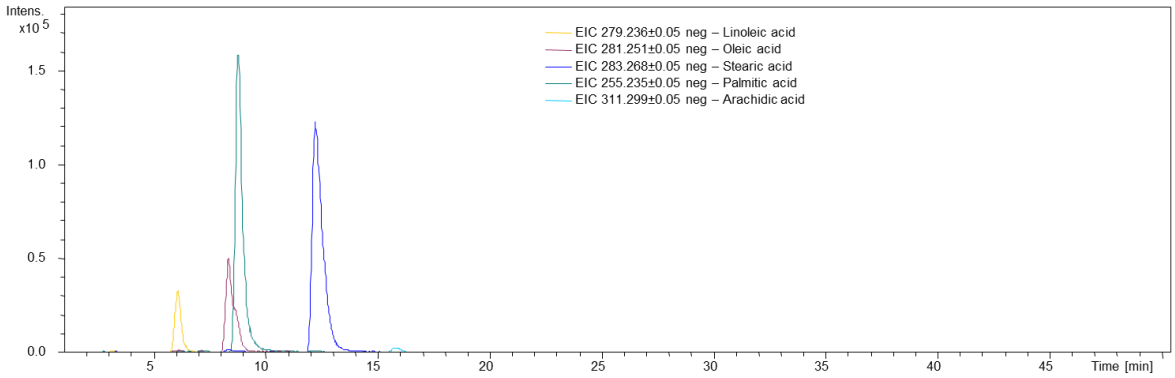


Figure 9.8 HPLC-MS extract ion chromatogram in negative mode of free fatty identified in cocoa lipid extract.

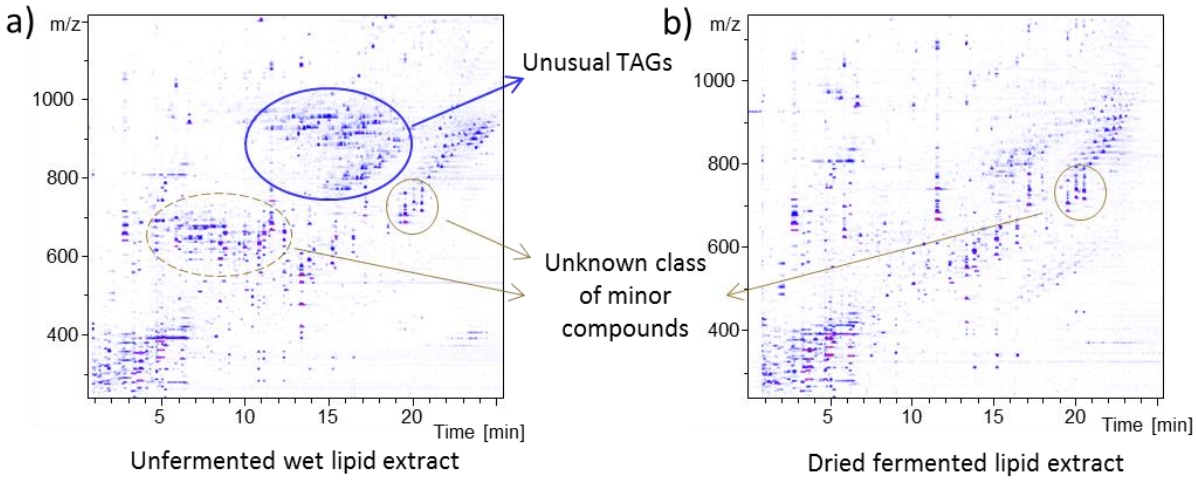


Figure 9.9 2D map of the LC-MS chromatogram in positive ion mode of fraction 4 minor components of the SPE lipid extract from **(a)** unfermented wet cocoa beans and **(b)** fermented dried cocoa beans. Blue circle indicates other unusual TAGs, light brown circle indicates other unknown lipid classes.

9.3.4 Molecular species fraction 5

Sterol esters glucosides and phospholipids are the most representative classes in fraction 5 (Fig. 9.10). Several molecules could be identified based on tandem MS data. Figure 9.11

illustrates the tandem mass spectrum in positive ion mode of sterol esters glucosides 16:0-Glc-Sitosterol (*m/z* 832.6) ammonia adducts, 18:0-Glc-Stigmasterol (*m/z* 858.7) ammonia adducts. As for sterol esters, the tandem MS of $[M+NH_4]^+$ precursor ions generated a MS^2 sterol fragment ion at *m/z* 383.4 and *m/z* 397.4 for the 2 sterol esters glucosides respectively (Fig. 9.11 (a) and (b)). The resulted neutral losses correspond to acyl group bonded to the glucose and ammonium adduct.

Two major subclasses of phospholipids were identified: phosphocholines, and phosphatidylethanolamines. Figure 9.12 (a) and (b) show the MS and MS^2 spectra of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-phosphatidylethanolamine under both positive ionization modes. Major fragment ion for phosphatidylcholine is derived from the loss of the polar head group choline $[C_5H_{13}NPO_4]^+$, *m/z* 184, which is characteristic for this class of phospholipids. Whereas for phosphatidylethanolamines, the MS^2 base peak at *m/z* 603.5, corresponding to $[OO]^+$ product ion, indicated that the polar head group 141 Da was lost as neutral loss. According to these fragmentation parameters, 17 phospholipids were identified. It worth mentioning that phospholipids were present only in dried fermented beans.

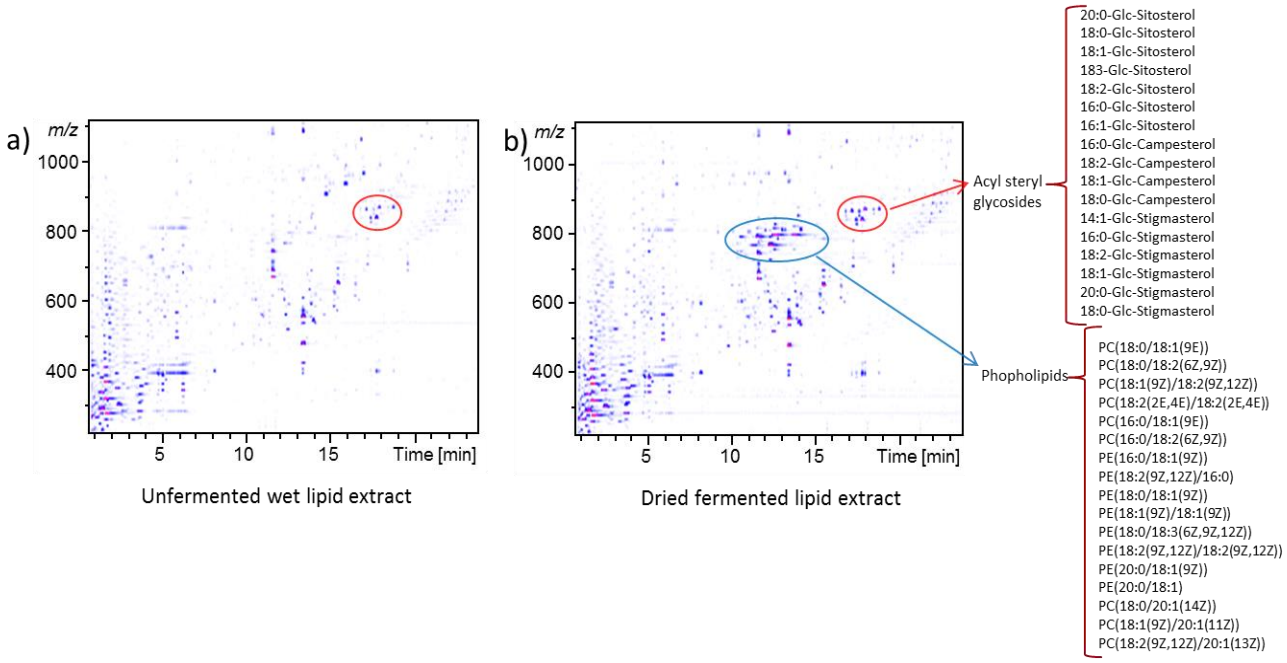


Figure 9.10 2D map of the LC-MS chromatogram in positive ion mode of fraction 5 minor components of the SPE lipid extract from **(a)** unfermented wet cocoa beans and **(b)** fermented dried cocoa beans. Red circle indicated sterol esters glucosides class and light blue circle indicates phospholipid class in cocoa.

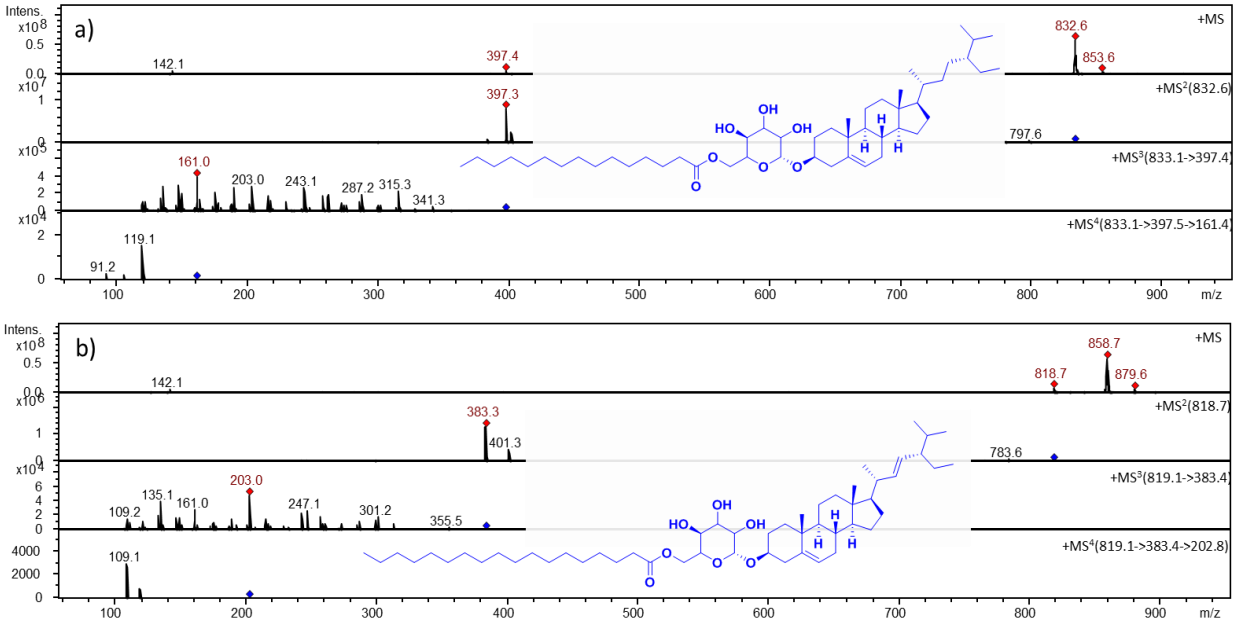


Figure 9.11 Positive ion mode tandem mass spectrum of sterol esters glucosides **(a)** 16:0-Glc-Sitosterol (*m/z* 832.6) ammonia adducts and **(b)** 18:0-Glc-Stigmasterol (*m/z* 858.7) ammonia adducts.

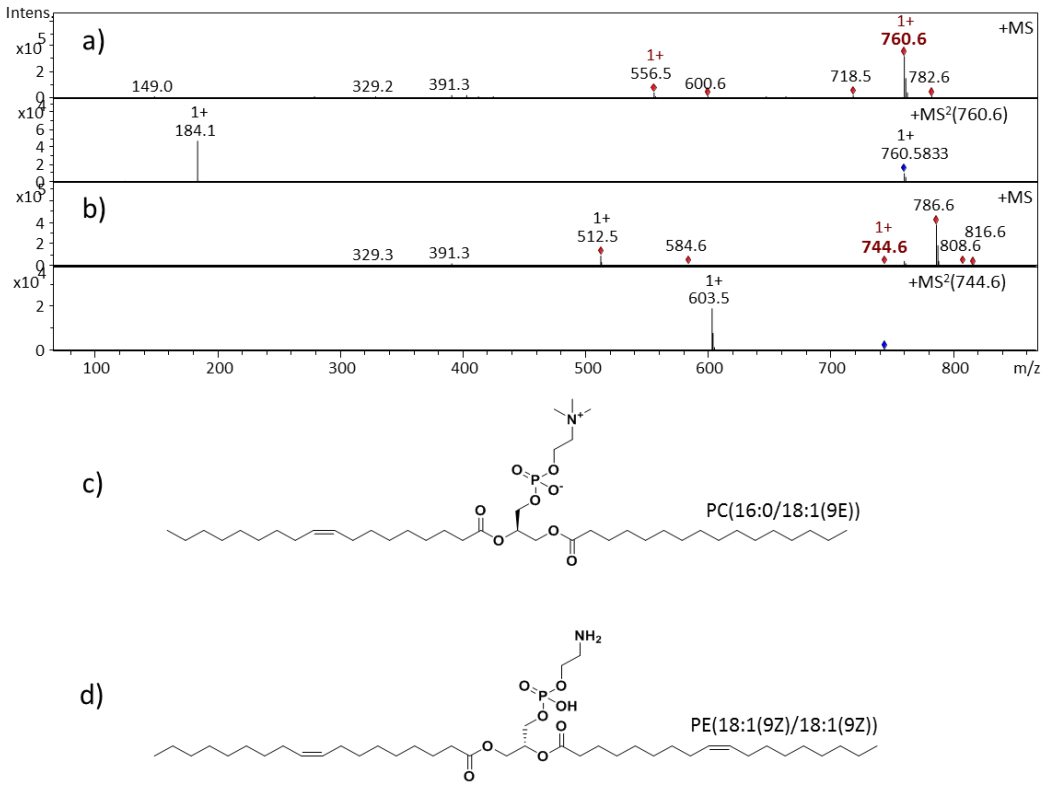


Figure 9.12 Positive ion mode tandem mass spectrum of phospholipids **(a)** 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, **(b)** 1,2-dioleoyl-*sn*-phosphatidylethanolamine, **(c)** and **(d)** their corresponding chemical structures.

9.4 Conclusions

In this chapter, structure elucidation of 62 minor components subdivided by lipid classes was achieved. Thus, fractionation allowed separation of minor lipid components from the major TAGs and therefore concentration enhancement, whereas HPLC-MS methods endorsed detection and identification of sterol esters, diacylglycerols, waxes, free fatty acids, sterol esters glucosides and phospholipids. Minor components have shown variation in occurrence in unfermented wet cocoa beans if compared to dried fermented cocoa beans. However, relative and absolute quantification of such differences is required and has to be considered as a focus of future work.

References

Cert, A., Moreda, W., & Pérez-Camino, M. C. (2000). Chromatographic analysis of minor constituents in vegetable oils. *Journal of Chromatography A*, *881*(1–2), 131–148. https://doi.org/10.1016/S0021-9673(00)00389-7

Christie, W. W., & Han, X. (2010). *Lipid Analysis, Fourth Edition: Isolation, Separation, Identification and Lipidomic Analysis* (4 edition). Bridgwater, England: Oily Press.

Kim, H. Y., & Salem, N. (1990). Separation of lipid classes by solid phase extraction. *Journal of Lipid Research*, *31*(12), 2285–2289.

Lipp, M., & Anklam, E. (1998). Review of cocoa butter and alternative fats for use in chocolate—Part A. Compositional data. *Food Chemistry*, *62*(1), 73–97. https://doi.org/10.1016/S0308-8146(97)00160-X

Lipp, M., Simoneau, C., Ulberth, F., Anklam, E., Crews, C., Brereton, P., Wiedmaier, C. (2001). Composition of Genuine Cocoa Butter and Cocoa Butter Equivalents. *Journal of Food Composition and Analysis*, *14*(4), 399–408. https://doi.org/10.1006/jfca.2000.0984

Chapter 10:

10 Comparative lipidomics of *Cylindrotheca closterium* and *Scenedesmus sp.* reveals differences in lipid production under nitrogen starvation

Song Wang[a], Diana Sirbu[b], Laurenz Thomsen[a], Nikolai Kuhnert[b], Matthias S. Ullrich[b]

[1]*Department of Physics and Earth Sciences, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany*

[2]*Department of Life Science and Chemistry, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany*

*Correspondence to:
Song Wang, s.wang@jacobs-university.de

Submitted to: Journal of Lipid Research.

Supplementary material is available on request. E-mail: n.kuhnert@jacobs-university.de, s.wang@jacobs-university.de

Abstract

Microalgae-derived biofuel is gaining increasing interests. However, factors, influencing lipid production from oleaginous algae, still require investigation. In the present study, the lipid production of two promising candidates—Scenedesmus sp. (Chlorophyceae) and Cylindrotheca closterium (Bacillariophyceae) under nitrogen starvation, was investigated by a method using liquid chromatography-electrospray ionization mass spectrometry techniques. By combining the alterations of total lipids, growth kinetics, fatty acids composition, and glycerolipids profiles, a comprehensive analysis of lipid synthesis in response to nitrogen stress were carried out at a molecular level. The cell concentration of Cylindrotheca closterium doubled within 24 h, whereas Scenedesmus sp. increased 10% at 24 h of nitrogen starvation. Triacylglycerols (TAGs) in Scenedesmus sp. increased continuously during nitrogen starvation and represented 11% of the dry biomass. In contrast, TAGs in C. closterium decreased significantly within 12 h nitrogen starvation and started to increase after 12 h, which accounted for 17% at 96 h. An increased digalactosyldiacylglycerols concentration in Scenedesmus sp. showed a more important role in keeping the integrity of photosynthetic systems in green algae than diatom. A rapid decrease of diacylglycerolstrimethylhomoserine concentration in Scenedesmus sp. within 12 h suggested its role as the first response to nitrogen starvation. 60 and 72 TAG molecules were identified in Scenedesmus sp. and C. closterium respectively providing the most abundant TAG profiles in green algae and diatom. The de novo synthesis of the lipids in Scenedesmus sp. under nitrogen starvation showed a preference in a “eukaryotic pathway”, while C. closterium relied on “prokaryotic pathway” to enhance TAG production.

Keywords: diatom; green algae; lipid; fatty acid; triacylglycerol; digalactosyldiacylglycerol.

10.1 Introduction

Due to the dramatically expanding global population, energy consumption will increase by 56% between 2010 and 2040 leading to rapid exhaustion of the limited fossil fuel stock within 30 years (Abomohra et al., 2016). The overexploitation of fossil fuels caused severe environmental concerns due to the emission of carbon dioxide, sulfur and nitrous oxides and poly-aromatic hydrocarbons. Biofuel, derived from biomass, is suggested as a potentially sustainable alternative to fossil fuels (Sharma and Singh, 2017). Microalgae represent a large group of photosynthetically active microorganisms distributed in various aquatic environments, such as freshwater, seawater, brackish water or even wastewater and readily adaptive to a wide range of abiotic parameters, such as temperature, light intensity, pH, and nutrients availability (Hu Q et al., 2008). Microalgae, are receiving increasing attention as a promising feedstock for biofuels production, owing to the following advantages over the 1st and 2nd generation of biomass sources: 1) the production of microalgae biomass requires neither arable land nor freshwater (Michael Hannon et al., 2010); 2) microalgae, due to a higher photosynthetic efficiency and CO2 concentration mechanism, yield higher biomass than terrestrial plants (Sayre, 2010) (Spalding, 2008); 3) microalgae can accumulate a higher lipid content under stress conditions (Gouveia and Oliveira, 2009); 4) microalgae, cultivated in wastewater, can remove nutrients and harmful compounds to produce a clean effluent to offset the cost of biofuel production (Randrianarison and Ashraf, 2017).

However, the high cost of algal biofuel production has, to date, industrialization inhibited. The selection of algae species with an increased lipid production could cut down the cost and make exploitation feasible (Chu, 2017). Stress strategies, such as modification of nutrient supply and environmental parameters, offer promising approaches, which were applied to algae cultivation to induce lipid synthesis and have been successfully demonstrated as a proof of principle (Benavente-Valdes et al., 2016). Among all the stress approaches, nitrogen starvation is most investigated, causing boosting of the lipid synthesis and offering insights into the lipid biosynthesis pathway in algae. For instance, higher efficiency of lipid production in Phaeodactylum tricornutum was obtained when nitrogen availability was limited in media (Frada et al., 2013). Furthermore, the biomass of green algae N. oleoabundans, with higher lipid content, was produced after the depletion of nitrogen, accompanied with the static cell division and decrement of chlorophyll a (Li et al., 2008). It has been reported that the lipid

content of Scenedesmus obliquus attained 22.4% on the dry biomass basis by 5-day nitrogen starvation (Ho et al., 2012).

Nevertheless, the lipid metabolisms of algae from different classes in response to nitrogen starvation have not yet been well understood. In most of the cases, researchers focused on total lipids content and fatty acids profile, neglecting detailed analysis at a molecular level. Comprehensive analysis of the changes of intact lipids, including storage lipids and membrane lipids under nitrogen starvation has only been carried out in several model species, such as Phaeodactylum tricornutum (Levitan et al., 2015), Nannochloropsis oceanica (Li et al., 2014), and Chlamydomonas reinhardtii (Yang et al., 2015). It has been reported that under nitrogen starvation nitrogen in half of the Phaeodactylum tricornutum proteins was scavenged to strengthen the nitrogen incorporation enzymes as well as photosynthetic carbon was reallocated into lipid biosynthesis pathway (Levitan et al., 2015). Moreover, varied fatty acid chains in triacylglycerols (TAGs) and diacylglycerols-trimethyl-homoserine (DGTS) were found in Chlamydomonas reinhardtii under nitrogen deprivation (Yang et al., 2015). Combination of transcriptomics and lipidomics analysis revealed the suppression of the de novo fatty acid biosynthesis pathway of Nannochloropsis oceanica, yet the pathway that redirected the carbon flux from protein to lipid and carbohydrate was upregulated in a nitrogen-free medium (Li et al., 2014). However, more studies on other oleaginous algae are necessary to reveal the lipid metabolism in algae and guide a rational manipulation of lipid biosynthesis.

In the present study, the changes of lipids in a freshwater green algae Scenedesmus sp. (Chlorophyceae) and a benthic diatom Cylindrotheca closterium (Bacillariophyceae) were determined in response to the nitrogen starvation. The Scenedesmus sp. was isolated from a municipal sewage water plant, which is highly tolerant to heavy metals, organic pollutants and wide thermal variation. Cylindrotheca closterium in this experiment, featuring a high growth rate and lipid content, was considered as a promising candidate for biofuel and high-value pigment production (Wang et al., 2015). On another note, both species could biosynthesize a wide arrange of poly-unsaturated fatty acids, essential to human health, such as α-Linolenic acid by Scenedesmus obliquus (Makulla, 2000) and eicosapentaenoic acid by C. closterium (Wang et al., 2015). The current experiment is designated to elucidate the variance of lipid metabolisms, with special emphasis on triacylglycerol, between marine diatom and freshwater green algae triggered by nitrogen stress at a molecular level.

10.2 Material and methods

10.2.1 Algae and culture condition

The stock solutions of Scenedesmus sp. and C. closterium were maintained in freshwater with Basal Bold Medium (BBM) and seawater enriched with f/2 medium nutrients (Guillard, 1975) respectively at 12/12 light/darkness circle and 40 μmol/m2/s light intensity. Algae used in nitrogen starvation experiment were harvested from a culture in a bag photobioreactor (PBR) patented by Phytolutions GmbH at 18/6 light regime, 100 μmol/m2/s. C. closterium in the bag PBR was cultured in f medium at 20±1 °C, whereas Scenedesmus sp. in BBM 25±2 °C. Algae, reaching the late exponential phase, were harvested by centrifugation at 2000 g. C. closterium was rinsed with seawater, while Scenedesmus sp. by ultra-pure water for three times to remove nitrogen. The algae were re-suspended in nitrogen-free medium bottle PBRs at the same environmental parameters to the bag PBRs. Algal biomass was harvested at time points of 0 h, 12 h, 24 h, 48 h and 96 h after inoculation with three biological replicates. Dry biomass was obtained by lyophilization for 48 h before lipid extraction. The growth performance was monitored by counting the cell concentration daily.

10.2.2 Chemicals and reagents

Ethanol gradient grade was purchased from Merck (Darmstadt, Germany); isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade), methanol (Rotisolv® HPLC ultra gradient grade), chloroform (Rotisolv® HPLC grade) and Tetra-dodecyl ammonium bromide were purchased from Carl Roth (Karlsruhe, Germany); dichloromethane 99,8% stabilized with amylene for synthesis was purchased from Panreac AppliChem (Darmstadt, Germany); ammonium formate LC-MS Ultra and formic acid (puriss., ≥ 98% (T) for mass spectrometry), potassium hydroxide (KOH), sulfuric acid (H_2SO_4), heptane (synthesis grade) and potassium chloride (KCl) were ordered from Carl Roth (Karlsruhe, Germany); molecular sieves pellets (AW-300, 1.6 mm) and Spelco Supelco 37 Component fatty acids methyl esterification (FAME) mix 1x1 mL were purchased from Sigma-Aldrich Chemie (Steinheim, Germany).

10.2.3 Total lipids extraction

Total lipids were extracted by a modified gravitational method. Approximately 100 mg of every sample was extracted by 9 mL of dichloromethane/methanol at the ratio of 1:1 in a glass vial. Extraction was assisted by a magnetic stirrer for 1h at room temperature. The mixture was centrifuged at 3000 g for 10 min. The extraction procedure was repeated once. The supernatant was combined in fresh vials. 4.5 mL KCl solution (0.88%) was added to the combined supernatant. An organic and an aqueous phase were separated by centrifugation at 3000 g for 5 min. The separation procedure was repeated once more with KCl solution. 3 mL organic phase was transferred to a pre-weighed test vial and evaporated by N_2. Total lipids content was calculated by subtracting the mass of the empty vials from that of vials with lipids. Chloroform was added to dissolve the lipids at a concentration of 5 mg/mL, which were stored at -20 °C for further analysis.

10.2.4 FAME preparation and GC analysis

500 µL of the lipid extract, dissolved in chloroform, was transferred in a glass vial for Fatty acid methyl ester (FAME) synthesis. 700 µL 10 N KOH and 5.3 mL methanol were added to the extract, stirred continuously at 50 °C for one hour. Subsequently, 580 µL 24 N H_2SO_4 was added when samples were cooled down at room temperature. The samples were stirred continuously at 50 °C for one hour. FAME was isolated with 2 mL heptane by vortex for 5 minutes and centrifugation at 3000 g for 3 minutes. Molecular sieves were added to remove the aqueous phase. FAME was subject to GC-FID for analysis. A capillary column VF-5ms, 30 m x 0.25 mm with an injector temperature of 265 °C and detector temperature of 300 °C was integrated to the GC. The GC used helium as a carrier gas at a flow rate of 40 mL/min. The split ratio was 1:50 with an injection volume of 1.0 µL. GC program started at 60 °C for 1 minute, followed by a second step of 17 °C /min temperature rate until 160 °C, a third step of 7 °C/min rate until 240 °C and a temperature rate of 5 °C/min until 300 °C with a hold time of 10 min. Fatty acids were assigned by Supelco 37 compounds fatty acid methyl ester (FAME) MIX standard and quantified by the calibration curves of the standard.

10.2.5 High-performance liquid chromatography

Lipids were fractionated by high-performance liquid chromatography (Agilent 1100 series, Waldbronn, Germany) with our previously established method (Sirbu et al., 2018) to which slight modifications on elution gradient were applied. One column Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles) was integrated to the HPLC. The temperature of the column oven was set to 35 °C. Each extract was diluted at the ratio of 1:22 in chloroform/ethanol (1:1) with 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and B of ethanol with 10 mmol/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. The gradient elution program started with A/B (90/10%) increased linearly to A/B (65/35%) in 4 min; followed by a second linear increasing to solvent A/B (50/50%) in 16 min; a third linear increase to solvent A/B (37/63%) in 2 min; a forth linear increase to solvent A/B (20/80%) in 23 min; a fifth step, linear increase to solvent B (100%) in 2 min; ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at A/B (90/10%) solvent for 10 min before reuse. Tetra-dodecyl ammonium bromide at the concentration of 0.0002 mg/mL, mass-to-charge (m/z) 690.7850, and retention time (R_t) 4.5 min was used as internal standard.

10.2.6 High-resolution mass spectrometry

High-resolution masses were acquired by a time of flight MicrOTOF Focus mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an ESI source as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas pressure of 2 Ba drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of *m/z* 200–1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out by the enhanced quadratic mode.

10.2.7 Tandem mass spectrometry

LC-tandem MS was carried out by using an Ion-Trap detector in positive ion mode equipped with an ESI source (Bruker Daltonics, Bremen, Germany). The full scan mass spectra were recorded in the range *m/z* 200-1200 operating in positive ion mode. Capillary temperature was

set to 350 °C, drying gas flow rate 10 L/min and nebulizer pressure 10 psi. Tandem mass spectra were acquired in Auto MS^n (smart fragmentation) using a ramping of the collision energy.

10.2.8 Statistical analysis

The individual lipid lists of the high-resolution masses were combined for peak matching and retention time (R_t) alignment using a *m/z* tolerance of $\Delta m/z = 0.0001$ and a R_t tolerance of 0.5 min for elution time <20 min and 1.0 min for an elution time ≥20 min. Extensive manual curation resulted in a set of 102 peaks for *C. closterium* and 93 peaks for *Scenedesmus sp.*. Lipid compounds could be uniquely assigned with *m/z* and R_t tolerances of $\Delta m/z = 0.01$ and $\Delta R_t = 0.8$ min. For further analyses, the peak areas were normalized to the peak area of the internal standard (Tetra-dodecyl ammonium bromide). Heat maps, as graphical representations for visualizing attribute values by class in a two-way matrix, as well as principal component analyses (PCA) were performed by Orange software. Microsoft Office Excel 2016 was used to process data and perform ANOVA single-factor analysis.

10.3 Results and discussion

10.3.1 Total lipid content and growth kinetics under nitrogen starvation

Our previous study indicated that *Scenedesmus sp.* could grow fast in wastewater, recover the nutrients producing clean effluent and display excellent thermo-tolerant property. *Cylindrotheca closterium* features for high specific growth rate and rapid cell sedimentation. Consequently, *Scenedesmus sp.* and *C closterium* were chosen in this experiment for lipids production owing to the favorable characteristics in cultivation or harvesting. Algae, accumulating biomass in bag PBRs, were transferred to bottles PBRs to induce lipid synthesis at the same abiotic parameters. Total lipids were determined gravimetrically following dichloromethane extraction. As indicated in Fig. 10.1 (a), a lipid content of 13% in dry biomass was synthesized in *Scenedesmus sp.* at 0 h. During nitrogen starvation, the total lipids content of *Scenedesmus sp.* increased gradually and culminated at 96 h, while no prominent changes were observed between 0 h and 48 h. *C. closterium* accumulated 20% lipid before the onset of nitrogen starvation, consistent with our previous results and indicating a stable lipid content

(Wang et al., 2015). Surprisingly, the lipid content of *C. closterium* decreased significantly within 12 h of nitrogen starvation and maintained at this level till 48 h. *C. closterium* showed a remarkable increment of lipids between 48 and 96 h, but no significant difference was found between the total lipids amount at 0 h as a negative control and 96 h of starvation. The growth kinetics in Fig. 10.1 (b) suggested the cell concentration of *Scenedesmus sp.* is 5-fold higher than *C. closterium* at the late exponential phase. By 24 h of nitrogen stress, the cell concentration of *Scenedesmus sp.* increased by approximately 12%, whereas *C. closterium* increased by more than 64% at 12 h, nearly doubled the concentration at 24 h and stopped growing at 48 h. Both *Scenedesmus sp.* and *C. closterium* continued growing in nitrogen-free media. However, the growth rate varied remarkably.

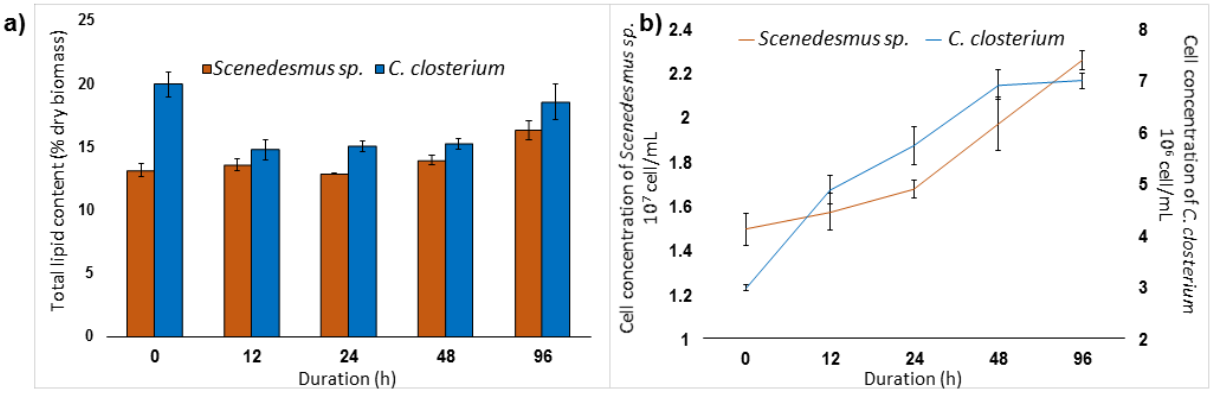


Figure 10.1 (a) Total lipids and **(b)** growth kinetics of *Scenedesmus sp.* and *C. closterium* under nitrogen starvation. Results were shown as means±SD, n=3.

Despite the absence of nitrogen, both species continued proliferating, especially *C. closterium* doubling the cell concentration at 24 h. This might be explained by assuming that algae in nutrients-sufficient environment tend to assimilate more nutrients, including nitrogen, to form an internal reservoir in vacuole which could support the growth in a nitrogen-scarce or even free environment (Lavín and Lourenço2, 2005). The existence of internal pool was confirmed in *Chlorella sp.* and *Nannochloropsis oculata* (Paes et al., 2016). It has been previously reported *Scenedesmus obliquus* could accumulate as much as 20% lipid of dry biomass after 5-day nitrogen starvation compared to 11% in a negative control, accompanied by an increase of carbohydrate and decrease of protein content (Ho et al., 2012). This literature prediction is in agreement with the lipid content of the *Scenedesmus sp.* after nitrogen starvation in this study. This could be interpreted by the fact that nitrogen was intimately

connected to the carbon metabolism. The reduced availability of nitrogen led to the re-allocation of photosynthetic carbon flux and proteins scavenged to increase the lipid synthesis (Alipanah et al., 2015). In response to nitrogen-starvation, *Phaeodactylum tricornutum*, as the model diatom, produced significantly more lipid after 48 h cultivation in nitrogen-free medium, but the cell growth was suppressed if compared to control after 48 h (Alipanah et al., 2015), which contrasted with the lipid metabolism displayed by *C. closterium* in the present study. This distinct lipid metabolism could possibly be explained by the rapid cellular reproduction within 12 h, which required a significant amount of energy. Therefore, lipids, compounds storage of energy, were consumed to provide energy due to the deficiency of photosynthetic ATP production.

10.3.2 Fatty acids profile under nitrogen starvation.

FAME analysis of lipid extraction was carried out and identified compounds were shown in Fig. 10.2 (a-d). It revealed that C16:3, C16:0 and C18:1 are the major fatty acids species in *Scenedesmus sp.* with C18:1 being the most abundant one and long-chain fatty acids (>20 C) in a minor amount at 0 h before nitrogen starvation (Fig. 10.2 (a)). Saturated fatty acids, such as C14:0, C16:0 and C18:0, increased significantly during nitrogen starvation with C16:0 and C18:0 increased by around 4-fold and 10-fold, respectively at 96 h, if compared to 0 h. Monounsaturated fatty acids C20:1 showed a 3-fold increase at 24 h, while C18:1 only displayed noticeable increase at 96 h. Polyunsaturated fatty acids, such as C16:3 and C18:3, and odd number fatty acids C15:0 and C17:0 decreased significantly after 12 h starvation with the exception of C18:2. C16:3 and C18:3 decreased to 67% and 37%, respectively at 96 h as opposed to 0 h. C18:2 increased immediately after the onset of nitrogen starvation. After 96 h nitrogen starvation, C16:0 and C18:1 accounted for 70% of the total fatty acids.

Five most abundant fatty acids (C14:0, C16:1, C16:0, C20:4 and C20:5) were synthesized by *C. closterium* with palmitoleic (C16:1) and palmitic acids (C16:0) as the most dominant species at 0 h (Fig. 10.2 (c)). Under nitrogen starvation, a 26% increase of saturated fatty acid C14:0 occurred at 96 h compared to 0 h, while C16:0 underwent a decrease within first 12 h and an increase after 12 h, following the tendency of total lipids. Monounsaturated fatty acids C15:1 and C16:1 increased in connection during the whole time-course, yet remarkable increments were only observed at 96 h ($p<0.05$). Polyunsaturated fatty acids (PUFA) and long-chain PUFA maintained a constant level till 48 h and decreased significantly at 96 h. C20:4 and

C20:5 reduced by 14% and 19%, respectively at 96 h compared to the negative control at 0 h. The odd number saturated fatty acids (C15:0 and C17:0), rarely reported previously, in *C. closterium* followed a decreasing trend. At 96 h, the two dominant fatty acids—C16:1 and C16:0 represented 65% of the total fatty acids.

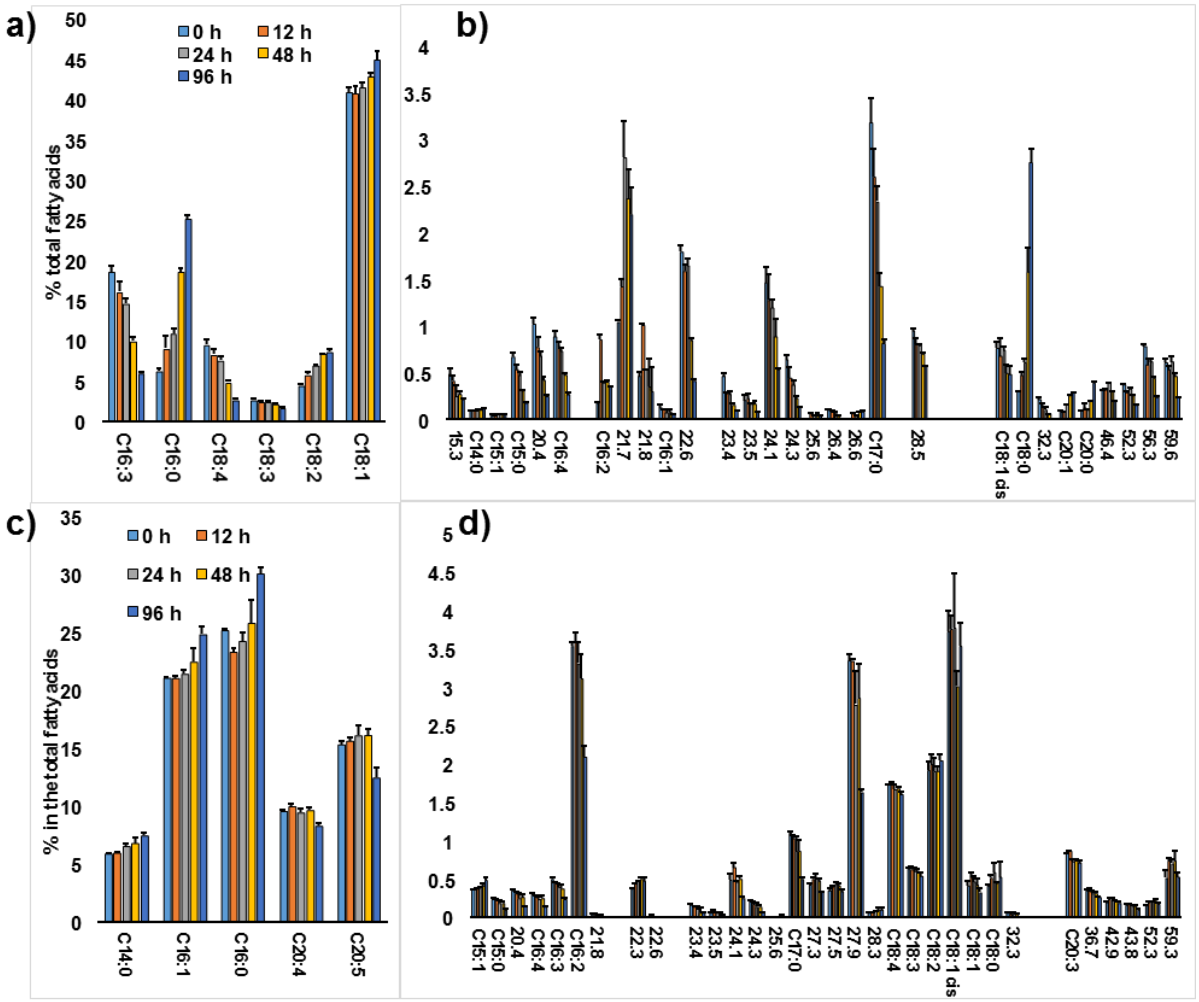


Figure 10.2 Fatty acids composition of *Scenedesmus sp.* and *C. closterium* under nitrogen starvation. **(a)** Major fatty acids in *Scenedesmus sp.*; **(b)** Minor fatty acids in *Scenedesmus sp.*; **(c)** Major fatty acids in *C. closterium*; **(d)** Minor fatty acids in *C. closterium*. Results were shown as means±SD, n=3. Compounds could be assigned by standards were named by the retention time.

Although *Scenedesmus sp.* and *C. closterium* were characterized by different fatty acids composition, the percentage of polyunsaturated fatty acids decreased in both species at 96 h of nitrogen starvation, but the ratio of saturated and monounsaturated fatty acids increased in response to a nitrogen-free environment. Similar alterations of fatty acids happened in

Chlorella zofingiensis under nitrogen starvation with an increased saturated and monounsaturated but a diminishing polyunsaturated fatty acid ratio (Zhu et al., 2015). Likewise, in another previous study, the synthesis of saturated fatty acid of *P. tricornutum* was elevated in a nitrogen-depleted medium at 96 h compared to nitrogen-replete medium (Yodsuwan et al., 2017). The decrease of polyunsaturated fatty could be considered as the consequence of the inhibition of the photosynthesis and degradation of the photosynthetic systems where PUFA are usually present. Since saturated/monounsaturated acyl chains are beneficial for biodiesel production, both species featuring high saturated/monounsaturated fatty acids contents, induced by nitrogen starvation, are suitable for biodiesel production (Islam et al., 2013).

10.3.3 Relative quantitative analysis of glycerolipids and pigments

Identification of lipid compounds was achieved by a high-resolution MicrOTOF mass spectrometry (MS) operating in positive mode. The study of the compounds fragmentation was conducted in an Ion-Trap MS. Moreover, the tandem MS data were compared with fragmentation pathways in literature and the database LIPID MAPS (http://www.lipidmaps.org). For high-resolution MS, calculation of molecular formulae with mass error < 4 ppm was accepted. Tandem MS data were interpreted as described by (Sirbu et al., 2018) and (Su et al., 2013). Relative quantification of all the identified compounds was performed in positive ion mode with respect to an internal standard (Tetra-dodecyl ammonium bromide). Spectra describing the fragmentation pattern of one example from each lipid class and tables (Appendix Table G.1 and G.2) with identified compounds of the individual species are given in Appendix. Additionally, representative chromatograms and their two-dimensional maps at 0 and 96 h for each species are in the Appendix (Fig. G.1).

Thanks to the development of lipidomics based on mass spectrometry, much more knowledge could be obtained on different groups of lipids in algae. 99 molecules, listed in Appendix Table G.1, were successfully annotated in *Scenedesmus sp.* total lipids and assigned to 9 classes: triacylglycerol (TAG) ≤3, TAG >3 double-bond, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG), diacylglyceryl-N, N, N- trimethylhomoserine (DGTS), lyso-DGTS and phosphatidylethanolamine (PE) in Fig. 10.3 (a). Before the start of nitrogen starvation, TAG (≤3 and >3) and plastidial lipids (MGDG, DGDG and PG) accounted for 1.8% and 5.1% of the dry biomass, respectively. After 96 h of

nitrogen starvation, the overall amount of TAG increased more than 6-fold more in *Scenedesmus sp.* with TAG ≤3 and >3 double-bonds increased 4-fold and 28-fold, respectively with respect to the counterparts at 0 h. Total TAGs accounted for 68% and 11% of the total lipids and biomass respectively at 96 h in *Scenedesmus sp.*. Pigments including chlorophyll a/b and pheophytin decreased significantly to one quarter at 96 h in response to nitrogen starvation if compared to 0 h, concomitant with the decrements of MGDG and PG. MGDG and PG decreased to one third and one fifth respectively at 96 h as a comparison to 0 h. Another essential plastidial lipid DGDG reached the maximum at 48 h and dropped at 96 h. The concentration of DGDG at 48 and 96 h was 4 and 3 times more than the negative control at 0 h, respectively. The plastidial membrane lipids (MGDG, DGDG and PG) comprised 39% of the total lipids at 0 h, as judged by the peak area, which dropped to 15% at 96 h. DGTS, lyso-DGTS and PE, as N-containing lipids, are crucial extra-plastidial membrane lipids. A significant decrease of DGTS and PE occurred at 12 and 24 h, respectively, while lyso-DGTS first increased at 24 h and subsequently reduced till 48 h.

92 lipid molecules, listed in Appendix Table G.2, were identified in *C. closterium* allocated into 6 groups: TAG ≤3 and >3 double-bond, MGDG, DGDG, sulfoquinovosyl diacylglycerol (SQDG) and pigments (Fig. 10.3 (b)). The TAG (≤3 and >3) and plastidial lipids (MGDG, DGDG and SQDG) represented 14% and 2% of the dry biomass respectively in *C. closterium* before subject to nitrogen-free media. Under nitrogen starvation, pigments decreased to one third at 96 h if compared to 0 h. The abundance of both TAGs classes decreased dramatically at 12 h and gradually increased after 12 h, with a significant enhancement observed at 96 h. Total TAG content increased by 20% at 96 h, with TAGs (≤ 3 double-bond) and TAGs (> 3 double-bond) increasing by 10% and 49% in comparison to 0 h. The TAG represented up to 90% and 17% of the total lipids and dry biomass at 96 h. Among the three plastidial membrane lipid classes (MGDG, DGDG and SQDG), the dominant class was MGDG decreased to one fourth at 96 h as opposed to 0 h. Concomitantly, DGDG dropped to half of the amount, while SQDG rise at 24 h and reduced at the 96 h than 0 h. Plastidial membrane lipids accounted for 10% and 4% of the total lipids at 0 and 96 h, respectively.

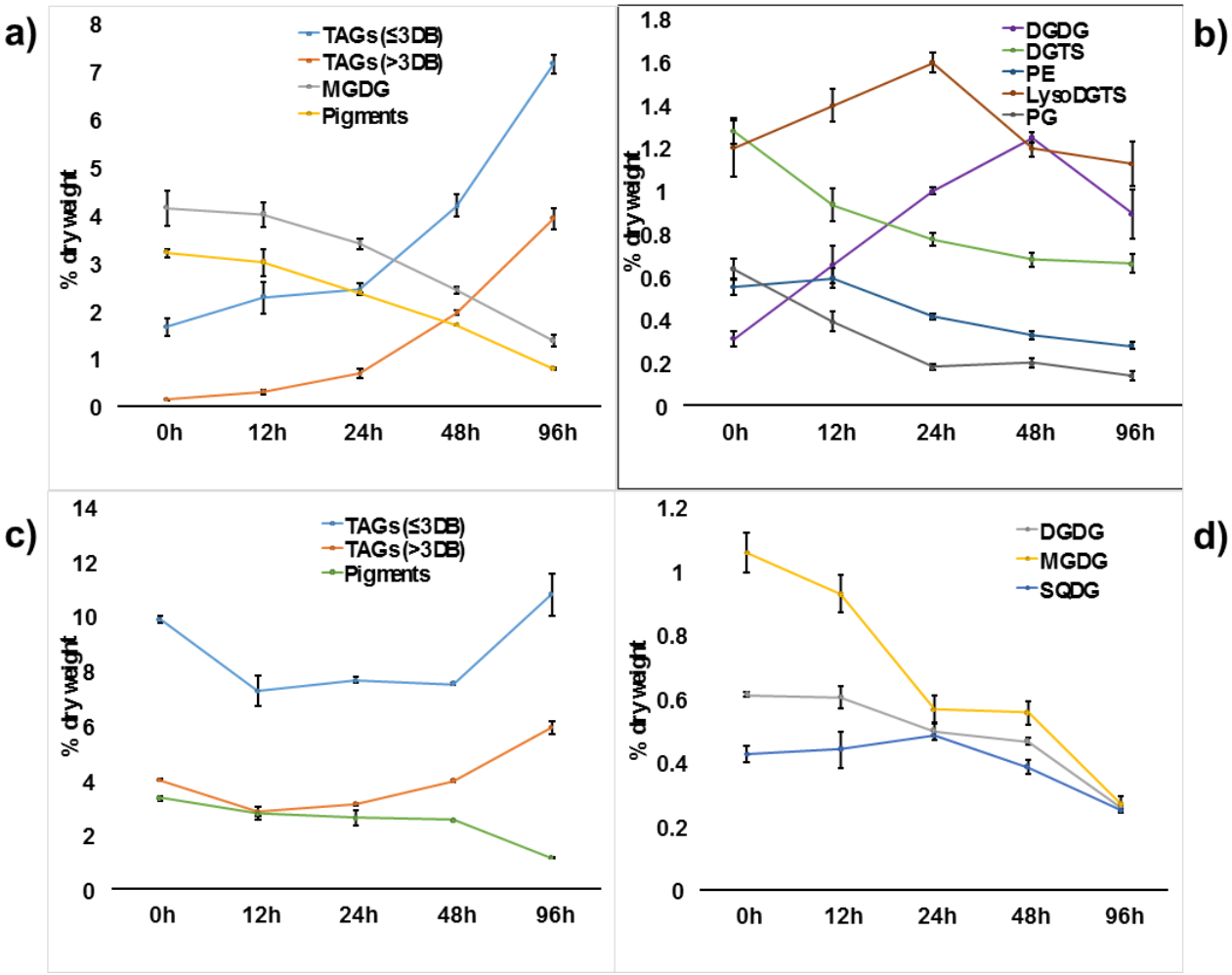


Figure 10.3 Changes of glycerolipids and pigments in *Scenedesmus sp.* and *C. closterium* under nitrogen starvation: **(a)** and **(b)** Glycerolipids and pigments in *Scenedesmus sp.*; **(c)** and **(d)** Glycerolipids and pigments in *C. closterium*. Results were shown as means±SD, n=3.

The results demonstrated that photosynthesis of *Scenedesmus sp.* and *C. closterium* was suppressed due to the reduction of photosynthetic pigments and decrease of plastidial membrane lipids in nitrogen-free media. The decrease of pigments was due to the degradation of the essential enzymes and structural protein in the photosynthetic pathway and meanwhile, the N in pigments, as N-rich compounds, were presumably redirected to protein synthesis under nitrogen starvation condition (Levitan et al., 2015).

In comparison to *Scenedesmus sp.*, *C. closterium* adopted a different lipid adaptation triggered by nitrogen starvation. *C. closterium* accumulated a higher TAG content at 0 h and as the proceeding of nitrogen starvation, TAG decreased to provide energy for the rapid cell division. On the contrary, *Scenedesmus sp.* accumulated very low amount TAGs at 0 h and

increased gradually. TAG only accounted for 1.8% of the dry biomass of *Scenedesmus sp.*, in a nutrients-replete environment, which indicated most of the photosynthetically fixed carbon was partitioned into protein and carbohydrate to enhance biomass. Neither *C. closterium* nor *Scenedesmus sp.* showed a slowdown in TAG accumulation at 96 h. Thus, further TAG increase could be expected by extending the stress process. In the previous study, *Coccomyxa subellipsoidea* could accumulate 13% TAG in dry biomass after 10-day nitrogen starvation (Allen et al., 2015). *Scenedesmus sp.* in the present study exhibited similar TAG content, which *C. closterium* displayed higher TAG content, and both species were characterized by favourable fatty acids composition (high saturated/monounsaturated fatty acids ratio), whereby this enabled them to be potential candidates suitable for biofuel production. Moreover, *Scenedesmus sp.* was more abundant in plastidial membrane lipids than *C. closterium*. As most of MGDG, DGDG and SQDG are mainly present in thylakoids membrane and have a substantial impact on the photosynthesis and growth, photosynthetic apparatus in *Scenedesmus sp.*is more developed in *C. closterium* (Peter Dormann et al., 1995). DGDG in *Scenedesmus sp.* increased within 48 h but decreased during the whole process in *C. closterium*. The ratio of MGDG/DGDG in both species decreased significantly. Decreased MGDG, along with increased DGDG, was reported previously in *Arabidopsis* (Gaude N et al., 2007) and *P. tricornutum* (Abida et al., 2015), yet in model algae, *Chlamydomonas reinhardtii* both MDGD and DGDG decreased under nitrogen starvation (Siaut et al., 2011). This could be explained DGDG, a bilayer-lipid, showed superiority over MGDG, a non-bilayer-lipid, in maintaining the integrity of thylakoids under nitrogen stress (Webb and Green, 1991). However, as the proceeding of nitrogen starvation, the more photosynthetic machinery was degraded, whereby the DGDG started to decrease at 48 h of nitrogen starvation. The explanation was supported by the declining MGDG/DGDG ratio. Consequently, we can hypothesize that the structure of the photosynthetic system in *C. closterium* is more subject to nitrogen stress than *Scenedesmus sp.*, due to the immediate reduction of DGDG.

10.3.4 Lipid and pigment molecules alteration

Two principal components analysis of LC-MS data of lipid extract taken at different time points were performed for each of two species based on the lipids and pigments identified (Fig. 10.4). PCA on *Scenedesmus sp.* showed clear separation of samples taken at each time series. The first principal component in the score plot is strongly correlated with the amount of TAG.

Significant changes occurred after 12 h of nitrogen starvation in *Scenedesmus sp.*. In contrast, *C. closterium* did not show a net separation between all the time series, but distinguishable changes of samples at 0 h and 96 h in comparison to the other 3 could be observed. Moreover, the grouping shown on the plots reflected the reproducibility of data and how close are the samples to each other in term of lipids profile.

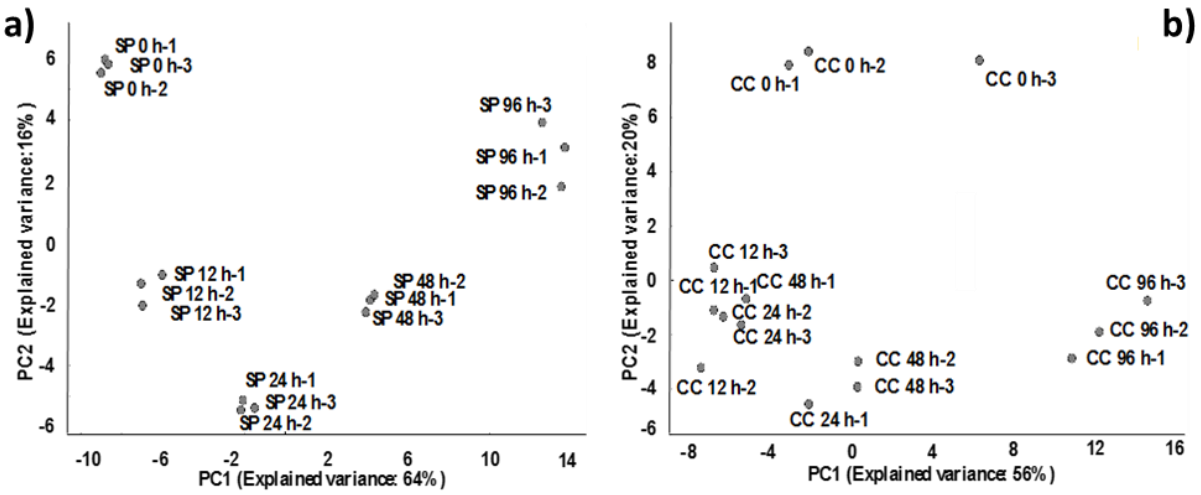


Figure 10.4 Principal component analysis of *Scenedesmus sp.* and *C. closterium* under nitrogen starvation: **(a)** Samples from *Scenedesmus sp.*; **(b)** Samples from *C. closterium.* Three biological replicates were performed at each time point.

The heat maps illustrated the alteration of the lipid and pigment molecules in *Scenedesmus sp.* (Fig. 10.5 (a)) and *C. closterium* (Fig. 10.5 (b)) in the time-course of nitrogen starvation. All the molecules are listed in Table G.1 for *Scenedesmus sp.* and G.2 for *C. closterium* in the Appendix. 60 and 72 TAG molecules were identified in *Scenedesmus sp.* and *C. closterium* respectively which give the most abundant TAG profiles in both green algae and diatom, although not comprehensive. Generally, TAGs with more than 3 double bonds were absent at 0 h and only present at 12 h of nitrogen starvation. Among the 60 TAGs in *Scenedesmus sp.*, TAG 50:1(16:0/16:0/18:1), 52:1 (18:0/16:0/18:1), 50:2 (14:0/18:1/18:1), 52:2 (16:0/18:1/18:1), 50:3 (16:0/16:0/18:3), 52:3(16:0/18:1/18:2), 54:3 (18:0/18:0/18:3), 52:4 (18:1/16:0/18:3) and 52:4 (16:0/18:2/18:2) are the dominant TAG species at 96 h. The abundance of the major TAGs (≤3 double bonds), increased by 8-20 folds at 96 h if compared to the counterparts at 0 h, except 52:1 (18:0/16:0/18:1) and 50:3 (16:0/16:0/18:3). 52:1 (18:0/16:0/18:1) increased 3-fold at 96 h compared to the negative control, while no 50:3

(16:0/16:0/18:3) was synthesized at 0 h. 52:4 (18:1/16:0/18:3) and 52:4 (16:0/18:2/18:2), containing >3 double-bond by approximately 60-fold at 96 h, if compared to TAGs at 0 h. With respect to plastidial membrane lipids, a more complex pattern was adopted. MGDG 34:7 (16:4/18:3), as the most abundant MGDG, decreased significantly at 12 h under nitrogen starvation. On the contrary, the other three MGDGs 34:6 (16:3/18:3), MGDG 34:6 (16:4/18:2) and MGDG 34:5 (16:3/18:2) started to decrease at 48 h. All DGDGs displayed the same tendency as the total DGDG amount culminating at 48 h and decreasing at 96 h. Of special note is that the dominant DGDG 34:3 (16:0/18:3) at 96 h increased during the whole process. All PG molecules underwent noteworthy reduction at 24 h. DGTS and PE are the major constituents of extra-plastidial membrane. PE 36:6 (C18:3/18:3), as the major PE at 0 h, decreased significantly at 12 h, while the other PEs started to decrease at 24 h. In contrast, most DGTS lipids with PUFA chains decremented dramatically at 12 h with an exception that no remarkable difference was found in the dominant DGTS 34:3 (16:0/18:3). The primary lyso-DGTS (18:3) fluctuated in a certain range during the whole process. DGTSs as a kind of N-containing lipids tended to be the first cellular reaction coping with nitrogen starvation, which is also confirmed in *Nannochloropsis* (Jing Li). DGTS 34:3 (16:0/18:3) and lyso-DGTS (18:3) might serve as the house-keeping lipid to maintain the whole integrity of cell membrane. Figure 10.6 illustrates the chemical structure of the most abundant lipid compounds in *Scenedesmus sp.*

72 TAG molecules have been assigned at 96 h based on high-resolution and tandem MS data. 8 TAG molecules 46:1 (14:0/16:1/16:0), 48:1 (16:0/16:1/16:0&16:0/18:1/14:0), 52:1 (16:0/18:1/18:0&14:0/18:1/20:0), 48:2 (16:0/16:1/16:1&14:0/18:1/16:1), 50:2 (16:0/18:1/16:1&14:0/18:1/18:1), 52:5 (16:0/16:0/20:5), 52:5 (16:0/16:1/20:4) and 52:6 (16:0/16:1/20:5) were found to be predominant species in *C. closterium*. All the major TAGs started to decrease in concentration at 12 h, while significant increase of each major TAG only took place at 96 h, if compared to the 0 h counterparts. 46:1 (14:0/16:1/16:0), 48:2 (16:0/16:1/16:1&14:0/18:1/16:1), 52:5 (16:0/16:0/20:5) and 52:6 (16:0/16:1/20:5) increased 40-60% at 96 h. MGDG species followed the same trend of the major MGDG 36:8 (16:3/20:5), which decreased significantly at 12 h. The two primary DGDG 36:7 (C16:2/C20:5) and 36:6 (C16:1/C20:5) stayed unchanged until 12 h and decreased after 12 h. The SQDG 32:1 (16:0/16:1) increased within 24 h before it decreased at 48 h. Figure 10.7 illustrates the chemical structure of the most abundant lipid compounds in *C. closterium.*

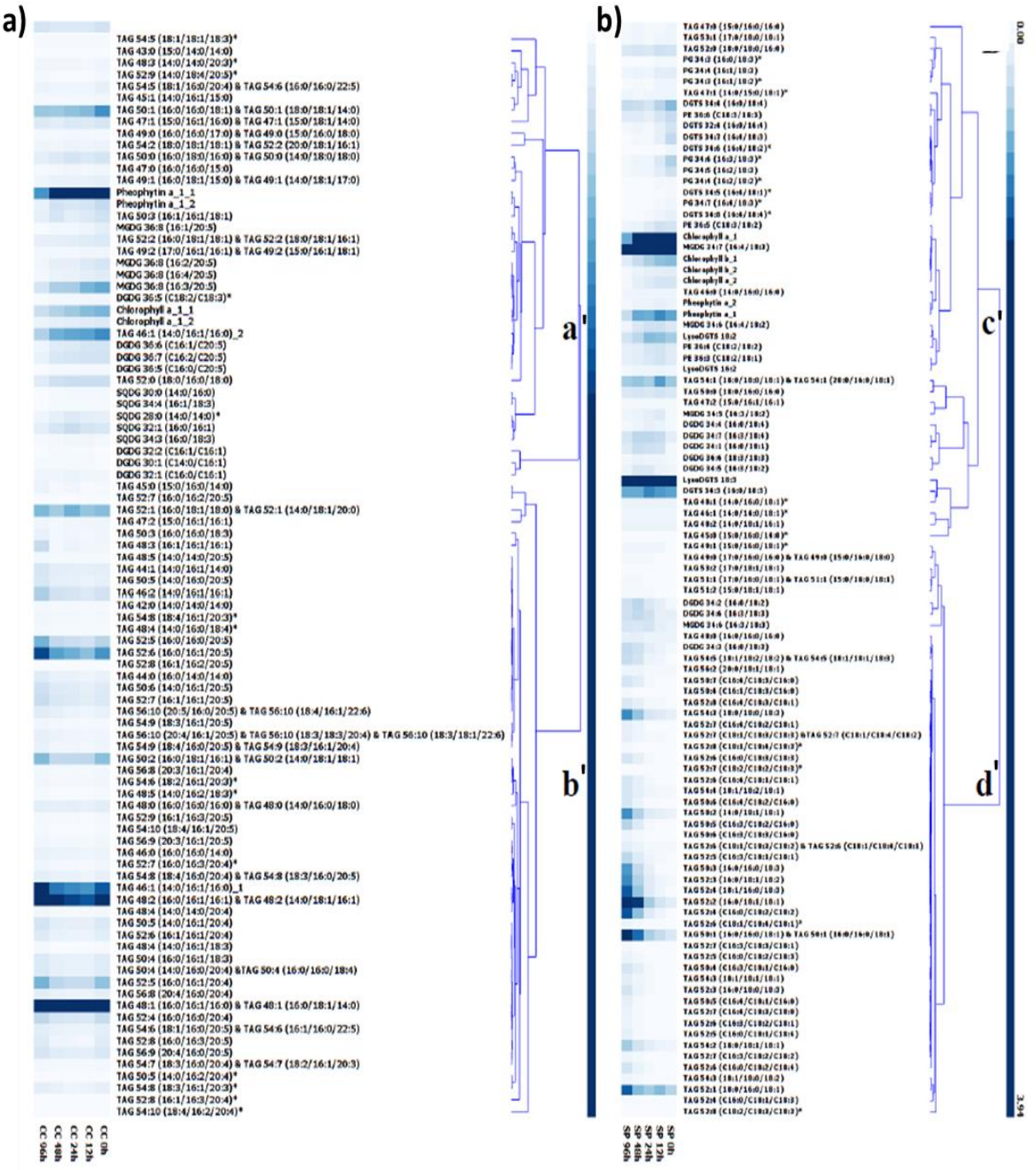
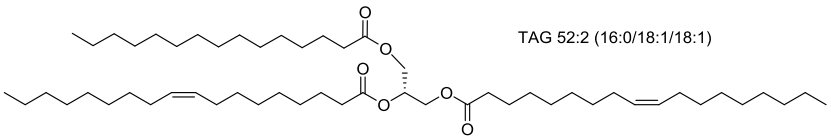
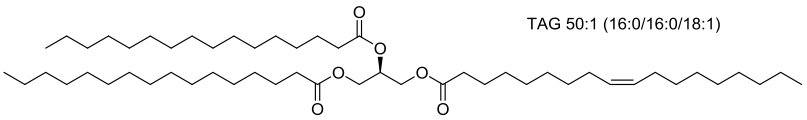


Figure 10.5 Heat map of glycerolipid and pigment molecules in *Scenedesmus sp.* and *C. closterium* under nitrogen starvation: **(a)** glycerolipid and pigment molecules from *C. closterium*; **(b)** glycerolipid and pigment molecules from *Scenedesmus sp.* All the identified molecules were primarily clustered in 4 groups: molecules in group **a'** and **c'** displayed a decreasing trend during the time course; molecules in group **b'** decreased at the beginning, then increased till 96 h; group **d'** exhibited an increasing trend. *compounds were tentatively identified due to the insufficient fragment information.



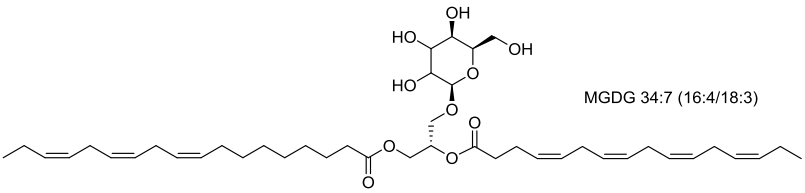
TAG 52:2 (16:0/18:1/18:1)

(*R*)-3-(pentadecanoyloxy)propane-1,2-diyl dioleate
Chemical Formula: $C_{54}H_{100}O_6$
Molecular Weight: 845.39



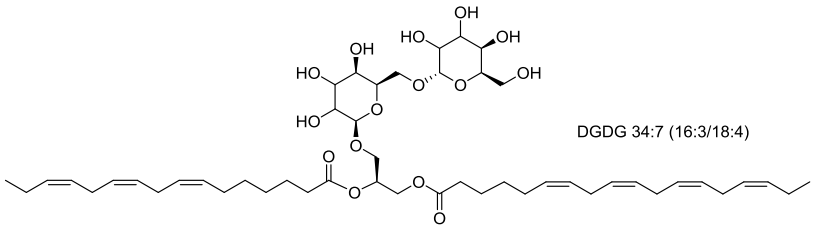
TAG 50:1 (16:0/16:0/18:1)

(*R*)-3-(oleoyloxy)propane-1,2-diyl dipalmitate
Chemical Formula: $C_{53}H_{100}O_6$
Molecular Weight: 833.38



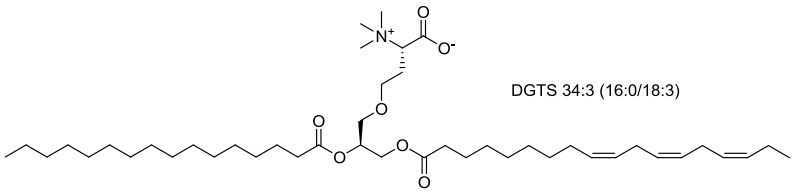
MGDG 34:7 (16:4/18:3)

(2*S*)-2-(((4*Z*,7*Z*,10*Z*,13*Z*)-hexadeca-4,7,10,13-tetraenoyl)oxy)-3-(((2*R*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)propyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate
Chemical Formula: $C_{43}H_{68}O_{10}$
Molecular Weight: 745.01



DGDG 34:7 (16:3/18:4)

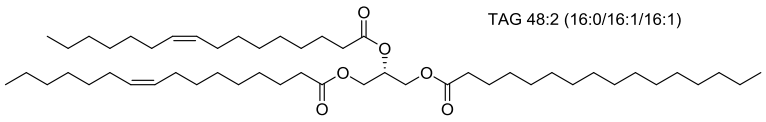
(2*S*)-2-(((7*Z*,10*Z*,13*Z*)-hexadeca-7,10,13-trienoyl)oxy)-3-(((2*R*,5*R*,6*R*)-3,4,5-trihydroxy-6-((((2*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)propyl (6*Z*,9*Z*,12*Z*,15*Z*)-octadeca-6,9,12,15-tetraenoate
Chemical Formula: $C_{49}H_{78}O_{15}$
Exact Mass: 906.53



DGTS 34:3 (16:0/18:3)

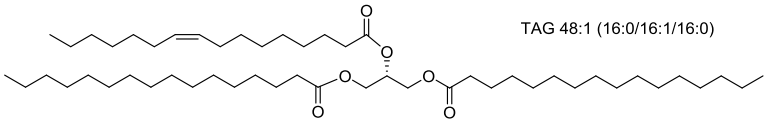
(*S*)-4-((*S*)-3-(((9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoyl)oxy)-2-(palmitoyloxy)propoxy)-2-(trimethylammonio)butanoate
Chemical Formula: $C_{44}H_{79}NO_7$
Exact Mass: 733.59

Figure 10.6 Chemical structures of the most abundant lipid compounds in *Scenedesmus sp.*



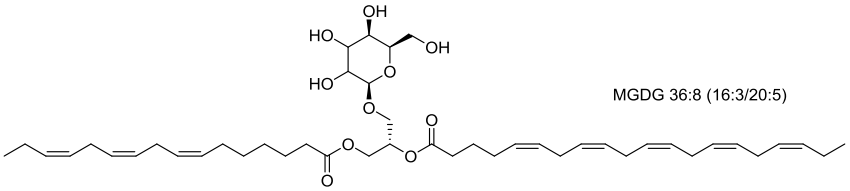
TAG 48:2 (16:0/16:1/16:1)

(*R*)-3-(palmitoyloxy)propane-1,2-diyl (9*Z*,9'*Z*)-bis(hexadec-9-enoate)
Chemical Formula: $C_{51}H_{94}O_6$
Exact Mass: 802.71



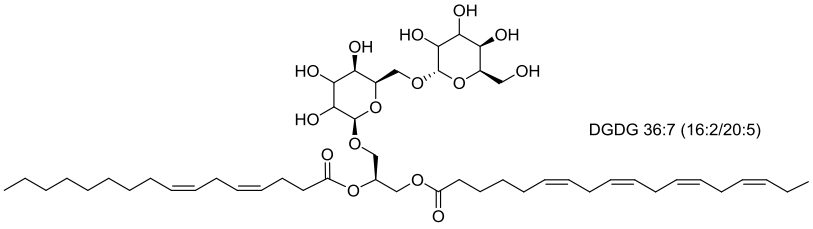
TAG 48:1 (16:0/16:1/16:0)

(*Z*)-2-(hexadec-9-enoyloxy)propane-1,3-diyl dipalmitate
Chemical Formula: $C_{51}H_{96}O_6$
Exact Mass: 804.72



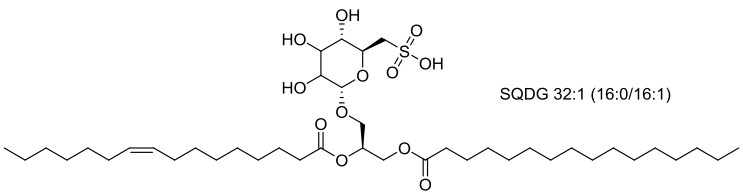
MGDG 36:8 (16:3/20:5)

(2*S*)-1-(((7*Z*,10*Z*,13*Z*)-hexadeca-7,10,13-trienoyl)oxy)-3-(((2*R*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)propan-2-yl (5*Z*,8*Z*,11*Z*,14*Z*,17*Z*)-icosa-5,8,11,14,17-pentaenoate
Chemical Formula: $C_{45}H_{70}O_{10}$
Exact Mass: 770.50



DGDG 36:7 (16:2/20:5)

(2*S*)-2-(((4*Z*,7*Z*)-hexadeca-4,7-dienoyl)oxy)-3-(((2*R*,5*R*,6*R*)-3,4,5-trihydroxy-6-((((2*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)propyl (6*Z*,9*Z*,12*Z*,15*Z*)-octadeca-6,9,12,15-tetraenoate
Chemical Formula: $C_{49}H_{80}O_{15}$
Exact Mass: 908.55



SQDG 32:1 (16:0/16:1)

((2*S*,3*S*,6*S*)-6-((*S*)-2-(((*Z*)-hexadec-9-enoyl)oxy)-3-(palmitoyloxy)propoxy)-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methanesulfonic acid
Chemical Formula: $C_{41}H_{76}O_{12}S$
Exact Mass: 792.51

Figure 10.7 Chemical structures of the most abundant lipid compounds in *C. closterium.*

As shown, the PUFA chains were mainly present in membrane lipids at 0 h in both species, while TAG with PUFA acyl chain increased significantly at 96 h. PUFA concentration was observed to decrease in FAME analysis, hence we suggested that PUFA acyl chains are recycled by TAGs in both algae, which was witnessed in a previous study (Goncalves et al., 2016). As the precursor of TAG *de novo* synthesis, DAG was synthesized by the acylation of the *sn-2* position of lysophosphatidic acid catalyzed by lysophosphatidic acid acyltransferases (LPAAT) (Nobusawa et al., 2017). LPAAT localized in different organelles showed varied preference. LPAAT in chloroplast showed preference to C16 moiety substrate – the "prokaryotic" pathway, while LPAAT in endoplasmic reticulum preferred C18 – the "eukaryotic" pathway (Allen et al., 2014). As shown in data of Table 10.1, although TAGs were synthesized in both pathways, the ratio of lipids from the two pathways differed. Before the start of nitrogen starvation, more TAGs were synthesized in "eukaryotic" pathway than "prokaryotic" pathway in *Scenedesmus sp*., but the ratio of TAG with C16/18 in *sn-2* position decreased significantly at 24 h in response to nitrogen starvation. In contrast, most of the TAGs were synthesized by "prokaryotic" pathway at 0 h in *C. closterium*. Moreover, the increased TAGs induced by nitrogen starvation were mainly contributed by "prokaryotic" pathway. Compared to the rapid increase of TAGs in *Scenedesmus sp*. significant increase only happened at 96 h in *C. closterium* revealing *Scenedesmus sp*. can switch to lipids production faster. DGDG molecules increased within 48 h in *Scenedesmus sp*., while all DGDG molecules decreased significantly at 24 h in *C. closterium*. Interestingly, the amount of DGDG 34:3 (16:0/18:3) was elevated at 96 h in *Scenedesmus sp*. indicating its essential function in photosystems of green algae (Sozer et al., 2011).

Table 10.1 The content of TAGs (% dry biomass) with C16 or C18 FA in the *sn-2* position and ratio of TAG with C16 and C18 in the *sn-2* position.

Time (h)	***Scenedesmus sp.***			***C. closterium***		
	% C16 *sn-2*	% C18 *sn-2*	Ratio of C16/C18 *sn-2*	% C16 *sn-2*	% C18 *sn-2*	Ratio of C16/C18 *sn-2*
0	0.75±0.06	0.99±0.15	0.76±0.1	11.89±0.1	1.75±0.06	6.79±0.16
12	1.08±0.16	1.46±0.15	0.74±0.04	8.66±0.63	1.28±0.08	6.77±0.21
24	1.19±0.06	1.88±0.17	0.64±0.04	9.25±0.20	1.42±0.04	6.53±0.21
48	2.00±0.11	4.11±0.18	0.49±0.01	10.22±0.10	1.01±0.05	10.09±0.57
96	3.49±0.17	7.53±0.23	0.46±0.01	14.84±1.36	1.41±0.04	10.53±1.17

If two or three molecules shared one mass/charge ratio, they were represented by the dominant one based on the fragment information in the calculation. Data were shown as mean±SD, n=3.

DGTS molecules in *Scenedesmus sp*. dropped immediately after nitrogen starvation indicating the sensitivity of DGTS to nitrogen stress.

From the results, we can draw a conclusion that *Scenedesmus sp*. and *C. closterium* adopted a different strategy to cope with nitrogen stress, specifically the TAG accumulation mechanism. The TAG content of *C. closterium* decreased at 12 h which might be induced by the rapid cell reproduction. This unique lipid metabolism that we discovered in *C. closterium*, is presumably due to its benthic characteristics. Diatoms are more competitive in absorbing nutrients than other phytoplankton. Large diatoms, in particular, tend to have a relatively larger vacuole than small diatom where nitrogen could be stored (Litchman, 2007) (Sicko-Goad et al., 1984) (Raven, 1987). The growth of *Scenedesmus sp.* in this study slowed down, but *C. closterium* duplicated rapidly in response to nitrogen starvation. More small cells of *C. closterium* were present at 96 h compared to algae as seen at 0 h from the observation under a microscope. *C. closterium* is a kind of large diatom, 30-40 um in length. It could be beneficial for ciliates defense, but at the same time, they will have a lower area/volume, compared to small cells, which will not be harmful in a nutrient-rich environment. Nevertheless, in nutrients limited environment, smaller cells are more favourable for nutrients uptake (Raven, 1998) (Banse, 1976). Therefore, *C. closterium* improved the capability of nitrogen assimilation by reproducing smaller cells. Likewise, algae under nitrogen stress tended to strengthen assimilation apparatus were noted down in several studies, which supported the hypothesis in principle (Levitan et al., 2015).

10.4 Conclusions

Freshwater green algae and marine benthic diatom exhibited different lipid metabolism in response to nitrogen starvation. A detailed comparative lipidomic analysis by LC-MS of both species, yielding information at the molecular level, was carried out for the first time. In contrast to a constant increase lipid content in *Scenedesmus sp.*, the lipids decreased to support a rapid duplication of *C. closterium* within 12 h but increased at 96 h. Nitrogen starvation induced an increased level of saturated/monounsaturated fatty acids but decreased the production of polyunsaturated fatty acids, which is beneficial for biofuel production. By providing the most abundant TAG profiles in green algae and diatom, more insights have been brought into the different metabolisms of lipid production. TAG increased by 6-fold and 20%

in *Scenedesmus sp.* and *C. closterium* at 96 h if compared to samples before nitrogen starvation, respectively. In comparison to the concomitant decrease of MGDG and DGDG in and *C. closterium*, DGDG in *Scenedesmus sp.* increased at 48 h indicating an essential role in maintaining the integrity of photosystems in green algae. *C. closterium* grew rapid at 12, resulting in a significant TAG decrease to provide energy for reproduction. The TAG of *C. closterium* attaining 17% of the dry biomass. The TAG synthesized by "eukaryotic pathway" in *Scenedesmus sp.* were strengthened, in contrast, increased TAG was synthesized by "prokaryotic," indicating the discrepancy of *de novo* TAG biosynthesis pathway.

References

Abida, H., Dolch, L.J., Mei, C., Villanova, V., Conte, M., Block, M.A., Finazzi, G., Bastien, O., Tirichine, L., Bowler, C., Rebeille, F., Petroutsos, D., Jouhet, J., Marechal, E., 2015. Membrane glycerolipid remodeling triggered by nitrogen and phosphorus starvation in Phaeodactylum tricornutum. Plant Physiol. 167(1), 118-136.

Abomohra, A.E.-F., Jin, W., Tu, R., Han, S.-F., Eid, M., Eladel, H., 2016. Microalgal biomass production as a sustainable feedstock for biodiesel: Current status and perspectives. Renewable and Sustainable Energy Reviews. 64, 596-606.

Alipanah, L., Rohloff, J., Winge, P., Bones, A.M., Brembu, T., 2015. Whole-cell response to nitrogen deprivation in the diatom Phaeodactylum tricornutum. J Exp Bot. 66(20), 6281-6296.

Allen, J.W., DiRusso, C.C., Black, P.N., 2015. Triacylglycerol synthesis during nitrogen stress involves the prokaryotic lipid synthesis pathway and acyl chain remodeling in the microalgae Coccomyxa subellipsoidea. Algal Research. 10, 110-120.

Allen, J.W., DiRusso, C.C., Black, P.N., 2014. Triglyceride quantification by catalytic saturation and LC–MS/MS reveals an evolutionary divergence in regioisometry among green microalgae. Algal Research. 5, 23-31.

Banse, K., 1976. Rates of growth, respiration and photosynthesis of unicellular algae as related to cell size - a review. J. Plycol. . 12, 135-140.

Benavente-Valdes, J.R., Aguilar, C., Contreras-Esquivel, J.C., Mendez-Zavala, A., Montanez, J., 2016. Strategies to enhance the production of photosynthetic pigments and lipids in chlorophycae species. Biotechnol Rep (Amst). 10, 117-125.

Chu, W.-L., 2017. Strategies to enhance production of microalgal biomass and lipids for biofuel feedstock. European Journal of Phycology. 52(4), 419-437.

Frada, M.J., Burrows, E.H., Wyman, K.D., Falkowski, P.G., 2013. Quantum requirements for growth and fatty acid biosynthesis in the marine diatom Phaeodactylum tricornutum (Bacillariophyceae) in nitrogen replete and limited conditions. Journal of Phycology. 49(2), 381-388.

Gaude N, Bréhélin C, Tischendorf G, Kessler F, P., D., 2007. Nitrogen deficiency in Arabidopsis affects galactolipidcomposition and gene expression and results inaccumulation of fatty acid phytyl esters. Plant J. 49(4), 729-739.

Goncalves, E.C., Wilkie, A.C., Kirst, M., Rathinasabapathi, B., 2016. Metabolic regulation of triacylglycerol accumulation in the green algae: identification of potential targets for engineering to improve oil yield. Plant Biotechnol J. 14(8), 1649-1660.

Gouveia, L., Oliveira, A.C., 2009. Microalgae as a raw material for biofuels production. J Ind Microbiol Biotechnol. 36(2), 269-274.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. . In: Smith, W.L. and Chanley, M.H., Eds., Culture of Marine Invertebrates Animals, Plenum Publishing, New York,, 29-60.

Ho, S.H., Chen, C.Y., Chang, J.S., 2012. Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N. Bioresour Technol. 113, 244-252.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, A., D., 2008. Microalgal triacylglycerols as feedstocks for biofuelproduction- perspectives and advances. Plant J. . 54(4), 621-639.

Islam, M., Magnusson, M., Brown, R., Ayoko, G., Nabi, M., Heimann, K., 2013. Microalgal Species Selection for Biodiesel Production Based on Fuel Properties Derived from Fatty Acid Profiles. Energies. 6(11), 5676-5702.

Lavín, P.L., Lourenço2, S.O., 2005. AN EVALUATION OF THE ACCUMULATION OF INTRACELLULAR INORGANIC NITROGEN POOLS BY MARINE MICROALGAE IN BATCH CULTURES. BRAZILIAN JOURNAL OF OCEANOGRAPHY. 53(1/2), 55-68.

Levitan, O., Dinamarca, J., Zelzion, E., Lun, D.S., Guerra, L.T., Kim, M.K., Kim, J., Van Mooy, B.A., Bhattacharya, D., Falkowski, P.G., 2015. Remodeling of intermediate metabolism in the diatom Phaeodactylum tricornutum under nitrogen stress. Proc Natl Acad Sci U S A. 112(2), 412-417.

Li, J., Han, D., Wang, D., Ning, K., Jia, J., Wei, L., Jing, X., Huang, S., Chen, J., Li, Y., Hu, Q., Xu, J., 2014. Choreography of Transcriptomes and Lipidomes of Nannochloropsis Reveals the Mechanisms of Oil Synthesis in Microalgae. Plant Cell. 26(4), 1645-1665.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C.Q., 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. Appl Microbiol Biotechnol. 81(4), 629-636.

Litchman, E., 2007. CHAPTER 16 – Resource Competition and the Ecological Success of Phytoplankton.Evolution of Primary Producers in the Sea. 351-375.

Makulla, A., 2000. Fatty Acid Composition of Scenedesmus obliquus- Correlation to Dilution Rates. Limnologica. 30, 162-168.

Michael Hannon, Javier Gimpel, Miller Tran, Beth Rasala, Mayfield, S., 2010. Biofuels from algae- challenges and potential. Biofuels. 1(5), 763-784.

Nobusawa, T., Hori, K., Mori, H., Kurokawa, K., Ohta, H., 2017. Differently localized lysophosphatidic acid acyltransferasescrucial for triacylglycerol biosynthesis in the oleaginous alga Nannochloropsis. Plant J. 90(3), 547-559.

Paes, C.R.P.S., Faria, G.R., Tinoco, N.A.B., Castro, D.J.F.A., Barbarino, E., Lourenco, S.O., 2016. Growth, nutrient uptake and chemical composition of Chlorella sp. and Nannochloropsis oculata under nitrogen starvation. Latin American Journal of Aquatic Research. 44(2), 275-292.

Peter Dormann, Susanne Hoffmann-Benning, l.B., Benning', C., 1995. lsolation and Characterization of an Arabidopsis Mutant Deficient in the Thylakoid Lipid Digalactosyl Diacylglycero. the Plant Cell. 7, 1801-1810.

Randrianarison, G., Ashraf, M.A., 2017. Microalgae: a potential plant for energy production. Geology, Ecology, and Landscapes. 1(2), 104-120.

Raven, J.A., 1987. The role of vacuoles. New Phytol. . 106, 357–422.

Raven, J.A., 1998. The twelfth Tansley Lecture. Small is beautiful: the picophytoplankton. Functional Ecology 12, 503-513.

Sayre, R., 2010. Microalgae: The Potential for Carbon Capture. BioScience. 60(9), 722-727.

Sharma, Y.C., Singh, V., 2017. Microalgal biodiesel: A possible solution for India's energy security. Renewable and Sustainable Energy Reviews. 67, 72-88.

Siaut, M., Cuiné, S., Cagnon, C., Fessler, B., Nguyen, M., Carrier, P., Beyly, A., Beisson, F., Triantaphylidès, C., Li-Beisson, Y., Peltier, G., 2011. Oil accumulation in the model green alga Chlamydomonas reinhardtii-characterization, variability between common laboratory strains and relationship with starch reserves. BMC Biotechnology. 11, 7.

Sicko-Goad, L.M., Schelske, C.L., Stoermer, E.F., 1984. Estimation of intracellular carbon and silica content of diatoms from natural assemblages using morphometric techniques. Limnol. Oceanogr. . 29, 1170-1178.

Sirbu, D., Corno, M., Ullrich, M.S., Kuhnert, N., 2018. Characterization of triacylglycerols in unfermented cocoa beans by HPLC-ESI mass spectrometry. Food Chem. 254, 232-240.

Sozer, O., Kis, M., Gombos, Z., Ughy, B., 2011. Proteins, glycerolipids and carotenoids in the functional photosystem II architecture. Front Biosci (Landmark Ed). . 16, 619-643.

Spalding, M.H., 2008. Microalgal carbon-dioxide-concentrating mechanisms: Chlamydomonas inorganic carbon transporters. J Exp Bot. 59(7), 1463-1473.

Wang, S., Zhang, L., Yang, G., Zhu, B., Pan, K., 2015. Purification of a diatom and its identification to *Cylindrotheca closterium*. J. Ocean Univ. China. 14(2), 357-361.

Webb, M.S., Green, B.R., 1991. Biochemical and biophysical properties of thylakoid acyl lipids. Biochimica et Biophysica Acta. 1060, 133-158.

Yang, D., Song, D., Kind, T., Ma, Y., Hoefkens, J., Fiehn, O., 2015. Lipidomic Analysis of Chlamydomonas reinhardtii under Nitrogen and Sulfur Deprivation. PLoS One. 10(9), e0137948.

Yodsuwan, N., Sawayama, S., Sirisansaneeyakul, S., 2017. Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom Phaeodactylum tricornutum. Agriculture and Natural Resources. 51(3), 190-197.

Zhu, S., Wang, Y., Shang, C., Wang, Z., Xu, J., Yuan, Z., 2015. Characterization of lipid and fatty acids composition of Chlorella zofingiensis in response to nitrogen starvation. J Biosci Bioeng. 120(2), 205-209.

General Conclusions

Unraveling the chemistry of cocoa butter constitutes the basis for understanding the crystallization properties of this fat. Nowadays, lipidomics offers a broad array of methods, but not all are appropriate for the analysis of cocoa lipids. Most, if not all previous studies on cocoa chemistry have used the standardized official methods of analysis and very little to no method optimization. Consequently, only few lipid compounds were described. Some studies have reported that minor components influence the physical properties of cocoa butter; for this reason, an adequate method for the analysis and evaluation of these compounds was necessary.

One of the main objectives of this thesis was to develop the most suitable methods for the analysis of lipids in a large number of cocoa bean samples from all the different stages of the industrial processing. In this regard, intense optimization was performed at the extraction, separation and detection levels. As a consequence, a long list of methods and tools for comprehensive chemical analysis of cocoa fat is presented in this thesis. Methods such as Soxhlet extraction method using dichloromethane as extraction solvent, thin layer chromatography separation and solid phase extraction methods for the separation of different lipid classes, high-resolution and tandem HPLC-MS method for lipid investigation at the molecular level, fatty acids methyl esters synthesis and GC-FID identification and spectrophotometric assays for color assessment and glycerol quantification. Furthermore, this lipidomics approach was successfully used to investigate the lipids in other seeds or oil containing plant material, like algae. The advantages of such methods are that they are easy to apply and they offer a comprehensive picture of all the compounds present in a lipid extract. Furthermore, these methods were applied to other plant material such as green algae and marine benthic diatom. Thus, this lipidomic approach helped to elucidate the lipids in algae and to observe their lipid metabolism in response to nitrogen starvation.

HPLC coupled with high resolution-MS and tandem MS enabled the characterization of 83 different TAGs in unfermented cocoa beans, 77 of which were positively dereplicated and 58 of which were reported for the first time in cocoa. The identification and characterization were based on retention time behavior, tandem MS fragmentation and two-dimensional *m/z* retention time maps as powerful visual representation tools for the identification of entire homologous series of TAGs. Moreover, the results showed that reversed-phase C18 column packing is not able to separate regioisomers of TAGs and therefore additional analytical tools, such as chiral

column packings, are required to fully elucidate the entire TAGs profile. At the same time, further studies need to be carried out in order to completely understand the influences of different regioisomeric TAGs compositions on the physical properties of cocoa butter.

Among the newly identified compounds, TAGs carrying a hydroxyl-containing side chain were characterized by tandem MS, derivatization, NMR, and comparison with surrogate standards. In view of that, many different methods were required to confirm the results obtained. These compounds are reported for the first time in cocoa and they showed to be present in high amount in unfermented fresh beans. Hence, further research is required to appraise their biological functions, physical characteristics, and potential industrial valorization.

The chemical composition showed an enormous variability of the TAGs composition within samples from different origins. However, statistical analysis results have shown that the softness/hardness can be predicted by taking into account only four di-unsaturated TAGs relative quantities. Therefore, a novel softness/hardness indicator tool was developed, which could be used by industry to predict the softness/hardness of cocoa butter in a faster and more affordable way. Nevertheless, a standard reference of the four TAGs would be expected as means of comparison, and further physical parameters as solid fat content are required to confirm the functionality of this tool.

A detailed analysis of fatty acids methyl esters showed significant differences among the samples of different origins and fermentation status. Fermented dried cocoa beans have exhibited higher levels of linoleic and palmitoleic acid. These findings were not reported before in cocoa, and they could be used in the future to optimize and tailor cocoa lipid profile with respect to innovative applications and health products.

The comprehensive analysis has shown that the fermentation process has a significant impact on the lipid profile of cocoa and the present contribution is reporting this observation for the first time in literature. There is a lot of potential to be exploited from this findings, although it is still challenging to elucidate the mechanisms operating behind these chemical modifications. Moreover, this study draws the attention towards the importance of understanding the impact of fermentation microbiota on the final chemical composition of cocoa butter. Consequently, the crystallization behavior of cocoa butter in different stages of the fermentation process is still to be investigated and correlated with the composition of TAGs and fermentative microorganisms.

Elucidation of fatty acids amides as markers of the alkalization provided more understandings into how the factory processing affects the lipid profile of the final product. Moreover, these compounds were associated with the poor crystallization of cocoa butter.

Concerning minor components, 62 compounds were positively identified. The prominent compounds among the minor constituents are diacylglycerols. Two regioisomers of diacylglycerols were detected, 1,2-acyl-*sn*-glycerol and 1,3-acyl-*sn*-glycerol; 1,2-acyl-*sn*-glycerol regioisomer was the most abundant molecular species. Other minor components could be identified such as sterol esters, waxes, free fatty acids, sterol esters glucosides, and phospholipids. Notwithstanding, there are still numerous minor components in the cocoa lipid extract, which require future identification and quantification. With the help of the improved tools presented in this thesis, a complete chemical elucidation of all the lipid classes is now possible, which will lead to a deeper understanding of the crystallization of cocoa butter.

To sum up, the results of this thesis point out towards two main factors influencing the chemical composition of CB: origin and environmental growing conditions determine the profile, which is subsequently shaped by the processing conditions. Future work is required to connect the findings of this thesis to the physical properties of cocoa butter and to allow substantial industrial and commercial impact.