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CHROMATOGRAPHIC AND MASS-SPECTROMETRIC ANALYSIS OF LIPIDS OF VERNIX CASEOSA

Chromatografická a hmotnostně-spektrometrická analýza lipidů novorozeneckého mázku

Doctoral Thesis

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Prohlášení

Prohlašuji, že jsem tuto dizertační práci vypracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, …….. ……

Mgr. Aneta Vavrušová

PREFACE

This PhD has been an incredible journey. I would first and foremost like to express my sincere gratitude and deep appreciation to my esteemed supervisor and guide doc. RNDr. Josef Cvačka, Ph.D., for his constant encouragement, valuable suggestions, and patience throughout the study period. My deepest thanks are also given to the Mass Spectrometry Group at IOCB, especially to RNDr. Vladimír Vrkoslav, Ph.D., for his guidance, great attention, and timely advice throughout my doctoral studies. I am grateful to my colleagues from Zentiva, namely RNDr. Jiří Břicháč, Ph.D. and Ing. Stanislav Kalášek, for giving me the opportunity to use the instrumentation from their laboratory to complete my research. I also wish to express my special thanks to Dr. Achille Cappiello and his Mass Spectrometry Group at the University of Urbino, Italy, for his guidance and excellent advice during my research internship. My sincere thanks also go to doc. RNDr. Zuzana Bosáková, CSc. for her professional advice. And last but not least, I would like to express my love to my husband, parents, and my sister. Without their constant support, encouragement and babysitting, this would not have been possible.

This thesis represents the result of my PhD studies from 2015 to 2020 at the Department of Analytical Chemistry, Faculty of Science, Charles University in Prague. I have had the great possibility to carry out the work in the modern laboratories of Mass Spectrometry Group at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences, Prague. Part of this study was conducted in the laboratories of Mass Spectrometry at Zentiva.

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ABSTRACT

Lipidomics is a rapidly expanding research field that has captured extensive attention worldwide in the past few years due to the increasing awareness of the crucial roles of lipids in biological systems. The aim of lipidomics is to comprehensively analyze all lipids, to study their structure, biological function within the cell as well as interactions of lipids with other molecules. A combination of advanced analytical techniques, such as extraction, chromatography, and mass spectrometry, is an effective tool for studying all aspects of lipidomics. This dissertation thesis is based on two journal publications and presents the application of analytical strategies based on chromatography and mass spectrometry for investigation and characterization of new lipid classes of vernix caseosa.

Firstly, the applicability of nonaqueous reversed-phase liquid chromatography atmospheric pressure ionization tandem mass spectrometry (LC-APCI-MS^2) for structural characterization of cholesteryl esters of ω-(*O*-acyl)-hydroxy fatty acids (Chl-ωOAHFAs) in vernix caseosa was investigated. For this purpose, a TLC chromatography method for the isolation of neutral Chl-ωOAHFAs from vernix caseosa was developed. Their general structure was established using a multi-step mass spectrometric approach requiring transesterification and derivatization steps. To get the structural information of Chl-ωOAHFAs individual species, a mass spectrometric method using controlled thermal decomposition and data-dependent fragmentation was carefully optimized. About three hundred molecular species of Chl-ωOAHFAs were identified and quantified.

Secondly, while exploring vernix caseosa, a structurally related subclass of Chl-ωOAHFAs lipids, namely ω-(*O*-acyl)-hydroxy fatty acids (ωOAHFAs), was discovered. OAHFAs were isolated from a sample of vernix caseosa by an optimized 2-step TLC on silica gel. Using high-performance liquid chromatography coupled-

electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS^2), we identified and quantified a group of more than 400 species. Both Chl-ωOAHFAs and ωOAHFAs have been detected in other biological materials, such as human meibum or skin. However, when investigating the structure of ωOAHFAs we revealed the presence of α-isomers of OAHFAs. Intact αOAHFAs have never been detected before in any biological material.

ABSTRAKT

Lipidomika je rychle se rozšiřující vědní obor, který zaznamenal v posledních letech vzrůstající pozornost, a to zejména díky zvyšujícímu se povědomí o stěžejních rolích lipidů v biologických systémech. Hlavním cílem lipidomiky je systematické studium všech lipidů, zahrnuje popis jejich struktury, biologických funkcí v buňce a rovněž popis interakcí lipidů s dalšími molekulami. Spojení pokročilých analytických metod, jako je extrakce, chromatografie a hmotnostní spektrometrie, umožňuje efektivním způsobem studovat všechny aspekty lipidomiky. Předkládaná dizertační práce je komentovaným souborem ke dvěma publikovaným pracím a zabývá se využitím separačních technik a hmotnostní spektrometrie pro detekci a strukturní charakterizaci nových lipidových tříd v novorozeneckém mázku.

V prvním případě bude popsáno použití techniky nevodné kapalinové chromatografie na reverzní fázi ve spojení s tandemovou hmotnostní spektrometrií s chemickou ionizací za atmosférického tlaku (LC-APCI-MS^2) pro strukturní charakterizaci cholesterol esterů ω-(*O*-acyl)-hydroxy mastných kyselin (Chl-ωOAHFAs) v novorozeneckém mázku. Pro účel izolace neutrálních Chl-ωOAHFAs byla vyvinuta metoda adsorpční tenkovrstvé chromatografie. Obecná struktura Chl-ωOAHFAs byla odvozena na základě analýzy jejich produktů transesterifikace a derivatizace. Ke zjištění struktury jednotlivých molekulových druhů byla optimalizována hmotnostně-spektrometrická metoda s využitím cílené termální degradace protonovaných molekul a datově závislá fragmentace vzniklých produktů. Tímto způsobem bylo identifikováno a kvantifikováno více než 300 molekulových druhů Chl-ωOAHFAs.

V druhém případě půjde o identifikaci a popis strukturně příbuzné podskupiny lipidů, a to (*O*-acyl)-hydroxy mastných kyselin (OAHFAs). OAHFAs byly izolovány

z novorozeneckého mázku pomocí dvoukrokové tenkovrstvé chromatografie. Pro jejich detekci a identifikaci byla optimalizována metoda kapalinové chromatografie a tandemové hmotnostní spektrometrie s elektrosprejovou ionizací (LC-ESI-MS^2). Touto technikou bylo možné identifikovat a stanovit více než 400 molekulových druhů. Chl-ωOAHFAs a ωOAHFAs byly již dříve identifikovány například v lidském meibu či kůži. Během analýzy ωOAHFAs v novorozeneckém mázku byla odhalena přítomnost α-izomerů OAHFAs. Intaktní αOAHFAs nebyly doposud detekovány v žádném biologickém materiálu.

CONTENTS

LIST OF PUBLICATIONS RELEVANT FOR THIS THESIS

I. Kalužíková A., Vrkoslav, V., Harazim, E., Hoskovec, M., Plavka, R., Buděšínský, M., Bosáková Z., and Cvačka, J. Cholesteryl esters of ω-(*O*-acyl)-hydroxy fatty acids in vernix caseosa. *J Lipid Res*. 2017; **58**(8):1579-1590.

II. Vavrušová, A., Vrkoslav, V., Plavka, R., Bosáková, Z., and Cvačka, J. Analysis of (*O*-acyl) alpha- and omega-hydroxy fatty acids in vernix caseosa by high-performance liquid chromatography-Orbitrap mass spectrometry. *Anal Bioanal Chem.* 2020; **412**(10): 2291-2302.

The publications are referred to in the text by their Roman numerals.

DECLARATION OF AUTHORSHIP

As a representative of the co-authors I declare that Aneta Vavrušová, M.Sc. participated in the publications listed below:

I. The experimental work was carried out by the author with contributions from others. The manuscript was written by the author with contributions from others.

participation: 85 %

II. The experimental work was carried out by the author. The manuscript was written by the author with contributions from others.

participation: 85 %

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doc. RNDr. Josef Cvačka, Ph.D.

ABBREVIATIONS

1,2-DDE	1,2-Diol diester
AC	Alternating current
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
CE	Capillary electrophoresis
Cer	Ceramide
Chl-ωOAHFA	Cholesteryl esters of ω-(*O*-acyl)-hydroxy fatty acid
CI	Chemical ionization
CID	Collision-induced dissociation
CRM	Charge residue model
DG	Diacylglycerol
DESI	Desorption electrospray ionization
DC	Direct current
EASI	Easy ambient sonic-spray ionization
ECN	Equivalent carbon number
EI	Electron ionization
ESI	Electrospray ionization
FA	Fatty acid
FAME	Fatty acid methyl ester
FTICR	Fourier transform ion cyclotron resonance
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GP	Glycerophospholipid
GL	Glycerolipid
HFA	Hydroxy fatty acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
IEM	Ion evaporation model
IMS	Ion mobility spectrometry
IT	Ion trap
LC	Liquid chromatography

LLE	Liquid-liquid extraction
MG	Monoacylglycerol
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MSI	Mass spectrometry imaging
MTBE	Methyl-*tert*-butyl ether
MUFA	Monounsaturated fatty acid
NMR	Nuclear magnetic resonance
NPLC	Normal-phase liquid chromatography
OAHFA	(*O*-acyl)-hydroxy fatty acid
PK	Polyketide
PR	Prenol
PUFA	Polyunsaturated fatty acid
Q	Quadrupole
QqQ	Triple quadrupole
R_F	Retardation factor
RPLC	Reversed-phase liquid chromatography
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SIMS	Secondary ion mass spectrometry
SP	Sphingolipid
SL	Saccharolipid
SPE	Solid-phase extraction
ST	Sterol
TG	Triacylglycerol
TEWL	Transepidermal water loss
TOF	Time-of-flight
TLC	Thin-layer chromatography

INTRODUCTION

1 Lipids and lipidomics

Lipids are essential natural compounds that contribute to structural integrity and regulate metabolic pathways in all living cells (1). They are involved in various important biological processes that living organisms require for surviving. The majority of lipids are the main constituents of membrane bilayers surrounding every cell and organelle (2, 3). Others act as precursors for lipid second messengers during signal transduction (4) or provide energy storage for many biological processes (5). In addition, research shows that lipid metabolism disorders are deeply associated with some human diseases, *e.g.*, obesity (6), diabetes (7), atherosclerosis (8), cancer (9), Alzheimer's diseases (10), and many more (11-13).

Lipids have been defined and classified by Fahy et al. (14, 15) as small hydrophobic or amphipathic molecules that originate entirely or in part by carbanion based condensations of ketoacyl thioesters (e.g., fatty acids, glycerolipids) and/or by carbocation based condensations of isoprene units (e.g., sterols, prenols). Based on this concept, lipids may be classified into eight primary categories (fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids, polyketides, sterol lipids, and prenol lipids). Examples of chemical structures for the eight categories are shown in **Fig 1**. Regarding saccharolipids and polyketides, there is no single general structure that represents the whole lipid class, therefore a representative structure for each class was selected and shown in **Fig 1**. Each category can be further divided into classes and subclasses for more precise identification.

Fatty acids (FA) and their functional derivatives such as fatty alcohols, fatty aldehydes, fatty amines, fatty amides, and esters are a structurally diverse group of molecules that are

widely distributed in natural fats, dietary oils, and living organisms (16, 17). These lipids have a relatively simple chemical structure and exist either as free lipids or as building blocks of more complex lipid classes, such as phospholipids or triacylglycerols. A basic FA molecule consists of a carboxylic functional group to which an aliphatic hydrocarbon chain is attached. The hydrocarbon chain in FAs can differ in its length, the degree of unsaturation, the position and geometry of the unsaturated bonds, and branching (18). According to the number of double bonds, FAs can be classified into saturated (no double bond), monounsaturated (one double bond), diunsaturated (two double bonds), and polyunsaturated (more than two double bonds). Depending on the geometry of the double bond in monounsaturated FAs (MUFA), they are described as *cis* or *trans* isomers. The *trans* MUFAs have been studied more deeply after they were discovered to have possible negative effects on human health (19). In the case of polyunsaturated FAs (PUFA), there exist a number of various isomers that combine *cis* and *trans* configuration and double bond positional isomerization (20).

Lipids that contain a glycerol group in their structures are divided into two categories, glycerolipids (GL) and glycerophospholipids (GP). GLs are composed of a glycerol backbone that is usually esterified to one, two, or three FA chains. The mono-substituted species are monoacylglycerols (MG), the di-substituted species are diacylglycerols (DG) and triacylglycerols (TG) are esters in which three molecules of FAs are linked to the three free hydroxyls in glycerol (21). TGs represent the main constituents of human and animal body fats and vegetable oils. DGs are a minor component of many seed oils and together with MGs are common food additives largely used as emulsifiers (22, 23). GPs differ from GLs by the presence of a distinct phosphoryl head group that is esterified to the same structural feature of a glycerol backbone with one or two FAs attached to it. The phosphoryl group is commonly linked at the *sn*-3 position of the glycerol moiety. By

adding different substituents to the phosphoryl group, GPs may be divided into subclasses such as phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserin (24).

Sterols (ST) represent a group of naturally occurring substances that are derived from C27-C30 secondary alcohols having a tetracyclic 1,2-cyclopentanoperhydrophenanthrene ring system and a side chain at C17 of at least seven carbon atoms (25). Considering the number and position of double bonds, variations in the side chain, and stereochemistry, more than 250 naturally occurring sterols have been identified. STs are widely distributed in plants (phytosterols) (26), animals (zoosterols) (27), yeast (28), and fungi (mycosterols) (29). Many phytosterols showed important health and nutrition effects and thus became industrially significant. The main dietary sources of phytosterols are unrefined vegetable oils, nut and olive oils, seeds, whole grains, and legumes (30). Essential zoosterol for all animal tissues, cholesterol, is an important structural membrane constituent. In addition, it serves as a precursor for a variety of products with specific biological activities such as steroid hormones, bile acids, vitamin D, etc. (31).

In terms of prenol lipids (PR), despite the fact that they share the same biosynthetic pathway through the polymerization of dimethylallyl pyrophosphate/isopentenyl pyrophosphate as STs, their final structure and function are fundamentally different. PRs include many structurally different bioactive compounds such as carotenoids that are important antioxidants and precursors of vitamin A, ubiquinones, vitamins E and K, etc. (14).

Sphingolipids (SP) are a complex family of compounds that share a long-chain nitrogenous base (sphingoid base) backbone in their structure. They mainly include simple free sphingoid bases, ceramides (Cer), and ceramide containing complex SPs (such as sphingomyelin, cerebrosides, sulfatides, etc.) (32). Cers are formed by linking a fatty acid

chain to a sphingoid base *via* an amide bond (33). Adding a sugar head group to the 1-hydroxyl group of Cer leads to the formation of glycosphingolipids, whereas the addition of phosphatidylcholine forms sphingomyelins (34, 35). The addition of sialic acid or sulfate group to the sugar moiety in GSL molecules leads to even more complex SPs, gangliosides, and sulfatides (36). Considering the structural variations and their location within the human body, all SP have unique and characteristic biological functions.

Saccharolipids (SL) are compounds in which fatty acyls are linked directly to a sugar backbone. In SLs, this sugar backbone replaces the glycerol backbone that is the main structural component of glycerolipids and glycerophospholipids (14). The most familiar examples of SLs are the acylated glucosamine precursors of the lipid A component of lipopolysaccharides in gram-negative bacteria (37).

The ultimate category is polyketides (PK). PKs are natural metabolites from plants, animals, and microbial sources with great structural diversity. They exhibit diverse biological functions such as antitumor, anti-inflammatory, antimicrobial, anti-cholesterol, etc. (14).

Additionally, lipids can be also classified into simple and complex depending on the number of products of hydrolysis. Simple lipids yield at most two types of distinct hydrolysis products (such as acylglycerols, fatty acids, and sterols). Generating three or more distinct entities upon hydrolysis, lipids such as glycerophospholipids and glycosphingolipids are thus referred to as complex (15).

Taking into account the variable chain length, the number of biochemical transformations including oxidation, reduction, substitution, and ring-formation as well as modifications with sugar residues and other functional groups, there is a rough estimation of approximately 200,000 discrete lipid molecules in nature (38).

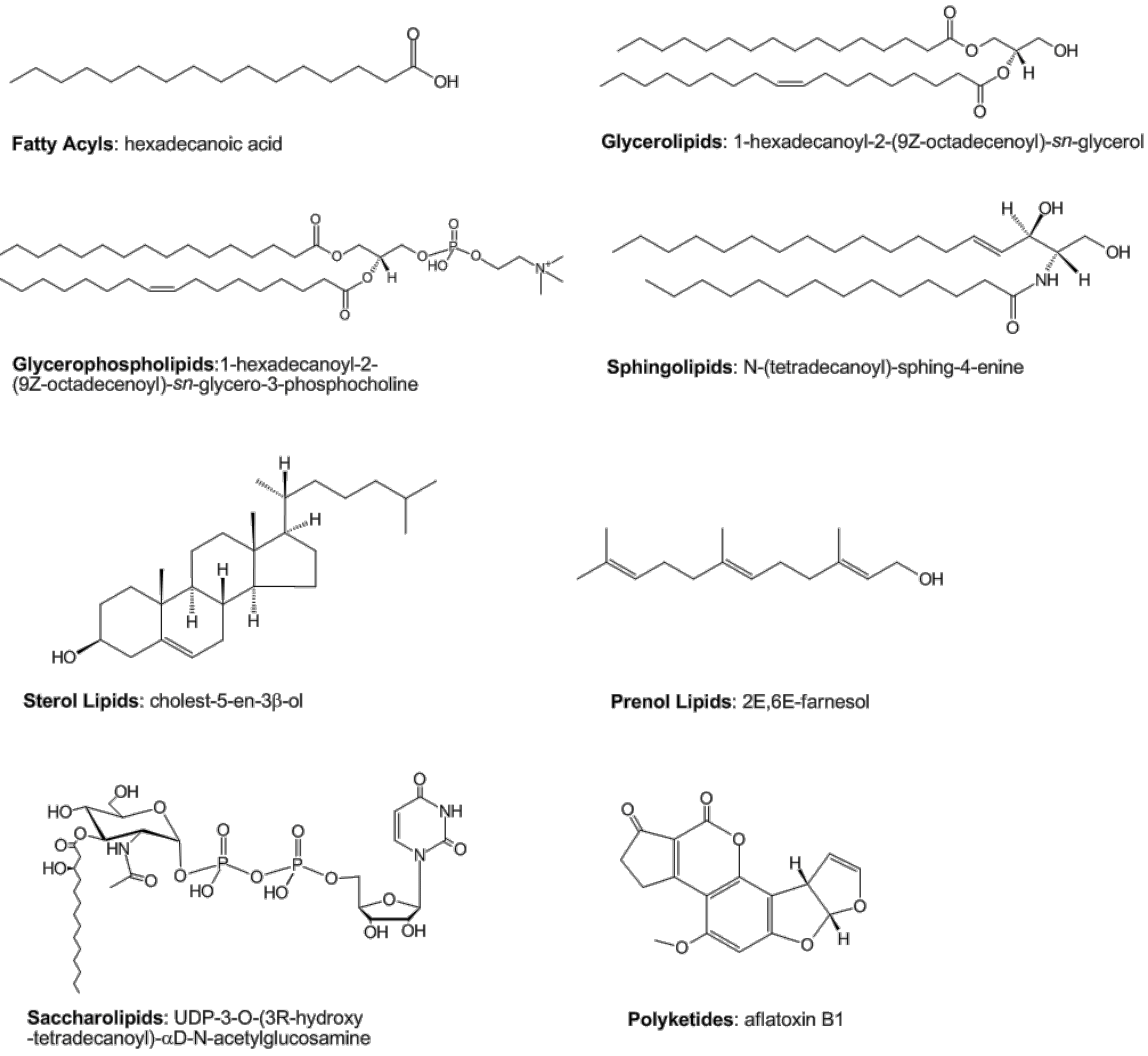

Figure 1. Examples of lipid categories. Representative structures from each of the 8 LIPID MAPS lipid categories (38).

Owing to the importance of their biological role, lipids were intensively studied during the period from the 1960s to 1980s (39, 40). Later, the focus of researchers moved towards the "omics" science in biology, where the primary aim is the comprehensive system-level bioanalysis of all gene products present in a specific biological sample, such as proteomics, genomics, metabolomics. More recently, with the rapid development of lipid research and the ongoing "omics" revolution, a new unique concept called "lipidomics" was firstly introduced in early 2000 (41, 42, 43). Lipidomics focuses on the full structural characterization of lipid molecular species as well as on metabolic pathways and interactions of lipids to establish their roles in biological systems (44). The biological importance of lipid metabolites is well known across many research disciplines. Maintenance of cellular and organismal lipid homeostasis is critical for life and any imbalance of lipid metabolism may have deleterious consequences resulting in severe lipid-associated disorders (45). Accordingly, lipidomics can be used as a powerful tool for the investigation of lipid alternations in tissues, cells, and biological fluids that facilitates elucidation of disease pathogenesis and the discovery of potential biomarkers for early diagnosis of diseases and potential drug efficiency (46).

Originally, analytical techniques such as thin-layer chromatography (TLC) (47), gas chromatography (GC) (48), electron ionization mass spectrometry (EI-MS) (49) and nuclear magnetic resonance (NMR) (50) have been used to study the lipidome in biological cells, tissues, and biofluids. More recently, modern advances such as reversed-phase liquid chromatography (RP-LC) (51), electrospray ionization (ESI) (52), and matrix-assisted laser desorption ionization mass spectrometry (MALDI) (53) have been employed that greatly contributed to the development of lipidomics. The technological progress in instrumentation and bioinformatics tools has driven the emergence and rapid expansion of lipidomics, especially in the past two decades. However, despite the novel analytical

approaches, the field of lipidomics still remains notably challenging and it is not surprising that, yet, there does not exist any complex technique applicable for lipidomic analysis to encompass the full lipidome. The main reason is primarily due to the complexity of lipids and the enormous number of chemically and structurally distinct individual lipid species.

The analytical strategies of lipidomics can be divided into two overlapping approaches depending on the aim of the research and experimental designs. Targeted and non-targeted (also known as global) lipidomics are two approaches with distinct features, advantages, and limitations (54). The targeted analysis is traditionally employed when the focus is on a single or a small number of pre-defined lipids within a specific lipid class. Targeted methods are required for low abundant lipid classes in biological samples, instable lipids, or lipids characterized by other physicochemical features that may limit the analytical procedure (55). The non-targeted lipid analysis aims at the rapid identification and relative quantification of every lipid in a sample simultaneously. The non-targeted lipid analysis generates overwhelming amounts of data that require complex data processing for acquiring meaningful biological information (56, 57). In contrast, targeted lipidomics is usually more specific and provides lipid-specific signals but prior knowledge of the target lipids in the sample is essential (59, 58). Different research aims require different analytical techniques. By way of example, LC/MS-based approaches are often performed for targeted lipid profiling in biological samples, while ‘shotgun lipidomics‘, which uses a direct infusion of lipid extracts into a mass spectrometer, is more feasible for non-targeted lipidomics (58).

A typical lipidomics workflow includes lipid extraction, mass spectrometry-based analytical technologies, and bioinformatics tools for valuable data interpretation (60).

Below is a flowchart that illustrates the serial of analytical approaches taken to perform a comprehensive analysis of lipids in any biological system (**Fig 2**).

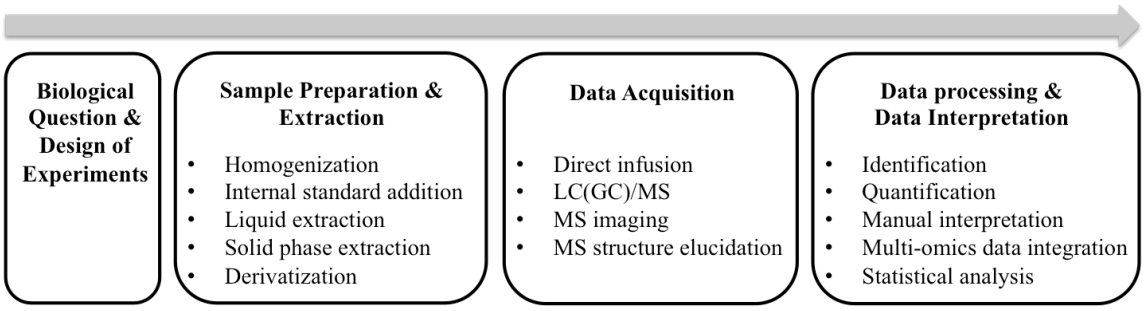

Figure 2. A typical workflow of steps in the lipidomics experiment.

2 Lipidomics techniques

2.1 Sample preparation techniques for lipid analysis

Careful sample preparation and lipid extraction are the primary steps in every lipidomics study. Lipids in nature usually exist as components of more complex biological matrixes (e.g. vernix caseosa, meibum, plasma, etc.) that contain many interferences (e.g. proteins, carbohydrates, amino acids, etc.). The major aim of these procedures is to quantitatively recover the analytes from the biological sample, reduce interferences that may contaminate the instruments or influence the sensitivity of the assay, and concentrate the target analytes (61, 62). Lipid extraction has experienced many advances going from conventional solvent extraction, to solid-phase extraction and finally to some more recent innovations such as supercritical fluid extraction.

The traditional liquid phase extraction (LLE) using organic solvents is a widely employed technique with the aim to efficiently extract solely lipids from the biological materials without changing their native structure. The efficiency of lipid extraction depends on the type of sample (e.g. tissue, cell, biological fluid, etc.), the lipid composition of the sample, and the partitioning of different lipids into the organic phase. A great number of lipid extraction methods have been developed throughout the years. A suitable procedure is usually chosen depending on the lipid composition of the analyzed sample (62). Historically, the most commonly used solvent systems for extracting lipids were described more than fifty years ago by Folch et al. and Bligh and Dyer (63, 64). The extractant is a mixture of chloroform and methanol in different ratios. Methanol added into the aqueous phase reduces the hydrophobic interactions among lipid molecules and weakens the hydrogen bonds and electrostatic interactions between lipids and proteins. Lipids are then

extracted into the organic (chloroform) phase as shown in **Fig 3**. Alternatively, chloroform is increasingly being replaced by other organic solvents, such as methyl-*tert*-butyl ether (MTBE) or 2-propanol for several reasons. Firstly, MTBE is less toxic than chloroform. Secondly, due to its lower density than water, MTBE with dissolved lipids forms the upper of the two phases, minimizing any loss of sample (65). Also, the number of different lipid classes in their extraction yields may differ significantly depending on the chosen extraction method. Therefore, chloroform/methanol protocols may not always be the most suitable choice, especially for more polar lipid classes (66). Finally, considering large-scale lipidomics that requires high sample throughput, the traditional two-phase system may be too time-consuming. For this purpose, simple monophasic extractions (67, 68) as well as ultrasound extractions (69) have recently been developed.

With the development of column techniques, a technique known as solid-phase extraction (SPE) with hydrophobic carrier materials has gained in importance. Lipids can be extracted from large volumes of biological fluids (e.g. serum, urine, etc.) based on their affinity towards a solid phase through which the sample is passed. Lipids bind to the sorbent and are then eluted with small volumes of organic solvents. The SPE cartridges could be packed with various polar (normal phase), non-polar (reversed-phase), ionic or polymeric materials. The variability of the sorbents makes this technique applicable to a wide range of different polarity lipids (70, 71). Besides lipid extraction, SPE can also be used for the fractionation of lipids (72).

The more recent technique, supercritical fluid chromatography (SFE), was introduced for its application for isolation of lipids from biological matrices. It has gained an increased interest mainly due to low toxicity, cost-effective, and non-inflammable supercritical CO_2 that is used as an extraction medium. The supercritical CO_2 is a low polarity extractant

which limits its application. Therefore, it is usually mixed with a small amount of a polar solvent (methanol, ethanol, water) to increase its polarity and hence enable analysis of more polar lipids (73, 74).

Apart from the three main extraction methods discussed above, several other methods based on novel technologies have been proposed. They are not used as often but improve the extraction processes by reducing the time of analysis, extraction temperature, or solvent requirements. These new technologies used for lipid extraction from different biological samples contain microwave-assisted extraction (75), dispersive liquid-liquid micro-extraction (76), and ultrasonic-assisted extraction (77).

In this thesis, a modified extraction method according to Bligh and Dyer was used to obtain the total lipid extract of vernix caseosa.

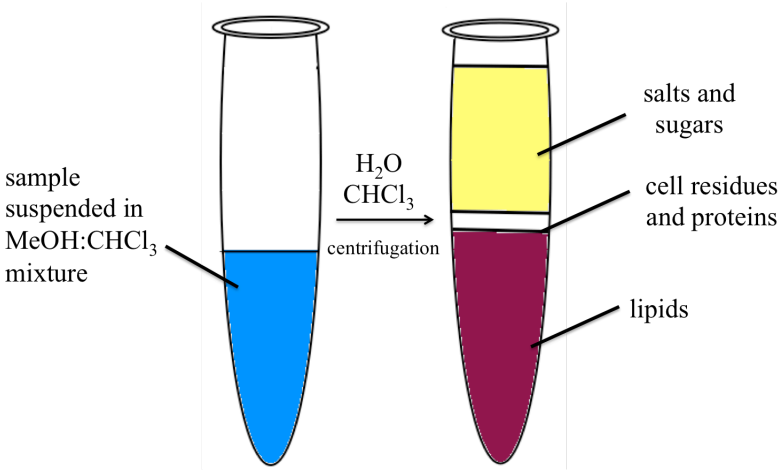

Figure 3. Scheme of LLE of lipids according to Bligh and Dyer (64). Lipids are separated and dissolved in the bottom organic phase while polar components (carbohydrates, salts, etc.) remain in the upper aqueous phase. The middle layer consists of dead cell residues and proteins.

2.2 Lipid separation and analysis

The structural diversity and complexity of lipid species lead to significant variations with respect to their physicochemical properties as polarity and solubility. This results in a great challenge when choosing optimal analytical/biochemical strategies for the identification and quantification of the various lipid species in the lipidome (55). The lipidomic analysis may include the determination of the total lipid content, separation, and structural characterization of individual lipid classes as well as identification and quantitative determination of a specific lipid class or individual lipid molecular species (78). Nowadays, there is a wide spectrum of techniques used for separating and analyzing lipids. The most commonly utilized are chromatography-based methods and mass spectrometry-based approaches. Spectroscopy-based methods and capillary electrophoresis are as well important in lipidomics. Usually, a combination of more than two methods is used since each approach has its strengths and weaknesses. Those used in this thesis will be discussed in detail.

2.2.1 Chromatography-based methods

Chromatographic techniques are sophisticated analytical tools for the analysis of complex biological samples that can be applied in the modern lipidomic analysis. There are many methods based on separation, the most commonly used being thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE).

Thin-layer chromatography

Thin-layer chromatography (TLC) is one of the oldest analytical methods that are still in use in everyday laboratory practice. The history of TLC dates back to the early 1950s (79,

80). In TLC, the components of the sample are separated by their interaction with the stationary phase which is arranged as a thin layer on a rigid support (such as a glass plate) and with the liquid mobile phase that moves along the stationary phase (81). In contrast to liquid chromatography (LC), TLC allows separation without or with minimal sample preparation. TLC is a fast, low-cost, and well-established technique that yields results quickly once the separation system has been carefully optimized (82). The method development in TLC is essential and includes several steps as there are numerous parameters affecting the separation. The most crucial is the selection of the stationary phase sorbent, the optimization of the mobile phase composition, and perhaps the choice of elution technique (horizontal, vertical, with filter paper, etc.). The most commonly used stationary phase for lipophilic compounds is silica gel; other sorbents like alumina, polyamide, or cellulose are also commercially available but used less frequently. The mobile phase is usually a mixture of organic solvents (hexane, diethyl ether, chloroform, ethyl acetate, etc.), sometimes with an addition of acids or bases (48).

Detection of lipids in TLC is usually performed off-line in a separate experiment. The detection of TLC-separated compounds under a UV lamp relies on spraying the plates with a fluorescent indicator (diluted solution of e.g. 2,7-dichlorofluorescein, rhodamine 6G, primuline, etc.) that makes the compounds visible as colorful spots. After visualization, the compounds can be identified by comparing the retardation factor values (R_F) of the analytes with those of the corresponding standards. Thus, if no standard compound is available, identification has to be done with a more specific technique, such as MS detection. The specific fluorescent dyes are non-destructive and the lipids can be scraped off the plates for further quantitation. The quantitative measurements can be done by a variety of spectroscopic methods and mass spectrometry techniques. TLC is applicable when smaller amounts of the sample need to be processed, while column chromatography

is more suitable for larger amounts of sample (48).

Gas chromatography

GC-based methods are essential and fundamental techniques in the field of lipidomics that are used to analyze volatile compounds by partitioning them between a mobile carrier gas and a stationary phase. The analyzed compounds are first vaporized in a heated inlet system and transferred into a GC column. Therefore, the analytes must be thermally stable with a relatively high vapor pressure, which is limiting for many lipids. For compounds without these properties, a derivatization step is required (83, 84). In GC, derivatization is a process by which a compound is chemically modified to increase its volatility. It not only improves the shape and height of the peak but also increases the sensitivity, selectivity, and overall stability of the compound. The most common derivatization reactions for GC used in lipid analysis are silylation, acylation, and alkylation/esterification (83, 85). GC coupled with mass spectrometry is a well-established technique for the analysis of fatty acid methyl esters (FAMEs) after the transesterification of the lipid sample. Fatty acid chains are firstly cleaved by hydrolysis and then esterified to produce FAMEs (85-88). In this way, the fatty acid composition can be described but the information on intact lipids is completely lost. GC exhibits good separation efficiency but extensive sample preparation and time-consuming analysis are required.

Liquid chromatography

Liquid chromatography (LC) is one of the most prominently used techniques in lipidomics due to its high sensitivity and capability for identification, quantification, and structure elucidation of lipid classes and molecular species in complex biological samples. Moreover, it does not require a derivatization step like most GC experiments that makes it

a more appealing method for lipidomics (89).

LC is a versatile technique that operates in various separation modes; the two most widely used are normal phase (NPLC) and reversed-phase (RPLC) chromatography (90). The retention mechanism on NPLC is achieved by surface adsorption of the analyte to the stationary phase by hydrophilic interactions (e.g. hydrogen bonding, dipole-related interactions). NPLC employs a polar stationary phase such as bare silica, amino-bonded or cyano-bonded silica, and a nonpolar non-aqueous mobile phase such as hexane, methanol, etc. NPLC is very challenging to operate because even a trace level of moisture in the mobile phase can significantly impact the chromatographic separation (91).

On the other hand, RPLC involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic interactions of the analytes from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. The most commonly used stationary phase is silica treated with organosilane [$R(CH_3)_2SiCl$], where R represents different bonded substituents (such as C18, C8, phenyl, etc.) to provide specific selectivity. Another type of RP stationary phase is polymer-based, such as polystyrene divinyl benzene. These polymeric materials promise better column stability under a wider range of pH and higher temperatures than silica-based columns. Usual RP mobile phases are any miscible combinations of water with various organic solvents such as acetonitrile and methanol. The addition of mobile phase additives such as ammonium acetate or formic acid can result in a change of the pH and ionic strength of the solution phase and therefore have a significant effect on the retention and also on selectivity and sensitivity of detection (92-94). LC has already proven to be highly efficient in analyzing lipids. NPLC is typically used to separate lipid classes based on the polar functional groups, while RPLC is especially suitable to resolve individual lipid species within the same lipid class based on their lipophilic composition.

Currently, NPLC is being replaced by hydrophilic interaction chromatography (HILIC) (95). This method was firstly introduced in 1990 (96) and since then, it has been gaining interest and utilization for analysis of extremely polar and hydrophilic compounds such as PLs and PLs-related metabolites (97-99). HILIC enables the separation of lipid classes based on the headgroup composition, similar to that employed in NPLC, while using a common RPLC binary mobile phase of water and acetonitrile. Compared to RPLC, in HILIC the polar compounds are more highly retained than the nonpolar compounds and the typical elution order is usually reverse to that found in RPLC with the less polar compounds eluting prior to the more polar ones. Therefore, it provides great selectivity for polar compounds. HILIC is readily compatible with ESI-MS and the high organic contents in the mobile phase when analytes leave the column can provide significantly increased sensitivity and improved peak shapes of the polar compounds compared to RPLC (99).

Apart from the above-mentioned separation systems, silver ion chromatography is another LC-based method that was well adopted in lipid analysis. Here, the principle of separation is based on the fact that π-electrons of unsaturated bonds in the fatty acyl residues of lipids form weak reversible polar complexes with silver ions. Thus, the total number, configuration, and position of double bonds determine the retention time of the molecules (100). In general, the greater the number of double bonds the stronger the complexation effect resulting in longer retention times. In lipid analysis, silver ion chromatography has found great use in the separation of fatty acids and triacylglycerols according to the number of double bonds (101). An important field of application of silver ion chromatography is the separation of configurational (*cis*/*trans*) or positional isomers of fatty acids (102). Moreover, it could be used in the determination of conjugated linoleic acid isomers in commercial dairy products. Nevertheless, silver ion chromatography also brings some drawbacks such as the possible formation of corrosive silver nitrate

precipitates which may lead to damage to the detection system (103).

Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) is a promising separation technique for fast and comprehensive lipidomics and represents a step forward towards green analytical chemistry (GAC) (104). SFC enables the simultaneous profiling of diverse lipids with a wide range of polarities due to the physicochemical properties of its mobile phase. The most widely used mobile phase in SFC is supercritical carbon dioxide (SC-CO_2). SC-CO_2 is chemically inert and has low critical pressure and temperature. Moreover, it possesses lower viscosity and higher molecular diffusivity than a liquid, thereby facilitating higher separation efficiency and shorter analysis time compared to standard HPLC. SC-CO_2 as a mobile phase has almost the same polarity as hexane which greatly limits its use in various applications involving more polar compounds. To adjust the polarity of SC-CO_2, a small amount of a polar organic modifier such as methanol is usually added to allow the elution of different polarity lipids and more particularly complex lipids (105, 106).

The lipidomic analysis deals with an enormous sample complexity and there exist numerous challenges that simply cannot be addressed with a single one-dimensional technique even with the possibility of high-resolution chromatographic techniques (107). Coupling two or more methods together may be required since each approach has its strengths and weaknesses. These hyphenated technologies are for example two-dimensional TLC (108), LC (109), GC (110), and online LC-GC (111). Such combinations of two different separation mechanisms provide improvement of the separation power and thus allow comprehensive lipid analysis. A common approach in lipidomics is a two-dimensional HPLC which allows separation of complex lipidomic mixtures. In such a system, HILIC is used in the first dimension to separate lipid classes

based on electrostatic forces related to the polarity of the compounds. RP-HPLC is used in the second dimension to obtain the structural information of individual molecular species based on the hydrophobic character of the molecules (112-114).

2.2.2 Spectroscopy-based methods

Two major spectroscopic techniques utilized in lipidomics are nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectroscopy (FT-IT).

Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is a broadly utilized non-destructive technique in lipidomics that uses static and oscillating magnetic fields to identify the resonance of nuclei in molecules. It has been widely exploited in studying lipid-protein interactions, identifying the structure of lipid membranes as well as lipid profiling over disease development (115, 116). Despite its high robustness, high analytical reproducibility and ability to provide direct quantitative information, it still possesses notable disadvantages in analyzing lipids. Compared to MS, it tends to have lower sensitivity and can detect only highly abundant analytes. Thus, trace levels of lipids in a biological system are difficult or impossible to measure. Moreover, when analyzing complex mixtures, the signals of lipids generated in NMR are superimposed making the interpretation rather complicated (117). So far, NMR has been most useful in detecting phospholipids. Molar quantities of phosphorus can easily be determined by ^{31}P NMR spectroscopy, which has been used for describing the structural organization of biological membrane systems where phospholipids are found in high abundance (118, 119) Nevertheless, the limitations and low resolving power of this technique make it less desired analytical tool in lipid analysis. In many cases, NMR is used as a secondary method for confirming the structure (120).

Furrier transform infrared spectroscopy

Furrier transform infrared spectroscopy (FT-IR) is a non-destructive and non-invasive spectroscopic method that offers advantages of short analysis time and simple sample preparation step. FT-IR is a robust technique suitable for routine analyses. Chemical information is obtained from the infrared spectrum of absorption or emission over a broad range of wavelengths at a time (121). It is not widely adopted in lipidomics, however, several specific applications have been reported such as analysis of PUFAs composition in various marine oils (122), semiquantitative determination of TGs in serum (123) or determination of the total phospholipid content of vegetable oils (124).

Raman spectroscopy

In addition to FT-IR and NMR spectroscopy, Raman spectroscopy enhances the list of available spectroscopic methods used in lipidomics. The main positives of this technique are that no sample preparation is required and that it can be applied to samples in any physical state including gases, liquids, gels, or crystals. However, its use is hindered by the high cost and complexity of the instrumentation required (125).

2.2.3 Mass spectrometry-based lipidomics

The development of lipidomics has been largely driven by rapid advances in technologies, mainly in mass spectrometry. MS has been the most important and widely used technique in lipid analysis due to its high level of sensitivity, molecular specificity, speed, and most importantly, its capability to generate structural information of the analyzed lipid molecules. MS-based techniques can vary in their analytical spectrum (targeted vs nontargeted lipidomics). They can also be different with respect to the necessary extraction step and/or chromatography prior to the mass spectrometric analysis (126). In principle,

lipids can be analyzed directly from complex biological matrices by MS imaging (127). Alternatively, lipids can be extracted from the biological materials with organic solvents and the produced lipid extract can be analyzed either by direct infusion shotgun lipidomics or by LC/MS-based lipidomics (109, 128).

Biological lipid extracts are typically very complex, consisting of diverse lipid classes, subclasses, and molecular species. Moreover, the vast dynamic range in the contents of individual species adds to the overall complexity. Therefore, for a reliable and accurate characterization of individual lipid species, it is essential to reduce the complexity of the lipid samples. Having a pure lipid extract without interferencies is particularly important for shotgun lipidomics because of the absence of pre-chromatographic separation prior to MS analysis. In shotgun lipidomics, the complexity may be reduced for example during sample preparation or by employing intrasource separation/selective ionization during the MS step or by both. In contrast, LC-based lipidomics achieves partial simplification of the lipid samples by utilizing separation science (129, 130).

Direct infusion MS or shotgun lipidomics involves injection (without prior fractionation) of the crude lipid extracts directly into the MS instrument. The identification and quantification of lipid species by shotgun analysis are based on the accurately determined masses, tandem mass spectrometry analysis, and/or specific multiple-dimensional mass spectrometry analysis (131, 132). A major advantage of this technique over LC-based MS analysis is that a mass spectrum of the individual molecular species can be acquired at a constant concentration of the solution during direct infusion. This feature allows to carry out specific precursor ion scans of the particular fragment ions or neutral loss scans of the neutrally lost fragments of interest on the lipid class/subclass or individual molecular species. These groups of scans determine the identity of the lipid species from the combination of the fragments, which act as building blocks. The most common building

blocks include glycerol, sphingoid bases, polar head groups, and fatty acyl substituents (133-135). The most widely used ion sources in shotgun MS analysis are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (135). In ESI-shotgun MS the sample as a gas or in solution is introduced into the instrument by direct infusion into the ionization source whereas in MALDI-shotgun MS the sample is placed onto a probe or plate and then directly inserted into the ionization region of the mass spectrometer.

MS is often used in combination with LC or GC (LC/MS or GC/MS). The most widely used method in chromatography-based lipidomics is the hyphenated technique of HPLC and electrospray-MS (59). Three major factors are generally considered essential for the successful development of an HPLC/MS lipidomics analysis. The first factor is the selection of a suitable column and optimization of the separation conditions (e.g. mobile phase and its gradient) to achieve the best separation of lipid classes and/or lipid molecular species. The second factor is the choice of appropriate coupling of the LC elution conditions with a mass spectrometer (e.g. ion strength of the eluting mobile phase) to avoid undesirable effects of ion suppression on ionization efficiency. And the third factor is the MS or MS/MS parameters settings to identify and quantify as many eluting lipids species as possible (48, 136). Shotgun lipidomics offers a straightforward and rapid analysis and is undoubtedly faster than LC/MS-based strategies (137). However, this approach is limited by its inability to resolve isobaric compounds. Also, a possible risk of ion suppression is limiting as it may cause decreased sensitivity of very low abundance lipids (59). Running an LC separation prior to MS decreases the number of competing analytes entering MS ion source and thus reduces the risk of ion suppression. Moreover, the possibility to set up suitable gradient conditions allows for better separation and identification of isobaric and isomeric lipid species (59).

Imaging of lipids by mass spectrometry (MSI) has recently gained great interest in lipidomics research. MSI methods generate ions directly from the surface of biological materials thus avoiding the extraction and separation steps (138, 139). It can be accomplished by several different ionization techniques compatible with MSI, namely desorption electrospray ionization (DESI), secondary ion mass spectrometry (SIMS), matrix-assisted laser desorption ionization (MALDI) and most recent easy ambient sonic spray ionization (EASI). Each ionization method has its own advantages and disadvantages, ranging from the character of the ionized molecules to the special resolution available (140). MALDI is the dominant technique for MSI, especially due to high speed, stability, and most importantly its ability to image a wide range of molecular weights and molecular species. MALDI is a soft ionization technique, in which ions are generated from a solid or solvent matrix by irradiation by a laser beam (141). It has been extensively applied to studying brain tissue (142-144). Compared to MALDI, the other techniques DESI, SIMS, and EASI benefit from minimal sample preparation and absence of interfering matrix peaks in the low-mass region since they do not require the presence of matrix (145). DESI can generate lipid ions directly from a tissue section (with typical section thickness 5 to 100 μm) under ambient conditions using electrically charged solvent (146). In SIMS, ions are produced by bombarding the sample surface (nanometers to sub-micrometers in diameter) with a focused primary ion beam (147). EASI is a simple and gentle ionization technique where ions are formed from a solvent stream that is mechanically distributed using a flow of N_2 at supersonic speeds (148).

MS is an analytical technique that identifies molecules based on their mass to charge ratio. It is used for both quantitative and qualitative analyses to identify unknown compounds within the sample, elucidate their structure, and determine their amount within the sample. In order to acquire readouts from a mass spectrometer, three major steps must occur during

the basic working process of the instrument. The first step in the mass spectrometric analysis involves the production of ions in the ionization source. In the second step, the generated ions are sorted and separated in the mass analyzer according to their mass to charge ratio. In the final event, the separated ions are detected (149).

Depending on the application, the ionization technique is chosen with respect to the physicochemical properties of the analyte as well as the desired degree of excitation of the analyte molecule. The ion sources are generally categorized into soft and hard ion sources. Common hard ionization techniques such as electron ionization (EI) are highly energetic and can cause extensive fragmentation of the analyte molecules in the ion source. A lower energy alternative to EI is chemical ionization (CI). It is considered a soft ionization technique in which ions are produced with minimal fragmentation of the analyte molecules. CI uses ion-molecule reactions to produce ions from the analyte. Both these ionization techniques are only suitable for analytes that are volatile and thermally stable because they require the introduction of the analytes into the gas phase. Non-volatile compounds, such as polar lipids, must be transferred directly from the condensed to the gas phase as occurs in electrospray ionization (ESI). Other soft ionization techniques commonly used in lipidomics encompass atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and matrix-assisted laser desorption/ionization (MALDI). Each technique that was mentioned is unique and, therefore, it is necessary to consider all advantages and disadvantages it may have, to decide which one is the most suitable for the specific lipid analysis (150, 151). This dissertation involves the use of EI, ESI, and APCI sources. Hence, these will be discussed in the following sections.

Ionization techniques

Electron ionization

In the EI ionization source, the electrons are produced from a resistively heated tungsten or rhenium filament and are energized by accelerating them through a potential of 70 V. The electron beam then interacts with a stream of neutral analyte molecules in the gas phase. Upon the interaction, the molecule loses an electron forming a radical cation $M^{+\cdot}$, called molecular ion (152).

$$M + e^- \rightarrow M^{+\cdot} + 2e^-$$

A scheme of the EI ionization source is shown in **Fig 4**. The interactions with high-energy electrons can leave analyte ions with excess energy. If this energy is sufficient, the molecular ions undergo fragmentation by bond cleavages and rearrangement processes to give lower mass ions and neutral species (152, 153). The produced fragments are structurally related to the analyte and can be used to confirm the structure of the target analyte. A great advantage of using standardized ionization energy of 70 eV is that the spectra are reproducible across different instruments enabling the creation of comprehensive spectral libraries for thousands of analytes. EI-MS was for the first time applied to fatty acid methyl esters (FAMEs) in 1960 and since then is still widely used (154).

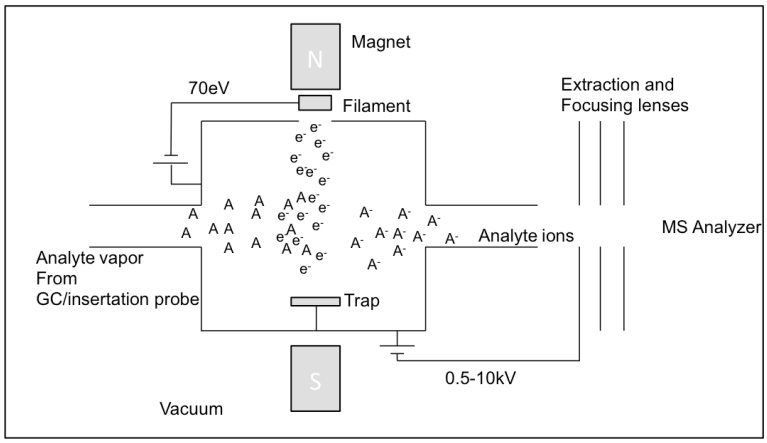

Figure 4. Scheme of EI source. Downloaded and modified from (https://www.chem.pitt.edu/facilities/mass-spectrometry/mass-spectrometry-introduction).

Electrospray ionization

The development of electrospray ionization has revolutionized the MS analysis of biomolecules, including lipids (109). ESI was for the first time applied in lipid analysis in 1991 and since then it has become the most frequently employed ionization technique in lipidomics (155).

ESI is performed by pumping an analyte that is dissolved in a polar solvent through a capillary (˜100 μm diameter) at μL/min range flow rates. A potential of several kV is applied to the capillary that creates an electric field between the capillary and the orifice of the mass spectrometer. Two competing forces, surface tension derived force and electrostatic Coulomb attraction towards the counter electrode, form the Taylor cone. When the applied field is sufficiently high, a fine jet of liquid emerges from the cone tip. The jet then breaks up into individual small charged droplets. As the charged droplets move towards the mass spectrometer, the solvent starts to evaporate. The solvent evaporation causes the surface of the droplets to grow smaller while the charge remains the same. Once the Rayleigh limit (a point when the Coulombic repulsion and the surface tension are balanced) is reached, a Rayleigh fission occurs and the large highly charged

droplets burst into smaller droplets with smaller number of charges. This process is repeated producing a cascade of progeny droplets until all of the solvent is evaporated (156). There currently exist two mechanisms that explain the final formation of gas-phase ions from these charged droplets; the ion evaporation model (IEM) and the charge residue model (CRM). According to the IEM, solvated ions are ejected from the charged droplet as dry ions at a sufficiently high electric field. This assumption has been verified to be applicable in explaining the generation of relatively small ions from ESI. The CRM claims that a series of solvent evaporation and Coulomb explosions split large droplets into many fine droplets containing only one solute molecule in each droplet, and finally the formation of dry ions with the evaporation of the rest of the solvent. This mechanism is more suitable for multiple-charged ions produced from large molecules with molecular weight over 3000 Da (157, 158) (see **Fig 5**).

ESI exhibits a distinct advantage to ionize polar molecules. Under typical ESI conditions, the full MS spectra produce singly-charged molecular-related ions, usually protonated molecular ions with the formula $[M + H]^+$ in the positive ionization mode and deprotonated molecular ions with the formula $[M - H]^-$ in the negative ionization mode, molecular adduct ions, and only a few fragments with small abundances. (150).

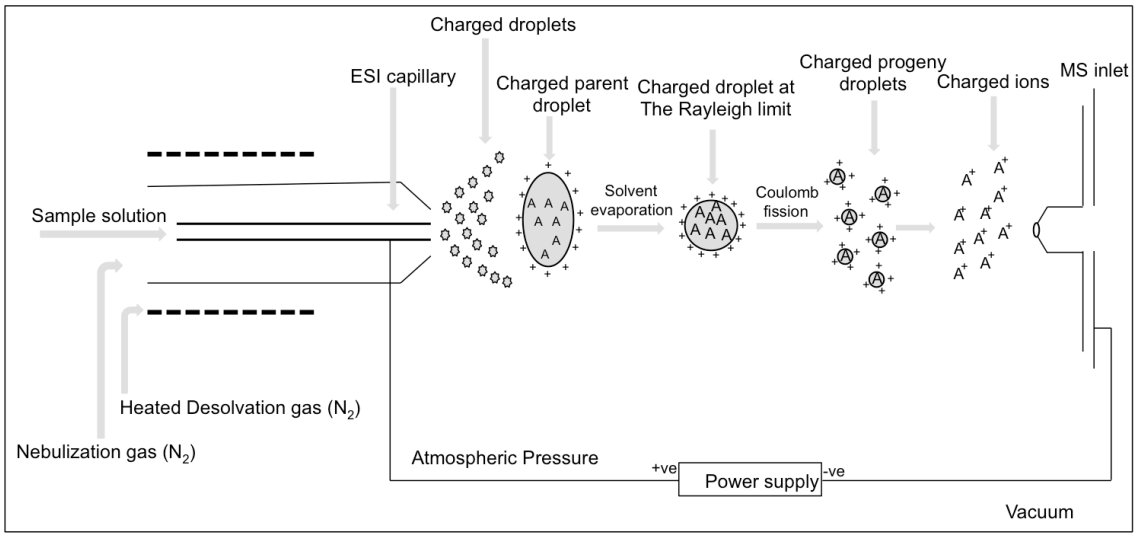

Figure 5. Scheme of ESI ionization process. Downloaded and modified from (https://www.chem.pitt.edu/facilities/mass-spectrometry/mass-spectrometry-introduction).

Atmospheric pressure chemical ionization

In atmospheric pressure chemical ionization (APCI), the analyte in solution or an LC elute is directly introduced to a pneumatic nebulizer where it is converted into a vapor by a nitrogen stream. Created droplets are then moved by the gas flow through a heated tube called a desolvation chamber and solvent and solute are volatilized. Then the analyte is carried through a corona discharge electrode for ionization through molecular-ion reactions in the gas phase (159). It is mainly used to ionize polar and relative non-polar compounds with medium molecular weight up to 1500 Da and typically generates mono-charged ions (**Fig 6**).

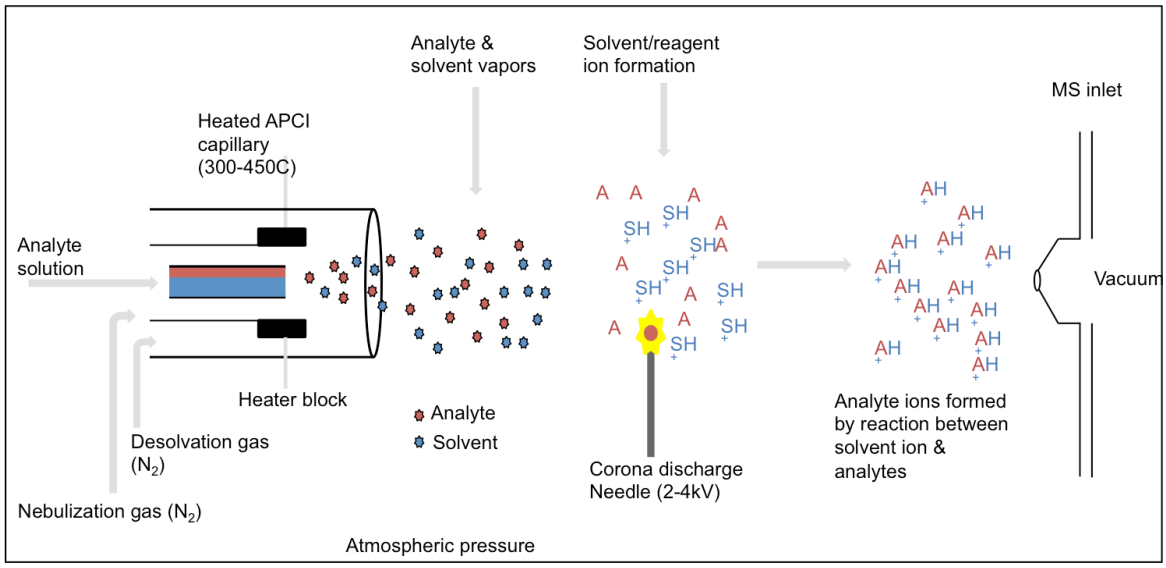

Figure 6. Scheme of APCI ionization process. Downloaded and modified from (https://www.chem.pitt.edu/facilities/mass-spectrometry/mass-spectrometry-introduction).

Mass analyzers

Once ions have been formed and introduced into a mass spectrometer, a mass analyzer is used to separate the ions based upon their mass to charge ratios by applying electric or magnetic fields. The analyzers based on the magnetic sector field played an important role in the past of MS but in nowadays lipidomics their application is very limited mainly due to its large size, the cost versus effectiveness, lower scan speed that may limit the LC/MS applications, and relatively complicated operation (160). Fourier transform ion cyclotron resonance (FTICR) analyzers also use magnetic fields but they provide high mass accuracy and resolution. The Orbitrap-based instruments have gained importance due to the instrumentation variability at a reasonable cost and become a common sight in analytical laboratories (161). Other commonly used analyzers in lipidomics include time-of-flight (TOF), quadrupole (Q), and ion trap (IT). Mass analyzers differ in their resolving power, mass accuracy, sensitivity, mass range, linear dynamic range, and scan speed (162). Many modern instruments feature several mass analyzers coupled together, such as triple

quadrupole (QqQ), for use in tandem mass spectrometry. Combinations of two and more analyzers of a different type, such as QqTOF, IT-TOF, IT-Orbitrap, are referred to as hybrid instruments and are also well established (163). Recently, ion mobility spectrometry (IMS) has attracted great attention in lipid analysis. IMS is a gas-phase electrophoretic technique that enables the separation of gaseous ions according to their size, shape, and charge in the presence of a weak electric field. IMS is compatible with modern mass spectrometers and thus can be integrated into conventional lipidomics MS workflows. Such combination results in improving the sensitivity, peak capacity, and compound identification (164).

Quadrupole mass analyzer

A quadrupole mass analyzer consists of four parallel (hyperbolic or cylindrical) rod electrodes that have direct current (DC) and radio frequency alternating current (AC) potentials applied to them. Ions produced in the ion source are then focused and passed into the middle space between the quadrupole rods. The AC and DC voltages are changed simultaneously such that only ions of a certain *m/z* follow a stable path through the quadrupole field while ions of all other *m/z* collide with one of the rods and are discharged. A quadrupole, therefore, operates as a mass filter allowing only ions with certain specific *m/z* value to reach the detector (156). Quadrupole mass spectrometers are nowadays standard equipment in MS laboratories as they are affordable and relatively uncomplicated to use (**Fig 7**).

Triple quadrupole instrument (QqQ) is a tandem mass spectrometer that is capable of performing MS/MS scans. It consists of two quadrupoles that function as mass filters (Q1 and Q3) and third quadrupole that acts as a collision cell “q” in a position between them. The analyte is fragmented through the interaction with the collision gas (usually nitrogen,

argon). The first quadrupole Q1 filters a specific set of precursor ions while in quadrupole Q2 these ions are fragmented by collision-induced dissociation to produce product ions. These product ions are then passed to the last quadrupole Q3 where only a certain set of ions may be allowed to pass into the detector. Such combination greatly improves the selectivity and sensitivity of the instrument enabling to perform qualitative and quantitative experiments (150, 164).

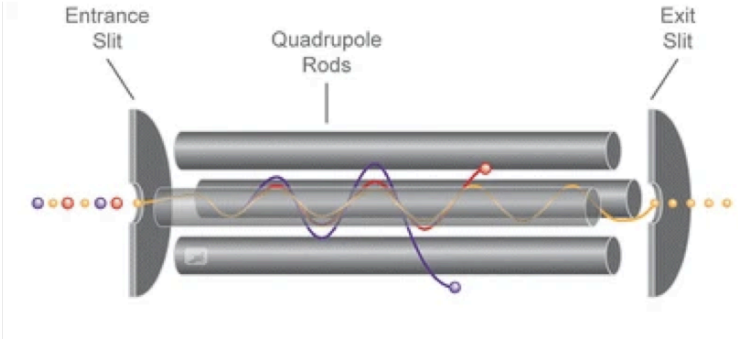

Figure 7. Quadrupole mass analyzer. Downloaded from (https://doi.org/10.1007/s00418-010-0753-3).

Ion trap mass analyzer

Three-dimensional ion trap (3D-IT) mass spectrometer consists of a central ring electrode and two end-cap electrodes. There is an RF potential applied to the ring electrode while the end-cap electrodes are maintained at ground potential. Ions of a broad *m/z* range are trapped due to the oscillating 3D electric field inside the ion trap. Increasing the amplitude of the RF potential destabilizes the trajectories of the ions, which is accompanied by ejecting them out of the trap for detection. 3D-IT spectrometers are simple to operate, inexpensive, sensitive, and capable of conducting multistage MS experiments for structural studies. A linear ion trap (2D-IT) is made of four parallel rods coupled with electrodes on each end and uses RF and DC potentials for mass separation and analysis. Such

configuration gives a 2D-IT a dual functionality - it can work as a selective quadrupole mass filter or an ion trap. Compared to 3D-IT, the linear ion trap has a higher trapping efficiency, and scan speed (150, 151, 156) (**Fig 8**).

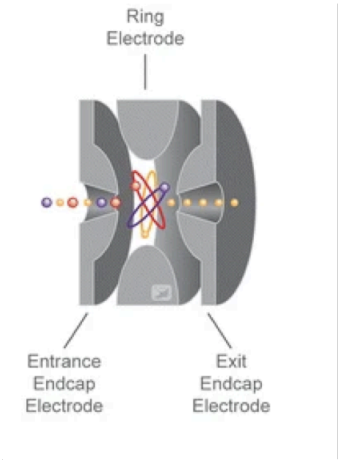

Figure 8. Ion trap mass analyzer. Downloaded from (https://doi.org/10.1007/s00418-010-0753-3).

Orbitrap

A new type of mass analyzer, known as the Orbitrap, works on the principle of orbital trapping of ions around an axial central electrode using an electrostatic field. The field is created from an axial spindle-like central electrode and an outer coaxial barrel-like electrode split into two halves. The trapped ions rotate around the central electrode and oscillate harmonically along its length. The *m/z* values of the trapped ions are related to the frequencies of their harmonic oscillations. The image current induced by the oscillating ions is recorded between the two axial halves of the outer electrode and converted by an FT to the individual frequencies and intensities, yielding the mass spectrum (**Fig 9**) (161).

The orbitrap has several desirable features, including high resolving power (modern instruments up to 1 000 000 FWMH at *m/z* 200), high mass accuracy, large space-charge capacity, high dynamic range (>10) compared to other trap instruments, good sensitivity

and *m/z* range up to 6000. A linear ion trap is combined with an Orbitrap to provide a hybrid tandem mass spectrometry system with a higher application range (150, 156).

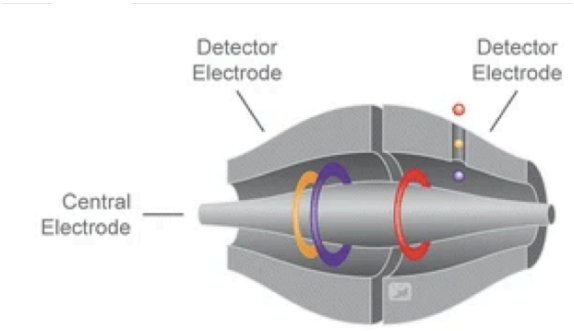

Figure 9. Orbitrap mass analyzer. Downloaded from (https://doi.org/10.1007/s00418-010-0753-3).

Mass detectors

The detector is the final component of a mass spectrometer. Typically, some type of electron multiplier is commonly used but other types of detectors including Faraday cups, or photomultipliers are also used. All these mentioned are destructive detectors where ions are discharged in order to produce a signal. They also cannot distinguish between ions of different masses and therefore require prior separation of ions according to their *m/z* values. The exception is FTICR and Orbitrap mass spectrometers where ions are not discharged during detection and the mass analyzer simultaneously function as a detector (151)

3 Vernix caseosa

At birth, babies experience significant transitional changes coming out from a warm, wet, sterile in utero environment to a cold, dry, bacteria-laden nursery. Skin and other major organs such as lungs, gut, and kidney undergo a systematic series of functional and structural development during late gestation. This prenatal maturation is necessary for a successful transition to postnatal life. Significant attention has been given to the development of human skin as the epithelial system provides a protective barrier from pathogens and damage between the internal and external environments of the organism. The epidermal barrier formation during late gestation is closely associated with the production of vernix caseosa on the fetal skin surface.

Vernix caseosa is a white, cheese-like, naturally occurring biofilm that coats the skin of the fetus during the last trimester of gestation and usually remains present on the skin during delivery (165). It is present in the amount that correlates with gestational age, gender, race, delivery mode, and the presence of meconium (166). Vernix caseosa is essential for skin development in utero as well as post-birth adaptation providing multiple functions (167-169). It has long been thought to be uniquely human; however, recently it was discovered coating the back of California sea lion fetus (170).

Recent research has focused on the biochemical and physiological roles of vernix caseosa. The results have shown that the application of vernix caseosa based formulations offers the potential for accelerating skin barrier recovery (171). These findings make vernix caseosa an attractive candidate to treat skin disorders with impaired skin barrier such as atopic dermatitis or to treat patients with altered skin integrity due to burn injuries (167).

Figure 10. A newborn covered with vernix caseosa. Downloaded from (https://cz.pinterest.com/pin/271834527479439026/)

3.1 Neonatal barrier development and vernix caseosa formation

The skin structure of the term newborn is histologically similar to that of adult individuals whereas premature infant skin was observed to have several unique features that greatly contributed to the understanding of fetal skin development. The outermost compartment of the skin, the epidermis, develops from embryonic ectoderm at about three weeks of gestation and consists of a single layer of undifferentiated cuboidal cells. At about four weeks it becomes two-layered. The outer protective cover of cells, the periderm, is found only in developing skin and is transiently present. Eventually, it undergoes a series of apoptotic cellular events as the epidermis becomes multilayered and the formation of stratum corneum, the outermost layer of flattened, non-nucleated skin cells, occurs. Periderm cells are replaced continuously and about twenty-four weeks it is completely shed and replaced by stratum corneum while the epidermis shows considerable progressive maturation (172, 173). The shed periderm cells are combined with sebum secretions from the sebaceous glands within the epithelial wall resulting in the formation of vernix caseosa (174, 175). The remaining period of gestation is focused on the structural and functional

maturation of both the epidermis and the dermis. Particularly, the dermis undergoes significant reorganization and growth. The layer of the stratum corneum thickens and epidermal glands are formed. Desquamated fetal corneocytes are mixed with the lipid matrix to form additional vernix material. The epidermal development is complete in utero at approximately thirty-four week gestational age (172, 173).

The production of vernix caseosa is thought to be associated with an adrenal-dependent mechanism leading to increased activity of sebaceous glands in the last trimmest of pregnancy. Recently, a specific endocrine-based mechanism for its formation in utero has been proposed (176).

Vernix caseosa is commonly observed in the creases and flexor surfaces of the newborn (mean surface coverage 38 +- 1.2 %) (177). Very low birth weight infants do not have stratum corneum fully developed and also lack of vernix (178). Usually, premature neonates are born with a generous amount of vernix whereas at term newborns it usually remains present only in the skin creases and hair as a result of an interaction with pulmonary surfactant in the amniotic fluid (179).

3.2 Structure and composition of vernix caseosa

Vernix caseosa is composed of about 80% water, 10% proteins, and the remaining 10% account for a complex mixture of lipids. It is a highly cellular material that consists of a non-lamellar lipid matrix containing hydrophilic desquamated corneocytes with no cell-to-cell desmosomal connections (176). In contrast, stratum corneum is comprised of mature corneocytes linked together *via* corneodesmosomes that are embedded in a finely organized lamellar matrix (180). Thus, the vernix morphology is often described as mobile "pasta and cheese" (181) whereas the stratum corneum structure resembles that of "bricks and mortar" (182).

3.2.1 Lipids

The first investigations of lipid composition date back to 1939 and since then there have been numerous studies on its composition (183). However, despite the great effort of researchers, the entire lipidome of vernix caseosa has not been described comprehensively yet. The main methods for studying the lipids of vernix caseosa are chromatography and mass spectrometry. They have shown that it consists of an extremely complex mixture of various lipids. Vernix caseosa lipids exist in intercellular space as free (extractable) components or they are bound to the cornified envelope. About 90% of the free lipids are nonpolar species where sterol esters, wax esters, diacylated diols, and triacylglycerols predominate. They are considered as products of sebaceous gland secretions. More polar components, such as free cholesterol, free fatty acids, and ceramides are the barrier lipids accounting for the remaining 10% of the total free lipids. The barrier lipids are generally products of stratum corneum development. The lipids covalently linked to the cornified envelope consist of fatty acids, ω-fatty acids, and ω-hydroxyceramides (184).

Numerous studies have investigated the individual lipid classes after being hydrolyzed. Hydrolysis enables monitoring of the fatty acid composition but unfortunately, the valuable information on the molecular structure of the intact compounds was lost. Vernix caseosa lipids are characterized by the presence of unusual fatty acyls. The aliphatic carbon chains can be extremely long, methyl-branched, with an odd number of carbon atoms and double bonds in unusual positions (185). In addition, α- and ω-hydroxylated fatty acyls are often detected (184).

3.2.2 Proteins

Proteins of vernix caseosa seem to have multiple origins. Some of them originate from the amniotic fluid that surrounds the fetus in utero. The amniotic fluid is also in close contact

with the fetal lungs and so a portion of the lung proteins may be as well found in vernix caseosa. Vernix caseosa of newborns may contain blood proteins such as hemoglobin through contamination during vaginal delivery. Finally, a great amount of vernix caseosa proteins are of dermal origin (181). A significant portion (approximately 39%) of the identified vernix caseosa proteins are components of innate immune protection already during fetal and neonatal life, others (approximately 29%) have direct antimicrobial properties (186).

Vernix caseosa protein fractions mainly consist of keratin, an epidermal-derived structural protein that forms the core components of the corneocytes. Vernix caseosa has been proved to have an antimicrobial activity indicating the presence of active antimicrobial proteins and peptides. The main families of mammalian antimicrobial peptides, the cathelicidins, and the defensins, were detected together with the well-known defense proteins lysozyme, lactoferrin, secretory leukocyte protease inhibitor and psoriasin (186-189). Furthermore, the collectin associated surfactant proteins A and D were demonstrated to be present (179). Hydrolysis of the protein fractions showed that amino acids glutamine and asparagine are present in great abundance (190). Glutamine is of significant importance when swallowed by the fetus because it is suggested to have an impact on fetal gut development (191).

3.3 Role and functions of vernix caseosa

During the third trimester of pregnancy, the maturing fetal lung produces and secrets increasing amounts of pulmonary surfactant that causes detachment of superficial vernix caseosa leading to amniotic fluid turbidity. The development of such turbidity is well known as a marker of fetal lung maturity (192). Vernix caseosa with amniotic fluid, swallowed by the fetus, has a potential effect on developing gut due to its high contents of glutamine and asparagine. Glutamine has already been described as a trophic factor for the

developing gut and is generally required by rapidly proliferating cells such as intestinal epithelium and lymphocytes (191, 193).

Vernix caseosa also acts as a skin barrier in utero allowing epidermal growth underneath it (181). Owing to its hydrophobic character, it protects the fetus from amniotic fluid maceration and loss of fluids and electrolytes or transepidermal water loss (TEWL) (167). In contrast, the high water content of vernix caseosa may have an effect of maintaining stratum corneum moisturization with controlled slow drying post-partum. Retaining vernix caseosa on the skin of a newborn after birth results in significantly more hydrated skin surface suggesting that it may facilitate skin hydration postnatally (177).

During delivery, it performs a protective biofilm function by minimizing friction as the fetus and the mother's genital tract are during the process of births exposed to the mechanical stress of the uterine contractions (167).

Previously published reports have described vernix caseosa as a possible antimicrobial protective barrier due to its hydrophobic and mechanical nature (194). More recently, several studies showed that vernix caseosa contains antimicrobial proteins/peptides and has a direct function in defense against bacteria. In addition to antimicrobial activity, many of these proteins enhanced the protective functions of vernix caseosa by exhibiting a clear antifungal activity, protease inhibition, or parasite inactivation. These findings confirmed the hypothesis that vernix caseosa is an important antimicrobial barrier that protects the fetus from chorioamnionitis in utero and prevents from neonatal infections during the critical time of bacterial colonization after birth (179, 186-189). Moreover, free fatty acids in vernix caseosa lipids were found to exhibit antibacterial activity. The lipids isolated from vernix caseosa also enhanced the activity of an antibacterial peptide LL-37 suggesting synergistic interactions between lipids and peptides present in vernix caseosa (189).

Finally, vernix caseosa is known to contain antioxidants α-tocopherol (Vitamin E) and melanin, which indicates that vernix caseosa may be important for coping with high oxidative stress of the skin at birth (195). Other benefits of vernix caseosa such as skin cleansing (196) or wound healing properties (197) were also reported.

All these multiple overlapping maturational functions suggest that vernix caseosa has an essential role in the development of skin in utero and adaptation post birth. The unique innate properties of vernix caseosa inspired many researchers to come up with analogous synthetic biofilms that closely mimic its structure and properties (198, 199). This may be of great advantage for the potential treatment of different skin disorders. The synthetic biofilms may prove useful for improving skin barrier in preterm infants where vernix caseosa is absent. Moreover, beneficial effects can be expected for applications in order to enhance wound healing in treating skin burn injuries. Patients with skin disorders, such as atopic dermatitis, where imbalances either in lipid composition or moisturization level occur may also be great candidates for the newly developed biofilms.

RESEARCH AIMS

The overall aim of this work was to contribute to the general knowledge of the lipid composition of vernix caseosa. New lipid classes were characterized in detail by high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry.

Specifically, the following tasks were involved in this research:

- optimization of a multistage sample pretreatment (including lipid extraction and TLC isolation) to obtain an enriched fraction of desired analytes from the biological sample of vernix caseosa
- preparation of in-house lipid standards of the studied lipid classes
- combination of various methods to confirm the intact structure of the newly discovered lipids of vernix caseosa
- development and optimization of HPLC separation methods for lipid classes and subclasses
- development and optimization of mass spectrometric methods for the analysis of intact lipid molecular species.

EXPERIMENTAL

1 Materials and instrumentation

Biological material

Vernix caseosa was collected from full-term healthy infants born at the General University Hospital in Prague as approved by the institutional review board. Immediately after the delivery, it was transferred into a sterile amber glass vials and stored at −80 °C until extraction. All vernix samples were recorded for information on gestation age, sex, delivery method, and part of the body that it was removed from. Samples contaminated with blood were discarded.

Lipids were extracted from vernix caseosa according to a slightly modified procedure published by Bligh and Dyer. When extracting vernix caseosa, a triphasic system is produced. The extra middle layer consisted mainly of corneocytes and dead cells from vernix caseosa and therefore needed to be reextracted. A detailed extraction protocol can be found in the publication I and II.

Chemicals and synthesis of in-house standards

A detailed list of chemicals used in this work is provided in the original publications (I and II). Detailed synthetic procedures of in-house standards are described below.

16-(Oleoyloxy)hexadecanoic acid

(Z)-Octadec-9-enoyl chloride (602 mg; 2 mmol) was added dropwise to a stirred solution of 16-hydroxyhexadecanoic acid (272 mg; 1 mmol) in 2 ml of anhydrous pyridine at 0 °C. After stirring for 96 hours at ambient temperature, the mixture was diluted with ether

(20 ml), washed with 10% HCl (2×20 ml), a saturated solution of $NaHCO_3$ (2×20 ml), brine (20 ml) and dried over sodium sulfate. The solvent was evaporated in vacuo and the oily residue was pre-purified by flash chromatography (15 g of Merck Kiesegel 60; mobile phase 5% of ethyl acetate in hexanes) to give 456 mg (85% yield) of 16-(oleoyloxy)hexadecanoic acid.

16-Cholesteryloxy-16-oxohexadecyl oleate

N,*N*'-Dicyclohexylcarbodiimide (DCC; 206 mg, 1 mmol) in 2 ml of anhydrous dichloromethane was added dropwise to a stirred and cooled (0 °C) solution of 16-(oleoyloxy)hexadecanoic acid (268 mg, 0.5 mmol), 4-dimethylaminopyridine (30 mg) and cholesterol (195 mg, 0.5 mmol) in 5 ml of anhydrous dichloromethane. After stirring for 20 hours at ambient temperature, the precipitated dicyclohexylurea was filtered off, the filtrate was diluted with dichloromethane (15 ml), washed with 3% HCl (2×10 ml), saturated solution of $NaHCO_3$ (2×10 ml), brine (10 ml) and dried over sodium sulfate. The solvent was evaporated in vacuo and the residual dark oil was purified by flash chromatography (10 g of Merck Kiesegel 60; mobile phase 3% of ethyl acetate in hexane) to give 210 mg (46% yield) of desired 16-cholesteryloxy-16-oxohexadecyl oleate.

16-(9Z-octadecenoyloxy)-hexadecanoic acid and 2-(9Z-octadecenoyloxy)-hexadecanoic acid

(*Z*)-octadec-9-enoyl chloride (2×602 mg) was added dropwise to a stirred solution of 16-hydroxyhexadecanoic acid (272 mg) or 2-hydroxyhexadecanoic acid (272 mg) in 2 ml of anhydrous pyridine at 0 °C. The reaction mixture was stirred for 96 hours at ambient temperature. Then, the mixture was diluted with ether (20 ml), washed with 10% HCl (2×20 ml), washed with a saturated solution of $KHCO_3$ (2×20 ml), washed with brine (20 ml) and dried over sodium sulfate. The solvent was evaporated under a stream of

nitrogen and the oily residue was pre-purified by flash chromatography (15 g of silica gel, purified cotton wool, and mobile phase 5% ethyl acetate in hexane).

2-(Octadecanoyloxy)-eicosanoic acid and 22-(hexadecanoyloxy)-docosanoic acid

Stearoyl chloride (14 mg) or palmitoyl chloride (14 mg) was added dropwise to a stirred solution of corresponding hydroxy fatty acid (2-hydroxy eicosanoic acid, 6.3 mg, or 22-hydroxy docosanoic acid, 6.3 mg) in 1 ml of anhydrous pyridine at 0 °C. The reaction mixture was stirred for 96 hours at ambient temperature. Then, the mixture was diluted with ether (5 ml), washed with 10% HCl (2×5 ml), washed with a saturated solution of $KHCO_3$ (2×5 ml), washed with brine (10 ml), and dried over anhydrous sodium sulfate. The solvent was evaporated under a stream of nitrogen and the oily residue was purified by flash chromatography using 15 g of silica gel and 5% ethyl acetate in hexane.

Preparation of TLC plates

The silica gel TLC plates were prepared as follows: ca 50 g of silica gel (Silica Gel 60G for thin-layer chromatography, Merck & Co., United States) were carefully added to 100 ml of distilled water in the 2 l conical flask while being slowly shaken by hand. When the mixture was homogenized, it was poured over the glass slides and let resting until dry in laboratory temperature (ca 48 hours).

The magnesium hydroxide plates were prepared as follows: ca 60 g of magnesium hydroxide (BioUltra, ≥ 99.0%, Sigma-Aldrich, United States) were transferred to a Petri dish and it was placed into an oven for two hours at 105 °C. Then the dry magnesium hydroxide was slowly added to 100 ml of distilled water in a 500ml beaker. The suspension was mixed by magnetic stirred for approximately 20 minutes to achieve

accurate homogenization. When the suspension was thoroughly homogenized, it was poured over the glass slides and let resting until dry in laboratory temperature (ca 48 hours).

Instrumentation

The GC/MS analyses were performed on a 7890N gas chromatograph coupled to a 5975C mass spectrometer, equipped with electron ionization and quadrupole analyzer (Agilent Technologies, Santa Clara, CA, United States).

Publication I: The HPLC/MS experiments to characterize cholesteryl esters of ω-(*O*-acyl)-hydroxy fatty acids in vernix caseosa were performed using an LTQ Orbitrap XL hybrid FT mass spectrometer equipped with an atmospheric pressure chemical ionization source and controlled by Xcalibur (all Thermo Fisher Scientific, San Jose, CA, United States). The spectrometer was coupled to an HPLC system consisting of a Rheos 2200 quarternary gradient pump (Flux Instruments, Reinach, Switzerland), a PAL HTS autosampler (CTC Analytics, Zwingen, Switzerland), and a DeltaChrom CTC 100 column oven (Watrex, Prague, Czech Republic).

Publication II: The HPLC/MS analyses to characterize (*O*-acyl) α- and ω-hydroxy fatty acids in vernix caseosa were conducted on a liquid chromatograph consisting of a 10390 Rheos Allegro UHPLC pump (Flux Instruments, Basel-Stadt, Switzerland), an HTS9 PAL autosampler (GenTech Scientific, Inc., Arcade, NY, USA), and a Jetstream 2 Plus column thermostat (ABL&E-JASCO Magyarország, Budapest, Hungary). The HPLC system was coupled to an LTQ Orbitrap XL hybrid FT mass spectrometer with electrospray ionization and controlled by Xcalibur (all Thermo Fisher Scientific, Waltham, MA, USA).

2 Results and discussion

This section summarizes and discusses the results of the research. Details can be found in the original publications I and II.

2.1 Cholesteryl esters of ω-(*O*-acyl)-hydroxy fatty acids in vernix caseosa

Cholesteryl esters of ω-(*O*-acyl)-hydroxy fatty acids (Chl-ωOAHFAs) were identified for the first time in vernix caseosa and characterized using chromatography and mass spectrometry. Chl-ωOAHFAs were isolated from the total lipid extract by a two-step semipreparative TLC using plates coated with silica gel and magnesium hydroxide. Their general structure was elucidated using high-resolution and tandem mass spectrometry of intact lipids and their transesterification and derivatization products. A carefully optimized method of non-aqueous reversed-phase HPLC coupled to mass spectrometer equipped with atmospheric pressure chemical ionization source was used for characterization of the Chl-ωOAHFAs molecular species. The analytes were detected as protonated molecules, and their structures were elucidated in the negative ion mode using controlled thermal decomposition and data-dependent fragmentation.

2.1.1 Isolation of Chl-ωOAHFAs

During the structural investigation of 1,2-diol diesters (1,2-DDEs) in vernix caseosa (200), the HPLC/APCI-MS analysis revealed the presence of so far unknown lipid class of vernix caseosa (**Fig 11**). These lipids eluted in reversed-phase HPLC with higher retention times than 1,2-DDEs. Later, the unknown lipid class was identified as Chl-ωOAHFAs. Their general structure elucidation was performed by colleagues from our MS research group at IOCB. The schematic flowchart of how the general structure was determined is shown in

Fig 12. and a detailed process can be found in publication I. After the general structure was known, the main aim of my research was to characterize the structures of these new lipids in detail.

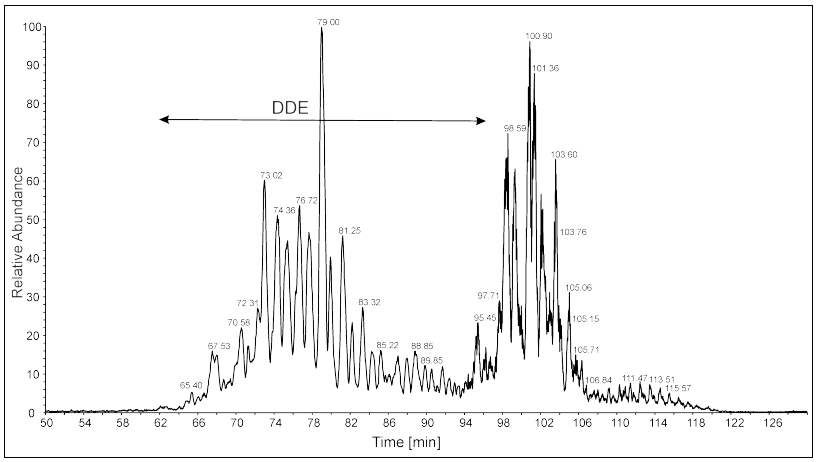

Figure 11. The HPLC/APCI-MS base peak chromatogram of the 1,2-DDE fraction isolated from vernix caseosa. The first cluster of chromatographic peaks corresponds to 1,2-DDE and the second group of peaks at higher retention times represents lipids later identified as Chl-ωOAHFAs.

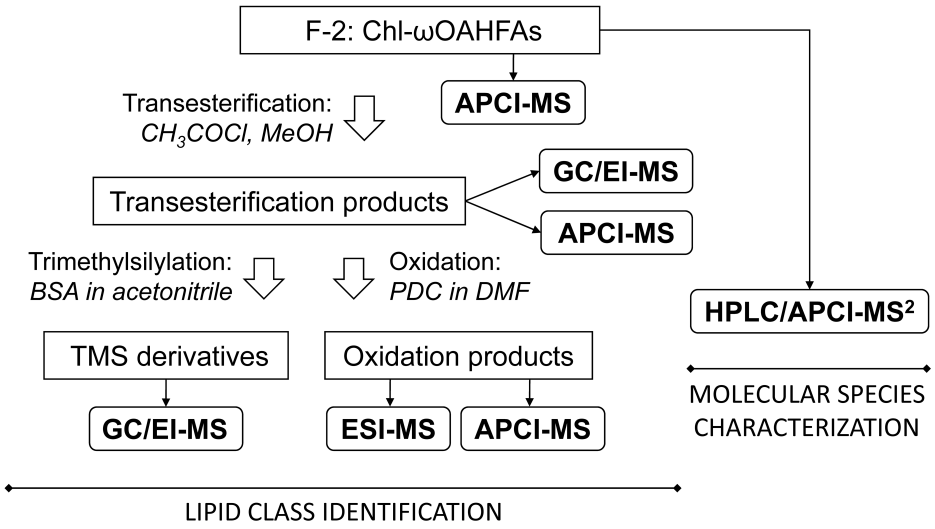

Figure 12. A flowchart showing the steps used during elucidation of the general structure of Chl-*ω*OAHFAs and identification of the molecular species within this lipid class.

Comprehensive characterization of individual molecular species requires considerable attention to isolation and purification in addition to consideration of mass spectrometric parameters. Thus, a two-step isolation procedure was developed. Firstly, the total lipid extract was fractionated using glass plates coated with silica gel and mobile phase hexane:diethyl ether (93:7, by vol) to isolate all diesters (1,2-DDE and Chl-ωOAHFAs). Chl-ωOAHFAs coeluted with 1,2-DDEs on silica gel. In order to isolate Chl-ωOAHFAs without interferences, it was necessary to develop a TLC to separate Chl-ωOAHFAs from 1,2-DDEs. Due to their similar polarities, common silica gel sorbents were not suitable for separation and thus magnesium-based materials were required (201, 202). Glass TLC plates coated with Florisil (activated magnesium silicate) and magnesium hydroxide were tested together with various compositions of mobile phases and developing parameters. Finally, glass plates coated with magnesium hydroxide (EXPERIMENTAL 1.Materials and instrumentation) and binary mobile phase hexane:ethyl acetate (99.95:0.05, by vol.) showed satisfactory separation (**Fig 13**). Each TLC plate was developed twice to focus the zones; in the first step to ¾ of the plate height and then, after air-drying, to the top. The zones were visualized under ultraviolet light after being sprayed with 0.05% primuline in ethanol.

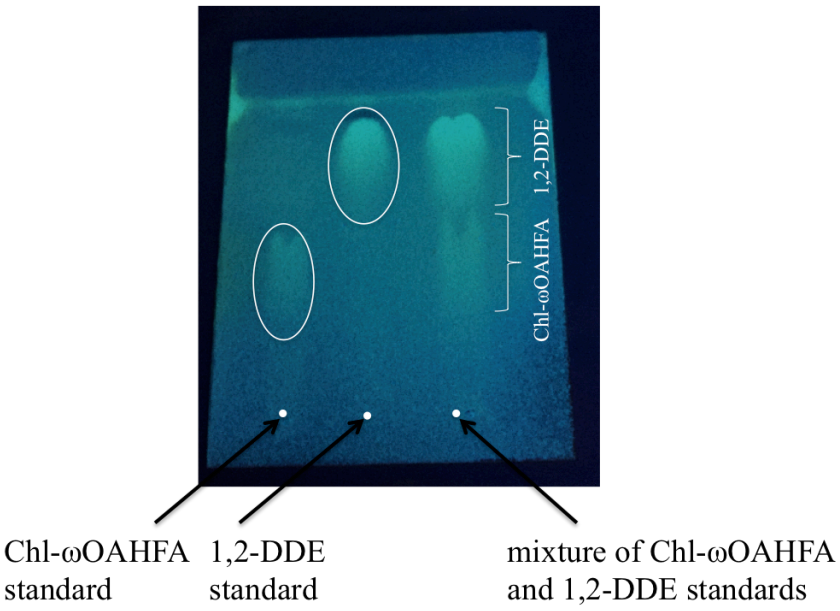

Figure 13. TLC separation of 1,2-DDE and Chl-ωOAHFA standards.

2.1.2 Optimization of HPLC/APCI-MS

The mass spectra and retention behavior of a synthetic standard 16-cholesteryloxy-16-oxohexadecyl oleate (18:1(*n*-9)/16:0-Chl) were studied with the aim to develop and optimize an HPLC/MS method for a comprehensive characterization of Chl-ωOAHFA molecular species. Cholesteryl esters of all kinds possess inherent instability in the positive ion mode which causes the cholesteryl esters to spontaneously fragment and produce intense ion of dehydrated cholesterol at *m/z* 369 (203). The APCI source operates at much higher temperatures than the ESI source causing thermally labile molecules to fragment in the region of the source before entering the analyzer. The APCI spectrum of 18:1(*n*-9)/16:0-Chl in the positive ion mode showed protonated molecule (*m/z* 905.6), an intensive signal of protonated and dehydrated cholesterol $[Chl + H - H_2O]^+$ (*m/z* 369.5), and fragments consistent with a neutral loss of dehydrated cholesterol $[M + H - Chl + H_2O]^+$ (*m/z* 537.5) and cholesterol $[M + H - Chl]^+$ (*m/z* 519.7) (**Fig 14**). The fragmentation MS^2 spectrum of protonated molecule resembled the full scan spectrum

with identical fragments. Consequently, the MS^3 spectra of $[M + H - Chl + H_2O]^+$ and $[M + H - Chl]^+$ were measured in order to obtain a structural information on the FAs and/or HFAs. Unfortunately, the fragmentation channels proceeded almost exclusively *via* the elimination of water. Therefore, positive ion mode proved to be unusable for the structure elucidation of Chl-ωOAHFAs.

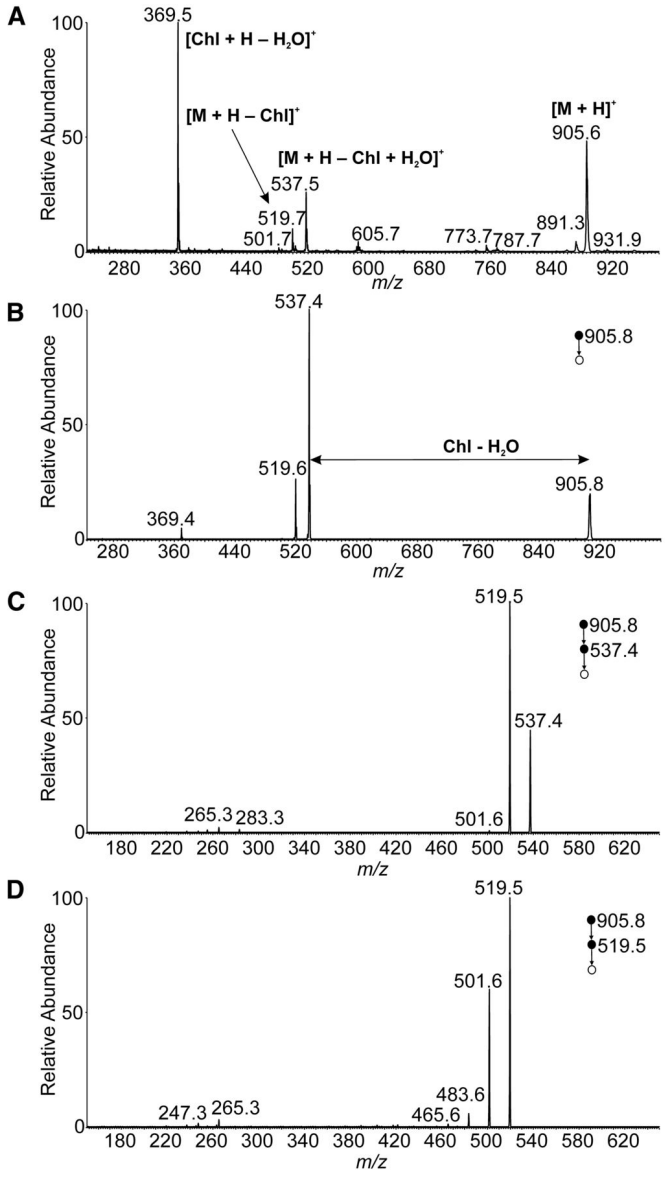

Figure 14. APCI mass spectra of 18:1(*n*-9)/16:0-Chl in the positive ion mode. Full scan spectrum (A). CID MS^2 spectrum of the protonated molecule (*m/z* 905.8; normalized collision energy 15%) (B). CID MS^3 spectrum of $[M + H – Chl + H2O]^+$ (*m/z* 537.4; normalized collision energy 16%) (C). CID MS3 spectrum of $[M + H – Chl]^+$ (*m/z* 519.5; normalized collision energy 16%) (D).

Then, the mass spectrometer was switched to the negative ion mode. As expected, the standard did not provide $[M - H]^-$ in the negative ion mode as it does not consist of any functional group that enables deprotonation. However, we observed that elevated temperature in the ion source induced thermal degradation to ωOAHFA that easily deprotonated in the corona discharge (**Fig 15**). The process of thermal decomposition required optimization of the APCI ion source parameters in order to increase detection sensitivity. The ion source temperature and the mobile phase flow rate, as well as ion optics voltages, were tuned to maximize signal of deprotonated ωOAHFA. The effect of the ion source temperature and mobile phase flow rate on the intensity of $[M - H - Chl + H_2O]^-$ is shown in **Fig 16**. The maximal signal of $[M - H - Chl + H_2O]^-$ was achieved when the ion source temperature reached 500 °C and the flow rate was at its lowest value 150 µl/min. It is known that the fragmentation of deprotonated ωOAHFAs ($[M - H - Chl + H_2O]^-$) gives structural information on FAs and ωHFAs. As expected, the MS^2 spectrum of 18:1(*n*-9)/16:0 (**Fig 15**) showed the same fragmentation pattern as in previously published work (204).

Complex optimization of the HPLC method was required in order to achieve the highest possible resolution for Chl-ωOAHFA molecular species in a reasonable time. No Chl-ωOAHFA standards were commercially available. Therefore, the separation conditions were optimized directly with the Chl-ωOAHFA isolated from vernix caseosa. Based on our previous experience with 1,2-DDEs (200), a non-aqueous reversed-phase system with the two Nova Pak C_{18} columns connected in series with a total length of 45 cm and a column oven set at 40 °C was selected. Given that the mobile phase also plays a role in separation efficiency, different binary solvents containing acetonitrile, ethyl acetate, methanol, acetone and 2-propanol, were studied. Sufficiently good peak-to-peak resolutions were achieved in systems combining acetonitrile with ethyl acetate and

methanol with acetone. However, methanol/acetone appeared to be an inappropriate solvent system for the separation of the Chl-ωOAHFA because of very long retention (110-140 min) and broad peaks that led to significant signal suppression. Finally, similar separation conditions to those used for 1,2-DDEs (200) were selected, i. e., a linear increase of ethyl acetate in acetonitrile in 140 min. Ultimately, the mobile phase flow rate was optimized to obtain sufficiently good performance for ionization. It was reduced from 0.6 to 0.15 ml/min in the elution window of Chl-ωOAHFAs to increase detection sensitivity Better detection sensitivity was achieved due to increased ionization efficiency that occurred at such low flow rate (0.15 ml/min). The effect of the flow rate on the intensity of $[M - H - Chl + H_2O]^-$ is shown in **Fig 16**.

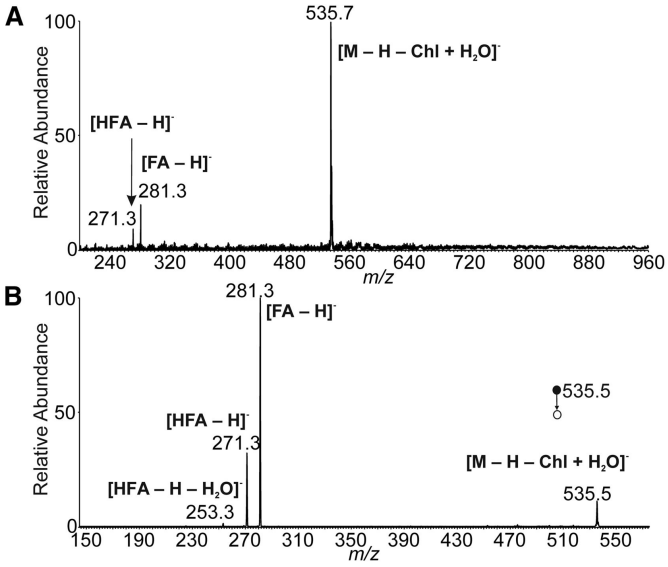

Figure 15. APCI mass spectra of 18:1(*n*-9)/16:0-Chl in the negative ion mode. Full scan spectrum (A). CID MS^2 spectrum of $[M - H - Chl + H_2O]^-$ (*m/z* 535.5; normalized collision energy 15%) (B).

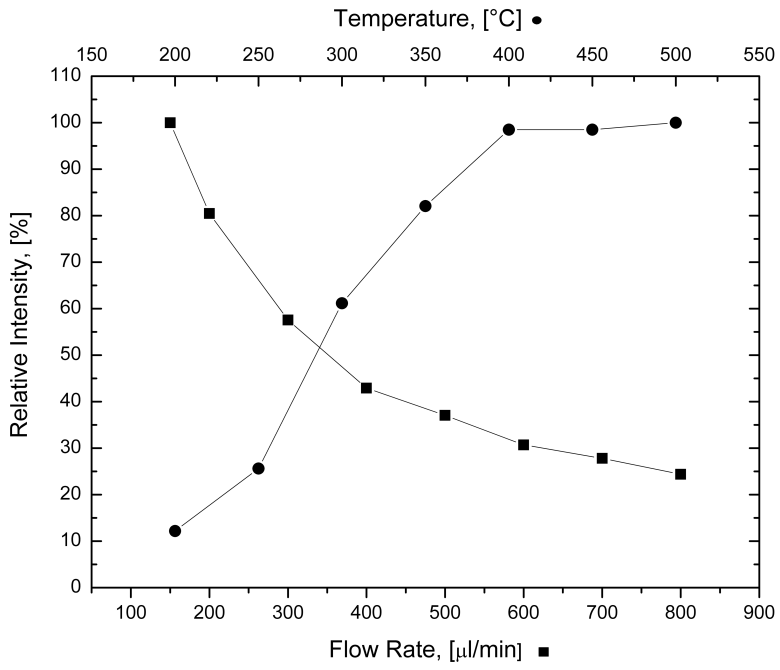

Figure 16. The effect of the ion source temperature and mobile phase flow rate on the intensity of $[M - H - Chl + H_2O]^-$ (*m/z* 535. 7, deprotonated OAHFA formed from 16:0/32:1-Chl in the ion source).

2.1.3 RP-HPLC/MS^2 of Chl-ωOAHFAs in vernix caseosa

The chromatographic data showed a high number of peaks partially overlapping in the base peak trace (**Fig 17**). The chromatograms reconstructed for *m/z* values of particular protonated molecules showed up to several peaks, obviously representing isomers. In general, Chl-ωOAHFAs eluted from the column in the order of increasing chain length following the equivalent carbon number concept. The ECN concept assumes the same effect of a double bond on the retention time of the analyte molecule as that of the shortening of the chain by two methylene groups. Thus, when the ECN values of each Chl-ωOAHFAs were plotted against retention time, all molecular species appeared on a band rising almost linearly with the retention time (at higher retention times the curve rose exponentially as the flow rate increased from 150 μl/min back to 600 μl/min) (**Fig 18**).

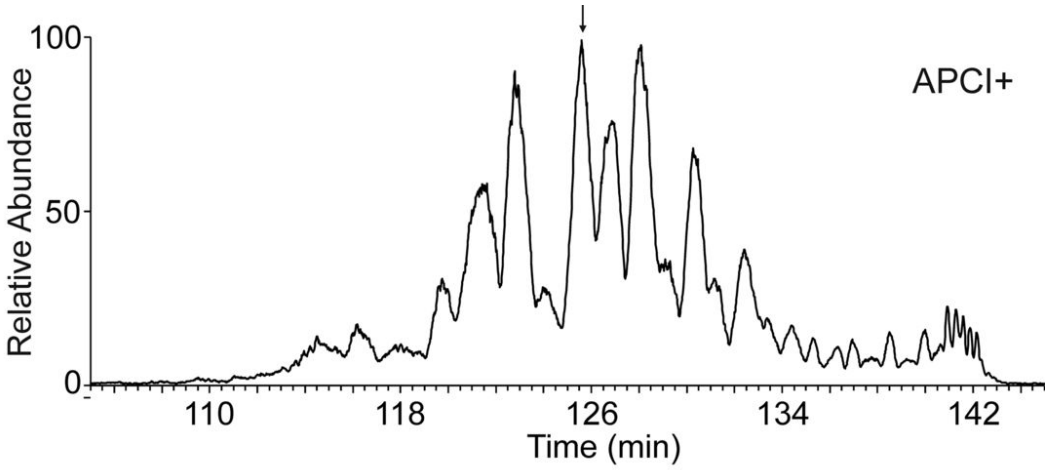

Figure 17. Positive ion base-peak RP-HPLC/MS chromatogram (m/z 1000–1300) of Chl-ωOAHFAs isolated from vernix caseosa.

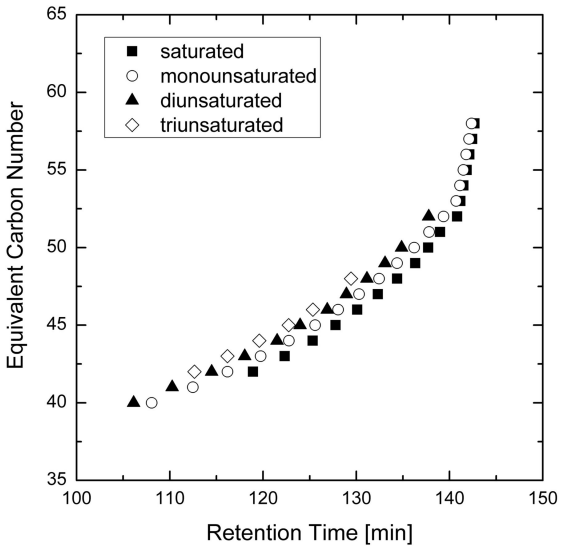

Figure 18. Plot of calculated equivalent carbon number (ECN) values versus retention times for the Chl-ωOAHFAs identified in vernix caseosa (ECN = CN − 2DB where CN is the total number of carbons and DB is the total number of double bonds in the ωOAHFA part of the molecules).

The APCI-tandem MS detection with data-dependent scanning was used to identify the molecular species of Chl-ωOAHFAs. The MS detection method consisted of three scanning events: (1) the high-resolution full MS scan event in the positive ion mode was used for determining the total number of carbons and double bonds in fatty chains and served for confirmation of the expected elemental composition; (2) the liner ion-trap full MS scan event in the negative ion mode (showing deprotonated ωOAHFAs) for further confirmation of the total number of carbons and double bonds in fatty chains and for data-dependent selection of precursors for MS/MS; and (3) the collision-induced dissociation (collision energy 21.5%) MS/MS scan event of the first most intense ion from the parent mass list in the negative ion mode that revealed FAs and ωHFAs. The deprotonated ωHFA ions ($[HFA - H]^-$) were always accompanied by less abundant water loss peaks ($[HFA - H - H_2O]^-$), which made it possible to distinguish them from deprotonated FA ions.

By manual interpretation of the data, we fully characterized 295 molecular species of Chl-ωOAHFAs in 59 chromatographic peaks. Additionally, Chl-ωOAHFAs in other 11 peaks were characterized by the total number of carbons and double bonds because the signal intensity was to low to be recognized for fragmentation in the data-dependent scan.

The relative proportions of identified molecular species were estimated from peak areas integrated in the chromatograms reconstructed for $[M + H]^+$ and relative intensities of deprotonated FA ions in MS^2 spectra. Lipid response factors depend on the number of double bonds and carbon chain length (205, 206). Therefore, as neither standards nor response factors were available for quantification, the relative proportions must be considered merely as an estimate. In this way, the results showed that the saturated Chl-ωOAHFAs corresponded to 11% of the total integrated signal, whereas monounsaturated, diunsaturated, and triunsaturated species accounted for 55, 30, and 4 %,

respectively.

The experiments showed that Chl-ωOAHFAs are estimated to account for ca 1 – 2 % of vernix caseosa lipids. The GC/EI-MS data of the transesterified sample of Chl-ωOAHFAs revealed that the sterol moiety in Chl-ωOAHFAs was exclusively represented by cholesterol. The most abundant ωHFAs in Chl-ωOAHFAs from vernix caseosa were 32:1, 34:1, and 30:1. Both straight-chain and methyl branched ωHFAs were detected. As regards the double bond position in unsaturated ωHFAs, it was demonstrated that the double bonds in 32:1 ωHFA are in two positions, *n*-7 (more abundant) and *n*-9 (less abundant). Concerning FAs in Chl-ωOAHFAs, the largest signals provided Chl-ωOAHFAs with 14:0, 15:0, 16:0, 16:1, and 18:1 chains, which is not surprising for a sample of vernix caseosa origin (207, 208). The distribution of FAs in Chl-ωOAHFAs was similar to the overall representation of FAs in the vernix caseosa lipidome reported earlier (209). However, the higher levels of 16:1 and 18:1 observed for Chl-ωOAHFAs has to be interpreted with caution because APCI-MS is somewhat more sensitive to unsaturated lipids (205, 206). The main species in vernix caseosa were 15:0/32:1-Chl, 14:0/32:1-Chl, 18:1/32:1-Chl, 16:1/32:1-Chl, and 16:0/32:1-Chl. Considering the intact molecules, this study showed the ratio of unsaturated and saturated Chl-ωOAHFAs as 89 %:10 %.

2.2 Analysis of (*O*-acyl) alpha- and omega-hydroxy fatty acids in vernix caseosa by high-performance liquid chromatography-Orbitrap mass spectrometry

Fatty acid esters of long-chain hydroxy fatty acids or (O-acyl)-hydroxy fatty acids (OAHFAs) were analyzed for the first time in vernix caseosa using chromatography and mass spectrometry. OAHFAs were isolated from the total lipid extract by a two-step semipreparative TLC using plates coated with silica gel. Their general structure was identified using high-resolution and tandem MS of intact lipids, and products of their transesterification and derivatization. Two isomeric lipid classes were identified: *O*-acyl esters of ω-hydroxy fatty acids (ωOAHFA) and *O*-acyl esters of α-hydroxy fatty acids (αOAHFAs). Chromatographic separation of OAHFA molecular species was achieved in the non-aqueous reversed-phase HPLC. The analytes were detected as deprotonated molecules by an Orbitrap mass spectrometer equipped with an electrospray ionization source. Their structures were characterized using data-dependent fragmentation in the negative ion mode.

2.2.1 Isolation of OAHFAs

Since Chl-ωOAHFAs were discovered in vernix caseosa, the question of whether intact ωOAHFAs are present as well arose. The lipidome of vernix caseosa is one of the most complex lipid mixtures, which makes the characterization of its minor components challenging. Due to the high complexity of the sample, it was necessary to perform a multi-stage sample pretreatment to obtain a fraction enriched with the desired analytes. In the first step, polar lipids were separated from nonpolar lipids (which exist in large excess in vernix caseosa) on pre-cleaned TLC silica gel plates using hexane:diethyl ether:acetic acid (80:20:1, by vol.) mobile phase. In the next step, the fraction of polar lipids was spiked with 10-POHSA for later quantification. Then, the sample was applied on TLC

plates with silica gel with an aim to isolate OAHFAs from the complex mixture of polar lipids using hexane:diethyl ether:acetic acid (70:30:2, by vol.) mobile phase. A small amount of acetic acid was added to the mobile phase to prevent the broadening of the zones of the lipids with the free –COOH group. The standard 18:1/*ω*-16:0 was used to mark the R_F of OAHFAs.

2.2.2 General structure elucidation

The mass spectra indicated that the desired lipids (possibly OAHFAs) eluted in the reversed-phase HPLC in 28-35 min range. For deprotonated molecules, the high-resolution ESI spectra revealed elemental compositions $C_nH_{2n-x}O_4^-$, where x = 3, 5, 7, or 9, depending on the degree of unsaturation. The MS/MS spectra were in agreement with the published spectra of ωOAHFAs (204). In order to determine the position of the branching ester group, OAHFAs were hydrolyzed and derivatized. For these experiments, OAHFAs were transesterified (210) and analyzed by high-temperature GC/EI-MS. As expected, the GC/EI-MS of the transesterified sample showed a rich mixture of FAMEs and free HFAs. This mixture was treated with $TMSCHN_2$ to produce HFAMEs, which were in the next step trimethylsilylated with *N*,*O*-bis(trimethylsilyl)acetamide. The spectra showed that the majority of the TMS derivatives were ωTMS derivatives with the hydroxy group at the terminal carbon. However, the spectra also revealed the presence of αTMS derivatives. Therefore, besides ωOAHFAs, a new subclass of fatty acid esters of hydroxy fatty acids, αOAHFAs, was likely present in vernix caseosa. To confirm the hypothesis, standards of OAHFAs (16:0/*ω*-22:1 and 18:0/*α*-20:1) were synthesized and their retention times compared to their equivalents naturally occurring in vernix caseosa. Their retention times turned out to be in excellent agreement with those of 38:0 OAHFAs observed in the chromatogram of vernix caseosa (**Fig 19**). All these results led us to the conclusion that

both ωOAHFAs and αOAHFAs are indeed present in vernix caseosa.

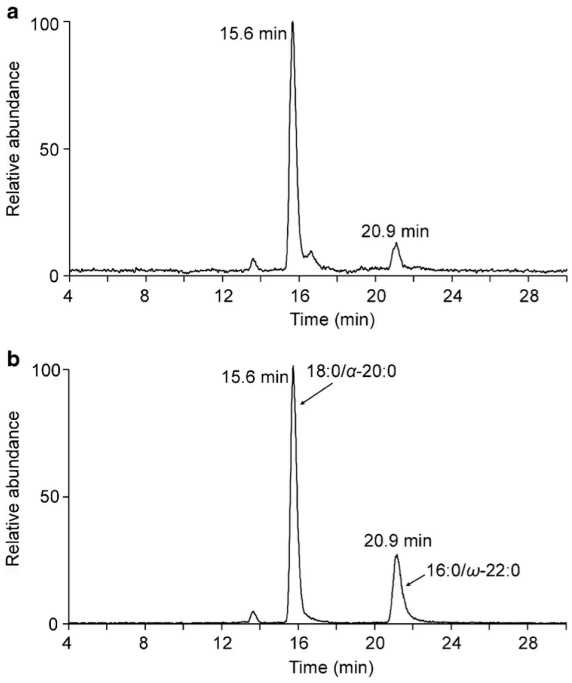

Figure 19. Chromatograms reconstructed for m/z 593.5 showing saturated OAHFA species with 38 carbons in their chains. Sample of OAHFAs isolated from vernix caseosa (A), and the same sample spiked with 2-(stearoyloxy)eicosanoic acid (18:0/α-20:0) and 22-(palmitoyloxy)docosanoic acid (16:0/ω-22:0) (B).

2.2.3 Optimization of HPLC/ESI-MS

The chromatography was carefully optimized directly with the lipid sample from vernix caseosa. A non-aqueous reversed-phase system with two Nova-Pak C18 columns connected in series with a total length of 45 cm was developed for the separation of OAHFAs molecular species. Even though the isolation procedure consisted of several steps, the OAHFAs fraction appeared to contain also unwanted lipids identified as free FAs. Therefore, the HPLC conditions were optimized with the aim to separate OAHFAs from contaminating lipids and achieve good chromatographic resolution of OAHFA molecular species. Based on the previous experience with separation of lipids from vernix

caseosa, the gradient conditions for the separation of OAHFAs were tested, including the methods previously suggested by Mori et al. (211) (binary gradient: water with 5 mmol/l ammonium acetate (A) and methanol:2-propanol (50:50) with 5 mmol/l ammonium acetate (B)). However, the gradient elution did not show any improvements compared to isocratic elution. Finally, we ended up using isocratic separation with a mobile phase containing propan-2-ol:methanol (1:1, by vol.) with the addition of 0.05% ammonium acetate. Lower flow rate than the optimum value was used (0.25 ml/min) to slow down the elution, which provided us with extra time to collect enough spectra for the structural characterization of the analytes. Since ESI is considered a concentration detector, there was no loss of sensitivity.

Mass spectra of ωOAHFAs have been previously published and thus their fragmentation is well known (204, 211). On the other hand, the fragmentation spectra of *α*OAHFAs have not been studied yet and therefore the difference of spectra between omega and alpha isomers was investigated. For this purpose, mass spectra of synthetic standards were studied. Firstly, the full scan Orbitrap ESI spectra of stearic acid esters of hydroxy palmitoleic acid standards (18:0/*ω*-16:1 and 18:0/*α*-16:1) were measured in the negative ion mode. They showed a strong signal of deprotonated molecules $[M - H]^-$ accompanied by fragments consistent with deprotonated stearic acids ($[FA - H]^-$) and deprotonated hydroxyl palmitic acids ($[HFA - H]^-$). In the spectrum of the alpha isomer, higher intensities of the $[FA - H]^-$ and $[HFA - H]^-$ fragments were observed compared to the omega isomer, likely because of the proximity of the ester group to the negative charge site. These fragments were also detected in the CID MS/MS spectra generated from $[M - H]^-$. Whereas $[FA - H]^-$ was the base peak in the spectrum of 18:0/*ω*-16:1, the most abundant ion in the spectrum of 18:0/*α*-16:1 was $[HFA - H]^-$. In addition, a water loss

peak ([HFA − H − H_2O]$^-$) was present in the fragmentation spectra of both isomers. However, the main difference between the ω- and α-isomers that made it possible to differentiate between them was a fragment consistent with formic acid loss from deprotonated hydroxy fatty acid ([HFA – H – HCOOH]$^-$). This specific fragment was only observed in the MS/MS spectrum of 18:0/α-16:1, but not in 18:0/ω-16:1 (**Fig 20**).

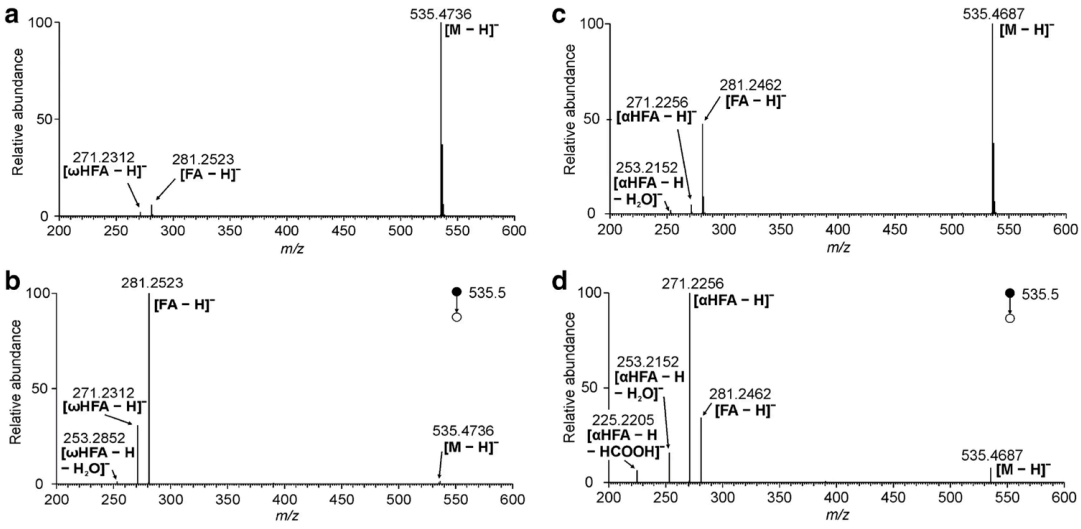

Figure 20. ESI mass spectra of 18:1/ω-16:0 and 18:1/α-16:0 standards in the negative ion mode taken from HPLC/MS(/MS) data. Full-scan spectrum of 18:1/ω-16:0 (A), CID MS2 spectrum of 18:1/ω-16:0 [M − H]$^-$ (*m/z* 535.5; normalized collision energy 25%) (B), full-scan spectrum of 18:1/α-16:0 (C), and CID MS2 spectrum of 18:1/α-16:0 [M − H]$^-$ (m/z 535.5; normalized collision energy 25%) (D).

2.2.4 RP-HPLC/MS^2 of OAHFAs in vernix caseosa

The chromatographic data of OAHFAs were quite complex and showed a high number of peaks partially overlapping in the base peak trace (**Fig 21a**). Isobaric OAHFAs were observed in reconstructed chromatograms for [M − H]$^-$ *m/z* values. Saturated OAHFAs provided up to four isomeric peaks arranged in two separate distributions (**Fig 21b**). The

peaks at shorter retention times were interpreted as αOAHFAs because of the presence of the specific $[HFA - H - HCOOH]^-$ fragments. The species with higher retention times corresponded to ωOAHFAs. Within both distributions, typically two peaks existed likely representing methyl branching isomers. Based on the experience with other lipids (200, 206, 212), the more retained species possibly contained straight chains, while those with shorter retention were methyl-branched. Unfortunately, no standards of methyl-branched OAHFAs were available to prove this hypothesis. Unsaturated OAHFAs showed only one distribution of closely eluting peaks in their reconstructed chromatograms. The unsaturated OAHFAs were solely ωOAHFAs isomers because no $[HFA - H - HCOOH]^-$ fragments were detected. Similarly to saturated OAHFAs, partially separated peaks were explained by methyl branching and possibly also by a retention time variability of isobaric species having the carbon atoms differently distributed between FA and HFA chains. The elution order of OAHFAs followed the ECN concept similarly to Chl-ωOAHFAs described in the previous chapter. When the ECN values were plotted against retention time (**Fig 22**), the molecular species were distributed in two bands, confirming the existence of two different lipid classes, i.e., αOAHFAs and ωOAHFAs. The band of αOAHFAs was shifted to higher ECN values, which corresponded well with the lower retention of αOAHFAs in the reversed-phase HPLC.

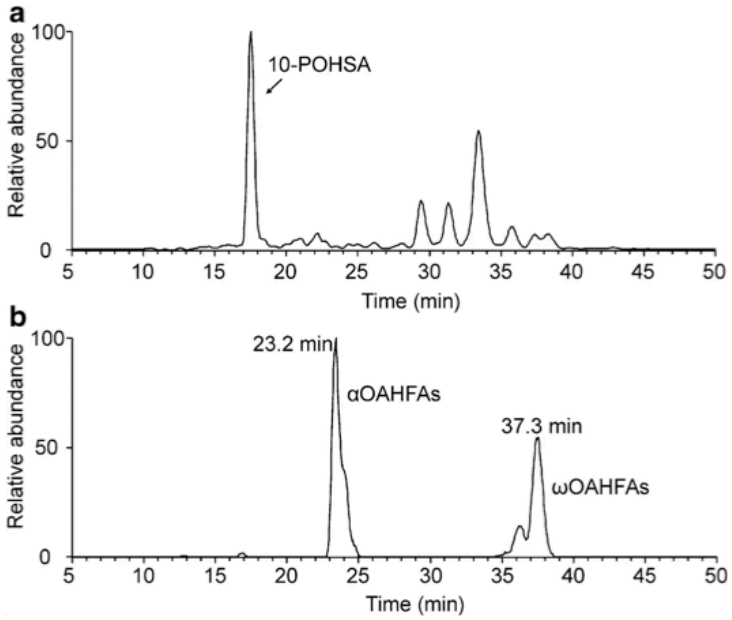

Figure 21. Base peak chromatograms of OAHFAs isolated from vernix caseosa with internal standard added (A) and reconstructed chromatograms for *m/z* 705.6766 showing saturated OAHFAs with 46 carbons (B).

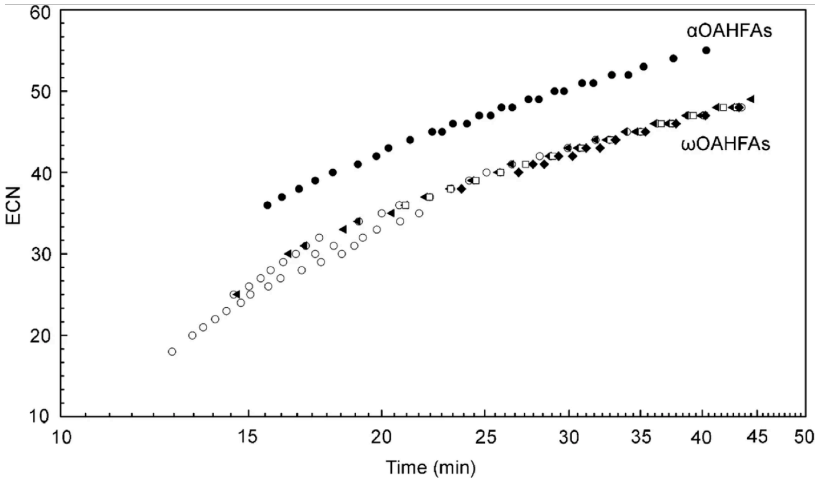

Figure 22. Plot of calculated equivalent carbon number (ECN) values versus retention times for OAHFAs identified in vernix caseosa (ECN = CN − 2DB where CN is the total number of carbons and DB is the total number of double bonds in OAHFAs). Black circles, saturated αOAHFAs species; white circles, saturated ωOAHFAs species; black triangles, monounsaturated ωOAHFAs species; white squares, diunsaturated ωOAHFAs species; and black rhombuses, triunsaturated ωOAHFAs species.

The molecular species of *ω*OAHFAs were detected and identified using ESI-tandem MS with data-dependent scanning. The high-resolution full scan spectra of deprotonated molecules were used for determining the total number of carbons and double bonds in OAHFAs. The data-dependent high-resolution CID (collision energy 25%) MS/MS spectra of the first most intense ion from the parent mass list in the negative ion mode served for characterization of individual molecular species using $[FA - H]^-$ and $[HFA - H]^-$ fragments, which were easily distinguished from each other based on their exact masses. The parent mass list was calculated for $[M - H]^-$ ions of all possible OAHFAs with the total number of carbons and double bonds in the range of 30-60 and 0-3, respectively. The presence of $[HFA – H – HCOOH]^-$ ions and retention data made it possible to deduce the position of ester branching site in OAHFAs (alpha or omega).

The HPLC/MS^2 data were interpreted manually. In total, 250 ωOAHFA molecular species in 47 chromatographic peaks and 104 αOAHFA molecular species in 18 chromatographic peaks were fully characterized. Additionally, another 63 chromatographic peaks gave 58 ωOAHFA molecular species and 5 αOAHFA molecular species, which were only partially characterized by the total number of carbons and double bonds.

Due to the presence of interfering lipids in the sample, an internal standard (10-POHSA) was used to quantify the molecular species of OAHFAs. A single-point calibration method was used for quantification. The response factors of all OAHFAs including the internal standards were considered equal. Generally, the response factors of lipids depend on the number of double bonds and carbon chain length (205, 206). Since neither standards of ω- and αOAHFAs nor response factors were available, the quantification must be to some extent considered biased. Peak areas for each OAHFA were obtained by integration of the appropriate reconstructed ion chromatograms for $[M - H]^-$ and quantified by comparison to the reconstituted ion peak of an internal standard. The relative proportions of the

individual molecular species with the same *m/z* value were estimated from the peak areas integrated in the chromatograms reconstructed for $[M - H]^-$ and the relative intensities of deprotonated HFA ions in MS^2 spectra. In this way, the results showed that the saturated ωOAHFAs corresponded to 9 % of the total integrated signal, whereas monounsaturated, diunsaturated, and triunsaturated species accounted for 10, 60, and 15 %, respectively. The saturated αOAHFAs corresponded to the remaining 6 % of the integrated signal. The *α*-isomers and *ω*-isomers corresponded to 6 and 94 % of OAHFAs, respectively.

The experiments showed that vernix caseosa lipids contained 0.003 % of αOAHFAs and 0.04 % of ωOAHFAs. ωOAHFAs turned out to be mostly diunsaturated species with ωHFAs 30:0, 29:0, 28:0, 32:1, 34:1, and FA 18:2. The highly abundant FA 18:2 was identified as linoleic acid (retention time and mass spectra were compared with methyl linoleate in GC/MS), which is commonly found in the human epidermis (213). The main ωOAHFAs species detected in vernix caseosa were 28:0/*ω*-18:2, 29:0/*ω*-18:2, 30:0/*ω*-18:2, 32:0/*ω*-18:2, and 30:0/*ω*-18:3. On the other hand. The composition of αOAHFAs appeared to be significantly different. The αOAHFA molecular species contained solely saturated aliphatic chains. Regarding αOAHFAs, the most abundant species contained αHFA 24:0 and a wide range of long-chain saturated FAs with 20-26 carbons. The main αOAHFAs in vernix caseosa were 21:0/*α*-24:0, 22:0/*α*-24:0, 23:0/*α*-24:0, 24:0/*α*-24:0, and 26:0/*α*-24:0. This was the first time when intact αOAHFAs were identified in a biological matrix. The results showed that ωOAHFAs were composed of 32 different ωHFAs with 16–36 carbons (up to 3 double bonds) and 28 different FAs with 12-24 (up to three double bonds), while αOAHFAs were composed of 12 saturated αHFAs with 16–29 carbons and 16 saturated FAs with 15-30 carbons.

2.3 Chl-ωOAHFAs and OAHFAs from vernix caseosa

Until now, ωOAHFAs have been detected in human skin (214), meibum (204, 215, 216), equine sperm (217), amniotic fluid (218), and as structural components of more complex lipids (215, 219-221). Free ωOAHFAs are essential amphiphilic compounds in human meibum that play an important role in stabilizing the tear film and thus prevent drying of the ocular surface (222). The complex mixture of meibomian ωOAHFAs contains molecular species composed mostly of monounsaturated FAs with 18 and 16 carbons and long-chain monounsaturated ωHFAs with 28-34 carbons (204, 215, 216). Many of the meibomian ωOAHFAs have also been described in equine sperm and amniotic fluid (217, 218). Similar ωOAHFAs were also observed as components of more complex Chl-ωOAHFAs in vernix caseosa and human meibum (215, 219). Whereas, free ωOAHFAs of vernix caseosa described in this work were mainly composed of long-chain saturated ωHFAs with 28-31 carbons to which linoleic acid (18:2) was esterified most predominantly. The ωHFA chains in vernix caseosa ωOAHFAs were closest to ωHFAs detected in vernix caseosa *O*-acylceramides EOS (220). Therefore, ωOAHFAs may be biosynthetically related to *O*-acylceramides EOS. The different composition of the ωOAHFA moieties in Chl-ωOAHFAs and *O*-acylceramides stems from their different origin. While ceramides are considered products of epidermal development, Chl-ωOAHFAs are of sebaceous origin (168).

In the contrary, the αOAHFAs molecular species differed significantly from ωOAHFAs as they were composed solely of saturated aliphatic chains. The most abundant αHFA in αOAHFAs was 24:0 and the ester-linked FAs were mostly long-chain FAs, namely 20:0, 21:0, 22:0, 24:0 and 26:0. Intact αOAHFAs have not been identified in any biological matrix yet. There are pieces of evidence that αOAHFAs exist in *O*-acylceramides and diester waxes, but no conclusive proof has been given so far (220).

Finally, the biological role of Chl-ωOAHFAs and OAHFAs in vernix caseosa remains to be clarified. Before OAHFAs were identified in vernix caseosa, Chl-ωOAHFAs were thought to possibly function as storage or inactivated form of ωOAHFAs, similarly to cholesterol esters of very long chain FAs and ωOAHFAs in meibum (204, 216). However, the different compositions of HFAs and FAs in Chl-ωOAHFAs and OAHFAs in vernix caseosa described in this work did not support this hypothesis. Thus, the function of Chl-ωOAHFAs remains elusive. Similarly to meibum, amphiphilic OAHFAs may act as surfactants contributing to the cohesiveness of vernix caseosa. They may provide interphase between nonpolar lipids of vernix caseosa and the aqueous environment of the amniotic fluid.

3 Conclusions

The thesis is focused on the analysis of new lipid classes in vernix caseosa by using liquid chromatography and mass spectrometry. This research, which comprises specialized targeted lipidomic analysis, shows how the field of lipidomics is used to aid in revealing the structures of unknown lipids in complex biological materials such as vernix caseosa. The results of this work confirm the enormous lipid complexity of the vernix caseosa lipidome and contribute to the overall knowledge of this unique natural biofilm. However, there still exist numerous low abundant lipid classes yet to be discovered. Therefore, in addition to studies on biochemical and physiological roles of vernix caseosa, the research should also focus on the identification of new lipid classes to add to the knowledge of vernix caseosa lipidome. The increased attention and understanding of this unique biofilm may bring potential applications for the treatment of preterm infants lacking functional skin or adult population suffering from skin diseases or injuries.

The presented method in the publication I encompasses a simple but versatile lipid extraction, two-step TLC isolation, powerful reverse-phase liquid chromatography, and detection with high-resolution APCI tandem mass spectrometry. This combination enabled quantification and comprehensive structural characterization of more than 300 molecular species of Chl-ωOAHFAs of vernix caseosa, by far the highest number of reported Chl-ωOAHFAs in biological samples so far. These diesters represent a new lipid class for vernix caseosa.

In publication II, a combination of multistage sample pre-treatment (including lipid extraction and TLC two-step isolation) and carefully optimized reversed-phase liquid chromatography coupled with high-resolution ESI tandem mass spectrometry served for identification and quantification of more than 400 molecular species of OAHFAs in vernix caseosa. Two structurally different subclasses of OAHFAs were discovered, ωOAHFAs

and αOAHFAs. In ωOAHFAs, the position of the ester group is on the terminal ω carbon whereas in αOAHFAs it is located on the second α carbon relative to a carboxylic acid. αOAHFAs represent a newly discovered lipid subclass that has not been detected in any biological matrix yet. Both αOAHFAs and ωOAHFAs are newly described lipids for vernix caseosa.

REFERENCES

1 Lehninger, A. L., Nelson. D. L., and Cox, M. M. 2013. Lehninger principles of biochemistry, 6th edition. New York, Worth Publishers.

2 Han, X. L., and Gross, R. W. 2005. Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass. Spectrom. Rev*. **24**: 367-412.

3 Wenk, M. R. 2010. Lipidomics: New Tools and Applications. *Cell.* **143**: 888-895.

4 Wymann, M. P., and Schneiter, R. 2008. Lipid signalling in disease. *Nat. Rev. Mol. Cell Bio.* **9**: 162-176.

5 Fujimoto, T., and Parton, R. G. 2011. Not just fat: the structure and function of the lipid droplet. *Cold Spring Harb. Perspect. Biol*. **3**(3): a004838.

6 Pietiläinen, K. H., Róg, T., Seppänen-Laakso, T., Virtue, S., Gopalacharyulu, P., Tang, J., et al. 2011. Association of Lipidome Remodeling in the Adipocyte Membrane with Acquired Obesity in Humans. *PLoS Biol.* **9**(6): e1000623.

7 Erion, D. M., Park, H. J., and Lee, H. Y. 2016. The role of lipids in the pathogenesis and treatment of type 2 diabetes and associated co-morbidities. *BMB Rep*. **49**(3): 139-148.

8 Burnett, J. R. Lipids, lipoproteins, atherosclerosis and cardiovascular disease. *Clin. Biochem. Rev*. **25**(1): 2.

9 Long, J., Zhang, C. J., Zhu, N., et al. 2018. Lipid metabolism and carcinogenesis, cancer development. *Am. J. Cancer Res*. **8**(5): 778-791.

10 Di Paolo, G., and Kim, T. W. 2011. Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat. Rev. Neurosci*. **12**(5): 284-296.

11 Oresic, M. 2012. Obesity and psychotic disorders: uncovering common mechanisms through metabolomics. *Dis. Model. Mech*. **5**(5): 614-620.

12 Giera, M., Ioan-Facsinay, A., Toes, R., et al. 2012. Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC-MS/MS. *Biochim. Biophys. Acta*. **1821**(11): 1415-1424.

13 Cheng, D., Jenner, A. M., Shui, G., et al. 2011. Lipid pathway alterations in Parkinson's disease primary visual cortex. *PLoS One*. **6**(2): e17299.

14 Fahy, E., Subramaniam, S., Brown, H. A., Glass, C. K., Merrill, A. H., Murphy, Jr., R. C., Raetz, C. R., Russell, D. W., Seyama, Y., Shaw, W., et al. 2005. A comprehensive classification system for lipids. *J. Lipid Res*. **46**: 839-862.

15 Fahy, E., Cotter, D., Sud, M., and Subramaniam, S. 2011 Lipid classification, structures and tools. *Biochim. Biophys. Acta*. **1811**(11): 637-647.

16 De Oliveira, M. A. L., Porto, B. L. S., Faria, I. D. L., de Oliveira, P. L., de Castro Barra, P. M., Castro, R. D. J. C., and Sato, R.T. Sato. 2014. 20 years of fatty acid analysis by capillary electrophoresis. *Molecules*. **19**: 14094-14113.

17 Chen, S. H., and Chuang, Y. J. 2002. Analysis of fatty acids by column liquid chromatography. *Anal. Chim. Acta*. **465**: 145-155.

18 Vance, D. E., and Vance, J. E. 2002. Biochemistry of Lipids, Lipoproteins and Membranes, 4th edition. New York. Elsevier Science.

19 Mjøs, S. A., and Haugsgjerd, B. O. 2011. Trans fatty acid analyses in samples of marine origin: the risk of false positives. *J. Agric. Food Chem*. **59**: 3520-353.

20 Wiktorowska-Owczarek, A., Berezińska, M., and Nowak, J. Z. 2015. PUFAs: Structures, Metabolism and Functions. *Adv. Clin. Exp. Med*. **24**(6): 931-94.

21 Stam, H., Schoonderwoerd K., and Hulsmann, W. C. 1987. Synthesis, storage and degradation of myocardial triglycerides. *Basic. Res. Cardiol.* **82 (Suppl. 1)**: 19-28.

22 Bates, P. D., and Browse, J. 2012. The significance of different diacylgycerol synthesis pathways on plant oil composition and bioengineering. *Front. Plant. Sci*. **3**: 147.

23 Qu, S., Du, Z., and Zhang, Y. 2015. Direct detection of free fatty acids in edible oils using supercritical fluid chromatography coupled with mass spectrometry. *Food Chem*. **170**: 463-469.

24 Hishikawa, D., Hashidate, T., Shimizu, T., and Shindou, H. 2014. Diversity and function of membrane glycerophospholipids generated by the remodeling pathway in mammalian cells *J. Lipid Res.* **55**(5): 799-807.

25 Abidi, S.L. 2001. Chromatographic analysis of plant sterols in foods and vegetable oils. *J. Chromatogr. A*. **935**(1-2): 173-201.

26 Horník, Š., Sajfrtová, M., Karban, J., Sýkora, J., Březinová, A., and Wimmer, Z. 2013. LC-NMR technique in the analysis of phytosterols in natural extracts. *J. Anal. Methods Chem*. 1-7.

27 Kanazawa, A. 2001. Sterols in marine invertebrates. *Fish. Sci.* **67:** 997-1007.

28 Zinser, E., Paltauf, F., and Daum, G. 1993. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J Bacteriol*. **175**(10): 2853-2858.

29 Rúbia, C., Corrêa, G., Rosane, M., Peralta, Bracht, A., and Ferreira, I. C. F. R. 2017. The emerging use of mycosterols in food industry along with the current trend of extended use of bioactive phytosterols. *Trends in Food Sc. Tech.* **67**: 19-

35.

30 Yang, R., Xue, L., Zhang, L., et al. 2019. Phytosterol Contents of Edible Oils and Their Contributions to Estimated Phytosterol Intake in the Chinese Diet. *Foods*. **8**(8): 334.

31 Berg, J. M., Tymoczko, J.L., and Stryer, L. 2002. Biochemistry, 5th edition. New York: W H Freeman.

32 Shaner, R. L., Allegood, J. C., Park, H., et al. 2009. Quantitative analysis of sphingolipids for lipidomics using triple quadrupole and quadrupole linear ion trap mass spectrometers. *J. Lipid Res*. **50**(8): 1692-1707.

33 Boiten, W., Absalah, S., Vreeken, R., Bouwstra, J., and van Smeden, J. 2016. Quantitative analysis of ceramides using a novel lipidomics approach with three dimensional response modeling. *Biochim. Biophys. Acta.* **1861**: 1652-1661.

34 Schnaar, R. L., and Kinoshita, T. 2017 Glycosphingolipids. 2017. In: Varki, A., Cummings, R. D., Esko, J. D., et al. Essentials of Glycobiology, 3rd edition. New York. Cold Spring Harbor Laboratory Press.

35 Slotte, J. P. 2013. Biological functions of sphingomyelins. *Prog. Lipid Res*. **52**(4): 424-37.

36 Ikeda, K., Shimizu, T., and Taguchi, R. J. 2008. Targeted analysis of ganglioside and sulfatide molecular species by LC/ESI-MS/MS with theoretically expanded multiple reaction monitoring. *J. Lipid Res*. **49**(12): 2678-2689.

37 Raetz, C. R. H., and Whitfield, C. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem*. **71**: 635-700.

38 Sud, M., Fahy, E., Cotter, D., et al. 2007. LMSD: LIPID MAPS structure database. *Nucleic Acids Res*. **35**: D527–D532.

39 Orešič, M., Hänninen, V. A., and Vidal-Puig, A. 2008. Lipidomics: a new window to biomedical frontiers. *Trends Biotechnol*. **26**: 647-652.

40 Shevchenko, A., and Simons, K. 2010, Lipidomics: coming to grips with lipid diversity. *Nat. Rev. Mol. Cell Biol*. **11**: 593-598.

41 Han, X. and Gross, R. W. 2003. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: A bridge to lipidomics. *J. Lipid Res.* **44**: 1071-1079.

42 Lagarde, M., Geloen, A., Record, M., Vance, D. and Spener, F. 2003. Lipidomics is emerging. Biochim. *Biophys. Acta*. **1634**: 61.

43 Lee, S. H., Williams, M. V., DuBois, R. N. and Blair, I. A. 2003. Targeted lipidomics using electron capture atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **17**: 2168-2176.

44 Spener, F., Lagarde, M., Géloên, A., and Record, M. 2003. Editorial: What is lipidomics? *Eur. J. Lipid Sci. Tech*. **105**: 481-482.

45 Ding, H. R., Wang, J. L., Ren, H. Z., and Shi, X. L. 2018. Lipometabolism and Glycometabolism in Liver Diseases. *Biomed. Res. Int*. **2018**: 1287127.

46 Yan, F., Zhao, H., and Zeng, Y. 2018. Lipidomics: a promising cancer biomarker. *Clin. Transl. Med*. **7**(1):21.

47 White, T., Bursten, S., Federighi, D., Lewis, R. A., and Nudelman, E. 1998. High-resolution separation and quantification of neutral lipid and phospholipid species in mammalian cells and sera by multi-one-dimensional thin-layer chromatography. *Anal. Biochem*. **258**(1): 109-117.

48 Christie, W. W. and Han, X. Lipid Analysis - Isolation, Separation, Identification and Lipidomic Analysis, 4th edition. Bridgewater, Oily Press, 2010.

49 Seppanen-Laakso, T., Laakso, I., and Hiltunen, R. 2002. Analysis of fatty acids by gas chromatography, and its relevance to research on health and nutrition. *Anal. Chim. Acta*. **465**: 39-62.

50 Meneses, P., and Glonek, T. 1988. High resolution 31P NMR of extracted phospholipids. *J Lipid Res*. **29**: 679-689.

51 Philips, F. C., Erdahl, W. L., Schmit, J. A., and Prvett, O. S. 1984. Quantitative analysis of triglyceride species of vegetable oils by high perfromance liquid chromatogrpahy via a flame ionization detector. *Lipids.* **19**(11): 880-887.

52 Milne, S., Ivanova, P., Forrester, J., and Alex Brown, H. 2006. Lipidomics: an anlysis of cellular lipids by ESI-MS. *Methods*. **39**(2): 92-103

53 Wang, Z., Cai, Y., Wang, Y., Zhou, X., Zhang, Y., and Lu, H. 2017. Improved MALDI imaging MS analysis of phospholipids using graphene oxide as new matrix. *Sci. Rep*. **7**: 44466.

54 Lee, H. C., and Yokomizo, T. 2018. Applications of mass spektrometry-based targeted and non targeted lipidomics. *Biochem. Biophys. Res. Com.* **504**(3): 576-581.

55 Lam, S. M., and Shui, G. 2013. Lipidomics as a principal tool for advancing biomedical research. *J. Genet. Genomics*. **40**: 375-390.

56 Contrepois, K., Mahmoudi, S., Ubhi, B.K. et al. 2018. Cross-Platform Comparison of Untargeted and Targeted Lipidomics Approaches on Aging Mouse Plasma. *Sci. Rep.* **8**: 17747.

57 Cajka, T., and Fiehn, O. 2016. Toward Merging Untargeted and Targeted Methods inMass Spectrometry-Based Metabolomics and Lipidomics. *Anal. Chem*. **88**(1): 524-545.

58 Han, X. Lipidomics: Comprehensive Mass Spectrometry of Lipids. John Wiley & Sons, Inc. 2016.

59 Hu, C., van der Heijden, R., Wang, M., van der Greef, J., Hankemeier, T., and Xu, G. 2009. Analytical strategies in lipidomics and applications in disease biomarker discovery. *J Chromatogr. B*. **877**: 2836-2846.

60 Züllig, T., Trötzmüller, M. and Köfeler, H.C. 2020. Lipidomics from sample preparation to data analysis: a primer. Anal. Bioanal. Chem. 412: 2191–2209.

61 Hanson, C. Recent Advances in Liquid-Liquid Extraction. New York, Pergamon Press, 1971.

62 Reis, A., Rudnitskaya, A., Blackburn, G. J., Mohd Fauzi, N., Pitt, A. R., and Spickett, C. M. 2013. A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. *J. Lipid Res*. **54**(7): 1812-1824.

63 Folch, J., Lees, M., and Sloane Stanley, G. H. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**: 497-509.

64 Bligh, E. G. and Dyer, W. J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*. **37**: 911-917.

65 Matyash, V., Liebisch, G., Kurzchalia, T. V., Shevchenko, A., and Schwudke, D. 2008. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J. Lipid Res*. **49**(5): 1137-1146.

66 Triebl, A., Weissengruber, S., Trötzmüller, M., Lankmayr, E., and Köfeler, H. 2016. Quantitative analysis of N -acylphosphatidylethanolamine molecular species in rat brain using solid-phase extraction combined with reversed-phase chromatography and tandem mass spectrometry. *J. Sep. Sc*. **39**(13): 2474-2480.

67 Pellegrino, R. M., Di Veroli, A., Valeri, A., Goracci, L., and Cruciani, G. 2014. LC/MS lipid profiling from human serum: a new method for global lipid extraction. *Anal. Bioanal. Chem*. **406**(30), 7937-7948.

68 Sarafian, M. H., Gaudin, M., Lewis, M. R., Martin, F., Holmes, E., Nicholson, J. K. and Dumas, M. 2014. Objective Set of Criteria for Optimization of Sample Preparation Procedures for Ultra-High Throughput Untargeted Blood Plasma Lipid Profiling by Ultra Performance Liquid Chromatography–Mass Spectrometry. *Anal. Chem*. **86**(12): 5766-5774.

69 Pizarro, C., Arenzana-Rámila, I., Pérez-del-Notario, N., Pérez-Matute, P., and González-Sáiz, J. M. 2013. Plasma Lipidomic Profiling Method Based on Ultrasound Extraction and Liquid Chromatography Mass Spectrometry. *Anal. Chem*. **85**(24): 12085-12092.

70 Hennion, M. C. 1999. Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr. A*. **856**(1-2): 3–54.

71 Ruiz-Gutierrez, V., and Perez-Camino, M. C. 2000. Update on solid-phase extraction for the analysis of lipid classes and related compounds. *J. of Chromatogr. A*. **885**(1-2): 321-341.

72 Fauland, A., Trötzmüller, M., Eberl, A., Afiuni-Zadeh, S., Köfeler, H., Guo, X., and Lankmayr, E. 2013. An improved SPE method for fractionation and identification of phospholipids. *J. Sep. Sc*. **36**(4): 744-751.

73 Eller, F. J., and King, J. W. 1996. Determination of fat content in foods by analytical SFE. *Seminars in food analysis*. **1**: 145-165.

74 Ong, C. P., Lee, H. K., Li, S. F. 1990. Supercritical fluid extraction and chromatography of cholesterol in food samples. *J. Chromatogr. A*. **515**: 509-513.

75 Medina, A. L., Oseas da Silva, M. A., Barbosa, H., Zezzi Arruda, M. A., Masaioli Jr., A., and Bragagnolo, N. 2015. Rapid microwave assisted extraction of meat lipids. *Food Res. International*. **78**: 124-130.

76 Rezaee, M., Assadi, Y., Hosseini, M. R. M., Aghaee, E., Ahmadi, F. and Berijani, S. 2006. Determination of organic compounds in water using dispersive liquid–liquid microextraction. *J. Chromatogr. A*. **1116**(1): 1-9.

77 Teng, H., Chen, L., Huang, Q., Wang, J., Lin, Q., Liu, M., et al. 2016. Ultrasonic-Assisted Extraction of Raspberry Seed Oil and Evaluation of Its Physicochemical Properties, Fatty Acid Compositions and Antioxidant Activities. *PLoS ONE*. **11**(4): e0153457.

78 McDonald, J. G., Ivanova, P. T., and Brown, H. A. Approaches to lipid analysis. Biochemistry of Lipids, Lipoproteins and Membranes, pp. 41-71. Amsterda, Elsevier, 2015.

79 Kirchner, J. G., Miller, J. M., and Keller, G. J. 1951. Separation and identification of some terpenes by new chromatographic technique. *Anal. Chem.* **23**(3): 420-425.

80 Stahl, E. 1956. Thin-layer chromatography; methods, influencing factors and an example of its use. *Pharmazie.* **11**(10): 633-637.

81 Mangold, H. K. 1964. Thin-layer chromatography of lipids. *J. Am. Oil Chem. Soc*. **41**(11): 762-773.

82 Fuchs, B., Süß, R., Teuber, K., Eibisch, M., and Schiller, J. 2011. Lipid analysis by thinlayerchromatography-A review of the current status. *J. Chromatogr. A*. **1218**(19): 2754-2774.

83 Grob R., and Barry E. Modern Practice of Gas Chromatography, Wiley-Interscience, 2004.

84 Hubschmann, H. Handbook of GC/MS: Fundamentals and Applications. Verlag, Wiley, 2001.

85 Apon, J. M. B., and Nicolaides, N. 1975. The determination of the position isomers of the methyl branched fatty acid methyl esters by capillary GC/MS. *J. Chromatogr. Sci.* **13**: 467-473.

86 Hallgren, B., Ryhage, R. and Stenhagen, E. 1959. The mass spectra of methyl oleate, methyl linoleate and methyl linolenate. *Acta Chem. Scand.* **13**: 845-847.

87 Quehenberger, O., Armando, A. M., Dennis, E. A. 2011. High sensitivity quantitative lipidomics analysis of fatty acids in biological samples by gas chromatography-mass spectrometry. *Biochim. Biophys. Acta*. **1811**(11): 648-656.

88 Chiu, H. H., Kuo, C. H. 2020. Gas chromatography-mass spektrometry-based analytical strategies for fatty acid analysis in biological samples. *J. Food Drug Anal*. **28**(1): 60-73.

89 Mossoba, M. M., et al. Lipid Analysis and Lipidomics: New Techniques and Applications. Champaign, AOCS Publishing, 2006.

90 Meyer, V. R. Practical high-performance liquid chromatography. 2nd ed. John Wiley & Sons, Ltd., 2010.

91 Buchgraber, M., Ulberth, F., Emons, H., and Anklam, E. 2004. Triacylglycerol profiling by using chromatographic techniques. *Eur. J. Lipid Sci. Technol*. **106**: 621-648.

92 Novakova, L., and Dousa, M. Moderni HPLC separace v teorii a praxi I. 2013.

93 Brouwers, J.F. 2011. Liquid chromatographic-mass spectrometric analysis of phospholipids. Chromatography, ionization and quantification. *Biochim. Biophys. Acta*. **1811**: 763-775.

94 Sommer, U., Herscovitz, H.,Welty, F.K. and Costello, C.E. 2006. LC-MS-basedmethodfor the qualitative and quantitative analysis of complex lipid mixtures. *J. Lipid Res*. **47**: 804-814.

95 Buszewski, B., and Noga, S. 2012. Hydrophilic interaction liquid chromatography (HILIC) - a powerful separation technique. *Anal. Bioanal. Chem*. **402**(1): 231-247.

96 Alpert, A. 1990. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr. A*. **499**: 177-196.

97 Cifkova, E., Hajek, R., Lisa, M. and Holcapek, M. 2016. Hydrophilic interaction liquid chromatography-mass spectrometry of (lyso)phosphatidic acids, (lyso)phosphatidylserines and other lipid classes. *J. chromatogr A*. **1439**: 65-73.

98 Schwalbe-Herrmann, M., Willmann, J., and Leibfritz, D. 2010. Separation of phospholipid classes by hydrophilic interaction chromatography detected by electrospray ionization mass spectrometry. *J. Chromatogr A*. **1217**(32): 5179-5183.

99 Lisa, M., Cifkova, E., and Holcapek, M. 2011. Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography-mass spectrometry. *J Chromatogr A*. **1218**(31): 5146-5156.

100 Buchgraber, M., Ulberth, F., Emons, H., and Anklam, E. 2004. Triacylglycerol profiling by using chromatographic techniques. *Eur. J. Lipid Sci. Technol.* **106**: 621-648.

101 Nikolova-Damyanova, B., and Momchilova, S. 2002. Silver ion HPLC for the analysis of positionally isomeric fatty acids. *J. Liq. Chromatogr. R. T.* **25**(13-15): 1947-1965.

102 Sun, C., Black, B.A., Zhao, Y.Y., Ganzle, M.G. and Curtis, J.M. 2013 Identificationof conjugated linoleic acid (CLA) isomers by silver ion-liquid chromatography/in-lineozonolysis/mass spectrometry (Ag°æLC/O3-MS). *Anal. Chem*. **85**: 7345-7352.

103 Holcapek, M., Dvorakova, H., Lisa, M., Giron, A.J., Sandra, P. and Cvacka, J. 2010. Regioisomeric analysis of triacylglycerols using silver-ion liquid chromatographyatmosphericpressure chemical ionization mass spectrometry: Comparison of five differentmass analyzers. *J. Chromatogr. A*. **1217**: 8186-8194.

104 Laboureur, L., Ollero, M., and Touboul, D. 2015. Lipidomics by Supercritical Fluid Chromatography. *Int. J. Mol. Sci*. **16**(6): 13868-13884.

105 Senorans, F. J., and Ibanez, E. 2002. Analysis of fatty acids in foods by supercritical fluid chromatography. *Anal. Chim. Acta*. **465**: 131-144.

106 Lesellier E., and West C. 2015. The many faces of packed column supercritical fluid chromatography - A critical review. *J. Chromatogr. A*. **1382C**: 2-46.

107 Hyötyläinen, T., and Orešič, M. 2015. Analytical lipidomics in metabolic and clinical research. *Trends Endocrinol. Metab*. **26**: 671-673.

108 Tyurina, Y. Y., Tyurin, V. A., Kapralova, V. I., Wasserloos, K., MOsher, M., Epperly, M. W., Greenberger, J. S., Pitt, B. R., and Kagan, V. E. 2011. Oxidative lipidomics of γ-radiation-induced lung injury: mass spectrometric characterization of cardiolipin and phosphatidylserine peroxidation. *Radiat. Res*. **175**: 610-621.

109 Cajka, T., and Fiehn, O. 2014. Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry. *Trends Analyt. Chem*. **61**: 192-206.

110 Quehenberger, O., Armando, A. M., and Dennis, E. A. 2011. High sensitivity quantitative lipidomics analysis of fatty acids in biological samples by gas chromatography-mass spectrometry. *Biochim. Biophys. Acta*. **1811**(11): 648-656.

111 Toledano, R. M., Cortés, J. M., Andini, J. C., Vázquez, A., and Villén, J. 2012. On-line derivatization with on-line coupled normal phase liquid chromatography-gas chromatography using the through oven transfer adsorption desorption interface:

Application to the analysis of total sterols in edible oils. *J. Chromatogr. A*. **1256**: 191-196.

112 Holcapek, M., Ovcacikova, M., Lisa, M., Cifkova, E., and Hajek, T. 2015. Continuous comprehensive two-deminesional liquid chromatography-electrospray ionization mass spectrometry of complex lipidomic samples. *Anal. Bioanal. Chem.* **407**: 5033-5043.

113 Schwaiger, M., Schoeny, H., El Abiead, Y., Hermann, G., Rampler, E., and Koellensperger, G. 2019. Merging metabolomics and lipidomics into one analytical run. *Analyst*. **244**: 220-229.

114 Berkecz, R., Tomosi, F., Kormoczi, T., Szegedi, V., Horvath, J., and Janaky, T. 2018. Comprehensive phospholipid and sphingomyelin profiling of different brain regions in mouse model of anxiety disorder using online two dimensional (HILIC/RP)-LC/MS method. *J. Pharm. Biomed. Anal*. **149**: 308-317.

115 Urbina, J. A., and J. S. Waugh. 1974. Proton-enhanced 13C nuclear magnetic resonanceof lipids and biomembranes. *Proc. Natl. Acad. Sci*. **71**(12): 5062-5067.

116 Jeyarajah, E. J., Cromwell, W. C., and Otvos, J. D. 2006. Lipoprotein Particle Analysisby Nuclear Magnetic Resonance Spectroscopy. *Clin. Lab. Med*. **26**(4): 847-870.

117 Carrasco-Pancorbo, A., Navas-Iglesias, N., and Cuadros-Rodríguez, L. 2009. From lipidanalysis towards lipidomics, a new challenge for the analytical chemistry of the21st century. Part I: Modern lipid analysis," TrAC – Trends. *Anal. Chem*. **28**(3): 263-278.

118 Sotirhos, N., Herslof, B., and Kenne, L. 1986. Quantitative analysis of phospholipids by 31P-NMR. *J. Lipid Res*. **27**: 386-392.

119 Culeddu, N., Bosco, M., Toffain, R., and Pollesello, P. 1999. 31P NMR analysis of phospholipids in crude extracts from different sources: improved efficiency of the solvent system. *Magn. Reson. Chem*. **36**(12): 907-912.

120 Sethi, S., and Brietzke, E. 2017. Recent advances in lipidomics: Analytical and clinicalperspectives. *Prostaglandins Other Lipid Mediat.* **128-129**: 8-16.

121 Jurowski, K., Kochan, K., Walczak, J., Baranska, M., Piekoszewski, W., and Buszewski, B. 2017. Analytical Techniques in lipidomics: state of the art. *Crit. Rev. Anal. Chem.* **47**(5): 418-437.

122 Vongsvivut, J., Miller, M. R., McNaughton, D., Heraud, P., and Barrow, C. J. 2014. Rapid discrimination and determination of polyunsaturated fatty acid composition in marine oils by FTIR spectroscopy and multivariate data analysis. *Food Bioprocess Tech*. **7**: 2410-2422.

123 Oleszko, A., Hartwich, J., Wójtowicz, A., et al. 2017. Comparison of FTIR-ATR and Raman spectroscopy in determination of VLDL triglycerides in blood serum

with PLS regression. Spectrochimica acta. Part A. *Mol. Biomol. Spectr*. **183**: 239-246.

124 Meng, X., Pan, Q., Ding, Y., and Jiang, J. 2014. Rapid determination of phospholipid concent of vegetable oils by FTIR spectroscopy combined with partial least-square regression. *Food Chem*. **147**: 272-278.

125 Wu, H., Volponi, J. V., Oliver, A. E., Parikh, A. N., Simmons, B. A., and Singh, S. 2011. In vivo lipidomics using single-cell Raman spectroscopy. *Proc. Natl. Acad. Sci*. **108**: 3809-3814.

126 Yang, K. and X. Han. 2016. Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends Biochem. Sc*. **41**(11): 954-969.

127 Murphy, R.C., J.A. Hankin, and R.M. Barkley. 2009. Imaging of lipid species by MALDI mass spectrometry. *J. Lipid Res*. **50**: S317-S322.

128 Ståhlman, M., et al. 2009. High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectrometry. *J. Chromatogr. B*. **877**(26): 2664-2672.

129 Harkewicz, R. and Dennis, E.A. 2011. Applications of Mass Spectrometry to Lipids and Membranes. *Annu. Rev. Biochem.* **80**(1): 301-325.

130 Yang, K., and Han, X. 2016. Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends biochem.l Sci.* **41**(11): 954-969.

131 Han, X., Yang, K., and Gross, R.W. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. Mass Spectrom. Rev., 31, 134-178 (2012).

132 Wang, M., Wang, C., Han, R.H., and Han, X. 2016. Novel advances in shotgun lipidomics for biology and medicine. *Prog. Lipid Res*. **61**: 83-108.

133 Brugger, B., Erben, G., Sandhoff, R., Wieland, F.T. and Lehmann, W.D. 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry. *Proc. Natl. Acad. Sci*. **94**: 2339-2344.

134 Welti, R., and Wang, X. 2004. Lipid species profiling: a high-throughput approach to identify lipid compositional changes and determine the function of genes involved in lipid metabolism and signaling. *Curr. Opin. Plant Biol*. **7**: 337-344.

135 Han, X., Yang, K., and Gross, R. W. 2012. Multi-dimensional mass spectrometry-based shotgun lipidomics and novel strategies for lipidomic analyses. *Mass Spectrom. Rev*. **31**(1): 134-178.

136 Niessen, W. M. A. Liquid Chromatography-Mass Spectrometry. New York, Marcel Dekker Inc., 1999.

137 Hsu, F. F. 2018. Mass spectrometry-based shotgun lipidomics - a critical review

from the technical point of view. *Anal. Bioanal. Chem*. **410**(25): 6387-6409.

138 Rohner, T. C., Staab, D., and Stoeckli, M. 2005. MALDI mass spectrometric imaging of biological tissue sections. *Mech. Ageing Dev*. **126**(1): 177-185.

139 McDonnell, L. A., and Heeren, R. M. A. 2007. Imaging mass spectrometry. *Mass Spectrom. Rev.* **26**(4): 606-643.

140 Buchberger, A. R., DeLaney, K., Johnson, J., and Li, L. 2018. Mass Spectrometry Imaging: A Review of Emerging Advancements and Future Insights. *Anal. Chem*. **90**(1): 240-265.

141 Bai, H. R., Wang, S. J., Liu, J. J., Gao, D., Jiang, Y. Y., Liu, H. X., and Cai, Z. W. 2016. Localization of ginsenosides in Panax ginseng with different age by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry imaging. *J. Chromatogr. B Analyt.l Technol. Biomed. Life Sci.* **1026**: 263-271.

142 Jackson, S. N., Wang, H. Y., and Woods, A. S. 2005. Direct profiling of lipid distribution in brain tissue using MALDI-TOFMS. *Anal. Chem*. **77**(14): 4523-4527.

143 Jackson, S. N., Ugarov, M., Egan, T., et al. 2007. MALDI-ion mobility-TOFMS imaging of lipids in rat brain tissue. *J. Mass Spectrom*. **42**(8): 1093-1098.

144 Hankin, J. A., Farias, S. E., Barkley, R. M., et al. (2011). MALDI Mass spectrometric imaging of lipids in rat brain injury models. *J. Am. Soc. Mass Spectrom*. **22**: 1014.

145 Gemperline, E., Chen, B., and Li, L. 2014. Challenges and recent advances in mass spectrometric imaging of neurotransmitters. *Bioanalysis*. **6**(4): 525-540.

146 Blanksby, S. J., and Mitchell, T. W. 2010. Advances in mass spectrometry for lipidomics. *Annu. Rev. Anal. Chem*. **3**: 433-465.

147 Gamble, L. J., and Anderton, C. R. 2016. Secondary Ion Mass Spectrometry Imaging of Tissues, Cells, and Microbial Systems. *Micros. Today*. **24**(2): 24-31.

148 Alberici, R. M., Vendramini, P. H., and Eberlin, M. N. 2017. Easy ambient sonic-spray ionization mass spektrometry for tissue imaging. *Anal. Methods*. **9**(34): 5029-5036.

149 Skoog, D. A., Holler, F. J., and Crouch, S. R. Principles of Instrumental Analysis, sixth Edition. Thomson Brooks/Cole, USA, 2007.

150 Cajka, T., Hajslova, J., and Mastovska K. Mass spectrometry and hyphenated instruments in food analysis. Handbook of Food Analysis Instruments. S. Otles (ed.), CRC Press, Taylor & Francis Group (in press).

151 de Hoffman, E., and Stroobant, V. Mass Spectrometry: Principles and Applications, 2nd ed. John Wiley and Sons, 2001.

152 Herbert, C. G., and Johnstone, R. A. W. Mass spectrometry basics. CRC Press, USA, 2003.

153 Lafferty, M. C. Mass spectrometry of organic ions. Academic Press, New York, 1963.

154 Ryhage, R., and Stenhagen, E. 1960. Mass spectrometry in lipid research. *J. Lipid Res*. **1**: 361-390.

155 Duffin, K. L., Henion, J. D., and Shieh, J. J. 1991. Electrospray and tandem mass spectrometric characterization of acylglycerol mixtures that are dissolved in nonpolar solvents. *Anal. Chem*. **63**(17): 1781-1788.

156 Gross, J. H. Mass Spectrometry. Springer, Germany, 2004.

157 Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., Ng, K. F., Suen, M. W. M., and Tai, H. L. 2003. Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. *Clin. Biochem. Rev*. **24**: 3-12.

158 Snyder, A. P. Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry. American Chemical Society, Washington DC, 1996.

159 Byrdwell, W. C. 2001. Atmospheric pressure chemical ionization mass spectrometry for analysis of lipids. *Lipids*. **36**(4): 327-346.

160 Fauland, A., Kofeler, H., Trotzmuller, M., Knopf, A., Hartler, J., Eberl, A., Chitraju,C., Lankmayr, E. and Spener, F. 2011. A comprehensive method for lipid profilingby liquid chromatography-ion cyclotron resonance mass spectrometry. *J. Lipid Res*. **52**: 2314-2322.

161 Zubarev, R. A., and Makarov, A. 2013. Orbitrap Mass Spectrometry. *Anal. Chem.* ***85***(11): 5288-5296.

162 Holcapek, M., Jirasko, R., and Lisa, M. 2012. Recent developments in liquid chromatography-mass spektrometry related techniques. *J. Chromatogr. A*. **1259**: 3-15. Gross, J. Mass Spectrometry. Heidelberg, Springer, 2004.

163 Glish, G. L., and Burinsky, D. J. 2006. Hybrid mass spectrometers for tandem mass spektrometry. *J. Am Soc. Mass Spectr*. **19**(2): 161-172.

164 Tu, J., Zhou, Z., Li, T., and Zhu, Z. 2019. The emerging role of ion mobility-mass spectrometry in lipidomics to facilitate lipid separation and identification. *Trend Anal. Chem.* **116**: 332-339.

165 Pickens, W. L., Warner, R. R., Boissy, Y. L., Boissy, R. E., and Hoath, S. B. 2000. Characterization of vernix caseosa: water content, morphology, and elemental analysis. *J. Invest. Dermatol.* **115**(5): 875-81.

166 Visscher, M., Narendran, V., Pickens, W. et al. 2005. Vernix Caseosa in Neonatal Adaptation. *J. Perinatol.* **25**: 440-446.

167 Haubrich, K. A. 2003. Role of vernix caseosa in the neonate: potential application in the adult population. *AACN Clin. Issues*. **14**: 457-464.

168 Hoath, S. B., W. L. Pickens, and M. O. Visscher. 2006. The biology of vernix caseosa. *Int. J. Cosmet. Sci.* **28**: 319-333.

169 Singh, G., and G. Archana. 2008. Unraveling the mystery of vernix caseosa. *Indian J. Dermatol*. **53**: 54-60.

170 Wang, D. H., Ran-Ressler, R., St Leger, J., Nilson, E., Palmer, L., Collins, R., et al. 2018. Sea lions develop human-like vernix caseosa delivering branched fats and squalene to the GI tract. *Sci Rep*. **8**(1): 7478.

171 Boiten, W. A., Berkers, T., Absalah, S., van Smeden, J., Lavrijsen, A. P. M., and Bouwstra, J. A. 2018. Applying a vernix caseosa based formulation accelerates skin barrier repair by modulating lipid biosynthesis. *J. Lipid Res*. **59**(2): 250-260.

172 Eichenfield, L. F., Frieden, I. J., Zaenglein, A., and MAthes, E. Neonatal and infant dermatology E-book. Elsevier Health Science. 2014.

173 Lawrence, F., Eichenfield, I., Frieden, J., and Nancy, B. 2008. Esterly Neonatal dermatology, 2nd ed. Saunders, Philadelphia, 2008.

174 Hardman, M. J., Moore, L., Ferguson, M. W. J., and Byrne, C. 1999. Barrier formation in the human fetus is patterned. *J. Invest. Derm*. **113**: 1106-1113.

175 Haubrich, K. A. 2003. Role of vernix caseosa in the neonates: potential application in the adult population. *AACN Clin. Iss.* **14**: 457-464.

176 Hoath, S. B., Narendran, V., and Visscher, M. O. 2001. The Biology and Role of Vernix. *Newborn Infant Nurs. Rev*. **1**(1): 53-58.

177 Visscher, M. O., Narendran, V., Pickens, W., Laruffa, A. A., Meinzen-Derr, J., Allen, K., and Hoath, S. 2005. Vernix caseosa in neonatal adaptation. *J. Perinatol*. **7**: 440-446.

178 Oranges, T., Dini, V., and Romanelli, M. 2015. Skin Physiology of the Neonate and Infant: Clinical Implications. *Adv. Wound Care (New Rochelle)*. **4**(10): 587-595.

179 Narendran, V., Hull, W. M., Akinbi, H. T., Whitsett, J. A., Pickens, W. L., Lambers, D. S., et al. 2000. Vernix caseosa contains surfactant proteins: Potential role in innate immune function in the fetus. *Pediatr. Res*. **47**(4):420a-a.

180 Pickens, W., Warner, R., Boissy, Y., Boissy, R., and Hoath, S. 2000. Characterization of vernix caseosa: water content, morphology, and elemental analysis. *J. Invest. Dermatol.* **115**: 875-881.

181 Singh, G., and Archana, G. 2008. Unraveling the mystery of vernix caseosa. *Indian. J. Dermatol*. **53**(2): 54-60.

182 Wickett, R. R., and Visscher, M. O. 2006. Structure and function of the epidermal barrier. *Am. J. Infection Control*. **34**(10): S98-S110.

183 Schmid, R. 1939. Notizen zur kenntnis der vernix caseosa. *Arch. Gynakol*. **168**:445–50.

184 Rissmann, R., Groenink, H. W. W., Weerheim, A. M., Hoath, S. B., Ponec, M., and Bouwstra, J. A. 2006. New insights into ultrastructure, lipid composition and organization of vernix caseosa. *J. Invest. Dermatol*. **126**: 1823-1833.

185 Nicolaides, N. 1974. Skin lipids: their biochemical uniqueness. *Science.* **186**(4158): 19-26.

186 Tollin, M., et al. 2006. Proteome Analysis of Vernix Caseosa. *Pediatr. Res*. **60**(4): 430-434.

187 Yoshio, H., Tollin, M., Gudmundsson, G. et al. 2003. Antimicrobial Polypeptides of Human Vernix Caseosa and Amniotic Fluid: Implications for Newborn Innate Defense. *Pediatr. Res.* **53:** 211-216.

188 Akinbi, H. T., Narendran, V., Pass, A. K., Markart, P., and Hoath, S. B. 2004. Host defense proteins in vernix caseosa and amniotic fluid. *Am. J. Obstet. Gynecol.* **191**: 2090-2096.

189 Tollin, M., et al. 2005. Vernix Caseosa as a Multi-Component Defence System Based onPolypeptides, Lipids and Their Interactions. *Cell. Mol. Life Sci.* **62**(19-20): 2390-2399.

190 Baker, S. M., Balo, N. N., and Abdel, A. F. T. 1995. Is vernix caseosa a protective material to the newborn?A biochemical approach. *Indian. J. Pediatr*. **62**(2): 237-9.

191 Buchman, A. L. 1996. Glutamine: is it a conditionally required nutrient for the human gastrointestinal system? *J. Am. Coll. Nutr*. **15**(3): 199-205.

192 Narendran, V., Wickett, R., Pickens, W. et al. 2000. Interaction Between Pulmonary Surfactant and Vernix: A Potential Mechanism for Induction of Amniotic Fluid Turbidity. *Pediatr. Res.* **48**: 120-124.

193 Li, J., Langkamp-Henken, B., Suzuki, K., and Stahlgren, L. H. 1994. Glutamine prevents parenteral nutrition induced increases in intestinal permeability. *J. Parent. Enter. Nutr*. **18**: 303-307.

194 Joglekar, V. M. 1980. Barrier properties of vernix caseosa. *Arch. Dis. Child*. **55**: 817-819.

195 Pickens, W., Zhou, Y., Wickett, R., Visscher, M., and Hoath, S. 2000. Antioxidant defense mechanisms in vernix caseosa: potential role of endogenous vitamin E. *Pediatr. Res*. **47**(4): 425A.

196 Moraille, R., Pickens, W. L., Visscher, M. O., and Hoath, S. B. 2005. A novel role for vernix caseosa as a skin cleanser. *Biol. Neonate*. **87**(1): 8-14.

197 Zhukov, W., E. Neverova, K. Nikitin and e. al. 1992. A comparative evaluation of the use of caseosa and solcoseryl in treating patients with trophic ulcers of the lower extremities. *Vestnik Khirurgii Imeni I. Grekova*. **148**: 339-341.

198 Tansirikongkol, A., VIsscher, M. O., and Wickett, R. R. 2007. Water-handeling properties of vernix caseosa and a synthetic analogue. *J. Cosmet. Sci.* **58**(6): 651-662.

199 Rissmann, R., Oudshoorn, M. H., Zwier, R., Ponec, M., Bouwstra, J. A., and Hennink, W. E. 2009. Mimicking vernix caseosa – preparation and characterization of synthetic biofilms. *Int. J. Pharm*. **372**(1-2): 59-65.

200 Šubčíková, L., Hoskovec, M., Vrkoslav, V., et al. 2015. Analysis of 1,2-diol diesters in vernix caseosa by high-performance liquid chromatography - atmospheric pressure chemical ionization mass spectrometry. *J Chromatogr A*. **1378**: 8-18.

201 Stewart, M. E., and Downing, D. T. 1981. Separation of Wax Esters from Steryl Esters by Chromatography on Magnesium Hydroxide. *Lipids.* **16**: 355-359.

202 Nicolaides, N. 1970. Magnesium Oxide as an Adsorbent for the Chromatographic Separation of Molecules According to their Degree of Flatness, e.g. the Separation of Wax Esters from Sterol Esters. *J. Chromatogr. Sci*. **8**:717-720.

203 Butovich, I. A. 2010. Fatty acid composition of cholesteryl esters of human meibomian gland secretion. *Steroids*. **75**(10): 726-733.

204 Butovich, I. A., Wojtowitz, J. C., and Molai, M. 2009. Human tear film and meibum. Very long chain wax esters and (O-acyl)-omega-hydroxy fatty acids of meibum. *J. Lipid Res*. **50**: 2471-2485.

205 Holčapek, M., Lísa, M., Jandera, P., and Kabátová, N. 2005. Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. *J. Sep. Sci.* **28***:* 1315-1333.

206 Vrkoslav, V., Urbanová, K., and Cvačka, J. 2010. Analysis of wax ester molecular species by high performance liquid chromatography/atmospheric pressure chemical ionisation mass spectrometry. *J. Chromatogr. A*. **1217***:* 4184-4194.

207 Haahti, E., Nikkari, T., Salmi, A. M., and Laaksonen, A. L. 1961. Fatty acids of vernix caseosa. *Scand. J. Clin. Lab. Invest.* **13***:* 70-73.

208 Mikova, R., Vrkoslav, V., Hanus, R., Hakova, E., Habova, Z, DOlezal, A., Plavka, R., Coufal, P., and Cvacka, J. 2014. Newborn boys and girls differ in the lipid composition of vernix caseosa. *Plos One*. **9**: e99173.

209 Hauff, S., and Vetter, W. 2010. Exploring the fatty acids of vernix caseosa in form of their methyl esters by off-line coupling of non-aqueous reversed phase high performance liquid chromatography and gas chromatography coupled to mass spectrometry. *J. Chromatogr. A*. **1217***:* 8270–8278.

210 Stransky, K., and Jursik, T. 1996. Simple quantitative transesterification of lipids, 1. Introduction. *Fett-Lipid*. **98**(2): 65-71.

211 Mori, N., Fukano, Y., Arita, R., Shirakawa, R., Kawazu, K., Nakamura, M., Amano, S. 2014 Rapid identification of fatty acids and (O-acyl)-*ω*-hydroxy fatty acids in human meibum by liquid chromatography/high-resolution mass spectrometry. *J. Chromatogr. A*. **1347**: 129-136.

212 Christie, W. W. Mass spectrometry of methyl esters: hydroxy fatty acids - trimethylsilyl derivatives. 2016. http://www.lipidhome.co.uk/ms/methesters/me-hydroxy-2/index.htm. Accessed 5 Jan 2017.

213 Chapkin, R. S., Ziboh, V. A. Marcelo, C. L., and Voorhees, J. J. (1986). Metabolism of essential fatty acids by human epidermal enzyme preparations: evidence of chain elongation. *J. Lipid. Res*., **27**: 945–954.

214 Hirabayashi, T., Anjo, T., Kaneko, A., Senoo, Y., Shibata, A., Takama, H., Yokoyama, K., Nishito, Y., Ono, T., Taya, C., Muramatsu, K., Fukami, K., Muñoz-Garcia, A., Brash, A. R., Ikeda, K., Arita, M., Akiyama, M., and Murakami, M. (2017). PNPLA1 has a crucial role in skin barrier function by directing acylceramide biosynthesis. *Nat. Commun*, **8**: 14609.

215 Chen, J., Green-Church, K. B., and Nichols, K. K. (2010). Shotgun lipidomic analysis of human Meibomian gland secretions with electrospray ionization mass spectrometry. *Invest. Ophthalmol. Vis. Sci*., **51**: 6220–6231.

216 Butovich, I. A. (2011). Lipidomics of human Meibomian gland secretions: chemistry, biophysics, and physiological role of Meibomian lipids. *Prog. Lipid Res.*, **50**: 278–301.

217 Wood, P. L., Scoggin, K., Ball, B. A., Troedsson, M. H., and Squires, E. L. (2016). Lipidomics of equine sperm and seminal plasma: identification of amphiphilic (O-acyl)-ω-hydroxy fatty acids. *Theriogenology*, **86**(5): 1212–1221.

218 Wood, P. L., Ball, B. A., Scoggin, K., Troedsson, M. H., and Squires, E. L. (2018). Lipidomics of equine amniotic fluid: identification of amphiphilic (O-acyl) ω-hydroxy fatty acids. *Theriogenology*, **105**: 120–125.

219 Kaluzikova, A., Vrkoslav, V., Harazim, E., Hoskovec, M., Plavka R., Budesinsky, M., Bosakova, Z., and Cvacka, J. (2017). Cholesteryl esters of ω-(O-acyl)0hydroxy fatty acids in vernix caseosa. J. Lipid Res., **58**(8): 1579–1590.

220 Oku, H., K. Mimura, Y. Tokitsu, K. Onaga, H. Iwasaki,and I. Chinen.
(2000). Biased distribution of the branched-chain fatty acids in ceramides of vernix

221 Opalka, L., Kovacik, A., Maixner, J., and Vavrova K. (2016). Omega-O-acyl ceramides in skin lipid membranes: effects of concentration, sphingoid base, and model complexity on microstructure and permeability. *Langmuir*, **32**(48): 12894–12904.

222 Schuett, B. S., and Millar, T. J. (2013). An investigation of the likely role of (O-acyl)- ω -hydroxy fatty acids in meibomian lipid films using (O-acyl) ω-hydroxy palmitic acid as a model. *Exp. Eye Res.*, **115**: 57–64.

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