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Development of a semi-targeted lipidomic method for clinical applications

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Abstract

Lipids are a diverse and ubiquitous group of biomolecules incorporating more than 180,000 different molecular species. In general, lipids are considered as amphipathic or hydrophobic small molecules that are produced partially or entirely by carbocation/carbanion-based condensations of isoprene/thioester units, respectively. According to structure variation, lipids are divided into eight categories: fatty acids (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), saccharolipids (SL), and polyketides (PK, derived from the condensation of ketoacyl subunits); and sterol lipids (ST) and prenol lipids (PR, derived from the condensation of isoprene subunits). The role of lipids has been traditionally associated with energy storage, structural components of cells and hormonal systems. The hydrophobic and hydrophilic portion of polar lipids makes them ideal for constituting the cellular membrane. In addition to constituting membranes, lipids provide membranes with the potential for budding, tubulation, fission and fusion, characteristics that are essential for cell division, biological reproduction and intracellular membrane trafficking. It is estimated that around 50% or more of the mass of most mammalian cell membranes is composed of lipids. The most abundant class of lipids in structuring cell membrane are glycerophospholipids, which accounts for more than 50 % of membrane lipids, and some other classes of lipids, like sphingolipids and non-polar lipids sterols, also constitute as main classes in the cell membrane. Lipids also play a major role in the human body for energy storage, and produce great energy upon oxidation from their relatively reduced state. Triacylglycerol and steryl esters play a principal role in storing caloric reserves and collecting fatty acid and sterol components for membrane synthesis.

Lipids have lately received great attention due to the critical roles lipid metabolism play in human diseases. It is illustrated that lipids promote the formation of inflammation, cancer, metabolic disease, Alzheimer and neurodegenerative disease. These are related to lipids function as the first and second messengers in signal transduction and molecular recognition processes. For example, membrane lipids are considered binding sites for cytoplasmic proteins and they can initiate a degradation process that gives rise to cleavage products that act as ligands or substrates for other signalling molecules. Also, cytoplasmic lipids can serve as ligands for proteins and allow them to associate intimately with the membrane. These lipids are highly regulated and have an impact on various physiological functions. For example, Ceramide and sphingosine promote apoptosis while sphingosine 1-phosphate stimulates cell growth and inhibits apoptosis. As a result, the role of sphingosine as a messenger of apoptosis is important. Many diseases result from aberrant communication between cells and thus the precise balance of signalling lipids and their effects can be employed to discover biomarkers of diseases. To date, considerable research has been conducted to explore the mechanism of lipid signalling and critical signalling of lipids that are directly related to these diseases. Lipid signalling (LS) transmit through

nuclear hormone receptors, such as peroxisome proliferator-activated receptors (PPARs), farnesoid X receptor (FXR) and liver X receptor (LXR). Lipids also convert to active chemokines or cytokines through lipid-modifying enzymes, such as phospholipase A2 (PLA2), cyclooxygenase (COX), 5-lipoxygenase (5-LO), sphingosine kinase (SphK), phosphatidylinositol 3-kinase (PI3K) and sphingomyelinase (SMase). To inhibit lipid signalling-promoting disease, numerous drugs have been developed to target nuclear receptors and lipid-modifying enzymes (for example, aspirin and other non-steroidal anti-inflammatory drugs block COX to treat inflammatory). However, the mechanism of lipid roles in some diseases, such as Alzheimer's and type II diabetes/ metabolic syndrome, remains an ongoing challenge.

Due to the potential of lipids related to many diseases, lipidomics research has grown rapidly in the last twenty years. Despite these considerable advantages, the exploration of critical lipid biomarkers in disease is still difficult due to the limitation of the analytical instrument. Nowadays, the most frequently used analyse instrument is liquid chromatography coupled to mass spectrometry (LC-MS), and it shows a remarkable improvement in identification sensitivity and resolution towards lipid biomarkers compared to thin layer chromatography(TLC), liquid chromatography(LC), nuclear magnetic resonance (NMR) and other instruments. To explore reliable lipid biomarkers, many clinical experiments have been conducted to identify lipids that exist in human plasma, and the concentration changes between patients and healthy controls. Quehenberger*et al.* defined the human plasma lipidome and their concentrations with different extraction methodologies for six main categories of lipids. Untargeted lipidomics concentrates on identifying all the lipids that appeared in the disease sample, which usually losses lipid identification and accurate concentration change of these lipids. On the other hand, targeted lipidomics present the concentration change of the lipids, but due to the instrument stability and experiment complexity, it usually restricts to one class of lipids and is unlikely to expand into hundreds of lipids.

To fill the gap between untargeted lipidomics and targeted lipidomics, a 'semi-targeted' lipidomics method is proposed to monitor the concentration fold change of hundreds of lipids in the human body. A semi-targeted approach will explore the relationship between different classes of lipids and potential pathways of certain diseases, as well as the relative change of each lipid at the same time. However, this method is to some extent ambiguous as the lack of reliable lipid identification can limit the analytical confidence in the profiling data. To overcome this problem, it was decided to use a comprehensive and confirmed list of lipids in human plasma that have been quantified in a widely accepted definitive published paper and build up an unambiguous lipid database. The plan is to optimise the method by comparing the extraction method, mobile phase proportion, gradient, running time and different columns. Therefore, a pre-defined list of lipids with known formula, exact mass and the fold change of these lipids was analysed to study specific diseases in order to monitor disease onset and

progress and to identify diagnostic lipid biomarkers. These biomarkers can be used as new targets for chemotherapy and the development of new drugs.

Declaration

I hereby declare that the content of this PhD thesis is the presentation of my original research work and efforts, and has not been submitted anywhere else for any other qualification. Where contributions of others are involved, every effort has been made to indicate clearly the extent of their contribution, with due reference to the literature and acknowledgement of collaborative research. The work was done under the supervision of Professor David Barrett and Dr. Dong-Hyun Kim, centre for analytical bioscience, school of Pharmacy, University of Nottingham, UK.

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List of Abbreviations

5-LO	5-lipoxygenase
AD	Alzheimer's disease
AGC	Automatic gain control
ANOVA	Analysis of variance
APCI	Atmosphere pressure chemical ionization
ASCA	Analysis of variance-Simultaneous Component Analysis
ATP	Adenosine triphosphate
BHT	3, 5-di-tert-butyl-4-hydroxytoluene
BMI	Body mass index
CACT	Carnitine acylcarnitine translocase
CAD	Coronary artery disease
CE	Collision energy
Cer	Ceramides
CerG1	Glucosylceramides
CGM	Continuous glucose monitoring
Ch	Cholesterol
CHCl3	Chloroform
ChE	Cholesteryl esters
CHO	Chinese hamster ovarian
CI	Chemical ionization
CL	Cardiolipin
CNS	Central nervous system
CON:CR	Control/caloric restriction
CoQ	Coenzyme
COX	Cyclooxygenase
CPT1	Carnitine palmitoyl transferase 1

CPT2	Carnitine palmitoyl transferase 2
CSCs	Cancer stem cells
CV	Coefficient of variation
CV	Coefficient of variation
DG	Diacylglycerols
DGDG	Glycosyldiacylglycerols
dhCer	Dihydroceramide
DM	Diabetes mellitus
dMePE	Dimethyl phosphatidylethanolamine
DXA	Dual-energy X-ray absorptiometry
EI	Electron ionization
ESI	Electrospray ionization
eTRF	Early time-restricted feeding
FA	Fatty acid
FAB	Fast atom bombardment
FAO	Fatty acid oxidation
FDA	Food Drug Administration
FDR	False discovery rate
FFA	Free fatty acid
FPG	Fasting plasma glucose
FXR	Farnesoid X receptor
GBM	Glioblastoma multiforme
GC	Gas chromatography
GL	Glycerolipids
GluCer	Glucosylceramide
GP	Glycerophospholipids
HDL	High-density lipoprotein

HILIC	Hydrophilic interaction liquid chromatography
HIV	Human immunodeficiency virus
HMDB	Human metabolome database
HPLC	High-performance liquid chromatography
HPLC-HRMS	High-performance liquid chromatography-tandem high-resolution mass spectrometry method
IDF	International diabetes federation
IDH1	Isocitrate dehydrogenase
ILCNC	International Lipid Classification and Nomenclature Committee
IS	Internal standard
LacCer	Lactosylceramide
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LDL	Low-density lipoprotein
LdMePE	Lysodimethylphosphatidylethanolamine
LGG	Low-grade glioma
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
LPA	Lysophosphatidic acids
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamines
LXR	Liver X receptor
MALDI	Matrix-assisted laser desorption ionisation
MeOH	Methanol
MG	Monoacylglycerols
MGDG	Glycosyldiacylglycerols

MRI	Magnetic Resonance Imaging
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether
NF	Neurofibrin
NMR	Nuclear magnetic resonance
NP-LC	Normal phase liquid chromatography
OPLSDA	Orthogonal-partial least square-discriminant analysis
OS	Overall survival
PA	Physical activity
PA	Phosphatidic acids
PC	Phosphatidylcholines
PC	Phosphatidylcholines
PCA	Principal Component Analysis
PCA	Principal component analysis
PE	Phosphatidylethanolamines
PFS	Progression-free survival
PG	Phosphatidylglycerols
PI	Phosphatidylinositols
PI3K	Phosphatidylinositol 3-kinase
PK	Polyketides
PLA2	Phospholipase A2
PPARs	Peroxisome proliferator-activated receptors
PR	Prenol lipids
PS	Phosphatidylserines
QA	Quality assurance
QC	Quality control

QMC	Queen's medical centre
RP-LC	Reversed-phase liquid chromatography
RPLC-MS	Reversed-phase liquid chromatography-mass spectrometry
RSD	Relative standard deviation
RT	Retention time
S1P	Sphingosine-1-phosphate
SFC	Supercritical fluid chromatography
SIE	Sitosteryl ester
SIM	Selected ion monitoring
SL	Saccharolipids
SM	Sphingomyelin
SMase	Sphingomyelinase
SO	Sphingosine
SP	Sphingolipids
SPE	Solid-phase extraction
SphK	Sphingosine kinase
SQDG	Glycosyldiacylglycerols
ST	Sterol lipids
StE	Stigmasterel ester
T2DM	Type 2 diabetes mellitus
TG	Triacylglycerols
TIC	Total ion count
TLC	Thin layer chromatography
TQMS	Triple quadruple tandem mass spectrometer
TSC	Tuberous sclerosis
UPLC/MS	Ultra-performance liquid chromatography coupled to mass spectrometry

VIP	Variable important in projection
ZyE	Zymosteryl ester

Chapter One

General introduction about lipidomics and analytical bioscience

1. Introduction

1.1. General introduction of lipids

Lipids are a large group of biomolecules, which have more than 180,000 different molecular species [1], including phospholipids, monoglycerides, diglycerides, fats, waxes, sterols and fat-soluble vitamins. All of them play a role in controlling cellular processes and signalling diseases [2]. In general, lipids are considered as amphipathic or hydrophobic small molecules that are produced partially or entirely by carbocation/carbanion-based condensations of isoprene/thioester units, respectively [3], [4].

The function of lipids can be generally divided into three parts. First, it can be used for energy storage as a result of its reduced state. Second, the hydrophobic and hydrophilic portion of polar lipids makes it ideal for constituting the cellular membrane. It is estimated that around 50% or more of the mass of most mammalian cell membranes is composed of lipids [5]. The amphipathic lipids not only help cells to communicate with the external environment and work as a barrier, but also help in maintaining an electrochemical gradient across the membrane and any alteration to its composition will greatly affect its function. For example, the addition of shorter and/or unsaturated fatty acid chains affects the ability of membrane lipids to pack against each other which render the membrane less rigid and more permeable[5], [6]. Finally, lipids can act as the first and second messengers in signal transduction and molecular recognition processes [7]. It has been reported that there are various changes in lipids and lipid metabolic pathways in many diseases, including cancer[8], inflammation[9], metabolic disease[10], cardiovascular disease[11], neurodegeneration[12] and many others. Thus, signalling lipids may play a key role in disease development and show huge potential in disease detecting. Besides the three major functions, lipids also play a role in anti-bacteria, anticancer and so on, the detail function of each class of lipids were shown in Table 1.1. Therefore, monitoring lipid change can provide valuable information that could improve our understanding regards different pathological conditions and guide the treatment therapy plan more efficiently.

1.2. Lipid classification

Lipids are a group of natural compounds that exist widely in the natural world, including plants, animals, bacteria, fungi, algae, and marine organisms. Fahy and colleagues have classified lipids according to their structures[13]. In this classification, lipids can be divided into eight categories and the main characteristics of each kind of lipid are shown below (Table 1.1).

In 2005, a comprehensive classification system for lipids was developed by the International Lipid Classification and Nomenclature Committee (ILCNC) which divides lipids into eight distinct categories [4], [13]. Each category is further divided into classes and subclasses. An example of each group and its chemical structure is shown in Figure 1.1.

According to structure variation, lipids are divided into eight categories: fatty acids (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), saccharolipids (SL), and polyketides (PK,

Table 1.1. Lipid categories, representations and functions (adapted from Fahy et al [13].)

lipid category	representations	functions
fatty acid (FA)	linoleic acid (LA, ω6 acid) and α-linolenic acid (ALA, ω3 acid)	plays an important role in the structure and function of cell membranes
glycerolipids (GL)	monoradylglycerols, diradylglycerols, triadylglycerols, glycosylmonoradylglycerols, glycosyldiradylglycerols, etc.	energy storage
glycerophospholipids (GP)	phosphatidylcholine (also known as PC, GPCho or lecithin), phosphatidylethanolamine (PE or GPEtn) and phosphatidylserine (PS or GPSer)	key component in cell membrane, involved in metabolism and cell signalling
sphingolipids (SP)	sphingomyelins (ceramide phosphocholines), ceramide phosphoethanolamines	signal transmission and cell recognition
saccharolipids (SL)	monogalactosyldiacylglycerols, digalactosyldiacylglycerols and sulfoquinovosyldiacylglycerols	component of membrane bilayers
polyketides (PK)	erythromycins, tetracyclines, avermectins, etc	Anti-microbial, anti-parasitic, and anti-cancer agents
sterol lipids (ST)	cholesterol and its derivatives	important component of membrane lipids, hormones and signalling molecules
prenol lipids (PR)	polyterpenes, quinones and hydroquinones, etc.	antibiotic, antifungal, anticancer, antiparasitic, cytostatic, coccidiostats, antichlesteremic, immunosuppressive, insecticide, and animal growth promote properties

Lipid categories and representative lipids of each group of lipids were shown above. The function of these lipids was also added to the table.

derived from the condensation of ketoacyl subunits); sterol lipids (ST) and prenol lipids (PR, derived from the condensation of isoprene subunits) [13]. The role of lipids has been traditionally associated with energy storage, structural components of cells and hormonal systems [14]. The hydrophobic and hydrophilic portion of polar lipids makes them ideal for constituting the cellular membrane. In addition to constituting membranes, lipids provide membranes with the potential for budding, tubulation, fission

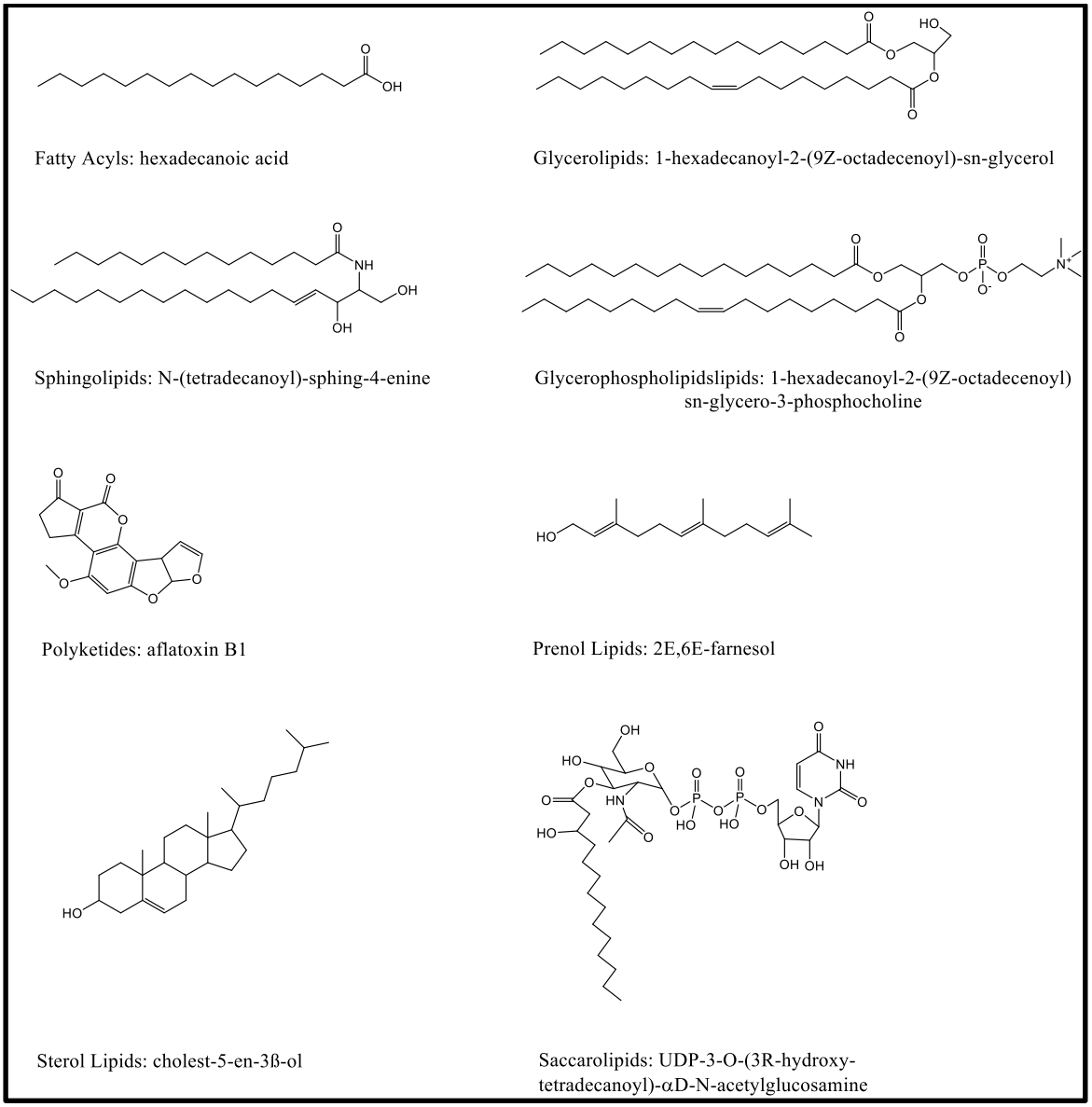


Figure 1.1. *representative structures for the eight lipid categories (adapted from Fahy et al* [4]*).*

and fusion, characteristics that are essential for cell division, biological reproduction and intracellular membrane trafficking. It is estimated that around 50% or more of the mass of most cell membranes is composed of lipids. The most abundant class of lipids in structuring cell membrane are glycerophospholipids, which accounts for more than 50 % of membrane lipids, and some other classes of lipids, like sphingolipids and non-polar lipids sterols, also constitute as main classes in the cell membrane [15]. Lipids also play a major role in the human body for energy storage, and produce great energy upon oxidation from their relatively reduced state. Triacylglycerol and sterol esters play a principal role in storing caloric reserves and collecting fatty acid and sterol components for membrane synthesis [16].

1.3. Lipid biochemical roles

Lipids play a number of key roles in living organisms, especially in energy storage, cellular membranes, endocrine actions and in cell signalling and transduction. For example, lipids play a critical role in cell function, especially in the formation of the bilayer membrane that acts as a barrier between cells and the outside environment or between internal organelles from the cytoplasm, it is estimated that more than 50% of the mass of cell membranes is composed of lipids [17], [18]. This lipid barrier helps in maintaining an electrochemical gradient across the membrane and any alteration to its composition will greatly affect its function. For example, the addition of shorter and/or unsaturated fatty acid chains affects the ability of membrane lipids to pack against each other which render the membrane more permeable [17], [19]. In addition, lipids play an important role in intracellular membrane transport by the generation of positive or negative curvature. Positive curvature of lipid monolayer is defined by a bulge in the direction of the polar heads and negative curvature is defined by a bulge in the opposite direction. Several mechanisms have been proposed to explain the generation of positive or negative curvatures including changes to the membrane lipid composition or by recruiting specialised proteins to the sites of fission and fusion where lipid head groups serve as attachment sites for peripheral membrane proteins which are necessary for curvature generation that affects several cell functions such as cell division and intracellular membrane trafficking [20]. Moreover, lipids are indispensable compounds in signal transduction between cells [21]. Membrane and cytoplasmic lipids can serve as binding sites for cytoplasmic proteins and they can initiate a degradation process that gives rise to cleavage products that act as ligands or substrates for other signalling molecules. These lipids are highly regulated and have an impact on various physiological functions. For example, Ceramide and sphingosine promote apoptosis while sphingosine 1-phosphate stimulates cell growth and inhibits apoptosis. As a result, the role of sphingosine as a messenger of apoptosis is important. Many diseases result from aberrant communication between cells and thus the precise balance of signalling lipids and their effects can be employed to discover biomarkers of disease.

1.4. Lipid and diseases

Lipids have lately received great attention due to the critical roles lipid metabolism plays in human diseases. It is illustrated that lipids promote the formation of inflammatory [22], cancer[23]–[25], metabolic disease [26], Alzheimer and neurodegenerative disease [27]. These are related to lipids function as the first and second messengers in signal transduction and molecular recognition processes. For example, membrane lipids are considered binding sites for cytoplasmic proteins and they can initiate a degradation process that gives rise to cleavage products that act as ligands or substrates for other signalling molecules. Also, cytoplasmic lipids can serve as ligands for proteins and allow them to associate intimately with the membrane. These lipids are highly regulated and have an impact on various physiological functions. For example, Ceramide and sphingosine promote apoptosis while sphingosine 1-phosphate stimulates cell growth and inhibits apoptosis. As a result, the role of sphingosine as a messenger of apoptosis is important [28]. Many diseases result from aberrant

communication between cells and thus the precise balance of signalling lipids and their effects can be employed to discover biomarkers of disease [29]–[31]. To date, considerable researches have been conducted to explore the mechanism of lipid signalling and critical signalling lipids that are directly related to these diseases [29], [32], [33]. Lipid signalling (LS) transmit through nuclear hormone receptors, such as peroxisome proliferator-activated receptors (PPARs) [34], farnesoid X receptor (FXR) and liver X receptor (LXR) [35]. Lipids also convert to active chemokines or cytokines through lipid-modifying enzymes, such as phospholipase A2 (PLA2), cyclooxygenase (COX), 5-lipoxygenase (5-LO), sphingosine kinase (SphK), phosphatidylinositol 3-kinase (PI3K) and sphingomyelinase (SMase) [2]. To inhibit lipid signalling-promoting disease, numerous drugs have been developed to target nuclear receptors and lipid-modifying enzymes (for example, aspirin and other non-steroidal anti-inflammatory drugs block COX to treat inflammatory). However, the mechanism of lipid roles in some diseases, such as Alzheimer's and type II diabetes/ metabolic syndrome, remains an ongoing challenge.

It is documented that perturbations in lipid metabolism are associated with several human diseases including diabetes, cancer, atherosclerosis, Alzheimer's and others [36]. For example, in patients with atherosclerosis, defects in cholesterol and lipid metabolism, especially in the low-density lipoprotein (LDL) receptor, induce a series of biological events that lead to the accumulation of plasma lipoproteins in the bloodstream which promotes the expression of chemokines and adhesion molecules that promote macrophage apoptosis and necrosis that eventually will provoke a series of cardiovascular events [36], [37]. Therefore, total blood cholesterol level has been used in assessing the risk of high cholesterol levels on the heart function. Also, it was shown that acute elevation in free fatty acid levels in rodents induces muscle insulin resistance within a few hours which is comparable to the one caused by prolonged high-fat diet-induced insulin resistance which promotes diabetes development. In addition, it is believed that a misbalance in lipid levels is associated with the development of Alzheimer's disease (AD) where it was reported that a reduction in phospholipid levels was observed in brain tissues from patients with AD this could be a result of decreased levels of the phospholipid precursors or/and an increase in phospholipid turnover and metabolism. This reduction in phospholipid level could influence cell viability by reducing the stability and permeability of the membrane, which most likely results in cellular dysfunction or cellular death. Also, sphingolipids, which are believed to be abundant in the CNS, play an essential role in signal transmission and even small changes in their levels within the brain might interfere with the neurodegenerative processes including AD, Parkinson's disease and HIV-dementia [38]. In the cancer research area, metabolic reprogramming is now established and documented in various tumour types [39]. Tumour cells must efficiently generate energy and biomass components to expand and therefore these cells express flexibility in their metabolic phenotype to meet the high energy requirement needed for their growth, and proliferation and to withstand harsh tumour environments [40]. Since lipids play several key roles in cellular functions and their importance is well-documented in the proliferation and growth of cancerous and noncancerous cells, several research

groups have inspected lipid alterations in cancerous cells to explore the disease, stratify the patients, discover potential biomarkers and set up therapeutic strategies through targeting the cancerous cell's lipid metabolism. Therefore, monitoring lipid species could provide valuable information that could improve our understanding regards different pathological conditions and guide the treatment therapy plan more efficiently.

1.5. Current Lipidomics research

Lipidomics, also viewed as a branch of metabolomics, concentrate on the biochemistry reaction of lipids and interactions between them (Figure 1.2). It includes the extraction of lipids from tissues and cells, analyses of lipids composition and proportion using analytical techniques, inspects the probable pathways inducing the lipid change, and finally finding out the candidate enzyme/protein targets and drug for the disease. Although of great consequence, the study of lipids has been impeded by analytical limitations[41]. It is partly because of the structural similarity between lipids. For example, some lipid groups, such as phospholipids, nearly are the same except for the difference in their head groups and acyl [42]. Thus, it is highly important to resolve the limitations of the analytical techniques used for lipidomics and try to improve the experimental methods toward different lipids.

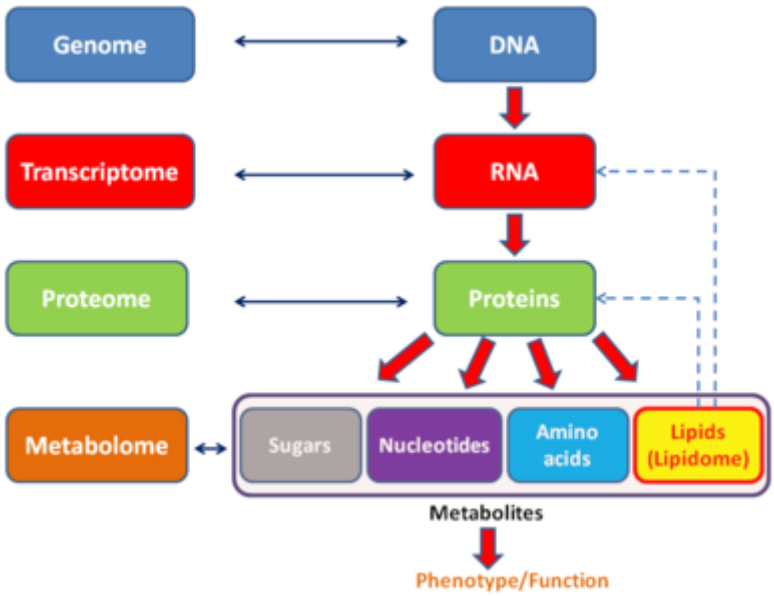


Figure 1.2. *The relationships between lipidomics with genomics, transcriptomics, proteomics and metabolomics. [19]*

Lipidomics can be divided into targeted and untargeted lipidomics, and both of them have advantages and limitations[43]. The untargeted approach has been used widely to perform global lipid profiling in a biological sample and to discover new biomarkers of disease and cell function [44]. Untargeted lipidomics concentrates on identifying all the lipids that appeared in the disease sample, which usually losses lipid identification and accurate concentration change of these lipids [45]–[47]. Targeted lipidomics, on the contrary, is hypothesis-driven, and focuses on identifying and quantifying a specific

set of lipid molecules based on existing knowledge of a biological system. It presents the concentration change of the lipids, but due to the instrument stability and experiment complexity, it usually restricts to one class of lipids and is unlikely to expand into hundreds of lipids [48], [49].To fill the gap between untargeted lipidomics and targeted lipidomics, a 'semi-targeted' lipidomics method is proposed to monitor the concentration fold change of hundreds of lipids in the human body. A pre-defined list of lipids with known formula, exact mass and the fold change of these lipids are analysed to study specific diseases to monitor disease onset and progress and to identify diagnostic lipid biomarkers. This method combines the advantage of untargeted lipidomics (identification of a big amount of lipids) and targeted lipidomics (quantification) and avoid the disadvantage of both methods. To ensure the accuracy of quantification, internal standards are always added, either as isotope dilution mass spectrometry, or one internal standard per lipid class [50]. However, due to the diversity of lipids, lipidomics method development is still a complex and challenging area.

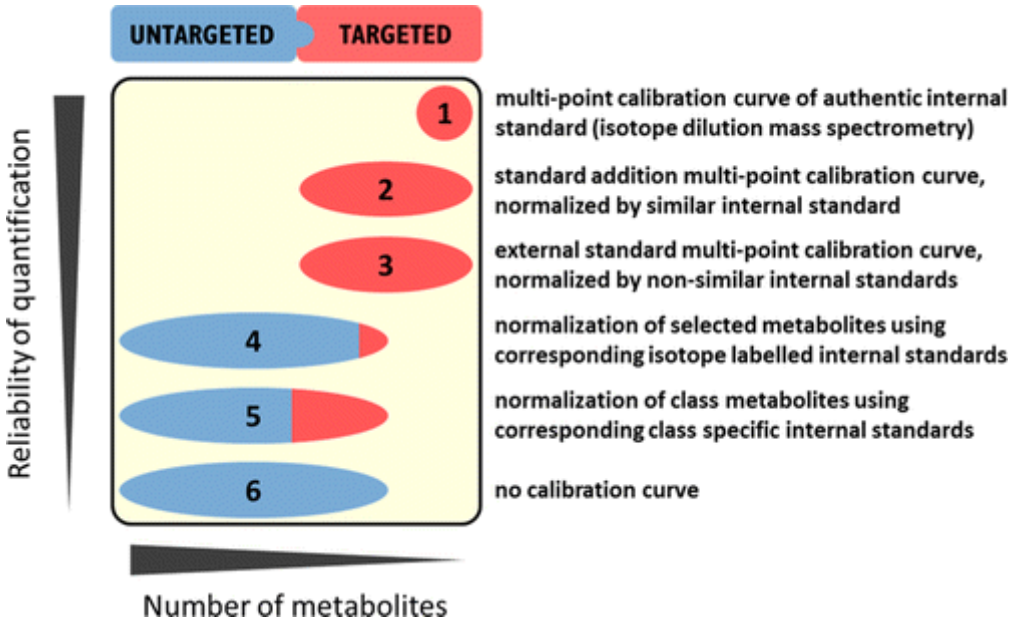


Figure 1.3. *The reliability of quantification of untargeted and targeted lipidomics with different normalization ways*[50].

1.6. Analytical techniques for lipidomics

Lots of approaches have been used for lipids analysis, such as chromatography, mass spectrometry, NMR, and some other techniques. Chromatography is easy to command; however, the mass resolution and sensitivity are limited in analysis, and isobaric compounds cannot be separated by this method. Nuclear magnetic resonance (NMR) stands out in direct measurement and non-destructive characteristics, but it also has shortcomings in sensitivity and resolution. Some main approaches for lipidomic analysis and their applications have been listed below (Table 1.2).

Table 1.2. Experimental approaches using extracts in lipidomics research

Experimental approach	Lipid classes	Advantages	Disadvantages
Chromatography			
Thin layer chromatography (TLC)	solvent systems established for most liquid classes.	Very established technique, technically relatively easy, and does not require sophisticated instrumentation, spot chromatograms allow for rapid screening of mutant extract libraries.	Low resolution and sensitivity limit many lipidomic applications; detection of lipids by iodine vapour and (class-specific) dyes and radioactivity.
High-performance liquid chromatography(HPLC)	Many lipids, including sterols, GP, TG, DG, FA and lipid headgroup derivatives.	Well established with worked-out reverse- and normal- phase conditions available; ease of automation; very quantitative.	Detection by refractive index or mass detector (lipids, as defined here, in general, do not absorb visible and UV light effectively); medium sensitivities in general.
Gas chromatography (GC)	Non-polar compounds such as TG; derivatized FA and sterols.	Very widely used for the determination of fatty acid composition, detection generally by MS.	Requires volatile compounds or derivatization of polar lipids.
Mass spectrometry			
ESI	polar compounds such as GP (more nonpolar compounds can be analysed by APCI)	Direct detection by m/z, high sensitivity and resolution, direct profiling of complex lipid mixtures, ease of automation, compatible with upfront LC separation.	Suppression of ionization. In particular in the case of crude extracts and when low-abundance species are to be analysed; absolute quantification requires considerable efforts (ie. Class and mass-dependent internal standards).
MALDI	Many lipids including complex glycolipids.	Direct detection by m/z, buffer and salt contaminants generally well tolerant can be combined with prior LC separation.	Suppression of ionization, in particular in the case of crude extracts and when low-abundance species are analysed; matrix backgrounds.
NMR	^{31}P for phospholipids, ^{1}H for all lipids.	Direct measurement; non-destructive; quantitative.	low sensitivity, spectra dominated by very abundant lipids (cholesterol, PC).

DG, diacylglycerol; ESI, electrospray ionization; FA, fatty acid; GP, glycerophospholipids; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TG, triacylglycerol.[41]

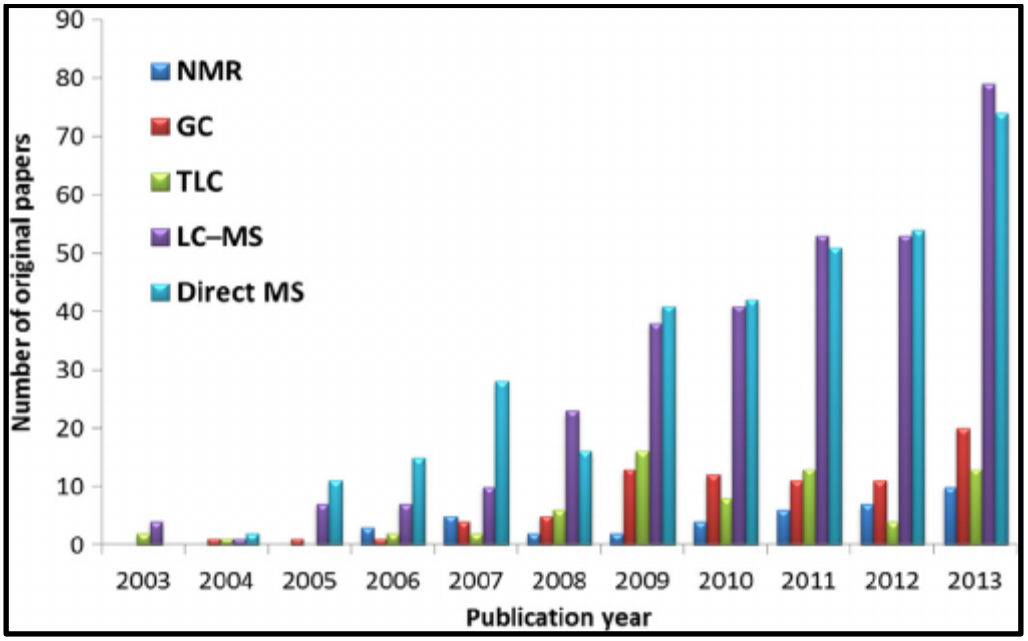


Figure 1.4. *The number of original papers published between 2003-2013, dedicated to lipidomics using different instrumental platforms* [51].

So far, the most efficient way for lipids analysis is mass spectrometry. It has an immense improvement in sensitivity and resolution compared to thin layer chromatography (TLC), liquid chromatography (LC), nuclear magnetic resonance (NMR) and other instruments, and is possible to measure various complex compounds simultaneously. In the mass spectrometry method, lipids are extracted from biological tissues through organic solvents, and then injected directly into mass spectrometry (named shotgun lipidomics [52], [53]) or separated by gas chromatography or liquid chromatography in advance. This method has been greatly accelerated by the Noble prize-winning research in the development of the soft ionization techniques of electrospray ionization (ESI)[54] and matrix-assisted laser desorption/ionization (MALDI) [55], respectively.

Due to the high sensitivity of MS measurements and its ability to analyse a wide range of molecules, the use of MS in lipid analysis has increased rapidly over the past few years. Figure 1.4 shows an increase in the number of MS-related lipidomics research publications compared to other techniques [51].

ESI, initially developed by Fenn in 1989, permits analysing large, non-volatile molecules straightway from the liquid phase without affecting the chemical structures of the analytes [56]. The principle of the ESI process is shown in Figure 1.5. The sample enters the ion source of the MS via a capillary tube at normal pressure. Usually, a high voltage (2–5 kV) is applied to the capillary relative to the entrance of the mass spectrometer that provokes an electric gradient along which the charged droplets travel. As they transport, the solvent evaporates and the intensity of the charges as well as the Coulombic repulsion within the droplet increase, which results in the dissociation of large droplets into smaller ones. This

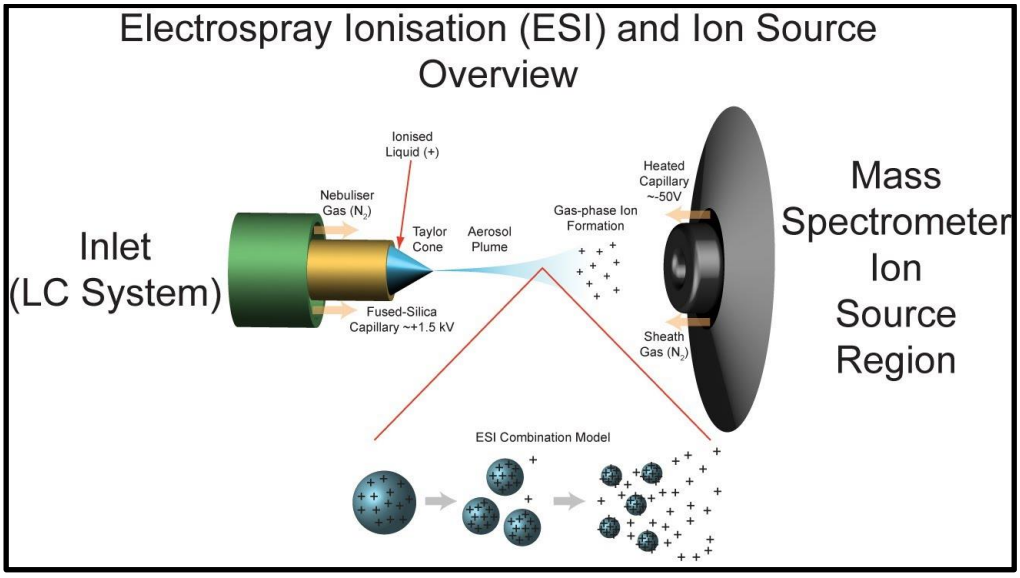


Figure 1.5. *Electrospray ionisation mass spectrometry* [57].

continues to happen until it ends up with charged individual molecules in the gaseous phase. Then they enter the mass spectrometry for separation and detection based on their mass-to-charge ratio [58].

The ionisation efficiency in MS varies from one lipid class to another [57]. For example, phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, and sphingomyelins are detected in better in positive mode while phosphatidylserine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, free fatty acids, phosphatidylethanolamine, and eicosanoids are detected better in negative mode [59]. Therefore, both modes are required in order to analyse the diverse lipid groups simultaneously with high sensitivity [57]. Neutral lipids (e.g., triacylglycerols) which are not easily ionised by ESI, can be seen in the positive mode as ammonium, lithium, or sodium adducts [59].

The shotgun approach was introduced in 2003 by Han and Gross [58]. In this approach, lipid extracts were introduced directly into the MS system without prior separation. Nevertheless, because no separation methods were applied, overlaps between different molecular species hinder the identification and quantification of low-abundant molecules. Moreover, ion suppression limits the use of this technology particularly when some lipid classes are predominant which results in ion suppression of low abundant lipid species [60].

Coupling a separation technology to the MS, like thin layer chromatography, gas or liquid chromatography helps in analysing a full range of phospholipids from a complex mixture which brings lipidomics to practice as well it increases the sensitivity and efficiency of the analysis [61]. Combining liquid chromatography (LC) with MS separates different lipid classes, reducing the ion suppression of co-eluting compounds and matrix effects, which can assist in the identification of isobaric lipid species. In addition, retention time based on an LC system provides valuable information for metabolite annotation and allows for more accurate quantification. [60], [62], [63].

1.6.1. Mass spectrometry

The working principle of a mass spectrometer is ionising chemicals and separating them according to their mass-to-charge ratio. A simplified mass spectrometer structure is shown in Figure 1.6. Typically, a mass spectrometer comprises three parts: an ion source, a mass analyser and a detector. A theoretical example of a mass spectrometer's internal structure is shown below:

Ion sources break down the sample in different ways, including chemical ionization, electron ionization, gas discharge ion source, photoionization, desorption ionization, spray ionization, thermal ionization, ambient ionization, etc. According to the fragmentation type, ionization can be classified as hard ionization and soft ionization[64]. Hard ionization, such as electron ionization (EI), can produce a large

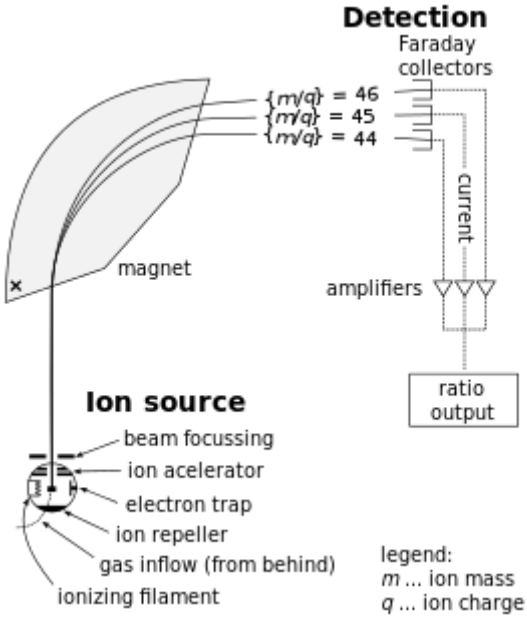


Figure 1.6. *A theoretical example of mass spectrometer structure [43].*

amount of energy to subject molecules and cause large degree of fragmentation and lower m/z fragment. In this case, mass spectrometer will acquire more complicated fragments and get more detailed results. Through analysing the results, structure and some unknown compounds can be determined through comparing with the mass spectral library. However, electron ionization also has limitations in coupling to HPLC (such as LC-MS), since the instrument tend to burn out rapidly in atmosphere pressure. But EI can be used for gaseous and highly volatile liquid samples, as it works in vacuum atmosphere[65].

Due to the potential of lipids related to many diseases, lipidomics research has grown rapidly in the last twenty years [14]. Despite these considerable advantages, the exploration of critical lipid biomarkers in disease is still difficult due to the limitation of the analytical instrument [27]. Nowadays, the most frequently used analyse instrument is liquid chromatography coupled to mass spectrometry (LC-MS), and it shows a remarkable improvement in identification sensitivity and resolution towards lipid

biomarkers compared to thin layer chromatography(TLC), liquid chromatography(LC), nuclear magnetic resonance (NMR) and other instruments [66].

Soft ionization, on the contrary, produces a small amount of energy in samples and causes softer fragmentation. Many ionization is included in this area, such as fast atom bombardment (FAB), electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI)[67], chemical ionization (CI), atmosphere pressure chemical ionization (APCI), etc.

Detector measures the number of ion groups in each area, and then calculates the separation mode of the sample and ensures the structure. Since the signal derived from the mass analyser is very trivial, a signal amplification detector is often needed. An ideal detector is expected to have some properties like fast time response, high amplification, low noise, high collection efficiency, long-term stability, low cost, etc[68]. Microchannel plate detector is a very common detector to be used in modern commercial instruments nowadays[69], but there are still many other genies of detectors [70].

According to the ion selection times, mass spectrometry can be classified into MS and MS/MS spectrometry. In the MS mode, ions were selected only once, and the example is the full scan mode of Exactive Orbitrap mass spectrometry. MS/MS mode, also named MS^2 or tandem mass spectrometry, means ionized ions were separated more than one time, which is accomplished by tandem in space or tandem in time. The representation of tandem in space is triple quadrupole mass spectrometry, and the selection was achieved by using a two-quadrupole filter. The representation of tandem in time is quadrupole ion trap mass spectrometry, which achieves multiple separations by trapping ions in the same place many times. The mechanism of MS mass spectrometry and different type of MS/MS mass spectrometry was compared below.

1.6.2. Triple quadrupole tandem mass spectrometry

Quadrupole mass spectrometer uses 4 parallel rods, called quadrupole field, to select some specific ions having a certain range of mass to charge ratio to pass through the system. By changing the potential on the rods, ions with various mass-to-charge ratios can be selected. A triple quadruple tandem mass spectrometer (TQMS) is composed of three quadrupole mass spectrometers and acts as a mass filter, collision chamber, and mass filter in series. The first quadrupole selects a chosen group of ions and transmits them to the second quadrupole, the second one is used for the collision chamber and breaks the ions into different fragments. The last one also works as a mass filter to select some desirable fragments for the detector.

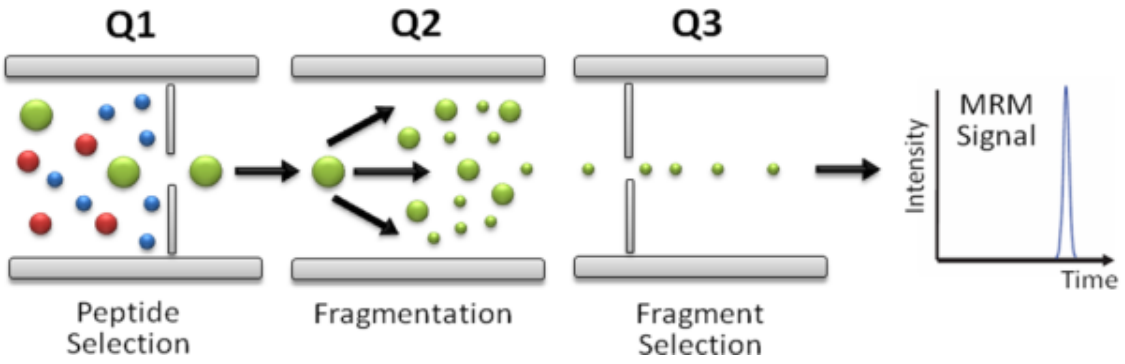


Figure 1.7. *The structure of triple quadrupole mass spectrometer*[71]

Triple quadrupole mass spectrometry suits targeted and quantitative mass spectrometry very well, since it only focusses on some specially selected fragments, which makes it highly efficient in these ions. In this mass spectrometer, the fragmentation process of some selected ions provides enhanced selectivity and discrimination than normal mass spectrometers with pretty good sensitivity. Typically, the selectivity of triple quadrupole mass spectrometry is less than 1000 with a resolution better than 1 amu[72]. Quattro Ultimate LC-MS/MS is a typical triple quadrupole tandem mass spectrometer, it shows outstanding performance on targeted analysis and monitoring multiple analytes simultaneously.

1.6.3. Orbitrap mass spectrometry

Orbitrap is a kind of mass analyser derived from the Kingdon trap in 2005 [73]. It has a structure similar to the Kingdon ion trap mass spectrometer with a central spindle electrode[74], [75], and also similar to a quadrupole ion trap but with a static electrostatic field. The working principle of orbitrap can be divided into trapping, injection, excitation and detection. In the orbitrap mass analyser, ions are trapped in the orbit and balanced by inertia and electrostatic attraction. Ions are injected by c-trap from an external ion source, then rotate in a spindle-like inner electrode, and finally detected according to their orbit (indicating different mass charge ratios). Ions are transferred into electrons when stroke to dynode, and then proliferated by electron multiplier into electrical signals that could be identified by the software. The Exactive orbitrap instrument, with the equipment of full scan, and mass accuracy of less than 3 ppM, shows an excellent performance both on quantitative analysis and qualitative analysis[73].

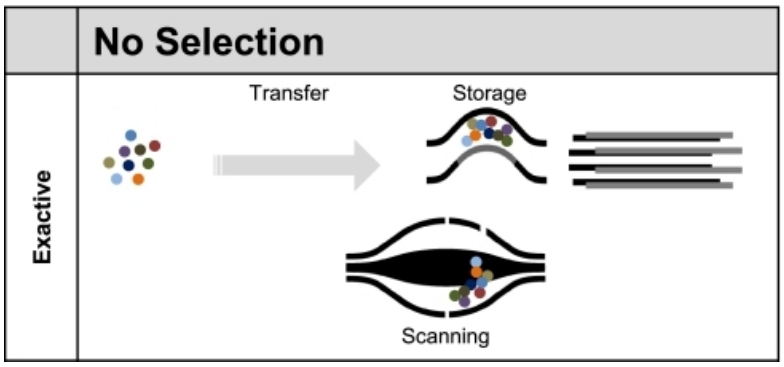


Figure 1.8. *the structure and mechanism of Orbitrap mass spectrometry* [76]

1.6.4. Quadrupole Orbitrap mass spectrometry

Orbitrap can be linked with a quadrupole mass analyser (Q Exactive instrument) to get the Quadrupole Orbitrap mass spectrometry. Ions are selected through the quadrupole mass filter, then injected into the C-trap and rotate in the spindle-like inner electrode, which enhances the quantitative analysing ability and high resolution and accurate mass (HRAM). Exactivepolarity switched between a positive and negative mode in 1 second were achieved by the instrument to get high-resolution LC-MS result.

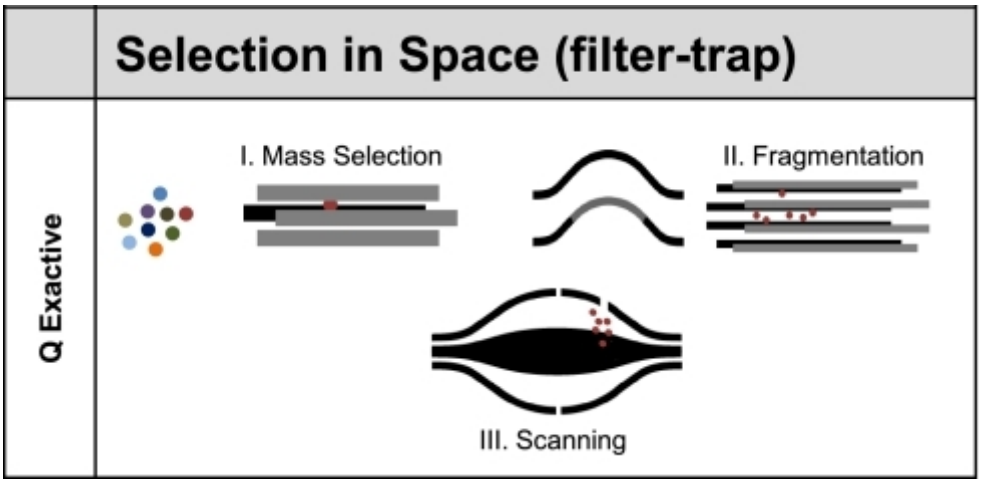


Figure 1.9. *the structure and mechanism of Quadrupole Orbitrap mass spectrometry*[76]

1.7. Normalisation of lipidomic data

Isotope dilution is a kind of internal standardisation method that add a known quantity of the isotopically labelled substance to the sample and then analyses the sample concentration by measuring the signal ratio of isotope to sample [77]. Isotope dilution replaces traditional internal standards into labelled samples, reduces the possibility of influencing the composition of the sample, and makes the analysing result more reliable.

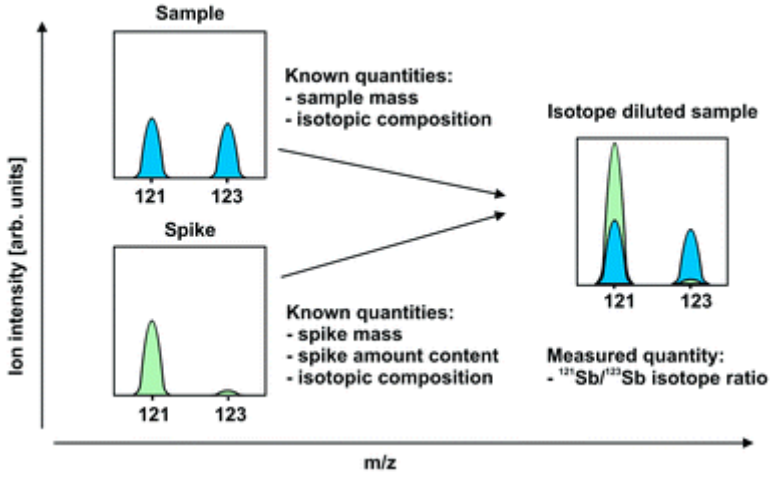


Figure 1.10. *Isotope dilution demonstration*[78]

It is impractical to select an internal standard for each lipid species, especially if we are interested in hundreds or thousands of species. In addition, it is difficult to use only one labelled standard because of the huge diversity of metabolite structures, concentration and composition and it is not practical to assume that all the metabolites /lipids with different masses and different physicochemical properties are subjected to the same amount of variation as the standard [79], [80]. Also, different lipid species such as ceramide, diacylglycerols, and sphingomyelins are overlapped, and it is not practical to use the same normalisation factor on these species [80]. In addition, non-polar lipid classes that do not have an ionizable head group (cholesterol esters, diacylglycerols, triacylglycerols, etc) do not manifest identical response factors even at low concentration and their response should be determined in advance with respect to their acyl chain length and their degree of unsaturation which complicate the analysis even more [81], [82].

Accordingly, several researchers are using more than one labelled standard and each standard represents a specific retention time. Various previous studies proved that at low concentrations, lipids in the same class possess the same polar head group and approximately have similar response factors [82]. This is because the ionisation efficiency of those species relies on the charged head group which is the same in each group [81]. Therefore, for better results, more than one standard should be included for each lipid class and this can increase the species that can be detected even when higher concentrations of lipids are analysed, and this alleviates the requirement of controlling the total lipid concentration. However, when various species are present in a mixture, it is not practical to include multiple internal standards to normalise each class with various degrees of unsaturation within the same class [83]. Also, the availability of commercial stable isotope-labelled standards and their costs limit the large-scale usage of this method [63]. So, isotopically labelled organism extracts seem as a better source of these internal standards.

To address these issues, the application of uniformly (U) ^{13}C-labelled biological organisms, such as bacteria, could be applied instead, as a source of internal standards [84]. By sustaining the growth of these organisms on one carbon source that could be replaced later with isotopically labelled form, in theory, a variety of isotopically labelled metabolites/lipids can be generated after a proper extraction procedure to the cells is applied. This approach has been widely used for both quantitative analyses in proteomics and metabolomics using ^{15}N- or/and ^{13}C sources and others [85], [86].

Ideally, the source of stable isotopes should be the same as the samples to ensure all metabolites are present. However, a different source of the isotopically labelled extract could be used for complex organisms [87]. For many organisms, ^{13}C labelling can be performed easily and at a reasonable cost [85]. Researchers have been using a labelled extract from different organisms such as E. coli, S. cerevisiae, P. pastors and others to normalise a selected number of metabolites which will help them report their absolute concentrations [88]–[90]. However, the labelled extract from human cells is not

commonly used, since the difficulty of keeping the solely ^{13}C condition in culturing cells. But considering the similarity between mammalian cells with human diseases, trying to get ^{13}C labelling extracts from mammalian cells might be a good practice.

1.8. Data processing

After an analytical run of biological samples on an LC-MS instrument, the 3D LC-MS data produced (in the form of peak intensities, mass-to-charge ratios, and the retention time of analytes) is usually obtained with a specifically written programme for the instrument, e.g., "XCalibur" for LC-MS Exactive, visually showing the spectra peaks (raw data). The raw data files are then pre-processed to generate 2D output files, which can later be used for further statistical analysis. Data pre-processing has played an important role in lipidomics research so far, with several bioinformatics tools assisting in the accurate processing of data. Computational platforms, such as "metAlign", "XCMS" and "mzMatch," were developed for a variety of pre-processing purposes, transferring raw data into a more organised, tabulated and accurate format [91]. "MetAlign" is intentionally designed to remove any background noise and outliers from the raw data, correct the baseline, perform peak matching and align retention times [92]. "XCMS" processing is found to be similar to "metAlign", but it also helps scientists with the identification of putative metabolites, structural characterisation and statistical analysis. "XCMS" has been used more regularly in human biofluid and tissue untargeted LC-MS lipidomics and metabolite profiling, as well as targeted analysis. The main advantage of "XCMS" over other pre-processing software is that it can correlate specific lipids to their biological origin and incorporate non-linear retention time alignment, helping to calculate relative metabolite ion intensities – useful in identifying potential biomarkers later on. "mzMatch" helps with data pre-processing further by matching and aligning peaks [93]. All pre-processing stages applied to the raw data are normally reported in detail when the studies are published, so that other scientists can replicate those steps again. This has been strongly recommended as a proposed minimum requirement by MSI.

However, even with advanced pre-processing tools being used, limitations still exist with the presence of background noise or artefact peaks often incorrectly recognised as lipids. Lipid identification algorithms, applied in those programmes, are hard to use without special training. Recently, a group of scientists in Molecular Parasitology, at Glasgow University has developed a bioanalytical programme called "IDEOM" that comprises several advanced pre-processing computational programmes, including "mzMatch" and "XCMS" tools, and lipid identification steps [94]. This was performed to improve comprehensive data processing further, particularly paying attention to noise filtering, correct identification of lipids and data visualisation. Putative lipids can be identified by matching their masses and retention times to authentic standards. Retention times of those authentic standards are uploaded into IDEOM before the main processing steps of the sample data. Therefore, the final list of putative metabolites is produced with the identification of confidence levels related to standards. Retention times

of authentic standards uploaded into the programme help to make a retention time prediction model, assisting with the confidence identification of putative metabolites [95]. IDEOM is designed as an Excel user-friendly interface, allowing for rapid data generation without the need for complicated bioinformatics skills. Additionally, IDEOM provides dual polarity data merging, targeted analysis and isotope tracking for data normalisation[94].

1.9. Statistical analysis

Once the pre-processing has been performed, statistical analysis can further help to detect significantly altered metabolites between distinct biological groups, helping scientists to find potential biomarkers or major changes in metabolic pathways in certain clinical conditions. The specific choice of statistical analysis methods depends on the design and aim of the study. There are usually two main statistical approaches involved in lipidomics and other omics studies: a univariate and multivariate analysis. The univariate approach assesses the statistical significance of each metabolite peak separately, using techniques such as t-test, sum test, false discovery rate (FDR) [96] and analysis of variance (ANOVA) [97]. T-test compares the means of two groups, for example, control vs treatment/disease, calculating p values for each metabolite to determine their significance – if the p-value is less than 0.05, the null hypothesis stating a lack of statistical significance is rejected and instead accepts that there is a significant difference for particular metabolites between the studied groups. In a large untargeted lipidomics study with a wide range of detected metabolites, a multiple hypothesis t-test for a large dataset may cause high false discoveries, even with a small p value threshold. Therefore, an FDR correction is usually performed on the metabolite dataset straight after the t-test to control and prevent a large total number of false discoveries [98]. Univariate statistical tests help to reduce a large number of measured metabolites to only those which show the strongest response under the investigated conditions. In one study, a two-way ANOVA test investigated medication-induced level changes of individual lipids and managed to demonstrate several statistically significant metabolites from patients with metastatic disease. However, the univariate analysis fails to discriminate between biological groups properly if there are only minor differences present at the single-molecule level. Multivariate analysis, on the other hand, discriminates between groups based on changes in single-molecule levels, as well as on dependency structures between the individual molecules.

Multivariate analysis is usually used to reduce the dimensionality (random variables) of lipidomics data and to see some general trends between groups in highly complex datasets, by dealing with multiple variables, rather than one variable as in univariate analysis. Multivariate analysis allows us to identify biologically relevant spectral features and biological class differences from the multivariate dataset [99]. This type of analysis is further categorised into supervised and unsupervised methods. The “Principal Component Analysis” (PCA) technique belongs to the unsupervised category that explains the variance of a dataset by analysing principal components (vectors of metabolite contributions), usually without

the knowledge of metabolite classes or groups – therefore called unsupervised. The technique focuses more on differences between samples, rather than between groups. PCA is often used as a starting step in multivariate data analysis and has been extensively applied in untargeted lipidomics studies where it showed good data visualisation, clustering of sample points and sample group discrimination [100]. One of the developed unsupervised techniques, ANOVA-Simultaneous Component Analysis (ASCA), combines ANOVA and PCA methods and is normally applied in datasets with complex designs, allowing variation in the data to be directly related to various design factors. This technique successfully showed the sample clustering and distribution of saliva metabolome in one of the studies, but the method is still under investigation [101], [102]. A supervised technique, such as "Orthogonal-partial least square-discriminant analysis" (OPLSDA), builds on the PCA model and uses the sample group information for classification purposes. This classification helps to discriminate between sample groups, improving clustering and separation between data groups in the model and helping to identify statistically significant metabolites [103]. However, this approach often misses some low-concentration lipids which are significantly changed, and which can be detected by univariate analysis. The issue of data over-fitting is also a major issue in supervised techniques [104]. So, it is very important to choose the appropriate samples for this type of analysis – human biofluid metabolite studies normally involve observation and analysis of several variables at a time, therefore they benefit from using multivariate analysis in search of biomarkers.

Both univariate and multivariate analysis approaches are regularly used in human lipidomics studies, in particular untargeted lipidomics, producing highly accurate data with clear observations between sample groups. This helps lipidomics research in the search for biomarkers and metabolic pathways associated with clinical diseases and other types of perturbation.

1.10. General aims and objectives of the thesis

This thesis presents a potential LC-MS-based method for semi-targeted lipidomics and applied the developed method to different clinical samples. In this thesis, the detailed experimental work will demonstrate how this method has been developed using a pre-defined list of lipids, validated using plasma samples and applied in clinical.

The main specific aims of this work are:

1. To develop a pre-defined semi-targeted lipidomics method for over three hundred lipids with good identification and relative quantification.

2. To validate the method with human plasma samples.

3. To apply the validated normalisation method on clinical samples in search of potential biomarkers.

Chapter Two

LC-MS method development for a semi-targeted lipidomic method

2.1. Introduction

2.1.1. LC-MS/MS method application in lipidomics

The disruption of lipid metabolism is known to be associated with many diseases, and clinical investigations using lipidomics analysis of biofluids and tissues have grown rapidly over recent years[14]. Despite these considerable advantages, the exploration of critical lipid biomarkers in disease is still difficult due to the limitation of the analytical instrument[27]. To explore reliable lipid biomarkers, many clinical experiments have been conducted to identify lipids that exist in human plasma, and the concentration changes between patients and healthy controls. Quehenberger*et al.* defined the human plasma lipidome and their concentrations with different extraction methodologies for six main categories of lipids [105].

Liquid chromatography coupled mass spectrometry (LC-MS) is the most frequently used method in lipidomics research compared to NMR, TLC and so on (see chapter 1). In the LC-MS method, there are three most important LC systems including reversed-phase LC (RP-LC), normal-phase LC (NP-LC), and hydrophilic interaction LC (HILIC). NP-LC and HILIC methods separate lipids according to their hydrophilic functionalities (the polarity of the lipid headgroup), while RP-LC separate lipids based on their hydrophobic functionalities[106]. Currently, RP-LC is the most efficient method, as a different number of carbons and saturation degree will influence lipids' hydrophobic properties.

In the RP-LC system, C18 and C8 columns are the most frequently used LC columns. These columns have a hydrophobic stationary phase and hydrophilic mobile phase, which will adsorb lipids once injected into the column as most of the lipids are hydrophobic. Through the run, the mobile phase will increase the hydrophobicity, and thus hydrophilic lipids will be eluted earlier, and the most hydrophobic lipids will be eluted last, in this way the column separate lipids gradually. The column property also influences the separation of lipids, narrow columns are more sensitive to flow rate change, and shorter columns decrease the pressure and allow for higher flow rates. A guard column is often used to protect the LC column by removing impurities.

The LC system is important to the separation of lipids by class, mass, polarity and isobaric lipids. This can be achieved by modifying the mobile phase composition, the gradient through the run, the running time and other parameters. A good LC method should elute most of the lipids from injects and has a competent separation and identification of closely eluted lipids. A longer run promises better lipid profile coverage, while it may harm the sample if it is a large clinical study[51]. Thus, a proper flow rate and running time need to be selected according to the column performance.

The extraction method in lipidomics served two roles: First, it reduces the complexity of the sample by getting rid of any unwanted nonlipid compounds, this also lessens contaminants and maintains the stability of the mass spectrometry. Second, it enriches the analytes and improves the signal-to-noise

(S/N) ratios. The most widely used extraction method in lipidomics is liquid-liquid extraction (LLE). The Folch method[107] and the Bligh and Dyer protocol[108] both rely on a ternary mixture of chloroform, methanol and water. After extraction, the organic chloroform/methanol phase contains the lipids and the aqueous phase contains the more hydrophilic compounds and salts. Some experiments add some acid to the extraction solvent, to neutralize the anionic lipids and thus improve solubility in the organic phase. Anionic lipids include phosphatidic acids (PA), LPA, phosphatidylinositols (PI), sphingosine-1-phosphate (S1P) and so on. However, the concentration of acid and extraction times need to be strictly controlled, to avoid hydrolysis artefacts such as generating lysophospholipids, leading to false positive data in the worst-case scenario[109]. Matyash et al. proposed another extraction method replacing chloroform with methyl tert-butyl ether (MTBE), by mixing MTBE with methanol and water in the ratio 5:1.5:1.25 (v/v/v), the upper organic phase retains the lipids while salts and hydrophilic compounds are enriched in the lower aqueous phase[110]. The extraction efficiency is comparable to the Bligh and Dyer protocol, but the handling is much easier since the upper layer (MTBE) is removed more efficiently by pipetting than the lower layer (chloroform). Additionally, MTBE is less harmful than chloroform, which is an important safety aspect for people exposed to these chemicals daily. A recently published comparison of chloroform and MTBE protocols suggests that the MTBE method is more efficient for glycerophospholipids, ceramides, and unsaturated fatty acids, while the chloroform protocol is superior for saturated fatty acids and plasmalogens[111].

There are also some other sample preparation methods used in lipidomics. Satomi et al. used a one-step alcohol extraction method and compared methanol, ethanol, 2-propanol, and acetonitrile to the MTBE method[112]. Alcohol extraction is a simple and quick sample preparation method for lipidomics analysis, but differences in the extraction efficiencies between the alcohols exist. MeOH extraction has a preference for hydrophilic lipids, whereas IPA extraction is inclined to hydrophobic lipids, such as TG. However, one-step extraction also increased the number of nonlipid compounds in the extract compared to two-phase systems, which may lead to increased instrument contamination and greater ion suppression effects. Solid-phase extraction (SPE) is also a commonly used extraction method for lipidomics. It includes the liquid phase and stationary phase and can be considered a simple liquid chromatography system. According to the cartridge type, SPE can be classified as normal-phase SPE, reversed-phase SPE and ion exchange SPE. SPE is usually used after the liquid-liquid extraction (LLE) method for sample preparation before loading to the LC-MS system. In the SPE method, the SPE cartridge need to be conditioned and equilibrated first, and then the sample solution (usually extracts from LLE) was loaded onto the cartridge. After loading the sample, the cartridge was washed and eluted with a different solvent. In the SPE method, different classes of lipids were isolated and concentrated with different elution, thus is very useful for a few precious samples that need to be analysed with very high coverage of lipid species or just looking for several classes of lipids[113], [114]. Nevertheless, the

SPE method looks highly sophisticated and laborious when looking for a big coverage of lipids with many classes involved[115].

Mobile phase modifiers are added into the mobile phase, to increase LC separation, ionization and detection of lipids. Several modifiers are typically used in lipidomics research: ammonium formate, ammonium acetate, formic acid and acetic acid. It has been reported that these modifiers have different performances in ESI (+) and ESI (-) modes, Ammonium acetate has a low signal intensity in positive mode, but has a high signal intensity and ionization efficiency in negative mode[116]. Thus, a combination of different modifiers can improve lipid detection in different ESI polarities.

Table 2.1. lipid class and ionization in ESI (+) and ESI (-) [51]

Lipid class	**Positive mode**	**Negative mode**
LPC, PC	$[M + H]^+$, $[M + Na]^+$	$[M–H]^–$, $[M + HCOO]^–$, $[M + CH_3COO]^–$
LPE, PE	$[M + H]^+$, $[M + Na]^+$	$[M–H]^–$
PG	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$
PI	$[M + H]^+$, $[M + NH_4]^+$	$[M–H]^–$
PS	$[M + H]^+$	$[M–H]^–$
PA		$[M–H]^–$
CE	$[M + NH_4]^+$	
SM	$[M + H]^+$	$[M + HCOO]^–$
Cholesterol	$[M–H_2O+H]^+$	
Cer, GluCer, LacCer	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$, $[M + HCOO]^–$
MG, DG, TG	$[M + NH_4]^+$, $[M + Na]^+$	
MGDG, DGDG, SQDG	$[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$
FA		$[M–H]^–$
CL	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$
Cer	$[M + H]^+$, $[M + NH_4]^+$, $[M + Na]^+$	$[M–H]^–$

LPC, lysophosphatidylcholine; PC, phosphatidylcholines; LPE, lyso-phosphatidylethanolamines; PE, phosphatidylethanolamines; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserines; PA, phosphatidic acid; CE, cholesterol esters; SM, sphingomyelin; Cer, Ceramides; GluCer, glucosylceramide; LacCer, Lactosylceramide; MG, Monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; MGDG, glycosyldiacylglycerols; DGDG, glycosyldiacylglycerols; SQDG, glycosyldiacylglycerols; FA, fatty acids; CL, Cardiolipin.

The ionisation efficiency in MS varies from one lipid class to another. For example, phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, and sphingomyelins are detected in positive mode while phosphatidylserine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, free fatty acids, phosphatidylethanolamine, and eicosanoids are detected in

negative mode. Therefore, both modes are required in order to analyse the diverse lipid groups simultaneously with high sensitivity. Neutral lipids (e.g., triacylglycerols) which are not easily ionised by ESI, can be seen in the positive mode as ammonium, lithium, or sodium adducts. The detail of the ionization and adducts of each lipid class is shown in Table 2.1.

2.1.2. Lipid identification

Complete structural elucidation requires several levels of identification including lipid class, the total number of carbon atoms and the total number of double bonds, the total number of carbon atoms and a total number of double bonds per acyl chain, location of the double bond and location of each acyl chain in the lipid backbone [117]. The main challenges in accurate lipid identification are the accurate fatty acyl composition and the positions of the double bonds which could be more functionally relevant [118]. These ultimate desires are not possible by simple MS-only measurements or by using conventional MS^2-based fragmentation methods. Also, Complete structural elucidation requires such as ozone-induced dissociation, high-energy collision-induced dissociation or multistage fragmentation approaches [117], [119], [120].

Researchers in the lipidomics field should not annotate features based on exact mass only as each *m/z* value can represent different lipids ions with different adducts, since the lipidome is diverse and enormous overlap in exact mass is expected between the species (65). Whereas identification according to the metabolite standards initiative is achieved only by matching retention time, full scan and product ion spectra with reference standards (66). Putative identification can be made without chemical reference, by matching the retention times (sometimes if the same method and instrument are used), full scan and product ion spectra of that specific peak with entries in different public/commercial spectral databases (67). However, in untargeted studies, manual annotation of all lipid species is unrealistic, therefore researchers usually relied on different software to annotate the lipid species (65). Different software has been developed either based on libraries of the fragmentation pattern of experimentally determined or computationally generated (in silico) MS/MS spectrum such as LipidMatch (68), Lipid Data Analyzer (69) LipidBlast (70), LipidSearch (71) and LipidXplorer (72). Although this software decreased time and effort in lipidomic studies, most of them require a few optimisation steps, and also, to set a few parameters in order to decrease the false identification. Therefore, the development of more advanced bioinformatics tools, improved algorithms for processing large-scale data, and more reliable structural annotation are vital to obtaining more reliable and comprehensive lipid profiling of untargeted lipidomics studies.

2.1.3. Current challenges and solutions for lipidomics

Although the LC-MS system is the most efficient method for lipidomics research, it still has many pitfalls to overcome. This includes lipid extraction, separation, MS detection, lipid identification and other challenges.

Ideally, sample preparation for lipidomics simultaneously accomplishes several tasks: extraction and isolation of lipids; removal of contaminating molecules; and preparation of the sample for MS analysis. An appropriate sample preparation minimizes loss of lipids, maximises ionization efficiency, is reproducible and ensures the longevity of instrumentation. Methods must also be suitable for the extraction of lipids from the biological matrix and be specific for lipids of interest[121]. Flash freezing and the use of enzyme inhibitors and anti-oxidants are effective ways to reduce the modification of the lipidome[122]. The most widely used extraction method is Folch's method (chloroform: methanol 2:1)[107], and there are several modified methods published later, like using an acidifier or using a different ratio of organic solvent[108]. Folch's method has a shortcoming in that uses halogenated solvents, which with health issues in the laboratory. Thus, another method using MTBE instead of chloroform was invented and is frequently used nowadays as well[110]. It reduces toxicity, but the MTBE also brings a strong flavour. Another difference between the two methods is, MTBE is on the upper layer, while chloroform is on the lower layer after extraction (both incorporate extracted lipids). For the lipid coverage, some papers consider these methods have a similar extraction efficiency, while some shows MTBE method generates less lipids than Folch method.

In the LC system, there are trade-offs between comprehensiveness, system complexity and time for optimization. Additionally, it should be noted that the ion response of an analyte can change, depending on the solvent composition at which it elutes from the column and enters the ion source. This poses challenges for accurate quantitation when an analyte's internal standard elutes with different solvent compositions. Evaluation of an ion response to solvent gradient can be evaluated by infusing the analyte into a t-junction post column and monitoring the ion signal throughout the gradient profile. Supercritical fluid chromatography (SFC) is also used as the mobile phase, and the most common is carbon dioxide. It has some advantages like cheap, low polarity, low viscosity, and can be used for fast separation of lipids[123].

Most of the mass spectrometers introduced over the last twenty years achieve full mass spectra acquisition (high-resolution mode) better than the targeted mass spectra acquisition, like multiple reaction monitoring (MRM) and selected ion monitoring (SIM). For MRM instruments, low dwell time is an important parameter because hundreds of transitions need to be collected within a short time. To get more information about lipids, MS/MS mode was used to do the full MS scan, activation through collision-induced dissociation and product ion detection at the same time. Polarity switching is also used to detect lipid ionization in positive and negative modes at the same time.

Raw data processing usually includes filtering, feature detection, alignment and normalization. Filtering methods process the raw signal intending to remove the noise or baseline. Feature detection is conducted to identify all signals caused by true ions and avoid the detection of false positives. This step may also involve a deconvolution method and isotope-pattern detection. Alignment methods are needed for correcting retention-time differences between runs and combining data from different samples. Normalization steps remove factors causing unwanted systematic bias in ion intensities between measurements or during sample-preparation steps. However, it frequently makes false positive or false negative peak detection, so miss some real peaks and include some noise peak. Another issue is different software generates largely different results, which makes it necessary to build up good laboratory guidelines in different groups[124].

Lipid identification is still the bottleneck in lipidomics research. Although online databases and computational tools have been developed for mass spectral lipid analysis (e.g., LipidQA, LIMSA, FAAT, lipID, LipidSearch, LipidView, LipidInspector, LipidXplorer, and ALEX), they do not provide stand-alone MS/MS spectral libraries. In this case, even though MS/MS databases were acquired and matched to the spectra, they still could not be identified because of the incomplete lipid library. Thus, a fully build up lipid library with all of the lipids and their structure, and MS/MS information is needed.

2.1.4. Semi-targeted lipidomics

Untargeted lipidomics concentrates on identifying all the lipids that appeared in the disease sample, which usually losses lipid identification and accurate concentration change of these lipids [45]–[47]. On the other hand, targeted lipidomics present the concentration change of the lipids, but due to the instrument stability and experiment complexity, it usually restricts to one class of lipids and is unlikely to expand into hundreds of lipids [48], [49].To fill the gap between untargeted lipidomics and targeted lipidomics, a ‘semi-targeted’ lipidomics method is proposed to monitor the concentration fold change of hundreds of lipids in the human body. A semi-targeted approach will explore the relationship between different classes of lipids and potential pathways of certain diseases, as well as the relative change of each lipid at the same time. However, this method is to some extent ambiguous as the lack of reliable lipid identification can limit the analytical confidence in the profiling data. To overcome this problem, it was decided to use a comprehensive and confirmed list of lipids in human plasma that have been quantified in widely accepted definitive published papers [105] and build up an unambiguous lipid database. The plan was to optimise the method by comparing the extraction method, mobile phase proportion, gradient, running time and different columns. Therefore, a pre-defined list of lipids with known formulas, exact mass and the fold change of these lipids will be analysed to study specific diseases to monitor disease onset and progress and to identify diagnostic lipid biomarkers. These biomarkers can be used as new targets for chemotherapy and the development of new drugs.

2.1.5. Aims and Objectives

The main aim of this chapter is to develop a sensitive, robust and reproducible LC-MS method for semi-targeted lipidomics analysis of human plasma. This method is expected to be able to identify most of the lipids that have been published in previous papers and to be suitable for application to clinical investigations to search for lipid biomarkers of disease progression.

To achieve this aim, the objectives of this chapter are:

- To build up a database with 300 to 400 lipids that have been previously identified in human plasma.
- To compare and select a suitable extraction method to enable recovery of all main lipid families to form human plasma.
- To develop and optimize a high-performance liquid chromatography-tandem high-resolution mass spectrometry method (HPLC-HRMS) for semi-targeted lipidomic analysis
- To investigate approaches to confirming the identity of lipids based on databases and specialized software.

2.2. Materials and Methods

2.2.1. Materials and Apparatus

2.2.1.1. Materials and chemicals

A Milli-Q water purification system (Millipore, MA, US) was used in the preparation of deionized water (18.2 MΩ). Acetonitrile, chloroform, ethyl acetate and hexane (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Methanol (LC-MS grade) and ammonium acetate were purchased from Sigma-Aldrich (Darmstadt, Germany). Isopropanol (LC-MS grade) was purchased from Acros Organics (New Jersey, USA). BHT 3, 5-Di-tert-butyl-4-hydroxytoluene (BHT) was purchased from Supelco (PA, USA), human plasma (donated from Queens Medical Centre Nottingham blood bank).

2.2.1.2. Apparatus

-80℃ freezer, Fisherbrand™ Wizard™ Infrared Vortex Mixer (Fisher Scientific, UK), Orbit™ Digital Microtube and Microplate Shakers (Labnet, USA), Harrier 18/80" Centrifuge rotor, Jouan Centrifugal Evaporator, Biostage SPE manifold, Techne Dri-Block heater (DB-3B), pipettes and tips, LC-MS vial with inserts and caps, Oasis SPE cartridge (Waters, UK), glass tube, Eppendorf, Accela liquid chromatography coupled with Orbitrap Quadrupole-Exactive mass spectrometer (Thermo Fisher

Scientific, Hemel Hempstead, UK), ACE Excel 3 SuperC18 Column(100×2.1mm id), ACE Excel 2 C18 (50×2.1mm id) and guard column were purchased from Advanced Chromatography Technologies.

2.2.2. Sample preparation method development

2.2.2.1. Sample preparation

Plasma donated from Queens Medical Centre was used as a method development sample. Deionized water was used to replace plasma in the extraction to prepare regent blanks. Extraction of lipids from plasma samples was carried out by forming organic and aqueous phases with different solvents, and most of the lipids were stored in the organic phase. Lipid extracts were loaded onto the LC-MS system for further lipid identification, and lipids in regent blank were excluded from identified lipid list.

2.2.2.2. Solid phase extraction (SPE) method

200 µl of plasma or H_2O (regent blank) was added into 400 µl of extract solvent (chloroform: methanol = 1:2), and then the mixture was vortexed for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper aqueous phase and a lower organic phase, as well as a protein layer in the middle. The lower organic phase was collected and mixed with the same volume of isopropanol (to increase compatibility), and then loaded onto preconditioned SPE cartridge (preconditioned with 2 ml of methanol for 2 min). After the first elution, a series of solvents (H_2O, methanol, isopropanol, ethyl acetate, chloroform and hexane for 10 ml each) were loaded onto an SPE cartridge and collected with different glass tubes. Collected eluents were evaporated under vacuum at room temperature using Juan's centrifugal evaporator (ordinary temperature) and reconstituted with 100 µl of isopropanol.

Table 2.2. The detail of the SPE cartridge

Barrel Size	6 cc
Brand	Oasis
Chemistry	HLB
Format/Type	Vac Cartridge
Mass Spec Compatibility	Mass Spec Compatible
Mode	Reversed-phase
Particle Size	30 µm
Pore Size	80Å
Sorbent Substrate	Copolymer
Sorbent Weight	200 mg
Units in Package	30/pkg

Water Wettable	Water Wettable
pH Range	0 - 14 pH

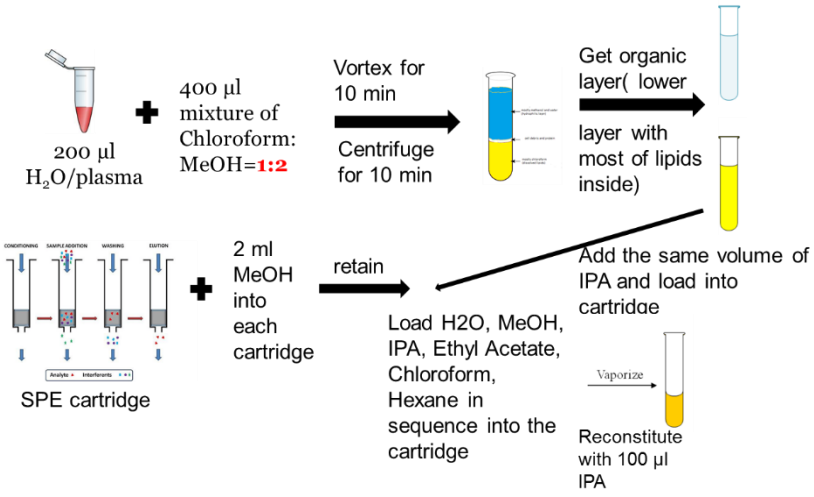


Figure 2.1. *The schematic of the solid phase extraction (SPE) method. Plasma was extracted with a mixture of chloroform and methanol, and the organic phase was collected after vortex and centrifuge. Then the organic phase was loaded onto the retained SPE cartridge, and different classes of lipids were eluted by adding different solvents. All of the eluents were evaporated and reconstituted with isopropanol afterwards.*

2.2.2.3. Folch method

50 µl of plasma (sample) or water (regent blank) was added into 500 µl of ice-cold (-20℃) mixture (chloroform: methanol = 2:1). After 10 min vortex, 500 µl of water was added to the mixture. The mixture was vortexed again for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper aqueous phase and a lower organic phase, as well as a protein layer in the middle. The lower organic phase was collected and evaporated under vacuum at room temperature using Juan's centrifugal evaporator. Then the sample was reconstituted with 100 µl of isopropanol and transferred into an LC-MS glass vial. In another version of the Folch method, the first extracted lower organic phase was collected into a new Eppendorf and 500 µl of ice-cold chloroform: methanol (2:1) was added into the previous aqueous and protein mixture. After 10 min of a vortex and 10 min of centrifuge, the new lower organic phase was combined with the previous organic phase together, and then evaporated under vacuum at room temperature using Juan centrifugal evaporator and reconstituted with 100 µl of isopropanol.

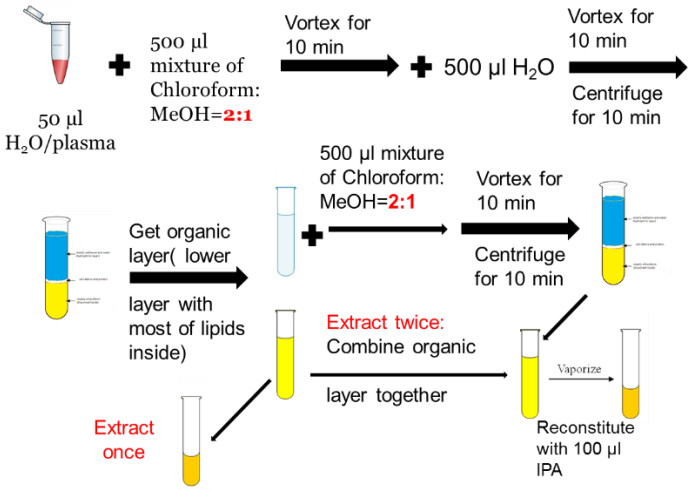


Figure 2.2. *The schematic of the Folch (once or twice extracted) extraction method. Plasma was extracted with a mixture of chloroform and methanol, and after vortex and centrifuge, the organic phase was collected, evaporated and reconstituted with isopropanol.*

2.2.2.4. Bligh and Dyer's method

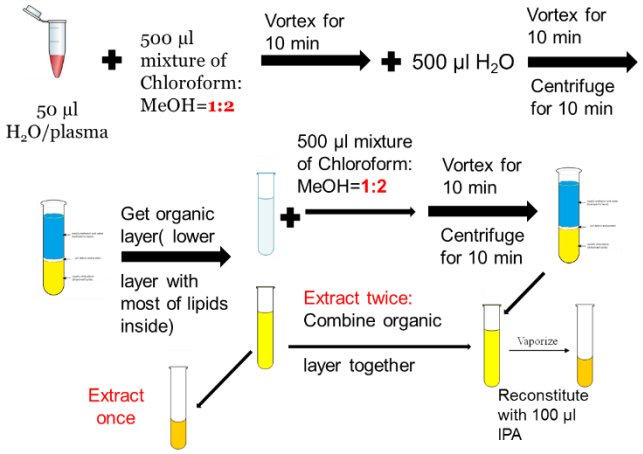


Figure 2.3. *The schematic of Bligh and Dyer (once or twice extracted) extraction method. Plasma was extracted with a mixture of chloroform and methanol, and after vortex and centrifuge, the organic phase was collected, evaporated and reconstituted with isopropanol.*

50 µl of plasma (sample) or water (regent blank) was added into 500 µl of ice-cold (-20℃) mixture (chloroform: methanol = 1:2). After 10 min vortex, 500 µl of water was added to the mixture. The mixture was vortexed again for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper aqueous phase and a lower organic phase, as well as a protein layer in the middle. The lower organic phase was collected and evaporated by a centrifugal evaporator. Then the sample was reconstituted with 100 µl of isopropanol and transferred into an LC-MS glass vial. In another version of the Bligh and Dyer method, the extracted lower organic phase was collected into a new Eppendorf and 500 µl of ice-cold chloroform: methanol (1:2) was added into the previous aqueous

and protein mixture. After 10 min of a vortex and 10 min of centrifuge, the new lower organic phase was combined with the previous organic phase together, and then evaporated under vacuum at room temperature using Juan centrifugal evaporator and reconstituted with 100 µl of isopropanol.

2.2.2.5. MTBE method

50 µl of plasma (sample) or water (regent blank) was added into 500 µl of ice-cold (-20℃) mixture (methanol: MTBE = 1:2). After 10 min vortex, 500 µl of water was added to the mixture. The mixture was vortexed again for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper organic phase and a lower aqueous phase, as well as a protein layer at the bottom. The upper organic phase was collected and evaporated by a centrifugal evaporator. Then the sample was reconstituted with 100 µl of isopropanol and transferred into an LC-MS glass vial. In another version of the MTBE method, the extracted upper organic phase was collected into a new Eppendorf and 500 µl of ice-cold methanol: MTBE (1:2) was added into the previous aqueous and protein mixture. After 10 min of a vortex and 10 min of centrifuge, the new organic phase was combined with the previous organic phase together, and then evaporated under vacuum at room temperature using Juan centrifugal evaporator and reconstituted with 100 µl of isopropanol.

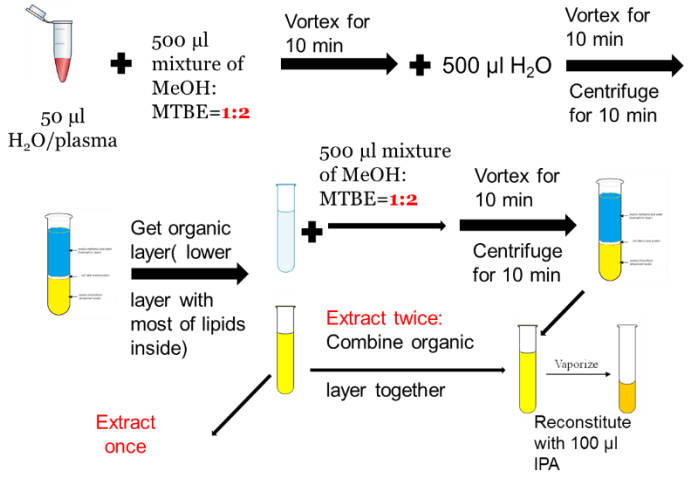


Figure 2.4. *The schematic of MTBE (once or twice extracted) extraction method. Plasma was extracted with a mixture of MTBE and methanol, and after vortex and centrifuge, the organic phase was collected, evaporated and reconstituted with isopropanol.*

2.2.3. LC-MS method development

2.2.3.1. LC-MS apparatus

An Accela high-speed LC-MS system was used which consists of a Quadrupole-Exactive mass analyser (Thermo Fisher Scientific, Hemel Hempstead, UK), Accela Quadrupole-Exactive autosampler, Accela Quadrupole-Exactive pump and Accela Quadrupole-Exactive column oven. The system was operated

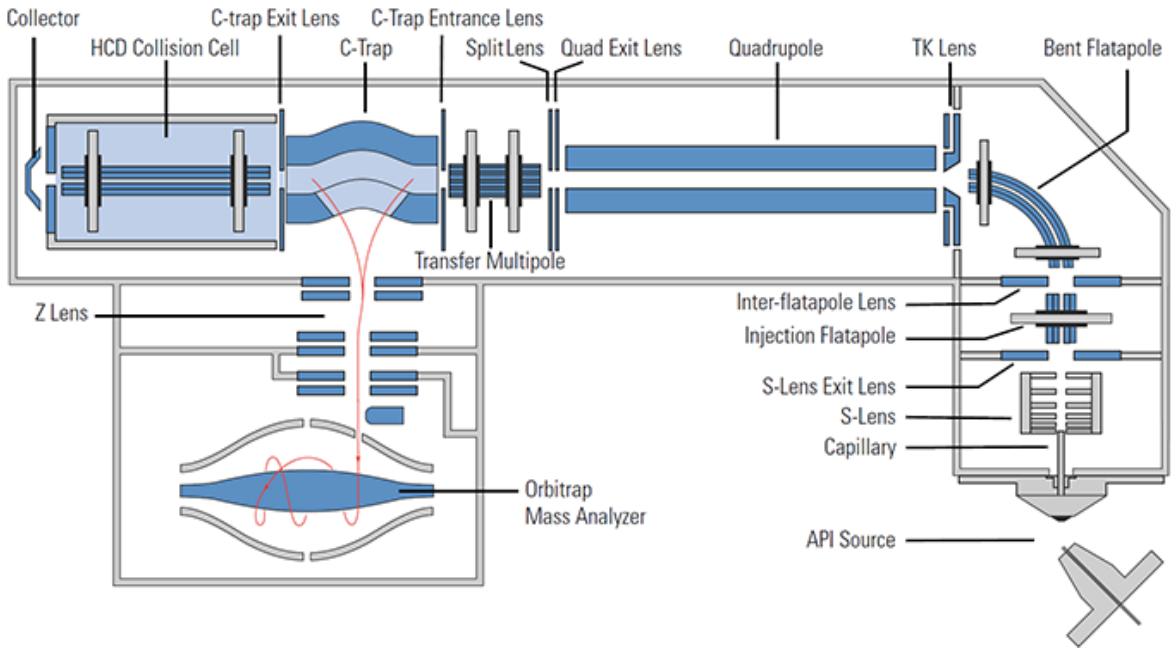


Figure 2.5. *A schematic diagram for Orbitrap Quadrupole-Exactive mass spectrometry (creative proteomics website).*

under Xcalibur software 3.0 (Thermo Fisher Scientific, USA). The mechanism of this MS system was shown in Figure 2.5.

2.2.3.2. Liquid chromatography condition modification

LC-MS method was modified based on a previously published article [125].LC–MS lipidomics analysis was conducted on an Accela high-speed liquid chromatography coupled to Quadrupole-Exactive mass spectrometry (Thermo Fisher Scientific, Hemel Hempstead, UK).LC–MS analysis was maintained at a temperature of 40 °C and a flow rate of 300 µL/min for chromatogram separation. The mobile phase consisted of (A) 60% of H2O, 40% of acetonitrile and 0.1% of ammonium acetate and (B) 80% of isopropanol, 10% of methanol, 10% of H2O and 0.1% of ammonium acetate. A binary gradient (40–100 % B) from channels A and B was used for 0–18 min, 100 % B for 18–27 min, a binary gradient (100 %-20% B) from 27 to 28 min and 20% of B for 28-30 min, with a total run time of 30 min for 10 cm column. The injection volume was 5 µL. All study samples and QC samples were analysed in a single continuous analytical run. Then this method was modified by comparing columns, mobile phase proportion, gradient, collision energy and other conditions that will influence lipid separation and identification.

2.2.3.2.1. Comparison of C18 (100 ×2.1 mm) and C18 (50 ×2.1 mm) columns

In this section, two different lengths of columns were compared to get a better lipid elution efficiency. A guard column was used to protect the LC column. ACE Excel 3 SuperC18 Column(100×2.1mm id)

and ACE Excel 2 C18 (50×2.1mm id) were chosen to evaluate the length influence on lipid elution. Both of the columns have a 2.1 mm id and 300 particle size, but in different column lengths (10 cm and 5 cm). The 30 min running time method was conducted on a 10 cm column, and a reduced 15 min running time was conducted on a 5 cm column, with all of the mobile phase percentage change time reduced to half correspondingly. A binary gradient (40–100 % B) from channels A and B was used for 0 to 18 min, 100 % B for 18 to 27 min, a binary gradient (100 %-20% B) from 27 to 28 min and 20% of B for 28 to 30 min, with a total run time of 30 min for 10 cm column. A binary gradient (40–100 % B) from channels A and B was used for 0 to 9 min, 100 % B for 9 to 13.5 min, a binary gradient (100 %-20% B) from 13.5 to 14 min and 20% of B for 14 to 15 min, with a total run time of 15 min for 5 cm column. The details of the time, mobile phase percentage and flow rate of the two columns are shown in table 1.

Table 2.3. The comparison of the two columns

Time of 10 cm (min)	Time of 5 cm (min)	A%	B%	Flow rate (µL/min)
0.0	0.0	60	40	300
2.0	1.0	55	45	300
18.0	9.0	0	100	300
27.0	13.5	0	100	300
28.0	14.0	80	20	300
30.0	15.0	80	20	300

A% and B% represent the percentage of mobile phases A and B. The constitution of mobile phase A: 60% of H2O, 40% of acetonitrile and 0.1% of ammonium acetate. The constitution of mobile phase B: 80% of isopropanol, 10% of methanol, 10% of H2O and 0.1% of ammonium acetate.

2.2.3.2.2. Comparison of mobile phase proportion

In this liquid chromatography system, the C18 column acts as a hydrophobic stationary phase and nonpolar analytes will be retained longer when the mobile phase elutes through the column. As lipids have different polarity by class, they will be eluted in a different order and thus separated. Polar lipids will be eluted earlier, and nonpolar lipids will be eluted later. Both mobile phases A and B are a mixture of different polarity solvents, and mobile phase A is more polar than mobile phase B. By changing the proportion of mobile phase A and B in the run, the percentage of mobile phase B increased and nonpolar lipids were eluted gradually.

A series of different proportions of mobile phases A and B were compared to study the mobile phase composition's influence on lipids elution. Guard column and ACE Excel 3 SuperC18 Column(100×2.1mm id) were used to conduct the mobile phase proportion comparison. The starting percentage of mobile phase B ranges from 40% to 50%, 60% and 70%, and then increased by 5% of

mobile phase B at 1 min (10 cm column) or 0.67 min (5 cm column). The proportion of mobile phase B became 100% at 9 min (10 cm column) or 6 min (5 cm column). From 13.5 min to 14 min (10 cm column) or 9 min to 9.33 min (5 cm column), the percentage of mobile phase B decreased from 100% to 20%, 50%, 60% and 70% respectively. The flow rate was a matinee at 300 µL/min. The details of the comparison of the proportion of mobile phases A and B were summarized in table 2 below.

Table 2.4. The comparison of the different proportions of mobile phases A and B.

Time of 10 cm (min)	Time of 5 cm (min)	B%				Flow rate (µL/min)
0.0	0.0	40	50	60	70	300
1.0	0.67	45	55	65	75	300
9.0	6.0	100	100	100	100	300
13.5	9.0	100	100	100	100	300
14.0	9.33	20	50	60	70	300
15.0	10.0	20	50	60	70	300

The percentage of mobile phase B started from 40%, 50%, 60% and 70% in the comparison, and then increased by 5% of mobile phase B at 1 min (10 cm column) or 0.67 min (5 cm column). The proportion of mobile phase B became 100% at 9 min (10 cm column) or 6 min (5 cm column). From 13.5 min to 14 min (10 cm column) or 9 min to 9.33 min (5 cm column), the percentage of mobile phase B decreased from 100% to 20%, 50%, 60% and 70% respectively.

2.2.3.2.3. Comparison of gradient

The separation of compounds in the column was achieved by changing the eluting solvent, and thus the change of the mobile phase needs to be investigated. In this section, the LC method started with a proportion of 60% mobile phase A and 40% mobile phase B, and gradually increased the percentage of mobile phase B until it became 100%. After keeping the mobile phase B at 100% for a while, decreased it to 20% in 0.5 min and keep it at 20% for 1 min. 0.1% of ammonium acetate was added to both mobile phases A and B for lipid ionization. It makes lipids into positive and negative ions and has NH_4+, H+, H- or CH_3COO- addicts according to their structure. The mobile phase B was also used for washing the autosampler between the injection.

As Table 2.5 shows, different gradients were compared by changing the slope of mobile phase B from 40% to 100%. To adapt the method for clinical applications, the running time was shortened to 15 min for a 10 cm column and 10 min for a 5 cm column. The starting percentage of mobile phase B is 40% and then increased to 45% of mobile phase B at 1 min (10 cm column) or 0.67 min (5 cm column). Then the mobile phase B increased to 100% at 9 min, 7 min, 5 min, and 3 min on the 10 cm column, or at 6 min, 5 min, 4 min, 3 min, and 2 min on the 5 cm column. Afterwards, the mobile phase B was kept at 100% until 13.5 min for the 10 cm column and 9 min for the 5 cm column. The mobile phase B was

decreased to 20% at 14 min on the 10 cm column and 9.33 min on the 5 cm column, and the proportion of mobile phase was kept at 20% until the end of the run, which is 15 min for 10 cm column and 10 min for 5 cm column.

Table 2.5. Method development on gradient change

Time (min) for 10 cm column				Time (min) for 5 cm column					A%	B%	Flow rate (µL/min)
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	60	40	300
1.0	1.0	1.0	1.0	0.67	0.67	0.67	0.67	0.67	55	45	300
9.0	7.0	5.0	3.0	6.0	5.0	4.0	3.0	2.0	0	100	300
13.5	13.5	13.5	13.5	9.0	9.0	9.0	9.0	9.0	0	100	300
14.0	14.0	14.0	14.0	9.33	9.33	9.33	9.33	9.33	80	20	300
15.0	15.0	15.0	15.0	10.0	10.0	10.0	10.0	10.0	80	20	300

All of the conditions (black number) were kept the same, and only the time that mobile phase B increased to 100% (red number) was changed to investigate the gradient influence on lipid elution and separation. For the 10 cm column, mobile phase B became 100% at 9, 7, 5, and 3 min, while for the 5 cm column, mobile phase B became 100% at 6, 5, 4, 3, and 2 min.

2.2.3.3. Mass spectrometry condition modification

Before conducting the mass spectrometry analysis, Pierce® solution was used for calibrating the instrument in positive and negative ionization modes separately, to increase the analysis accuracy. Data were acquired simultaneously in full scan ion mode (*m/z* 100–1500, resolution 70,000, AGC target 3e6) in both positive and negative electrospray ionisation (ESI) mode. Data were also acquired in tandem MS/MS mode at data-dependent MS/MS scans with top 5 precursor ions per scan (resolution 17,500, AGC target 1e5). The capillary temperature and heater temperature were maintained at 256.25 °C and 412.5 °C respectively in both positive and negative modes. The maximum scan injects time and micro scans were 100 ms and 1 ms respectively. MS was performed in simultaneous ESI (+) and ESI(−) full-scan modes with spray voltages of 3.5 (ESI+) and 4.0 kV (ESI−).

A list of lipids (594 lipids) with absolute mass, formula, polarity, adduct type and name that were pre-defined from human plasma[105] was loaded to the MS method as an included list. When ionised lipids were injected into the mass spectrometer, they were fragmented by electrospray ionization (ESI). As lipids can be fragmented into different size fragments according to the collision energy, different collision energy was compared to get the best identification of lipids. When collision energy is not high enough, some lipids (like fatty acids) cannot be fragmented and thus could not be identified, when collision energy is too high, lipids could be fragmented into many small parts, which is also hard to identify. In this section, the collision energy was increased from 35 to 200 gradually, and the identified number of lipids in each extraction method and class was summarized in section 2.3.4.

2.2.4. Data analysis

Table 2.6. LipidSearch™ parameter used for lipid identification.

Search	Parameter
Settings	Q-Exactive
Search type	Product
Experiment type	LC-MS
Precursor tolerance	5 ppm
Product tolerance	5 ppm
Intensity threshold	1.0%
Execute Quantitation	On
m/z tolerance (quantitation)	−/+ 5.0 ppm
RT range (min.) (quantitation)	−/+ 0.5
Top rank filter	On
Main node filter	Main isomer peak
m-Score threshold	5.0 (1.0) *
c-Score threshold	2.0
FA priority	On
ID Quality Filter	A, B, C and D
Target class	ALL lipid classes
Ion adducts (pos)	H+, NH4+
Ion adducts (neg)	H-, CH3COO +
S/N	2

**m-Score threshold was selected as 1 when the lipids profile of different species was explored in order to include as much as possible of that species' lipidome to get a better comparison between the studied species.*

LC–MS raw data from the analysis were acquired with Xcalibur v3.0 software (Thermo Scientific, Hemel Hempstead UK). The lipid peaks were shown in the Xcalibur Qual browser. The ionised lipid mass and m/z result were used to identify and verify the lipid peak. By comparing the m/z value and lipid fragmentation ions, we can confirm the lipid isomer. By comparing the lipid peak shape and intensity in different ionization modes and with different addicts, the best ionization mode and adducts were chosen to identify the specific lipids.

Chromatographic raw data contain a 3D data set consisting of *m/z*, retention time and ion signal intensity. These data were then processed with LipidSearch v5.1 (MitsuiKnowledge Industry, Tokyo, Japan) to align the replicates and control results to get the extracted lipid formula. LipidSearch™ is a lipid identification software for lipidomics data by comparing their m/z and fragmentation patterns with an internal library that contains more than one million lipid ions and their predicted fragment ions from fatty acid chains and head groups. The library contains 19 main lipid classes and 71 subclasses. The

software can also be used to align lipid data obtained from multiple LC-MS samples. LipidSearch™ parameters used for lipids identifications and alignment across samples are listed in Tables 2.6 and 2.7.

Table 2.7. LipidSearch™ parameters used for alignment.

Search	**Parameter**
Experiment type	LC-MS/MS
Alignment Method	mean
R.T. Tolerance	0.5 min
Calculate the unassigned peak area	On
Filter type	New filter
Top rank filter	On
Main node filter	Main isomer peak
m-Score threshold	5.0 (1.0) *
ID quality filter	A, B, C and D

**m-Score threshold was selected as 1 when the lipids profile of different species was explored to include as much as possible of that species' lipidome to get a better comparison between the studied species.*

2.3. Results and Discussion

2.3.1. Defining a comprehensive list of lipids in human blood plasma

The human lipidome has been characterised by many different analytical methods and a comprehensive overview has been published previously [105], [126] listing the classification and individual lipids in each class identified in human plasma. These lipids were categorized into different classes according to the commonly used lipid classification system [4], [13]. The formula and monoisotopic mass of each lipid was added according to Lipid Maps and HMDB databases. The fully identified lipid list is shown in the appendix (Table A) with the confirmed identities processed by LipidSearch software. This list was subsequently used as an original lipid database uploaded onto the mass spectrometry inclusion list for developing the semi-quantitative lipidomic analysis and for clinical sample analysis. Table 2.8 is a summarised lipid database to show the number of lipids included from previous databases and those which were confirmed in this work by LC-MS analysis in each class.

The classification of lipids, the number of lipids summarized from published literature and the number of lipids identified in this LC-MS method was shown in Table 2.8. As the literature is a summarization of identified lipids with different extraction and LC-MS methods [3], [4], it is more conclusive than a single method. The LC-MS method used in this chapter shows a broad coverage of lipids in different classes, with good sensitivity and reproducibility. From the LipidSearch result, there are also many lipids identified but not in this lipid list, and they were added to the final lipid list (Table A).

Table 2.8. Lipid database summary

Lipid Class	lipid numbers	Identified number in this method
Diacylglyceride	28	5
Ceramides	41	0
Cholesteryl ester	22	13
CoQ	2	0
Eicosanoids	76	3
Fatty acids	31	6
Glycerophospholipids	135	63
N-acyl-PS	2	0
Sphingoid bases	6	0
Total (free and esterified) sterol	13	1
Triacylglyceride	18	17
Grand Total	374	108

2.3.2. Workflow

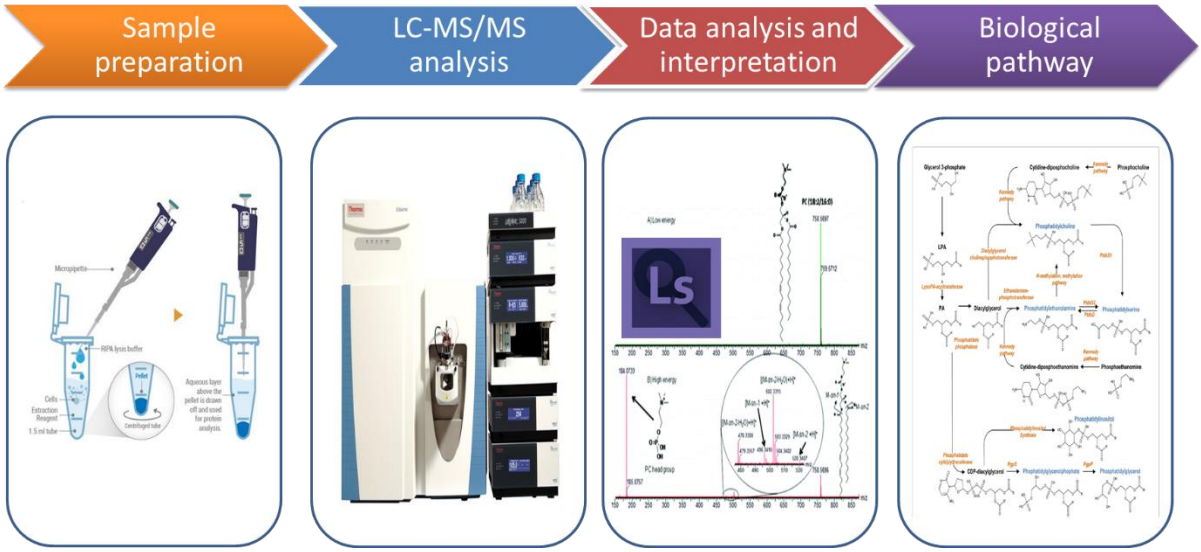


Figure 2.6. *The schematic of lipidomics study. Lipidomics, no matter whether untargeted, targeted or semi-targeted lipidomics, share a similar workflow to this figure. The sample was extracted and loaded on the LC-MS techniques, afterwards using professional software to analyse the result to find potential biomarkers.*

2.3.3. Efficiency of extraction

Different liquid-liquid extraction solvents and different extraction times were compared to select a proper extraction method for clinical samples. The lipid extraction efficiency with different methods was shown in Table 2.9. The results were collected through LipidSearch software. There are several classic extraction methods used broadly for lipidomics, including the Folch method (chloroform: methanol=2:1) [107] and Bligh and Dyer method (chloroform: methanol=1:2) [108]. These two methods use different ratios of methanol and chloroform to get a good cover of the total classes of lipids. Another methanol and tert-butyl methyl ether (MTBE) method (methanol: MTBE=1:2) shows a comparison result with Bligh and Dyer method and is easier to collect the organic layer since the light density of MTBE solvent [110]. The extraction efficiency of these several prominent extraction methods has been compared for lipid classes in human LDL [127], and in this study, these extraction methods were compared by identified lipid numbers in total and h class.

As Table 2.9 shows, the SPE method has the best coverage of extracted lipids, the Folch method, Bligh and Dyer method and MTBE method do not reveal a significant difference in identified lipid numbers. Due to the SPE method is time-consuming and the results are variable, which will influence clinical sample stability and reproducibility [128], a quicker and more convenient liquid-liquid extraction method need to be selected. As the result shows, the Folch method, Bligh and Dyer method and MTBE method have different performances on the class of lipids, for example, the Folch method and Bligh and Dyer method display a better coverage on phospholipids, while the MTBE method is better at triglyceride coverage. The result also shows extraction twice is slightly beneficial for the extraction, however, this benefit could be compensated by the conduction complexity with hundreds of clinical samples and result instability. Based on the experimental data, to shorten the extraction times and get a good lipid extraction efficiency at the same time, the Folch method (chloroform: methanol (2:1)) extraction once was chosen for the modified LC-MS method.

Table 2.9. Identified lipid number in each class with different extraction methods

Extraction solvent	Lipid Class																			Grand Total
	Cer	ChE	DG	dMePE	FA	LdMePE	LPC	LPE	PA	PC	PE	PG	PI	PS	SIE	SM	So	StE	TG	ZyE	
CHCl3: MeOH (1:2)	13	16	18		1	1	26	6	1	62	41	1	5	13		13	2		37	1	257
CHCl3: MeOH (1:2) *2	15	13	19	3	1	1	32	7	1	69	40		11	19		15	2	1	37	2	288
CHCl3: MeOH (2:1) *2	16	15	23	3		1	35	9		66	41		7	17		13	2	1	36	2	287
CHCl3: MeOH (2:1)	17	10	19	2	1	1	31	6	1	69	41		6	18		14	2		33	2	273
MeOH: MTBE (1:2)	12	9	20			1	19	5		52	36		6	6	1	7			62		236
MeOH: MTBE (1:2) *2	9	9	19	1		1	21	7		56	38	2	7	4		7			58		239
SPE	18	20	38	2	2	2	43	12	2	81	51	1	8	18		15	3		82	5	403

Cer-Ceramide, ChE-Cholesteryl ester, DG-Diacylglycerol, dMePE- Dimethyl phosphatidylethanolamine, FA-Fatty acids, LdMePE-lysodimethylphosphatidylethanolamine, LPC-lysophosphatidylcholine, LPE-lysophosphatidylethanolamine, PA- Phosphatidic acid, PC- Phosphatidylcholines, PE-Phosphatidylethanolamine, PG-Phosphatidylglycerol, PI-Phosphatidylinositol, PS-Phosphatidylserine, SIE-Sitosteryl ester, SM-Sphingomyelin, So-Sphingosine, StE-Stigmasterel ester, TG-Triglyceride, ZyE-Zymosteryl ester. CHCl3-Chloroform, MeOH-Methanol, MTBE-methyl tert-butyl ether.

**2 implies this method extracted the sample twice, and the detailed extraction procedure was written in section 2.2.2. CHCl3: MeOH (1:2) is Bligh and Dyer method extracted once, CHCl3: MeOH (1:2) *2 is Bligh and Dyer method extracted twice. CHCl3: MeOH (2:1) is Folch method extracted once, CHCl3: MeOH (2:1) *2 is the Folch method extracted twice. MeOH: MTBE (1:2) is MTBE method extracted once, MeOH: MTBE (1:2) *2 is the MTBE method extracted twice. SPE is solid phase extraction.*

The identified lipid number is calculated based on the result exported by LipidSearch software.6 replicates were used for SPE and other extraction methods.

2.3.4. Comparison and optimisation of LC-MS conditions

To compare the lipid elution efficiency difference according to LC-MS condition changes, different C18 columns (ACE Excel 3 SuperC18 Column(100×2.1mm id), ACE Excel 2 C18 (50×2.1mm id)) were chosen to compare as well as different gradients, mobile phase proportion, running time, etc. Investigation of a suitable gradient LC-MS method was the primary step in the method development stage. The mobile phase consisted of (A) 60% of H_2O, 40% of acetonitrile and 0.1% of ammonium acetate and (B) 80% of isopropanol, 10% of methanol, 10% of H_2O and 0.1% of ammonium acetate. The gradient started with 40% mobile phase B, changing to 100% mobile phase B at 9 min and maintained at 100% mobile phase B to 13.5 min, and finally to the initial conditions at 15 min. This primary LC-MS condition was further developed by changing time from 40% mobile phase B to 100% mobile phase B, the starting percentage of mobile phase B, as well as total running time. By changing these conditions, the composition of the mobile phase in the column was changed and the retention time of lipids was changed accordingly.

Table 2.10. Identified lipid numbers in different LC-MS conditions

LC-MS method	Identified lipid number	
	10cm	5cm
10min(2min) 40% of B		271
10min(3min) 40% of B		269
10min(4min) 40% of B		274
10min(5min) 40% of B		286
40% of B 10min(6min)		285
50% of B 10min(6min)		303
60% of B 10min(6min)		310
70% of B 10min(6min)		302
15min 40% of B		329
15min(3min) 40% of B	325	
15min(5min) 40% of B	347	
15min(7min) 40% of B	325	
40% of B 15min(9min)	312	
50% of B 15min(9min)	364	
60% of B 15min(9min)	350	
70% of B 15min(9min)	356	
30min 40% of B	421	

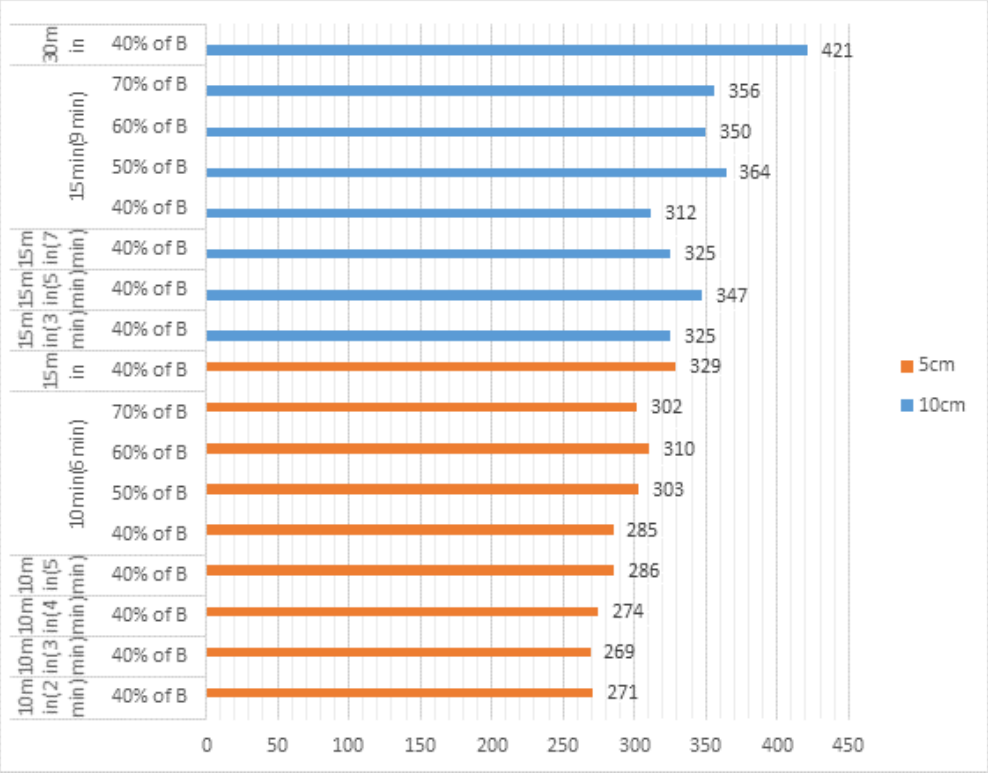


Figure 2.7*. Identified lipid numbers in different LC-MS conditions. Blue bars are elution running on a 10cm column, and orange bars are elution running on a 5cm column. The highest identified lipid number is 421, on a 10 cm column with 30min run. In 15min running time, starting from 50% of mobile phase B (364 lipids) and gradient reaching 100% of mobile phase B at 5min (347 lipids) have higher identified lipid numbers than other conditions. In a 10min running time, starting from 60% of mobile phase B (310 lipids) and gradient reaching 100% of mobile phase B at 5min (286 lipids) have higher identified lipid numbers than other conditions.*

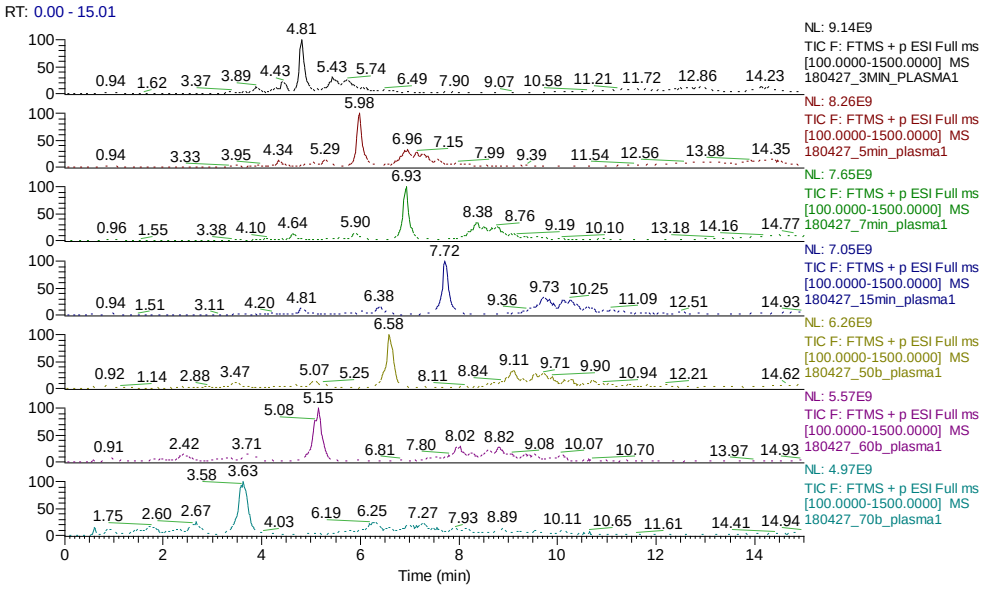


Figure 2.8. *TIC positive mode for different methods.*

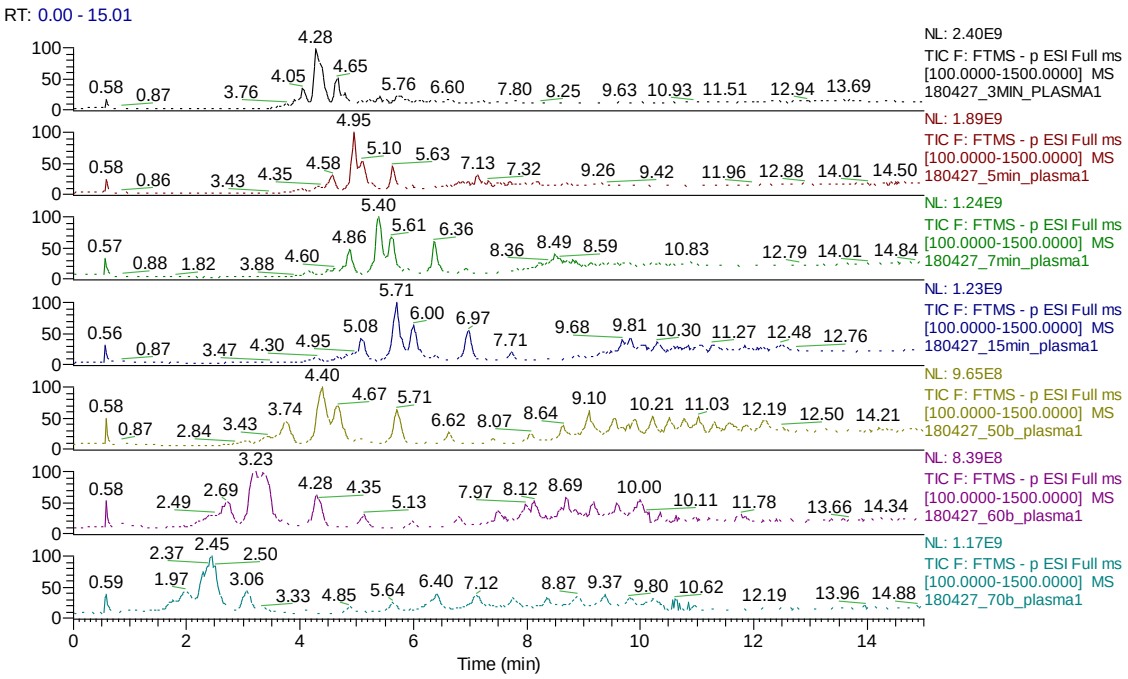


Figure 2.9. *TIC negative mode for different extraction methods.*

The eluted lipid number was chosen as a representative of elution efficiency. As Figures 2.8 and 2.9 show, increased column length and running time lead to more eluted lipids, this result is compatible with the findings from Hongji Liu [129]. In general, a 5 cm column elutes fewer lipids than 10 cm columns with the same method, it might be because of the shorter running time on the short column. Previous literature reported similar results and demonstrated the influence of column length also affected by the particle size. While increasing the length of a column packed with the 1.7 μm material helped improve peptide peak capacity at the whole range of the tested gradient lengths (24–432 min), little improvement in peak capacity was observed with the increase of the length of a column packed with the 3 μm material unless a gradient longer than 50 min was carried out [129]. Thus, a 10 cm column was chosen in later clinical applications to get a better peak separation and lipid identification, unless a shorter running time is necessary. Gradients and mobile phase proportions influence lipid elution as well, the final gradient and mobile phase proportions were chosen to get the best compound elution according to previous results [130]–[133]. 30 min run separated the most lipids compared to other methods, however, considering this method is built up for clinical studies, a long-running time may hamper samples stability, thus a shorter running time is considered. According to eluted lipid number, starting with 50% of mobile phase B and from the starting point to 100% of mobile phase B in 5 min (10 cm column) shows the best lipid elution in the comparison.

2.3.5. Optimisation of mass spectrometry detection

According to section 2.2.3.3., different collision energy (CE) was chosen to explore lipid fragmentation. As Table 2.11 shows, the collision energy was set at 35, 45, 55, 65, 80, 100, 150 and 200 V, and different lipid class shows different sensitivity to CE.

Table 2.11. The comparison of collision energy intensity and identified lipids by class

Count of Lipids	Collision Energy intensity							
	35	45	55	65	80	100	150	200
Cer	12	9	7					
ChE	14	11						
DG	24	13	4	4	4	4	4	4
dMePE	2	2	2					
FA	1	27						
LdMePE	1	1	1					
LPC	21	23	20	21	21	21		
LPE	4	5	4					
PA	1	1	1	1		2	2	1
PC	61	51	56	54	56	56	32	35
PE	47	43	40	34	36	42	43	37
phSM				1		1		
PI	7	7	7					
PIP2		1						
PS	18	24	7					
SM	9	4	7	5	3	7	1	1
TG	39	20	23	15	9	9	3	4
ZyE	4							
Grand Total	265	242	179	135	129	142	85	82

Some lipid classes were identified more when collision increased and then decreased onwards (like FA, LPC, PS), and other lipid classes get less identified lipids when collision energy increased. Since the different classes of lipids have different sensitivity to collision energy, different collision energy was chosen to get the most lipids in each class.

Table 2.12. Collision energy and fragmented lipid class

Collision energy (CE)	Extraction method			Main classes of lipids
	Folch	Bligh and Dyer	MTBE	
35	265	292	277	PE, PC, LPC, DG, TG, SM, phSM, Cer, StE, ChE, LPE, LdMePE, PS, PI, dMePE, FA, ZyE, PG
45	242	283	231	PE, PC, LPC, DG, TG, SM, phSM, Cer, StE, ChE, LPE, LdMePE, PS, PI, dMePE, FA, PIP2
55	179	186	166	PE, PC, PA, LPC, DG, TG, SM, phSM, Cer, StE, ChE, LPE, LdMePE, PS, PI, dMePE, FA
65	135	163	128	PE, PC, PA, LPC, DG, TG, SM, phSM, Cer, StE
80	129	140	130	PE, PC, LPC, DG, TG, SM, phSM, Cer
100	142	144	122	PE, PC, PA, LPC, DG, TG, SM, phSM, Cer
150	85	85	75	PE, PC, PA, DG, TG, SM, Cer
200	82	86	83	PE, PC, PA, DG, TG, SM

It presents the identified lipid class change in different CE and the influence of CE on different extraction methods. For all the three extraction methods, when CE increased from 35 to 200, the identified lipid number decreased gradually, thus for most of the lipid classes, CE35 was chosen, and for some specific lipid classes (FA, LPC, PS, etc), a higher CE was chosen to get most of the lipids.

2.3.6. Extracted ion chromatograms of some early and late eluted lipids in different methods

Extracted ion chromatography shows the chromatography of lipids with a certain mass. Due to the structural difference, lipids elute at a different time in the elution. To ensure all the lipid classes were eluted in the modified liquid chromatography method, the early and late eluted lipids were chosen to justify the elution competence in different methods. Lipids were chosen according to the generated SPE lipid list and retention time accordingly. Extracted ion chromatograms of these lipids were performed with Xcalibur software to check the peak shape as well as the retention time. The spectrum was also used to identify the lipids.

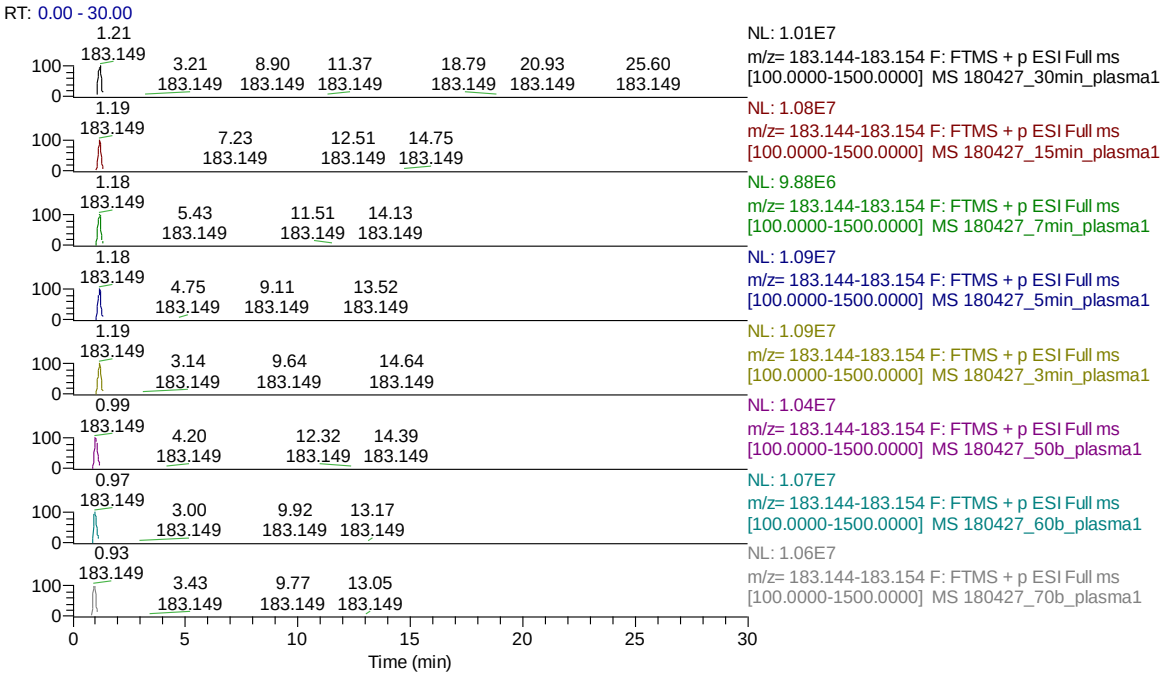


Figure 2.10. *An early eluted fatty acid ($C_{11}H_{18}O_2$) in different LC-MS conditions. The LC-MS condition from above to bottom are: 30 min (40% of B start, 18 min to 100% B), 15 min (40% of B start, 9 min to 100% B), 15 min (40% of B start, 7 min to 100% B), 15 min (40% of B start, 5 min to 100% B), 15 min (40% of B start, 3 min to 100% B), 15 min (50% of B start, 9 min to 100% B), 15 min (60% of B start, 9 min to 100% B), 15 min (70% of B start, 9 min to 100% B), all of them are in positive mode. As figure 6 shows, the elution of these lipids shows quite similar retention time and asymmetric peak shape in all the compared LC-MS methods, which can be used as an indication of good lipid elution coverage on early eluted lipids.*

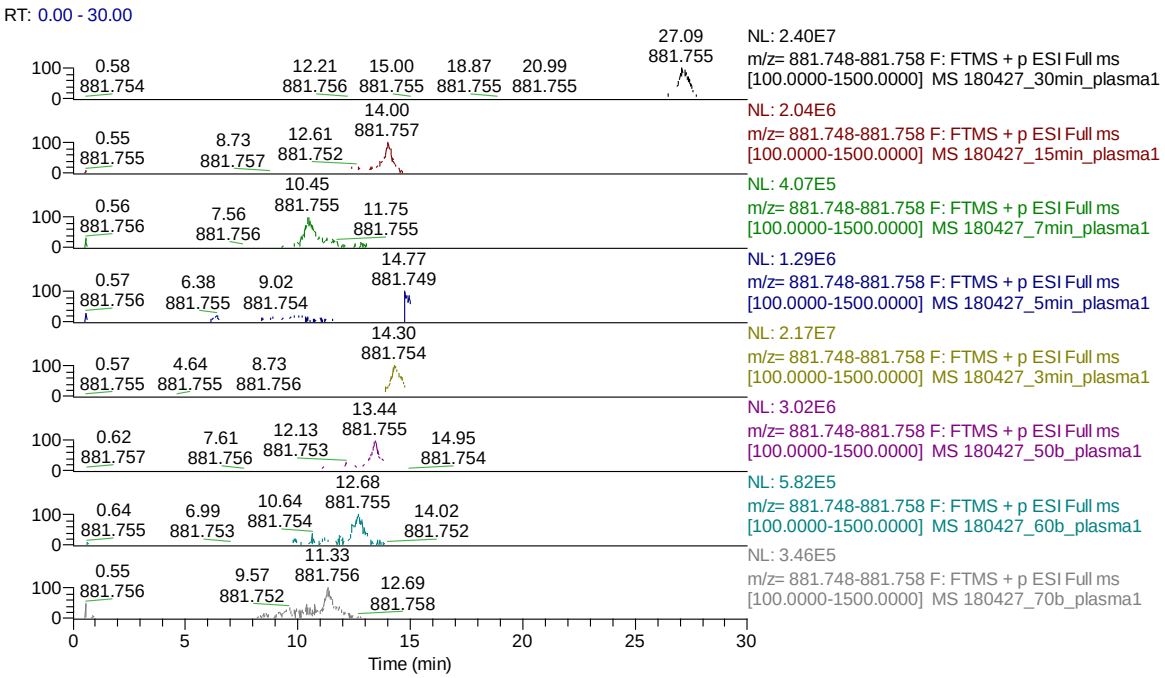


Figure 2.11. *A late eluted lipid (1-alkyl-2-acylglycerophosphocholines, $C_{52}H_{100}NO_7P$) in different LC-MS methods. The LC-MS condition from above to bottom are: 30 min (40% of B start, 18 min to 100% B), 15 min (40% of B start, 9 min to 100% B), 15 min (40% of B start, 7 min to 100% B), 15 min (40% of B start, 5 min to 100% B), 15 min (40% of B start, 3 min to 100% B), 15 min (50% of B start, 9 min to 100% B), 15 min (60% of B start, 9 min to 100% B), 15 min (70% of B start, 9 min to 100% B), all of them are in positive mode.*

As Figure 2.11 shows, the elution of the lipid shows a quite similar retention time and good peak shape in all the compared LC-MS methods, which can be used as an indication of good lipid elution coverage on late eluted lipids.

2.3.7. Total ion chromatogram for the chosen LC method

The developed LC-MS method was expected to have a proper lipid elution that incorporates different classes of lipids, and a symmetric peak shape for eluted lipids. Also, the short running time was considered for efficient clinical applications. To fulfil these requirements, a 15 min running time on a 10 cm C18 column was chosen with a 300 µL/min flow rate. A starting percentage of 50 % of mobile phase B was chosen and increased to 100% of B at 5 min. After keeping at 100 % of mobile phase B until 13.5 min, the gradient drops back to 50 % of B at 14 min and is maintained at the same gradient for 1 min. The developed method was shown in Table 2.13.

Table 2.13. The developed LC condition for the 10 cm column

Time (min)	A%	B%	Flow rate (µL/min)
0	50	50	300
1	45	55	300
5	0	100	300
13.5	0	100	300
14	50	50	300
15	50	50	300

To have a further understanding of the lipid elution on the developed LC-MS method, the total ion chromatograms (TIC) were generated on negative and positive modes. The elution of different classes of lipids was monitored on the chromatogram. A good retention time difference is proof of good elution efficiency in this method.

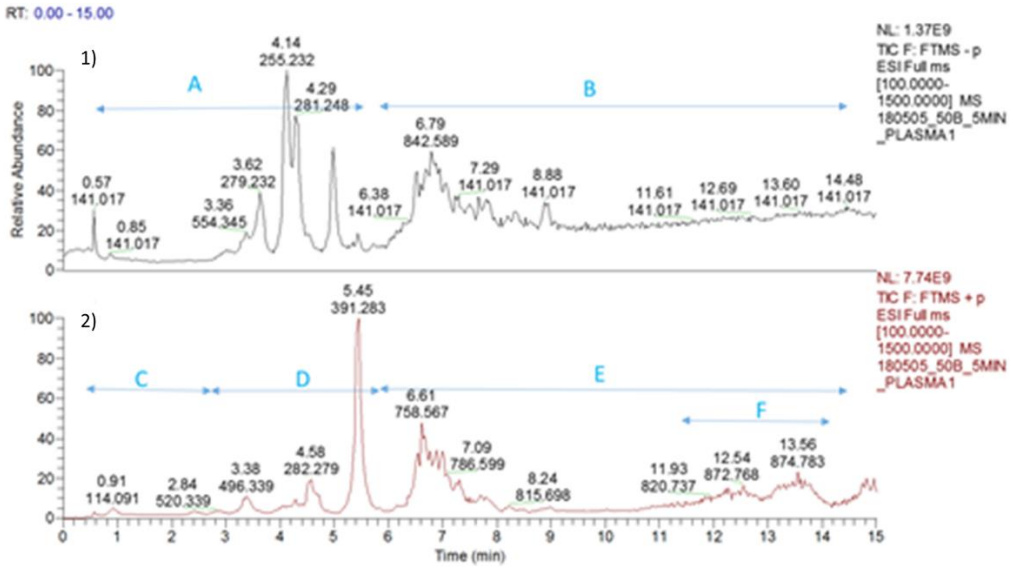


Figure 2.12. *Total ion chromatograms of plasma lipids extracted with chloroform/methanol (2:1) in both negative (1) and positive (2) modes using the chosen LC-MS method (Table 5). The composition of mobile phase A is 60% of H_2O, 40% of acetonitrile and 0.1% of ammonium acetate and mobile phase B is 80% of isopropanol, 10% of methanol, 10% of H_2O and 0.1% of ammonium acetate. The total run time is 15 min. This figure shows different classes of lipids elution through the column. A - fatty acids, B-phosphatidylcholines, phosphatidylethanolamines, phosphatidylserines, sphingomyelins, diacylglycerides, triacylglycerides, C – fatty acids, D – lyso-phosphatidylcholines, eicosanoids, E – phosphatidylcholines, phosphatidyl ethanolamines, phosphatidylserines, sphingomyelins, diacylglycerides, F – triacylglycerides.*

2.3.8. The separation of closely eluted lipids

To validate the peak separation of lipids in a narrow range of retention time, a series of lipids was chosen for comparison. The extracted ion chromatograms of various lipids in both positive and negative modes with final optimised LC conditions demonstrated sharp peak shapes with better resolution. The clear lipid and peak identification show a good lipid separation.

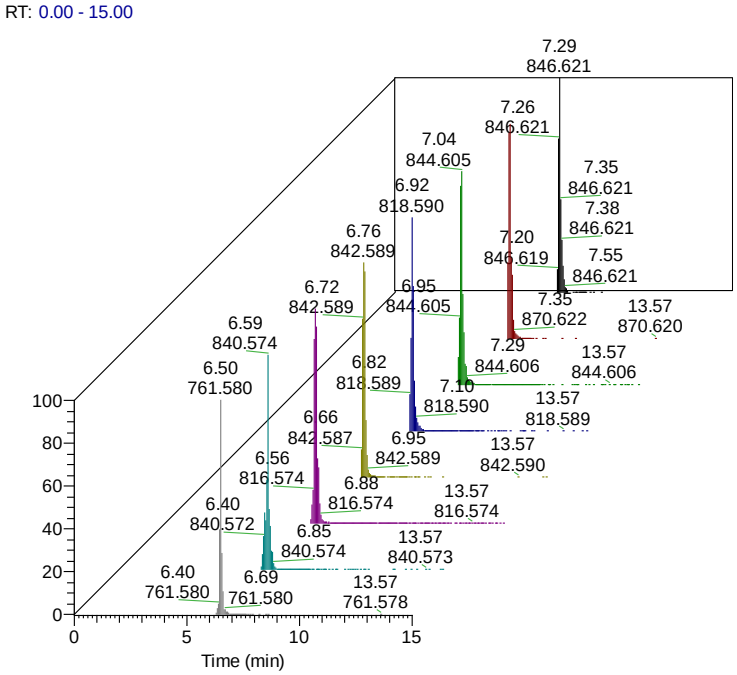


Figure 2.13. *Extracted ion chromatograms of different lipids in negative mode are shown above, all the lipids are well separated, and the peak shape was symmetrical. The LC condition is shown in table 4. The chosen lipid from below to upper are: PC (34:0) (761.580, 6.50 min), TG (51:4) (840.574, 6.59 min), TG (49:2) (816.574, 6.72 min), TG (51:3) (842.589, 6.76 min), TG (49:1) (818.590, 6.92 min), TG (51:2) (844.605, 7.04 min), TG (53:3) (870.621, 7.26 min), TG (51:1) (846.621, 7.29 min).*

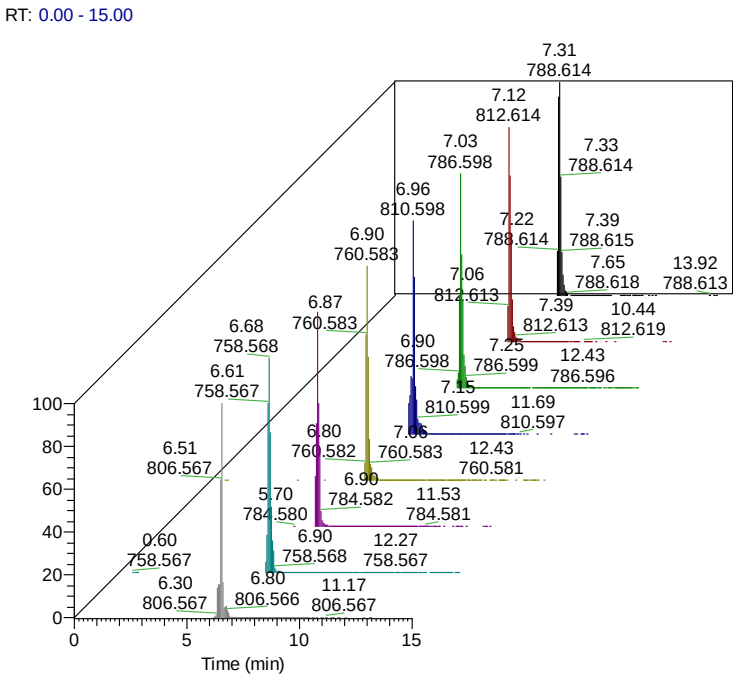


Figure 2.14. *Extracted ion chromatograms of different lipids in positive mode are shown above, all the lipids are well separated, and the peak shape was symmetrical. The LC condition is shown in table 4. The chosen lipid from below to upper are: TG (48:0) (806.567, 6.51 min), TG (45:3) (758.567, 6.61 min), TG (47:4) (784.584, 6.68 min), TG (45:2) (760.583, 6.90 min), TG (49:5) (810.599, 6.96 min), TG (47:3) (786.599, 7.03 min), TG (49:4) (812.614, 7.12 min), TG (47:2) (788.615, 7.31 min).*

2.4. Conclusion

In this study, the focus was on developing a semi-targeted method to identify more than 300 lipids from different classes and analyse the concentration change of these lipids in patients. For this purpose, solid-phase extraction was used to build up a lipid library from the plasma sample and has improved the coverage significantly than the liquid-liquid extraction method. Different liquid-liquid extraction methods were compared for later clinical application. The liquid chromatography method was developed by modifying the gradient, mobile phase proportion, running time and changing columns. The mass spectrometry condition was developed through choosing the proper parameters. As a result, the chloroform/methanol (2:1) extraction method was chosen for future extraction work, and 15 min run time on a C18 column (10 cm) was developed and has shown a good lipid elution efficiency. The modified method presents a significant increase in identified lipid number compared to the starting LC-MS method, as well as better separation of lipid peaks in similar retention time. In addition, this method will be advantageous for lipidomics studies of cellular responses and pathologyand should be useful for detecting minor lipid mediators such as LPA, LPS, and their precursors.

Chapter Three

LC-MS-based semi-targeted lipidomic method normalisation and validation

3.1 Introduction

3.1.1 LC-MS validation

The concept of validation was first introduced by the U.S. Food and Drug Administration (FDA) in the 1970s to assess the quality of pharmaceutical products [134]. The FDA defines validation as "the establishment of documentary evidence that provides a high degree of assurance that a particular process will consistently produce a product that meets its intended specifications and quality attributes" [135]. Validation is the process of establishing documented evidence of a procedure, process, or activity performed in testing and then maintaining desired compliance at all stages in production. In the pharmaceutical industry, in addition to the final testing and compliance of the product, it is very important to ensure that the process continues to produce the desired results. The desired results are defined in terms of the specifications of the process results. Therefore, the qualification of systems and equipment is part of the validation process. Validation is a requirement of food, drug and pharmaceutical regulatory agencies such as the US FDA and their Good Manufacturing Practice guidelines. In other words, validation confirms that a properly designed system, such as a new semi-targeted lipidomics method, can provide a high degree of assurance that it is properly evaluated and suitable for its intended use before implementation [136]. In addition to evaluating normalization techniques, the validation tested various methods used to identify potential biomarkers, metabolic pathways, and other relevant information[137].

Method validation has received considerable attention in the literature, and the FDA has several guidelines for analytical and bioanalytical aspects, which are listed below. The first is a validation of analytical methods (this guidance provides recommendations to applicants on the submission of analytical procedures, validation, data, and samples to support documentation of the identity, strength, quality, purity, and potency of drug substances and drug products)[135], and the second is a validation of bioanalytical methods (this guidance applies to bioanalytical procedures used in human or nonhuman clinical, pharmacological, and toxicological studies and bioanalytical-based bioanalytical methods such as chromatography, immunology, and microbiology) for human or nonhuman clinical, pharmacological, and toxicological studies and preclinical studies based on bioanalytical procedures[136]. The above guidelines focus on seven common parameters that should be considered during the validation of bioanalytical methods to determine the "fitness" of the method. The definitions of these seven parameters are included in Table 3.1. The limit of detection (LOD), the limit of quantification (LOQ), linearity, and range require information on the exact concentration of the analyte in the sample, so they are important parameters for target lipidomics validation, but are not very appropriate in non-target lipidomics or semi-target lipidomics validation, as these studies usually do not examine the true concentration of the lipid, but rather focus on the identification and relative concentration variation between samples.

Table 3.1. Parameters for analytical method validation [137]

Criteria	Definitions
Accuracy	The accuracy of an analytical procedure is expressed in terms of the closeness between the values accepted as conventional true values or accepted reference values and the original values found.
Precision	The precision of an analytical procedure is defined as the closeness of agreement between a series of analytical measurements obtained from multiple sampling of the same homogeneous sample under the same developed conditions. Precision can be considered at two levels: repeatability and intermediate precision Repeatability: Repeatability can be defined as the accuracy under the same operating conditions over a short period of time, which can be performed within the same day. Repeatability is also referred to as intra-assay accuracy Intermediate precision: Intermediate precision can be defined as the precision performed within-laboratories variations, including different days, different analysts, different equipment, etc. Repeatability is also referred to as inter-assay precision
Specificity	Specificity is expressed as the ability to evaluate analytes indisputably in the presence of components that might be expected to be present. In general, these components may include impurities, degradants, substrates, etc.
Limit of detection (LOD)	The detection limit of an individual analytical procedure is the lowest amount of analyte that can be detected in a sample, but not necessarily the exact quantitative value.
Limit of quantitation (LOQ)	The limit of quantification for an individual analytical procedure is the minimum amount of analyte in the sample that can be quantified with appropriate precision and accuracy.
Linearity	The linearity of an analytical procedure is its ability to obtain test results (over a specific range) that are proportional to the concentration (amount) of analytes in the sample.
Range	The range of an analytical procedure is the interval between the upper and lower limits of the concentration (quantity) of the analyte in the sample, for which the procedure has been demonstrated to have appropriate precision, accuracy and linearity.

3.1.2. Validation parameters in semi-targeted lipidomics

3.1.2.1. Precision

In the semi-targeted lipidomics validation, relative standard deviation (%RSD), otherwise known as the coefficient of variation (CV) is used to examine the stability and repeatability of the method. The %CV (RSD) calculation formula is shown below:

$$RSD = \frac{S}{\overline{x}} \times 100\% = \frac{\sqrt{\frac{\sum_{i=1}^{n}(x_i-\overline{x})^2}{n-1}}}{\overline{x}} \times 100\%,$$

$$\overline{x} = \frac{x_1 + x_2 + \ldots + x_n}{n} = \frac{\sum_{i=1}^{n} x_i}{n}. \quad , \quad SD = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{x})^2}{n-1}},$$

FDA has proposed useful criteria for bioanalytical methods validation to calculate the relative standard deviation (RSD) of the compounds present in QCs and clinical samples. It anticipates the RSD lower than 15% in quality control (QC) and below 30% in clinical samples (80% of the sample meets the criteria) [136]. To calculate the relative standard deviation, at least three replicates need to be injected and analysed in the LC-MS run, preferably 6 replicates or more were used for RSD calculation.

3.1.2.2. Selectivity and Specificity

During technique improvement, the sponsor ought to check that the substance being estimated is the expected analyte to limit or keep away from the impedance. Selectivity of the technique is regularly exhibited by dissecting clear examples of the suitable organic framework (e.g., plasma) from numerous sources. Contingent upon the expected utilization of the examination, the effect of haemolyzed tests, lipemic tests, or tests from exceptional populaces can be remembered for the selectivity evaluation. When utilizing fluid chromatography/mass spectrometry (LC/MS) techniques, the support or candidate ought to decide the impacts of the framework on particle concealment, particle improvement, or extraction productivity. Inside principles ought to be surveyed to stay away from obstruction with the analyte. Possible meddling substances in a natural framework incorporate endogenous network parts like metabolites, disintegration items – and from the genuine review – corresponding drugs and other xenobiotics. If a stabilizer or compound inhibitor is utilized during test assortment, the support ought to assess the potential for impedance on the quantitation of the analyte. Patrons should make a logical judgment about the need to survey these (and some other) likely impediments during technique advancement.

Ion suppression in LC-MS and LC-MS/MS refers to a reduction in detector response, or the apparent effect of competing ionization efficiencies as ionization sources, of other endogenous or exogenous (e.g., plasticizers from plastic tubes[138], mobile phase additives) substances that are not removed from

the sample matrix during sample preparation. Strictly speaking, ion suppression is not a problem unless the interfering compound elutes simultaneously with the analyte of interest. In cases where ion suppression does co-elute with the analyte, the effects on important analytical parameters including precision, accuracy, and detection limits (analytical sensitivity) can be extensive and severely limit the validity of the analytical results. Several methods can be used to eliminate ion suppression effects, such as modifying chromatographic separations, using appropriate sample preparation protocols, reducing analyte concentrations by reducing injection volumes and lowering flow rates, normalizing analytes with internal standards, etc[139].

3.1.2.3. Quality Control

Quality control (QC) is used to assess the precision and accuracy of the assay and the stability of the sample. QCs should be prepared in the same matrix as the study samples that will be assayed with the validated method. During method development, it is recommended that newly prepared QCs be used for precision and accuracy analysis, as stability data are generally not available at this time. During method validation, QCs assess the performance of the method and the stability of the analytes. Performance QC is included in the validation run to determine the precision and accuracy of the method. Stability QC assesses the stability of the analyte under various stress conditions. QC should be performed in a blank matrix without interference or matrix effects. QC is used for targeted lipidomics studies to check the precision and accuracy of the study, but it can also be used for non-targeted or semi-targeted lipidomics analyses to check data quality. It is necessary to include QC samples in semi-quantitative lipidomics studies to improve the quality of the data obtained and to confirm that the data collected do not show large differences due to the experimental procedure (sample preparation, injection, instrumentation, etc.).

3.1.3 Normalisation

Normalization is used to reduce variation from non-biological sources (e.g., instrument batch effects) while maintaining biological variation. Depending on the method, experimental measurements often take a long time and may even be interrupted. Therefore, systematic errors can sneak into such measurements, for example, drift in instrument sensitivity over time, batch effects such as changes in pH and concentration in solvents, or slight temperature fluctuations. Ignoring such errors can reduce the statistical power of significant differences between detected analytes and the phenotype of interest[140]. Several normalization methods have been used to reduce random errors in non-targeted analyses, including the application of scale factors[141], quality control samples[142], reference samples[143] or by using an IS or mixtures of IS [144], [145]. The most popular normalization methods are internal standard-based normalization and QC-based normalization. The internal standard-based normalization method suppresses systematic errors by using internal standards for correction. Several

different software tools are available for internal standard-based normalization methods, such as B-MIS[146], NOMIS [80], and CCMN [147]. As mentioned above, such methods have been integrated into LDA 2 and LipidMatch Normalizer. Another popular method is QC-based normalization. These QCs are usually pooled biological samples that contain all characteristics of the investigated biological sample.QC measurements are performed approximately every 10 samples and are used to normalize the data. There are several methods available for QC-based normalization, such as Batch Normalizer (based on LOESS regression)[148], StatTarget (a Vectro-machine-based normalization method) [149], and the systematic error removal using random forest (SERRF [150]) technique.

However, all the methods mentioned before except the use of IS are considered post-acquisition normalisation methods which can correct instrumental variations introduced during analysis. The use of IS can be considered as a post- and pre-acquisition normalisation method depending on the time when the IS was added to the samples [151]. Therefore, if the IS or the IS mixture was added before sample extraction, it can be used to correct variations introduced from the beginning during sample preparation, storage and throughout the analysis. However, In semi-targeted lipidomic studies, it is difficult to use only one labelled standard because of the huge diversity of metabolite structures, concentration and composition, in addition, it is impractical to assume that all the metabolites with different masses and physicochemical properties are subject to the same amount of variation as the standard [143]. Also, in lipidomics studies, different lipid species such as Cer, DG and SM can overlap due to inadequate chromatographic separation, and it is not practical to use the same normalisation factor on these species. In addition, non-polar lipid classes that do not have ionisable head groups (like ChE, DG, TG) do not manifest identical response factors even at low concentrations and their response should be determined in advance with respect to their acyl chain length and their degree of unsaturation which make the analysis even more complicated [152]. Therefore, in order to obtain better results, more than one standard should be included in the analysis and preferably one IS for each lipid species or one for each lipid class. However, the cost and the availability of IS limit the use of this approach in semi-targeted analysis, hence, there has been significant recent interest in generating comprehensive mixtures of isotopically labelled IS in vivo to evaluate and correct instrumental variations introduced during samples preparation, storage and analysis [145], [153]. However, the ability of these labelled IS to reduce variations in complex biological samples has not been fully evaluated with respect to lipidomics studies.

3.1.4 Aims and Objectives

The main aim of this chapter is to normalise and validate the developed semi-targeted lipidomics method using internal standards and human plasma. This method is expected to have good repeatability and selectivity, to achieve this aim, the objectives of this chapter are:

- To use quality control (QC) between the run and examine the stability of the method.

- To use SPLASH as a series of internal standards for different classes of lipids and test the repeatability of the internal standard in the plasma sample.
- To check the normalization efficiency by comparing the relative standard deviation between the unnormalized and normalized results.
- To evaluate the method repeatability by checking the RSD of peak area and retention time for each lipid and compare the result with the FDA requirements.
- To assess the ion suppression effect of the internal standard on the sample and check the selectivity of the method.

3.2. Materials and Methods

3.2.1. Materials and Apparatus

3.2.1.1. Materials and chemicals

A Milli-Q water purification system (Millipore, MA, US) was used in the preparation of deionized water (18.2 MΩ). Acetonitrile and chloroform (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Methanol (LC-MS grade) and ammonium acetate were purchased from Sigma-Aldrich (Darmstadt, Germany). Isopropanol (LC-MS grade) was purchased from Acros Organics (New Jersey, USA). BHT (3, 5-di-tert-butyl-4-hydroxytoluene) was purchased from Supelco (PA, USA), and human plasma was donated from Queens Medical Centre Nottingham blood bank. SPLASH® Lipidomix® Mass Spec Standard was purchased from Avanti (Darmstadt, Germany).

3.2.1.2. Apparatus

-80℃ freezer, Fisherbrand™ Wizard™ Infrared Vortex Mixer (Fisher Scientific, UK), Orbit™ Digital Microtube and Microplate Shakers (Labnet, USA), Harrier 18/80" Centrifuge rotor, Jouan Centrifugal Evaporator, Biostage SPE manifold, Techne Dri-Block heater (DB-3B), pipettes and tips, LC-MS vial with inserts and caps, Eppendorf, Accela liquid chromatography coupled with Orbitrap Quadrupole-Exactive mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK), ACE Excel 3 SuperC18 Column(100×2.1mm id) and guard column were purchased from Advanced Chromatography Technologies.

3.2.2. Sample preparation

Plasma donated from Queens Medical Centre was used for this LC-MS method validation. 50 µl of plasma (sample) or water (regent blank) was added into 500 µl of ice-cold (-20℃) extraction solvent mixture. The extraction solvent mixture was prepared by mixing organic solvent chloroform and methanol (2:1), and 10 µl SPLASH internal standard was added into 490 µl of organic solvent mixture to get the extraction solvent mixture. After 10 min vortex, 500 µl of water was added to the mixture.

The mixture was vortexed again for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper aqueous phase and a lower organic phase, as well as a protein layer in the middle. The lower organic phase was collected and evaporated by a centrifugal evaporator. Then the sample was reconstituted with 100 µl of isopropanol and transferred into an LC-MS glass vial. The quality control (QC) was prepared by mixing all the plasma extracts and well vortexed.

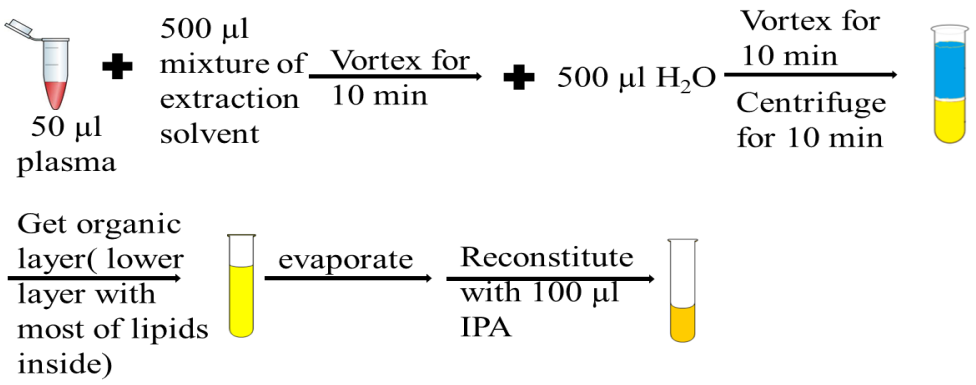


Figure 3.1. *The schematic of the Folch extraction method. Plasma was extracted with a mixture of chloroform and methanol (2:1), and after vortex and centrifuge, the organic phase was collected, evaporated and reconstituted with isopropanol.*

3.2.3. LC-MS method

LC–MS lipidomics analysis was conducted on an Accela high-speed liquid chromatography coupled to a Quadrupole-Exactive mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK). LC–MS analysis was maintained at a temperature of 40 °C and a flow rate of 300 µL/min on a 10 cm C18 column. The mobile phase consisted of (A) 60% of H2O, 40% of acetonitrile and 0.1% of ammonium acetate and (B) 80% of isopropanol, 10% of methanol, 10% of H2O and 0.1% of ammonium acetate. A starting percentage of 50 % of mobile phase B was chosen and increased to 100% of B at 5 min. After keeping at 100 % of B until 13.5 min, the gradient drops back to 50 % of B at 14 min and is maintained at the same gradient for 1 min. The injection volume was 5 µL. All study samples and QC samples were analysed in a single continuous analytical run. Data were acquired simultaneously in full scan ion mode (m/z 100–1500, resolution 25,000) in both positive and negative electrospray ionisation modes. A list of lipids with absolute mass, formula, polarity, adduct type and name that were pre-defined from human plasma was loaded to the MS method (see appendix Table A). The capillary temperature and heater temperature were maintained at 350 and 300 °C respectively in both positive and negative modes. The maximum scan injects time and micro scans were 100 s and 1 s respectively. MS was performed in simultaneous ESI (+) and ESI− full-scan modes with spray voltages of 4.5 (ESI+) and 3.5 kV (ESI−) and capillary voltages of 40 (ESI+) and −30V (ESI−).

The LC-MS validation sequence was shown in Table 3.2. First, The LC-MS run started with three blank samples, 100 µL of isopropanol was used as a blank sample as the QC and plasma sample was reconstituted with isopropanol. Second, three reagent blank samples were conducted, they were prepared by replacing the plasma with water and following the sample extraction procedure written in section 3.2.2. Third, SPLASH internal standard solution was diluted with isopropanol in a ratio of 1:10, which is also the final IS concentration in the plasma extracts. Forth, 9 injections of pooled QC were analysed, the first three injections were used for equilibrating the instrument, and the next 6 injections were used for RSD calculation. Fifth, 6 replicates of plasma extracts were analysed to test the extraction stability. Finally, one blank sample (isopropanol) was used to wash the column and instrument.

Table 3.2. The LC-MS run in the validation experiment

Sample type	Number	Note
Blank	3	Isopropanol
Reagent blank	3	extracts without plasma
IS only (QC-blank)	3	10 µL SPLASH diluted with 90 µL isopropanol
Pooled QC	9	A mixture of 6 plasma sample
Randomized plasma sample	6	6 replicates of plasma extracts
Blank	1	Isopropanol

3.2.4. Internal standard identification and RSD check

The SPLASH® Lipidomix® internal standard kit is a mixture of lipid internal standards, and the components of these internal standards and their concentration, formula and mass were shown in Table 3.3. This SPLASH IS mixture includes most of the important lipid classes, and the concentration ratio between each internal standard simulatess the lipid concentration in the human plasma. All internal standards in the SPLASH kit are deuterated (d7 or d9). The ionization mode, addictionion and retention time of the internal standard were found through the LC-MS run. The best ionization mode and addicts were chosen by comparing the peaks in both positive and negative modes, as well as different addicts for each internal standard. The retention time of these peaks was documented correspondingly. Afterwards, the relative standard deviation (%CV, or RSD) of internal standard peak area and retention time were calculated for each sample type in the LC-MS run (reagent blank, QC-blank, QC, plasma), to determine the repeatability of the internal standard in this LC-MS method.

Table 3.3. The SPLASH internal standard product details and identification in the sample

Internal Standards	Target Concentration (µg/mL)	Formula	Exact mass
15:0-18:1(d7) PC	160	$C_{41}H_{73}D_7NO_8P$	752.606
15:0-18:1(d7) PE	5	C38H67D7NO8P	710.559
15:0-18:1(d7) PS	5	C39H67D7NO10P	754.548
15:0-18:1(d7) PG	30	C39H68D7O10P	741.553
15:0-18:1(d7) PI	10	C42H72D7O13P	829.570
15:0-18:1(d7) PA	7	C36H62D7O8P	667.517
18:1(d7) LPC	25	C26H45D7NO7P	528.392
18:1(d7) LPE	5	C23H39D7NO7P	486.345
18:1(d7) Chol Ester	350	C45H71D7O2	657.644
18:1(d7) MG	2	C21H33D7O4	363.337
15:0-18:1(d7) DG	10	C36H61D7O5	587.551
15:0-18:1(d7)-15:0 TG	55	C51H89D7O6	811.765
18:1(d9) SM	30	C41H72D9N2O6P	737.64
Cholesterol (d7)	100	C27H39D7O	393.399

The SPLASH internal standards were shown above. The concentration, formula and exact mass of each internal standard in the IS mixture were attached to the table.

3.2.5. Representative lipid list selection and IS allocation

Before checking the repeatability and sample normalisation efficiency of each identified lipid in the method, a representative lipid list was chosen to examine the method's validity. Around 20 lipids from each lipid class were selected for normalisation and repeatability tests. All selected lipids present a clear and reproducible peak shape in each injection, and they were published in the human plasma sample. The ionization mode adducts and retention time of the selected lipids were marked, and the detail were shown in Table 3.4. The internal standard allocation to each lipid is based on the lipid class, adduct and retention time, the same lipid class would be preferable, but some lipids were allocated to internal standards from other lipid classes if the internal standard from this lipid class does not exist in SPLASH, or lipid ionization mode and addict different from the internal standard in the same lipid class. Finally, all allocated internal standards have the same ionization mode and adducts as the lipid, as well as the close retention time.

The finalised representative lipid list was loaded onto Tracefinder software for internal standard allocation and lipid peak area quantification. The original and normalised selected lipid peak area and retention time were recorded for both plasma and quality control samples. The relative standard deviation of each lipid was calculated using 6 replicates with the %RSD formula mentioned in section 3.1.2.1. The lipids peak that exists in the reagent blank and QC-blank (internal standard diluted samples) sample was used to delete analytes outside of plasma samples.

Table 3.4 The representative lipid list and internal standard allocation for each lipid class

Selected lipids	**Formula**	**Exact mass**	**adduct**	**Internal standard**	**RT(min) (Xcalibur)**
FA (16:0)	C16H32O2	256.2397	-H	18:1(d7) LPE	0.602
LPC(18:2)	C26H50NO7P	519.3319	+H	18:1(d7) LPC	2.93
LPE(18:2)	C23H44O7NP	477.2855	-H	18:1(d7) LPE	3.067
So(d18:1)	C18H37O2N	299.2824	+H	18:1(d7) LPC	3.422
LPE(16:0)	C21H44NO7P	453.285	-H	18:1(d7) LPE	3.55
PG(30:1)	C36H69O9P	676.4679	+H	15:0-18:1(d7) PG	5.964
PI(16:0/16:1)	C41H77O13P	808.5096	+H	15:0-18:1(d7) PI	6.131
PE(16:0/20:5)	C41H72O8NP	737.4996	+H	15:0-18:1(d7) PE	6.5
PI(18:0/20:4)	C47H83O13P	886.5571	+H	15:0-18:1(d7) PI	6.534

PA(27:6/22:6)	C52H79O8P	862.5513	-H	15:0-18:1(d7) PA	6.566
PE(16:0/20:4)	C41H74NO8P	739.5147	+H	15:0-18:1(d7) PE	6.732
PS(44:5)	C50H88O10NP	893.6146	+H	15:0-18:1(d7) PS	6.96
PC(17:4/18:0)	C43H78O8NP	767.5465	+H	15:0-18:1(d7) PC	7.09
SM(d22:1/20:2)	C47H91O6N2P	810.6615	+H	18:1(d9) SM	7.444
DG(16:0/18:1)	C37H70O5	594.5223	+NH4	15:0-18:1(d7) DG	7.951
Cer(d16:1/22:0)	C38H75O3N	593.5747	+H	18:1(d9) SM	7.974
SM(d18:1/25:1)	C48H95O6N2P	826.6928	+H	18:1(d9) SM	8.031
DG(18:0/16:0)	C37H72O5	596.538	+NH4	15:0-18:1(d7) DG	8.422
TG(16:1/16:1/16:1)	C51H92O6	800.6894	+NH4	15:0-18:1(d7)-15:0 TG	11.66
TG(16:0/16:1/16:1)	C51H94O6	802.705	+NH4	15:0-18:1(d7)-15:0 TG	12.765
ChE(20:5)	C47H74O2	670.5689	+NH4	18:1(d7) Chol Ester	13.386
ChE(18:3)	C45H74O2	646.5689	+NH4	18:1(d7) Chol Ester	13.948

Around 20 lipids from each lipid class were selected for sample normalisation efficiency and repeatability test. All selected lipids show a clear and reproducible peak in each injection, and the ionization mode, adduct and retention time were marked for each class. The allocation of each lipid class is based on the adduct and retention time, and all the allocated internal standard has the same ionization mode and adducts as the lipid, and retention time are close.

3.2.6. Whole database lipids identification and IS allocation

After checking the method validity by a selected lipid list, the whole list of identified lipids from the semi-targeted method was used for validation check. The ionization mode, adduct and retention time of the lipids were marked, and the detail is shown in the appendix Table C. The internal standard allocation to each lipid is based on the lipid class, adduct and retention time, the same lipid class would be preferable, but some lipids were allocated to internal standards from other lipid classes if the internal standard from this lipid class does not exist in SPLASH, or lipid ionization mode and addict different from the internal standard in the same lipid class. Finally, all allocated internal standards have the same ionization mode and adducts as the lipid, as well as the close retention time.

The finalised representative lipid list was loaded onto Tracefinder software for internal standard allocation and lipid peak area quantification. The original and normalised selected lipid peak area and retention time were recorded for both plasma and quality control samples. The relative standard deviation of each lipid was calculated using 6 replicates with the %RSD formula mentioned in section 3.1.2.1. The lipids peak that exists in the reagent blank and QC-blank (internal standard diluted samples) sample was used to delete analytes outside of plasma samples.

3.2.8. Raw data processing

LC–MS raw data from the analysis of extracts of study and control samples were acquired with Xcalibur v2.1 software (Thermo Scientific, Hemel Hempstead UK). Chromatographic raw data contain a 3D data set consisting of m/z, retention time and ion signal intensity. These data were processed with LipidSearch (v5.1) to align the replicates and control result and get the extracted lipid formula. The detail of the LipidSearch parameter was shown in Table 2.6 and Table 2.7. The identified lipid list from LipidSearch with allocated internal standards were processed by Tracefinder (Thermo Scientific, Hemel Hempstead UK) following the official protocols.

3.3 Results and Discussion

3.3.1. SPLASH internal standard identification

The SPLASH internal standard mixture includes 14 isotopically labelled internal standards from different lipid classes. All internal standards were identified except cholesterol (d7). The long-time storage of the SPLASH sample could be the reason that cause instability of the internal standard, which leads to cholesterol (d7) being unfounded in the analysis. It is also possible that the cholesterol (d7) has a later retention time compared to other lipids, and it was eluted after the LC-MS run. The concentration, formula, exact mass as well as addict and retention time detail of each internal standard was attached in Table 3.5. The internal standard ionization mode and adduct were checked with XCalibur and Tracefinder, and ionized mass was calculated using XCalibur.

Table 3.5. The SPLASH internal standard ionization, adduct and retention time details

Internal standards	Target Concentration (µg/mL)	Formula	Exact mass	adduct	Ionized mass	RT(15min)
15:0-18:1(d7) PC	160	C41H73D7NO8P	752.606	+H	753.613	6.77
15:0-18:1(d7) PE	5	C38H67D7NO8P	710.559	+H	711.566	6.83

15:0-18:1(d7) PS	5	C39H67D7NO10P	754.548	-H	753.542	6.71
15:0-18:1(d7) PG	30	C39H68D7O10P	741.553	+H	742.561	6.37
15:0-18:1(d7) PI	10	C42H72D7O13P	829.570	-H	828.562	6.28
15:0-18:1(d7) PA	7	C36H62D7O8P	667.517	-H	666.510	6.65
18:1(d7) LPC	25	C26H45D7NO7P	528.392	+H	529.399	3.57
18:1(d7) LPE	5	C23H39D7NO7P	486.345	-H	485.338	3.72
18:1(d7) Chol Ester	350	C45H71D7O2	657.644	+NH4	675.678	8.09
18:1(d7) MG	2	C21H33D7O4	363.337	+NH4	381.372	4.76
15:0-18:1(d7) DG	10	C36H61D7O5	587.551	+NH4	605.585	7.69
15:0-18:1(d7)-15:0 TG	55	C51H89D7O6	811.765	+NH4	829.799	13.93
18:1(d9) SM	30	C41H72D9N2O6P	737.64	+H	738.647	6.58
Cholesterol (d7)	100	C27H39D7O	393.399	+NH4	411.433	NA

The SPLASH internal standard in the sample was shown above. All internal standards were identified except Cholesterol (d7). The concentration, formula and exact mass of each internal standard in the IS mixture were attached to the table. The internal standard ionization mode and adduct were checked with XCalibur and Tracefinder, and ionized mass was calculated as well. The peak and retention time for each internal standard was identified and marked in the table.

3.3.2. SPLASH internal standard RSD result

The peak area and retention time RSD% result of internal standards was shown in Table 3.6 and Table 3.7. Four sample types were used for RSD verification, and the detail of each sample type was shown in Table 3.2. As the table shows, the RSD% in different sample types were calculated, such as reagent blank, plasma extracts, IS diluted blank and quality control (a mixture of all the plasma extracts). For peak area RSD in Table 3.6, the QC sample has a low RSD in most of the internal standards, and only one internal standard (15:0-18:1(d7) PA) RSD is higher than 15%. According to the FDA requirement for the RSD in bioanalytical samples [136], the RSD of the QC peak area meet the criteria. Thus, the

RSD result in the QC illustrates the developed semi-targeted method is repeatable. The RSD in plasma extract samples represents experimental stability before analysis, including the stability of extraction method, freeze-thaw, long-term storage, stock solution, autosampler and others [154][155]. The standard of RSD about stability is also within a 15% difference, and according to plasma RSD results, most of the internal standard RSD below 15% or around 15%, only two internal standards (15:0-18:1(d7) PA and 15:0-18:1(d7) PS) has a higher RSD than 15%. Both of these two internal standards have outliers that peak area is much higher than peaks in other replicates, After ignoring the outliers, the result of these two internal standards is much lower (15:0-18:1(d7) PA 16.54%, 15:0-18:1(d7) PS 13.11%).The retention time RSD in all the sample types is much lower compared to peak area RSD. Most of them are below 1%, and only one internal standard RSD is around 1%, thus the method has a stable elution time and good repeatability in retention time.

Table 3.6. The internal standard RSD results in blank, plasma and QC (peak area)

Internal standards	Formula	Blank	Plasma	QC-blank	QC
18:1(d7) LPC	C26H45D7NO7P	23.46%	5.09%	2.11%	2.86%
18:1(d7) LPE	C23H39D7NO7P	26.31%	8.54%	1.58%	2.51%
18:1(d7) MG	C21H33D7O4	24.93%	13.59%	18.40%	11.96%
15:0-18:1(d7) PI	C42H72O13PD7	16.56%	15.10%	3.01%	5.69%
15:0-18:1(d7) PG	C39H68D7O10P	7.71%	14.90%	2.78%	14.08%
15:0-18:1(d7) PS	C39H67D7NO10P	15.06%	21.18%	5.28%	6.57%
18:1(d9) SM	C41H72D9N2O6P	17.88%	3.97%	1.62%	1.87%
15:0-18:1(d7) PC	C41H73D7NO8P	11.36%	3.87%	1.32%	1.20%
15:0-18:1(d7) PE	C38H67D7NO8P	11.44%	5.80%	1.41%	4.99%
15:0-18:1(d7) PA	C36H62O8PD7	10.82%	94.33%	6.11%	35.10%
15:0-18:1(d7) DG	C36H61D7O5	11.24%	3.94%	1.50%	4.66%
18:1(d7) Chol Ester	C45H71D7O2	55.52%	16.15%	1.52%	5.86%
15:0-18:1(d7)-15:0 TG	C51H89 D7O6	7.65%	6.53%	5.97%	4.97%

The internal standard RSD results in blank, plasma, QC-blank and QC was shown in the table above. It is expected that most of the RSD result was below 15%. As the table shows, most of the RSD is below 15%, and some of them have a very high RSD, since there are some unidentified or much bigger peaks in some replicates.

Table 3.7. The internal standard RSD results in blank, plasma and QC (retention time)

Internal standards	Formula	Blank	Plasma	QC-blank	QC
18:1(d7) LPC	C26H45D7NO7P	0.10%	0.18%	0.20%	0.10%
18:1(d7) LPE	C23H39D7NO7P	0.23%	0.20%	0.14%	0.22%
18:1(d7) MG	C21H33D7O4	1.07%	0.36%	0.43%	0.26%
15:0-18:1(d7) PI	C42H72O13PD7	0.13%	0.11%	0.08%	0.17%
15:0-18:1(d7) PG	C39H68D7O10P	0.10%	0.17%	0.14%	0.18%
15:0-18:1(d7) PS	C39H67D7NO10P	0.18%	0.23%	0.19%	0.15%
18:1(d9) SM	C41H72D9N2O6P	0.13%	0.14%	0.08%	0.11%
15:0-18:1(d7) PC	C41H73D7NO8P	0.08%	0.13%	0.10%	0.10%
15:0-18:1(d7) PE	C38H67D7NO8P	0.09%	0.21%	0.13%	0.11%
15:0-18:1(d7) PA	C36H62O8PD7	0.54%	1.15%	0.62%	0.79%
15:0-18:1(d7) DG	C36H61D7O5	0.09%	0.12%	0.10%	0.14%
18:1(d7) Chol Ester	C45H71D7O2	0.28%	0.23%	0.33%	0.20%
15:0-18:1(d7)-15:0 TG	C51H89 D7O6	0.05%	0.08%	0.10%	0.10%

The internal standard RSD results in blank, plasma, QC-blank and QC were shown above. It is expected that most of the RSD result was below 15%. As the table shows, all of the below 15%, so it has a stable elution time and good repeatability.

3.3.3. Representative lipid identification and RSD result

The RSD of the selected lipid list was shown in Table 3.8 and Table 3.9. Both quality control and plasma samples were analysed to examine the repeatability and stability of the method. The unnormalized and normalized RSD results were compared to check the normalisation efficiency for the lipids. The SPLASH internal standard was used for normalization, and reagent blank and QC-blank samples were used to obliterate impurities and lipids from the environment [156]. As Table 3.8 shows, in a total of 22 lipids, 19 lipids have peak area RSD below 15%, meaning the method is repeatable in different injections. In the plasma sample, there are also 19 lipids has an RSD below 15% no matter unnormalized or normalized, thus the method stability is also verified, showing the extraction method, storage condition and other situations are stable for analysis. In Table 3.9, all the retention time RSD

are below 2%, and most of them below 1% no matter whether unnormalized or normalized, showing good repeatability on elution time.

For the normalization efficiency, some lipids show a better RSD after normalization, but many lipids do not support this. When looking into the lipids that RSD is higher after normalization, all the lipids that do not have an internal standard in SPLASH and are allocated to internal standards from other classes have a higher RSD after normalization. The similarity between the lipids and the internal standards may influence the normalization efficiency. Ideally, each lipid should be assigned to its isotopic pair, and researchers have proved allocating each analyte an internal standard are useful for normalisation [80]. But due to the large number of lipids that are targeted in the study, it is hard to generate or buy internal standards for each lipid. Another possibility is the variety of internal standards in each sample. For example, 15:0-18:1(d7) PG has a quite high RSD compared to other internal standards, which could influence the normalization efficiency by adding internal standard variation to the lipids. To check the normalization efficiency for all the identified lipids, the validation method was applied to the whole lipid list.

Table 3.8. The lipid peak area RSD result with or without normalisation

Selected lipids	**Formula**	**plasma**		**QC**	
		unnormalized	**normalized**	**unnormalized**	**normalized**
FA (16:0)	C16H32O2	9.00%	13.11%	22.64%	24.08%
LPC(18:2)	C26H50NO7P	4.87%	4.95%	0.71%	2.56%
LPE(18:2)	C23H44O7NP	7.34%	4.26%	2.02%	1.39%
So(d18:1)	C18H37O2N	4.03%	7.38%	1.92%	3.05%
LPE(16:0)	C21H44NO7P	11.35%	5.05%	1.93%	3.28%
PG(30:1)	C36H69O9P	12.95%	18.78%	27.82%	19.76%
PI(16:0/16:1)	C41H77O13P	18.11%	6.57%	4.10%	2.34%
PE(16:0/20:5)	C41H72O8NP	6.13%	10.92%	8.72%	7.21%
PI(18:0/20:4)	C47H83O13P	17.32%	5.26%	11.47%	5.49%
PA(27:6/22:6)	C52H79O8P	57.85%	77.96%	33.54%	66.56%
PE(16:0/20:4)	C41H74NO8P	3.96%	7.06%	4.88%	5.26%
PS(44:5)	C50H88O10NP	88.00%	17.43%	1.80%	6.77%

PC(17:4/18:0)	C43H78O8NP	3.07%	5.34%	4.01%	4.03%
SM(d22:1/20:2)	C47H91O6N2P	6.39%	7.39%	4.35%	5.14%
DG(16:0/18:1)	C37H70O5	5.51%	7.31%	8.24%	3.42%
Cer(d16:1/22:0)	C38H75O3N	5.15%	8.04%	5.15%	5.21%
SM(d18:1/25:1)	C48H95O6N2P	6.00%	7.38%	9.37%	9.78%
DG(18:0/16:0)	C37H72O5	5.94%	7.92%	3.85%	1.55%
TG(16:1/16:1/16:1)	C51H92O6	6.94%	11.13%	0.66%	5.63%
TG(16:0/16:1/16:1)	C51H94O6	5.20%	9.49%	0.63%	5.05%
ChE (20:5)	C47H74O2	2.81%	13.28%	0.91%	5.33%
ChE (18:3)	C45H74O2	3.13%	14.69%	3.20%	6.18%

Table 3.9. The lipid retention time RSD result with or without normalisation

Mixture Components	**Formula**	**plasma**		**QC**	
		unnormalized	**normalized**	**unnormalized**	**normalized**
FA (16:0)	C16H32O2	0.59%	0.68%	0.59%	0.64%
LPC(18:2)	C26H50NO7P	0.25%	0.16%	0.25%	0.30%
LPE(18:2)	C23H44O7NP	0.35%	0.42%	0.21%	0.34%
So(d18:1)	C18H37O2N	0.31%	0.42%	0.42%	0.51%
LPE(16:0)	C21H44NO7P	0.33%	0.39%	0.23%	0.32%
PG(30:1)	C36H69O9P	0.57%	0.57%	0.50%	0.48%
PI(16:0/16:1)	C41H77O13P	0.15%	0.17%	0.17%	0.26%
PE(16:0/20:5)	C41H72O8NP	0.11%	0.27%	0.14%	0.20%
PI(18:0/20:4)	C47H83O13P	0.13%	0.13%	0.18%	0.22%
PA(27:6/22:6)	C52H79O8P	1.24%	1.54%	0.37%	0.80%
PE(16:0/20:4)	C41H74NO8P	0.20%	0.33%	0.10%	0.15%
PS(44:5)	C50H88O10NP	0.15%	0.30%	0.13%	0.21%

PC(17:4/18:0)	C43H78O8NP	0.12%	0.17%	0.11%	0.18%
SM(d22:1/20:2)	C47H91O6N2P	0.12%	0.13%	0.10%	0.13%
DG(16:0/18:1)	C37H70O5	0.12%	0.17%	0.08%	0.15%
Cer(d16:1/22:0)	C38H75O3N	0.09%	0.13%	0.11%	0.16%
SM(d18:1/25:1)	C48H95O6N2P	0.18%	0.13%	0.16%	0.13%
DG(18:0/16:0)	C37H72O5	0.08%	0.15%	0.08%	0.16%
TG(16:1/16:1/16:1)	C51H92O6	0.09%	0.14%	0.11%	0.14%
TG(16:0/16:1/16:1)	C51H94O6	0.07%	0.11%	0.09%	0.15%
ChE(20:5)	C47H74O2	0.06%	0.21%	0.08%	0.22%
ChE(18:3)	C45H74O2	0.08%	0.25%	0.07%	0.18%

3.3.4. Whole database lipid normalisation and RSD result

The RSD of the whole identified lipid list was shown in the appendix Table C and Table D. Both quality control and plasma samples were analysed to examine the repeatability and stability of the method. The unnormalized and normalized RSD results were compared to check the normalisation efficiency for the lipids. The SPLASH internal standard was used for normalization, and reagent blank and QC-blank samples were used to obliterate impurities and lipids from the environment [156], [157]. As Table C shows, in a total of 353 identified lipids, 264 lipids have peak area RSD below 15%. After deleting the missing peak values and abnormal values, there are 296 lipids RSD below 15%, meaning the method is repeatable in different injections. In the plasma sample, there are 271 lipids have an RSD below 15% and 282 lipids below 15% after deleting outliers, thus the method stability is also verified, showing the extraction method, storage condition and other situations are stable for analysis. In Table D, for the whole 353 identified lipids, 329 lipids retention time RSD below 15%. After deleting the missing peak values and abnormal values, there are 351 lipids RSD below 15%, meaning the method is repeatable in different injections. In the plasma sample, there are 338 lipids that have a retention time RSD below 15% and 351 lipids below 15% after deleting outliers, thus the method stability is also verified, showing the extraction method, storage condition and other situations are stable for analysis. For the two lipids that have higher retention time RSD, PG (40:6) and TG (54:8) have many replicates unidentified, and there are less than three identified peaks for RSD calculation, which makes the result RSD higher than 200%. Most of the lipids that have a very high peak area RSD (like RSD>100%) have the same problem as PG (40:6) and TG (54:8).

In the QC normalization step, there are 137 lipids peak area RSD was lessened out of 353 lipids after normalization and most of the lipids that RSD are similar to the unnormalized QC result. The lipid normalization efficiency is decent when there are internal standards from the same lipid class, and lipids that allocate to internal standards from other classes still are a major part that shows an increased RSD after normalisation. For the retention time normalization, as most of the retention time RSD below 1% before and after normalization, it is hard to say the normalization effect. In the result table, there are some lipids attached with an asterisk like *1*, which represents the isobaric peak with the same formula. The LipidSearch software did much work on identifying the isobaric lipids, but the software still has some shortcomings in identification. The XCalibur software was also used to check the extracted ion chromatography, m/z and fragment peak to identify the missing lipids from the LipidSearch result. There are 110 lipids added to the LipidSearch identified list and later loaded onto Tracefinder software. However, Tracefinder is a quantification software and could not show any information about the peak, thus there are some isobaric lipids were identified by LipidSearch and manually, but there are some peaks that could not show the isobaric detail and labelled as *1*, *2* and so on.

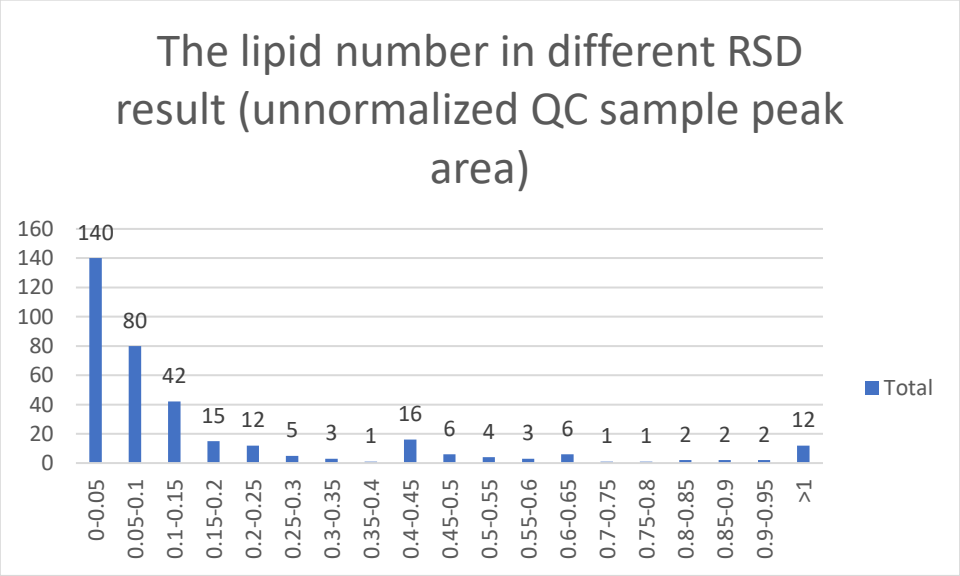


Figure 3.2. *The peak area RSD for unnormalized QC samples. The bar chart shows the lipid number in different RSD ranges. The x-axis is the RSD value from 0 to >1 (over 100%), and the y-axis is the lipid number in this RSD range. The left three bars are lipids with RSD <15% and in total 262 lipids.*

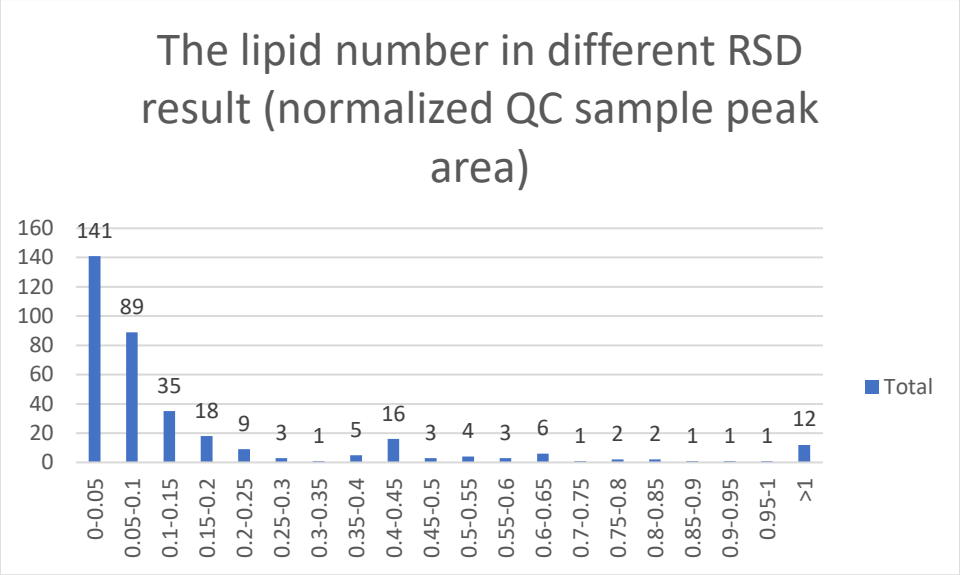


Figure 3.3*. The peak area RSD for normalized QC samples. The bar chart shows the lipid number in different RSD ranges. The x-axis is the RSD value from 0 to >1 (over 100%), and the y-axis is the lipid number in this RSD range. The left three bars are lipids with RSD <15% and in total 265 lipids.*

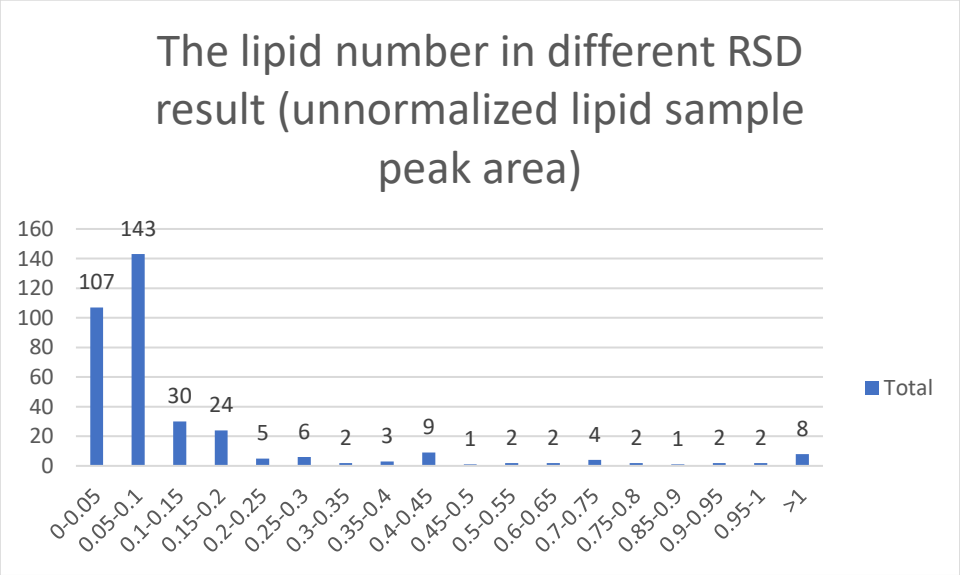


Figure 3.4. *The peak area RSD for unnormalized lipid samples. The bar chart shows the lipid number in different RSD ranges. The x-axis is the RSD value from 0 to >1 (over 100%), and the y-axis is the lipid number in this RSD range. The left three bars are lipids with RSD <15% and in total 280 lipids.*

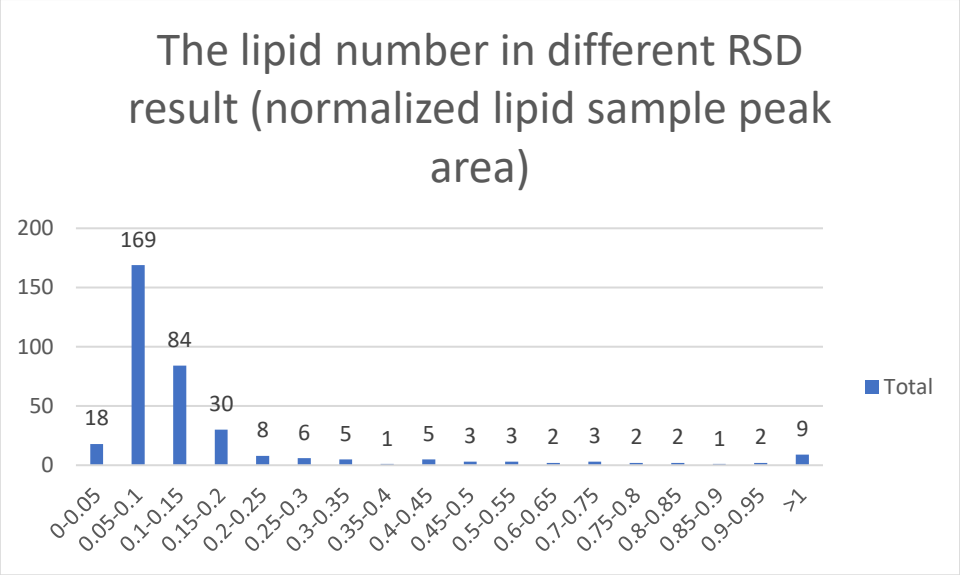


Figure 3.5. *The peak area RSD for normalized lipid samples. The bar chart shows the lipid number in different RSD ranges. The x-axis is the RSD value from 0 to >1 (over 100%), and the y-axis is the lipid number in this RSD range. The left three bars are lipids with RSD <15% and in total 271 lipids.*

3.3.5. Ion suppression of internal standard in plasma extracts

Table 3.10. The ion suppression effect on internal standards peak area

Internal standards	Formula	Blank	Plasma	QC-blank	QC
18:1(d7) LPC	C26H45D7NO7P	▲ 610592031	▼ 3.35E+08	▲ 585890070	▼ 3.22E+08
18:1(d7) LPE	C23H39D7NO7P	▲ 27522003	▲ 28716452	▼ 22230182	▲ 27155180
18:1(d7) MG	C21H33D7O4	▼ 2206302.5	▲ 9167446	▼ 2923732.7	▲ 8350523
15:0-18:1(d7) PI	C42H72O13PD7	▼ 29038976	▲ 46890920	▼ 28245865	▲ 46554787
15:0-18:1(d7) PG	C39H68D7O10P	▲ 36800328	▼ 18484689	▲ 39593142	▼ 19246289
15:0-18:1(d7) PS	C39H67D7NO10P	▲ 21346416	▼ 7687223	▲ 17883592	▼ 7093207
18:1(d9) SM	C41H72D9N2O6P	▲ 1.514E+09	▼ 6.86E+08	▲ 1.348E+09	▼ 6.79E+08
15:0-18:1(d7) PC	C41H73D7NO8P	▲ 1.116E+10	▼ 4.12E+09	▲ 9.4E+09	▼ 4.03E+09
15:0-18:1(d7) PE	C38H67D7NO8P	▲ 226529528	▼ 63669478	▲ 190390458	▼ 65268415
15:0-18:1(d7) PA	C36H62O8PD7	▲ 63677978	▼ 1561217	▲ 44764431	▼ 1223493
15:0-18:1(d7) DG	C36H61D7O5	▲ 681752191	▼ 4.34E+08	▲ 601256080	▼ 4.26E+08
18:1(d7) Chol Ester	C45H71D7O2	▲ 460432201	▼ 1.67E+08	▬ 353049021	▼ 1.66E+08
15:0-18:1(d7)-15:0 TG	C51H89 D7O6	▲ 2.901E+09	▬ 2.78E+09	▼ 2.708E+09	▬ 2.79E+09

Table 3.10. *The ion suppression effect on internal standards was shown by the mean of the peak area. The reagent blank sample was compared with the plasma sample, and the QC-blank sample was compared with the QC sample. The green arrow represents a higher peak area, the red arrow represents a lower peak area and the orange horizontal line means the peak area is similar to the compared pair.*

The ion suppression effect is a common phenomenon that causes inaccurate quantification of the analytes, it is related to ionisation source (like ESI), particles in the sample matrix and other factors [139], [158]. To study whether ion suppression has influenced the quantification of lipids in this semi-targeted lipidomic method, internal standard peak areas in different sample types were used for the review. Table 3.10 presents the mean peak area of each internal standard in different sample types. As the amount of the added internal standards in each sample is the same, it is expected to see a roughly similar mean peak area in different sample types without an ion suppression effect.

However, as the result table shows, the plasma matrix (plasma sample and QC sample) has a lower peak area for most of the internal standards compared to the blank sample (reagent blank and QC-blank). There are also three internal standards showing the plasma matrix increases the peak area of the internal standards (18:1(d7) LPE, 18:1(d7) MG and 15:0-18:1(d7) PI). There are many attempts to minimise ion suppression, including enhanced specimen clean-up, chromatographic changes, reagent modifications, and effective internal standardization [158]. Thomas and other people have used ^{13}C labelled internal standards to avoid co-eluting problems, and it shows a better effect than ^{2}H labelled internal standards [159].

3.4 Conclusion

As stated before, the stability and repeatability of the LC-MS method are important to the lipidomic method development and application. In this chapter, several validation parameters were reviewed for all identified lipids, and the result proves the developed method is in an acceptable range of stability and repeatability, as well as selectivity. Quality control was added between the injections, to check the LC-MS stability. The 6 plasma extraction samples confirm the stability of the extraction method, freeze-thaw, storage condition, human impact and other factors are stable and meet the FDA criteria. For the lipids with a high-level RSD, different reasons were found, and outliers or missing peaks are the main problems that cause the high RSD. Except this, most of the lipids have a clear peak and reasonable RSD.

The SPLASH internal standard was added into the method and normalise the lipids by class. The ion suppression effect study has demonstrated that there is an ion suppression effect in the LC-MS run and adding internal standards could lessen the influence on the quantification of lipids. The normalisation result shows many lipids' RSD decreased after normalisation, but lipids allocating to internal standards from other lipid classes or internal standards itself have a quite high RSD shows the RSD increased after normalisation.

In summary, the method stability, repeatability and other parameters all meet the clinical analysis standard, and the normalisation could help the variation in general. Thus, the method is considered a proper method for relatively quantifying over three hundred lipids and could be used on clinical plasma samples.

Chapter Four

Investigation of possible differences in blood lipid biomarkers in patients with different stages of glioblastoma

4.1. Introduction

4.1.1. General background and classification of brain tumour

Glioma is a kind of tumour originating from the brain and spinal glial cells that constitute the central nervous system (CNS). In brain physiology, the main function of glial cells is to isolate neurons, which plays an important role in the regulation of the central nervous system to maintain their homeostasis and protect them from external damage [160]. Glial cells, especially astrocytes, also play an important role in the central nervous system, controlling the release and role of chemical regulators involved in a variety of central nervous system mechanisms (GABA, acetylcholine, and arachidonic acid metabolites). Although nerve signal transmission is much faster than glial cells, it is also more important. Although astrocytes do not conduct action potentials or send signals to other parts of the body, they are very similar to neurons and play an important role in communication in the brain. When tumours are detected in glial cells, leading to uncontrolled cell proliferation, they may be benign or malignant. Unfortunately, gliomas account for about 80% of all malignant brain tumours, so they are more likely to occur than benign brain tumours [161]. They are classified according to the type of cells they develop and graded according to their pathogenicity and location within the cells [162]. Astrocytes are the main types of glial cells involved in cerebral glioma, which can cause astrocytoma and glioblastoma multiforme. Other glial cells affected were oligodendrocytes (oligodendrogliomas) and ependymal cells (ependymomas). Gliomas are further classified according to grade: low-grade gliomas are well differentiated, and cell proliferation is slightly out of control, usually leading to benign tendencies. As a result, low-grade gliomas are easier to treat, but the grade increases over time. On the other hand, high-grade glioma cells are poorly differentiated (anaplastic), which is called malignant brain tumour, also known as cancer. High-grade gliomas are difficult to treat and have a poor prognosis. For example, according to research, in GBM, the grading system is divided into four types: Grade 1 (the least advanced disease in which tumour cells appear to be close to normal), grade 2 (low grade), and grades 3 and 4 (high grade) [163]. Grade I gliomas are the least invasive grade and are associated with low proliferative potential lesions. 154 cases can be cured by surgery. The median survival time is 7-15 years. Grade III and IV gliomas are highly malignant and invasive [164]. The median survival time after diagnosis is 3.5-10 years and 1-2 years respectively [165]. Finally, the classification of gliomas depends on the affected brain region (location), that is, the region below or above the tentorial membrane, which separates the cerebellum from the brain. The most common brain cancer is GBM tumour, which is usually defined as invasive, high-grade (according to who study, grade 4), invasive and heterogeneous tumour. Figure 4.1 shows the MRI scan of the brain affected by GBM in the early stage and its progress one month later (left figure), as well as the asmri scan of the progress of low-grade astrocytoma in different patients in the same period (right figure). It can be seen that in a GBM MRI scan, the rapid growth of cancer cells is obvious in the centre of the brain, but it is not obvious in

a low-level MRI scan, which indicates that low-level glioma is more difficult to detect and may need further analysis to confirm.

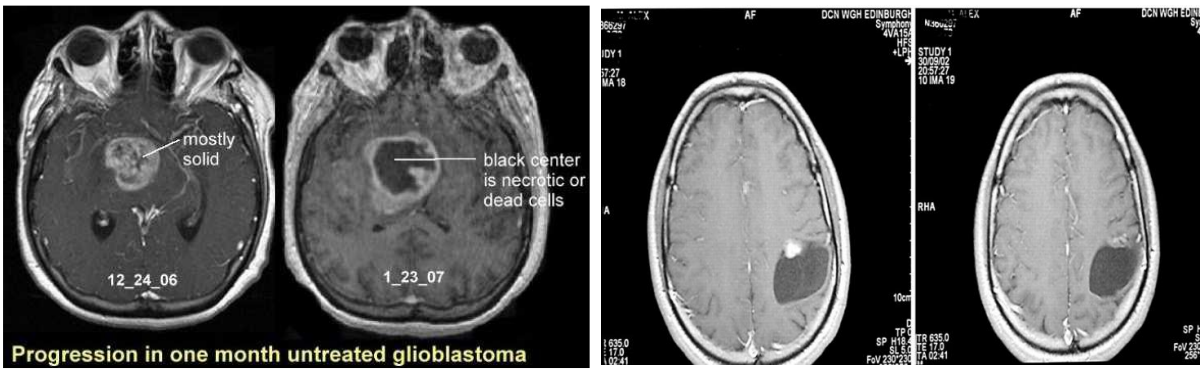


Figure 4.1.*GBM gliomas (grade 4) in the adult male brain progressed for more than one month (left figure), and low grade 2 astrocytomas in the adult female brain progressed for more than one month (right figure)* [166].

4.1.2. Low-grade glioma: epidemiology, aetiology and symptoms

As mentioned earlier, low-grade gliomas (LGGs) are the slowest-growing tumour types (grades 1 to 2), usually occurring in young, healthy adults and children. They usually form in astrocytes, such as hairy cell astrocytoma (grade 1) and oligodendroglioma (grade 2). Although LGG's initial cell proliferation is low, uncontrolled and sometimes asymptomatic, if not treated, it can lead to a considerable incidence rate, leading to death. As time goes on, LGG will develop into a more severe high-grade glioma [167]. In the past 15 to 20 years, according to the data of Cancer Research UK, the incidence rate of LGGs among British adults has increased from 2000 cases to 3000 cases each year [168]. LGGs account for almost 15% of all confirmed primary brain tumours annually, and the incidence rate is increasing among young white males. However, the proportion of adults suffering from low-grade gliomas in the whole British population is not very large, only about 3%, that is, 30 out of every 1000 people suffer from low-grade gliomas. In the United States, for example, between 2010 and 2014, the annual incidence rate of LGGs seems to be significantly lower than that of high-grade gliomas, whether male or female [169]. According to Cancer Research UK, there were 11432 brain tumour deaths (all types) in the UK in 2016, with a 10-year survival rate of 14% per year. This suggests that although eggs are not as severe as high-grade tumours (such as GBM), they can easily develop into cancerous types and therefore need immediate treatment.

As for most brain tumours, it is not clear what is the main cause of LGGs. Scientists have been studying the possible sources of glioma pathogenesis. There is a theory that electromagnetic radiation [170] from mobile phones, power cords [171], microwave radio frequency radiation [172] and some food additives such as aspartame [173] (sweetener) may cause the pathogenesis of diseases, although these factors are still under study. It is well known that LGG can occur at any time in life, most commonly in children and young people. The risk of any type of glioma (including LGG) increases with age [174]. The family

history of any type of glioma or some other diseases can also induce LGG. Many genes are found to be related to the progress of the disease, such as isocitrate dehydrogenase (IDH1) gene mutation, neurofibromin (NF1 and NF2) gene mutation and neurofibromatosis, tuberous sclerosis (TSC1 and TSC2) gene mutation and tuberous sclerosis. It is suggested that IDH1 and IDH2 gene mutations play an important role in its pathogenesis.

LGG originates from the inside of the brain and usually does not spread to the outside of the brain, but grows into normal brain tissue. When the tumour grows and expands locally, it can destroy the connections between brain cells, leading to serious brain problems, even if the tumour develops slowly. The most common symptom of [175] is usually epileptic seizure, which occurs in almost 80% of LGG patients. Epileptic seizures are usually caused by disorganized electrical activity in the brain, followed by involuntary movement, and muscle loss of control, severe seizures are loss of consciousness and language. Other clinical symptoms and signs associated with LGG included headache, mood swings, memory and visual loss [176]. Sometimes, individuals diagnosed with LGG may be asymptomatic for a long time without any neurological or cognitive changes, but they still need continuous monitoring through imaging and spectral analysis to show the presence of tumours.

4.1.3. High-grade glioma: epidemiology, aetiology and symptoms

Malignant glioma is a devastating nervous system disease with a poor prognosis. Its clinical manifestations are progressive functional and cognitive impairment [177]. Extracranial metastasis is rare [178], especially in the case of no previous surgical damage to the dura mater and skull, the dura mater and skull are considered to be the seeds of tumour cells in extracranial space [179]. Most of the reported cases of extracranial metastasis involve leptomeningeal spread to the spine [180], although metastasis of the liver, skin, spleen, lung, peritoneum and lymph nodes may also occur [181]–[184]. High-grade gliomas are particularly insensitive to radiation and genotoxic drugs [185]. Unlike many other non-neurological malignancies, local control of gliomas always fails. Most patients eventually died of local disease progression, recurrence of important brain regions or uncontrollable intracranial hypertension [186]. Surgery is the first-line treatment for glioma. However, gliomas recur at the resection margin in 90% of patients [187]. Many studies have reported that the blood-brain barrier allows the existence of monoclonal antibodies (mAbs) both at the baseline level and in the case of malignant tumours, which provides a theoretical basis for the use of bevacizumab [13], anti-CD40 mAb [14] and anti-CD25 mAb[188] in the treatment of intracranial malignant tumours [189]. Many clinical trials have evaluated the mAb-based strategy for the treatment of malignant glioma, and the implementation of this strategy seems imminent and optimistic [189]. The vast majority of gliomas recur within 2 cm of the primary tumour area [181]; therefore, the early correct diagnosis of tumour recurrence and the differentiation of treatment-related changes are very important for the timely and adequate treatment of gliomas [190].

Malignant glioma is a devastating neurological disease with a poor prognosis. Its clinical presentation is characterized by progressive functional and cognitive impairment [161]. Extracranial metastases are relatively rare [162], especially in the absence of previous surgical injury to the dura mater and skull, which are thought to seed tumour cells in the extracranial space [163]. Most reported cases of extracranial metastases to involve meningeal spread in the spine[164], but metastases to the liver, skin, spleen, lung, peritoneum and lymph nodes may also occur[165]-[168]. High-grade gliomas are particularly insensitive to radiation and genotoxic drugs [169]. Unlike many other non-neurological malignancies, local control of gliomas always fails. Most patients eventually die from local disease progression, recurrence in important brain regions, or uncontrolled intracranial hypertension [170]. Surgery is the first-line treatment for gliomas. However, gliomas recur at the margin of resection in 90% of patients [171]. Many studies have reported that the blood-brain barrier allows the presence of monoclonal antibodies (mAb) at baseline levels and in the presence of malignancy, which provides a rationale for the use of bevacizumab [13], anti-CD40 mAb [14] and anti-CD25 mAb [172] for the treatment of intracranial malignancies [173]. Many clinical trials have evaluated mAb-based treatment strategies for malignant gliomas, and the implementation of this strategy seems imminent and optimistic [173]. The vast majority of gliomas recur within 2 cm of the primary tumour area [165]; therefore, early and correct diagnosis of tumour recurrence and differentiation of treatment-related changes are important for the timely and adequate treatment of gliomas [174].

Neurosurgeons and oncologists generally believe that malignant gliomas do not metastasize outside the central nervous system. However, this concept has gradually been proven to be incorrect. It is reported that the extracranial metastasis of malignant glioma occurs in about 0.5% of cases [191]. This low incidence rate may be related to the short life span of glioma patients and may also be related to the biological barriers that prevent tumour cell infiltration and survival outside the neural environment. These disorders may include (1) lack of lymphatic system in the brain and spinal cord, leading to systemic spread; (2) dense dura mater around the intracranial vein, preventing tumour cell penetration; (3) lack of trophoblastic matrix in other organs to promote the survival and proliferation of glioblastoma cells [178]. However, with the development of glioma treatment (such as microsurgery, radiotherapy, chemotherapy and other adjuvant therapy), more and more extracranial metastases have been reported in the literature [178], [181].

According to literature reports, the average age of patients with malignant glioma was 38-42.2 years, the median survival time was 10.5 months, and the interval between the diagnosis of primary malignant glioma and extracranial metastasis was 1-24 months [182]. The pathological types were pleomorphic glioblastoma (case 1) and anaplastic astrocytoma (case 2). According to previous reports, the most common site of extracranial metastasis is in the spinal canal (i.e. along the nerve axis) [192], followed by vertebrae, lung, liver and lymph nodes; however, it has also been reported that metastasis occurs in the spleen, scalp, skin, paranasal sinuses, eyes, parotid gland, small intestine, pancreas, kidney, bone, pleura and peritoneum [193]–[196]. Histologically, the primary tumour is usually pleomorphic

glioblastoma, although anaplastic astrocytoma [197], oligodendroglioma [195], gliosarcoma[196], medulloblastoma [197] and pleomorphic xanthoastrocytoma[180] have also been reported. The main routes of extracranial metastasis include cerebrospinal fluid shunt, blood and lymphatic system [198]. Although the mechanism of extracranial metastasis is still unclear, iatrogenic diffusion after operation may be the main cause of extracranial metastasis [198], [199]. The metastasis of glioma occurs along the nerve propagation path or is implanted into the peritoneum through a ventriculoperitoneal shunt [200], [201]. In addition, radiotherapy can lead to the transformation of glioblastoma into sarcomatoid metaplasia and then form the ability to penetrate the vascular wall [197]. The diagnostic standard system of extracranial metastasis of malignant glioma was established. Weiss [202] put forward the diagnostic criteria for extracranial metastasis of primary central nervous system tumour: (1) the clinical history strongly suggests primary central nervous system tumour; (2) the pathological manifestations of metastasis are consistent with the characteristics of the intracranial primary tumour, although there is a certain degree of the anaplastic primary tumour is acceptable; (3) comprehensive autopsy or systemic examination is carried out to exclude the possibility of metastasis He had a primary tumour.

At present, there is no standardized palliative treatment for extracranial metastases. Most patients received radiotherapy and/or chemotherapy, and there was no significant difference in the clinical efficacy of these treatments. Radiotherapy is mainly a palliative treatment to relieve the symptoms caused by local metastasis; however, no matter what treatment method is chosen, the prognosis of patients is very poor. Most of the affected patients will die within a few months.

As mentioned earlier, the prognosis of patients with malignant glioma extracranial metastasis is extremely poor, and the life expectancy is less than 6 months [203], [204]. From the literature, the average interval between the diagnosis of extracranial metastasis and death is only 4.4 months. The diagnosis interval between intracranial lesions and extracranial metastasis was 1-60 months. Lu et al. [178] reported that for primary glioblastoma, the time interval from symptom onset to diagnosis of primary glioblastoma was 2.5 months, the time interval from diagnosis to detection of extracranial metastasis was 8.5 months, and the time interval from metastasis to death was 1.5 to 2.8 months. From 1940 to 2009, the time interval from the discovery of extracranial metastasis to death increased by 0.7 months every 10 years (95% confidence interval, 0.5-1.0 months). The use of MRI was associated with increased overall survival, but no similar correlation was observed in age, gender or location of primary glioblastoma.

Although surgery is the first choice for the treatment of malignant glioma, it can destroy the blood-brain barrier and make tumour cells form extracranial metastasis. Therefore, surgeons should be careful to ensure the principle of non-tumour surgery and keep the dura intact. According to the literature [204], following the following principles can eliminate or reduce the extracranial metastasis of malignant glioma: (1) in addition to tumour resection, the operator should protect and reduce the damage to the surrounding tissue as the goal, and should strive to achieve tumour free surgery, to strengthen the concept of preventing tumour metastasis. (2) The operative field should be flushed with normal saline

and/or chemotherapeutic drugs, and the dura mater should be carefully sutured to maintain its integrity. (3) Patients with high malignancy should receive radiotherapy and/or chemotherapy. (4) Patients can accept ventriculoperitoneal shunt and install a filter device to reduce intraperitoneal metastasis. (5) Postoperative concurrent chemoradiotherapy can reduce the chance of intracranial recurrence and extracranial metastasis.

4.1.4. Current method and achievement

At present, it is a great challenge to develop a treatment method suitable for any type of glioma, and even to design and evaluate clinical trials showing the effectiveness of the treatment. Every therapeutic drug or technology needs complex treatment procedures, high cost, safe and successful resection of tumours without any side effects and improve overall quality of life of patients [205]. At present, surgical resection of the tumour is one of the main methods of treatment, especially since it has been proven to improve the epilepsy control of hospitalized patients [206]. Some studies have shown that, in general, surgical treatment is safe and effective for cancer patients, and the survival rate is higher than non-surgical treatment [207]. They concluded that the survival rate tended to increase with the expansion of surgical resection. Some advanced techniques in surgery enable surgeons to remove tumours to the maximum extent without affecting their health, and significantly improve the life expectancy of individuals [208]. Biopsy, as mentioned earlier, is part of this type of surgery, in which removal of potentially cancerous brain tissue and immediate diagnosis are at greater risk than surgery. In most patients with brain tumours, the surgery is performed regularly, sometimes without complete anaesthesia and sedation - depending on the location of the tumour in the brain [209].

Other treatments include radiotherapy and chemotherapy. The role of radiotherapy in the treatment of patients with brain tumours is still unclear. Some clinical studies have investigated the overall effect of high-dose and low-dose radiation response in adult patients. In one study, patients who received radiotherapy after surgery (after Biosystemic surgery) had no significant difference in overall survival compared with a single surgery. There was no significant difference in survival rate between low dose and high dose (58% and 59% survival rates, respectively) [210]. In addition, in another randomized clinical study, the incidence of radiation neurotoxicity (adverse effects on the nervous system) of high-dose and low-dose treatment was higher, with 5% and 2.5% respectively [211]. Another study showed that long-term radiotherapy resulted in significant cognitive impairment in nearly half of the subjects (patients), affecting their memory [212]. Therefore, there is still uncertainty about whether patients will use radiotherapy, and the risks and complications are higher than the benefits. Chemotherapy, on the other hand, has been used regularly for all types of brain cancer, more in high-grade gliomas, and certain drugs with low doses of LGGs. Oncologists mainly focus on a drug called temozolomide, an alkylating agent, which is used as an anticancer drug for glioma, mainly for high-grade GBM and low-grade astrocytoma [213]. Compared with other alkylating agents such as procarbazine or lomustine, this drug shows obvious advantages, because it can more effectively cross the blood-brain barrier, is more

effective on tumour cells, and shows less toxic and adverse reactions [214]. In the phase 2 clinical study of astrocytoma grade 2 cancer, the drug has successfully demonstrated excellent pharmacological and anticancer properties - of the 46 patients tested with the drug, 61% achieved complete or partial drug responses with a significant reduction in the number of tumour cells, and 78% showed an additional 22 months of prolonged survival [215]. A phase 2 clinical study of temozolomide in the treatment of LGG patients (astrocytomas) after surgery showed that, as expected, the survival rate of 250 participants was significantly improved in the last 3 years before LGG became a high-grade type of drug no longer effective [216]. Compared with studies using temozolomide alone or other alkylated drugs (vincristine), temozolomide was used in high-risk LGGs studies Radiotherapy with oxazoline or other alkylating agents (vincristine) did not show an overall improvement in survival, again indicating that chemotherapy did not significantly reduce cancer cells [217], [218].

The management of glioma treatment is constantly changing, and new strategies are being proposed to balance the risk and benefits of reducing tumours. Considering the current effective evidence of glioma treatment, the best treatment strategy may be surgical resection combined with chemotherapy (combined with drugs such as temozolomide) and delayed radiotherapy as far as possible.

4.1.5. Biomarker in brain tumour

The latest progress in next-generation sequencing methods has led to the identification of GBM-specific molecular characteristics, to better understand the molecular pathogenesis of this disease [213]. Therefore, some potential diagnostic biomarkers have been proposed as predictive biomarkers. Diagnostic biomarkers can make tumour classification more accurate; prognostic biomarkers can inform possible cancer outcomes (e.g., disease recurrence, disease progression, and overall survival), and predictive biomarkers can help adjust treatment strategies according to the specific biological characteristics of patients, thus promoting patient management. Some molecular markers are still under evaluation, but some are usually tested as part of the routine clinical inquiry of GBM patients, including O6 methylguanine DNA methyltransferase (Mgmt), isocitrate dehydrogenase (IDH), epidermal growth factor receptor (EGFR), VEGF, tumour suppressor protein TP53, phosphatase and tensin homolog (PTEN) p16INK4a gene, phospholipid metabolites, cancer stem cells and recent imaging biomarkers (Table 4.1). A detailed description of these molecular characteristics must contribute to more personalized treatment. The development of a new generation of anti-GBM therapies, such as small molecule inhibitor receptors for growth factors, vaccines, antibody-based drug conjugates and inhibitors that recently block immune checkpoints [219].

4.1.6. Aims and Objectives

The main aim of this chapter is to apply the developed semi-targeted lipidomics method to human brain tumour blood samples. The brain tumour sample was classified into LGG and GBM types according to the sample grade. This method is expected to have good repeatability and selectivity on the clinical sample and identify the significantly different lipids in a different stage of brain tumour (GBM and

LGG) and look for potential biomarkers between GBM and LGG.To achieve this aim, the objectives of this chapter are:

- To use SPLASH as a series of internal standards for different classes of lipids and test the normalization efficiency by comparing the relative standard deviation between the unnormalized and normalized results.
- To evaluate the method repeatability by checking the RSD of peak area and retention time for each lipid and compare the result with the FDA requirements.
- To use univariate and multivariate methods to analyze the identified lipids and compare the relative concentration between sample types (GBM and LGG) to check the significantly of different lipids.

Table 4.1. Major biomarkers relevant to the management of patients with glioblastoma. [220]

Type of biomarker	EGFR mutation/ amplification	MGMT promotor methylation	IDH1/IDH2 mutation	Imaging
Diagnostic	EGFRvIII highly correlates with glioma subtypes. Real-time monitoring via typing of microvesicles with EGFR-specific RNA.	Help to distinguish true progression and pseudoprogression in patients with newly diagnosed GBM treated with surgery followed by radiochemotherapy	Differentiate between primary and secondary GBM. IDH-mutant diffuse gliomas and nonanaplastic reactive gliosis distinction.	Detection of specific molecular abnormalities. For example, EGFRvIII, MGMT promotor methylation, and 2-HG which correlate with IDH mutation.
Development status	+/under evaluation	Under evaluation	+	+
Prognostic	Better prognosis with (i) EGFRvIII + Ki64 20% or less, (ii) EGFRvIII + normal PTEN, (iii) EGFRvIII + methylated MGMT promotor	Better OS and PFS (probably with IDH mutations) in malignant gliomas treated with radio- and/or chemotherapy.	Better OS and PFS	MRI: the extent of tumour oedema and necrosis has a negative correlation with OS. PET: 11C-MET uptake is associated with poorer patient survival.
Development status	+/under evaluation	+	+	Under evaluation
Predictive	Possible biomarker for vaccine-based treatment.	Predicts response to chemotherapy with alkylation agents and radiotherapy. Correlate with better response to TMZ in (i) newly diagnosed GBM with TMZ as a first-line treatment, (ii) recurrent GBM, and (iii) elderly patients.	IDH1 mutation is independently associated with complete resection in patients with malignant gliomas treated with surgery. Complete surgical resection is associated with improved survival in patients with IDH1 mutation. The absenceence of mutation suggests a predictive role of MGMT promotor methylation for PFS in patients treated with chemotherapy.	Functional Diffusion Maps (fDMs) predict PFS and OS in patients treated with radiochemotherapy. ADC predicts a better response to bevacizumab combined with chemotherapy. Association between hypoxia level (measured by 18F-FIMSO and radiotherapy response.
Development status	Under evaluation		Under evaluation	Under evaluation

OS, overall survival; PFS, progression-free survival.

4.2. Materials and Methods

4.2.1. Materials and Apparatus

4.2.1.1. Materials and Chemicals

A Milli-Q water purification system (Millipore, MA, US) was used in the preparation of deionized water (18.2 MΩ). Acetonitrile, chloroform, ethyl acetate and hexane (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Methanol (LC-MS grade) and ammonium acetate were purchased from Sigma-Aldrich (Darmstadt, Germany). Isopropanol (LC-MS grade) was purchased from Acros Organics (New Jersey, USA). BHT (3, 5-di-tert-butyl-4-hydroxytoluene) was purchased from Supelco (PA, USA), and human plasma (donated from Queens Medical Centre Nottingham blood bank). SPLASH® Lipidomix®Mass Spec Standard was purchased from Avanti (Darmstadt, Germany). A brain tumour blood sample was collected from Queens Medical Centre, Nottingham.

4.2.1.2. Apparatus

-80℃ freezer, Fisherbrand™ Wizard™ Infrared Vortex Mixer (Fisher Scientific, UK), Orbit™ Digital Microtube and Microplate Shakers (Labnet, USA), Harrier 18/80" Centrifuge rotor, Jouan Centrifugal Evaporator, Biostage SPE manifold, Techne Dri-Block heater (DB-3B), pipettes and tips, LC-MS vial with inserts and caps, Oasis SPE cartridge (Waters, UK), glass tube, Eppendorf, Accela liquid chromatography coupled with Orbitrap Quadrupole-Exactive mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK), ACE Excel 3 SuperC18 Column(100×2.1mm id), and guard column were purchased from Advanced Chromatography Technologies.

4.2.2. Sample preparation

Brain tumour blood samples were collected from 20 patients by Dr Ruman Rahman group and treated at the Queen's Medical Centre (QMC) within the UK NHS Trust centre. There are 10 patients classified as GBM (grade 4) and 10 patients classified as LLG (grade 2) according to the glioma classification. After surgery, the sample was snap frozen and stored at –80°C for 10 min. The use of fresh frozen human samples was approved by the National Research Ethics Service Committee in East Midlands, UK (ethics approval reference 11/EM/0076). The clinical and pathological characteristics of patients are described in Table 4.2.

Table 4.2. Clinical and Pathological Data from 20 patients.

High Grade	Age	Sex	Grade	Histology	Tumour Site	IDH-1	ATRX
GBM69	62	M	IV	GBM	Right frontal	WT	WT
GBM70	38	M	IV	GBM	Right temporal	WT	WT

GBM71	76	M	IV	GBM	Left parietal	WT	WT
GBM73	70	M	IV	GBM	Right temporal	WT	WT
GBM77	72	M	IV	GBM	Right Frontal	WT	WT
GBM78	64	M	IV	GBM	Right temporal	WT	WT
GBM80	52	M	IV	GBM	Right parietal	WT	WT
GBM82	74	M	IV	GBM	Right frontal	WT	WT
GBM83	52	F	IV	GBM	Right occipital	WT	WT
GBM85	41	M	IV	GBM	Right parietal	WT	WT

Low Grade	Age	Sex	Grade	Histology	Tumour Site	IDH-1	ATRX
LGG5	37	F	II	Astrocytoma	Left frontal	R132H	Loss
LGG6	52	F	II	Astrocytoma	Left Frontal	R132H	Mixed
LGG7	27	F	II	Astrocytoma	Right frontal	R132H	Intact
LGG10	35	F	II	Astrocytoma	Left temporal	R132H	Intact
LGG11	19	F	II	Astrocytoma	Left frontal	R132H	Mutant
LGG12	32	M	II	Astrocytoma	Left frontal	R132H	Intact
LGG16	45	M	II	Astrocytoma	Left Parietal	R132H	Mutant
LGG19	33	M	II	Astrocytoma	Left temporal	R132H	Intact
LGG24	31	M	II	Astrocytoma	Left frontal	R132H	Intact
LGG26	45	M	II	Astrocytoma	Right temporal	R132H	Mutant

4.2.3. Sample extraction

Brain tumour blood samples, plasma from Queens Medical Centre and quality control were used for this LC-MS method validation. The sample extraction protocol was shown in Figure 4.2. In the plasma extraction, 50 µl of plasma (sample) or water (regent blank) was added into 500 µl of ice-cold (-20℃) extraction solvent mixture. The extraction solvent mixture was prepared by mixing organic solvent

chloroform and methanol (2:1), and 10 µl SPLASH internal standard was added into 490 µl of the organic solvent mixture to get the extraction solvent mixture. After 10 min vortex, 500 µl of water was added to the mixture. The mixture was vortexed again for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper aqueous phase and a lower organic phase, as well as a protein layer in the middle. The lower organic phase was collected and evaporated by a centrifugal evaporator. Then the sample was reconstituted with 100 µl of isopropanol and transferred into an LC-MS glass vial. In the brain tumour blood extraction, a blood sample was centrifuged (13000g, 4℃) for 10 min to collect plasma in the upper layer, and the cell membrane at the bottom was disposed of. Afterwards, the brain tumour plasma was collected, and the extraction procedure is the same as the plasma extraction procedure above. The quality control (QC) was prepared by mixing all the plasma extracts and well vortexed.

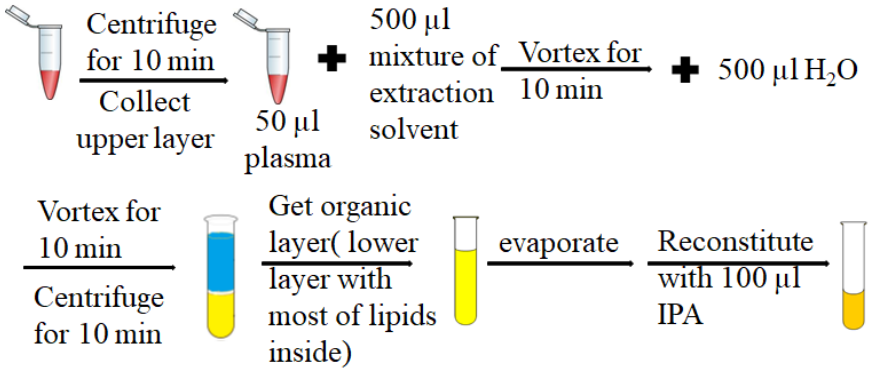


Figure 4.2. *The schematic of brain tumour blood extraction method. The blood sample was centrifuged to get the plasma sample at the upper layer. Plasma was extracted with a solvent mixture, and after vortex and centrifuge, the organic phase was collected, evaporated and reconstituted with isopropanol.*

4.2.4. LC-MS analysis

LC–MS lipidomics analysis was conducted on an Accela high-speed liquid chromatography coupled to Quadrupole-Exactive mass spectrometry (Thermo Fisher Scientific, Hemel Hempstead, UK). LC–MS analysis was maintained at a temperature of 40 °C and a flow rate of 300 µL/min on a 10 cm C18 column. The mobile phase consisted of (A) 60% of H2O, 40% of acetonitrile and 0.1% of ammonium acetate and (B) 80% of isopropanol, 10% of methanol, 10% of H2O and 0.1% of ammonium acetate. A starting percentage of 50 % of mobile phase B was chosen and increased to 100% of B at 5 min. After keeping at 100 % of B until 13.5 min, gradient drops back to 50 % of B at 14 min and maintained at same gradient for 1 min. The injection volume was 5 µL. All study samples and QC samples were analysed in a single continuous analytical run. Data were acquired simultaneously in full scan ion mode (m/z 100–1500, resolution 25,000) in both positive and negative electrospray ionisation modes. A list of lipids with absolute mass, formula, polarity, adduct type and name that were pre-defined from human plasma was loaded to the MS method (see appendix Table A). The capillary temperature and heater

temperature were maintained at 350 and 300 °C respectively in both positive and negative modes. The maximum scan inject time and micro scans were 100 s and 1 s respectively. MS was performed in simultaneous ESI (+) and ESI− full-scan modes with spray voltages of 4.5 (ESI+) and 3.5 kV (ESI−) and capillary voltages of 40 (ESI+) and −30V (ESI−).

The LC-MS sequence was shown in Table 4.3. First, The LC-MS run started with three blank samples, 100 µL of isopropanol was used as a blank sample as the QC and plasma sample was reconstituted with isopropanol. Second, one reagent blank sample without internal standard was continued, this sample was prepared by replacing the plasma with water and following the sample extraction procedure without adding SPLASH internal standard (replaced with mixed organic solvent). Third, three reagent blank samples were conducted, and they were prepared by replacing the plasma with water and following the sample extraction procedure written in section 3.2.2. Forth,6 injections of pooled QC were analysed, and they were used for equilibrating the instrument. Fifth, 6 plasma extracts and 20 brain tumour blood extracts were conducted, and the order was randomized by Excel. Sixth, 6 replicates of pooled QC were analysed to test the instrument stability. Finally, one blank sample (isopropanol) was used to wash the column and instrument.

Table 4.3. The LC-MS run in the brain tumour application

Sample type	Number	Note
Blank	3	Isopropanol
Blank_no_IS	1	Extracts without plasma and IS
Reagent Blank	3	Extracts without plasma
Pooled QC	6	For equilibration
plasma_3	1	
plasma_4	1	
GBM_77	1	
LGG_26	1	
plasma_6	1	
LGG_11	1	
GBM_85	1	
LGG_10	1	
plasma_5	1	
LGG_6	1	
LGG_19	1	
plasma_1	1	
GBM_78	1	
LGG_24	1	
LGG_5	1	
LGG_7	1	
plasma_2	1	
GBM_73	1	

GBM_71	1	
GBM_80	1	
LGG_16	1	
GBM_82	1	
GBM_70	1	
LGG_12	1	
GBM_69	1	
GBM_83	1	
Pooled QC	6	For RSD check (QC)
Blank	1	To wash the column and instrument

4.2.5. Data analysis

LC–MS raw data from the analysis of extracts of study and control samples were acquired with Xcalibur v2.1 software (Thermo Scientific, Hemel Hempstead UK). Chromatographic raw data contain a 3D data set consisting of m/z, retention time and ion signal intensity. These data were processed with LipidSearch (v5.1) to align the replicates and control results and get the extracted lipid formula. The detail of the LipidSearch parameter was shown in Table 2.6 and Table 2.7. The identified lipid list from LipidSearch with allocated internal standards was processed by Tracefinder (Thermo Scientific, Hemel Hempstead UK) following the official protocols.

The quality of the lipidomic analysis was assessed by calculating percentage RSD values (normalization). For multivariate analysis, PCA and OPLS-DA plots were constructed, comparing LGG and GBM groups. VIP score analysis was done in everyOPLS-DA plot, choosing lipids with a score of 1.0 or above and Q2 <0.5 only. Univariate analysis was performed on the same grouped data as used in multivariate analysis, and the parameters for t-test and FDR statistical correction were chosen (p-value of less than 0.05, FDR rate 5%). These lists were then compared to their corresponding multivariate VIP score lists to produce final lists of statistically significant lipids. The fold changes obtained from univariate analysis were used to decide whether a potential biomarker was elevated or decreased during the “disease” condition.

4.3. Results and Discussion

4.3.1. Identified lipids and RSD in patients

LipidSearch and Tracefinder results revealed 260 putatively identified lipids over (241 in common between LGG and GBM groups). The identified lipids in the patient's brain blood sample were summarized in Table 4.4, and lipid classifications, the number of lipids in each class and the average RSD for each lipid class were shown as well. Due to the weak reproducibility of eicosanoids, it lacks three replicate results, thus the RSD is shown as NA. As Table 4.4 shows, most of the classes of lipids have an RSD lower than 30%, which is considered as qualified for clinical sample (in FDA requirement,

clinical sample RSD should be lower than 30%, at least 80% of the analytes meet the criteria). The whole list of identified lipids and their peak area RSD in each group was shown in the Appendix Table F, and the RSD in LGG, GBM, QC and plasma also meet the criteria, thus the result is reproducible and can be analysed in other analysis methods.

Table 4.4. The identified lipids summary in brain tumour blood sample

Lipid class	**Count of Lipid Class**	**Average RSD/RSD**
Ceramides (Cer)	23	4.41%
Cholesterol Esters (ChE)	15	18.59%
Cholesterols	1	12.80%
Coenzyme (CoQ)	2	26.03%
Diacylglycerols (DG)	25	17.66%
Eicosanoids	2	NA
Fatty Acids (FA)	28	2.66%
Fatty Esters	1	1.55%
Glycerophosphocholines (PC)	50	3.35%
Glycerophosphoethanolamines (PE)	13	7.55%
Glycerophosphoglycerols (PG)	10	37.66%
Glycerophosphoinositols (PI)	20	5.01%
Glycerophosphoserines (PS)	10	14.73%
Monoacylglycero-phosphocholines (LPC)	9	1.58%
Monoacylglycerophospho-ethanolamines (LPE)	7	3.10%
Sphingoid bases	5	22.36%
Sphingomyelins (SM)	8	15.98%
Triracylglycerols (TG)	31	3.74%
Grand Total	260	10.19%

4.3.2. LC-MS analytical performance of the sample

Analytical runs of brain tissue samples have generated clear spectra with distinctive metabolite peaks, high TIC values ($\geqslant$ E8) and no background noise or carry-over effects. The spectra of QC and plasma samples also showed clear peaks with high TIC values, demonstrating good analytical stability of sample metabolites and reproducibility. The shape of the spectra peaks of QC samples was found to be similar to the clinical sample spectra, with some peaks identical, showing that QC samples contained the common metabolite peaks as the actual samples.

4.3.3. Normalization by SPLASH internal standard

Once the normalisation analysis had been applied to datasets of brain tumour samples, percentage RSD values of peak height intensities of every lipid were calculated in each LGG and GBM group, in the same way, that the data were normalised in validation experiments in chapter three. Out of 260 lipids, on average, around 30% of metabolites were normalised successfully (the reduced RSD after normalization) in each group. There was no significant difference in the number or nature of successfully normalized lipids between the data groups. Further quality of normalised data can be observed through multivariate PCA and OPLS-DA plots (see the next section), where the data points were observed to be clustered and data groups were well separated, showing reduced data variation.

4.3.4. Univariate analysis – t-test

T-test and false discovery rate correction were used for univariate analysis. The whole t-test result table was attached in Appendix Table G. The commonly used t-test correction method, Bonferroni correction and false discovery rate correction result were calculated and summarized in the table as well. Considering the Bonferroni correction is too stringent, several authors have advocated the use of the false discovery rate (FDR) for the problem of testing multiple hypotheses simultaneously [221]–[223], and thus FDR correction was used in conjugate with fold change to decide significant difference lipids in this thesis. The volcano plot used to visualize the t-test result was shown in Figure 4.3 below. To qualify as a significant difference, the t-test result should fulfil fold change >2 and q value <0.05 (FDR corrected t-test result), and the qualified result shown in the upper left and upper right in the volcano plot. As figure 4.3 shows, no lipids are sitting in these two areas, which signifies that there are no lipids considered significant differences between GBM and LGG groups in this clinical study.

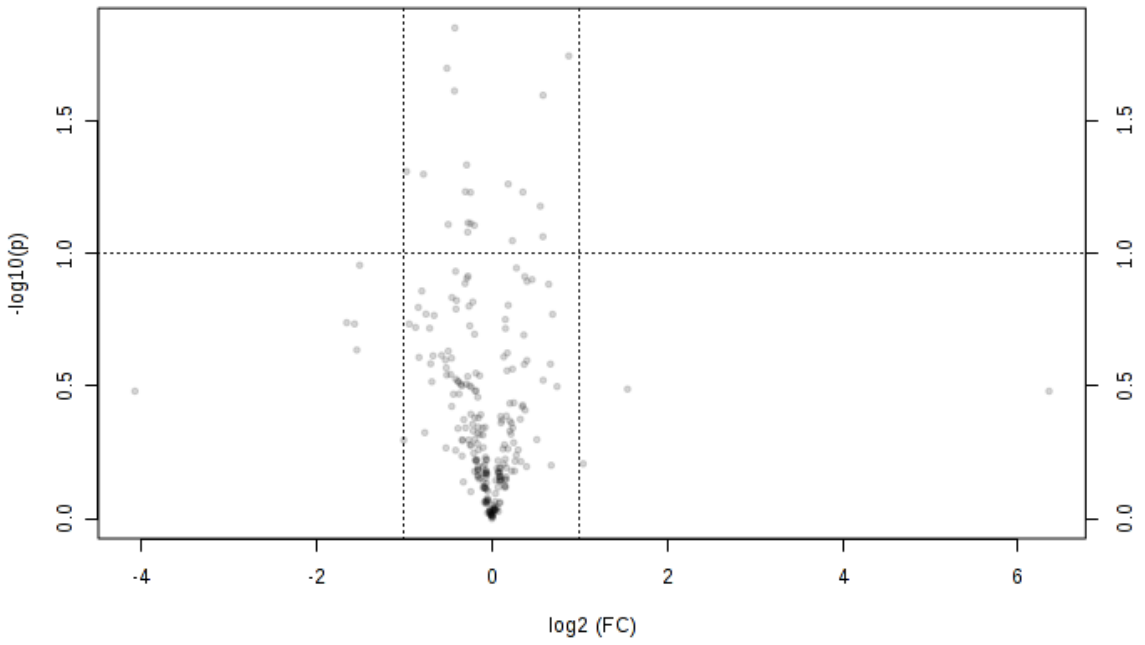


Figure 4.3. *The volcano plot for t-test result. The X-axis represents the log2 value of fold change, and the Y-axis represents the –log10 value of the t-test result with FDR correction. The area with fold change >2 and q value (corrected t-test result <0.05) was considered a significant difference.*

4.3.5. Multivariate analysis -- PCA result

Principal Component Analysis (PCA) graph was used for multivariate analysis, and different sample types (including GBM groups, LGG groups, QC samples, plasma samples, blank without IS and blank samples) were used to analyse the relationship between them. The data points that are closely located in the PCA graph mean the relationship between them is closer and data (identified lipids and their concentration) are similar. As Figure 4.4 shows, the blank, QC and plasma sample group was clustered together, which represents good reproducibility. The blank without IS points outside of the ellipse means it has a big difference compared with other groups, and this could be explained by the missing plasma lipids and internal standard in the sample. The GBM and LGG points are roughly clustered when compared with other groups, but the data points in these two groups are not clustered closely as the groups mentioned above. This result illustrates that the result of the brain tumour patient data (GBM, LGG group) has a big difference compared with healthy people (plasma and QC sample) and blank samples, but the difference in the patient groups are also quite high, and this result is also supported by the OPLS-DA result in the next section.

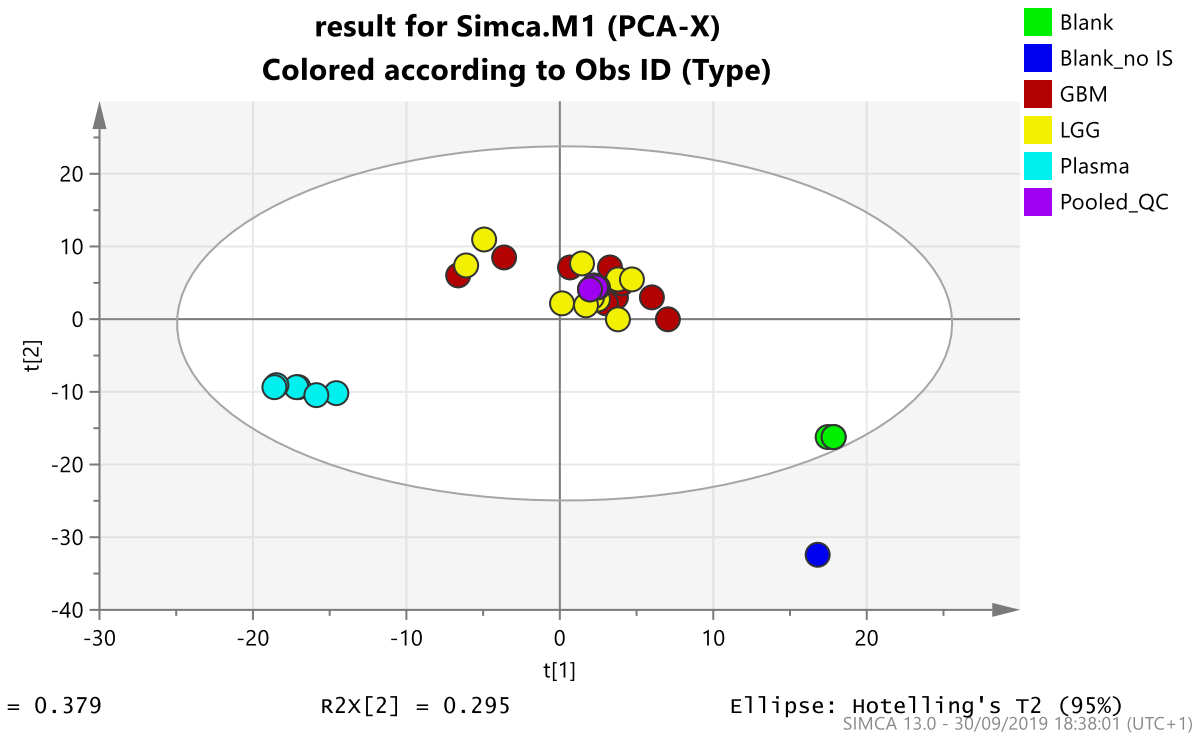


Figure 4.4. *The PCA result for GBM, LGG, QC, blank and plasma samples.*

4.3.6. Multivariate analysis -- OPLS-DA result

The OPLS-DA was also used for multivariate analysis in this study to investigate the relationship between GBM and LGG groups. Similar to the PCA plot, OPLS-DA results could also be visualized through the data point inter and intra groups. As Figure 4.5 shows, although GBM and LGG group shows a grouped tendency, the data points in both group are scattered, which means a high variance in the group. This could be the reason why no lipids in the clinical sample show a significant difference in the t-test result since the variance in the group could be higher than the inter-group difference. The Q2 and VIP value could also be used for OPLS-DA result analysis. The Q2 value represents the prediction ability of the OPLS-DA model, and Q2 > 0.5 was considered a predictive model. The Q2 value of the OPLS-DA result is negative, which means these two groups are not qualified for a different check. The VIP score (Variable important in projection) can be used to measure the impact of different metabolite accumulation differences on the classification and discrimination of samples in each group, and VIP $\geqslant$ 1 is the common screening criteria for different metabolites. The VIP result of all of the lipids was shown in Appendix Table G, and there are 87 lipids with a VIP score $\geqslant$ 1. Thus, the OPLS-DA result shows, the difference between the two groups is not clear and the significant difference in lipids is not reliable as the Q2 value is below 0.5. There are some findings from other researchers, for example, Hua Zhao identified five metabolites, namely uracil, arginine, lactate, cystamine, and ornithine, significantly differed between high- and low-grade glioma patients in both the discovery and

validation cohorts [224]. Thus, a further study with a larger amount of patient participants would help with biomarker investigation.

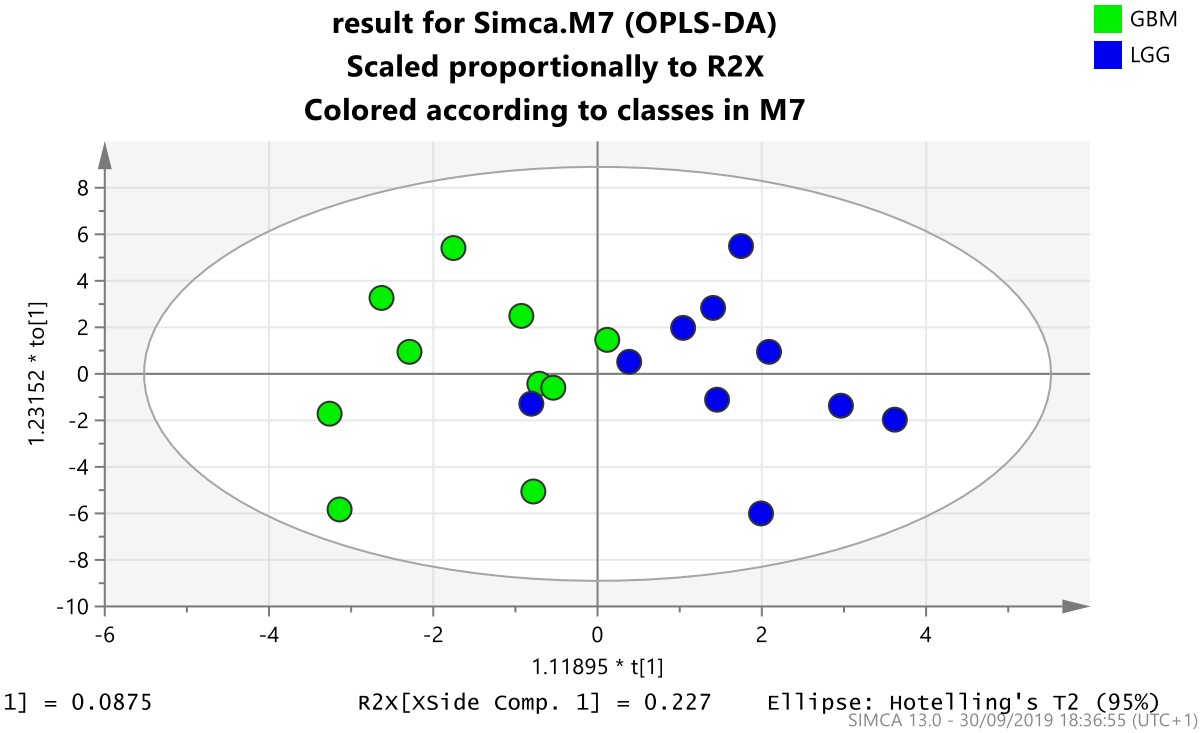


Figure 4.5. *The OPLS-DA result for GBM and LGG samples. The green spot represents 10 patient data in the GBM group, the blue represents 10 patient data in the LGG group.*

4.3.7. Heatmap

The heatmap is another visualization tool to represent lipid concentration in each sample by a different colour. As Figure 4.8 shows, the lipid relative concentrations in all the samples in GBM and LGG were compared using different colours. The GBM and LGG group of samples were classified by a red and green bar on the top of the figure, and the concentration of the lipids was signified from deep red to deep blue (red means lipid concentration is higher, while blue means the concentration is lower). As the figure shows, the concentration of the same lipid could be highly variable in different samples, for example in LGG_10 there are lots of highly concentrated lipids compared to other samples. However, another phenomenon could be seen is the big variance in the same group, which means the lipid relative concentration variance was also high in the same group, and this is the reason why GBM and LGG samples were considered closely related on the top of the figure (colour bar) and samples in the same group are not classified together. Thus, the heatmap illustrates the big difference in lipid concentration in different samples, and they are useful data to find potential biomarkers between LGG and GBM groups, but it also shows the possibility of the single sample variation and big variance in the same patient group.

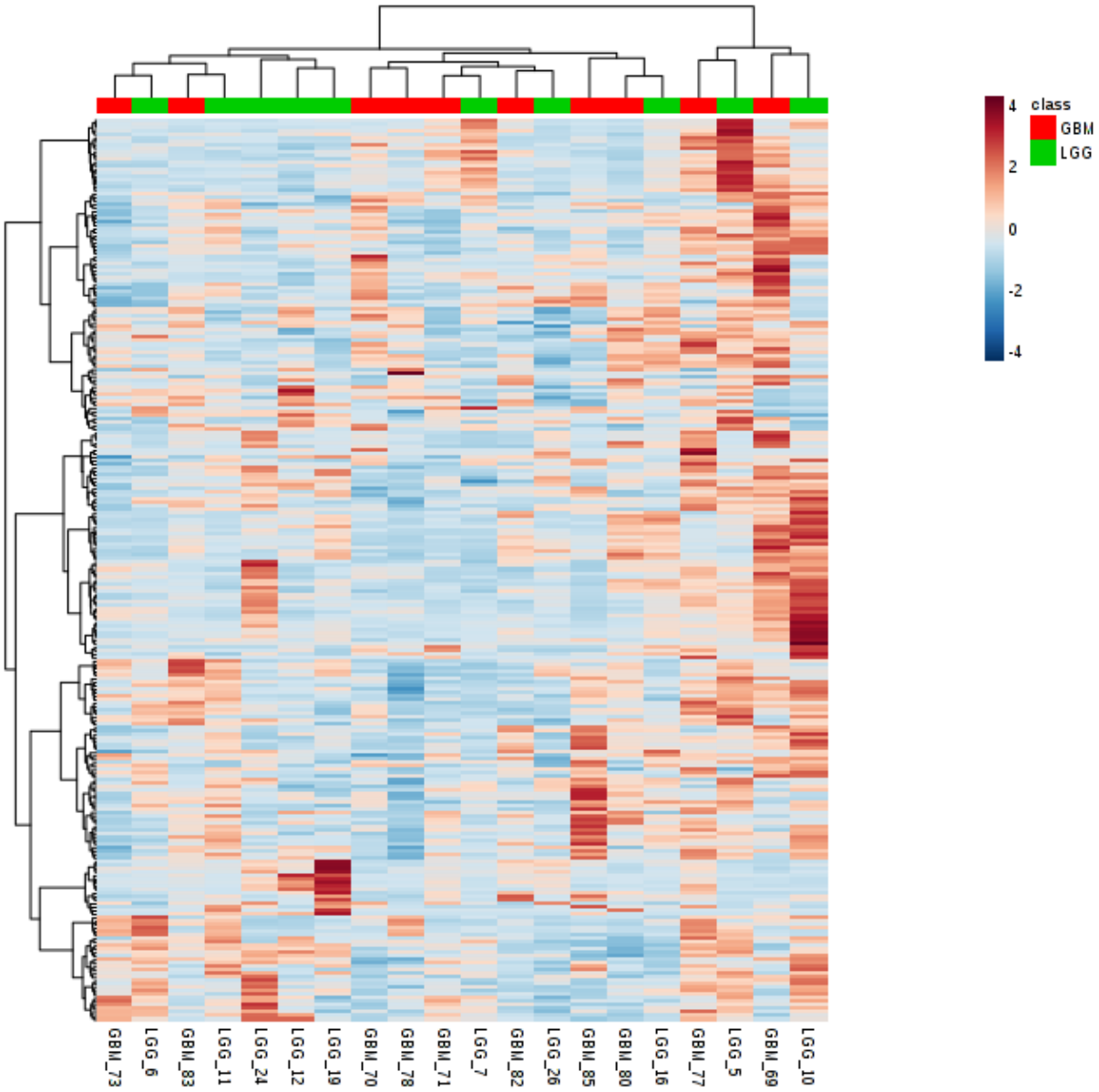


Figure 4.8. *The heatmap result of GBM and LGG samples. The green bar represents the LGG group, and the red bar represents the GBM group. Different colour embodies different concentrations of lipids, and blue means lower concentration, red means higher concentration.*

4.4. Conclusion

In this chapter, the developed LC-MS method was applied to the brain tumour blood samples, several validation parameters were reviewed for all identified lipids, and the result proves the developed method is in an acceptable range of stability and repeatability, as well as selectivity. Quality control was added to equilibrate the instrument and check the LC-MS stability. The 6 plasma extraction samples confirm the stability of the extraction method, freeze-thaw, storage condition, human impact and other factors are stable and meet the FDA criteria. For the lipids with a high-level RSD, different reasons were found,

and outliers or missing peaks are the main problems that cause the high RSD. Except this, most of the lipids have a clear peak and reasonable RSD.

The SPLASH internal standard was added to the method to normalise the lipids by class. The normalisation result shows many lipids' RSD decreased after normalisation, but lipids allocating to internal standards from other lipid classes or internal standards themselves have a quite high RSD showing the RSD increased after normalisation. The result supports the usefulness of adding internal standards in the sample, but the normalization result could be better if adding more internal standards for the lipids.

For the clinical application result, there are putatively 260 lipids found in the sample and 241 lipids appeared in both groups, the big amount of lipids with good identifications (class 1) and relative concentration information presents many useful data in the brain tumour samples. In the univariate test, the t-test and its correction were calculated, but the result did not meet the significant difference criteria (fold change > 2 and q value < 0.05). In the multivariate test, PCA and OPLS-DA were used to check the relationship between inter and intra-sample groups. The PCA result shows the identified lipids have a big variance among different groups, but the OPLS-DA result shows the identified lipids also have a big variance in GBM and LGG groups themselves and no lipids are qualified as significantly different lipids ($Q2 > 0.5$ and VIP $\geqslant 1$). The heatmap graph visualizes the relative concentration of all the identified lipids in each sample, and the huge difference in the concentration of some lipids in different samples proves that there are potential biomarkers but negated because of big variance in the GBM and LGG groups.

Several reasons cause no significant difference in lipids found between GBM and LGG samples. This might be due to the limited number of patients included in the analysis. The ideal experimental design should include spatially resolved samples from a larger number of patients to address the level of inter-tumoral variations which is challenging to achieve. In addition, the sample preparation method needs to be carefully examined and conducted, to decrease the possibility of adding more variables.

In summary, the method stability, repeatability and other parameters all meet the clinical analysis standard, and the normalisation could help the variation in general. The clinical application identified hundreds of lipids for analysis, and it shows a significant difference between the patient sample and healthy people. Between GBM and LGG sample, there are some lipids that show a huge difference between the group, however, they are not considered as significantly different lipids as the variance in the group are too high. Thus, the method is considered a proper method for relatively quantifying lipids on brain tumour samples, but there are more efforts needed to find potential biomarkers in it.

Chapter Five

Application of semi-targeted lipidomics method to feeding-related insulin and anabolic study

5.1. Introduction

5.1.1. Obesity: aetiology, epidemiology and symptoms

Obesity refers to the physical state in which excessive body fat accumulation has a negative impact on health, which may lead to shorter life expectancy and various health problems [225]. Obesity is related to heredity and lifestyle: some people may be less likely to get fat because of their genes, but no matter who they are, anyone can get fat, but conversely, anyone can lose weight. It is the main preventable cause of death in the world, and it is also one of the most important public health problems in the 21st century [226]. At present, the prevalence of obesity in adults and children is rising, and it occurs more often in women than in men. In 2015, 600 million adults (13%) and 42 million children under the age of five had obesity worldwide. It is stigmatized in many parts of the modern world, especially in western countries, although obesity is often regarded as a symbol of wealth and fertility at other times in history, and still in some parts of the world [227], [228]. In 2013, several medical associations, including the American Medical Association and the American Heart Association, defined obesity as a disease [229].

In adults, overweight and obesity are defined according to body mass index (BMI), which refers to weight (kg) divided by height 2 (m). Table 1 shows the categories of BMI. Healthy BMI ranged from 18.5 to 24.9 kg / m2. Overweight is defined as having a body mass index between 25 and 29.9 kg / m2, and obesity is defined as having a body mass index of 30 kg / m2 or more. 5 obesity can be further subdivided according to the subclass of body mass index, as shown in Table 1. Waist circumference can be combined with BMI to assess an individual's health risk.

Table 5.1. Categories of BMI and disease risk [a] relative to normal weight and waist circumference [230]

	BMI (kg/m2)	**Obesity Class**	**Men<102 cm (<40 in) Women <88 cm (<35 in)**	**>102 cm (>40 in) >88 cm (>35 in)**
Underweight	< 18.5		—	—
Normal [b]	18.5–24.9		—	—
Overweight	25.0–29.9		Increased	High
Obesity	30.0–34.9	I	High	Very high
	35.0–39.9	II	Very high	Very high
Extreme obesity	40	III	Extremely high	Extremely high

[a] Disease risk for type 2 diabetes, hypertension, and CVD. [b] Increased waist circumference can also be a marker for increased risk even in persons of normal weight. Data from Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary [231].

5.1.2. Obesity-related disease

Obesity can increase the chance of cardiovascular disease, type 2 diabetes, cancer, degenerative arthritis and other diseases [227]. The main causes of obesity often include excessive calorie intake, lack of exercise and physical problems. Other factors such as genetic defects, endocrine abnormalities, drug effects and mental diseases may also cause obesity. Due to the energy required to maintain weight gain, obese people consume more energy than normal people [232].

5.1.2.1. Diabetes

The relationship between obesity and type 2 diabetes is so close that Sims and his colleagues created the term "diabetes" in the 1970s when they found that in young men without a family history of diabetes, the body mass index increased to 28. 0 kg/m2 after overfeeding for 6 months, and the levels of fasting insulin, glucose and insulin showed a reversible increase [233]. Stevens and colleagues [234] showed that about 90% of type 2 diabetic patients had a higher BMI than 23. 0 kg/m2. Early weight gains [235] significantly increased the risk of diabetes, especially in children and those with a family history of diabetes, abdominal obesity, and with gestational diabetes. In Japan, being overweight will greatly increase the risk of diabetes. 50% of Japanese aged 70 years with a body mass index of 28.0kg/m2 have diabetes.

5.1.2.2. Cancer and reproductive abnormalities

Obesity is one of the most important preventable causes of cancer known. About 10% of cancer deaths among non-smokers are related to obesity. The WHO International Cancer Institute [236] estimates that overweight and non-exercise account for 1/4 to 1/3 of breast, colon, endometrial, renal and esophageal cancers. Potential mechanisms are difficult to define. Gastric acid reflux, due to abdominal hypertrophy, leads to esophageal and colon cancer, which is related to hyperinsulinemia. Breast cancer seems to be related to the abnormal high concentration of free estrogen in postmenopausal obese women. This is due to the transformation of sex hormones in adipose tissue by aromatase and the decrease in plasma steroid binding globulin concentration. These changes may also explain the tendency of endometrial cancer and may be related to the association between overweight and prostate cancer.

High estrogen concentration will also interfere with the feedback regulation of the hypothalamic optic axis, disrupt normal reproductive function, and lead to irregular and usually anovulatory cycles, The greater the degree of obesity, the more far-reaching the impact on ovarian function. Now the greater the possibility of obesity, accounting for 6% of primary infertility [237]. In men, similar changes are now considered to be the cause of the increase in impotence and infertility, and abdominal obesity has once again become a special risk [238].

These sex hormone disorders are usually accompanied by hirsutism and polycystic ovary syndrome. They are characterized by severe insulin resistance and estrogen-producing androgen, partly due to the high aromatase activity of adipocytes in obese patients. However, this poorly defined syndrome

responds to weight loss and changes in essential fatty acid intake, as well as the treatment of insulin resistance [239]–[241].

Changes in hormone concentrations associated with obesity have adverse effects on pregnancy; The risk of hospitalization is four to seven times higher than that of non-obese women. The obesity report of the US health secretary [242] stressed that the risk of preeclampsia, more common gestational diabetes, difficulties in childbirth and delivery, higher cesarean delivery rate and increased risk of maternal and infant mortality increased by three to ten times. Infants are at greater risk of neural tube defects and macrosomia.

5.1.3. Current method and achievement

The treatment of obesity includes behavioural therapy, drug therapy and surgery. Behavioural therapy is the most important treatment, that is, to achieve the purpose of weight loss by changing eating habits, eating structure and exercise [243]. Reasonable diet control can significantly reduce body weight in the short term [244], but it is often quite difficult to maintain the results of weight loss. It often needs to be combined with exercise and a lifestyle of low-calorie diet [245], [246]. There seems to be no difference in the health benefits of various low-carbohydrate and low-fat diets [247]. These diet strategies also are roughly the same as [248] in controlling the risk of heart disease and diabetes. The long-term weight loss success rate and lifestyle change rate are not high, about 2-20% [249]. Generally, only 20% of people can maintain their weight for a long time [250]. Further investigation found that 67% of patients who lost more than 10% of their weight could maintain their weight or continue to lose weight within the next year [251]. Patients with an average weight loss of more than 3kg or more than 3% of their total weight can maintain this level within 5 years [252].

The benefits of weight loss are obvious. In a prospective study, active weight loss, regardless of the degree of weight loss, can reduce the mortality caused by any cause by 20% [253]. To control weight gain during pregnancy and improve maternal and infant health, it is effective to adjust diet and lifestyle [254]. If patients with heart disease have obesity and other disease risk factors, they will be recommended to receive intensive behavioural counselling [255].

Diet control methods are generally divided into the following four categories: a low-fat diet, a low-carbohydrate diet, a low-calorie diet and a very low-calorie diet [244]. A meta-analysis of six randomized controlled trials found that the main dieting methods (low calorie, low carbohydrate and low fat) had no difference in weight loss effect and could successfully reduce two to four kilograms [244].

There are three weight-loss drugs on the market that have been proven to be able to be used for a long time, namely orlistat (Roche fresh), lorcaserin (belviq) and pentamidine and topiramate compound (qsymia) [256]. Orlistat can lose a small amount of weight, with an average of about 2.9 kg in one to

four years [257], but it has a high chance of gastrointestinal side effects [257], and its impact on the kidney has also been questioned. The other two drugs are only available in the United States [258]. On average, lorcaserin can reduce 3.1 kg of body weight (3% of body weight) more than a placebo in one year [259], but it may cause heart valve problems [258]. Fentadine and topiramate also have some weight loss effects [189], but the use of these two drugs is also associated with heart problems [258]. At present, there is no data to clarify the impact of these weight-loss drugs on the long-term complications of obesity, such as cardiovascular disease and death [256].

The most effective treatment for obesity is bariatric surgery [260]. After surgery, severely obese people can lose weight for a long time, improve obesity-related problems [261], and reduce mortality. One study pointed out that surgery can reduce the weight of patients by 14% - 25% in ten years (depending on the surgical method) and can also reduce mortality by 29% compared with the general weight loss method [262]. However, the incidence of surgical complications is 17%, and 7% of patients need a second operation [262]. Because of the cost and risk of surgery, researchers are looking for less invasive but equally effective treatments, such as inserting space-occupying objects (balloons) into the stomach to reduce gastric volume.

5.1.4. Justification of the study

Most nutritional health studies focus on changing the amount and/or type of food consumed. Recent studies have shown that the temporal distribution of nutrient intake (timing nutrition) can also play a role in regulating the health effects of a specific diet. For example, limiting the daily energy intake (EI) window to between 4 and 10 hours, called time-restricted feeding (TRF), can produce beneficial metabolic effects independent of energy balance [263], [264] in rodents, including preventing excessive weight gain due to high-fat and high sugar diet, reducing serum triglycerides, fasting insulin concentration and liver fat content, and improving glucose tolerance. However, key differences in adaptive metabolic responses to fasting between rodents and humans, including hepatic glycogen depletion rates [265], [266], may limit the translatability of these findings.

Studies on the effects of TRF on humans have mainly focused on the adaptation of resistance training [267]–[269] and/or only Fasting Metabolism, energy balance or weight loss [270]–[272]. In a feasibility study, overweight individuals received 16 weeks of TRF during which they were asked to reduce their daily eating time to 10-12 hours, thereby significantly reducing weight and improving self-reported sleep satisfaction and energy levels [273]. In addition, glucose tolerance in overweight/obese adults also improved after TRF treatment [274]–[276]. Short-term TRF has also been shown to reduce appetite markers and increase the 24-hour fat oxidation rate in overweight individuals [277]. Recently, the administration of 12-week 10-hour TRF in patients with metabolic syndrome improved cardiac metabolic health indicators, including blood pressure and circulating lipids [278]. Currently, studies in this area rarely use detailed metabolic measurements during the postprandial period (including most of

the time awake). In addition, no study examined the effect of TRF on skeletal muscle metabolism, which plays an important role in the treatment of oral glucose load [279]. In addition, improved skeletal muscle anabolic sensitivity compared to the energy-matched control diet of people undergoing resistance training may be the basis for the preferential reduction of fat mass observed during TRF [267].

Another consideration is the optimal timing of the EI window. Diurnal changes in metabolic function, including glucose tolerance, were established decades ago [280], [281]. Recent randomized crossover studies have shown that there is a good acute metabolic response to foods eaten earlier in the day [282], [283], and there is evidence that it may also be beneficial to transfer more proportion of EI in the long term to earlier in the day [284], [285]. Early restricted feeding (eTRF) is a dietary strategy that combines these methods by shifting EI to early in the day and prolonging the time of night fasting. A recent randomized controlled trial of supervised and controlled feeding showed that 5 weeks of eTRF improved systemic insulin sensitivity in prediabetic patients and was not associated with weight loss [276]. However, after the matched fasting time, the metabolic response has not been compared, and the role of eTRF in the free-living environment has not been tested. This includes physical activity (PA) levels and blood glucose variability objectively measured using a continuous glucose monitor (CGM).

The main purpose of this study was to compare the effects of 2-week free-living eTRF and energy-matching (restrictive) control intervention on systemic and skeletal muscle insulin and anabolic sensitivity in healthy young men. The secondary objective was to evaluate the changes in body composition and PA pattern after 2 weeks of intervention. It is speculated that eTRF will provide beneficial metabolic benefits during the postprandial period in healthy people compared with the control group

5.1.5. Aims and Objectives

The primary aim of this study was to compare the effects of 2 weeks of free-living eTRF and an energy-matched (restricted) control intervention on markers of whole-body and skeletal muscle insulin and anabolic sensitivity in healthy young men. The secondary aims were to assess the changes in body composition and patterns of PA following 2 weeks of intervention. It was hypothesized that, compared with the control, eTRF will confer favourable metabolic benefits during the postprandial period in healthy humans.

The main aim of this chapter is to apply the developed semi-targeted lipidomics method to human obesity plasma samples and test the hypothesis that dietary will influence obesity and impair the skeletal muscle protein synthetic response to dietary protein ingestion. The obesity plasma sample was classified into TRF pre and post-intervention groups, and CR pre and post-intervention groups according to the feeding type and intervention. This method is expected to have good repeatability and selectivity on the clinical samples and identify the significantly different lipids in a different types of samples so that looking for potential biomarkers between them. To achieve this aim, the objectives of this chapter are:

- To evaluate the method repeatability by checking the RSD of peak area and retention time for each lipid and compare the result with the FDA requirements.
- To use univariate and multivariate methods to analyze the identified lipids and compare the relative concentration between different sample types to find the significantly different lipids.

5.2. Materials and Methods

5.2.1. Materials and Apparatus

5.2.1.1. Materials and Chemicals

A Milli-Q water purification system (Millipore, MA, US) was used in the preparation of deionized water (18.2 MΩ). Acetonitrile, chloroform, ethyl acetate and hexane (HPLC grade) were purchased from Fisher Scientific (Loughborough, UK). Methanol (LC-MS grade) and ammonium acetate were purchased from Sigma-Aldrich (Darmstadt, Germany). Isopropanol (LC-MS grade) was purchased from Acros Organics (New Jersey, USA). BHT (3, 5-di-tert-butyl-4-hydroxytoluene) was purchased from Supelco (PA, USA), and human plasma (donated from Queens Medical Centre Nottingham blood bank). SPLASH® Lipidomix® Mass Spec Standard was purchased from Avanti (Darmstadt, Germany). Clinical feeding study plasma samples were collected from Queens Medical Centre, Nottingham.

5.2.1.2. Apparatus

-80℃ freezer, Fisherbrand™ Wizard™ Infrared Vortex Mixer (Fisher Scientific, UK), Orbit™ Digital Microtube and Microplate Shakers (Labnet, USA), Harrier 18/80" Centrifuge rotor, Jouan Centrifugal Evaporator, Biostage SPE manifold, Techne Dri-Block heater (DB-3B), pipettes and tips, LC-MS vial with inserts and caps, Oasis SPE cartridge (Waters, UK), glass tube, Eppendorf, Accela liquid chromatography coupled with Orbitrap Quadrupole-Exactive mass spectrometer (Thermo Fisher Scientific, Hemel Hempstead, UK), ACE Excel 3 SuperC18 Column(100×2.1mm id), and guard column were purchased from Advanced Chromatography Technologies.

5.2.2. Sample preparation

Clinical samples were collected from Professor Kostas Tsintzas's group and treated at the Queen's Medical Centre (QMC) within the UK NHS Trust centre. This study was approved by the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (Ref. No. 19–1705) and performed at the David Greenfield Human Physiology Unit, University of Nottingham. It was registered at clinicaltrials.gov as NCT03969745 and met the regulations outlined in the Declaration of Helsinki. Informed and written consent was obtained from all individuals before enrolment in the study. A CONSORT flow diagram outlining the study protocol is displayed in Figure 5.1.

This study was performed on 16 healthy young men [age 23 ± 1 year; BMI (kg-m-2) 24.0 ± 0.6; mean ± SEM]. Eligibility was assessed at a medical screening, which included anthropometric measurements,

blood pressure assessment, 12-lead ECG, and blood samples for routine screening. Inclusion criteria included men between 18 and 35 years of age recruited from universities and the general public, with a BMI between 18 and 27 and a moderate activity level (PAL) between 1.6 and 1.99 (PAL = total energy expenditure/basal metabolic rate). Exclusion criteria included any metabolic, endocrine, or cardiovascular health condition; use of prescription medications that may affect cardiovascular and/or metabolic function; smoking; regular heavy alcohol consumption; irregular breakfast consumption (<5 days/week); and an Eating Attitudes Test-26 score >20. All participants underwent a separate medical screening, which included completing a health and physical exercise questionnaire, measuring 12 main electrocardiograms and blood pressure, and drawing blood samples for routine screening. Individuals with cardiovascular disease, diabetes, musculoskeletal disease, smoking and excessive drinking (>28 units per week) were excluded.

Acute feeding study subjects (or time restricted feeding, TRF) and short-term study subjects (daily caloric restriction, CR) were included in the study, and the detail of the subjects were shown below. After surgery, the samples were snap frozen and stored at –80°C within 10 min. Clinical and pathological characteristics of patients are described in Table 5.1. Sixteen healthy males (aged 23 ± 1 y; BMI 24.0 ± 0.6 kg·m−2) were assigned to 2 groups that underwent either 2 weeks of eTRF (n = 8) or control/caloric restriction (CON:CR; n = 8) diet. The eTRF diet was consumed ad libitum and the intervention was conducted before the CON:CR, in which the diet was provided to match the reduction in EI and body weight observed in eTRF. During eTRF, daily EI was restricted to between 08:00 and 16:00, which prolonged the overnight fast by ∼5 h. The metabolic responses to a carbohydrate/protein drink were assessed pre- and post-interventions following a 12-h overnight fast.

Table 5.1. Experimental design for TRF and CR study.

Stage	Time-restricted feeding (TRF study)	Daily caloric restriction (CR study)
pre-intervention	8 samples	8 samples
post-intervention	8 samples	8 samples

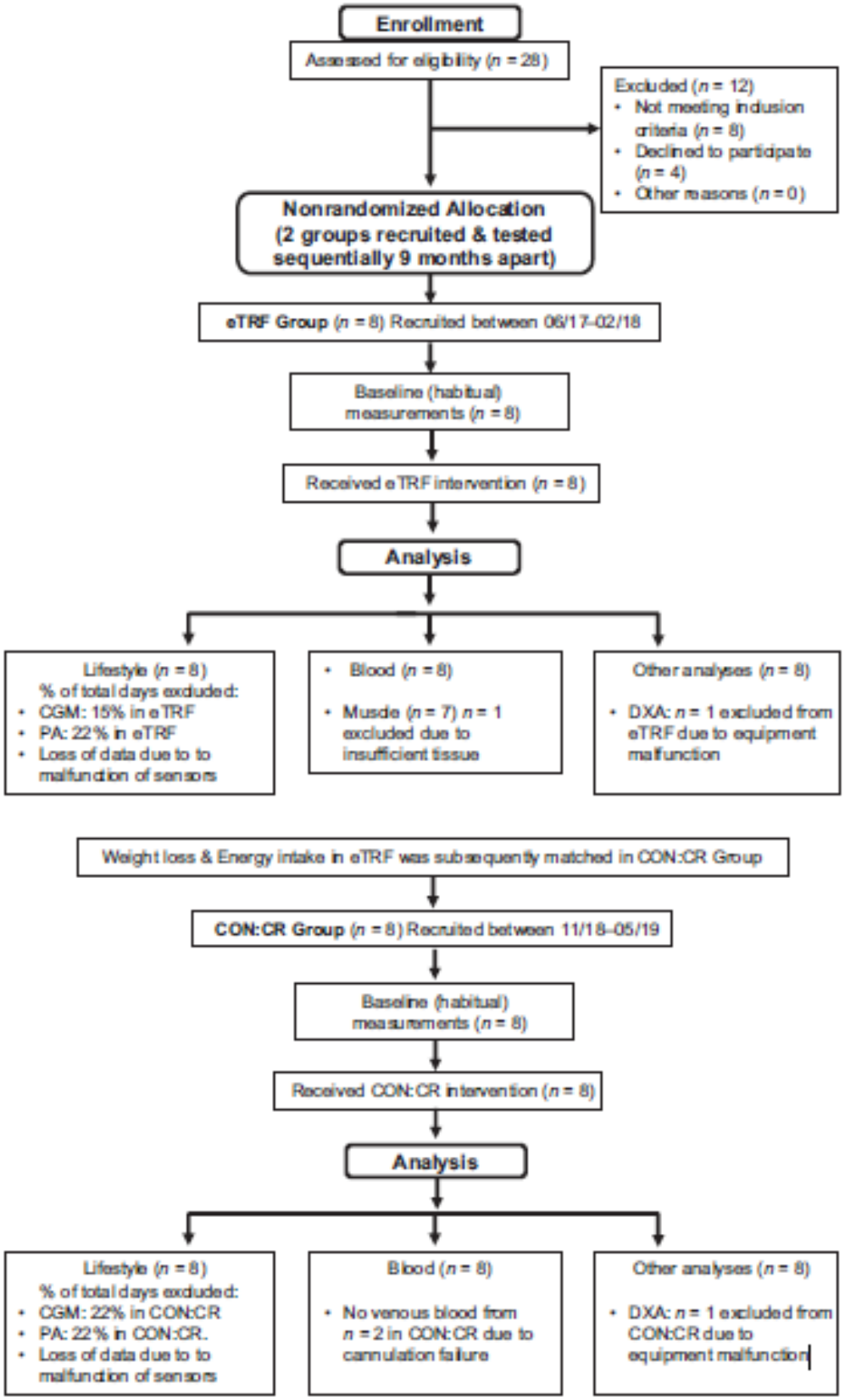


Figure 5.1. *CONSORT flow diagram of study protocol* [286]. *CGM, continuous glucose monitoring; CON:CR, control/caloric restriction; DXA, dual-energy X-ray absorptiometry; eTRF, early time-restricted feeding; PA, physical activity.*

5.2.3. Sample extraction

Clinical samples, plasma and quality control were extracted using the Folch method. The sample extraction protocol was shown in Figure 5.2. In the plasma extraction,50 µl of plasma (sample) or water (regent blank) was added into 500 µl of ice-cold (-20℃) extraction solvent mixture. The extraction solvent mixture was prepared by mixing organic solvent chloroform and methanol (2:1), and 10 µl SPLASH internal standard was added into 490 µl of the organic solvent mixture to get the extraction solvent mixture. After 10 min vortex, 500 µl of water was added to the mixture. The mixture was vortexed again for 10 min and centrifuged (13000g, 4℃) for 10 min. Afterwards, the sample was separated into an upper aqueous phase and a lower organic phase, as well as a protein layer in the middle. The lower organic phase was collected and evaporated by a centrifugal evaporator. Then the sample was reconstituted with 100 µl of isopropanol and transferred into an LC-MS glass vial. The quality control (QC) was prepared by mixing all the plasma extracts and well vortexed.

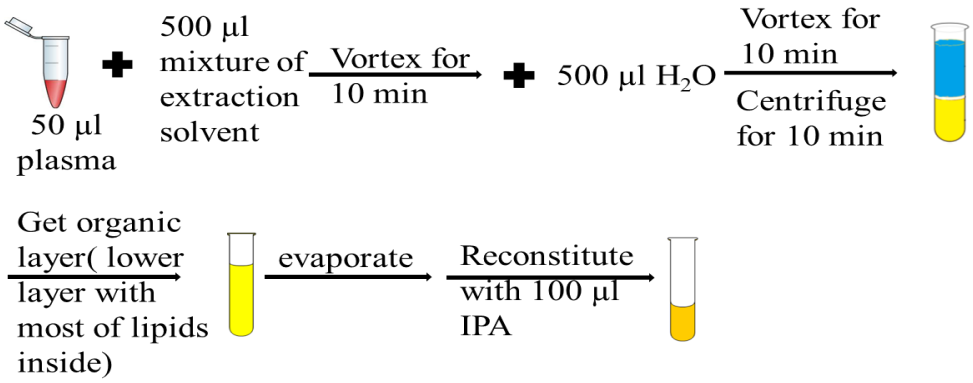


Figure 5.2. *The schematic of the plasma extraction method. Plasma was extracted with a solvent mixture, and after vortex and centrifuge, the organic phase was collected, evaporated and reconstituted with isopropanol.*

5.2.4. LC-MS analysis

LC–MS lipidomics analysis was conducted on an Accela high-speed liquid chromatography coupled to Quadrupole- Exactive mass spectrometry (Thermo Fisher Scientific, Hemel Hempstead, UK). LC–MS analysis was maintained at a temperature of 40 °C and a flow rate of 300 µL/min on a 10 cm C18 column. The mobile phase consisted of (A) 60% of H2O, 40% of acetonitrile and 0.1% of ammonium acetate and (B) 80% of isopropanol, 10% of methanol, 10% of H2O and 0.1% of ammonium acetate. A starting percentage of 50 % of mobile phase B was chosen and increased to 100% of B at 5 min. After keeping at 100 % of B until 13.5 min, the gradient drops back to 50 % of B at 14 min and is maintained at the same gradient for 1 min. The injection volume was 5 µL. All study samples and QC samples were analysed in a single continuous analytical run. Data were acquired simultaneously in full scan ion mode (m/z 100–1500, resolution 25,000) in both positive and negative electrospray ionisation modes. A list of lipids with absolute mass, formula, polarity, adduct type and name that were pre-defined from human

plasma was loaded to the MS method (see appendix Table A). The capillary temperature and heater temperature were maintained at 350 and 300 °C respectively in both positive and negative modes. The maximum scan injects time and micro scans were 100 s and 1 s respectively. MS was performed in simultaneous ESI (+) and ESI− full-scan modes with spray voltages of 4.5 (ESI+) and 3.5 kV (ESI−) and capillary voltages of 40 (ESI+) and −30V (ESI−).

The LC-MS sequence was shown in Table 5.2. First, The LC-MS run started with three blank samples, 100 µL of isopropanol was used as a blank sample as the QC and plasma sample was reconstituted with isopropanol. Second, one reagent blank sample without internal standard was continued, this sample was prepared by replacing the plasma with water and following the sample extraction procedure without adding SPLASH internal standard (replaced with mixed organic solvent). Third, three reagent blank samples were conducted, and they were prepared by replacing the plasma with water and following the sample extraction procedure written in section 3.2.2. Forth,6 injections of pooled QC were analysed, and they were used for equilibrating the instrument. Fifth, 6 plasma extracts, 4 pooled QC and 32 clinical sample extracts were conducted, and the plasma sample and QC sample were inserted into the list of clinical extracts to test the instrument stability and CV. Sixth, 6 replicates of pooled QC were analysed to test the instrument stability. Finally, one blank sample (isopropanol) was used to wash the column and instrument.

Table 5.2. The LC-MS run in the obesity application

Sample type	Number	Note
Blank	3	Isopropanol
Blank_no_IS	1	Extracts without plasma and IS
Regant Blank	3	Extracts without plasma
PooledQC	6	For equilibration
plasma_1	1	For CV check
1A	1	TRF pre-intervention
1B	1	TRF post-intervention
2A	1	TRF pre-intervention
2B	1	TRF post-intervention
3A	1	TRF pre-intervention
3B	1	TRF post-intervention
PooledQC_1	1	Stability and CV check
plasma_2	1	CV check
4A	1	TRF pre-intervention
4B	1	TRF post-intervention
5A	1	TRF pre-intervention
5B	1	TRF post-intervention
6A	1	TRF pre-intervention
6B	1	TRF post-intervention

PooledQC_2	1	Stability and CV check
plasma_3	1	CV check
7A	1	TRF pre-intervention
7B	1	TRF post-intervention
8A	1	TRF pre-intervention
8B	1	TRF post-intervention
9A	1	CR pre-intervention
9B	1	CR post-intervention
PooledQC_3	1	Stability and CV check
plasma_4	1	CV check
10A	1	CR pre-intervention
10B	1	CR post-intervention
11A	1	CR pre-intervention
11B	1	CR post-intervention
12A	1	CR pre-intervention
12B	1	CR post-intervention
PooledQC_4	1	Stability and CV check
plasma_5	1	CV check
13A	1	CR pre-intervention
13B	1	CR post-intervention
14A	1	CR pre-intervention
14B	1	CR post-intervention
15A	1	CR pre-intervention
15B	1	CR pos-intervention
16A	1	CR pre-intervention
16B	1	CR post-intervention
plasma_6		CV check
PooledQC_(5-10)	6	For RSD check (QC)
Blank	1	To wash the column and instrument

5.2.5. Data analysis

LC–MS raw data from the analysis of extracts of study and control samples were acquired with Xcalibur v2.1 software (Thermo Scientific, Hemel Hempstead UK). Chromatographic raw data contain a 3D data set consisting of m/z, retention time and ion signal intensity. These data were processed with LipidSearch (v5.1) to align the replicates and control results and get the extracted lipid formula. The detail of the LipidSearch parameter was shown in Table 2.6 and Table 2.7. The identified lipid list from LipidSearch with allocated internal standards was processed by Tracefinder (Thermo Scientific, Hemel Hempstead UK) following the official protocols.

The quality of the lipidomic analysis was assessed by calculating percentage RSD values (normalization). For multivariate analysis, PCA and OPLS-DA plots were constructed to compare pre and post-intervention for each study or compare the two studies with the same intervention type (pre or

post). VIP score analysis was done in every OPLS-DA plot, choosing lipids with a score of 1.0 or above and Q2 <0.5 only. Univariate analysis was performed on the same grouped data as used in multivariate analysis, and the parameters for T-test and FDR statistical correction were chosen (p-value of less than 0.05, FDR rate 5%). These lists were then compared to their corresponding multivariate VIP score lists to produce final lists of statistically significant lipids. The fold changes obtained from the univariate analysis were used to decide whether a potential biomarker was elevated or decreased during the "disease" condition.

5.3. Results and Discussion

5.3.1. Identified lipids and CV in patients

LipidSearch and Tracefinder results revealed 273 putatively identified lipids in different study groups. The identified lipids in a clinical sample were summarized in Table 5.3, and lipid classifications, the number of lipids in each class and the average CV for each lipid class were shown as well. As the table shows, most of the lipid classes have a mean CV of around 30% (including the outliers that have missing peaks in the replicates and have CV higher than 100%), which is considered as qualified for clinical sample (in FDA requirement, clinical sample CV should be lower than 30%, at least 80% of the analytes meet the criteria). The whole list of identified lipids and their peak area CV in each group was shown in Appendix Table I, and the CV in TRF, CR, QC and plasma also meet the criteria, thus the result is reproducible and can be analysed in other analyses methods. The appendix table result was visualized with a box plot in Figure 5.3.

Table 5.3. The identified lipids summary in the obesity sample

Lipid Class	Count of Lipid Class	Average CV/RSD
Ceramides	23	37.34%
Cholesterol Esters	14	32.86%
Cholesterols	1	43.61%
Coenzyme	18	23.84%
Diacylglycerols	9	36.02%
Eicosanoids	2	35.51%
Fatty Acids	27	35.01%
Fatty Esters	1	34.54%
Glycerophosphocholines	60	31.31%
Glycerophosphoethanolamines	19	37.85%
Glycerophosphoglycerols	9	39.37%
Glycerophosphoinositols	19	39.54%
Glycerophosphoserines	7	30.83%
Monoacylglycero-phosphocholines	11	35.33%
Monoacylglycerophospho-ethanolamines	8	34.68%
Sphingoid bases	4	48.50%

Sphingomyelins	8	42.07%
Triradylglycerols	33	34.99%
Grand Total	273	35.28%

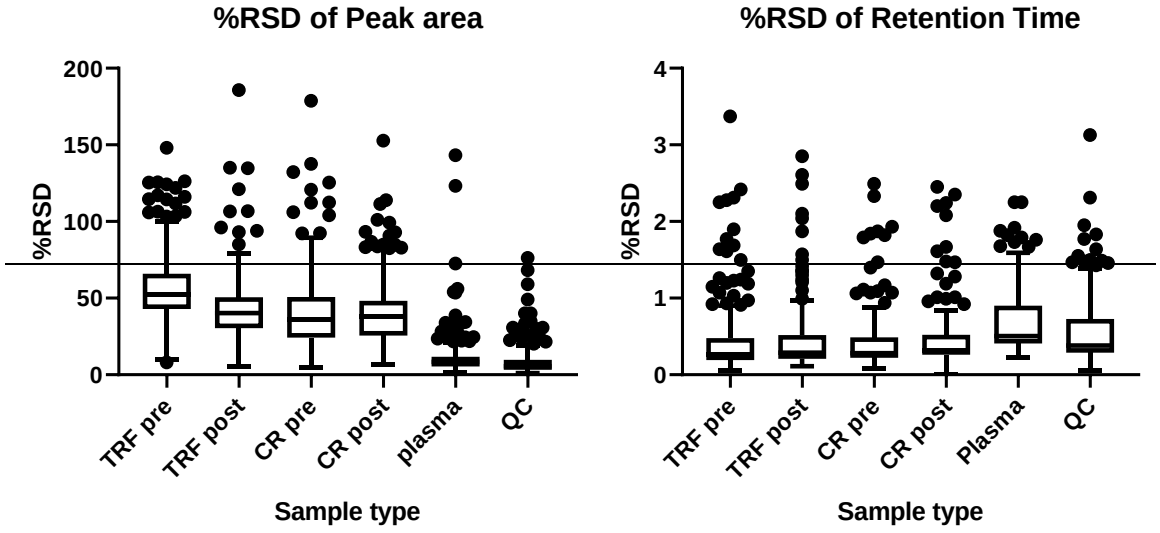


Figure 5.3. *The box plot of RSD for each sample group. The left figure shows the RSD of the peak area, and the right figure shows the RSD of retention time.*

5.3.2. LC-MS analytical performance of the sample

Analytical runs of clinical samples in both studies (TRF and CR) have generated clear spectra with distinctive metabolite peaks, high TIC values ($\geqslant$ E9) and no background noise or carryover effects. The spectra of QC and plasma samples also showed clear peaks with high TIC values, demonstrating good analytical stability of sample metabolites and reproducibility. The shape of the spectra peaks of QC samples was found to be similar to the clinical sample spectra, with some peaks identical, showing that QC samples contained the common metabolite peaks as the actual samples.

5.3.3. Univariate analysis – t-test

T-test and false discovery rate correction were used for univariate analysis. The whole t-test result table was attached in Appendix Table J (pre-TRF vs. pre-CR) and Table K (post-TRF vs. post-CR). The commonly used t-test correction method, BF correction and FDR correction result were calculated and summarized in the table as well. As the table shows, although there are many lipids that show significant differences after the student t-test, there are no lipids qualified after the Bonferroni correction or FDR correction. There are some significant differences between pre and post-intervention, and the volcano plot was used to visualize the t-test result in the study (TRF pre vs. Post-intervention, CR pre vs. Post-intervention) and the result was shown in Figure 5.4 and Figure 5.5 below. To qualify as a significant

difference, the t-test result should fulfil fold change >2 and q value <0.05 (FDR corrected t-test result), and the qualified result shown in the upper left and upper right in the volcano plot. As the figure shows, in the TRF study, there are several significantly different lipids identified and labelled between pre and post-intervention, but there are no significantly different lipids identified in the CR study.

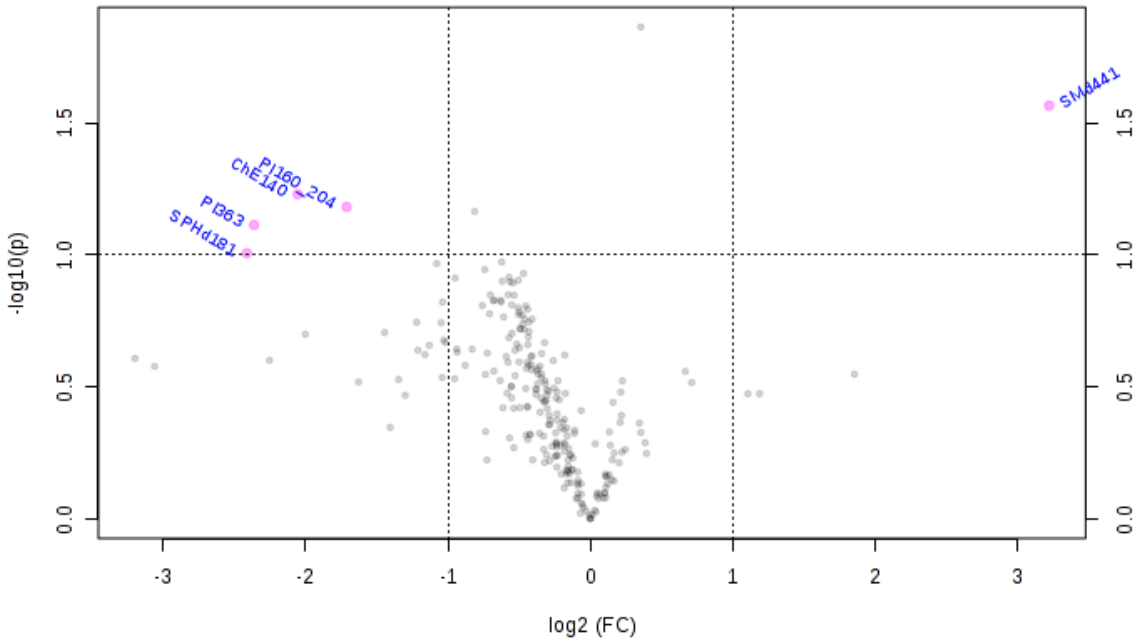


Figure 5.4. *The volcano plot for t-test result (TRF pre vs post-intervention). The X-axis represents the log2 value of fold change, and the Y-axis represents the –log10 value of the t-test result with FDR correction. The area with fold change >2 and q value (corrected t-test result <0.05) was considered a significant difference.*

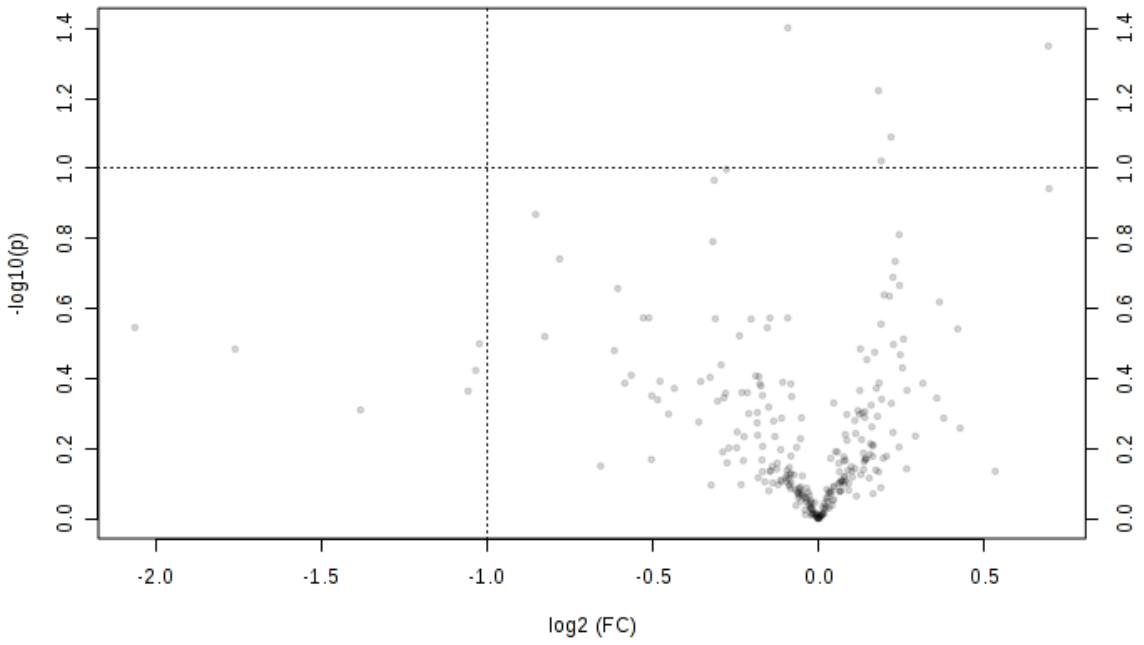


Figure 5.5. *The volcano plot for t-test result (CR pre vs post-intervention). The X-axis represents the log2 value of fold change, and the Y-axis represents the –log10 value of the t-test result with FDR correction. The area with fold change >2 and q value (corrected t-test result <0.05) was considered a significant difference.*

5.3.4. Multivariate analysis -- PCA result

Principal Component Analysis (PCA) graph was used for multivariate analysis, and different sample types (including TRF pre and post-intervention groups, CR pre and post-intervention groups, QC samples, plasma samples, blank without IS and blank samples) were used to analyse the relationship between them. The data points that are closely located in the PCA graph mean the relationship between them is closer and data (identified lipids and their concentration) are similar. As Figure 5.6 shows, blank and plasma sample groups were clustered together, which represents good reproducibility. The QC samples also show a good cluster, except a QC sample (purple) has a big difference compared with other QC data points. The plasma group points are located outside of the ellipse meaning it has a big difference compared with other groups, and this could be explained by the different subjects and missing plasma lipids in the sample. The TRF and CR points are roughly clustered when compared with other groups, but the data points in these four groups are not clustered closely as the groups mentioned above. This result illustrates that the result of the clinical data (TRF pre and post-intervention groups, CR pre and post-intervention groups) has a big difference compared with healthy people (plasma) and blank samples, but the difference in the clinical samples is also quite high, and this result is also supported by the OPLS-DA result in the next section.

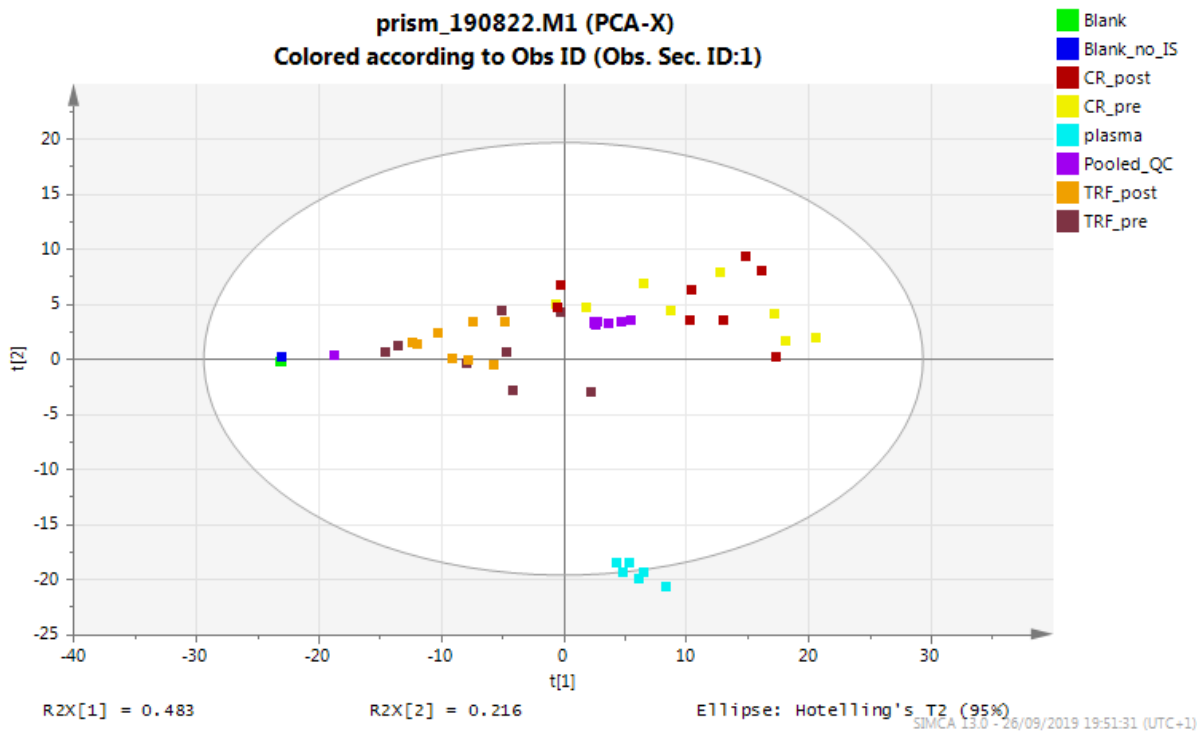


Figure 5.6. *The PCA result for TRF pre and post-intervention groups, CR pre and post-intervention groups, pooled QC, blank, blank without internal standard and plasma samples.*

5.3.5. Multivariate analysis -- OPLS-DA result

The OPLS-DA was also used for multivariate analysis in this study to investigate the relationship between different clinical groups. Similar to the PCA plot, the OPLS-DA result could also be visualized through the data point inter and intra groups. As Figure 5.8 and Figure 5.9 show, although different sample group shows a grouped tendency, the data points in both groups are scattered, which means a high variance in the group. This could be the reason why no lipids in the clinical sample show a significant difference in the t-test result since the variance in the group could be higher than the inter-group difference. The Q2 and VIP value could also be used for OPLS-DA result analysis. The Q2 value represents the prediction ability of the OPLS-DA model, and Q2 > 0.5 was considered a predictive model. The Q2 value of the OPLS-DA result is negative, which means these two groups are not qualified for a different check. The VIP score (Variable important in projection) can be used to measure the impact of different metabolite accumulation differences on the classification and discrimination of samples in each group, and VIP $\geqslant$ 1 is the common screening criteria for different metabolites. The VIP result of all of the lipids was shown in Appendix Table G, and there are 87 lipids with a VIP score $\geqslant$ 1. Thus, the OPLS-DA result shows, the difference between the two groups is not clear, and the significant difference in lipids is not reliable as the Q2 value is below 0.5.

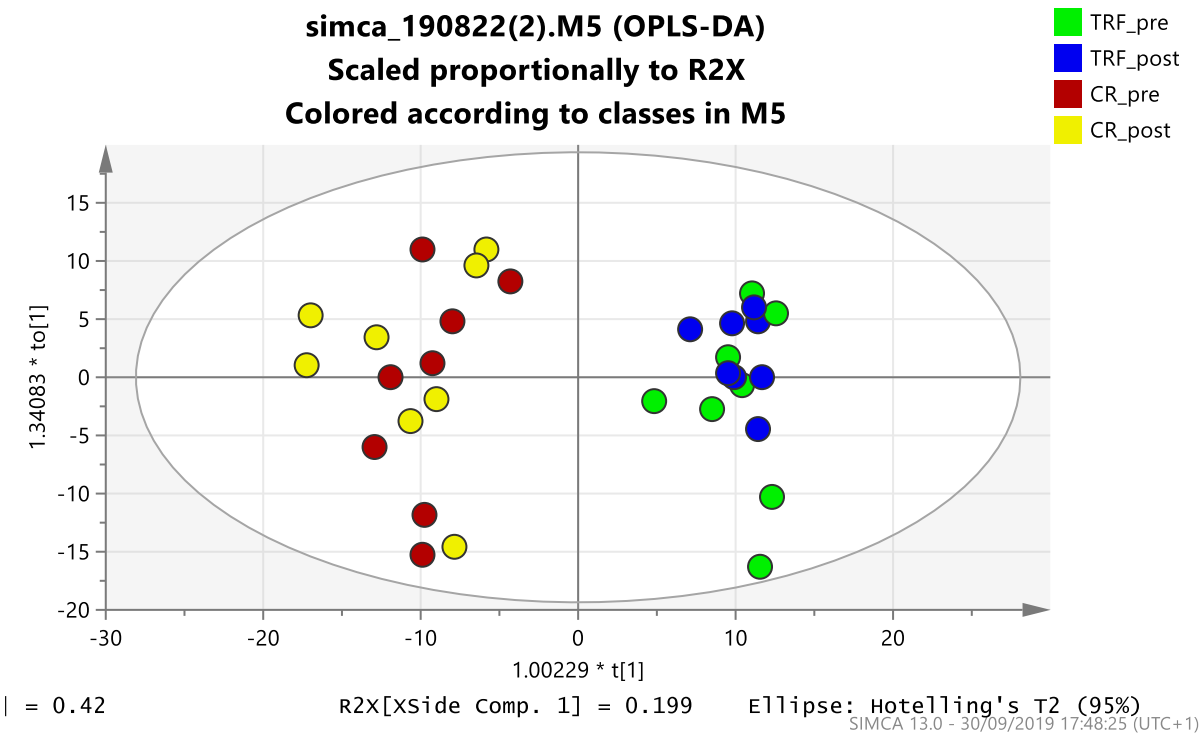


Figure 5.7. *The OPLS-DA result for TRF pre and post-intervention groups, CR pre and post-intervention groups samples. The green spot represents 8 patient data in the TRF pre-intervention group, the blue data points represent 8 patient data in the TRF post-intervention group. The red spot represents 8 patient data in the CR pre-intervention group, the yellow data points represent 8 patient data in the CR post-intervention group.*

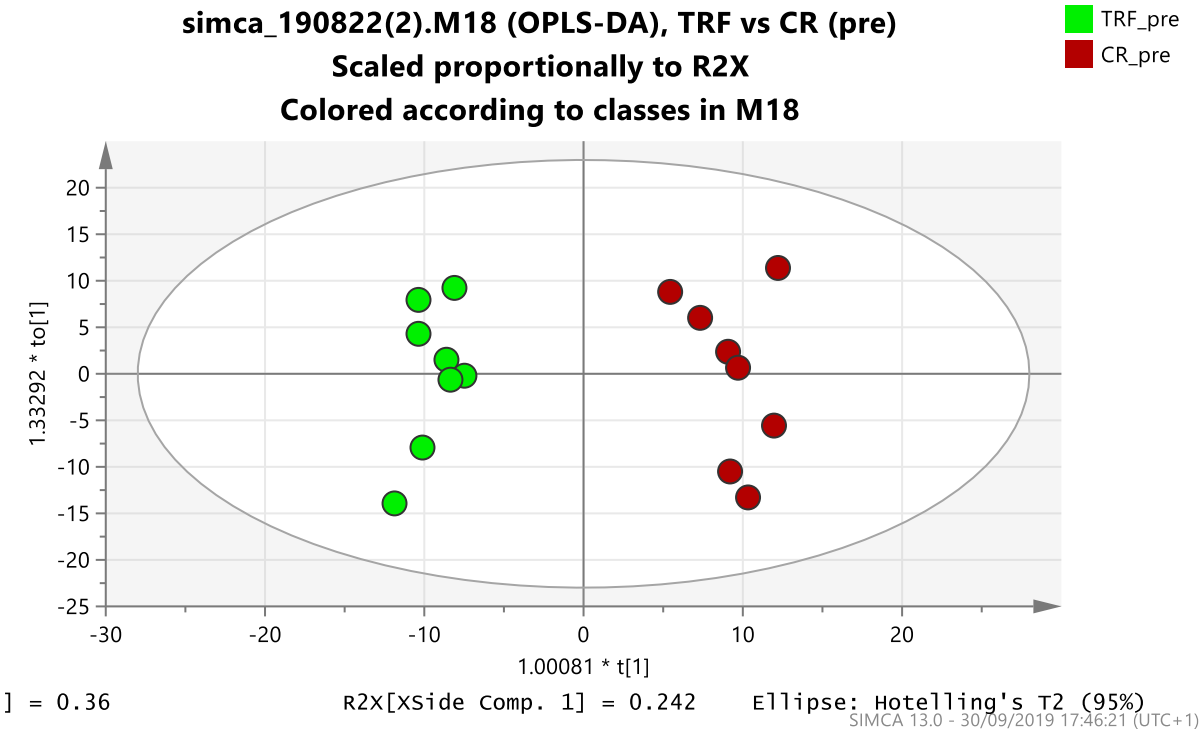


Figure 5.8. *The OPLS-DA result for TRF and CR pre-intervention groups. The green spot represents 8 patient data in the TRF pre-intervention group, the red spot represents 8 patient data in the CR pre-intervention group.*

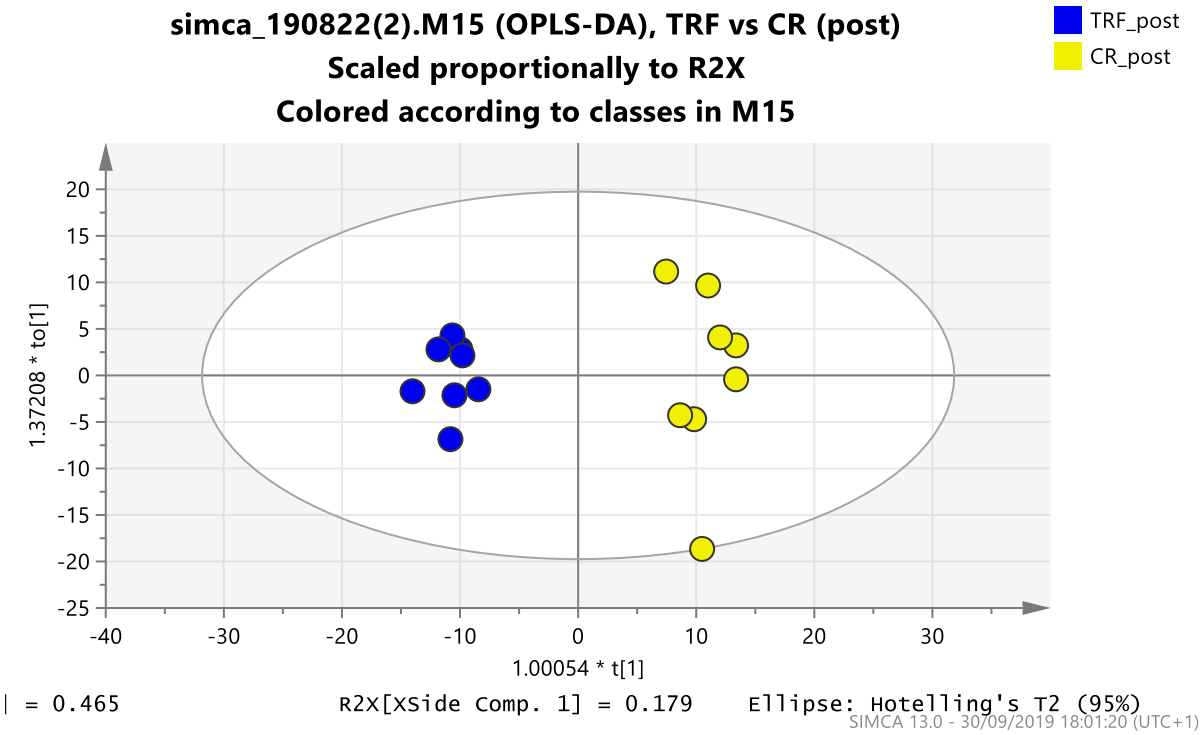


Figure 5.9. *The OPLS-DA result for TRF and CR post-intervention groups. The blue data points represent 8 patient data in the TRF post-intervention group, and the yellow data points represent 8 patient data in the CR post-intervention group.*

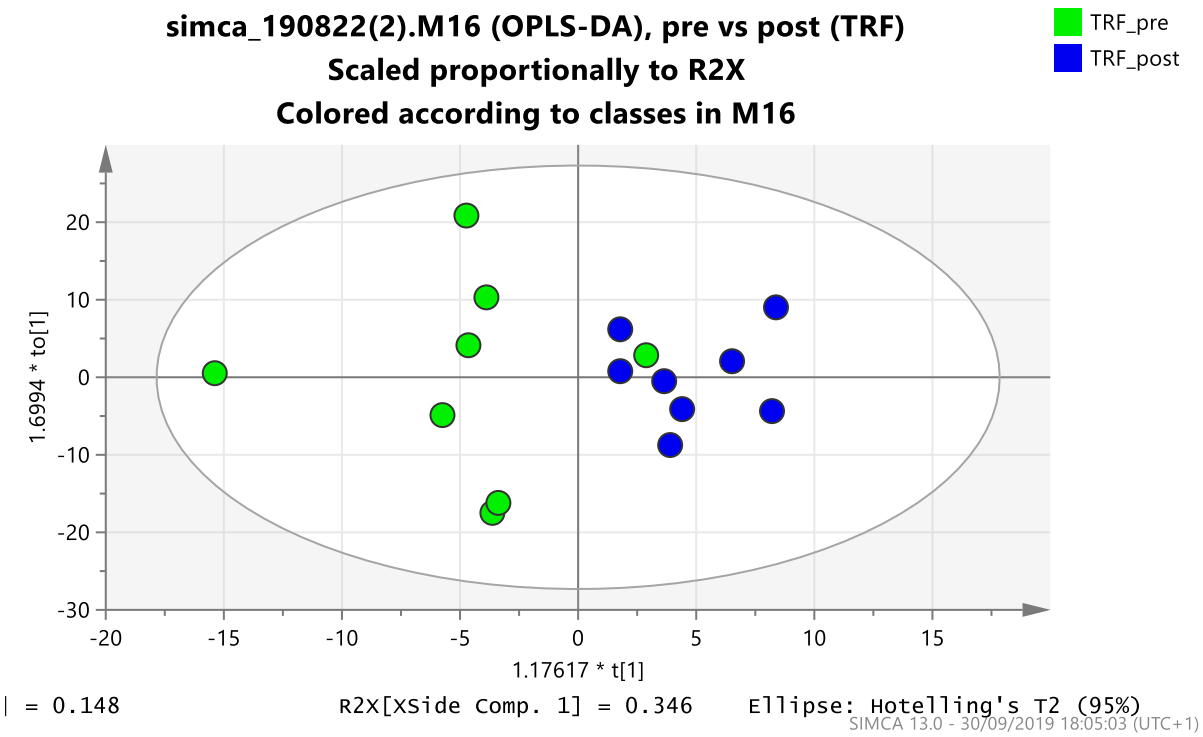


Figure 5.10. *The OPLS-DA result for TRF pre and post-intervention groups. The green spot represents 8 patient data in the TRF pre-intervention group, the blue data points represent 8 patient data in the TRF post-intervention group.*

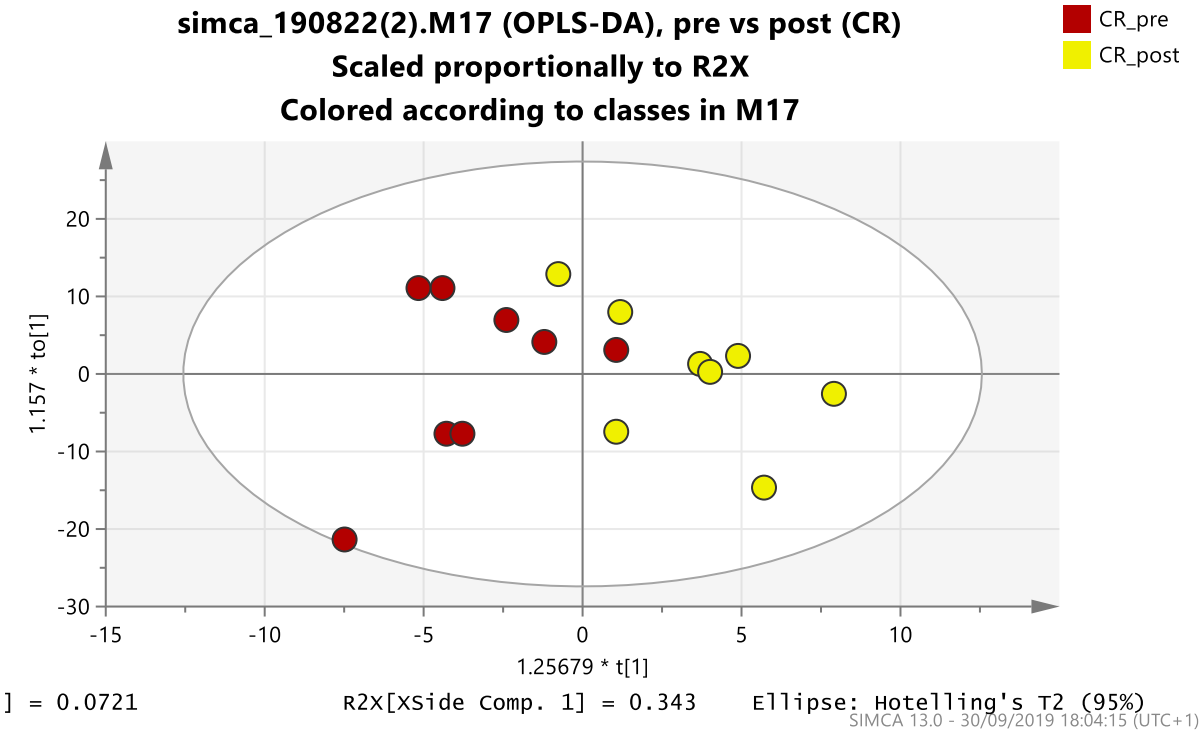


Figure 5.11. *The OPLS-DA result for CR pre and post-intervention groups samples. The red spot represents 8 patient data in the CR pre-intervention group, the yellow data points represent 8 patient data in the CR post-intervention group.*

5.3.6. Heatmap

The heatmap is another visualization tool to represent lipid concentration in each sample by a different colour. As Figure 5.14 and Figure 5.15 show, the lipid relative concentration in all the samples between TRF pre-intervention and TRF post-intervention, CR pre-intervention and CR post-intervention were compared using different colours. A different group of samples were classified by a red and green bar on the top of the figure, and the concentration of the lipids was signified from deep red to deep blue (red means lipid concentration is higher, while blue means the concentration is lower). As the figure shows, the concentration of the same lipid could be highly variable in different samples, for example in TRF pre 4 and TRF pre 6, there are lots of highly concentrated lipids compared to other samples. However, another phenomenon could be seen is the big variance in the same group, which means the lipid relative concentration variance was also high in the same group, and this is the reason why pre and post-samples were considered closely related on the top of the figure (colour bar) and samples in the same group are not classified together. Thus, the heatmap illustrates the big difference in lipid concentration in different samples, and they are useful data to find potential biomarkers between each sample group, but it also shows the possibility of the single sample variation and big variance in the same patient group.

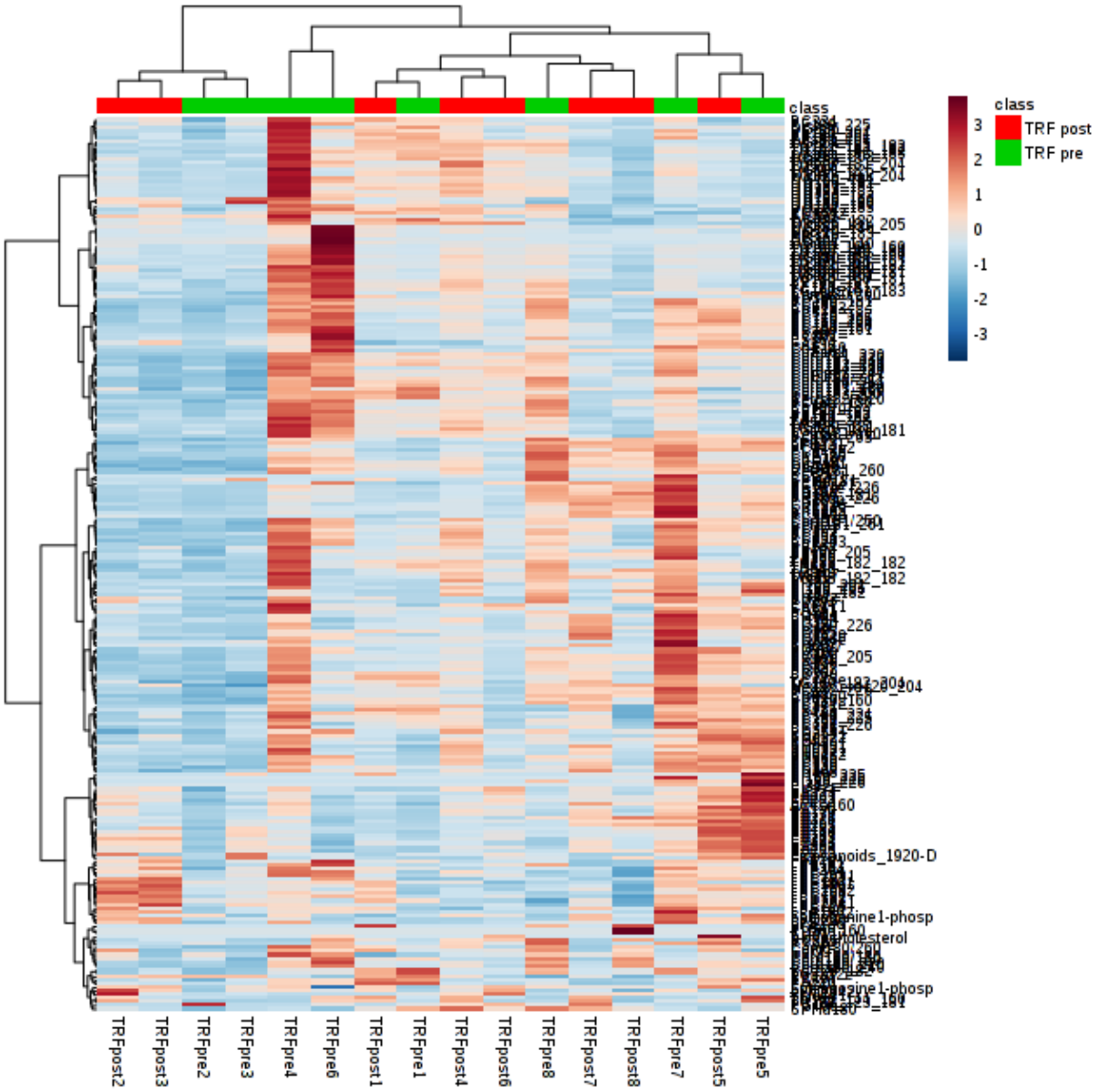


Figure 5.12. *The heatmap result of TRF pre-intervention and TRF post-intervention samples. The green bar represents the TRF pre-intervention group, and the red bar represents the TRF post-intervention group. Different colour embodies different concentrations of lipids, and blue means lower concentration, red means higher concentration.*

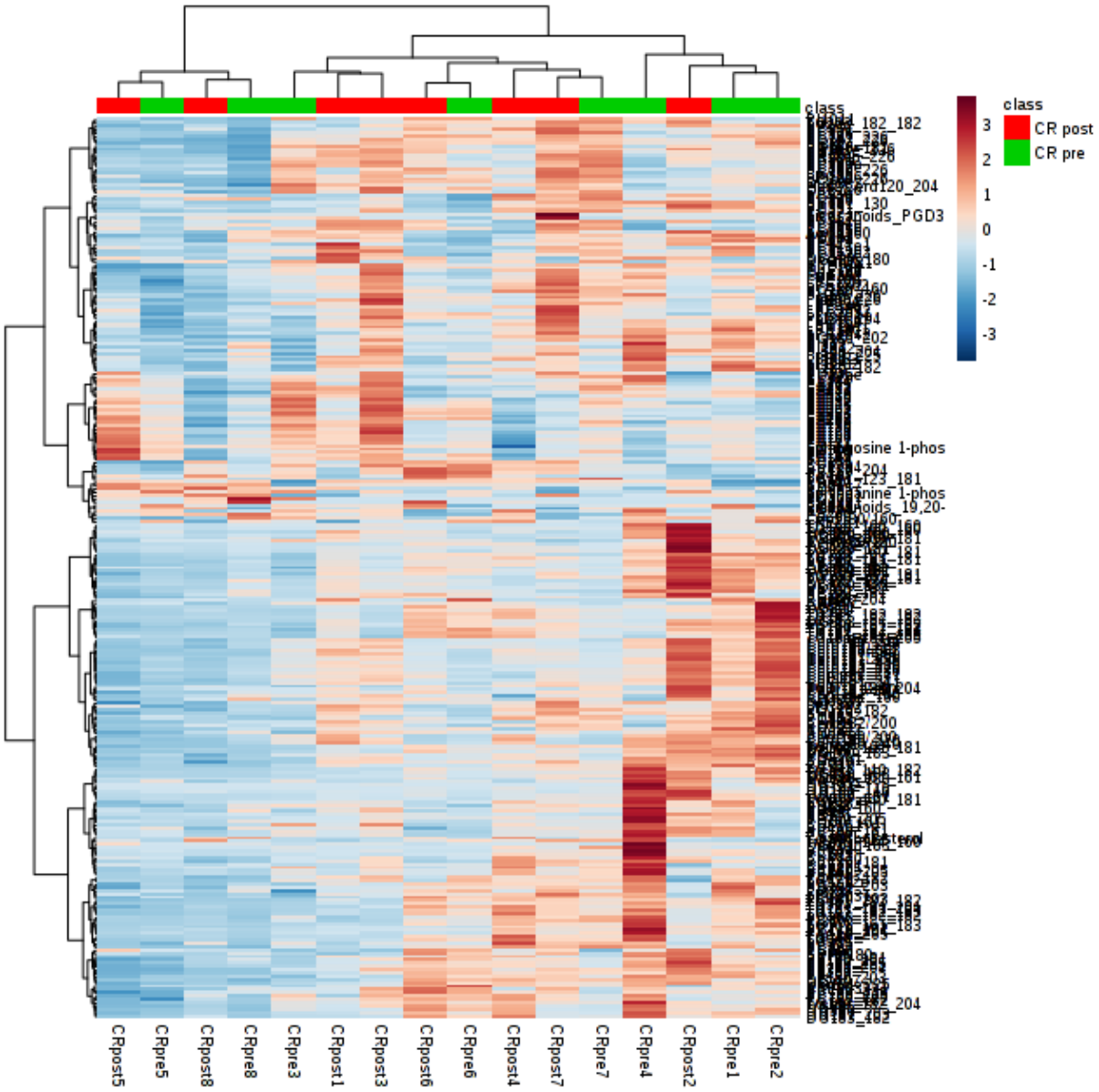


Figure 5.13. *The heatmap result of CR pre-intervention and CR post-intervention samples. The green bar represents the CR pre-intervention group, and the red bar represents the CR post-intervention group. Different colour embodies different concentrations of lipids, and blue means lower concentration, red means higher concentration.*

5.4. Conclusion

In this chapter, the developed LC-MS method was applied to the feeding study clinical samples, several validation parameters were reviewed for all identified lipids, and the result proves the developed method is in an acceptable range of stability and repeatability, as well as selectivity. The SPLASH internal standard was added to the method to normalise the lipids by class. Quality control was added to equilibrate the instrument and check the LC-MS stability. The 6 plasma extraction samples confirm the stability of the extraction method, freeze-thaw, storage condition, human impact and other factors are

stable and meet the FDA criteria. For the lipids with a high-level CV, different reasons were found, and outliers or missing peaks are the main problems that cause the high CV. Except this, most of the lipids have a clear peak and reasonable CV.

In the feeding study clinical application result, there are putatively 273 lipids found in the sample, the big amount of lipids with good identifications (class 1) and relative concentration information presents many useful data in the feeding study samples. In the univariate test, t-test and FDR correction were calculated, although there are many lipids shows a significant difference after the student t-test(pre-TRF vs. pre-CR and post-TRF vs. post-CR), there are no lipids qualified after Bonferroni correction or FDR correction. There is some significant difference between pre and post-intervention, and the volcano plot was used to visualize the t-test result in the study (TRF pre vs. Post-intervention, CR pre vs. Post-intervention). In the multivariate test, PCA and OPLS-DA were used to check the relationship between inter and intra-sample groups. The PCA result shows blank, QC and plasma sample groups were clustered together, which represents good reproducibility. In the clinical sample group, identified lipids are roughly clustered but are not compact as in the QC sample group, which represents a big variance in the group. The OPLS-DA result shows the identified lipids also have a big variance in TRF pre vs. Post-intervention and CR pre vs. Post-intervention group and no lipids are qualified as significantly different lipids ($Q2 > 0.5$ and $VIP \geqslant 1$). The heatmap graph visualizes the relative concentration of all the identified lipids in the pre-TRF vs. pre-CR and post-TRF vs. post-CR groups, and the huge difference in the concentration of some lipids in different samples proves that there are potential biomarkers between these groups.

In summary, the method stability, repeatability and other parameters all meet the clinical analysis standard, and the normalisation could help the variation in general. The clinical application identified hundreds of lipids for analysis, and it shows a significant difference between time-restricted feeding and caloric restriction samples. Between time-restricted feeding and caloric restriction sample, there are some lipids that show a huge difference between the group, however, they are not considered as significantly different lipids as the variance in the group is too high. Thus, the method is considered a proper method for relatively quantifying lipids on brain tumour samples, but there are more efforts needed to find potential biomarkers in it.

Chapter Six

Conclusions and future work

6.1. Summary

The role of lipids has been traditionally associated with energy storage, structural components of cells and hormonal systems [27]. The information is strengthened by a number of human diseases that are linked with abnormal lipid metabolism including obesity, atherosclerosis, diabetes and fatty liver disease[12], [36], [287]. Therefore, the study of changes in lipid metabolites of specific molecular species biological samples will be useful for the discovery of bioactive lipids related to metabolic dysfunctions or diseases. Quantitative and qualitative analysis of lipids are both essential for the identification of specific biomarkers, thus combining the targeted and untargeted lipid analysis will be a good option for the method development in this experiment [288].

In this thesis, a proposed LC-MS method with normalisation for semi-quantitative (or semi-targeted) lipidomics has been applied in several clinical studies. The main aim of this project was to demonstrate the effectiveness of this LC-MS method in improving the accuracy and quality of lipidomic data for a wide range of polar and non-polar lipids in the human samples and quantify the concentration change of pre-defined lipids which have been reported previously. Great attention to detail was applied in the development of the method, validation using SPLASH lipid internal standard, and application of the method to a range of clinical human blood and plasma samples with different diseases to obtain highly accurate data on potential biomarkers involved in specific metabolic pathways.

According to experimental design, the thesis could be roughly separated into two parts: 1. method development and validation (chapters 2 and 3) and 2. Clinical applications (chapters 4 and 5). Chapter 2 illustrates the concept of developing the LC-MS method and the critical steps to fulfil the aim. Chapter 3 uses a few validation parameters to validate the method using human plasma, to make sure the method meets the requirement. Chapters 4 and 5 use brain tumour blood and feeding study plasma as a clinical sample, and apply the developed LC-MS method to these samples, in order to investigate the lipid change in the study.

6.2. Method development and validation

Liquid chromatography-mass spectrometry (LC-MS)-based lipid profiling can provide a rapid snapshot of disease status and progression from abnormal lipid metabolism by monitoring lipid changes [289], [290]. Therefore, untargeted lipidomics has been widely used in applications to investigate human health and diseases [154], [291]–[293]. However, a key limitation of this untargeted technique is the lack of a reliable lipid identification method for the profiling data and accurate measurement of structurally diverse lipid species in a biological sample. Ambiguous identification and inaccurate quantitative measurement of lipids can make it difficult to discover biological and clinical significance [294]–[296].

Therefore, in the method development part, to achieve unambiguous identification and obtain accurate quantitative lipidomics data, the focus was on developing a semi-targeted method to identify more than 300 lipids from different classes and modifying the method for better lipid coverage, separation and identification. For this purpose, solid-phase extraction was used to build up a lipid library from the plasma sample and has improved the coverage significantly than the liquid-liquid extraction method. Different liquid-liquid extraction methods were compared to get good coverage of different classes of lipids for later clinical application. The liquid chromatography method was developed by modifying gradient, mobile phase proportion, running time and changing columns, and the modified LC method shows a decent separation of closely eluted lipids and isomers. The mass spectrometry condition was developed by choosing the proper parameters for lipid fragmentation and ionization. As a result, the chloroform/methanol (2:1) extraction method (Folch method) was chosen for future extraction work, and 15 min run time on a C18 column (10 cm) was developed and has shown a good quality lipid elution efficiency. The modified method presents a significant increase in identified lipid number compared to the starting LC-MS method, as well as better separation of lipid peaks in similar retention time.

After method development, the next step is to validate the developed method and check the reproducibility and sensitivity of different plasma samples. As the stability and repeatability of the LC-MS method are important to the lipidomic method development and application. Thus, in chapter 3, several validation parameters were reviewed for all identified lipids, and the result proves the developed method is in an acceptable range of stability and repeatability, as well as selectivity. Quality control was added between the injections, to check the LC-MS stability. The 6 plasma extraction samples confirm the stability of the extraction method, freeze-thaw, storage condition, human impact and other factors are stable and meet the FDA criteria. For the lipids with a high-level CV, different reasons were found, and outliers or missing peaks are the main problems that cause the high CV. Except this, most of the lipids have a clear peak and reasonable CV.

The SPLASH internal standard was added into the method and normalise the lipids by class. The ion suppression effect study has demonstrated that there is an ion suppression effect in the LC-MS run and adding internal standards could lessen the influence on the quantification of lipids. The normalisation result shows many lipids' CV decreased after normalisation, but lipids allocating to internal standards from other lipid classes or internal standards themselves have a quite high CV showing the CV increased after normalisation. In summary, the method stability, repeatability and other parameters all meet the clinical analysis standard, and the normalisation could help the variation in general. Thus, the method is considered a proper method for relatively quantifying over three hundred lipids and could be used on clinical plasma samples.

6.3. The clinical applications of the developed LC-MS method

After developing and validating the semi-targeted lipidomics method, several clinical samples were applied to the method to verify the method's efficiency and explore lipid biomarkers. In chapter 4, the developed LC-MS method was applied to the brain tumour blood samples, several validation parameters were reviewed for all identified lipids, and the result proves the developed method is in an acceptable range of stability and repeatability, as well as selectivity. Quality control was added to equilibrate the instrument and check the LC-MS stability. The 6 plasma extraction samples confirm the stability of the extraction method, freeze-thaw, storage condition, human impact and other factors are stable and meet the FDA criteria. For the lipids with a high-level CV, different reasons were found, and outliers or missing peaks are the main problems that cause the high CV. Except this, most of the lipids have a clear peak and reasonable CV. The SPLASH internal standard was added to the method to normalise the lipids by class. The normalisation result shows many lipids' CV decreased after normalisation, but lipids allocating to internal standards from other lipid classes or internal standards themselves have a quite high CV showing the CV increased after normalisation. The result supports the usefulness of adding internal standards in the sample, but the normalization result could be better if adding more internal standards for the lipids.

For the brain tumour clinical application result, there are putatively 260 lipids found in the sample and 241 lipids appeared in both groups, the big amount of lipids with good identifications (class 1) and relative concentration information presents many useful data in the brain tumour samples. In the univariate test, the t-test and its correction were calculated, but the result did not meet the significant difference criteria (fold change > 2 and q value < 0.05). In the multivariate test, PCA and OPLS-DA were used to check the relationship between inter and intra-sample groups. The PCA result shows the identified lipids have a big variance among different groups, but the OPLS-DA result shows the identified lipids also have a big variance in GBM and LGG groups themselves and no lipids are qualified as significantly different lipids ($Q2 > 0.5$ and $VIP \geqslant 1$). The heatmap graph visualizes the relative concentration of all the identified lipids in each sample, and the huge difference in the concentration of some lipids in different samples proves that there are potential biomarkers but negated because of big variance in the GBM and LGG groups.

There are several reasons that cause no significant different in lipids found between GBM and LGG samples. This might be due to the limited number of patients included in the analysis. The ideal experimental design should include spatially resolved samples from a larger number of patients to address the level of inter-tumoral variations which is challenging and difficult to achieve. In addition, the sample preparation method needs to be carefully examined and conducted, to decrease the possibility of adding more variables.

In chapter 5, the developed LC-MS method was applied to the feeding study clinical samples, several validation parameters were reviewed for all identified lipids, and the result proves the developed method is in an acceptable range of stability and repeatability, as well as selectivity. The SPLASH internal standard was added to the method to normalise the lipids by class. Quality control was added to equilibrate the instrument and check the LC-MS stability. The 6 plasma extraction samples confirm the stability of the extraction method, freeze-thaw, storage condition, human impact and other factors are stable and meet the FDA criteria. For the lipids with a high-level CV, different reasons were found, and outliers or missing peaks are the main problems that cause the high CV. Except this, most of the lipids have a clear peak and reasonable CV.

In the feeding study clinical application result, there are putatively 273 lipids found in the sample, the big amount of lipids with good identifications (class 1) and relative concentration information presents many useful data in the feeding study samples. In the univariate test, t-test and FDR correction were calculated, although there are many lipids shows a significant difference after the student t-test(pre-TRF vs. pre-CR and post-TRF vs. post-CR), there are no lipids qualified after Bonferroni correction or FDR correction. There is some significant difference between pre and post-intervention, and the volcano plot was used to visualize the t-test result in the study (TRF pre vs. Post-intervention, CR pre vs. Post-intervention). In the multivariate test, PCA and OPLS-DA were used to check the relationship between inter and intra-sample groups. The PCA result shows blank, QC and plasma sample groups were clustered together, which represents good reproducibility. In the clinical sample group, identified lipids are roughly clustered but are not compact as in the QC sample group, which represents a big variance in the group. The OPLS-DA result shows the identified lipids also have a big variance in TRF pre vs. Post-intervention and CR pre vs. Post-intervention group and no lipids are qualified as significantly different lipids ($Q2 > 0.5$ and $VIP \geqslant 1$). The heatmap graph visualizes the relative concentration of all the identified lipids in pre-TRF vs. pre-CR and post-TRF vs. post-CR groups, and the huge difference in the concentration of some lipids in different samples proves that there are potential biomarkers between these groups.

In summary, the method stability, repeatability and other parameters all meet the clinical analysis standard, and the normalisation could help the variation in general. The clinical application identified hundreds of lipids for analysis, and it shows a significant difference between the patient sample and healthy people or different clinical groups. Between GBM and LGG sample, there are some lipids that show a huge difference between the group, however, they are not considered as significantly different lipids as the variance in the group are too high. In the feeding study sample, this phenomenon also happens between TRF and CR groups, but there are some significantly different lipids found in TRF and CR groups before and after the intervention. Thus, the method is considered a proper method for relatively quantifying lipids on brain tumour samples, but there are more efforts needed to find potential biomarkers in it.

6.4. Future Work

In this thesis, the semi-targeted lipidomics method has been established to achieve accurate identification of more than 300 lipids from different classes and their relative concentration changes between the sample. The SPLASH internal standard has shown some improvement in the normalisation of lipids, but many lipids could not be normalised properly as the lack of structural-related internal standards. Thus, one of the future work goals would be to use 13C internal standards to achieve good coverage of lipids and improve the normalisation efficiency in the experiment. The 13C internal standards can be bought directly from the company, but due to the lipid difference in distinct people and the big amount of lipids comprised in the body, it is expensive to buy hundreds of 13C internal standards to normalise the lipids. A feasible way is generating the internal standards through bacterial or mammalian cells which contain most of the lipids in the human body. For example, 20% U-13C glucose instead of unlabelled glucose was used to culture bacteria, then the bacteria were extracted to collect 13C labelled lipids. As bacterial and mammalian cells incorporate most lipids in humans, hundreds of 13C lipid internal standards could be acquired through this method.

Another future goal is increasing the clinical sample scope to prove the result is validated. In chapter 4, there is a list of lipids that have shown significant differences between LGG and GBM sample groups, but they were not considered as potential biomarkers as the two groups were not well scattered and were cross related. One of the reasons for this phenomenon is the sample number is too small-scale (only 10 samples in each group and 20 samples in total), thus the group difference is not clearly shown, and the result could not be considered as significantly different lipids in this situation. Through increasing the sample scale, there is more related information between the groups shown, and the significantly different lipids could be verified whether they are potential biomarkers.

Furthermore, the developed method could be applied to a wider range of mammalian or human biofluids and tissues. Biofluid samples such as saliva, cerebrospinal fluid and body sweat also contain important metabolites which are significantly changed by diseases, so is worth applying the method to them. Application to a variety of tissues would also be beneficial, such as liver tissue, heart and kidney which are constantly affected by a variety of serious diseases. Thus, applying the method to other clinical samples would be interesting to observe potential lipid biomarkers in these diseases, and can test the method's efficiency. In clinical studies, to fully validate the potential biomarkers, it would be useful to combine the targeted metabolomics approach with LC-MS/MS instrumentation for those clinical samples. That would give more qualitative and quantitative information about the reported biomarkers.

In summary, the semi-targeted lipidomics method for over three hundred lipids has been developed and validated in this thesis, and SPLASH internal standard was added to improve the lipidomic data. Using this method, over two hundred lipids related to disease could be identified with good quality continuously. The method's repeatability and reproducibility were validated through percentage RSD

calculations and multivariate and univariate statistical analysis. As the result shows, the developed method is stable in different applications. This can be of benefit by providing further understanding of the biological processes related to the study in question.

References

[1] J. Dehairs, R. Derua, N. Rueda-Rincon, and J. V. Swinnen, "Lipidomics in drug development," *Drug Discovery Today: Technologies*. 2015, doi: 10.1016/j.ddtec.2015.03.002.

[2] M. P. Wymann and R. Schneiter, "Lipid signalling in disease," *Nat Rev Mol Cell Biol*, vol. 9, no. 2, pp. 162–176, Feb. 2008.

[3] E. Fahy, D. Cotter, M. Sud, and S. Subramaniam, "Lipid classification, structures and tools," *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1811, no. 11, pp. 637–647, 2011, doi: 10.1016/j.bbalip.2011.06.009.

[4] E. Fahy *et al.*, "A comprehensive classification system for lipids," *J. Lipid Res.*, vol. 46, no. 5, pp. 839–862, 2005, doi: 10.1194/jlr.E400004-JLR200.

[5] F. Vella, "The cell. A molecular approach," *Biochem. Educ.*, 1998, doi: 10.1016/s0307-4412(98)00065-x.

[6] S. Klemin, "Molecular Biology of the Cell, Molecular Biology of the Cell: The Problems Book," *Yale J. Biol. Med.*, 2008.

[7] G. Van Meer, D. R. Voelker, and G. W. Feigenson, "Membrane lipids: Where they are and how they behave," *Nature Reviews Molecular Cell Biology*, vol. 9, no. 2. pp. 112–124, 2008, doi: 10.1038/nrm2330.

[8] C. R. Santos and A. Schulze, "Lipid metabolism in cancer," *FEBS J.*, vol. 279, no. 15, pp. 2610–2623, 2012, doi: 10.1111/j.1742-4658.2012.08644.x.

[9] B. D. Levy, C. B. Clish, B. Schmidt, K. Gronert, and C. N. Serhan, "Lipid mediator class switching during acute inflammation: Signals in resolution," *Nature Immunology*, vol. 2, no. 7. pp. 612–619, 2001, doi: 10.1038/89759.

[10] C. Harrison, "Obesity and diabetes: Targeting lipid transport in diabetes," *Nat Rev Drug Discov*, vol. 11, no. 12, p. 908, Dec. 2012.

[11] "fish-consumption,-fish-oil,-omega-3-fatty-acids,-and-cardiovascular-disease." .

[12] R. Sultana, M. Perluigi, and D. Allan Butterfield, "Lipid peroxidation triggers neurodegeneration: A redox proteomics view into the Alzheimer disease brain," *Free Radic. Biol. Med.*, vol. 62, pp. 157–169, 2013, doi:

http://dx.doi.org/10.1016/j.freeradbiomed.2012.09.027.

[13] E. Fahy *et al.*, "Update of the LIPID MAPS comprehensive classification system for lipids," *J. Lipid Res.*, vol. 50, no. Suppl, pp. S9–S14, Apr. 2009, doi: 10.1194/jlr.R800095-JLR200.

[14] M. R. Wenk, "Lipidomics: New Tools and Applications," *Cell*, vol. 143, no. 6, pp. 888–895, Dec. 2010, doi: http://dx.doi.org/10.1016/j.cell.2010.11.033.

[15] G. van Meer, D. R. Voelker, and G. W. Feigenson, "Membrane lipids: where they are and how they behave," *Nat Rev Mol Cell Biol*, vol. 9, no. 2, pp. 112–124, Feb. 2008.

[16] W. Dowhan, M. Bogdanov, and E. Mileykovskaya, "Functional roles of lipids in membranes," in *Biochemistry of Lipids, Lipoproteins and Membranes*, 2008, pp. 1–37.

[17] P. B. Gahan, "The cell: a molecular approach (3rd edn) G. M. Cooper and R. E. Hausman, Palgrave-Macmillans Global Academic Publishing, 713 pp., ISBN 0-87893-214-3 (2004)," *Cell Biochem. Funct.*, 2005, doi: 10.1002/cbf.1157.

[18] H. T. McMahon and J. L. Gallop, "Membrane curvature and mechanisms of dynamic cell membrane remodelling," *Nature*, vol. 438, no. 7068. pp. 590–596, 2005, doi: 10.1038/nature04396.

[19] B. Alberts, J. A, and Lewis J, "Analyzing Protein Structure and Function," *Mol. Biol. Cell*, 2002.

[20] H. T. McMahon and J. L. Gallop, "Membrane curvature and mechanisms of dynamic cell membrane remodelling," *Nature*. 2005, doi: 10.1038/nature04396.

[21] G. van Meer, D. R. Voelker, and G. W. Feigenson, "Nature Reviews | Molecular Cell Biology PA Structural Signalling," *Nat. Rev. Mol. …*, 2008.

[22] S. Yedgar, M. Krimsky, Y. Cohen, and R. J. Flower, "Treatment of inflammatory diseases by selective eicosanoid inhibition: a double-edged sword?," *Trends in Pharmacological Sciences*, vol. 28, no. 9. pp. 459–464, 2007, doi: 10.1016/j.tips.2007.07.005.

[23] Y. Samuels *et al.*, "High Frequency of Mutations of the PIK3CA Gene in Human Cancers," *Science (80-.).*, vol. 304, no. 5670, p. 554, 2004, doi: 10.1126/science.1096502.

[24] J. D. Carpten *et al.*, "A transforming mutation in the pleckstrin homology domain of AKT1 in cancer," *Nature*, vol. 448, no. 7152, pp. 439–444, 2007, doi: 10.1038/nature05933.

[25] N. Miled *et al.*, "Mechanism of two classes of cancer mutations in the phosphoinositide 3-kinase catalytic subunit," *Science (80-.).*, vol. 317, no. 5835, pp. 239–242, 2007, doi: 10.1126/science.1135394.

[26] M. Furuhashi *et al.*, “Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2,” *Nature*, vol. 447, no. 7147, pp. 959–965, 2007, doi: 10.1038/nature05844.

[27] M. R. Wenk, “The emerging field of lipidomics,” *Nat. Rev. Drug Discov.*, vol. 4, no. 7, pp. 594–610, 2005, doi: 10.1038/nrd1776.

[28] O. Cuvillier, “Sphingosine in apoptosis signaling,” *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, vol. 1585, no. 2–3. pp. 153–162, 2002, doi: 10.1016/S1388-1981(02)00336-0.

[29] M. P. Wymann, M. Zvelebil, and M. Laffargue, “Phosphoinositide 3-kinase signalling - Which way to target?,” *Trends in Pharmacological Sciences*, vol. 24, no. 7. pp. 366–376, 2003, doi: 10.1016/S0165-6147(03)00163-9.

[30] T. Baumruker, F. Bornancin, and A. Billich, “The role of sphingosine and ceramide kinases in inflammatory responses,” *Immunology Letters*, vol. 96, no. 2. pp. 175–185, 2005, doi: 10.1016/j.imlet.2004.09.001.

[31] K. M. Eyster, “The membrane and lipids as integral participants in signal transduction: lipid signal transduction for the non-lipid biochemist,” *AJP Adv. Physiol. Educ.*, vol. 31, no. 1, pp. 5–16, 2007, doi: 10.1152/advan.00088.2006.

[32] M. Laffargue *et al.*, “Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function.,” *Immunity*, vol. 16, no. 3, pp. 441–451, 2002, doi: 10.1016/S1074-7613(02)00282-0.

[33] S. Spiegel and S. Milstien, “Sphingosine-1-phosphate: an enigmatic signalling lipid,” *Nat Rev Mol Cell Biol*, vol. 4, no. 5, pp. 397–407, May 2003.

[34] S. Kersten, B. Desvergne, and W. Wahli, “Roles of PPARS in health and disease,” *Nature*, vol. 405, no. 6785, pp. 421–424, 2000, doi: 10.1038/35013000.

[35] A. C. Calkin and P. Tontonoz, “Transcriptional integration of metabolism by the nuclear sterol-activated receptors LXR and FXR,” *Nature Reviews Molecular Cell Biology*, vol. 13, no. 4. pp. 213–224, 2012, doi: 10.1038/nrm3312.

[36] F. R. Maxfield and I. Tabas, “Role of cholesterol and lipid organization in disease,” *Nature*, vol. 438, no. 7068. pp. 612–621, 2005, doi: 10.1038/nature04399.

[37] P. Libby and J. S. Pober, “Chronic Rejection Review is the actual incidence of chronic rejection as a cause,” 2001.

[38] M. P. McGowan, “Pathophysiology of Severe Familial Hypercholesterolemia,” *J. Clin. Lipidol.*, 2012, doi: 10.1016/j.jacl.2012.10.008.

[39] D. E. Weinberg, K. Nakanishi, D. J. Patel, and D. P. Bartel, “The inside-out mechanism of dicers from budding yeasts,” *Cell*, 2011, doi: 10.1016/j.cell.2011.06.021.

[40] S. Vasseur and F. Guillaumond, “LDL Receptor: An open route to feed pancreatic tumor cells,” *Mol. Cell. Oncol.*, 2016, doi: 10.1080/23723556.2015.1033586.

[41] M. R. Wenk, “The emerging field of lipidomics,” *Nat Rev Drug Discov*, vol. 4, no. 7, pp. 594–610, Jul. 2005.

[42] K. Dettmer, P. A. Aronov, and B. D. Hammock, “Mass spectrometry-based metabolomics,” *Mass Spectrom. Rev.*, vol. 26, no. 1, pp. 51–78, 2007, doi: 10.1002/mas.20108.

[43] L. D. Roberts, A. L. Souza, R. E. Gerszten, and C. B. Clish, “Targeted Metabolomics,” in *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., 2001.

[44] Z. Wang *et al.*, “Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease.,” *Nature*, vol. 472, no. 7341, pp. 57–63, Apr. 2011, doi: 10.1038/nature09922.

[45] M. Lísa, E. Cífková, and M. Holčapek, “Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography-mass spectrometry,” *J. Chromatogr. A*, vol. 1218, no. 31, pp. 5146–5156, 2011, doi: 10.1016/j.chroma.2011.05.081.

[46] Z. Haoula *et al.*, “Lipidomic analysis of plasma samples from women with polycystic ovary syndrome,” *Metabolomics*, pp. 657–666, 2014, doi: 10.1007/s11306-014-0726-y.

[47] S. Ravipati, D. R. Baldwin, H. L. Barr, A. W. Fogarty, and D. A. Barrett, “Plasma lipid biomarker signatures in squamous carcinoma and adenocarcinoma lung cancer patients,” *Metabolomics*, vol. 11, no. 6, pp. 1600–1611, 2015, doi: 10.1007/s11306-015-0811-x.

[48] V. C. Tam *et al.*, “XLipidomic profiling of influenza infection identifies mediators that induce and resolve inflammation,” *Cell*, vol. 154, no. 1, 2013, doi: 10.1016/j.cell.2013.05.052.

[49] Y. Kihara *et al.*, “Targeted lipidomics reveals mPGES-1-PGE2 as a therapeutic target for multiple sclerosis,” *Proc. Natl. Acad. Sci.*, vol. 106, no. 51, pp. 21807–21812, 2009, doi: 10.1073/pnas.0906891106.

[50] T. Cajka and O. Fiehn, “Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics,” *Anal. Chem.*, vol. 88, no. 1, pp. 524–545, Jan. 2016, doi: 10.1021/acs.analchem.5b04491.

[51] T. Cajka and O. Fiehn, “Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry,” *TrAC - Trends in Analytical Chemistry*, vol. 61. pp. 192–206, 2014, doi: 10.1016/j.trac.2014.04.017.

[52] A. Shevchenko and K. Simons, “Lipidomics: coming to grips with lipid diversity,” *Nat Rev Mol Cell Biol*, vol. 11, no. 8, pp. 593–598, Aug. 2010.

[53] W. J. Griffiths and Y. Wang, “Mass spectrometry: from proteomics to metabolomics and lipidomics,” *Chem. Soc. Rev.*, vol. 38, no. 7, pp. 1882–1896, 2009, doi: 10.1039/B618553N.

[54] J. B. Fenn, “Electrospray Wings for Molecular Elephants (Nobel Lecture),” *Angew. Chemie Int. Ed.*, vol. 42, no. 33, pp. 3871–3894, 2003, doi: 10.1002/anie.200300605.

[55] K. Tanaka, “The Origin of Macromolecule Ionization by Laser Irradiation (Nobel Lecture),” *Angew. Chemie Int. Ed.*, vol. 42, no. 33, pp. 3860–3870, 2003, doi: 10.1002/anie.200300585.

[56] J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong, and C. M. Whitehouse, “Electrospray ionization for mass spectrometry of large biomolecules,” *Science (80-.).*, vol. 246, no. 4926, pp. 64–71, 1989, doi: 10.1126/science.2675315.

[57] T. Yamada, T. Uchikata, S. Sakamoto, Y. Yokoi, E. Fukusaki, and T. Bamba, “Development of a lipid profiling system using reverse-phase liquid chromatography coupled to high-resolution mass spectrometry with rapid polarity switching and an automated lipid identification software,” *J. Chromatogr. A*, vol. 1292, pp. 211–218, 2013, doi: 10.1016/j.chroma.2013.01.078.

[58] X. Han and R. W. Gross, “Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples,” *Mass Spectrom. Rev.*, vol. 24, no. 3, pp. 367–412, 2005, doi: 10.1002/mas.20023.

[59] K. Schmelzer, E. Fahy, S. Subramaniam, and E. A. Dennis, “The Lipid Maps Initiative in Lipidomics,” *Methods in Enzymology*, vol. 432. pp. 171–183, 2007, doi: 10.1016/S0076-6879(07)32007-7.

[60] O. L. Knittelfelder, B. P. Weberhofer, T. O. Eichmann, S. D. Kohlwein, and G. N. Rechberger, “A versatile ultra-high performance LC-MS method for lipid profiling,” *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, vol. 951–952, no. 1, pp. 119–128, 2014, doi: 10.1016/j.jchromb.2014.01.011.

[61] J. B. German, L. A. Gillies, J. T. Smilowitz, A. M. Zivkovic, and S. M. Watkins, “Lipidomics and lipid profiling in metabolomics,” *Current Opinion in Lipidology*, vol. 18, no. 1. pp. 66–71, 2007, doi: 10.1097/MOL.0b013e328012d911.

[62] S. S. Bird, V. R. Marur, M. J. Sniatynski, H. K. Greenberg, and B. S. Kristal, “Lipidomics Profiling by High-Resolution LC - MS and High-Energy Collisional Dissociation Fragmentation: Focus on Characterization of Mitochondrial Cardiolipins and

Monolysocardiolipins.," *Anal. Chem.*, vol. 83, no. 3, pp. 940–949, 2011, doi: dx.doi.org/10.1021/ac102598u.

[63] Z. Lei, D. V. Huhman, and L. W. Sumner, "Mass spectrometry strategies in metabolomics," *Journal of Biological Chemistry*, vol. 286, no. 29. pp. 25435–25442, 2011, doi: 10.1074/jbc.R111.238691.

[64] R. G. Pearson, "Hard and Soft Acids and Bases," *J. Am. Chem. Soc.*, 1963, doi: 10.1021/ja00905a001.

[65] R. A. Klein, "Mass spectrometry of the phosphatidylcholines: dipalmitoyl, dioleoyl, and stearoyl-oleoyl glycerylphosphorylcholines.," *J. Lipid Res.*, 1971, doi: 10.1016/s0022-2275(20)39520-1.

[66] H. C. Köfeler, A. Fauland, G. N. Rechberger, and M. Trötzmüller, "Mass Spectrometry Based Lipidomics: An Overview of Technological Platforms," *Metabolites*, vol. 2, no. 1, pp. 19–38, 2012, doi: 10.3390/metabo2010019.

[67] B. Fuchs, R. Süß, and J. Schiller, "An update of MALDI-TOF mass spectrometry in lipid research," *Prog. Lipid Res.*, vol. 49, no. 4, pp. 450–475, Oct. 2010, doi: http://dx.doi.org/10.1016/j.plipres.2010.07.001.

[68] S. Medhe, "Mass Spectrometry: Detectors Review," *Chem. Biomol. Eng.*, 2018.

[69] F. Dubois, R. Knochenmuss, R. Zenobi, A. Brunelle, C. Deprun, and Y. Le Beyec, "A comparison between ion-to-photon and microchannel plate detectors," *Rapid Commun. Mass Spectrom.*, 1999, doi: 10.1002/(SICI)1097-0231(19990515)13:9<786::AID-RCM566>3.0.CO;2-3.

[70] X. W. Zhang, D. Wei, Y. Yap, L. Li, S. Y. Guo, and F. Chen, "MASS SPECTROMETRY REVIEWS," *Mass spectrometry-based "omics" technologies in cancer diagnostics*. 2007.

[71] A. Schmidt, P. Picotti, and R. Aebersold, "Systembiologie Proteomanalyse und Systembiologie," *BioSpektrum*, 2008.

[72] S. Nishiumi *et al.*, "Investigations in the possibility of early detection of colorectal cancer by gas chromatography/triple-quadrupole mass spectrometry," *Oncotarget*, 2017, doi: 10.18632/oncotarget.15081.

[73] Q. Hu, R. J. Noll, H. Li, A. Makarov, M. Hardman, and R. Graham Cooks, "The Orbitrap: a new mass spectrometer," *J. Mass Spectrom.*, vol. 40, no. 4, pp. 430–443, 2005, doi: 10.1002/jms.856.

[74] T. Geiger, J. Cox, and M. Mann, "Proteomics on an Orbitrap Benchtop Mass Spectrometer

Using All-ion Fragmentation," *Mol. Cell. Proteomics*, vol. 9, no. 10, pp. 2252–2261, Oct. 2010, doi: 10.1074/mcp.M110.001537.

[75] A. Makarov *et al.*, "Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer," *Anal. Chem.*, 2006, doi: 10.1021/ac0518811.

[76] A. Michalski *et al.*, "Mass Spectrometry-based Proteomics Using Q Exactive, a High-performance Benchtop Quadrupole Orbitrap Mass Spectrometer," *Mol. Cell. Proteomics*, vol. 10, no. 9, p. M111.011015, Sep. 2011, doi: 10.1074/mcp.M111.011015.

[77] M. J. T. Milton and R. I. Wielgosz, "Uncertainty in SI-traceable measurements of amount of substance by isotope dilution mass spectrometry," *Metrologia*, 2000, doi: 10.1088/0026-1394/37/3/3.

[78] J. Vogl, "Characterisation of reference materials by isotope dilution mass spectrometry," *J. Anal. At. Spectrom.*, vol. 22, no. 5, pp. 475–492, 2007, doi: 10.1039/B614612K.

[79] Y. Wu and L. Li, "Sample normalization methods in quantitative metabolomics," *Journal of Chromatography A*. 2016, doi: 10.1016/j.chroma.2015.12.007.

[80] M. Sysi-aho, M. Katajamaa, and L. Yetukuri, "Normalization method for metabolomics data using optimal selection of multiple internal standards," vol. 17, pp. 1–17, 2007, doi: 10.1186/1471-2105-8-93.

[81] M. Wang, C. Wang, and X. Han, "Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry—What, how and why?," *Mass Spectrometry Reviews*. 2017, doi: 10.1002/mas.21492.

[82] X. Han and R. W. Gross, "Quantitative analysis and molecular species fingerprinting of triacylglyceride molecular species directly from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry," *Anal. Biochem.*, 2001, doi: 10.1006/abio.2001.5178.

[83] A. Zacarias, D. Bolanowski, and A. Bhatnagar, "Comparative measurements of multicomponent phospholipid mixtures by electrospray mass spectroscopy: Relating ion intensity to concentration," *Anal. Biochem.*, 2002, doi: 10.1016/S0003-2697(02)00209-9.

[84] S. Yang, J. C. Hoggard, M. E. Lidstrom, and R. E. Synovec, "Comprehensive discovery of 13C labeled metabolites in the bacterium Methylobacterium extorquens AM1 using gas chromatography-mass spectrometry," *J. Chromatogr. A*, 2013, doi: 10.1016/j.chroma.2013.08.059.

[85] A. D. Hegeman *et al.*, "Stable isotope assisted assignment of elemental compositions for

metabolomics," *Anal. Chem.*, 2007, doi: 10.1021/ac070346t.

[86] K. Li, X. Wang, V. R. Pidatala, C. P. Chang, and X. Cao, "Novel quantitative metabolomic approach for the study of stress responses of plant root metabolism," *J. Proteome Res.*, 2014, doi: 10.1021/pr5007813.

[87] A. Chokkathukalam, D. H. Kim, M. P. Barrett, R. Breitling, and D. J. Creek, "Stable isotope-labeling studies in metabolomics: New insights into structure and dynamics of metabolic networks," *Bioanalysis*. 2014, doi: 10.4155/bio.13.348.

[88] M. R. Mashego *et al.*, "MIRACLE: Mass Isotopomer Ratio Analysis of U-13C-Labeled Extracts. A New Method for Accurate Quantification of Changes in Concentrations of Intracellular Metabolites," *Biotechnol. Bioeng.*, 2004, doi: 10.1002/bit.10907.

[89] D. H. Kim, F. Achcar, R. Breitling, K. E. Burgess, and M. P. Barrett, "LC–MS-based absolute metabolite quantification: application to metabolic flux measurement in trypanosomes," *Metabolomics*, 2015, doi: 10.1007/s11306-015-0827-2.

[90] A. De Ghellinck *et al.*, "Production and analysis of perdeuterated lipids from Pichia pastoris cells," *PLoS One*, 2014, doi: 10.1371/journal.pone.0092999.

[91] X. Wei *et al.*, "Data preprocessing method for liquid chromatography-mass spectrometry based metabolomics," *Anal. Chem.*, 2012, doi: 10.1021/ac3016856.

[92] A. Lommen and H. J. Kools, "MetAlign 3.0: Performance enhancement by efficient use of advances in computer hardware," *Metabolomics*, 2012, doi: 10.1007/s11306-011-0369-1.

[93] J. Boccard and S. Rudaz, "Extracting knowledge from ms clinical metabolomic data: Processing and analysis strategies," in *Methods in Molecular Biology*, 2018.

[94] D. J. Creek, A. Jankevics, K. E. V. Burgess, R. Breitling, and M. P. Barrett, "IDEOM: An Excel interface for analysis of LC-MS-based metabolomics data," *Bioinformatics*, 2012, doi: 10.1093/bioinformatics/bts069.

[95] D. J. Creek, A. Jankevics, R. Breitling, D. G. Watson, M. P. Barrett, and K. E. V. Burgess, "Toward global metabolomics analysis with hydrophilic interaction liquid chromatography-mass spectrometry: Improved metabolite identification by retention time prediction," *Anal. Chem.*, 2011, doi: 10.1021/ac2021823.

[96] F. Matsuda *et al.*, "Assessment of metabolome annotation quality: A method for evaluating the false discovery rate of elemental composition searches," *PLoS One*, 2009, doi: 10.1371/journal.pone.0007490.

[97] C. Bertinetto, J. Engel, and J. Jansen, "ANOVA simultaneous component analysis: A tutorial

review," *Analytica Chimica Acta: X*. 2020, doi: 10.1016/j.acax.2020.100061.

[98] D. I. Broadhurst and D. B. Kell, "Statistical strategies for avoiding false discoveries in metabolomics and related experiments," *Metabolomics*, 2006, doi: 10.1007/s11306-006-0037-z.

[99] B. Worley and R. Powers, "Multivariate Analysis in Metabolomics," *Curr. Metabolomics*, 2013, doi: 10.2174/2213235x11301010092.

[100] J. Zhang *et al.*, "Metabonomics research of diabetic nephropathy and type 2 diabetes mellitus based on UPLC-oaTOF-MS system," *Anal. Chim. Acta*, 2009, doi: 10.1016/j.aca.2009.02.027.

[101] J. J. Jansen, H. C. J. Hoefsloot, J. Van Der Greef, M. E. Timmerman, J. A. Westerhuis, and A. K. Smilde, "ASCA: Analysis of multivariate data obtained from an experimental design," *J. Chemom.*, 2005, doi: 10.1002/cem.952.

[102] D. J. Vis, J. A. Westerhuis, A. K. Smilde, and J. van der Greef, "Statistical validation of megavariate effects in ASCA," *BMC Bioinformatics*, 2007, doi: 10.1186/1471-2105-8-322.

[103] J. Boccard and D. N. Rutledge, "A consensus orthogonal partial least squares discriminant analysis (OPLS-DA) strategy for multiblock Omics data fusion," *Anal. Chim. Acta*, 2013, doi: 10.1016/j.aca.2013.01.022.

[104] M. N. Triba *et al.*, "PLS/OPLS models in metabolomics: The impact of permutation of dataset rows on the K-fold cross-validation quality parameters," *Molecular BioSystems*. 2015, doi: 10.1039/c4mb00414k.

[105] O. Quehenberger *et al.*, "Lipidomics reveals a remarkable diversity of lipids in human plasma," *J. Lipid Res.*, vol. 51, no. 11, pp. 3299–3305, 2010, doi: 10.1194/jlr.M009449.

[106] K. Sandra and P. Sandra, "Lipidomics from an analytical perspective," *Curr. Opin. Chem. Biol.*, vol. 17, no. 5, pp. 847–853, 2013, doi: 10.1016/j.cbpa.2013.06.010.

[107] J. Folch, M. Lees, and G. H. S. Stanley, "A simple method for the isolation and purification of total lipids from animal animal tissues," *J. Biol. Chem.*, vol. 226, pp. 497–509, 1957.

[108] E. G. Bligh, W. J. Dyer, A. R. Method, O. F. Total, and L. Extraction, "A rapid method of total lipid extraction and purification," *Filtration*, vol. 37, no. 8, pp. 911–917, 1959, doi: dx.doi.org/10,1139/cjm2014-0700.

[109] A. Triebl, M. Trötzmüller, A. Eberl, P. Hanel, J. Hartler, and H. C. Köfeler, "Quantitation of phosphatidic acid and lysophosphatidic acid molecular species using hydrophilic interaction liquid chromatography coupled to electrospray ionization high resolution mass spectrometry," *J. Chromatogr. A*, vol. 1347, pp. 104–110, 2014, doi: 10.1016/j.chroma.2014.04.070.

[110] V. Matyash, G. Liebisch, T. V Kurzchalia, A. Shevchenko, and D. Schwudke, “Lipid extraction by methyl-terf-butyl ether for high-throughput lipidomics,” *Journal of Lipid Research*, vol. 49, no. 5. pp. 1137–1146, 2008, doi: 10.1194/jlr.D700041-JLR200.

[111] M. M. Malagon, “Author ’ s Accepted Manuscript INFLUENCE OF SAMPLE PREPARATION ON LIPIDOMICS ANALYSIS OF POLAR LIPIDS IN ADIPOSE TISSUE,” *Talanta*, 2017, doi: 10.1016/j.talanta.2017.09.017.

[112] Y. Satomi, M. Hirayama, and H. Kobayashi, “One-step lipid extraction for plasma lipidomics analysis by liquid chromatography mass spectrometry,” *J. Chromatogr. B*, 2017, doi: 10.1016/j.jchromb.2017.08.020.

[113] A. Fauland, M. Tr, A. Eberl, S. Afiuni-zadeh, X. Guo, and E. Lankmayr, “An improved SPE method for fractionation and identification of phospholipids,” pp. 744–751, 2013, doi: 10.1002/jssc.201200708.

[114] R. Hájek, R. Jirásko, M. Lísa, E. Cífková, and M. Holcapek, “Hydrophilic Interaction Liquid Chromatography – Mass Spectrometry Characterization of Gangliosides in Biological Samples Hydrophilic Interaction Liquid Chromatography – Mass Spectrometry Characterization of Gangliosides in Biological Samples,” 2017, doi: 10.1021/acs.analchem.7b03523.

[115] T. Züllig, M. Trötzmüller, and H. C. Köfeler, “Lipidomics from sample preparation to data analysis : a primer,” 2019.

[116] T. Cajka and O. Fiehn, “Increasing lipidomic coverage by selecting optimal mobile-phase modifiers in LC–MS of blood plasma,” *Metabolomics*, vol. 12, no. 2, pp. 1–11, 2016, doi: 10.1007/s11306-015-0929-x.

[117] S. H. J. Brown, T. W. Mitchell, and S. J. Blanksby, “Analysis of unsaturated lipids by ozone-induced dissociation,” *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. 2011, doi: 10.1016/j.bbalip.2011.04.015.

[118] Y. H. Rustam and G. E. Reid, “Analytical Challenges and Recent Advances in Mass Spectrometry Based Lipidomics,” *Analytical Chemistry*. 2018, doi: 10.1021/acs.analchem.7b04836.

[119] B. L. J. Poad *et al.*, “Ozone-induced dissociation on a modified tandem linear ion-trap: Observations of different reactivity for isomeric lipids,” *J. Am. Soc. Mass Spectrom.*, 2010, doi: 10.1016/j.jasms.2010.08.011.

[120] H. T. Pham, A. J. Trevitt, T. W. Mitchell, and S. J. Blanksby, “Rapid differentiation of isomeric lipids by photodissociation mass spectrometry of fatty acid derivatives,” *Rapid*

Commun. Mass Spectrom., 2013, doi: 10.1002/rcm.6503.

[121] S. Furse, M. R. Egmond, and J. A. Killian, "Isolation of lipids from biological samples," *Mol. Membr. Biol.*, vol. 32, no. 3, pp. 55–64, 2015, doi: 10.3109/09687688.2015.1050468.

[122] J. P. Koelmel *et al.*, "Examining heat treatment for stabilization of the lipidome," *Bioanalysis*, vol. 10, no. 5, pp. 291–305, 2018, doi: 10.4155/bio-2017-0209.

[123] M. Lísa, E. Cífková, M. Khalikova, M. Ovčačíková, and M. Holčapek, "Lipidomic analysis of biological samples: Comparison of liquid chromatography, supercritical fluid chromatography and direct infusion mass spectrometry methods," *J. Chromatogr. A*, vol. 1525, pp. 96–108, 2017, doi: 10.1016/j.chroma.2017.10.022.

[124] G. Gürdeniz, M. Kristensen, T. Skov, and L. O. Dragsted, "The effect of LC-MS data preprocessing methods on the selection of plasma biomarkers in fed vs. fasted rats," *Metabolites*, vol. 2, no. 1, pp. 77–99, 2012, doi: 10.3390/metabo2010077.

[125] S. Ravipati, D. R. Baldwin, H. L. Barr, A. W. Fogarty, and D. A. Barrett, "Plasma lipid biomarker signatures in squamous carcinoma and adenocarcinoma lung cancer patients," *Metabolomics*, vol. 11, no. 6, pp. 1600–1611, 2015, doi: 10.1007/s11306-015-0811-x.

[126] J. A. Bowden *et al.*, "Harmonizing lipidomics: NIST interlaboratory comparison exercise for lipidomics using SRM 1950-Metabolites in Frozen Human Plasma.," *J. Lipid Res.*, vol. 58, no. 12, pp. 2275–2288, Oct. 2017, doi: 10.1194/jlr.M079012.

[127] A. Reis, A. Rudnitskaya, G. J. Blackburn, N. M. Fauzi, A. R. Pitt, and C. M. Spickett, "A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL," *J. Lipid Res.*, vol. 54, no. 7, pp. 1812–1824, 2013, doi: 10.1194/jlr.M034330.

[128] G. J. Maranata, N. O. Surya, and A. N. Hasanah, "Optimising factors affecting solid phase extraction performances of molecular imprinted polymer as recent sample preparation technique," *Heliyon*. 2021, doi: 10.1016/j.heliyon.2021.e05934.

[129] H. Liu, J. W. Finch, M. J. Lavallee, R. A. Collamati, C. C. Benevides, and J. C. Gebler, "Effects of column length, particle size, gradient length and flow rate on peak capacity of nano-scale liquid chromatography for peptide separations," *J. Chromatogr. A*, vol. 1147, no. 1, pp. 30–36, Apr. 2007, doi: 10.1016/j.chroma.2007.02.016.

[130] C. West, J. Melin, H. Ansouri, and M. M. Metogo, "Unravelling the effects of mobile phase additives in supercritical fluid chromatography. Part I: Polarity and acidity of the mobile phase," *J. Chromatogr. A*, 2017, doi: 10.1016/j.chroma.2017.02.066.

[131] Y. Liang *et al.*, "Effect of mobile phase additives on qualitative and quantitative analysis of

ginsenosides by liquid chromatography hybrid quadrupole-time of flight mass spectrometry," *J. Chromatogr. A*, 2013, doi: 10.1016/j.chroma.2013.04.001.

[132] Z. Hao, B. Xiao, and N. Weng, "Impact of column temperature and mobile phase components on selectivity of hydrophilic interaction chromatography (HILIC)," *Journal of Separation Science*. 2008, doi: 10.1002/jssc.200700624.

[133] V. Agrahari, M. Bajpai, and S. Nanda, "Essential concepts of mobile phase selection for Reversed phase HPLC," *Res. J. Pharm. Technol.*, 2013.

[134] J. Agalloco, "Validation: an unconventional review and reinvention.," *PDA J. Pharm. Sci. Technol.*, vol. 49, no. 4, pp. 175–179, 1995.

[135] H. Services, "Guidance for Industry Process Validation : General Principles and Practices," no. January, 2011.

[136] F. D. A. Cder, "Bioanalytical Method Validation Guidance for Industry," no. May, 2018.

[137] S. Naz, M. Vallejo, A. García, and C. Barbas, "Method validation strategies involved in non-targeted metabolomics," *J. Chromatogr. A*, 2014, doi: 10.1016/j.chroma.2014.04.071.

[138] H. Mei *et al.*, "Investigation of matrix effects in bioanalytical high- performance liquid chromatography / tandem mass spectrometric assays : application to drug discovery," pp. 97–103, 2003, doi: 10.1002/rcm.876.

[139] A. Furey, M. Moriarty, V. Bane, B. Kinsella, and M. Lehane, "Talanta Ion suppression ; A critical review on causes , evaluation , prevention and applications," *Talanta*, vol. 115, pp. 104–122, 2013, doi: 10.1016/j.talanta.2013.03.048.

[140] J. N. Sampson *et al.*, "Metabolomics in Epidemiology : Sources of Variability in Metabolite Measurements and Implications," pp. 631–641, 2013, doi: 10.1158/1055-9965.EPI-12-1109.

[141] R. A. Van Den Berg, H. C. J. Hoefsloot, J. A. Westerhuis, A. K. Smilde, and M. J. Van Der Werf, "Centering , scaling , and transformations : improving the biological information content of metabolomics data," vol. 15, pp. 1–15, 2006, doi: 10.1186/1471-2164-7-142.

[142] M. R. N. R. T *et al.*, "Evaluation of Normalization Methods for Analysis of LC-MS Data," pp. 610–617, 2012.

[143] M. Chen, R. S. P. Rao, Y. Zhang, C. X. Zhong, and J. J. Thelen, "A modified data normalization method for GC-MS-based metabolomics to minimize batch variation," pp. 1–7, 2014.

[144] B. D. Bennett, J. Yuan, E. H. Kimball, and J. D. Rabinowitz, "Absolute quantitation of

intracellular metabolite concentrations by an isotope ratio-based approach," vol. 3, no. 8, pp. 1299–1311, 2008, doi: 10.1038/nprot.2008.107.

[145] S. Neubauer *et al.*, "U 13 C cell extract of Pichia pastoris – a powerful tool for evaluation of sample preparation in metabolomics," pp. 3091–3105, 2012, doi: 10.1002/jssc.201200447.

[146] A. K. Boysen, K. R. Heal, L. T. Carlson, and A. E. Ingalls, "Best-matched internal standard normalization in liquid chromatography-mass spectrometry metabolomics applied to environmental samples," 2017, doi: 10.1021/acs.analchem.7b04400.

[147] H. Redestig, A. Fukushima, H. Stenlund, T. Moritz, and M. Arita, "Compensation for Systematic Cross-Contribution Improves Normalization of Mass Spectrometry Based Metabolomics Data," vol. 81, no. 19, pp. 7974–7980, 2009.

[148] S. Wang, C. Kuo, and Y. J. Tseng, "Batch Normalizer: A Fast Total Abundance Regression Calibration Method to Simultaneously Adjust Batch and Injection Order Effects in Liquid Chromatography/Time-of-Flight Mass Spectrometry-Based Metabolomics Data and Comparison with Current Calibration Met," 2013.

[149] H. Luan, F. Ji, Y. Chen, and Z. Cai, "statTarget: a streamlined tool for signal drift correction and interpretations of quantitative mass spectrometry-based omics data," *Anal. Chim. Acta*, 2018, doi: 10.1016/j.aca.2018.08.002.

[150] S. Fan *et al.*, "Systematic Error Removal using Random Forest (SERRF) for Normalizing Large-Scale Untargeted Lipidomics Data Systematic Error Removal using Random Forest (SERRF) for Normalizing Large-Scale Untargeted Lipidomics Data," 2019, doi: 10.1021/acs.analchem.8b05592.

[151] D. Broadhurst *et al.*, "Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies," *Metabolomics*, vol. 14, no. 6, pp. 1–17, 2018, doi: 10.1007/s11306-018-1367-3.

[152] M. Wang, C. Wang, R. H. Han, and X. Han, *Novel advances in shotgun lipidomics for biology and medicine*. Elsevier B.V., 2015.

[153] E. Rampler, C. Coman, G. Hermann, A. Sickmann, R. Ahrends, and G. Koellensperger, "LILY-lipidome isotope labeling of yeast : in vivo synthesis of 13 C labeled reference lipids for quanti fi cation by mass spectrometry †," 2017, doi: 10.1039/c7an00107j.

[154] L. Righetti *et al.*, "A novel approach based on untargeted lipidomics reveals differences in the lipid pattern among durum and common wheat," *Food Chem.*, 2018, doi:

10.1016/j.foodchem.2017.08.020.

[155] N. Osel, T. P. Parfant, A. Kristl, and R. Roškar, “Stability-indicating analytical approach for stability evaluation of lactoferrin,” *Pharmaceutics*, 2021, doi: 10.3390/pharmaceutics13071065.

[156] A. Tan, N. Boudreau, and A. Lévesque, “Internal standards for quantitative LC-MS bioanalysis,” in *LC-MS in Drug Bioanalysis*, 2012.

[157] U. Magnusson, B.; Örnemark, *The Fitness for Purpose of Analytical Methods*. 2014.

[158] T. M. Annesley, “Ion suppression in mass spectrometry,” *Clinical Chemistry*. 2003, doi: 10.1373/49.7.1041.

[159] T. Berg and D. H. Strand, “13C labelled internal standards-A solution to minimize ion suppression effects in liquid chromatography-tandem mass spectrometry analyses of drugs in biological samples?,” *J. Chromatogr. A*, 2011, doi: 10.1016/j.chroma.2011.10.081.

[160] J. A. Schwartzbaum, J. L. Fisher, K. D. Aldape, and M. Wrensch, “Epidemiology and molecular pathology of glioma,” *Nat. Clin. Pract. Neurol.*, vol. 2, no. 9, pp. 494–503, 2006, doi: 10.1038/ncpneuro0289.

[161] Q. T. Ostrom *et al.*, “CBTRUS Statistical Report: Primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014,” *Neuro. Oncol.*, 2017, doi: 10.1093/neuonc/nox158.

[162] D. N. Louis, E. C. Holland, and J. G. Cairncross, “Glioma classification: A molecular reappraisal,” *Am. J. Pathol.*, 2001, doi: 10.1016/S0002-9440(10)61750-6.

[163] C. Watts, *Emerging concepts in neuro-oncology*. 2013.

[164] P. Wesseling and D. Capper, “WHO 2016 Classification of gliomas,” *Neuropathology and Applied Neurobiology*. 2018, doi: 10.1111/nan.12432.

[165] W. Sage *et al.*, “Local alkylating chemotherapy applied immediately after 5-ALA guided resection of glioblastoma does not provide additional benefit,” *J. Neurooncol.*, 2018, doi: 10.1007/s11060-017-2649-8.

[166] E. Ozdemir-Kaynak, A. A. Qutub, and O. Yesil-Celiktas, “Advances in glioblastoma multiforme treatment: New models for nanoparticle therapy,” *Frontiers in Physiology*. 2018, doi: 10.3389/fphys.2018.00170.

[167] A. Mahajan and A. Paulino, *Radiation oncology for pediatric CNS tumors*. 2017.

[168] “Comprehensive, Integrative Genomic Analysis of Diffuse Lower-Grade Gliomas,” *N. Engl. J.*

Med., 2015, doi: 10.1056/nejmoa1402121.

[169] Q. T. Ostrom *et al.*, "Sex-specific glioma genome-wide association study identifies new risk locus at 3p21.31 in females, and finds sex-differences in risk at 8q24.21," *Sci. Rep.*, 2018, doi: 10.1038/s41598-018-24580-z.

[170] S. J. Hepworth, M. J. Schoemaker, K. R. Muir, A. J. Swerdlow, M. J. A. Van Tongeren, and P. A. McKinney, "Mobile phone use and risk of glioma in adults: Case-control study," *Br. Med. J.*, 2006, doi: 10.1136/bmj.38720.687975.55.

[171] M. Wrensch, M. Yost, R. Miike, G. Lee, and J. Touchstone, "Adult glioma in relation to residential power frequency electromagnetic field exposures in the San Francisco Bay area," *Epidemiology*, 1999, doi: 10.1097/00001648-199909000-00010.

[172] B. V.S., P. K., . R. G. K., B. V., G. J., and S. J., "Mobile phone use and risk of brain neoplasms and other cancers: Prospective study," *Int. J. Epidemiol.*, 2013.

[173] M. Marinovich, C. L. Galli, C. Bosetti, S. Gallus, and C. La Vecchia, "Aspartame, low-calorie sweeteners and disease: Regulatory safety and epidemiological issues," *Food and Chemical Toxicology*. 2013, doi: 10.1016/j.fct.2013.07.040.

[174] A. Corell, L. Carstam, A. Smits, R. Henriksson, and A. S. Jakola, "Age and surgical outcome of low-grade glioma in Sweden," *Acta Neurol. Scand.*, 2018, doi: 10.1111/ane.12973.

[175] D. van Nieuwenhuizen *et al.*, "Cognitive functioning and functional brain networks in postoperative WHO grade I meningioma patients," *J. Neurooncol.*, 2018, doi: 10.1007/s11060-018-2987-1.

[176] J. Lahti, T. Saunamäki, J. Salo, A. Niemelä, and M. Jehkonen, "Cognitive Impairment and Recovery in Meningiomas and Low-Grade Gliomas," *J. Behav. Brain Sci.*, 2018, doi: 10.4236/jbbs.2018.88029.

[177] E. L. Diamond, G. W. Corner, A. De Rosa, W. Breitbart, and A. J. Applebaum, "Prognostic awareness and communication of prognostic information in malignant glioma: a systematic review," *Journal of neuro-oncology*. 2014, doi: 10.1007/s11060-014-1487-1.

[178] M. Lun, E. Lok, S. Gautam, E. Wu, and E. T. Wong, "The natural history of extracranial metastasis from glioblastoma multiforme," *J. Neurooncol.*, 2011, doi: 10.1007/s11060-011-0575-8.

[179] T. M. Forsyth, W. L. Bi, M. Abedalthagafi, I. F. Dunn, and E. A. Chiocca, "Extracranial growth of glioblastoma multiforme," *J. Clin. Neurosci.*, 2015, doi: 10.1016/j.jocn.2015.03.018.

[180] S. Amitendu, S. K. D. Mak, J. M. Ling, and W. H. Ng, “A single institution experience of the incidence of extracranial metastasis in glioma,” *J. Clin. Neurosci.*, 2012, doi: 10.1016/j.jocn.2011.08.040.

[181] A. Elena *et al.*, “Extraneural metastases in glioblastoma patients: two cases with YKL-40-positive glioblastomas and a meta-analysis of the literature,” *Neurosurgical Review*. 2016, doi: 10.1007/s10143-015-0656-9.

[182] M. Piccirilli, G. M. F. Brunetto, G. Rocchi, F. Giangaspero, and M. Salvati, “Extra central nervous system metastases from cerebral glioblastoma multiforme in elderly patients. Clinico-pathological remarks on our series of seven cases and critical review of the literature,” *Tumori*, 2008, doi: 10.1177/030089160809400109.

[183] M. C. Robert and M. E. Wastie, “Glioblastoma multiforme: A rare manifestation of extensive liver and bone metastases,” *Biomed. Imaging Interv. J.*, 2008, doi: 10.2349/biij.4.1.e3.

[184] T. L. Beaumont, W. J. Kupsky, G. R. Barger, and A. E. Sloan, “Gliosarcoma with multiple extracranial metastases: Case report and review of the literature,” *J. Neurooncol.*, 2007, doi: 10.1007/s11060-006-9295-x.

[185] S. Mehta *et al.*, “The Central Nervous System-Restricted Transcription Factor Olig2 Opposes p53 Responses to Genotoxic Damage in Neural Progenitors and Malignant Glioma,” *Cancer Cell*, 2011, doi: 10.1016/j.ccr.2011.01.035.

[186] H. C. Bock *et al.*, “First-line treatment of malignant glioma with carmustine implants followed by concomitant radiochemotherapy: A multicenter experience,” *Neurosurg. Rev.*, 2010, doi: 10.1007/s10143-010-0280-7.

[187] L. Hamard, D. Ratel, L. Selek, F. Berger, B. van der Sanden, and D. Wion, “The brain tissue response to surgical injury and its possible contribution to glioma recurrence,” *Journal of Neuro-Oncology*. 2016, doi: 10.1007/s11060-016-2096-y.

[188] P. E. Fecci *et al.*, “Systemic anti-CD25 monoclonal antibody administration safely enhances immunity in murine glioma without eliminating regulatory T cells.,” *Clin. Cancer Res.*, 2006, doi: 10.1158/1078-0432.CCR-06-0053.

[189] K. S. Chen and D. A. Mitchell, “Monoclonal antibody therapy for malignant glioma,” *Adv. Exp. Med. Biol.*, 2012, doi: 10.1007/978-1-4614-3146-6_10.

[190] A. Santra, R. Kumar, P. Sharma, C. Bal, P. K. Julka, and A. Malhotra, “Detection of recurrence in glioma: A comparative prospective study between Tc-99m GHA SPECT and F-18 FDG PET/CT,” *Clinical Nuclear Medicine*. 2011, doi: 10.1097/RLU.0b013e318217aee0.

[191] B. Pasquier, D. Pasquier, A. N'golet, M. H. Panh, and P. Couderc, "Extraneural metastases of astrocytomas and glioblastomas clinicopathological study of two cases and review of literature," *Cancer*, 1980, doi: 10.1002/1097-0142(19800101)45:1<112::AID-CNCR2820450121>3.0.CO;2-9.

[192] R. L. Carter, "The Spread of Tumours in the Human Body," *J. Clin. Pathol.*, 1974, doi: 10.1136/jcp.27.5.432-c.

[193] S. S. Schönsteiner *et al.*, "Rare phenomenon: Liver metastases from glioblastoma multiforme," *J. Clin. Oncol.*, 2011, doi: 10.1200/JCO.2011.35.9232.

[194] G. Li *et al.*, "Occipital anaplastic oligodendroglioma with multiple organ metastases after a short clinical course: A case report and literature review," *Diagn. Pathol.*, 2014, doi: 10.1186/1746-1596-9-17.

[195] C. C. Lee, J. S. Jiang, E. T. S. Chen, H. Yokoo, Y. H. Pan, and M. D. Tsai, "Cytologic diagnosis of a metastatic oligodendroglioma in a pleural effusion: A case report," *Acta Cytol.*, 2006, doi: 10.1159/000326011.

[196] F. B. Mesfin, E. M. Deshaies, R. Patel, S. Weaver, P. Spurgas, and A. J. Popp, "Metastatic gliosarcoma with a unique presentation and progression: Case report and review of the literature," *Clin. Neuropathol.*, 2010, doi: 10.5414/npp29147.

[197] J. E. Kim, C. Y. Kim, D. G. Kim, and H. W. Jung, "Implantation metastasis along the stereotactic biopsy tract in anaplastic astrocytoma: A case report," *J. Neurooncol.*, 2003, doi: 10.1023/A:1022581526527.

[198] S. Frank *et al.*, "Metastatic glioblastoma cells use common pathways via blood and lymphatic vessels," *Neurol. Neurochir. Pol.*, 2009.

[199] S. Ueda, T. Mineta, K. Suzuyama, M. Furuta, T. Shiraishi, and K. Tabuchi, "Biologic characterization of a secondary glioblastoma with extracranial progression and systemic metastasis.," *Neuro. Oncol.*, 2003, doi: 10.1215/s1522851702000169.

[200] A. Narayan, G. Jallo, and T. A. G. M. Huisman, "Extracranial, peritoneal seeding of primary malignant brain tumors through ventriculo-peritoneal shunts in children: Case report and review of the literature," *Neuroradiology Journal*. 2015, doi: 10.1177/1971400915609348.

[201] R. Barajas *et al.*, "Metastatic Diffuse Intrinsic Pontine Glioma to the Peritoneal Cavity Via Ventriculoperitoneal Shunt: Case Report and Literature Review," *J. Neurol. Surg. Reports*, 2015, doi: 10.1055/s-0035-1547365.

[202] L. Weiss, "A metastasizing ependymoma of the cauda equina," *Cancer*, 1955, doi:

10.1002/1097-0142(1955)8:1<161::AID-CNCR2820080123>3.0.CO;2-K.

[203] H. B. Newton, M. K. Rosenblum, and R. W. Walker, “Extraneural metastases of infratentorial glioblastoma multiforme to the peritoneal cavity,” *Cancer*, 1992, doi: 10.1002/1097-0142(19920415)69:8<2149::AID-CNCR2820690822>3.0.CO;2-G.

[204] F. Zustovich, A. Della Puppa, R. Scienza, P. Anselmi, C. Furlan, and G. Cartei, “Metastatic oligodendrogliomas: A review of the literature and case report,” *Acta Neurochir. (Wien).*, 2008, doi: 10.1007/s00701-008-1507-z.

[205] F. K. Aarsen, H. R. Van Dongen, P. F. Paquier, M. Van Mourik, and C. E. Catsman-Berrevoets, “Long-term sequelae in children after cerebellar astrocytoma surgery,” *Neurology*, 2004, doi: 10.1212/01.WNL.0000120549.77188.36.

[206] A. S. Jakola *et al.*, “Surgical resection versus watchful waiting in low-grade gliomas,” *Ann. Oncol.*, 2017, doi: 10.1093/annonc/mdx230.

[207] M. J. McGirt, I. M. Goldstein, K. L. Chaichana, M. E. Tobias, K. F. Kothbauer, and G. I. Jallo, “Extent of surgical resection of malignant astrocytomas of the spinal cord: Outcome analysis of 35 patients,” *Neurosurgery*, 2008, doi: 10.1227/01.NEU.0000335070.37943.09.

[208] N. Sanai and M. S. Berger, “Glioma extent of resection and its impact on patient outcome,” *Neurosurgery*. 2008, doi: 10.1227/01.neu.0000318159.21731.cf.

[209] H. Duffau, “Is non-awake surgery for supratentorial adult low-grade glioma treatment still feasible?,” *Neurosurgical Review*. 2018, doi: 10.1007/s10143-017-0918-9.

[210] A. B. M. F. Karim *et al.*, “A randomized trial on dose-response in radiation therapy of low-grade cerebral glioma: European organization for research and treatment of cancer (EORTC) study 22844,” *Cancer/Radiotherapie*. 1997, doi: 10.1016/S1278-3218(97)89774-0.

[211] E. Shaw *et al.*, “Prospective randomized trial of low-versus high-dose radiation therapy in adults with supratentorial low-grade glioma: Initial report of a North Central Cancer Treatment Group/Radiation Therapy Oncology Group/Eastern Cooperative Oncology Group Study,” *J. Clin. Oncol.*, 2002, doi: 10.1200/JCO.2002.09.126.

[212] L. Douw *et al.*, “Cognitive and radiological effects of radiotherapy in patients with low-grade glioma: long-term follow-up,” *Lancet Neurol.*, 2009, doi: 10.1016/S1474-4422(09)70204-2.

[213] K. Aldape, G. Zadeh, S. Mansouri, G. Reifenberger, and A. von Deimling, “Glioblastoma: pathology, molecular mechanisms and markers,” *Acta Neuropathol.*, vol. 129, no. 6, pp. 829–848, 2015, doi: 10.1007/s00401-015-1432-1.

[214] J. H. Sherman *et al.*, “Impact of temozolomide chemotherapy on seizure frequency in patients

with low-grade gliomas: Clinical article," *J. Neurosurg.*, 2011, doi: 10.3171/2010.12.JNS101602.

[215] J. A. Quinn *et al.*, "Phase II trial of temozolomide in patients with progressive low-grade glioma," *J. Clin. Oncol.*, 2003, doi: 10.1200/JCO.2003.01.009.

[216] G. Kaloshi *et al.*, "Temozolomide for low-grade gliomas: Predictive impact of 1p/19q loss on response and outcome," *Neurology*, 2007, doi: 10.1212/01.wnl.0000262034.26310.a2.

[217] E. G. Shaw *et al.*, "Randomized trial of radiation therapy plus procarbazine, lomustine, and vincristine chemotherapy for supratentorial adult low-grade glioma: Initial results of RTOG 9802," *J. Clin. Oncol.*, 2012, doi: 10.1200/JCO.2011.35.8598.

[218] R. Stupp *et al.*, "Radiotherapy plus Concomitant and Adjuvant Temozolomide for Glioblastoma," *N. Engl. J. Med.*, 2005, doi: 10.1056/nejmoa043330.

[219] S. K. Carlsson, S. P. Brothers, and C. Wahlestedt, "Emerging treatment strategies for glioblastoma multiforme," *EMBO Mol. Med.*, vol. 6, no. 11, pp. 1359–1370, 2014, doi: 10.15252/emmm.201302627.

[220] W. Szopa, T. A. Burley, G. Kramer-Marek, and W. Kaspera, "Diagnostic and therapeutic biomarkers in glioblastoma: Current status and future perspectives," *Biomed Res. Int.*, vol. 2017, 2017, doi: 10.1155/2017/8013575.

[221] B. Efron, R. Tibshirani, J. D. Storey, and V. Tusher, "Empirical bayes analysis of a microarray experiment," *J. Am. Stat. Assoc.*, 2001, doi: 10.1198/016214501753382129.

[222] J. D. Storey, J. E. Taylor, and D. Siegmund, "Strong control, conservative point estimation and simultaneous conservative consistency of false discovery rates: A unified approach," *J. R. Stat. Soc. Ser. B Stat. Methodol.*, 2004, doi: 10.1111/j.1467-9868.2004.00439.x.

[223] D. Ghosh, W. Chen, and T. Raghunathan, "The false discovery rate: a variable selection perspective," *J. Stat. Plan. Inference*, 2006, doi: 10.1016/j.jspi.2004.10.024.

[224] H. Zhao *et al.*, "Metabolomics profiling in plasma samples from glioma patients correlates with tumor phenotypes," *Oncotarget*, 2016, doi: 10.18632/oncotarget.7974.

[225] WHO, "Obesity : Preventing and Managing The Global Epidemic: Technical Report Series," *WHO*, 2015. .

[226] J. K. Dibaise and A. E. Foxx-Orenstein, "Role of the gastroenterologist in managing obesity," *Expert Review of Gastroenterology and Hepatology*. 2013, doi: 10.1586/17474124.2013.811061.

[227] D. W. Haslam and W. P. T. James, “Obesity,” in *Lancet*, 2005, doi: 10.1016/S0140-6736(05)67483-1.

[228] R. Woodhouse, “Obesity in art - A brief overview,” *Frontiers of Hormone Research*. 2007, doi: 10.1159/000115370.

[229] A. Pollack, “A.M.A. Recognizes Obesity as a Disease - The New York Times,” *The New York Times*, 2013. .

[230] N. S. Mitchell, V. A. Catenacci, H. R. Wyatt, and J. O. Hill, “Obesity: Overview of an epidemic,” *Psychiatric Clinics of North America*. 2011, doi: 10.1016/j.psc.2011.08.005.

[231] F. X. Pi-Sunyer *et al.*, “Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: Executive summary,” *American Journal of Clinical Nutrition*. 1998, doi: 10.1093/ajcn/68.4.899.

[232] J. P. Adams and P. G. Murphy, “Obesity in anaesthesia and intensive care,” *Br. J. Anaesth.*, 2000, doi: 10.1093/bja/85.1.91.

[233] E. A. H. Sims, E. Danforth, and E. S. Horton, “Endocrine and metabolic effects of experimental obesity in man,” *Recent Prog. Horm. Res.*, 1973, doi: 10.1016/b978-0-12-571129-6.50016-6.

[234] J. Stevens *et al.*, “Sensitivity and specificity of anthropometrics for the prediction of diabetes in a biracial cohort,” *Obes. Res.*, 2001, doi: 10.1038/oby.2001.94.

[235] S. G. Wannamethee and A. G. Shaper, “Weight change and duration of overweight and obesity in the incidence of type 2 diabetes,” *Diabetes Care*, 1999, doi: 10.2337/diacare.22.8.1266.

[236] IARC, “IARC Handbooks of Cancer Prevention Cervix Cancer Screening,” 2005.

[237] K. Biberoglu, “Prevention of endometriosis: Is it possible?,” *J. Endometr.*, 2012.

[238] K. Esposito *et al.*, “Effect of lifestyle changes on erectile dysfunction in obese men: A randomized controlled trial,” *J. Am. Med. Assoc.*, 2004, doi: 10.1001/jama.291.24.2978.

[239] J. S. E. Laven, B. Imani, M. J. C. Eijkemans, and B. C. J. M. Fauser, “New approach to polycystic ovary syndrome and other forms of anovulatory infertility,” *Obstetrical and Gynecological Survey*. 2002, doi: 10.1097/00006254-200211000-00022.

[240] R. Pasquali, F. Casimirri, and V. Vicennati, “Weight control and its beneficial effect on fertility in women with obesity and polycystic ovary syndrome.,” *Human reproduction (Oxford, England)*. 1997, doi: 10.1093/humrep/12.suppl_1.82.

[241] T. Wang *et al.*, “Dietary α-Linolenic Acid-Rich Flaxseed Oil Exerts Beneficial Effects on

Polycystic Ovary Syndrome Through Sex Steroid Hormones—Microbiota—Inflammation Axis in Rats," *Front. Endocrinol. (Lausanne).*, 2020, doi: 10.3389/fendo.2020.00284.

[242] Office Of The Surgeon General, "The Surgeon General's Call to Action To Prevent and Decrease Overweight and Obesity: Overweight in Children and Adolescents," *Heal. San Fr.*, 2001.

[243] D. C. W. Lau, J. D. Douketis, K. M. Morrison, I. M. Hramiak, A. M. Sharma, and E. Ur, "2006 Canadian clinical practice guidelines on the management and prevention of obesity in adults and children [summary].," *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*. 2007, doi: 10.1503/cmaj.061409.

[244] V. Martínez-Vizcaíno, C. Álvarez-Bueno, and I. Cavero-Redondo, "Diet in the management of weight loss," *Nutrients*. 2021, doi: 10.3390/nu13041306.

[245] S. M. Shick, R. R. Wing, M. L. Klem, M. T. McGuire, J. O. Hill, and H. Seagle, "Persons successful at long-term weight loss and maintenance continue to consume a low-energy, low-fat diet," *J. Am. Diet. Assoc.*, 1998, doi: 10.1016/S0002-8223(98)00093-5.

[246] D. F. Tate, R. W. Jeffery, N. E. Sherwood, and R. R. Wing, "Long-term weight losses associated with prescription of higher physical activity goals. Are higher levels of physical activity protective against weight regain?," *Am. J. Clin. Nutr.*, 2007, doi: 10.1093/ajcn/85.4.954.

[247] B. C. Johnston *et al.*, "Comparison of weight loss among named diet programs in overweight and obese adults: A meta-analysis," *JAMA - J. Am. Med. Assoc.*, 2014, doi: 10.1001/jama.2014.10397.

[248] C. E. Naude, A. Schoonees, M. Senekal, T. Young, P. Garner, and J. Volmink, "Low carbohydrate versus isoenergetic balanced diets for reducing weight and cardiovascular risk: A systematic review and meta-analysis," *PLoS ONE*. 2014, doi: 10.1371/journal.pone.0100652.

[249] R. R. Wing and S. Phelan, "Long-term weight loss maintenance.," *The American journal of clinical nutrition*. 2005, doi: 10.1093/ajcn/82.1.222s.

[250] G. L. Blackburn and W. A. Walker, "Science-based solutions to obesity: what are the roles of academia, government, industry, and health care?," *The American journal of clinical nutrition*. 2005, doi: 10.1093/ajcn/82.1.207s.

[251] E. C. Weiss, D. A. Galuska, L. Kettel Khan, C. Gillespie, and M. K. Serdula, "Weight Regain in U.S. Adults Who Experienced Substantial Weight Loss, 1999-2002," *Am. J. Prev. Med.*, 2007, doi: 10.1016/j.amepre.2007.02.040.

[252] J. W. Anderson, E. C. Konz, R. C. Frederich, and C. L. Wood, “Long-term weight-loss maintenance: A meta-analysis of US studies,” *Am. J. Clin. Nutr.*, 2001, doi: 10.1093/ajcn/74.5.579.

[253] D. F. Williamson, E. Pamuk, M. Thun, D. Flanders, T. Byers, and C. Heath, “Prospective study of intentional weight loss and mortality in never-smoking overweight US white women aged 40-64 years,” *Am. J. Epidemiol.*, 1995, doi: 10.1093/oxfordjournals.aje.a117386.

[254] S. Thangaratinam *et al.*, “Effects of interventions in pregnancy on maternal weight and obstetric outcomes: Meta-analysis of randomised evidence,” *BMJ*, 2012, doi: 10.1136/bmj.e2088.

[255] M. L. LeFevre, “Behavioral counseling to promote a healthful diet and physical activity for cardiovascular disease prevention in adults with cardiovascular risk factors: U.S. preventive services task force recommendation statement,” *Annals of Internal Medicine*. 2014, doi: 10.7326/M14-1796.

[256] S. Z. Yanovski and J. A. Yanovski, “Long-term drug treatment for obesity: A systematic and clinical review,” *JAMA - Journal of the American Medical Association*. 2014, doi: 10.1001/jama.2013.281361.

[257] D. Rucker, R. Padwal, S. K. Li, C. Curioni, and D. C. W. Lau, “Long term pharmacotherapy for obesity and overweight: Updated meta-analysis,” *Br. Med. J.*, 2007, doi: 10.1136/bmj.39385.413113.25.

[258] S. M. Wolfe, “When EMA and FDA decisions conflict: differences in patients or in regulation?,” *BMJ*, 2013, doi: 10.1136/bmj.f5140.

[259] H. E. Bays, “Lorcaserin: Drug profile and illustrative model of the regulatory challenges of weight-loss drug development,” *Expert Rev. Cardiovasc. Ther.*, 2011, doi: 10.1586/erc.10.22.

[260] J. L. Colquitt, K. Pickett, E. Loveman, and G. K. Frampton, “Surgery for weight loss in adults. Cochrane Database of Systematic Reviews.,” *Cochrane Collab.*, 2014.

[261] S. H. Chang, C. R. T. Stoll, J. Song, J. E. Varela, C. J. Eagon, and G. A. Colditz, “The effectiveness and risks of bariatric surgery an updated systematic review and meta-analysis, 2003-2012,” *JAMA Surgery*. 2014, doi: 10.1001/jamasurg.2013.3654.

[262] L. Sjöström *et al.*, “Effects of Bariatric Surgery on Mortality in Swedish Obese Subjects,” *N. Engl. J. Med.*, 2007, doi: 10.1056/nejmoa066254.

[263] M. Hatori *et al.*, “Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet,” *Cell Metab.*, 2012, doi: 10.1016/j.cmet.2012.04.019.

[264] A. Chaix, A. Zarrinpar, P. Miu, and S. Panda, “Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges,” *Cell Metab.*, 2014, doi: 10.1016/j.cmet.2014.11.001.

[265] C. Chen, P. F. Williams, G. J. Cooney, I. D. Caterson, and J. R. Turtle, “The effects of fasting and refeeding on liver glycogen synthase and phosphorylase in obese and lean mice,” *Horm. Metab. Res.*, 1992, doi: 10.1055/s-2007-1003285.

[266] D. L. Rothman, I. Magnusson, L. D. Katz, R. G. Shulman, and G. I. Shulman, “Quantitation of hepatic glycogenolysis and gluconeogenesis in fasting humans with 13C NMR,” *Science (80-.).*, 1991, doi: 10.1126/science.1948033.

[267] T. Moro *et al.*, “Effects of eight weeks of time-restricted feeding (16/8) on basal metabolism, maximal strength, body composition, inflammation, and cardiovascular risk factors in resistance-trained males,” *J. Transl. Med.*, 2016, doi: 10.1186/s12967-016-1044-0.

[268] G. M. Tinsley *et al.*, “Time-restricted feeding plus resistance training in active females: A randomized trial,” *Am. J. Clin. Nutr.*, 2019, doi: 10.1093/ajcn/nqz126.

[269] G. M. Tinsley *et al.*, “Time-restricted feeding in young men performing resistance training: A randomized controlled trial†,” *Eur. J. Sport Sci.*, 2017, doi: 10.1080/17461391.2016.1223173.

[270] R. Antoni, T. M. Robertson, M. D. Robertson, and J. D. Johnston, “A pilot feasibility study exploring the effects of a moderate time-restricted feeding intervention on energy intake, adiposity and metabolic physiology in free-living human subjects,” *J. Nutr. Sci.*, 2018, doi: 10.1017/jns.2018.13.

[271] S. D. Anton *et al.*, “The effects of time restricted feeding on overweight, older adults: A pilot study,” *Nutrients*, 2019, doi: 10.3390/nu11071500.

[272] K. Gabel, K. K. Hoddy, and K. A. Varady, “Safety of 8-h time restricted feeding in adults with obesity,” *Appl. Physiol. Nutr. Metab.*, 2019, doi: 10.1139/apnm-2018-0389.

[273] S. Gill and S. Panda, “A Smartphone App Reveals Erratic Diurnal Eating Patterns in Humans that Can Be Modulated for Health Benefits,” *Cell Metab.*, 2015, doi: 10.1016/j.cmet.2015.09.005.

[274] A. T. Hutchison *et al.*, “Time-Restricted Feeding Improves Glucose Tolerance in Men at Risk for Type 2 Diabetes: A Randomized Crossover Trial,” *Obesity*, 2019, doi: 10.1002/oby.22449.

[275] H. Jamshed, R. A. Beyl, D. L. D. Manna, E. S. Yang, E. Ravussin, and C. M. Peterson, “Early time-restricted feeding improves 24-hour glucose levels and affects markers of the circadian

clock, aging, and autophagy in humans," *Nutrients*, 2019, doi: 10.3390/nu11061234.

[276] E. F. Sutton, R. Beyl, K. S. Early, W. T. Cefalu, E. Ravussin, and C. M. Peterson, "Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes," *Cell Metab.*, 2018, doi: 10.1016/j.cmet.2018.04.010.

[277] E. Ravussin, R. A. Beyl, E. Poggiogalle, D. S. Hsia, and C. M. Peterson, "Early Time-Restricted Feeding Reduces Appetite and Increases Fat Oxidation But Does Not Affect Energy Expenditure in Humans," *Obesity*, 2019, doi: 10.1002/oby.22518.

[278] M. J. Wilkinson *et al.*, "Ten-Hour Time-Restricted Eating Reduces Weight, Blood Pressure, and Atherogenic Lipids in Patients with Metabolic Syndrome," *Cell Metab.*, 2020, doi: 10.1016/j.cmet.2019.11.004.

[279] D. Kelley *et al.*, "Skeletal muscle glycolysis, oxidation, and storage of an oral glucose load," *J. Clin. Invest.*, 1988, doi: 10.1172/JCI113489.

[280] I. T. Campbell, R. J. Jarrett, and H. Keen, "Diurnal and seasonal variation in oral glucose tolerance: Studies in the antarctic," *Diabetologia*, 1975, doi: 10.1007/BF00429838.

[281] N. J. Aparicio *et al.*, "Circadian variation of the blood glucose, plasma insulin and human growth hormone levels in response to an oral glucose load in normal subjects," *Diabetes*, 1974, doi: 10.2337/diab.23.2.132.

[282] C. Bandín *et al.*, "Meal timing affects glucose tolerance, substrate oxidation and circadian-related variables: A randomized, crossover trial," *Int. J. Obes.*, 2015, doi: 10.1038/ijo.2014.182.

[283] S. Bo *et al.*, "Is the timing of caloric intake associated with variation in diet-induced thermogenesis and in the metabolic pattern? A randomized cross-over study," *Int. J. Obes.*, 2015, doi: 10.1038/ijo.2015.138.

[284] M. Garaulet, P. Gómez-Abellán, J. J. Alburquerque-Béjar, Y. C. Lee, J. M. Ordovás, and F. A. J. L. Scheer, "Timing of food intake predicts weight loss effectiveness," *Int. J. Obes.*, 2013, doi: 10.1038/ijo.2012.229.

[285] D. Jakubowicz, M. Barnea, J. Wainstein, and O. Froy, "High Caloric intake at breakfast vs. dinner differentially influences weight loss of overweight and obese women," *Obesity*, 2013, doi: 10.1002/oby.20460.

[286] R. Jones *et al.*, "Two weeks of early time-restricted feeding (eTRF) improves skeletal muscle insulin and anabolic sensitivity in healthy men," *Am. J. Clin. Nutr.*, 2020, doi:

10.1093/ajcn/nqaa192.

[287] S. Ravipati, D. R. Baldwin, H. L. Barr, A. W. Fogarty, and D. A. Barrett, “Plasma lipid biomarker signatures in squamous carcinoma and adenocarcinoma lung cancer patients,” *Metabolomics*, vol. 11, no. 6, 2015, doi: 10.1007/s11306-015-0811-x.

[288] Y. H. Rustam and G. E. Reid, “Analytical Challenges and Recent Advances in Mass Spectrometry Based Lipidomics Analytical Challenges and Recent Advances in Mass Spectrometry Based Lipidomics,” 2017, doi: 10.1021/acs.analchem.7b04836.

[289] B. Jenkins, M. Ronis, and A. Koulman, “Lc–ms lipidomics: Exploiting a simple high-throughput method for the comprehensive extraction of lipids in a ruminant fat dose-response study,” *Metabolites*, 2020, doi: 10.3390/metabo10070296.

[290] J. Liu, M. Zhao, Y. Zhu, X. Wang, L. Zheng, and Y. Yin, “LC-MS-Based Metabolomics and Lipidomics Study of High-Density-Lipoprotein-Modulated Glucose Metabolism with an apoA-I Knockout Mouse Model,” *J. Proteome Res.*, 2019, doi: 10.1021/acs.jproteome.8b00290.

[291] W. Hewelt-Belka, Á. Kot-Wasik, P. Tamagnini, and P. Oliveira, “Untargeted lipidomics analysis of the cyanobacterium synechocystis sp. Pcc 6803: Lipid composition variation in response to alternative cultivation setups and to gene deletion,” *Int. J. Mol. Sci.*, 2020, doi: 10.3390/ijms21238883.

[292] J. Clària *et al.*, “Untargeted lipidomics uncovers lipid signatures that distinguish severe from moderate forms of acutely decompensated cirrhosis,” *J. Hepatol.*, 2021, doi: 10.1016/j.jhep.2021.06.043.

[293] W. Hewelt-Belka, J. Nakonieczna, M. Belka, T. Bączek, J. Namieśnik, and A. Kot-Wasik, “Untargeted Lipidomics Reveals Differences in the Lipid Pattern among Clinical Isolates of Staphylococcus aureus Resistant and Sensitive to Antibiotics,” *J. Proteome Res.*, 2016, doi: 10.1021/acs.jproteome.5b00915.

[294] C. Giles, R. Takechi, V. Lam, S. S. Dhaliwal, and J. C. L. Mamo, “Contemporary lipidomic analytics: opportunities and pitfalls,” *Prog. Lipid Res.*, vol. 71, pp. 86–100, 2018, doi: 10.1016/j.plipres.2018.06.003.

[295] J. E. Hunter, M. J. Frada, H. F. Fredricks, A. Vardi, and B. A. S. Van Mooy, “Targeted and untargeted lipidomics of Emiliania huxleyi viral infection and life cycle phases highlights molecular biomarkers of infection, susceptibility, and ploidy,” *Front. Mar. Sci.*, 2015, doi: 10.3389/fmars.2015.00081.

[296] T. Xu, C. Hu, Q. Xuan, and G. Xu, “Recent advances in analytical strategies for mass spectrometry-based lipidomics,” *Anal. Chim. Acta*, 2020, doi: 10.1016/j.aca.2020.09.060.

Appendix

Table A. Lipid list generated through SPE method with inclusion of pre-defined lipid list

Index	Compound Name	Lipid class	Formula	Monoisopopic mass
1	Cer(d18:1/26:0)	Cer	C44 H87 O3 N1	677.6686
2	Cer(d17:0/26:1)	Cer	C43 H85 O3 N1	663.6529
3	Cer(d42:4)	Cer	C42 H77 O3 N1	643.5903
4	Cer(d18:2/24:1)	Cer	C42 H79 O3 N1	645.606
5	Cer(d18:1/24:1)	Cer	C42 H81 O3 N1	647.6216
6	Cer(d18:1/24:0)	Cer	C42 H83 O3 N1	649.6373
7	Cer(d18:0/24:0)	Cer	C42 H85 O3 N1	651.6529
8	Cer(d17:1/24:1)	Cer	C41 H79 O3 N1	633.606
9	Cer(d15:0/26:1)	Cer	C41 H81 O3 N1	635.6216
10	Cer(d18:2/22:0)	Cer	C40 H77 O3 N1	619.5903
11	Cer(d18:1/22:0)	Cer	C40 H79 O3 N1	621.606
12	Cer(d18:0/22:0)	Cer	C40 H81 O3 N1	623.6216
13	Cer(d17:1/22:0)	Cer	C39 H77 O3 N1	607.5903
14	Cer(d18:1/16:0)	Cer	C34 H67 O3 N1	537.5121
15	Cer(d16:1/22:0)	Cer	C38 H75 O3 N1	593.5747
16	Cer(d16:0/22:0)	Cer	C38 H77 O3 N1	595.5903
17	Cer(d18:1/18:0)	Cer	C36 H71 O3 N1	565.5434
18	Cer(d35:0)	Cer	C35 H71 O3 N1	553.5434
19	ChE(22:6)	ChE	C49 H76 O2	696.5845
20	ChE(16:1)	ChE	C43 H74 O2	622.5689
21	ChE(16:0)	ChE	C43 H76 O2	624.5845
22	ChE(15:0)	ChE	C42 H74 O2	610.5689
23	ChE(20:4)	ChE	C47 H76 O2	672.5845
24	ChE(20:5)	ChE	C47 H74 O2	670.5689
25	ChE(20:4)	ChE	C47 H76 O2	672.5845
26	ChE(20:4)	ChE	C47 H76 O2	672.5845
27	ChE(18:3)	ChE	C45 H74 O2	646.5689
28	ChE(20:3)	ChE	C47 H78 O2	674.6002
29	ChE(18:2)	ChE	C45 H76 O2	648.5845
30	ChE(18:3)	ChE	C45 H74 O2	646.5689
31	ChE(18:2)	ChE	C45 H76 O2	648.5845
32	ChE(18:2)	ChE	C45 H76 O2	648.5845
33	ChE(18:2)	ChE	C45 H76 O2	648.5845
34	ChE(18:2)	ChE	C45 H76 O2	648.5845

35	ChE(18:2)	ChE	C45 H76 O2	648.5845
36	ChE(18:2)	ChE	C45 H76 O2	648.5845
37	ChE(18:1)	ChE	C45 H78 O2	650.6002
38	ChE(18:2)	ChE	C45 H76 O2	648.5845
39	DG(38:7)	DG	C41 H66 O5	638.491
40	DG(18:1/20:5)	DG	C41 H68 O5	640.5067
41	DG(16:0/22:6)	DG	C41 H68 O5	640.5067
42	DG(18:2/20:4)	DG	C41 H68 O5	640.5067
43	DG(40:7)	DG	C43 H70 O5	666.5223
44	DG(18:1/22:5)	DG	C43 H72 O5	668.538
45	DG(18:1/22:5)	DG	C43 H72 O5	668.538
46	DG(34:3)	DG	C37 H66 O5	590.491
47	DG(16:0/18:3)	DG	C37 H66 O5	590.491
48	DG(14:0/20:4)	DG	C37 H64 O5	588.4754
49	DG(16:1/18:3)	DG	C37 H64 O5	588.4754
50	DG(16:0/18:1)	DG	C37 H70 O5	594.5223
51	DG(18:0/16:0)	DG	C37 H72 O5	596.538
52	DG(16:0/18:3)	DG	C37 H66 O5	590.491
53	DG(16:0/18:2)	DG	C37 H68 O5	592.5067
54	DG(16:0/16:1)	DG	C35 H66 O5	566.491
55	DG(16:0/16:0)	DG	C35 H68 O5	568.5067
56	DG(14:0/18:3)	DG	C35 H62 O5	562.4597
57	DG(14:0/18:2)	DG	C35 H64 O5	564.4754
58	DG(16:0/14:0)	DG	C33 H64 O5	540.4754
59	DG(12:0/18:2)	DG	C33 H60 O5	536.4441
60	DG(16:1/14:0)	DG	C33 H62 O5	538.4597
61	DG(18:1/20:3)	DG	C41 H72 O5	644.538
62	DG(18:1/20:3)	DG	C41 H72 O5	644.538
63	DG(18:1/20:4)	DG	C41 H70 O5	642.5223
64	DG(18:1/20:4)	DG	C41 H70 O5	642.5223
65	DG(16:1/20:4)	DG	C39 H66 O5	614.491
66	DG(36:5)	DG	C39 H66 O5	614.491
67	DG(20:1/18:1)	DG	C41 H76 O5	648.5693
68	DG(18:1/20:2)	DG	C41 H74 O5	646.5536
69	DG(18:2/18:2)	DG	C39 H68 O5	616.5067
70	DG(18:1/18:3)	DG	C39 H68 O5	616.5067
71	DG(16:0/20:4)	DG	C39 H68 O5	616.5067
72	DG(18:3/18:2)	DG	C39 H66 O5	614.491

73	DG(18:0/18:0)	DG	C39 H76 O5	624.5693
74	DG(18:0/18:1)	DG	C39 H74 O5	622.5536
75	DG(18:1/18:1)	DG	C39 H72 O5	620.538
76	DG(18:1/18:2)	DG	C39 H70 O5	618.5223
77	dMePE(16:0/18:1)	dMePE	C41 H80 O8 N1 P1	745.5622
78	dMePE(18:0/18:2)	dMePE	C43 H82 O8 N1 P1	771.5778
79	FA(18:2)	FA	O2 H32 C18	280.2402
80	FA(16:1)	FA	O2 H30 C16	254.2246
81	LdMePE(16:0)	LdMePE	C23 H48 O7 N1 P1	481.3168
82	LdMePE(16:0)	LdMePE	C23 H48 O7 N1 P1	481.3168
83	LPC(20:5)	LPC	C28 H48 O7 N1 P1	541.3168
84	LPC(20:5)	LPC	C28 H50 O7 N1 P1	543.3325
85	LPC(20:5)	LPC	C30 H52 O7 N1 P1	569.3481
86	LPC(20:5)	LPC	C28 H48 O7 N1 P1	541.3168
87	LPC(20:5)	LPC	C30 H52 O7 N1 P1	569.3481
88	LPC(20:5)	LPC	C30 H52 O7 N1 P1	569.3481
89	LPC(20:5)	LPC	C30 H50 O7 N1 P1	567.3325
90	LPC(22:5)	LPC	C30 H52 O7 N1 P1	569.3481
91	LPC(20:3)	LPC	C28 H52 O7 N1 P1	545.3481
92	LPC(20:0)	LPC	C28 H58 O7 N1 P1	551.3951
93	LPC(20:3)	LPC	C28 H52 O7 N1 P1	545.3481
94	LPC(20:3)	LPC	C28 H52 O7 N1 P1	545.3481
95	LPC(20:4)	LPC	C28 H50 O7 N1 P1	543.3325
96	LPC(20:3)	LPC	C28 H52 O7 N1 P1	545.3481
97	LPC(20:4)	LPC	C28 H50 O7 N1 P1	543.3325
98	LPC(20:4)	LPC	C28 H50 O7 N1 P1	543.3325
99	LPC(22:6)	LPC	C30 H50 O7 N1 P1	567.3325
100	LPC(22:6)	LPC	C30 H50 O7 N1 P1	567.3325
101	LPC(22:6)	LPC	C30 H50 O7 N1 P1	567.3325
102	LPC(22:6)	LPC	C30 H50 O7 N1 P1	567.3325
103	LPC(15:1)	LPC	C23 H46 O7 N1 P1	479.3012
104	LPC(15:0)	LPC	C23 H48 O7 N1 P1	481.3168
105	LPC(15:0)	LPC	C23 H48 O7 N1 P1	481.3168
106	LPC(16:0p)	LPC	C24 H50 O6 N1 P1	479.3376
107	LPC(16:0)	LPC	C24 H50 O7 N1 P1	495.3325
108	LPC(16:0)	LPC	C24 H50 O7 N1 P1	495.3325
109	LPC(15:2)	LPC	C23 H44 O7 N1 P1	477.2855
110	LPC(18:2)	LPC	C26 H50 O7 N1 P1	519.3325

111	LPC(18:2)	LPC	C26 H50 O7 N1 P1	519.3325
112	LPC(18:1)	LPC	C26 H52 O7 N1 P1	521.3481
113	LPC(18:1)	LPC	C26 H52 O7 N1 P1	521.3481
114	LPC(18:3)	LPC	C26 H48 O7 N1 P1	517.3168
115	LPC(19:1)	LPC	C27 H54 O7 N1 P1	535.3638
116	LPC(18:3)	LPC	C26 H48 O7 N1 P1	517.3168
117	LPC(18:3)	LPC	C26 H48 O7 N1 P1	517.3168
118	LPC(17:0)	LPC	C25 H52 O7 N1 P1	509.3481
119	LPC(17:4)	LPC	C25 H44 O7 N1 P1	501.2855
120	LPC(16:1)	LPC	C24 H48 O7 N1 P1	493.3168
121	LPC(16:1)	LPC	C24 H48 O7 N1 P1	493.3168
122	LPC(18:0)	LPC	C26 H54 O7 N1 P1	523.3638
123	LPC(18:1)	LPC	C26 H52 O7 N1 P1	521.3481
124	LPC(17:4)	LPC	C25 H44 O7 N1 P1	501.2855
125	LPC(18:0)	LPC	C26 H54 O7 N1 P1	523.3638
126	LPE(18:0)	LPE	C23 H48 O7 N1 P1	481.3168
127	LPE(18:1)	LPE	C23 H46 O7 N1 P1	479.3012
128	LPE(18:1)	LPE	C23 H46 O7 N1 P1	479.3012
129	LPE(18:2)	LPE	C23 H44 O7 N1 P1	477.2855
130	LPE(18:2)	LPE	C23 H44 O7 N1 P1	477.2855
131	LPE(20:4)	LPE	C25 H44 O7 N1 P1	501.2855
132	LPE(20:4)	LPE	C25 H44 O7 N1 P1	501.2855
133	LPE(22:6)	LPE	C27 H44 O7 N1 P1	525.2855
134	LPE(16:0)	LPE	C21 H44 O7 N1 P1	453.2855
135	LPE(16:0)	LPE	C21 H44 O7 N1 P1	453.2855
136	LPE(16:0)	LPE	C21 H44 O7 N1 P1	453.2855
137	LPE(18:0)	LPE	C23 H48 O7 N1 P1	481.3168
138	PA(29:2/15:0)	PA	C47 H89 O8 N0 P1	812.6295
139	PA(27:2/20:4)	PA	C50 H87 O8 N0 P1	846.6139
140	PC(40:5)	PC	C48 H86 O8 N1 P1	835.6091
141	PC(40:6)	PC	C48 H84 O8 N1 P1	833.5935
142	PC(40:2)	PC	C48 H92 O8 N1 P1	841.6561
143	PC(40:4)	PC	C48 H88 O8 N1 P1	837.6248
144	PC(40:6p)	PC	C48 H84 O7 N1 P1	817.5985
145	PC(40:7)	PC	C48 H82 O8 N1 P1	831.5778
146	PC(40:6)	PC	C48 H84 O8 N1 P1	833.5935
147	PC(18:0/22:6)	PC	C48 H84 O8 N1 P1	833.5935
148	PC(40:8)	PC	C48 H80 O8 N1 P1	829.5622

149	PC(40:8)	PC	C48 H80 O8 N1 P1	829.5622
150	PC(40:7)	PC	C48 H82 O8 N1 P1	831.5778
151	PC(40:8)	PC	C48 H80 O8 N1 P1	829.5622
152	PC(42:10)	PC	C50 H80 O8 N1 P1	853.5622
153	PC(42:11)	PC	C50 H78 O8 N1 P1	851.5465
154	PC(40:9)	PC	C48 H78 O8 N1 P1	827.5465
155	PC(40:9)	PC	C48 H78 O8 N1 P1	827.5465
156	PC(44:3p)	PC	C52 H98 O7 N1 P1	879.7081
157	PC(42:9)	PC	C50 H82 O8 N1 P1	855.5778
158	PC(42:9)	PC	C50 H82 O8 N1 P1	855.5778
159	PC(42:7p)	PC	C50 H86 O7 N1 P1	843.6142
160	PC(44:3p)	PC	C52 H98 O7 N1 P1	879.7081
161	PC(37:2)	PC	C45 H86 O8 N1 P1	799.6091
162	PC(37:3)	PC	C45 H84 O8 N1 P1	797.5935
163	PC(37:4)	PC	C45 H82 O8 N1 P1	795.5778
164	PC(37:4)	PC	C45 H82 O8 N1 P1	795.5778
165	PC(36:5)	PC	C44 H78 O8 N1 P1	779.5465
166	PC(36:6)	PC	C44 H76 O8 N1 P1	777.5309
167	PC(36:6)	PC	C44 H76 O8 N1 P1	777.5309
168	PC(36:6)	PC	C44 H76 O8 N1 P1	777.5309
169	PC(37:6)	PC	C45 H78 O8 N1 P1	791.5465
170	PC(38:2)	PC	C46 H88 O8 N1 P1	813.6248
171	PC(38:3)	PC	C46 H86 O8 N1 P1	811.6091
172	PC(38:3)	PC	C46 H86 O8 N1 P1	811.6091
173	PC(37:4)	PC	C45 H82 O8 N1 P1	795.5778
174	PC(37:5)	PC	C45 H80 O8 N1 P1	793.5622
175	PC(37:5)	PC	C45 H80 O8 N1 P1	793.5622
176	PC(37:6)	PC	C45 H78 O8 N1 P1	791.5465
177	PC(38:5)	PC	C46 H82 O8 N1 P1	807.5778
178	PC(38:5)	PC	C46 H82 O8 N1 P1	807.5778
179	PC(38:6)	PC	C46 H80 O8 N1 P1	805.5622
180	PC(38:5)	PC	C46 H82 O8 N1 P1	807.5778
181	PC(38:4)	PC	C46 H84 O8 N1 P1	809.5935
182	PC(38:4)	PC	C46 H84 O8 N1 P1	809.5935
183	PC(38:4p)	PC	C46 H84 O7 N1 P1	793.5985
184	PC(38:4)	PC	C46 H84 O8 N1 P1	809.5935
185	PC(39:6)	PC	C47 H82 O8 N1 P1	819.5778
186	PC(39:5)	PC	C47 H84 O8 N1 P1	821.5935

187	PC(40:10)	PC	C48 H76 O8 N1 P1	825.5309
188	PC(39:7)	PC	C47 H80 O8 N1 P1	817.5622
189	PC(38:6e)	PC	C46 H82 O7 N1 P1	791.5829
190	PC(38:6)	PC	C46 H80 O8 N1 P1	805.5622
191	PC(38:7)	PC	C46 H78 O8 N1 P1	803.5465
192	PC(38:7)	PC	C46 H78 O8 N1 P1	803.5465
193	PC(32:2)	PC	C40 H76 O8 N1 P1	729.5309
194	PC(33:0)	PC	C41 H82 O8 N1 P1	747.5778
195	PC(32:0)	PC	C40 H80 O8 N1 P1	733.5622
196	PC(32:1)	PC	C40 H78 O8 N1 P1	731.5465
197	PC(34:2e)	PC	C42 H82 O7 N1 P1	743.5829
198	PC(34:3)	PC	C42 H78 O8 N1 P1	755.5465
199	PC(34:1)	PC	C42 H82 O8 N1 P1	759.5778
200	PC(34:2)	PC	C42 H80 O8 N1 P1	757.5622
201	PC(18:0/15:2)	PC	C41 H78 O8 N1 P1	743.5465
202	PC(34:0)	PC	C42 H84 O8 N1 P1	761.5935
203	PC(33:1)	PC	C41 H80 O8 N1 P1	745.5622
204	PC(33:2)	PC	C41 H78 O8 N1 P1	743.5465
205	PC(35:4)	PC	C43 H78 O8 N1 P1	767.5465
206	PC(35:4)	PC	C43 H78 O8 N1 P1	767.5465
207	PC(35:3)	PC	C43 H80 O8 N1 P1	769.5622
208	PC(35:3)	PC	C43 H80 O8 N1 P1	769.5622
209	PC(35:2)	PC	C43 H82 O8 N1 P1	771.5778
210	PC(35:1)	PC	C43 H84 O8 N1 P1	773.5935
211	PC(34:4)	PC	C42 H76 O8 N1 P1	753.5309
212	PC(34:3)	PC	C42 H78 O8 N1 P1	755.5465
213	PC(36:5)	PC	C44 H78 O8 N1 P1	779.5465
214	PC(17:4/19:0)	PC	C44 H80 O8 N1 P1	781.5622
215	PC(36:4)	PC	C44 H80 O8 N1 P1	781.5622
216	PC(36:4)	PC	C44 H80 O8 N1 P1	781.5622
217	PC(36:3)	PC	C44 H82 O8 N1 P1	783.5778
218	PC(36:2)	PC	C44 H84 O8 N1 P1	785.5935
219	PC(36:1)	PC	C44 H86 O8 N1 P1	787.6091
220	PC(35:5)	PC	C43 H76 O8 N1 P1	765.5309
221	PE(18:0p/18:2)	PE	C41 H78 O7 N1 P1	727.5516
222	PE(18:1/18:2)	PE	C41 H76 O8 N1 P1	741.5309
223	PE(18:1p/18:2)	PE	C41 H76 O7 N1 P1	725.5359
224	PE(18:2/18:2)	PE	C41 H74 O8 N1 P1	739.5152

225	PE(16:0/20:4)	PE	C41 H74 O8 N1 P1	739.5152
226	PE(16:0e/20:4)	PE	C41 H76 O7 N1 P1	725.5359
227	PE(37:3)	PE	C42 H78 O8 N1 P1	755.5465
228	PE(37:4)	PE	C42 H76 O8 N1 P1	753.5309
229	PE(18:0/20:3)	PE	C43 H80 O8 N1 P1	769.5622
230	PE(38:4)	PE	C43 H78 O8 N1 P1	767.5465
231	PE(18:0/20:4)	PE	C43 H78 O8 N1 P1	767.5465
232	PE(38:4)	PE	C43 H78 O8 N1 P1	767.5465
233	PE(18:1/20:4)	PE	C43 H76 O8 N1 P1	765.5309
234	PE(18:0/20:5)	PE	C43 H76 O8 N1 P1	765.5309
235	PE(16:0/22:6)	PE	C43 H74 O8 N1 P1	763.5152
236	PE(16:0/22:6)	PE	C43 H74 O8 N1 P1	763.5152
237	PE(39:4)	PE	C44 H80 O8 N1 P1	781.5622
238	PE(16:0p/22:6)	PE	C43 H74 O7 N1 P1	747.5203
239	PE(39:5e)	PE	C44 H80 O7 N1 P1	765.5672
240	PE(39:5)	PE	C44 H78 O8 N1 P1	779.5465
241	PE(18:0/22:5)	PE	C45 H80 O8 N1 P1	793.5622
242	PE(18:0/22:4)	PE	C45 H82 O8 N1 P1	795.5778
243	PE(18:0p/22:6)	PE	C45 H78 O7 N1 P1	775.5516
244	PE(18:0/22:6)	PE	C45 H78 O8 N1 P1	791.5465
245	PE(41:4)	PE	C46 H84 O8 N1 P1	809.5935
246	PE(18:1p/22:6)	PE	C45 H76 O7 N1 P1	773.5359
247	PE(41:6)	PE	C46 H80 O8 N1 P1	805.5622
248	PE(41:5)	PE	C46 H82 O8 N1 P1	807.5778
249	PE(43:5)	PE	C48 H86 O8 N1 P1	835.6091
250	PE(41:6p)	PE	C46 H80 O7 N1 P1	789.5672
251	PE(43:6p)	PE	C48 H84 O7 N1 P1	817.5985
252	PE(43:6)	PE	C48 H84 O8 N1 P1	833.5935
253	PE(18:0e)	PE	C23 H48 O7 N1 P1	481.3168
254	PE(16:0e)	PE	C21 H44 O7 N1 P1	453.2855
255	PE(16:0e)	PE	C21 H44 O7 N1 P1	453.2855
256	PE(16:0/18:1)	PE	C39 H76 O8 N1 P1	717.5309
257	PE(23:2p)	PE	C28 H52 O7 N1 P1	545.3481
258	PE(18:0p)	PE	C23 H46 O7 N1 P1	479.3012
259	PE(18:0e)	PE	C23 H48 O7 N1 P1	481.3168
260	PE(18:0/18:2)	PE	C41 H78 O8 N1 P1	743.5465
261	PE(18:0/18:1)	PE	C41 H80 O8 N1 P1	745.5622
262	PE(16:0p/18:2)	PE	C39 H74 O7 N1 P1	699.5203

263	PE(16:0/18:2)	PE	C39 H74 O8 N1 P1	715.5152
264	PE(43:7)	PE	C48 H82 O8 N1 P1	831.5778
265	PE(43:7)	PE	C48 H82 O8 N1 P1	831.5778
266	PE(43:8)	PE	C48 H80 O8 N1 P1	829.5622
267	PE(43:7p)	PE	C48 H82 O7 N1 P1	815.5829
268	PE(45:10)	PE	C50 H80 O8 N1 P1	853.5622
269	PE(43:8)	PE	C48 H80 O8 N1 P1	829.5622
270	PE(46:3)	PE	C51 H96 O8 N1 P1	881.6874
271	PE(45:9)	PE	C50 H82 O8 N1 P1	855.5778
272	PG(18:0/18:1)	PG	C42 H81 O10 N0 P1	776.5567
273	PI(18:0/22:6)	PI	C49 H83 O13 N0 P1	910.5571
274	PI(16:0/18:1)	PI	C43 H81 O13 N0 P1	836.5415
275	PI(18:0/18:1)	PI	C45 H85 O13 N0 P1	864.5728
276	PI(16:0/18:2)	PI	C43 H79 O13 N0 P1	834.5258
277	PI(16:0/20:4)	PI	C45 H79 O13 N0 P1	858.5258
278	PI(18:0/18:2)	PI	C45 H83 O13 N0 P1	862.5571
279	PI(18:0/20:4)	PI	C47 H83 O13 N0 P1	886.5571
280	PI(18:0/20:3)	PI	C47 H85 O13 N0 P1	888.5728
281	PS(40:3)	PS	C46 H84 O10 N1 P1	841.5833
282	PS(40:3)	PS	C46 H84 O10 N1 P1	841.5833
283	PS(40:4)	PS	C46 H82 O10 N1 P1	839.5676
284	PS(41:6)	PS	C47 H80 O10 N1 P1	849.552
285	PS(42:3)	PS	C48 H88 O10 N1 P1	869.6146
286	PS(42:3)	PS	C48 H88 O10 N1 P1	869.6146
287	PS(42:4)	PS	C48 H86 O10 N1 P1	867.5989
288	PS(42:5)	PS	C48 H84 O10 N1 P1	865.5833
289	PS(36:0)	PS	C42 H82 O10 N1 P1	791.5676
290	PS(37:0)	PS	C43 H84 O10 N1 P1	805.5833
291	PS(37:2)	PS	C43 H80 O10 N1 P1	801.552
292	PS(38:2)	PS	C44 H82 O10 N1 P1	815.5676
293	PS(39:4)	PS	C45 H80 O10 N1 P1	825.552
294	PS(39:4)	PS	C45 H80 O10 N1 P1	825.552
295	PS(40:2)	PS	C46 H86 O10 N1 P1	843.5989
296	PS(44:5)	PS	C50 H88 O10 N1 P1	893.6146
297	PS(44:4)	PS	C50 H90 O10 N1 P1	895.6302
298	PS(42:5)	PS	C48 H84 O10 N1 P1	865.5833
299	SM(d43:2)	SM	C48 H95 O6 N2 P1	826.6928
300	SM(d44:6)	SM	C49 H89 O6 N2 P1	832.6458

301	SM(d41:2)	SM	C46 H91 O6 N2 P1	798.6615
302	SM(d22:1/18:1)	SM	C45 H89 O6 N2 P1	784.6458
303	SM(d22:1/18:0)	SM	C45 H91 O6 N2 P1	786.6615
304	SM(d22:1/16:0)	SM	C43 H87 O6 N2 P1	758.6302
305	SM(d43:2)	SM	C48 H95 O6 N2 P1	826.6928
306	SM(d43:1)	SM	C48 H97 O6 N2 P1	828.7084
307	SM(d22:1/20:2)	SM	C47 H91 O6 N2 P1	810.6615
308	SM(d17:1/24:3)	SM	C46 H87 O6 N2 P1	794.6302
309	SM(d34:2)	SM	C39 H77 O6 N2 P1	700.5519
310	SM(d20:1/16:0)	SM	C41 H83 O6 N2 P1	730.5989
311	SM(d35:2)	SM	C40 H79 O6 N2 P1	714.5676
312	SM(d35:1)	SM	C40 H81 O6 N2 P1	716.5832
313	SM(d35:1)	SM	C40 H81 O6 N2 P1	716.5832
314	So(d17:1)	So	C17 H35 O2 N1	285.2668
315	So(d17:1)	So	C17 H35 O2 N1	285.2668
316	So(d17:1)	So	C17 H35 O2 N1	285.2668
317	TG(16:0/16:0/20:4)	TG	C55 H98 O6	854.7363
318	TG(16:0/18:1/18:3)	TG	C55 H98 O6	854.7363
319	TG(16:0/18:2/18:2)	TG	C55 H98 O6	854.7363
320	TG(16:0/16:0/20:4)	TG	C55 H98 O6	854.7363
321	TG(10:0/18:3/24:0)	TG	C55 H100 O6	856.752
322	TG(16:0/18:1/18:2)	TG	C55 H100 O6	856.752
323	TG(16:0/16:0/20:3)	TG	C55 H100 O6	856.752
324	TG(52:4)	TG	C55 H98 O6	854.7363
325	TG(16:0/18:1/18:1)	TG	C55 H102 O6	858.7676
326	TG(16:0/18:1/18:1)	TG	C55 H102 O6	858.7676
327	TG(16:0/16:0/20:3)	TG	C55 H100 O6	856.752
328	TG(16:0/16:0/20:3)	TG	C55 H100 O6	856.752
329	TG(18:0/16:0/18:1)	TG	C55 H104 O6	860.7833
330	TG(16:0/18:1/18:1)	TG	C55 H102 O6	858.7676
331	TG(16:0/18:1/18:1)	TG	C55 H102 O6	858.7676
332	TG(16:0/18:1/18:1)	TG	C55 H102 O6	858.7676
333	TG(18:0/16:0/18:1)	TG	C55 H104 O6	860.7833
334	TG(18:0/16:0/18:1)	TG	C55 H104 O6	860.7833
335	TG(18:0/16:0/18:1)	TG	C55 H104 O6	860.7833
336	TG(18:0/16:0/18:1)	TG	C55 H104 O6	860.7833
337	TG(18:4/16:0/16:0)	TG	C53 H94 O6	826.705
338	TG(18:4/16:0/16:0)	TG	C53 H94 O6	826.705

339	TG(18:0/16:0/18:1)	TG	C55 H104 O6	860.7833
340	TG(14:0/14:0/22:5)	TG	C53 H92 O6	824.6894
341	TG(16:0/16:0/18:3)	TG	C53 H96 O6	828.7207
342	TG(16:0/16:1/18:1)	TG	C53 H98 O6	830.7363
343	TG(14:0/18:2/18:2)	TG	C53 H94 O6	826.705
344	TG(16:0/16:0/18:3)	TG	C53 H96 O6	828.7207
345	TG(16:0/16:0/18:1)	TG	C53 H100 O6	832.752
346	TG(16:0/16:0/18:1)	TG	C53 H100 O6	832.752
347	TG(16:0/16:0/18:1)	TG	C53 H100 O6	832.752
348	TG(16:0/16:0/18:1)	TG	C53 H100 O6	832.752
349	TG(16:0/17:3/22:1)	TG	C58 H104 O6	896.7833
350	TG(16:1/16:1/23:3)	TG	C58 H102 O6	894.7676
351	TG(54:7)	TG	C57 H96 O6	876.7207
352	TG(16:1/16:1/22:5)	TG	C57 H96 O6	876.7207
353	TG(16:0/18:2/20:4)	TG	C57 H98 O6	878.7363
354	TG(16:0/18:1/20:5)	TG	C57 H98 O6	878.7363
355	TG(16:0/18:1/20:4)	TG	C57 H100 O6	880.752
356	TG(18:2/18:2/18:2)	TG	C57 H98 O6	878.7363
357	TG(16:0/18:1/20:4)	TG	C57 H100 O6	880.752
358	TG(16:0/18:1/20:4)	TG	C57 H100 O6	880.752
359	TG(16:0/18:1/20:4)	TG	C57 H100 O6	880.752
360	TG(18:1/18:2/18:2)	TG	C57 H100 O6	880.752
361	TG(16:0/18:1/20:3)	TG	C57 H102 O6	882.7676
362	TG(16:1/16:1/22:3)	TG	C57 H100 O6	880.752
363	TG(18:0/18:1/18:3)	TG	C57 H102 O6	882.7676
364	TG(18:1/18:1/18:2)	TG	C57 H102 O6	882.7676
365	TG(18:0/18:1/18:3)	TG	C57 H102 O6	882.7676
366	TG(18:0/16:0/20:4)	TG	C57 H102 O6	882.7676
367	TG(20:0/16:1/18:3)	TG	C57 H102 O6	882.7676
368	TG(16:0/18:1/20:3)	TG	C57 H102 O6	882.7676
369	TG(18:1/18:1/18:1)	TG	C57 H104 O6	884.7833
370	TG(17:0/17:0/20:3)	TG	C57 H104 O6	884.7833
371	TG(18:0/18:1/18:1)	TG	C57 H106 O6	886.7989
372	TG(18:0/18:1/18:1)	TG	C57 H106 O6	886.7989
373	TG(16:1/16:1/21:2)	TG	C56 H100 O6	868.752
374	TG(16:0/16:1/20:5)	TG	C55 H94 O6	850.705
375	TG(30:6/11:0/11:0)	TG	C55 H94 O6	850.705
376	TG(6:0/22:6/24:0)	TG	C55 H94 O6	850.705

377	TG(16:0/16:0/20:5)	TG	C55 H96 O6	852.7207
378	TG(16:1/18:2/18:2)	TG	C55 H96 O6	852.7207
379	TG(52:5)	TG	C55 H96 O6	852.7207
380	TG(16:0/16:0/20:4)	TG	C55 H98 O6	854.7363
381	TG(18:0/16:0/16:0)	TG	C53 H102 O6	834.7676
382	TG(18:0/16:0/16:0)	TG	C53 H102 O6	834.7676
383	TG(16:0/16:1/16:1)	TG	C51 H94 O6	802.705
384	TG(16:0/14:0/18:3)	TG	C51 H92 O6	800.6894
385	TG(16:0/16:0/18:1)	TG	C53 H100 O6	832.752
386	TG(16:0/16:0/18:1)	TG	C53 H100 O6	832.752
387	TG(18:0/16:0/16:0)	TG	C53 H102 O6	834.7676
388	TG(18:0/16:0/16:0)	TG	C53 H102 O6	834.7676
389	TG(4:0/20:4/22:5)	TG	C49 H76 O6	760.5642
390	TG(16:0/14:0/18:1)	TG	C51 H96 O6	804.7207
391	TG(36:1e)	TG	C39 H74 O5	622.5536
392	TG(56:6)	TG	C59 H102 O6	906.7676
393	TG(18:1/18:1/20:4)	TG	C59 H102 O6	906.7676
394	TG(18:1/17:3/21:2)	TG	C59 H102 O6	906.7676
395	TG(18:1/18:1/20:4)	TG	C59 H102 O6	906.7676
396	TG(27:4/8:0/21:3)	TG	C59 H100 O6	904.752
397	TG(56:7)	TG	C59 H100 O6	904.752
398	TG(26:4/15:1/15:2)	TG	C59 H100 O6	904.752
399	ZyE(0:0)	ZyE	C27 H44 O1	384.3392
400	ZyE(0:0)	ZyE	C27 H44 O1	384.3392
401	ZyE(0:0)	ZyE	C27 H44 O1	384.3392
402	ZyE(18:2)	ZyE	C45 H74 O2	646.5689
403	ZyE(18:2)	ZyE	C45 H74 O2	646.5689

Table B. The whole identified lipid information in validation and internal standard allocation

Identified lipids	Formula	Internal Standard	Adduct	RT
AEA(16:0)	C18H37NO2	18:1(d7) LPC	M+H	4.274
AcCa(16:0)	C23H45NO4	18:1(d7) LPC	M+H	3.177
Sphinganine 1-phosphate (d18:0-P)	C18H40NO5P	18:1(d7) LPC	M+H	4.36
Sphingosine 1-phosphate (d18:1-P)	C18H38NO5P	18:1(d7) LPC	M+H	3.76
7-oxocholesterol	C27H44O2	18:1(d7) LPC	M+H	3.18
7-oxocholesterol	C27H44O3	18:1(d7) LPC	M+H	5.21
Campesterol	C28H48O	18:1(d7) LPC	M+H	4.19
SPH(d18:1)	C18H37NO2	18:1(d7) LPC	M+H	3.415
SPH(d18:0)	C18H39NO2	18:1(d7) LPC	M+H	3.586
SPHP(d21:1)	C21H44NO5P	18:1(d7) LPE	M+CH3COO	3.436
Lauric acid	C12H24O2	18:1(d7) LPE	M-H	2.07
Myristic acid	C14H28O2	18:1(d7) LPE	M-H	3.1
Pentadecanoic acid	C15H30O2	18:1(d7) LPE	M-H	3.63
cis-9-palmitoleic acid	C16H30O2	18:1(d7) LPE	M-H	3.38
Margaric acid	C17H34O2	18:1(d7) LPE	M-H	4.61
10Z-heptadecenoic acid	C17H32O2	18:1(d7) LPE	M-H	3.91
Stearic acid	C18H36O2	18:1(d7) LPE	M-H	5.04
Oleic acid	C18H34O2	18:1(d7) LPE	M-H	4.37
Linoleic acid	C18H32O2	18:1(d7) LPE	M-H	3.71
a/y-linolenic acid	C18H30O2	18:1(d7) LPE	M-H	3.09
Stearidonic acid	C18H28O2	18:1(d7) LPE	M-H	2.51
Arachidic acid	C20H40O2	18:1(d7) LPE	M-H	5.73
Arachidic acid	C20H40O2	18:1(d7) LPE	M-H	5.31
11,14-eicosadienoic acid	C20H36O2	18:1(d7) LPE	M-H	4.58
11,14,17-eicosatrienoic acid	C20H34O2	18:1(d7) LPE	M-H	4.05
Arachidonic acid	C20H32O2	18:1(d7) LPE	M-H	3.58
5,8,11,14,17-eicosapentaenoic acid	C20H30O2	18:1(d7) LPE	M-H	2.93
Behenic acid	C22H44O2	18:1(d7) LPE	M-H	6.34
cis-erucic acid	C22H42O2	18:1(d7) LPE	M-H	5.79
13,16-docosadienoic acid	C22H40O2	18:1(d7) LPE	M-H	5.33
13,16,19-docosatrienoic acid	C22H38O2	18:1(d7) LPE	M-H	4.92
7Z,10Z,13Z,16Z-docosatetraenoic acid	C22H36O2	18:1(d7) LPE	M-H	4.37
7,10,13,16,19-docosapentaenoic acid	C22H34O2	18:1(d7) LPE	M-H	3.8
4, 7,10,13,16,19-docosahexaenoic acid	C22H32O2	18:1(d7) LPE	M-H	3.33
Lignoceric acid	C24H48O2	18:1(d7) LPE	M-H	6.3
cis-selacholeic acid	C24H46O2	18:1(d7) LPE	M-H	6.34
Cerotic acid	C26H52O2	18:1(d7) LPE	M-H	7.03
2,3-dinor-11ß-PGF2a	C18H30O5	18:1(d7) LPE	M-H	0.88
12-HHTrE	C17H28O3	18:1(d7) LPE	M-H	1.22
PGD3	C20H30O5	18:1(d7) LPE	M-H	3.37
12,13-DiHOME	C18H34O4	18:1(d7) LPE	M-H	2.48

19,20-DiHDPA	C22H34O4	18:1(d7) LPE	M-H	3.71
FA(16:0)	O2H32C16	18:1(d7) LPE	M-H	4.173
FA(23:0)	O2H46C23	18:1(d7) LPE	M-H	6.298
Cer(d18:1_16:0)	C34H67NO3	18:1(d9) SM	M+H	7.093
Cer(d18:1_18:0)	C36H71NO3	18:1(d9) SM	M+H	7.514
Cer(d16:1_22:0)	C38H75NO3	18:1(d9) SM	M+H	7.974
Cer(d18:2_22:0)	C40H77NO3	18:1(d9) SM	M+H	8.052
Cer(d18:1_22:0)	C40H79NO3	18:1(d9) SM	M+H	8.497
Cer(d18:0_22:0)	C40H81NO3	18:1(d9) SM	M+H	8.706
Cer(d18:2_23:0)	C41H79NO3	18:1(d9) SM	M+H	8.316
Cer(d18:1_23:0)	C41H81NO3	18:1(d9) SM	M+H	8.788
Cer(d18:2_24:1)	C42H79NO3	18:1(d9) SM	M+H	8.055
Cer(d18:1_24:1)	C42H81NO3	18:1(d9) SM	M+H	8.59
Cer(d18:0_24:1)	C42H83NO3	18:1(d9) SM	M+H	9.11
Cer(d18:0_24:0)	C42H85NO3	18:1(d9) SM	M+H	9.372
Cer(d18:1_26:1)	C44H85NO3	18:1(d9) SM	M+H	9.141
Cer(d18:1_26:0)	C44H87NO3	18:1(d9) SM	M+H	9.873
Cer(d18:0/16:0)	C34H69NO3	18:1(d9) SM	M+H	7.26
Cer(d18:0/18:0)	C36H73NO3	18:1(d9) SM	M+H	7.68
Cer(d18:0/20:0)	C38H77NO3	18:1(d9) SM	M+H	8.15
Cer(d18:0/26:0)	C44H89NO3	18:1(d9) SM	M+H	10.12
Cer(d18:1/19:0)	C37H73NO3	18:1(d9) SM	M+H	7.74
Cer(d18:1/25:0)	C43H85NO3	18:1(d9) SM	M+H	9.44
Cer(d18:2/20:0)	C38H73NO3	18:1(d9) SM	M+H	7.56
Cer(d18:2/24:2)	C42H77NO3	18:1(d9) SM	M+H	8.53
DG(16:1_14:0)	C33H62O5	15:0-18:1(d7) DG	M+NH4	7.103
DG(16:0_14:0)	C33H64O5	15:0-18:1(d7) DG	M+NH4	7.445
DG(14:0_18:3)	C35H62O5	15:0-18:1(d7) DG	M+NH4	6.926
DG(14:0_18:2)	C35H64O5	15:0-18:1(d7) DG	M+NH4	7.195
DG(16:0_16:1)	C35H66O5	15:0-18:1(d7) DG	M+NH4	7.499
DG(16:0_16:0)	C35H68O5	15:0-18:1(d7) DG	M+NH4	7.891
DG(16:1_18:3)	C37H64O5	15:0-18:1(d7) DG	M+NH4	7.127
DG(16:0_18:3)	C37H66O5	15:0-18:1(d7) DG	M+H	6.319
DG(16:1_18:2)	C37H66O5	15:0-18:1(d7) DG	M+NH4	7.36
DG(34:3)	C37H66O5	15:0-18:1(d7) DG	M+H	7.904
DG(16:0_18:2)	C37H68O5	15:0-18:1(d7) DG	M+NH4	7.604
DG(16:0_18:1)	C37H70O5	15:0-18:1(d7) DG	M+NH4	7.948
DG(16:0_18:1)	C37H70O5	15:0-18:1(d7) DG	M+NH4	8.894
DG(18:0_16:0)	C37H72O5	15:0-18:1(d7) DG	M+NH4	8.42
DG(18:3_18:2)	C39H66O5	15:0-18:1(d7) DG	M+NH4	7.162
DG(18:1_18:3)	C39H68O5	15:0-18:1(d7) DG	M+H	6.375
DG(18:2_18:2)	C39H68O5	15:0-18:1(d7) DG	M+NH4	7.365
DG(18:1_18:2)	C39H70O5	15:0-18:1(d7) DG	M+NH4	7.661
DG(36:3)	C39H70O5	15:0-18:1(d7) DG	M+H	8.437

DG(18:1_18:1)	C39H72O5	15:0-18:1(d7) DG	M+NH4	8.002
DG(18:0_18:1)	C39H74O5	15:0-18:1(d7) DG	M+NH4	8.482
DG(18:0_18:0)	C39H76O5	15:0-18:1(d7) DG	M+NH4	9.033
DG(18:1_20:3)	C41H72O5	15:0-18:1(d7) DG	M+NH4	7.84
DG(18:1_20:3)	C41H72O5	15:0-18:1(d7) DG	M+NH4	7.986
DG(18:1_22:5)	C43H72O5	15:0-18:1(d7) DG	M+NH4	7.628
1,2-DG(30:2)	C33H60O5	15:0-18:1(d7) DG	M+NH4	6.86
1,2-DG(38:0)	C41H80O5	15:0-18:1(d7) DG	M+NH4	8.26
1,2-DG(38:2)	C41H76O5	15:0-18:1(d7) DG	M+NH4	13.96
1,2-DG(38:3)	C41H74O5	15:0-18:1(d7) DG	M+NH4	7.68
1,2-DG(38:3)	C41H74O5	15:0-18:1(d7) DG	M+NH4	8.13
1,2-DG(38:3)	C41H74O5	15:0-18:1(d7) DG	M+NH4	12.87
1,2-DG(40:4)	C43H76O5	15:0-18:1(d7) DG	M+NH4	13.36
1,2-DG(40:7)	C43H70O5	15:0-18:1(d7) DG	M+NH4	7.47
LPC(16:1)	C24H48NO7P	18:1(d7) LPC	M+H	2.643
LPC(16:0)	C24H50NO7P	18:1(d7) LPC	M+H	3.424
LPC(18:2)	C26H50NO7P	18:1(d7) LPC	M+H	2.937
LPC(18:1)	C26H52NO7P	18:1(d7) LPC	M+H	3.601
LPC(18:0)	C26H54NO7P	18:1(d7) LPC	M+H	4.33
LPC(20:4)	C28H50NO7P	18:1(d7) LPC	M+H	2.843
LPC(20:4)	C28H50NO7P	18:1(d7) LPC	M+H	3.597
LPC(20:3)	C28H52NO7P	18:1(d7) LPC	M+H	3.284
LPC(20:3)	C28H52NO7P	18:1(d7) LPC	M+H	4.328
LPC(22:6)	C30H50NO7P	18:1(d7) LPC	M+H	2.718
LPC(22:5)	C30H52NO7P	18:1(d7) LPC	M+H	3.032
LPE(16:0)	C21H44NO7P	18:1(d7) LPE	M-H	3.551
LPE(18:2)	C23H44NO7P	18:1(d7) LPE	M-H	3.065
LPE(18:0)	C23H48NO7P	18:1(d7) LPE	M-H	3.4
LPE(18:0)	C23H48NO7P	18:1(d7) LPE	M-H	4.43
LPE(18:1)	C23H46NO7P	18:1(d7) LPE	M-H	3.73
LPE(20:4)	C25H44NO7P	18:1(d7) LPE	M-H	2.95
LPE(22:1)	C27H54NO7P	18:1(d7) LPE	M+H	4.01
LPE(22:6)	C27H44NO7P	18:1(d7) LPE	M-H	2.83
PC(30:1)	C38H74NO8P	15:0-18:1(d7) PC	M+H	6.232
PC(18:2_13:0)	C39H74NO8P	15:0-18:1(d7) PC	M+H	6.766
PC(18:1_13:0)	C39H76NO8P	15:0-18:1(d7) PC	M+H	7.01
PC(31:1)	C39H76NO8P	15:0-18:1(d7) PC	M+H	7.185
PC(31:0)	C39H78NO8P	15:0-18:1(d7) PC	M+H	6.736
PC(14:0_18:2)	C40H76NO8P	15:0-18:1(d7) PC	M+H	6.326
PC(16:0_16:1)	C40H78NO8P	15:0-18:1(d7) PC	M+H	6.602
PC(33:5)	C41H72NO8P	15:0-18:1(d7) PC	M+H	6.494
PC(33:4)	C41H74NO8P	15:0-18:1(d7) PC	M+H	6.729
PC(33:3)	C41H76NO8P	15:0-18:1(d7) PC	M+H	6.306
PC(33:3)	C41H76NO8P	15:0-18:1(d7) PC	M+H	6.807

PC(34:3e)	C42H80NO7P	15:0-18:1(d7) PC	M+H	6.908
PC(33:2)	C41H78NO8P	15:0-18:1(d7) PC	M+H	6.512
PC(34:2e)	C42H82NO7P	15:0-18:1(d7) PC	M+H	6.956
PC(33:1)	C41H80NO8P	15:0-18:1(d7) PC	M+H	6.801
PC(33:1)	C41H80NO8P	15:0-18:1(d7) PC	M+H	7.129
PC(34:1e)	C42H84NO7P	15:0-18:1(d7) PC	M+H	7.255
PC(33:0)	C41H82NO8P	15:0-18:1(d7) PC	M+H	7.113
PC(33:0)	C41H82NO8P	15:0-18:1(d7) PC	M+H	7.382
PC(14:0_20:5)	C42H74NO8P	15:0-18:1(d7) PC	M+CH3COO	6.042
PC(14:0_20:4)	C42H76NO8P	15:0-18:1(d7) PC	M+H	6.29
PC(34:3)	C42H78NO8P	15:0-18:1(d7) PC	M+H	5.614
PC(34:3)	C42H78NO8P	15:0-18:1(d7) PC	M+H	6.497
PC(34:2)	C42H80NO8P	15:0-18:1(d7) PC	M+H	0.613
PC(34:2)	C42H80NO8P	15:0-18:1(d7) PC	M+H	6.727
PC(16:0_18:1)	C42H82NO8P	15:0-18:1(d7) PC	M+H	6.98
PC(18:0_16:0)	C42H84NO8P	15:0-18:1(d7) PC	M+CH3COO	7.322
PC(35:5)	C43H76NO8P	15:0-18:1(d7) PC	M+H	6.252
PC(35:4)	C43H78NO8P	15:0-18:1(d7) PC	M+H	7.09
PC(36:4e)	C44H82NO7P	15:0-18:1(d7) PC	M+H	6.909
PC(35:3)	C43H80NO8P	15:0-18:1(d7) PC	M+H	6.613
PC(35:2)	C43H82NO8P	15:0-18:1(d7) PC	M+H	6.885
PC(35:1)	C43H84NO8P	15:0-18:1(d7) PC	M+H	7.172
PC(36:6)	C44H76NO8P	15:0-18:1(d7) PC	M+H	6.219
PC(16:0_20:5)	C44H78NO8P	15:0-18:1(d7) PC	M+H	6.439
PC(36:4)	C44H80NO8P	15:0-18:1(d7) PC	M+H	0.613
PC(18:2_18:2)	C44H80NO8P	15:0-18:1(d7) PC	M+H	6.486
PC(36:4)	C44H80NO8P	15:0-18:1(d7) PC	M+H	6.651
PC(36:4)	C44H80NO8P	15:0-18:1(d7) PC	M+H	7.141
PC(16:0_20:3)	C44H82NO8P	15:0-18:1(d7) PC	M+H	6.821
PC(36:2)	C44H84NO8P	15:0-18:1(d7) PC	M+H	0.614
PC(18:0_18:2)	C44H84NO8P	15:0-18:1(d7) PC	M+H	7.098
PC(35:1CHO)	C43H82NO9P	15:0-18:1(d7) PC	M+H	5.665
PC(18:0_18:1)	C44H86NO8P	15:0-18:1(d7) PC	M+H	7.376
PC(34:2+OO)	C42H80NO10P	15:0-18:1(d7) PC	M+H	4.964
PC(34:2+OO)	C42H80NO10P	15:0-18:1(d7) PC	M+H	5.471
PC(38:7e)	C46H80NO7P	15:0-18:1(d7) PC	M+H	6.793
PC(37:6)	C45H78NO8P	15:0-18:1(d7) PC	M+H	6.407
PC(38:6e)	C46H82NO7P	15:0-18:1(d7) PC	M+H	6.89
PC(37:5)	C45H80NO8P	15:0-18:1(d7) PC	M+H	6.545
PC(38:5e)	C46H84NO7P	15:0-18:1(d7) PC	M+H	6.927
PC(37:4)	C45H82NO8P	15:0-18:1(d7) PC	M+H	6.854
PC(38:4e)	C46H86NO7P	15:0-18:1(d7) PC	M+H	7.316
PC(37:1)	C45H88NO8P	15:0-18:1(d7) PC	M+H	7.591
PC(38:7)	C46H78NO8P	15:0-18:1(d7) PC	M+H	6.389

PC(38:7)	C46H78NO8P	15:0-18:1(d7) PC	M+H	6.635
PC(16:0_22:6)	C46H80NO8P	15:0-18:1(d7) PC	M+H	6.591
PC(16:0_22:5)	C46H82NO8P	15:0-18:1(d7) PC	M+H	6.822
PC(18:0_20:4)	C46H84NO8P	15:0-18:1(d7) PC	M+H	7.028
PC(18:0_20:3)	C46H86NO8P	15:0-18:1(d7) PC	M+H	7.203
PC(36:4+OO)	C44H80NO10P	15:0-18:1(d7) PC	M+H	4.83
PC(36:4+OO)	C44H80NO10P	15:0-18:1(d7) PC	M+H	5.61
PC(18:0_20:2)	C46H88NO8P	15:0-18:1(d7) PC	M+H	7.464
PC(36:3+2O)	C44H82NO10P	15:0-18:1(d7) PC	M+H	5.632
PC(36:2+2O)	C44H84NO10P	15:0-18:1(d7) PC	M+H	5.911
PC(39:6)	C47H82NO8P	15:0-18:1(d7) PC	M+H	6.78
PC(18:2_22:6)	C48H80NO8P	15:0-18:1(d7) PC	M+H	6.373
PC(18:1_22:6)	C48H82NO8P	15:0-18:1(d7) PC	M+H	6.618
PC(18:0_22:6)	C48H84NO8P	15:0-18:1(d7) PC	M+H	6.96
PC(40:5)	C48H86NO8P	15:0-18:1(d7) PC	M+H	6.588
PC(18:0_22:5)	C48H86NO8P	15:0-18:1(d7) PC	M+H	7.081
PC(38:6+OO)	C46H80NO10P	15:0-18:1(d7) PC	M+H	4.743
PC(38:6+OO)	C46H80NO10P	15:0-18:1(d7) PC	M+H	5.537
PC(18:0_22:4)	C48H88NO8P	15:0-18:1(d7) PC	M+H	7.321
PC(38:4+OO)	C46H84NO10P	15:0-18:1(d7) PC	M+H	5.581
PC(38:4+OO)	C46H84NO10P	15:0-18:1(d7) PC	M+H	6.067
PC(22:0_18:2)	C48H92NO8P	15:0-18:1(d7) PC	M+H	7.956
PC(44:5e)	C52H96NO7P	15:0-18:1(d7) PC	M+H	8.248
PE(16:0_18:2)	C39H74NO8P	15:0-18:1(d7) PE	M-H	6.786
PE(16:0_18:1)	C39H76NO8P	15:0-18:1(d7) PE	M-H	7.012
PE(18:0_16:0)	C39H78NO8P	15:0-18:1(d7) PE	M-H	7.337
PE(16:0_20:5)	C41H72NO8P	15:0-18:1(d7) PE	M-H	6.501
PE(16:0_20:4)	C41H74NO8P	15:0-18:1(d7) PE	M+H	6.552
PE(16:0_20:4)	C41H74NO8P	15:0-18:1(d7) PE	M-H	6.736
PE(36:3)	C41H76NO8P	15:0-18:1(d7) PE	M+H	6.051
PE(18:1_18:2)	C41H76NO8P	15:0-18:1(d7) PE	M-H	6.843
PE(36:2)	C41H78NO8P	15:0-18:1(d7) PE	M-H	6.394
PE(36:2)	C41H78NO8P	15:0-18:1(d7) PE	M-H	6.846
PE(18:0_18:2)	C41H78NO8P	15:0-18:1(d7) PE	M-H	7.118
PE(18:0_18:1)	C41H80NO8P	15:0-18:1(d7) PE	M-H	7.392
PE(16:1e_22:6)	C43H74NO7P	15:0-18:1(d7) PE	M-H	6.85
PE(16:0p_22:6)	C43H74NO7P	15:0-18:1(d7) PE	M+H	6.856
PE(38:1e)	C43H86NO7P	15:0-18:1(d7) PE	M+H	6.617
PE(38:6)	C43H74NO8P	15:0-18:1(d7) PE	M+H	6.641
PE(40:7e)	C45H78NO7P	15:0-18:1(d7) PE	M+H	7.006
PE(18:0p_22:6)	C45H78NO7P	15:0-18:1(d7) PE	M+H	7.218
PE(43:4)	C48H88NO8P	15:0-18:1(d7) PE	M+H	10.937
PE(32:1)	C37H72NO8P	15:0-18:1(d7) PE	M-H	5.89
PE(32:1)	C37H72NO8P	15:0-18:1(d7) PE	M-H	6.66

PE(32:1)	C37H72NO8P	15:0-18:1(d7) PE	M-H	6.85
PE(40:7)	C45H76NO8P	15:0-18:1(d7) PE	M-H	5.97
PE(40:7)	C45H76NO8P	15:0-18:1(d7) PE	M-H	6.69
PE(40:7)	C45H76NO8P	15:0-18:1(d7) PE	M-H	6.82
PE(42:5)	C47H84NO8P	15:0-18:1(d7) PE	M+H	6.9
PE(42:7)	C47H80NO8P	15:0-18:1(d7) PE	M-H	6.15
PG(16:0_18:1)	C40H77O10P	15:0-18:1(d7) PG	M-H	6.854
PG(34:2)	C40H75O10P	15:0-18:1(d7) PG	M-H	6.34
PG(36:1)	C42H81O10P	15:0-18:1(d7) PG	M-H	6.88
PG(36:1)	C42H81O10P	15:0-18:1(d7) PG	M-H	7.22
PG(36:2)	C42H79O10P	15:0-18:1(d7) PG	M-H	6.88
PG(36:3)	C42H77O10P	15:0-18:1(d7) PG	M-H	6.34
PG(36:4)	C42H75O10P	15:0-18:1(d7) PG	M-H	6.31
PG(36:5)	C42H73O10P	15:0-18:1(d7) PG	M+H	6.29
PG(38:4)	C44H79O10P	15:0-18:1(d7) PG	M+H	6.93
PG(38:6)	C44H75O10P	15:0-18:1(d7) PG	M+H	6.51
PG(40:6)	C46H79O10P	15:0-18:1(d7) PG	M+H	6.95
PG(40:8)	C46H75O10P	15:0-18:1(d7) PG	M+H	5.5
Hex2Cer(d12:0_20:4)	C44H77NO13	15:0-18:1(d7) PI	M+CH3COO	6.528
PI(16:0_16:1)	C41H77O13P	15:0-18:1(d7) PI	M-H	6.129
PI(16:0_16:0)	C41H79O13P	15:0-18:1(d7) PI	M-H	6.414
PI(34:2)	C43H79O13P	15:0-18:1(d7) PI	M-H	6.225
PI(34:1)	C43H81O13P	15:0-18:1(d7) PI	M-H	6.47
PI(16:0_20:4)	C45H79O13P	15:0-18:1(d7) PI	M-H	6.196
PI(36:3)	C45H81O13P	15:0-18:1(d7) PI	M-H	6.331
PI(18:0_18:2)	C45H83O13P	15:0-18:1(d7) PI	M-H	6.563
PI(16:0_22:6)	C47H79O13P	15:0-18:1(d7) PI	M-H	6.143
PI(18:0_20:4)	C47H83O13P	15:0-18:1(d7) PI	M-H	6.537
PI(18:0_22:6)	C49H83O13P	15:0-18:1(d7) PI	M-H	6.483
PI(18:0_22:5)	C49H85O13P	15:0-18:1(d7) PI	M-H	6.569
PI(22:1_18:2)	C49H89O13P	15:0-18:1(d7) PI	M-H	6.419
PI(34:0)	C43H83O13P	15:0-18:1(d7) PI	M-H	6.21
PI(36:0)	C45H87O13P	15:0-18:1(d7) PI	M-H	6.59
PI(36:1)	C45H85O13P	15:0-18:1(d7) PI	M-H	6.31
PI(36:1)	C45H85O13P	15:0-18:1(d7) PI	M-H	6.82
PI(36:5)	C45H77O13P	15:0-18:1(d7) PI	M-H	5.97
PI(38:2)	C47H87O13P	15:0-18:1(d7) PI	M-H	6.37
PI(38:3)	C47H85O13P	15:0-18:1(d7) PI	M-H	6.66
PI(38:5)	C47H81O13P	15:0-18:1(d7) PI	M-H	6.27
PI(40:4)	C49H87O13P	15:0-18:1(d7) PI	M-H	6.31
PI(40:4)	C49H87O13P	15:0-18:1(d7) PI	M-H	6.88
PS(34:0)	C40H78NO10P	15:0-18:1(d7) PS	M+H	6.381
PS(42:3)	C48H88NO10P	15:0-18:1(d7) PS	M-H	6.084
PC(32:0)	C40H80NO8P	15:0-18:1(d7) PC	M+H	6.92

PC(36:0)	C44H88NO8P	15:0-18:1(d7) PC	M+H	7.77
PS(32:1)	C38H72NO10P	15:0-18:1(d7) PS	M-H	5.97
PS(34:1)	C40H76NO10P	15:0-18:1(d7) PS	M-H	5.92
PS(34:2)	C40H74NO10P	15:0-18:1(d7) PS	M-H	6.75
PS(36:0)	C42H82NO10P	15:0-18:1(d7) PS	M-H	6.59
PS(36:2)	C42H78NO10P	15:0-18:1(d7) PS	M+H	5.16
PS(36:2)	C42H78NO10P	15:0-18:1(d7) PS	M-H	6.1
PS(38:4)	C44H78NO10P	15:0-18:1(d7) PS	M-H	5.46
PS(38:4)	C44H78NO10P	15:0-18:1(d7) PS	M-H	6.05
PS(38:5)	C44H76NO10P	15:0-18:1(d7) PS	M+H	5.09
PS(40:4)	C46H82NO10P	15:0-18:1(d7) PS	M-H	6.43
SM(d32:2)	C37H73N2O6P	18:1(d9) SM	M+H	5.765
SM(d35:1)	C40H81N2O6P	18:1(d9) SM	M+H	6.764
SM(d37:1)	C42H85N2O6P	18:1(d9) SM	M+H	7.165
SM(d39:1)	C44H89N2O6P	18:1(d9) SM	M+H	7.617
SM(d41:7)	C46H81N2O6P	18:1(d9) SM	M+H	6.326
SM(t40:7)	C45H79N2O7P	18:1(d9) SM	M+H	6.761
SM(d43:2)	C48H95N2O6P	18:1(d9) SM	M+H	8.025
SM(d44:1)	C49H99N2O6P	18:1(d9) SM	M+H	8.996
TG(12:1e_6:0_16:0)	C37H70O5	15:0-18:1(d7)-15:0 TG	M+H	7.959
TG(12:1e_6:0_18:4)	C39H66O5	15:0-18:1(d7)-15:0 TG	M+H	6.119
TG(12:1e_6:0_18:2)	C39H70O5	15:0-18:1(d7)-15:0 TG	M+H	7.667
TG(12:1e_6:0_20:5)	C41H68O5	15:0-18:1(d7)-15:0 TG	M+H	7.669
TG(12:1e_6:0_20:4)	C41H70O5	15:0-18:1(d7)-15:0 TG	M+NH4	7.586
TG(16:0_14:4_16:0)	C49H86O6	15:0-18:1(d7)-15:0 TG	M+NH4	10.018
TG(18:1_12:3_18:1)	C51H88O6	15:0-18:1(d7)-15:0 TG	M+NH4	10.356
TG(16:1_14:0_18:2)	C51H92O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.656
TG(16:0_16:1_16:1)	C51H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.771
TG(16:0_16:0_16:1)	C51H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.788
TG(16:0_14:0_18:1)	C51H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.001
TG(16:0_16:0_16:0)	C51H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	7.656
TG(52:12e)	C55H84O5	15:0-18:1(d7)-15:0 TG	M+NH4	12.744
TG(16:1_16:1_18:3)	C53H92O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.191
TG(16:1_16:1_18:3)	C53H92O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.398
TG(16:0_14:0_20:5)	C53H92O6	15:0-18:1(d7)-15:0 TG	M+H	12.732
TG(16:0_16:1_18:3)	C53H94O6	15:0-18:1(d7)-15:0 TG	M+H	8.874
TG(14:0_18:2_18:2)	C53H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.967
TG(16:0_16:0_18:3)	C53H96O6	15:0-18:1(d7)-15:0 TG	M+H	6.324
TG(16:1_16:1_18:1)	C53H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.928
TG(16:0_16:1_18:1)	C53H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.25
TG(16:0_16:0_18:1)	C53H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	6.888
TG(16:0_16:0_18:1)	C53H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	7.597
TG(18:0_16:0_16:0)	C53H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	9.239
TG(48:2+3O)	C51H94O9	15:0-18:1(d7)-15:0 TG	M+H	11.961

TG(16:1_18:2_18:3)	C55H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.611
TG(16:0_16:1_20:5)	C55H94O6	15:0-18:1(d7)-15:0 TG	M+H	12.941
TG(16:0_16:0_20:5)	C55H96O6	15:0-18:1(d7)-15:0 TG	M+H	0.585
TG(16:1_18:2_18:2)	C55H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.358
TG(16:0_16:0_20:5)	C55H96O6	15:0-18:1(d7)-15:0 TG	M+H	14.272
TG(16:0_18:2_18:2)	C55H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.274
TG(18:0_16:0_18:3)	C55H100O6	15:0-18:1(d7)-15:0 TG	M+H	9.245
TG(16:0_18:1_18:2)	C55H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.57
TG(16:0_18:1_18:1)	C55H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	6.452
TG(18:3+OO_16:0_16:0)	C53H96O8	15:0-18:1(d7)-15:0 TG	M+NH4	8.72
TG(18:0_16:0_18:1)	C55H104O6	15:0-18:1(d7)-15:0 TG	M+NH4	9.54
TG(54:8)	C57H94O6	15:0-18:1(d7)-15:0 TG	M+H	12.289
TG(18:3_18:2_18:2)	C57H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.109
TG(16:0_18:2_20:5)	C57H96O6	15:0-18:1(d7)-15:0 TG	M+H	13.289
TG(18:2_18:2_18:2)	C57H98O6	15:0-18:1(d7)-15:0 TG	M+H	0.586
TG(18:2_18:2_18:2)	C57H98O6	15:0-18:1(d7)-15:0 TG	M+H	5.972
TG(18:2_18:2_18:2)	C57H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.441
TG(18:2_18:2_18:2)	C57H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.015
TG(18:1_18:2_18:2)	C57H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.45
TG(18:1_18:2_18:2)	C57H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.213
TG(18:0_18:1_18:3)	C57H102O6	15:0-18:1(d7)-15:0 TG	M+H	9.588
TG(18:1_18:1_18:2)	C57H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.745
TG(18:1_18:1_18:1)	C57H104O6	15:0-18:1(d7)-15:0 TG	M+NH4	7.701
TG(18:1_18:1_18:1)	C57H104O6	15:0-18:1(d7)-15:0 TG	M+NH4	8.37
TG(18:0_18:0_18:3)	C57H104O6	15:0-18:1(d7)-15:0 TG	M+H	12.184
TG(18:0_18:1_18:1)	C57H106O6	15:0-18:1(d7)-15:0 TG	M+NH4	9.779
TG(18:2+OO_16:0_18:1)	C55H100O8	15:0-18:1(d7)-15:0 TG	M+NH4	9.323
TG(17:0_18:1_20:4)	C58H102O6	15:0-18:1(d7)-15:0 TG	M+H	8.538
TG(55:4)	C58H104O6	15:0-18:1(d7)-15:0 TG	M+H	10.85
TG(56:9)	C59H96O6	15:0-18:1(d7)-15:0 TG	M+H	13.048
TG(16:0_18:3_22:4)	C59H100O6	15:0-18:1(d7)-15:0 TG	M+H	8.836
TG(18:1_18:2_20:4)	C59H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.125
TG(20:3_18:2_18:2)	C59H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.756
TG(20:5+OO_16:0_18:3)	C57H94O8	15:0-18:1(d7)-15:0 TG	M+H	8.562
TG(20:5+OO_16:0_18:3)	C57H94O8	15:0-18:1(d7)-15:0 TG	M+H	8.801
TG(18:1_18:1_20:4)	C59H102O6	15:0-18:1(d7)-15:0 TG	M+H	7.729
TG(18:1_18:1_20:4)	C59H102O6	15:0-18:1(d7)-15:0 TG	M+H	8.665
ChE(16:1)	C43H74O2	18:1(d7) Chol Ester	M+NH4	14.925
ChE(16:0)	C43H76O2	18:1(d7) Chol Ester	M+NH4	8.096
ChE(18:3)	C45H74O2	18:1(d7) Chol Ester	M+NH4	9.169
ChE(18:3)	C45H74O2	18:1(d7) Chol Ester	M+H	9.398
ChE(18:3)	C45H74O2	18:1(d7) Chol Ester	M+NH4	13.943
ChE(18:1)	C45H78O2	18:1(d7) Chol Ester	M+NH4	8.261
ChE(18:0)	C45H80O2	18:1(d7) Chol Ester	M+NH4	10.991

ChE(20:5)	C47H74O2	18:1(d7) Chol Ester	M+H	9.356
ChE(20:5)	C47H74O2	18:1(d7) Chol Ester	M+NH4	13.38
ChE(20:4)	C47H76O2	18:1(d7) Chol Ester	M+H	9.688
ChE(20:4)	C47H76O2	18:1(d7) Chol Ester	M+NH4	14.629
ChE(20:3)	C47H78O2	18:1(d7) Chol Ester	M+NH4	6.426
ChE(22:6)	C49H76O2	18:1(d7) Chol Ester	M+H	6.314
ChE(22:6)	C49H76O2	18:1(d7) Chol Ester	M+H	8.916
ChE(22:6)	C49H76O2	18:1(d7) Chol Ester	M+NH4	14.019
ChE(22:6)	C49H76O2	18:1(d7) Chol Ester	M+NH4	14.662
ChE(22:6)	C49H76O2	18:1(d7) Chol Ester	M+NH4	14.803
ChE(14:0)	C41H72O2	18:1(d7) Chol Ester	M+NH4	14.84
ChE(15:0)	C42H74O2	18:1(d7) Chol Ester	M+NH4	8.07
ChE(15:0)	C42H74O3	18:1(d7) Chol Ester	M+NH4	8.56
ChE(16:2)	C43H72O2	18:1(d7) Chol Ester	M+NH4	13.65
ChE(17:0)	C44H78O2	18:1(d7) Chol Ester	M+NH4	9.18
ChE(17:1)	C44H76O2	18:1(d7) Chol Ester	M+NH4	8.56
ChE(20:0)	C47H84O2	18:1(d7) Chol Ester	M+NH4	11.01
ChE(20:1)	C47H82O2	18:1(d7) Chol Ester	M+NH4	8.26
ChE(22:0)	C49H88O2	18:1(d7) Chol Ester	M+H	8.46
CoQ9	C54H82O4	18:1(d7) Chol Ester	M+NH4	10.18
Co(Q10)	C59H90O4	18:1(d7) Chol Ester	M+NH4	11.731

Table C. The identified lipid peak area RSD result before and after normalisation

lipids (peak area)	**plasma**		**QC**	
	unnormalised	normalised	unnormalised	normalised
AcCa(16:0)	5.07%	8.27%	1.28%	2.30%
AEA(16:0)	16.42%	17.39%	9.20%	7.72%
Cer(d16:1_22:0)	5.15%	8.04%	5.15%	5.21%
Cer(d18:0_22:0)	5.74%	7.76%	1.69%	2.47%
Cer(d18:0_24:0)	6.33%	8.13%	2.69%	2.76%
Cer(d18:0_24:1)	5.48%	7.64%	1.81%	2.79%
Cer(d18:1_16:0)	5.16%	6.50%	2.36%	2.28%
Cer(d18:1_18:0)	4.84%	6.47%	6.80%	7.53%
Cer(d18:1_22:0)	5.59%	8.54%	3.28%	4.09%
Cer(d18:1_23:0)	4.42%	6.46%	1.96%	2.91%
Cer(d18:1_24:1)	5.08%	7.90%	2.62%	3.60%
Cer(d18:1_26:0)	4.95%	7.31%	7.41%	7.90%
Cer(d18:1_26:1)	16.05%	18.64%	13.61%	13.68%
Cer(d18:2_22:0)	4.21%	7.20%	5.61%	5.69%
Cer(d18:2_23:0)	4.78%	7.70%	1.96%	1.52%
Cer(d18:2_24:1)	4.65%	5.08%	6.79%	7.28%
ChE(16:0)	5.55%	14.86%	40.47%	40.56%

ChE(16:1)	6.81%	14.39%	7.06%	8.30%
ChE(18:0)	10.05%	19.89%	40.73%	40.98%
ChE(18:1)	5.70%	16.49%	40.43%	40.46%
ChE(18:3)	4.65%	13.17%	3.34%	3.43%
ChE(18:3) *1*	4.02%	13.13%	1.18%	5.35%
ChE(18:3) *2*	2.61%	14.30%	0.57%	5.62%
ChE(20:3)	5.85%	16.56%	40.50%	40.45%
ChE(20:4)	5.02%	13.60%	3.80%	8.72%
ChE(20:4) *1*	2.38%	14.16%	1.88%	5.76%
ChE(20:5)	4.73%	12.92%	1.81%	6.54%
ChE(20:5) *1*	2.81%	13.25%	0.91%	5.68%
ChE(22:6)	42.72%	41.92%	51.35%	51.06%
ChE(22:6) *1*	3.65%	14.63%	3.04%	5.69%
ChE(22:6) *2*	3.54%	14.14%	0.92%	5.34%
Co(Q10)	4.72%	14.40%	2.37%	4.42%
DG(14:0_18:2)	3.08%	5.70%	6.32%	1.87%
DG(14:0_18:3)	14.36%	14.94%	10.60%	7.17%
DG(16:0_14:0)	6.17%	8.60%	8.23%	3.42%
DG(16:0_16:0)	4.82%	6.92%	6.15%	1.53%
DG(16:0_16:1)	4.52%	6.89%	7.54%	2.72%
DG(16:0_18:1)	5.51%	7.31%	8.24%	3.42%
DG(16:0_18:2)	4.97%	6.53%	6.37%	2.03%
DG(16:0_18:3)	6.78%	8.62%	9.11%	12.24%
DG(16:1_14:0)	4.60%	5.16%	9.87%	6.92%
DG(16:1_18:2)	5.27%	7.16%	7.92%	3.38%
DG(16:1_18:3)	12.38%	13.01%	7.13%	5.13%
DG(18:0_16:0)	5.94%	7.92%	3.85%	1.55%
DG(18:0_18:0)	31.04%	31.71%	48.54%	41.69%
DG(18:0_18:1)	5.76%	7.79%	3.17%	2.21%
DG(18:1_18:1)	5.10%	7.02%	10.28%	5.26%
DG(18:1_18:2)	4.35%	5.86%	6.26%	2.18%
DG(18:1_18:3)	7.09%	6.82%	9.19%	12.08%
DG(18:1_20:3)	5.72%	7.63%	8.96%	4.27%
DG(18:1_20:3) *1*	5.72%	7.63%	8.96%	4.27%
DG(18:1_22:5)	7.75%	9.34%	9.62%	6.54%
DG(18:2_18:2)	4.05%	6.27%	6.91%	2.27%
DG(18:3_18:2)	4.67%	3.92%	5.56%	3.68%
DG(34:3)	51.09%	53.16%	80.46%	80.93%
DG(36:3)	145.52%	147.88%	94.19%	95.29%
FA(16:0)	4.55%	9.51%	1.23%	2.99%
FA(23:0)	4.10%	8.33%	11.72%	13.05%
Hex2Cer(d12:0_20:4)	17.98%	5.95%	12.36%	6.16%
LPC(16:0)	7.01%	5.12%	1.97%	1.19%
LPC(18:0)	4.77%	5.14%	2.62%	4.68%

LPC(18:1)	5.62%	4.94%	2.54%	0.71%
LPC(18:2)	4.87%	4.95%	0.71%	2.56%
LPC(20:3)	5.20%	6.05%	1.82%	1.55%
LPC(20:4)	4.62%	5.54%	0.90%	2.65%
LPC(22:5)	3.91%	5.94%	1.20%	3.06%
LPE(16:0)	11.35%	5.05%	1.93%	3.28%
LPE(18:2)	7.34%	4.26%	2.02%	1.39%
PC(14:0_18:2)	4.15%	7.11%	3.65%	4.08%
PC(14:0_20:4)	4.47%	6.98%	5.52%	6.13%
PC(14:0_20:5)	8.13%	8.95%	6.09%	6.75%
PC(16:0_16:1)	3.14%	6.59%	2.66%	2.83%
PC(16:0_18:1)	1.82%	5.15%	2.06%	2.56%
PC(16:0_20:3)	2.98%	5.70%	2.32%	2.60%
PC(16:0_20:5)	3.10%	5.99%	2.06%	2.74%
PC(16:0_22:5)	4.01%	6.50%	3.44%	3.88%
PC(16:0_22:6)	2.60%	5.82%	2.65%	2.97%
PC(18:0_16:0)	5.14%	8.59%	4.31%	4.00%
PC(18:0_18:1)	5.98%	8.94%	5.57%	5.64%
PC(18:0_18:2)	2.52%	5.17%	1.45%	1.57%
PC(18:0_20:2)	9.53%	11.61%	10.08%	9.43%
PC(18:0_20:3)	3.06%	5.69%	2.78%	2.88%
PC(18:0_20:4)	2.22%	4.57%	1.19%	1.52%
PC(18:0_22:4)	2.95%	6.30%	4.45%	4.05%
PC(18:0_22:5)	2.59%	5.68%	2.31%	2.86%
PC(18:0_22:6)	1.70%	4.27%	1.57%	1.66%
PC(18:1_13:0)	3.80%	6.24%	5.06%	5.08%
PC(18:1_22:6)	21.39%	23.64%	22.01%	21.79%
PC(18:2_13:0)	4.62%	6.91%	5.21%	5.05%
PC(18:2_18:2)	2.32%	4.93%	1.48%	1.88%
PC(18:2_22:6)	5.64%	7.72%	9.46%	9.45%
PC(22:0_18:2)	7.14%	9.33%	5.18%	5.68%
PC(30:1)	5.53%	8.42%	5.95%	6.08%
PC(31:0)	17.23%	17.89%	22.57%	22.82%
PC(31:1)	3.80%	6.24%	5.06%	5.08%
PC(33:0)	14.44%	14.96%	6.55%	7.08%
PC(33:0) *1*	14.44%	14.96%	6.55%	7.08%
PC(33:1)	4.35%	7.23%	1.43%	1.21%
PC(33:1) *1*	5.68%	8.13%	7.69%	7.34%
PC(33:2)	3.40%	6.73%	3.20%	2.94%
PC(33:3)	12.53%	12.39%	19.67%	19.71%
PC(33:3) *1*	4.66%	5.80%	6.95%	6.95%
PC(33:4)	3.96%	5.81%	4.88%	5.01%
PC(34:1e)	3.18%	6.38%	4.11%	3.73%
PC(34:2) *1*	2.04%	5.12%	1.25%	1.44%

PC(34:2+OO)	8.88%	11.62%	8.28%	8.54%
PC(34:2+OO) *1*	7.70%	9.76%	12.35%	12.65%
PC(34:2e)	3.72%	6.71%	2.81%	2.49%
PC(34:3) *1*	3.31%	6.35%	1.72%	2.01%
PC(34:3e)	3.16%	5.46%	2.40%	2.03%
PC(35:1)	2.92%	6.29%	2.01%	2.13%
PC(35:1CHO)	11.06%	12.01%	21.79%	21.93%
PC(35:2)	2.20%	4.84%	1.92%	1.60%
PC(35:3)	18.36%	21.22%	8.31%	8.76%
PC(35:4)	3.07%	5.34%	4.01%	4.03%
PC(35:5)	7.31%	10.17%	7.25%	7.96%
PC(36:2+2O)	4.07%	6.28%	11.42%	10.80%
PC(36:3+2O)	6.79%	7.84%	13.35%	13.41%
PC(36:4) *1*	2.32%	4.93%	1.48%	1.88%
PC(36:4) *2*	2.32%	4.93%	1.48%	1.88%
PC(36:4+OO)	7.87%	10.73%	6.12%	6.53%
PC(36:4+OO) *1*	9.69%	10.43%	12.12%	12.04%
PC(36:4e)	3.35%	4.99%	2.69%	2.54%
PC(36:6)	8.57%	10.22%	11.32%	11.90%
PC(37:1)	7.64%	8.96%	8.62%	9.05%
PC(37:4)	10.71%	10.95%	3.55%	4.20%
PC(37:5)	26.87%	27.29%	9.51%	9.17%
PC(37:6)	4.21%	6.19%	5.23%	5.42%
PC(38:4+OO)	4.98%	7.56%	10.14%	10.49%
PC(38:4+OO) *1*	5.65%	7.77%	10.41%	10.64%
PC(38:4e)	3.61%	6.69%	5.90%	6.33%
PC(38:5e)	1.74%	4.79%	1.92%	2.17%
PC(38:6+OO)	7.00%	9.98%	7.73%	8.04%
PC(38:6+OO) *1*	10.70%	10.89%	14.17%	14.33%
PC(38:6e)	3.00%	3.99%	3.79%	3.89%
PC(38:7)	12.22%	12.42%	22.21%	22.94%
PC(38:7) *1*	135.82%	135.29%	220.92%	219.80%
PC(38:7e)	3.11%	5.20%	5.82%	5.91%
PC(39:6)	7.37%	8.01%	8.37%	9.04%
PC(40:5)	2.59%	5.68%	2.30%	2.86%
PC(44:5e)	5.07%	7.96%	11.55%	11.72%
PE(16:0_18:1)	6.40%	9.97%	8.74%	7.90%
PE(16:0_18:2)	6.62%	10.90%	13.09%	11.53%
PE(16:0_20:4)	3.96%	7.06%	4.88%	5.26%
PE(16:0_20:4) *1*	5.39%	9.52%	13.83%	11.66%
PE(16:0_20:5)	6.00%	9.66%	10.91%	8.42%
PE(16:0p_22:6)	4.56%	3.68%	5.91%	5.04%
PE(16:1e_22:6)	9.94%	13.87%	12.65%	11.23%
PE(18:0_16:0)	6.58%	9.53%	7.25%	11.55%

PE(18:0_18:1)	3.96%	8.96%	3.96%	5.23%
PE(18:0_18:2)	5.65%	10.06%	7.52%	6.70%
PE(18:0p_22:6)	4.82%	6.82%	8.67%	8.12%
PE(18:1_18:2)	8.57%	12.84%	13.07%	11.21%
PE(36:2) *1*	5.37%	9.91%	7.39%	6.56%
PE(36:3)	12.53%	16.15%	19.67%	17.76%
PE(38:1e)	216.62%	212.17%	182.83%	178.05%
PE(38:6)	3.03%	6.34%	4.93%	4.93%
PE(40:7e)	4.82%	6.82%	8.67%	8.12%
PE(43:4)	79.14%	81.59%	50.93%	52.72%
PG(16:0_18:1)	9.56%	15.83%	12.42%	10.78%
PI(16:0_16:0)	20.64%	9.80%	10.59%	7.44%
PI(16:0_16:1)	18.11%	6.57%	4.10%	2.34%
PI(16:0_20:4)	16.15%	5.93%	4.44%	2.73%
PI(16:0_22:6)	17.77%	7.57%	7.41%	4.01%
PI(18:0_18:2)	17.73%	5.54%	10.69%	4.71%
PI(18:0_20:4)	17.32%	5.26%	11.47%	5.49%
PI(18:0_22:5)	15.55%	9.98%	15.88%	9.79%
PI(18:0_22:6)	15.33%	4.14%	7.26%	2.33%
PI(22:1_18:2)	12.99%	16.71%	26.41%	19.08%
PI(34:1)	18.26%	6.35%	8.63%	3.30%
PI(34:2)	17.40%	6.65%	4.69%	2.36%
PI(36:3)	20.39%	7.65%	5.01%	3.27%
PS(34:0)	13.55%	19.34%	13.70%	17.11%
PS(42:3)	3.74%	17.47%	2.70%	6.56%
SM(d32:2)	5.31%	7.54%	9.77%	9.92%
SM(d35:1)	10.54%	9.32%	2.10%	3.30%
SM(d37:1)	5.69%	5.58%	3.56%	4.39%
SM(d39:1)	7.40%	7.83%	6.18%	6.68%
SM(d41:7)	6.74%	9.23%	5.35%	5.98%
SM(d43:2)	6.00%	7.38%	9.37%	9.78%
SM(d44:1)	9.96%	9.01%	11.55%	11.96%
SM(t40:7)	41.61%	42.88%	64.82%	64.79%
SPH(d18:0)	6.78%	10.40%	2.83%	2.35%
SPH(d18:1)	4.15%	8.35%	2.92%	4.48%
SPHP(d21:1)	10.56%	7.98%	2.42%	3.16%
TG(12:1e_6:0_16:0)	9.11%	12.19%	9.94%	9.08%
TG(12:1e_6:0_18:2)	5.99%	9.46%	9.17%	8.41%
TG(12:1e_6:0_18:4)	6.96%	10.13%	11.28%	11.75%
TG(12:1e_6:0_20:4)	4.60%	7.41%	5.80%	4.83%
TG(12:1e_6:0_20:5)	93.80%	92.83%	155.35%	155.44%
TG(14:0_18:2_18:2)	5.81%	9.94%	0.56%	1.47%
TG(16:0_14:0_18:1)	4.90%	9.13%	1.19%	0.70%
TG(16:0_14:0_20:5)	96.15%	101.05%	46.33%	45.66%

TG(16:0_14:4_16:0)	7.60%	11.70%	7.90%	8.67%
TG(16:0_16:0_16:0)	9.49%	11.57%	40.40%	40.43%
TG(16:0_16:0_16:1)	4.97%	9.17%	1.26%	0.92%
TG(16:0_16:0_18:1)	8.36%	10.01%	40.45%	40.48%
TG(16:0_16:0_18:1) *1*	7.33%	9.29%	40.49%	40.49%
TG(16:0_16:0_18:3)	73.93%	76.52%	93.88%	93.31%
TG(16:0_16:0_20:5) *1*	129.03%	130.17%	60.64%	60.13%
TG(16:0_16:1_16:1)	5.20%	9.32%	0.63%	1.61%
TG(16:0_16:1_18:1)	3.65%	7.90%	0.93%	0.90%
TG(16:0_16:1_18:3)	5.26%	6.95%	6.96%	7.74%
TG(16:0_16:1_20:5)	124.71%	123.58%	105.93%	105.38%
TG(16:0_18:1_18:1)	7.38%	9.35%	40.43%	40.44%
TG(16:0_18:1_18:2)	3.52%	7.96%	1.50%	2.22%
TG(16:0_18:2_18:2)	4.53%	8.76%	0.69%	0.98%
TG(16:0_18:2_20:5)	25.83%	27.09%	109.92%	108.83%
TG(16:0_18:3_22:4)	6.23%	10.16%	2.51%	3.12%
TG(16:1_14:0_18:2)	6.92%	10.98%	0.79%	1.90%
TG(16:1_16:1_18:1)	4.35%	8.54%	0.64%	1.39%
TG(16:1_16:1_18:3)	7.13%	11.14%	2.89%	3.73%
TG(16:1_16:1_18:3) *1*	7.24%	11.30%	2.99%	3.86%
TG(16:1_18:2_18:2)	5.07%	9.09%	0.85%	1.59%
TG(16:1_18:2_18:3)	7.05%	11.02%	1.18%	1.96%
TG(17:0_18:1_20:4)	26.40%	27.83%	56.45%	57.06%
TG(18:0_16:0_16:0)	7.85%	10.27%	40.38%	40.39%
TG(18:0_16:0_18:1)	7.37%	9.79%	40.40%	40.41%
TG(18:0_16:0_18:3)	71.77%	70.84%	60.63%	60.70%
TG(18:0_18:1_18:1)	7.08%	9.33%	40.42%	40.43%
TG(18:0_18:1_18:3)	43.23%	44.31%	63.27%	63.03%
TG(18:1_12:3_18:1)	7.77%	11.58%	7.79%	8.55%
TG(18:1_18:1_18:1)	6.54%	8.74%	40.40%	40.42%
TG(18:1_18:1_18:2)	3.92%	8.09%	3.38%	4.02%
TG(18:1_18:1_20:4)	44.45%	44.91%	63.07%	62.81%
TG(18:1_18:1_20:4) *1*	87.98%	88.32%	85.23%	85.65%
TG(18:1_18:2_18:2)	4.48%	8.93%	1.46%	1.58%
TG(18:1_18:2_18:2) *1*	2.90%	6.60%	2.94%	3.26%
TG(18:1_18:2_20:4)	5.79%	9.80%	3.89%	3.82%
TG(18:2_18:2_18:2) *1*	12.14%	14.90%	52.36%	52.27%
TG(18:2_18:2_18:2) *2*	7.13%	10.37%	3.86%	4.35%
TG(18:2_18:2_18:2) *3*	5.94%	9.39%	3.46%	3.33%
TG(18:2+OO_16:0_18:1)	5.72%	8.91%	3.38%	3.75%
TG(18:3_18:2_18:2)	5.94%	10.06%	0.60%	1.41%
TG(18:3+OO_16:0_16:0)	9.05%	12.50%	4.62%	5.55%
TG(20:3_18:2_18:2)	5.69%	9.32%	3.56%	3.84%
TG(20:5+OO_16:0_18:3)	6.40%	9.74%	21.26%	19.96%

TG(20:5+OO_16:0_18:3) *1*	6.40%	9.74%	21.26%	19.96%
TG(48:2+3O)	2.36%	6.51%	3.48%	2.87%
TG(52:12e)	6.55%	7.85%	5.07%	5.47%
TG(54:8)	189.17%	192.40%	282.84%	282.84%
TG(55:4)	16.92%	17.26%	41.65%	41.67%
TG(56:9)	9.16%	6.49%	20.58%	19.68%
1,2-DG(30:2)	14.75%	14.46%	33.73%	35.28%
1,2-DG(38:0)	25.84%	26.39%	58.09%	58.22%
1,2-DG(38:2)	30.22%	30.27%	20.67%	22.78%
1,2-DG(38:3)	20.71%	21.61%	44.51%	38.30%
1,2-DG(38:3) *1*	8.49%	10.22%	18.73%	13.18%
1,2-DG(38:3) *2*	63.25%	60.45%	114.04%	110.24%
1,2-DG(40:4)	64.29%	64.47%	75.61%	75.97%
1,2-DG(40:7)	5.48%	7.00%	8.49%	4.19%
10Z-heptadecenoic acid	5.74%	11.51%	5.00%	6.37%
11,14,17-eicosatrienoic acid/Bishomo-?-linolenic acid/5,8,11-eicosatrienoic acid	4.95%	10.57%	2.58%	4.32%
11,14-eicosadienoic acid	4.53%	9.72%	2.16%	4.32%
12,13-DiHOME	5.92%	10.84%	2.08%	4.08%
12-HHTrE	10.65%	14.94%	2.96%	3.29%
13,16,19-docosatrienoic acid	8.09%	11.01%	11.02%	12.09%
13,16-docosadienoic acid	6.02%	11.05%	14.22%	15.34%
19,20-DiHDPA	5.01%	10.30%	4.86%	6.16%
2,3-dinor-11-PGF2a	9.13%	13.05%	1.73%	2.61%
4, 7,10,13,16,19-docosahexaenoic acid	5.80%	10.09%	1.66%	3.46%
5,8,11,14,17-eicosapentaenoic acid	5.94%	10.05%	1.72%	3.55%
7,10,13,16,19-docosapentaenoic acid	5.60%	10.67%	4.49%	5.99%
7-oxocholesterol	8.34%	12.71%	2.69%	2.70%
7-oxocholesterol *1*	44.19%	44.33%	28.90%	29.78%
7Z,10Z,13Z,16Z-docosatetraenoic acid	6.03%	10.22%	1.34%	2.87%
a/y-linolenic acid	5.62%	10.12%	1.87%	3.51%
Arachidic acid	4.81%	10.06%	18.83%	19.61%
Arachidic acid *1*	4.67%	10.18%	15.56%	16.56%
Arachidonic acid	5.51%	9.98%	2.44%	3.91%
Behenic acid	4.45%	9.02%	12.15%	13.33%
Campesterol	77.01%	77.63%	41.63%	42.34%
CE(14:0)	47.21%	51.16%	74.93%	75.80%
CE(15:0)	6.89%	16.87%	21.52%	16.92%
CE(15:0) *1*	4.79%	16.77%	45.67%	36.13%
CE(16:2)	15.69%	24.31%	16.61%	15.82%
CE(17:0)	16.38%	19.02%	28.70%	23.46%
CE(17:1)	50.59%	51.81%	132.01%	119.31%
CE(20:0)	90.32%	90.39%	122.49%	122.34%

CE(20:1)	15.19%	18.37%	47.24%	46.10%
CE(22:0)	39.97%	47.98%	42.18%	43.57%
Cer(d18:0/16:0)	17.06%	18.82%	28.86%	28.11%
Cer(d18:0/18:0)	14.97%	16.43%	16.64%	16.05%
Cer(d18:0/20:0)	12.87%	15.50%	10.85%	10.35%
Cer(d18:0/26:0)	8.74%	8.51%	5.72%	5.78%
Cer(d18:1/19:0)	35.11%	32.96%	34.51%	34.99%
Cer(d18:1/25:0)	4.98%	7.85%	2.54%	3.17%
Cer(d18:2/20:0)	20.27%	21.81%	15.84%	16.23%
Cer(d18:2/24:2)	4.79%	8.49%	45.67%	45.00%
Cerotic acid	3.71%	9.54%	1.50%	3.40%
cis-9-palmitoleic acid	5.83%	10.36%	1.94%	3.88%
cis-erucic acid	5.10%	9.16%	16.41%	17.46%
cis-selacholeic acid	5.41%	10.69%	11.37%	12.61%
CoQ9	11.40%	20.82%	10.89%	13.88%
Lauric acid	6.94%	11.56%	1.19%	3.21%
Lignoceric acid	7.88%	9.77%	5.55%	6.63%
Linoleic acid	5.33%	10.16%	2.44%	3.89%
LPE(18:0)	7.59%	5.33%	2.09%	2.43%
LPE(18:0) *1*	9.29%	5.06%	2.92%	1.97%
LPE(18:1)	8.46%	6.02%	1.46%	3.00%
LPE(20:4)	6.55%	5.47%	1.60%	3.31%
LPE(22:1)	10.66%	12.14%	2.27%	3.54%
LPE(22:6)	5.99%	6.29%	1.84%	2.85%
Margaric acid	5.63%	8.54%	5.72%	5.82%
Myristic acid	6.30%	11.46%	1.32%	3.29%
Oleic acid	4.69%	9.62%	1.08%	3.36%
PC(32:0)	2.46%	5.61%	2.32%	2.25%
PC(36:0)	12.27%	13.39%	6.13%	6.34%
PE(32:1)	28.75%	25.37%	52.31%	53.89%
PE(32:1) *1*	9.10%	5.56%	17.32%	6.83%
PE(32:1) *2*	9.10%	5.56%	17.32%	6.83%
PE(40:7)	9.43%	3.84%	4.87%	11.52%
PE(40:7) *1*	5.98%	4.69%	16.88%	4.99%
PE(40:7) *2*	5.98%	4.69%	16.88%	4.99%
PE(42:5)	16.92%	19.44%	21.81%	26.40%
PE(42:7)	6.02%	5.31%	7.40%	8.38%
Pentadecanoic acid	5.79%	10.19%	1.99%	2.23%
PG(34:2)	18.32%	10.93%	11.99%	8.39%
PG(36:1)	8.61%	11.44%	12.01%	4.52%
PG(36:1) *1*	8.61%	11.44%	12.01%	4.52%
PG(36:2)	10.27%	13.77%	14.01%	5.89%
PG(36:3)	42.04%	32.89%	31.06%	24.58%
PG(36:4)	36.75%	26.86%	45.37%	38.27%

PG(36:5)	71.93%	73.57%	86.91%	71.39%
PG(38:4)	121.15%	121.04%	158.06%	137.34%
PG(38:6)	40.61%	47.54%	64.82%	56.72%
PG(40:6)	228.24%	233.30%	282.84%	282.84%
PG(40:8)	4.04%	13.10%	20.72%	13.64%
PGD3	70.28%	72.03%	80.48%	82.63%
PI(34:0)	3.25%	15.23%	2.95%	7.08%
PI(36:0)	3.46%	15.32%	1.20%	4.71%
PI(36:1)	5.70%	17.13%	2.60%	6.16%
PI(36:1) *1*	19.44%	7.50%	7.22%	4.30%
PI(36:5)	19.73%	13.13%	15.06%	14.28%
PI(38:2)	7.59%	17.41%	2.31%	5.84%
PI(38:3)	12.35%	6.54%	8.91%	9.87%
PI(38:5)	16.52%	7.04%	5.12%	3.24%
PI(40:4)	4.81%	14.25%	9.43%	11.11%
PI(40:4) *1*	41.54%	39.73%	38.53%	37.54%
PS(32:1)	41.59%	47.06%	56.26%	64.36%
PS(34:1)	6.60%	16.27%	14.40%	15.50%
PS(34:2)	97.98%	83.54%	155.82%	154.05%
PS(36:0)	3.60%	17.65%	1.38%	8.36%
PS(36:2)	10.12%	20.62%	12.25%	15.04%
PS(36:2) *1*	1.99%	16.35%	3.60%	10.46%
PS(38:4)	10.85%	16.27%	23.48%	24.27%
PS(38:4) *1*	9.23%	17.31%	4.17%	11.28%
PS(38:5)	13.24%	23.92%	12.12%	14.32%
PS(40:4)	3.28%	17.47%	0.97%	8.90%
Sphinganine 1-phosphate (d18:0-P)	27.95%	31.53%	25.08%	24.78%
Sphingosine 1-phosphate (d18:1-P)	10.20%	11.84%	12.22%	13.02%
Stearic acid	5.94%	10.16%	14.45%	15.58%
Stearidonic acid	6.14%	10.66%	1.70%	3.78%

Table D. The identified lipid retention time RSD result before and after normalisation.

lipids (retention time)	**plasma**		**QC**	
	unnormalised	normalised	unnormalised	normalised
AcCa(16:0)	0.34%	0.36%	0.33%	0.36%
AEA(16:0)	0.24%	0.34%	0.28%	0.28%
Cer(d16:1_22:0)	0.09%	0.13%	0.11%	0.16%
Cer(d18:0_22:0)	0.11%	0.17%	0.11%	0.17%
Cer(d18:0_24:0)	0.10%	0.16%	0.08%	0.16%
Cer(d18:0_24:1)	0.11%	0.14%	0.10%	0.10%
Cer(d18:1_16:0)	0.20%	0.23%	0.18%	0.25%
Cer(d18:1_18:0)	0.11%	0.10%	0.09%	0.14%

Cer(d18:1_22:0)	0.10%	0.15%	0.08%	0.06%
Cer(d18:1_23:0)	0.12%	0.14%	0.13%	0.16%
Cer(d18:1_24:1)	0.09%	0.16%	0.19%	0.15%
Cer(d18:1_26:0)	0.09%	0.17%	0.06%	0.14%
Cer(d18:1_26:1)	0.66%	0.63%	0.48%	0.52%
Cer(d18:2_22:0)	0.14%	0.14%	0.11%	0.18%
Cer(d18:2_23:0)	0.12%	0.22%	0.13%	0.11%
Cer(d18:2_24:1)	0.08%	0.13%	0.06%	0.08%
ChE(16:0)	0.20%	0.40%	40.41%	40.41%
ChE(16:1)	0.17%	0.18%	0.12%	0.20%
ChE(18:0)	0.31%	0.23%	40.41%	40.41%
ChE(18:1)	0.25%	0.30%	40.41%	40.41%
ChE(18:3)	0.14%	0.31%	0.08%	0.21%
ChE(18:3) *1*	0.12%	0.28%	0.13%	0.18%
ChE(18:3) *2*	0.10%	0.27%	0.07%	0.18%
ChE(20:3)	0.22%	0.26%	40.41%	40.41%
ChE(20:4)	0.04%	0.22%	0.09%	0.18%
ChE(20:4) *1*	0.10%	0.29%	0.08%	0.26%
ChE(20:5)	0.14%	0.20%	0.11%	0.26%
ChE(20:5) *1*	0.06%	0.21%	0.08%	0.22%
ChE(22:6)	0.64%	0.67%	40.41%	40.41%
ChE(22:6) *1*	0.09%	0.26%	0.08%	0.22%
ChE(22:6) *2*	0.06%	0.26%	0.08%	0.25%
Co(Q10)	0.06%	0.25%	0.10%	0.22%
DG(14:0_18:2)	0.12%	0.21%	0.12%	0.17%
DG(14:0_18:3)	0.32%	0.35%	0.24%	0.28%
DG(16:0_14:0)	0.13%	0.16%	0.17%	0.19%
DG(16:0_16:0)	0.12%	0.17%	0.07%	0.12%
DG(16:0_16:1)	0.11%	0.18%	0.14%	0.22%
DG(16:0_18:1)	0.12%	0.17%	0.08%	0.15%
DG(16:0_18:2)	0.12%	0.14%	0.09%	0.07%
DG(16:0_18:3)	0.15%	0.11%	0.16%	0.25%
DG(16:1_14:0)	0.16%	0.23%	0.18%	0.21%
DG(16:1_18:2)	0.10%	0.06%	0.12%	0.21%
DG(16:1_18:3)	0.22%	0.23%	0.24%	0.26%
DG(18:0_16:0)	0.08%	0.15%	0.08%	0.16%
DG(18:0_18:0)	0.16%	0.15%	0.22%	0.32%
DG(18:0_18:1)	0.10%	0.17%	0.06%	0.16%
DG(18:1_18:1)	0.11%	0.15%	0.10%	0.18%
DG(18:1_18:2)	0.11%	0.05%	0.14%	0.17%
DG(18:1_18:3)	0.16%	0.19%	0.22%	0.22%
DG(18:1_20:3)	0.27%	0.35%	0.48%	0.39%
DG(18:1_20:3) *1*	0.27%	0.35%	0.48%	0.39%
DG(18:1_22:5)	0.16%	0.20%	0.18%	0.18%

DG(18:2_18:2)	0.14%	0.23%	0.15%	0.21%
DG(18:3_18:2)	0.23%	0.21%	0.18%	0.24%
DG(34:3)	1.81%	1.86%	1.67%	1.72%
DG(36:3)	106.92%	106.92%	1.63%	1.65%
FA(16:0)	0.20%	0.32%	0.22%	0.22%
FA(23:0)	0.20%	0.22%	0.13%	0.20%
Hex2Cer(d12:0_20:4)	0.13%	0.13%	0.18%	0.22%
LPC(16:0)	0.21%	0.18%	0.22%	0.22%
LPC(18:0)	0.17%	0.25%	0.17%	0.17%
LPC(18:1)	0.18%	0.13%	0.27%	0.29%
LPC(18:2)	0.25%	0.16%	0.25%	0.30%
LPC(20:3)	0.36%	0.44%	0.16%	0.19%
LPC(20:4)	0.18%	0.22%	0.31%	0.32%
LPC(22:5)	0.33%	0.37%	0.34%	0.33%
LPE(16:0)	0.33%	0.39%	0.23%	0.32%
LPE(18:2)	0.35%	0.42%	0.21%	0.34%
PC(14:0_18:2)	0.15%	0.22%	0.15%	0.23%
PC(14:0_20:4)	0.18%	0.22%	0.16%	0.12%
PC(14:0_20:5)	0.19%	0.24%	0.11%	0.15%
PC(16:0_16:1)	0.14%	0.14%	0.11%	0.00%
PC(16:0_18:1)	0.11%	0.15%	0.13%	0.15%
PC(16:0_20:3)	0.17%	0.16%	0.10%	0.00%
PC(16:0_20:5)	0.14%	0.15%	0.17%	0.19%
PC(16:0_22:5)	0.19%	0.16%	0.10%	0.00%
PC(16:0_22:6)	0.11%	0.16%	0.19%	0.25%
PC(18:0_16:0)	0.13%	0.24%	0.15%	0.12%
PC(18:0_18:1)	0.18%	0.21%	0.12%	0.10%
PC(18:0_18:2)	0.12%	0.17%	0.09%	0.13%
PC(18:0_20:2)	0.17%	0.23%	0.23%	0.23%
PC(18:0_20:3)	0.13%	0.19%	0.10%	0.13%
PC(18:0_20:4)	0.11%	0.15%	0.14%	0.09%
PC(18:0_22:4)	0.10%	0.19%	0.19%	0.18%
PC(18:0_22:5)	0.22%	0.22%	0.18%	0.21%
PC(18:0_22:6)	0.13%	0.20%	0.17%	0.24%
PC(18:1_13:0)	0.17%	0.22%	0.13%	0.18%
PC(18:1_22:6)	0.18%	0.19%	0.11%	0.16%
PC(18:2_13:0)	0.13%	0.23%	0.14%	0.17%
PC(18:2_18:2)	0.11%	0.18%	0.22%	0.25%
PC(18:2_22:6)	0.13%	0.18%	0.13%	0.10%
PC(22:0_18:2)	0.13%	0.20%	0.08%	0.13%
PC(30:1)	0.11%	0.19%	0.23%	0.25%
PC(31:0)	0.18%	0.19%	0.17%	0.23%
PC(31:1)	0.17%	0.22%	0.13%	0.18%
PC(33:0)	0.23%	0.25%	0.19%	0.18%

PC(33:0) *1*	0.23%	0.25%	0.19%	0.18%
PC(33:1)	0.13%	0.23%	0.11%	0.16%
PC(33:1) *1*	0.11%	0.15%	0.14%	0.20%
PC(33:2)	0.20%	0.26%	0.16%	0.21%
PC(33:3)	0.17%	0.26%	0.22%	0.25%
PC(33:3) *1*	0.15%	0.22%	0.11%	0.16%
PC(33:4)	0.20%	0.24%	0.10%	0.08%
PC(34:1e)	0.16%	0.18%	0.18%	0.23%
PC(34:2) *1*	0.11%	0.16%	0.19%	0.27%
PC(34:2+OO)	0.26%	0.32%	0.14%	0.14%
PC(34:2+OO) *1*	0.15%	0.15%	0.14%	0.13%
PC(34:2e)	0.12%	0.20%	0.17%	0.17%
PC(34:3) *1*	0.11%	0.15%	0.14%	0.08%
PC(34:3e)	0.12%	0.16%	0.10%	0.08%
PC(35:1)	0.14%	0.20%	0.16%	0.16%
PC(35:1CHO)	0.28%	0.28%	0.52%	0.58%
PC(35:2)	0.10%	0.16%	0.13%	0.07%
PC(35:3)	0.14%	0.14%	0.11%	0.00%
PC(35:4)	0.12%	0.17%	0.11%	0.18%
PC(35:5)	0.12%	0.16%	0.13%	0.15%
PC(36:2+2O)	0.17%	0.22%	0.12%	0.17%
PC(36:3+2O)	0.21%	0.30%	0.21%	0.25%
PC(36:4) *1*	0.11%	0.18%	0.22%	0.25%
PC(36:4) *2*	0.11%	0.18%	0.22%	0.25%
PC(36:4+OO)	0.19%	0.20%	0.16%	0.16%
PC(36:4+OO) *1*	0.41%	0.44%	0.25%	0.32%
PC(36:4e)	0.15%	0.17%	0.14%	0.20%
PC(36:6)	0.15%	0.15%	0.62%	0.64%
PC(37:1)	0.16%	0.21%	0.10%	0.07%
PC(37:4)	0.25%	0.24%	0.11%	0.05%
PC(37:5)	0.23%	0.33%	0.20%	0.21%
PC(37:6)	0.16%	0.15%	0.17%	0.18%
PC(38:4+OO)	0.16%	0.24%	0.21%	0.20%
PC(38:4+OO) *1*	0.20%	0.15%	0.09%	0.10%
PC(38:4e)	0.14%	0.20%	0.13%	0.10%
PC(38:5e)	0.15%	0.05%	0.11%	0.08%
PC(38:6+OO)	0.26%	0.30%	0.48%	0.43%
PC(38:6+OO) *1*	1.02%	0.96%	0.80%	0.73%
PC(38:6e)	0.10%	0.16%	0.13%	0.07%
PC(38:7)	0.16%	0.20%	0.21%	0.20%
PC(38:7) *1*	5.11%	5.03%	3.39%	3.39%
PC(38:7e)	0.15%	0.17%	0.17%	0.24%
PC(39:6)	0.19%	0.20%	0.26%	0.24%
PC(40:5)	0.22%	0.22%	0.18%	0.21%

PC(44:5e)	0.12%	0.23%	0.10%	0.19%
PE(16:0_18:1)	0.12%	0.28%	0.10%	0.15%
PE(16:0_18:2)	0.10%	0.26%	0.09%	0.15%
PE(16:0_20:4)	0.20%	0.33%	0.10%	0.15%
PE(16:0_20:4) *1*	0.47%	0.50%	0.33%	0.36%
PE(16:0_20:5)	0.14%	0.24%	0.14%	0.10%
PE(16:0p_22:6)	0.17%	0.23%	0.10%	0.05%
PE(16:1e_22:6)	0.13%	0.28%	0.20%	0.23%
PE(18:0_16:0)	0.15%	0.24%	0.18%	0.22%
PE(18:0_18:1)	0.16%	0.26%	0.12%	0.17%
PE(18:0_18:2)	0.13%	0.23%	0.11%	0.15%
PE(18:0p_22:6)	0.15%	0.23%	0.12%	0.16%
PE(18:1_18:2)	0.15%	0.26%	0.13%	0.08%
PE(36:2) *1*	0.13%	0.23%	0.11%	0.15%
PE(36:3)	0.17%	0.27%	0.22%	0.28%
PE(38:1e)	138.06%	138.07%	106.91%	106.91%
PE(38:6)	0.18%	0.24%	0.11%	0.00%
PE(40:7e)	0.15%	0.23%	0.12%	0.16%
PE(43:4)	2.61%	2.62%	0.47%	0.44%
PG(16:0_18:1)	0.13%	0.21%	0.20%	0.25%
PI(16:0_16:0)	0.13%	0.08%	0.14%	0.20%
PI(16:0_16:1)	0.15%	0.17%	0.17%	0.26%
PI(16:0_20:4)	0.14%	0.09%	0.15%	0.10%
PI(16:0_22:6)	0.14%	0.08%	0.08%	0.17%
PI(18:0_18:2)	0.15%	0.24%	0.12%	0.24%
PI(18:0_20:4)	0.13%	0.13%	0.18%	0.22%
PI(18:0_22:5)	0.11%	0.14%	0.14%	0.26%
PI(18:0_22:6)	0.16%	0.10%	0.16%	0.17%
PI(22:1_18:2)	0.19%	0.26%	0.16%	0.30%
PI(34:1)	0.17%	0.18%	0.17%	0.22%
PI(34:2)	0.18%	0.10%	0.12%	0.12%
PI(36:3)	0.13%	0.17%	0.20%	0.21%
PS(34:0)	0.13%	0.28%	0.16%	0.26%
PS(42:3)	0.15%	0.26%	0.19%	0.22%
SM(d32:2)	0.16%	0.12%	0.23%	0.22%
SM(d35:1)	0.15%	0.20%	0.14%	0.17%
SM(d37:1)	0.14%	0.22%	0.20%	0.25%
SM(d39:1)	0.12%	0.16%	0.07%	0.07%
SM(d41:7)	0.13%	0.23%	0.17%	0.23%
SM(d43:2)	0.18%	0.13%	0.16%	0.13%
SM(d44:1)	0.13%	0.18%	0.07%	0.09%
SM(t40:7)	0.88%	0.81%	0.52%	0.54%
SPH(d18:0)	0.25%	0.33%	0.23%	0.20%
SPH(d18:1)	0.31%	0.42%	0.42%	0.51%

SPHP(d21:1)	0.27%	0.42%	0.15%	0.32%
TG(12:1e_6:0_16:0)	0.12%	0.10%	0.08%	0.13%
TG(12:1e_6:0_18:2)	0.13%	0.15%	0.18%	0.16%
TG(12:1e_6:0_18:4)	0.27%	0.30%	0.22%	0.27%
TG(12:1e_6:0_20:4)	0.14%	0.13%	0.10%	0.16%
TG(12:1e_6:0_20:5)	40.49%	40.49%	3.07%	3.06%
TG(14:0_18:2_18:2)	0.30%	0.28%	0.26%	0.23%
TG(16:0_14:0_18:1)	0.07%	0.03%	0.08%	0.15%
TG(16:0_14:0_20:5)	1.07%	1.06%	0.67%	0.67%
TG(16:0_14:4_16:0)	0.10%	0.09%	0.14%	0.15%
TG(16:0_16:0_16:0)	0.24%	0.25%	0.30%	0.33%
TG(16:0_16:0_16:1)	0.07%	0.03%	0.08%	0.15%
TG(16:0_16:0_18:1)	0.17%	0.18%	40.41%	40.41%
TG(16:0_16:0_18:1) *1*	0.30%	0.28%	2.23%	2.23%
TG(16:0_16:0_18:3)	40.48%	40.47%	40.60%	40.60%
TG(16:0_16:0_20:5) *1*	61.73%	61.73%	1.16%	1.17%
TG(16:0_16:1_16:1)	0.07%	0.11%	0.09%	0.15%
TG(16:0_16:1_18:1)	0.24%	0.27%	0.19%	0.17%
TG(16:0_16:1_18:3)	0.23%	0.21%	0.26%	0.34%
TG(16:0_16:1_20:5)	106.91%	106.91%	61.73%	61.73%
TG(16:0_18:1_18:1)	2.02%	2.03%	1.80%	1.80%
TG(16:0_18:1_18:2)	0.07%	0.06%	0.11%	0.14%
TG(16:0_18:2_18:2)	0.05%	0.09%	0.07%	0.14%
TG(16:0_18:2_20:5)	0.16%	0.19%	1.58%	1.57%
TG(16:0_18:3_22:4)	0.10%	0.12%	0.16%	0.19%
TG(16:1_14:0_18:2)	0.09%	0.13%	0.11%	0.14%
TG(16:1_16:1_18:1)	0.07%	0.04%	0.05%	0.09%
TG(16:1_16:1_18:3)	0.48%	0.47%	0.62%	0.61%
TG(16:1_16:1_18:3) *1*	0.48%	0.47%	0.62%	0.61%
TG(16:1_18:2_18:2)	0.14%	0.15%	0.13%	0.19%
TG(16:1_18:2_18:3)	0.06%	0.08%	0.09%	0.06%
TG(17:0_18:1_20:4)	0.61%	0.62%	40.41%	40.41%
TG(18:0_16:0_16:0)	0.26%	0.25%	0.73%	0.72%
TG(18:0_16:0_18:1)	0.19%	0.22%	0.15%	0.17%
TG(18:0_16:0_18:3)	1.69%	1.66%	40.41%	40.41%
TG(18:0_18:1_18:1)	0.22%	0.22%	0.94%	0.94%
TG(18:0_18:1_18:3)	3.47%	3.50%	3.44%	3.38%
TG(18:1_12:3_18:1)	0.12%	0.13%	0.31%	0.35%
TG(18:1_18:1_18:1)	0.29%	0.32%	0.11%	0.13%
TG(18:1_18:1_18:2)	0.09%	0.13%	0.13%	0.14%
TG(18:1_18:1_20:4)	1.17%	1.19%	40.43%	40.42%
TG(18:1_18:1_20:4) *1*	61.72%	61.72%	40.41%	40.41%
TG(18:1_18:2_18:2)	0.10%	0.09%	0.17%	0.19%
TG(18:1_18:2_18:2) *1*	0.06%	0.09%	0.07%	0.11%

TG(18:1_18:2_20:4)	0.06%	0.06%	0.09%	0.12%
TG(18:2_18:2_18:2) *1*	0.98%	1.00%	40.43%	40.42%
TG(18:2_18:2_18:2) *2*	0.22%	0.20%	0.16%	0.21%
TG(18:2_18:2_18:2) *3*	0.12%	0.15%	0.06%	0.14%
TG(18:2+OO_16:0_18:1)	0.07%	0.05%	0.15%	0.19%
TG(18:3_18:2_18:2)	0.04%	0.09%	0.13%	0.18%
TG(18:3+OO_16:0_16:0)	0.09%	0.11%	0.06%	0.11%
TG(20:3_18:2_18:2)	0.11%	0.08%	0.08%	0.17%
TG(20:5+OO_16:0_18:3)	1.23%	1.25%	0.96%	0.98%
TG(20:5+OO_16:0_18:3) *1*	1.23%	1.25%	0.96%	0.98%
TG(48:2+3O)	0.34%	0.37%	0.15%	0.17%
TG(52:12e)	0.17%	0.18%	0.14%	0.19%
TG(54:8)	185.19%	185.19%	282.84%	282.84%
TG(55:4)	0.79%	0.79%	40.41%	40.41%
TG(56:9)	0.17%	0.20%	1.27%	1.22%
1,2-DG(30:2)	0.38%	0.30%	0.20%	0.28%
1,2-DG(38:0)	0.71%	0.76%	1.12%	1.15%
1,2-DG(38:2)	0.47%	0.53%	0.63%	0.57%
1,2-DG(38:3)	0.12%	0.14%	0.12%	0.07%
1,2-DG(38:3) *1*	0.17%	0.17%	0.13%	0.21%
1,2-DG(38:3) *2*	0.69%	0.73%	82.81%	82.81%
1,2-DG(40:4)	0.36%	0.38%	0.47%	0.50%
1,2-DG(40:7)	0.20%	0.21%	0.12%	0.22%
10Z-heptadecenoic acid	0.26%	0.32%	0.26%	0.19%
11,14,17-eicosatrienoic acid/Bishomo-?-linolenic acid/5,8,11-eicosatrienoic acid	0.19%	0.35%	0.22%	0.32%
11,14-eicosadienoic acid	0.11%	0.18%	0.20%	0.20%
12,13-DiHOME	0.37%	0.42%	0.58%	0.55%
12-HHTrE	1.18%	1.27%	0.78%	0.95%
13,16,19-docosatrienoic acid	0.15%	0.25%	0.26%	0.31%
13,16-docosadienoic acid	0.16%	0.21%	0.12%	0.22%
19,20-DiHDPA	0.19%	0.28%	0.20%	0.19%
2,3-dinor-11-PGF2a	0.39%	0.38%	0.60%	0.79%
4, 7,10,13,16,19-docosahexaenoic acid	0.28%	0.43%	0.19%	0.34%
5,8,11,14,17-eicosapentaenoic acid	0.16%	0.29%	0.24%	0.22%
7,10,13,16,19-docosapentaenoic acid	0.13%	0.19%	0.24%	0.24%
7-oxocholesterol	0.34%	0.41%	0.33%	0.41%
7-oxocholesterol *1*	2.50%	2.44%	0.99%	1.00%
7Z,10Z,13Z,16Z-docosatetraenoic acid	0.12%	0.15%	0.19%	0.35%
a/y-linolenic acid	0.37%	0.47%	0.27%	0.42%
Arachidic acid	0.12%	0.20%	0.18%	0.35%
Arachidic acid *1*	0.12%	0.20%	0.18%	0.35%

Arachidonic acid	0.18%	0.13%	0.33%	0.21%
Behenic acid	0.13%	0.19%	0.16%	0.21%
Campesterol	61.75%	61.74%	0.43%	0.40%
CE(14:0)	0.26%	0.40%	40.41%	40.41%
CE(15:0)	0.08%	0.22%	0.21%	0.32%
CE(15:0) *1*	0.15%	0.14%	0.08%	0.21%
CE(16:2)	0.32%	0.37%	0.43%	0.39%
CE(17:0)	0.37%	0.28%	0.17%	0.25%
CE(17:1)	0.34%	0.48%	0.38%	0.32%
CE(20:0)	82.81%	82.81%	106.91%	106.91%
CE(20:1)	0.87%	0.85%	40.41%	40.41%
CE(22:0)	0.46%	0.33%	0.56%	0.60%
Cer(d18:0/16:0)	0.44%	0.41%	0.42%	0.44%
Cer(d18:0/18:0)	0.16%	0.19%	0.29%	0.29%
Cer(d18:0/20:0)	0.14%	0.19%	0.19%	0.20%
Cer(d18:0/26:0)	0.14%	0.15%	0.21%	0.19%
Cer(d18:1/19:0)	0.17%	0.21%	0.13%	0.19%
Cer(d18:1/25:0)	0.69%	0.70%	0.17%	0.20%
Cer(d18:2/20:0)	0.11%	0.15%	0.14%	0.14%
Cer(d18:2/24:2)	0.15%	0.17%	0.08%	0.06%
Cerotic acid	0.16%	0.22%	0.15%	0.20%
cis-9-palmitoleic acid	0.29%	0.31%	0.31%	0.34%
cis-erucic acid	0.14%	0.26%	0.13%	0.25%
cis-selacholeic acid	0.15%	0.27%	0.13%	0.17%
CoQ9	0.08%	0.23%	0.13%	0.26%
Lauric acid	0.22%	0.31%	0.45%	0.42%
Lignoceric acid	0.12%	0.27%	0.17%	0.28%
Linoleic acid	0.19%	0.28%	0.25%	0.25%
LPE(18:0)	0.26%	0.40%	0.27%	0.28%
LPE(18:0) *1*	0.17%	0.26%	0.16%	0.21%
LPE(18:1)	0.14%	0.22%	0.25%	0.25%
LPE(20:4)	0.18%	0.31%	0.28%	0.35%
LPE(22:1)	0.40%	0.49%	0.36%	0.40%
LPE(22:6)	0.16%	0.29%	0.31%	0.27%
Margaric acid	0.19%	0.35%	0.16%	0.23%
Myristic acid	0.34%	0.47%	0.27%	0.35%
Oleic acid	0.12%	0.15%	0.19%	0.23%
PC(32:0)	0.13%	0.17%	0.14%	0.20%
PC(36:0)	0.11%	0.16%	0.13%	0.14%
PE(32:1)	1.14%	1.21%	1.23%	1.28%
PE(32:1) *1*	0.16%	0.27%	0.17%	0.22%
PE(32:1) *2*	0.16%	0.27%	0.17%	0.22%
PE(40:7)	0.59%	0.53%	0.53%	0.52%
PE(40:7) *1*	0.74%	0.74%	0.56%	0.55%

PE(40:7) *2*	0.74%	0.74%	0.56%	0.55%
PE(42:5)	0.26%	0.33%	0.16%	0.23%
PE(42:7)	0.17%	0.23%	0.09%	0.10%
Pentadecanoic acid	0.32%	0.38%	0.13%	0.27%
PG(34:2)	0.22%	0.17%	0.20%	0.17%
PG(36:1)	0.16%	0.24%	0.12%	0.20%
PG(36:1) *1*	0.16%	0.24%	0.12%	0.20%
PG(36:2)	0.16%	0.25%	0.16%	0.21%
PG(36:3)	1.06%	1.02%	0.59%	0.70%
PG(36:4)	0.22%	0.30%	0.23%	0.26%
PG(36:5)	40.41%	40.41%	0.39%	0.42%
PG(38:4)	61.75%	61.75%	61.76%	61.76%
PG(38:6)	0.89%	0.90%	0.53%	0.55%
PG(40:6)	185.16%	185.17%	282.84%	282.84%
PG(40:8)	0.17%	0.24%	0.23%	0.29%
PGD3	40.47%	40.47%	40.47%	40.48%
PI(34:0)	0.19%	0.19%	0.19%	0.30%
PI(36:0)	0.11%	0.08%	0.14%	0.23%
PI(36:1)	0.22%	0.23%	0.16%	0.21%
PI(36:1) *1*	0.17%	0.27%	0.13%	0.24%
PI(36:5)	0.30%	0.22%	0.19%	0.12%
PI(38:2)	0.17%	0.22%	0.14%	0.29%
PI(38:3)	0.32%	0.26%	0.25%	0.34%
PI(38:5)	0.21%	0.20%	0.12%	0.20%
PI(40:4)	0.16%	0.20%	0.29%	0.24%
PI(40:4) *1*	0.71%	0.69%	0.45%	0.41%
PS(32:1)	0.63%	0.67%	0.71%	0.74%
PS(34:1)	0.22%	0.27%	0.27%	0.19%
PS(34:2)	61.73%	61.74%	138.01%	138.01%
PS(36:0)	0.17%	0.26%	0.16%	0.16%
PS(36:2)	0.12%	0.24%	0.19%	0.21%
PS(36:2) *1*	0.15%	0.26%	0.23%	0.19%
PS(38:4)	0.56%	0.69%	0.64%	0.65%
PS(38:4) *1*	0.19%	0.37%	0.11%	0.21%
PS(38:5)	0.38%	0.54%	0.40%	0.44%
PS(40:4)	0.19%	0.33%	0.16%	0.17%
Sphinganine 1-phosphate (d18:0-P)	0.76%	0.74%	0.29%	0.25%
Sphingosine 1-phosphate (d18:1-P)	0.22%	0.25%	0.19%	0.25%
Stearic acid	0.17%	0.21%	0.15%	0.21%
Stearidonic acid	0.39%	0.52%	0.25%	0.24%

Table E. The whole identified lipid list in brain tumour application and internal standard allocation

Identified lipids	Formula	Internal Standard	Adduct	RT
15:0-18:1(d7)PC+H	C41H73D7NO8P		M+H	6.73
15:0-18:1(d7)PC+CH3COO	C41H73D7NO8P		M+CH3COO	6.78
15:0-18:1(d7)PE-H	C38H67D7NO8P		M-H	6.78
15:0-18:1(d7)PS-H	C39H67D7NO10P		M-H	6.3
15:0-18:1(d7)PG+H	C39H68D7O10P		M+H	6.31
15:0-18:1(d7)PI-H	C42H72O13PD7		M-H	6.23
15:0-18:1(d7)PA-H	C36H62O8PD7		M-H	4.93
18:1(d7)LPC+H	C26H45D7NO7P		M+H	3.51
18:1(d7)LPE-H	C23H39D7NO7P		M-H	3.62
18:1(d7)LPE+H	C23H39D7NO7P		M+H	3.69
18:1(d7)ChE+NH4	C45H71D7O2		M+NH4	14.69
18:1(d7)MG+H	C21H33D7O4		M+NH4	4.8
15:0-18:1(d7)DG+NH4	C36H61D7O5		M+NH4	7.58
15:0-18:1(d7)-15:0TG+NH4	C51H89D7O6		M+NH4	13.07
18:1(d9) SM+H	C41H72D9N2O6P		M+H	6.51
Cholesterol (d7)+H	C27H39D7O		M+H	6.35
7-oxocholesterol	C27H44O3	18:1(d7) LPC	M+H	5.57
AcCa(16:0)	C23H45NO4	18:1(d7) LPC	M+H	3.11
Cer(d16:1_22:0)	C38H75NO3	18:1(d9) SM	M+H	7.84
Cer(d18:0/16:0)	C34H69NO3	18:1(d9) SM	M+H	7.26
Cer(d18:0/18:0)	C36H73NO3	18:1(d9) SM	M+H	7.68
Cer(d18:0/20:0)	C38H77NO3	18:1(d9) SM	M+H	8.15
Cer(d18:0/26:0)	C44H89NO3	18:1(d9) SM	M+H	9.87
Cer(d18:0_22:0)	C40H81NO3	18:1(d9) SM	M+H	8.52
Cer(d18:0_24:0)	C42H85NO3	18:1(d9) SM	M+H	9.14
Cer(d18:0_24:1)	C42H83NO3	18:1(d9) SM	M+H	8.91
Cer(d18:1/19:0)	C37H73NO3	18:1(d9) SM	M+H	7.74
Cer(d18:1/25:0)	C43H85NO3	18:1(d9) SM	M+H	9.44
Cer(d18:1_16:0)	C34H67NO3	18:1(d9) SM	M+H	7.01
Cer(d18:1_18:0)	C36H71NO3	18:1(d9) SM	M+H	7.42
Cer(d18:1_22:0)	C40H79NO3	18:1(d9) SM	M+H	8.34
Cer(d18:1_23:0)	C41H81NO3	18:1(d9) SM	M+H	8.97
Cer(d18:1_24:1)	C42H81NO3	18:1(d9) SM	M+H	8.34
Cer(d18:1_26:0)	C44H87NO3	18:1(d9) SM	M+H	9.63
Cer(d18:1_26:1)	C44H85NO3	18:1(d9) SM	M+H	8.91
Cer(d18:2/20:0)	C38H73NO3	18:1(d9) SM	M+H	7.56
Cer(d18:2/24:2)	C42H77NO3	18:1(d9) SM	M+H	7.57
Cer(d18:2_22:0)	C40H77NO3	18:1(d9) SM	M+H	7.92
Cer(d18:2_23:0)	C41H79NO3	18:1(d9) SM	M+H	8.07
Cer(d18:2_24:1)	C42H79NO3	18:1(d9) SM	M+H	7.92
ChE(14:0)	C41H72O2	18:1(d7) Chol Ester	M+NH4	13.65

ChE(15:0)	C42H74O2	18:1(d7) Chol Ester	M+NH4	8.07
ChE(16:0)	C43H76O2	18:1(d7) Chol Ester	M+NH4	6.19
ChE(16:1)	C43H74O2	18:1(d7) Chol Ester	M+NH4	13.65
ChE(16:2)	C43H72O2	18:1(d7) Chol Ester	M+NH4	12.97
ChE(17:0)	C44H78O2	18:1(d7) Chol Ester	M+NH4	8.96
ChE(17:1)	C44H76O2	18:1(d7) Chol Ester	M+NH4	8.01
ChE(18:0)	C45H80O2	18:1(d7) Chol Ester	M+NH4	9.49
ChE(18:1)	C45H78O2	18:1(d7) Chol Ester	M+NH4	6.31
ChE(18:3)	C45H74O2	18:1(d7) Chol Ester	M+NH4	13.12
ChE(20:0)	C47H84O2	18:1(d7) Chol Ester	M+NH4	11.01
ChE(20:1)	C47H82O2	18:1(d7) Chol Ester	M+NH4	14.78
ChE(20:3)	C47H78O2	18:1(d7) Chol Ester	M+NH4	14.11
ChE(20:4)	C47H76O2	18:1(d7) Chol Ester	M+NH4	13.53
ChE(20:5)	C47H74O2	18:1(d7) Chol Ester	M+NH4	12.77
ChE(22:6)	C49H76O2	18:1(d7) Chol Ester	M+NH4	13.18
CoQ10	C59H90O4	18:1(d7) Chol Ester	M+NH4	11.33
CoQ9	C54H82O4	18:1(d7) Chol Ester	M+NH4	8.44
DG(30:2)	C33H60O5	15:0-18:1(d7) DG	M+NH4	6.78
DG(38:0)	C41H80O5	15:0-18:1(d7) DG	M+NH4	8.26
DG(38:3)	C41H74O5	15:0-18:1(d7) DG	M+NH4	8.01
DG(40:7)	C43H70O5	15:0-18:1(d7) DG	M+NH4	7.47
DG(14:0_18:2)	C35H64O5	15:0-18:1(d7) DG	M+NH4	7.245
DG(14:0_18:3)	C35H62O5	15:0-18:1(d7) DG	M+NH4	6.977
DG(16:0_14:0)	C33H64O5	15:0-18:1(d7) DG	M+NH4	7.517
DG(16:0_16:0)	C35H68O5	15:0-18:1(d7) DG	M+NH4	8.012
DG(16:0_16:1)	C35H66O5	15:0-18:1(d7) DG	M+NH4	7.62
DG(16:0_18:1)	C37H70O5	15:0-18:1(d7) DG	M+NH4	7.81
DG(16:0_18:2)	C37H68O5	15:0-18:1(d7) DG	M+NH4	7.696
DG(16:1_14:0)	C33H62O5	15:0-18:1(d7) DG	M+NH4	7.168
DG(16:1_18:3)	C37H64O5	15:0-18:1(d7) DG	M+NH4	7.189
DG(18:0_16:0)	C37H72O5	15:0-18:1(d7) DG	M+NH4	8.42
DG(18:0_18:0)	C39H76O5	15:0-18:1(d7) DG	M+NH4	8.84
DG(18:0_18:1)	C39H74O5	15:0-18:1(d7) DG	M+NH4	8.629
DG(18:1_18:1)	C39H72O5	15:0-18:1(d7) DG	M+NH4	8.12
DG(36:4)	C39H68O5	15:0-18:1(d7) DG	M+NH4	7.26
DG(18:1_20:3)	C41H72O5	15:0-18:1(d7) DG	M+NH4	7.87
DG(18:1_22:5)	C43H72O5	15:0-18:1(d7) DG	M+NH4	7.51
DG(18:3_18:2)	C39H66O5	15:0-18:1(d7) DG	M+NH4	7.04
DG(16:0_18:3)	C37H66O5	15:0-18:1(d7) DG	M+NH4	7.2
DG(36:3)	C39H70O5	15:0-18:1(d7) DG	M+NH4	7.54
DG(38:5)	C41H70O5	15:0-18:1(d7) DG	M+NH4	7.48
DG(38:6)	C41H68O5	15:0-18:1(d7) DG	M+NH4	7.17
Eicosanoids_19,20-DiHDPA	C22H34O4	18:1(d7) LPE	M-H	3.6
Eicosanoids_PGD3	C20H30O5	18:1(d7) LPE	M-H	3.25

FA(12:0)	C12H24O2	18:1(d7) LPE	M-H	2.07
FA(14:0)	C14H28O2	18:1(d7) LPE	M-H	3.1
FA(15:0)	C15H30O2	18:1(d7) LPE	M-H	3.63
FA(16:0)	C16H32O2	18:1(d7) LPE	M-H	4.06
FA(16:1)	C16H30O2	18:1(d7) LPE	M-H	3.38
FA(17:0)	C17H34O2	18:1(d7) LPE	M-H	4.61
FA(17:1)	C17H32O2	18:1(d7) LPE	M-H	3.91
FA(18:0)	C18H36O2	18:1(d7) LPE	M-H	5.04
FA(18:1)	C18H34O2	18:1(d7) LPE	M-H	4.37
FA(18:2)	C18H32O2	18:1(d7) LPE	M-H	3.71
FA(18:3)	C18H30O2	18:1(d7) LPE	M-H	3.09
FA(18:4)	C18H28O2	18:1(d7) LPE	M-H	2.51
FA(20:0)	C20H40O2	18:1(d7) LPE	M-H	5.73
FA(20:2)	C20H36O2	18:1(d7) LPE	M-H	4.58
FA(20:3)	C20H34O2	18:1(d7) LPE	M-H	4.05
FA(20:4)	C20H32O2	18:1(d7) LPE	M-H	3.58
FA(20:5)	C20H30O2	18:1(d7) LPE	M-H	2.93
FA(22:0)	C22H44O2	18:1(d7) LPE	M-H	6.34
FA(22:1)	C22H42O2	18:1(d7) LPE	M-H	5.79
FA(22:2)	C22H40O2	18:1(d7) LPE	M-H	5.33
FA(22:3)	C22H38O2	18:1(d7) LPE	M-H	4.92
FA(22:4)	C22H36O2	18:1(d7) LPE	M-H	4.37
FA(22:5)	C22H34O2	18:1(d7) LPE	M-H	3.8
FA(22:6)	C22H32O2	18:1(d7) LPE	M-H	3.33
FA(23:0)	C23H46O2	18:1(d7) LPE	M-H	6.2
FA(24:0)	C24H48O2	18:1(d7) LPE	M-H	6.74
FA(24:1)	C24H46O2	18:1(d7) LPE	M-H	6.34
FA(26:0)	C26H52O2	18:1(d7) LPE	M-H	7.03
Hex2Cer(d12:0_20:4)	C44H77NO13	15:0-18:1(d7) PI	M+CH3COO	6.46
LPC(16:0)	C24H50NO7P	18:1(d7) LPC	M+H	3.36
LPC(16:1)	C24H48NO7P	18:1(d7) LPC	M+H	2.5
LPC(18:0)	C26H54NO7P	18:1(d7) LPC	M+H	4.27
LPC(18:1)	C26H52NO7P	18:1(d7) LPC	M+H	3.51
LPC(18:2)	C26H50NO7P	18:1(d7) LPC	M+H	2.83
LPC(20:3)	C28H52NO7P	18:1(d7) LPC	M+H	3.18
LPC(20:4)	C28H50NO7P	18:1(d7) LPC	M+H	2.78
LPC(22:5)	C30H52NO7P	18:1(d7) LPC	M+H	2.98
LPC(22:6)	C30H50NO7P	18:1(d7) LPC	M+H	2.61
LPE(16:0)	C21H44NO7P	18:1(d7) LPE	M-H	3.45
LPE(18:0)	C23H48NO7P	18:1(d7) LPE	M-H	4.38
LPE(18:1)	C23H46NO7P	18:1(d7) LPE	M-H	3.62
LPE(18:2)	C23H44NO7P	18:1(d7) LPE	M-H	2.99
LPE(20:4)	C25H44NO7P	18:1(d7) LPE	M-H	2.92
LPE(22:1)	C27H54NO7P	18:1(d7) LPE	M+H	4.01

LPE(22:6)	C27H44NO7P	18:1(d7) LPE	M-H	2.81
PC(14:0_18:2)	C40H76NO8P	15:0-18:1(d7) PC	M+H	6.326
PC(14:0_20:4)	C42H76NO8P	15:0-18:1(d7) PC	M+H	6.29
PC(14:0_20:5)	C42H74NO8P	15:0-18:1(d7) PC	M+H	5.95
PC(16:0_16:1)	C40H78NO8P	15:0-18:1(d7) PC	M+H	6.602
PC(16:0_18:1)	C42H82NO8P	15:0-18:1(d7) PC	M+H	6.98
PC(16:0_20:3)	C44H82NO8P	15:0-18:1(d7) PC	M+H	6.821
PC(16:0_20:5)	C44H78NO8P	15:0-18:1(d7) PC	M+H	6.439
PC(16:0_22:5)	C46H82NO8P	15:0-18:1(d7) PC	M+H	6.822
PC(16:0_22:6)	C46H80NO8P	15:0-18:1(d7) PC	M+H	6.5
PC(18:0_16:0)	C42H84NO8P	15:0-18:1(d7) PC	M+H	7.2
PC(18:0_18:1)	C44H86NO8P	15:0-18:1(d7) PC	M+H	7.26
PC(18:0_18:2)	C44H84NO8P	15:0-18:1(d7) PC	M+H	7.098
PC(18:0_20:2)	C46H88NO8P	15:0-18:1(d7) PC	M+H	7.37
PC(18:0_20:3)	C46H86NO8P	15:0-18:1(d7) PC	M+H	7.13
PC(18:0_20:4)	C46H84NO8P	15:0-18:1(d7) PC	M+H	6.95
PC(18:0_22:4)	C48H88NO8P	15:0-18:1(d7) PC	M+H	7.23
PC(18:0_22:5)	C48H86NO8P	15:0-18:1(d7) PC	M+H	6.98
PC(18:0_22:6)	C48H84NO8P	15:0-18:1(d7) PC	M+H	6.96
PC(18:1_13:0)	C39H76NO8P	15:0-18:1(d7) PC	M+H	6.36
PC(18:1_22:6)	C48H82NO8P	15:0-18:1(d7) PC	M+H	6.54
PC(18:2_13:0)	C39H74NO8P	15:0-18:1(d7) PC	M+CH3COO	6.26
PC(18:2_18:2)	C44H80NO8P	15:0-18:1(d7) PC	M+H	6.57
PC(18:2_22:6)	C48H80NO8P	15:0-18:1(d7) PC	M+H	6.31
PC(22:0_18:2)	C48H92NO8P	15:0-18:1(d7) PC	M+H	7.78
PC(30:1)	C38H74NO8P	15:0-18:1(d7) PC	M+H	6.16
PC(31:0)	C39H78NO8P	15:0-18:1(d7) PC	M+H	6.63
PC(32:0)	C40H80NO8P	15:0-18:1(d7) PC	M+H	6.92
PC(33:0)	C41H82NO8P	15:0-18:1(d7) PC	M+H	7.01
PC(33:1)	C41H80NO8P	15:0-18:1(d7) PC	M+H	6.73
PC(33:2)	C41H78NO8P	15:0-18:1(d7) PC	M+H	6.44
PC(33:3)	C41H76NO8P	15:0-18:1(d7) PC	M+CH3COO	6.36
PC(33:4)	C41H74NO8P	15:0-18:1(d7) PC	M+H	6.66
PC(34:1e)	C42H84NO7P	15:0-18:1(d7) PC	M+H	7.13
PC(34:2)	C42H80NO8P	15:0-18:1(d7) PC	M+H	6.63
PC(34:2e)	C42H82NO7P	15:0-18:1(d7) PC	M+H	7.1
PC(34:3)	C42H78NO8P	15:0-18:1(d7) PC	M+H	6.41
PC(34:3e)	C42H80NO7P	15:0-18:1(d7) PC	M+H	6.82
PC(35:1)	C43H84NO8P	15:0-18:1(d7) PC	M+H	7.07
PC(35:2)	C43H82NO8P	15:0-18:1(d7) PC	M+H	6.79
PC(35:3)	C43H80NO8P	15:0-18:1(d7) PC	M+H	7.13
PC(35:4)	C43H78NO8P	15:0-18:1(d7) PC	M+H	7.09
PC(35:5)	C43H76NO8P	15:0-18:1(d7) PC	M+H	6.73
PC(36:0)	C44H88NO8P	15:0-18:1(d7) PC	M+H	7.77

PC(36:4e)	C44H82NO7P	15:0-18:1(d7) PC	M+H	6.82
PC(36:6)	C44H76NO8P	15:0-18:1(d7) PC	M+H	6.13
PC(37:1)	C45H88NO8P	15:0-18:1(d7) PC	M+H	7.51
PC(37:4)	C45H82NO8P	15:0-18:1(d7) PC	M+H	6.76
PC(37:5)	C45H80NO8P	15:0-18:1(d7) PC	M+H	7.04
PC(37:6)	C45H78NO8P	15:0-18:1(d7) PC	M+H	6.34
PC(38:4e)	C46H86NO7P	15:0-18:1(d7) PC	M+H	7.23
PC(38:5e)	C46H84NO7P	15:0-18:1(d7) PC	M+H	6.82
PC(38:6e)	C46H82NO7P	15:0-18:1(d7) PC	M+H	6.89
PC(38:7)	C46H78NO8P	15:0-18:1(d7) PC	M+H	6.22
PC(38:7e)	C46H80NO7P	15:0-18:1(d7) PC	M+H	6.76
PC(39:6)	C47H82NO8P	15:0-18:1(d7) PC	M+H	6.78
PC(44:5e)	C52H96NO7P	15:0-18:1(d7) PC	M+H	8.07
PE(16:0_18:1)	C39H76NO8P	15:0-18:1(d7) PE	M-H	6.95
PE(16:0_18:2)	C39H74NO8P	15:0-18:1(d7) PE	M-H	6.74
PE(16:0_20:4)	C41H74NO8P	15:0-18:1(d7) PE	M-H	6.74
PE(16:0_20:5)	C41H72NO8P	15:0-18:1(d7) PE	M-H	6.43
PE(16:1e_22:6)	C43H74NO7P	15:0-18:1(d7) PE	M-H	6.79
PE(18:0_16:0)	C39H78NO8P	15:0-18:1(d7) PE	M-H	7.28
PE(18:0_18:1)	C41H80NO8P	15:0-18:1(d7) PE	M-H	7.28
PE(18:0_18:2)	C41H78NO8P	15:0-18:1(d7) PE	M-H	7.03
PE(18:0p_22:6)	C45H78NO7P	15:0-18:1(d7) PE	M-H	7.13
PE(18:1_18:2)	C41H76NO8P	15:0-18:1(d7) PE	M-H	6.78
PE(32:1)	C37H72NO8P	15:0-18:1(d7) PE	M-H	6.58
PE(38:1e)	C43H86NO7P	15:0-18:1(d7) PE	M-H	7.97
PE(38:6)	C43H74NO8P	15:0-18:1(d7) PE	M-H	6.57
PE(40:7)	C45H76NO8P	15:0-18:1(d7) PE	M-H	6.63
PE(42:5)	C47H84NO8P	15:0-18:1(d7) PE	M-H	6.62
PE(42:7)	C47H80NO8P	15:0-18:1(d7) PE	M-H	6.06
PG(16:0_18:1)	C40H77O10P	15:0-18:1(d7) PG	M+H	6.78
PG(34:2)	C40H75O10P	15:0-18:1(d7) PG	M+H	6.39
PG(36:1)	C42H81O10P	15:0-18:1(d7) PG	M+H	7.12
PG(36:2)	C42H79O10P	15:0-18:1(d7) PG	M+H	6.81
PG(36:3)	C42H77O10P	15:0-18:1(d7) PG	M+H	6.34
PG(36:4)	C42H75O10P	15:0-18:1(d7) PG	M+H	6.31
PG(36:5)	C42H73O10P	15:0-18:1(d7) PG	M+H	6.25
PG(38:4)	C44H79O10P	15:0-18:1(d7) PG	M+H	6.93
PG(38:6)	C44H75O10P	15:0-18:1(d7) PG	M+H	6.51
PG(40:6)	C46H79O10P	15:0-18:1(d7) PG	M+H	6.95
PG(40:8)	C46H75O10P	15:0-18:1(d7) PG	M+H	5.38
PI(16:0_16:0)	C41H79O13P	15:0-18:1(d7) PI	M-H	6.3
PI(16:0_16:1)	C41H77O13P	15:0-18:1(d7) PI	M-H	6.09
PI(16:0_20:4)	C45H79O13P	15:0-18:1(d7) PI	M-H	6.15
PI(16:0_22:6)	C47H79O13P	15:0-18:1(d7) PI	M-H	6.09

PI(18:0_18:2)	C45H83O13P	15:0-18:1(d7) PI	M-H	6.49
PI(18:0_20:4)	C47H83O13P	15:0-18:1(d7) PI	M-H	6.46
PI(18:0_22:5)	C49H85O13P	15:0-18:1(d7) PI	M-H	6.52
PI(18:0_22:6)	C49H83O13P	15:0-18:1(d7) PI	M-H	6.46
PI(22:1_18:2)	C49H89O13P	15:0-18:1(d7) PI	M-H	5.67
PI(34:0)	C43H83O13P	15:0-18:1(d7) PI	M-H	6.78
PI(34:1)	C43H81O13P	15:0-18:1(d7) PI	M-H	6.47
PI(34:2)	C43H79O13P	15:0-18:1(d7) PI	M-H	6.18
PI(36:0)	C45H87O13P	15:0-18:1(d7) PI	M-H	6.59
PI(36:1)	C45H85O13P	15:0-18:1(d7) PI	M-H	6.78
PI(36:3)	C45H81O13P	15:0-18:1(d7) PI	M-H	6.26
PI(36:5)	C45H77O13P	15:0-18:1(d7) PI	M-H	5.97
PI(38:2)	C47H87O13P	15:0-18:1(d7) PI	M-H	6.37
PI(38:3)	C47H85O13P	15:0-18:1(d7) PI	M-H	6.66
PI(38:5)	C47H81O13P	15:0-18:1(d7) PI	M-H	6.27
PI(40:4)	C49H87O13P	15:0-18:1(d7) PI	M-H	6.78
PS(32:1)	C38H72NO10P	15:0-18:1(d7) PS	M-H	5.36
PS(34:0)	C40H78NO10P	15:0-18:1(d7) PS	M-H	6.18
PS(34:1)	C40H76NO10P	15:0-18:1(d7) PS	M-H	6.52
PS(34:2)	C40H74NO10P	15:0-18:1(d7) PS	M-H	6.75
PS(36:0)	C42H82NO10P	15:0-18:1(d7) PS	M-H	6.59
PS(36:2)	C42H78NO10P	15:0-18:1(d7) PS	M-H	6.55
PS(38:4)	C44H78NO10P	15:0-18:1(d7) PS	M-H	6.55
PS(38:5)	C44H76NO10P	15:0-18:1(d7) PS	M-H	6.31
PS(40:4)	C46H82NO10P	15:0-18:1(d7) PS	M-H	6.43
PS(42:3)	C48H88NO10P	15:0-18:1(d7) PS	M-H	6.96
SM(d32:2)	C37H73N2O6P	18:1(d9) SM	M+H	5.68
SM(d35:1)	C40H81N2O6P	18:1(d9) SM	M+H	6.7
SM(d37:1)	C42H85N2O6P	18:1(d9) SM	M+H	7.07
SM(d39:1)	C44H89N2O6P	18:1(d9) SM	M+H	7.48
SM(d41:7)	C46H81N2O6P	18:1(d9) SM	M+H	6.25
SM(d43:2)	C48H95N2O6P	18:1(d9) SM	M+H	7.87
SM(d44:1)	C49H99N2O6P	18:1(d9) SM	M+H	8.78
SM(t40:7)	C45H79N2O7P	18:1(d9) SM	M+H	6.76
SPH(d18:0)	C18H39NO2	18:1(d7) LPC	M+H	3.51
SPH(d18:1)	C18H37NO2	18:1(d7) LPC	M+H	3.415
Sphinganine 1-phosphate (d18:0-P)	C18H40NO5P	18:1(d7) LPC	M-H	4.98
Sphingosine 1-phosphate (d18:1-P)	C18H38NO5P	18:1(d7) LPC	M-H	4.38
SPHP(d21:1)	C21H44NO5P	18:1(d7) LPC	M+CH3COO	4.38
TG(50:4)	C53H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.54
TG(16:0_14:0_18:1)	C51H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.12

TG(16:1_16:1_18:3)	C53H92O6	15:0-18:1(d7)-15:0 TG	M+NH4	10.8
TG(16:0_14:4_16:0)	C49H86O6	15:0-18:1(d7)-15:0 TG	M+NH4	9.68
TG(16:0_16:0_16:0)	C51H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.08
TG(16:0_16:0_18:1)	C53H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.15
TG(16:1_18:2_18:2)	C55H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.85
TG(16:0_16:1_16:1)	C51H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.23
TG(16:0_16:1_18:1)	C53H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.24
TG(16:1_18:2_18:3)	C55H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.15
TG(16:0_18:1_18:1)	C55H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.31
TG(16:0_18:1_18:2)	C55H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.44
TG(16:0_18:2_18:2)	C55H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.66
TG(18:3_18:2_18:2)	C57H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.07
TG(20:3_18:2_18:2)	C59H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.57
TG(16:1_14:0_18:2)	C51H92O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.29
TG(16:1_16:1_18:1)	C53H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.4
TG(17:0_18:1_20:4)	C58H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.55
TG(18:0_16:0_16:0)	C53H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	7.53
TG(18:0_16:0_18:1)	C55H104O6	15:0-18:1(d7)-15:0 TG	M+NH4	7.82
TG(18:1_18:1_18:1)	C57H104O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.46
TG(18:0_18:1_18:1)	C57H106O6	15:0-18:1(d7)-15:0 TG	M+NH4	8.01
TG(18:1_18:1_18:2)	C57H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.55
TG(18:1_12:3_18:1)	C51H88O6	15:0-18:1(d7)-15:0 TG	M+NH4	10.08
TG(18:1_18:1_20:4)	C59H102O6	15:0-18:1(d7)-15:0 TG	M+NH4	13.36
TG(18:1_18:2_18:2)	C57H100O6	15:0-18:1(d7)-15:0 TG	M+NH4	12.77
TG(18:2_18:2_18:2)	C57H98O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.85
TG(52:12e)	C55H84O5	15:0-18:1(d7)-15:0 TG	M+NH4	12.12
TG(54:8)	C57H94O6	15:0-18:1(d7)-15:0 TG	M+NH4	10.42

TG(55:4)	C58H104O6	15:0-18:1(d7)-15:0 TG	M+NH4	14.25
TG(56:9)	C59H96O6	15:0-18:1(d7)-15:0 TG	M+NH4	11.01

Table F. The identified lipid peak area RSD result before and after normalisation

Lipid	GBM		LGG		Pooled QC		plasma	
	unnormalised	normalised	unnormalised	normalised	unnormalised	normalised	unnormalised	normalised
7-oxocholesterol	23.98%	33.83%	29.37%	34.63%	13.83%	12.80%	107.92%	110.76%
AcCa(16:0)	38.88%	28.89%	28.54%	17.49%	1.38%	1.55%	12.40%	10.05%
Cer(d16:1_22:0)	23.78%	26.69%	18.55%	23.53%	2.35%	2.47%	11.19%	10.67%
Cer(d18:0/16:0)	28.51%	34.24%	29.84%	39.76%	9.96%	10.23%	17.39%	18.56%
Cer(d18:0/18:0)	67.18%	60.68%	47.90%	49.12%	4.70%	4.85%	7.84%	7.87%
Cer(d18:0/20:0)	66.81%	60.74%	43.60%	40.87%	2.96%	2.88%	8.17%	8.71%
Cer(d18:0/26:0)	47.55%	42.01%	26.55%	26.08%	1.09%	2.02%	10.34%	11.32%
Cer(d18:0_22:0)	41.51%	39.08%	30.48%	26.88%	3.51%	4.37%	9.94%	8.22%
Cer(d18:0_24:0)	48.23%	42.88%	26.04%	25.58%	2.76%	3.34%	7.56%	7.20%
Cer(d18:0_24:1)	22.74%	23.24%	12.73%	21.33%	0.95%	1.71%	8.36%	7.77%
Cer(d18:1/19:0)	55.80%	54.47%	32.18%	33.77%	18.13%	17.62%	42.23%	40.18%
Cer(d18:1/25:0)	22.31%	25.51%	24.46%	28.11%	1.06%	1.47%	7.94%	7.44%
Cer(d18:1_16:0)	19.57%	20.30%	17.03%	22.58%	4.39%	3.87%	9.00%	10.42%
Cer(d18:1_18:0)	21.02%	20.91%	20.44%	28.10%	1.83%	2.58%	7.02%	6.47%
Cer(d18:1_22:0)	17.36%	22.87%	15.23%	20.65%	2.03%	2.66%	7.64%	6.96%
Cer(d18:1_23:0)	20.41%	27.27%	24.33%	28.35%	1.81%	3.21%	9.88%	9.03%
Cer(d18:1_24:1)	21.81%	25.10%	20.19%	28.88%	2.73%	4.19%	8.35%	8.22%
Cer(d18:1_26:0)	26.02%	22.16%	12.51%	18.81%	2.08%	2.96%	10.94%	10.43%
Cer(d18:1_26:1)	28.23%	24.96%	20.47%	29.66%	1.65%	1.75%	11.72%	11.96%
Cer(d18:2/20:0)	24.89%	30.40%	32.18%	39.98%	6.75%	7.01%	8.15%	10.32%
Cer(d18:2/24:2)	45.76%	50.82%	27.62%	32.43%	10.49%	10.61%	50.63%	50.00%
Cer(d18:2_22:0)	23.76%	25.58%	19.42%	25.47%	1.63%	3.23%	8.05%	8.09%
Cer(d18:2_23:0)	25.36%	30.01%	20.84%	27.07%	2.23%	2.46%	8.36%	8.25%

Cer(d18:2_24:1)	27.51%	32.21 %	25.59%	34.64 %	1.68%	2.58%	7.44%	6.77%
ChE(14:0)	60.12%	48.80 %	92.53%	87.07 %	10.77%	9.21%	59.75%	67.71 %
ChE(15:0)	31.62%	45.04 %	27.29%	41.96 %	2.94%	6.51%	9.96%	9.40%
ChE(16:0)	59.79%	66.13 %	63.73%	70.41 %	28.82%	29.25 %	42.44%	49.41 %
ChE(16:1)	90.56%	73.68 %	59.15%	77.03 %	4.17%	4.19%	9.37%	10.57 %
ChE(16:2)	97.34%	94.59 %	97.95%	98.15 %	48.84%	43.36 %	244.95%	244.95 %
ChE(17:0)	37.66%	29.04 %	68.54%	84.37 %	34.77%	38.51 %	4.34%	2.71%
ChE(17:1)	58.46%	62.62 %	56.42%	66.15 %	52.37%	49.49 %	#DIV/0!	#DIV/ 0!
ChE(18:0)	132.58%	137.01 %	147.45%	149.83 %	31.36%	30.24 %	159.23%	166.38 %
ChE(18:1)	41.28%	40.99 %	54.24%	66.28 %	10.40%	15.43 %	29.04%	28.12 %
ChE(18:3)	56.70%	50.45 %	31.69%	28.87 %	2.44%	6.08%	7.07%	9.26%
ChE(20:1)	46.88%	48.93 %	26.08%	32.31 %	20.03%	21.97 %	42.42%	43.17 %
ChE(20:3)	41.62%	31.99 %	35.75%	38.91 %	2.64%	5.39%	5.75%	10.20 %
ChE(20:4)	27.11%	18.04 %	24.66%	25.74 %	1.99%	6.11%	7.16%	9.65%
ChE(20:5)	49.27%	41.41 %	44.54%	35.44 %	0.92%	6.97%	8.16%	9.55%
ChE(22:6)	45.37%	48.03 %	36.84%	36.32 %	2.48%	6.07%	8.62%	9.70%
CoQ10	36.22%	39.61 %	26.46%	35.70 %	3.16%	5.21%	8.98%	9.58%
CoQ9	64.90%	62.57 %	70.30%	84.00 %	47.80%	46.85 %	59.66%	62.49 %
DG(30:2)	316.23%	316.23 %	41.95%	27.51 %	#DIV/0!	#DIV/ 0!	14.57%	14.05 %
DG(38:0)	64.38%	65.75 %	41.83%	44.51 %	14.58%	16.19 %	75.46%	76.82 %
DG(38:3)	36.25%	38.82 %	62.45%	67.92 %	8.20%	7.17%	9.22%	8.75%
DG(40:7)	64.56%	66.13 %	47.02%	49.91 %	7.84%	8.80%	6.82%	3.19%
DG(14:0_18:2)	58.28%	61.22 %	78.35%	83.79 %	9.00%	8.21%	5.62%	3.38%
DG(14:0_18:3)	51.11%	57.53 %	316.23%	316.23 %	244.95%	244.95 %	17.07%	18.18 %
DG(16:0_14:0)	78.80%	81.04 %	79.80%	79.23 %	10.37%	9.66%	6.46%	3.88%
DG(16:0_16:0)	60.97%	65.08 %	56.81%	55.03 %	3.62%	3.41%	7.60%	4.67%
DG(16:0_16:1)	65.83%	67.46 %	73.64%	80.91 %	3.82%	4.43%	7.17%	3.39%
DG(16:0_18:2)	41.95%	47.82 %	43.75%	50.25 %	1.82%	2.24%	7.13%	4.11%
DG(16:1_14:0)	112.66%	109.50 %	74.24%	79.62 %	24.59%	25.32 %	2.14%	4.01%

DG(16:1_18:3)	113.69%	123.03 %	72.04%	72.16 %	28.40%	28.43 %	5.15%	4.87%
DG(18:0_16:0)	57.35%	63.50 %	48.46%	49.06 %	2.18%	2.16%	8.28%	6.09%
DG(18:0_18:0)	62.83%	69.23 %	44.89%	43.14 %	4.78%	4.61%	7.60%	7.45%
DG(18:0_18:1)	52.82%	59.86 %	50.83%	57.80 %	2.01%	2.69%	9.02%	6.58%
DG(18:1_18:1)	35.18%	41.45 %	60.24%	69.97 %	1.04%	1.73%	8.96%	6.75%
DG(18:1_20:3)	35.23%	37.53 %	40.65%	47.44 %	5.77%	4.48%	7.19%	4.80%
DG(18:1_22:5)	61.28%	61.72 %	62.18%	66.58 %	13.67%	13.07 %	10.32%	7.66%
DG(18:3_18:2)	87.27%	91.17 %	69.43%	69.12 %	10.56%	10.76 %	3.77%	2.28%
DG(34:1)	45.82%	51.21 %	49.25%	56.13 %	1.11%	2.24%	8.47%	6.09%
DG(34:3)	47.42%	53.28 %	48.53%	55.69 %	5.02%	5.97%	6.59%	3.38%
DG(36:3)	41.85%	46.39 %	50.39%	56.10 %	1.61%	1.49%	7.93%	5.49%
DG(36:4)	56.94%	61.28 %	54.75%	55.02 %	2.21%	2.65%	7.31%	3.38%
DG(38:5)	40.24%	46.39 %	44.25%	42.28 %	4.97%	5.07%	9.76%	6.79%
DG(38:6)	37.61%	39.80 %	42.51%	37.45 %	6.95%	8.10%	5.78%	3.36%
Eicosanoids_19,20-DiHDPA	37.72%	25.44 %	10.45%	28.94 %	244.95%	244.95 %	#DIV/0!	#DIV/ 0!
Eicosanoids_PGD3	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!	244.95%	244.95 %
FA(12:0)	39.62%	32.39 %	46.08%	71.52 %	3.73%	2.39%	18.62%	13.46 %
FA(14:0)	31.58%	26.38 %	52.81%	69.74 %	0.95%	2.69%	14.89%	11.80 %
FA(15:0)	31.91%	32.69 %	33.48%	43.46 %	1.71%	2.64%	12.04%	9.33%
FA(16:0)	22.22%	29.66 %	38.84%	55.84 %	1.30%	1.43%	10.41%	9.25%
FA(16:1)	54.08%	54.62 %	103.28%	120.77 %	1.19%	2.66%	14.96%	12.91 %
FA(17:0)	23.44%	29.98 %	23.62%	35.77 %	1.04%	2.13%	13.86%	11.17 %
FA(17:1)	46.13%	46.73 %	76.62%	92.97 %	1.96%	1.44%	14.73%	11.27 %
FA(18:0)	14.44%	35.15 %	15.72%	27.09 %	1.36%	2.35%	8.67%	6.50%
FA(18:1)	45.98%	49.63 %	71.90%	90.06 %	1.10%	2.44%	12.33%	12.01 %
FA(18:2)	47.92%	54.76 %	50.95%	70.31 %	1.07%	1.38%	13.39%	11.49 %
FA(18:3)	56.01%	60.27 %	69.91%	92.96 %	1.17%	2.42%	16.80%	14.36 %
FA(18:4)	32.81%	55.49 %	48.08%	73.69 %	2.48%	4.33%	17.56%	13.58 %
FA(20:0)	18.43%	39.74 %	16.62%	26.46 %	1.12%	2.31%	9.66%	4.99%

FA(20:2)	47.28%	50.96 %	44.76%	61.36 %	1.12%	1.20%	17.66%	14.56 %
FA(20:3)	23.38%	42.27 %	44.11%	52.88 %	1.02%	2.15%	13.58%	12.35 %
FA(20:4)	14.95%	39.67 %	26.70%	45.47 %	1.04%	1.66%	13.33%	11.40 %
FA(20:5)	51.87%	44.58 %	42.52%	58.91 %	1.92%	2.83%	18.52%	15.79 %
FA(22:0)	38.17%	52.48 %	39.71%	32.74 %	1.74%	2.14%	7.71%	12.17 %
FA(22:1)	31.43%	39.42 %	32.63%	47.82 %	1.68%	3.09%	10.67%	9.09%
FA(22:2)	35.26%	44.22 %	33.03%	47.28 %	4.60%	6.23%	14.09%	8.10%
FA(22:3)	33.75%	36.53 %	44.83%	57.18 %	3.09%	3.29%	18.95%	17.09 %
FA(22:4)	30.41%	38.96 %	43.55%	60.44 %	2.03%	3.28%	14.62%	13.19 %
FA(22:5)	29.02%	40.91 %	41.11%	62.32 %	1.01%	1.56%	14.28%	11.81 %
FA(22:6)	40.86%	40.98 %	33.73%	57.68 %	1.09%	2.65%	14.27%	12.00 %
FA(23:0)	62.44%	80.39 %	38.92%	41.83 %	2.83%	3.58%	10.99%	14.36 %
FA(24:0)	20.97%	54.21 %	21.19%	30.42 %	1.50%	1.91%	5.06%	11.32 %
FA(24:1)	14.74%	37.50 %	27.51%	38.12 %	2.00%	2.55%	12.44%	15.01 %
FA(26:0)	26.98%	53.94 %	20.10%	38.23 %	4.07%	5.69%	5.03%	12.72 %
Hex2Cer(d12:0_20:4)	48.24%	125.85 %	59.60%	81.55 %	2.94%	3.32%	25.92%	27.96 %
LPC(16:0)	38.15%	33.01 %	31.22%	12.97 %	1.59%	1.46%	11.96%	4.90%
LPC(16:1)	68.03%	55.33 %	48.95%	30.69 %	2.30%	2.67%	13.89%	7.28%
LPC(18:0)	29.25%	53.94 %	27.52%	21.04 %	1.57%	1.46%	13.48%	7.47%
LPC(18:1)	48.51%	40.17 %	42.29%	23.93 %	1.59%	1.39%	12.91%	6.83%
LPC(18:2)	56.53%	41.37 %	37.05%	27.80 %	1.60%	0.90%	14.97%	8.47%
LPC(20:3)	65.16%	46.91 %	52.76%	35.07 %	2.71%	2.29%	13.86%	8.19%
LPC(20:4)	43.97%	27.75 %	33.54%	24.92 %	1.42%	0.68%	15.22%	9.20%
LPC(22:5)	49.05%	35.54 %	27.56%	30.09 %	2.46%	2.07%	14.54%	8.77%
LPC(22:6)	45.76%	31.51 %	27.42%	25.73 %	0.79%	1.30%	15.75%	10.66 %
LPE(16:0)	33.85%	41.49 %	36.62%	21.26 %	2.55%	2.84%	11.40%	5.03%
LPE(18:0)	31.09%	56.17 %	38.05%	22.93 %	0.85%	1.21%	12.67%	8.97%
LPE(18:1)	39.79%	45.78 %	50.13%	34.62 %	1.38%	1.55%	13.60%	8.10%
LPE(18:2)	44.32%	47.69 %	45.91%	30.77 %	1.80%	1.64%	13.88%	9.01%

LPE(20:4)	29.74%	32.29 %	37.88%	27.07 %	1.40%	1.11%	13.82%	8.56%
LPE(22:1)	45.75%	34.04 %	50.46%	34.70 %	11.57%	11.10 %	19.15%	13.15 %
LPE(22:6)	29.74%	26.25 %	29.21%	27.76 %	1.98%	2.27%	16.04%	11.52 %
PC(14:0_18:2)	43.94%	58.41 %	36.34%	46.54 %	2.48%	3.42%	11.64%	10.70 %
PC(14:0_20:4)	41.13%	49.69 %	35.17%	44.09 %	2.10%	2.54%	12.22%	11.33 %
PC(14:0_20:5)	60.32%	68.18 %	36.65%	37.56 %	2.86%	3.99%	13.00%	12.92 %
PC(16:0_16:1)	50.77%	59.56 %	43.77%	55.26 %	1.22%	2.65%	6.63%	5.98%
PC(16:0_18:1)	12.37%	22.81 %	15.22%	24.93 %	2.74%	2.12%	2.99%	4.05%
PC(16:0_20:3)	14.87%	27.08 %	18.93%	28.67 %	1.10%	1.43%	5.38%	5.12%
PC(16:0_20:5)	42.87%	50.36 %	31.08%	25.16 %	2.24%	3.49%	8.69%	7.94%
PC(16:0_22:5)	15.16%	24.03 %	16.68%	15.45 %	0.98%	1.99%	5.03%	5.12%
PC(16:0_22:6)	27.37%	25.94 %	18.98%	21.05 %	1.23%	2.76%	8.28%	7.61%
PC(18:0_16:0)	23.72%	19.67 %	17.27%	13.85 %	5.20%	5.88%	5.95%	4.67%
PC(18:0_18:1)	17.76%	26.73 %	18.13%	23.09 %	2.14%	1.42%	6.85%	6.36%
PC(18:0_20:2)	22.81%	33.80 %	22.87%	25.76 %	2.44%	2.02%	10.78%	10.01 %
PC(18:0_20:3)	22.58%	32.79 %	38.93%	49.38 %	1.93%	3.06%	5.83%	5.93%
PC(18:0_20:4)	18.34%	20.31 %	19.40%	20.97 %	2.74%	2.73%	4.34%	5.62%
PC(18:0_22:4)	21.45%	32.33 %	23.65%	27.76 %	4.73%	4.13%	5.17%	4.79%
PC(18:0_22:5)	21.74%	33.58 %	23.72%	24.02 %	3.49%	3.25%	5.55%	5.99%
PC(18:0_22:6)	35.40%	31.87 %	32.66%	29.64 %	3.23%	2.79%	3.53%	4.88%
PC(18:1_13:0)	14.20%	15.50 %	20.59%	23.65 %	3.39%	2.97%	7.26%	8.67%
PC(18:1_22:6)	29.09%	28.99 %	22.10%	20.18 %	4.54%	6.22%	5.65%	5.86%
PC(18:2_18:2)	12.29%	23.80 %	10.54%	15.95 %	1.62%	1.96%	5.80%	6.09%
PC(18:2_22:6)	32.97%	39.16 %	18.40%	15.58 %	3.21%	4.01%	12.10%	11.34 %
PC(22:0_18:2)	31.82%	42.13 %	16.26%	13.07 %	2.43%	4.24%	11.69%	10.43 %
PC(30:1)	72.60%	84.17 %	57.47%	67.69 %	2.26%	2.93%	11.42%	10.24 %
PC(32:0)	13.15%	16.95 %	8.74%	13.55 %	2.51%	1.81%	6.20%	6.21%
PC(33:0)	25.05%	20.40 %	13.88%	16.53 %	2.17%	2.70%	2.79%	4.16%
PC(33:4)	22.92%	13.99 %	16.86%	21.34 %	2.38%	1.00%	5.39%	6.14%

PC(34:1e)	12.41%	18.86 %	14.12%	23.05 %	1.75%	2.00%	7.37%	7.55%
PC(34:2)	16.08%	30.04 %	6.24%	17.48 %	1.59%	0.80%	4.05%	4.12%
PC(34:2e)	29.82%	39.56 %	17.70%	17.93 %	3.13%	2.70%	6.08%	6.25%
PC(34:3)	33.83%	43.20 %	25.62%	34.95 %	1.77%	3.25%	9.41%	8.55%
PC(34:3e)	42.10%	53.85 %	20.86%	21.90 %	3.37%	2.32%	5.92%	5.52%
PC(35:1)	21.85%	20.54 %	13.59%	19.70 %	1.35%	2.87%	5.45%	4.48%
PC(35:2)	23.04%	25.10 %	20.35%	22.06 %	2.01%	1.87%	5.06%	5.51%
PC(35:3)	56.36%	66.47 %	73.66%	85.66 %	13.12%	13.61 %	5.26%	5.48%
PC(35:4)	19.32%	16.27 %	14.48%	20.46 %	3.28%	2.83%	3.98%	4.62%
PC(35:5)	20.35%	15.46 %	15.06%	18.46 %	1.69%	1.07%	5.95%	5.59%
PC(36:0)	316.23%	316.23 %	316.23%	316.23 %	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!
PC(36:4e)	18.34%	26.62 %	17.76%	18.86 %	2.51%	2.92%	5.40%	5.68%
PC(36:6)	38.43%	44.10 %	24.31%	33.03 %	4.78%	5.91%	9.79%	8.59%
PC(37:1)	15.69%	23.94 %	20.37%	17.04 %	1.39%	3.20%	8.18%	7.65%
PC(37:4)	49.57%	42.49 %	39.71%	36.53 %	3.79%	3.19%	9.46%	9.18%
PC(37:5)	14.63%	15.25 %	13.91%	19.83 %	1.45%	2.33%	4.00%	5.99%
PC(37:6)	55.08%	51.30 %	46.46%	42.30 %	7.95%	7.85%	7.32%	6.07%
PC(38:4e)	23.33%	28.77 %	20.53%	23.39 %	1.56%	1.54%	5.36%	5.79%
PC(38:5e)	15.20%	19.56 %	16.26%	17.93 %	2.94%	1.87%	6.15%	7.17%
PC(38:6e)	22.49%	24.06 %	21.12%	21.35 %	2.33%	0.93%	5.83%	5.89%
PC(38:7)	31.20%	42.74 %	17.34%	18.26 %	2.89%	3.37%	9.84%	8.73%
PC(38:7e)	38.47%	34.09 %	45.33%	40.51 %	5.32%	5.69%	3.67%	3.93%
PC(39:6)	65.58%	65.66 %	66.77%	58.59 %	12.86%	11.68 %	7.99%	10.19 %
PC(44:5e)	32.30%	42.48 %	29.31%	31.80 %	2.72%	2.97%	14.23%	12.97 %
PE(16:0_20:5)	36.09%	37.56 %	33.73%	39.60 %	1.33%	7.15%	13.72%	13.60 %
PE(16:1e_22:6)	19.72%	13.14 %	29.16%	32.04 %	5.03%	2.38%	14.74%	5.88%
PE(18:0_16:0)	12.54%	9.48%	31.58%	37.56 %	7.38%	11.56 %	18.80%	18.68 %
PE(18:0_18:1)	10.99%	19.96 %	15.23%	20.24 %	1.36%	5.71%	7.31%	12.70 %
PE(18:0_18:2)	18.90%	28.94 %	13.66%	20.54 %	2.21%	6.54%	8.39%	12.93 %

PE(18:0p_22:6)	20.42%	15.19 %	23.91%	30.63 %	2.51%	7.12%	10.28%	12.63 %
PE(18:1_18:2)	19.78%	24.54 %	15.53%	18.88 %	4.41%	2.20%	14.68%	5.33%
PE(32:1)	33.40%	38.66 %	24.01%	28.81 %	4.00%	5.51%	14.61%	11.23 %
PE(38:1e)	31.43%	33.32 %	55.08%	53.70 %	10.76%	15.06 %	131.15%	132.55 %
PE(38:6)	26.97%	24.02 %	19.43%	26.70 %	0.95%	5.66%	14.93%	5.94%
PE(40:7)	33.29%	30.53 %	31.66%	29.79 %	6.50%	10.64 %	9.65%	8.70%
PE(42:5)	16.04%	15.37 %	10.14%	11.96 %	11.44%	12.28 %	6.00%	13.41 %
PE(42:7)	22.30%	20.32 %	14.88%	13.17 %	5.71%	6.38%	8.47%	17.64 %
PG(16:0_18:1)	23.90%	42.92 %	43.85%	107.77 %	6.05%	27.21 %	4.81%	11.26 %
PG(34:2)	78.11%	81.16 %	116.67%	153.57 %	19.94%	23.05 %	105.65%	107.53 %
PG(36:1)	24.53%	50.76 %	31.68%	108.60 %	3.79%	27.11 %	55.69%	60.69 %
PG(36:2)	45.95%	85.28 %	33.81%	105.23 %	5.20%	26.28 %	32.16%	34.32 %
PG(36:3)	71.65%	113.65 %	88.10%	130.47 %	73.67%	77.02 %	244.95%	244.95 %
PG(36:5)	25.52%	33.29 %	52.11%	62.60 %	62.86%	56.03 %	16.90%	23.33 %
PG(38:4)	316.23%	316.23 %	#DIV/0!	#DIV/ 0!	14.24%	26.94 %	#DIV/0!	#DIV/ 0!
PG(38:6)	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!
PG(40:6)	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!	#DIV/0!	#DIV/ 0!
PG(40:8)	#DIV/0!	#DIV/ 0!	316.23%	316.23 %	244.95%	244.95 %	12.72%	19.60 %
PI(16:0_16:0)	40.40%	19.47 %	46.60%	24.30 %	3.10%	2.87%	30.23%	12.70 %
PI(16:0_16:1)	38.08%	32.78 %	78.44%	49.71 %	4.47%	5.17%	26.13%	11.00 %
PI(16:0_20:4)	51.23%	32.41 %	63.14%	29.52 %	2.47%	3.08%	24.61%	7.75%
PI(16:0_22:6)	37.14%	39.23 %	58.18%	32.45 %	4.12%	4.40%	34.60%	23.30 %
PI(18:0_18:2)	52.12%	33.70 %	51.19%	21.65 %	3.95%	4.12%	24.64%	10.69 %
PI(18:0_20:4)	47.19%	22.48 %	59.50%	25.07 %	2.10%	1.35%	25.14%	11.07 %
PI(18:0_22:5)	69.21%	45.25 %	76.98%	51.15 %	16.04%	14.92 %	35.15%	18.68 %
PI(18:0_22:6)	33.80%	22.41 %	56.51%	30.33 %	3.26%	2.63%	30.01%	16.38 %
PI(22:1_18:2)	14.84%	35.26 %	8.61%	68.51 %	3.11%	3.51%	18.29%	18.96 %
PI(34:0)	56.27%	33.06 %	54.57%	34.84 %	6.12%	7.21%	244.95%	244.95 %
PI(34:1)	39.52%	19.98 %	52.17%	26.84 %	3.59%	4.33%	23.26%	7.28%

PI(34:2)	44.10%	28.82 %	50.54%	21.79 %	2.67%	3.02%	24.07%	7.03%
PI(36:0)	26.95%	40.74 %	19.71%	81.23 %	1.87%	1.52%	7.51%	15.68 %
PI(36:1)	62.93%	38.13 %	49.46%	21.39 %	4.22%	5.01%	32.66%	17.35 %
PI(36:3)	60.69%	37.90 %	56.89%	24.99 %	3.04%	3.48%	20.60%	9.07%
PI(36:5)	31.59%	24.71 %	48.16%	29.93 %	4.27%	5.16%	99.10%	97.79 %
PI(38:2)	31.50%	50.64 %	19.38%	84.57 %	2.78%	3.49%	11.08%	15.32 %
PI(38:3)	67.83%	42.11 %	88.67%	65.05 %	3.90%	5.06%	25.28%	10.80 %
PI(38:5)	47.39%	25.53 %	53.19%	20.67 %	2.90%	3.12%	27.00%	11.04 %
PI(40:4)	81.36%	57.94 %	77.43%	50.62 %	16.61%	16.67 %	109.33%	91.27 %
PS(32:1)	63.29%	84.14 %	110.38%	82.81 %	54.18%	52.68 %	#DIV/0!	#DIV/ 0!
PS(34:0)	63.76%	85.04 %	69.43%	77.17 %	3.18%	9.86%	12.46%	15.18 %
PS(34:1)	79.12%	73.58 %	65.83%	53.77 %	15.06%	14.86 %	#DIV/0!	#DIV/ 0!
PS(34:2)	38.98%	52.40 %	91.22%	79.77 %	17.89%	16.86 %	244.95%	244.95 %
PS(36:0)	53.88%	82.01 %	46.57%	89.78 %	3.26%	8.38%	5.90%	15.12 %
PS(36:2)	66.01%	59.67 %	101.91%	78.42 %	7.02%	12.98 %	17.86%	21.85 %
PS(38:4)	51.84%	21.06 %	50.62%	20.24 %	2.13%	9.56%	#DIV/0!	#DIV/ 0!
PS(38:5)	41.55%	13.91 %	47.65%	24.44 %	1.77%	7.06%	58.98%	45.07 %
PS(40:4)	43.31%	58.61 %	30.26%	96.87 %	1.58%	7.61%	10.82%	15.94 %
PS(42:3)	18.02%	34.93 %	20.39%	78.43 %	1.05%	7.45%	3.55%	17.67 %
SM(d32:2)	22.04%	27.99 %	37.08%	42.50 %	3.90%	3.23%	14.97%	12.25 %
SM(d35:1)	28.03%	21.29 %	19.89%	24.73 %	2.38%	3.52%	9.47%	11.14 %
SM(d37:1)	37.96%	33.64 %	24.77%	29.27 %	5.77%	6.30%	7.97%	6.39%
SM(d39:1)	18.38%	19.74 %	19.38%	20.23 %	2.40%	3.64%	9.82%	8.81%
SM(d41:7)	39.22%	48.74 %	30.91%	34.21 %	8.39%	8.74%	14.04%	15.23 %
SM(d43:2)	26.45%	20.91 %	26.31%	28.78 %	1.73%	3.01%	10.37%	8.99%
SM(d44:1)	27.52%	16.92 %	18.02%	20.17 %	1.03%	2.05%	15.86%	15.63 %
SM(t40:7)	48.21%	51.32 %	70.07%	69.52 %	96.27%	97.34 %	24.41%	25.04 %
SPH(d18:0)	32.65%	56.27 %	52.42%	57.12 %	4.71%	4.19%	11.76%	12.42 %
SPH(d18:1)	49.91%	57.48 %	74.76%	62.04 %	8.50%	8.68%	15.60%	16.04 %

Sphinganine 1-phosphate (d18:0-P)	50.19%	47.77 %	63.76%	68.45 %	86.40%	86.60 %	86.67%	91.91 %
Sphingosine 1-phosphate (d18:1-P)	27.73%	32.38 %	44.75%	55.24 %	9.01%	9.07%	24.43%	25.78 %
SPHP(d21:1)	28.77%	50.89 %	41.04%	29.46 %	2.77%	3.28%	18.10%	10.80 %
TG(16:0_14:0_18:1)	55.69%	69.78 %	52.92%	79.18 %	0.73%	0.32%	6.30%	4.86%
TG(16:0_14:4_16:0)	82.45%	102.52 %	84.84%	85.31 %	2.88%	2.57%	10.00%	8.65%
TG(16:0_16:0_16:0)	73.38%	84.70 %	70.32%	68.92 %	1.20%	1.39%	5.69%	8.99%
TG(16:0_16:0_18:1)	43.84%	66.28 %	36.88%	57.20 %	0.71%	1.06%	3.03%	5.33%
TG(16:0_16:1_16:1)	47.90%	66.28 %	63.33%	96.19 %	1.15%	0.64%	6.08%	5.41%
TG(16:0_16:1_18:1)	30.93%	56.54 %	45.15%	77.01 %	0.77%	0.97%	5.49%	5.15%
TG(16:0_18:1_18:1)	28.47%	50.62 %	37.50%	65.30 %	0.27%	0.97%	3.02%	4.55%
TG(16:0_18:2_18:2)	38.27%	57.42 %	37.76%	58.58 %	0.67%	1.09%	4.76%	5.01%
TG(16:1_14:0_18:2)	46.74%	69.88 %	65.83%	98.04 %	0.70%	1.08%	8.50%	6.16%
TG(16:1_16:1_18:1)	24.77%	50.97 %	50.77%	81.76 %	1.24%	0.72%	5.03%	4.45%
TG(17:0_18:1_20:4)	30.90%	47.01 %	45.50%	56.83 %	3.92%	3.62%	8.67%	6.05%
TG(18:0_16:0_16:0)	74.48%	73.17 %	107.49%	99.65 %	27.48%	26.74 %	53.47%	55.46 %
TG(18:0_16:0_18:1)	59.85%	59.60 %	82.69%	74.83 %	13.00%	12.32 %	55.18%	55.48 %
TG(18:0_18:1_18:1)	56.70%	55.24 %	69.10%	64.11 %	6.06%	5.98%	54.90%	53.41 %
TG(18:1_12:3_18:1)	57.85%	77.85 %	56.60%	61.99 %	4.75%	5.38%	10.02%	9.05%
TG(18:1_18:1_20:4)	15.91%	28.47 %	37.17%	49.15 %	1.69%	2.02%	4.58%	3.40%
TG(18:1_18:2_18:2)	43.49%	70.02 %	44.59%	56.47 %	0.78%	1.11%	5.72%	4.79%
TG(18:2_18:2_18:2)	53.89%	81.91 %	59.69%	64.71 %	0.66%	1.20%	5.41%	4.76%
TG(50:4)	41.25%	63.76 %	60.44%	88.21 %	1.30%	0.84%	8.14%	5.71%
TG(50:5)	54.84%	76.69 %	53.16%	77.50 %	1.31%	1.83%	9.73%	6.89%
TG(52:12e)	81.59%	81.31 %	92.22%	114.83 %	30.36%	30.72 %	32.20%	37.86 %
TG(52:3)	32.68%	48.37 %	36.47%	59.97 %	1.45%	1.54%	3.87%	4.74%
TG(52:5)	44.56%	66.97 %	42.57%	63.97 %	1.05%	0.98%	6.46%	5.53%
TG(52:6)	50.94%	71.32 %	46.41%	67.83 %	0.67%	0.80%	8.78%	6.31%
TG(54:3)	33.79%	49.83 %	46.37%	65.93 %	0.79%	1.06%	4.79%	5.47%
TG(54:4)	40.70%	61.08 %	47.03%	62.43 %	0.90%	1.51%	3.42%	5.03%

TG(54:7)	58.21%	84.89 %	60.42%	66.62 %	0.71%	0.97%	8.74%	6.90%
TG(54:8)	58.71%	82.26 %	60.23%	64.63 %	1.57%	1.61%	10.46%	7.32%
TG(55:4)	23.34%	35.76 %	53.45%	64.51 %	2.06%	2.57%	32.15%	32.19 %
TG(56:7)	25.98%	33.34 %	33.52%	43.41 %	1.23%	0.98%	4.76%	4.57%
TG(56:9)	50.95%	60.87 %	44.85%	43.06 %	0.92%	1.21%	9.87%	6.64%

Table G. The univariate and multivariate result of identified lipids (deleting outliers)

Identified lipids	**t-test**	**BF correction**	**FDR correction**	**fold change**	**VIP**
7-oxocholesterol	0.525922	5.259220738	0.985066	1.104185106	0.683101
AcCa(16:0)	0.201676	2.016761277	0.985066	0.871493496	1.35137
Cer(d16:1_22:0)	0.623323	6.233230475	0.985066	0.94548552	0.5302
Cer(d18:0/16:0)	0.69556	6.955600737	0.985066	1.068246445	0.42297
Cer(d18:0/18:0)	0.982997	9.829971349	>0.999999	1.005154639	0.023086
Cer(d18:0/20:0)	0.761671	7.616706417	0.989933	0.931790985	0.328104
Cer(d18:0/26:0)	0.760341	7.603409442	0.989933	1.050225564	0.329988
Cer(d18:0_22:0)	0.63531	6.353097386	0.985066	0.931061807	0.512098
Cer(d18:0_24:0)	0.772772	7.72771968	0.992639	0.95540797	0.312417
Cer(d18:0_24:1)	0.483142	4.83141549	0.985066	0.931179775	0.754313
Cer(d18:1/19:0)	0.310617	3.106166327	0.985066	0.815762113	1.08234
Cer(d18:1/25:0)	0.124105	1.24104996	0.985066	0.820984585	1.61085
Cer(d18:1_16:0)	0.886738	8.86738266	>0.999999	1.013422819	0.154246
Cer(d18:1_18:0)	0.803246	8.03245901	>0.999999	1.027992278	0.269651
Cer(d18:1_22:0)	0.454553	4.54553374	0.985066	0.928327269	0.803649
Cer(d18:1_23:0)	0.32928	3.29280462	0.985066	0.882049321	1.04252
Cer(d18:1_24:1)	0.698787	6.987868376	0.985066	0.95349563	0.418275
Cer(d18:1_26:0)	0.585454	5.854538697	0.985066	0.950307091	0.588332
Cer(d18:1_26:1)	0.733115	7.331149422	0.989933	0.958312898	0.368758
Cer(d18:2/20:0)	0.71546	7.154601146	0.985066	1.060232221	0.39413
Cer(d18:2/24:2)	0.595227	5.952270906	0.985066	1.111637704	0.573185
Cer(d18:2_22:0)	0.67911	6.791104423	0.985066	0.953031597	0.44702
Cer(d18:2_23:0)	0.41624	4.162400177	0.985066	0.89927104	0.872359
Cer(d18:2_24:1)	0.957384	9.573836889	>0.999999	1.008247423	0.057883
ChE(14:0)	0.317935	3.179351737	0.985066	1.673981191	1.06656
ChE(15:0)	0.92685	9.268504501	>0.999999	1.018480493	0.09944
ChE(16:0)	0.661818	6.618176452	0.985066	0.871979287	0.472523
ChE(16:1)	0.754908	7.549083712	0.989933	1.112884504	0.337691
ChE(18:1)	0.941537	9.415365607	>0.999999	0.98156682	0.079439
ChE(18:3)	0.466644	4.666443123	0.985066	1.152696209	0.782597
ChE(20:1)	0.727854	7.278535036	0.989933	0.937451737	0.376299

ChE(20:3)	0.415417	4.154174996	0.985066	0.873664749	0.873871
ChE(20:4)	0.05864	0.586395018	0.985066	0.81105309	1.94762
ChE(20:5)	0.427491	4.274906326	0.985066	1.152644731	0.851848
ChE(22:6)	0.150452	1.504524127	0.985066	0.756158317	1.51257
CoQ10	0.765056	7.650555308	0.989933	0.950207469	0.323315
DG(38:0)	0.56542	5.654197532	0.985066	0.866146124	0.619725
DG(38:3)	0.530066	5.300663519	0.985066	0.847175278	0.676351
DG(40:7)	0.050419	0.504187976	0.985066	0.582152062	2.00808
DG(14:0_18:2)	0.377045	3.770451325	0.985066	0.728312679	0.946284
DG(16:0_14:0)	0.906133	9.061333542	>0.999999	1.043531576	0.127709
DG(16:0_16:0)	0.858677	8.586774558	>0.999999	0.953183521	0.192794
DG(16:0_16:1)	0.503431	5.034308834	0.985066	0.791025641	0.720176
DG(16:0_18:2)	0.635128	6.35127696	0.985066	0.899063476	0.512372
DG(16:1_14:0)	0.790149	7.90148915	>0.999999	0.846464646	0.28798
DG(16:1_18:3)	0.541342	5.413418694	0.985066	0.694605809	0.65811
DG(18:0_16:0)	0.93726	9.372599852	>0.999999	0.980018526	0.08526
DG(18:0_18:0)	0.421183	4.211833347	0.985066	1.252602359	0.863311
DG(18:0_18:1)	0.868097	8.680970209	>0.999999	0.956680015	0.179832
DG(18:1_18:1)	0.453769	4.537686316	0.985066	0.811960133	0.805026
DG(18:1_20:3)	0.500327	5.003270003	0.985066	0.874475891	0.725354
DG(18:1_22:5)	0.049234	0.492338128	0.985066	0.509202454	2.01741
DG(18:3_18:2)	0.261174	2.611742954	0.985066	1.58893617	1.19545
DG(34:1)	0.598127	5.981270759	0.985066	0.877962625	0.56871
DG(34:3)	0.305581	3.055814285	0.985066	0.769415149	1.09333
DG(36:3)	0.699759	6.997588534	0.985066	0.912904556	0.416863
DG(36:4)	0.378894	3.788942668	0.985066	1.272341813	0.942703
DG(38:5)	0.466059	4.660593135	0.985066	0.862787356	0.78361
DG(38:6)	0.549223	5.492233683	0.985066	0.899960143	0.645462
FA(12:0)	0.524478	5.24477535	0.985066	0.843174847	0.685459
FA(14:0)	0.248227	2.48226759	0.985066	0.726967048	1.22725
FA(15:0)	0.521234	5.212341905	0.985066	0.891778913	0.690765
FA(16:0)	0.762552	7.625516307	0.989933	0.939393939	0.326857
FA(16:1)	0.110869	1.108688292	0.985066	0.35166794	1.66594
FA(17:0)	0.668134	6.681344875	0.985066	1.065878378	0.46318
FA(17:1)	0.24318	2.431797827	0.985066	0.627962085	1.23993
FA(18:0)	0.272699	2.726992883	0.985066	1.177066451	1.16797
FA(18:1)	0.45646	4.564602361	0.985066	0.764716737	0.800311
FA(18:2)	0.693989	6.939891188	0.985066	1.117647059	0.425259
FA(18:3)	0.865629	8.656286726	>0.999999	0.940841866	0.183226
FA(18:4)	0.339164	3.391635045	0.985066	0.738126774	1.02197
FA(20:0)	0.122414	1.224140454	0.985066	1.297093649	1.61765
FA(20:2)	0.660981	6.609807411	0.985066	0.892498679	0.473763
FA(20:3)	0.597938	5.979375869	0.985066	0.889983274	0.569002
FA(20:4)	0.439866	4.398658889	0.985066	0.857959543	0.829615

FA(20:5)	0.964135	9.641346448	>0.999999	0.989350373	0.048708
FA(22:0)	0.018083	0.180828152	0.985066	1.837511606	2.36858
FA(22:1)	0.436565	4.365645529	0.985066	1.166666667	0.835514
FA(22:2)	0.544647	5.446468208	0.985066	1.134379786	0.652796
FA(22:3)	0.961643	9.616434639	>0.999999	0.989394609	0.052094
FA(22:4)	0.605312	6.053120318	0.985066	0.884436946	0.557663
FA(22:5)	0.650226	6.502258562	0.985066	0.894505074	0.489754
FA(22:6)	0.311335	3.113345574	0.985066	0.782604293	1.08078
FA(23:0)	0.710584	7.105841359	0.985066	1.118176365	0.401172
FA(24:0)	0.086591	0.865907944	0.985066	1.496631376	1.78081
FA(24:1)	0.922717	9.227169726	>0.999999	1.01665936	0.105074
FA(26:0)	0.261966	2.619664662	0.985066	1.290816327	1.19354
Hex2Cer(d12:0_20:4)	0.502718	5.02717874	0.985066	1.426497838	0.721364
LPC(16:0)	0.538247	5.382468738	0.985066	0.933679707	0.663099
LPC(16:1)	0.47974	4.797400539	0.985066	1.162775864	0.760105
LPC(18:0)	0.315075	3.150751759	0.985066	0.837225275	1.0727
LPC(18:1)	0.367409	3.674086696	0.985066	1.152650177	0.965108
LPC(18:2)	0.695921	6.959214011	0.985066	1.06589428	0.422444
LPC(20:3)	0.621458	6.214580141	0.985066	1.099673203	0.533029
LPC(20:4)	0.784649	7.846488435	>0.999999	0.967765043	0.2957
LPC(22:5)	0.862042	8.620415903	>0.999999	1.026307832	0.188162
LPC(22:6)	0.480831	4.808308699	0.985066	0.91217498	0.758245
LPE(16:0)	0.902743	9.027426858	>0.999999	1.018481848	0.132342
LPE(18:0)	0.715805	7.158050386	0.985066	1.075471698	0.393632
LPE(18:1)	0.066525	0.665245335	0.985066	1.466599361	1.8954
LPE(18:2)	0.365564	3.655641349	0.985066	1.189370554	0.968743
LPE(20:4)	0.409432	4.094321972	0.985066	1.120797812	0.884916
LPE(22:1)	0.476089	4.760889908	0.985066	0.893800813	0.766344
LPE(22:6)	0.918254	9.182537307	>0.999999	0.98763524	0.111162
PC(14:0_18:2)	0.641385	6.413848524	0.985066	1.120464952	0.502974
PC(14:0_20:4)	0.865055	8.650552179	>0.999999	0.964316797	0.184015
PC(14:0_20:5)	0.130846	1.308461664	0.985066	1.566903685	1.5844
PC(16:0_16:1)	0.516201	5.162007946	0.985066	1.188072537	0.699031
PC(16:0_18:1)	0.276694	2.766941504	0.985066	1.127128849	1.15861
PC(16:0_20:3)	0.972964	9.729642606	>0.999999	0.995981919	0.036712
PC(16:0_20:5)	0.12569	1.256898351	0.985066	1.374153138	1.60455
PC(16:0_22:5)	0.288802	2.888020448	0.985066	0.907675195	1.13076
PC(16:0_22:6)	0.08324	0.832395936	0.985066	0.825266612	1.79847
PC(18:0_16:0)	0.078532	0.785323097	0.985066	0.870109546	1.82418
PC(18:0_18:1)	0.672775	6.727746739	0.985066	0.953564728	0.456336
PC(18:0_20:2)	0.329803	3.298032439	0.985066	0.876101861	1.04142
PC(18:0_20:3)	0.403107	4.031069806	0.985066	0.846734072	0.896685
PC(18:0_20:4)	0.046492	0.464917388	0.985066	0.819277108	2.03966
PC(18:0_22:4)	0.129923	1.299230257	0.985066	0.808651645	1.58797

PC(18:0_22:5)	0.187527	1.875270734	0.985066	0.839579224	1.39281
PC(18:0_22:6)	0.020132	0.20132339	0.985066	0.700745474	2.3344
PC(18:1_13:0)	0.156898	1.568982466	0.985066	1.138075314	1.49039
PC(18:1_22:6)	0.014186	0.141857937	0.985066	0.745997233	2.44321
PC(18:2_18:2)	0.598318	5.983183353	0.985066	0.953212031	0.568415
PC(18:2_22:6)	0.024487	0.244871237	0.985066	0.74378921	2.2701
PC(22:0_18:2)	0.944792	9.447916762	>0.999999	0.990286363	0.075009
PC(30:1)	0.606454	6.064540344	0.985066	1.20179667	0.555911
PC(32:0)	0.452152	4.521518573	0.985066	0.948871409	0.807865
PC(33:0)	0.956557	9.565569934	>0.999999	0.995286724	0.059007
PC(33:4)	0.191917	1.919174202	0.985066	1.1133829	1.37973
PC(34:1e)	0.665212	6.652117818	0.985066	0.959090909	0.467499
PC(34:2)	0.662062	6.620620576	0.985066	1.050644291	0.472161
PC(34:2e)	0.644704	6.447039946	0.985066	1.068269231	0.498003
PC(34:3)	0.853639	8.536388162	>0.999999	0.967695449	0.199739
PC(34:3e)	0.75148	7.514799179	0.989933	0.943557583	0.342561
PC(35:1)	0.435034	4.350336065	0.985066	1.074862637	0.838257
PC(35:2)	0.654309	6.543090009	0.985066	0.952986726	0.483672
PC(35:3)	0.576071	5.760714809	0.985066	1.211893764	0.602976
PC(35:4)	0.411584	4.11583613	0.985066	1.071554459	0.880936
PC(35:5)	0.177645	1.776451838	0.985066	1.111804767	1.42302
PC(36:4e)	0.076883	0.768828594	0.985066	0.827865831	1.83346
PC(36:6)	0.835264	8.352641781	>0.999999	0.963906582	0.225128
PC(37:1)	0.679319	6.793187857	0.985066	0.962043796	0.446714
PC(37:4)	0.290991	2.909914474	0.985066	0.825622776	1.12581
PC(37:5)	0.245398	2.453980808	0.985066	1.098609904	1.23434
PC(37:6)	0.146787	1.467873555	0.985066	0.729418222	1.52548
PC(38:4e)	0.348104	3.481040076	0.985066	0.894234937	1.00369
PC(38:5e)	0.059036	0.590355924	0.985066	0.84440678	1.94487
PC(38:6e)	0.152486	1.524855962	0.985066	0.859831461	1.5055
PC(38:7)	0.451209	4.512094771	0.985066	0.896674584	0.809523
PC(38:7e)	0.117006	1.170059331	0.985066	0.750246305	1.63984
PC(39:6)	0.23361	2.336100988	0.985066	0.708632479	1.26445
PC(44:5e)	0.720612	7.206117875	0.98568	1.062908497	0.386707
PE(16:0_20:5)	0.127631	1.2763098	0.985066	1.321299639	1.59689
PE(16:1e_22:6)	0.282956	2.829559311	0.985066	0.880785575	1.14412
PE(18:0_16:0)	0.956197	9.561969919	>0.999999	0.993328391	0.059497
PE(18:0_18:1)	0.089688	0.896878187	0.985066	1.175725027	1.76494
PE(18:0_18:2)	0.113666	1.136662185	0.985066	1.213901276	1.65392
PE(18:0p_22:6)	0.971787	9.717865204	>0.999999	0.996208254	0.038312
PE(18:1_18:2)	0.237415	2.374148208	0.985066	1.129740519	1.25463
PE(32:1)	0.025439	0.25438761	0.985066	1.49862543	2.25727
PE(38:1e)	0.203158	2.03157845	0.985066	1.286912752	1.34714
PE(38:6)	0.674652	6.746517864	0.985066	1.049662571	0.453573

PE(40:7)	0.671797	6.717966843	0.985066	1.059724349	0.457777
PE(42:5)	0.054905	0.549051645	0.985066	1.137527264	1.97423
PE(42:7)	0.598766	5.987656613	0.985066	0.960079051	0.567726
PG(16:0_18:1)	0.184598	1.845979009	0.985066	0.51977644	1.40165
PG(34:2)	0.182493	1.824931158	0.985066	0.317106549	1.40806
PG(36:1)	0.304553	3.045528614	0.985066	0.621923475	1.09559
PG(36:2)	0.24641	2.464101928	0.985066	0.562065541	1.2318
PG(38:4)	0.330565	3.305649313	0.985066	#DIV/0!	1.03983
PI(16:0_16:0)	0.425182	4.251823974	0.985066	1.082767978	0.856032
PI(16:0_16:1)	0.917429	9.174288234	>0.999999	1.019671169	0.112287
PI(16:0_20:4)	0.999103	9.991029886	>0.999999	0.99929627	0.001218
PI(16:0_22:6)	0.318794	3.187937257	0.985066	0.849470499	1.06472
PI(18:0_18:2)	0.641467	6.414670008	0.985066	1.06212766	0.502851
PI(18:0_20:4)	0.931829	9.318285997	>0.999999	0.990705394	0.092657
PI(18:0_22:5)	0.454335	4.543349825	0.985066	1.178726483	0.804032
PI(18:0_22:6)	0.158215	1.582145432	0.985066	0.834220532	1.48594
PI(22:1_18:2)	0.297154	2.971544576	0.985066	0.750658828	1.11197
PI(34:0)	0.688578	6.885777401	0.985066	1.063934903	0.433154
PI(34:1)	0.934671	9.346706835	>0.999999	1.00896861	0.088786
PI(34:2)	0.914295	9.142950263	>0.999999	1.013104013	0.116563
PI(36:0)	0.2422	2.421998526	0.985066	0.672162334	1.24242
PI(36:1)	0.545075	5.450754937	0.985066	1.091326963	0.652108
PI(36:3)	0.971676	9.716756081	>0.999999	1.005279188	0.038462
PI(36:5)	0.058856	0.588561884	0.985066	1.277223608	1.94612
PI(38:2)	0.169425	1.694251205	0.985066	0.593776425	1.44903
PI(38:3)	0.253238	2.532379907	0.985066	1.319224257	1.21482
PI(38:5)	0.404821	4.048213541	0.985066	0.915777653	0.893485
PI(40:4)	0.936515	9.365150007	>0.999999	0.980830671	0.086274
PS(34:0)	0.608117	6.081172441	0.985066	1.256838366	0.553364
PS(34:1)	0.169798	1.697982005	0.985066	1.6160407	1.44783
PS(34:2)	0.949252	9.492522494	>0.999999	0.965632858	0.068941
PS(36:0)	0.867507	8.675070796	>0.999999	1.066867268	0.180643
PS(36:2)	0.750116	7.501155881	0.989933	1.104670985	0.344501
PS(38:4)	0.603446	6.034462344	0.985066	1.050130054	0.560526
PS(38:5)	0.644389	6.443886221	0.985066	1.042361743	0.498475
PS(40:4)	0.928648	9.286475401	>0.999999	1.03275582	0.096991
PS(42:3)	0.171362	1.713620414	0.985066	0.633001988	1.44282
SM(d32:2)	0.847855	8.478546921	>0.999999	0.968831169	0.207719
SM(d35:1)	0.668161	6.681610187	0.985066	0.955831608	0.463141
SM(d37:1)	0.671608	6.71608053	0.985066	0.941219691	0.458055
SM(d39:1)	0.077197	0.771970523	0.985066	0.844620015	1.83168
SM(d41:7)	0.715084	7.150843172	0.985066	1.073057236	0.394672
SM(d43:2)	0.121927	1.219271842	0.985066	0.827630897	1.61962
SM(d44:1)	0.71627	7.162703977	0.985066	1.031185884	0.392961

SPH(d18:0)	0.853452	8.534515053	>0.999999	0.953285827	0.199997
SPH(d18:1)	0.706938	7.069375227	0.985066	0.902614044	0.406449
Sphingosine 1-phosphate (d18:1-P)	0.981655	9.816553651	>0.999999	1.004878049	0.024908
SPHP(d21:1)	0.766753	7.667530785	0.989933	0.946296296	0.320915
TG(16:0_14:0_18:1)	0.506735	5.067351569	0.985066	0.792768792	0.71468
TG(16:0_14:4_16:0)	0.579461	5.794611299	0.985066	0.790161257	0.597674
TG(16:0_16:0_16:0)	0.869323	8.693228535	>0.999999	1.059548978	0.178146
TG(16:0_16:0_18:1)	0.692933	6.929334295	0.985066	0.895259096	0.426797
TG(16:0_16:1_16:1)	0.260542	2.605417387	0.985066	0.615905245	1.19698
TG(16:0_16:1_18:1)	0.287269	2.87268592	0.985066	0.699882307	1.13425
TG(16:0_18:1_18:1)	0.422564	4.225643728	0.985066	0.8007034	0.860793
TG(16:0_18:2_18:2)	0.338454	3.384536067	0.985066	0.771766392	1.02344
TG(16:1_14:0_18:2)	0.190084	1.900840649	0.985066	0.546808984	1.38517
TG(16:1_16:1_18:1)	0.138754	1.38753824	0.985066	0.574502421	1.55458
TG(17:0_18:1_20:4)	0.652634	6.526341412	0.985066	0.897849462	0.486165
TG(18:0_16:0_16:0)	0.300718	3.007178901	0.985066	1.499011858	1.10405
TG(18:0_16:0_18:1)	0.389511	3.895114858	0.985066	1.300488238	0.922329
TG(18:0_18:1_18:1)	0.932274	9.322744115	>0.999999	1.023510972	0.092049
TG(18:1_12:3_18:1)	0.252444	2.524442541	0.985066	0.692676687	1.21678
TG(18:1_18:1_20:4)	0.162188	1.621884619	0.985066	0.752941176	1.47266
TG(18:1_18:2_18:2)	0.91693	9.169300446	>0.999999	1.030595813	0.112967
TG(18:2_18:2_18:2)	0.549573	5.495734955	0.985066	1.231748727	0.644903
TG(50:4)	0.159862	1.598622673	0.985066	0.558978102	1.48041
TG(50:5)	0.19155	1.915498865	0.985066	0.611258697	1.38082
TG(52:3)	0.303161	3.031614225	0.985066	0.763885301	1.09865
TG(52:5)	0.285797	2.857970824	0.985066	0.721192053	1.13761
TG(52:6)	0.270068	2.700680429	0.985066	0.697228145	1.17418
TG(54:3)	0.504295	5.042951824	0.985066	0.831948566	0.718737
TG(54:4)	0.878366	8.78366407	>0.999999	0.957993999	0.165726
TG(54:7)	0.659811	6.598111458	0.985066	1.169139466	0.475498
TG(54:8)	0.761167	7.611668066	0.989933	1.10922205	0.328818
TG(55:4)	0.603784	6.037838429	0.985066	0.880092533	0.560008
TG(56:7)	0.077934	0.779343215	0.985066	0.707813998	1.82752
TG(56:9)	0.315529	3.155290853	0.985066	0.7893488	1.07172

Table H. The whole identified lipid list in fasting application and internal standard allocation

Identified lipids	Formula	Internal Standard	Adduct	RT
15:0-18:1(d7)PC+H	C41H73D7NO8P		M+H	6.54
15:0-18:1(d7)PC+CH3COO	C41H73D7NO8P		M+CH3COO	6.56
15:0-18:1(d7)PE-H	C38H67D7NO8P		M-H	6.59
15:0-18:1(d7)PS-H	C39H67D7NO10P		M-H	5.94
15:0-18:1(d7)PG+H	C39H68D7O10P		M+H	5.95

15:0-18:1(d7)PG+NH4	C39H68D7O10P		M+NH4	5.99
15:0-18:1(d7)PG-H	C39H68D7O10P		M-H	5.95
15:0-18:1(d7)PI-H	C42H72O13PD7		M-H	5.87
15:0-18:1(d7)PA-H	C36H62O8PD7		M-H	4.81
18:1(d7)LPC+H	C26H45D7NO7P		M+H	3.36
18:1(d7)LPE-H	C23H39D7NO7P		M-H	3.47
18:1(d7)LPE+H	C23H39D7NO7P		M+H	3.47
18:1(d7)ChE+NH4	C45H71D7O2		M+NH4	13.99
18:1(d7)MG+H	C21H33D7O4		M+NH4	4.76
15:0-18:1(d7)DG+NH4	C36H61D7O5		M+NH4	7.35
15:0-18:1(d7)-15:0TG+NH4	C51H89D7O6		M+NH4	12.07
18:1(d9) SM+H	C41H72D9N2O6P		M+H	6.37
Cholesterol (d7)+H	C27H39D7O		M+H	6.13
7-oxocholesterol	C27H44O3	18:1(d7)LPC+H	M+H	3.07
AcCa(16:0)	C23H45NO4	18:1(d7)LPC+H	M+H	2.97
Cer(d16:1_22:0)	C38H75NO3	18:1(d9) SM+H	M+H	7.61
Cer(d18:0/16:0)	C34H69NO3	18:1(d9) SM+H	M+H	6.99
Cer(d18:0/18:0)	C36H73NO3	18:1(d9) SM+H	M+H	7.35
Cer(d18:0/20:0)	C38H77NO3	18:1(d9) SM+H	M+H	7.74
Cer(d18:0/26:0)	C44H89NO3	18:1(d9) SM+H	M+H	9.27
Cer(d18:0_22:0)	C40H81NO3	18:1(d9) SM+H	M+H	8.21
Cer(d18:0_24:0)	C42H85NO3	18:1(d9) SM+H	M+H	8.7
Cer(d18:0_24:1)	C42H83NO3	18:1(d9) SM+H	M+H	8.52
Cer(d18:1/19:0)	C37H73NO3	18:1(d9) SM+H	M+H	7.44
Cer(d18:1/25:0)	C43H85NO3	18:1(d9) SM+H	M+H	8.68
Cer(d18:1_16:0)	C34H67NO3	18:1(d9) SM+H	M+H	6.88
Cer(d18:1_18:0)	C36H71NO3	18:1(d9) SM+H	M+H	7.21
Cer(d18:1_22:0)	C40H79NO3	18:1(d9) SM+H	M+H	8.03
Cer(d18:1_23:0)	C41H81NO3	18:1(d9) SM+H	M+H	8.28
Cer(d18:1_24:1)	C42H81NO3	18:1(d9) SM+H	M+H	8.03
Cer(d18:1_26:0)	C44H87NO3	18:1(d9) SM+H	M+H	9.08
Cer(d18:1_26:1)	C44H85NO3	18:1(d9) SM+H	M+H	8.5
Cer(d18:2/20:0)	C38H73NO3	18:1(d9) SM+H	M+H	7.29
Cer(d18:2/24:2)	C42H77NO3	18:1(d9) SM+H	M+H	8.05
Cer(d18:2_22:0)	C40H77NO3	18:1(d9) SM+H	M+H	7.69
Cer(d18:2_23:0)	C41H79NO3	18:1(d9) SM+H	M+H	7.88
Cer(d18:2_24:1)	C42H79NO3	18:1(d9) SM+H	M+H	7.69
ChE(14:0)	C41H72O2	18:1(d7)ChE+NH4	M+NH4	12.55
ChE(15:0)	C42H74O2	18:1(d7)ChE+NH4	M+NH4	7.69
ChE(16:0)	C43H76O2	18:1(d7)ChE+NH4	M+NH4	13.88
ChE(16:1)	C43H74O2	18:1(d7)ChE+NH4	M+NH4	12.58
ChE(17:0)	C44H78O2	18:1(d7)ChE+NH4	M+NH4	8.59
ChE(17:1)	C44H76O2	18:1(d7)ChE+NH4	M+NH4	8.08
ChE(18:0)	C45H80O2	18:1(d7)ChE+NH4	M+NH4	15.78

ChE(18:1)	C45H78O2	18:1(d7)ChE+NH4	M+NH4	14.12
ChE(18:3)	C45H74O2	18:1(d7)ChE+NH4	M+NH4	11.88
ChE(20:1)	C47H82O2	18:1(d7)ChE+NH4	M+NH4	15.78
ChE(20:3)	C47H78O2	18:1(d7)ChE+NH4	M+NH4	13.06
ChE(20:4)	C47H76O2	18:1(d7)ChE+NH4	M+NH4	12.25
ChE(20:5)	C47H74O2	18:1(d7)ChE+NH4	M+NH4	11.36
ChE(22:6)	C49H76O2	18:1(d7)ChE+NH4	M+NH4	11.82
CoQ10	C59H90O4	18:1(d7)ChE+NH4	M+NH4	10.23
CoQ9	C54H82O4	18:1(d7)ChE+NH4	M+NH4	9.17
DG(30:2)	C33H60O5	15:0-18:1(d7)DG+NH4	M+NH4	6.56
DG(38:0)	C41H80O5	15:0-18:1(d7)DG+NH4	M+NH4	12.83
DG(38:3)	C41H74O5	15:0-18:1(d7)DG+NH4	M+NH4	7.69
DG(38:5)	C41H70O5	15:0-18:1(d7)DG+NH4	M+NH4	7.26
DG(38:6)	C41H68O5	15:0-18:1(d7)DG+NH4	M+NH4	6.99
DG(40:7)	C43H70O5	15:0-18:1(d7)DG+NH4	M+NH4	7.18
DG(14:0_18:2)	C35H64O5	15:0-18:1(d7)DG+NH4	M+NH4	6.94
DG(14:0_18:3)	C35H62O5	15:0-18:1(d7)DG+NH4	M+NH4	6.64
DG(16:0_14:0)	C33H64O5	15:0-18:1(d7)DG+NH4	M+NH4	7.13
DG(16:0_16:0)	C35H68O5	15:0-18:1(d7)DG+NH4	M+NH4	7.53
DG(16:0_16:1)	C35H66O5	15:0-18:1(d7)DG+NH4	M+NH4	7.21
DG(16:0_18:1)	C37H70O5	15:0-18:1(d7)DG+NH4	M+NH4	7.59
DG(16:0_18:2)	C37H68O5	15:0-18:1(d7)DG+NH4	M+NH4	7.29
DG(16:1_14:0)	C33H62O5	15:0-18:1(d7)DG+NH4	M+NH4	6.88
DG(16:1_18:3)	C37H64O5	15:0-18:1(d7)DG+NH4	M+NH4	6.85
DG(18:0_16:0)	C37H72O5	15:0-18:1(d7)DG+NH4	M+NH4	7.96
DG(18:0_18:0)	C39H76O5	15:0-18:1(d7)DG+NH4	M+NH4	8.43
DG(18:0_18:1)	C39H74O5	15:0-18:1(d7)DG+NH4	M+NH4	7.99
DG(18:1_18:1)	C39H72O5	15:0-18:1(d7)DG+NH4	M+NH4	7.64
DG(36:4)	C39H68O5	15:0-18:1(d7)DG+NH4	M+NH4	7.05
DG(18:1_20:3)	C41H72O5	15:0-18:1(d7)DG+NH4	M+NH4	7.53
DG(18:1_22:5)	C43H72O5	15:0-18:1(d7)DG+NH4	M+NH4	7.29
DG(18:3_18:2)	C39H66O5	15:0-18:1(d7)DG+NH4	M+NH4	6.88
DG(16:0_18:3)	C37H66O5	15:0-18:1(d7)DG+NH4	M+NH4	6.97
DG(36:3)	C39H70O5	15:0-18:1(d7)DG+NH4	M+NH4	7.32
Eicosanoids_19,20-DiHDPA	C22H34O4	18:1(d7)LPE-H	M-H	3.17
Eicosanoids_PGD3	C20H30O5	18:1(d7)LPE-H	M-H	2.89
FA(12:0)	C12H24O2	18:1(d7)LPE-H	M-H	1.69
FA(14:0)	C14H28O2	18:1(d7)LPE-H	M-H	2.65
FA(15:0)	C15H30O2	18:1(d7)LPE-H	M-H	3.12
FA(16:0)	C16H32O2	18:1(d7)LPE-H	M-H	3.74
FA(16:1)	C16H30O2	18:1(d7)LPE-H	M-H	2.89
FA(17:0)	C17H34O2	18:1(d7)LPE-H	M-H	4.28
FA(17:1)	C17H32O2	18:1(d7)LPE-H	M-H	3.4
FA(18:0)	C18H36O2	18:1(d7)LPE-H	M-H	4.83

FA(18:1)	C18H34O2	18:1(d7)LPE-H	M-H	4.01
FA(18:2)	C18H32O2	18:1(d7)LPE-H	M-H	3.19
FA(18:3)	C18H30O2	18:1(d7)LPE-H	M-H	2.56
FA(18:4)	C18H28O2	18:1(d7)LPE-H	M-H	2.06
FA(20:2)	C20H36O2	18:1(d7)LPE-H	M-H	4.26
FA(20:3)	C20H34O2	18:1(d7)LPE-H	M-H	3.6
FA(20:4)	C20H32O2	18:1(d7)LPE-H	M-H	3.06
FA(20:5)	C20H30O2	18:1(d7)LPE-H	M-H	2.46
FA(22:0)	C22H44O2	18:1(d7)LPE-H	M-H	6.15
FA(22:1)	C22H42O2	18:1(d7)LPE-H	M-H	5.63
FA(22:2)	C22H40O2	18:1(d7)LPE-H	M-H	5.1
FA(22:3)	C22H38O2	18:1(d7)LPE-H	M-H	4.6
FA(22:4)	C22H36O2	18:1(d7)LPE-H	M-H	4.01
FA(22:5)	C22H34O2	18:1(d7)LPE-H	M-H	3.29
FA(22:6)	C22H32O2	18:1(d7)LPE-H	M-H	2.79
FA(23:0)	C23H46O2	18:1(d7)LPE-H	M-H	6.15
FA(24:0)	C24H48O2	18:1(d7)LPE-H	M-H	6.62
FA(24:1)	C24H46O2	18:1(d7)LPE-H	M-H	6.15
FA(26:0)	C26H52O2	18:1(d7)LPE-H	M-H	6.84
Hex2Cer(d12:0_20:4)	C44H77NO13	15:0-18:1(d7)PI-H	M+H	6.34
LPC(16:0)	C24H50NO7P	18:1(d7)LPC+H	M+H	3.18
LPC(16:1)	C24H48NO7P	18:1(d7)LPC+H	M+H	2.43
LPC(18:0)	C26H54NO7P	18:1(d7)LPC+H	M+H	4.07
LPC(18:1)	C26H52NO7P	18:1(d7)LPC+H	M+H	3.36
LPC(18:2)	C26H50NO7P	18:1(d7)LPC+H	M+H	2.71
LPC(20:3)*1	C28H52NO7P	18:1(d7)LPC+H	M+H	3.04
LPC(20:3)*2	C28H52NO7P	18:1(d7)LPC+H	M+H	4.07
LPC(20:4)*1	C28H50NO7P	18:1(d7)LPC+H	M+H	2.61
LPC(20:4)*2	C28H50NO7P	18:1(d7)LPC+H	M+H	3.36
LPC(22:5)	C30H52NO7P	18:1(d7)LPC+H	M+H	2.66
LPC(22:6)	C30H50NO7P	18:1(d7)LPC+H	M+H	2.43
LPE(16:0)	C21H44NO7P	18:1(d7)LPE-H	M-H	3.33
LPE(18:0)*1	C23H48NO7P	18:1(d7)LPE-H	M-H	3.19
LPE(18:0)*2	C23H48NO7P	18:1(d7)LPE-H	M-H	4.12
LPE(18:1)	C23H46NO7P	18:1(d7)LPE-H	M-H	3.49
LPE(18:2)	C23H44NO7P	18:1(d7)LPE-H	M-H	2.86
LPE(20:4)	C25H44NO7P	18:1(d7)LPE-H	M-H	2.65
LPE(22:1)	C27H54NO7P	18:1(d7)LPE-H	M-H	6.59
LPE(22:6)	C27H44NO7P	18:1(d7)LPE-H	M-H	2.6
PC(14:0_18:2)	C40H76NO8P	15:0-18:1(d7)PC+H	M+H	6.1
PC(14:0_20:4)	C42H76NO8P	15:0-18:1(d7)PC+H	M+H	6.08
PC(14:0_20:5)	C42H74NO8P	15:0-18:1(d7)PC+H	M+H	6.1
PC(16:0_16:1)	C40H78NO8P	15:0-18:1(d7)PC+H	M+H	6.37
PC(16:0_18:1)	C42H82NO8P	15:0-18:1(d7)PC+H	M+H	6.71

PC(16:0_20:3)	C44H82NO8P	15:0-18:1(d7)PC+H	M+H	6.58
PC(16:0_20:5)	C44H78NO8P	15:0-18:1(d7)PC+H	M+H	6.21
PC(16:0_22:5)	C46H82NO8P	15:0-18:1(d7)PC+H	M+H	6.45
PC(38:5)	C46H82NO8P	15:0-18:1(d7)PC+H	M+H	6.86
PC(16:0_22:6)	C46H80NO8P	15:0-18:1(d7)PC+H	M+H	6.34
PC(18:0_16:0)	C42H84NO8P	15:0-18:1(d7)PC+H	M+H	7.03
PC(18:0_18:1)	C44H86NO8P	15:0-18:1(d7)PC+H	M+H	7.08
PC(18:0_18:2)	C44H84NO8P	15:0-18:1(d7)PC+H	M+H	6.83
PC(18:0_20:2)	C46H88NO8P	15:0-18:1(d7)PC+H	M+H	7.15
PC(18:0_20:3)	C46H86NO8P	15:0-18:1(d7)PC+H	M+H	6.91
PC(18:0_20:4)	C46H84NO8P	15:0-18:1(d7)PC+H	M+H	6.77
PC(18:0_22:4)	C48H88NO8P	15:0-18:1(d7)PC+H	M+H	7
PC(18:0_22:5)	C48H86NO8P	15:0-18:1(d7)PC+H	M+H	6.79
PC(18:0_22:6)	C48H84NO8P	15:0-18:1(d7)PC+H	M+H	6.69
PC(18:1_13:0)	C39H76NO8P	15:0-18:1(d7)PC+H	M+H	6.21
PC(18:1_22:6)	C48H82NO8P	15:0-18:1(d7)PC+H	M+H	6.4
PC(40:7)	C48H82NO8P	15:0-18:1(d7)PC+H	M+H	6.77
PC(18:2_13:0)	C39H74NO8P	15:0-18:1(d7)PC+H	M+H	6.52
PC(18:2_18:2)	C44H80NO8P	15:0-18:1(d7)PC+H	M+H	6.42
PC(18:2_22:6)	C48H80NO8P	15:0-18:1(d7)PC+H	M+H	6.31
PC(22:0_18:2)	C48H92NO8P	15:0-18:1(d7)PC+H	M+H	7.53
PC(30:1)	C38H74NO8P	15:0-18:1(d7)PC+H	M+H	6.02
PC(31:0)	C39H78NO8P	15:0-18:1(d7)PC+H	M+H	6.52
PC(32:0)	C40H80NO8P	15:0-18:1(d7)PC+H	M+H	6.66
PC(33:0)	C41H82NO8P	15:0-18:1(d7)PC+H	M+H	6.79
PC(33:1)	C41H80NO8P	15:0-18:1(d7)PC+H	M+H	6.54
PC(33:2)	C41H78NO8P	15:0-18:1(d7)PC+H	M+H	6.29
PC(33:3)	C41H76NO8P	15:0-18:1(d7)PC+H	M+H	6.59
PC(33:4)	C41H74NO8P	15:0-18:1(d7)PC+H	M+H	6.49
PC(34:1e)	C42H84NO7P	15:0-18:1(d7)PC+H	M+H	6.97
PC(34:2)	C42H80NO8P	15:0-18:1(d7)PC+H	M+H	6.45
PC(34:2e)*1	C42H82NO7P	15:0-18:1(d7)PC+H	M+H	6.69
PC(34:2e)*2	C42H82NO7P	15:0-18:1(d7)PC+H	M+H	6.94
PC(34:3)	C42H78NO8P	15:0-18:1(d7)PC+H	M+H	6.34
PC(34:3e)	C42H80NO7P	15:0-18:1(d7)PC+H	M+H	6.66
PC(35:1)	C43H84NO8P	15:0-18:1(d7)PC+H	M+H	6.88
PC(35:2)	C43H82NO8P	15:0-18:1(d7)PC+H	M+H	6.63
PC(35:3)	C43H80NO8P	15:0-18:1(d7)PC+H	M+H	6.37
PC(35:4)	C43H78NO8P	15:0-18:1(d7)PC+H	M+H	6.24
PC(35:5)*1	C43H76NO8P	15:0-18:1(d7)PC+H	M+H	6.27
PC(35:5)*2	C43H76NO8P	15:0-18:1(d7)PC+H	M+H	6.88
PC(36:0)	C44H88NO8P	15:0-18:1(d7)PC+H	M+H	7.39
PC(36:4e)	C44H82NO7P	15:0-18:1(d7)PC+H	M+H	6.66
PC(36:6)	C44H76NO8P	15:0-18:1(d7)PC+CH3COO	M+CH3COO	5.99

PC(37:1)	C45H88NO8P	15:0-18:1(d7)PC+H	M+H	7.24
PC(37:4)	C45H82NO8P	15:0-18:1(d7)PC+H	M+H	6.58
PC(37:5)	C45H80NO8P	15:0-18:1(d7)PC+CH3COO	M+CH3COO	6.33
PC(37:6)	C45H78NO8P	15:0-18:1(d7)PC+H	M+H	6.18
PC(38:4e)	C46H86NO7P	15:0-18:1(d7)PC+H	M+H	7.03
PC(38:5e)	C46H84NO7P	15:0-18:1(d7)PC+H	M+H	6.69
PC(38:6e)	C46H82NO7P	15:0-18:1(d7)PC+H	M+H	6.63
PC(38:7)	C46H78NO8P	15:0-18:1(d7)PC+CH3COO	M+CH3COO	6.06
PC(38:7e)	C46H80NO7P	15:0-18:1(d7)PC+H	M+H	6.54
PC(39:6)	C47H82NO8P	15:0-18:1(d7)PC+H	M+H	6.52
PC(44:5e)	C52H96NO7P	15:0-18:1(d7)PC+H	M+H	7.81
PE(16:0_18:1)	C39H76NO8P	15:0-18:1(d7)PE-H	M-H	6.76
PE(16:0_18:2)	C39H74NO8P	15:0-18:1(d7)PE-H	M-H	6.53
PE(16:0_20:4)	C41H74NO8P	15:0-18:1(d7)PE-H	M-H	6.51
PE(16:0_20:5)	C41H72NO8P	15:0-18:1(d7)PE-H	M-H	6.25
PE(16:1e_22:6)	C43H74NO7P	15:0-18:1(d7)PE-H	M-H	6.59
PE(18:0_18:1)	C41H80NO8P	15:0-18:1(d7)PE-H	M-H	7.09
PE(18:0_18:2)	C41H78NO8P	15:0-18:1(d7)PE-H	M-H	6.86
PE(18:0_22:5)	C45H80NO8P	15:0-18:1(d7)PE-H	M-H	6.84
PE(18:0p_22:6)	C45H78NO7P	15:0-18:1(d7)PE-H	M-H	6.74
PE(18:1_18:2)	C41H76NO8P	15:0-18:1(d7)PE-H	M-H	6.56
PE(32:1)	C37H72NO8P	15:0-18:1(d7)PE-H	M-H	6.41
PE(18:0_20:3)	C43H80NO8P	15:0-18:1(d7)PE-H	M-H	6.94
PE(18:0_20:4)	C43H78NO8P	15:0-18:1(d7)PE-H	M-H	6.83
PE(18:1_20:4)	C43H76NO8P	15:0-18:1(d7)PE-H	M-H	6.54
PE(38:6)	C43H74NO8P	15:0-18:1(d7)PE-H	M-H	6.41
PE(39:6)	C44H76NO8P	15:0-18:1(d7)PE-H	M-H	6.56
PE(40:7)	C45H76NO8P	15:0-18:1(d7)PE-H	M-H	6.47
PE(42:5)	C47H84NO8P	15:0-18:1(d7)PE-H	M-H	6.5
PE(42:7)	C47H80NO8P	15:0-18:1(d7)PE-H	M-H	5.94
PG(16:0_18:1)	C40H77O10P	15:0-18:1(d7)PG+NH4	M+H	6.6
PG(36:1)	C42H81O10P	15:0-18:1(d7)PG+NH4	M+H	6.92
PG(36:2)	C42H79O10P	15:0-18:1(d7)PG-H	M-H	6.59
PG(36:3)	C42H77O10P	15:0-18:1(d7)PG+NH4	M+NH4	5.24
PG(36:5)	C42H73O10P	15:0-18:1(d7)PG+NH4	M+H	6.14
PG(38:4)	C44H79O10P	15:0-18:1(d7)PG+NH4	M+NH4	5.35
PG(38:6)	C44H75O10P	15:0-18:1(d7)PG+NH4	M+H	6.21
PG(40:6)	C46H79O10P	15:0-18:1(d7)PG+NH4	M+H	6.63
PG(40:8)	C46H75O10P	15:0-18:1(d7)PG+NH4	M+H	6.24
PI(16:0_16:0)	C41H79O13P	15:0-18:1(d7)PI-H	M-H	5.99
PI(16:0_16:1)	C41H77O13P	15:0-18:1(d7)PI-H	M-H	6.09
PI(16:0_20:4)	C45H79O13P	15:0-18:1(d7)PI-H	M-H	5.78
PI(16:0_22:6)	C47H79O13P	15:0-18:1(d7)PI-H	M-H	5.72
PI(18:0_18:2)	C45H83O13P	15:0-18:1(d7)PI-H	M-H	6.12

PI(18:0_20:4)	C47H83O13P	15:0-18:1(d7)PI-H	M-H	6.09
PI(18:0_22:5)	C49H85O13P	15:0-18:1(d7)PI-H	M-H	6.12
PI(18:0_22:6)	C49H83O13P	15:0-18:1(d7)PI-H	M-H	6.01
PI(34:0)	C43H83O13P	15:0-18:1(d7)PI-H	M-H	5.99
PI(34:1)	C43H81O13P	15:0-18:1(d7)PI-H	M-H	6.03
PI(34:2)	C43H79O13P	15:0-18:1(d7)PI-H	M-H	5.78
PI(36:0)	C45H87O13P	15:0-18:1(d7)PI-H	M-H	6.35
PI(36:1)	C45H85O13P	15:0-18:1(d7)PI-H	M-H	6.33
PI(36:3)	C45H81O13P	15:0-18:1(d7)PI-H	M-H	5.85
PI(38:2)	C47H87O13P	15:0-18:1(d7)PI-H	M-H	6.15
PI(38:3)	C47H85O13P	15:0-18:1(d7)PI-H	M-H	6.22
PI(38:5)	C47H81O13P	15:0-18:1(d7)PI-H	M-H	5.86
PI(40:4)*1	C49H87O13P	15:0-18:1(d7)PI-H	M-H	6.09
PI(40:4)*2	C49H87O13P	15:0-18:1(d7)PI-H	M-H	6.28
PS(34:0)	C40H78NO10P	15:0-18:1(d7)PS-H	M+H	5.95
PS(34:2)	C40H74NO10P	15:0-18:1(d7)PS-H	M+H	4.68
PS(36:0)	C42H82NO10P	15:0-18:1(d7)PS-H	M-H	6.38
PS(36:2)	C42H78NO10P	15:0-18:1(d7)PS-H	M-H	5.85
PS(38:4)	C44H78NO10P	15:0-18:1(d7)PS-H	M+H	5.26
PS(40:4)	C46H82NO10P	15:0-18:1(d7)PS-H	M-H	6.19
PS(42:3)	C48H88NO10P	15:0-18:1(d7)PS-H	M-H	6.78
SM(d32:2)	C37H73N2O6P	18:1(d9) SM+H	M+H	5.58
SM(d35:1)	C40H81N2O6P	18:1(d9) SM+H	M+H	6.62
SM(d37:1)	C42H85N2O6P	18:1(d9) SM+H	M+H	6.88
SM(d39:1)	C44H89N2O6P	18:1(d9) SM+H	M+H	7.24
SM(d41:7)	C46H81N2O6P	18:1(d9) SM+H	M-H	6.56
SM(d43:2)	C48H95N2O6P	18:1(d9) SM+H	M+H	7.53
SM(d44:1)	C49H99N2O6P	18:1(d9) SM+H	M+H	9.98
SM(t40:7)	C45H79N2O7P	18:1(d9) SM+H	M+H	6.42
SPH(d18:0)	C18H39NO2	18:1(d7)LPC+H	M+H	3.51
SPH(d18:1)	C18H37NO2	18:1(d7)LPC+H	M+H	3.415
Sphinganine 1-phosphate (d18:0-P)	C18H40NO5P	18:1(d7)LPE-H	M-H	4.81
Sphingosine 1-phosphate (d18:1-P)	C18H38NO5P	18:1(d7)LPE-H	M-H	4.01
TG(50:4)	C53H94O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.6
TG(16:0_14:0_18:1)	C51H96O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	12.11
TG(16:1_16:1_18:3)	C53H92O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	9.98
TG(16:0_14:4_16:0)	C49H86O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	7.42
TG(16:0_16:0_16:0)	C51H98O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	13.24
TG(16:0_16:0_18:1)	C53H100O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	13.38
TG(16:1_18:2_18:2)	C55H96O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.8
TG(16:0_16:1_16:1)	C51H94O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	11.19
TG(16:0_16:1_18:1)	C53H98O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	12.25
TG(16:1_18:2_18:3)	C55H94O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.15
TG(16:0_18:1_18:1)	C55H102O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	13.49

TG(16:0_18:1_18:2)	C55H100O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	12.48
TG(16:0_18:2_18:2)	C55H98O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	11.54
TG(18:3_18:2_18:2)	C57H96O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.23
TG(20:3_18:2_18:2)	C59H100O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	11.88
TG(18:1_18:1_20:5)	C59H100O6	15:0-18:1(d7)-15:0TG+NH4	M+H	12.66
TG(16:1_14:0_18:2)	C51H92O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.45
TG(16:1_16:1_18:1)	C53H96O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	11.33
TG(17:0_18:1_20:4)	C58H102O6	15:0-18:1(d7)-15:0TG+NH4	M+H	14.4
TG(18:0_16:0_16:0)	C53H102O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	14.75
TG(18:0_16:0_18:1)	C55H104O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	14.94
TG(18:1_18:1_18:1)	C57H104O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	13.72
TG(18:0_18:1_18:1)	C57H106O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	15.11
TG(18:1_18:1_18:2)	C57H102O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	12.63
TG(18:1_12:3_18:1)	C51H88O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	7.42
TG(18:1_18:1_20:4)	C59H102O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	12.27
TG(18:1_18:2_18:2)	C57H100O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	11.65
TG(18:2_18:2_18:2)	C57H98O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.88
TG(16:0_18:2_20:4)	C57H98O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	11.36
TG(52:12e)	C55H84O5	15:0-18:1(d7)-15:0TG+NH4	M+NH4	7.53
TG(54:8)	C57H94O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.15
TG(55:4)	C58H104O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	13.17
TG(56:9)	C59H96O6	15:0-18:1(d7)-15:0TG+NH4	M+NH4	10.23

Table I. The identified lipid peak area CV result

lipid	TRF pre	TRF post	CR pre	CR post	plasma	QC
7-oxocholesterol	282.84%	207.50 %	157.30%	127.26 %	72.56%	39.10%
AcCa(16:0)	47.01%	21.36%	20.05%	29.91%	12.22%	3.15%
Cer(d16:1_22:0)	49.34%	40.08%	43.56%	41.67%	12.58%	12.14%
Cer(d18:0/16:0)	33.25%	26.49%	28.68%	30.97%	15.99%	27.61%
Cer(d18:0/18:0)	41.32%	34.93%	29.58%	65.60%	8.62%	34.87%
Cer(d18:0/20:0)	50.48%	22.93%	41.83%	45.88%	11.88%	13.37%
Cer(d18:0/26:0)	41.45%	27.02%	55.49%	48.77%	5.78%	10.13%
Cer(d18:0_22:0)	53.99%	44.54%	39.24%	44.08%	7.97%	11.33%
Cer(d18:0_24:0)	51.65%	46.13%	41.27%	48.54%	4.19%	8.18%
Cer(d18:0_24:1)	49.27%	39.01%	31.96%	43.03%	8.19%	11.39%
Cer(d18:1/19:0)	79.02%	75.42%	120.76%	111.46 %	20.94%	30.81%
Cer(d18:1/25:0)	57.03%	57.25%	55.80%	57.74%	7.81%	9.58%
Cer(d18:1_16:0)	21.93%	18.31%	15.78%	25.33%	9.17%	5.77%
Cer(d18:1_18:0)	55.23%	41.01%	25.23%	36.38%	8.42%	14.21%
Cer(d18:1_22:0)	49.80%	38.68%	38.18%	46.19%	12.90%	12.36%
Cer(d18:1_23:0)	49.60%	38.37%	39.10%	48.29%	9.29%	9.58%
Cer(d18:1_24:1)	44.45%	32.15%	30.80%	38.28%	11.29%	12.40%
Cer(d18:1_26:0)	45.67%	41.04%	43.59%	46.27%	7.59%	8.04%
Cer(d18:1_26:1)	55.61%	39.36%	43.16%	44.16%	6.59%	11.84%
Cer(d18:2/20:0)	47.45%	44.05%	41.89%	33.04%	7.26%	16.56%
Cer(d18:2/24:2)	52.73%	38.45%	30.62%	35.81%	2.95%	5.84%
Cer(d18:2_22:0)	47.00%	40.51%	44.28%	44.41%	11.47%	12.84%
Cer(d18:2_23:0)	44.56%	36.36%	45.43%	46.60%	12.71%	12.14%
Cer(d18:2_24:1)	43.66%	26.54%	33.89%	37.65%	8.66%	12.57%
ChE(14:0)	93.33%	218.31 %	190.93%	173.34 %	NA	18.75%
ChE(15:0)	48.12%	27.87%	35.17%	38.06%	14.59%	12.84%
ChE(16:0)	48.21%	41.58%	18.09%	21.85%	2.17%	1.78%
ChE(16:1)	84.42%	64.10%	58.31%	48.44%	10.11%	10.06%
ChE(17:0)	54.69%	40.28%	58.32%	42.09%	53.64%	5.61%
ChE(17:1)	96.05%	58.38%	77.18%	70.50%	10.37%	13.22%
ChE(18:0)	97.36%	96.08%	44.88%	54.50%	28.24%	21.65%
ChE(18:1)	44.72%	34.77%	16.84%	20.28%	2.90%	2.45%
ChE(18:3)	47.56%	40.20%	46.06%	28.81%	3.04%	2.27%
ChE(20:1)	186.28%	125.02 %	60.08%	80.45%	47.02%	20.30%
ChE(20:3)	48.57%	42.15%	30.50%	28.45%	6.13%	2.26%
ChE(20:4)	38.22%	31.63%	24.23%	22.35%	9.29%	1.72%
ChE(20:5)	80.80%	58.70%	51.16%	49.80%	3.53%	1.46%
ChE(22:6)	62.60%	50.91%	41.75%	38.98%	3.40%	3.58%

CoQ10	56.89%	40.32%	19.67%	21.22%	2.67%	2.47%
CoQ9	93.46%	67.22%	70.03%	43.08%	10.70%	12.15%
DG(30:2)	208.32%	150.49 %	231.84%	252.81 %	9.93%	#DIV/0 !
DG(38:0)	32.40%	29.09%	11.05%	17.26%	9.05%	9.22%
DG(38:3)	96.90%	72.52%	64.40%	45.61%	8.35%	13.96%
DG(40:7)	83.59%	49.27%	49.54%	53.16%	11.37%	7.08%
DG(14:0_18:2)	85.07%	48.57%	87.39%	82.99%	4.49%	4.86%
DG(14:0_18:3)	154.89%	81.60%	186.45%	167.58 %	14.36%	44.59%
DG(16:0_14:0)	148.01%	47.13%	137.58%	152.79 %	6.13%	29.90%
DG(16:0_16:0)	65.57%	32.22%	45.11%	74.50%	10.45%	8.33%
DG(16:0_16:1)	103.32%	54.03%	80.47%	90.77%	5.63%	4.51%
DG(16:0_18:1)	86.61%	53.50%	38.82%	63.58%	9.27%	8.71%
DG(16:0_18:2)	79.49%	50.18%	26.81%	37.34%	6.50%	4.89%
DG(16:0_18:3)	73.13%	48.95%	58.87%	53.49%	5.18%	4.30%
DG(16:1_14:0)	168.42%	54.19%	228.75%	225.91 %	5.05%	45.70%
DG(16:1_18:3)	90.99%	77.79%	178.82%	69.64%	5.23%	76.21%
DG(18:0_16:0)	47.27%	29.25%	41.40%	56.67%	9.53%	9.76%
DG(18:0_18:0)	52.53%	30.90%	41.07%	40.54%	21.25%	6.08%
DG(18:0_18:1)	95.01%	59.37%	39.55%	92.87%	11.72%	9.89%
DG(18:1_18:1)	85.23%	66.98%	38.28%	45.02%	9.05%	10.74%
DG(18:1_20:3)	69.09%	53.45%	51.46%	42.65%	8.55%	9.52%
DG(18:1_22:5)	92.17%	48.52%	38.21%	43.89%	5.97%	4.61%
DG(18:3_18:2)	55.25%	42.14%	77.08%	75.29%	3.26%	15.35%
DG(36:3)	73.26%	53.77%	62.14%	43.63%	9.31%	8.47%
DG(36:4)	57.61%	40.97%	79.22%	49.12%	4.15%	3.62%
DG(38:5)	53.73%	32.66%	41.91%	36.33%	7.30%	9.41%
DG(38:6)	42.51%	16.86%	48.30%	45.27%	7.13%	4.73%
Eicosanoids_19,20-DiHDPA	70.94%	106.64 %	66.62%	33.34%	56.05%	33.35%
Eicosanoids_PGD3	#DIV/0!	#DIV/0 !	#DIV/0!	282.84 %	#DIV/0!	#DIV/0 !
FA(12:0)	55.44%	41.51%	23.17%	42.05%	10.54%	5.66%
FA(14:0)	56.43%	44.43%	20.75%	52.87%	8.58%	3.90%
FA(15:0)	42.14%	35.79%	16.91%	33.65%	11.77%	2.62%
FA(16:0)	40.14%	20.40%	18.06%	37.31%	6.28%	3.00%
FA(16:1)	62.64%	46.71%	30.38%	64.47%	10.09%	2.35%
FA(17:0)	40.97%	24.78%	16.80%	26.01%	8.99%	5.08%
FA(17:1)	63.39%	42.89%	20.45%	43.33%	12.31%	4.38%
FA(18:0)	20.78%	8.46%	16.68%	24.60%	8.17%	4.02%
FA(18:1)	43.04%	18.74%	20.62%	40.98%	5.59%	2.31%
FA(18:2)	56.21%	36.28%	22.70%	42.99%	8.29%	2.71%
FA(18:3)	64.33%	42.37%	21.93%	43.36%	7.31%	2.31%

FA(18:4)	49.38%	45.49%	30.49%	34.53%	5.13%	2.78%
FA(20:2)	51.24%	24.17%	24.83%	43.25%	7.11%	3.43%
FA(20:3)	46.18%	29.41%	20.96%	37.94%	6.35%	2.11%
FA(20:4)	46.89%	30.52%	30.43%	40.35%	8.74%	1.56%
FA(20:5)	77.57%	54.71%	38.48%	43.46%	5.75%	2.32%
FA(22:0)	21.59%	21.20%	15.62%	20.84%	8.90%	4.39%
FA(22:1)	54.74%	27.24%	29.27%	38.09%	7.24%	4.07%
FA(22:2)	47.31%	18.44%	26.24%	38.24%	7.09%	6.64%
FA(22:3)	37.53%	20.49%	24.32%	35.17%	7.26%	2.58%
FA(22:4)	37.31%	19.95%	27.34%	34.88%	7.20%	1.85%
FA(22:5)	53.95%	30.82%	30.19%	35.45%	8.16%	3.20%
FA(22:6)	67.08%	55.90%	44.81%	43.19%	8.47%	1.53%
FA(23:0)	26.06%	22.13%	31.99%	41.50%	8.06%	4.49%
FA(24:0)	17.66%	23.02%	14.77%	20.07%	7.24%	4.17%
FA(24:1)	38.81%	17.52%	21.03%	25.33%	9.07%	5.05%
FA(26:0)	32.19%	24.64%	29.51%	26.24%	4.78%	7.48%
Hex2Cer(d12:0_20:4)	67.98%	32.05%	39.26%	26.11%	8.41%	14.91%
LPC(16:0)	15.53%	27.69%	26.64%	26.86%	11.42%	1.04%
LPC(16:1)	33.52%	24.12%	40.63%	30.13%	13.57%	2.40%
LPC(18:0)	12.99%	30.79%	27.74%	31.48%	13.59%	1.04%
LPC(18:1)	17.86%	28.73%	24.30%	25.27%	10.87%	1.56%
LPC(18:2)	20.49%	26.66%	28.51%	26.82%	12.56%	1.67%
LPC(20:3)*1	43.30%	36.89%	31.44%	27.81%	12.34%	3.06%
LPC(20:3)*2	18.26%	34.92%	25.01%	33.36%	9.86%	2.47%
LPC(20:4)*1	27.88%	38.58%	20.26%	23.66%	10.77%	1.93%
LPC(20:4)*2	26.74%	40.32%	29.01%	28.79%	18.38%	4.86%
LPC(22:5)	45.12%	58.27%	20.93%	22.83%	9.34%	4.34%
LPC(22:6)	53.64%	39.58%	44.04%	38.91%	12.00%	5.52%
LPE(16:0)	67.94%	50.98%	45.31%	47.17%	14.39%	7.67%
LPE(18:0)*1	33.51%	68.32%	33.22%	26.78%	24.99%	16.13%
LPE(18:0)*2	79.11%	46.56%	35.72%	44.07%	10.46%	7.32%
LPE(18:1)	63.74%	61.80%	39.28%	31.56%	14.45%	5.84%
LPE(18:2)	62.92%	45.36%	36.35%	32.09%	10.17%	5.21%
LPE(20:4)	33.75%	33.85%	17.32%	26.72%	13.63%	7.63%
LPE(22:1)	10.26%	10.41%	4.76%	6.49%	7.61%	4.44%
LPE(22:6)	62.50%	37.57%	33.17%	43.44%	11.47%	5.73%
PC(14:0_18:2)	58.70%	58.25%	68.70%	43.51%	7.31%	3.07%
PC(14:0_20:4)	50.68%	64.53%	85.85%	49.46%	7.34%	6.90%
PC(14:0_20:5)	54.67%	56.05%	67.03%	41.35%	8.42%	5.53%
PC(16:0_16:1)	65.17%	40.43%	52.57%	41.13%	5.28%	5.96%
PC(16:0_18:1)	29.82%	19.99%	13.58%	13.55%	2.21%	2.49%
PC(16:0_20:3)	35.05%	29.31%	19.05%	15.48%	5.48%	3.03%
PC(16:0_20:5)	57.70%	43.02%	63.15%	39.78%	6.80%	7.52%
PC(16:0_22:5)	31.34%	26.07%	20.03%	19.01%	1.92%	4.09%

PC(16:0_22:6)	39.58%	27.48%	26.37%	24.33%	3.73%	4.91%
PC(18:0_16:0)	36.47%	29.68%	15.48%	17.98%	6.06%	5.13%
PC(18:0_18:1)	46.45%	29.28%	21.57%	21.77%	5.20%	5.25%
PC(18:0_18:2)	30.17%	19.83%	11.45%	11.25%	3.69%	2.53%
PC(18:0_20:2)	43.14%	26.70%	23.46%	19.17%	5.17%	5.95%
PC(18:0_20:3)	50.38%	42.74%	21.87%	29.36%	8.54%	5.28%
PC(18:0_20:4)	26.78%	28.09%	15.40%	18.78%	2.07%	1.33%
PC(18:0_22:4)	35.72%	31.39%	12.25%	18.02%	7.44%	6.06%
PC(18:0_22:5)	32.76%	25.79%	12.47%	16.92%	6.01%	2.08%
PC(18:0_22:6)	35.94%	24.65%	24.72%	28.07%	4.44%	2.02%
PC(18:1_13:0)	65.88%	67.93%	43.62%	40.81%	6.75%	6.49%
PC(18:1_22:6)	37.35%	22.23%	31.75%	28.82%	2.86%	2.29%
PC(18:2_13:0)	50.84%	30.02%	38.51%	61.33%	5.02%	7.12%
PC(18:2_18:2)	21.32%	19.59%	12.71%	10.83%	2.76%	1.60%
PC(18:2_22:6)	49.60%	34.44%	40.01%	39.13%	9.35%	5.76%
PC(22:0_18:2)	53.76%	32.46%	24.48%	24.20%	15.99%	6.36%
PC(30:1)	96.34%	78.99%	112.29%	43.56%	8.08%	8.13%
PC(31:0)	44.02%	50.58%	30.34%	27.57%	6.47%	5.77%
PC(32:0)	33.70%	25.99%	13.68%	13.69%	6.99%	3.35%
PC(33:0)	50.60%	47.34%	27.71%	20.06%	6.43%	2.21%
PC(33:1)	49.46%	46.92%	26.26%	29.82%	7.03%	2.72%
PC(33:2)	49.62%	39.86%	35.98%	23.25%	7.49%	2.30%
PC(33:3)	48.25%	40.53%	56.31%	54.65%	9.54%	14.13%
PC(33:4)	37.32%	27.08%	24.47%	40.22%	6.63%	3.98%
PC(34:1e)	38.72%	23.93%	15.51%	22.41%	8.33%	7.45%
PC(34:2)	27.80%	19.23%	10.28%	11.01%	1.30%	2.19%
PC(34:2e)*1	53.59%	38.91%	33.57%	40.37%	3.83%	6.78%
PC(34:2e)*2	44.05%	30.80%	17.51%	23.32%	8.45%	5.49%
PC(34:3)	45.50%	40.81%	45.04%	31.79%	6.14%	3.84%
PC(34:3e)	48.42%	37.41%	19.11%	22.86%	8.01%	4.05%
PC(35:1)	47.89%	44.76%	20.88%	20.96%	6.83%	4.87%
PC(35:2)	39.06%	34.42%	33.82%	17.82%	9.11%	5.37%
PC(35:3)	44.65%	49.11%	42.66%	28.17%	5.58%	7.00%
PC(35:4)	36.95%	48.54%	23.62%	23.71%	9.95%	5.83%
PC(35:5)*1	30.79%	37.07%	37.35%	41.26%	33.90%	20.32%
PC(35:5)*2	54.93%	55.99%	37.62%	86.79%	23.62%	16.19%
PC(36:0)	43.21%	34.26%	19.54%	25.73%	13.66%	8.19%
PC(36:4e)	39.40%	28.64%	12.65%	11.88%	5.11%	3.95%
PC(36:6)	52.18%	57.95%	71.36%	76.26%	10.16%	30.82%
PC(37:1)	51.62%	39.35%	24.93%	21.73%	8.70%	8.52%
PC(37:4)	37.73%	43.18%	23.02%	22.75%	10.69%	4.38%
PC(37:5)	44.33%	41.44%	27.59%	32.93%	4.94%	7.22%
PC(37:6)	51.41%	42.27%	50.67%	41.54%	10.21%	4.92%
PC(38:4e)	41.37%	34.04%	15.21%	16.73%	6.54%	5.01%

PC(38:5)	8.02%	5.55%	6.59%	10.02%	3.54%	1.97%
PC(38:5e)	37.47%	24.80%	15.44%	13.99%	4.77%	3.10%
PC(38:6e)	59.27%	33.49%	23.65%	19.02%	6.94%	3.75%
PC(38:7)	55.98%	34.54%	56.02%	53.52%	9.00%	8.30%
PC(38:7e)	61.27%	40.64%	32.88%	19.04%	5.37%	6.84%
PC(39:6)	56.96%	43.13%	42.33%	41.12%	11.67%	7.27%
PC(40:7)	30.26%	22.07%	22.94%	27.85%	5.35%	5.21%
PC(44:5e)	43.00%	28.38%	12.24%	20.52%	11.45%	11.60%
PE(16:0_18:1)	55.38%	33.52%	46.07%	41.95%	9.75%	8.33%
PE(16:0_18:2)	63.82%	36.43%	52.88%	71.59%	20.11%	17.57%
PE(16:0_20:4)	49.14%	29.68%	41.10%	47.12%	19.96%	16.28%
PE(16:0_20:5)	61.67%	36.32%	104.16%	56.93%	9.94%	11.60%
PE(16:1e_22:6)	76.50%	60.36%	25.88%	31.79%	14.90%	8.76%
PE(18:0_18:1)	58.63%	40.02%	51.22%	46.73%	11.57%	8.15%
PE(18:0_18:2)	61.72%	42.62%	43.75%	60.57%	11.05%	11.90%
PE(18:0_20:3)	77.44%	57.63%	49.42%	47.59%	8.66%	7.24%
PE(18:0_20:4)	53.27%	37.38%	29.83%	40.70%	10.81%	7.07%
PE(18:0_22:5)	90.67%	66.21%	56.42%	73.71%	13.85%	22.58%
PE(18:0p_22:6)	62.07%	49.66%	37.06%	32.49%	10.32%	7.17%
PE(18:1_18:2)	56.83%	42.78%	55.11%	63.15%	15.09%	11.56%
PE(18:1_20:4)	54.67%	36.63%	44.93%	48.21%	16.38%	15.24%
PE(32:1)	215.11%	282.84 %	173.80%	127.79 %	14.06%	70.36%
PE(38:6)	51.36%	22.74%	43.58%	48.84%	9.63%	5.55%
PE(39:6)	111.82%	56.22%	63.06%	61.62%	27.57%	14.46%
PE(40:7)	54.53%	25.94%	52.11%	55.94%	24.61%	9.82%
PE(42:5)	31.89%	22.57%	11.30%	10.86%	7.24%	5.19%
PE(42:7)	56.27%	47.90%	31.17%	25.12%	21.82%	13.79%
PG(16:0_18:1)	57.79%	50.30%	25.56%	23.86%	13.51%	6.75%
PG(36:1)	63.94%	55.56%	41.24%	26.33%	14.87%	9.19%
PG(36:2)	73.91%	48.17%	36.19%	35.87%	12.59%	7.15%
PG(36:3)	76.51%	46.49%	18.69%	19.75%	5.81%	2.65%
PG(36:5)	45.45%	42.80%	64.46%	84.67%	33.30%	49.10%
PG(38:4)	91.58%	34.67%	22.18%	21.10%	5.91%	3.65%
PG(38:6)	84.18%	46.27%	68.95%	59.39%	7.25%	26.14%
PG(40:6)	154.52%	138.46 %	192.59%	193.44 %	7.18%	33.73%
PG(40:8)	52.65%	61.68%	40.93%	43.32%	28.33%	19.11%
PI(16:0_16:0)	#DIV/0!	282.84 %	101.78%	43.00%	61.63%	25.19%
PI(16:0_16:1)	#DIV/0!	#DIV/0 !	102.52%	56.04%	244.95 %	15.50%
PI(16:0_20:4)	92.52%	135.00 %	31.50%	33.43%	54.02%	5.70%
PI(16:0_22:6)	185.69%	#DIV/0 !	36.02%	42.73%	244.95 %	10.72%

PI(18:0_18:2)	114.39%	93.84%	33.72%	21.29%	33.75%	7.89%
PI(18:0_20:4)	84.86%	54.69%	21.93%	28.58%	30.17%	8.84%
PI(18:0_22:5)	198.59%	131.37 %	29.71%	23.43%	55.75%	8.68%
PI(18:0_22:6)	160.57%	208.05 %	28.50%	39.10%	64.36%	11.93%
PI(34:0)	58.77%	62.00%	80.26%	84.84%	15.58%	23.72%
PI(34:1)	113.36%	116.64 %	52.38%	28.83%	45.94%	11.22%
PI(34:2)	213.09%	282.84 %	43.05%	25.26%	66.41%	3.43%
PI(36:0)	43.17%	30.23%	27.77%	24.45%	5.77%	3.18%
PI(36:1)	55.86%	39.59%	43.01%	26.30%	13.63%	24.27%
PI(36:3)	121.90%	185.72 %	43.64%	25.08%	72.57%	3.36%
PI(38:2)	42.29%	30.73%	35.05%	32.55%	12.70%	19.40%
PI(38:3)	116.08%	134.71 %	33.43%	21.17%	38.69%	14.89%
PI(38:5)	#DIV/0!	#DIV/0 !	35.56%	26.26%	244.95 %	4.34%
PI(40:4)*1	75.28%	51.13%	52.14%	38.18%	21.02%	59.08%
PI(40:4)*2	#DIV/0!	#DIV/0 !	40.16%	25.97%	#DIV/0!	20.00%
PS(34:0)	282.84%	185.27 %	76.91%	11.92%	9.69%	9.60%
PS(34:2)	100.05%	106.73 %	14.36%	26.04%	34.40%	13.27%
PS(36:0)	65.78%	39.49%	57.38%	39.02%	4.81%	1.45%
PS(36:2)	124.32%	121.12 %	132.19%	82.10%	14.86%	13.07%
PS(38:4)	125.36%	93.16%	34.13%	20.38%	8.09%	8.72%
PS(40:4)	54.13%	39.18%	64.90%	39.70%	8.17%	8.38%
PS(42:3)	25.60%	27.22%	18.16%	20.56%	3.47%	3.81%
SM(d32:2)	38.99%	40.01%	33.95%	25.02%	12.56%	4.35%
SM(d35:1)	41.49%	28.90%	24.08%	17.28%	12.23%	3.80%
SM(d37:1)	65.36%	35.10%	29.00%	20.59%	11.25%	4.43%
SM(d39:1)	52.49%	39.33%	35.53%	28.16%	17.82%	10.97%
SM(d41:7)	#DIV/0!	282.84 %	92.75%	29.19%	6.71%	6.78%
SM(d43:2)	71.99%	47.95%	40.68%	35.72%	21.97%	10.99%
SM(d44:1)	150.51%	101.42 %	273.01%	162.93 %	#DIV/0!	63.85%
SM(t40:7)	215.13%	239.32 %	134.94%	177.34 %	88.33%	89.44%
SPH(d18:0)	79.62%	53.49%	59.33%	71.64%	33.55%	29.17%
SPH(d18:1)	124.97%	207.02 %	108.28%	88.10%	38.45%	63.98%
Sphinganine 1-phosphate (d18:0-P)	43.51%	28.52%	39.64%	47.56%	24.39%	40.01%

Sphingosine 1-phosphate (d18:1-P)	24.55%	10.25%	8.92%	27.38%	11.33%	9.61%
TG(16:0_14:0_18:1)	117.09%	54.52%	84.93%	82.61%	2.13%	2.22%
TG(16:0_14:4_16:0)	114.59%	72.63%	112.36%	83.33%	123.26 %	40.03%
TG(16:0_16:0_16:0)	125.52%	42.37%	73.17%	99.27%	3.75%	1.90%
TG(16:0_16:0_18:1)	73.23%	43.30%	34.46%	49.18%	1.39%	2.04%
TG(16:0_16:1_16:1)	106.43%	57.68%	80.70%	69.07%	3.59%	4.01%
TG(16:0_16:1_18:1)	66.05%	47.03%	37.05%	45.86%	2.04%	1.47%
TG(16:0_18:1_18:1)	50.82%	46.94%	20.17%	25.40%	1.43%	2.19%
TG(16:0_18:1_18:2)	43.42%	37.05%	29.13%	25.94%	1.88%	1.19%
TG(16:0_18:2_18:2)	46.54%	37.38%	45.33%	35.30%	2.73%	2.70%
TG(16:0_18:2_20:4)	36.30%	31.35%	54.35%	36.16%	3.44%	11.01%
TG(16:1_14:0_18:2)	106.21%	57.14%	92.46%	67.57%	5.46%	4.06%
TG(16:1_16:1_18:1)	62.55%	48.22%	47.53%	43.81%	2.86%	2.45%
TG(16:1_16:1_18:3)	68.21%	52.59%	92.18%	73.90%	5.73%	3.75%
TG(16:1_18:2_18:2)	57.83%	50.09%	60.68%	53.84%	3.64%	5.33%
TG(16:1_18:2_18:3)	56.16%	46.99%	74.96%	74.87%	5.90%	3.32%
TG(17:0_18:1_20:4)	84.57%	85.01%	41.52%	45.78%	11.83%	7.30%
TG(18:0_16:0_16:0)	126.17%	48.12%	74.59%	113.98 %	6.51%	2.72%
TG(18:0_16:0_18:1)	103.42%	61.45%	45.49%	83.74%	4.46%	2.72%
TG(18:0_18:1_18:1)	82.51%	75.14%	42.05%	50.55%	4.41%	2.51%
TG(18:1_12:3_18:1)	105.86%	63.30%	125.35%	93.17%	143.24 %	68.33%
TG(18:1_18:1_18:1)	53.65%	58.92%	43.63%	32.32%	3.65%	3.16%
TG(18:1_18:1_18:2)	47.31%	48.74%	58.90%	39.25%	4.77%	3.98%
TG(18:1_18:1_20:4)	47.37%	32.49%	33.53%	37.82%	2.33%	1.93%
TG(18:1_18:1_20:5)	32.32%	42.61%	42.48%	27.54%	9.51%	8.97%
TG(18:1_18:2_18:2)	55.79%	51.15%	76.72%	51.97%	3.13%	3.04%
TG(18:2_18:2_18:2)	59.62%	53.79%	106.12%	71.08%	3.95%	5.75%
TG(18:3_18:2_18:2)	52.23%	34.80%	65.16%	74.70%	4.59%	3.95%
TG(20:3_18:2_18:2)	76.52%	51.29%	43.20%	47.10%	3.46%	3.53%
TG(50:4)	68.85%	55.01%	78.15%	58.64%	4.30%	4.71%
TG(52:12e)	54.61%	33.03%	25.08%	25.24%	22.18%	7.30%
TG(54:8)	51.02%	32.61%	89.46%	101.06 %	4.63%	4.31%
TG(55:4)	74.12%	72.82%	77.82%	43.46%	2.93%	3.85%
TG(56:9)	54.56%	41.84%	78.67%	80.68%	5.39%	3.01%

Table J. The univariate and multivariate result of identified lipids (pre TRF vs CR)

lipid	T test	BF correction	q value (FDR correction)	VIP	fold change
7-oxocholesterol	0.179325	1.434599	0.167042	0.601335	1.414
AcCa(16:0)	0.000326	0.002609	0.000969	1.33376	4.724
Cer(d16:1_22:0)	0.110869	0.886953	0.11325	0.704459	1.702
Cer(d18:0/16:0)	0.50849	4.067921	0.402715	0.303605	0.6785
Cer(d18:0/18:0)	0.649208	5.193663	0.482292	0.209757	0.4648
Cer(d18:0/20:0)	0.079043	0.632343	0.087718	0.768501	1.894
Cer(d18:0/26:0)	0.050158	0.40126	0.059352	0.845652	2.143
Cer(d18:0_22:0)	0.015648	0.125183	0.022797	1.00762	2.75
Cer(d18:0_24:0)	0.007854	0.062828	0.012799	1.08524	3.099
Cer(d18:0_24:1)	0.00789	0.063123	0.012799	1.08475	3.096
Cer(d18:1/19:0)	0.69025	5.522	0.504444	0.183938	0.4069
Cer(d18:1/25:0)	0.017766	0.142131	0.025068	0.992017	2.687
Cer(d18:1_16:0)	0.005494	0.043953	0.009407	1.12127	3.278
Cer(d18:1_18:0)	0.003783	0.030265	0.007085	1.15624	3.466
Cer(d18:1_22:0)	0.024328	0.194625	0.03247	0.951415	2.522
Cer(d18:1_23:0)	0.00551	0.044083	0.009407	1.12098	3.281
Cer(d18:1_24:1)	0.003127	0.025014	0.005881	1.17313	3.565
Cer(d18:1_26:0)	0.006967	0.055737	0.011491	1.09761	3.159
Cer(d18:1_26:1)	0.009041	0.072327	0.014134	1.07029	3.028
Cer(d18:2/20:0)	0.059167	0.473337	0.067752	0.818703	2.054
Cer(d18:2/24:2)	0.002842	0.02274	0.005538	1.18135	3.612
Cer(d18:2_22:0)	0.041991	0.335931	0.050785	0.873394	2.24
Cer(d18:2_23:0)	0.023023	0.184187	0.031335	0.958748	2.555
Cer(d18:2_24:1)	0.004061	0.032488	0.007415	1.1498	3.433
ChE(14:0)	0.602003	4.816025	0.455206	0.240207	0.5336
ChE(15:0)	0.005335	0.042676	0.009311	1.12413	3.293
ChE(16:0)	0.000575	0.004596	0.001518	1.29946	4.428
ChE(16:1)	0.1253	1.002401	0.126553	0.67968	1.63
ChE(17:0)	0.058801	0.470409	0.067752	0.819737	2.057
ChE(17:1)	0.03256	0.260481	0.040935	0.91103	2.372
ChE(18:0)	0.000506	0.004049	0.001379	1.30744	4.493
ChE(18:1)	0.000237	0.001897	0.00074	1.35163	4.899
ChE(18:3)	0.015719	0.12575	0.022797	1.00707	2.747
ChE(20:1)	0.002393	0.019143	0.005002	1.19582	3.697
ChE(20:3)	0.000477	0.003816	0.001298	1.31111	4.532
ChE(20:4)	0.001826	0.014607	0.003935	1.21762	3.836
ChE(20:5)	0.196378	1.571028	0.182984	0.579957	1.353
ChE(22:6)	0.037055	0.29644	0.045868	0.89219	2.301
CoQ10	0.000117	0.000936	0.000404	1.38794	5.277

CoQ9	0.005239	0.041911	0.009234	1.12588	3.302
DG(30:2)	0.565838	4.526707	0.434728	0.264182	0.5881
DG(38:0)	0.000222	0.001779	0.000714	1.35511	4.928
DG(38:3)	0.60262	4.820961	0.455206	0.239803	0.5327
DG(40:7)	0.773227	6.185817	0.54514	0.133188	0.2938
DG(14:0_18:2)	0.997274	7.97819	0.65674	0.001582	0.003478
DG(14:0_18:3)	0.296058	2.36846	0.255651	0.474051	1.085
DG(16:0_14:0)	0.735331	5.882649	0.526685	0.156155	0.3449
DG(16:0_16:0)	0.37064	2.96512	0.308489	0.408334	0.925
DG(16:0_16:1)	0.804259	6.434071	0.560425	0.114599	0.2526
DG(16:0_18:1)	0.913741	7.309928	0.617585	0.050133	0.1103
DG(16:0_18:2)	0.101177	0.809414	0.105208	0.722398	1.758
DG(16:0_18:3)	0.852352	6.818819	0.58487	0.086103	0.1896
DG(16:1_14:0)	0.541584	4.332668	0.42209	0.280628	0.6257
DG(16:1_18:3)	0.927056	7.416447	0.624218	0.042374	0.09321
DG(18:0_16:0)	0.053009	0.424075	0.062288	0.836759	2.113
DG(18:0_18:0)	0.083835	0.670678	0.090794	0.757802	1.861
DG(18:0_18:1)	0.883025	7.064203	0.601327	0.068085	0.1498
DG(18:1_18:1)	0.724312	5.794499	0.52539	0.162896	0.3593
DG(18:1_20:3)	0.993224	7.945795	0.656478	0.003931	0.008645
DG(18:1_22:5)	0.978959	7.831672	0.651842	0.012209	0.02685
DG(18:3_18:2)	0.544972	4.359774	0.422306	0.278311	0.6204
DG(36:3)	0.59717	4.777363	0.453136	0.243376	0.5442
DG(36:4)	0.578668	4.629344	0.442892	0.255606	0.5682
DG(38:5)	0.230352	1.842814	0.207202	0.540709	1.254
DG(38:6)	0.974111	7.792887	0.651025	0.015023	0.03304
Eicosanoids_19,20-DiHDPA	0.05466	0.437278	0.063398	0.831774	2.097
Eicosanoids_PGD3	#DIV/0!	#DIV/0!			
FA(12:0)	0.082025	0.656202	0.089373	0.761789	1.874
FA(14:0)	0.30838	2.467042	0.263034	0.462581	1.056
FA(15:0)	0.490058	3.920466	0.393617	0.316703	0.7082
FA(16:0)	0.460607	3.684858	0.374781	0.338133	0.7592
FA(16:1)	0.660762	5.286097	0.487462	0.202432	0.4472
FA(17:0)	0.617831	4.942649	0.463043	0.229898	0.5098
FA(17:1)	0.767242	6.137934	0.54514	0.136795	0.3042
FA(18:0)	0.104818	0.838546	0.107196	0.715522	1.737
FA(18:1)	0.6531	5.224799	0.482457	0.207285	0.4607
FA(18:2)	0.488805	3.910438	0.393617	0.317602	0.7116
FA(18:3)	0.297179	2.37743	0.255651	0.472996	1.083
FA(18:4)	0.152002	1.216019	0.146134	0.638592	1.515
FA(20:2)	0.730563	5.844502	0.526685	0.159068	0.3496
FA(20:3)	0.542276	4.338208	0.42209	0.280154	0.6245
FA(20:4)	0.516087	4.128694	0.40533	0.298272	0.6659
FA(20:5)	0.258084	2.064669	0.225234	0.511312	1.179

FA(22:0)	0.733895	5.871161	0.526685	0.157032	0.3468
FA(22:1)	0.614162	4.913298	0.461984	0.232278	0.5156
FA(22:2)	0.814111	6.512889	0.5651	0.108733	0.2396
FA(22:3)	0.868892	6.951135	0.593952	0.076374	0.1681
FA(22:4)	0.893542	7.148339	0.602985	0.061929	0.1424
FA(22:5)	0.374658	2.997262	0.309836	0.405017	0.9184
FA(22:6)	0.205705	1.645643	0.188915	0.568774	1.326
FA(23:0)	0.022458	0.179661	0.031121	0.962026	2.564
FA(24:0)	0.938121	7.504967	0.629311	0.035933	0.07904
FA(24:1)	0.004912	0.039297	0.008884	1.13204	3.332
FA(26:0)	0.027892	0.223139	0.03571	0.932816	2.455
Hex2Cer(d12:0_20:4)	0.989084	7.912672	0.656478	0.006333	0.01308
LPC(16:0)	1.13E-06	9.04E-06	0.000013	1.54574	8.123
LPC(16:1)	0.000152	0.001219	0.000498	1.37487	5.132
LPC(18:0)	2.2E-06	1.76E-05	0.000019	1.5295	7.673
LPC(18:1)	7.31E-07	5.85E-06	0.00001	1.55543	8.436
LPC(18:2)	8.26E-06	6.61E-05	0.000049	1.49197	6.826
LPC(20:3)*1	5.25E-05	0.00042	0.000219	1.42429	5.725
LPC(20:3)*2	4.32E-07	3.45E-06	0.000009	1.56636	8.824
LPC(20:4)*1	6.95E-07	5.56E-06	0.00001	1.55654	8.486
LPC(20:4)*2	2.1E-06	1.68E-05	0.000019	1.53068	7.709
LPC(22:5)	8.22E-07	6.58E-06	0.00001	1.55289	8.356
LPC(22:6)	0.002708	0.021662	0.005434	1.18549	3.634
LPE(16:0)	2.75E-05	0.00022	0.000123	1.45031	6.099
LPE(18:0)*1	4.41E-06	3.53E-05	0.000032	1.51076	7.222
LPE(18:0)*2	4.11E-06	3.29E-05	0.000031	1.51272	7.267
LPE(18:1)	1.13E-05	9.06E-05	0.000057	1.48179	6.63
LPE(18:2)	9.18E-06	7.34E-05	0.000051	1.48863	6.76
LPE(20:4)	1.91E-09	1.53E-08	<0.000001	1.64023	13.57
LPE(22:1)	0.001162	0.009298	0.002493	1.25166	4.087
LPE(22:6)	8.63E-06	6.9E-05	0.00005	1.49059	6.798
PC(14:0_18:2)	0.026407	0.211253	0.03463	0.940334	2.478
PC(14:0_20:4)	0.046553	0.372426	0.055779	0.857446	2.183
PC(14:0_20:5)	0.080664	0.645309	0.088458	0.764831	1.883
PC(16:0_16:1)	0.144183	1.153462	0.141457	0.650076	1.548
PC(16:0_18:1)	0.00038	0.003038	0.001107	1.32488	4.64
PC(16:0_20:3)	0.001219	0.009755	0.00274	1.24818	4.033
PC(16:0_20:5)	0.054356	0.43485	0.063398	0.832683	2.099
PC(16:0_22:5)	0.006039	0.048316	0.010292	1.11198	3.228
PC(16:0_22:6)	0.017571	0.140572	0.025068	0.99339	2.688
PC(18:0_16:0)	1.1E-05	8.83E-05	0.000057	1.48262	6.637
PC(18:0_18:1)	0.000956	0.00765	0.002257	1.26551	4.164
PC(18:0_18:2)	0.000256	0.002044	0.000768	1.34754	4.87
PC(18:0_20:2)	0.000407	0.003254	0.00115	1.32079	4.612

PC(18:0_20:3)	0.000451	0.003608	0.00126	1.31454	4.556
PC(18:0_20:4)	0.001014	0.008108	0.002364	1.26143	4.134
PC(18:0_22:4)	1.99E-06	1.59E-05	0.000019	1.53214	7.732
PC(18:0_22:5)	4.3E-05	0.000344	0.000187	1.43261	5.843
PC(18:0_22:6)	0.029292	0.234339	0.037584	0.926007	2.424
PC(18:1_13:0)	0.023975	0.191799	0.032166	0.953369	2.531
PC(18:1_22:6)	0.012586	0.100692	0.019254	1.03338	2.854
PC(18:2_13:0)	0.014251	0.114007	0.02135	1.01884	2.797
PC(18:2_18:2)	9.83E-05	0.000787	0.000373	1.39624	5.377
PC(18:2_22:6)	0.000905	0.007238	0.002216	1.26935	4.193
PC(22:0_18:2)	0.000787	0.006296	0.002021	1.27886	4.264
PC(30:1)	0.133416	1.06733	0.132362	0.666604	1.594
PC(31:0)	0.067673	0.541386	0.076518	0.795909	1.98
PC(32:0)	0.00014	0.001116	0.00047	1.37931	5.174
PC(33:0)	0.006652	0.053217	0.011177	1.10231	3.182
PC(33:1)	0.031125	0.248997	0.03952	0.917462	2.394
PC(33:2)	0.025983	0.207861	0.034201	0.942538	2.492
PC(33:3)	0.040554	0.324428	0.049597	0.878689	2.257
PC(33:4)	0.002592	0.020737	0.005296	1.18916	3.656
PC(34:1e)	7.72E-06	6.17E-05	0.000048	1.49409	6.862
PC(34:2)	0.001945	0.015563	0.004131	1.21261	3.805
PC(34:2e)*1	0.044035	0.35228	0.053208	0.866097	2.212
PC(34:2e)*2	8.48E-05	0.000678	0.000338	1.40314	5.456
PC(34:3)	0.005161	0.041286	0.009069	1.12732	3.316
PC(34:3e)	0.0279	0.223196	0.03571	0.932781	2.454
PC(35:1)	0.000874	0.00699	0.0022	1.27175	4.205
PC(35:2)	0.006739	0.053915	0.011223	1.10099	3.175
PC(35:3)	0.071236	0.56989	0.080388	0.786982	1.95
PC(35:4)	0.003	0.024004	0.00576	1.17671	3.581
PC(35:5)*1	0.004108	0.032866	0.007461	1.14873	3.424
PC(35:5)*2	0.780734	6.245869	0.548282	0.128675	0.2838
PC(36:0)	0.000329	0.00263	0.000969	1.3333	4.72
PC(36:4e)	9.82E-05	0.000786	0.000376	1.39631	5.361
PC(36:6)	0.07566	0.605283	0.084486	0.776339	1.919
PC(37:1)	0.000136	0.001092	0.000463	1.3804	5.192
PC(37:4)	0.011294	0.090353	0.017409	1.04576	2.914
PC(37:5)	0.254498	2.035982	0.224282	0.514999	1.188
PC(37:6)	0.102817	0.822533	0.106607	0.71928	1.747
PC(38:4e)	1.09E-05	8.71E-05	0.000057	1.48308	6.637
PC(38:5)	4.29E-06	3.43E-05	0.000031	1.51152	7.261
PC(38:5e)	0.000298	0.002388	0.000938	1.33883	4.754
PC(38:6e)	0.023587	0.188697	0.031905	0.955541	2.539
PC(38:7)	0.149014	1.192115	0.144031	0.642932	1.527
PC(38:7e)	0.696066	5.568524	0.507779	0.180322	0.3966

PC(39:6)	0.505968	4.04774	0.402715	0.305384	0.6832
PC(40:7)	0.012522	0.100175	0.019127	1.03398	2.862
PC(44:5e)	5.3E-06	4.24E-05	0.000035	1.50545	7.108
PE(16:0_18:1)	0.00272	0.021764	0.005434	1.18509	3.632
PE(16:0_18:2)	0.003004	0.024034	0.00576	1.1766	3.581
PE(16:0_20:4)	0.000605	0.004838	0.00157	1.29618	4.402
PE(16:0_20:5)	0.096516	0.772126	0.102181	0.73146	1.782
PE(16:1e_22:6)	0.000105	0.000842	0.000378	1.39301	5.336
PE(18:0_18:1)	0.008767	0.070138	0.013948	1.07359	3.043
PE(18:0_18:2)	0.009741	0.077925	0.015179	1.0622	2.987
PE(18:0_20:3)	0.026123	0.208986	0.034281	0.941804	2.487
PE(18:0_20:4)	0.000577	0.004613	0.001518	1.29922	4.427
PE(18:0_22:5)	0.018646	0.149166	0.025986	0.985962	2.66
PE(18:0p_22:6)	0.003906	0.031246	0.007233	1.15335	3.45
PE(18:1_18:2)	0.00283	0.022639	0.005538	1.18173	3.611
PE(18:1_20:4)	0.000896	0.007168	0.002216	1.27002	4.188
PE(32:1)	0.240975	1.927797	0.214467	0.5292	1.224
PE(38:6)	0.0021	0.016797	0.004428	1.2065	3.764
PE(39:6)	0.017334	0.138675	0.024931	0.995076	2.698
PE(40:7)	0.008893	0.071147	0.014025	1.07206	3.036
PE(42:5)	0.001073	0.008582	0.002441	1.2574	4.104
PE(42:7)	5.88E-05	0.00047	0.00024	1.41943	5.661
PG(16:0_18:1)	0.023007	0.184054	0.031335	0.958843	2.552
PG(36:1)	0.033706	0.269648	0.041897	0.906049	2.356
PG(36:2)	0.000825	0.006596	0.002069	1.2757	4.244
PG(36:3)	8.25E-09	6.6E-08	<0.000001	1.62573	12.12
PG(36:5)	0.316966	2.535727	0.268793	0.454748	1.038
PG(38:4)	5.24E-08	4.2E-07	0.000002	1.60231	10.47
PG(38:6)	0.790321	6.322567	0.552856	0.122926	0.271
PG(40:6)	0.841448	6.731584	0.5796	0.092535	0.2038
PG(40:8)	0.462038	3.696304	0.374781	0.337076	0.7562
PI(16:0_16:0)	0.014783	0.118266	0.021965	1.01446	2.779
PI(16:0_16:1)	0.015377	0.123012	0.022659	1.00974	2.759
PI(16:0_20:4)	3.62E-07	2.9E-06	0.000008	1.56981	8.95
PI(16:0_22:6)	1.73E-06	1.38E-05	0.000017	1.53562	7.842
PI(18:0_18:2)	8.21E-07	6.57E-06	0.00001	1.55291	8.383
PI(18:0_20:4)	3.97E-09	3.17E-08	<0.000001	1.63337	12.78
PI(18:0_22:5)	1.83E-07	1.47E-06	0.000005	1.58236	9.467
PI(18:0_22:6)	1.08E-07	8.61E-07	0.000003	1.5913	9.886
PI(34:0)	0.136055	1.088444	0.134396	0.662471	1.582
PI(34:1)	9.68E-05	0.000774	0.000373	1.39699	5.384
PI(34:2)	1.26E-05	0.000101	0.000061	1.47818	6.564
PI(36:0)	0.018234	0.145875	0.02547	0.988767	2.675
PI(36:1)	0.000106	0.000848	0.000381	1.39268	5.32

PI(36:3)	1.48E-05	0.000118	0.00007	1.47278	6.468
PI(38:2)	0.000946	0.007569	0.002257	1.26625	4.169
PI(38:3)	7.47E-07	5.98E-06	0.00001	1.55498	8.424
PI(38:5)	1.46E-06	1.17E-05	0.000015	1.53963	7.954
PI(40:4)*1	0.092172	0.737377	0.098635	0.740189	1.808
PI(40:4)*2	5.84E-06	4.67E-05	0.000038	1.50255	7.042
PS(34:0)	0.002488	0.019905	0.005142	1.19259	3.677
PS(34:2)	4.98E-11	3.99E-10	<0.000001	1.66529	17.85
PS(36:0)	0.218646	1.749164	0.197775	0.553787	1.29
PS(36:2)	0.171338	1.370702	0.162122	0.61181	1.442
PS(38:4)	2.73E-06	2.19E-05	0.000022	1.52388	7.534
PS(40:4)	0.096452	0.771615	0.102181	0.731586	1.781
PS(42:3)	0.001717	0.013737	0.003613	1.22242	3.886
SM(d32:2)	4.59E-05	0.000367	0.000196	1.42992	5.802
SM(d35:1)	5.06E-06	4.05E-05	0.000035	1.50677	7.115
SM(d37:1)	9.5E-06	7.6E-05	0.000051	1.4875	6.752
SM(d39:1)	2.65E-05	0.000212	0.000121	1.4516	6.126
SM(d41:7)	0.008658	0.069262	0.013897	1.07494	3.05
SM(d43:2)	0.000102	0.00082	0.000378	1.39429	5.345
SM(d44:1)	0.353132	2.825052	0.296664	0.42303	0.9604
SM(t40:7)	0.357989	2.863911	0.299345	0.418913	0.9505
SPH(d18:0)	0.257068	2.056543	0.225234	0.512353	1.182
SPH(d18:1)	0.772488	6.179907	0.54514	0.133633	0.2948
Sphinganine 1-phosphate (d18:0-P)	0.350785	2.80628	0.296076	0.42503	0.9653
Sphingosine 1-phosphate (d18:1-P)	0.10389	0.831117	0.107196	0.717259	1.739
TG(16:0_14:0_18:1)	0.8345	6.675996	0.576754	0.096642	0.2134
TG(16:0_14:4_16:0)	0.223825	1.790603	0.202451	0.547947	1.272
TG(16:0_16:0_16:0)	0.67989	5.439117	0.499949	0.190404	0.4194
TG(16:0_16:0_18:1)	0.148726	1.189807	0.144031	0.643354	1.527
TG(16:0_16:1_16:1)	0.508023	4.064185	0.402715	0.303934	0.6802
TG(16:0_16:1_18:1)	0.281365	2.250921	0.243879	0.488104	1.122
TG(16:0_18:1_18:1)	0.033898	0.27118	0.042383	0.905229	2.347
TG(16:0_18:1_18:2)	0.050244	0.401949	0.059352	0.845379	2.143
TG(16:0_18:2_18:2)	0.061608	0.492868	0.070897	0.811928	2.026
TG(16:0_18:2_20:4)	0.091047	0.728379	0.09835	0.742497	1.813
TG(16:1_14:0_18:2)	0.388162	3.105299	0.319609	0.394006	0.8918
TG(16:1_16:1_18:1)	0.132673	1.06138	0.132116	0.66778	1.598
TG(16:1_16:1_18:3)	0.203973	1.631786	0.188058	0.570825	1.333
TG(16:1_18:2_18:2)	0.100647	0.805175	0.105208	0.723413	1.759
TG(16:1_18:2_18:3)	0.129293	1.03434	0.129501	0.673177	1.614
TG(17:0_18:1_20:4)	0.511647	4.093173	0.403379	0.301385	0.6735
TG(18:0_16:0_16:0)	0.631993	5.055947	0.471641	0.220761	0.4892

TG(18:0_16:0_18:1)	0.422514	3.380114	0.345694	0.366891	0.8288
TG(18:0_18:1_18:1)	0.155115	1.240919	0.147999	0.634132	1.504
TG(18:1_12:3_18:1)	0.463409	3.707275	0.374781	0.336065	0.7549
TG(18:1_18:1_18:1)	0.147271	1.178172	0.143723	0.645491	1.535
TG(18:1_18:1_18:2)	0.298678	2.389423	0.255697	0.471589	1.079
TG(18:1_18:1_20:4)	0.079671	0.63737	0.088181	0.767072	1.888
TG(18:1_18:1_20:5)	0.554047	4.432375	0.427048	0.272138	0.6072
TG(18:1_18:2_18:2)	0.1722	1.377603	0.16235	0.610663	1.438
TG(18:2_18:2_18:2)	0.208487	1.667895	0.190386	0.565502	1.318
TG(18:3_18:2_18:2)	0.123792	0.990335	0.125752	0.682174	1.637
TG(20:3_18:2_18:2)	0.776109	6.208875	0.54514	0.131454	0.2946
TG(50:4)	0.161716	1.293727	0.15409	0.624869	1.476
TG(52:12e)	0.001057	0.008454	0.002436	1.25847	4.112
TG(54:8)	0.249402	1.995214	0.221006	0.520294	1.201
TG(55:4)	0.178663	1.429304	0.167042	0.602191	1.416
TG(56:9)	0.240109	1.920875	0.214467	0.530126	1.227

Table K. The univariate and multivariate result of identified lipids (post TRF vs CR)

lipid	t test	BF correction	q value (FDR correction)	VIP	fold change
7-oxocholesterol	0.832262	6.658094	0.407746	0.08559	0.2158
AcCa(16:0)	0.001455	0.011638	0.00154	1.07908	3.949
Cer(d16:1_22:0)	0.007527	0.060214	0.006295	0.95201	3.12
Cer(d18:0/16:0)	0.938059	7.504476	0.451162	0.031425	0.07912
Cer(d18:0/18:0)	0.303553	2.428422	0.166764	0.408043	1.068
Cer(d18:0/20:0)	0.014219	0.113752	0.010982	0.890363	2.799
Cer(d18:0/26:0)	0.009666	0.077329	0.007692	0.92875	2.994
Cer(d18:0_22:0)	0.003662	0.029299	0.003536	1.01273	3.482
Cer(d18:0_24:0)	0.003422	0.027373	0.003328	1.01803	3.516
Cer(d18:0_24:1)	0.002231	0.017846	0.002236	1.0498	3.736
Cer(d18:1/19:0)	0.265535	2.124279	0.148361	0.440114	1.16
Cer(d18:1/25:0)	0.004337	0.034693	0.004096	0.99926	3.394
Cer(d18:1_16:0)	0.005425	0.043401	0.004913	0.9807	3.285
Cer(d18:1_18:0)	0.000664	0.005309	0.000871	1.12718	4.352
Cer(d18:1_22:0)	0.004383	0.035064	0.004096	0.998398	3.39
Cer(d18:1_23:0)	0.002043	0.016348	0.00212	1.056	3.775
Cer(d18:1_24:1)	0.000799	0.006388	0.001009	1.11645	4.256
Cer(d18:1_26:0)	0.000949	0.007596	0.001133	1.10607	4.167
Cer(d18:1_26:1)	0.001183	0.009462	0.001339	1.09243	4.054
Cer(d18:2/20:0)	0.001407	0.011259	0.001515	1.08125	3.966
Cer(d18:2/24:2)	0.000316	0.00253	0.00044	1.16682	4.748
Cer(d18:2_22:0)	0.005938	0.047507	0.005288	0.972969	3.234

Cer(d18:2_23:0)	0.003749	0.029993	0.003593	1.01089	3.47
Cer(d18:2_24:1)	0.001009	0.008075	0.001169	1.10233	4.147
ChE(14:0)	0.620205	4.961641	0.313204	0.199513	0.5068
ChE(15:0)	0.001289	0.010311	0.001422	1.08695	4.011
ChE(16:0)	6.71E-05	0.000537	0.000121	1.23506	5.593
ChE(16:1)	0.006181	0.04945	0.005375	0.969495	3.219
ChE(17:0)	0.001083	0.008665	0.001248	1.09796	4.1
ChE(17:1)	0.018901	0.151209	0.014345	0.859911	2.653
ChE(18:0)	0.00038	0.003042	0.00052	1.15745	4.643
ChE(18:1)	9.84E-06	7.87E-05	0.000024	1.29861	6.725
ChE(18:3)	0.000149	0.001188	0.000238	1.20232	5.146
ChE(20:1)	0.00454	0.036317	0.004198	0.995535	3.374
ChE(20:3)	2.46E-05	0.000197	0.00005	1.2707	6.166
ChE(20:4)	0.000192	0.001533	0.000289	1.19085	5.014
ChE(20:5)	0.123013	0.984103	0.07495	0.59713	1.643
ChE(22:6)	0.00605	0.048398	0.005288	0.971362	3.235
CoQ10	2.55E-06	2.04E-05	0.000009	1.33299	7.586
CoQ9	0.001419	0.011355	0.001515	1.08069	3.961
DG(30:2)	0.636472	5.091778	0.320187	0.190363	0.4831
DG(38:0)	0.000291	0.002328	0.00042	1.17097	4.784
DG(38:3)	0.122668	0.981346	0.07495	0.597633	1.643
DG(40:7)	0.565843	4.526745	0.290216	0.230807	0.5881
DG(14:0_18:2)	0.64457	5.15656	0.323023	0.18584	0.4715
DG(14:0_18:3)	0.602813	4.822505	0.306781	0.209399	0.5324
DG(16:0_14:0)	0.715547	5.724376	0.355876	0.147022	0.3719
DG(16:0_16:0)	0.466333	3.730666	0.243942	0.291733	0.7489
DG(16:0_16:1)	0.146506	1.172051	0.087837	0.564936	1.537
DG(16:0_18:1)	0.104941	0.839528	0.066033	0.624934	1.733
DG(16:0_18:2)	0.001292	0.010332	0.001436	1.08682	4.001
DG(16:0_18:3)	0.322455	2.579644	0.17641	0.392983	1.026
DG(16:1_14:0)	0.818655	6.549242	0.402582	0.092638	0.2336
DG(16:1_18:3)	0.367495	2.939963	0.196147	0.359032	0.9313
DG(18:0_16:0)	0.724977	5.799819	0.359206	0.141962	0.359
DG(18:0_18:0)	0.857159	6.857273	0.415295	0.072753	0.1833
DG(18:0_18:1)	0.121041	0.96833	0.074644	0.600019	1.65
DG(18:1_18:1)	0.126719	1.013753	0.076634	0.591786	1.624
DG(18:1_20:3)	0.26303	2.104239	0.147621	0.442322	1.166
DG(18:1_22:5)	0.179412	1.435298	0.105452	0.525273	1.413
DG(18:3_18:2)	0.837908	6.703264	0.408986	0.082673	0.2084
DG(36:3)	0.165927	1.327417	0.098483	0.540885	1.46
DG(36:4)	0.40499	3.239918	0.214914	0.332496	0.8538
DG(38:5)	0.012461	0.099691	0.009739	0.903846	2.866
DG(38:6)	0.514461	4.115692	0.26594	0.261587	0.6689
Eicosanoids_19,20-DiHDPA	0.396949	3.175594	0.21101	0.338071	0.8738

Eicosanoids_PGD3	0.334282	2.674256	0.182121		1
FA(12:0)	0.084075	0.672596	0.053976	0.661615	1.869
FA(14:0)	0.298186	2.385486	0.163927	0.412418	1.083
FA(15:0)	0.50503	4.04024	0.263918	0.267385	0.6785
FA(16:0)	0.108926	0.871411	0.068517	0.61853	1.709
FA(16:1)	0.652929	5.223431	0.325657	0.181194	0.4604
FA(17:0)	0.285814	2.286511	0.157963	0.422687	1.111
FA(17:1)	0.568325	4.546596	0.291812	0.229352	0.5799
FA(18:0)	0.010949	0.087588	0.008107	0.916705	2.964
FA(18:1)	0.097313	0.778501	0.06243	0.637686	1.776
FA(18:2)	0.133829	1.070633	0.080443	0.581827	1.593
FA(18:3)	0.103441	0.827527	0.064448	0.627388	1.749
FA(18:4)	0.042427	0.339413	0.03015	0.761685	2.233
FA(20:2)	0.189727	1.517812	0.109584	0.513848	1.379
FA(20:3)	0.186041	1.48833	0.108021	0.517882	1.391
FA(20:4)	0.342444	2.739556	0.184627	0.377604	0.9872
FA(20:5)	0.064668	0.517347	0.043777	0.70218	2.006
FA(22:0)	0.541122	4.328975	0.278625	0.245454	0.6264
FA(22:1)	0.359838	2.878704	0.192844	0.364632	0.9467
FA(22:2)	0.160152	1.281213	0.095581	0.54783	1.483
FA(22:3)	0.166513	1.332102	0.098483	0.540189	1.459
FA(22:4)	0.198524	1.588192	0.113569	0.50442	1.351
FA(22:5)	0.064597	0.516774	0.043777	0.702345	2.005
FA(22:6)	0.079091	0.632729	0.052542	0.671324	1.893
FA(23:0)	0.054526	0.436208	0.037432	0.72705	2.102
FA(24:0)	0.973167	7.785333	0.466339	0.013604	0.03424
FA(24:1)	0.000623	0.004982	0.00082	1.13079	4.388
FA(26:0)	0.007333	0.058668	0.00615	0.954361	3.135
Hex2Cer(d12:0_20:4)	0.473961	3.791689	0.245615	0.286859	0.7402
LPC(16:0)	1.74E-06	1.39E-05	0.000007	1.34142	7.818
LPC(16:1)	4.47E-06	3.58E-05	0.000012	1.31956	7.213
LPC(18:0)	1.06E-05	8.48E-05	0.000026	1.2965	6.664
LPC(18:1)	1.18E-06	9.47E-06	0.000005	1.34951	8.114
LPC(18:2)	3.61E-06	2.89E-05	0.000012	1.32479	7.35
LPC(20:3)*1	4.07E-06	3.26E-05	0.000012	1.32188	7.274
LPC(20:3)*2	1.28E-05	0.000103	0.000029	1.29094	6.539
LPC(20:4)*1	2.95E-06	2.36E-05	0.00001	1.32958	7.484
LPC(20:4)*2	4.03E-06	3.23E-05	0.000012	1.32211	7.28
LPC(22:5)	4.35E-06	3.48E-05	0.000012	1.32023	7.231
LPC(22:6)	0.000836	0.006688	0.001029	1.11373	4.233
LPE(16:0)	4.29E-05	0.000344	0.000082	1.25165	5.84
LPE(18:0)*1	5.39E-07	4.31E-06	0.000003	1.36456	8.658
LPE(18:0)*2	3.23E-05	0.000259	0.000064	1.26158	6.004
LPE(18:1)	7.93E-07	6.35E-06	0.000004	1.3574	8.381

LPE(18:2)	1.29E-06	1.03E-05	0.000005	1.34782	8.043
LPE(20:4)	2.22E-07	1.77E-06	0.000002	1.37952	9.321
LPE(22:1)	0.005807	0.046459	0.005599	0.974889	3.189
LPE(22:6)	7.92E-05	0.000634	0.000139	1.22858	5.493
PC(14:0_18:2)	0.001413	0.011302	0.001515	1.08099	3.963
PC(14:0_20:4)	0.002046	0.016366	0.00212	1.05591	3.775
PC(14:0_20:5)	0.009155	0.073236	0.007374	0.93391	3.021
PC(16:0_16:1)	0.005984	0.04787	0.005288	0.972311	3.239
PC(16:0_18:1)	1.01E-05	8.04E-05	0.000024	1.29801	6.729
PC(16:0_20:3)	1.81E-05	0.000144	0.000037	1.2806	6.355
PC(16:0_20:5)	0.004045	0.032361	0.00385	1.00486	3.432
PC(16:0_22:5)	0.000468	0.00374	0.000639	1.14656	4.523
PC(16:0_22:6)	0.001214	0.009713	0.001358	1.09078	4.043
PC(18:0_16:0)	1.26E-06	1.01E-05	0.000005	1.3483	8.079
PC(18:0_18:1)	4.08E-06	3.26E-05	0.000012	1.32186	7.269
PC(18:0_18:2)	7.64E-07	6.12E-06	0.000004	1.35811	8.386
PC(18:0_20:2)	6.61E-07	5.29E-06	0.000003	1.36083	8.516
PC(18:0_20:3)	4.27E-05	0.000342	0.000081	1.25184	5.85
PC(18:0_20:4)	0.000206	0.001651	0.000314	1.18744	4.963
PC(18:0_22:4)	5.28E-07	4.23E-06	0.000003	1.36493	8.671
PC(18:0_22:5)	1.99E-06	1.59E-05	0.000007	1.33854	7.735
PC(18:0_22:6)	0.004908	0.039264	0.004483	0.989103	3.334
PC(18:1_13:0)	0.000839	0.006709	0.001029	1.11355	4.231
PC(18:1_22:6)	0.000744	0.005952	0.000963	1.12059	4.29
PC(18:2_13:0)	0.023058	0.184466	0.017136	0.837459	2.559
PC(18:2_18:2)	3.83E-06	3.06E-05	0.000012	1.3234	7.313
PC(18:2_22:6)	0.000227	0.001819	0.000343	1.18289	4.91
PC(22:0_18:2)	6.47E-05	0.000517	0.00012	1.23649	5.607
PC(30:1)	0.001167	0.009336	0.001332	1.09329	4.061
PC(31:0)	0.008357	0.066859	0.006779	0.942424	3.067
PC(32:0)	5.48E-05	0.000438	0.000105	1.24276	5.69
PC(33:0)	9.58E-05	0.000766	0.000161	1.22095	5.387
PC(33:1)	0.001048	0.00838	0.001244	1.10004	4.109
PC(33:2)	9.28E-05	0.000743	0.000158	1.22222	5.405
PC(33:3)	0.014155	0.113237	0.010982	0.890833	2.801
PC(33:4)	0.002745	0.021956	0.002709	1.03473	3.628
PC(34:1e)	4.2E-06	3.36E-05	0.000012	1.32114	7.286
PC(34:2)	0.000165	0.001324	0.000256	1.19752	5.093
PC(34:2e)*1	0.006544	0.052348	0.005599	0.96451	3.19
PC(34:2e)*2	2.75E-05	0.00022	0.000055	1.26705	6.1
PC(34:3)	0.000121	0.000966	0.000193	1.21126	5.275
PC(34:3e)	0.001999	0.015991	0.002092	1.05754	3.789
PC(35:1)	5.96E-06	4.77E-05	0.000016	1.31223	7.03
PC(35:2)	1.42E-05	0.000113	0.000031	1.28798	6.495

PC(35:3)	0.00108	0.008643	0.001244	1.09812	4.105
PC(35:4)	7.11E-05	0.000569	0.000127	1.2328	5.552
PC(35:5)*1	0.053545	0.428357	0.037198	0.729632	2.108
PC(35:5)*2	0.610628	4.885026	0.309558	0.204943	0.5208
PC(36:0)	1.63E-05	0.00013	0.000035	1.2838	6.41
PC(36:4e)	4.04E-07	3.23E-06	0.000002	1.36963	8.925
PC(36:6)	0.179904	1.439229	0.105452	0.524719	1.412
PC(37:1)	1.23E-06	9.88E-06	0.000005	1.34866	8.071
PC(37:4)	0.000467	0.003733	0.000634	1.14667	4.534
PC(37:5)	0.025562	0.204494	0.01875	0.825408	2.498
PC(37:6)	0.003027	0.024219	0.002954	1.02739	3.58
PC(38:4e)	2.18E-07	1.74E-06	0.000002	1.3798	9.302
PC(38:5)	5.99E-06	4.79E-05	0.000016	1.31211	7.013
PC(38:5e)	7.63E-07	6.11E-06	0.000004	1.35814	8.385
PC(38:6e)	7.05E-05	0.000564	0.000127	1.23317	5.555
PC(38:7)	0.047069	0.376553	0.033227	0.747619	2.177
PC(38:7e)	0.074331	0.59465	0.048699	0.681019	1.941
PC(39:6)	0.087351	0.698807	0.056561	0.655455	1.838
PC(40:7)	0.009346	0.074771	0.007471	0.93195	3.012
PC(44:5e)	1.12E-06	8.97E-06	0.000005	1.35061	8.129
PE(16:0_18:1)	0.000299	0.002395	0.000427	1.16956	4.769
PE(16:0_18:2)	0.005888	0.047105	0.00528	0.973701	3.245
PE(16:0_20:4)	0.000336	0.002691	0.000461	1.16371	4.712
PE(16:0_20:5)	0.006297	0.050379	0.00544	0.967871	3.21
PE(16:1e_22:6)	9.83E-06	7.87E-05	0.000024	1.29863	6.709
PE(18:0_18:1)	0.000759	0.006074	0.000968	1.11941	4.282
PE(18:0_18:2)	0.006132	0.049059	0.005375	0.970184	3.219
PE(18:0_20:3)	0.000836	0.00669	0.001029	1.11372	4.232
PE(18:0_20:4)	0.00028	0.002242	0.00041	1.17281	4.803
PE(18:0_22:5)	0.008331	0.066644	0.006779	0.942722	3.069
PE(18:0p_22:6)	8.36E-05	0.000669	0.000147	1.22643	5.454
PE(18:1_18:2)	0.002101	0.016806	0.002141	1.05405	3.762
PE(18:1_20:4)	0.000315	0.002518	0.000441	1.16707	4.741
PE(32:1)	0.05081	0.406483	0.035676	0.737018	2.136
PE(38:6)	0.000905	0.007243	0.001091	1.10896	4.196
PE(39:6)	0.001242	0.009934	0.001382	1.08934	4.03
PE(40:7)	0.002695	0.021559	0.002681	1.03608	3.637
PE(42:5)	0.000114	0.000912	0.000189	1.21371	5.291
PE(42:7)	1.18E-05	9.42E-05	0.000027	1.29346	6.606
PG(16:0_18:1)	0.002095	0.016759	0.002141	1.05425	3.764
PG(36:1)	0.00016	0.00128	0.000256	1.19903	5.1
PG(36:2)	4.11E-05	0.000329	0.000081	1.25318	5.858
PG(36:3)	8.03E-09	6.43E-08	<0.000001	1.42062	12.14
PG(36:5)	0.841356	6.73085	0.409148	0.080893	0.2039

PG(38:4)	7.95E-09	6.36E-08	<0.000001	1.42072	12.15
PG(38:6)	0.120436	0.96349	0.074591	0.600912	1.654
PG(40:6)	0.263087	2.104694	0.147621	0.442272	1.166
PG(40:8)	0.90308	7.224637	0.435935	0.049236	0.124
PI(16:0_16:0)	1.27E-05	0.000102	0.000029	1.29117	6.558
PI(16:0_16:1)	0.000178	0.001426	0.000275	1.19416	5.047
PI(16:0_20:4)	7.15E-07	5.72E-06	0.000004	1.35936	8.455
PI(16:0_22:6)	1.15E-05	9.21E-05	0.000027	1.29412	6.62
PI(18:0_18:2)	2.6E-09	2.08E-08	<0.000001	1.43056	13.2
PI(18:0_20:4)	1.1E-07	8.81E-07	<0.000001	1.38997	9.871
PI(18:0_22:5)	9.02E-09	7.21E-08	<0.000001	1.4195	12.03
PI(18:0_22:6)	4.36E-06	3.49E-05	0.000012	1.32018	7.229
PI(34:0)	0.219448	1.755586	0.124734	0.483034	1.286
PI(34:1)	1.21E-07	9.67E-07	0.000001	1.38864	9.763
PI(34:2)	2.27E-08	1.81E-07	<0.000001	1.40988	11.2
PI(36:0)	0.000936	0.00749	0.001114	1.10692	4.18
PI(36:1)	2.38E-07	1.9E-06	0.000002	1.37839	9.266
PI(36:3)	2.07E-08	1.66E-07	<0.000001	1.41086	11.27
PI(38:2)	0.000145	0.001162	0.000235	1.20331	5.158
PI(38:3)	2.4E-09	1.92E-08	<0.000001	1.43121	13.29
PI(38:5)	3.69E-08	2.95E-07	<0.000001	1.40423	10.77
PI(40:4)*1	0.007914	0.063314	0.006535	0.947439	3.095
PI(40:4)*2	3.21E-08	2.57E-07	<0.000001	1.4059	10.89
PS(34:0)	1.05E-12	8.43E-12	<0.000001	1.46837	23.72
PS(34:2)	5.42E-08	4.33E-07	<0.000001	1.39948	10.45
PS(36:0)	0.007726	0.061809	0.006486	0.949636	3.102
PS(36:2)	0.023481	0.187849	0.017618	0.835355	2.542
PS(38:4)	3.96E-09	3.17E-08	<0.000001	1.42706	12.83
PS(40:4)	0.014334	0.114672	0.010998	0.889527	2.795
PS(42:3)	0.000661	0.00529	0.000889	1.12739	4.336
SM(d32:2)	2.62E-06	2.1E-05	0.000009	1.33234	7.562
SM(d35:1)	1.11E-07	8.9E-07	<0.000001	1.38982	9.896
SM(d37:1)	8.27E-08	6.62E-07	<0.000001	1.39392	10.1
SM(d39:1)	1.69E-06	1.35E-05	0.000006	1.34205	7.857
SM(d41:7)	1.38E-07	1.11E-06	0.000001	1.38669	9.688
SM(d43:2)	1.78E-05	0.000143	0.000037	1.28099	6.355
SM(d44:1)	0.401772	3.214177	0.212712	0.334719	0.8647
SM(t40:7)	0.349465	2.795723	0.188052	0.372324	0.968
SPH(d18:0)	0.34582	2.766563	0.186857	0.375057	0.9756
SPH(d18:1)	0.024579	0.196634	0.018233	0.830024	2.518
Sphinganine 1-phosphate (d18:0-P)	0.081676	0.653406	0.053693	0.666235	1.876
Sphingosine 1-phosphate (d18:1-P)	0.281512	2.252096	0.156621	0.42632	1.12

TG(16:0_14:0_18:1)	0.126625	1.012997	0.076634	0.591921	1.624
TG(16:0_14:4_16:0)	0.240026	1.920207	0.135897	0.463236	1.227
TG(16:0_16:0_16:0)	0.081888	0.655106	0.053693	0.665822	1.875
TG(16:0_16:0_18:1)	0.008462	0.0677	0.006779	0.941266	3.067
TG(16:0_16:1_16:1)	0.059476	0.475806	0.040646	0.714522	2.051
TG(16:0_16:1_18:1)	0.035376	0.283008	0.025723	0.785446	2.327
TG(16:0_18:1_18:1)	0.002292	0.018333	0.002321	1.04787	3.713
TG(16:0_18:1_18:2)	0.004269	0.034151	0.003993	1.00054	3.41
TG(16:0_18:2_18:2)	0.007287	0.058296	0.00615	0.954932	3.136
TG(16:0_18:2_20:4)	0.00464	0.037122	0.004276	0.993738	3.361
TG(16:1_14:0_18:2)	0.054707	0.437657	0.037517	0.726577	2.098
TG(16:1_16:1_18:1)	0.015027	0.120213	0.011502	0.884602	2.769
TG(16:1_16:1_18:3)	0.070583	0.564661	0.047557	0.688976	1.957
TG(16:1_18:2_18:2)	0.02998	0.239837	0.021932	0.806188	2.414
TG(16:1_18:2_18:3)	0.078552	0.628416	0.052456	0.6724	1.897
TG(17:0_18:1_20:4)	0.097361	0.778892	0.06243	0.637602	1.774
TG(18:0_16:0_16:0)	0.098529	0.78823	0.06243	0.635607	1.773
TG(18:0_16:0_18:1)	0.040887	0.327093	0.029354	0.766606	2.252
TG(18:0_18:1_18:1)	0.011324	0.090591	0.008987	0.91339	2.909
TG(18:1_12:3_18:1)	0.731541	5.852331	0.360907	0.138451	0.3505
TG(18:1_18:1_18:1)	0.037481	0.299846	0.027088	0.778005	2.297
TG(18:1_18:1_18:2)	0.113021	0.90417	0.070127	0.612119	1.692
TG(18:1_18:1_20:4)	0.02434	0.19472	0.018233	0.83117	2.521
TG(18:1_18:1_20:5)	0.460386	3.683089	0.241722	0.295561	0.7593
TG(18:1_18:2_18:2)	0.053174	0.425389	0.037119	0.730618	2.112
TG(18:2_18:2_18:2)	0.083291	0.666329	0.054192	0.663113	1.864
TG(18:3_18:2_18:2)	0.099152	0.793214	0.063262	0.634548	1.763
TG(20:3_18:2_18:2)	0.181451	1.451605	0.106815	0.522981	1.401
TG(50:4)	0.042376	0.339008	0.03015	0.761845	2.232
TG(52:12e)	0.000249	0.001994	0.000368	1.17849	4.867
TG(54:8)	0.201072	1.608573	0.114811	0.501741	1.341
TG(55:4)	0.024981	0.19985	0.018426	0.828119	2.51
TG(56:9)	0.196823	1.574585	0.113569	0.506221	1.352