Characterization of the metabolic changes in chicken liver due to exposure of perfluorooctane sulfonate (PFOS) during the embryo development

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by

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Abstract

Perfluoroalkyl substances (PFASs) are anthropogenic compounds that have been classed as persistent organic pollutants (POPs) and are found in both commercial and industrial products. PFASs have been detected in different environmental matrices and have been found to bioaccumulate in all trophic levels. The adverse effects that are associated with PFAS exposure include reduced body weight, increased liver weight, hepatocellular hypertrophy, a decrease in serum cholesterol and triglycerides. This project aims to characterize the metabolic changes in lipid metabolism in the liver after exposure to one of the well-studied PFASs, the perfluorooctane sulfonate (PFOS), during the embryo development using the domestic chicken as a model organism.

The characterization of the metabolic changes was done by conducting both quantitative lipidomic analysis and semi-quantitative global profiling on extracted lipids from liver homogenates from a former related project looking at fatty acid profiles. The extracted lipids were analyzed using UHPLC/Q-TOF-MS. In the quantitative analysis, the PFOS-treated groups (0.1 ug/g and 1.0 ug/g) exhibited higher lipid concentrations when compared with the solvent control group (5% DMSO) and the untreated group leading to the conclusion that PFOS exposure disrupts the lipid metabolism. When comparing the lipid concentrations between the two PFOS-treated groups (0.1 ug/g and 1.0 ug/g), the majority of the lipids exhibited higher lipid concentrations in the 1.0 ug/g PFOS-treated groups leading to the conclusion that the effect PFOS has on the lipid metabolism is dose dependent. In the global profiling analysis, 63 lipids showed significant differences when comparing the solvent control group with samples either treated with 0.1 ug/g PFOS or 1.0 ug/g PFOS.

KEYWORDS | PFCs, PFOS, Lipidomics, Lipid metabolism, UHPLC/Q-TOF-MS

List of abbreviations

PFASs - Perfluoroalkyl substances
POPs - Persistent organic pollutants
PFAAs - Perfluorinated alkyl acids
PFOS - Perfluorooctane sulfonate
PPARα - Peroxisome proliferator-activated receptor alpha
FA - Fatty acids
GL - Glycerolipids
GP - Glycerophospholipids
SP - Sphingolipids
ST - Sterol lipids
PR - Prenol lipids
SL - Saccharolipids
PK - Polyketides
MeOH - Methanol
CH_3Cl - Chloroform
MTBE - Methyl tert-butyl ether
NMR - Nuclear magnetic resonance
MS - Mass spectrometry
GC-MS - Gas chromatography-mass spectrometry
CE-MS - Capillary electrophoresis-mass spectrometry
LC-MS - Liquid chromatography-mass spectrometry
TLC - Thin liquid chromatography
ESI - Electrospray ionisation
CE - Cholesteryl ester
PC - Phosphatidylcholine
LysoPC - Lysophosphatidylcholine
TGs - Triglycerides
Cers - Ceramides
PE - Phosphatidylethanolamine
LysoPE - Lysophosphatidylethanolamine
SMs - Sphingomyelins
DGs - Diglycerides
HPLC - High Performance Liquid Chromatography
IPA - 2-propanol
THF - Tetrahydrofuran
ACN - Acetonitrile
H_2O - Water
UHPLC - Ultra-High Performance Liquid Chromatography
UHPLC/Q-TOF-MS - Ultra-High Performance Liquid Chromatography/Quadruple Time-of-Flight-Mass Spectrometry
RSD - Relative standard deviation
FDR - False discovery rates

1. Introduction

1.1 | Background

Perfluoroalkyl substances

Perfluoroalkyl substances (PFASs) are anthropogenic compounds that consist of a completely fluorine-saturated carbon chain (C_4–C_{14}) with different charged moieties (e.g. carboxylate, sulfonate, or phosphonate) [1, 2]. The chemical stability of perfluoroalkyl substances is the result of the strength of the carbon-fluorine bond, which leads to these compounds having a resistance to chemical and biological changes under environmental conditions, e.g. hydrolysis, photolysis, biodegradation, and metabolism. The stable chemical structure makes these compounds persistent and some of them have the potential to bioaccumulate in organisms and induce long-term health effects [1-3, 5-7].

PFASs are special in the sense that these compounds are both lipophilic and hydrophilic in nature due to their chemical structure being both partly lipophilic and partly hydrophilic [1]. As a result, PFASs have been used in both industrial and commercial products, e.g. surfactants, fire-fighting foams, emulsifiers, pharmaceuticals, and pesticides [2, 6].

Perfluorinated alkyl acids (PFAAs) are a subgroup of PFASs and classified as persistent organic pollutants (POPs). These compounds have been found in different environmental matrices (e.g. freshwater lakes, fish, birds, mammals) including humans. Perfluorooctane sulfonate (PFOS), an eight-carbon long compound with a terminal sulfonate moiety, is a PFAA and the end-stage metabolite which has also been found in all trophic levels. It has been shown that PFOS bioaccumulates in higher trophic levels of the food chain [1, 3, 4, 8, 9].

Perfluoroalkyl substances have been manufactured and used for over half a century, however due to their potential health effects and observed persistence in both humans and wildlife; PFOS were phased out in 2002 by most countries except for China which still manufactures and uses products containing PFOS. In 2009 at the Stockholm Convention, PFOS was listed as one of the nine new POPs under Annex B of the Stockholm Convention [2, 6].

The adverse effects of PFOS exposure has mainly been studied in rats and to some extent in chickens as well. The effects reported in the studies using the domestic chicken as a model have been consistent with the studies using the rat as a toxicological model. Organisms exposed to PFOS have shown adverse effects such as reduced body weight, hepatocellular hypertrophy, a decrease in serum cholesterol and triglycerides, increased liver weight, peroxisome proliferations, increase in ß-oxidation of fatty acids, several cytochrome P-450 mediated reactions, inhibition of the secretion of low-density lipoproteins, cholesterol from the liver and increased activity of lipid and xenobiotic metabolizing enzymes [2-4, 8, 10, 11]. Studies have indicated that PFOS and other PFAAs are peroxisome proliferator-activated receptor alpha (PPARα)-agonists and their role as a PPARα-agonist is believed to be the cause of the hepatotoxicity, as the structure of PFOS is similar to those of fatty acids leading to the possibility that PFOS binds to PPARα and disturbs its regulatory activity [1, 2].

PPARα is one of the major regulatory nuclear receptors whose role is to regulate the expression of the genes involved in fatty acid and cholesterol transportation and metabolism in the cell as well as DNA replication and cellular proliferation [1]. The PPARα is expressed in organs such as the brain, lungs, kidney, heart, intestine, adipose tissue and liver [12].

As previously stated PFASs have the ability to bioaccumulate in the organism, different PFASs bioaccumulate in different body compartments. PFOS accumulates more in liver tissues while PFOA accumulate more in blood and kidney tissue. Chicken, which is used as an avian model in toxicological studies, has a tendency to accumulate higher concentration of PFOS in both blood plasma and liver tissue compared to other farm animals (e.g., cattle, pigs) [10, 11, 13].

Lipids

Lipids are an heterogenous group of hydrophobic biomolecules that possess several biological functions in terms of being the structural components of cell membranes; they are also involved in energy storage in adipose tissue and muscle tissue, transport in and out of the cell, cell communication and signaling in the cell to maintain homeostasis [14-16]. They also play a large role in the body's metabolism and disturbances in the lipid metabolism, which may arise due to any type of disturbances of the lipid metabolism that has been related to the pathogenesis of many severe diseases such as obesity, diabetes, atherosclerosis, and cancer-related diseases [14, 15, 17].

Lipids are a heterogenous group of biomolecules; they can be classified into eight different categories based on the their polarity and backbone structure. The eight categories are (1) fatty acids (FA), (2) glycerolipids (GL), (3) glycerophospholipids (GP), (4) sphingolipids (SP), (5) sterol lipids (ST), (6) prenol lipids (PR), (7) saccharolipids (SL), and (8) polyketides (PK) [16, 17].

1.2 | Metabolomics and lipidomics

Metabolomics

Metabolomics, also referred to as metabonomics, is a quickly developing scientific discipline that is defined as “the comprehensive quantitative and qualitative analysis of all metabolites in cell, tissue, or biofluids following a genetic modification or physiological stimulus” [18]. This scientific discipline has allowed scientists to obtain metabolic fingerprints that can be used to determine the metabolic changes caused by exposure to a xenobiotic chemical as well as to quantify the amount of the xenobiotic compound and its resulting metabolites [19]. Metabolomics is not limited to only one scientific research field but employs a multidisciplinary research techniques from different scientific fields which includes advanced analytical chemistry and statistical analysis, biochemistry, medicine/life sciences, nutritional, agricultural or environmental sciences [20].

The major application areas for metabolomics are in biomarker discovery in medical and life sciences, plant/food and environmental sciences [20, 21]. The analytical strategies for metabolomics research aims to characterize the whole metabolite profile of the samples under study, and then relate their concentration to features or properties of the sample [20].

Lipidomics

Lipidomics focuses on the analysis of the overall lipid composition/metabolism of a system. It can be defined as a branch of metabolomics in the sense that it is fully dedicated to fully characterizing the lipid species within the cells, tissues, and biofluids as well as their biological role

and regulation of lipid metabolism [15, 22]. This field of study is, however, limited in its development by two parts, (1) the unclear definition of and diversity of lipid species and (2) inadequate analytical technology; despite these limitations lipidomics has dramatically gained importance in many fields of bioscience [14, 22].

Lipidomics can also be used in biomarker discovery for different diseases as well as for identification of lipid-related pathways which are altered in various physiological conditions [23]. Due to these applications, lipidomics is not only an emerging discipline in medicine but also in fundamental research on model organisms [14].

There are two major analytical approaches for analysis samples in lipidomics — targeted and non-targeted analyses. In the targeted approach, a number of predetermined lipid species are analyzed and the signals obtained are used to establish the relative abundance of the expected endogenous lipids in the sample. In the non-targeted approach, a global analysis of all measurable lipids present in a sample is preformed. The non-targeted approach has some advantages over the targeted approach, one of these advantages is that novel and interesting molecules can be discovered and the only limitations imposed on this approach are the sample preparation and analytical techniques used [15].

1.3 | Lipid extraction techniques

In a lipidomic analysis, the most common extraction methods are the two traditionally used Folch et al. method and Bligh and Dyer method and the relatively new Matyash et al. method [15, 24]. These extraction methods for lipid extraction are based on liquid-liquid extraction principle where two immiscible phases separate due to their density resulting in an upper and lower phase [16]. Both Folch et al. and Bligh and Dyer methods are chloroform-based extraction methods where Folch et al. method uses a chloroform:methanol ratio of 2:1 whilst Bligh and Dyer method uses a chloroform:methanol ratio of 1:2 [25].

The Matyash et al. extraction method uses methyl-*tert*-butyl ether (MTBE) in place of chloroform, the use of MTBE provides numerous advantages over the traditional chloroform/MeOH extraction methods as it provides a more improved efficiency, higher throughput and safety in terms of reducing hazardous chemical use as chloroform has known carcinogenic effects that involve a considerable health risk for laboratory personnel plus the decomposition of chloroform yields phosgene and hydrochloric acid which inflicts chemical modification of labile lipid species [24, 26]. Chen et al. demonstrated that using more water in the Matyash et al. extraction method provides an aqueous phase of a typical 80% extraction [24].

Another advantage with using MTBE instead of chloroform is that the density of MTBE is lower than the water/MeOH phase which leads to the upper phase (organic phase) lays on top of the water/MeOH phase (aqueous phase) and proteins accumulates at the bottom of the extraction tube enabling easier and cleaner access to the upper non-polar (organic) fraction and lower polar (aqueous) fraction [24, 27]. The organic phase being the upper phase minimizes pipette contamination and leads to easier integration with robotics and automation [24].

However, combining extraction with chloroform-based mixtures with robotic sample preparation units is challenging as the extractant is located at the bottom of the extraction tube in the chloroform phase. The chloroform phase being on the bottom leads to possible contamination of

the needle during the collection of the extract, another major concern is the handling of lipid extract in low volumes of volatile chloroform/MeOH in sample evaporation [23].

A crucial part in every extraction method is the ratio of the solvents used and in order to ensure good separation of the phases, the ratio of solvents must be optimal [2]. In addition to the solvent ratio, the ratio of solvent to sample is also important as it should be 25 % w/v sample/ solvent to allow satisfactory recovery of lipids [23].

1.4 | Analytical techniques

In metabolomics, the usual analytical techniques used are nuclear magnetic resonance spectroscopy (NMR), mass spectrometry in combination with gas chromatography (GC-MS), capillary electrophoresis (CE-MS), and especially liquid chromatography-mass spectrometry (LC-MS) [21]. LC-MS provides both good separation capacity and sensitivity towards most compounds and is one of the most frequently used analytical platforms. Separation using a reversed-phase column is the routine analysis used for metabolomic analysis due to its robust operation and usability [19-20].

The complexity of lipidomics is the result of the lipids complexity and due to this there are numerous analytical approaches that can be taken to perform a lipidomic analysis [14]. These analytical techniques consists of thin-layer chromatography (TLC), gas chromatography (GC), and liquid chromatography (LC), both GC and LC are usually coupled to a mass spectrometer as it provides good sensitivity, specificity and dynamic range [17, 28].

In order to obtain maximal information from the sample, both metabolomics and lipidomics uses LC-MS as it has been proven to be a sensitive instrument that can be used to characterize, identify, and quantify a large number of compounds in a biological sample [17]. LC-MS also has several advantages over direct infusion techniques, such as more reliable identification of individual lipid species, even at trace levels, separation of isomers and isobars, or reduced ion-suppression effects. They also permit more effective separation, and reduce analysis time and solvent consumption [29].

For most non-targeted lipidomic analysis, the separation is performed on a reversed-phase column using high-resolution MS with an ESI interface [23]. The two most common columns used for essentially all reversed-phase LC-MS method are C_8 or C_{18} columns. For the gradient elution the temperature are usually set to 40-55°C at a flow-rate of 1.0-0.5 mL/min. The choice of mobile phase modifier is a crucial choice as it is important for achieving both a good separation and MS detection. The most commonly used modifiers are ammonium acetate or ammonium formate at a concentration of 5-10 mM and formic acid or acetic acid at a percentage of 0.05-1.0 % [3].

Due to the diverse nature of lipids there are two important parameters to take into consideration are elevated temperature and solvent strength. In terms of elevated temperature, this parameter enhances the elution of strongly retained lipids such as TGs and CEs and in terms of using strong enough solvents ensures that all analytes are eluted from the column, in order to avoid memory effects and contamination. In reversed-phase HPLC, the commonly used solvents are 2-propanol (IPA) or tetrahydrofuran (THF) used together with acetonitrile (ACN) or methanol (MeOH). When using either only acetonitrile or methanol, which is possible in principle, it requires that elevated temperatures and/or very short columns are used [23].

Something typical for UHPLC analyses are the use of short columns with a length of 5-10 cm and an inner diameter of 1-2.1 mm which allows for separation in less than 15-20 min. Shortening the column length from 10 cm to 5 cm does not reduce the analysis time substantially but it does reduce the resolution. Ionization is different for different lipid classes; for more polar lipids ESI is the best method for ionization and for neutral and non-polar lipids other methods such as atmospheric pressure chemical ionization and atmospheric pressure photoionisation can also be applied [23]

For lipidomics, the most common detection method used is mass spectrometry (MS) due to its high sensitivity and specificity. The goal when using MS is to capture multiple structural and functional classes of lipids with minimal bias for a specific lipid class or species in a single analysis [23].

In addition to actual chemical analysis the crucial aspects in the analytical workflow include also data processing, data mining, and identification. Different software programs can be utilized to perform different functions that make up the three important aspects in the analytical workflow. These functions consists of peak detection, filtering, alignment, normalization, gap filling etc. Different functions focus on different aspects, peak detection and filtering focus on detecting real chromatographic peaks in each data file while peak alignment focuses on locating and listing detected peaks found in the sample files [23]. One common software program that can be used for identifying metabolites is MZMine®, which provides the different function listed above and many more [17].

1.5 | Objective

The main objective of this project is to characterize how the lipid metabolism in the liver changes when exposed to perfluorooctane sulfonate (PFOS) during embryo development using the domestic chicken (*Gallus gallus domesticus*) as a model.

2. Method and materials

2.1 | Chemicals, internal standards and sample preparation

For this project only liquid chromatography grade solvents used, these solvents were either from Honeywell (Morris Plains, NJ, USA), Fisher Scientific (Waltham, MA, USA) or Sigma-Aldrich (St. Louis, MS, USA) solvents. Mass spectrometry grade ammonium acetate (Sigma-Aldrich, St. Louis, MO, USA) and reagent grade formic acid (Sigma-Aldrich, St. Louis, MO, USA) were used as mobile phase modifiers. The lipid standards used for both internal standards and calibration standards were from Avanti Polar Lipids Inc. (Alabaster, AL, USA).

The internal lipid standards used were triglyceride TG(16:0/16:0/16:0-^{13}C3), lysophosphatidylcholine LysoPC(17:0), sphingomyelin SM(d18:1/17:0), ceramide Cer(d18:1/17:0), phosphatidylcholine PC(16:0/d31-18:1), triglyceride TG(17:0/17:0/17:0) and phosphatidylcholine PC(17:0) and the calibration lipid standards used were lysophosphatidylcholine LysoPC(18:0), cholesteryl ester CE(18:1(9Z)), triglyceride TG(16:0/16:0/16:0), phosphatidylcholine PC(16:0), triglyceride TG(18:0/18:0/18:0), cholesteryl ester CE(18:0), lysophosphatidylcholine LysoPC(18:1), lysophosphatidylethanolamine LysoPE(18:1), phosphatidylcholine PC(16:0/18:1), ceramide Cer(d18:1)/18:1(9Z)), phosphatidylcholine PC(18:0/18:0), phosphatidylethanolamine PE(16:0/18:1), cholesteryl ester CE(18:2(9Z, 12Z)), and cholesteryl ester CE(16:0).

For the internal lipid standard mix, the concentration was 7000 ng/mL and for the calibration lipid standards the concentrations were 0, 100, 500, 1000, 10000 ng/mL. The solvents used preparing the internal standard mixes and calibration standard mixes were CH_3Cl:MeOH (2:1, v/v).

As this project was a continuation on a previous bachelor thesis project titled "Metabolomic study of the effects of perfluorinated compounds on the fatty acid metabolism during the development of *Gallus gallus domesticus*", no liver homogenization was required as this had already been done in the previous project [31]. The chicken embryo were treated in four different ways; the first group was untreated (LS 1-4); the second group was treated with 5% DMSO as a solvent control (LS 5-8); the third group was treated with 0.1 ug/g PFOS in 5% DMSO (LS 9-12); and lastly the fourth group was treated with 1.0 ug/g PFOS in 5% DMSO (LS 13-16).

2.2 | Method testing/comparison

As the non-targeted lipidomics method, provided by the supervisor, used for this project was already validated, only an external calibration curve using with internal lipid standards was done with the use of the calibration lipid standards. A comparison between the Folch et al. method and the Chen et al. method (a modified Matyash et al.) was done and from this comparison of lipid extraction method the choice of extraction method for the liver homogenate samples was chosen. As the Folch et al. method required the chloroform to be evaporated before the samples were reconstituted in an identical solvent volume of 2:1 (v/v) CH_3Cl:MeOH, the Chen et al. method was chosen as the extraction method as it did not require any evaporation step leading to shorter laboration time and minimizing the amount of solvent used.

2.3 | Lipid extraction

16 liver homogenates (four untreated samples, four solvent control samples, four 0.1 ug/g PFOS-treated samples, and four 1.0 ug/g PFOS-treated samples) obtained from the previous bachelor thesis project was used in this study. For the liquid-liquid MTBE extraction, the extraction method used in this project was a modified Matyash et al. extraction protocol conceived by Chen et al. [27]. Every extraction was performed in triplicates for the evaluation of the precision and accuracy of the extraction method and the extraction was done over a week.

10 uL of each liver homogenate was transferred to new 15 mL plastic tubes. To each sample both 400 uL of ice-cold 75% MeOH and 200 uL 7 ppm internal standard mix was added. The sample were then bath sonicated using a Bandelin Sonorex Digitec (Berlin, Germany) for 10 minutes in order to break the cells. After this 1 mL MTBE was added and the samples were shaken for 1 hour to allow for optimal contact between the extraction solvent and liver homogenate.

After the agitation step, 250 uL HPLC-grade water was added to induce phase separation and the mixture let stand for 10 minutes. After the phase separation step was concluded, the samples were then centrifuged using a Sigma 3-16L Centrifuge (SciQuip, Newton, Shropshire, UK) for 15 minutes at 8,000 x g at room temperature. The two phases were transferred separately to new Eppendorf tubes before one aliquot of 200 uL was taken and transferred to an LC vial and stored at -20°C until analysis.

For the method blank, the same extraction procedure used for the samples was followed with the only difference being no sample was added to the method blank.

2.4 | Quality control and sample pooling

All samples were spiked with an internal standard mix to account for any variation such as recovery loss and any matrix effect during the analytical analysis.

After the aliquots for analysis were stored, the remaining aliquots of the sample's triplicates were pooled into one sample. From the pooled samples, three aliquots of 200 uL were analyzed. A workflow of this is shown in Figure 1. The sample pooling in this project was done to check if the analytical instrument gave reliable data.

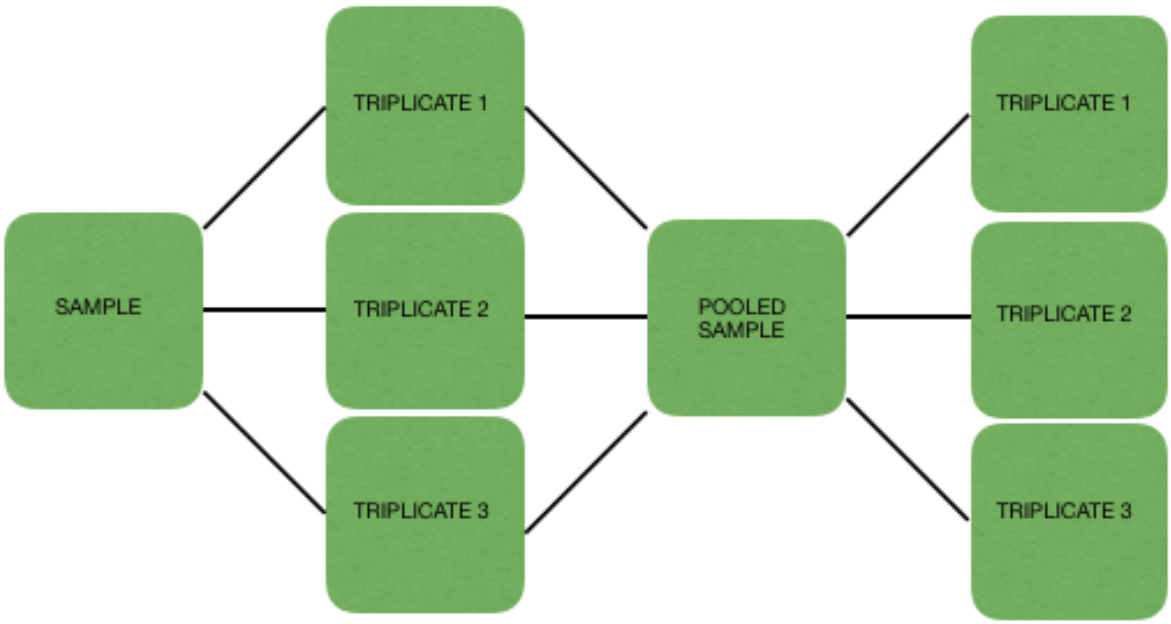

Figure 1 | Workflow over the sample pooling.

2.5 | Lipidomic analysis by UHPLC/Q-TOF-MS

The chromatographic separation was performed on a 1290 Infinity UHPLC system from Agilent Technologies (Santa Clara, CA, USA) coupled to a mass spectrometer, 6545 quadrupole time-of-flight (Q-TOF) from Agilent Technologies (Santa Clara, CA, USA) interfaced with a dual jet stream electrospray (dual ESI) ion source. The chromatographic separation was done on a reversed phase ACQUITY UPLC® BEH C18 column (2.1 mm x 100 mm, particle size 1.7 µm) by Waters (Milford, MA, USA). The mobile phases, flow rate and elution gradient can be found in Table 1. The mass spectrometric detection parameters can be found in Table 2.

For the analysis, the samples were analyzed in a randomized order to minimize any bias during analytical analysis. A calibration curve of 0-10 000 ng/mL was done before analysis. Every sixth sample in the sample run was a solvent blank (ACN) and this was done to ensure that any retained lipids on the column was flushed out. All data obtained from the lipidomic analysis was processed using MzMine 2.21.

Tables 1 and 2 show the conditions used in UHPLC and MS respectively.

UHPLC Conditions	
Mobile Phase A	H_2O with 10 mM ammonium acetate and 1.0% formic acid
Mobile Phase B	ACN/IPA (1:1) with 10 mM ammonium acetate and 1.0 % formic acid
Flow rate	0.4 mL/min
Elution gradient	1. At 0 min 65 % A and 35 % B 2. At 2.0 min 20 % A and 80 % B 3. At 7.0 min-21.50 0 % A and 100 % B 4. At 21.50 back to 65 % A and 35 % B

MS Conditions	
Ionization mode (ESI)	Positive
Capillary voltage (V)	3643
Nozzle voltage (V)	1500
Nebuliser gas (N_2) (psi)	21
Drying gas (N_2) flow rate (L/min)	10
Temperature (°C)	193

3. Results

In this project, a combination of quantitative analysis of selected target lipids with semiquantitative profiling of the whole lipidome in the analysis of liver samples obtained from chicken embryos after treatment with PFOS were performed.

3.1 | Quantitative analysis

For the quantitative analysis, all calibration curves had an r^2-value of 0.998 or above, the calibration curves are shown in Appendix 1. During the data processing, not all calibration and internal lipid standards used in the calibration and internal standard solutions were detected. The detected internal lipid standard were LysoPC(17:0), SM(d18:1/17:0), Cer(d18:1/17:0), TG(17:0/17:0/17:0), and PC(17:0) and the detected calibration lipid standards were LysoPC(18:0), TG(16:0/16:0/16:0), TG(18:0/18:0/18:0), LysoPE(18:1), PC(16:0/18:1), PC(18:0/18:0), and PE(16:0/18:1), and CE. Two of the calibration lipids, TG(16:0/16:0/16:0) and TG(18:0/18:0/18:0), were detected in the calibration blank.

Some lipids were detected in the method blank; they were PC(18:0/18:0), TG(16:0/16:0/16:0) and TG(18:0/18:0/18:0). The detected peaks for PC(18:0/18:0) and TG(18:0/18:0/18:0) were above the limit of quantification (LOQ) while TG(16:0/16:0/16:0) was below LOQ. For PC(18:0/18:0), the PC(18:0/18:0) concentration detected in the method blank was slightly lower than the PC(18:0/18:0) concentrations detected in the liver samples (figure 6). The detected TG(18:0/18:0/18:0) concentration in the method blank was higher than the TG(18:0/18:0/18:0) concentrations detected in the liver homogenate samples (figure 8).

The general metabolic changes seen in this experiment were similar for all lipids (see figures 2-8), where the average lipid concentrations were higher in the PFOS-treated groups. The lipids that exhibited the highest lipid concentration in the 1.0 ug/g PFOS-treated group were LysoPE(18:1), CE, PE(16:0/18:1), TG(16:0/16:0/16:0), and TG(18:0/18:0/18:0). The lipids that exhibited the highest lipid concentration in the 0.1 ug/g PFOS-treated group were LysoPC(18:0) and PC(18:0/18:0). Each of the lipid's average lipid concentration, standard deviation, and relative standard deviation for the four different groups (untreated, solvent control, 0.1 ug/g PFOS, and 1.0 ug/g PFOS) is shown in Appendix 2.

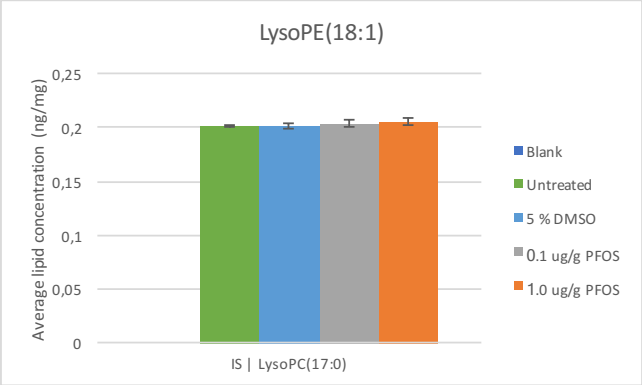

Figure 2 | The average LysoPE(18:1) concentration in the liver tissue samples and standard deviation over the four differently treated groups.

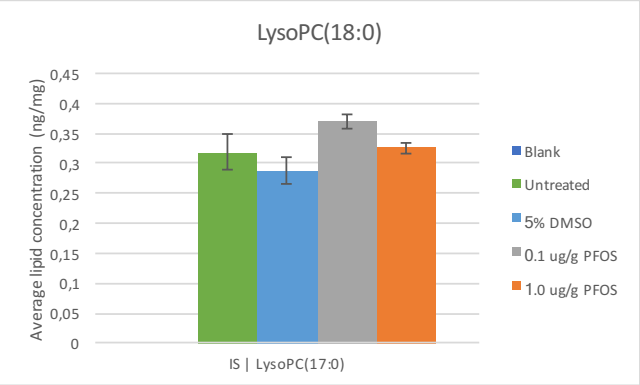

Figure 3 | The average LysoPC(18:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups.

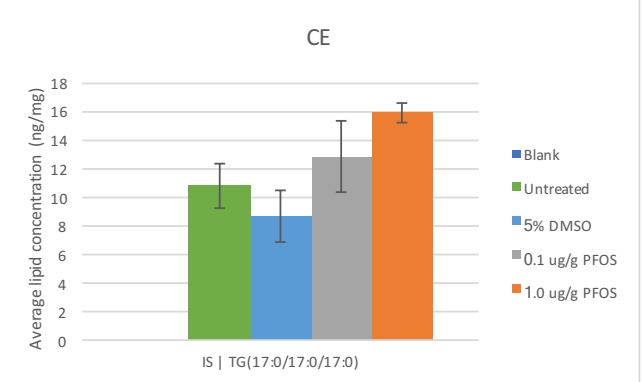

Figure 4 | The average CE concentration in the liver tissue samples and standard deviation over the four differently treated groups.

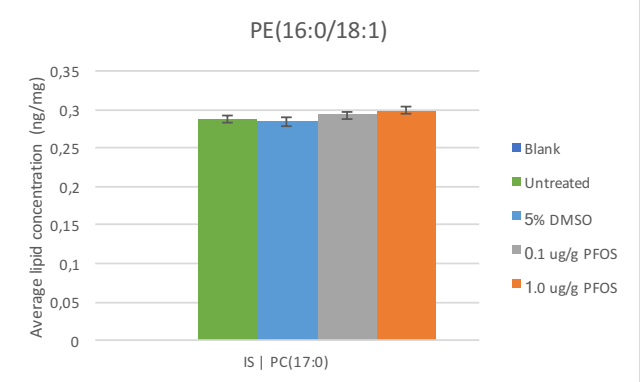

Figure 5 | The average PE(16:0/18:1) concentration in the liver tissue samples and standard deviation over the four differently treated groups.

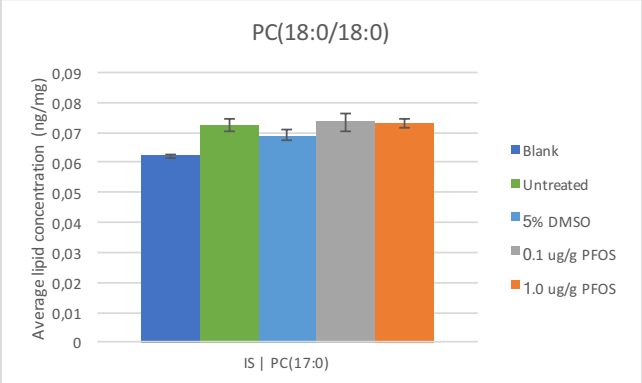

Figure 6 | The average PC(18:0/18:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups.

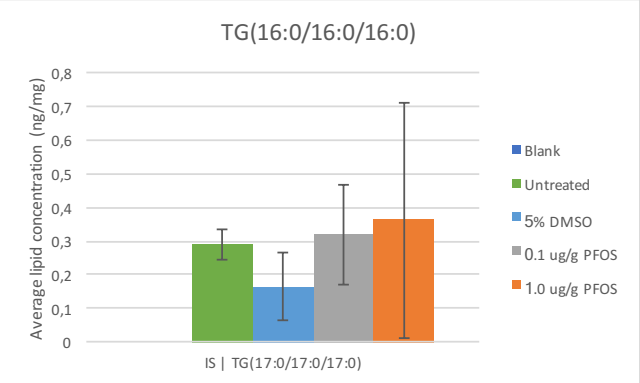

Figure 7 | The average TG(16:0/16:0/16:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups.

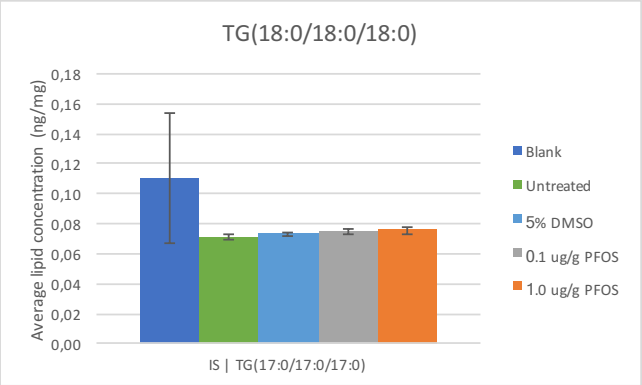

Figure 8 | The average TG(18:0/18:0/18:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups.

16 pooled samples were measured in triplicates during this work, representing the four groups (untreated, solvent control, 0.1 ug/g PFOS, and 1.0 ug/g PFOS), were analyzed. As expected the general metabolic changes were similar to the changes seen in the liver homogenate samples. In where the average lipid concentration was higher in the samples treated with PFOS (figures 9-15). For the following lipids, LysoPE(18:1), CE, PE(16:0/18:1), and PC(18:0/18:0), the highest concentration of the respective lipids was found in the pooled group treated with 1.0 ug/g PFOS. The highest LysoPC(18:0) and TG(18:0/18:0/18:0) concentrations were found in the 0.1 ug/g PFOS-treated pooled group. For TG(16:0/16:0/16:0), there was no discernible trend that could be seen for the metabolic change are shown in figure 14.

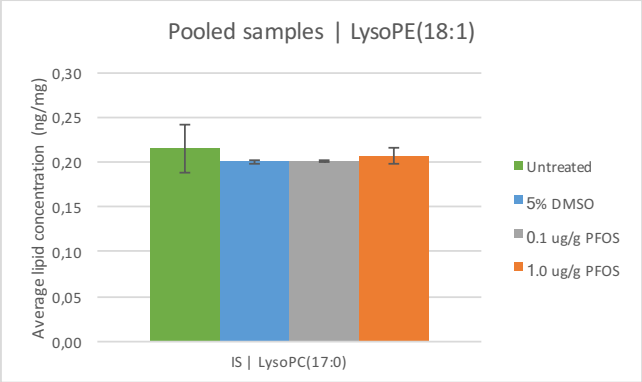

Figure 9 | The average LysoPE(18:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples

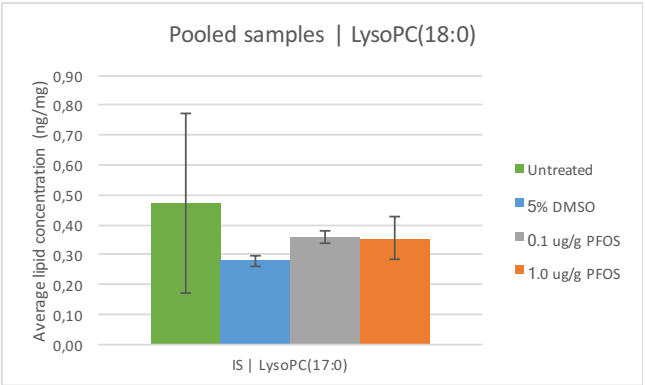

Figure 10 | The average LysoPC(18:1) concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples .

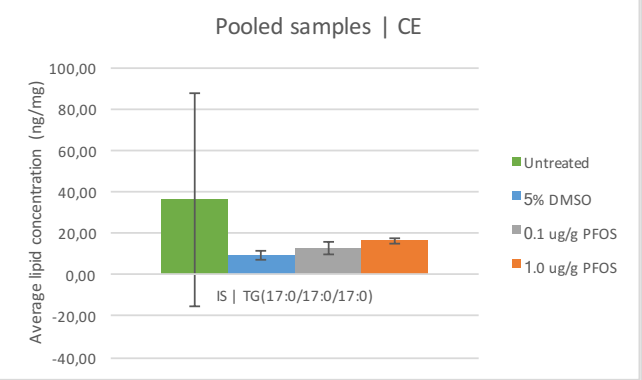

Figure 11 | The average CE concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples.

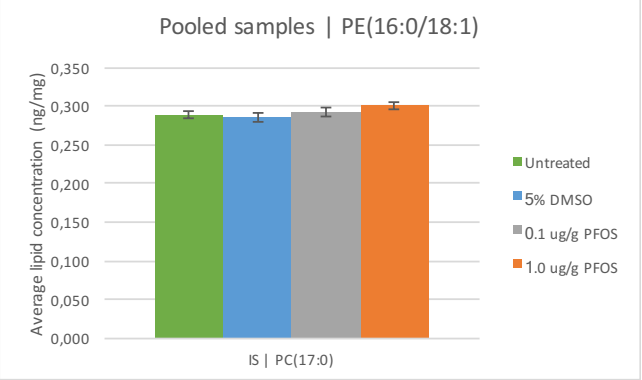

Figure 12 | The average PE(16:0/18:1) concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples.

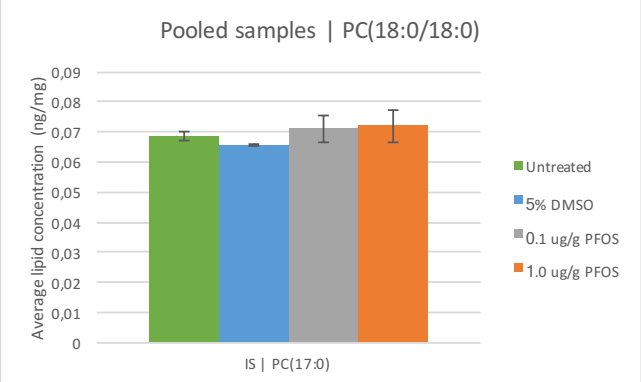

Figure 13 | The average PC(18:0/18:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples.

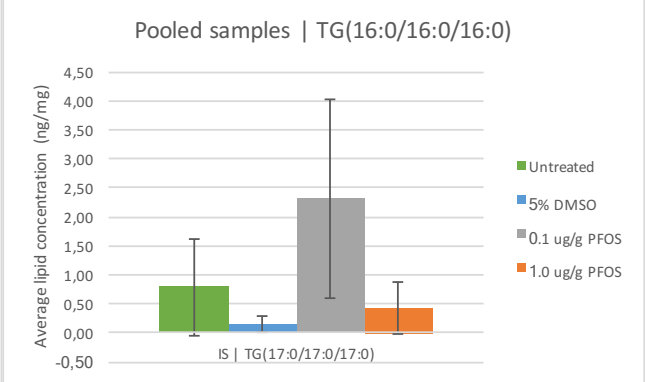

Figure 14 | The average TG(16:0/16:0/16:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples.

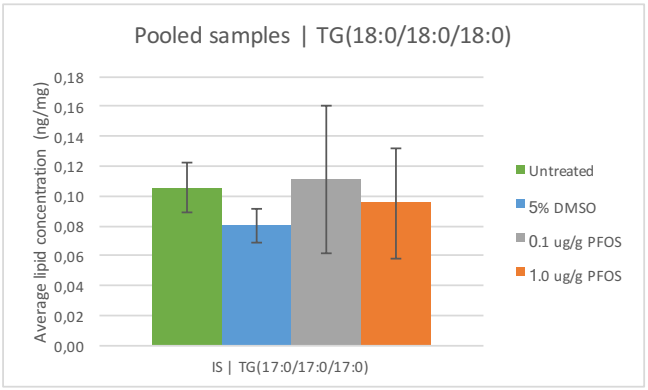

Figure 15 | The average TG(18:0/18:0/18:0) concentration in the liver tissue samples and standard deviation over the four differently treated groups in the pooled samples.

The metabolic changes for TG(18:0/18:0/18:0) differed in the pooled samples when compared to the metabolic changes observed in the individual liver homogenate samples. The highest TG(18:0/18:0/18:0) concentration was found in the 0.1 ug/g PFOS-treated pooled group and not the 1.0 ug/g PFOS-treated pooled group (which was the case for the liver homogenate samples). Another deviation for TG(18:0/18:0/18:0) was that the untreated pooled group exhibited a higher TG(18:0/18:0/18:0) concentration compared to the 1.0 ug/g PFOS-treated pooled group. TG(18:0/18:0/18:0) overall had high RSD-values, the 0.1 ug/g PFOS-treated pooled group had a RSD-value of 44.9% and the solvent control pooled group had a RSD value of 14.6% and the RSD-values for the untreated pooled group and 1.0 ug/g PFOS-treated pooled group fell between these two (Table 17 in Appendix 3).

When looking at the results within in the different groups (i.e. untreated, solvent control, 0.1 ug/g PFOS, and 1.0 ug/g PFOS), for the 16 pooled samples there were some deviating samples. For both LysoPE(18:1) and LysoPC(18:0), one of the four untreated pooled samples had a higher lipid concentration (Table 3). For CE, one of the four pooled samples in the untreated, solvent control, and the 1.0 ug/g PFOS-treated group deviated from the rest (Table 3). For TG(18:0/18:0/18:0), one of the four pooled samples in the untreated group, 0.1 ug/g PFOS-treated group, and 1.0 ug/g PFOS-treated group deviated from the rest of the samples. These deviations, which are shown in Table 3, lead to higher standard deviation within the groups which in turn leads to uncertain results.

Table 3 | The deviating samples for each group and lipid for the pooled samples. Higher denotes that the deviating sample had a higher lipid concentration when compared to the other three of the sample in the treatment group and Lower denotes that the deviating sample had a lower lipid concentration when compared to the other three of the samples in the treatment group. N/A denotes that there were no deviating samples in the treatment group. To see the lipid concentration for all four groups, see Appendix 3.

Deviations	Untreated	Solvent control	0.1 ug/g PFOS	1.0 ug/g PFOS
LysoPE(18:1)	Higher	n/a	n/a	n/a
LysoPC(18:0)	Higher	n/a	n/a	n/a
CE	Higher	Higher	n/a	Lower
TG(18:0/18:0/18:0)	Lower	Higher	Lower	Lower

The presence of deviating samples (n=4 for untreated, n=2 for solvent control, n=1 for 0.1 ug/g PFOS, and n=2 for 1.0 ug/g PFOS) was not the only parameter that skewed the data (leading to a higher standard deviation). Some of the lipids were not detected at all in one or more of the samples. No LysoPE(18:1) was detected in two of the four 0.1 ug/g PFOS samples and one of the four 1.0 ug/g PFOS samples. No PC(18:0/18:0) was detected in two of the four 0.1 ug/g PFOS samples. No TG(18:0/18:0/18:0) was detected in one of the four 0.1 ug/g PFOS samples and one of the four 1.0 ug/g PFOS samples.

3.2 | Global profiling

A semiquantitative global profiling analysis was also conducted on the liver samples. Within this analysis, the lipids detected in the liver samples were identified with the use of a customized database provided by one of the supervisors. The lipids identified in this step were cholesteryl esters (CEs), diglycerides (DGs), lysophosphatidylcholines (LysoPCs), phosphatidylacholines (PCs), phosphatidylethanolamines (PEs), sphingomyelins (SMs), and triglycerides (TGs). In total, 337 lipids were identified (see Appendix 4). In addition, large number of unknown lipids were also detected.

Only the identified lipids from the semiquantitative global profiling analysis were taken for the statistical analyses. Student's T-test, combined with calculation of false discovery rate (FDR) using the Benjamini-Hochberg procedure was applied for the characterization of the differences between groups. For statistical significance, threshold for p values < 0.05 combined with FDR values <1.0, were used. The p-values and FDR-values for the differently treated groups are shown in Appendix 5-7. The goal of the statistical analyses was to identify those lipids that showed significant difference between the solvent control and PFOS-treated and verify that the differences were driven by the PFOS treatment, as the solvent PFOS had been dissolved in was DMSO.

A comparison between the untreated group with the solvent control group showed that 63 lipids showed significant differences (Table 18). These 63 lipids were then used as a basis when comparing the solvent control group with the PFOS-treated groups. When comparing the solvent control group with the PFOS-treated groups, the 0.1 ug/g PFOS-treated group had 37 out of 63 lipids with a p-value below 0.05 and FDR-value below 0.1 (Table 20 in Appendix 6) and the 1.0 ug/g PFOS-treated group had 55 of the 63 lipids with a p-value below 0.05 and FDR-value below 0.1 (Table 20 in Appendix 6).

In general, most TGs with less saturation, i.e. TGs where the fatty acid chains attached to the glycerol head group were saturated or had only 1 or 2 double bonds were increased after the treatment, and similar trend was seen for most of the CEs. Several TGs that contained polyunsaturated TGs (e.g. TG(54:7)) showed significant decrease, particularly after treatment with the high concentration of PFOS. The treatment of high concentration of PFOS resulted also significant decrease in several LysoPCs, particularly plasmalogen LysoPCs.

In order to see how the differently treated groups correlated to each other, both regular heat map was made and a clustered one (using hierarchal clustering) using MeV 4.9.0, these are shown in Appendix 8.

4. Discussion

All calibration curves, are shown in Appendix 1, had an r^2-value better than 0.998 and thus, the calculated concentration shown in figures 1-14 plus Appendix 2 and 3 can be considered sufficiently accurate. The calibration range was 0 to 10000 ng/mL. Two of the calibration lipids were detected in the calibration blank, these two lipids were both triglycerides (TGs). The reason for why these two lipids were detected could be attributed to contamination in the calibration blank.

As previously stated in 3.1 Quantitative analysis, not all calibration lipid standards and internal lipid standards were detected during the data processing. This can be attributed to the fact that the parameters used during the data processing were not optimized and the novelty of the software program used.

For the liver samples, the overall metabolic changes for all lipids was the same in where the highest lipid concentration were found in either of the PFOS-treated groups. For the majority of the lipids, the highest lipid concentration was found in the group treated with 1.0 ug/g PFOS. However, there were two lipids that deviated from the trend in where the group treated with 1.0 ug/g PFOS had the highest concentration and these lipids were LysoPC(18:0) and PC(18:0/18:0).

For the 16 pooled samples, there was no discernible trend within the groups or between the groups for TG(16:0/16:0/16:0) and all RSD-values were above 74.65%, with the untreated group having a RSD-value of 106.69%, 5% DMSO-treated group having 92.07%, 0.1 µg/g PFOS-treated group having 74.65% and 1.0 µg/g PFOS-treated group having 108.20%. With no discernible trend and high RSD-values, no quantitative answers can be made. When looking at the results for TG(16:0/16:0/16:0) in the liver homogenate samples and not the pooled samples, there is a discernible trend to be seen. However, the RSD-values were high in this case as well with the lowest RSD-value being 15,9% for the untreated group and the highest RSD-value being 96,0% for the 1.0 ug/g PFOS-treated group. With high RSD-values like these, the quantitative results obtained are not reliable in both the liver homogenate and pooled samples. A reason for why these results varied so much could be due to TG(16:0/16:0/16:0)'s non-polar nature, in where it sticks to the non-polar column leading to analytical variation.

Another deviation for the pooled samples was the case of some missing lipid peaks, where LysoPE(18:1), PC(18:0/18:0), and TG(18:0/18:0/18:0) were not detected in some of the PFOS-treated samples. Why none of these lipids were not detected in these samples could been due to data processing in where these peaks were not big enough in relation to the noise level used. However, even though these lipids were missing, these missing peaks were used in the calculations in order to obtain representative data.

In the analysis, a large number of different lipids were identified. The different lipids detected within this analysis were CEs, DGs, LysoPCs, PCs, PEs, SMs, and TGs. The statistical analyses done on the identified lipids showed that there were a large number of lipids that changed due to the treatment with PFOS, and changes were dose dependent. This dose dependency was seen when the solvent control group, the 0.1 ug/g PFOS-treated group, and the 1.0 ug/g PFOS-treated groups were compared in terms of lipid concentration. The higher PFOS dose, in general, resulted in higher lipid concentration. The largest differences were observed in the samples that had been treated with the higher concentration of PFOS.

Heat maps, see Appendix 8, was also constructed to see the correlation between the different liver samples and a clustered heat map was done to see which liver samples were closely related to each other to see if the liver samples in the same group clustered together.In the clustered heat map, the majority of the liver samples that belong to the same treated group did cluster together. However, there were some liver samples that deviated from their clustered, i.e. one of the 0.1 ug/g PFOS triplicates was not clustered with the rest of the 0.1 ug/g PFOS group. Why these deviating samples were not clustered together with the rest of the group could be due to data processing or analytical variation. All the 1.0 ug/g PFOS samples clustered together.

Possible challenges in the work are due to various experimental errors. When it comes to analyzing biological materials such as lipids in different tissues, the biggest contamination source is the person conducting the experiment as the lipids in this work are endogenous to humans. As TGs were detected in the calibration blank and TGs and PC(18:0/18:0) detected in the method blanks, another possible contamination source may be that the surface area in the fume hood was not properly cleaned from when the actual lipid extraction was conducting, meaning that if a pipette tip touched the fume hood surface without the person noticing it could transfer any lipids that was adhered to the metal surface. Something that could minimize these experimental errors is the use of aseptic techniques, in where by sterilizing the work surface, equipment and gloves the risk of contaminations are reduced.

The quantitative analysis suggested a decrease in ß-oxidation in the liver samples treated with PFOS, as these samples had a higher lipid concentration in comparison with the solvent control group. Generally, the highest lipid concentration was found in the samples treated with 1.0 ug/g PFOS. This suggests that livers exposed to higher PFOS concentrations exhibit a greater down-regulation of the lipid metabolism, which in turn leads to a decreased ß-oxidation of lipids that results in higher lipid concentration in the livers. In the quantitative analysis, all lipids had ≤ 18 carbon atoms in its molecular backbone and these lipids are metabolized in the mitochondria by ß-oxidation. This suggests a decrease in the mitochondrial ß-oxidation and in turn a down-regulation of the mitochondrial lipid metabolism.

When looking at CE and TG(18:0/18:0/18:0) in the quantitative analysis, for example, the effect PFOS has on the lipid metabolism is quite evident. However, it is more evident for CE than it is for TG(18:0/18:0/18:0). The PFOS-treated samples have higher lipid concentration when compared to the solvent control group, and the higher PFOS dose results in higher lipid concentration in general in the liver samples. As other studies have pointed out, PFOS is an PPARα-agonist that disturbs the PPARα's regulatory activity, which includes the transport of fatty acids and cholesterol. By disrupting the transportation of these compounds, they accumulate in the liver resulting in higher lipid concentrations. This accumulation plus the decrease in ß-oxidation which in

turn leads to down-regulation of lipid metabolism can lead to increased liver weight, which is one of the adverse effects of PFOS exposure.

In the global profiling analysis on the other hand, there were lipids with ≤22 carbon atoms in its molecular backbone structure and lipids with ≥22 carbon atoms in its molecular backbone structure. The lipids with ≥ 22 carbon atoms in its molecular backbone structure are metabolized in the peroxisomes by ß-oxidation. Global lipidomics showed that PFOS treatments resulted in decreasing levels of particularly TGs with long-chain fatty acids, and several plasmalogens, suggesting increased peroxisomal ß-oxidation.

When comparing TGs with short-chain fatty acids to TGs with long-chain fatty acids, the impact of the PFOS-treatment is different. For the TGs with short-chain fatty acids, the effect that the PFOS-treatment had resulted in increased levels of these TGs whilst for the TGs with long-chain fatty acids the treatment resulted in decreased levels of these TGs.

The metabolic changes from the PFOS treatment differed in the TGs with short-chain fatty acids and the TGs with long-chain fatty acids. The effect the PFOS treatment had on the TGs with short-chain fatty acids was a decrease in the mitochondrial ß-oxidation which resulted in increased TG levels for these TGs. On the other hand, the effect the PFOS treatment had on the TGs with long-chain fatty acids was a increase in the peroxisomal ß-oxidation which resulted in decreased TG levels for these TGs. This suggests that PFOS treatment decreases the mitochondrial ß-oxidation and increases the peroxisomal ß-oxidation.

In order to confirm the results obtained in this project, future experiments are needed where more samples are analyzed to get a larger data set as 16 samples is quite a small data set and also looking at other PFOS concentrations. More experiments can be done to compare the impact PFOS treatment has on mitochondrial ß-oxidation and peroxisomal ß-oxidation.

5. Conclusion

In the quantitative analysis, the overall trend for the liver samples and QC samples followed the hypothesized trend of the PFOS-treated groups having the highest lipid concentration due to PFOS disturbing the lipid metabolism. Some lipids in the global lipidomics showed also decreasing levels of specific lipids after PFOS-treatment. This leads to the overall conclusion that PFOS disrupts the lipid metabolism, especially the ß-oxidation of the lipids, however, the effect on lipid metabolism is dose dependent.

6. Acknowledgements

I would like to thank both of my supervisors (Dr. Tuulia Hyötyläinen, Ph.D and Dr. Nikolai Scherbak, Ph.D) who have guided and helped me through this project. I would also thank Dr. Dawei Geng, Ph.D, and Cecilia Carlsson, who with me were frustrated every time we try to wrangle and fix the UHPLC/Q-TOF-MS machine every time it decided that it was not going to work properly. Some people that I cannot forget to thank are my classmates who have been there when everything felt like it was going wrong and made me laugh.

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Appendix

Appendix 1 | Calibration curves

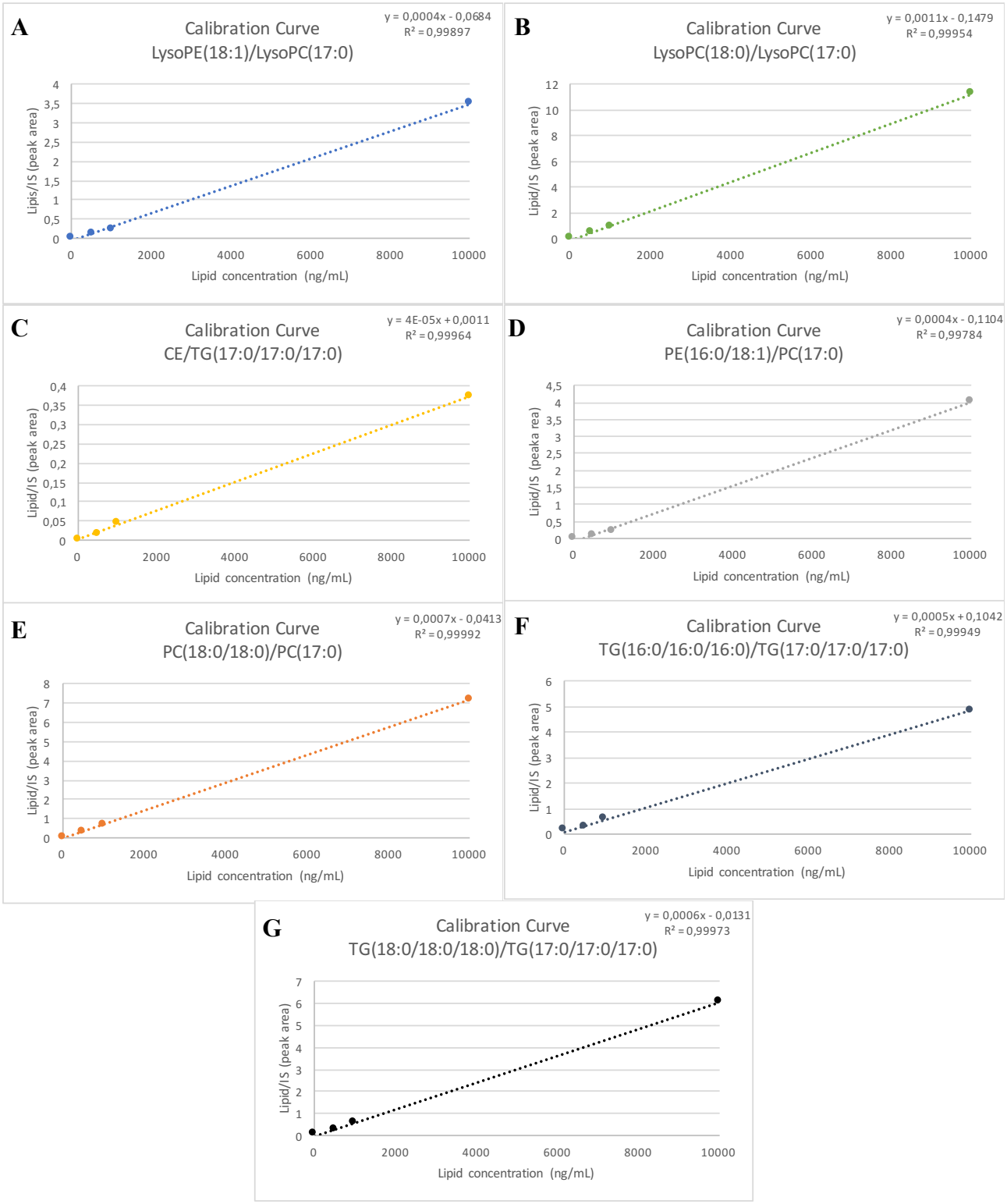

Figure 16 | The calibration curves for the different lipid species (A) LysoPE, (B) LysoPC, (C) CE, (D) PE, (E) PC, (F) TG(16:0/16:0/16:0), and (G) TG(18:0/18:0/18:0)

Appendix 2 | Tables over average concentration, standard deviation, and RSD for the liver samples

Table 4 | The average lipid concentration, STDEV and RSD for LysoPE(18:1)

LysoPE(18:1)/LysoPC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	0	0	0
Untreated	0,201	0,000582	0,290
5% DMSO	0,202	0,00235	1,17
0.1 µg/g PFOS	0,204	0,00299	1,47
1.0 µg/g PFOS	0,205	0,00330	1,61

Table 5 | The average lipid concentration, STDEV and RSD for LysoPC(18:0)

LysoPC(18:0)/LysoPC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	0	0	0
Untreated	0,319	0,0296	9,31
5% DMSO	0,288	0,0235	8,16
0.1 µg/g PFOS	0,370	0,0125	3,38
1.0 µg/g PFOS	0,326	0,00878	2,69

Table 6 | The average lipid concentration, STDEV and RSD for CE

CE/TG(17:0/17:0/17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	0	0	0
Untreated	10,8	1,55	14,4
5% DMSO	8,68	1,83	21,0
0.1 µg/g PFOS	12,8	2,49	19,4
1.0 µg/g PFOS	15,9	0,66	4,17

Table 7 | The average lipid concentration, STDEV and RSD for PE(16:0/18:1)

PE(16:0/18:1)/PC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	0	0	0
Untreated	0,288	0,00493	1,71
5% DMSO	0,285	0,00577	2,03
0.1 µg/g PFOS	0,292	0,00533	1,82
1.0 µg/g PFOS	0,299	0,00508	1,70

Table 8 | The average lipid concentration, STDEV and RSD for PC(18:0/18:0)

PC(18:0/18:0)/PC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	0,0624	0,000603	16,6
Untreated	0,0725	0,00236	3,25
5% DMSO	0,0685	0,00159	2,32
0.1 µg/g PFOS	0,0734	0,00277	3,77
1.0 µg/g PFOS	0,0731	0,00170	2,33

Table 9 | The average lipid concentration, STDEV and RSD for TG(16:0/16:0/16:0)

TG(16:0/16:0/16:0)/ TG(17:0/17:0/17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	Under LOQ	Under LOQ	Under LOQ
Untreated	0,289	0,0458	15,9
5% DMSO	0,126	0,100	79,8
0.1 µg/g PFOS	0,321	0,148	46,2
1.0 µg/g PFOS	0,364	0,349	96,0

Table 10 | The average lipid concentration, STDEV and RSD for TG(18:0/18:0/18:0)

TG(18:0/18:0/18:0)/ TG(17:0/17:0/17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Blank	0,110	0,0437	81,1
Untreated	0,0712	0,00208	2,92
5% DMSO	0,0732	0,00139	1,90
0.1 µg/g PFOS	0,0747	0,00178	2,38
1.0 µg/g PFOS	0,0757	0,00220	2,90

Appendix 3 | Tables over average concentrations, standard deviation, and RSD for the pooled samples

Table 11 | The average lipid concentration, STDEV and RSD for LysoPE(18:1)

Pooled | LysoPE(18:1)/ LysoPC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	0,215	0,0273	12,7
5% DMSO	0,201	0,00185	0,920
0.1 µg/g PFOS	0,201	0,000789	0,393
1.0 µg/g PFOS	0,206	0,00893	4,32

Table 12 | The average lipid concentration, STDEV and RSD for LysoPC(18:0)

Pooled | LysoPE(18:1)/ LysoPC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	0,215	0,0273	12,7
5% DMSO	0,201	0,00185	0,920
0.1 µg/g PFOS	0,201	0,000789	0,393
1.0 µg/g PFOS	0,206	0,00893	4,32

Table 13 | The average lipid concentration, STDEV and RSD for CE

Pooled | CE/ TG(17:0/17:0/17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	36,2	51,3	142
5% DMSO	9,29	2,06	22,2
0.1 µg/g PFOS	12,9	3,17	24,6
1.0 µg/g PFOS	16,1	1,52	9,43

Table 14 | The average lipid concentration, STDEV and RSD for PE(16:0/18:1)

Pooled | PE(16:0/18:1)/ PC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	0,288	0,00469	1,63
5% DMSO	0,285	0,00586	2,06
0.1 µg/g PFOS	0,292	0,00687	2,35
1.0 µg/g PFOS	0,300	0,00427	1,42

Table 15 | The average lipid concentration, STDEV and RSD for PC(18:0/18:0)

Pooled | PC(18:0/18:0)/ PC(17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	0,0686	0,00146	2,13
5% DMSO	0,0656	0,000407	0,621
0.1 µg/g PFOS	0,0711	0,00440	6,19
1.0 µg/g PFOS	0,0719	0,00508	7,06

Table 16 | The average lipid concentration, STDEV and RSD for TG(16:0/16:0/16:0)

Pooled | TG(16:0/16:0/16:0)/ TG(17:0/17:0/17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	0,784	0,836	107
5% DMSO	0,121	0,132	110
0.1 µg/g PFOS	2,30	1,71	74,7
1.0 µg/g PFOS	0,415	0,448	108

Table 17 | The average lipid concentration, STDEV and RSD for TG(18:0/18:0/18:0)

Pooled | TG(18:0/18:0/18:0)/ TG(17:0/17:0/17:0)	Average concentration (ng/mg)	Average STDEV	Average RSD (%)
Untreated	0,106	0,0163	15,4
5% DMSO	0,0801	0,0117	14,6
0.1 µg/g PFOS	0,111	0,0498	44,9
1.0 µg/g PFOS	0,0950	0,0371	39,0

Appendix 4 | All identified lipids in the semiquantitative global profiling analysis

Table 18 | All lipids identified in the global profiling analysis

Identified lipids				
CE(18:1)	PC(36:2)	PC(O-32:1)	TG(18:1/12:0/18:1) or TG(18:2/16:0/14:0)	TG(52:6)
CE(18:1) + Unknown CE(669,6358)	PC(36:2)	PC(O-34:3)	TG(18:1/12:0/18:1) or TG(18:2/16:0/14:0)	TG(53:2)
CE(18:1) + Unknown CE(669,6358)	PC(36:2)	PC(O-34:3)	TG(18:1/18:1/16:0)	TG(53:2)
CE(18:1) + Unknown CE(669,6358)	PC(36:3)	PC(O-38:5)	TG(18:1/18:1/18:1)	TG(53:2)
CE(18:2)	PC(36:3)	PC(O-38:6)	TG(18:1/18:1/18:1)	TG(53:2)
CE(18:2)	PC(36:3)	PC(O-38:6)	TG(18:1/18:1/22:6)	TG(53:2)
CE(20:4)	PC(36:3)	PC(O-38:6)	TG(18:1/18:1/22:6)	TG(53:2)
CE(20:5)	PC(36:3)	PC(O-38:6)	TG(18:1/18:1/22:6)	TG(53:4)
CE(20:5)	PC(36:4)	PC(O-38:6)	TG(18:1/18:1/22:6)	TG(53:5)
DG(18:0/20:4)	PC(36:4)	PC(O-40:4)	TG(18:1/18:2/18:2)	TG(54:0)
DG(18:0/20:4)	PC(36:4)	PC(O-40:4)	TG(18:1/18:2/18:2)	TG(54:0)
DG(18:0/20:4)	PC(36:5)	PC(O-40:4)	TG(18:2/18:1/16:0)	TG(54:1)
DG(18:0/20:4)	PC(36:5)	PC(O-40:4)	TG(18:2/18:1/18:1)	TG(54:1)
Fragment: CE signature ion	PC(37:1)	PC(O-40:5)	TG(18:2/18:1/18:1)	TG(54:2)
Fragment: CE signature ion	PC(37:1)	PC(O-40:5)	TG(18:2/18:1/18:1)	TG(54:2)
Fragment: CE signature ion	PC(37:2)	PC(O-40:5)	TG(18:2/18:1/18:1)	TG(54:2)
Fragment: CE signature ion	PC(37:2)	PC(O-40:6)	TG(18:2/18:2/18:2) or TG(18:3/18:2/18:1)	TG(54:2)
Fragment: CE signature ion	PC(37:2)	PC(O-40:6)	TG(18:2/18:2/18:2) or TG(18:3/18:2/18:1)	TG(54:2)
Fragment: CE signature ion	PC(37:2)	PC(P-18:0/22:6)	TG(18:2/18:2/18:2) or TG(18:3/18:2/18:1)	TG(54:3)
LPC(16:0)	PC(37:3)	PC(P-18:0/22:6)	TG(18:2/18:2/18:2) or TG(18:3/18:2/18:1)	TG(54:3)
LPC(16:0)	PC(37:3)	PC(P-18:0/22:6)	TG(18:2/22:5/16:0)	TG(54:4)
LPC(16:0)	PC(37:4)	PE(34:2)	TG(18:2/22:5/16:0)	TG(54:4)
LPC(16:0)	PC(37:4)	PE(36:4)	TG(18:2/22:5/16:0)	TG(54:4)
LPC(16:0e)	PC(37:4)	PE(36:4)	TG(37:0)	TG(54:4)
LPC(16:0e)	PC(37:4)	PE(36:4)	TG(45:0)	TG(54:4)
LPC(16:0p)	PC(37:5)	PE(36:4)	TG(47:0)	TG(54:4)
LPC(16:0p)	PC(37:5)	PE(O-16:0/22:6) or PE(P-18:0/20:5)	TG(47:1)	TG(54:5)
LPC(16:1)	PC(37:5)	PE(O-16:0/22:6) or PE(P-18:0/20:5)	TG(47:2)	TG(54:5)
LPC(18:0)	PC(38:2)	PE(O-38:5) or PE(P-38:4)	TG(48:0)	TG(54:5)

Identified lipids

LPC(18:0)	PC(38:2)	PI(18:0/20:4)	TG(48:1)	TG(54:6)
LPC(18:1)	PC(38:2)	SM(d18:0/16:0)	TG(48:1)	TG(54:6)
LPC(18:1)	PC(38:3)	SM(d18:0/22:0)	TG(48:3)	TG(54:6)
LPC(18:2)	PC(38:3)	SM(d18:0/22:0)	TG(48:3)	TG(54:7)
LPC(20:3)	PC(38:3)	SM(d18:0/22:0)	TG(49:0)	TG(54:7)
LPC(20:3)	PC(38:3)	SM(d18:0/22:0)	TG(49:1)	TG(54:7)
LPC(20:4)	PC(38:3)	SM(d18:1/24:0) or SM(d18:0/24:1)	TG(49:1)	TG(55:5)
LPC(20:4)	PC(38:3)	SM(d18:1/24:0) or SM(d18:0/24:1)	TG(49:1)	TG(55:5)
LPC(22:6)	PC(38:3)	SM(d18:2/24:1)	TG(49:2)	TG(56:2)
PC(16:0e/18:1(9Z))	PC(38:4)	SM(d18:2/24:1)	TG(49:3)	TG(56:2)
PC(31:0)	PC(38:4)	SM(d18:2/24:1)	TG(49:3)	TG(56:3)
PC(32:0)	PC(38:4)	SM(d18:2/24:1)	TG(50:0)	TG(56:3)
PC(32:1)	PC(38:5)	SM(d18:2/24:1)	TG(50:0)	TG(56:4)
PC(32:1)	PC(38:5)	SM(d34:1)	TG(50:1)	TG(56:4)
PC(32:2)	PC(38:5)	SM(d36:1)	TG(50:1)	TG(56:4)
PC(33:0)	PC(38:5)	SM(d38:1)	TG(50:2)	TG(56:4)
PC(33:0)	PC(38:6)	SM(d39:1)	TG(50:3)	TG(56:5)
PC(33:1)	PC(38:6)	SM(d40:1)	TG(50:3)	TG(56:5)
PC(33:1)	PC(39:6)	SM(d40:1)	TG(50:5)	TG(56:5)
PC(33:1)	PC(39:6)	SM(d40:2)	TG(50:5)	TG(56:5)
PC(34:1)	PC(39:6)	SM(d41:1)	TG(51:1)	TG(56:5)
PC(34:2)	PC(39:6)	SM(d41:2)	TG(51:1)	TG(56:5)
PC(34:3)	PC(40:4)	SM(d42:2)	TG(51:2)	TG(56:6)
PC(34:3)	PC(40:4)	SM(d42:2)	TG(51:2)	TG(56:6)
PC(34:3)	PC(40:5)	SM(d42:2)	TG(51:2)	TG(56:6)
PC(34:3)	PC(40:5)	SM(d42:2)	TG(51:2)	TG(56:8)
PC(35:1)	PC(40:5)	TG(14:0/16:0/18:1)	TG(51:2)	TG(56:9)
PC(35:2)	PC(40:5)	TG(14:0/18:1/18:1)	TG(51:2)	TG(56:9)
PC(35:2)	PC(40:5)	TG(14:0/18:2/18:2)	TG(51:3)	TG(56:9)
PC(35:3)	PC(40:6)	TG(16:0/18:0/18:1)	TG(51:3)	TG(58:6)
PC(35:3)	PC(40:6)	TG(16:0/18:2/18:2)	TG(52:0)	TG(58:6)
PC(35:4)	PC(40:7)	TG(16:0/18:2/18:2)	TG(52:2)	TG(58:6)

Identified lipids

PC(35:4)	PC(40:7)	TG(16:0/18:2/18:3)	TG(52:3)	TG(58:9)
PC(35:4)	PC(40:7)	TG(16:0/18:2/18:3)	TG(52:4)	TG(58:9)
PC(36:1)	PC(40:8)	TG(16:0/22:5/18:1) or TG(20:4/18:1/18:1)	TG(52:4)	TG(58:9)
PC(36:1)	PC(40:8)	TG(16:0/22:5/18:1) or TG(20:4/18:1/18:1)	TG(52:4)	TG(58:9)
PC(36:1)	PC(40:8)	TG(16:0/22:5/18:1) or TG(20:4/18:1/18:1)	TG(52:4)	
PC(36:1)	PC(42:5)	TG(17:0/17:0/17:0)	TG(52:5)	
PC(36:2)	PC(O-32:1)	TG(17:0/17:0/17:0)	TG(52:5)	

Appendix 5 | p-values and FDR (false discovery rates) for the untreated group compared with the other three treated groups

Table 19 | p≥0.05, p-values below 0.05 are *italicized*, and FDR (False discovery rate) ≥0.1, FDR-values below 0.1 are **bolded**.

Name	**Untreated | Average peak area**	**p-value | untreated vs 5% DMSO**	**FDR | untreated vs 5% DMSO**	**p-value | untreated vs 0.1 µg/g PFOS**	**FDR | untreated vs 0.1 µg/g PFOS**	**p-value | untreated vs 1.0 µg/g PFOS**	**FDR | untreated vs 1.0 µg/g PFOS**
PC(O-40:6)	0,27	0,22	**0,092**	*0,000072*	**0,0035**	*4,6E-09*	**1,0E-06**
TG(56:5)	0,019	0,098	**0,031**	*0,0040*	**0,059**	*1,6E-08*	**2,7E-06**
PC(40:5)	0,024	0,19	**0,056**	*0,0010*	**0,02**	*1,1E-07*	**9,6E-06**
TG(54:6)	0,0062	0,47	**0,043**	0,13	0,40	*1,3E-07*	**1,0E-05**
SM(d18:0/22:0)	0,024	0,34	**0,098**	*0,00074*	**0,016**	*1,9E-07*	**1,3E-05**
PC(36:4)	0,023	0,13	**0,078**	*0,00057*	**0,014**	*2,4E-07*	**1,5E-05**
PC(36:1)	0,022	0,54	**0,023**	*0,00055*	**0,014**	*3,2E-07*	**1,8E-05**
TG(56:6)	2,20	0,10	**0,078**	*0,015*	0,14	*7,4E-07*	**3,6E-05**
TG(45:0)	0,0054	0,73	**0,08**	*0,00035*	**0,011**	*1,7E-06*	**6,3E-05**
LPC(16:0e)	0,064	0,11	0,15	0,32	0,40	*1,9E-06*	**6,9E-05**
PC(32:0)	0,040	0,40	0,14	*0,00051*	**0,014**	*3,3E-06*	**1,0E-04**
TG(56:5)	0,064	0,34	**0,050**	*0,0051*	**0,071**	*3,8E-06*	**1,1E-04**
PC(38:5)	0,34	0,075	**0,023**	0,13	0,40	*1,1E-05*	**2,4E-04**
LPC(18:1)	0,084	0,15	**0,016**	0,33	0,40	*2,2E-05*	**4,7E-04**
TG(56:5)	0,053	0,46	0,20	0,90	0,40	*2,5E-05*	**5,2E-04**
DG(18:0/20:4)	0,23	0,47	0,40	*0,026*	0,22	*3,0E-05*	**6,0E-04**
TG(37:0)	0,0058	0,056	**0,018**	0,62	0,40	*3,7E-05*	**7,1E-04**
SM(d42:2)	0,0080	0,20	0,40	*0,00068*	**0,015**	*4,5E-05*	**8,3E-04**
TG(18:2/18:1/18:1)	0,0078	1,00	**0,035**	0,33	0,40	*4,6E-05*	**8,3E-04**
TG(50:5)	0,0066	0,18	**0,099**	0,43	0,40	*5,5E-05*	**9,4E-04**
TG(53:2)	0,023	0,44	**0,065**	0,33	0,40	*1,0E-04*	**1,7E-03**
TG(53:2)	0,0057	0,58	**0,0020**	*0,00011*	**0,0051**	*1,1E-04*	**0,0017**
LPC(18:0)	0,017	0,21	**0,089**	0,32	0,40	*1,9E-04*	**0,0027**

Name	Untreated | Average peak area	p-value | untreated vs 5% DMSO	FDR | untreated vs 5% DMSO	p-value | untreated vs 0.1 µg/g PFOS	FDR | untreated vs 0.1 µg/g PFOS	p-value | untreated vs 1.0 µg/g PFOS	FDR | untreated vs 1.0 µg/g PFOS
TG(49:3)	0,19	0,36	0,17	0,66	0,40	*2,5E-04*	**0,0031**
DG(18:0/20:4)	0,19	0,36	**0,010**	0,65	0,40	*2,5E-04*	**0,0031**
PC(P-18:0/22:6)	0,052	0,05	0,31	*0,020*	0,18	*5,6E-04*	**0,0063**
PC(35:3)	0,00039	0,52	**0,042**	0,18	0,40	*6,1E-04*	**0,0067**
PC(36:2)	0,052	0,14	**0,089**	0,59	0,40	*0,0011*	**0,011**
TG(54:3)	0,023	0,064	**0,065**	0,33	0,40	*0,0012*	**0,013**
TG(56:9)	0,0054	0,56	0,40	*0,00024*	**0,0085**	*0,0014*	**0,014**
PC(37:2)	0,015	0,47	**0,027**	*0,0027*	**0,042**	*0,0016*	**0,015**
PC(O-32:1)	0,43	0,33	0,12	0,095	0,40	*0,0020*	**0,019**
TG(54:0)	0,016	0,16	0,13	0,075	0,40	*0,0020*	**0,019**
TG(49:1)	0,015	0,16	**0,090**	0,19	0,40	*0,0020*	**0,019**
PC(38:3)	0,020	0,075	**0,078**	0,051	0,33	*0,0031*	**0,029**
TG(51:3)	0,070	0,16	**0,092**	0,15	0,40	*0,0038*	**0,035**
PC(33:0)	0,017	0,73	0,40	0,35	0,40	*0,0051*	**0,045**
SM(d41:1)	0,55	0,21	**0,010**	0,067	0,40	*0,0054*	**0,047**
TG(54:2)	0,014	0,66	**0,0037**	*0,0082*	0,10	*0,0067*	**0,054**
PE(O-38:5) or PE(P-38:4)	0,059	0,21	**0,057**	0,074	0,40	*0,0068*	**0,054**
PC(38:2)	0,030	0,19	0,12	*0,029*	0,23	*0,0078*	**0,061**
PC(36:5)	0,0064	0,77	0,31	0,49	0,40	*0,0090*	**0,069**
TG(50:3)	0,013	0,76	0,27	*0,0015*	**0,027**	*0,0098*	**0,073**
LPC(18:0)	0,022	0,076	**0,038**	0,33	0,40	*0,010*	**0,073**
SM(d18:0/22:0)	0,031	0,99	**0,048**	*0,043*	0,30	*0,010*	**0,073**
TG(47:1)	0,41	0,092	**0,051**	0,64	0,40	*0,010*	**0,073**
PC(38:6)	0,11	0,099	**0,039**	0,28	0,40	*0,010*	**0,073**
TG(48:3)	0,060	0,30	0,14	0,61	0,40	*0,011*	**0,078**
TG(56:5)	0,057	0,22	0,21	0,45	0,40	*0,012*	**0,082**
PC(38:4)	0,088	0,19	0,12	0,062	0,38	*0,013*	**0,082**

Name	**Untreated | Average peak area**	**p-value | untreated vs 5% DMSO**	**FDR | untreated vs 5% DMSO**	**p-value | untreated vs 0.1 µg/g PFOS**	**FDR | untreated vs 0.1 µg/g PFOS**	**p-value | untreated vs 1.0 µg/g PFOS**	**FDR | untreated vs 1.0 µg/g PFOS**
TG(54:6)	0,23	0,30	**0,030**	0,26	0,40	*0,013*	**0,083**
TG(53:2)	0,026	0,72	**0,043**	*0,000055*	**0,0029**	*0,013*	**0,083**
TG(50:1)	0,083	0,067	**0,075**	0,31	0,40	*0,015*	**0,095**
CE(18:1) + Unknown CE(669,6358)	0,044	0,16	0,37	*0,00077*	**0,016**	*0,020*	0,12
TG(54:7)	0,0091	0,057	**0,055**	*0,0064*	**0,083**	*0,020*	0,12
TG(52:5)	0,12	0,92	0,15	*0,00041*	**0,012**	*0,023*	0,14
LPC(20:3)	0,025	0,65	**0,030**	*0,00047*	**0,013**	*0,028*	0,16
TG(54:0)	0,0052	0,55	0,32	*0,00021*	**0,0085**	0,082	0,35
PE(36:4)	0,025	0,46	0,40	*0,0020*	**0,034**	0,11	0,40
Fragment: CE signature ion	0,024	0,27	0,15	*0,0037*	**0,057**	0,11	0,40
TG(51:1)	4,79	0,082	**0,016**	*0,0011*	**0,022**	0,12	0,40
PC(36:4)	0,013	0,13	0,40	*0,0075*	**0,094**	0,61	0,40
PC(34:3)	0,034	0,64	0,23	*0,0049*	**0,071**	0,86	0,40

Appendix 6 | p-values and FDR (false discovery rates) for the DMSO-treated group with the two PFOS-treated groups

Table 20 | p≥0.05, p-values below 0.05 are *italicized*, and FDR (False discovery rate) ≥0.1, FDR-values below 0.1 are **bolded**.

Name	**5% DMSO | Average peak area**	**p-value | DMSO vs 0.1 µg/g PFOS**	**FDR | DMSO vs 0.1 µg/g PFOS**	**p-value | DMSO vs 1.0 µg/g PFOS**	**FDR | DMSO vs 1.0 µg/g PFOS**
PC(O-40:6)	0,256545528	*2,3E-05*	**0,00019**	*5,3E-09*	**9,1E-07**
TG(56:5)	0,018935718	*3,5E-04*	**0,00019**	*8,3E-10*	**1,9E-07**
PC(40:5)	0,024947359	*1,3E-04*	**0,00024**	*2,7E-08*	**2,6E-06**
TG(54:6)	0,006593842	*4,3E-02*	**0,00037**	*1,9E-07*	**1,1E-05**
SM(d18:0/22:0)	0,025633445	*2,5E-04*	**0,00043**	*1,6E-07*	**1,0E-05**
PC(36:4)	0,024441886	*3,6E-05*	**0,00045**	*1,8E-08*	**2,0E-06**
PC(36:1)	0,02443508	*2,3E-03*	**0,00052**	*4,8E-06*	**1,8E-04**
TG(56:6)	2,256980473	*8,9E-04*	**0,00053**	*1,5E-08*	**2,0E-06**
TG(45:0)	0,005803279	*1,4E-03*	**0,001**	*1,1E-05*	**3,4E-04**
LPC(16:0e)	2,320520534	3,5E-01	**0,0011**	*1,5E-03*	**9,9E-03**
PC(32:0)	0,042530085	*2,5E-04*	**0,0011**	*2,9E-06*	**1,2E-04**
TG(56:5)	0,068401684	*4,3E-03*	**0,0011**	*2,0E-05*	**5,2E-04**
PC(38:5)	0,345062275	*5,3E-03*	**0,0020**	*1,5E-07*	**1,0E-05**
LPC(18:1)	1,564184265	3,5E-01	**0,0023**	*8,7E-04*	**0,0070**
TG(56:5)	0,052990645	5,0E-01	**0,0023**	*4,3E-04*	**0,0045**
DG(18:0/20:4)	0,243252188	*2,4E-02*	**0,0023**	*1,2E-04*	**0,0019**
TG(37:0)	0,005446921	*2,1E-02*	**0,0023**	*2,3E-04*	**0,0032**
SM(d42:2)	0,008014086	*5,1E-03*	**0,0023**	*4,1E-04*	**0,0045**
TG(18:2/18:1/18:1)	0,191697763	3,5E-01	**0,0029**	*1,1E-04*	**0,0018**
TG(50:5)	0,006245299	5,5E-02	**0,0030**	*5,7E-04*	**0,0051**
TG(53:2)	0,543767235	3,5E-01	**0,0030**	*8,7E-05*	**0,0015**
TG(53:2)	0,00639171	*4,2E-04*	**0,0030**	*3,5E-04*	**0,0043**
LPC(18:0)	0,180491036	3,5E-01	**0,0032**	*1,2E-03*	**0,0085**
TG(49:3)	0,191487196	8,2E-01	**0,0032**	*2,2E-04*	**0,0032**
DG(18:0/20:4)	0,191400207	8,2E-01	**0,0032**	*2,2E-04*	**0,0032**
PC(P-18:0/22:6)	0,051825945	7,4E-01	**0,0039**	*1,7E-04*	**0,0027**

Name	**5% DMSO | Average peak area**	**p-value | DMSO vs 0.1 µg/g PFOS**	**FDR | DMSO vs 0.1 µg/g PFOS**	**p-value | DMSO vs 1.0 µg/g PFOS**	**FDR | DMSO vs 1.0 µg/g PFOS**
PC(35:3)	0,000407768	5,0E-01	**0,0040**	*1,5E-03*	**0,0099**
PC(36:2)	0,056971159	7,9E-02	**0,0043**	*3,6E-04*	**0,0043**
TG(54:3)	0,425915758	3,5E-01	**0,0043**	*4,5E-04*	**0,0045**
TG(56:9)	0,005786204	*5,0E-05*	**0,0043**	*3,8E-03*	**0,021**
PC(37:2)	0,015598754	4,6E-01	**0,0049**	*1,2E-02*	**0,053**
PC(O-32:1)	0,468207396	*3,1E-02*	**0,0050**	*1,1E-03*	**0,0083**
TG(54:0)	0,01602964	6,0E-01	**0,0051**	*5,8E-04*	**0,0051**
TG(49:1)	0,014315482	9,5E-01	**0,0056**	*9,8E-04*	**0,0077**
PC(38:3)	0,02216047	*4,4E-03*	**0,0056**	*0,0011*	**0,0082**
TG(51:3)	0,063118577	5,0E-01	**0,0057**	*0,036*	0,12
PC(33:0)	0,016481388	1,8E-01	**0,0061**	*0,0028*	**0,016**
SM(d41:1)	0,573696449	*1,5E-02*	**0,0070**	*0,0021*	**0,013**
TG(54:2)	0,01529581	*4,4E-02*	**0,0079**	0,069	0,20
PE(O-38:5) or PE(P-38:4)	0,061004404	*1,8E-02*	**0,0079**	*0,0030*	**0,017**
PC(38:2)	0,032377903	*8,8E-03*	**0,0079**	*0,0033*	**0,019**
PC(36:5)	0,006017487	7,8E-01	**0,0079**	*0,012*	**0,052**
TG(50:3)	0,01438405	*2,4E-03*	**0,0079**	*0,013*	**0,057**
LPC(18:0)	1,0348058	3,5E-01	**0,0080**	*0,00047*	**0,0046**
SM(d18:0/22:0)	0,031179284	1,5E-01	**0,0091**	0,062	0,19
TG(47:1)	0,434387648	2,0E-01	**0,0092**	*0,0013*	**0,0091**
PC(38:6)	0,114429599	*1,2E-02*	**0,0092**	*0,00044*	**0,0045**
TG(48:3)	0,061791394	1,5E-01	**0,0098**	*0,0027*	**0,016**
TG(56:5)	0,056052414	8,4E-02	**0,0098**	*0,000033*	**0,00073**
PC(38:4)	0,099678751	*1,6E-02*	**0,011**	*0,0061*	**0,031**
TG(54:6)	0,237910549	1,0E-01	**0,011**	*0,015*	**0,063**
TG(53:2)	0,027915333	*2,5E-03*	**0,011**	*0,015*	**0,062**
TG(50:1)	0,086677707	*6,7E-03*	**0,015**	*0,00070*	**0,0059**
CE(18:1) + Unknown CE(669,6358)	0,044235307	*2,3E-02*	**0,015**	*0,0086*	**0,041**
TG(54:7)	0,009194574	6,5E-02	**0,017**	0,37	0,40

Name	**5% DMSO | Average peak area**	**p-value | DMSO vs 0.1 µg/g PFOS**	**FDR | DMSO vs 0.1 µg/g PFOS**	**p-value | DMSO vs 1.0 µg/g PFOS**	**FDR | DMSO vs 1.0 µg/g PFOS**
TG(52:5)	0,120793854	*1,0E-03*	**0,020**	*0,028*	**0,097**
LPC(20:3)	0,027247924	*3,2E-04*	**0,020**	*0,017*	**0,068**
TG(54:0)	0,005474563	*6,9E-04*	**0,057**	0,052	0,16
PE(36:4)	0,02915859	*1,2E-04*	**0,072**	*0,016*	**0,066**
Fragment: CE signature ion	0,029325245	*3,1E-03*	**0,078**	*0,046*	0,15
TG(51:1)	5,230024417	*2,5E-04*	**0,089**	*0,009*	**0,044**
PC(36:4)	0,014052956	*2,2E-04*	0,40	0,098	0,26
PC(34:3)	0,037529698	*1,9E-03*	0,40	0,67	0,40

Appendix 7 | p-values and FDR (false discovery rates) for 0.1 µg/g PFOS-treated group compared with the 1.0 µg/g PFOS-treated group

Table 21 | p≥0.05, p-values below 0.05 are *italicized*, and FDR (False discovery rate) ≥0.1, FDR-values below 0.1 are **bolded**.

Name	1.0 µg/g PFOS | Average peak area	1.0 µg/g PFOS | Average peak area	p-value | 1.0 µg/g PFOS vs 0.1 µg/g PFOS	B-H | 1.0 µg/g PFOS vs 0.1 µg/g PFOS
PC(O-40:6)	0,44	0,54	*0,013*	0,12
TG(56:5)	0,031	0,038	*0,033*	0,25
PC(40:5)	0,035	0,041	*0,016*	0,13
TG(54:6)	0,0078	0,012	*0,0000044*	**0,00075**
SM(d18:0/22:0)	0,036	0,041	*0,047*	0,29
PC(36:4)	0,035	0,039	0,055	0,32
PC(36:1)	0,033	0,043	*0,0045*	**0,057**
TG(56:6)	3,2	3,7	*0,046*	0,29
TG(45:0)	0,0075	0,0091	*0,013*	0,12
LPC(16:0e)	2,7	0,11	0,33	0,40
PC(32:0)	0,059	0,064	0,20	0,40
TG(56:5)	0,092	0,11	*0,035*	0,25
PC(38:5)	0,48	0,63	*0,0039*	**0,052**
LPC(18:1)	1,8	0,11	0,33	0,40
TG(56:5)	0,057	0,075	*0,000010*	**0,00093**
DG(18:0/20:4)	0,31	0,39	*0,014*	0,12
TG(37:0)	0,0047	0,015	*0,000029*	**0,0014**
SM(d42:2)	0,011	0,012	0,47	0,40
TG(18:2/18:1/18:1)	0,22	0,020	0,36	0,40
TG(50:5)	0,0039	0,019	*0,000027*	**0,0014**
TG(53:2)	0,63	0,010	0,32	0,40
TG(53:2)	0,0083	0,0095	0,21	0,40
LPC(18:0)	0,21	0,024	0,34	0,40
TG(49:3)	0,19	0,099	*0,00040*	**0,0084**
DG(18:0/20:4)	0,19	0,099	*0,00040*	**0,0084**
PC(P-18:0/22:6)	0,054	0,024	*0,000061*	**0,0026**
PC(35:3)	0,00060	0,0043	*0,0015*	**0,025**

Name	1.0 µg/g PFOS | Average peak area	1.0 µg/g PFOS | Average peak area	p-value | 1.0 µg/g PFOS vs 0.1 µg/g PFOS	B-H | 1.0 µg/g PFOS vs 0.1 µg/g PFOS
PC(36:2)	0,074	0,10	*0,0075*	**0,076**
TG(54:3)	0,49	0,012	0,32	0,40
TG(56:9)	0,0077	0,0029	*0,0000036*	**0,00075**
PC(37:2)	0,017	0,025	*0,011*	0,10
PC(O-32:1)	0,60	0,72	0,12	0,40
TG(54:0)	0,017	0,0077	*0,00026*	**0,0064**
TG(49:1)	0,015	0,020	*0,0014*	**0,024**
PC(38:3)	0,031	0,047	*0,024*	0,19
TG(51:3)	0,090	0,13	0,29	0,40
PC(33:0)	0,020	0,028	*0,018*	0,15
SM(d41:1)	0,85	0,95	0,30	0,40
TG(54:2)	0,019	0,017	0,49	0,40
PE(O-38:5) or PE(P-38:4)	0,089	0,099	0,30	0,40
PC(38:2)	0,046	0,049	0,64	0,40
PC(36:5)	0,0059	0,029	*0,0076*	**0,076**
TG(50:3)	0,019	0,017	0,39	0,40
LPC(18:0)	1,2	0,038	0,33	0,40
SM(d18:0/22:0)	0,038	0,040	0,51	0,40
TG(47:1)	0,50	0,68	*0,0066*	**0,074**
PC(38:6)	0,16	0,19	0,073	0,39
TG(48:3)	0,077	0,098	*0,034*	0,25
TG(56:5)	0,069	0,080	0,19	0,40
PC(38:4)	0,14	0,19	0,13	0,40
TG(54:6)	0,30	0,34	0,23	0,40
TG(53:2)	0,035	0,013	*0,000014*	**0,0011**
TG(50:1)	0,11	0,13	0,058	0,33
CE(18:1) + Unknown CE(669,6358)	0,054	0,025	*0,00033*	**0,0072**
TG(54:7)	0,011	0,010	0,19	0,40
TG(52:5)	0,16	0,086	*0,000053*	**0,0024**
LPC(20:3)	0,043	0,038	0,33	0,40

Name	1.0 µg/g PFOS | Average peak area	1.0 µg/g PFOS | Average peak area	p-value | 1.0 µg/g PFOS vs 0.1 µg/g PFOS	B-H | 1.0 µg/g PFOS vs 0.1 µg/g PFOS
TG(54:0)	0,0074	0,0038	*0,00020*	**0,0059**
PE(36:4)	0,048	0,038	0,088	0,40
Fragment: CE signature ion	0,043	0,035	0,10	0,40
TG(51:1)	8,1	6,8	0,054	0,32
PC(36:4)	0,017	0,015	0,12	0,40
PC(34:3)	0,049	0,035	*0,0018*	**0,028**

Appendix 8 | Heat maps

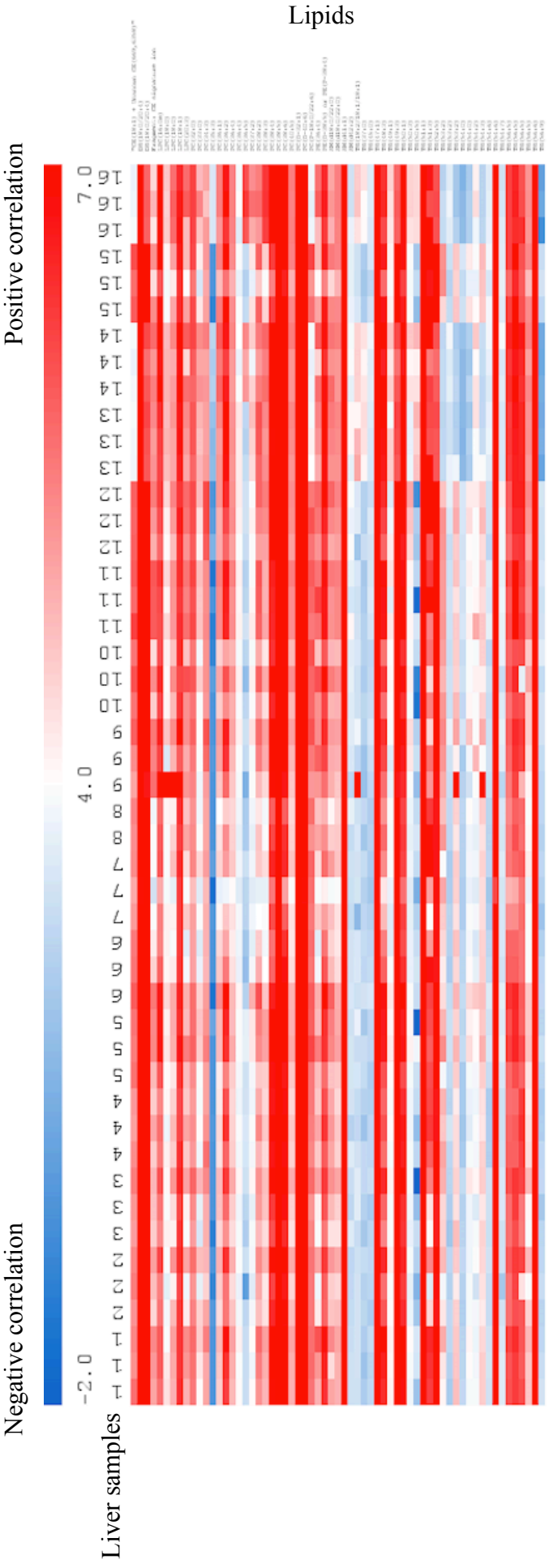

Figure 17 | The heat map over the liver samples

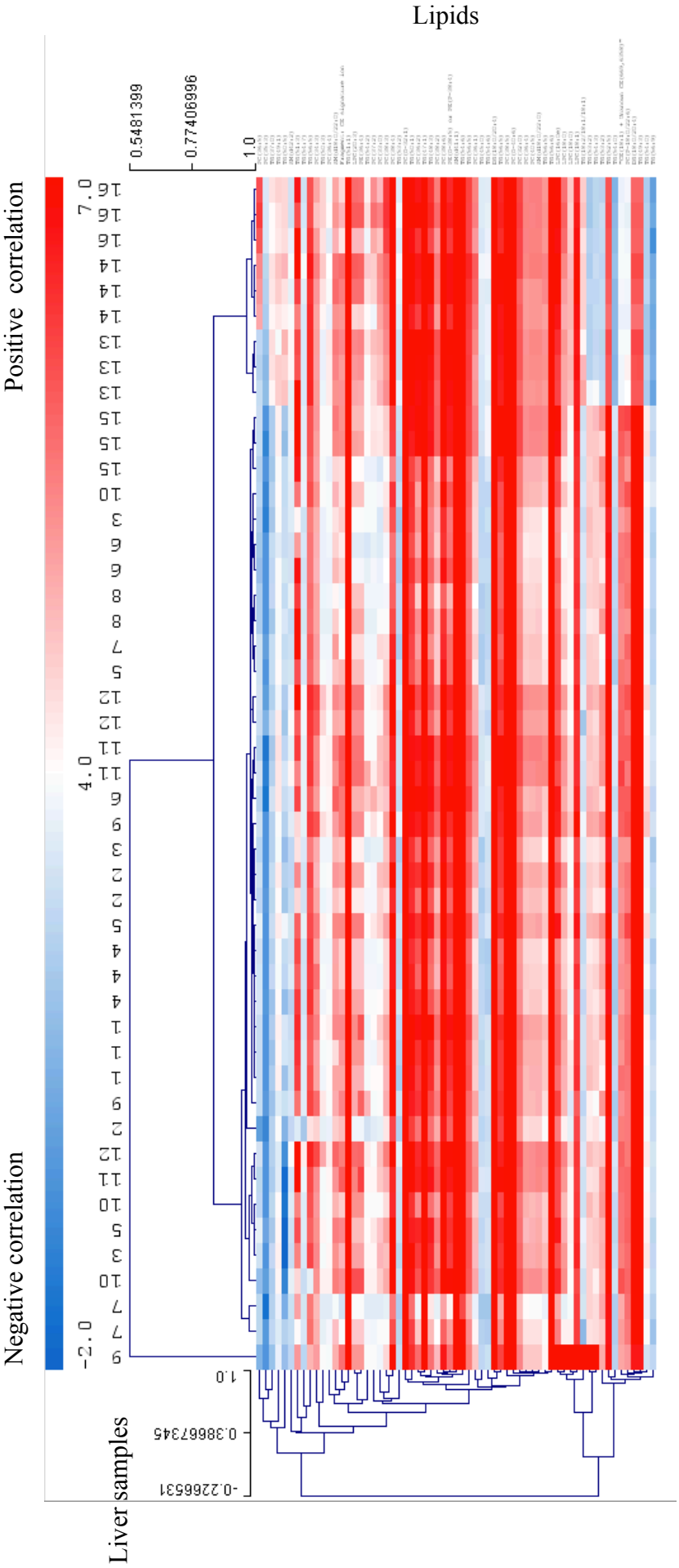

Figure 18 | The clustered heat map over the liver samples