Characterizing Triacylglycerol in Cell Culture:

Can LCMS replace HPTLC when assessing the effect of metformin in salmon *in-vitro* fatty liver model?

Monirul Hoque Pasha

Master Degree Thesis

for

The Erasmus Mundus Master in Quality in Analytical Laboratories (EMQAL)

University of Bergen

Bergen, Norway

September 2017

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September 2017

Dedication

To my beloved wife and dearly loved only son,

… Without whom this thesis could come to the daylight atleast a month before…

Contents

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Bergen, September 2017

List of Abbreviations

APCI	Atmospheric-Pressure Chemical Ionization
ATP	Adenosine Triphosphate
BHT	Butylated Hydroxytoluene
CI	Chemical Ionization
DAG	Diacylglycerol
ECN	Equivalent Carbon Number
EDTA	Ethylenediaminetetraacetic Acid
EI	Electron Ionization
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization of the United Nations
FBS	Foetal Bovine Serum
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
ICS	International Chemometrics Society
LCMS	Liquid Chromatography Mass Spectrometry
LLE	Liquid-Liquid Extraction
LSSR	Least Squares Spectral Resolution
MALDI	Matrix-Assisted Laser Desorption Ionization
MATLAB	Matrix Laboratory
MS	Mass Spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
OA	Oleic Acid
PBS	Phosphate Buffered Saline
PC	Principal Component
PCA	Principal Component Analysis
PLS	Partial Least Squares
SEM	Standard Error of Mean
SIMCA	Soft Independent Modeling of Class Analogy
SPE	Solid Phase Extraction
TAG	Triacylglycerol
TLC	Thin Layer Chromatography
UPLCMS	Ultra Performance Liquid Chromatography Mass Spectrometry
VBA	Visual Basic for Applications
WHO	World Health Organization

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Abstract

Fatty liver, a health complication for aquacultured fish is developed by the deposition of triacylglycerol (TAG) within liver cells. Metformin has been proven to be an effective drug for ameliorating fatty liver in human and rodent model; however, its action in the fish model is yet unknown. The preliminary aim of the present thesis was producing an oleic acid-induced *in-vitro* salmon fatty liver with excessive TAG accumulation and assessing whether metformin might reduce the TAG level. The cells were analyzed by high performance thin layer chromatography (HPTLC) that indicated the capacity of metformin to attenuate fatty liver.

HPTLC has been generally used as a conventional and reliable technique for determining the level of TAG in biological samples. But the determination of individual TAG species and positional distribution of fatty acids on the backbone of TAG demand employing additional method(s) because of the inability of HPTLC to do so. However, due to cost-cutting approach, modern laboratories are in need of finding means to simultaneously measure TAG level and elucidate TAG structure in the same sample by a single technique.

Liquid chromatography mass spectrometry (LCMS) has been widely used for structural elucidation of TAG; however, the ability of LCMS to measure the TAG level in salmon liver was not vastly studied. The present thesis, then principally aimed at evaluating the potential of LCMS as an alternative to HPTLC for quantitative analysis of TAG in cell cultures. MATLAB based Chrombox D and QueryTAG algorithmic software tools with several strategies were employed to examine the LCMS chromatogram and spectrum. An MS Excel-based macro-enabled tool has been developed for rapid and easy processing of Chrombox D data for multiple samples. The multivariate chemometric analysis was applied in further data exploration to understand significant effects of the variables. Most of the data analysis strategies indicated that LCMS can reproduce the outcome patterns of HPTLC for some treatments with the experimental condition for one hour with metformin exposure followed by incubation with oleic acid up to 48 hours. Therefore, it can be postulated that LCMS is a potential alternative to the traditional HPTLC for TAG analysis in modern analytical and research laboratories.

Key words: Triacylglycerol, Fatty liver, Metformin, LCMS, HPTLC, Chrombox D, QueryTAG, Multivariate analysis, PLS, PCA.

Chapter 1 Introduction

Fish is not only delicious and healthy for human consumption but also the most resource efficient animal protein available to humankind [1] providing nearly 16% of the animal protein consumed by the world's population [2]. In the past, wild fisheries have been the primary resource for fish, but, due to full and over-exploitation; nearly 85% of wild-caught fishes are depleted. Consequently, as a profitable and ecologically viable alternative, aquaculture industries around the globe have thriven to meet the increasing demand that fulfilling nearly half of the world's demand for fish consumption [3]. Thus, fisheries sectors are greatly contributing to achieving world's food security and improving nutrition in line with the Millennium Development Goal (MDG) - Sustainable Development Goal (SDG) 2 reported by FAO [4].

However, one of the major factors hugely affecting the business of aquaculture production and the overall economy with growing fish farming has been the fish diseases. Apart from many other challenges, the aquaculture industries are struggling to control and prevent fish diseases and the scientific communities are striving ceaselessly to invent appropriate measures and thereby, contriving to develop updated and effective techniques to address the issue.

1.1 Fatty liver

Fatty liver is one of the major chronic health problems for most long-term captive fishes and might be a health concern for aqua-cultured marine fishes as well. Fatty liver is developed by the deposition of triacylglycerol (TAG) within hepatocytes- the major cells in the liver, responsible for its function [5]. This generally may arise from defective fatty acid metabolism that may be due to energy intake and combustion imbalance, by mitochondrial damage, by insulin resistance, or by impairment of receptors and enzymes involved [6]. Once developed, it can lead to many health complications such as liver degeneration [7]. Apart from degrading the flesh quality, fatty liver disease often makes the fishes immuno-compromised and prone to suffer epizootics (a contagious disease event in animal population, analogous to an epidemic in humans), such as vibriosis [8], a systemic fish disease caused by bacteria *Vibrio* spp. [9].

Excessive feeding of energy-rich diets to the farmed fish can result in fatty degeneration of the liver [10]. Again too little of several nutrients can result in fish changing their lipid storage pattern. In particular, too little of the amino acid methionine, marine omega-3 fatty acids, and phospholipids in the fish feed increases the storage of lipids in the liver of Atlantic salmon [11].

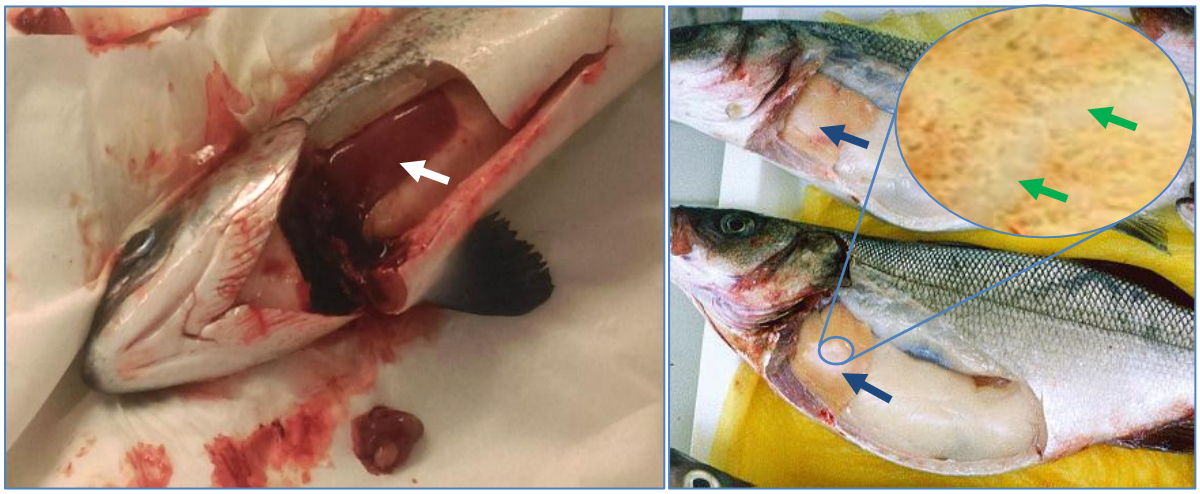


Figure 1.1 Difference between normal and fatty liver is marked by deposition of fat. The left picture shows a typical and normal salmon liver (indicated by white arrow) (photo taken in cell and molecular biology lab at NIFES). The right one depicts a pale, fatty liver (indicated by blue arrows) result from overfeeding with high-energy diets in sea bass where the foci and larger areas of the liver parenchyma have been replaced by fatty tissue (on inset enlarged view- indicated by green arrows) (photo source: http://www.vetcare.gr/ARTPRES/pics_santiago_143_msw10.htm)

Fatty liver is commonly seen in adult fish as they grow considerably slower than younger whose growth rate is faster enough to minimize fat deposition. The main source of deposited fat is animal or fish-diet, which is taken up in high amount. In fish, the deposition of fat occurs in various tissues, especially in the liver as shown in Figure 1.1 and unlike in mammals, this fat is not readily usable to fish during times of starvation [7]. It is yet unknown the required level of lipid or fat to be stored in the fish liver for developing liver degeneration and inflammation whereas, in the case of humans and rodents, it requires more than 5% of lipids to constitute liver damage leading to health challenges [11].

1.2 Triacylglycerol

A triacylglycerol, in short TAG (in other names, triglyceride or triacylglyceride) is a tri-ester derived from one glycerol and three fatty acids as shown in Figure 1.2. TAGs are neutral lipids that serve as a source and storage of energy in mammalian cells. They are the main constituents of body fat in humans and other animals, as well as plant fat [12].

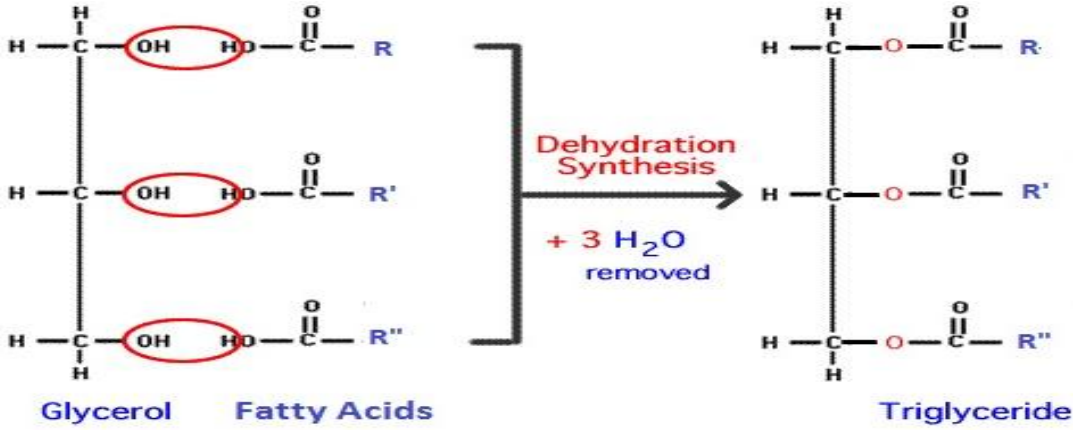


Figure 1.2 Formation of TAG through esterification from one glycerol and three fatty acids.

Amongst many different types of TAGs, the mains are saturated – all three fatty acids on its backbone are saturated and thereby having a higher melting point and more likely of being solid at room temperature, and unsaturated – at least one double bond in any of three fatty acids and thereby having a lower melting point and more likely of being liquid at room temperature.

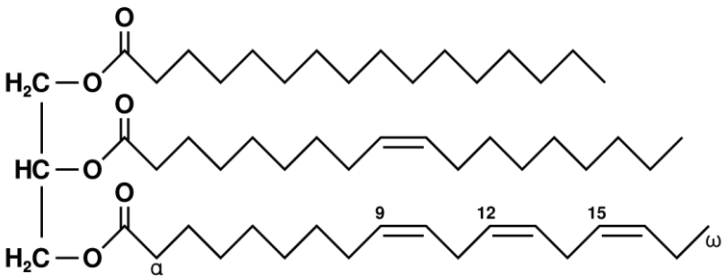


Figure 1.3 Example of an unsaturated TAG (C55H98O6). Left part: glycerol; right part, from top to bottom: palmitic acid, oleic acid, alpha-linolenic acid.

The glycerol molecule shows a plane of symmetry. On the other hand, the two primary hydroxyl groups esterified with different acids produce an asymmetric glyceride that is optically active. For the reason of the difficulty arisen in applying conventional D/L (dextrorotatory/levorotatory) systems to the complex mixtures of TAGs found in the nature, the "stereospecific numbering" (*sn*) system as recommended by an IUPAC-IUB commission is considered as an alternative system of nomenclature which is nowadays appreciated by the biochemical scientific communities [13]. This system allows glycerol to be stereospecifically numbered (*sn*-glycerol) from top to bottom in the L-form of its Fischer projection (as shown in Figure 1.3) where the two primary hydroxyl groups are designated as *sn*-1 and *sn*-3 and the secondary one is marked at position *sn*-2 [14, 15].

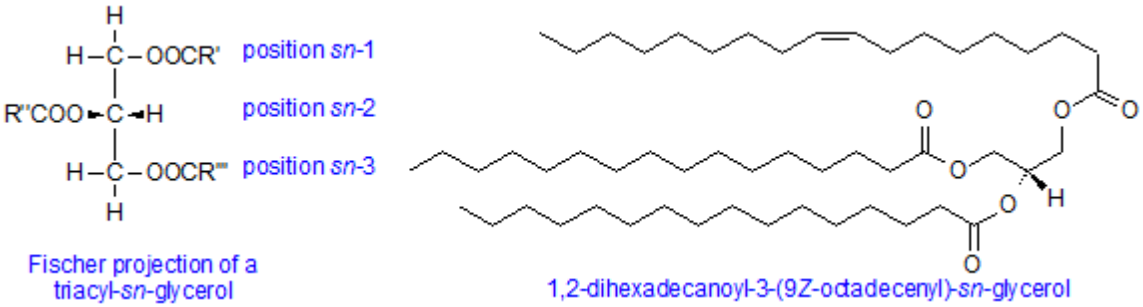


Figure 1.4 Schematic diagram of TAG molecule showing different stereospecific positions and three different fatty acids on its backbone, namely palmitic acid, oleic acid and alpha-linolenic acid.

TAGs contain three fatty acids at positions *sn*-1, *sn*-2, and *sn*-3 that may vary to yield a large diversity of TAGs [14, 15]. The number of possible TAG species increases with the different combination of fatty acids in three different positions, for example with three different fatty acid constituents, the number of possible TAGs rises to ten excluding isomers and eighteen including isomers [12].

1.2.1 Triacylglycerol metabolism and Fatty liver disease in fish

Like other animals, fish also use TAG as the primary energy depot. Fish have the unique capability of metabolizing TAG readily and, subsequently, during starvation or lack of food, can survive for long periods of time. A typical example is the many weeks of migration by salmon in their return upstream to spawn while fish use the stored TAGs to enable body processes to continue during the strenuous journey [16]. This is why; wild fish generally do not accumulate TAG for a long time. Unlike the wild fish, the farmed or aqua-cultured fish such as salmon do not encounter any situations that lead to metabolizing stored TAGs and as a consequence, promoting increased storage of TAG in the liver of which over time might cause fatty liver disease as described in man and rodents. Studies elsewhere reported that in farmed or aqua-cultured salmon, the greater levels of total lipid including TAG accumulation were found than in wild salmon [17, 18].

1.3 Chemometrics - multivariate analysis

The term ‘Chemo’ refers to chemistry and the ‘metrics’ refers to mathematical or statistical methods. Chemometrics is a discipline of chemistry where optimal mathematical or statistical methods are applied to translate complex chemical data into relevant and meaningful information [19].

The definition of chemometrics by The International Chemometrics Society (ICS) is: “Chemometrics is the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods” [20].

Chemometrics is very useful to remove redundant data, reduce unrelated variation among analytical signals and build models to predict newer data set. As chemometrics requires multiple variables to interpret the data, chemometricians also call it multivariate analysis. Multivariate analysis is the set of statistical or mathematical methods that perform on a certain data set containing multiple measurements (variables) and samples (objects) to analyze the interactions between them to get multiple predictions. One main objective of the multivariate analysis is to decompose mixed and complex data structure into its components. Multivariate analysis can be employed for several purposes categorized mainly into three – exploratory data analysis, classification and discrimination, and regression modeling. Commonly employed multivariate techniques include principal component analysis (PCA),

soft independent modeling of class analogy (SIMCA) and partial least squares (PLS) [21]. PCA is used for exploration of analytical data and classification to extract sources of variation in the order of significance and identify the outliers among data set. In the present thesis, the emphasis is given on PLS for regression coefficient and PCA for the purpose of exploratory data analysis.

In chemometrics, PLS regression has become a standard tool for modeling linear relations between multivariate measurements [22]. It reduces the independent variables to a smaller set of uncorrelated components. The regression coefficient is a projected scatter plot of the coefficients that are derived from the unstandardized regression displaying the sign and the magnitude of the relationship between independent and dependent variables [23].

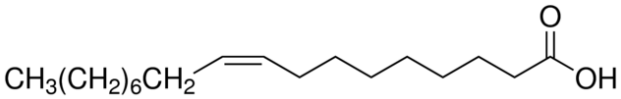
The main idea of PCA is to reduce the dimensionality of a data set consisting of a large number of interrelated variables retaining higher variation in the data set. This is achieved by transforming to a new set of uncorrelated variables that retain most of the variation in all the original variables. These new variables are called ‘principal components’ (PCs) [24]. The PCs are given as vectors of loadings and scores where they represent a basis for respectively the variable space and the object space. Plotting the objects on the loading vectors and the variables on the score vectors shows the relationships between respectively objects and variables. The reproduced variable space and object space can be combined into one plot – a biplot [21] The first PC is the major axis of the points in the p-dimensional space that explains maximum variation in the data whereas, the second PC is perpendicular to the first PC and defines the next largest amount of variation that left unexplained by the first PC. Graphical representation of PCs obtained can be used to look for meaningful and relevant information and to distribution and classification patterns of the data [14, 15, 21].

Chapter 2 Assessment of impact of metformin in fatty liver model using oleic acid to induce TAG accumulation in primary hepatocytes isolated from Atlantic salmon

2.1 Background

2.1.1 Oleic acid

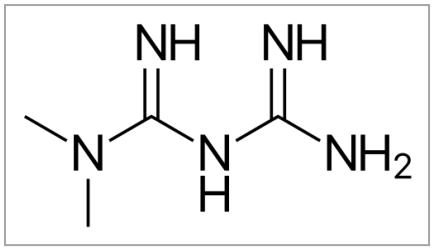
Oleic acid is a monounsaturated omega-9 fatty acid with a molecular formula of $C_{18}H_{34}O_2$ and a molecular weight of 282.46 g/mol [25]. It is the most widely distributed and abundant fatty acid in nature. It is mainly found in various animal and plant fats and oils in glycerol ester i.e. triacylglycerol form [26]. In chemical terms, oleic acid is named as *cis*-9-octadecenoic acid and abbreviated with a lipid number of 18:1 cis-9. The term "oleic" refers to olive oil that is mostly composed of oleic acid.



In cell culture systems, oleic acid serves as a long-term energy supplier as the NADPH and ATP derived energy usually stores in fatty acids. The stored energy is released upon the degradation of oleic acid. Oleic acid is esterified to a glycerol backbone to form a group of compounds known as mono-, di- and triacylglycerols [27]. Oleic acid shows the fatty acid specific effect on lipid synthesis in animal cell and supplementation with oleic acid leads to triacylglycerol accumulation [28]. Addition of oleic acid at 300 µM concentration in cell culture increased the amount of total cellular triacylglycerol at 20-40% [29].

2.1.2 Metformin

Metformin is the most widely used medication for diabetes that is taken orally [30]. It is the first-line medication for the treatment of type 2 diabetes and listed as WHO recommended most effective and safe medicine needed for the health system, especially type 2 diabetes [31]. Metformin is used along with diet and exercise to reduce blood sugar levels in patients with type 2 diabetes [32]. The chemical name and 2-D structure of metformin is given below:



Metformin; 1,1-Dimethylbiguanide; 657-24-9; Glucophage; Glumetza; Dimethylbiguanide

Source: https://pubchem.ncbi.nlm.nih.gov/compound/metformin#section=2D-Structure

In mammals and other animals, metformin has been used as an effective drug with reportedly several actions, including lowering blood glucose by suppressing gluconeogenesis [33, 34], stimulating glycolysis [35], preventing hepatic steatosis [36, 37], and ameliorating hepatic inflammation [37].

Though the complete mode of action of metformin is yet to be revealed, one of the potential mechanisms of action that has been proposed is activation of AMP-activated protein kinase (AMPK) enzyme [38] that plays an important role in changing energy metabolism to increase ATP production [39].

Nevertheless, the impact of metformin on the fish species in cellular lipid metabolism and inhibition of TAG synthesis is still incompletely understood. It has been reported that in rainbow trout dietary metformin showed a hypoglycemic effect due to induction of hepatic lipogenesis leading to fatty acid synthesis [40] and inability to improve glucose homeostasis [41]. Hertz et al. (1989) reported that metformin inhibits gluconeogenesis and surmised that the mode of action of metformin is similar in fish and mammals [42]. In mammals, metformin has been found having an impact on fatty liver disease. Metformin inhibits lipid deposition in skeletal muscle through fatty acid oxidation [43] and prevents fatty liver disease in mice [44, 45].

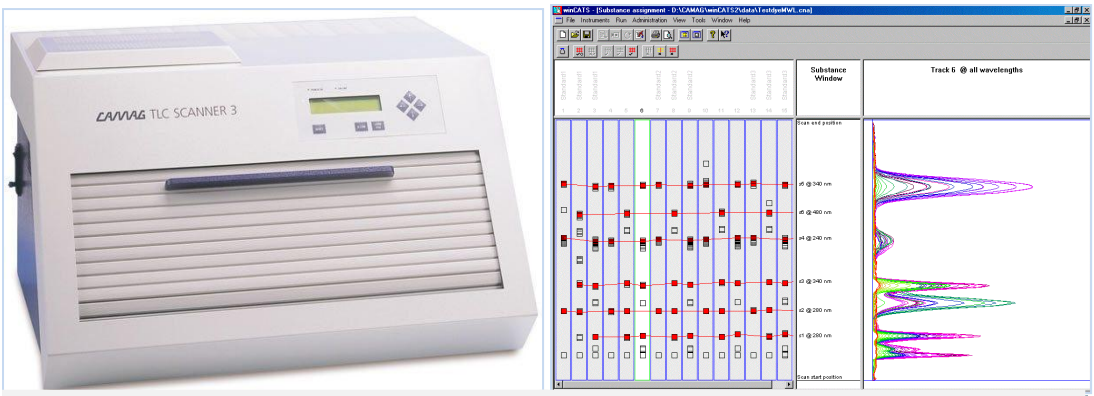
Metformin is not used *in vivo* in aquaculture or farmed and wild fishes due to its side effects. Studies have suggested that exposure to metformin at environmentally relevant concentrations causes potential endocrine disruption in adult male fish [46], metformin from wastewater contaminant causes intersex and reduced fecundity in fish [47] and feminizes male minnows and affects fertility [48].

2.1.3 High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) has the principle of separation is adsorption. The mobile phase solvent flows through the plate because of capillary action. The components move according to their affinities towards the adsorbent in the plate normally silica that works as the stationary phase [49]. The component that has the higher affinity towards the stationary phase travels slower than the components with lesser affinity. Thus the components are separated on a chromatographic plate. The separation of the components are spotted and finally visualized by a scanner with, for instance, non-destructive UV light. Quantification is based on comparing chromatograph with standard run on the same plate [50].

An HPTLC system for high sample throughput analysis includes sample application, chromatogram development, and evaluation steps that involve at least one instrument for every step. As illustrated in Figure 2.1, a CAMAG HPTLC system is comprised of a CAMAG Automatic TLC Sampler (ATS4), an Automated Multiple Development (AMD2) Chamber, a TLC Scanner 3 and a Viewing cabinet with Camag's integrated winCATS software, which incorporates all steps of the instrumental process [51].

	
CAMAG Automatic TLC Sampler (ATS4) Pre developed TLC plate is placed to imprint the sample using automatic sampler	CAMAG Automated Multiple Development (AMD2) Chamber Plate development with mobile phase solvents (Polar solvents and Neutral solvents) are done here



CAMAG TLC Scanner 3

TLC plate is scanned here using UV lamp to spot the bands of components in the sample separated due to the adsorption chromatography

CAMAG viewing and documentation software winCATS

Scanned image is analyzed by the winCATS software installed in the computer connected to the system and the presence of different components in samples are studied and quantified based on band intensity of standard.

Figure 2.1 HPTLC system for quantitative analyses including evaluation with TLC Scanner 3 and image documentation, including winCATS software for control of instruments. Illustrations were collected from http://www.camag.com/en/tlc_hptlc/complete_systems/advanced_systems/HPTLC_System_for_quantitative_analyses_high.cfm

2.2 Significance of the study

Fatty liver is one of the health complications for farmed or aqua-cultured salmon. This issue remains ethically a concern for salmon health. Metformin has been known to have the capability of ameliorating fatty liver in human and other mammals. The *in-vivo* use of metformin is not permissible in many countries including Norway; therefore, the *in-vitro* trial of metformin might be useful to acquire valuable information on its impact on the salmon fatty liver.

2.3 Objective

One objective of this thesis was to assess the effect of metformin in an oleic acid-induced *in-vitro* fatty liver model using salmon liver cell. Some distinct strategies as mentioned below were followed to achieve the objective:

- Producing *in-vitro* fatty liver model by inducing accumulation of TAGs by oleic acid and treating with metformin.
- Monitoring the levels of TAGs by HPTLC.
- Employing multivariate chemometric strategies to comprehensively elucidate the effect of metformin on fatty liver development.

2.4 Materials and Method for liver cell preparation

2.4.1 Sample, reagents and culture medium

The salmon liver cells were cultured and harvested at cell and molecular biology lab at NIFES during June-July 2016 and immediately after preparation, cells were frozen at -80 °C for long-term preservation until analysis.

- Liver cells were isolated from six healthy Atlantic salmon (Salmo salar) with average body weight of 500 g obtained from Bergen Aquarium located in Nordness.
- L-15 medium was supplemented with 10% foetal bovine serum (FBS) (BioWhittaker, cat#14e801F), pen/strep (50U/mL, Bio- Whittaker, cat#17-602E), 2% 2 mM glutamaxTM 100x (Gibco, cat#35056) and was designated complete medium (cL-15). Washed liver cells were re-suspended in cL-15 medium and counted using a Bürcher chamber and 0.4% trypan blue solution (BioWhittaker, cat#17-942E).
- Each insert (ThinCerts™ 0.4 u, # 657641, Greiner bio-one) and wells of six-well culture plates were coated with laminin (1-2 mg/cm2, Sigma L2020) for 24 hours at room temperature. The laminin solution was then removed and the wells were allowed to dry before adding the liver cell suspensions.

2.4.2 Isolation of liver cells

The fish were anaesthetized by metacaine (MS222, 0.5 g/10 L) and the livers were perfused with a 0.09 MHepes buffer containing 1.4 M NaCl, 0.067 M KCl and 0.03 M EDTA, pH 7.4 at a flow of 4 ml/min until free of blood. Thereafter the livers were digested with collagenase

(0.1% collagenase type IV was dissolved in the 0.9 M Hepes buffer as used for perfusion). The isolated cells were harvested in 10 ml 10% phosphate-buffered saline buffer (PBS buffer: 0.002 M KH2PO4, 0.02 M Na2HPO, 0.03 M KCl and 0.14 M NaCl, pH 7.4), filtrated through a 100 mm mesh cell strainer and washed twice in the PBS buffer, re-suspended in cL-15 medium before the viability of the isolated cells was assessed. All centrifugations were done at 50×g for 5 min. The isolations of cells were done with sterile equipments and buffers. The liver cell isolation protocol followed here was published elsewhere [52, 53].

2.4.3 Cell cultures and harvesting cells

For cultures, the liver cells count of 0.8×10^6 cells per square centimeter were added to six-well culture plates (Costar, cat#3335) and cL-15 medium was added to a final volume of 2 ml. From each fish, total 14 culture wells were prepared by several combinations of two concentrations of metformin (analytical grade), purchased from Sigma-Aldrich (St. Louis, MO, USA) and two concentrations of oleic acid (water-soluble powder, suitable for cell culture) from Sigma-Aldrich (St. Louis, MO, USA). The two incubation setup for metformin and oleic acid are briefly described in the following section. Induced, treated and untreated liver cells were harvested at 24 or 48 hours post oleic acid addition. The medium was removed and the cells were washed in PBS before cells were collected in 1.5 ml Eppendorf tubes and frozen at -80 °C until the extraction of triacylglycerol for chemical analysis The liver cell culture and harvesting protocol followed has been published elsewhere [52, 53].

2.5 Experimental

2.5.1 Reagents and standards

Chloroform, diethyl ether, methyl acetate, potassium chloride, copper(I) acetate, ortho-phosphoric acid, isohexane, butylated hydroxytoluene (BHT), acetic acid, hexane and methanol (HPLC grade > 99.9%) used for LLE and HPTLC were from Merck (Darmstadt, Germany). Isopropanol used for HPTLC was from Kemetyl (Norway). De-ionized and purified water in a Milli-Q system was used throughout the experiments (Millipore, Milford, USA). The lipid standard for HPTLC analysis including the TAG (trilinolenin) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.5.2 Experimental design for sample production

The salmon liver cells were cultured, treated and harvested in accordance with a pre-planned experimental design. A total of 84 cell cultures were conditioned from 6 fish where each fish produced a total of 14 samples. 7 samples were incubated for 1 hour with metformin and following 47 hours with oleic acid whereas the rest 7 samples got 24 hours incubation with metformin followed by further 24 hours incubation with oleic acid. 4 of each 7 samples were prepared in combination of treatment with metformin at two different concentrations (0.1 and 10 mM) and inducement with oleic acid at two different concentrations (0.2 and 10 mM). Two samples out of each 7 samples contain no metformin but oleic acid at concentrations levels of 0.2 and 10 mM and the rest one sample had been exposed to neither metformin nor oleic acid and served as control.

That is, half of the cultures were incubated for 1h with metformin that later received more 47 hours incubation after addition of oleic acid and the rest half were incubated for 24 hours with metformin that incubated further 24 hours after addition of oleic acid. Untreated cultures were considered as controls. For each of 6 fish, cell culturing conditions and treatments are summarized in Table 2.1.

Table 2.1 Cell culture wells with cell type and conditions; metformin (Metf) at 0.1 and 10 mM and oleic acid (OA) at 0.2 and 0.4 mM additions.

Treatments	**Experimental Condition X**	**Experimental Condition Y**
	Incubation for 1 h with Metf + 47 h with OA	Incubation for 24 h with Metf + 24 h with OA
	$X_{Metf/OA\ (mM/mM)}$	$Y_{Metf/OA\ (mM/mM)}$
Liver cell control	$X_{0/0}$	$Y_{0/0}$
Liver cell control + 0.2 mM OA	$X_{0/0.2}$	$Y_{0/0.2}$
Liver cell + 0.1mM Metf + 0.2 mM OA	$X_{0.1/0.2}$	$Y_{0.1/0.2}$
Liver cell + 10mM Metf + 0.2 mM OA	$X_{10/0.2}$	$Y_{10/0.2}$
Liver cell control + 0.4 mM OA	$X_{0/0.4}$	$Y_{0/0.4}$
Liver cell + 0.1mM Metf + 0.4 mM OA	$X_{0.1/0.4}$	$Y_{0.1/0.4}$
Liver cell + 10mM Metf + 0.4 mM OA	$X_{10/0.4}$	$Y_{10/0.4}$

2.5.3 Sample preparation and liquid-liquid extraction

During preparation for analysis, the liver cell was added to an equal volume of glass pellets (2-3 pieces), suspended in 1 ml of chloroform with 0.01% BHT (as an antioxidant to prevent rancidity of TAG) and vortex-mixed twice for 30 seconds. The sample was left at 4 °C overnight. Next day, the sample was centrifuged at 13000×g for 10 min. Glass pellets were sedimented at the bottom and cell debris was layered at the top whereas in the middle the bright chloroform phase was seen and aspirated carefully using micropipette without disturbing top layer. The bright chloroform phase was collected into previously labeled 10 ml vial, allowed to dry under a stream of nitrogen and submitted to the liquid-liquid extraction**.**

The LLE protocol has been published elsewhere [54] and slightly modified for TAG extraction from salmon liver cells. Briefly, the dried residue is dissolved in successive 2 ml aliquots of methanol, hexane and 0.2 ml of water (10:10:1 v/v), vortex-mixed for 30 s, centrifuged at 3000×g for 3 min. A clear separation between upper hexane layer and lower methanol phase was observed and the upper hexane layer was collected that ideally contains the TAGs. Aliquots of 2 ml of methanol and 0.2 ml of water were further added into the collected hexane phase, vortex-mixed and centrifuged at 3000×g for 3 min. After phase separation, the hexane phase washed one more time with successive 2 ml aliquots of methanol and 0.2 ml water. The final hexane layer was collected into 10 ml Falcon centrifuge tube (VWR, Radnor, PA, USA) and dried under a stream of nitrogen, weighed and redissolved in chloroform at 5 mg/ml. A general diagram of the LLE procedure is shown in Figure 2.2.

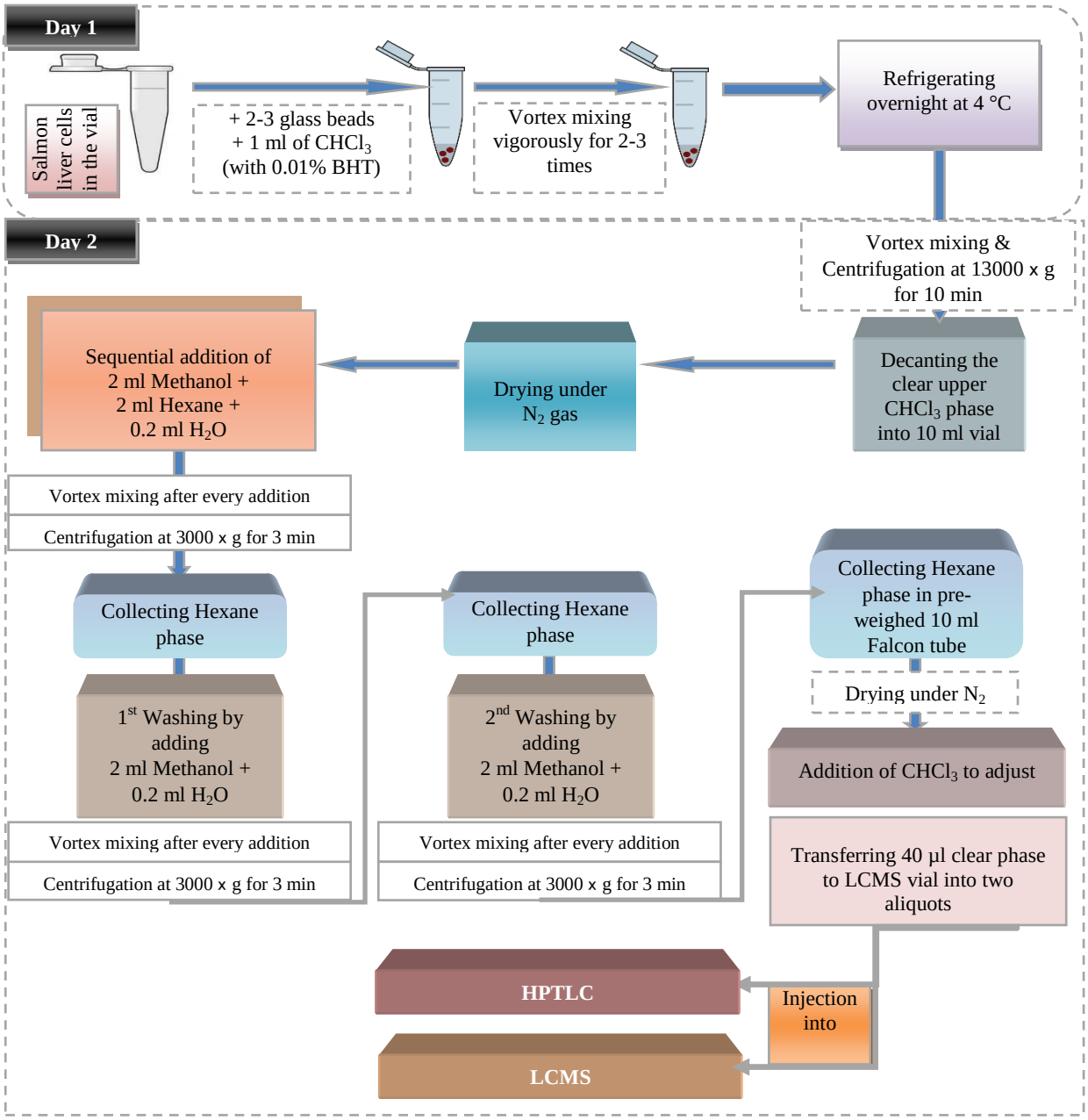


Figure 2.2 Liquid-liquid extraction procedure

2.5.4 High performance thin layer chromatography instrumentation

The HPTLC protocol is part of the methods developed by NIFES for determining lipid classes in oils, tissue and biological fluids and archived as method number MET.NÆR.01-25. 25. The precision of the method was lower than 15% of the coefficient of variation (CV=100 × σ/µ), the recovery was between 80% and 105% and the limits of quantification for the TAG was 0.024 mg/ml.

Briefly, the sample redissolved in chloroform at 5 mg/ml is submitted for HPTLC analysis. The various standards used for HPTLC were individually diluted to 0.1 mg/ml by adding chloroform (0.01% BHT). The HPTLC plates 20×10 cm and silica 60 were from Merck (Darmstadt, Germany). The plate was pre-cleaned by eluting the polar solution (KCl: methanol: chloroform: isopropanol: methyl acetate, 9:10:25:25:25, v/v) way up to the top of the plate in a 20×10 cm glass tank. The plate was dried and activated in an oven at 110 ° C for 30 min. Standards and samples (1µl each) were applied to the plate with a digital microdispenser (ATS4, Camag, Switzerland). Lipids were first eluted with a polar solution in an automatic development chamber (AMD2, Camag, Switzerland) until the elution goes up to 48 mm. After 30 min, the plate was wiped and neutral lipids were further eluted with a neutral solution (isohexane:diethyl ether:acetic acid, 80:20:1.5, v/v) up to 88 mm. The plate was dried for 20 min. After removing the plate from the development chamber, it was dipped into a glass tank with developing solution (3% copper (I) acetate and 8% ortho-phosphoric acid) and developed for about 10 seconds. The liquid was drained and dried in an oven at 160 °C for 15 min. The Plate was cooled at room temperature and scanned by a D lamp (Scanner3, Camag, Switzerland) at 350nm. TAGs in the sample were identified by comparing with the standard band.

Concentrations of the chromatographed compounds were determined automatically from the intensity of the absorption via peak areas using winCATS Planar Chromatography Manager version 1.3.3 (Camag, Switzerland). The weight (*W*) in mg TAG/ sample was calculated by the expression:

$$W = \frac{(y - b) \times f}{a \times m} \ldots\ldots\ldots\ldots Equation\ 1$$

Where *y* is the corrected area of the absorption peaks, *a* and *b* are the slope and intercept of the calibration curve respectively, *f* is the dilution factor and *m* is the amount of sample.

2.6 Results and Discussion

2.6.1 Analysis of HPTLC derived data

The amounts of total TAG in each sample obtained after performing HPTLC by using Equation 1 are arranged in Table 2.2. In total 60 samples of six biologically similar fish with 10 different treatments were analyzed, where each treatment (including control) had six replicates. Three samples ($X_{10/0.4}$ for fish 3 and $Y_{0.1/0.2}$ for fish 1 and 2) were found having response below the limit of detection. This might have happened due to over dilution and therefore, were not estimated and included in this analysis. The six controls for fish in two different experimental conditions ($X_{0/0}$ and $Y_{0/0}$) showed a variable amount of TAG. But ideally, they should have responded similarly as both had been subjected to 48 hours incubation with neither any treatment by metformin nor inducement by oleic acid.

Table 2.2 Total amount of TAGs (in mg) per sample determined by HPTLC analysis where X denotes 1 hour treatment with metformin (Metf) and following 47 hours inducement with oleic acid (OA) whilst Y denotes 24 hours treatment with metformin and following 24 hours inducement with oleic acid. The subscripted numbers indicate the concentrations of metformin (numerator) and oleic acid (denominator) at 0, 0.1, or 10 mM for metformin and 0, 0.2, 0.4 mM for oleic acid.

Treatment$_{Met/OA}$	**$X_{0/0}$**	**$X_{0/0.4}$**	**$X_{10/0.4}$**	**$Y_{0/0}$**	**$Y_{0/0.2}$**	**$Y_{0.1/0.2}$**	**$Y_{10/0.2}$**	**$Y_{0/0.4}$**	**$Y_{0.1/0.4}$**	**$Y_{10/0.4}$**
(mM/mM)	1h Metf + 47h OA			24h Metf + 24h OA						
Fish 1	0.47	2.66	1.52	**1.42**	0.27	ND	0.74	0.32	0.93	1.78
Fish 2	0.43	1.43	1.21	0.33	0.12	ND	0.02	0.29	0.26	0.88
Fish 3	**1.90**	1.76	0.35	**1.36**	**1.42**	**1.98**	0.56	**2.17**	1.31	1.19
Fish 4	0.08	0.68	ND	0.22	0.28	0.26	0.26	0.47	0.73	0.47
Fish 5	0.08	0.40	0.19	0.11	0.16	0.12	0.14	0.34	0.21	0.42
Fish 6	0.35	0.76	0.54	0.38	0.64	0.46	0.64	0.47	0.70	0.44
Mean	0.55	1.28	0.76	0.64	0.48	0.70	0.39	0.68	0.69	0.86
SEM	0.28	0.34	0.26	0.24	0.20	0.43	0.12	0.30	0.17	0.22

Metf- Metformin, OA- Oleic acid, SEM= the standard error of the mean, ND- not detected, Bold entries- outlying values by Iglewicz and Hoaglin's robust test for multiple outliers.

The standard error of the mean (SEM) was calculated by the following expression:

$$SEM = \frac{SD}{\sqrt{n}} \ldots\ldots\ldots\ldots Equation\ 2$$

Where SD is the Standard deviation and n is the total number of samples.

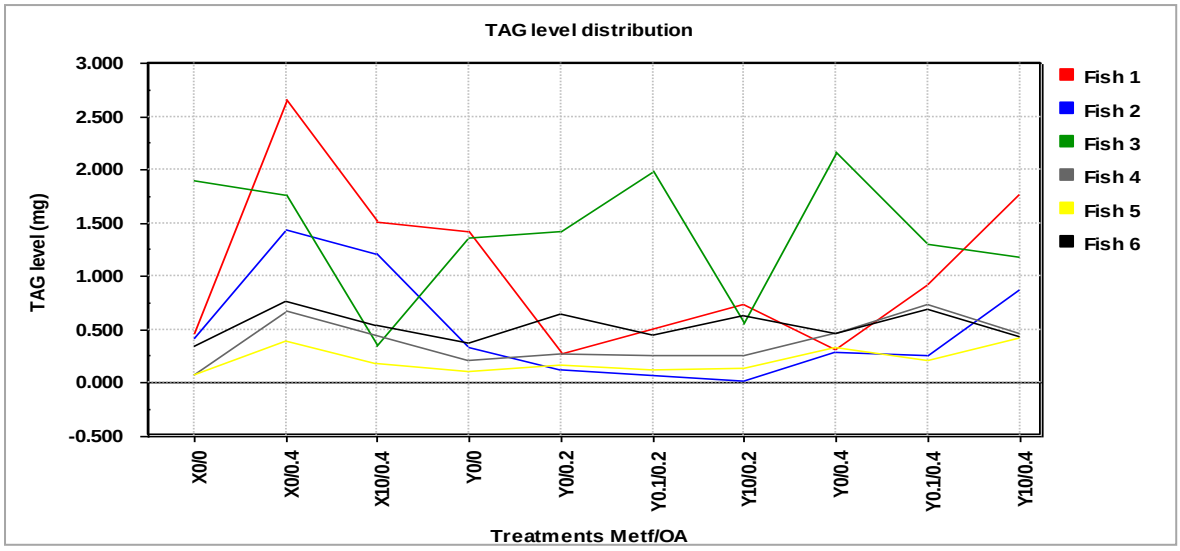


Figure 2.3 Standard plot showing overall distribution of TAG levels in six fish samples in response to ten treatments including controls measured by HPTLC.

The standard plot in Figure 2.3 showed the distribution of the total amount of TAGs for all six similar fish (deemed as biological replicates) in regards to ten different treatments including controls. An uneven distribution of the amount of TAG in response to individual treatment for all biological replicates was observed. The controls and other treatments were seen having higher variation amongst six fish. Specifically, fish 3 displayed exceptionally higher response, whereas fish 4 and 5 showed substantially too low. Despite belonging to the same species and been reared in the same farm (habitat), fish (especially fish 1, 2 and 3) exhibited biological variability in their response to the oleic acid and metformin. This might have occurred due to the variability in their cellular and molecular mechanism of TAG metabolism.

The TAG amounts of controls from six biological replicates were averaged to calculate the mean value and plotted in Figure 2.4. Though $X_{0/0}$ (1h Metf + 47h OA) and $Y_{0/0}$ (24h Metf +

24h OA) represented as controls for two different experimental conditions, they were treated similarly and incubated for the same period of time. Although both the controls were expected to have similar levels of intracellular TAG, the control $Y_{0/0}$ showed apparently higher than control $X_{0/0}$ however the difference is statistically insignificant (p= 0.8121).

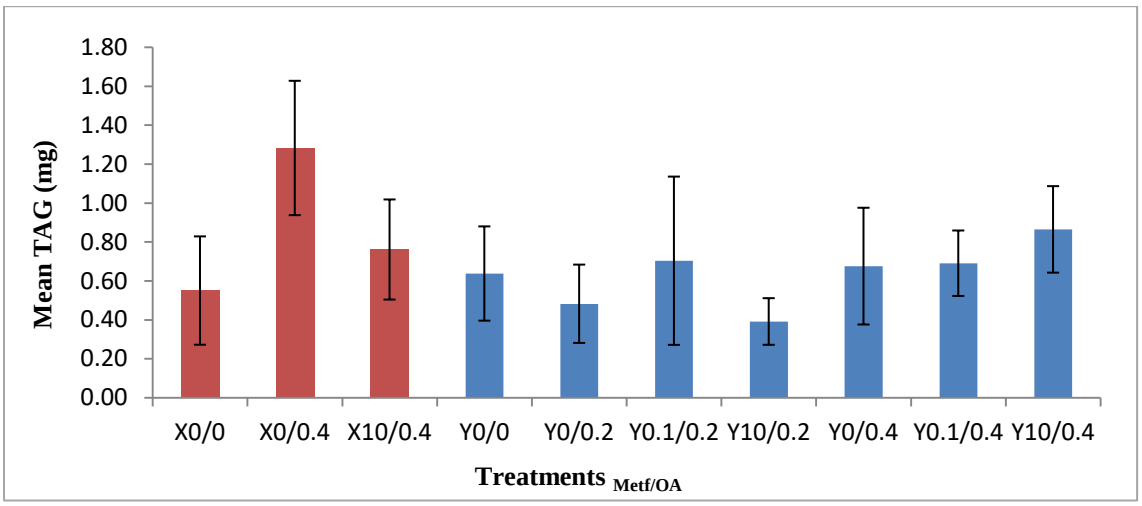


Figure 2.4 Mean value of total TAG (±SEM for n=6 fish) in response to 10 different treatments analyzed by HPTLC where red and blue color bars depicted the experimental condition respectively X and Y.

The normal plots were drawn for controls in Figure 2.5, depicting some extreme values far from the fit line. Control $Y_{0/0}$ for fish 1 and both controls ($X_{0/0}$ and $Y_{0/0}$) for fish 3 displayed deviations from the fit line indicating the presence of outliers in the data set.

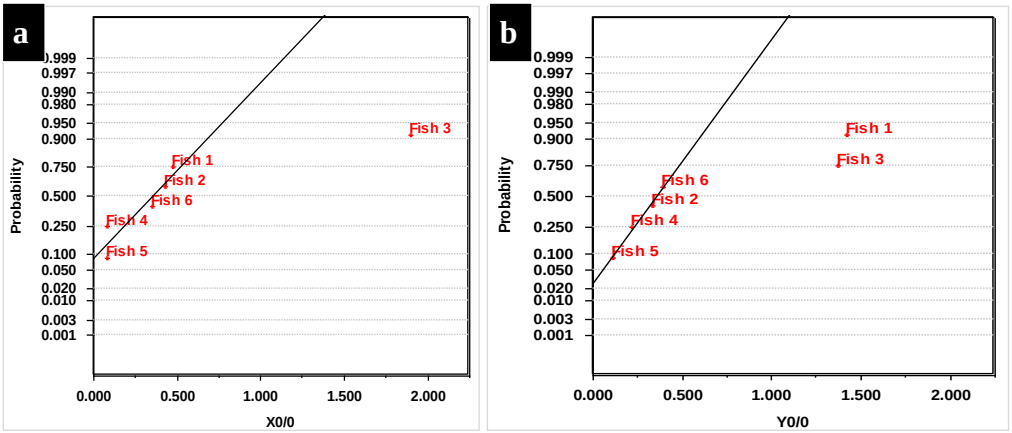


Figure 2.5 The normal probability plot showing a normal distribution pattern of a) control for 1h Metf + 47h OA and b) control for 24h Metf + 24h OA.

Similarly, Figure 2.3 indicated that many of the treatments especially for fish 1, fish 2 and fish 3 appeared with uneven distribution pattern. This indicates the presence of outliers that may, in fact, be due to the non-normality of the data rather than the presence of outliers leading to non-normality [55]. Therefore, in order to get reliable results, the elimination of outliers from the data set was conducted while the data normalization was conducted for PCA analysis to remove the variable effect of size factors on the data. The Iglewicz and Hoaglin's robust test for multiple outliers (two-sided test) based on modified Z-score method was employed to detect outliers. For non-normal and particularly small sample size data, the modified Z-score method is reliable since the parameters used to calculate the modified Z-score are minimally affected by the outliers [56]. The calculation is based on outlier resistant estimators, the median of absolute deviation about the median as follows [57]:

$$\text{Modified Z} - \text{score} = \frac{0.6745x(\text{Xi} - \text{Xm})}{\text{Median}\{|\text{Xi} - \text{Xm}|\}} \ldots\ldots\ldots\ldots Equation\ 3$$

Where X_i is the observations and X_m is the sample median.

Figure 2.6, created by pruned data set indicated that, for the experimental condition X (1h Metf + 47h OA), 0.4 mM oleic acid has induced TAG formation ($X_{0/0.4}$) that the TAG level increase was more than three times (357%) as compared to the control ($X_{0/0}$). The treatment $X_{10/0.4}$ (with 10mM metformin in the presence of 0.4mM oleic acid) reduced TAG level nearly half (41%) as compared to $X_{0/0.4}$, though, the reduction was statistically insignificant (p=0.2740) and expected to be equal or less than the level of control ($X_{0/0}$). Significance test was done based on unpaired t-test for unequal variances [58, 59]. Thus, the treatments for 1 hour incubation with metformin at 10mM concentration and subsequent 47 hours incubation with oleic acid at 0.4mM concentration have indicated that metformin retarded the TAG accumulation to a certain level. However, the reason for less reduction might be inadequate incubation period with metformin.

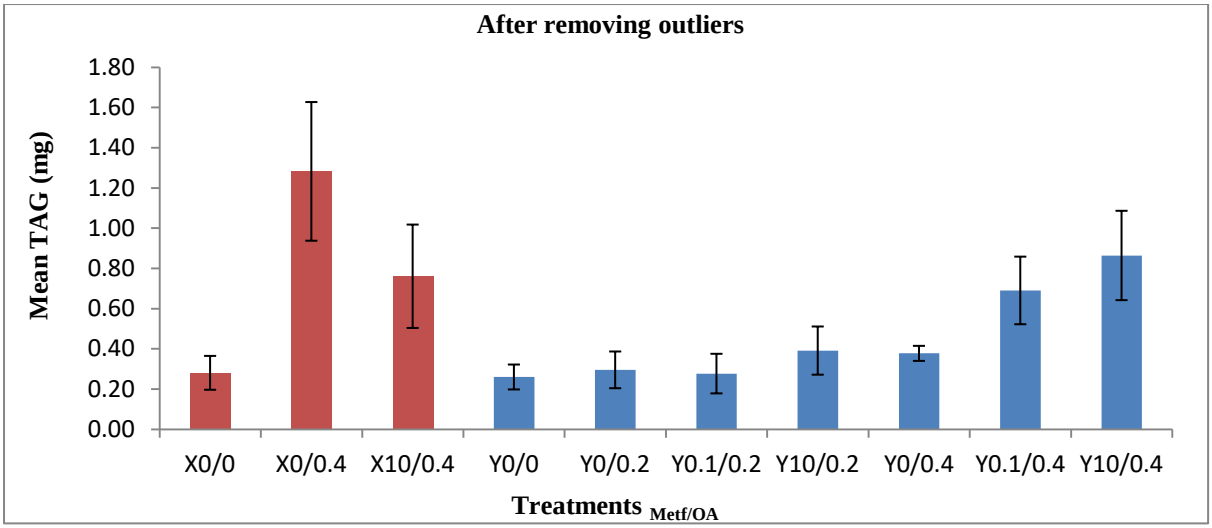


Figure 2.6 Mean TAGs (±SEM; after pruning the outliers) in response to 10 different treatments analyzed by HPTLC where red and blue color bars depicted the experimental condition respectively X and Y.

On the other hand, for the experimental condition Y (24h Metf + 24h OA), the 0.2mM and 0.4mM oleic acid ($Y_{0/0.2}$ and $Y_{0/0.4}$) increased fairly less TAG level (respectively in 14% and 45%). This implies that the 24 hours incubation with oleic acid was insufficient to induce production of higher level of TAG. However, the subsequent treatments in combination with the different concentration of metformin, unfortunately, did not show the expected outcomes. The treatments $Y_{0.1/0.2}$ and $Y_{10/0.2}$ (0.1 and 10 mM metformin in the presence of 0.2 mM oleic acid) showed respectively 6% reduction and 32% increase of TAG levels as compared to $Y_{0/0.2}$ and the treatments $Y_{0.1/0.4}$ and $Y_{10/0.4}$ (0.1 and 10 mM metformin in the presence of 0.4 mM oleic acid) showed respectively 83% and 129% increase of TAG level as compared to $Y_{0/0.4}$, which was not in accordance with the speculation. The treatments in experimental condition Y were unable to show any agreement to conclude upon possible effect of metformin. This might have happened because of the metformin treatment for 24 hours making the cells saturated and in result, cells were degraded or became inactive to metabolize TAG while induced with oleic acid.

2.6.2 Multivariate analysis

2.6.2.1 Partial least square regression analysis

For partial least square regression (PLS) analysis, a data matrix for 10 treatments with three independent variables and one dependent variable (as shown in Table 2.3) was prepared and fed into the pattern recognition software Sirius 9.0 for calculating regression coefficients.

Table 2.3 The data matrix with three independent variables at original levels such as: 1) metformin at 0.1, 0, 10 mM, 2) oleic acid at 0.2, 0, 0.4 mM, 3) experimental condition at (1h Metf + 47h OA) and (24h Metf + 24h OA) where the mean TAG was recorded as dependent variable.

	Metf (mM)	OA (mM)	EC (time)	TAG Level (mg/sample)
X0/0	0	0	1	0.28
X0/0.4	0	0.4	1	1.28
X10/0.4	10	0.4	1	0.76
Y0/0	0	0	2	0.26
Y0/0.2	0	0.2	2	0.30
Y0.1/0.2	0.1	0.2	2	0.28
Y10/0.2	10	0.2	2	0.39
Y0/0.4	0	0.4	2	0.68
Y0.1/0.4	0.1	0.4	2	0.69
Y10/0.4	10	0.4	2	0.86

Metf- metformin, OA-oleic acid and EC- experimental condition

Since the experimental condition is qualitative, numeric value ‘1’ and ‘2’ were assigned respectively. Discrepancies due to quantitative values with different units and qualitative entries were removed through standardization that was done by multiplying each variable with the inverse of its standard deviation making equal variance [60].

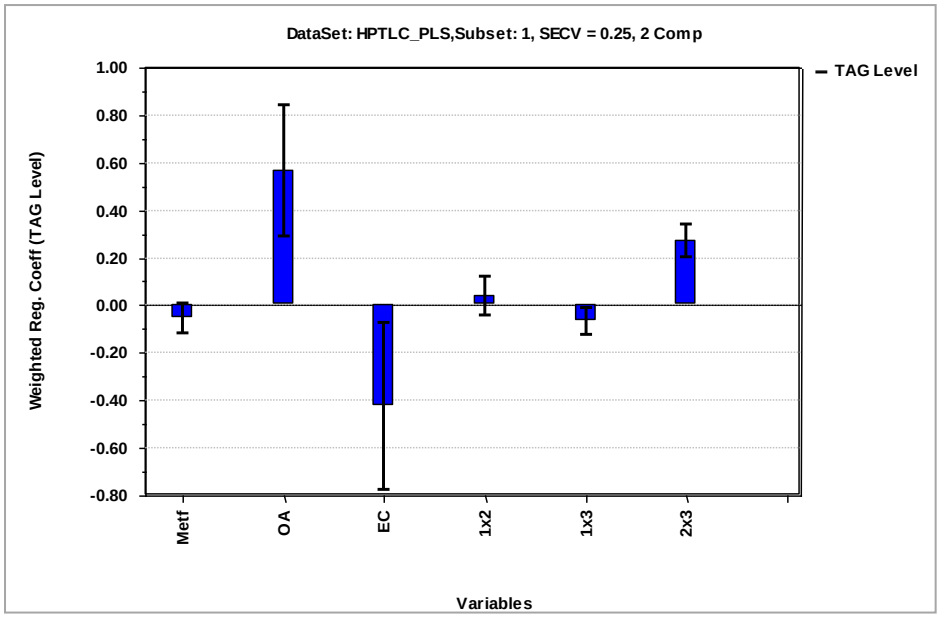


Figure 2.7 Regression coefficient showing the main and interaction effects of variables on TAG level analyzed by HPTLC.

PLS regression in Figure 2.7 showed that metformin, the interaction between metformin and oleic acid (1×2), and the interaction between metformin and experimental condition (EC) (1×3) have less significant impact on the TAG level. Oleic acid and its interaction with experimental condition (2×3) showed positive effect meaning that higher level would yield more TAG. Despite the larger uncertainty, the negative effect of the experimental condition indicated that increased incubation time for metformin but decreased for oleic acid would yield reduced TAG level. Virtually, this was observed otherwise in Figure 2.6 - connoting the incubation time as a crucial factor. Though the variable, metformin showed less significant effect, the negative trend has indicated its effect of TAG reduction.

2.6.2.2 Principal component analysis

For principal component analysis (PCA), a 6×10 data matrix consisting 6 rows corresponding to 6 fish and 10 columns corresponding to the 10 treatments' value (as shown in Table 2.2) was fed into the Sirius 9.0 for exploratory analysis.

As suggested by Figure 2.5, data normalization was done prior to data exploration by PCA. The data were normalized to a constant sum which transformed the data to a relative scale as such variables for each fish object were summed to 100 % and made total weight to 1.

A mean centering was also performed so that the origin of data is moved to the center of gravity, which helps PCA to describe the data better. A total of five PCs were extracted in a decreasing order of variance respectively 56.8%, 19.8%, 12.8%, 6.5% and 4.1% of the information. As PC1 and PC2 expressed most of the information, they were used to create the score and loading plots in addition to biplot and scores dendrogram shown in Figure 2.8.

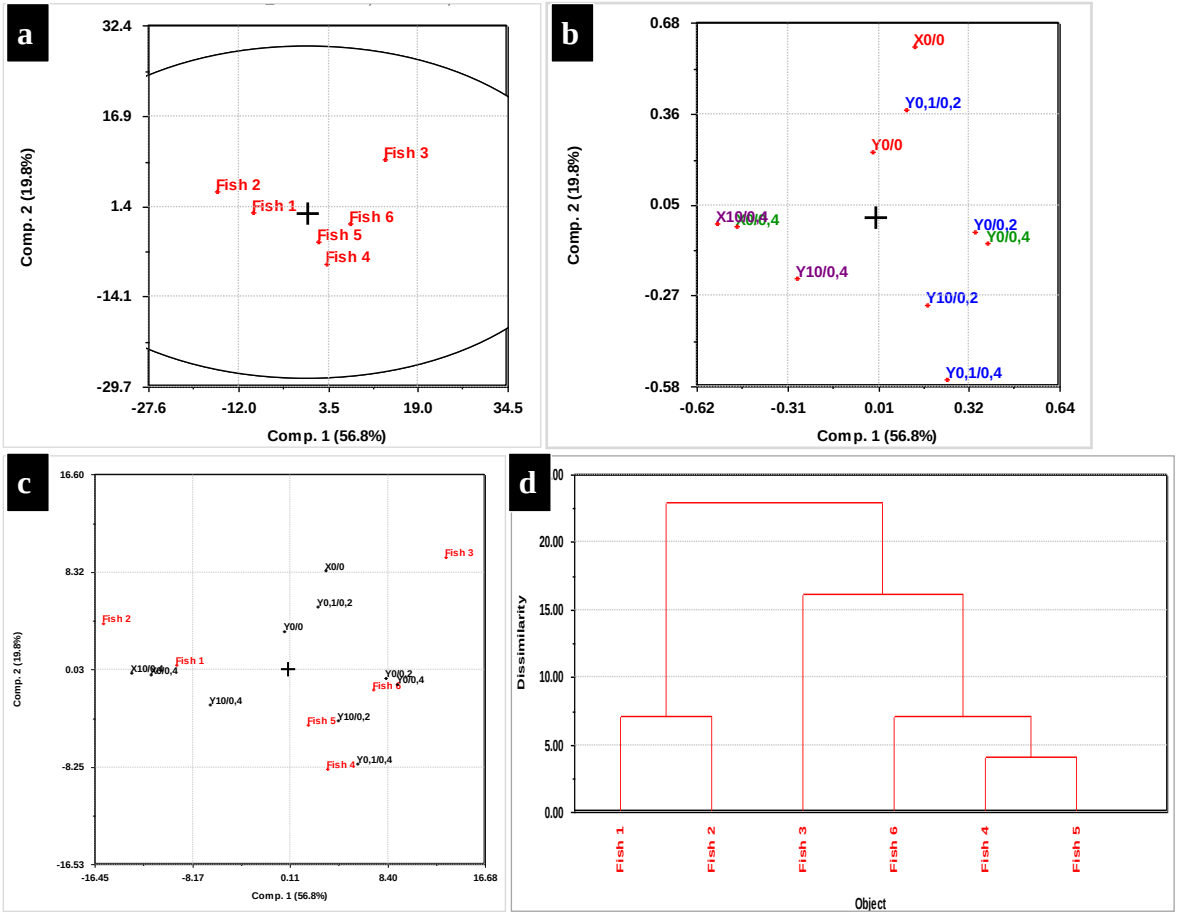


Figure 2.8 a) Score plot showing the picture of the relationship between 6 fish b) Loading plot showing the picture of the relationship between 10 variable treatments, c) Biplot - the combination of score and loading vectors deducing the interrelationship between fish and variable treatments and d) Scores dendrogram explaining distance of dissimilarity and clustering between the fish.

Score plot displayed (Figure 2.8 a) the relationship between all six fish based on their variable treatment behavior. It was also seen that fish 3 was remarkably different than the rest whereas fish 1 and 2 were the next candidates for greater dispersion from other fish. The togetherness and central tendency of fish 4, 5 and 6 stipulated the higher similitude amidst their response pattern. This picture indicated the variations among fish as discussed in section 2.6.1. But the score plot contained all the fish inside the Hotelling T^2 limits, confirming that in regards to all treatments, no fish should distinguishingly be considered as an extreme outlier and therefore, removal of any of the objects for the further data analysis would have been unmerited [61].

Depicting the interrelationship between different treatments, the loading plot (Figure 2.8 b) displayed that both controls ($X_{0/0}$ and $Y_{0/0}$) shown in red color grouped in the top right quarter of the plane whereas metformin-treated samples ($X_{10/0.4}$ and $Y_{10/0.4}$) in violet grouped in the down left quarter. These indicated that the treatments in two experimental conditions had a nearly similar effect on controls and 10 mM metformin-treated samples. On the other hand, 0.4 mM oleic acid-induced samples ($X_{0/0.4}$ and $Y_{0/0.4}$) in green placing in two opposite segments indicated that inducement with oleic acid was notably affected by the incubation period.

In the biplot, the objects and the variables were plotted on the same axes where the normalized object scores and variable loadings on each PC were scaled proportionally to the root of the variance accounted for by that PC. The biplot in Figure 2.8 c) helped to understand the contribution of variable treatments for individual variability attained among the fish. It depicted that the treatment $X_{0/0}$, $Y_{0.1/0.2}$ and $Y_{0/0}$ as the prime contributor for the fish 3 being exceptional which were previously identified as outliers (Figures 2.3 and 2.5).

The variability among the fish was also visualized from scores dendrogram (Figure 2.8 d) showing the relative distance of dissimilarity based on the Euclidean distance and the clustering pattern. All fish grouped into two major clusters whereas, fish 1 and 2 belonged to one cluster and the rest belonged to another. The earlier identified most exceptional fish 3, in spite of belonging to the second group, showed higher order dissimilarity with fish 4, 5 and 6.

As the loading plot in Figure 2.8 (b) suggested a variation of incubation period over the treatment outcome, the data matrix was reformed as shown in Table 2.4, such a way that the individual treatment acted as an object and the experimental condition as a variable.

Table 2.4 The data matrix showing the individual treatment as an object and the experimental condition as a variable.

Treatment		**Mean TAG**	
		Incubation for 1 h with Metf + 47 h with OA	Incubation for 24 h with Metf + 24 h with OA
		X	**Y**
Liver cell control	$\mathbf{K_{0/0}}$	0.55	0.64
Liver cell control+0.2 mM OA	$\mathbf{K_{0/0.2}}$	-	0.48
Liver cell+0.1mM Metf+0.2 mM OA	$\mathbf{K_{0.1/0.2}}$	-	0.70
Liver cell+10mM Metf+0.2 mM OA	$\mathbf{K_{10/0.2}}$	-	0.39
Liver cell control+0.4 mM OA	$\mathbf{K_{0/0.4}}$	1.28	0.68
Liver cell+0.1mM Metf+0.4 mM OA	$\mathbf{K_{0.1/0.4}}$	-	0.69
Liver cell+10mM Metf+0.4 mM OA	$\mathbf{K_{10/0.4}}$	0.76	0.86

K- Treatment, X- Mean TAG obtained in first experimental condition, Y- Mean TAG obtained in second condition.

Since the PCA was plotted with only two variables (X and Y), PC1 and PC2 have explained variance nearly 100%. The expected grouping among the treatments were observed in score plot and scores dendrogram in Figure 2.9 a) and d) where treatments with 0.4 mM oleic acid ($K_{0/0.4}$) formed one group, controls ($K_{0/0}$) and treatments with 10 mM metformin plus 0.4 mM oleic acid ($K_{10/0.4}$) together formed another group and the rest of the treatments remained in separated groups. This grouping pattern demonstrated that 0.4 mM oleic acid ideally induced TAG accumulation making the treatments separated from others and the presence of 10 mM metformin considerably reduced the TAG to the level of controls making the treatments in one group with controls. The rest treatments grouped together in another due to their insignificant relative dissimilarities i.e. those have responded indifferently to the presence of metformin and oleic acid. Figure 2.9 b) displayed the clear segregation of two experimental conditions indicating the significant variable effect of condition on the treatment outcomes. Biplot in Figure 2.9 c) again asserted that X condition (1h Metf + 47h OA) had been the main contributor for making the treatment $K_{0/0.4}$ different than others.

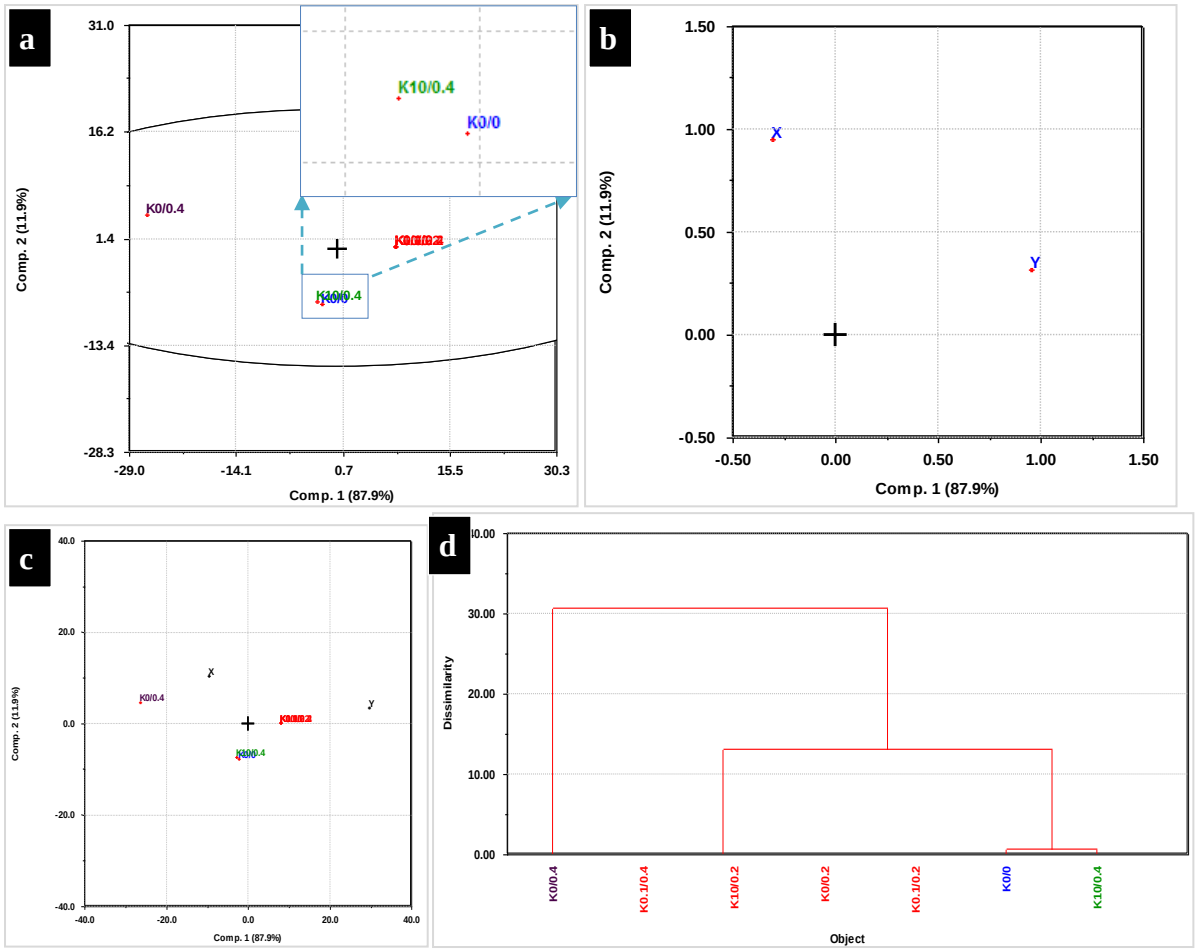


Figure 2.9 The normalized reformed data matrix for PCA showed a) score plot of the treatments' variability while inset showing enlarged view of group of $K_{0/0}$ and $K_{10/0.4}$ b) loading plot indicating variation in the effect of two experimental conditions c) biplot – with combining treatments and conditions and d) the scores dendrogram showed the pattern of dissimilarity.

2.7 Conclusions

As indicated by HPTLC result, metformin has the potentiality of attenuating fatty liver conditions by reducing TAG accumulation in *in-vitro* salmon liver cells induced. A significant effect of metformin was observed in fatty liver development in the experimental condition of 1 hour incubation with metformin and following 47 hours incubation with oleic acid. Metformin showed a significant effect on the cell cultures treated with metformin at 10 mM for 1 hour followed by inducement with oleic acid at 0.4 mM for 47 hours. However, the treatments with metformin for 24 hours incubation followed by further 24 hours incubation with oleic acid have not persuaded any conclusive effect of metformin on the fatty liver model.

PLS regression coefficient exhibited a trend of reducing TAG as increasing the concentration of metformin despite, less significant. PLS also revealed valuable information about the effect of the incubation period and the interaction of incubation period with metformin. Therefore, careful adjustment of the incubation period for metformin would be greatly recommended.

PCA confirmed the point that the experimental condition of 1 hour incubation with metformin and following 47 hours incubation with oleic acid is better to produce expected outcome as compared to the other condition. In addition, PCA extracted the information that the lower concentrations of oleic acid (0.2 mM) and metformin (0.1 mM) are unable to show significant effect on the *in-vitro* salmon liver cells. Moreover, PCA revealed the dissimilarity pattern among the salmon fish and highlighted potential biological variability in TAG metabolism among the fish.

Chapter 3 Evaluation of liquid chromatography mass spectrometry - as an alternative technique to the conventional high performance thin layer chromatography for quantitative analysis and characterization of triacylglycerol in cell culture

3.1 Background

HPTLC is the most advanced form of thin layer chromatography (TLC) that is widely used for qualitative and quantitative lipid analysis [50]. In comparing with TLC, HPTLC has been advanced through the introduction of automation in sample application (loading), plate development, detection and documentation and in consequence, the separation efficiency and resolution has appreciably been enhanced facilitating more accurate quantitative measurements [51].

For determination of the level of TAG in biological samples, HPTLC has widely been used as a conventional, rapid and reliable technique in analytical laboratories since long. Whatsoever, it is inapplicable for advanced TAG studies especially identifying the individual TAG species, determining the intensity of individual TAG species, and the structural elucidation of TAG and the positional distribution of fatty acids on the backbone of TAG, for those, the analytical laboratories require additional technique(s) in addition to HPTLC.

In lipidomics, for individual TAG species identification, amongst the reported analytical methods, GCMS using ionization technique such as electron ionization (EI), cold electron ionization (Cold EI) and chemical ionization (CI) coupled to single or tandem mass spectrometers [62–66] and LCMS using soft ionization techniques, for example, chemical ionization (CI), atmospheric-pressure chemical ionization (APCI), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI) coupled to single or tandem mass spectrometers [67–71] have been reported most frequently.

The positional distribution of fatty acids in TAG can be analyzed by gas chromatography (GC) [72], nuclear magnetic resonance (NMR) [73–75], High performance liquid chromatography (HPLC) [76, 77], Ultra performance liquid chromatography mass spectrometry (UPLCMS) [78], Liquid chromatography mass spectrometry (LCMS) [79, 80],

spectroscopic and spectrometric method [81], and various chemical [82], enzymatic methods [83] reported elsewhere.

In the case of GCMS, TAGs are normally hydrolyzed and methylated to form fatty acid methyl esters that are finally analyzed [84] and hence, much vital information on the actual structure and concentration/level of the various parent TAGs are missed [85].

Again in LCMS, analysis using soft ionization, ionization efficiency of an analyte is changed due to the presence of co-eluting components that causes ion suppression in lipid analysis. For example, the detection of TAGs is highly suppressed by the presence of phospholipids in positive ion electrospray mode (+ESI) [54]. But this challenge is overcome by separation of mixture component of lipids, in this case, the TAGs (neutral lipids) are separated from polar lipids especially phospholipids prior to injecting to LCMS by applying either solid phase extraction (SPE) [86, 87] or liquid-liquid extraction (LLE) [54] or thin layer chromatography [87].

Since LCMS is nowadays used in lipidomics for structural elucidation of TAG [88], it could also be used for simultaneously measuring the level of total TAG in the biological sample and individual TAG species. Hence, LCMS could potentially be substituting the conventional HPTLC in analysis and characterization of TAG in biological samples.

3.1.2 Liquid chromatography mass spectrometry

LCMS is an analytical technique that involves use of a high performance liquid chromatography (HPLC) system to separate individual components in the complex mixture and combines with mass spectrometry (MS) to, upon ionization of the molecules, analyze and detect the ions on the basis of their mass-to-charge (m/z) ratios [89].

HPLC is used for physical separation of components in a liquid mixture based on their interaction and chemical affinity with two immiscible phases, i.e., stationary and mobile phase. The basic components of the HPLC are solvents as mobile phase, solvent delivery pump, sample injection manager, packed column as the stationary phase and detection system or coupling with other hyphenated systems [90, 91]. Figure 3.1 shows the basic structure of an LCMS. In LCMS, an ionization interface couples HPLC with MS system where the molecules are ionized. The interface also serves as an ion source for MS that measures the

mass-to-charge ratios of the ions. MS has basic components – 1) ion source, for example, APCI, ESI, MALDI; 2) mass analyzer, for example, time of flight (ToF), quadruple, ion trap, etc. The ion source converts and fragments the neutral sample molecules into gas-phase ions that are subsequently analyzed by the mass analyzer that sorts the ions based on their mass/charge by applying electric and magnetic field. The ion current is detected and assessed by the detector that amplifies the ion current to calculate the abundances of each mass-resolved ion [92]. For increased sensitivity and specificity and to gain more structural information on the components in the mixture, two mass analyzers can be used that is called LC Tandem MS or LCMS/MS [93].

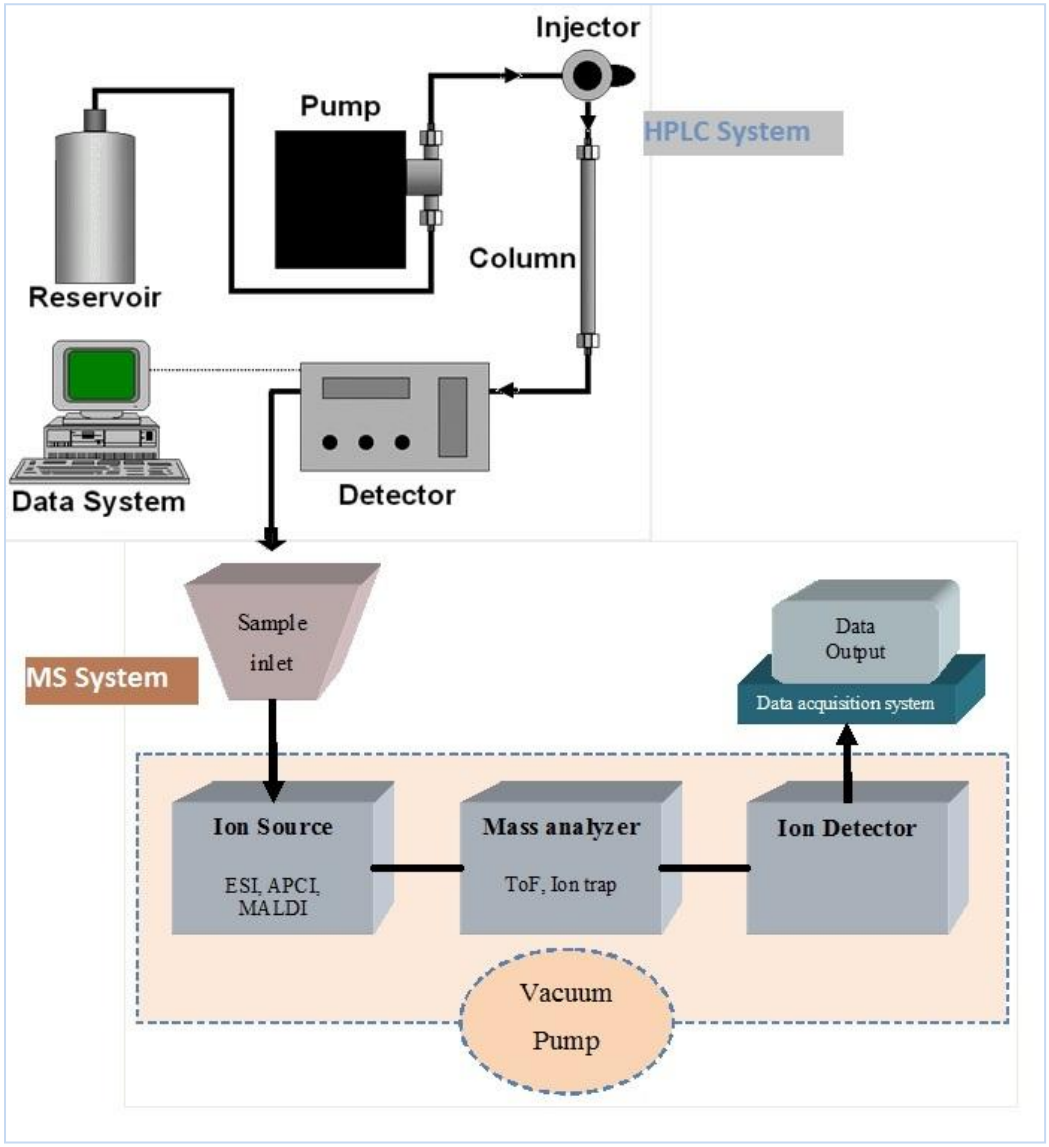


Figure 3.1 Diagram of an LCMS system

3.2 Significance of the study

Modern analytical and research laboratories having the approach of cost minimization in delivering services or conducting researches always look for saving valuables resources- time and money. The laboratories using HPTLC, for determination of total TAG in biological samples, are required to use another technique for the structural elucidation of TAG in the same sample. So finding a means to simultaneously measure TAG level and elucidate TAG structure in the same biological sample by a single technique would be of great importance today. Due to the many advantages and popularity in lipidomics, LCMS might be a good candidate in this regard.

3.3 Objective

The second objective of this thesis was to evaluate the potential of LCMS to replace the conventional HPTLC for quantitative analysis of TAG and characterizing TAG in cell culture. Specific strategies were taken into account such as:

- Analysing the samples by LCMS combining with different software tools and strategies
- Employing multivariate chemometrics strategies to elucidate comprehensive information in order to compare the outcomes of HPTLC and LCMS analysis.

3.4 Experimental

3.4.1 Reagents

Methanol (HPLC grade, =99.9 %) and butylated hydroxytoluene (BHT) (=99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade, =99.9 %) was from Honeywell (Israel). Hexane (LC grade, =98 %), ammonium acetate (mass spectrometry grade, =99%) and chloroform were from Merck (Darmstadt, Germany). Isopropanol used for HPLC was from Kemetyl (Norway). L-serine was purchased from Ajinomoto U.S.A. Inc. De-ionized water was used throughout the experiment and purified in a Milli-Q system (Millipore, Milford, USA). The TAG (trilinolenin) standard for LCMS analysis was purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.4.2 Sample preparation and liquid-liquid extraction

Sample preparation and liquid-liquid extraction were described in Section 2.5.3. The same samples used for HPTLC analysis were saved in another aliquot for analysis by LC-ESI-MS/MS.

3.4.3 Liquid chromatography ion-trap mass spectrometry instrumentation

The TAG analysis was carried out by using an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface, a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 25 µl injection volumes. The Zorbax Eclipse-C8 RP 150 × 4.6 mm, 5 µm (Agilent Technologies, Palo Alto, CA) was kept in the column compartment at 40 °C and the solvent system in gradient mode consisted of methanol:acetonitrile (40:60, v/v) (A) and methanol:acetonitrile:water (45:30:25, v/v) (B). Both solvents (A and B) contained 2.5 mM ammonium acetate and 10 µM L-serine. The mobile phase was delivered at a flow rate of 0.8 ml/min and UV detection was set at 254 nm. The following gradient program was used: initial 10 min (0-10 min) at condition 40 % A and 60 % B that was ramped in 15 min (10-25 min) to 100 % A and remained unchanged at this concentration up to 85 min (25-85 min) and then returned to the initial condition in 2 min (85-87 min) where it was held for 3 min up to end of run (87-90 min). The ESI source was operated in positive ion mode and the ESI conditions were: capillary voltage -4000 V, nebulizer gas 50 psi, dry gas 8 L/min and dry temperature 350 °C. The MS conditions were: skimmer 40 V, capillary exit 158.5 V, octopole 1 and 2 (both in DC) 12 and 2.5 V, respectively, octopole RF 200 Vpp, lens 1 and 2 at -5 and -60 V, respectively and trap drive 74.1 V. The ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option. Data acquisition and processing were controlled by the software MSD trap control version 5.3 (Agilent Technologies, Inc. Santa-Clara, USA).

3.4.4 LCMS data deconvolution for automated characterization of TAG

Though LCMS represents a powerful tool for the analysis of lipids, manual data calculation and interpretation is a tedious, time-consuming and complicated process (as seen in Figure 3.2) that is regarded as a bottleneck for the identification, characterization and quantification of lipids, especially TAG [78, 94]. Therefore, a least square regression approach based Chrombox D and a computational algorithm based QueryTAG software tools were used for automatated data deconvolution.

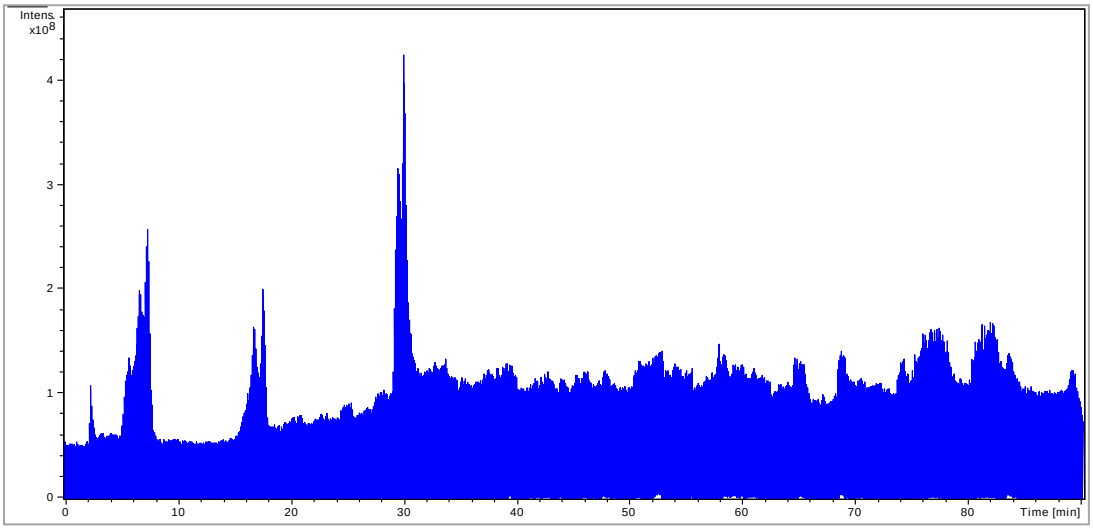


Figure 3.2 LCMS derived total ion chromatogram

3.4.4.1 LC-ESI-MS data exportation for analysis by Chrombox D

The LCMS raw data were read in chemstation (LC/MSD Trap Software version 5.3 (©Agilent Technologies, Inc. 2005, Bruker Daltonik GmbH Inc., Billerica, MA, USA) and TIC + MS were exported to NetCDF files for data analysis into Chrombox D (Code revisions -16-01) (http://www.chrombox.org/d/) running under MATLAB (Natick, MA, USA). The Chrombox D was previously developed by Zeng et al. (2013) for LCMS analysis with particular focus on analyses of polar lipid classes which also allows analyzing neutral lipids such as TAG [94].

The software setup and library creation described by Zeng et al. (2013) was readjusted and tuned for TAG identification in this study. Briefly, the following list of fatty acids from the carbon number 14 to 26 as known composition of fatty acid in TAG in salmon fish liver [95], was applied in creating the library: 14:0, 14:1, 16:0, 16:1, 16:2, 18:0, 18:1, 18:2, 18:3, 18:4,

20:0, 20:1, 20:2, 20:3, 20:4, 20:5, 22:0, 22:1, 22:2, 22:3, 22:4, 22:5, 22:6, 24:0, 24:1, 24:2, 26:0 and 26:1 that filtered 214 possible TAG spectra. In order to get upward rounded mass, the masses were binned to unit resolution with mass offset +0.2 Da. After removal of isomers, the complete report was appeared with a list of ions of 198 TAG species. Quantification of the amount of TAG was based on least squares spectral resolution (LSSR) for that the threshold was set to absolutely 2%. Recalculation generated a report in MS Excel format that was used for further data analysis.

3.4.4.2 LC-ESI-MS/MS data exportation for analysis by QueryTAG

The LCMS raw data from chemstation was read to get TIC+ All (MS + MSn) and converted into netCDF and ASCII files by LC/MSD Trap Software version 5.3 (©Agilent Technologies, Inc. 2005, Bruker Daltonik GmbH Inc., Billerica, MA, USA). The netCDF and ASCII files were then transferred as input files into QueryTAG software – an automated TAG prediction algorithm for identification of TAG species. The QueryTAG is a MATLAB based algorithmic software previously developed by Zeng et al. (2010) which could automatically give the elucidation results of TAG structures without manually introducing data into the algorithm [88].

3.5 Results and Discussion

During the initial experiment a TAG standard was analyzed by infusing into the LCMS system trilinolenin (molecular weight of ~873.36 g/mol) that displayed predominant presence of sodiated adduct ions [Trilinolenin + Na]$^+$ at ~m/z 896. At the same time, insignificant presence of ammoniated [Trilinolenin + NH_4]$^+$ adduct ions at ~m/z 891 were observed at some retention points whilst the protonated [Trilinolenin + H]$^+$ adduct at ~m/z 874 was not found as seen in Figure 3.3. It has been reported that despite not adding any sodium to the samples or solvents, positive ion electrospray produces high abundance of sodiated triacylglycerol ions in addition to less abundant ammoniated and protonated molecules. Sodium in the samples or solvents may come from external sources such as from the HPLC-grade solvents, glassware, and the standards [96]. Figure 3.3 also showed a dubious presence of a mass spectrum ion at ~910 m/z that was [Triolein + Na^+] which might have come from either the probable impurity of the standard (since it was not the purest one) or the residual of previous sample.

Furthermore, the QueryTAG analysis exhibited the presence of only sodium adduct ions in all samples, despite the identificaiton of ammoniated and protonated molecules by QueryTAG in similar samples and solvent system were reported elsewhere [54].

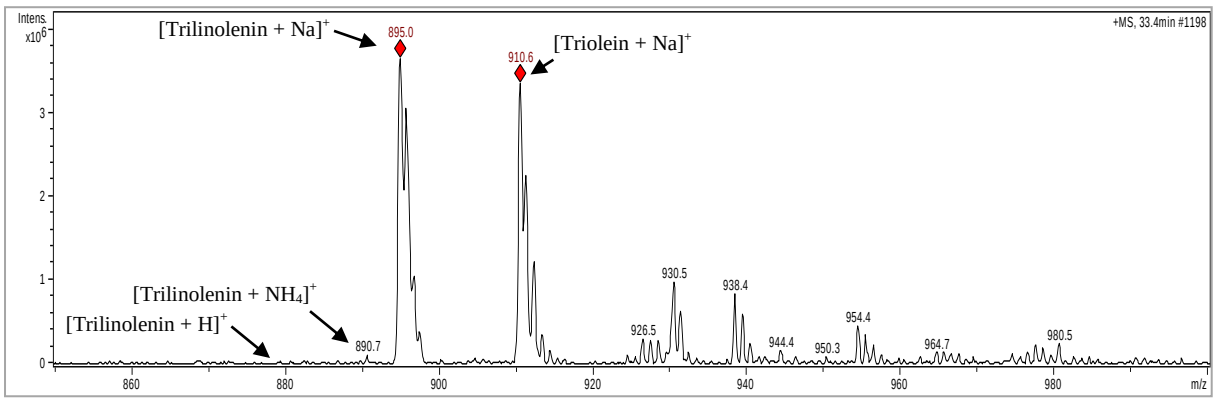


Figure 3.3 Positive ion electrospray mass spectra of the sodiated adduct of trilinolenin standard.

Therefore, in the case of Chrombox D software setup in Section 3.4.4.1, only adducts of sodium [Na^+] were chosen to create the library of [TAG + Na]$^+$ that finally after auto-removal of isomers, appeared with a list of [TAG+ Na]$^+$ ions of 198 TAG species.

3.5.1 Determination by Chrombox D

3.5.1.1 Data Analysis Strategy I (Total TAG Abundance)

The report generated by the Chrombox D software displayed a complete list of TAG species varying in the number of carbon atoms from 42 to 78 and number of double bonds from 0 to 13 in the three acyl chains as shown in Table 3.1.

Table 3.1 An example of Chrombox D generated data of LCMS analyzed sample

Least squares spectral resolution

Sample No #

Spectrum: 0.01 - 89.94 min

Identity	Calc RI	Base peak	Amount	**Identity**	Calc RI	Base peak	Amount
G[3] t42:3	36	740	7.48E+06	**G[3] t54:5**	44	904	-8.64E+06
G[3] t42:2	38	742	3.80E+06	**G[3] t54:4**	46	906	2.97E+07
G[3] t42:1	40	744	2.06E+07	**G[3] t54:3**	48	908	8.31E+06
G[3] t42:0	42	746	6.10E+05	**G[3] t54:2**	50	910	1.49E+08
G[3] t44:4	36	766	4.75E+07	**G[3] t54:1**	52	912	8.68E+07
G[3] t44:3	38	768	2.68E+07	**G[3] t54:0**	54	914	-2.00E+07
G[3] t44:2	40	770	-8.55E+06	**G[3] t56:13**	30	916	-1.40E+07
G[3] t44:1	42	772	-1.80E+07	**G[3] t56:12**	32	918	1.10E+05
G[3] t44:0	44	774	-3.32E+06	**G[3] t56:11**	34	920	1.24E+07
G[3] t46:6	34	790	-1.20E+07	**G[3] t56:10-G[3] t64:4**	Deleted just to show a shortened version of the table		
G[3] t46:5	36	792	4.96E+07	**G[3] t64:3**	58	1048	1.98E+08
G[3] t46:4	38	794	1.65E+07	**G[3] t64:2**	60	1050	4.06E+08
G[3] t46:3	40	796	-1.70E+07	**G[3] t64:1**	62	1052	1.23E+07
G[3] t46:2	42	798	-2.20E+07	**G[3] t66:10**	46	1062	-3.40E+07
G[3] t46:1	44	800	-2.20E+07	**G[3] t66:9**	48	1064	5.72E+07
G[3] t46:0	46	802	-9.00E+06	**G[3] t66:8**	50	1066	4.51E+07
G[3] t48:7	34	816	-2.00E+07	**G[3] t66:7**	52	1068	1.04E+08
G[3] t48:6	36	818	-7.17E+06	**G[3] t66:6**	54	1070	1.26E+07
G[3] t48:5	38	820	-2.40E+07	**G[3] t66:5**	56	1072	5.31E+06
G[3] t48:4	40	822	-1.00E+07	**G[3] t66:4**	58	1074	8.01E+06
G[3] t48:3	42	824	9.77E+06	**G[3] t66:3**	60	1076	1.88E+06
G[3] t48:2	44	826	-2.30E+07	**G[3] t66:2**	62	1078	-1.50E+07
G[3] t48:1	46	828	-1.30E+07	**G[3] t66:1**	64	1080	-2.40E+07
G[3] t48:0	48	830	-6.22E+06	**G[3] t66:0**	66	1082	1.23E+08
G[3] t50:7	36	844	-2.70E+07	**G[3] t68:13**	42	1084	-3.40E+07
G[3] t50:3	44	852	-3.50E+07	**G[3] t68:4**	60	1102	-2.80E+07
G[3] t52:1	50	884	-3.50E+07	**G[3] t68:3**	62	1104	-1.50E+07
G[3] t54:9	36	896	-3.70E+07				
G[3] t54:6	42	902	-2.40E+07	Correlation measured/calculated spectrum: 0.9002			

The data from Chrombox D were processed using another macro-enabled Microsoft Excel-based tool (described in Appendix A). The total abundance of TAG in every sample was found by summing up individual TAG species and the species with less than 2% to the total abundance were excluded for further calculation and PCA analysis (tabulated in Table 3.2) since those less abundant TAG species are less likely to be present in the samples due to the treatments.

Table 3.2 Total amount of TAGs (intensity) per sample determined by LCMS -Chrombox D analysis where X denotes 1 hour treatment with metformin (Metf) and following 47 hours inducement with oleic acid (OA) whilst Y denotes 24 hours treatment with metformin and following 24 hours inducement with oleic acid and the subscripted numbers indicate the concentrations of metformin (numerator) and oleic acid (denominator) at 0, 0.1, or 10 mM for metformin and 0, 0.2, 0.4 mM for oleic acid. Mean value and SEM by equation 2 were calculated.

	X0/0	**X0/0.2**	**X0.1/0.2**	**X10/0.2**	**X0/0.4**	**X0.1/0.4**	**X10/0.4**
Fish 1	1.7E+10	1.9E+09	3.0E+09	2.3E+09	3.3E+09	2.2E+09	1.3E+09
Fish 2	2.8E+09	1.6E+09	1.7E+09	1.9E+09	4.6E+09	1.6E+09	1.8E+09
Fish 3	9.9E+09	6.6E+09	9.1E+09	6.1E+09	8.1E+09	8.4E+09	8.3E+09
Fish 4	7.5E+08	1.0E+09	1.2E+09	1.0E+09	1.1E+09	9.8E+08	9.4E+08
Fish 5	6.5E+08	1.2E+09	1.4E+09	1.2E+09	1.3E+09	1.8E+09	1.6E+09
Fish 6	5.8E+07	7.9E+08	4.5E+08	5.8E+08	7.8E+08	8.1E+08	1.0E+09
Mean	5.2E+09	2.2E+09	2.8E+09	2.2E+09	3.2E+09	2.6E+09	2.5E+09
SEM	2.8E+09	9.0E+08	1.3E+09	8.2E+08	1.2E+09	1.2E+09	1.2E+09
	Y0/0	**Y0/0.2**	**Y0.1/0.2**	**Y10/0.2**	**Y0/0.4**	**Y0.1/0.4**	**Y10/0.4**
Fish 1	1.2E+09	2.3E+09	2.0E+09	8.5E+08	2.4E+09	1.3E+09	1.7E+09
Fish 2	3.0E+09	1.9E+09	2.1E+09	1.4E+09	1.4E+09	1.3E+09	2.4E+09
Fish 3	4.9E+09	3.9E+09	3.6E+09	3.7E+09	4.4E+09	2.6E+09	2.1E+09
Fish 4	1.1E+09	1.1E+09	1.3E+09	1.1E+09	1.1E+09	1.3E+09	8.7E+08
Fish 5	1.0E+09	1.4E+09	1.4E+09	1.0E+09	1.1E+09	1.4E+09	8.4E+08
Fish 6	5.2E+08	9.1E+08	9.5E+08	8.0E+08	4.3E+08	7.3E+08	5.8E+08
Mean	2.0E+09	1.9E+09	1.9E+09	1.5E+09	1.8E+09	1.4E+09	1.4E+09
SEM	6.9E+08	4.5E+08	3.8E+08	4.6E+08	5.8E+08	2.5E+08	3.1E+08

Figure 3.4 showed an uneven distribution of TAG level in response to individual treatment where controls and some other treatments displayed higher variation around six fish and fish 3 became exceptional one in terms of TAG intensity. The overall observation was found very similar to the result found in HPTLC analysis in Figure 2.3. Unlike HPTLC, the control for fish 1 displayed abnormally higher intensity. It might have happened due to a standard sample run immediately before the injection of this control into LCMS, which strongly recommends the necessity of allowing adequate time for column cleaning properly.

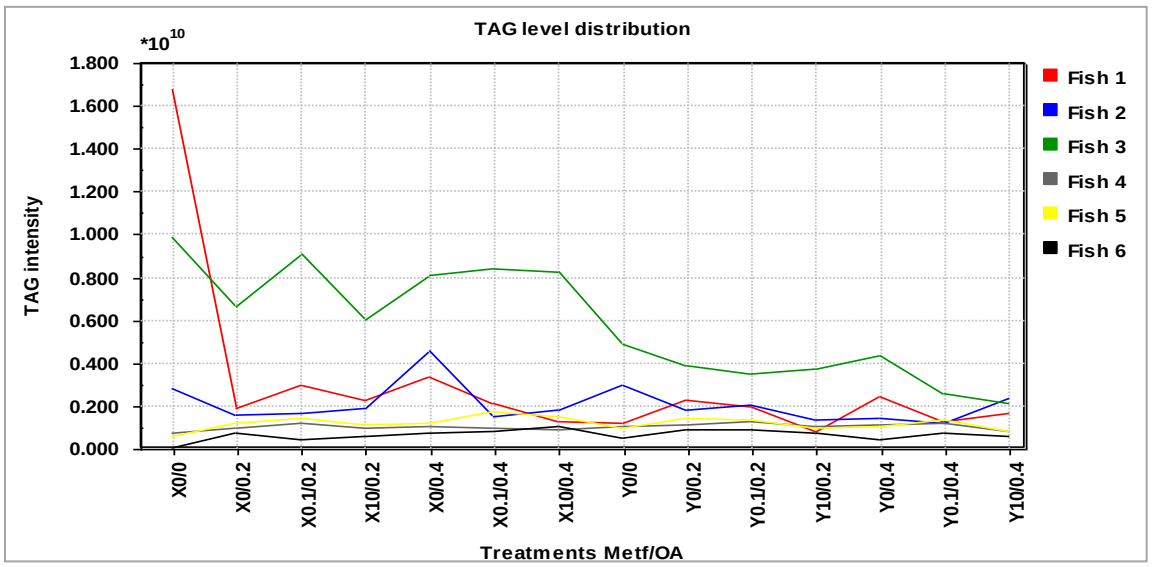


Figure 3.4 Standard plot for the distribution of TAG level for all six biologically similar fish in regards to fourteen different treatments including two controls analyzed by LCMS - Chrombox D.

Though a total of 84 samples (6 fish x14 treatments) were injected into LCMS but, due to the shortage of resources, 60 samples (6 fish x10 treatments) were analyzed by HPTLC. In order to avoid the complexity in method comparison, this chapter discussed only those common 60 samples whereas, 4 treatments ($X_{0/0.2}$, $X_{0.1/0.2}$, $X_{10/0.2}$ and $X_{0.1/0.4}$) from each fish were disregarded.

Control samples in both experimental conditions were tested for the normality of the data. The probability plots in Figure 3.5 showed that, for the first control, fish 1 and 3 and for second control, fish 2 and 3 deviated from the trend line. Therefore, further data analyses

were done removing the outliers by modified Z score method as described in Section 2.6.1 and the principal component analysis needed data normalization to reduce variability among samples.

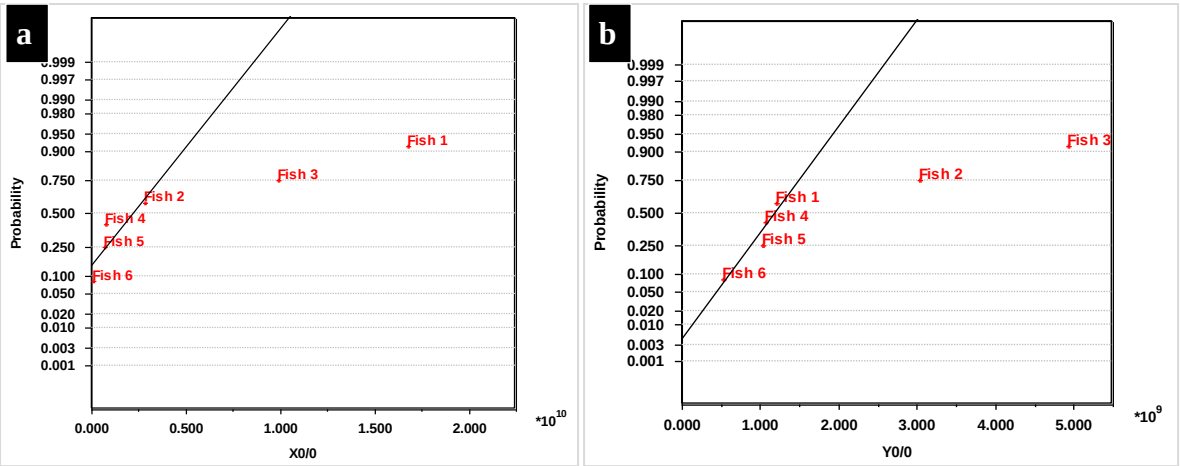


Figure 3.5 Normal plot of controls of both experimental conditions analyzed by LCMS - Chrombox D.

As Figure 3.6 showed, in the experimental condition X (1h Metf + 47h OA), the treatment with 0.4 mM oleic acid ($X_{0/0.4}$) induced nearly two times higher (199%) TAG formation as compared to the control ($X_{0/0}$) and the treatment with 10 mM metformin plus 0.4 mM oleic acid ($X_{10/0.4}$) reduced TAG level more than half (58%) as compared to $X_{0/0.4}$. Similar results for these treatments were also found in HPTLC analysis described in Section 2.6.1 and Figure 2.6.

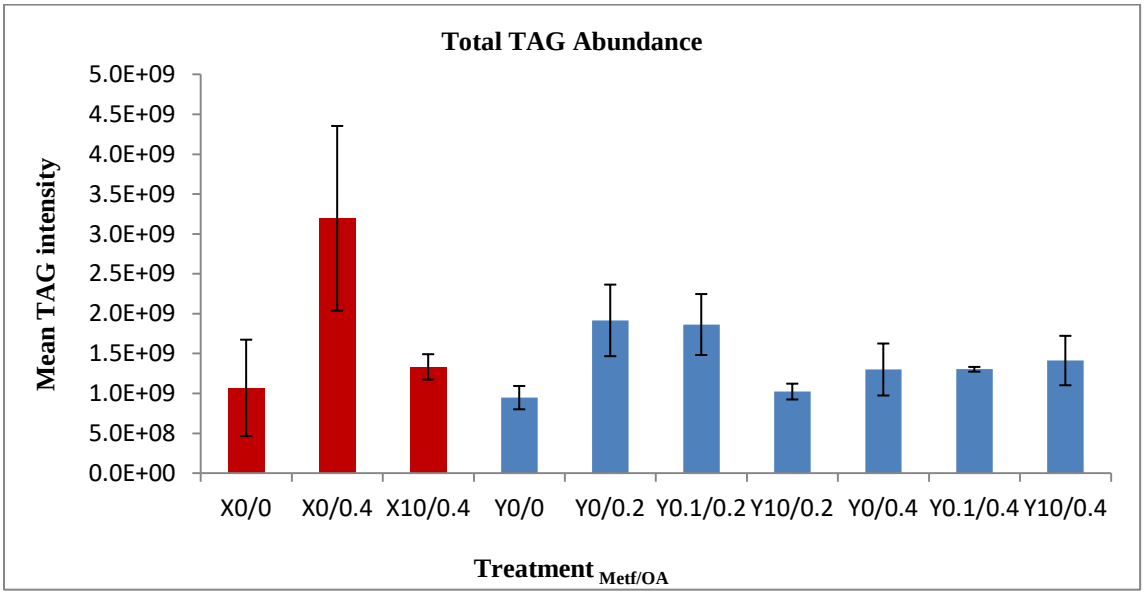


Figure 3.6 Mean of the total TAGs (±SEM; after pruning the outliers) in response to 10 different treatments analyzed by LCMS - Chrombox D where red and blue color bars depicted the experimental condition respectively X and Y.

For the experimental condition Y (24h Metf + 24h OA), the treatments $Y_{0/0.2}$ and $Y_{0/0.4}$ (induced with respectively 0.2mM and 0.4mM oleic acid) exhibited increase of TAG accumulation respectively 102% and 37% as compared to the control $Y_{0/0}$. Though the treatment $Y_{0.1/0.2}$ and $Y_{10/0.2}$ showed TAG reduction respectively 3% and 47% as compared to treatment $Y_{0/0.2,}$ the treatment $Y_{0.1/0.4}$ and $Y_{10/0.4}$ showed whatsoever no reduction (respectively 0% and -9%) than treatment $Y_{0/0.4,}$ which was not coherent to the assumption. That is, the overall outcome is alike to the HPTLC results discussed in Section 2.6.1 and Figure 2.6. In contrast, the treatments $Y_{0/0}$, $Y_{0/0.2}$, $Y_{0.1/0.2}$ and $Y_{10/0.2}$ expectedly displayed the effect of metformin, which was not determined by HPTLC. This point has strongly indicated the capability of LCMS to extract more accurate and reliable results than HPTLC.

3.5.1.2 Multivariate analysis

3.5.1.2.1 Partial least square regression analysis

As described in the Section 2.6.2.1, a similar data matrix with same three independent variables and the mean TAG analyzed by LCMS - Chrombox D as the dependent variable was prepared for PLS regression analysis by Sirius 9.0.

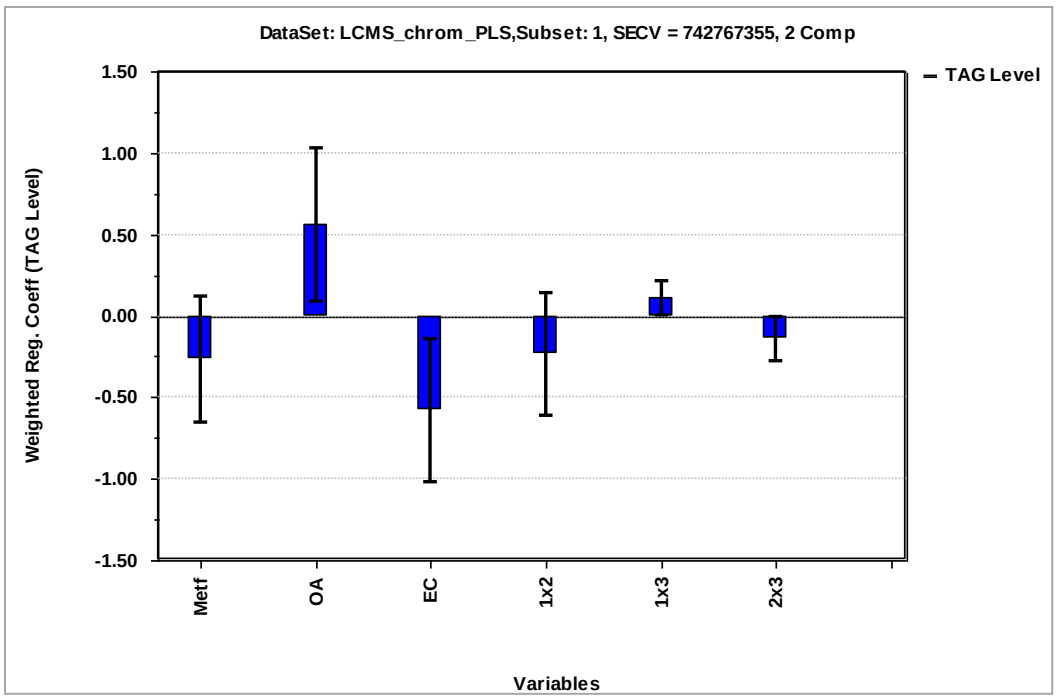


Figure 3.7 Regression coefficient showing the main and interaction effects of variables on TAG level analyzed by LCMS - Chrombox D.

PLS regression for LCMS - Chrombox D data displayed almost similar findings to the HPTLC (Section 2.6.2.1 and Figure 2.7). Figure 3.7 showed that the effect of metformin and all interactions (1×2), (1×3) and (2×3) were less significant to the model. Oleic acid showed positive effect proving that higher level would yield more TAG. Experimental condition (EC) showed negative effect indicating that increasing incubation time with metformin but decreasing with oleic acid would yield reduced TAG level. Despite larger uncertainty and less significant effect, the trend of metformin was indicating a negative effect on TAG accumulation.

3.5.1.2.2 Principal component analysis

For PCA analysis, the 6×10 data matrix was submitted to PCA for data exploration and PC1 and PC2 were considered for extracting information. The score plot (Figure 3.8 a) showed the highest variation in fish 1 and fish 2, and the biplot (Figure 3.8 c) showed that the observed variability was mainly due to the controls. The loading plot (Figure 3.8 b) showed the variability among the treatments where as controls were different than each other and the score dendrogram (Figure 3.8 d) showed the relative dissimilarity among the samples. PCA for these data supported the similarity in outcomes from HPTLC data.

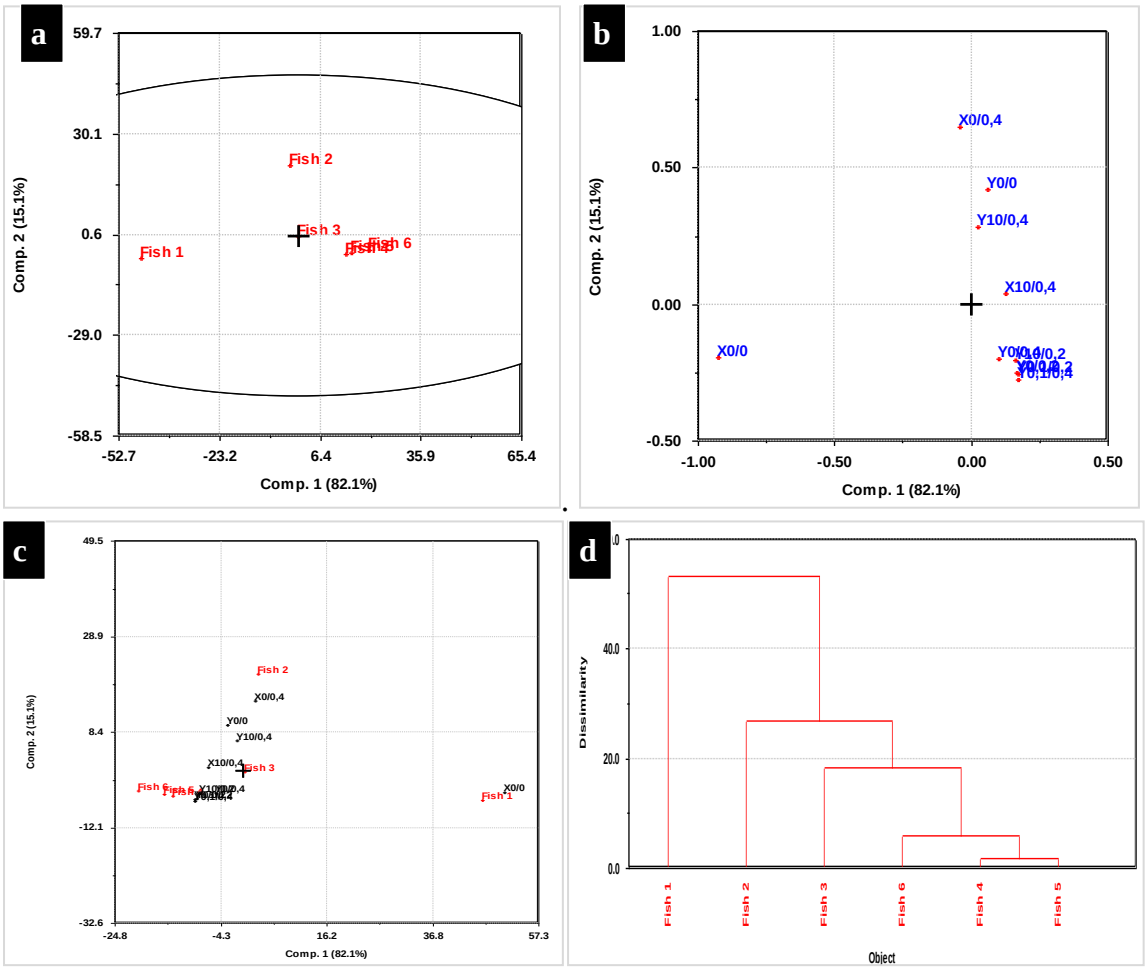


Figure 3.8 The normalized data matrix for PCA showed a) score plot of the sample variability b) loading plot indicating variation due to the treatment variability c) biplot – showing the impact of treatment on individual objects to be different than each other, and d) the score dendrogram showed the pattern of dissimilarity among samples.

3.5.1.3 Data Analysis Strategy II (Selective TAG Abundance)

The idea behind this strategy was to first extract specific TAG species which were exclusively present in 0.4 mM oleic acid-induced samples. It was assumed that the samples, induced with a high concentration of oleic acid ideally generated a high amount of TAGs and metformin-treated samples reduced the TAG level. Therefore, only those extracted TAG species were considered as indicator for measuring TAG abundance in the whole sample set.

The Chrombox D reports for 0.4 mM oleic acid-induced samples ($X_{0/0.4}$ and $Y_{0/0.4}$) were evaluated to extract specific TAG species which were commonly existing in those 12 samples. The rest TAG species were justifiably disregarded because of their presence not due to the inducement by oleic acid but rather to their intracellular synthesis (before oleic acid inducement) as occurred in control samples.

Table 3.3 A total of 27 TAG species extracted from the treatments induced with 0.4 mM oleic acid.

TAG 54:2	TAG 58:6	TAG 60:6
TAG 56:8	TAG 58:4	TAG 60:5
TAG 56:7	TAG 58:3	TAG 62:12
TAG 56:5	TAG 60:13	TAG 62:7
TAG 56:3	TAG 60:12	TAG 62:5
TAG 56:2	TAG 60:10	TAG 62:4
TAG 58:11	TAG 60:9	TAG 62:3
TAG 58:9	TAG 60:8	TAG 64:9
TAG 58:8	TAG 60:7	TAG 64:3

Expressed as TAG CN:DB, where CN= total carbon number and DB= total double bonds in all three fatty acyl chains.

The total TAG intensity in the rest of the samples was re-extracted by applying 27 TAG species (Table 3.3). The isolated 27 TAG species with their intensities for all 60 samples with the high concentration oleic acid treated samples are shown in Appendix B. Mean values for 6 fish in regards to 10 treatment were calculated; outlier removed and plotted to see whether the results were in the similar pattern with HPTLC results.

There was no outlier detected in the first four treatments and thus an insignificant increase or decrease of TAG intensity was observed in those cases as shown in Figure 3.9. In

experimental condition X (1h Metf + 47h OA), 0.4 mM oleic acid-induced higher TAG formation as compared to the control ($X_{0/0}$) (24%) and 10 mM metformin in the presence of 0.4 mM oleic acid ($X_{10/0.4}$) insignificantly reduced the TAG level to 21% as compared to $X_{0/0.4}$.

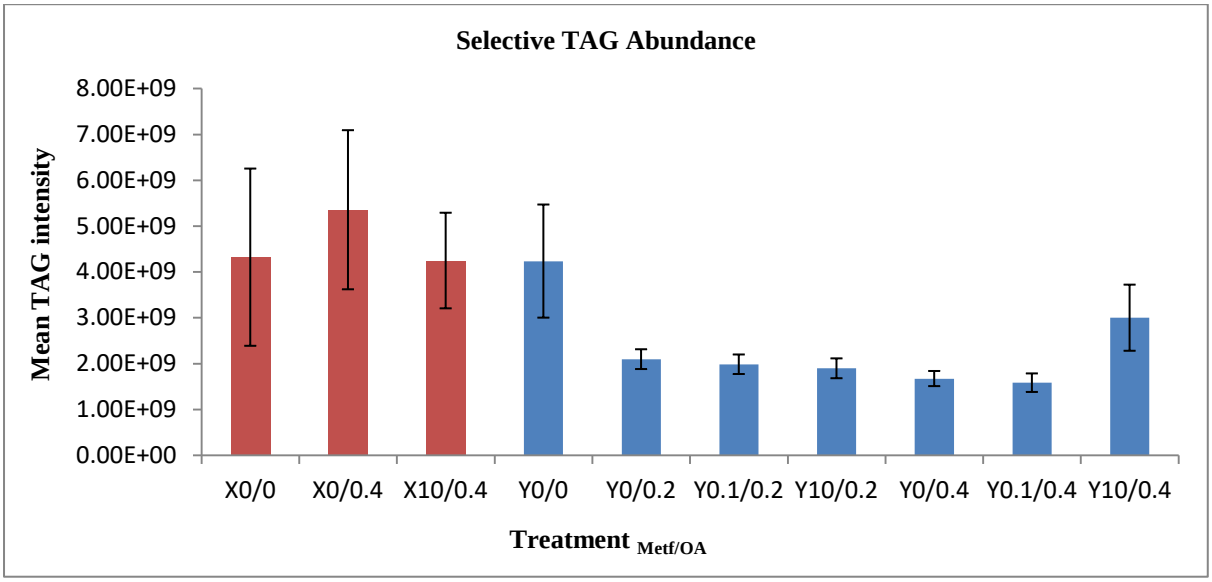


Figure 3.9 Mean of the selective TAGs (±SEM; after pruning the outliers) in response to 10 different treatments analyzed by LCMS - Chrombox D where red and blue color bars depicted the experimental condition respectively X and Y.

This selective TAG abundance strategy although showed a pattern somewhat similar to the total TAG abundance strategy (Section 3.5.1.1) for both experimental conditions, this strategy may not be very useful for quantitative analysis of TAG and interpreting the effect of metformin. Nonetheless, it might be useful to characterize specific samples with the presence of the selective TAG and to see how the individual TAG species varies in response to different treatments. Hence, 27 isolated TAG species were taken as variable to see their distribution pattern throughout all 10 treatments by multivariate analysis.

3.5.1.4 Multivariate analysis

3.5.1.4.1 Principal component analysis

A 10×27 matrix was formed to submit into PCA for data exploration applying data normalization with mean centering. The normal probability test of the data set was done and shown in Figure 3.10.

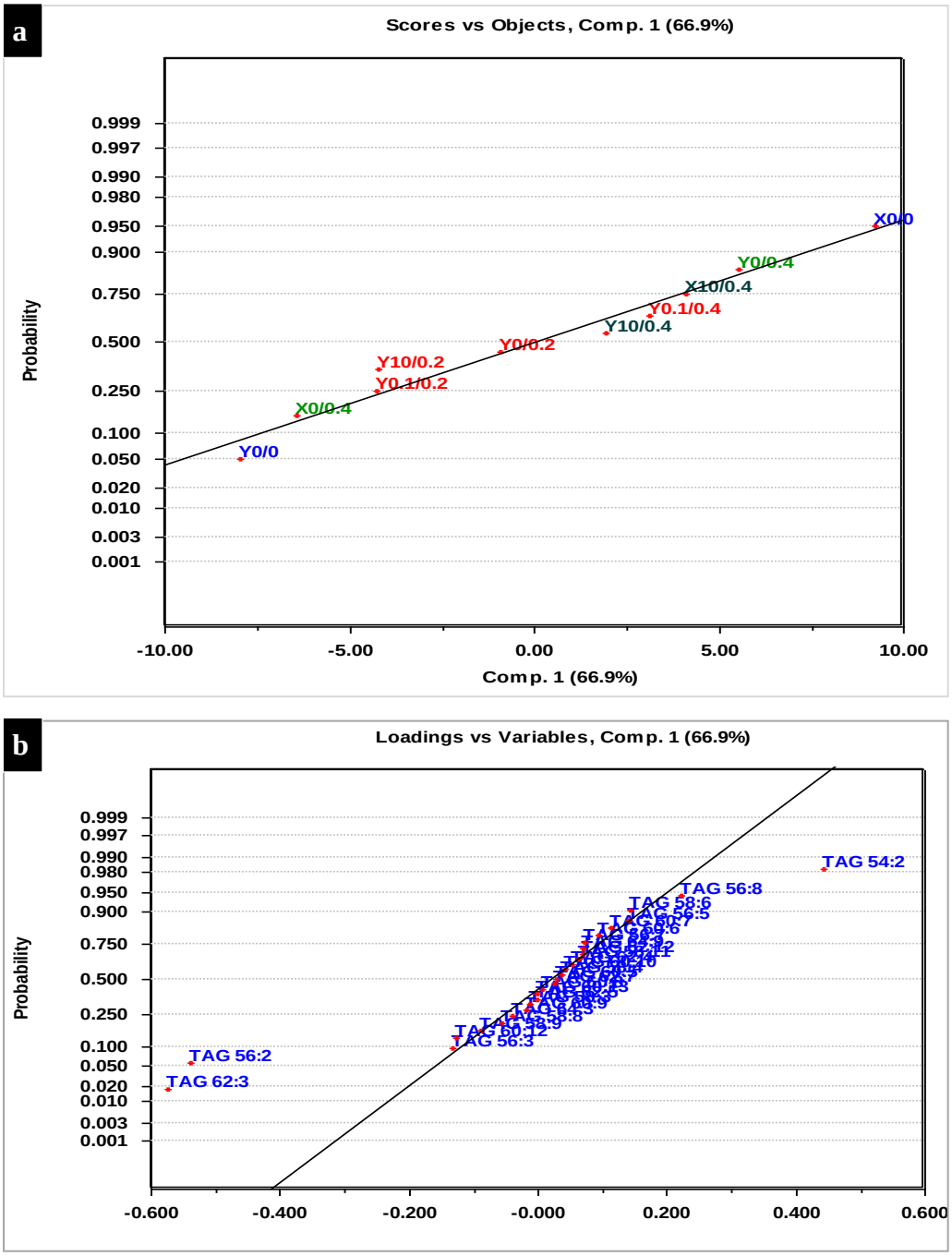
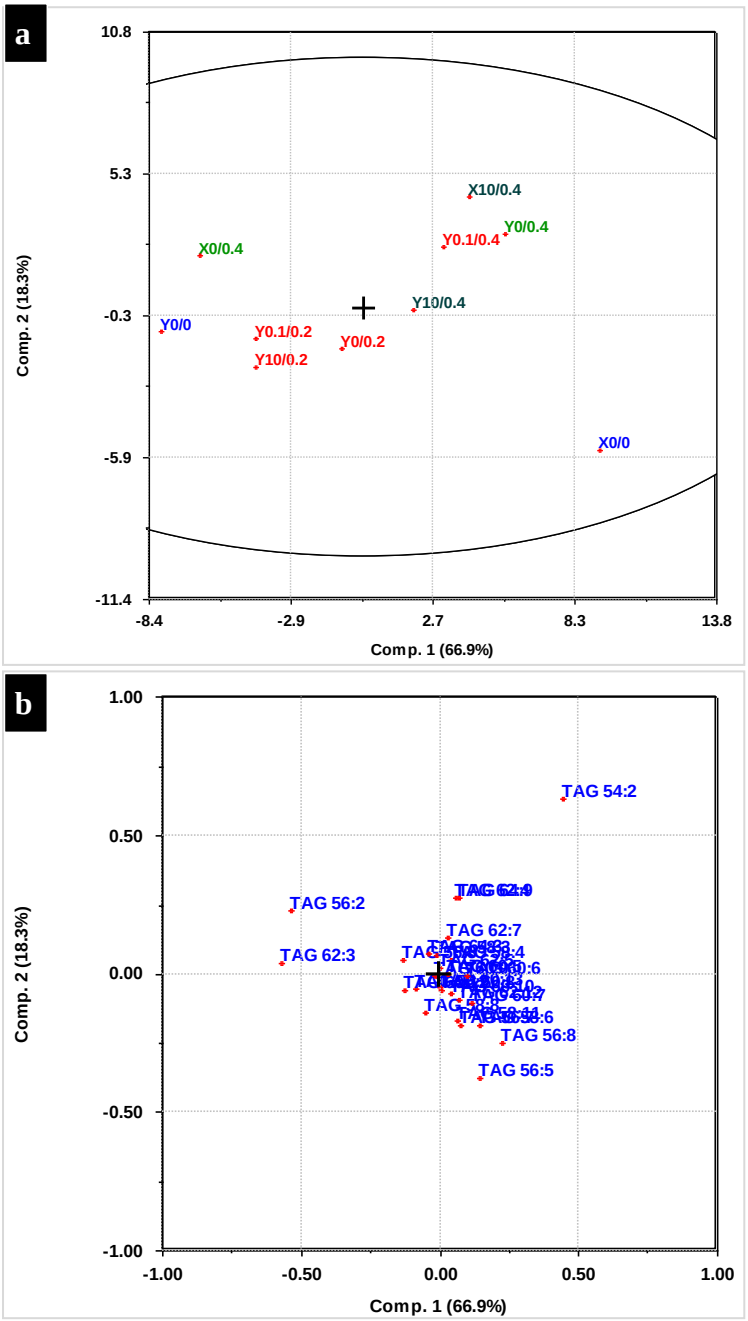


Figure 3.10 The treatments (a) and TAG species (b) showing the normal probability distribution pattern.

Figure 3.10 a) depicted that the treatments after data normalization showed normal distribution as expected while two controls placed in two opposite position indicating high variation in their relative intensities. In the case of Figure 3.10 b), the normal plot for 27 isolated TAG species showed clear deviations of three TAG species namely TAG 54:2, TAG 56:2 and TAG 62:3. TAG 54:2 showed the highest abundance where TAG 56:2 and TAG

62:3 showed the lowest. PCA revealed that TAG 54:2 may serve as predominant TAG species in oleic acid-induced samples. TAG 54:2 can most likely be the triacylglycerol with two monounsaturated C18 fatty acid i.e. oleic acid and one saturated C18 fatty acid i.e. stearic acid.



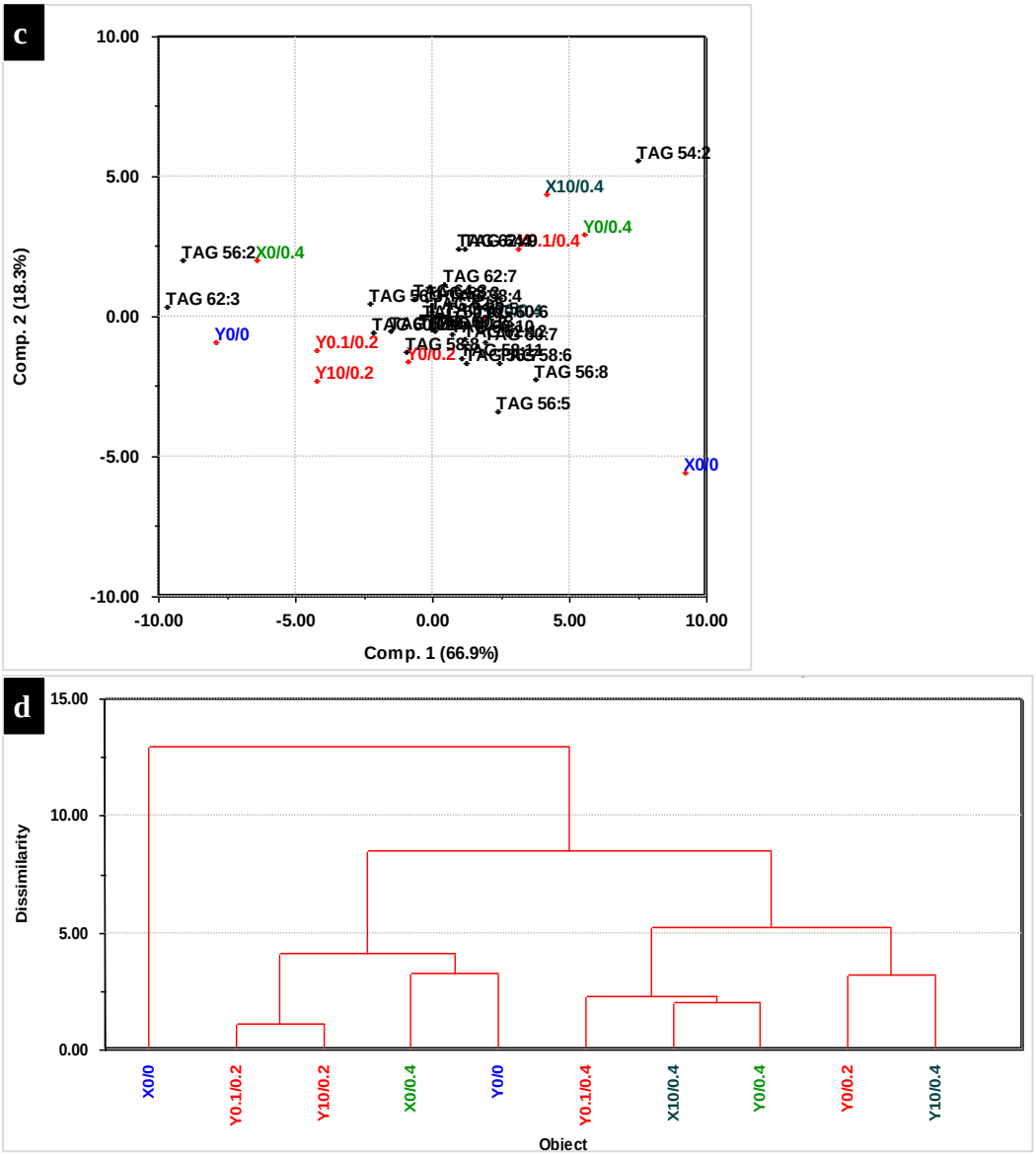


Figure 3.11 PCA for the normalized data showed a) score plot of the treatments' variability b) loading plot indicating variation due to the abundance variability of TAG species c) biplot – showing the impact of TAG abundance on individual treatment variability and d) the scores dendrogram showed the pattern of dissimilarity among treatments.

The score plot in Figure 3.11 revealed that the controls are extraordinary similar happened in case of HPTLC data, while loading plot and biplot revealed TAG54:2, TAG56:2 and TAG62:3 are distinctly positioned than others suggesting those as principal indicators for some treatments being different. Scores dendrogram treatments in first experimental condition ($X_{0/0}$, $X_{0/0.4}$ and $X_{10/0.4}$) showed significant dissimilarity indicating expected impact while treatments for second experimental condition remained unexplainable. This result also supported the findings from HPTLC analysis in Section 2.6.2.1.

3.5.2 Determination by QueryTAG

The identified TAG species for a sample as an example, their corresponding intensity, equivalent carbon number (ECN) and fatty acid in three positions (sn-1, sn-2, and sn-3) of TAG backbone were shown in increasing order of equivalent carbon number (ECN) in Table 3.4.

Table 3.4 An example of QueryTAG generated data for test samples, where sn- positions, ECN, and intensity are highlighted in golden.

'Rt'	'sn-1'	'sn-2'	'sn-3'	'Single bonds'	'Double bonds'	'ECN'	'Precursor ion'	'Adduct type'	'MW'	'MW'	'MW'	'Scanning point'	'Intensity'
'DFE'	'18:1n'	'EPA'	'DHA'	15	12	36	975,503	'Na'	302,52362	282,50769	328,55408	1350	184436,13
'CDC'	'DHA'	'18:1n'	'DHA'	15	13	36	1001,4	'Na'	282,43417	328,48788	328,48788	1383	369233,25
'DFE'	'EPA'	'18:1n'	'DHA'	15	12	36	975,797	'Na'	282,76996	328,57062	302,64423	1402	154303,66
'BFF'	'18:00'	'DHA'	'DHA'	16	12	38	1002,37	'Na'	328,4621	328,4621	284,19873	1452	425695,19
'AFE'	'DHA'	'18:1n'	'22:5n'	16	12	38	1002,31	'Na'	282,22797	328,24677	330,18408	1458	492253,94
'ACB'	'18:1n'	'DHA'	'22:5n'	16	12	38	1002,92	'Na'	328,87497	282,84927	330,86563	1467	254083,27
'CFE'	'18:2n'	'EPA'	'18:1n'	17	8	40	927,772	'Na'	302,87497	280,65292	282,48752	1518	202968,47
'DFD'	'18:1n'	'DHA'	'18:1n'	18	8	42	953,524	'Na'	328,1615	282,38037	282,38037	1554	193224,77
'BFB'	'18:1n'	'18:3n'	'18:1n'	19	5	44	904,648	'Na'	278,67151	282,5755	282,5755	1705	350826,09
'BCC'	'18:2n'	'18:2n'	'18:1n'	19	5	44	904,648	'Na'	280,58734	280,58734	282,5755	1705	437695
'AFC'	'18:2n'	'18:3n'	'18:00'	19	5	44	904,648	'Na'	278,67151	280,58734	284,25287	1705	413208,41
'AGC'	'18:1n'	'EPA'	'18:00'	19	6	44	930,766	'Na'	302,73328	282,84686	284,30573	1743	1714951,5
'CFC'	'18:1n'	'20:4n'	'18:1n'	19	6	44	931,706	'Na'	304,2561	282,77222	282,77222	1998	336903,25
'BCC'	'18:1n'	'18:1n'	'22:5n'	19	7	44	957,197	'Na'	282,16092	282,16092	331,0032	2001	206317,31
'AFC'	'18:1n'	'20:4n'	'18:00'	20	5	46	932,117	'Na'	304,57336	282,1485	283,93762	2010	945310,38
'AGF'	'18:00'	'18:1n'	'20:4n'	20	5	46	932,147	'Na'	282,16104	304,58914	284,01584	2038	665766,75
'BBB'	'18:1n'	'18:1n'	'18:1n'	21	3	48	909,728	'Na'	282,89481	282,89481	282,89481	2527	390449,13
'DGF'	'18:1n'	'18:2n'	'20:1n'	21	4	48	935,762	'Na'	280,68661	310,47086	282,6658	2865	259913,34
'ACC'	'18:1n'	'18:1n'	'18:00'	22	2	50	910,628	'Na'	282,86237	282,86237	284,38953	2529	1043815,6
'BEB'	'18:1n'	'20:1n'	'18:1n'	22	3	50	936,31	'Na'	310,98303	282,18665	282,18665	2857	506680,53
'ABA'	'18:1n'	'18:00'	'18:1n'	22	2	50	910,921	'Na'	284,83853	282,89481	282,89481	3081	202275,63

The total intensity of TAGs in every sample and the mean value with SEM by equation 2 were calculated and tabulated in Table 3.5.

Table 3.5 Total amount of TAGs (intensity) per sample determined by LCMS - QueryTAG analysis where X denotes 1 hour treatment with metformin (Metf) and following 47 hours inducement with oleic acid (OA) whilst Y denotes 24 hours treatment with metformin and following 24 hours inducement with oleic acid and the subscripted numbers indicate the concentrations of metformin (numerator) and oleic acid (denominator) at 0, 0.1, or 10 mM for metformin and 0, 0.2, 0.4 mM for oleic acid.

	X0/0	**X0/0.2**	**X0.1/0.2**	**X10/0.2**	**X0/0.4**	**X0.1/0.4**	**X10/0.4**
Fish 1	7.8E+07	1.7E+06	4.2E+06	4.3E+05	4.0E+06	1.8E+06	1.7E+06
Fish 2	5.5E+06	4.5E+05	1.7E+06	2.5E+06	1.0E+07	1.2E+06	2.8E+06
Fish 3	2.0E+07	1.7E+07	2.0E+07	1.6E+07	1.9E+07	2.1E+07	2.7E+07
Fish 4	7.1E+05	3.3E+06	3.2E+06	2.8E+06	2.3E+06	5.0E+06	3.3E+06
Fish 5	6.4E+04	2.4E+06	1.4E+06	2.3E+06	5.8E+06	1.8E+06	3.3E+06
Fish 6	2.8E+05	2.3E+06	1.2E+06	1.5E+06	3.0E+06	3.7E+06	4.1E+06
Mean	1.7E+07	4.6E+06	5.3E+06	4.3E+06	7.3E+06	5.8E+06	7.1E+06
SEM	1.3E+07	2.6E+06	3.0E+06	2.5E+06	2.5E+06	3.2E+06	4.0E+06
	Y0/0	**Y0/0.2**	**Y0.1/0.2**	**Y10/0.2**	**Y0/0.4**	**Y0.1/0.4**	**Y10/0.4**
Fish 1	2.2E+06	1.9E+06	2.9E+06	1.6E+06	1.2E+06	1.1E+06	3.0E+06
Fish 2	1.4E+07	1.7E+06	3.0E+06	5.4E+05	1.6E+06	8.9E+05	ND
Fish 3	1.1E+07	2.5E+07	8.9E+06	5.0E+06	1.6E+07	1.2E+07	8.9E+06
Fish 4	3.2E+06	5.1E+06	4.6E+06	1.7E+06	2.5E+06	6.6E+06	1.9E+06
Fish 5	2.1E+06	6.7E+06	1.3E+06	2.1E+06	1.2E+06	ND	1.4E+06
Fish 6	2.3E+06	1.9E+06	1.7E+06	2.1E+05	1.5E+06	2.0E+06	9.1E+05
Mean	5.7E+06	7.1E+06	3.7E+06	1.9E+06	4.1E+06	4.5E+06	3.2E+06
SEM	2.1E+06	3.8E+06	1.1E+06	7.0E+05	2.4E+06	2.1E+06	1.5E+06

The standard plot was created in Figure 3.12 with the value in Table 3.5 that showed the distribution of level of TAGs for all six biologically similar fish in regards to fourteen different treatments including controls. An uneven distribution of TAG level in response to individual treatment was observed where controls and some other treatments displayed higher variation amongst six fish. Fish 3 became exceptional one in terms of intensity of TAG as also seen in case of Chrombox D analysis and unlike HPTLC but LCMS- Chrombox D, the control for fish 1 displayed abnormally higher intensity (Section 3.5.1). The overall

observation was found very similar to the result found in HPTLC analysis in Figure 2.3 and LCMS- Chrombox D analysis in Figure 3.4.

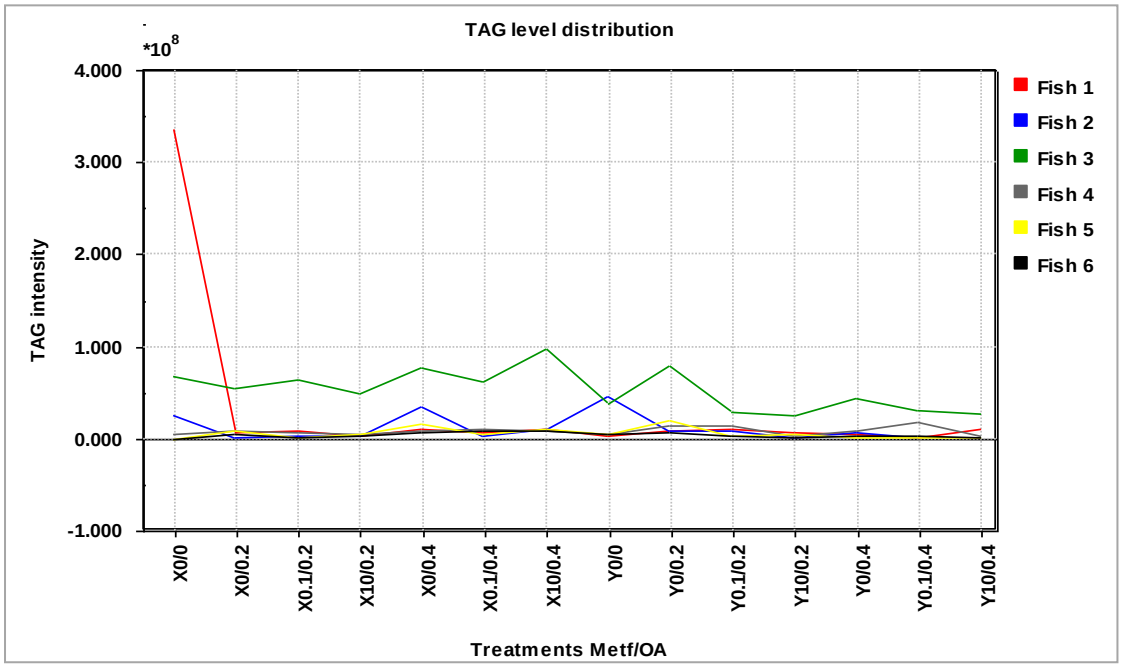


Figure 3.12 Standard plot for the distribution of TAG level for all six biologically similar fish in regards to fourteen different treatments including two controls analyzed by LCMS - QueryTAG.

To avoid complexity, ten treatments similar to the HPTLC were considered for further data analysis. Total TAG intensity for individual samples was counted to plot the bar diagram in Figure 3.13 that showed at the experimental condition X (1h Metf + 47h OA), the control ($X_{0/0}$) showed the highest intensity of TAG accumulation ($X_{0/0.4}$) that was unexpected, despite the higher standard error. But the treatment with 10 mM metformin in the presence of 0.4 mM oleic acid ($X_{10/0.4}$) showed a reduction of TAG nearly 36% as compared to $X_{0/0.4}$.

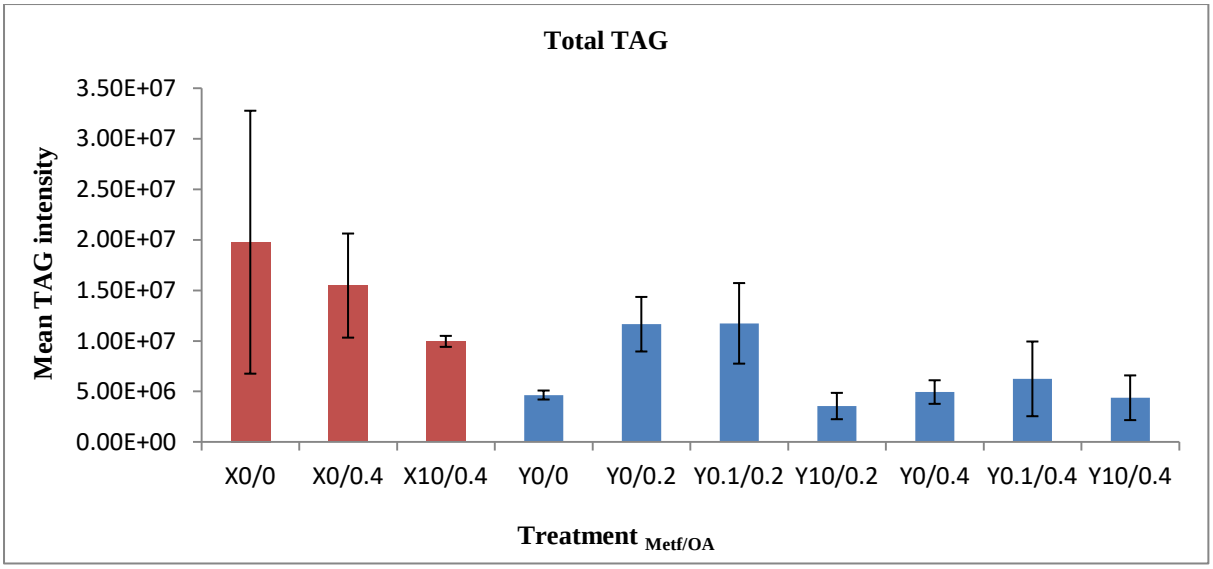


Figure 3.13 Mean of the total TAGs (±SEM; after pruning the outliers) in response to 10 different treatments analyzed by LCMS - QueryTAG where red and blue color bars depicted the experimental condition respectively X and Y.

The experimental condition Y (24h Metf + 24h OA) did not show any meaningful result in case of 0.4 mM oleic acid induced treatments likewise to the HPTLC in Section 2.6.1 & LCMS - Chrombox D in Section 3.5.1.1. However, the treatments $Y_{0/0}$, $Y_{0/0.2}$, $Y_{0.1/0.2}$ and $Y_{10/0.2}$ expectedly displayed the effect of metformin, which was also determined in LCMS-Chrombox in Section in 3.5.1.1 and Figure 3.6 but not determined by HPTLC in Section 2.6.1 and Figure 2.6. Therefore, it is also indicating the capability of LCMS to extract more accurate and reliable results than HPTLC.

Further data analysis strategies were explored to see the ability of QueryTAG to reproduce outcomes from HPTLC and LCMS - Chrombox D.

3.5.2.1 Data Analysis Strategy I (OA at *sn*-positions of TAG)

As the samples were induced with oleic acid in two concentrations, it was expected to have the high abundance of oleic acid on the backbone of TAG structure as compared to control and metformin-treated samples. Therefore, the presence of oleic acid (18:1n) in three positions (sn-1, sn-2, and sn-3) of TAG was explored using MS Excel datasheet and extracted corresponding intensity resulting in TAG formation. The mean intensity of TAG was thereby calculated and plotted in Figure 3.14.

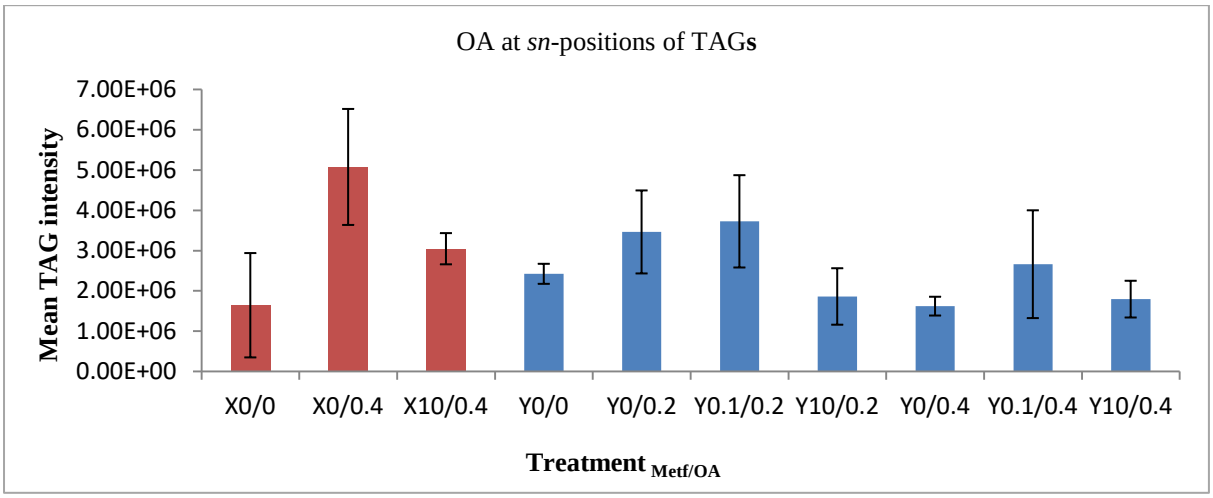


Figure 3.14 Mean intensity of TAGs (±SEM; after pruning the outliers) in response to 10 different treatments analyzed by LCMS - QueryTAG while considering intensity due to the presence of oleic acid in at least one of sn-1, 2 and 3 positions. The red and blue color bars depicted the experimental condition respectively X and Y.

The condition X (1h Metf + 47h OA), 0.4mM oleic acid-induced TAG formation ($X_{0/0.4}$) nearly 209% as compared to the control ($X_{0/0}$) and treatment with 10 mM metformin in the presence of 0.4 mM oleic acid ($X_{10/0.4}$) reduced level of TAG nearly 40% as compared to $X_{0/0.4}$. This finding showed the similarity with the HPTLC results (Section 2.6.1 and Figure 2.3) and LCMS - Chrombox D (Section 3.5.1.1 and Figure 3.6). The experimental condition Y (24h Metf + 24h OA) did not show any meaningful result in case of 0.4 mM oleic acid induced treatments likewise to the HPTLC & LCMS - Chrombox D. However, the treatments $Y_{0/0}$, $Y_{0/0.2}$ and $Y_{10/0.2}$ expectedly displayed the effect of metformin, which was also determined in LCMS- Chrombox but not determined by HPTLC. This strategy also strengthens the indication of the capability of LCMS to extract more accurate and reliable results than HPTLC.

3.5.2.2 Multivariate Analysis

3.5.2.2.1 Partial least square regression analysis

Similar to the Section 2.6.2.1 and 3.5.1.2.1, a data matrix with three independent variables and the mean TAG analyzed by LCMS - QueryTAG as the dependent variable was prepared for PLS regression analysis by Sirius 9.0.

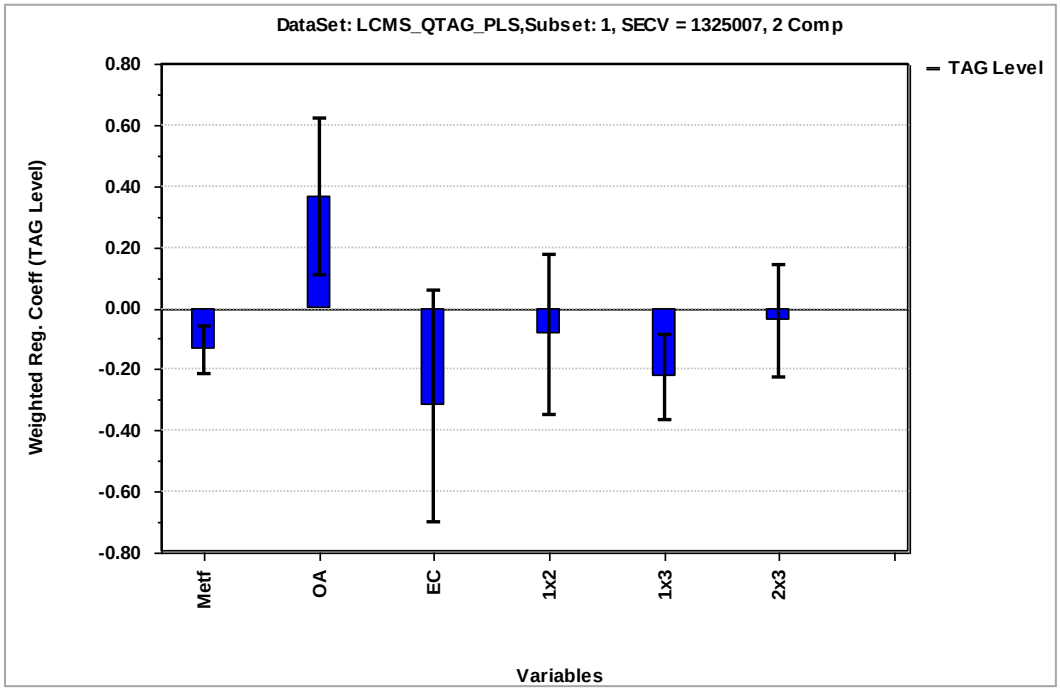


Figure 3.15 Regression coefficient showing the main and interaction effects of variables of variables on TAG level analyzed by LCMS - QueryTAG.

Like Section 3.5.1.2.1, PLS regression for LCMS - QueryTAG data displayed closely similar findings to the HPTLC (Section 2.6.2.1 and Figure 2.7). As seen in Figure 3.15, the effect of metformin and all interactions (1×2), (1×3) and (2×3) were less significant. Oleic acid showed positive effect proving higher level yielding more TAG. Experimental condition (EC) showed negative effect indicating that increasing incubation time with metformin but decreasing with oleic acid would yield reduced TAG level, however, the higher uncertainty indicated a less significant effect. This was also seen in interaction effect of metformin and experimental condition (1×3). The effect of metformin, despite less significant, the trend indicated a negative effect on TAG accumulation as occurred in LCMS-Chrombox (Figure 3.7).

3.5.2.2.2 Principal component analysis

The normalized data with 6×10 matrix was submitted to PCA for data exploration. The PC1 and PC2 expressing variance respectively 80.1% and 12.0% were considered for extracting score and loading plots in addition to biplot and scores dendrogram as shown in Figure 3.16. Score plot in Figure 3.16 a) showed the highest variation in fish 1 and fish 2 and the biplot in Figure 3.16 c) showed that the observed variation happened mainly due to the effect of controls. The scores dendrogram in Figure 3.16 d) showed the relative dissimilarity among the samples. That is, the PCA for these data showed the similarity in outcome from HPTLC data in Section 2.6.2.1 and LCMS - Chrombox D in Section 3.5.1.2.

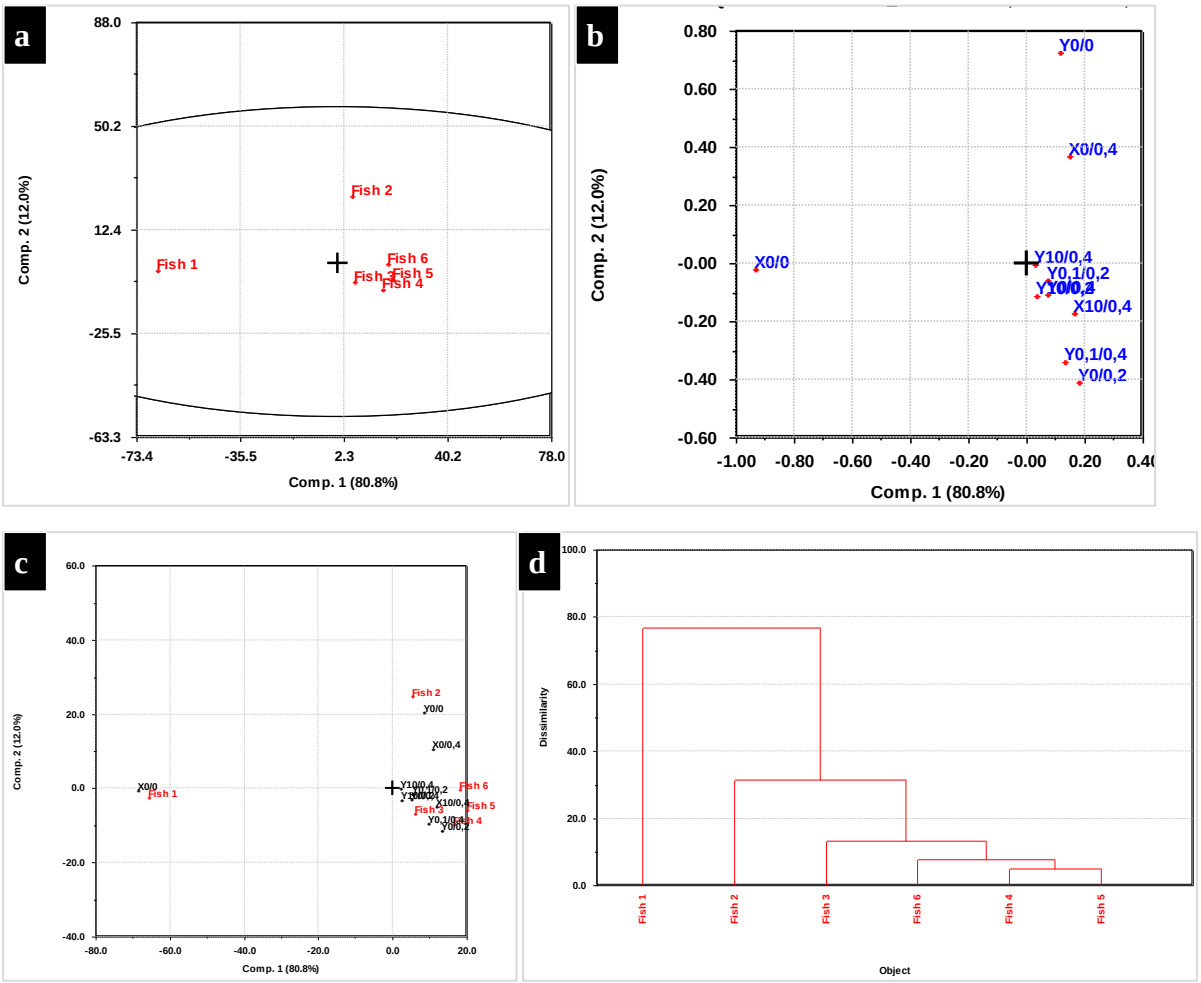


Figure 3.16 the normalized data matrix for PCA showed a) score plot of the sample variability b) loading plot indicating variation due to the treatment variability c) biplot – showing the impact of treatment on individual objects to be different than each other and d) the scores dendrogram showed the pattern of dissimilarity among samples.

3.5.2.3 Data Analysis Strategy II (ECN value)

In chromatography, many of studies have reported the usefulness of equivalent carbon number (ECN) for characterizing properties of specific analyte or metabolite in samples [97, 98] and discrimination analysis [14]. Some studies have used ECN for the tentative identification of TAG [99]. Therefore ECN has been considered as the key factor for this strategy. ECN can be calculated by following formula.

$$ECN = CN - (2 \times DB) \dots\dots\dots Equation\ 4$$

Where *CN* represents the total carbon number and DB the number of double bonds in the TAG species.

The QueryTAG raw data files (as seen in Table 3.4) displayed the range of ECN from 30 to 54. The frequencies of same ECN were summed up for similar individual treatments (replicates) and tabulated in table 3.6.

Table 3.6 Summed ECN value of 6 fish in response to ten individual treatments by LCMS-QueryTAG analysis.

ECN	X0/0	X0/0.4	X10/0.4	Y0/0	Y0/0.2	Y0.1/0.2	Y10/0.2	Y0/0.4	Y0.1/0.4	Y10/0.4
30	5	2	5	1	0	0	0	0	2	1
32	9	1	6	0	0	0	0	0	1	0
34	5	0	3	2	0	0	0	0	0	0
36	25	9	5	4	4	3	1	4	1	3
38	36	0	8	0	6	3	3	6	3	4
40	34	7	8	6	8	4	1	7	2	2
42	49	14	9	12	9	3	1	4	0	0
44	51	13	16	11	13	8	2	6	2	2
46	39	6	16	5	9	1	1	8	4	3
48	25	6	14	8	9	6	1	6	5	1
50	29	21	21	21	17	17	15	16	15	14
52	12	24	19	17	14	13	6	15	9	13
54	3	5	5	4	9	7	6	7	2	2
Sum	322	108	135	91	98	65	37	79	46	45
Mean	25	8	10	7	8	5	3	6	4	3
SD	16.8	7.7	6.0	6.6	5.4	5.2	4.2	5.0	4.2	4.6
CV	0.7	0.9	0.6	0.9	0.7	1.0	1.5	0.8	1.2	1.3

Table 3.6 showed that the control $X_{0/0}$ of the experimental condition X (1h Metf + 47h OA) had higher mean value of ECN with a high standard deviation as compared to other treatments. But the coefficient variance for the treatments were low (less than 1) that meant though the mean is higher, the variation around it is lower [100].

Inverse of mean square (multiplying to 100 %) was calculated to plot the bar diagram in figure 3.17 that showed for the experimental condition X (1h Metf + 47h OA), 0.4 mM oleic acid-induced treatment increased TAG (789%) as compared to the control ($X_{0/0}$) and treatment with 10 mM metformin in the presence of 0.4 mM oleic acid ($X_{10/0.4}$) displayed reduced in response to 36% as compared to $X_{0/0.4}$. This finding exhibited the similarity with the HPTLC results (Section 2.6.1 and Figure 2.6) and LCMS - Chrombox D (Section 3.5.1.1 and Figure 3.6). The response pattern for the experimental condition Y remained unresolved similar to the findings in the case of HPTLC.

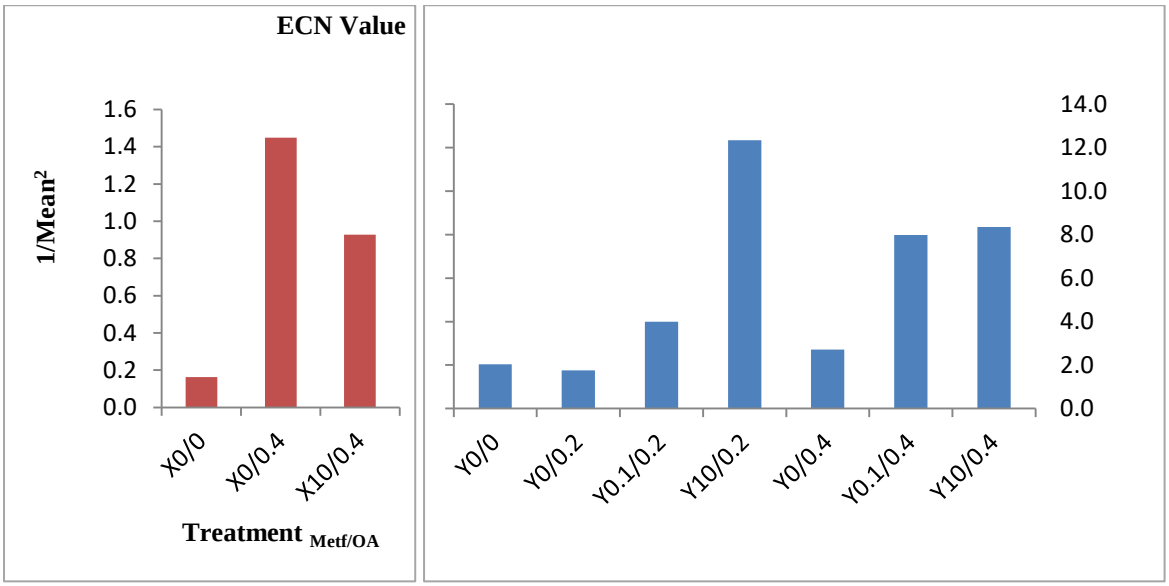
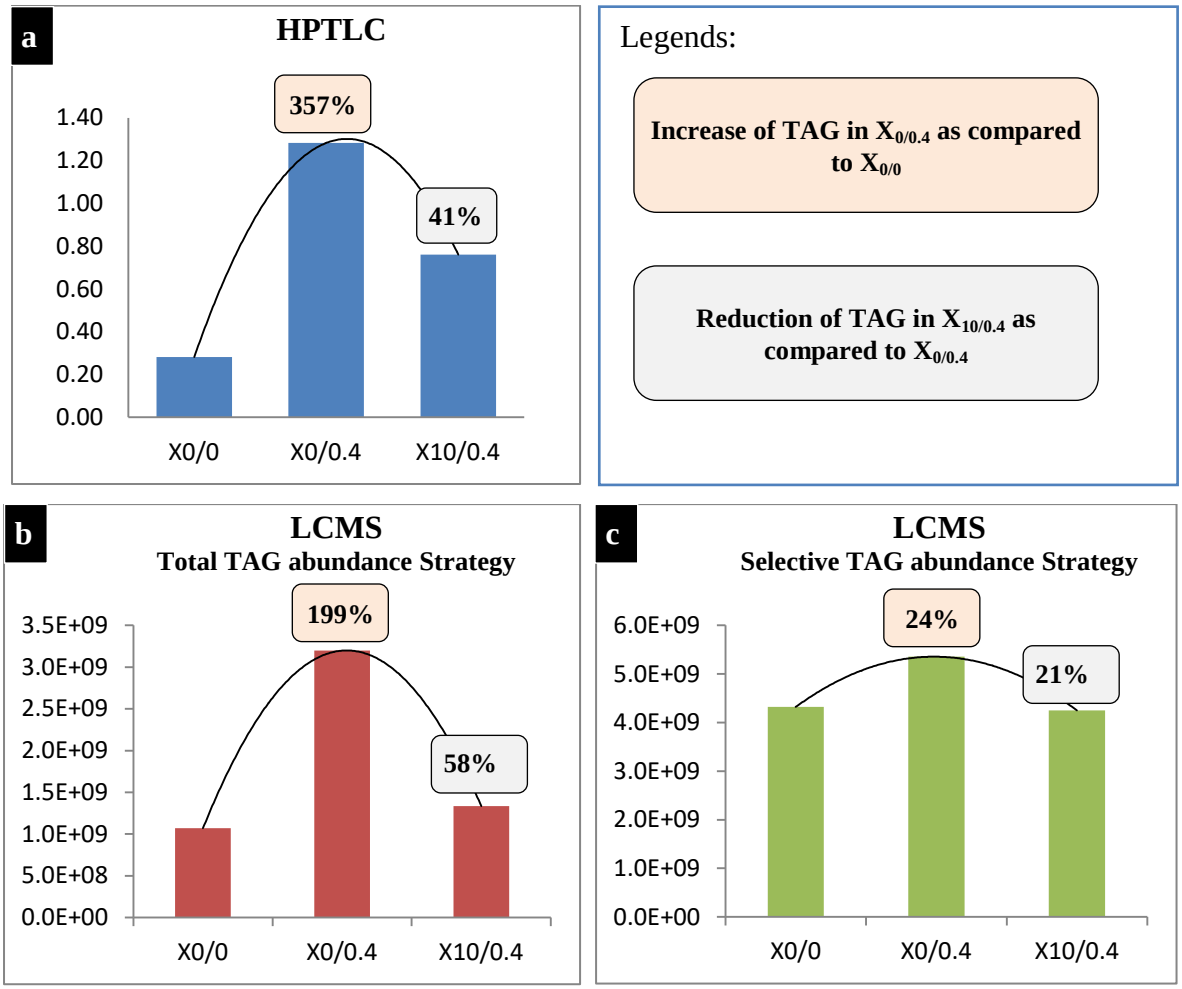


Figure 3.17 The bar plot showing the response (correlated to TAG level) in terms of ECN value versus treatments in two experimental conditions.

3.6 Comparison amongst outcomes of different strategies

The comparison of the results for the experimental condition X (1h Metf + 47h OA) between HPTLC and LCMS techniques along with different data analysis strategies were simplified and shown in Figure 3.18. In terms of reduction of TAG, the metformin-treated samples as compared to the oleic acid-induced samples showed 41% in HPTLC, while 58% and 21% for two strategies of LCMS - Chrombox D, and 40% and 36% for two strategies of LCMS - QueryTAG. As observed in Figure 3.18 (f), the TAG accumulation and reduction trend in case of LCMS-Chrombox D- total TAG abundance strategy and LCMS-QueryTAG- OA at sn-positions strategy displayed pattern similarity and proximity in trend to the HPTLC and thus these strategies could be more effective for TAG quantification by LCMS. Therefore, they were furthered compared by PLS regression and PCA.



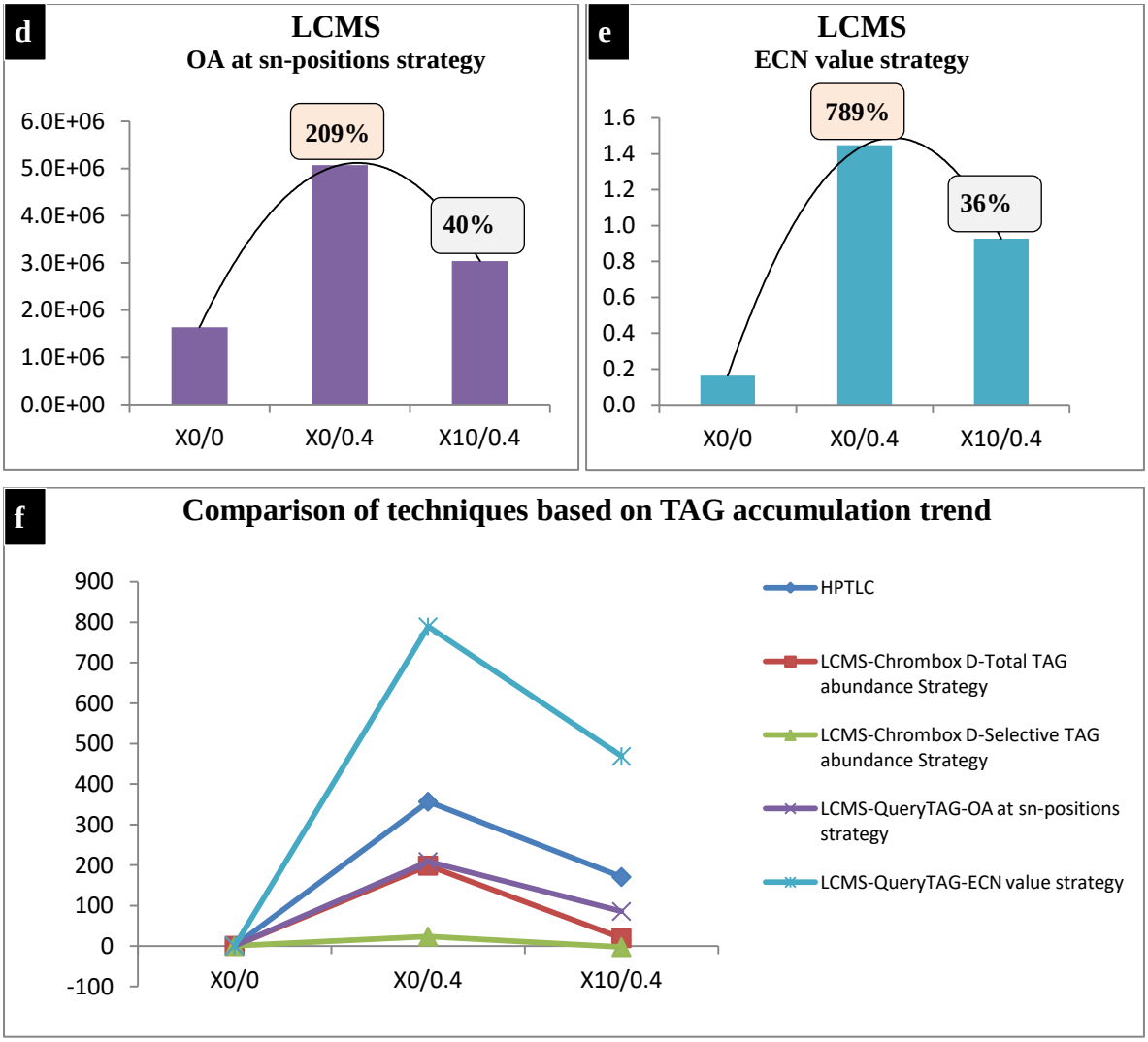


Figure 3.18 Comparison of the responses of the treatments in the condition X (1h Metf + 48h OA) found from a) HPTLC technique, b) LCMS - Chrombox D with total TAG abundance strategy, c) LCMS - Chrombox D with selective TAG abundance strategy, d) LCMS - QueryTAG with OA at sn-positions strategy, e) LCMS - QueryTAG with ECN value strategy and f) TAG accumulation trend from all techniques and strategies plotted together.

In Figure 3.19, PLS regression coefficient plots extracted from the data produced by HPTLC and LCMS -Chrombox D and LCMS- QueryTAG were aligned to compare which displayed high resemblance amongst the pattern of regression coefficients (for metformin, oleic acid, and experimental conditions) in response to TAG level. All the three main effects of variables had nearly similar regression coefficient and three interaction effects showed little dissimilarities; however, they were less significant for TAG quantification.

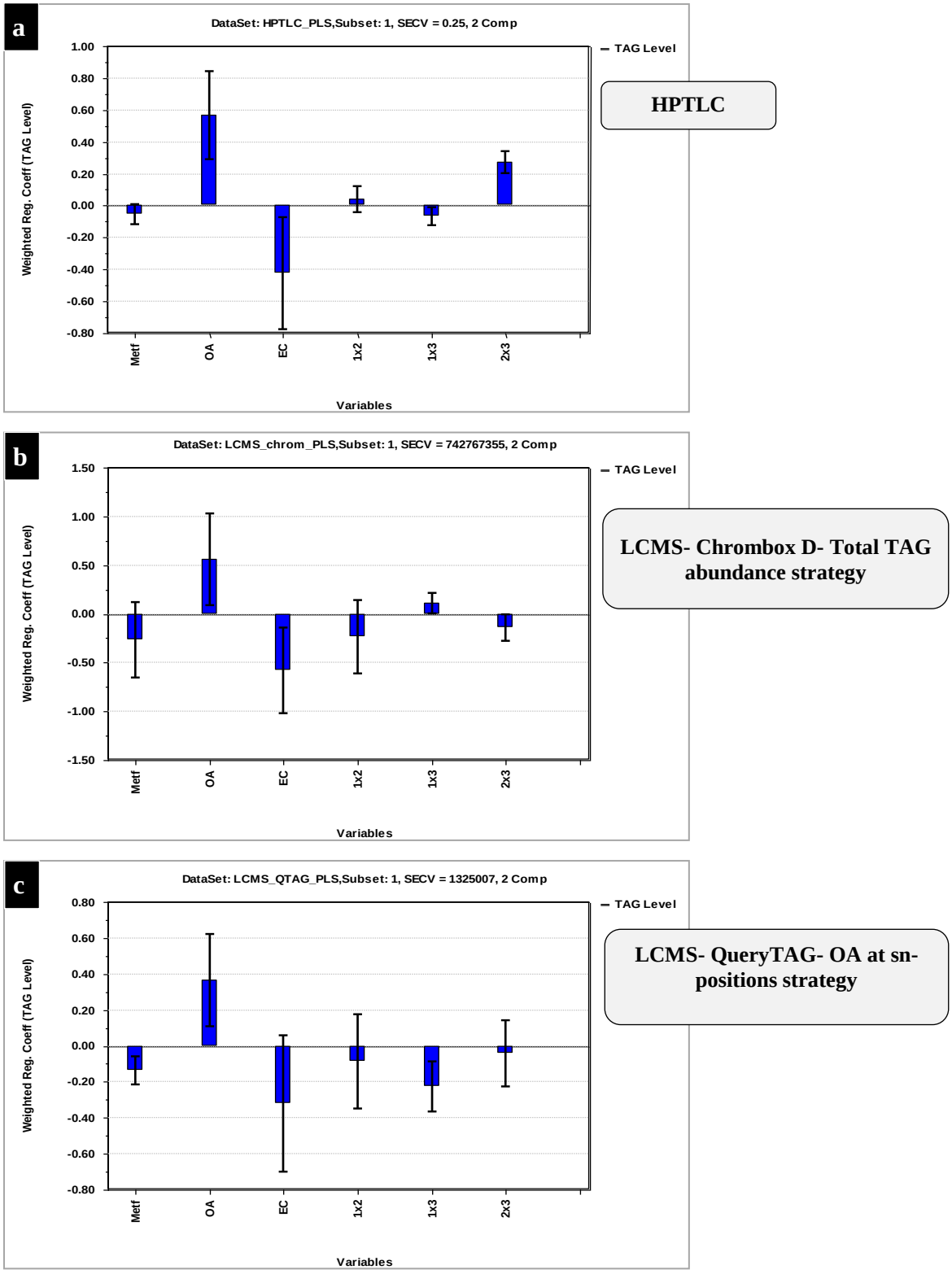


Figure 3.19 Comparison among the bar graph of PLS regression coefficient elucidated from HPTLC (a) and LCMS by Chrombox D (b) and QueryTAG (c) data analyses for the main factor effects and their interaction effects on the outcome of response.

PCA revealed the similar pattern of grouping among the fish replicates that generated from the data produced by HPTLC and LCMS techniques as seen in Figure 3.20. The scores dendrograms for LCMS - Chrombox D and LCMS - QueryTAG displayed exactly similar pattern that has a minor difference with the HPTLC. HPTLC made two major groups with fish 1 and 2 in one and the rest in another while the dissimilarity between fish 1 and 2 are smaller than LCMS analyses. Still, the rest of the dissimilarity pattern based on Euclidean distance remained almost similar.

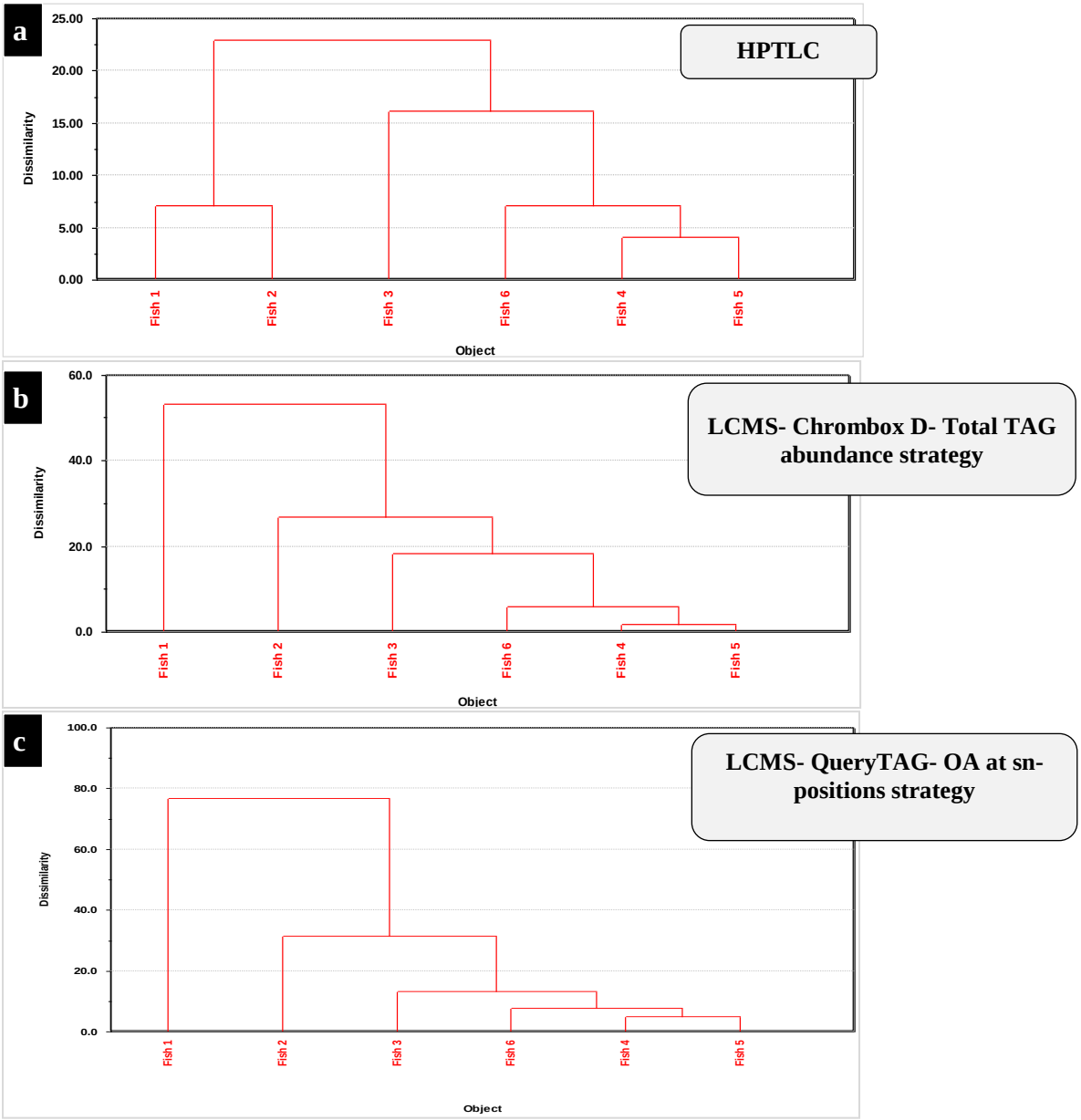


Figure 3.20 Comparison among the scores dendrograms found in HPTLC (a) and LCMS by Chrombox D (b) and QueryTAG (c) data analyses that indicated the similarity/dissimilarity in clustering pattern of fish samples.

3.7 Conclusions

HPTLC and LCMS techniques with different data analysis strategies were rigorously compared. The LCMS technique accompanied by multiple data analysis strategies (Chrombox D and QueryTAG) suggests it as an effective and good alternative to HPTLC for the analysis of TAG in Salmon liver cells. . HPTLC and LCMS results were in agreement for at least experiments labeled as $X_{Metf/OA}$= $X_{0/0}$; $X_{0/0.4}$; and $X_{10/0.4}$.

In case of TAG reduction by metformin, the two QueryTAG data analysis strategies (OA at *sn*-positions of TAG and ECN value) showed pattern similarity; however OA at *sn*-positions strategy displayed close accord with the HPTLC results, indicating this could be useful for TAG quantification. Similarly, the total TAG abundance strategy for Chrombox D showed close agreement with HPTLC and thus this strategy could also be effective for TAG quantification. The selective TAG abundance strategy may be useful for characterizing TAG species such as the presence of specific TAGs, variability, and predominance of TAG species in specific biological samples.

It was also observed that LCMS with both data analysis tools (Chrombox D and QueryTAG) could have detected the expected outcomes in the experimental condition, 24 hours treatment with metformin and following 24 hours inducement with 0.2 mM oleic acid, which was not detected by HPTLC. This point highlighted the power of LCMS to extract more accurate and reliable results than HPTLC.

After a critical comparative analysis, it is to conclude that LCMS can be a potential alternative to replace cumbersome and expensive HPTLC technique in the quantitative analysis of TAG, while moreover, LCMS can characterize TAG in biological samples.

Chapter 4 Concluding remarks and future recommendations

This thesis has successfully evaluated the potential of metformin on the *in-vitro* produced salmon liver cell in changing TAG metabolism. It was interesting to see that metformin treatment for 24 hours did not show any significant impact on the cell cultures induced by oleic acid for 24 hours. This situation might have happened due to the longer exposure with metformin and shorter exposure with oleic acid. For future studies, it is particularly recommended to adjust the incubation period for both metformin and oleic acid.

In this study, due to time and resource constraints, it was not possible to analyze four of the treatments ($X_{Metf/OA}$= $X_{0/0.2}$, $X_{0.1/0.2}$, $X_{10/0.2}$, $X_{0.1/0.2}$) by HPTLC and to compare with LCMS. This study strongly suggests that analyzing those treatments by HPTLC might be important to acquire valuable information in comparing the results with LCMS.

LCMS data analyzed by Chrombox D and QueryTAG showed mostly similar results to HPTLC. Therefore, both Chrombox D and QueryTAG data analysis are equally capable to be good tools in analyzing LCMS data. However, Chrombox D is unable to elucidate the positional distribution of fatty acids on the TAG backbone whilst QueryTAG serves as a standalone tool for this purpose. On the other hand, Chrombox D can be applied to a wide range of lipid analysis including TAG whereas QueryTAG is the algorithmic software tool intended only for TAG analysis. In Chrombox D analysis, it is possible to choose relevant fatty acids and possible ionization adducts formation during library creation for TAG analysis allowing users to have a robust system and to eliminate endogenous data signals from noises. In both ESI positive or negative mode, QueryTAG is able to identify the presence and abundance of ion adducts whereas Chrombox D remains dependent on user preference in selecting appropriate ion adducts. So, the combination of both software tools in TAG analysis would give complete, accurate and more authentic information of the biological sample in case of lipid analysis. However, Chrombox D algorithm allows the flexibility to analyze wider compound classes, it is strongly recommended for proper validation for each type of lipids; for instance, TAG data analysis requires prior validation of this software by the data of TAG standards.

Another recommendation for future work is to use a set of TAG standard to prepare calibration curve so that the intensities of TAGs by LCMS can be converted into real amount or concentration of TAGs in the sample.

This study was limited to TAG analyses in oleic acid-induced *in-vitro* salmon fatty liver cells, nonetheless, the findings might be applicable for the TAG analysis in other kinds of samples than salmon liver cells.

The research outcomes would greatly contribute solving research questions related to the choice of LCMS over HPTLC. This would also facilitate the analytical and research laboratories in taking decision of installing only LCMS instead of HPTLC for quantifying and characterizing TAGs in biological samples that, in result, would save valuable resources. For instance, NIFES is planning to purchase a new instrument for TAG analysis and this research would be an invaluable input for its decision-making.

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Appendix A: An Excel-based tool for rapid and easy processing of Chrombox D extracted LCMS data

A.1 Introduction

Chrombox D is useful for LCMS analysis for quantification of various lipid classes including neutral lipids such as diacylglycerol (DAG), TAG and polar lipids such as phospholipids, glycolipids etc. But, challenges associated with data analysis, in particular, is the simplified comparison of quantitative variation of specific lipid of interest in multiple biological samples.

Chrombox D presents a full list of possible species of individual lipid quantitatively from single analysis based on least squares spectral resolution [94]. It is hard to compare the presence and/or level of individual species of a lipid in multiple samples at the same time.

A Microsoft Excel template with a collection of Visual Basic for Applications (VBA) macros might enable rapid and simple Chrombox D data filtering and sorting, and automated processing with a particular focus on allowing species based quantitative comparability and the total amount of individual lipid in multiple samples.

A.2 Visual Basic for Applications

VBA is a scripting (macro) language based on Visual Basic, a high-level programming language developed by Microsoft. VBA macro provides the capability to perform interactive calculations and different functions based on the result of logic function in Excel and other Microsoft programs [101]. VBA code normally can only run within a host application such as Excel, rather than as a standalone program [102]. The coded VBA macro program for meeting our objective is written as follows:

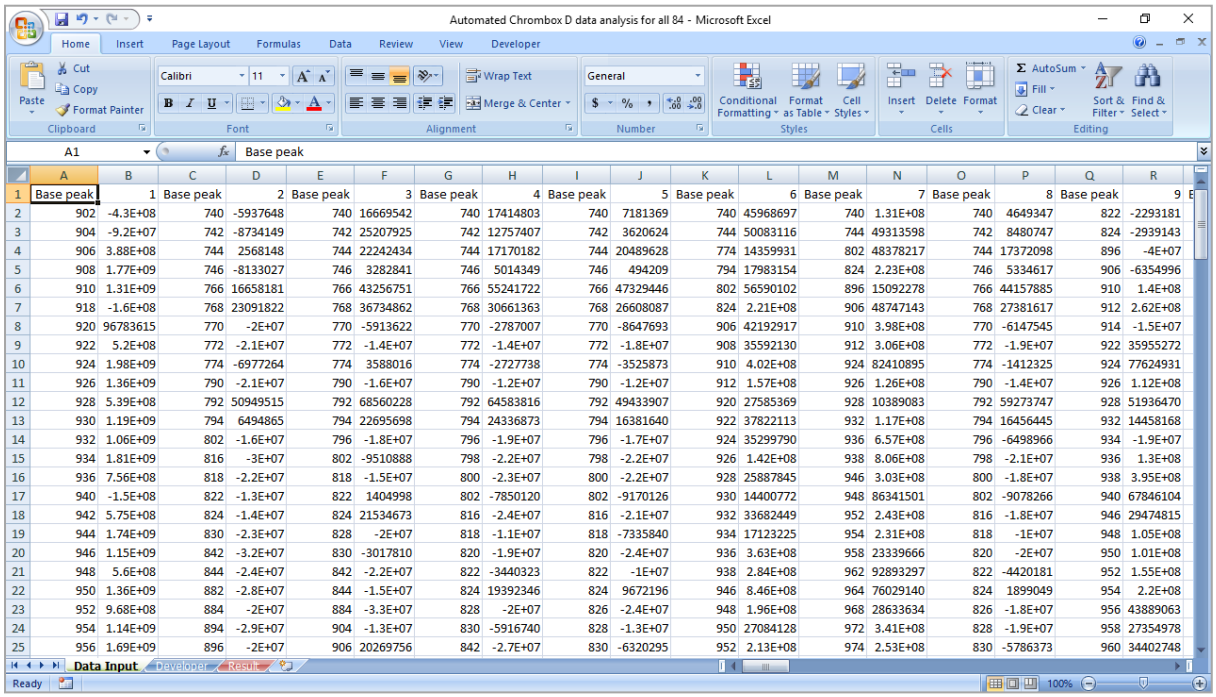
```
Sub makro1()

Dim analyteRows As Integer
Dim analyteArray  As Variant
Dim analyteValueArray As Variant
Dim analyteNumber As Integer
Dim listArray As Variant
Dim k As Integer
Dim counter As Integer
Dim analyteName As String
Dim numberColumns As Integer
Dim maxRowsInArea As Variant


Worksheets("Data Input").Activate
numberColumns = Cells(1, Columns.Count).End(xlToLeft).Column    ' finds number of columns

Worksheets("Developer").Activate
analyteRows = (Cells(Rows.Count, 1).End(xlUp).Row) 'finds number of rows in C column
listArray = Range(Cells(1, 1), Cells(analyteRows, 1)).Value


k = 1
counter = 2

Do While k < numberColumns


   Worksheets("Data Input").Activate
   analyteName = Cells(1, k + 1).Value
   maxRowsInArea = (Cells(Rows.Count, k).End(xlUp).Row)
   analyteArray = Range(Cells(2, k), Cells(maxRowsInArea, k)).Value
   analyteValueArray = Range(Cells(2, k + 1), Cells(maxRowsInArea, k + 1)).Value
   analyteNumber = UBound(analyteArray)

   Worksheets("Developer").Activate
   Cells(1, counter).Value = analyteName


   For j = 1 To analyteRows
      For i = 1 To analyteNumber
         If analyteArray(i, 1) = listArray(j, 1) Then
            Worksheets("Developer").Activate
            Cells(j, counter).Value = analyteValueArray(i, 1)
         End If
      Next i
   Next j


k = k + 2
counter = counter + 1

Loop
```

A.3 Methods

Opening the Excel template provides a spreadsheet platform on which the individual Chrombox D output data (only ‘base peak’ and ‘amount’ columns) are to be inserted one after another along the columns. The ‘Amount’ column can be renamed by the sample name manually to see the sample name in the final result sheet. A pre-written VBA project (Section 4.2) is set and saved in the macro that upon the run, processes data into an easily interpretable format. The whole process is described in following steps. TAG in the salmon liver cell is considered as an example of lipid classes, where the base peak of the TAG compound is 740 to 1250. For other TAG from different sources or other types of lipid classes, this range can be set according to the known base peak of lipid classes of interest.

Step 1:

The “Data Input” sheet is used for the input of Chrombox D generated data from multiple samples as shown in Figure A.1.



Base peak	1	Base peak	2	Base peak	3	Base peak	4	Base peak	5	Base peak	6	Base peak	7	Base peak	8	Base peak	9
902	-4.3E+08	740	-5937648	740	16669542	740	17414803	740	7181369	740	45968697	740	1.31E+08	740	4649347	822	-2293181
904	-9.2E+07	742	-8734149	742	25207925	742	12757407	742	3620624	744	50083116	744	49313598	742	8480747	824	-2939143
906	3.88E+08	744	2568148	744	22242434	744	17170182	744	20489628	774	14359931	802	48378217	744	17372098	896	-4E+07
908	1.77E+09	746	-8133027	746	3282841	746	5014349	746	494209	794	17983154	824	2.23E+08	746	5334617	906	-6354996
910	1.31E+09	766	16658181	766	43256751	766	55241722	766	47329446	802	56590102	896	15092278	766	44157885	910	1.4E+08
918	-1.6E+08	768	23091822	768	36734862	768	30661363	768	26608087	824	2.21E+08	906	48747143	768	27381617	912	2.62E+08
920	96783615	770	-2E+07	770	-5913622	770	-2787007	770	-8647693	906	42192917	910	3.98E+08	770	-6147545	914	-1.5E+07
922	5.2E+08	772	-2.1E+07	772	-1.4E+07	772	-1.4E+07	772	-1.8E+07	908	35592130	912	3.06E+08	772	-1.9E+07	922	35955272
924	1.98E+09	774	-6977264	774	3588016	774	-2727738	774	-3525873	910	4.02E+08	924	82410895	774	-1412325	924	77624931
926	1.36E+09	790	-2.1E+07	790	-1.6E+07	790	-1.2E+07	790	-1.2E+07	912	1.57E+08	926	1.26E+08	790	-1.4E+07	926	1.12E+08
928	5.39E+08	792	50949515	792	68560228	792	64583816	792	49433907	920	27585369	928	10389083	792	59273747	928	51936470
930	1.19E+09	794	6494865	794	22695698	794	24336873	794	16381640	922	37822113	932	1.17E+08	794	16456445	932	14458168
932	1.06E+09	802	-1.6E+07	796	-1.8E+07	796	-1.9E+07	796	-1.7E+07	924	35299790	936	6.57E+08	796	-6498966	934	-1.9E+07
934	1.81E+09	816	-3E+07	802	-9510888	798	-2.2E+07	798	-2.2E+07	926	1.42E+08	938	8.06E+08	798	-2.1E+07	936	1.3E+08
936	7.56E+08	818	-2.2E+07	818	-1.5E+07	800	-2.3E+07	800	-2.2E+07	928	25887845	946	3.03E+08	800	-1.8E+07	938	3.95E+08
940	-1.5E+08	822	-1.3E+07	822	1404998	802	-7850120	802	-9170126	930	14400772	948	86341501	802	-9078266	940	67846104
942	5.75E+08	824	-1.4E+07	824	21534673	816	-2.4E+07	816	-2.1E+07	932	33682449	952	2.43E+08	816	-1.8E+07	946	29474815
944	1.74E+09	830	-2.3E+07	828	-2E+07	818	-1.1E+07	818	-7335840	934	17123225	954	2.31E+08	818	-1E+07	948	1.05E+08
946	1.15E+09	842	-3.2E+07	830	-3017810	820	-1.9E+07	820	-2.4E+07	936	3.63E+08	958	23339666	820	-2E+07	950	1.01E+08
948	5.6E+08	844	-2.4E+07	842	-2.2E+07	822	-3440323	822	-1E+07	938	2.84E+08	962	92893297	822	-4420181	952	1.55E+08
950	1.36E+09	882	-2.8E+07	844	-1.5E+07	824	19392346	824	9672196	946	8.46E+08	964	76029140	824	1899049	954	2.2E+08
952	9.68E+08	884	-2E+07	884	-3.3E+07	828	-2E+07	826	-2.4E+07	948	1.96E+08	968	28633634	826	-1.8E+07	956	43889063
954	1.14E+09	894	-2.9E+07	904	-1.3E+07	830	-5916740	828	-1.3E+07	950	27084128	972	3.41E+08	828	-1.9E+07	958	27354978
956	1.69E+09	896	-2E+07	906	20269756	842	-2.7E+07	830	-6320295	952	2.13E+08	974	2.53E+08	830	-5786373	960	34402748

Figure A.1: Sreenshot ‘Data input’ sheet containing Chrombox D data for TAG species from multiple samples.

Step 2:

By selecting ‘Developer’ tab from the top menu bar of excel, macros tool box can be opened as shown in Figure A.2.

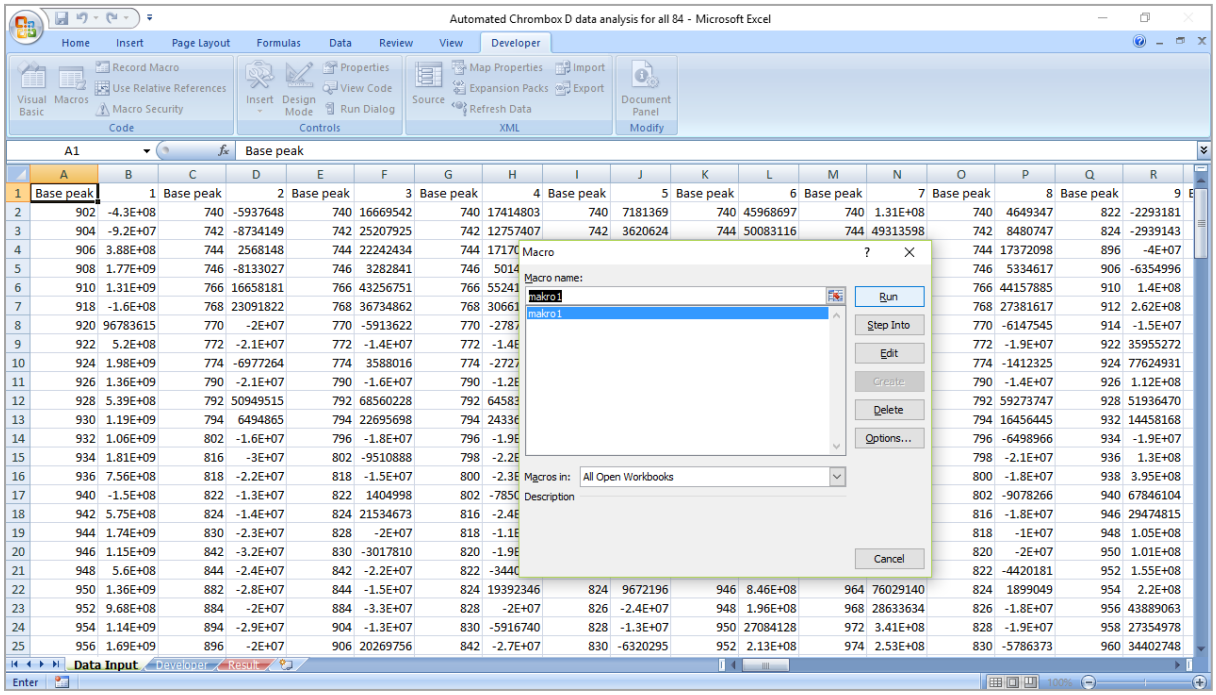


Figure A.2: Screenshot of ‘Data input’ sheet showing the option where to find and run macro for data processing.

Step 3:

Running macro by clicking the button in macro option opens a new sheet named “Developer” and aligns all individual TAG species for all the samples according to their relevant base peak in LCMS and the corresponding amount/intensity as shown in Figure A.3.

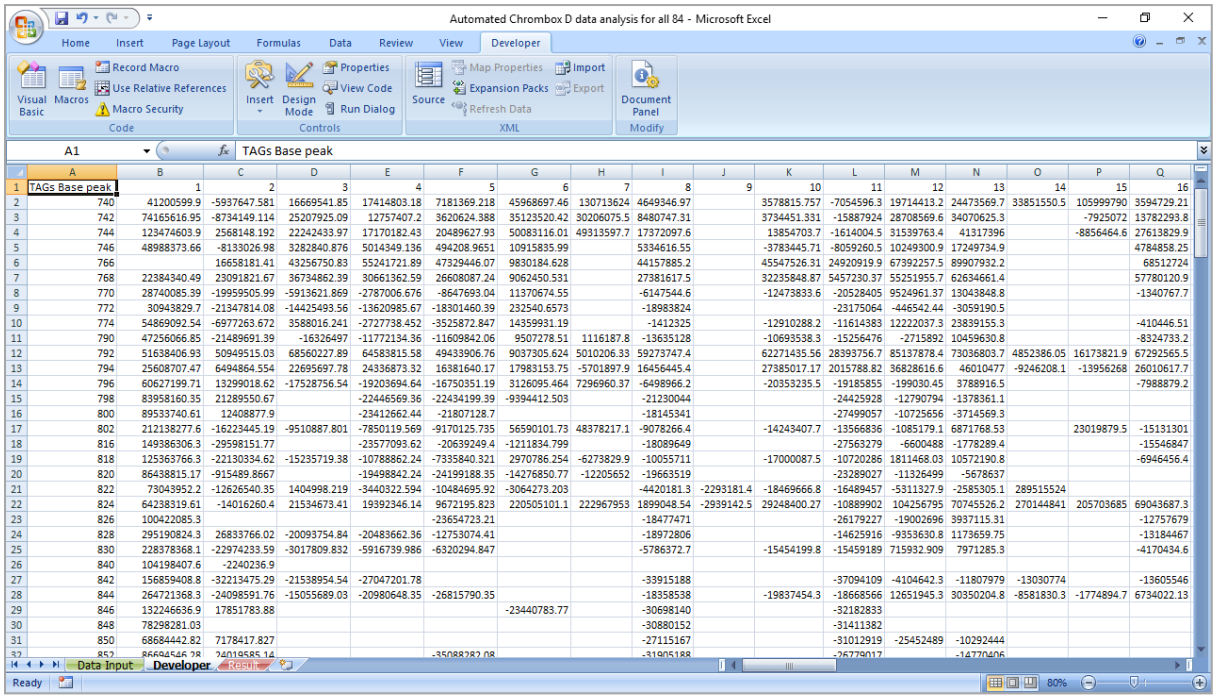


Figure A.3: Screenshot of 'Developer' sheet where the amounts of TAG species are aligned to corresponding base peak for all the samples.

Step 4:

As the Chrombox D gives results with the amount/intensity in positive and negative value, for the calculation of total intensity/amount or comparison of individual species of TAG, it is required to omit the negative values. At this step, the Excel is programmed for automatically omit the negative values and turned them into '0' (zero). It is also possible to apply blank subtraction to get a net result of the samples through eliminating sample blank data. This Excel-based user-friendly tool can be manipulated at this stage based on user interest and demand.

A.4 Results

The final Excel sheet "Result" as shown in Figure A.4 appeared with the whole picture that shows the total amount of TAG in every sample, TAG species corresponding to the base peak and bar-diagram showing the comparative intensity of the total TAG in different samples. The data from the "Result" sheet can be used for any further analysis or interpretation. The

bar-chart can be used to compare the total lipid (in this case, TAG) present in multiple samples.

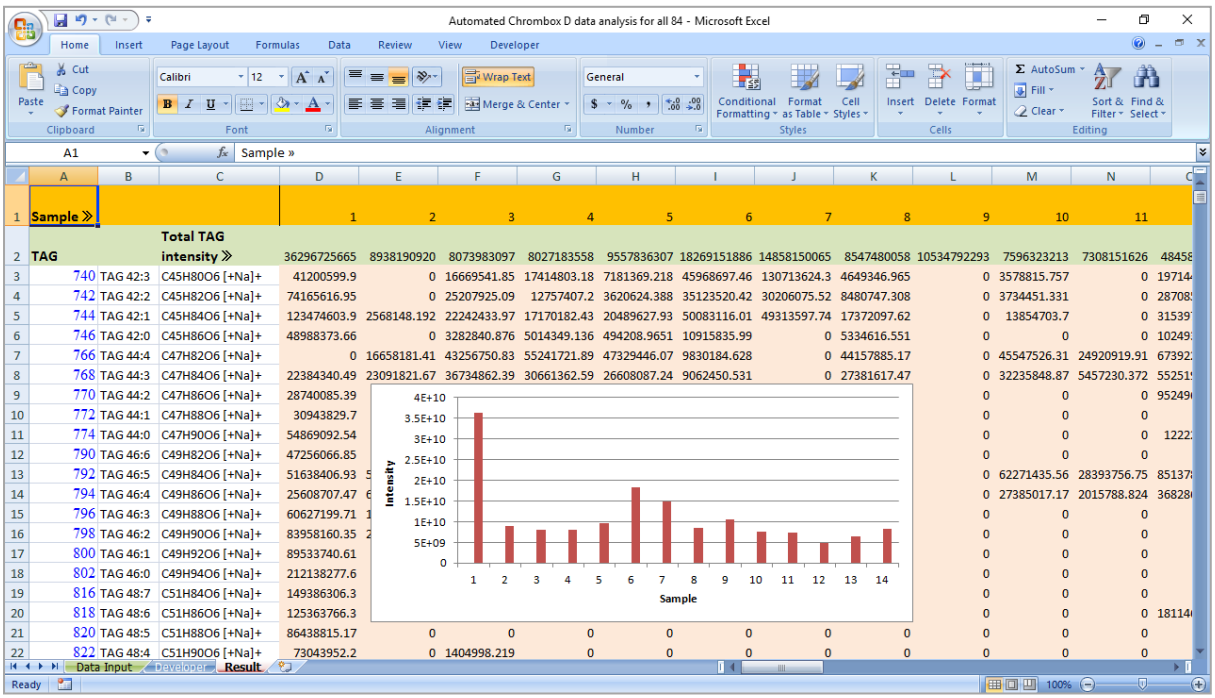


Figure A.4: Screenshot of the 'Result; sheet displaying the individual value of TAG species, total amount/intensity of TAGs in the samples, and their comparative analysis by bar-chart.

A.5 Conclusions

This Excel macro-enabled workbook provides a user-friendly tool for simplifying the Chrombox D generated lipid data sets without the need for specialist bioinformatics skills, which helps avoid hectic and time-consuming manual data processing for every sample one by one and allows for the quick production of interactive results of biological samples.

Appendix B:

Table B.1 Intensity of isolated 27 TAG species significant to the treatment with high concentration of oleic acid

	Treatment	TAG 54:2	TAG 56:8	TAG 56:7	TAG 56:5	TAG 56:3	TAG 56:2	TAG 58:11	TAG 58:9	TAG 58:8	TAG 58:6	TAG 58:4	TAG 58:3	TAG 60:13	TAG 60:12
Fish 1	X0/0.4	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09
Fish 2	X0/0.4	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09
Fish 3	X0/0.4	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10
	Y0/0.4	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09
Fish 4	X0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09
Fish 5	X0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09
Fish 6	X0/0.4	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09
	Y0/0.4	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09
	Treatment	**TAG 60:10**	**TAG 60:9**	**TAG 60:8**	**TAG 60:7**	**TAG 60:6**	**TAG 60:5**	**TAG 62:12**	**TAG 62:7**	**TAG 62:5**	**TAG 62:4**	**TAG 62:3**	**TAG 64:9**	**TAG 64:3**	**Total Intensity**
Fish 1	X0/0.4	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	6E+09	**6E+09**
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	**2E+09**
Fish 2	X0/0.4	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	**1E+10**
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	**2E+09**
Fish 3	X0/0.4	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	1E+10	**1E+10**
	Y0/0.4	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	7E+09	**7E+09**
Fish 4	X0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	**2E+09**
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	**2E+09**
Fish 5	X0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	**2E+09**
	Y0/0.4	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	2E+09	**2E+09**
Fish 6	X0/0.4	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	**1E+09**
	Y0/0.4	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	1E+09	**1E+09**

Table B.2 Intensity of isolated 27 TAG species in all 84 liver cell culture samples

		TAG 54:2	TAG 56:8	TAG 56:7	TAG 56:5	TAG 56:3	TAG 56:2	TAG 58:11	TAG 58:9	TAG 58:8	TAG 58:6	TAG 58:4	TAG 58:3	TAG 60:13	TAG 60:12
Fish 1	X0/0	1E+09	1E+09	5E+08	1E+09	8E+08	0E+00	6E+08	1E+09	1E+09	9E+08	8E+07	3E+07	8E+08	7E+08
Fish 1	X0.1/0.2	2E+08	2E+08	5E+07	8E+07	2E+08	3E+07	4E+07	9E+07	9E+07	7E+07	2E+07	5E+07	6E+07	2E+07
Fish 1	X10/0.2	8E+07	1E+08	5E+07	9E+07	7E+07	9E+07	9E+07	1E+08	7E+07	1E+08	4E+07	5E+07	9E+07	1E+08
Fish 1	X0/0.2	9E+07	9E+07	5E+07	9E+07	7E+07	1E+08	9E+07	1E+08	9E+07	1E+08	5E+07	6E+07	9E+07	1E+08
Fish 1	X0.1/0.4	1E+08	1E+08	6E+07	1E+08	5E+07	2E+08	1E+08	1E+08	1E+08	7E+07	3E+07	6E+07	7E+07	2E+08
Fish 1	X10/0.4	4E+08	1E+08	3E+07	3E+07	4E+08	3E+08	2E+08	2E+08	1E+08	4E+07	1E+08	8E+07	3E+08	8E+07
Fish 1	X0/0.4	4E+08	1E+08	1E+07	1E+08	7E+08	8E+08	9E+07	2E+08	2E+08	2E+07	9E+07	8E+07	3E+08	3E+08
Fish 1	Y0/0.2	1E+08	1E+08	6E+07	9E+07	1E+08	1E+08	1E+08	1E+08	1E+08	9E+07	3E+07	4E+07	9E+07	1E+08
Fish 1	Y0/0	1E+08	1E+08	5E+07	1E+07	1E+08	4E+08	1E+08	2E+08	2E+08	3E+07	4E+07	5E+07	7E+07	5E+08
Fish 1	Y0/0.4	1E+08	1E+08	6E+07	7E+07	4E+07	2E+08	7E+07	8E+07	1E+08	8E+07	3E+07	5E+07	1E+08	1E+08
Fish 1	Y0.1/0.2	2E+08	1E+08	5E+07	9E+07	8E+07	2E+08	4E+07	6E+07	1E+08	6E+07	6E+06	2E+07	4E+07	2E+08
Fish 1	Y0.1/0.4	6E+07	6E+07	3E+07	5E+07	4E+07	5E+07	5E+07	5E+07	5E+07	4E+07	5E+07	4E+07	5E+07	6E+07
Fish 1	Y10/0.2	3E+07	6E+07	3E+07	8E+07	3E+07	1E+08	5E+07	6E+07	5E+07	5E+07	5E+07	4E+07	4E+07	5E+07
Fish 1	Y10/0.4	2E+08	1E+08	4E+07	0E+00	2E+08	4E+08	7E+07	2E+08	3E+08	3E+07	7E+07	7E+07	2E+08	2E+08
Fish 2	X0/0	2E+08	2E+08	8E+07	0E+00	5E+08	7E+08	1E+08	5E+08	5E+08	1E+07	3E+08	2E+08	2E+08	2E+08
Fish 2	X0.1/0.2	1E+08	1E+08	4E+07	6E+07	6E+07	1E+08	1E+08	9E+07	7E+07	8E+07	2E+07	5E+07	7E+07	8E+07
Fish 2	X10/0.2	1E+08	1E+08	4E+07	6E+07	4E+07	1E+08	2E+07	5E+07	8E+07	5E+07	1E+07	3E+07	8E+07	1E+08
Fish 2	X0/0.2	7E+07	9E+07	3E+07	6E+07	6E+07	6E+07	1E+08	7E+07	6E+07	7E+07	1E+07	3E+07	6E+07	4E+07
Fish 2	X0.1/0.4	7E+07	9E+07	4E+07	7E+07	3E+07	1E+08	9E+07	7E+07	1E+08	7E+07	2E+07	4E+07	6E+07	1E+08
Fish 2	X10/0.4	4E+08	4E+08	1E+08	0E+00	5E+08	4E+08	0E+00	5E+08	3E+08	1E+07	3E+08	7E+07	2E+08	8E+07
Fish 2	X0/0.4	3E+08	2E+08	1E+08	4E+07	1E+09	2E+09	3E+08	1E+09	1E+09	3E+07	2E+08	9E+07	5E+08	1E+09
Fish 2	Y0/0.2	6E+07	1E+08	6E+07	9E+07	5E+07	2E+08	1E+08	8E+07	9E+07	7E+07	1E+07	3E+07	8E+07	1E+08
Fish 2	Y0/0	2E+08	2E+08	1E+08	3E+07	8E+08	2E+09	2E+08	1E+09	8E+08	0E+00	2E+08	7E+07	5E+08	4E+08
Fish 2	Y0/0.4	6E+07	8E+07	5E+07	7E+07	1E+07	1E+08	8E+07	6E+07	9E+07	5E+07	3E+07	4E+07	5E+07	1E+08
Fish 2	Y0.1/0.2	9E+07	1E+08	4E+07	8E+07	3E+07	2E+08	7E+07	5E+07	9E+07	6E+07	2E+07	2E+07	7E+07	9E+07
Fish 2	Y0.1/0.4	5E+07	7E+07	3E+07	4E+07	2E+07	5E+07	5E+07	5E+07	6E+07	6E+07	3E+07	2E+07	6E+07	7E+07
Fish 2	Y10/0.2	1E+08	1E+08	4E+07	6E+07	3E+07	1E+08	7E+07	5E+07	6E+07	5E+07	3E+07	3E+07	7E+07	1E+08
Fish 2	Y10/0.4	3E+08	4E+08	9E+07	0E+00	3E+08	3E+08	2E+08	5E+08	2E+08	0E+00	2E+08	1E+07	2E+08	8E+07
Fish 3	X0/0	1E+09	7E+08	4E+08	5E+08	4E+08	0E+00	2E+08	3E+08	3E+08	2E+08	8E+07	1E+08	1E+08	4E+07
Fish 3	X0.1/0.2	1E+09	6E+08	3E+08	4E+08	7E+08	4E+08	9E+07	4E+08	5E+08	1E+08	1E+08	4E+08	2E+08	9E+07
Fish 3	X10/0.2	1E+09	6E+08	2E+08	2E+08	9E+08	8E+08	1E+07	6E+08	7E+08	8E+07	6E+07	5E+08	3E+08	2E+08
Fish 3	X0/0.2	1E+09	5E+08	2E+08	3E+08	7E+08	5E+08	1E+06	4E+08	4E+08	3E+07	3E+06	3E+08	1E+08	1E+07
Fish 3	X0.1/0.4	2E+09	6E+08	3E+08	3E+08	8E+08	7E+08	7E+07	5E+08	6E+08	1E+08	1E+08	5E+08	3E+08	2E+08
Fish 3	X10/0.4	2E+09	4E+08	2E+08	2E+08	7E+08	8E+08	1E+07	5E+08	6E+08	2E+08	1E+08	3E+08	4E+08	3E+08

Fish 3	X0/0.4	2E+09	7E+08	2E+08	2E+08	1E+09	1E+09	3E+07	7E+08	7E+08	2E+08	2E+08	4E+08	5E+08	3E+08
Fish 3	Y0/0.2	7E+08	4E+08	2E+08	2E+08	9E+08	8E+08	7E+07	6E+08	5E+08	2E+08	2E+08	3E+08	5E+08	3E+08
Fish 3	Y0/0	5E+08	4E+08	2E+08	2E+08	8E+08	8E+08	4E+07	6E+08	5E+08	1E+08	2E+08	3E+08	4E+08	2E+08
Fish 3	Y0/0.4	2E+09	4E+08	1E+08	9E+07	6E+08	4E+08	2E+07	3E+08	3E+08	2E+08	2E+08	2E+08	3E+08	2E+08
Fish 3	Y0.1/0.2	5E+08	3E+08	1E+08	1E+08	7E+08	8E+08	1E+07	5E+08	4E+08	1E+08	1E+08	3E+08	4E+08	3E+08
Fish 3	Y0.1/0.4	1E+09	3E+08	5E+07	9E+07	5E+08	5E+08	4E+07	3E+08	3E+08	2E+08	2E+08	2E+08	3E+08	2E+08
Fish 3	Y10/0.2	3E+08	3E+08	8E+07	1E+08	6E+08	8E+08	3E+07	4E+08	4E+08	2E+08	1E+08	2E+08	4E+08	3E+08
Fish 3	Y10/0.4	8E+08	4E+08	8E+07	1E+08	4E+08	4E+08	3E+07	3E+08	2E+08	2E+08	1E+08	2E+08	3E+08	2E+08
Fish 4	X0/0	1E+07	1E+07	2E+07	0E+00	1E+07	2E+07	3E+05	1E+07	2E+07	2E+06	4E+06	0E+00	2E+07	2E+07
Fish 4	X0.1/0.2	6E+07	1E+08	1E+08	5E+07	1E+08	2E+08	4E+07	1E+08	2E+08	2E+07	4E+07	5E+07	4E+07	5E+07
Fish 4	X10/0.2	4E+07	9E+07	1E+08	5E+07	1E+08	2E+08	3E+07	1E+08	1E+08	1E+07	3E+07	4E+07	4E+07	4E+07
Fish 4	X0/0.2	4E+07	7E+07	7E+07	4E+07	1E+08	2E+08	3E+07	2E+08	2E+08	1E+07	4E+07	5E+07	5E+07	5E+07
Fish 4	X0.1/0.4	3E+07	6E+07	7E+07	4E+07	2E+08	2E+08	3E+07	2E+08	2E+08	3E+07	5E+07	5E+07	3E+07	6E+07
Fish 4	X10/0.4	3E+07	6E+07	6E+07	4E+07	2E+08	3E+08	4E+07	3E+08	3E+08	2E+07	6E+07	6E+07	5E+07	8E+07
Fish 4	X0/0.4	3E+07	4E+07	5E+07	2E+07	2E+08	2E+08	9E+06	2E+08	2E+08	6E+06	4E+07	4E+07	4E+07	4E+07
Fish 4	Y0/0.2	4E+07	7E+07	8E+07	6E+07	2E+08	3E+08	3E+07	2E+08	2E+08	1E+07	4E+07	4E+07	4E+07	7E+07
Fish 4	Y0/0	5E+07	7E+07	7E+07	4E+07	2E+08	2E+08	2E+07	2E+08	2E+08	6E+06	5E+07	5E+07	4E+07	6E+07
Fish 4	Y0/0.4	5E+07	7E+07	8E+07	3E+07	2E+08	2E+08	2E+07	2E+08	1E+08	2E+06	4E+07	5E+07	3E+07	4E+07
Fish 4	Y0.1/0.2	2E+07	7E+07	8E+07	6E+07	2E+08	4E+08	2E+07	3E+08	3E+08	7E+06	4E+07	5E+07	4E+07	7E+07
Fish 4	Y0.1/0.4	1E+07	4E+07	7E+07	4E+07	2E+08	3E+08	2E+07	3E+08	3E+08	1E+07	6E+07	6E+07	5E+07	6E+07
Fish 4	Y10/0.2	3E+07	5E+07	7E+07	4E+07	2E+08	3E+08	4E+07	3E+08	3E+08	3E+07	7E+07	5E+07	5E+07	6E+07
Fish 4	Y10/0.4	1E+07	3E+07	5E+07	8E+06	1E+08	2E+08	2E+07	2E+08	2E+08	1E+07	5E+07	5E+07	4E+07	4E+07
Fish 5	X0/0	3E+07	7E+07	7E+07	3E+07	5E+07	5E+07	1E+08	7E+07	4E+07	3E+07	2E+07	7E+06	3E+07	4E+07
Fish 5	X0.1/0.2	1E+08	1E+08	1E+08	5E+07	1E+08	1E+08	2E+08	1E+08	1E+08	3E+07	4E+07	6E+07	4E+07	7E+07
Fish 5	X10/0.2	7E+07	8E+07	8E+07	3E+07	1E+08	2E+08	8E+07	2E+08	1E+08	3E+07	3E+07	2E+07	5E+07	5E+07
Fish 5	X0/0.2	1E+08	1E+08	1E+08	6E+07	2E+08	2E+08	2E+08	2E+08	2E+08	5E+07	6E+07	7E+07	7E+07	9E+07
Fish 5	X0.1/0.4	1E+08	1E+08	1E+08	5E+07	2E+08	2E+08	2E+08	3E+08	2E+08	3E+07	4E+07	6E+07	6E+07	8E+07
Fish 5	X10/0.4	1E+08	1E+08	1E+08	6E+07	2E+08	3E+08	1E+08	3E+08	2E+08	3E+07	5E+07	4E+07	7E+07	1E+08
Fish 5	X0/0.4	9E+07	1E+08	1E+08	3E+07	2E+08	3E+08	5E+07	3E+08	3E+08	3E+07	5E+07	4E+07	4E+07	6E+07
Fish 5	Y0/0.2	2E+08	1E+08	1E+08	6E+07	2E+08	3E+08	1E+08	3E+08	2E+08	4E+07	6E+07	4E+07	9E+07	1E+08
Fish 5	Y0/0	1E+08	9E+07	8E+07	3E+07	2E+08	2E+08	6E+07	2E+08	2E+08	2E+07	4E+07	3E+07	4E+07	6E+07
Fish 5	Y0/0.4	1E+08	1E+08	1E+08	4E+07	1E+08	1E+08	5E+07	3E+08	2E+08	3E+07	5E+07	4E+07	3E+07	4E+07
Fish 5	Y0.1/0.2	1E+08	1E+08	1E+08	6E+07	3E+08	2E+08	8E+07	3E+08	2E+08	4E+07	5E+07	4E+07	7E+07	9E+07
Fish 5	Y0.1/0.4	6E+07	7E+07	8E+07	3E+07	2E+08	2E+08	3E+07	3E+08	3E+08	2E+07	4E+07	3E+07	4E+07	4E+07
Fish 5	Y10/0.2	8E+07	9E+07	9E+07	6E+07	2E+08	2E+08	8E+07	3E+08	2E+08	4E+07	6E+07	4E+07	8E+07	8E+07
Fish 5	Y10/0.4	7E+07	8E+07	8E+07	4E+07	1E+08	1E+08	3E+07	3E+08	2E+08	4E+07	4E+07	4E+07	5E+07	5E+07
Fish 6	X0/0	3E+07	9E+06	1E+07	1E+07	2E+07	3E+07	1E+07	3E+07	3E+07	2E+07	2E+07	2E+07	2E+07	2E+07
Fish 6	X0.1/0.2	5E+06	2E+07	3E+07	3E+07	9E+07	1E+08	9E+06	1E+08	1E+08	2E+07	5E+07	5E+07	2E+07	4E+07
Fish 6	X10/0.2	2E+06	2E+07	3E+07	4E+07	1E+08	2E+08	1E+07	2E+08	2E+08	2E+07	4E+07	4E+07	3E+07	6E+07

Fish 6	X0/0.2	3E+06	2E+07	3E+07	4E+07	1E+08	2E+08	1E+07	2E+08	2E+08	2E+07	5E+07	5E+07	3E+07	5E+07
Fish 6	X0.1/0.4	6E+06	2E+07	3E+07	5E+07	2E+08	2E+08	9E+06	2E+08	2E+08	1E+07	5E+07	5E+07	3E+07	6E+07
Fish 6	X10/0.4	0E+00	2E+07	3E+07	7E+07	1E+08	2E+08	1E+07	3E+08	3E+08	2E+07	4E+07	4E+07	4E+07	1E+08
Fish 6	X0/0.4	4E+05	2E+07	3E+07	5E+07	1E+08	2E+08	8E+06	2E+08	2E+08	1E+07	4E+07	4E+07	3E+07	5E+07
Fish 6	Y0/0.2	7E+05	1E+07	3E+07	6E+07	2E+08	3E+08	7E+06	3E+08	2E+08	1E+07	4E+07	4E+07	3E+07	7E+07
Fish 6	Y0/0	2E+06	2E+07	3E+07	4E+07	1E+08	2E+08	9E+06	2E+08	2E+08	2E+07	5E+07	4E+07	3E+07	5E+07
Fish 6	Y0/0.4	1E+07	3E+07	4E+07	3E+07	1E+08	1E+08	1E+07	1E+08	1E+08	2E+07	5E+07	5E+07	2E+07	3E+07
Fish 6	Y0.1/0.2	0E+00	1E+07	3E+07	4E+07	2E+08	2E+08	7E+06	2E+08	3E+08	2E+07	5E+07	4E+07	3E+07	7E+07
Fish 6	Y0.1/0.4	2E+06	2E+07	3E+07	3E+07	1E+08	2E+08	8E+06	2E+08	2E+08	2E+07	5E+07	4E+07	3E+07	5E+07
Fish 6	Y10/0.2	0E+00	2E+07	3E+07	5E+07	2E+08	2E+08	8E+06	2E+08	2E+08	2E+07	4E+07	4E+07	3E+07	6E+07
Fish 6	Y10/0.4	5E+06	3E+07	5E+07	3E+07	1E+08	1E+08	1E+07	2E+08	2E+08	2E+07	4E+07	4E+07	2E+07	3E+07

		TAG 54:2	TAG 56:8	TAG 56:7	TAG 56:5	TAG 56:3	TAG 56:2	TAG 58:11	TAG 58:9	TAG 58:8	TAG 58:6	TAG 58:4	TAG 58:3	TAG 60:13
Fish 1	X0/0	1E+09	1E+09	5E+08	1E+09	8E+08	0E+00	6E+08	1E+09	1E+09	9E+08	8E+07	3E+07	8E+08
Fish 1	X0.1/0.2	2E+08	2E+08	5E+07	8E+07	2E+08	3E+07	4E+07	9E+07	9E+07	7E+07	2E+07	5E+07	6E+07
Fish 1	X10/0.2	8E+07	1E+08	5E+07	9E+07	7E+07	9E+07	9E+07	1E+08	7E+07	1E+08	4E+07	5E+07	9E+07
Fish 1	X0/0.2	9E+07	9E+07	5E+07	9E+07	7E+07	1E+08	9E+07	1E+08	9E+07	1E+08	5E+07	6E+07	9E+07
Fish 1	X0.1/0.4	1E+08	1E+08	6E+07	1E+08	5E+07	2E+08	1E+08	1E+08	1E+08	7E+07	3E+07	6E+07	7E+07
Fish 1	X10/0.4	4E+08	1E+08	3E+07	3E+07	4E+08	3E+08	2E+08	2E+08	1E+08	4E+07	1E+08	8E+07	3E+08
Fish 1	X0/0.4	4E+08	1E+08	1E+07	1E+08	7E+08	8E+08	9E+07	2E+08	2E+08	2E+07	9E+07	8E+07	3E+08
Fish 1	Y0/0.2	1E+08	1E+08	6E+07	9E+07	1E+08	1E+08	1E+08	1E+08	1E+08	9E+07	3E+07	4E+07	9E+07
Fish 1	Y0/0	1E+08	1E+08	5E+07	1E+07	1E+08	4E+08	1E+08	2E+08	2E+08	3E+07	4E+07	5E+07	7E+07
Fish 1	Y0/0.4	1E+08	1E+08	6E+07	7E+07	4E+07	2E+08	7E+07	8E+07	1E+08	8E+07	3E+07	5E+07	1E+08
Fish 1	Y0.1/0.2	2E+08	1E+08	5E+07	9E+07	8E+07	2E+08	4E+07	6E+07	1E+08	6E+07	6E+06	2E+07	4E+07
Fish 1	Y0.1/0.4	6E+07	6E+07	3E+07	5E+07	4E+07	5E+07	5E+07	5E+07	5E+07	4E+07	5E+07	4E+07	5E+07
Fish 1	Y10/0.2	3E+07	6E+07	3E+07	8E+07	3E+07	1E+08	5E+07	6E+07	5E+07	5E+07	5E+07	4E+07	4E+07
Fish 1	Y10/0.4	2E+08	1E+08	4E+07	0E+00	2E+08	4E+08	7E+07	2E+08	3E+08	3E+07	7E+07	7E+07	2E+08
Fish 2	X0/0	2E+08	2E+08	8E+07	0E+00	5E+08	7E+08	1E+08	5E+08	5E+08	1E+07	3E+08	2E+08	2E+08
Fish 2	X0.1/0.2	1E+08	1E+08	4E+07	6E+07	6E+07	1E+08	1E+08	9E+07	7E+07	8E+07	2E+07	5E+07	7E+07
Fish 2	X10/0.2	1E+08	1E+08	4E+07	6E+07	4E+07	1E+08	2E+07	5E+07	8E+07	5E+07	1E+07	3E+07	8E+07
Fish 2	X0/0.2	7E+07	9E+07	3E+07	6E+07	6E+07	6E+07	1E+08	7E+07	6E+07	7E+07	1E+07	3E+07	6E+07
Fish 2	X0.1/0.4	7E+07	9E+07	4E+07	7E+07	3E+07	1E+08	9E+07	7E+07	1E+08	7E+07	2E+07	4E+07	6E+07
Fish 2	X10/0.4	4E+08	4E+08	1E+08	0E+00	5E+08	4E+08	0E+00	5E+08	3E+08	1E+07	3E+08	7E+07	2E+08
Fish 2	X0/0.4	3E+08	2E+08	1E+08	4E+07	1E+09	2E+09	3E+08	1E+09	1E+09	3E+07	2E+08	9E+07	5E+08
Fish 2	Y0/0.2	6E+07	1E+08	6E+07	9E+07	5E+07	2E+08	1E+08	8E+07	9E+07	7E+07	1E+07	3E+07	8E+07
Fish 2	Y0/0	2E+08	2E+08	1E+08	3E+07	8E+08	2E+09	2E+08	1E+09	8E+08	0E+00	2E+08	7E+07	5E+08
Fish 2	Y0/0.4	6E+07	8E+07	5E+07	7E+07	1E+07	1E+08	8E+07	6E+07	9E+07	5E+07	3E+07	4E+07	5E+07
Fish 2	Y0.1/0.2	9E+07	1E+08	4E+07	8E+07	3E+07	2E+08	7E+07	5E+07	9E+07	6E+07	2E+07	2E+07	7E+07
Fish 2	Y0.1/0.4	5E+07	7E+07	3E+07	4E+07	2E+07	5E+07	5E+07	5E+07	6E+07	6E+07	3E+07	2E+07	6E+07

Fish 2	Y10/0.2	1E+08	1E+08	4E+07	6E+07	3E+07	1E+08	7E+07	5E+07	6E+07	5E+07	3E+07	3E+07	7E+07
Fish 2	Y10/0.4	3E+08	4E+08	9E+07	0E+00	3E+08	3E+08	2E+08	5E+08	2E+08	0E+00	2E+08	1E+07	2E+08
Fish 3	X0/0	1E+09	7E+08	4E+08	5E+08	4E+08	0E+00	2E+08	3E+08	3E+08	2E+08	8E+07	1E+08	1E+08
Fish 3	X0.1/0.2	1E+09	6E+08	3E+08	4E+08	7E+08	4E+08	9E+07	4E+08	5E+08	1E+08	1E+08	4E+08	2E+08
Fish 3	X10/0.2	1E+09	6E+08	2E+08	2E+08	9E+08	8E+08	1E+07	6E+08	7E+08	8E+07	6E+07	5E+08	3E+08
Fish 3	X0/0.2	1E+09	5E+08	2E+08	3E+08	7E+08	5E+08	1E+06	4E+08	4E+08	3E+07	3E+06	3E+08	1E+08
Fish 3	X0.1/0.4	2E+09	6E+08	3E+08	3E+08	8E+08	7E+08	7E+07	5E+08	6E+08	1E+08	1E+08	5E+08	3E+08
Fish 3	X10/0.4	2E+09	4E+08	2E+08	2E+08	7E+08	8E+08	1E+07	5E+08	6E+08	2E+08	1E+08	3E+08	4E+08
Fish 3	X0/0.4	2E+09	7E+08	2E+08	2E+08	1E+09	1E+09	3E+07	7E+08	7E+08	2E+08	2E+08	4E+08	5E+08
Fish 3	Y0/0.2	7E+08	4E+08	2E+08	2E+08	9E+08	8E+08	7E+07	6E+08	5E+08	2E+08	2E+08	3E+08	5E+08
Fish 3	Y0/0	5E+08	4E+08	2E+08	2E+08	8E+08	8E+08	4E+07	6E+08	5E+08	1E+08	2E+08	3E+08	4E+08
Fish 3	Y0/0.4	2E+09	4E+08	1E+08	9E+07	6E+08	4E+08	2E+07	3E+08	3E+08	2E+08	2E+08	2E+08	3E+08
Fish 3	Y0.1/0.2	5E+08	3E+08	1E+08	1E+08	7E+08	8E+08	1E+07	5E+08	4E+08	1E+08	1E+08	3E+08	4E+08
Fish 3	Y0.1/0.4	1E+09	3E+08	5E+07	9E+07	5E+08	5E+08	4E+07	3E+08	3E+08	2E+08	2E+08	2E+08	3E+08
Fish 3	Y10/0.2	3E+08	3E+08	8E+07	1E+08	6E+08	8E+08	3E+07	4E+08	4E+08	2E+08	1E+08	2E+08	4E+08
Fish 3	Y10/0.4	8E+08	4E+08	8E+07	1E+08	4E+08	4E+08	3E+07	3E+08	2E+08	2E+08	1E+08	2E+08	3E+08
Fish 4	X0/0	1E+07	1E+07	2E+07	0E+00	1E+07	2E+07	3E+05	1E+07	2E+07	2E+06	4E+06	0E+00	2E+07
Fish 4	X0.1/0.2	6E+07	1E+08	1E+08	5E+07	1E+08	2E+08	4E+07	1E+08	2E+08	2E+07	4E+07	5E+07	4E+07
Fish 4	X10/0.2	4E+07	9E+07	1E+08	5E+07	1E+08	2E+08	3E+07	1E+08	1E+08	1E+07	3E+07	4E+07	4E+07
Fish 4	X0/0.2	4E+07	7E+07	7E+07	4E+07	1E+08	2E+08	3E+07	2E+08	2E+08	1E+07	4E+07	5E+07	5E+07
Fish 4	X0.1/0.4	3E+07	6E+07	7E+07	4E+07	2E+08	2E+08	3E+07	2E+08	2E+08	3E+07	5E+07	5E+07	3E+07
Fish 4	X10/0.4	3E+07	6E+07	6E+07	4E+07	2E+08	3E+08	4E+07	3E+08	3E+08	2E+07	6E+07	6E+07	5E+07
Fish 4	X0/0.4	3E+07	4E+07	5E+07	2E+07	2E+08	2E+08	9E+06	2E+08	2E+08	6E+06	4E+07	4E+07	4E+07
Fish 4	Y0/0.2	4E+07	7E+07	8E+07	6E+07	2E+08	3E+08	3E+07	2E+08	2E+08	1E+07	4E+07	4E+07	4E+07
Fish 4	Y0/0	5E+07	7E+07	7E+07	4E+07	2E+08	2E+08	2E+07	2E+08	2E+08	6E+06	5E+07	5E+07	4E+07
Fish 4	Y0/0.4	5E+07	7E+07	8E+07	3E+07	2E+08	2E+08	2E+07	2E+08	1E+08	2E+06	4E+07	5E+07	3E+07
Fish 4	Y0.1/0.2	2E+07	7E+07	8E+07	6E+07	2E+08	4E+08	2E+07	3E+08	3E+08	7E+06	4E+07	5E+07	4E+07
Fish 4	Y0.1/0.4	1E+07	4E+07	7E+07	4E+07	2E+08	3E+08	2E+07	3E+08	3E+08	1E+07	6E+07	6E+07	5E+07
Fish 4	Y10/0.2	3E+07	5E+07	7E+07	4E+07	2E+08	3E+08	4E+07	3E+08	3E+08	3E+07	7E+07	5E+07	5E+07
Fish 4	Y10/0.4	1E+07	3E+07	5E+07	8E+06	1E+08	2E+08	2E+07	2E+08	2E+08	1E+07	5E+07	5E+07	4E+07
Fish 5	X0/0	3E+07	7E+07	7E+07	3E+07	5E+07	5E+07	1E+08	7E+07	4E+07	3E+07	2E+07	7E+06	3E+07
Fish 5	X0.1/0.2	1E+08	1E+08	1E+08	5E+07	1E+08	1E+08	2E+08	1E+08	1E+08	3E+07	4E+07	6E+07	4E+07
Fish 5	X10/0.2	7E+07	8E+07	8E+07	3E+07	1E+08	2E+08	8E+07	2E+08	1E+08	3E+07	3E+07	2E+07	5E+07
Fish 5	X0/0.2	1E+08	1E+08	1E+08	6E+07	2E+08	2E+08	2E+08	2E+08	2E+08	5E+07	6E+07	7E+07	7E+07
Fish 5	X0.1/0.4	1E+08	1E+08	1E+08	5E+07	2E+08	2E+08	2E+08	3E+08	2E+08	3E+07	4E+07	6E+07	6E+07
Fish 5	X10/0.4	1E+08	1E+08	1E+08	6E+07	2E+08	3E+08	1E+08	3E+08	2E+08	3E+07	5E+07	4E+07	7E+07
Fish 5	X0/0.4	9E+07	1E+08	1E+08	3E+07	2E+08	3E+08	5E+07	3E+08	3E+08	3E+07	5E+07	4E+07	4E+07
Fish 5	Y0/0.2	2E+08	1E+08	1E+08	6E+07	2E+08	3E+08	1E+08	3E+08	2E+08	4E+07	6E+07	4E+07	9E+07
Fish 5	Y0/0	1E+08	9E+07	8E+07	3E+07	2E+08	2E+08	6E+07	2E+08	2E+08	2E+07	4E+07	3E+07	4E+07

Fish 5	Y0/0.4	1E+08	1E+08	1E+08	4E+07	1E+08	1E+08	5E+07	3E+08	2E+08	3E+07	5E+07	4E+07	3E+07
Fish 5	Y0.1/0.2	1E+08	1E+08	1E+08	6E+07	3E+08	2E+08	8E+07	3E+08	2E+08	4E+07	5E+07	4E+07	7E+07
Fish 5	Y0.1/0.4	6E+07	7E+07	8E+07	3E+07	2E+08	2E+08	3E+07	3E+08	3E+08	2E+07	4E+07	3E+07	4E+07
Fish 5	Y10/0.2	8E+07	9E+07	9E+07	6E+07	2E+08	2E+08	8E+07	3E+08	2E+08	4E+07	6E+07	4E+07	8E+07
Fish 5	Y10/0.4	7E+07	8E+07	8E+07	4E+07	1E+08	1E+08	3E+07	3E+08	2E+08	4E+07	4E+07	4E+07	5E+07
Fish 6	X0/0	3E+07	9E+06	1E+07	1E+07	2E+07	3E+07	1E+07	3E+07	3E+07	2E+07	2E+07	2E+07	2E+07
Fish 6	X0.1/0.2	5E+06	2E+07	3E+07	3E+07	9E+07	1E+08	9E+06	1E+08	1E+08	2E+07	5E+07	5E+07	2E+07
Fish 6	X10/0.2	2E+06	2E+07	3E+07	4E+07	1E+08	2E+08	1E+07	2E+08	2E+08	2E+07	4E+07	4E+07	3E+07
Fish 6	X0/0.2	3E+06	2E+07	3E+07	4E+07	1E+08	2E+08	1E+07	2E+08	2E+08	2E+07	5E+07	5E+07	3E+07
Fish 6	X0.1/0.4	6E+06	2E+07	3E+07	5E+07	2E+08	2E+08	9E+06	2E+08	2E+08	1E+07	5E+07	5E+07	3E+07
Fish 6	X10/0.4	0E+00	2E+07	3E+07	7E+07	1E+08	2E+08	1E+07	3E+08	3E+08	2E+07	4E+07	4E+07	4E+07
Fish 6	X0/0.4	4E+05	2E+07	3E+07	5E+07	1E+08	2E+08	8E+06	2E+08	2E+08	1E+07	4E+07	4E+07	3E+07
Fish 6	Y0/0.2	7E+05	1E+07	3E+07	6E+07	2E+08	3E+08	7E+06	3E+08	2E+08	1E+07	4E+07	4E+07	3E+07
Fish 6	Y0/0	2E+06	2E+07	3E+07	4E+07	1E+08	2E+08	9E+06	2E+08	2E+08	2E+07	5E+07	4E+07	3E+07
Fish 6	Y0/0.4	1E+07	3E+07	4E+07	3E+07	1E+08	1E+08	1E+07	1E+08	1E+08	2E+07	5E+07	5E+07	2E+07
Fish 6	Y0.1/0.2	0E+00	1E+07	3E+07	4E+07	2E+08	2E+08	7E+06	2E+08	3E+08	2E+07	5E+07	4E+07	3E+07
Fish 6	Y0.1/0.4	2E+06	2E+07	3E+07	3E+07	1E+08	2E+08	8E+06	2E+08	2E+08	2E+07	5E+07	4E+07	3E+07
Fish 6	Y10/0.2	0E+00	2E+07	3E+07	5E+07	2E+08	2E+08	8E+06	2E+08	2E+08	2E+07	4E+07	4E+07	3E+07
Fish 6	Y10/0.4	5E+06	3E+07	5E+07	3E+07	1E+08	1E+08	1E+07	2E+08	2E+08	2E+07	4E+07	4E+07	2E+07