

CHARACTERIZATION OF SKELETAL MUSCLE LIPIDS IN OBESE MICE  
LINES

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LINES**

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## ABSTRACT

### CHARACTERIZATION OF SKELETAL MUSCLE LIPIDS IN OBESE MICE LINES

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Obesity becomes an epidemic health problem in developing and developed countries, which arises due to stable life style and increase in the consumption of high fat diets. Obesity is generally accompanied with various clinical disorders, such as insulin resistance, type II diabetes, hypertension, dyslipidemia and cardiovascular diseases.

This study aims to characterize and quantify different lipid classes in *longissimus dorsi* (LD) and *quadriceps* (Q) skeletal muscles of control (DBA/2J), obese Berlin fat mouse inbred (BFMI) and Berlin muscle mouse inbred (BMMI) lines, which display high fat and high muscle content, respectively. These mouse lines were special due to their phenotypes, especially BFMI lines, which displayed spontaneous and strong obesity. These lines, more specifically BFMI860 and BFMI861, were also special due to their possibility of being an animal model of cardiovascular diseases and metabolic syndrome, since they also displayed insulin resistance. For separation,

identification and quantification of various lipids of these lines, a novel method was developed which gives better separation of main lipid classes via using high performance liquid chromatography (HPLC) coupled to an evaporative light scattering detector (ELSD). Addition of triethylamine (TEA) to the solvents being used, and altering the parameters of HPLC and ELSD instruments, and also the gradient elution, provided a better separation with an enhanced resolution.

This method has been applied to the lipid extracts obtained from longissimus dorsi (LD) and quadriceps (Q) skeletal muscles of control (DBA/2J), obese Berlin fat mouse inbred (BFMI) and Berlin muscle mouse inbred (BMMI). In this method, a binary gradient elution composed of n-Hexane, isopropanol, methanol, acetic acid and triethylamine was applied to the samples. All interested lipid classes, namely triglyceride (TG), cholesteryl ester (CO), cholesterol (C), 1-oleoyl-rac-glycerol (MG), phosphatidylcholine (PC) and cardiolipin (CLPN), all of which have been known to have a role in obesity, insulin resistance, and cardiovascular diseases, were separated, identified and quantified via this novel method. According to the results, among BFMI lines, BFMI860 and BFMI861 lines and BMMI806, among BMMI lines, are worth to study obesity. Especially, the former ones may also become animal models for cardiovascular diseases and metabolic syndrome.

**Keywords:** Method development, HPLC-ELSD, Obesity, Muscle tissues, BFMI, BMMI.

## ÖZ

### OBEZ FARE IRKLARINDA İSKELET KAS YAĞLARININ KARAKTERİZASYONU

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Obezite gelişmiş ve gelişmekte olan ülkelerde durağan hayat tarzı ve özellikle yüksek yağ içerikli beslenme alışkanlığından ötürü ortaya çıkmıştır. Epidemik bir hal alan obezite, genellikle insülin direnci, tip II diyabet, hipertansiyon, dislipidemi ve kardiovasküler hastalıklar gibi patolojik durumlarla beraber gözükmektedir.

Bu çalışmada, özel olarak üretilen kontrol (DBA/2J), yüksek yağ içeriğine sahip Berlin lipit inbred (BFMI) ve yüksek kas içeriğine sahip olan Berlin kas inbred (BMMI) fare modellerinin longissimus dorsi (LD) ve kuadriceps (Q) iskelet kas dokularındaki lipit profillerinin nitel ve nicel olarak belirlenmesi amaçlanmıştır. Bu fareler, özellikle BFMI860 ve BFMI861 fare hatları, spontan obezite göstermeleri açısından özeldirler. Bu amaç doğrultusunda, evaporatif ışık saçılımlı dedektörün (ELSD) bağlı olduğu yüksek performanslı sıvı kromatografisi (HPLC)

kullanılmıştır. Literatürde bulunan ve denenilen metotların ilgilenilen lipitlerin bulunmasında yeterli olmamasından dolayı, yeni bir metot geliştirilmiştir. Bu metotta, trietilaminin (TEA) eklenmesi, ELS dedektörünün ve HPLC instrümanının parametlerinin değiştirilmesi ve yeni bir gradiyent uygulanmasıyla, ilgilenilen lipitlerin açıkça ayrıştığı görülmüştür.

Geliştirilen bu metot, kontrol (DBA/2J), yüksek yağ içeriğine sahip Berlin lipit inbred (BFMI) ve yüksek kas içeriğine sahip olan Berlin kas inbred (BMMI) fare modellerinin longissimus dorsi (LD) ve kuadriceps (Q) iskelet kas dokularına uygulanmıştır. n-Hekzan, isopropanol, metanol, asetik asit ve trietilaminden oluşan ikili bir gradiyent akışı kullanılarak, obezite, insülin direnci ve kardiyovasküler hastalıklarında etkileri olduğu bilinen kolesteril ester (CO), trigliserit (TG), 1-oleoil-rak-gliserol (MG), kolesterol (C), fosfatidilkolin (PC) ve kardiyolipin (CLPN) lipitleri, bu metotla başarıyla ayrışmıştır. Elde edilen sonuçlara göre, BFMI860, BFMI861 ve BMMI806 fare hatlarının obezite çalışmalarına uygun olduğu görülmüştür. Özellikle BFMI860 ve BFMI861 fare hatlarının sadece obezite çalışmaları için değil, kardiyovasküler hastalıklar ve metabolik sendromu çalışmalarında da model olabilecekleri anlaşılmıştır.

**Anahtar Kelimeler:** Metot geliştirme, HPLC-ELSD, Obezite, İskelet kası, BFMI, BMMI

*To my beloved family,*

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## LIST OF ABBREVIATIONS

HPLC	High-performance liquid chromatography
ELSD	Evaporative light scattering dedector
BFMI	Berlin Fat Mouse Inbred
BMMI	Berlin Muscle Mouse Inbred
LD	Longissimus Dorsi
Q	Quadriceps
TG	Triglyceride
CO	Cholesteryl oleate
C	Cholesterol
MG	Monoglyceride
PC	Phosphatidylcholine
CLPN	Cardiolipin

# CHAPTER 1

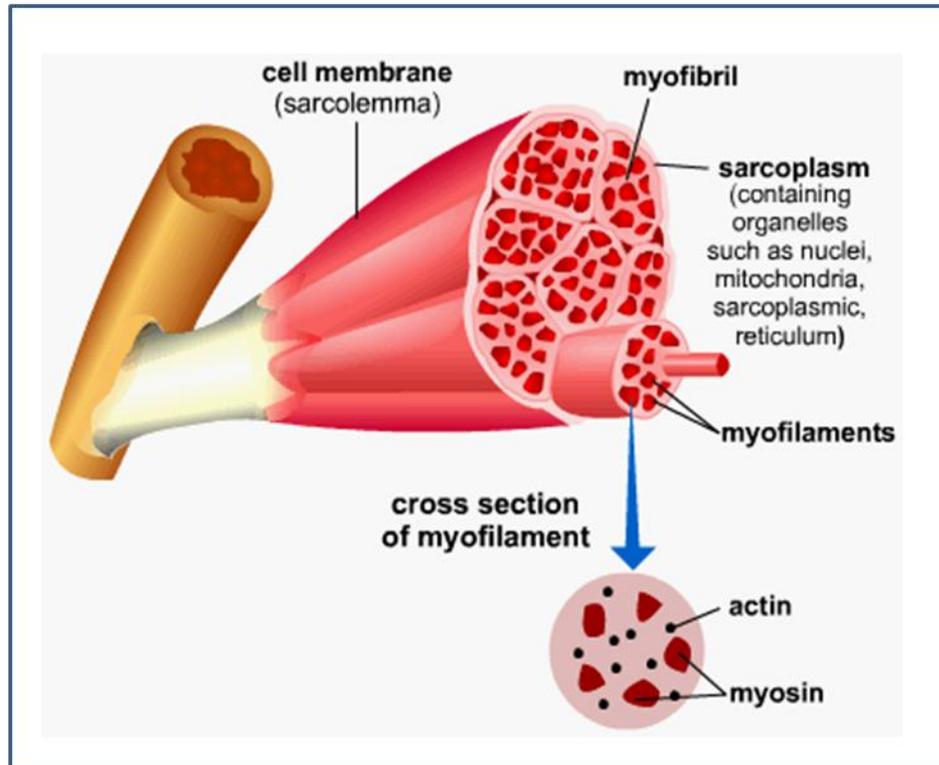
## INTRODUCTION

### 1.1 Skeletal Muscle Tissue

Skeletal muscle tissue has acquired its name because it is bound to the skeleton by tendons, and makes the skeleton move. It is also called striated muscle, since series of light and dark bands, or striations are observed, when observing the fiber under light microscope. Skeletal muscles are voluntarily controlled, and can generate great power (Greisheimer & Wiedeman, 1972). This specific muscle tissue functions in the movement, posture and also heat production, due to its contractions (Carola et al, 1992; Ono et al, 1995).

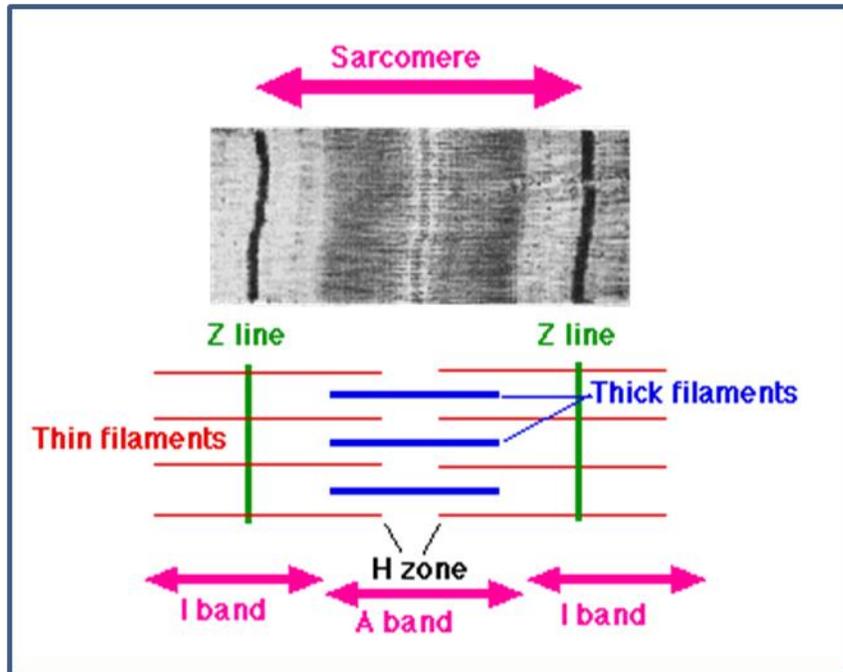
#### 1.1.1 Cellular Structure and Organization of Skeletal Muscle Tissue

Skeletal striated muscle tissues are composed of special cells called muscle fibers, which have long cylindrical shape and numerous nuclei. Plasma membrane and cytoplasm of muscle fibers are called sarcolemma and sarcoplasm, respectively. Within sarcoplasm, many parallel-found myofibrils which composed of thin and thick myofilaments and numerous mitochondria organelles are found to satisfy their continuous energy need. While thick myofilaments are almost composed of only myosin, thin myofilaments are composed of actin, troponin and tropomyosin proteins (Vander et al, 1998) (Figure 1.1).



**Figure 1.1.** Structural organization of a striated muscle (<http://www.s-cool.co.uk/a-level/biology/movement-and-support-in-animals/revise-it/muscles-and-movement>).

A sarcomere, which is the fundamental unit of muscle contraction, is made up of a specific section of a muscle fiber that is from one Z line to the other Z line. There are different zones on a sarcomere. Overlapping of thick myofilaments and thin myofilaments produces A bands which appear as dark bands. Within A band, there are H zone and M line, H zone is only composed of thick filaments and M line connects these thick filaments. Only thin myofilaments are present within I band (Carola et al, 1992; Vander et al, 1998) (Figure 1.2).



**Figure 1.2.** Structure of a sarcomere. Where A bands appear dark strands, I bands appear as light (<http://en.wikipedia.org/wiki/File:Sarcomere.gif#file>).

Molecular mechanism of contraction is based on sarcomeres. A mechanism called sliding-filament mechanism explained the cross-bridge movement of actin and myosin proteins during contraction. Generation of force induces shortening of a skeletal muscle fiber, therefore overlapping actin and myosin myofilaments in each sarcomere move past each other, propelled by movements of the cross bridges. There is no change in the lengths of thin and thick myofilaments during shortening process, while they are sliding. During contraction, conformational changes occur within sarcomere except A bands: I bands and H band get smaller, Z bands are pulled towards the center of sarcomere (Carola et al, 1992; Scott et al, 2001; Shier et al, 2010; Vander et al, 1998).

### **1.1.2 Types of skeletal muscle**

Skeletal muscle types differ according to their functions, according to their location, according to their enzyme activity and according to their energy metabolism.

#### **1.1.2.1 Type I Fibers**

Type I fibers are red, oxidative and slow-contractile fibers which function in slow and sustained activities. The dark or red appearance of type I fiber is due to high amounts of myoglobin and richness in its capillary content, which contribute to the fiber's oxidative energy metabolism (Scott et al, 2001). This oxidative fiber type is able to maintain continuous activity, and energy is derived from lipid oxidation (Collins, 1997). Slow-twitch fiber also differs from enzyme activity, where myosin ATPase activity is found to be slow when compared to fast-twitch muscle fibers. Activity of this enzyme was found to be related to the speed of muscle shortening, where low myosin ATPase activity is an indicator of slow muscle shortening (Barany, 1967; Scott et al, 2001).

#### **1.1.2.2 Type II Fibers**

Type II fibers can be described as fast-twitch muscle fibers, which can generate short bursts of speed and strength, but exhaust more quickly when compared to type I fibers. Another difference between these two fibers is that, type II fibers use mostly glycogen as energy source. Myosin ATPase activity is higher in fast-twitch muscle fibers, which indicates their faster rate of shortening during contraction. Type II fibers are subdivided into as Type IIa and Type IIb muscle fibers. Former one can be described as red, fast-twitch and oxidative, and the latter one as white, fast-twitch and glycolytic (Barany, 1967; Carola et al, 1992; Collins, 1997; Scott et al, 2001).

*Longissimus dorsi* (LD) is the main back muscle, which functions as maintaining posture (Hamelin et al, 2007; Ono et al, 1995). *Quadriceps* (Q) is the large muscle group found in front of thigh (Carola et al, 1992; Vander et al, 1998). Each individual muscle within the body is composed of all three fiber types; according to the need, the most suitable one starts to contract.

## **1.2 Obesity**

Overweight and obesity are becoming major health problems and epidemic in developed and developing countries. According to 2008 report of *World Health Organization* (WHO), there are 1.4 billion overweight adult individuals worldwide of whom 500 million are obese (WHO Report, 2008).

Overweight and obesity are described as abnormal or excessive fat accumulation within the body that presents health risk. Having a body mass index (BMI), which calculated by dividing a person's weight in kilograms by the square of individual's height in meters, higher than 25 kg/m<sup>2</sup> and 30 kg/m<sup>2</sup> is considered to be overweight and obese, respectively.

The growing prevalence of obesity, or overweight, is related to various factors. These factors such as excessive consumption of nutrient poor, more energy-dense, high sugar, high calorie and high saturated fat contentful food, stationary life-style, and genetic inheritance contribute in developing obesity. Although, genes themselves are pretty important in an individual's tendency to gain weight, energy balance, therefore being obese or lean, depends on energy intake and expenditure.

What makes obesity so dangerous and worth to study is the obesity-induced or obesity-accompanied diseases. There are various pathological states which are related to obesity, and present health risks, and even death.

### **1.2.1 Obesity Related Diseases and Disease States**

According to reports of WHO, obese and overweight individuals display higher risk factors for other diseases and disease states, including insulin resistance, type II diabetes, cardiovascular diseases, and even metabolic syndrome.

Insulin resistance is defined as the incapability of insulin hormone to reduce the blood glucose level, due to dysfunctioning or unresponsiveness of insulin receptors on the target tissues, like fat and muscle tissues (Kelley & Goodpaster, 2001). It was found that obesity is one of the major causes for insulin resistance due to accumulation of excess lipids, especially within skeletal muscle tissues, which is the main tissue of insulin action (Bogardus et al, 1985; DeFronzo et al, 1981; Goodpaster et al, 2000). Also incidence of type II diabetes mellitus has been known to engage with the incidence of obesity, due to insulin resistance. A study performed by Stevens and his colleagues showed that 90% of subjects with type II diabetes were also overweight or obese (Stevens et al, 2001). Diabetes risk increases if the individual was a juvenile obese (Haslam & James, 2005; Wannamethee & Shaper, 1999).

Cardiovascular diseases, as the name implies, is a class of diseases that appear in the heart or in blood vessels. Increase in BMI values, as mentioned above, is an indicator of being overweight or obese. As an individual's BMI increases, excess accumulation of lipids, which is called dyslipidemia, arises (Haslam & James, 2005). In dyslipidemic and obese subjects, which accompanied with diabetes and hypertension, incidence of cardiovascular disease increases (Caccamo et al, 2010; Haslam & James, 2005; Khaodhriar et al, 1999; Lean, 2000; Reaven et al, 2004).

Metabolic syndrome is defined as the combination of various diseases, like insulin resistance, obesity, type II diabetes, hypertension, hyperlipidemia and cardiovascular diseases. Obesity and increments in both circulating and abdominal lipid content

induce insulin resistance, and therefore diabetes and also cardiovascular diseases. All these states are associated with metabolic syndrome, and combination of even two of them makes an individual more predisposed to have metabolic syndrome (Khaodhiar et al, 1999; Lottenberg et al, 2012; Reaven, 2011).

### **1.2.2 Experimental Models of Obesity**

The need to understand the mechanisms and to find a treatment of obesity and its related diseases is a must due to their growing epidemic. For these purposes, animal model studies are necessary and priceless. There are many rodent models for obesity; some have monogenic mutations which induces alterations in energy metabolism through absence of a specific hormone, like leptin deficient *ob/ob* mice or through a null mutation; some display obesity due to polygenic mutation; and some have obesity through diet induction (Kanasaki & Koya, 2011; Reed, 2008; Speakman et al, 2008). Except special examples of obesity based on deficiency of a specific protein, like leptin-deficient adults in the study conducted by Licinio and co-workers (Licinio et al, 2004), human obesity is generally due to complex genetic background, and such animal models are actually more relevant to human obesity.

#### **1.2.2.1 Berlin Mouse Inbred Lines**

In this study, the examined animal models were DBA/2J, Berlin Fat Mouse Inbred (BFMI) and Berlin Muscle Mouse Inbred (BMMI). The founder pet of BFMI and BMMI mice were obtained from a random pet shop of Berlin.

Mice were chosen first according to their low muscle/protein content, and afterwards high fat content. No mutations were presented in the founder of this line, or in other generations. After 58 generations, those mice were named as BFMI, since they displayed obesity (Wagener et al, 2006). Since obesity of BFMI lines (BFMI852, BFMI856, BFMI860 and BFMI861) was not induced by any monogenic mutations,

or even diet, these lines were chosen to investigate the complex genetic background of human obesity.

Afterwards so-called BMMI mice were chosen according to their high muscle/protein content, low fat content, but high body weight. Among BMMI lines, while BMMINN806 line was wild-type in myostatin, *Mstn*<sup>-/-</sup> myostatin mutation was introduced to BMMICC826 and BMMICC866 lines (Karst et al, 2011). Therefore, the latter two did have unlimited muscle growth since myostatin is a growth limiting factor in muscle development (Slack, 1997).

Although, the forefathers of DBA/2J and Berlin inbred mouse lines were not the same, DBA/2J mouse line was used as a control. In the current study, use of DBA/2J line as a control was plausible, since it is one of the commercially available inbred line that is widely used as reference in different studies (Kanasaki & Koya, 2011; Speakman et al, 2008; Toye et al, 2005), and since it is susceptible to generate obesity with the induction of high-fat diet (HFD), among 43 inbred mouse lines (Svenson et al, 2007). In the current study, characterization and quantification of the lipid profiles of different skeletal muscle tissues from 10 week-old BFMI and BMMI mice was aimed. In order to obtain reliable results from a characterization study, a control group has to be used. The use of DBA/2J was plausible in order to observe the quantitative alterations of the lipid contents of special-bred mouse lines, and to conclude the most suitable line which generated more remarkable obesity-induced changes in comparison to a control line which is more susceptible to generate obesity.

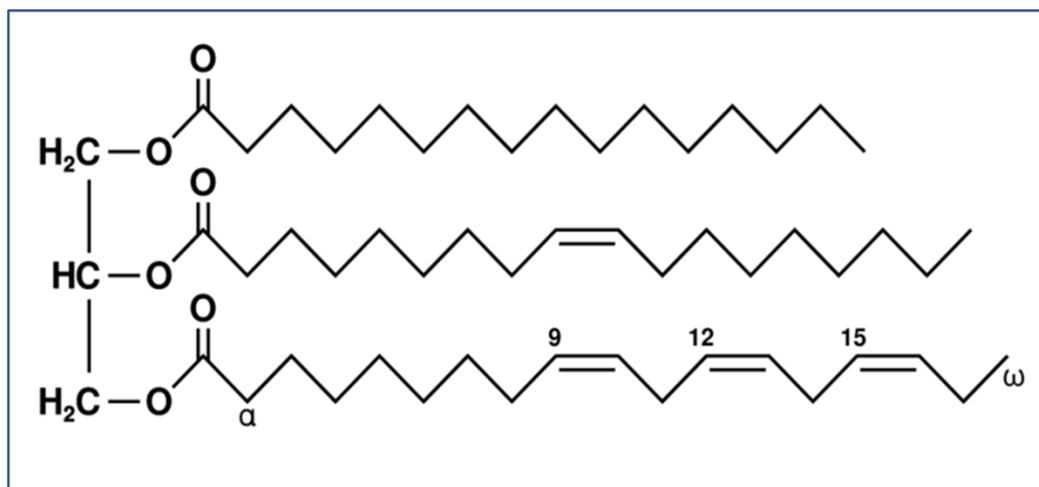
### **1.3 Lipids**

Lipids are naturally-occurring compounds including simple lipids, steroids, phospholipids, waxes and sphingolipids, which are distinguished from other naturally-occurring molecules by their solubility in apolar solvents (Christie, 1987).

Every lipid class is essential for maintaining biological functions, like cell energy storage, cell signaling, membrane functionality, and even survival and apoptosis (Christie, 1987; Hannun & Linares, 1993; Joshi et al, 2009). Among various types of lipids, only studied ones will be mentioned here

### 1.3.1 Triglyceride

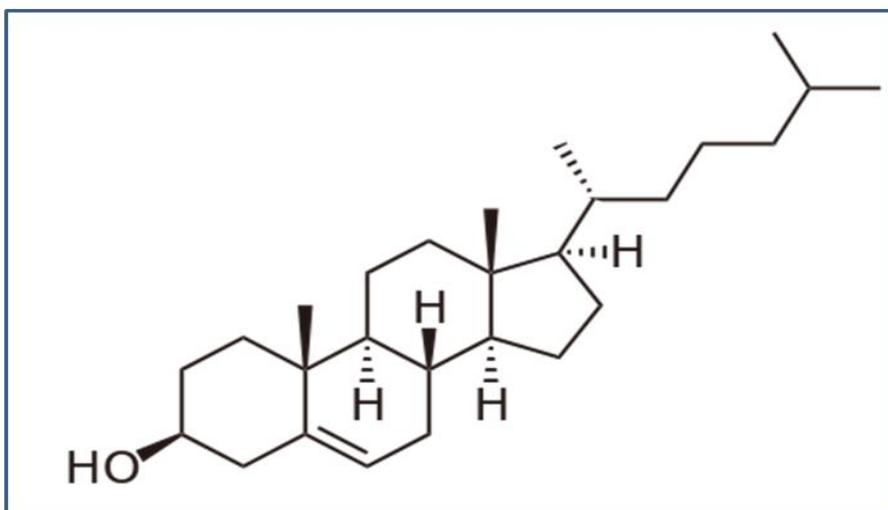
Triglyceride, or triacylglycerol, is composed of one molecule of glycerol and three molecules of fatty acids, as its name implies (Figure 1.3). It is one of the most known simple lipids, which synthesized via enzyme systems. It has been known that, triglycerides are involved in some pathological states, like obesity, insulin resistance and therefore, indirectly type II diabetes mellitus. For these reasons, this molecule is studied in most detailed among lipids.



**Figure 1.3.** A triglyceride molecule. Left part is glycerol and right part is fatty acids ([http://en.wikipedia.org/wiki/File:Fat\\_triglyceride\\_shorthand\\_formula.PNG](http://en.wikipedia.org/wiki/File:Fat_triglyceride_shorthand_formula.PNG)).

### 1.3.2 Cholesterol

Cholesterol is another abundant lipid member which is classified in steroid group (Christie, 1987) (Figure 1.4). This lipid is synthesized within a cell, since it has crucial roles in maintaining membrane fluidity, and being a precursor molecule of steroid hormones, like cortisol, aldosterone, estrogen, testosterone, and progesterone (Christie, 1987; Hu et al, 2010; Simons & Eehalt, 2002). Cholesterol level and metabolism are known to be altered in some pre-pathologic states, like insulin resistance and atherosclerosis (Barter et al, 2003; Gustavsson et al, 1999; Pike, 2003; Vainio et al, 2002). Therefore, cholesterol, likewise triglyceride, is one of the most studied lipids.

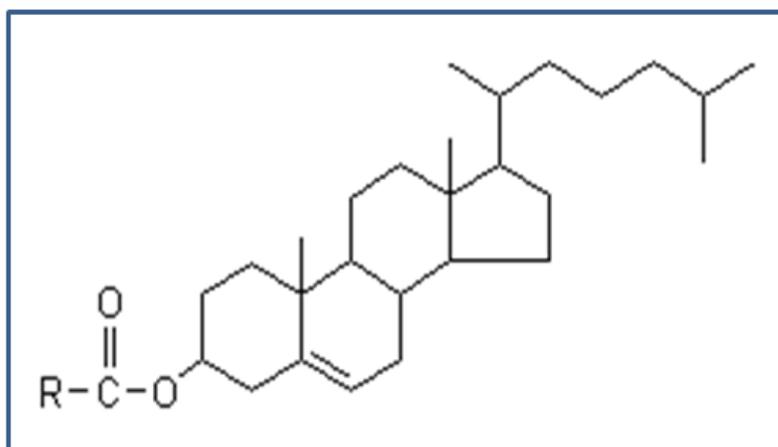


**Figure 1.4.** A cholesterol molecule. It has a tetracyclic ring structure which one of them has a double bond (<http://en.wikipedia.org/wiki/File:Cholesterol.svg>).

### 1.3.3 Cholesteryl Ester

Cholesteryl ester, as its name implies, is the esterified form of cholesterol, which is catalyzed by an enzyme called lecithin cholesterol acyltransferase (LCAT) (Christie,

1987; Li et al, 2011). Instead of cholesterol's hydroxyl group, cholesteryl ester has an ester group bound to the tetracyclic ring structure (Figure 1.5). Enzymes, LCAT and cholesteryl ester transport protein (CETP) which shuttles cholesteryl ester and triglycerides between lipoproteins (Fielding & Fielding, 1995; Tall, 1993), related to cholesteryl ester lipid, are involved in atherosclerosis and obesity (Arai et al, 1994; Chapman et al, 2010; Hayashibe et al, 1997; MacLean et al, 2003; Magkos et al, 2009). Therefore, analysis of this lipid is very important to reveal a part of the mechanisms of these diseases.

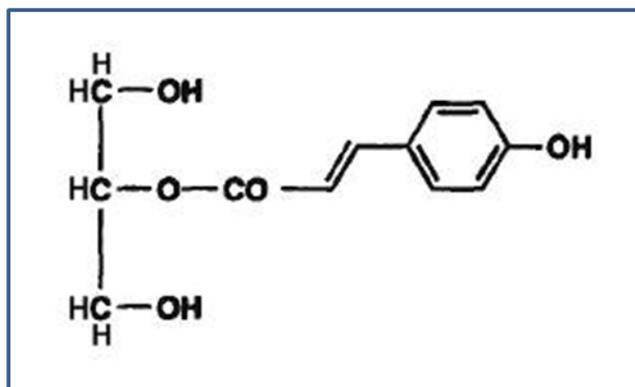


**Figure 1.5.** A cholesteryl ester molecule. Instead of hydroxyl group of cholesterol, ester group is present

(<http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/lipoprot.htm>).

### 1.3.4 Monoglyceride

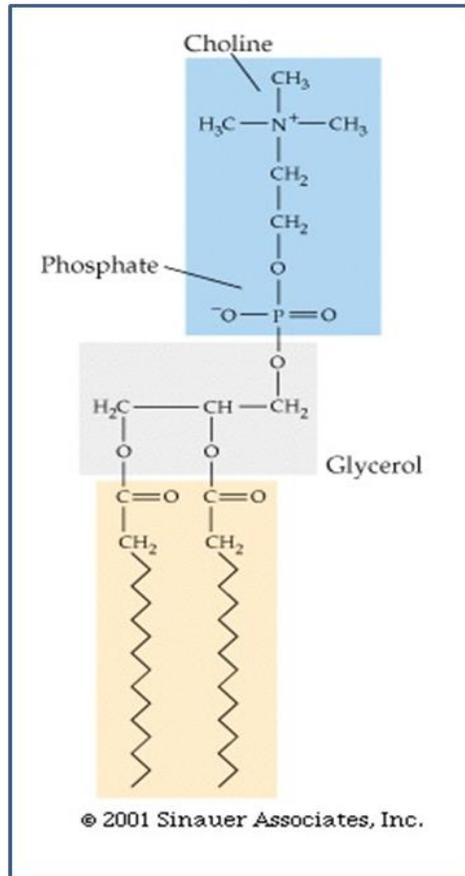
Monoglyceride, or monoacylglycerol, is composed of one molecule fatty acid and one molecule of glycerol (Figure 1.6). This lipid is found to be present in trace amounts in fresh animal and plant tissues (Christie, 1987). Monoglyceride is formed via the hydrolysis of triglyceride by lipase enzymes (Khoo et al, 1974; Saadeh et al, 2012; Schittmayer & Birner-Gruenberger, 2009; Wang & Eckel, 2009).



**Figure 1.6.** A monoglyceride molecule. Left part is the glycerol molecule, and right part is the fatty acid (<http://www.cyberlipid.org/glycer/glyc0002.htm>).

### 1.3.5 Phosphatidylcholine

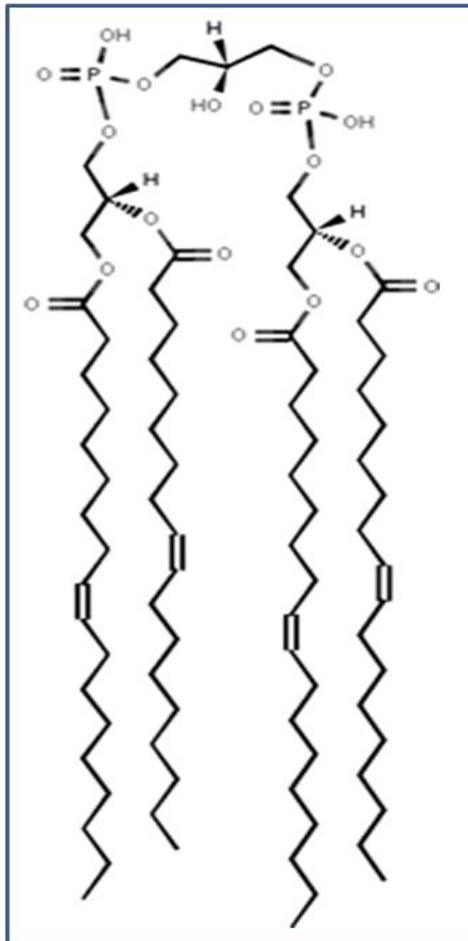
Phosphatidylcholine, also known as 1,2-Diacyl-sn-glycero-3-phosphrylcholine or as its commercial name lecithin, is one of the most studied phospholipids in biological and chemical studies (Christie, 1987). As its name indicates, it is composed of a choline head group, a phosphate group, a glycerol and two fatty acids (Figure 1.7). Being one of the most abundant components of cellular membranes of animal tissues, and playing important roles in cell signaling, synthesis of many molecules, and membrane integrity, this phospholipid has attracted attention from a long time (Christoffersen et al, 2003; Clore et al, 2000; Goto-Inoue et al, 2012; Grimm et al, 2011; Kent & Carman, 1999; Rauch & Loughna, 2005).



**Figure 1.7.** Molecular structure of phosphatidylcholine. It is composed of a choline head group, a phosphate group, a molecule of glycerol and two fatty acids (<http://www.columbia.edu/cu/biology/courses/c2005/purves6/figure03-21.jpg>).

### 1.3.6 Cardiolipin

Another glycerophospholipid type investigated in this study is cardiolipin, which is also known as diphosphatidylglycerol. As can be seen in Figure 1.8, lipid is composed of two phosphate groups, two glycerol molecules and four fatty acids. This lipid is almost found only in the inner membranes of mitochondria organelles of all living things (Christie, 1987; Joshi et al, 2009). Cardiolipin is an essential phospholipid and worth to analyze deeply due to its functions in mitochondrial energy metabolism, protein transport, aging, senescence and apoptosis (Claypool, 2009; Joshi et al, 2009; McMillin & Dowhan, 2002).



**Figure 1.8.** Structure of a cardiolipin molecule, which composed of two phosphatidylglycerol molecules (Kiebish et al, 2008).

Analysis of lipids and lipid metabolism has become a growing and improving field in recent years, since any disturbances or alterations in lipid metabolism are known to be both causative and outcome agents of diseases. Therefore, efficient and rapid methods to analyze these molecules have been of great importance. However, analyzing lipids is not easy due to their characteristic diversity. To be able to separate and identify each lipid class in a single mixture with different size, charge and polarity a gradient elution is required. HPLC is therefore, a handy tool in analyzing lipids, as will be mentioned in the next section.

## **1. 4 Chromatography**

Chromatography is a broad range of separations method used in separating, identifying and analyzing a specific molecule in a mixture. Separation is basically based on partitioning behavior of two phases: A mobile phase which the interested mixture is dissolved in and which is forced to flow through stationary bed, and a stationary phase, where the molecules of interest are held (Bobbitt et al, 1968).

Chromatography was first developed by a Russian botanist, Mikhail Tswett to separate plant pigments, in 1903 (Ditz, 2012). For more than 100 years, chromatography science has improved, so that lipids, proteins, vitamins, and even DNA fragments can be separated via different techniques (Christie, 1987; Yamakawa et al, 1996).

Various chromatographic techniques have been developed according to the interested molecules' properties. Adsorption chromatography, ion-exchange chromatography, thin layer chromatography, and affinity chromatography are the common separations techniques which used in different experimental designs (Christie, 1987; Snyder & Kirkland, 1979).

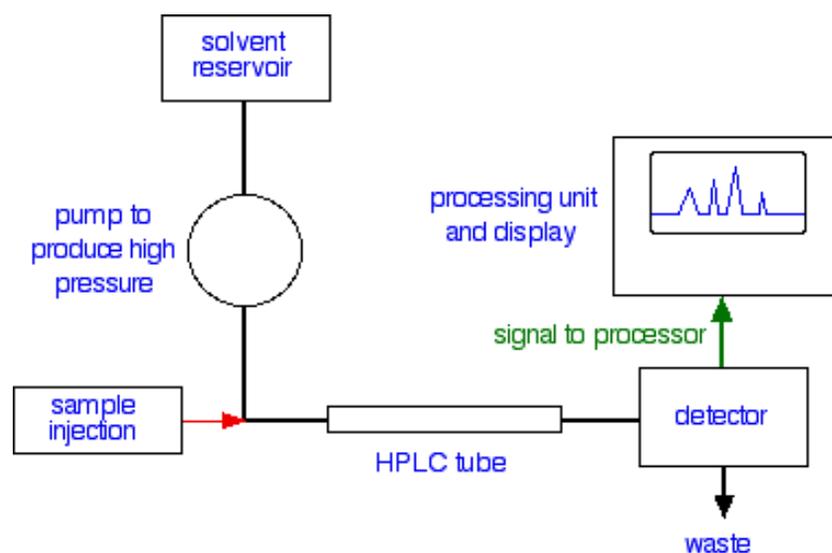
### **1.4.1 High-Performance Liquid Chromatography (HPLC)**

High-performance liquid chromatography (or high-pressure liquid chromatography) is a form of advanced and automated partitioning chromatography used to separate, identify and quantify individual compounds rapidly, in a complex mixture with high sensitivity and high reproducibility.

This chromatographic technique, as its name implies, uses high pressure to flush the mobile phase through the stationary phase (Figure 1.9). As the interested mixture migrates through the stationary phase, which in this study is a column, the mixture

starts to resolve and individual molecules within the complex mixture are held. The resolution rate is crucially important, and is dependent on the degree of interaction between analytes and the stationary phase. When the rate of resolution is insufficient, it is a must to change, to optimize the parameters of the elution conditions in order to enhance the selectivity between analytes, mobile phase and stationary phase.

Information, like retention time or peak area, obtained from HPLC are specific to the analyte. Retention time of a molecule changes according to the mobile phase solvents, parameters of HPLC and detectors being used, and of course to its stationary phase. However, retention time of a molecule is characteristic for the analyte, if the same instrument and same parameters have been used. Another important parameter, which gives quantitative data, is the peak area. Peak area is proportional to the amount of the analyte.



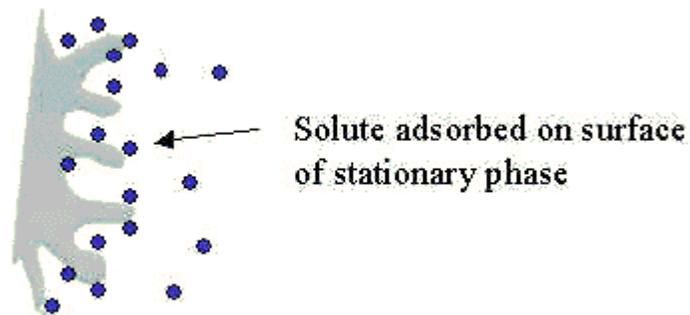
**Figure 1.9.** Working principle of a high-performance liquid chromatography instrument (<http://www.chemguide.co.uk/analysis/chromatography/hplc.html>).

## **1.4.2 HPLC and Lipids**

The detection and analysis of lipids have been of great importance in recent years, since they are involved in many essential biological functions and any alterations in lipid metabolism are known to be both causative and outcome of pathological states. Therefore, detecting healthy and pathological states of lipids, and also analyzing and quantifying of the lipid classes in each state have been an essential field which continuously grows and improves. Among separation and detection methods, HPLC technique has become one of the major tools. There are various HPLC-based chromatographic techniques which are used in the separation, detecting and analyzing of lipid compounds (Christie, 1987).

### **1.4.2.1 Adsorption Chromatography**

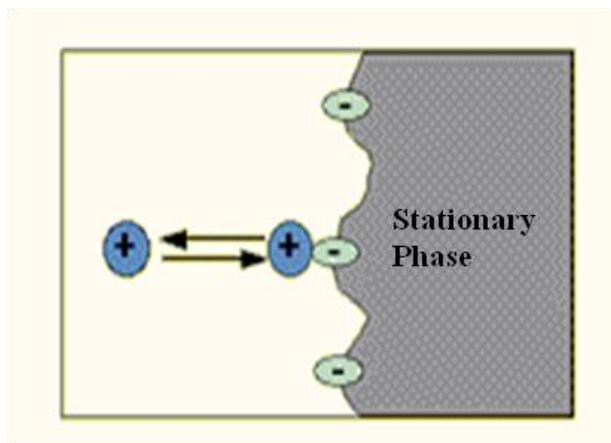
In adsorption chromatography, interactions between analyte and stationary phase take place on the surface of the stationary phase, which is also called as adsorbent (Bobbitt et al, 1968) (Figure 1.10). Adsorbent materials are defined as normal-phase and reverse-phase depending on their polarity properties, and generally silica-based materials have been used. In normal-phase chromatography, the stationary phase is polar (hydrophilic) and mobile phase is apolar (hydrophobic). Analytes with polar character retain longer on polar stationary phase, than apolar analytes do, which means apolar molecules elute earlier than polar molecules. Normal-phase adsorption chromatography is best used while separating and detecting molecules readily soluble in apolar solvents. In reverse-phase chromatography, the stationary phase is apolar and the mobile phase is polar. These two adsorption chromatography techniques are widely used in lipid class separation (Avalli & Contarini, 2005; Boselli et al, 2008; Brouwers et al, 1999; Christie, 1985; Lutzke & Braughler, 1990; Maraschiello et al, 1996; Olsson et al, 2012).



**Figure 1.10.** Adsorption chromatography. Analyte interacts with the surface of the stationary phase (<https://www.msu.edu/~gallego7/MassSpect/Chromatography.htm>).

#### 1.4.2.2 Ion-Exchange Chromatography

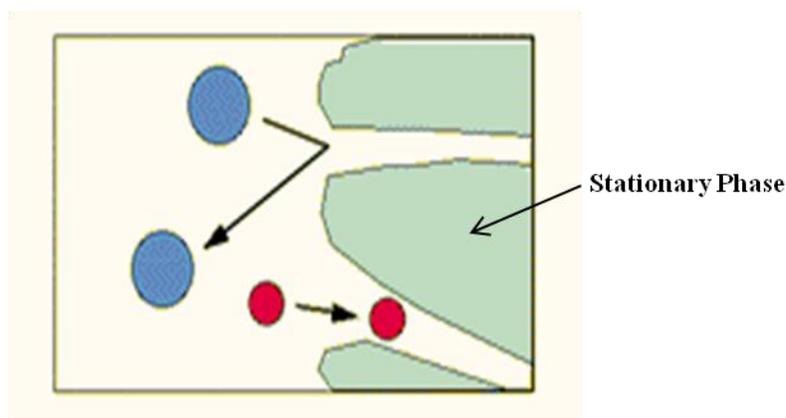
In ion-exchange chromatography, separation is based on the opposite charges of stationary phase and analyte (Roos, 1999) (Figure 1.11). This technique is suitable, when the analyte molecules are charged. Ion-exchange chromatography is generally applied on charged molecule class separation (Bandi et al, 1982).



**Figure 1.11.** Ion-exchange chromatography. Surface of the stationary phase is ionically charged, opposite charged of the analyte (<http://elchem.kaist.ac.kr/jhkwak/AnalChem/09/04/gif2.htm>).

### 1.4.2.3 Size Exclusion Chromatography

In size exclusion chromatography, also known as gel-permeation chromatography, molecules are separated according to their molecular size. While larger molecules elute quicker, smaller molecules reside within the stationary phase and elute later (Figure 1.12). This kind of chromatography is most suitable in the separation of especially proteins, and lipids accompanied with proteins, like lipoproteins or membrane lipids (Grabielle-Madelmont et al, 2003; Kunji et al, 2008; Rogner, 1999).



**Figure 1.12.** Size exclusion chromatography. Stationary phase has controlled pore size, which makes the separations based on molecular size (<http://elchem.kaist.ac.kr/jhkwak/AnalChem/09/04/gif2.htm>).

### 1.4.3 HPLC Detectors

Without a detector, only separation of visible molecules could be possible. To separate, to identify and to analyze interested molecules, HPLC instrument needs a detector which is suitable for the analytes' physical, chemical and spectroscopic properties. Various detectors have been developed according to the desired molecules' properties.

#### **1.4.3.1 UV/Visible (UV/Vis) Detectors**

UV/Vis detectors are the most widely used detectors, due to their relative inexpensiveness. Working principle of this detector is to respond to substances which absorb light. In lipid analysis, this detector has been used, however if the mobile phase solvents do have chromophores or if the interested analyte does have low chromophore activity, results will be unreliable (Christie, 1987).

#### **1.4.3.2 Refractive Index (RI) Detectors**

RI detectors are known to be the least sensitive detectors which respond to substances that refract light (Christie, 1987). This detector is mostly abandoned due to its insensitivity or its inability to be used in gradient elutions.

#### **1.4.3.3 Mass Spectrometry (MS) Detectors**

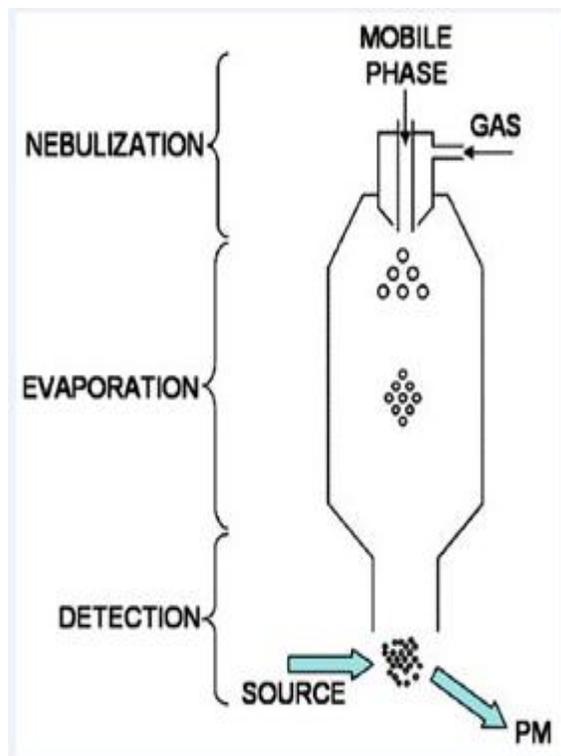
MS detector coupled to HPLC is one of the most powerful analytical techniques in chromatography. With this detector, not only quantitative data but also, structural information and the empirical formula can be obtained of an unknown compound (Christie, 1987; Di Stefano et al, 2012).

#### **1.4.3.4 Evaporative Light Scattering (ELS) Detectors**

ELS detectors are universal detectors which respond to substances that scatter light. This detector has been widely used in lipid analysis in recent years, due to its response to all compounds which are less volatile within the mobile phase. The working principle of ELS detector is to measure the scattered light intensity from unevaporated part, which is the interested compound, of the migrating mobile phase. Every molecule which is not evaporated or degraded within the detector emits signal,

and intensity of this signal gives information about the amount of the molecule. There are three main processes occur within ELS detection (Figure 1.13):

- Nebulization: In this step, mobile phase is nebulized, and becomes aerosol, and enters to drift tube accompanied by nebulizer gas which is in this study, pure nitrogen.
- Evaporation: With the presence of the nebulizer gas, aerosol enters to a heated drift tube. In there, solvent is completely evaporated, remaining only the analytes.
- Detection: The analytes emerge from drift tube, and pass through a light beam, where detection occurs upon the scattered light via either a photomultiplier tube or a laser diode (Christie, 1987).



**Figure 1.13.** Working principle of evaporative light scatter detector (<http://www.cyberlipid.org/elsd/elsd0001.htm>).

Due to its response to all compounds in the mobile phase, its independence on spectroscopic properties of the interested molecules and its applicability for gradient elutions, ELS detection provides several advantages over other detection techniques being used in lipid profile analysis (Bravi et al, 2006; Christie, 1987).

#### **1.4.3.4.1 Data Acquisition and Processing**

In data acquisition and processing, to obtain the calibration curves of injected standards are essential. At least three different concentrations of a single standard have to be injected, to draw a calibration curve. A calibration curve is represented by

a quadratic equation, as follows (Empower 2 Data Acquisition and Processing Theory Guide):

$$y = A + Bx + Cx^2$$

where:

$y$  = Response of the standard peak calculated by the software,

$x$  = Amount or concentration of the standard,

A, B, and C = Polynomial coefficients of the curve.

To determine the amount or the concentration of the standard, equation below has being used:

$$x = \frac{-B \pm \sqrt{B^2 - 4C(A - y)}}{2C}$$

Coefficient of determination ( $R^2$ ) value is important while obtaining calibration curves. If  $R^2$  value is off the 'goodness of fit' boundaries, a numerical value lower than 0.99, then the calibration curve cannot be used. This value is calculated as follows:

$$R^2 = 1 - \frac{(S_y)^2}{\sigma_y^2}$$

where:

$R^2$  = Coefficient of determination,

$S_y$  = Standard error of estimate of y on x,

$\sigma_y^2$  = Standard variance.

To be able to calculate the analyte, however there are some limitations in chromatography, which are called ‘limit of detection (LOD)’ and ‘limit of quantification (LOQ)’. These limitations are based on the strength of the signal. If signal-to-noise ratio is higher than 3 units, then the analyte can be identified or determined. And if the signal-to-noise ratio is higher than 10, then the analyte can be quantified.

### **1.5 Aim of the Study**

The first aim of this study is to develop a novel and a sensitive method for separating, identifying and quantifying the main lipid classes applicable to not only skeletal muscle tissues, but also other tissues via using a normal-phase HPLC-ELSD system. Since procedures inspired from literature are not compatible with the system and since use of these procedures are not enough to obtain interested lipids, it is a necessity to develop a new method according to the samples and according to the separation module.

Another aim of the current study is to investigate the lipid profiles of different skeletal muscle tissues, which are *Longissimus Dorsi* (LD) and *Quadriceps* (Q) from special-bred mouse lines, Berlin Inbred mice. These mouse lines display polygenic, spontaneous obesity, which is more relevant to human obesity, than the monogenic models. In addition to spontaneous obesity, these lines may also demonstrate diminished insulin sensitivity. Since skeletal muscle tissue is essential in lipid and carbohydrate metabolism, and since pathological abnormalities related to obesity and insulin resistance, primarily occur within this tissue, lipid level alterations were investigated via using an advanced, automated and reliable partitioning chromatography, namely HPLC.

## CHAPTER 2

### MATERIALS AND METHODS

In order to obtain maximum amount of lipid from a muscle tissue of a mouse, firstly, different lipid extraction methods were tried. Classic Folch method, modified Folch method, Bligh and Dyer method and Lutzke method were performed to choose the most effective extraction method in our study (Bligh & Dyer, 1959; Iverson et al, 2001; Lutzke & Braughler, 1990). After the decision of extraction procedure, different elution methods obtained from literature were tried on HPLC-ELSD instrument. Since, procedures obtained from literature did not fit to the instrument; a new elution method was developed according to the column, detector, and separation module.

#### **2.1. Part I: Method Development**

##### **2.1.1 Materials**

HPLC-grade isooctane, methanol, isopropanol, water, tetrahydrofurane, chloroform, acetonitrile, and n-hexane were used as high-performance liquid chromatography mobile phase solvents and concentrated sulfuric acid ( $H_2SO_4$ ), phosphoric acid ( $H_3PO_4$ ) and absolute ethanol were obtained from Merck (Merck Company, Darmstadt, Germany). Olive oil was bought from a random market. Standard lipids cholesteryl oleate (CO), cholesterol (C), phosphatidylcholine (PC) and cardiolipin (CLPN); and sodium chloride (NaCl), sodium bicarbonate ( $NaHCO_3$ ), potassium chloride (KCl), magnesium chloride ( $MgCl_2$ ), calcium chloride ( $CaCl_2$ ), HEPES dry

powder, lyophilized powder of BSA and vanillin were from Sigma (Sigma Chemical Company, Saint Louis, Missouri, USA). Pure nitrogen gas was used as nebulizer gas (Oksan Collective Company, Ankara, Turkey).

### **2.1.2 Lipid Extraction**

To obtain maximum lipid content from a scarce amount of a mouse muscle tissue, choosing an effective lipid extraction method was important. In order to determine the final extraction method, different methods were tried before using the actual mouse muscle tissues.

#### **2.1.2.1 Folch Method**

Muscle tissue of a random mouse which was present beforehand, was homogenized via classical Folch method (Folch et al, 1957). Briefly, 50 mg of muscle tissue was cut and homogenized by using ULTRA-TURRAX T25 (IKA-Werke GmbH&CO, Staufen, Germany) at the speed of 10000 g/min for 30 seconds with the solvents of chloroform and methanol (2:1, v/v) to a final volume where the solvents were used twenty times the volume of the muscle sample. The homogenate was agitated for 20 minutes in an orbital shaker at room temperature. The homogenate was filtered using filter paper to get rid of solid particles. The solvent was washed with 0.2 volume of 0.9% NaCl solution. The mixture was vortexed for few seconds. Afterwards, it was centrifuged at 2000 rpm for five minutes at room temperature. Upper phase was then removed. Lipids in the lower phase were dried under nitrogen gas. Sample was stored at -20 °C till total lipid determination. Three replicates were done.

#### **2.1.2.2 Modified Folch Method**

Muscle tissue of a random mouse was homogenized as described in Iverson *et al.* (Iverson et al, 2001). Difference between classic Folch method and modified Folch

method, is to use 30 volumes of chloroform and methanol (2:1, v/v) instead of 20 volumes. Rest of the procedure was performed as described in Folch method. Three replicates were done.

### **2.1.2.3 Lutzke Method**

50 mg of muscle tissue of a random mouse -which was also used in Folch extraction method- was homogenized following Lutzke method (Lutzke & Braugler, 1990). Firstly, Krebs-Henseleit buffer solution was prepared. Solvent was composed of three different stock solutions: Stock solution A: NaCl; stock solution B: KCl, NaHCO<sub>3</sub> and MgCl<sub>2</sub> .6H<sub>2</sub>O; stock solution C: CaCl<sub>2</sub> . 2H<sub>2</sub>O. Distilled water was poured onto three different stock solutions which were used in equal amounts. Then 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 0.25% BSA (bovine serum albumin) were added to the solution. After filtration, pH of the mixture was adjusted to 7.35, and stored at 4°C. Tissue was homogenized in ice-cold Krebs-Henseleit buffer solution (1:5, w/v; mg/μl) via using ULTRA-TURRAX T25. The homogenate was diluted with Krebs-Henseleit buffer solution, to a final volume of ten times the volume of the muscle sample. Later, 2 ml methanol was added in Krebs buffer. After vortexing the sample, 4 ml of chloroform was poured. The mixture was centrifuged at 2000 rpm for five minutes at 4 °C, after a second vortex stage. The lower phase was collected and dried under nitrogen gas. Sample was put in -20 °C until total lipid determination. Three replicates were performed.

### **2.1.2.4 Bligh and Dyer Method**

About 50 mg of muscle tissue of a random mouse was homogenized and its lipids were extracted according to Bligh and Dyer method (Bligh & Dyer, 1959; Iverson et al, 2001). Firstly, 40 μl of water was added since the tissue was dry. Then, 150 μl of chloroform and methanol was poured (1:2, v/v). After addition of solvents, homogenization was performed for one minute, using ULTRA-TURRAX T25. After

pouring another 50 µl of chloroform, second homogenization was done for 30 seconds. Later, the mixture was filtered using filtration paper to recover the liquid phase. After addition of 50 µl of water, mixture kept at room temperature for 30 minutes to obtain a biphasic layer. Centrifugation was done for five minutes at 2000 rpm, at room temperature. After centrifugation, the lower phase was collected and dried under nitrogen gas. Sample was kept at -20 °C until total lipid determination experiments. Three replicates were done.

### **2.1.3 Total Lipid Determination**

Total lipid contents of the tried methods were determined, to decide the optimal lipid extraction method.

Total lipid content was determined colorimetrically, following Frings method (Frings & Dunn, 1970). Standard olive oil was prepared by adding 700 mg of olive oil in 100 ml absolute ethanol. And the other reagent, phosphovanillin reagent was prepared by dissolving 1.2 g of vanillin within 200 ml water and 800 ml concentrated phosphoric acid. This reagent stored in a dark bottle.

100 µl of muscle tissue lipid extractions of random mice, obtained from different methods -i.e. Folch method, modified Folch method, Lutzke method, and Bligh and Dyer method (Bligh & Dyer, 1959; Folch et al, 1957; Iverson et al, 2001; Lutzke & Braugler, 1990), and 100 µl standard olive oil and 100 µl water and 2 ml of concentrated sulfuric acid were mixed in a test tube and heated in boiling water. After 10 minutes in boiling water, test tubes were cooled to ambient temperature. Then, 100 µl from each mixture was transferred to another test tube. Onto them, 6 ml of phosphovanillin reagent was added. Absorbance values were measured at 540 nm by Varian Cary 300 Bio UV-Visible Spectrophotometer (Harlow Scientific, Arlington, USA). Total lipid content was calculated as follows:

$$\text{Total lipid (mg/dL)} = (\text{OD}_{\text{Sample}}/\text{OD}_{\text{Standard}}) \times 700 \text{ mg/dL}$$

Water was used as a blank. All replicates of each muscle tissue lipid extracts were used to make sure of the results.

#### **2.1.4 HPLC-ELSD Optimization Studies**

In order to find the best separation method, various methods inspired from literature were tried (Descalzo et al, 2003; Lutzke & Braugher, 1990; Perona & Ruiz-Gutierrez, 2005). Lipid extracts obtained from extraction methods, were injected to the instrument following these procedures.

A Waters Alliance e2695 Separations Module coupled to a Waters 2424 ELS detector (Waters, Milford, USA). System consisted of a Waters Spherisorb normal-phase silica column (100 x 4.6 mm I.D) with 5  $\mu\text{m}$  particle size (Waters, Milford, USA).

Lipid extracts were injected to the instrument following the separation method developed by Perona and Ruiz-Gutierrez (Perona & Ruiz-Gutierrez, 2005). While the column temperature was varied between 30 ° C and 40 ° C, sample temperature was kept constant at 25 ° C. Ternary gradient elution was applied in the method. The gradient elution of the mobile phase solvents, namely n-Hexane, isopropanol and methanol is shown in Table 2.1. Temperature of the drift tube was set to 50 ° C, and nebulizer was set to heating mode with the power level 60%. The flow rate was 1 ml/min. Samples and standards were dissolved in chloroform and methanol (2:1, v/v).

**Table 2.1.** Ternary gradient elution scheme. Solvent A is n-Hexane; solvent B is isopropanol and solvent C is methanol.

<b>Time (min)</b>	<b>Flow (ml/min)</b>	<b>A (%)</b>	<b>B (%)</b>	<b>C (%)</b>
<b>0</b>	1.0	100	0	0
<b>3.00</b>	1.0	97	3	0
<b>17.00</b>	1.0	90	10	0
<b>22.00</b>	1.0	0	40	60
<b>28.00</b>	1.0	0	40	60
<b>30.00</b>	1.0	100	0	0

Another method, inspired from the literature was tried for the extracted lipid samples, to find the optimal separation method (Descalzo et al, 2003). While the column temperature was varied between 30 ° C and 40 ° C, the sample temperature was set to a temperature of 25 ° C. Quaternary gradient elution was performed (Table 2.2). Mobile phase solvents were composed of methanol, acetonitrile, n-Hexane, isopropanol and water. Drift tube temperature was set to 70 ° C, and nebulizer to heating mode with the power level of 60%. Samples and standards were dissolved in chloroform and methanol (2:1, v/v).

**Table 2.2.** Quaternary gradient elution scheme. Solvent A is methanol; solvent B is isopropanol, n-Hexane and water (63:35:2, v/v/v), solvent C is isopropanol and n-Hexane (40:60, v/v) and solvent D acetonitrile.

<b>Time (min)</b>	<b>Flow (ml/min)</b>	<b>A (%)</b>	<b>B (%)</b>	<b>C (%)</b>	<b>D (%)</b>
<b>0</b>	1.0	0	0	100	0
<b>5.00</b>	1.0	0	0	0	100
<b>10.00</b>	0.8	45	0	0	55
<b>13.00</b>	0.8	45	0	0	55
<b>13.10</b>	1.0	60	0	0	40
<b>15.00</b>	1.0	80	0	0	20
<b>17.00</b>	1.0	100	0	0	0
<b>22.00</b>	1.0	100	0	0	0
<b>24.00</b>	0.8	0	100	0	0
<b>25.00</b>	0.8	0	100	0	0
<b>30.00</b>	0.8	0	0	100	0

To investigate the most efficient separation method, for the interested lipids, one other method was also tried following the procedure developed by Lutzke and Braughler (Lutzke & Braughler, 1990). While the column temperature was varied between 30 and 40 ° C, the sample temperature kept at 25 ° C. Table 2.3 demonstrates the ternary gradient elution. Mobile phase solvents were isooctane, tetrahydrofurane, isopropanol, n-Hexane, chloroform and water. Drift tube temperature was set to 70 ° C, and nebulizer to heating mode with the power level of

60%. Samples and standards were dissolved in isooctane and chloroform in the ratio of 1 to 1.

**Table 2.3.** Ternary gradient elution scheme. Solvent A is isooctane and tetrahydrofuran (99:1, v/v); solvent B is isopropanol and chloroform (80:20, v/v) and solvent C is isopropanol and water (50:50, v/v).

<b>Time (min)</b>	<b>Flow (ml/min)</b>	<b>A (%)</b>	<b>B (%)</b>	<b>C (%)</b>
<b>5.00</b>	2.0	100	0	0
<b>6.00</b>	2.0	100	0	0
<b>10.00</b>	2.0	80	20	0
<b>14.90</b>	1.6	44	52	4
<b>15.00</b>	1.5	42.5	52	5.5
<b>25.00</b>	1.5	32.5	52	15.5
<b>31.70</b>	1.5	29	52	19
<b>31.80</b>	1.5	30	70	0
<b>37.30</b>	2.0	100	0	0

## **2.2. Part II: HPLC-ELSD STUDIES**

### **2.2.1 Materials**

Lipids glyceryl trioleate (TG), cholesteryl oleate (CO), cholesterol (C), 1-mono-oleoyl-rac-glycerol (MG), phosphatidylcholine (PC) and cardiolipin (CLPN) which were used as standards ; and sodium chloride (NaCl) salt were purchased from

Sigma (Sigma Chemical Company, Saint Louis, Missouri, USA). Solvents isopropanol, methanol, n-hexane, water and chloroform used both in lipid extraction and high-performance liquid chromatography studies were HPLC-grade and obtained from Merck (Merck Company, Darmstadt, Germany). Acetic acid (AA) and triethylamine (TEA) used in studies were in the highest purity and from Merck (Merck Company, Darmstadt, Germany). All mobile phase solvents were degassed with ultrasonicator before use. Pure nitrogen gas was used as nebulizer gas (Oksan Collective Company, Ankara, Turkey).

### **2.2.2 Preparation of Experimental Animals**

Control DBA/2J, obese BFMI and muscle BMMI mice were obtained from Humboldt University of Berlin. All experimental procedures were approved by the German Animal Welfare Authorities (approval no. G0171/10). The forefathers of these animals were purchased from random pet shops in Berlin, Germany. Obese BFMI lines were firstly chosen according to their low protein content, and then according to their high fat content (Wagener et al, 2006). Muscle BMMI lines were selected due to their high protein content and high body weight. Each mouse which specified according to their high fat or high protein content, was bred till generation 58. From these mice, obese BFMI lines (BFMI852, BFMI856, BFMI860, and BFMI861) and muscle BMMI lines (BMMINN806, BMMICC826, and BMMICC866) were derived and produced (Meyer et al, 2009; Wagener et al, 2006).

Male mice were maintained under conventional conditions and controlled lighting with a 12h:12h light/dark cycle at an ambient temperature of 22±2 °C and humidity of 65%. All mice had *ad libitum* access to food and water. After 3 weeks, mice were fed with standard breeding diet (SBD) (V1534-000, ssniff R/M-H, Ssniff Spezialdiäten GmbH, Soest/Germany). SBD is composed of 58% of carbohydrate, 33% of proteins and 9% of fat, which has 12.8 MJ/kg metabolizable energy.

DBA/2J (n=6), BFMI (n=6, from each line) and BMMI (n=6, from each line) mice were decapitated at the end of 10<sup>th</sup> week, and their Longissimus Dorsi (LD) and Quadriceps (Q) muscles were snap-frozen in liquid nitrogen and stored in -80 °C until use.

### **2.2.3 HPLC-ELSD Studies**

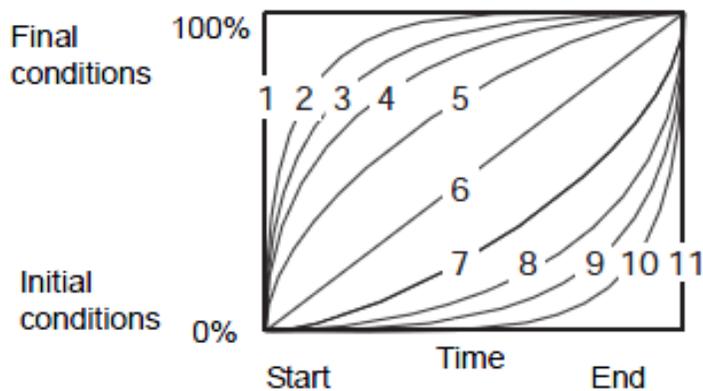
#### **2.2.3.1 Lipid Homogenization and Extraction**

Lipids from LD and Q skeletal muscle tissues of control, obese and muscle mouse lines were homogenized and extracted following classical Folch method (Folch et al, 1957). Skeletal muscle tissues were first cut in the amount of  $29.2 \pm 0.8$  mg. Samples were homogenized by using ULTRA-TURRAX T25 (IKA-Werke GmbH&CO, Staufen, Germany) at the speed of 13000 1/min for a minute with the solvents of chloroform and methanol (2:1, v/v) to a final volume where the solvents were used twenty times the volume of the muscle samples. The obtained homogenate was agitated for forty minutes in an orbital shaker at room temperature. Afterwards, the homogenate was centrifuged at 2000 rpm for five minutes at room temperature, in order to recover the liquid phase. Recovered homogenate was washed with 0.2 volume of 0.9% NaCl solution. After vortexing the mixture for ten seconds, it was centrifuged at 2500 rpm for ten minutes at room temperature. Upper phase was removed and re-centrifuged at 2500 rpm to recover any lipids. Lower phases both obtained from two centrifugation steps were collected in a test tube and dried under nitrogen gas, thus lipids were able to adhere to the inner wall of the test tube. Samples were stored at -20 °C until use.

#### **2.2.3.2 HPLC Instrumentation**

Throughout the whole study, a Waters Alliance e2695 Separations Module with quaternary liquid chromatographic pump was used which ensured to mix and pump

mobile phase solvents through the column (Waters, Milford, USA). The instrumentation allows both gradient and isocratic elution, adjusted either by manually or automatically. The HPLC system consisted of a silica filling normal-phase Agilent Polaris-Si (150 x 4.6 mm I.D) column with 3  $\mu\text{m}$  particle size (Agilent Technologies, Santa Clara, USA). The column temperature was maintained constant at 22 °C. A binary gradient elution was performed and the gradient curve value was 6, thus the gradient change kept linear as it is demonstrated in Figure 2.1. Solvent A consisted of n-Hexane, and solvent B consisted of isopropanol (IPA), methanol (MeOH), acetic acid (AA) and triethylamine (TEA) (60:40:0.2:0.1, by mw). As shown in table 2.4, the gradient started at 100% of A, and kept constant at the first 3 minutes of the run. Solvent A decreased to 90% in next 2 minutes, and to 60% in 12 minutes. Mobile phase A was held at 0% for the next 5 minutes, and then it reached back to 90% in 3 minutes. Then in the next 2 minutes, gradient percentage of A returned to the start point (Olsson et al, 2012). Each run was designed to last 35 minutes per sample, the first 28 minutes of the elution was for to separate lipids, while the last 7 minutes for the regeneration of the column prior to the next injection. The flow rate was 0.8 ml/min throughout.



**Figure 2.1.** Curve shapes for gradient profiles (Waters e2695 Separations Module Operator’s Guide).

**Table 2.4.** Binary gradient elution scheme.

<b>Time (min)</b>	<b>Flow (ml/min)</b>	<b>A (%)</b>	<b>B(%)</b>	<b>Curve</b>
<b>0</b>	0.8	100	0	6
<b>3.00</b>	0.8	100	0	6
<b>5.00</b>	0.8	90	10	6
<b>17.00</b>	0.8	60	40	6
<b>18.00</b>	0.8	0	100	6
<b>23.00</b>	0.8	0	100	6
<b>26.00</b>	0.8	90	10	6
<b>28.00</b>	0.8	100	0	6

For detection, a Waters 2424 ELS detector was used (Waters, Milford, USA). The drift tube temperature was set to 70 °C, and the nebulizer parameter was adjusted to the cooling mode. The data rate was 5 point per seconds (pps). The pressure of pure nitrogen gas was 60 psi (4.13 bar). Prior to injections, the instrument was washed with each mobile phase solvents at least 35 minutes (<http://www.nestgrp.com/pdf/colcare.pdf>). After washing step, it was calibrated and conditioned with the developed method for at least four times. Each standard and sample was dissolved in chloroform and methanol in the ratio of 2 to 1. From each standard lipid and sample at least five replicates were done, and the average chromatograms were obtained using these replicates. For calibration curve plotting and data analysis, Empower 2 Chromatography Data Software was used (Waters, Milford, USA).

#### 2.2.4 Statistical Analysis

The results were expressed as ‘mean  $\pm$  standard error of mean (SEM)’. The analysis of DBA/2J, BFMI and BMMI muscle samples was first done with ANOVA test, due to the abundance of the animal groups. And to find which means were significantly different from one group to another, Tukey’ s test was used as a post test, . A ‘p’value less than or equal to 0.05 was considered to be statistically significant (for ex.  $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ ). The degrees of significance were denoted with ‘\*’, ‘†’, ‘‡’, ‘#’, ‘¥’, ‘§’ and ‘□’ if the results were significant relative to DBA/2J, BMMI806, BMMI826, BMMI866, BFMI852, BFMI856, and BFMI860, respectively.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1. Part I: Method Development

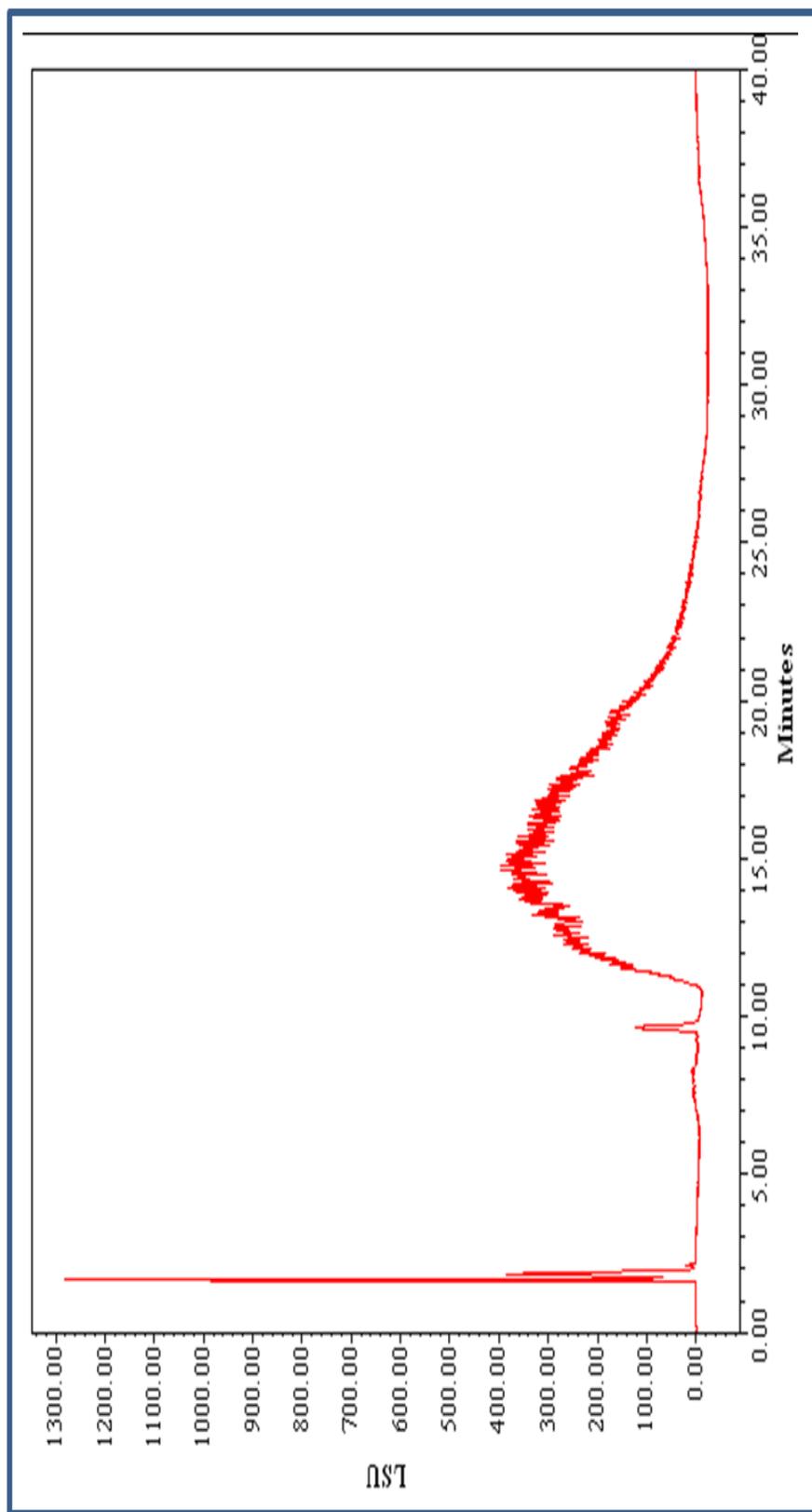
As mentioned in ‘Materials&Methods’ part, classic Folch, modified Folch, Bligh&Dyer and Lutzke lipid extraction methods, were performed in order to choose the most efficient procedure (Bligh & Dyer, 1959; Folch et al, 1957; Iverson et al, 2001; Lutzke & Braugher, 1990). In order to find out the yields of these tried methods, total lipid contents of different extraction methods were determined. Calculated total lipid amounts were given in Table 3.1, of each tried method. As it can be clearly seen, the highest total lipid content was determined via using classic Folch method (Folch et al, 1957) as the extraction method. Since estimation of lipid content in the method of Bligh&Dyer’s decreases as the content of lipids increases, this method was not preferred (Iverson et al, 2001). Therefore, in the current study, for the tissue samples, classic Folch method was preferred to be used.

**Table 3.1.** Total lipid amounts of each method.

<b>Extraction Methods</b>	<b>Classic Folch Method</b>	<b>Modified Folch Method</b>	<b>Lutzke Method</b>	<b>Bligh&amp;Dyer Method</b>
<b>Total Lipid (mg/dL)</b>	15.44 ± 0.47	14.61 ± 0.64	13.70 ± 0.68	7.91 ± 1.30

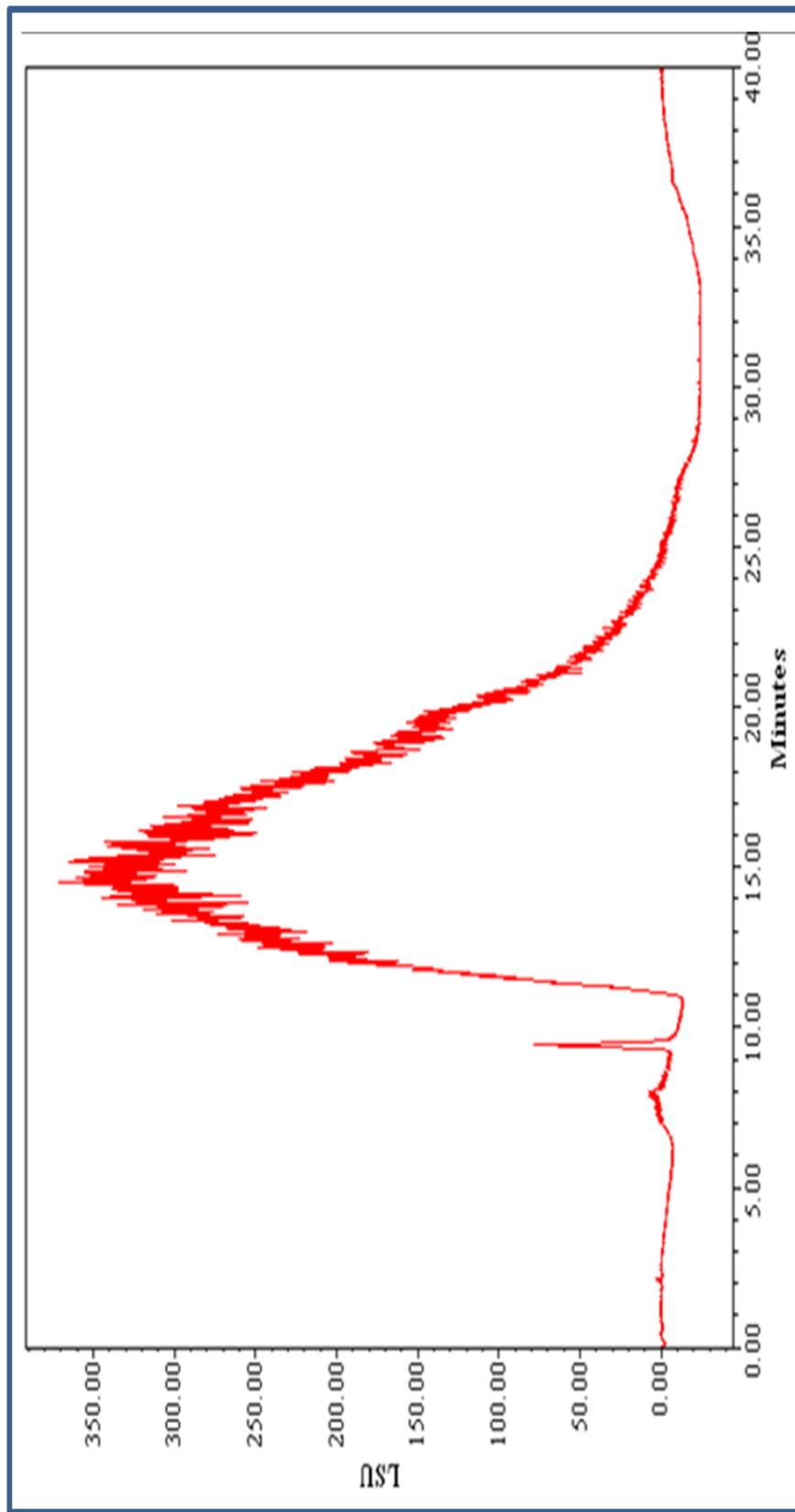
Although the most efficient extraction method was chosen, random mouse muscle lipid extracts were run through the instrument, according to the gradient elutions as described in detail, in 'Materials&Method' chapter.

Firstly, Descalzo method with the mobile phase solvents, methanol, acetonitrile, n-Hexane, isopropanol and water was tried (Descalzo et al, 2003). Solvent A was methanol; solvent B was isopropanol, n-Hexane and water (63:35:2, v/v/v); solvent C was isopropanol and n-Hexane (40:60, v/v) and solvent D was only acetonitrile. As can be seen in the Figure 3.1, use of water as a solvent blocked the signal of the interested lipids, in a way the signals of the lipids could not be seen.



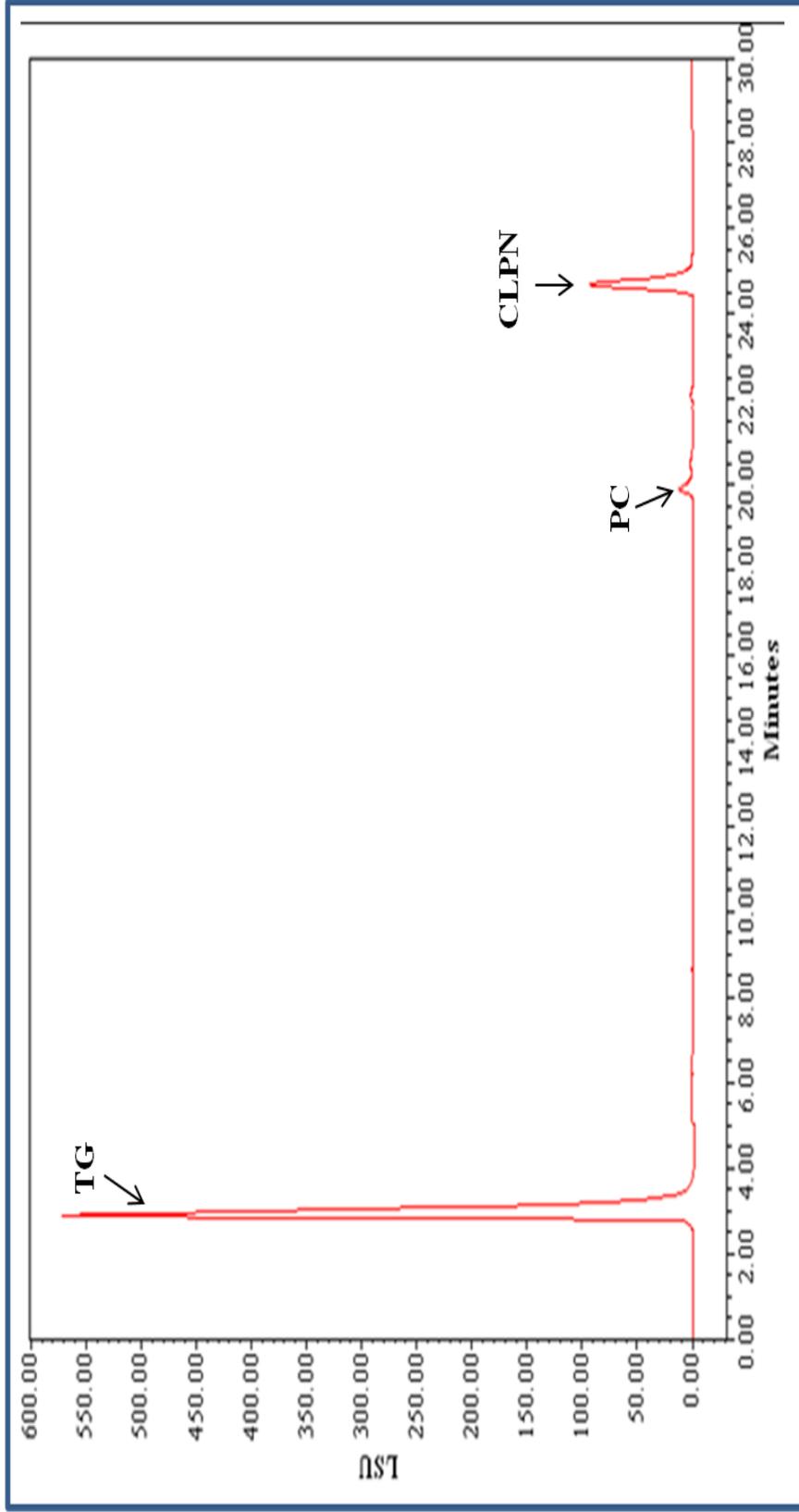
**Figure 3.1.** A representative chromatogram of the trial of Descalzo method. Mobile phase solvents composed of n-Hexane, water, isopropanol, methanol, and acetonitrile were used.

The separation method developed by Lutzke and Braughler were applied to the lipid extracts (Lutzke & Braughler, 1990). In this method, mobile phase solvents were isooctane, tetrahydrofurane, isopropanol, n-Hexane, chloroform and water. A ternary gradient was applied, with the solvent A was isooctane and tetrahydrofurane (99:1, v/v); solvent B was composed of isopropanol and chloroform (80:20, v/v); and solvent C was isopropanol and water (50:50, v/v). Use of water as a mobile phase, again blocked the signal of detector, as it can be seen in Figure 3.2. However, isooctane and tetrahydrofurane mobile phases caused greater problems, like increasing the backpressure, abortion of the run and wearing out the pieces of the instrument. These three solvents were abandoned because of the incompatibility with the separation instrument and the detector.



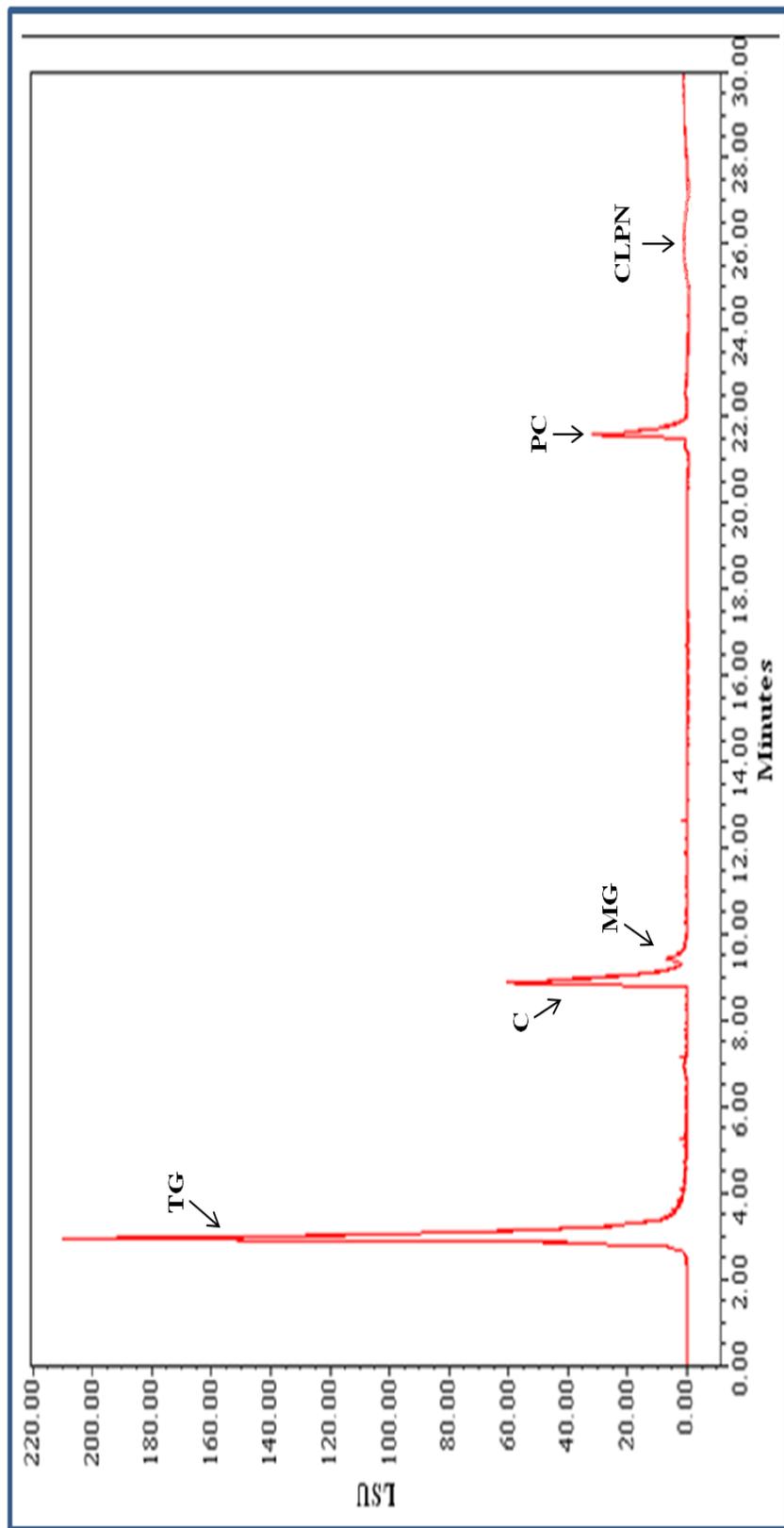
**Figure 3.2.** A representative chromatogram of the gradient elution trial of Lutzke method. Mobile phase solvents composed of isooctane, tetrahydrofurane, water, isopropanol, and chloroform were used.

After trials of above mentioned methods, another elution method was performed according to Perona and Ruiz-Gutierrez method (Perona & Ruiz-Gutierrez, 2005). A ternary gradient elution was applied with the mobile phase solvents of methanol, isopropanol and n-hexane. This method was promising since the solvents were compatible with the instrument and they caused relatively lower backpressure. Figure 3.3 shows a representative chromatogram of this procedure. Glyceryl trioleate (TG), phosphatidylcholine (PC) and cardiolipin (CLPN) lipids were eluted; however other main lipid components of a muscle mouse tissue were absent.



**Figure 3.3.** A representative chromatogram of the gradient elution trial following Perona and Ruiz-Gutierrez method. Mobile phase solvents composed of n-Hexane, methanol and isopropanol were used.

In order to obtain better separation and also to see the lipids of interest more clearly, the above mentioned method was developed further. For this purpose, in addition to n-Hexane, methanol, and isopropanol, which did not block the signals of lipids, triethylamine (TEA) was decided to be used. In addition to TG, PC and CLPN lipids, in order to detect other main lipids such as cholesterol (C) and 1-oleoyl-rac-glycerol (MG), triethylamine (TEA) was added into the solvent methanol, so that elution and the resolution of the lipids were enhanced (Figure 3.4).

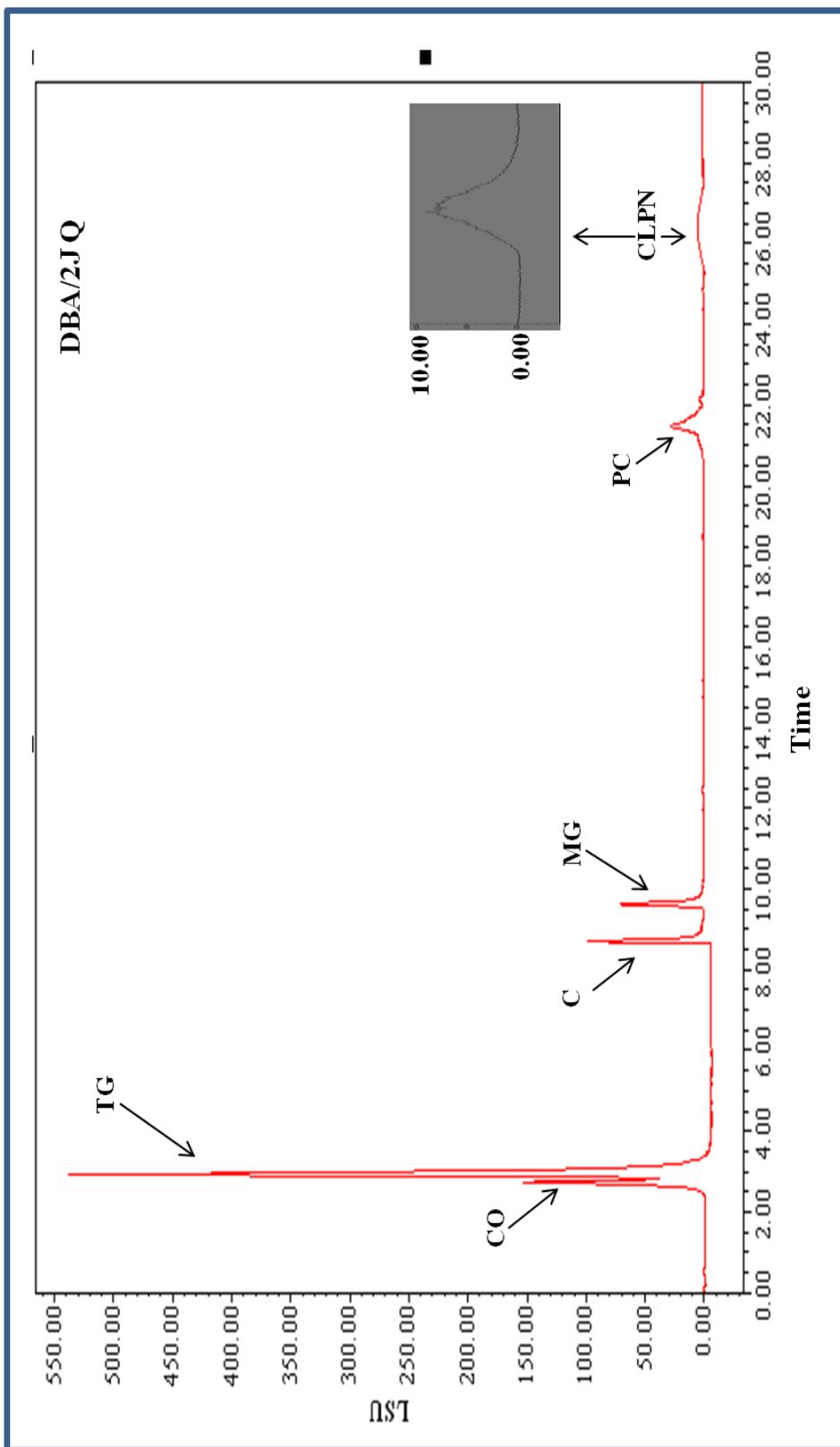


**Figure 3.4.** A representative chromatogram of the gradient elution with the addition of TEA following Perona and Ruiz-Gutierrez method.

With the addition of TEA, triglyceride, monoglyceride, cholesterol, cardiolipin and phosphatidylcholine lipid classes were eluted. However; signal-to-noise ratio was found to be dramatically high. To overcome this problem, flow rate of nitrogen gas was increased to 60 psi from 30 psi and drift tube temperature was increased to 70°C from 50°C. Also the gradient elution was changed as described in ‘Materials & Methods’ chapter, to separate more lipid classes and to obtain stronger elution (Olsson et al, 2012). Use of only n-Hexane, but nothing else, in the first three minutes provided a clear elution of apolar lipids, cholesteryl oleate and triglyceride.

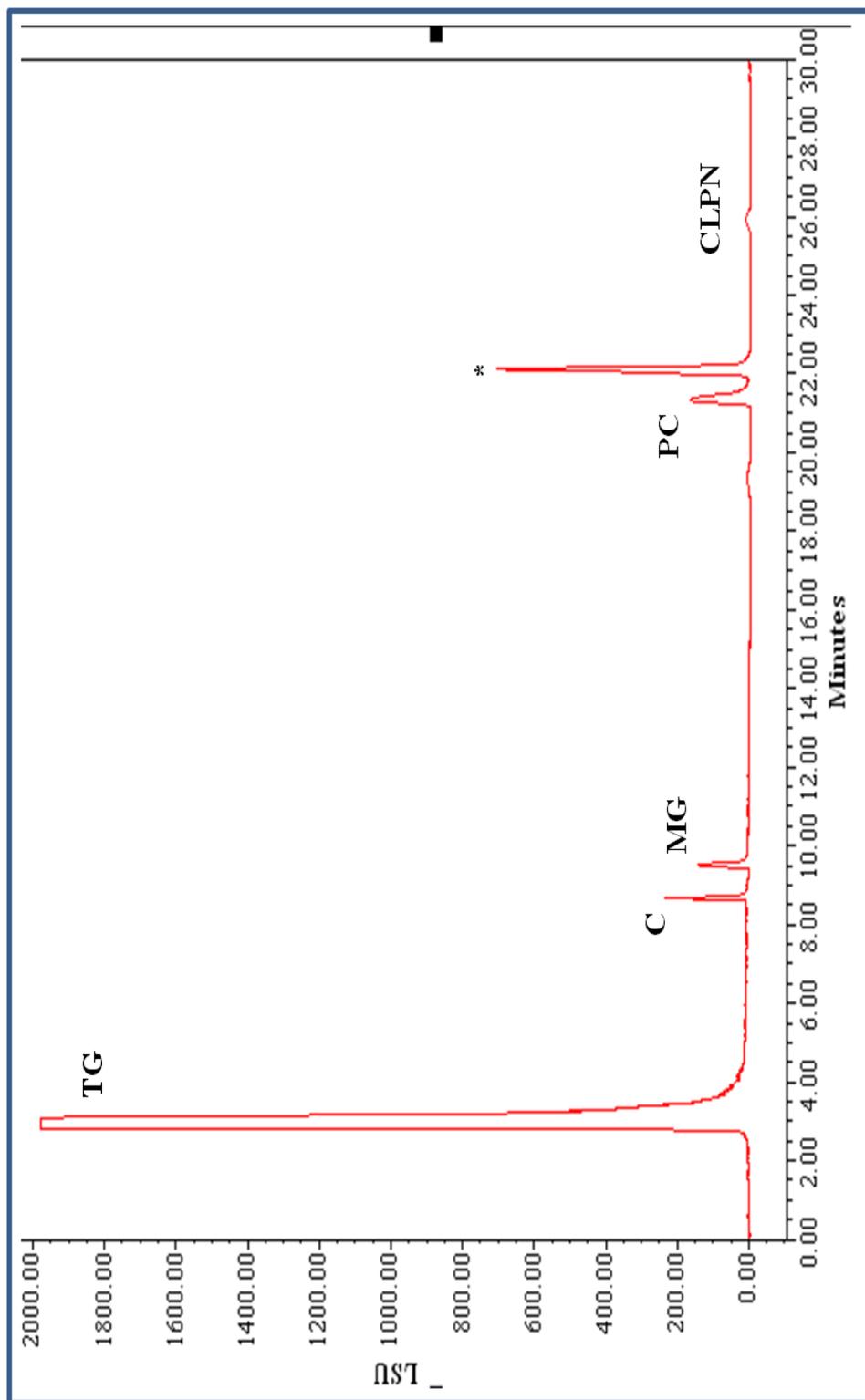
Since the length and the particle size of the column have also important effects on the results, the column was also altered. Separations were performed using another column with the length of 150 mm and particle size of 3µm, instead of a column with the length of 100 mm and particle size of 5 µm. As can be seen in Figure 3.5, this column alteration provided clear elution of lipids which were overlapping, like cholesteryl oleate and triglyceride, or which had retention times very close, like monoglyceride and cholesterol.

This figure displays the success of the newly developed method. Extracted lipids from *quadriceps* (Q) muscle of a mouse from control DBA/2J line were separated clearly, without any overlapping, as it can be clearly seen from the representative chromatogram.



**Figure 3.5.** A representative HPLC-ELSD chromatogram of Q muscle of DBA/2J.

This newly developed method was also tried on an adipose tissue, to see if this method is applicable to other kinds of tissues. As can be seen in Figure 3.6, with this method, lipids were also detected in another tissue, besides skeletal muscle tissues.



**Figure 3.6.** A representative HPLC-ELSD chromatogram of an adipose tissue of a random mouse.

### 3.2. Part II: HPLC-ELSD Studies

To identify and quantify lipids extracted from LD and Q muscles of control DBA/2J, obese BFMI and muscle BMMI mice, firstly different concentrations of standard lipids cholesteryl oleate (CO), glyceryl trioleate (TG), cholesterol (C), 1-oleoyl-rac-glycerol (MG), phosphatidylcholine (PC) and cardiolipin (CLPN) were injected to the instrument. In this way, retention times which are specific to each standard were found. Table 3.2 shows the retention times of each standard, according to their appearance order. In figures 3.7 – 3.12, chromatograms of standards are demonstrated.

**Table 3.2.** Retention time of lipid standards.

Standards	CO	TG	C	MG	PC	CLPN
<b>Retention Time (min)</b>	2.734±0.01	2.872±0.03	8.761±0.03	9.681±0.02	20.302±0.80	26.360±0.87

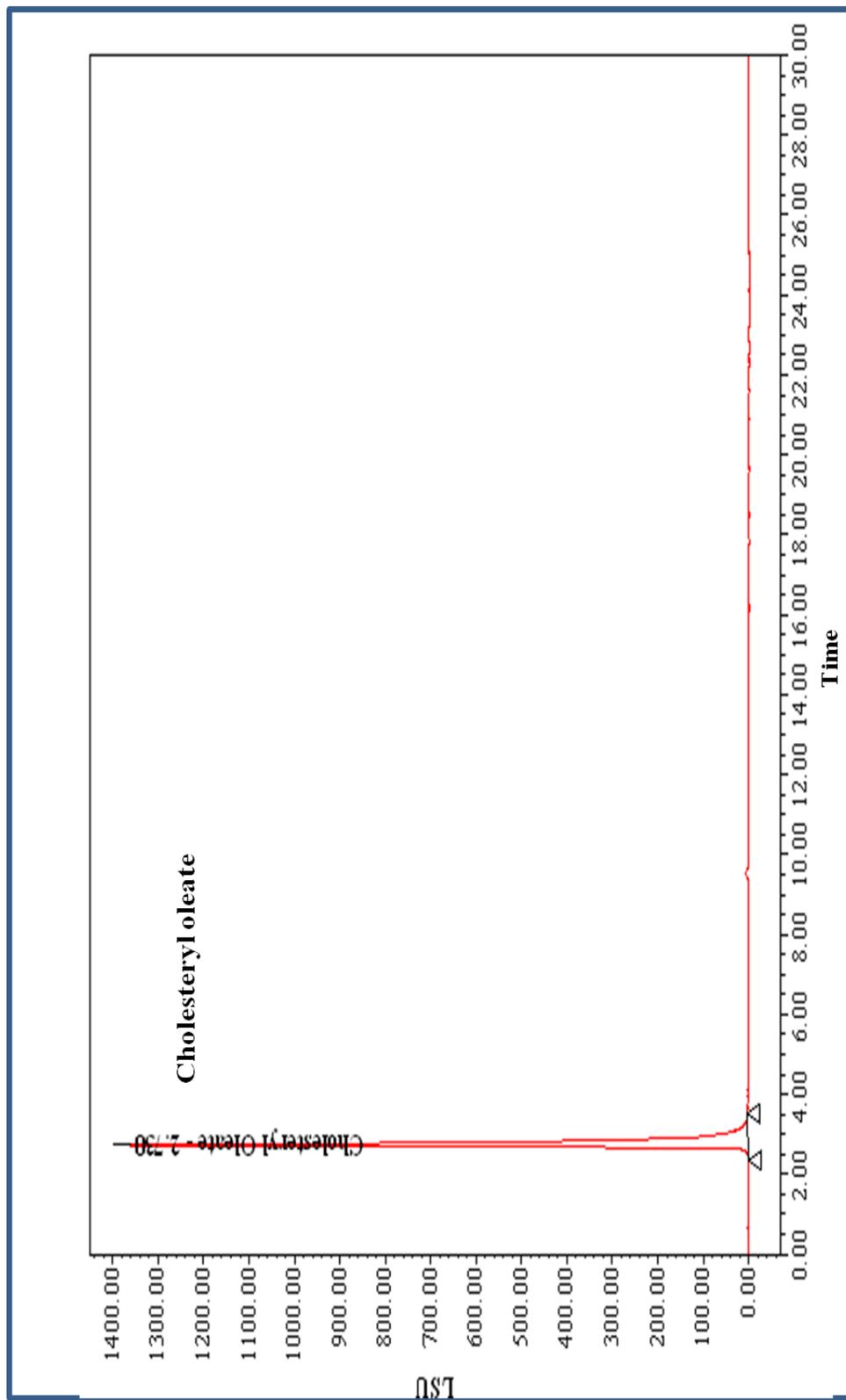
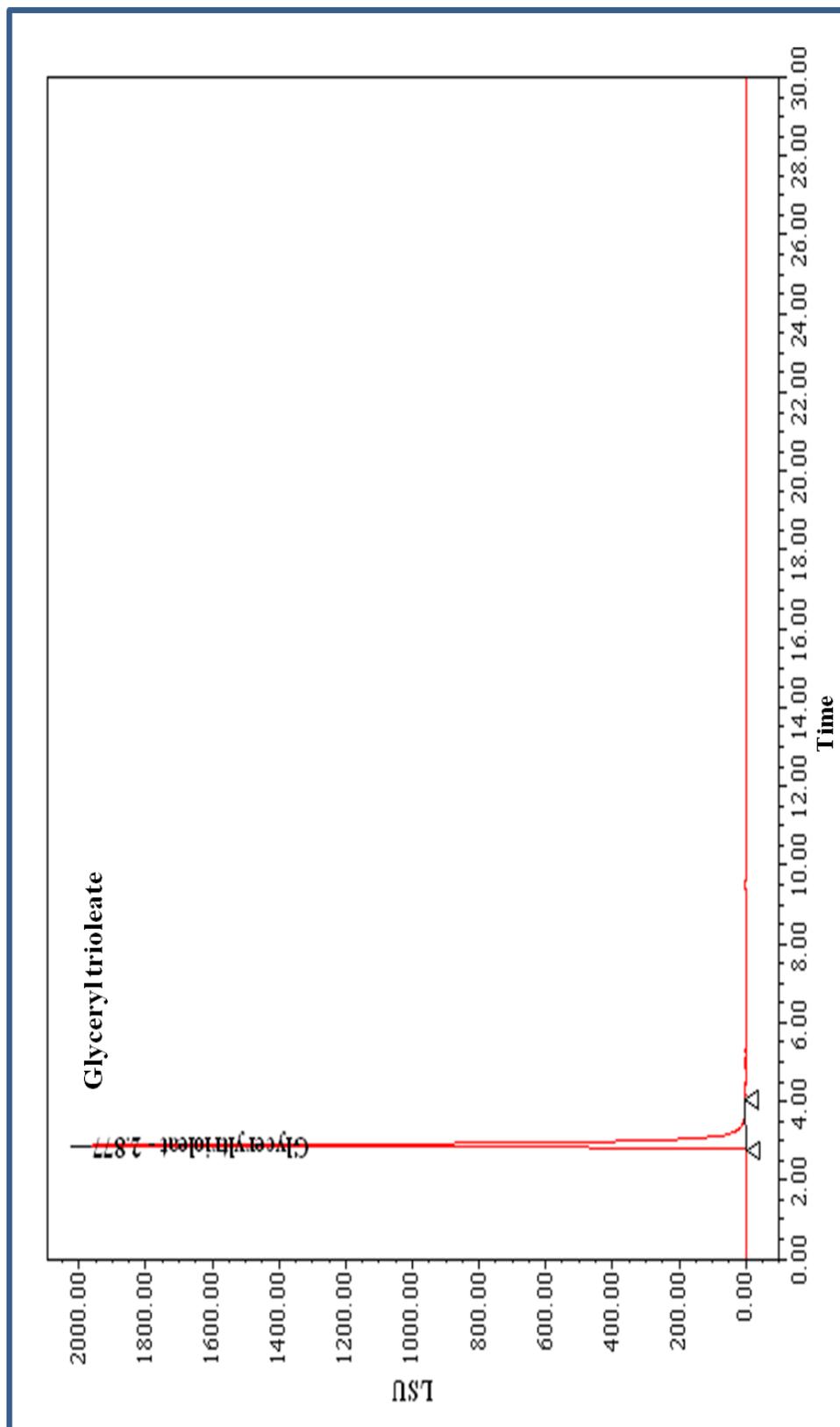
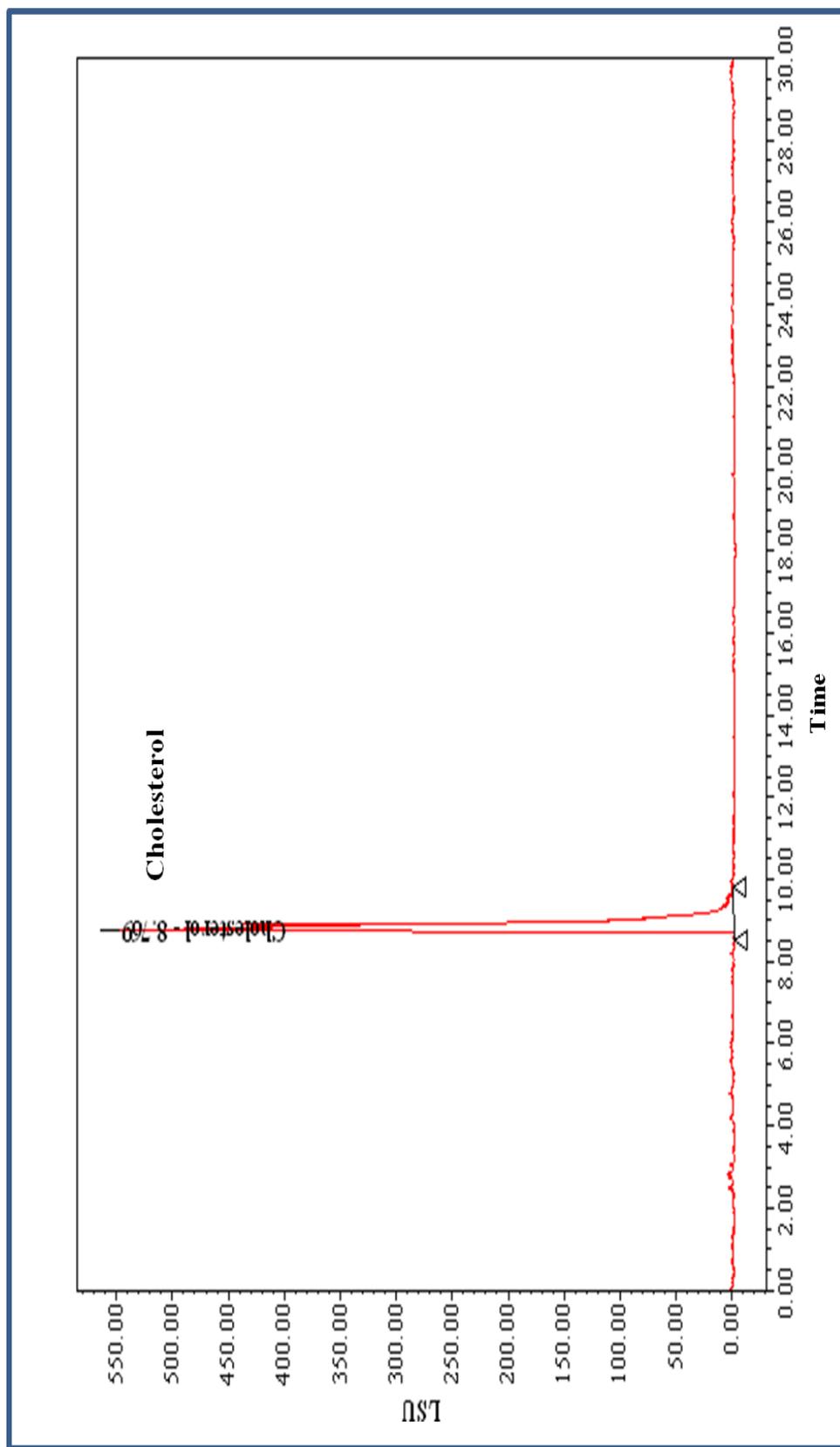


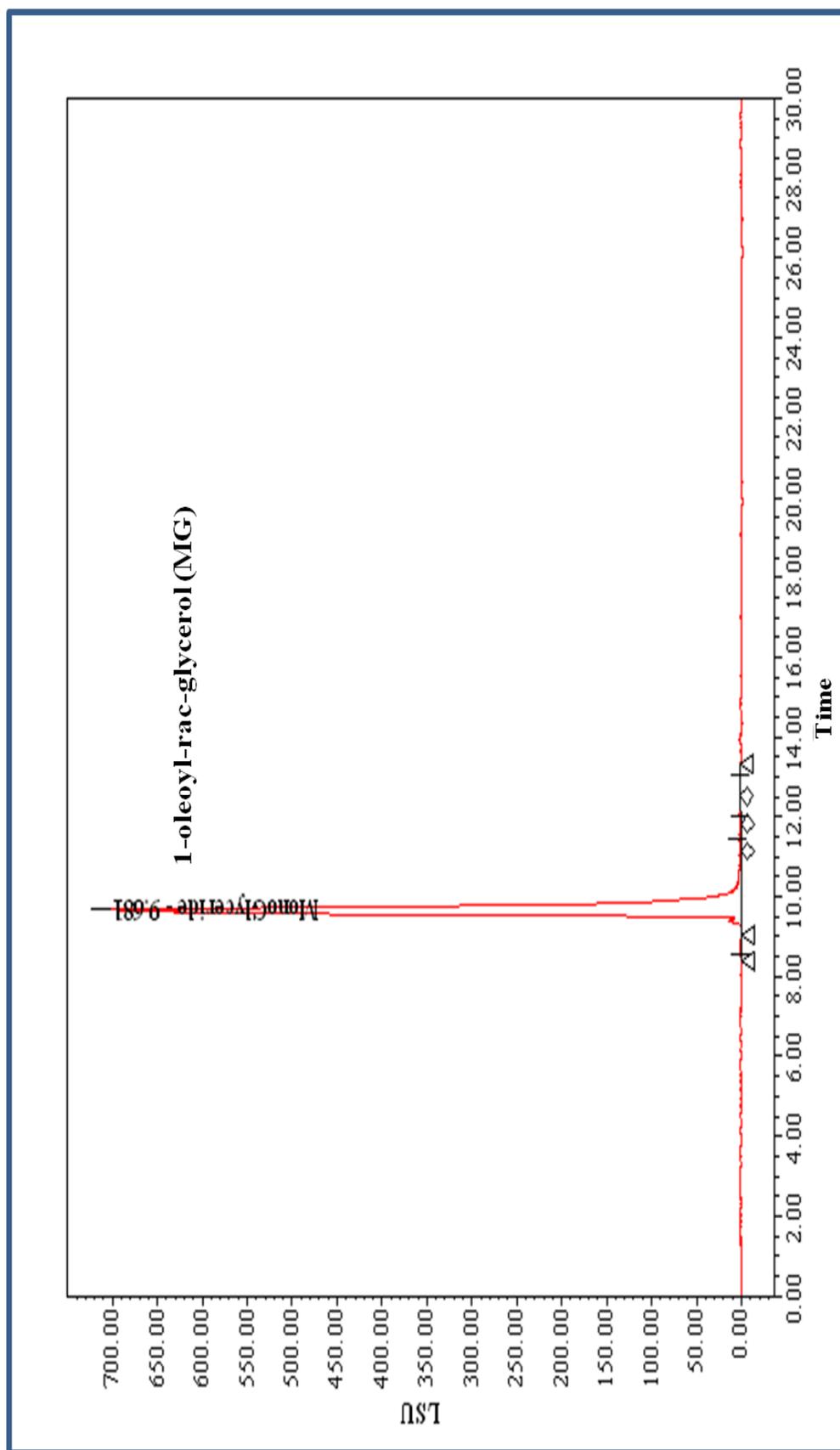
Figure 3.7. HPLC-ELSD chromatogram of the standard lipid cholesteryl oleate (CO).



**Figure 3.8.** HPLC-ELSD chromatogram of the standard lipid glyceryl trioleate (TG).



**Figure 3.9.** HPLC-ELSD chromatogram of the standard lipid cholesterol (C).



**Figure 3.10.** HPLC-ELSD chromatogram of the standard lipid 1-oleoyl-rac-glycerol (MG).

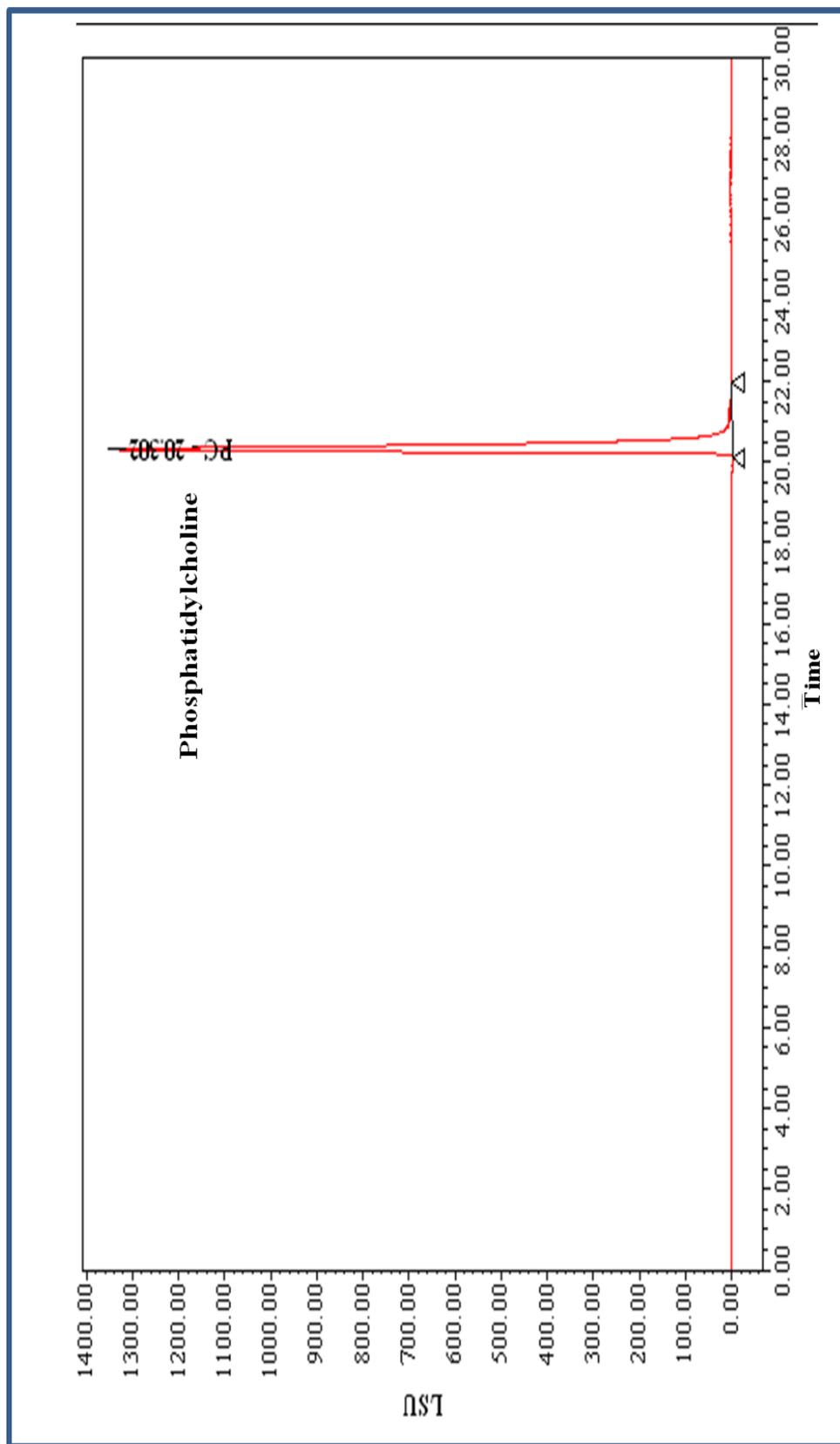
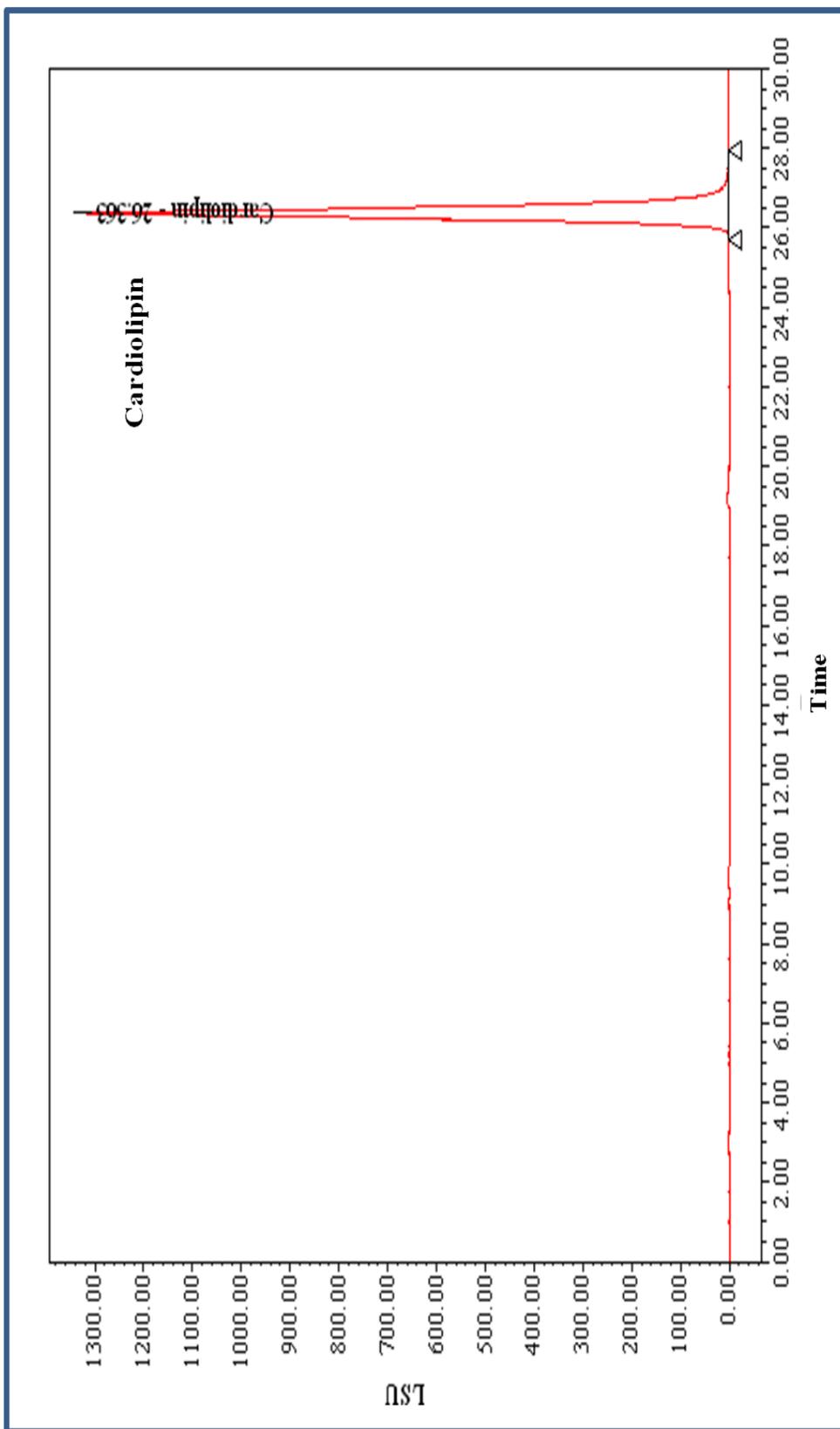
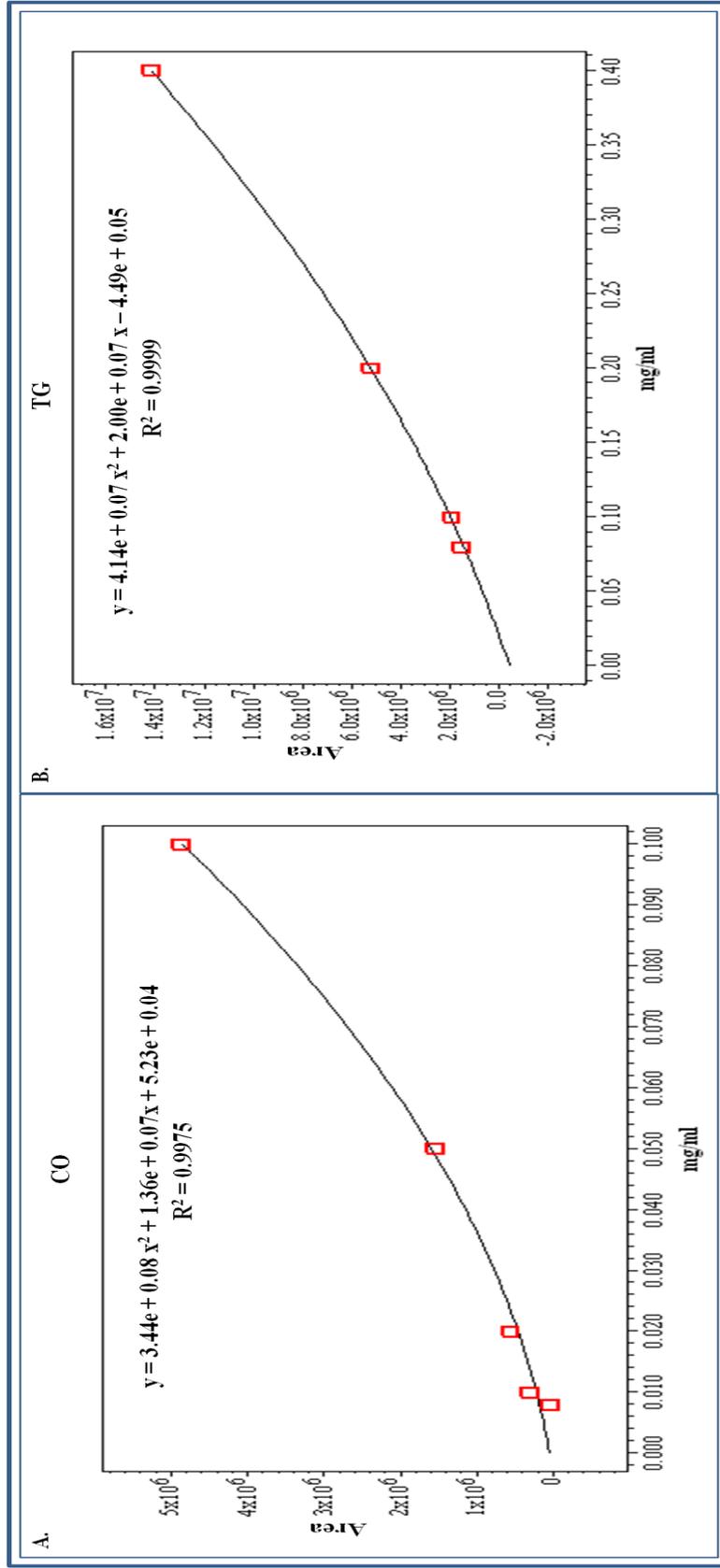


Figure 3.11. HPLC-ELSD chromatogram of the standard lipid phosphatidylcholine (PC).

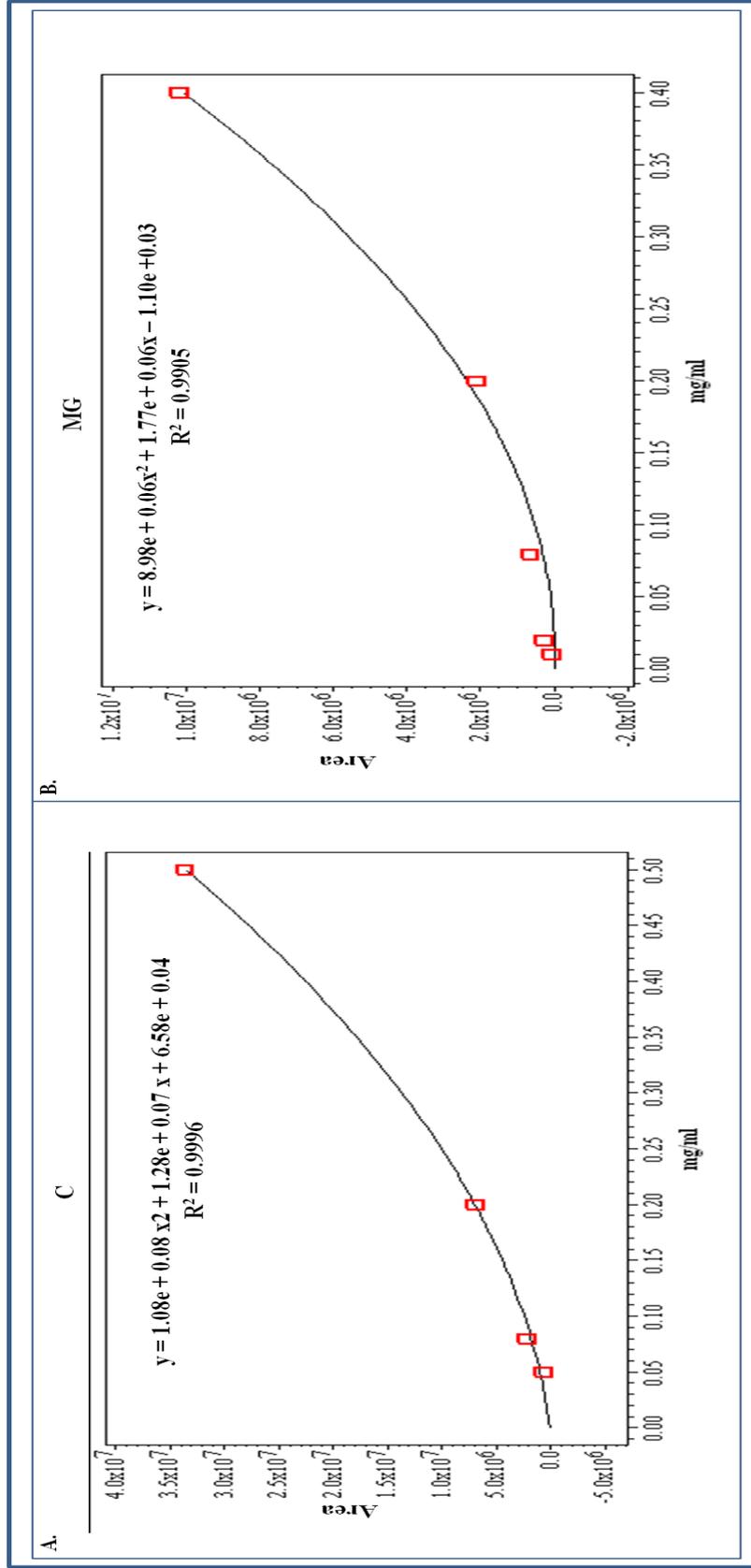


**Figure 3.12.** HPLC-ELSD chromatogram of the standard lipid cardiolipin (CLPN).

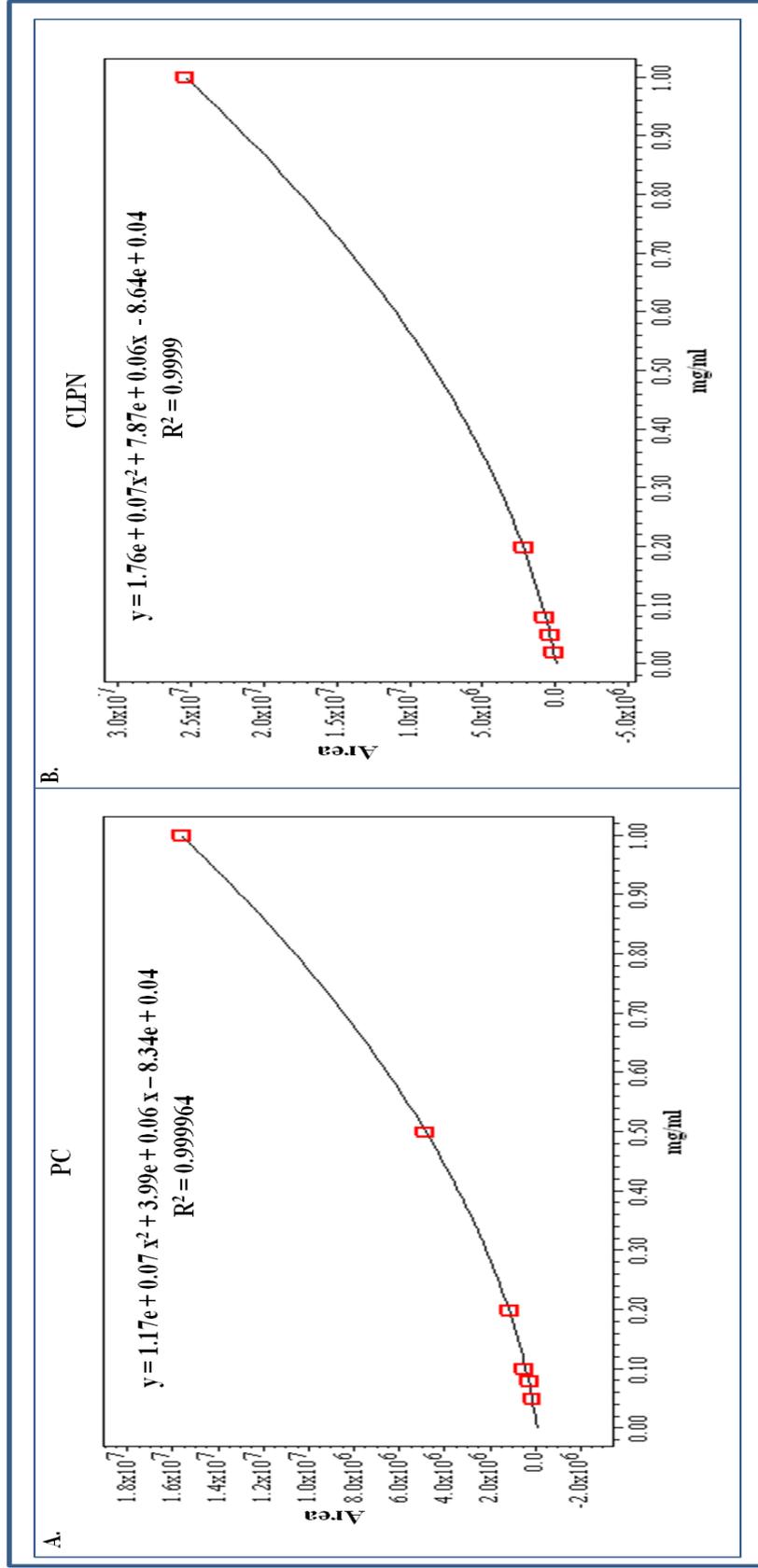
In order to quantify the lipids homogenized and extracted from LD and Q skeletal muscles of DBA/2J, BFMI and BMMI mice, standard calibration curves were drawn using Waters Empower Pro 2 software. For this purpose, standards were injected at different concentrations. Figures 3.13 - 3.15 show the standard calibration curves. Coefficients of determination ( $R^2$ ) of all standards were found to be higher than 0.99, which indicate the goodness of fit of all injected standards.



**Figure 3.13.** Calibration curves of standard lipids (A) Cholesteryl oleate (CO), (B) Glyceryl trioleate (TG). Red squares refer to the areas of the injected concentrations. R<sup>2</sup> values are **0.9975** and **0.9999**, respectively.

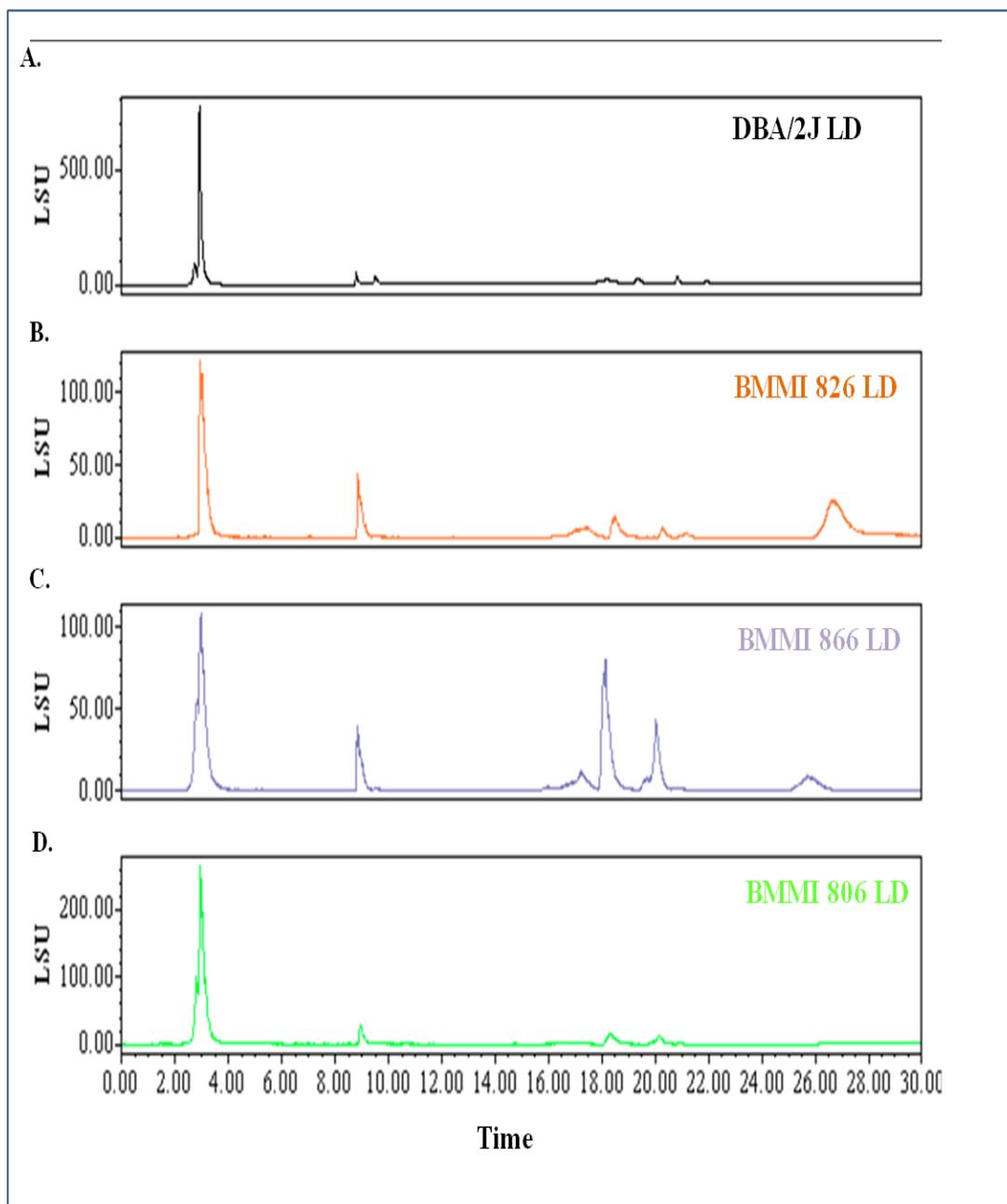


**Figure 3.14.** Calibration curves of standard lipids (A) Cholesterol (C), (B) 1-oleoyl-rac-glycerol (MG). Red squares refer to the areas of the injected concentrations.  $R^2$  values are **0.9996** and **0.9905**, respectively.

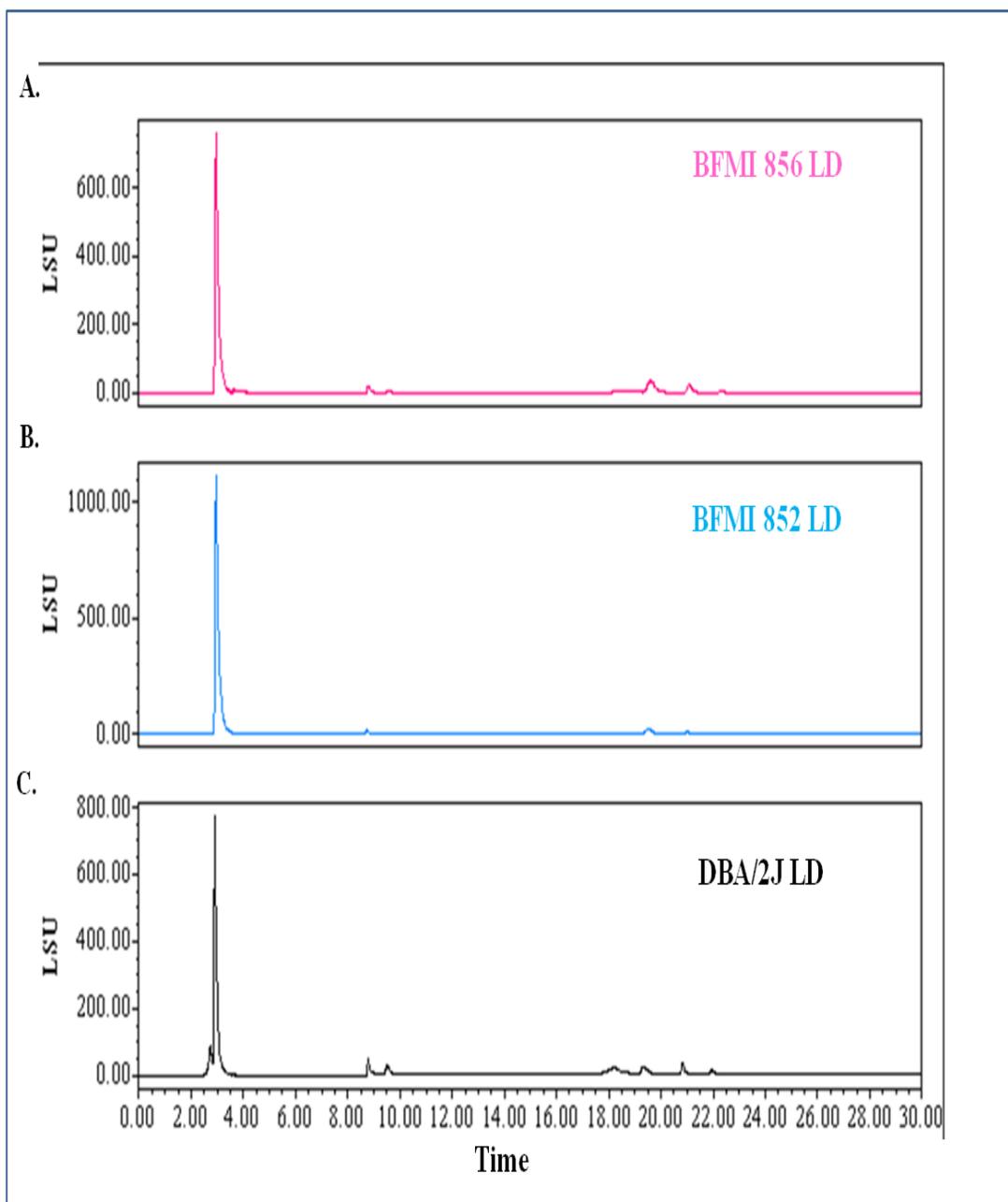


**Figure 3.15.** Calibration curves of standard lipids (A) Phosphatidylcholin (PC), (B) Cardiolipin (CLPN). Red squares refer to the areas of the injected concentrations.  $R^2$  values are **0.999964** and **0.9999**, respectively.

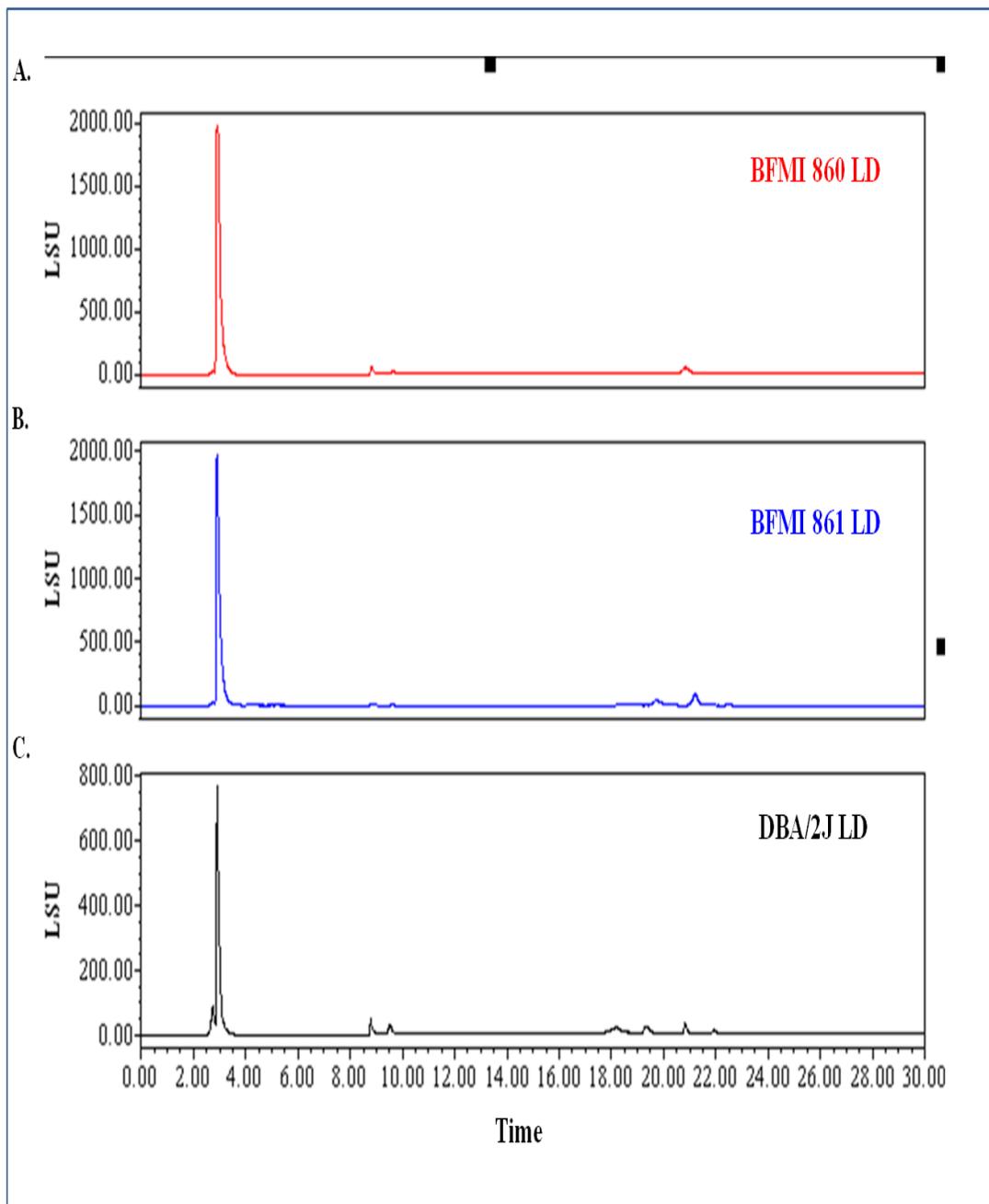
Lipids of DBA/2J, BFMI and BMMI mice were separated, identified and quantified via sensitive HPLC-ELSD system (Figures 3.16-3.18). As it can be seen in figures 3.19-3.21, regarding to all mouse lines, the highest lipid content was found in BFMI mouse lines. Within BFMI lines, the notable high total lipid contents were found in BFMI860 and BFMI861 mouse lines, respectively (Figures 3.18 and 3.21). BFMI852 and BFMI856 mouse lines displayed less lipid content, when they were compared to other BFMI lines, BFMI860 and BFMI861. BMMI806 mouse line was found to have the highest total lipid content within BMMI lines (Figures 3.16 and 3.19). Figure 3.22 shows the difference between the lipid contents, extracted from LD muscles of a BFMI and a BMMI mouse line. From chromatograms, these results can be seen qualitatively. Chromatogram of control mouse line was taken as a reference result.



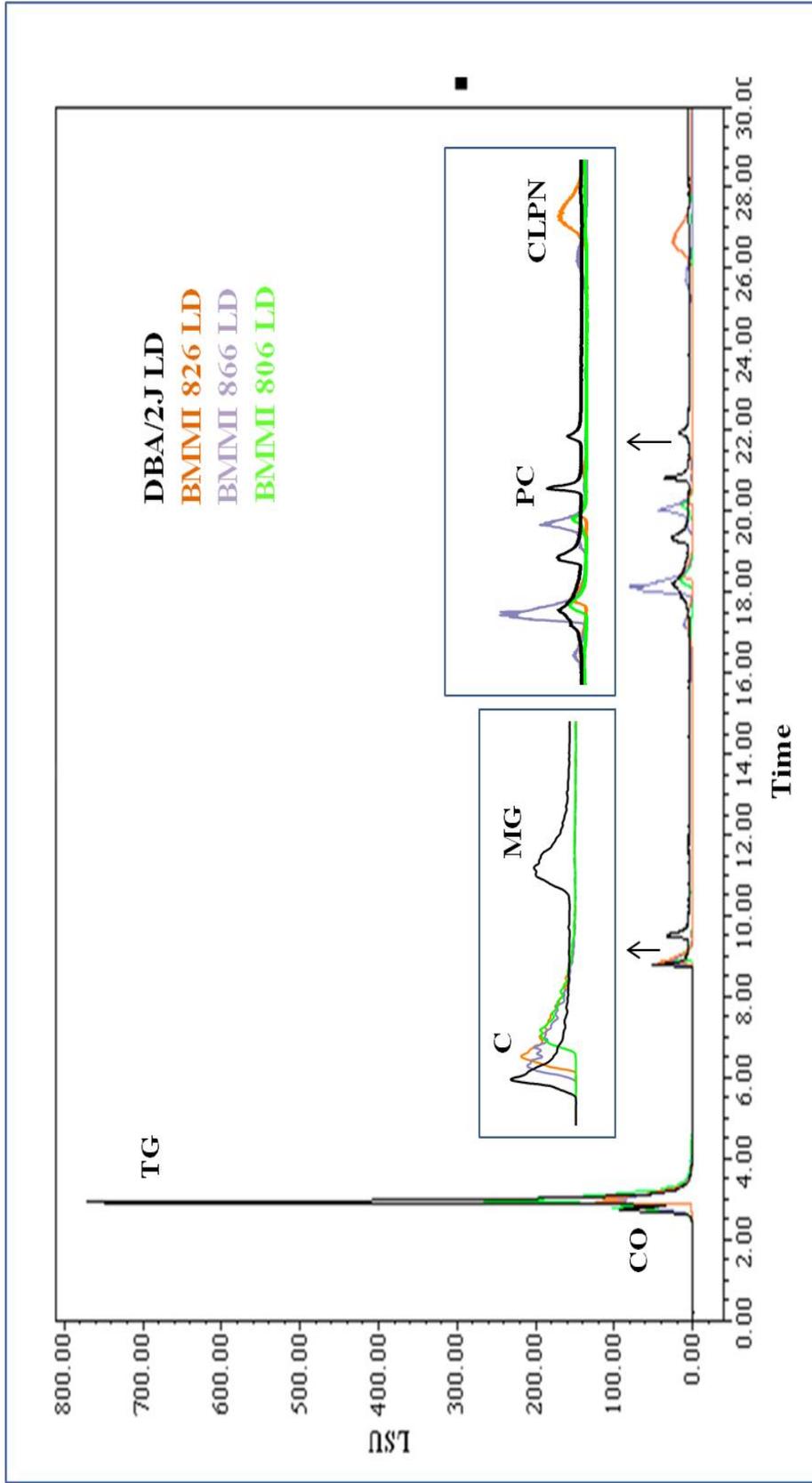
**Figure 3.16.** Chromatograms of lipids from LD muscle of (A) DBA/2J, (B) BMMI826, (C) BMMI866, and (D) BMMI806 obtained by HPLC-ELSD.



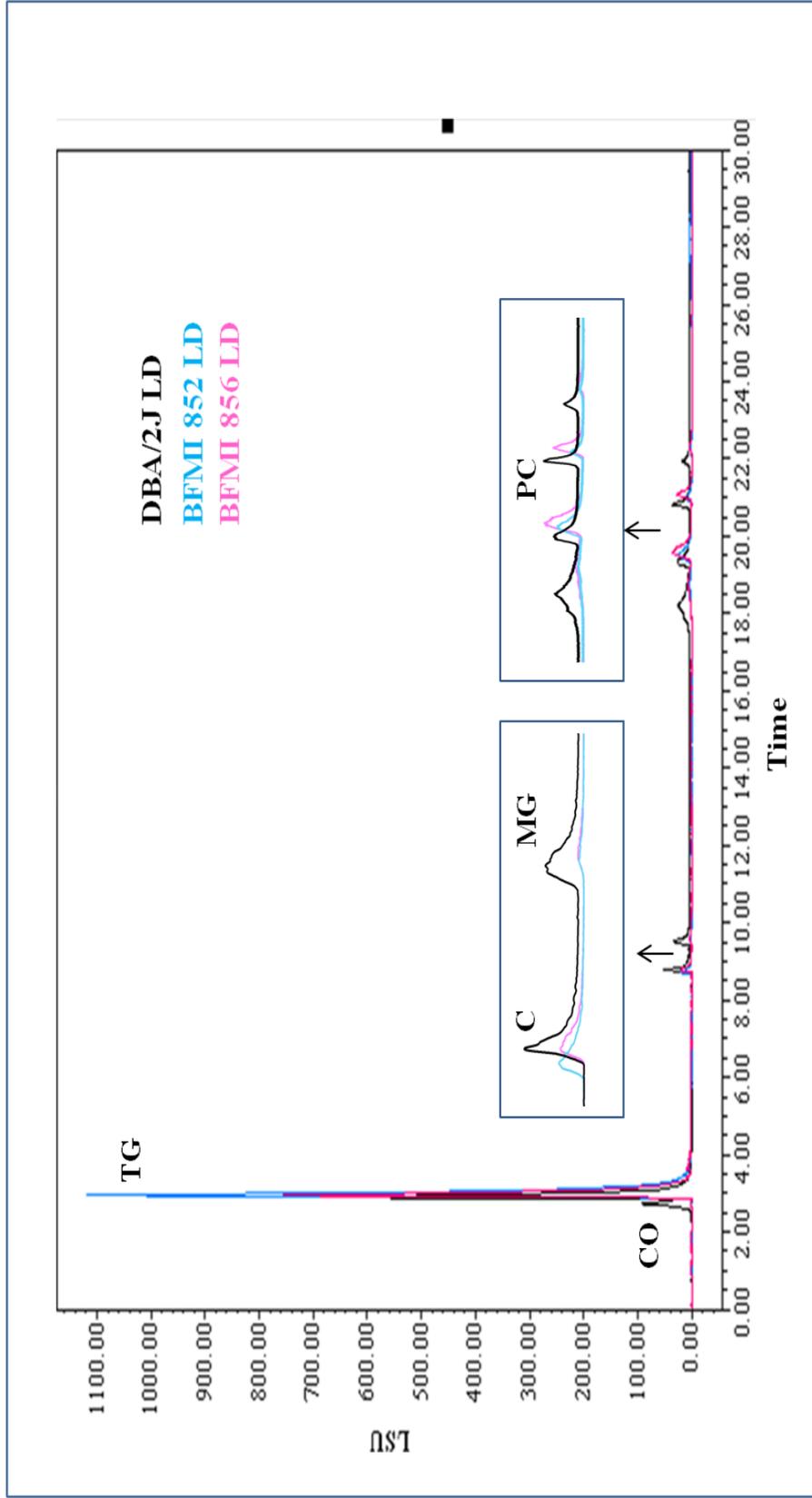
**Figure 3.17.** Chromatograms of lipids from LD muscle of (A) BFMI856, (B) BFMI852, and (C) DBA/2J obtained by HPLC-ELSD.



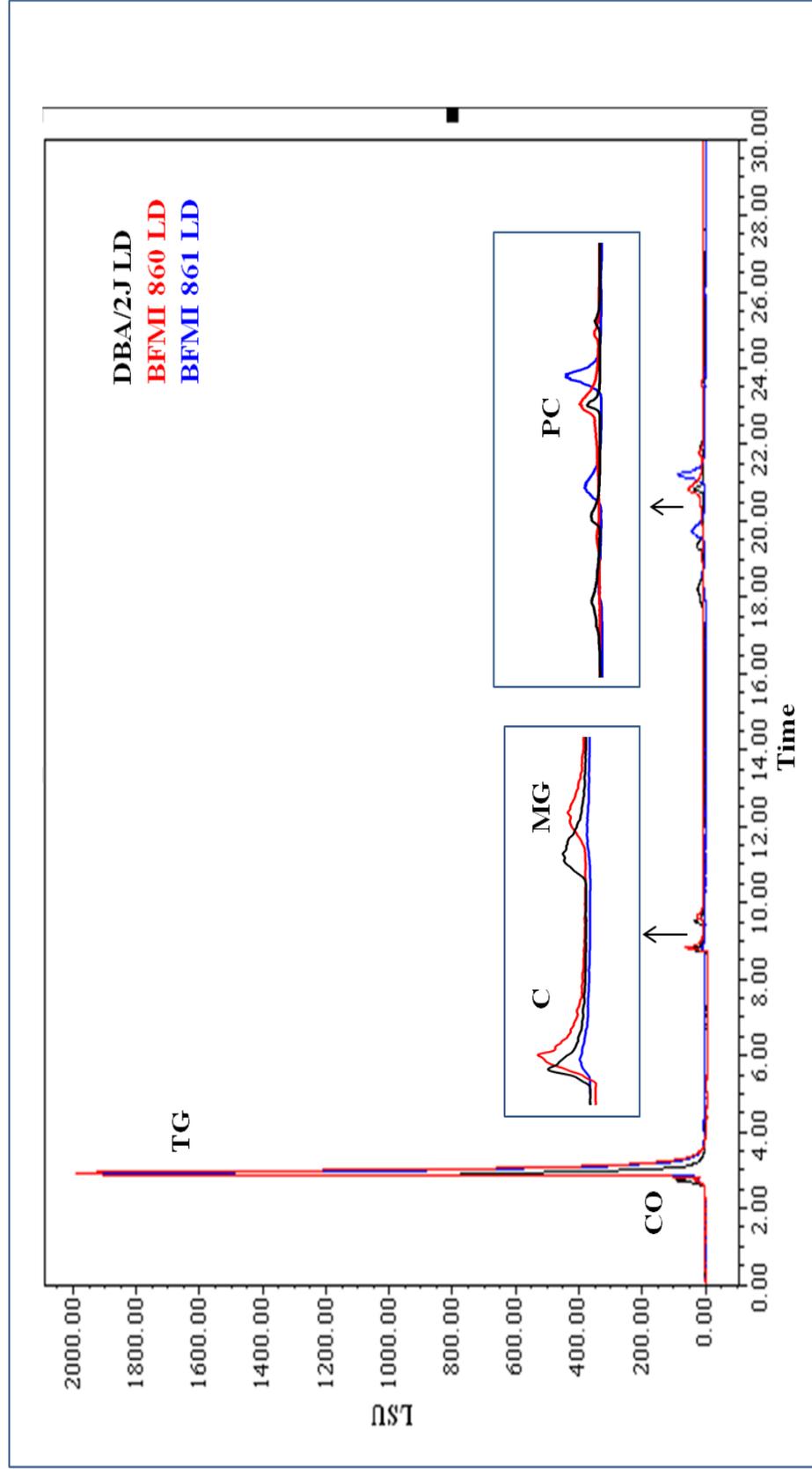
**Figure 3.18.** Chromatograms of lipids from LD muscle of (A) BFMI860, (B) BFMI861, and (C) DBA/2J obtained by HPLC-ELSD.



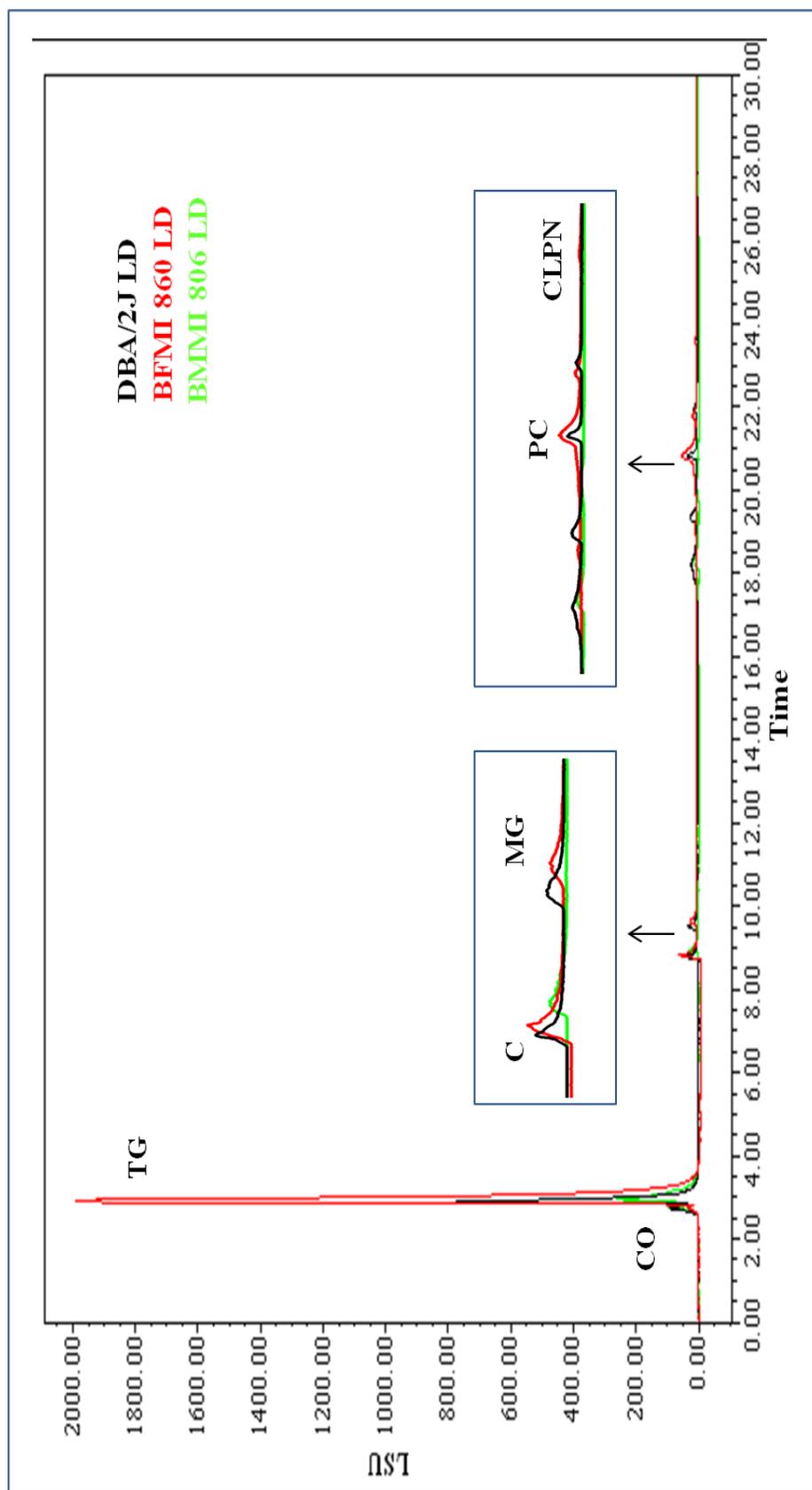
**Figure 3.19.** Comparative HPLC-ELSD chromatogram of the lipids from LD muscles of DBA/2J, BMMI806, BMMI826 and BMMI866. Same control sample was used as to be a reference.



**Figure 3.20.** Comparative HPLC-ELSD chromatogram of the lipids from LD muscles of DBA/2J, BFMI852 and BFMI856. Same control sample was used as to be a reference.



**Figure 3.21.** Comparative HPLC-ELSD chromatogram of the lipids from LD muscles of LD muscles of DBA/2J, BFM1860 and BFM1861. Same control sample was used as to be a reference chromatogram.



**Figure 3.22.** Comparative HPLC-ELSD chromatogram of the lipids from LD muscles of DBA/2J, BFM1860 and BM1806. Same control sample was used as to be a reference chromatogram.

Qualitative results can be seen from chromatograms. Using standard calibration curves, lipid concentrations of each sample was analyzed quantitatively via using Waters Empower2 Pro software. CO, TG, C, MG, PC and CLPN lipids from LD and Q muscles of each sample were quantified statistically (Figures 3.23-3.30).

Figure 3.23 demonstrates TG concentrations of the extracted lipids from both LD and Q skeletal muscles of control, BFMI and BMMI mouse lines. As it can be seen from the figure, all BFMI lines displayed at least two times higher amount of TG lipid when compared to BMMI lines, due to their physiologic state and complex genetic background. Since the quantity of simple lipids, especially TG can alter dramatically according to the dietary or physiological state of the animal, this result of TG concentration is expected (Christie, 1987).

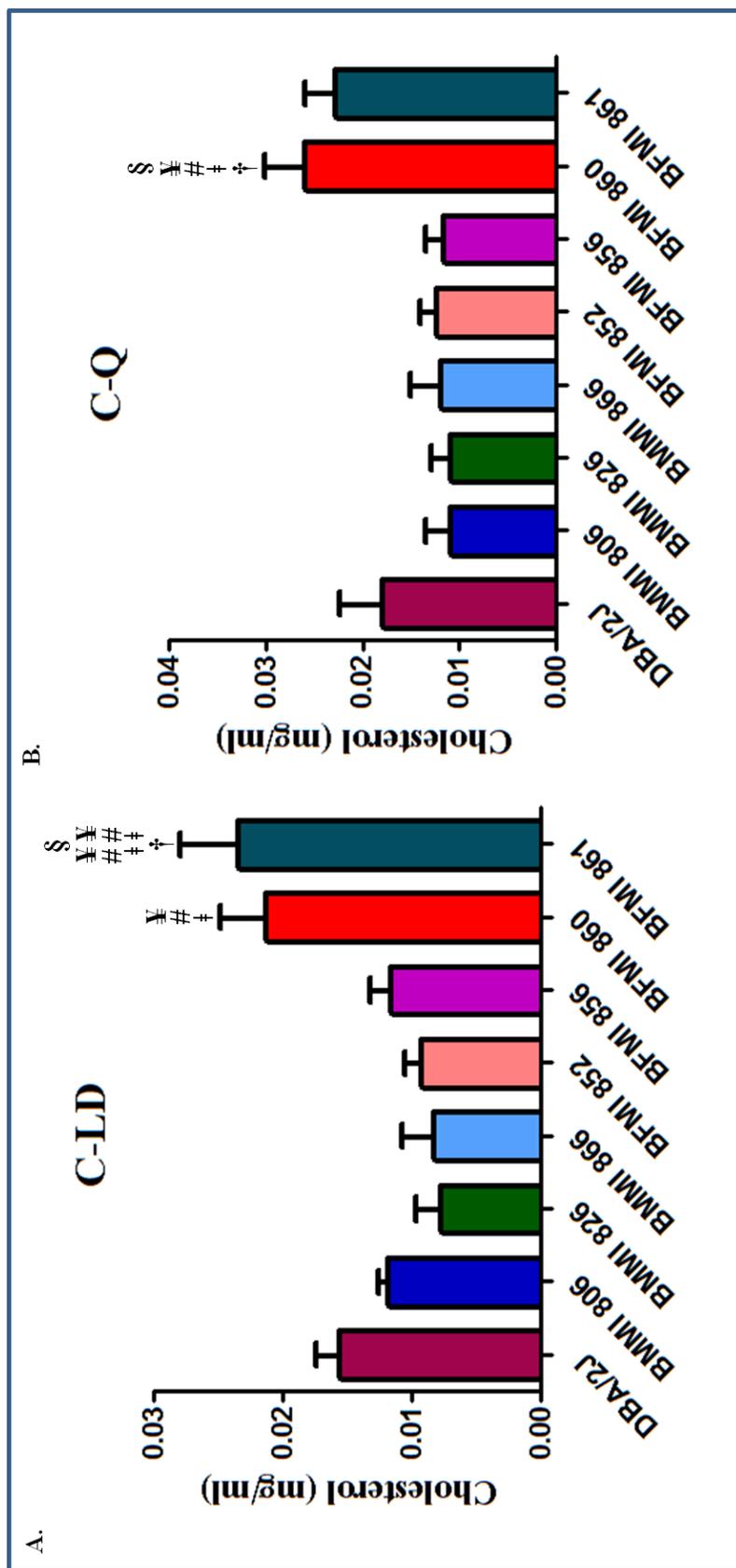
Within BFMI lines, especially BFMI860 and BFMI861 lines were found to have the highest TG. In other BFMI lines (BFMI852 and BFMI856), TG concentration was not found to be higher as BFMI860 and BFMI861. It is known that there is a link between obesity, muscle triglyceride storage and insulin resistance (Goodpaster et al, 2001; Kelley & Goodpaster, 2001; Pan et al, 1997). Especially, an increase in skeletal muscle triglyceride level indicates severe insulin resistance, and accumulation of this lipid increases in obesity (Goodpaster et al, 2000). In fact, in diabetic and pre-diabetic individuals, insulin resistance states are closely related to excess lipid availability, high circulating plasma free fatty acids and triglyceride and increment in intramyocellular triglyceride accumulation (Stein, 2004). Having at least two times higher TG concentrations, obese BFMI lines might have high tendency to have insulin resistance. Especially, the lines BFMI860 and BFMI861 which showed significant results not only to BMMI lines but also other BFMI lines, might be more predisposed to insulin resistance. Indeed, these lines were found to be insulin resistant (Hantschel et al, 2011; Wagener et al, 2006). In the light of these results, it can be deduced that, not BFMI852 or BFMI856, but BFMI860 and BFMI861 lines might be more predisposed to obesity-induced type II diabetes.

Regarding to muscle BMMI mouse lines, BMMI806 showed higher TG concentration compared to other BMMI lines, BMMI826 and BMMI866, which were both myostatin mutants. Higher TG level within BMMI806 clearly reveals the effect of the mutation on myostatin gene. Since myostatin acts as a growth limiting factor in the development of muscle tissue (Slack, 1997), individuals of wild-type BMMI806 line did not have larger muscles and higher numbers of muscle fibers, as did other BMMI lines in the studies. Mice of this specific line displayed higher intramuscular fat content when compared to BMMI826 and BMMI866 lines, which had unlimited muscle growth due to  $Mstn^{-/-}$  myostatin mutation. This result confirms the findings where animals with myostatin mutation have significant increase in muscle mass and decrease in fat mass (McPherron & Lee, 2002). And from this result, it can be deduced that, myostatin mutant lines BMMI826 and BMMI866 might be less prone to have diet-induced and genetic obesity, while BMMI806 line might be more predisposed to obesity and also, type II diabetes. This result confirms newly found data about the relation between myostatin mutation, obesity and type II diabetes (Guo et al, 2012; Guo et al, 2009).



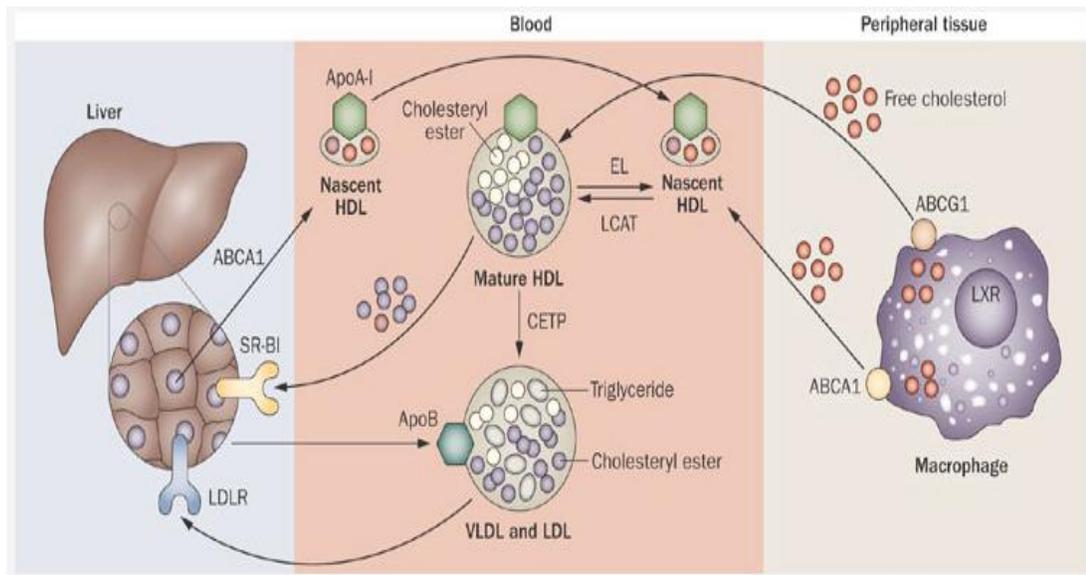
Free cholesterol (C) is a ubiquitous lipid which is found abundantly in animal tissues. Cholesterol is the most well known member of steroids (Christie, 1987). It has various functions like being a precursor to steroid hormones, like estrogen, testosterone, progesterone, cortisol and aldosterone and maintaining membrane fluidity (Christie, 1987; Hu et al, 2010; Simons & Ehehalt, 2002). Interacted with sphingomyelin, cholesterol forms lipid rafts. These microdomains of plasma membrane are crucial for maintaining cell membrane fluidity and also cell signaling (Calder & Yaqoob, 2007; Pike, 2003; Simons & Ehehalt, 2002). It has been well known that insulin receptors are located within a special lipid raft type, called caveolae (Gustavsson et al, 1999; Habegger et al, 2012; Parpal et al, 2001). Any disruption of these lipid rafts triggers dysfunctioning of insulin receptors, therefore diminishing the action of insulin-stimulated glucose uptake (Gustavsson et al, 1999; Pike, 2003; Vainio et al, 2002).

In recent study, cholesterol from LD and Q skeletal muscles were found to be significantly higher in BFMI860 and BFMI861 lines (Figure 3.24). While BFMI860 mice displayed remarked differences in both LD and Q skeletal muscle tissues, BFMI861 mice showed a dramatic significance only in their LD muscle. In the light of literature and the results, high concentrations of C in both lines might be an indicator of decreasing insulin sensitivity, and therefore increased tendency to be obesity-induced type II diabetes. In BMMI mice, no significant results were seen, though BMMI806 line displayed higher amounts of cholesterol levels than other muscle inbred mice, and even other obese inbred mice.



**Figure 3.24.** C (mg/ml) concentrations obtained from **A**) LD skeletal muscle, and **B**) Q skeletal muscle, of control, BFMI and BMMI mouse lines fed with SBD, **n=6** from each line (The degrees of significance were denoted with ‘\*’, ‘†’, ‘‡’, ‘§’, ‘¥’, ‘#’, ‘\$’ and ‘α’ if the results were significant relative to DBA/2J, BMMI806, BMMI826, BMMI866, BFMI852, BFMI856, and BFMI860, respectively). (The results were expressed as ‘mean ± standard error of mean’).

Cholesteryl ester (CO) is the esterified form of cholesterol (Christie, 1987). An enzyme called lecithin cholesterol acyltransferase (LCAT) converts free cholesterol to cholesteryl ester primarily in HDL and to a lower extent in LDL (Li et al, 2011). This enzyme is crucial in regulating the metabolism of high-density lipoprotein (HDL) and HDL-cholesterol levels in plasma (Li et al, 2011; Ng, 2012), which makes it related to another enzyme called cholesterol ester transfer protein (CETP) that functions as shuttle of TG and CO molecules between lipoproteins (Berti et al, 2003) (Figure 3.25). Recent studies show that LCAT deficient mice displayed protection against insulin resistance and obesity (Li et al, 2011; Ng, 2012). In the light of these studies, obese individuals may have higher LCAT activity, therefore higher cholesteryl ester in their body. In fact, a study conducted on non-diabetic obese and lean individuals, LCAT concentration was found greater nearly 30% in obese subjects than lean ones (Magkos et al, 2009).

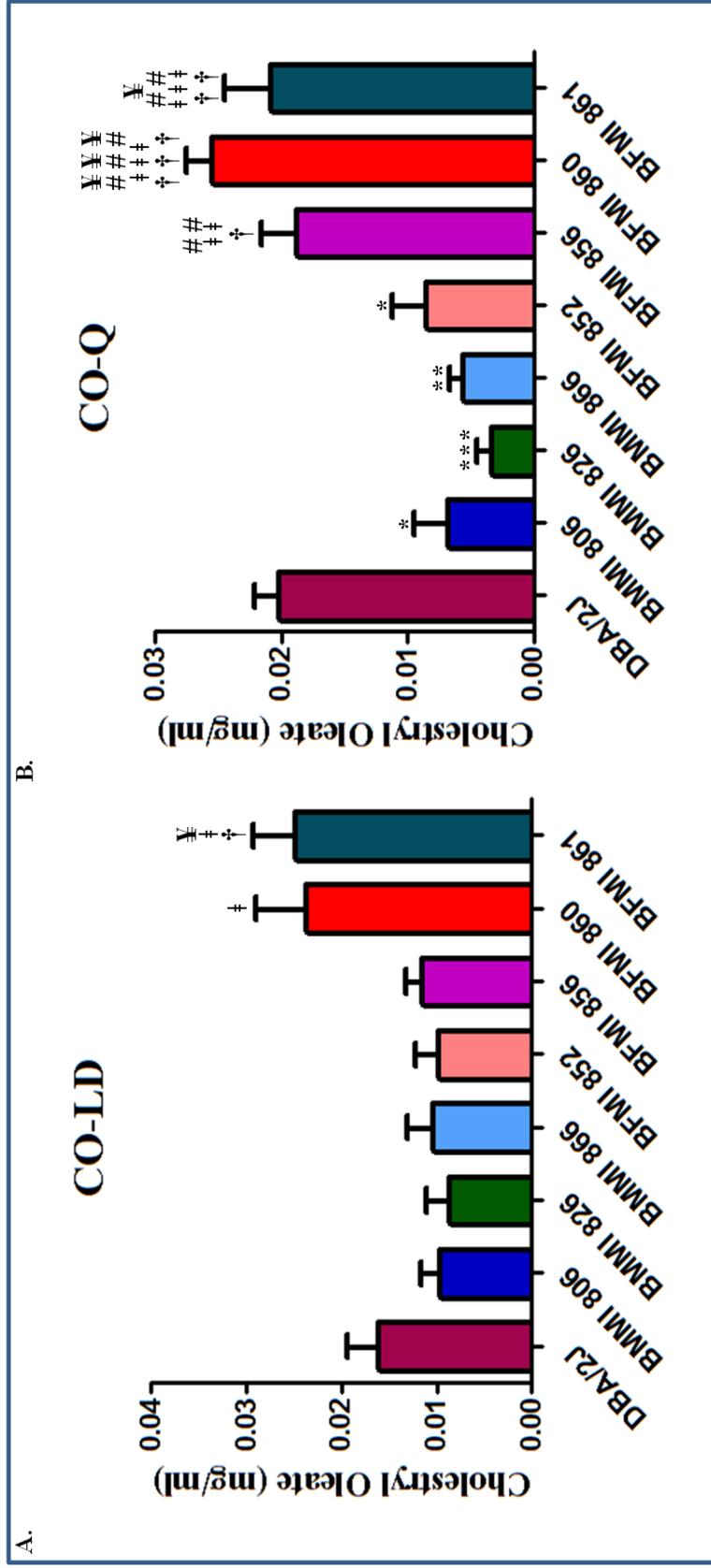


**Figure 3.25.** Mechanism of formation of mature HDL and the actions of the enzymes CETP and LCAT (Duffy & Rader, 2009).

Another crucial protein in cholesteryl ester metabolism is cholesteryl ester transfer protein (CETP) which shuttles triglycerides and cholesteryl esters between lipoproteins (Fielding & Fielding, 1995; Tall, 1993). It has been known that obesity is accompanied with increased levels of CETP, and increased levels of this glycoprotein induces reduction in the level of HDL-cholesterol complex –which is known as “good cholesterol”- and increase in triglyceride amount in plasma (Arai et al, 1994; Chapman et al, 2010; Hayashibe et al, 1997; MacLean et al, 2003; Magkos et al, 2009). And also CETP levels were found to be increased in non-diabetic insulin resistant subjects (Borggreve et al, 2003; Riemens et al, 2001).

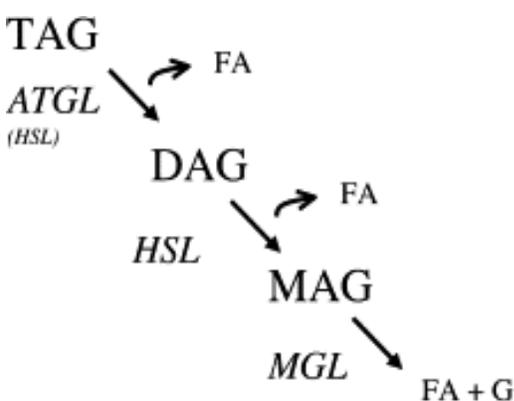
In this study, especially obese BFMI860 and BFMI861 lines displayed significantly higher amounts of CO both in their LD and Q muscle tissues compared to BMMI lines (Figure 3.26). Especially in the Q muscle, significance of this result was observed clearly. Other inbred mouse lines however, did not show any significant levels of CO. Since BFMI860 and BFMI861 lines exhibited stronger obese phenotypes, this result was expected. With the obtained results and literature knowledge, BFMI860 and BFMI861 mice may be more prone to display obesity-induced insulin resistance.

Regarding to muscle BMMI lines, as it can be seen from the figure, their CO concentration did not differ dramatically, though BFMI806 had slightly higher CO content in their skeletal muscle tissue. This result was also expected due to its normal functioning myostatin gene. As mentioned above, subjects who have myostatin mutation have increased muscle mass and decreased fat mass (McPherron & Lee, 2002).



**Figure 3.26.** CO (mg/ml) concentrations obtained from **A**) LD skeletal muscle, and **B**) Q skeletal muscle, of control, BMMI and BMMI mouse lines fed with SBD,  $n=6$  from each line (The degrees of significance were denoted with ‘\*’, ‘†’, ‘‡’, ‘§’, ‘¥’, ‘¤’, ‘§’, and ‘α’ if the results were significant relative to DBA/2J, BMMI806, BMMI826, BMMI866, BMMI852, BMMI856, and BMMI860, respectively). (The results were expressed as ‘mean  $\pm$  standard error of mean’).

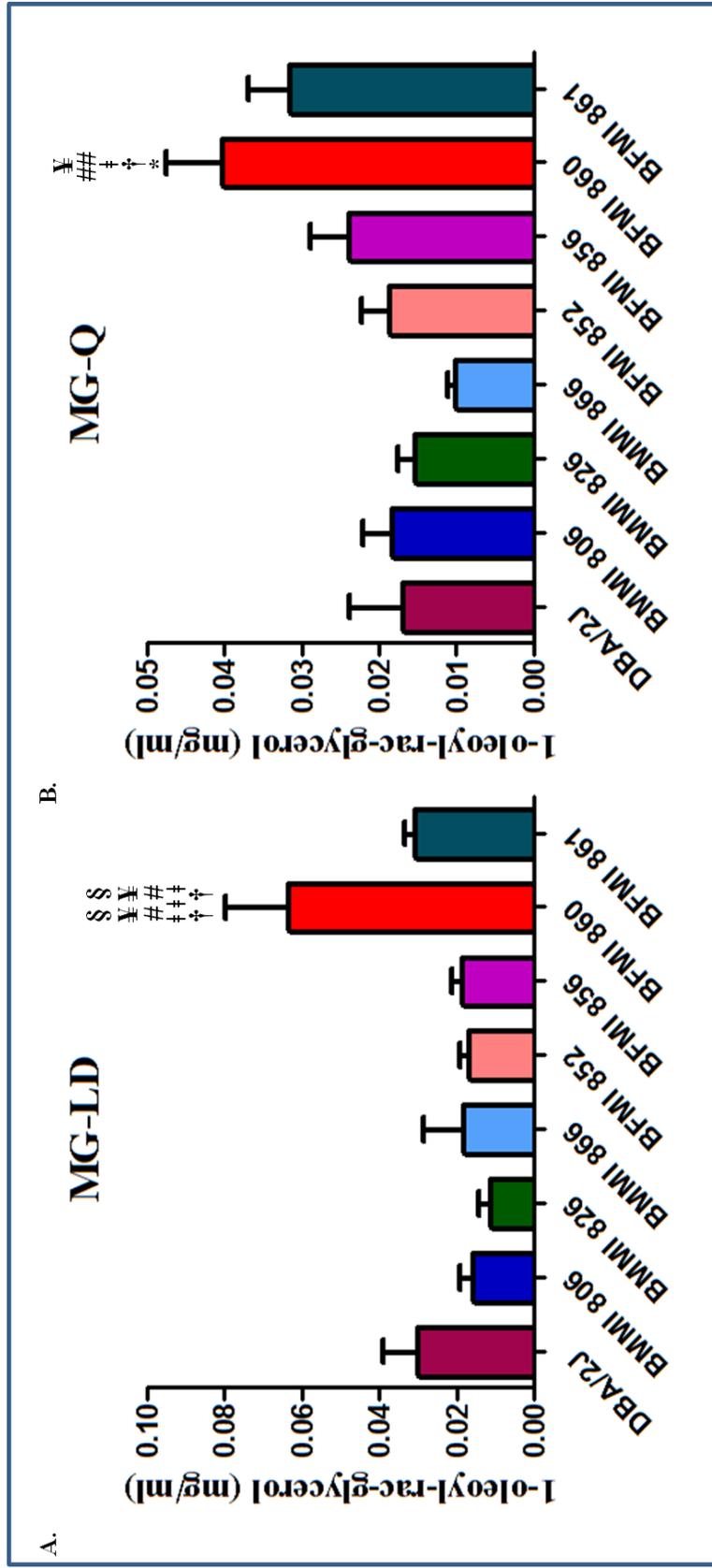
Monoacylglycerols which found in trace amounts in fresh animal tissues, are composed of one molecule fatty acid and one molecule of glycerol (Christie, 1987). This lipid is formed via the degradation of triglycerides first into diglycerides and then monoglycerides by digestive lipases (Figure 3.26) (Khoo et al, 1974; Saadeh et al, 2012; Schittmayer & Birner-Gruenberger, 2009; Wang & Eckel, 2009). After formation of monoglyceride, lipid can be stored in skeletal muscles (Wang & Eckel, 2009). Furthermore, this lipid can again be hydrolyzed to fatty acid and glycerol, as it can be seen from Figure 3.27. A study conducted on the deficiency of this lipid's lipase enzyme, monoglyceride lipase, shows the presence of improvement in insulin resistance, though not in diet-induced obesity (Taschler et al, 2011), which indicates this lipid and its lipase enzyme contribute into insulin resistance states.



**Figure 3.27.** Synthesis of monoglyceride molecules from triglycerides (Jocken & Blaak, 2008).

In Figure 3.28, MG concentration of LD and Q muscles of control, BFMI and BMMI mouse lines is shown. In both muscle types, especially in LD muscle, concentration of this lipid was significantly higher in BFMI860 among other BFMI and BMMI lines. This MG content of this line may be due to excess concentration of TG which is a precursor molecule of MG. In LD muscle of other lines, however no remarked difference was observed, not even in BFMI861 line. Also in Q muscle, the content of

MG of BFMI860 line was found to be the highest of all lines. This high amount of MG may also be due to high content of TG in this line.



**Figure 3.28.** MG (mg/ml) concentrations obtained from **A**) LD skeletal muscle, and **B**) Q skeletal muscle, of control, BFMI and BMMI mouse lines fed with SBD, **n=6** from each line (The degrees of significance were denoted with ‘\*’, ‘†’, ‘‡’, ‘#’, ‘¥’, ‘§’, ‘\$’ and ‘¤’ if the results were significant relative to DBA/2J, BMMI826, BMMI866, BFMI852, BFMI856, and BFMI860, respectively). (The results were expressed as ‘mean ± standard error of mean’).

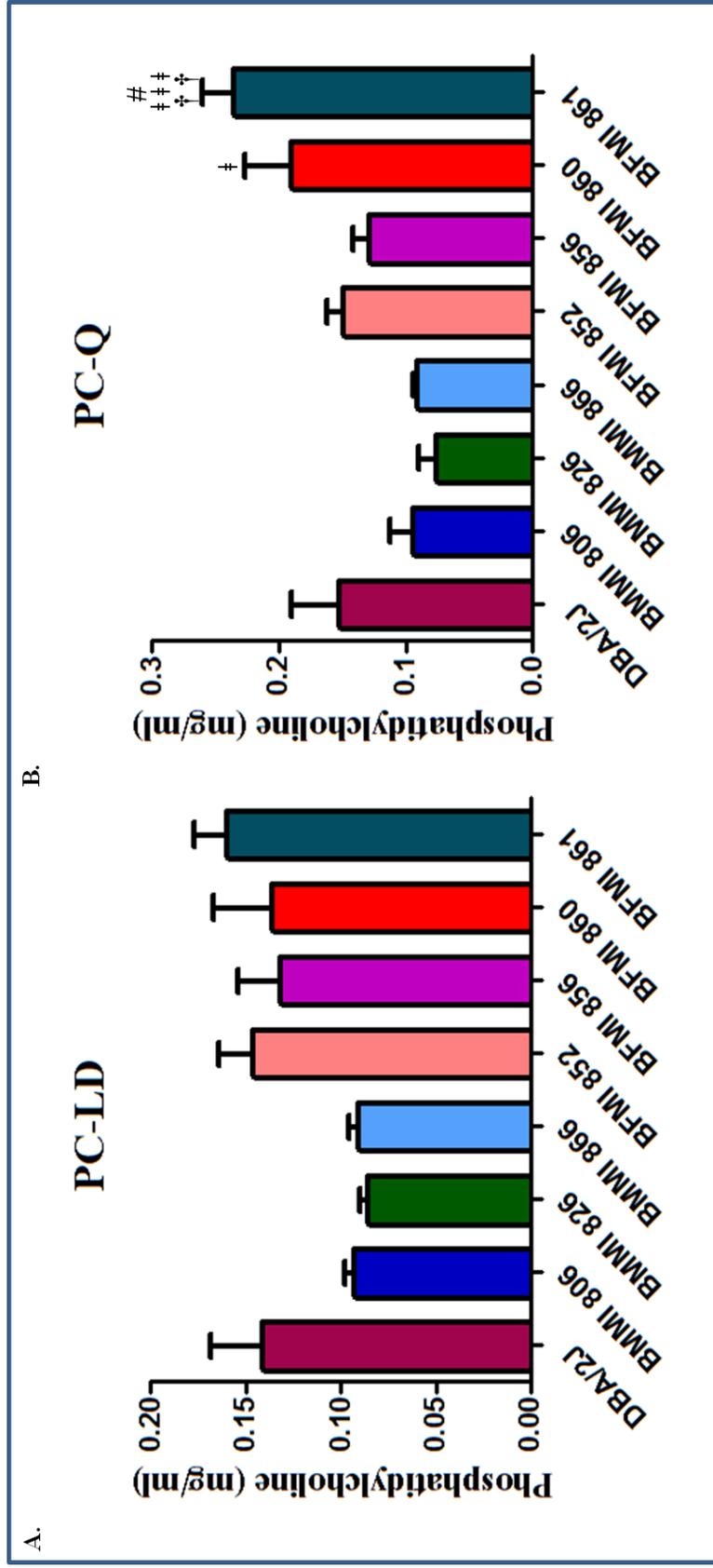
Phosphatidylcholine is one of the major components of cellular membrane phospholipids in eukaryotic cells which plays an important role in various functions like, cell signaling, membrane integrity and being a precursor of some bioactive lipids, such as lysophosphatidylcholine, phosphatidic acid, diacylglycerol, and arachidonic acid (Christie, 1987; Goto-Inoue et al, 2012; Kent & Carman, 1999). Although, this lipid is ubiquitous in all kinds of membranes, greater levels of phosphatidylcholine were found in obese *ob/ob* mice, not in skeletal muscle tissue but in cardiac muscle tissue (Christoffersen et al, 2003). Furthermore, studies showed that, alterations in the levels and composition of phosphatidylcholine change the response of skeletal muscle tissues to insulin, therefore induces insulin resistance (Clore et al, 2000; Rauch & Loughna, 2005).

In this study, PC content of control, obese and muscle inbred mice was calculated. As can be seen from Figure 3.29, although no significant results were obtained regarding the PC content in LD muscle type, there was an obvious increase all BFMI lines compared to BMMI lines. Most probably, this increment may be due to obese phenotypes of BFMI lines. Within BFMI lines, the content of the lipid was almost unchanged. Within BMMI lines the content was not changed either.

In Q muscles, however, a remarked difference in the levels of PC was observed in BFMI860 and BFMI861, but especially in BFMI861 line, when compared to BMMI lines. Significant results of PC contents of BFMI860 and BFMI861 lines, but not other BFMI lines, may arise from their severe obese phenotypes. Regarding to BMMI lines, PC levels did not change within lines.

Since phosphatidylcholine is the most abundant phospholipid type in all kind of membranes and since the lipid composition of membranes does rarely change from tissue to tissue (Christie, 1987), absence of significant results within BFMI lines, and within BMMI lines, both in LD and Q muscles were plausible. High amount of PC in

BFMI860 and BFMI861 lines may further indicate their diminished insulin sensitivity.

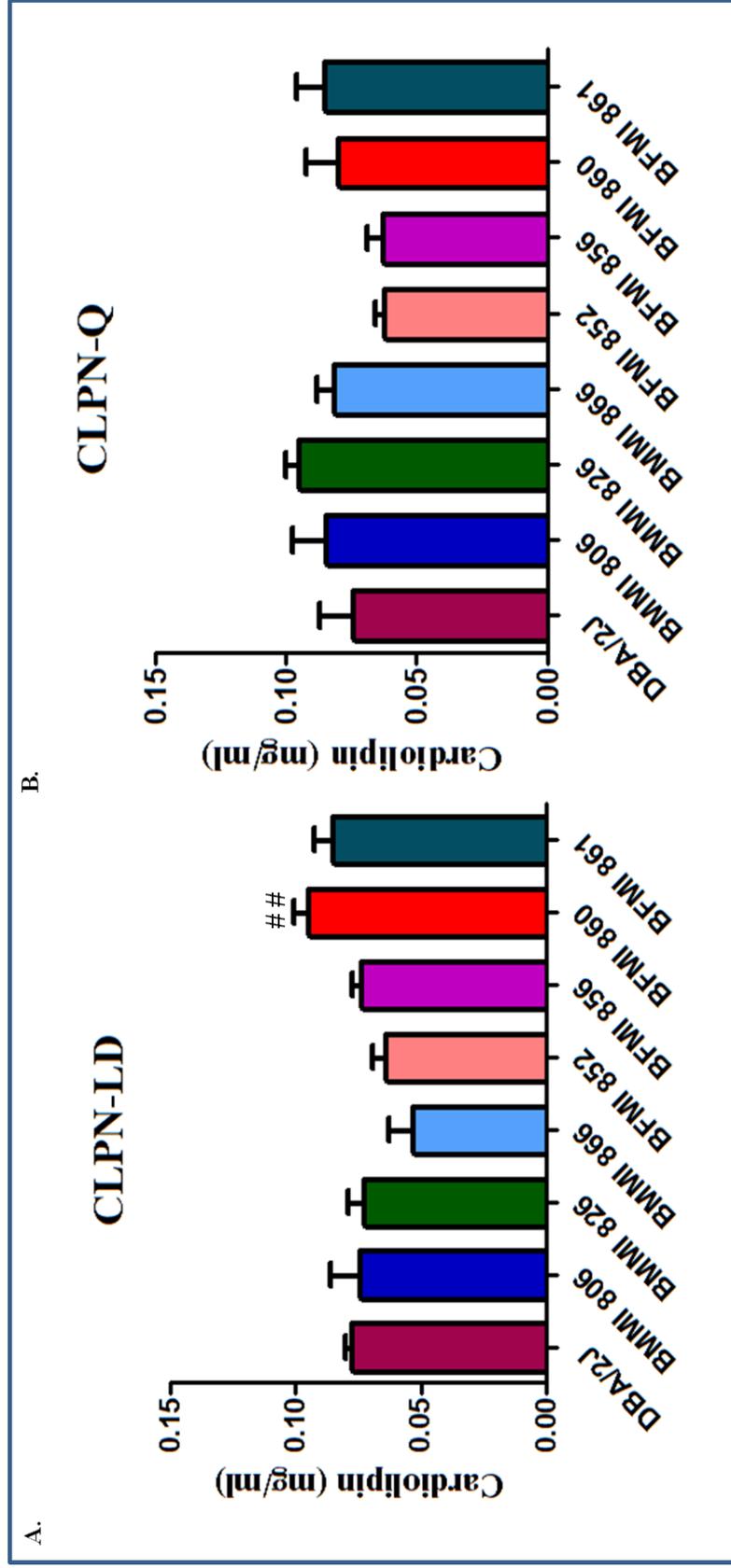


**Figure 3.29.** PC (mg/ml) concentrations obtained from A) LD skeletal muscle, and B) Q skeletal muscle, of control, BFMI and BMMI mouse lines fed with SBD,  $n=6$  from each line (The degrees of significance were denoted with ‘\*’, ‘†’, ‘#’, ‘‡’, ‘§’, ‘\$’ and ‘α’ if the results were significant relative to DBA/2J, BMMI806, BMMI826, BMMI866, BFMI852, BFMI856, and BFMI860, respectively). (The results were expressed as ‘mean  $\pm$  standard error of mean’).

Cardiolipin, also known as diphosphatidylglycerol, is composed of two molecules of phosphatidyl and one molecule of glycerol, and is a fingerprint of the presence of mitochondria (Christie, 1987; Joshi et al, 2009). This particular lipid is ubiquitously present in the inner membrane of mitochondria organelles of all mammalian tissues and also in all living things, even prokaryotes, which functions not only in mitochondrial bioenergetics, but also in protein transport, aging and apoptosis (Claypool, 2009; Joshi et al, 2009; McMillin & Dowhan, 2002).

Since CLPN is a mitochondrial lipid, its presence in both tissues was expected. Interestingly, in all mouse lines, whether belong to the control, muscle or obese, the content of this lipid was found to be the almost same, in both skeletal tissues, except BMMI866 (Figure 3.30). In fact, these findings confirm studies conducted about the relation between mitochondrial lipid content and skeletal muscle tissues (Ritov et al, 2010; Stefanyk et al, 2010). The former study revealed the similarity of cardiolipin content between lean and obese individuals, as the HPLC-ELSD results below display. And the latter study showed the similarity of cardiolipin content between different skeletal muscle types, which also HPLC-ELSD results indicate.

Only significant result was found between BMMI866 and BFMI860. The obese mouse line displayed significantly higher result when compared to the muscle mouse line.



**Figure 3.30.** CLPN (mg/ml) concentrations obtained from **A**) LD skeletal muscle, and **B**) Q skeletal muscle, of control, BFMI and BMMI mouse lines fed with SBD, **n=6** from each line (The degrees of significance were denoted with ‘\*’, ‘†’, ‘#’, ‘¥’, ‘\$’, ‘&’ and ‘α’ if the results were significant relative to DBA/2J, BMMI806, BMMI826, BMMI866, BMMI852, BMMI856, and BMMI860, respectively). (The results were expressed as ‘mean ± standard error of mean’).

Regarding to the muscles types, as it can be seen from the bar diagram figures, except cholesterol (C), phosphatidylcholine (PC) and cardiolipin (CLPN) lipids, LD skeletal muscle tissue was found to contain higher amount of lipids. Monoglyceride (MG), cholesteryl oleate (CO), but especially triglyceride (TG) lipids were found to be dramatically greater in *longissimus dorsi* (LD) skeletal muscle tissue, when compared to *quadriceps* (Q) skeletal muscle tissue. Since there is a link between obesity and muscle triglyceride accumulation, finding greater concentrations of TG, especially in BFMI860 and BFMI861, indicates that these lines displayed more obese phenotypes, and their LD muscle tissue was more affected. Being a product molecule of triglyceride, monoglyceride showed parallel results in both tissues, of course in fewer amounts. As it can be seen from the Figures 3.19 and 3.21, while CO was found higher in LD, level of C was found lower; and vice versa is true for the Q muscle. This result may indicate a greater cholesterol esterification rate in LD than in Q muscle. Another membrane phospholipid, phosphatidylcholine was found significantly higher only in Q muscles of BFMI860 and BFMI861 mice, which may be correlated to these lines' strong obese phenotypes. Other lines were found to contain similar amount of PC, in both tissues. Since, CLPN is an inner mitochondrial membrane phospholipid, and essential for maintaining life, the similar values among both in inbred lines, and both in tissues were expected.

## CHAPTER 4

### CONCLUSION

Optimization process is essential while using chromatography. Procedures copied from literature rarely fit to the instrument itself. Therefore, generally a new method according to the column, detector, separation module, and of course to the samples has to be developed. The results obtained from the ‘Method Development’ part of this study display the effectiveness of the newly developed method for lipid separation, from muscle tissues. Main lipid classes, namely triglyceride (TG), cholesteryl ester (CO), free cholesterol (C), monoglyceride (MG), phosphatidylcholine (PC) and cardiolipin (CLPN) were separated clearly.

In obesity studies, quantification of lipids is generally done by chromatography methods. In the second part of the study, *Longissimus Dorsi* (LD) and *Quadriceps* (Q) skeletal muscle tissues from special-bred mouse lines, control DBA/2J, obese BFMI and muscle BMMI lines, were analyzed and quantified. Among all mouse lines, BFMI860 and BFMI861 mice, but especially BFMI860, were found to have the highest amount of lipids.

Greatest TG lipid accumulation within LD and Q muscle tissues was observed in BFMI860 and BFMI861 lines. Other BFMI lines also displayed high amounts of TG when compared to BMMI lines, but not as BFMI860 and BFMI861. Among BMMI lines, myostatin wild-type BMMI806 line displayed higher amount of TG.

CO and C lipid levels were also found to be significantly greater in BFMI860 and BFMI861 lines, in both tissues. Only MG levels displayed higher concentration in BFMI860 in both tissues. Although, no significant results were observed among BMMI lines, BMMI806 mice showed relatively higher amounts of lipids than other BMMIs, in LD and Q muscles.

In LD tissues, PC contents were not altered in same inbred lines. BFMI mice were found to have higher PC values compared to BMMI lines, but they did not display any remarked differences among themselves. In Q tissues however, BFMI860 and BFMI861 lines, but especially BFMI861, showed significant results in comparison to BMMI lines.

CLPN content displayed almost no difference among control, obese and muscle lines. Similar concentrations were observed both in LD and Q muscles.

Regarding all these results, it can be deduced that BMMI806, BFMI860 and BFMI861 lines can be used as spontaneous, polygenic obese animal models, since they displayed dramatic increments in critical lipids. Furthermore, lipid quantification results obtained from HPLC-ELSD studies demonstrated that, BFMI860 and BFMI861 mouse lines may be applicable animal models in obesity-induced type II diabetes, due to TG and C, or cardiovascular diseases, due to C and CO, or even metabolic syndrome, due to all of the lipids.

## REFERENCES

Arai T, Yamashita S, Hirano K, Sakai N, Kotani K, Fujioka S, Nozaki S, Keno Y, Yamane M, Shinohara E, et al. (1994) Increased plasma cholesteryl ester transfer protein in obese subjects. A possible mechanism for the reduction of serum HDL cholesterol levels in obesity. *Arterioscler Thromb* **14**: 1129-1136

Avalli A, Contarini G (2005) Determination of phospholipids in dairy products by SPE/HPLC/ELSD. *J Chromatogr A* **1071**: 185-190

Bandi ZL, Moslen MT, Reynolds ES (1982) Ion-exchange chromatography of phosphatides and glycolipids on quaternary triethylammonium glycopease covalently bound to controlled pore glass. *J Chromatogr* **269**: 93-101

Barany M (1967) ATPase activity of myosin correlated with speed of muscle shortening. *J Gen Physiol* **50**: Suppl:197-218

Barter PJ, Brewer HB, Jr., Chapman MJ, Hennekens CH, Rader DJ, Tall AR (2003) Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. *Arterioscler Thromb Vasc Biol* **23**: 160-167

Berti JA, Casquero AC, Patricio PR, Bighetti EJ, Carneiro EM, Boschero AC, Oliveira HC (2003) Cholesteryl ester transfer protein expression is down-regulated in hyperinsulinemic transgenic mice. *J Lipid Res* **44**: 1870-1876

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**: 911-917

Bobbitt JM, Schwarting AE, Gritter RJ (1968) *Introduction to Chromatography*, New York: Reinhold Book Corporation.

Bogardus C, Lillioja S, Mott DM, Hollenbeck C, Reaven G (1985) Relationship between degree of obesity and in vivo insulin action in man. *Am J Physiol* **248**: E286-291

Borggreve SE, De Vries R, Dullaart RP (2003) Alterations in high-density lipoprotein metabolism and reverse cholesterol transport in insulin resistance and type 2 diabetes mellitus: role of lipolytic enzymes, lecithin:cholesterol acyltransferase and lipid transfer proteins. *Eur J Clin Invest* **33**: 1051-1069

Boselli E, Pacetti D, Curzi F, Frega NG (2008) Determination of phospholipid molecular species in pork meat by high performance liquid chromatography-tandem mass spectrometry and evaporative light scattering detection. *Meat Sci* **78**: 305-313

Bravi E, Perretti G, Montanari L (2006) Fatty acids by high-performance liquid chromatography and evaporative light-scattering detector. *J Chromatogr A* **1134**: 210-214

Brouwers JF, Vernooij EA, Tielens AG, van Golde LM (1999) Rapid separation and identification of phosphatidylethanolamine molecular species. *J Lipid Res* **40**: 164-169

Caccamo G, Bonura F, Vitale G, Novo G, Evola S, Evola G, Grisanti MR, Novo S (2010) Insulin resistance and acute coronary syndrome. *Atherosclerosis* **211**: 672-675

Calder PC, Yaqoob P (2007) Lipid rafts--composition, characterization, and controversies. *J Nutr* **137**: 545-547

Carola R, Noback CR, Harley JP (1992) Muscle Tissue. In *HUMAN ANATOMY*, 10. New York: McGraw-Hill, Inc

Chapman MJ, Le Goff W, Guerin M, Kontush A (2010) Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. *Eur Heart J* **31**: 149-164

Christie WW (1985) Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. *J Lipid Res* **26**: 507-512

Christie WW (1987) *High-performance Liquid Chromatography and Lipids*, 1 edn. Oxford: Pergamon Press.

Christoffersen C, Bollano E, Lindegaard ML, Bartels ED, Goetze JP, Andersen CB, Nielsen LB (2003) Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. *Endocrinology* **144**: 3483-3490

Claypool SM (2009) Cardiolipin, a critical determinant of mitochondrial carrier protein assembly and function. *Biochim Biophys Acta* **1788**: 2059-2068

Clore JN, Harris PA, Li J, Azzam A, Gill R, Zuelzer W, Rizzo WB, Blackard WG (2000) Changes in phosphatidylcholine fatty acid composition are associated with altered skeletal muscle insulin responsiveness in normal man. *Metabolism* **49**: 232-238

Collins RC (1997) Red and white brain. *Ann N Y Acad Sci* **835**: 250-254

DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP (1981) The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* **30**: 1000-1007

Descalzo AM, Insani EM, Pensel NA (2003) Light-scattering detection of phospholipids resolved by HPLC. *Lipids* **38**: 999-1003

Di Stefano V, Avellone G, Bongiorno D, Cunsolo V, Muccilli V, Sforza S, Dossena A, Drahos L, Vekey K (2012) Applications of HPLC-MS for food analysis. *J Chromatogr A*

Ditz R (2012) Separation Technologies 2030 - Are 100 Years of Chromatography Enough? *Chem Ing Tech* **84**: 875-879

Duffy D, Rader DJ (2009) Update on strategies to increase HDL quantity and function. *Nat Rev Cardiol* **6**: 455-463

Empower 2 Data Acquisition and Processing Theory Guide. In Waters (ed.), Milford.

Fielding CJ, Fielding PE (1995) Molecular physiology of reverse cholesterol transport. *J Lipid Res* **36**: 211-228

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

Frings CS, Dunn RT (1970) A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *Am J Clin Pathol* **53**: 89-91

Goodpaster BH, He J, Watkins S, Kelley DE (2001) Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* **86**: 5755-5761

Goodpaster BH, Theriault R, Watkins SC, Kelley DE (2000) Intramuscular lipid content is increased in obesity and decreased by weight loss. *Metabolism* **49**: 467-472

Goto-Inoue N, Manabe Y, Miyatake S, Ogino S, Morishita A, Hayasaka T, Masaki N, Setou M, Fujii NL (2012) Visualization of dynamic change in contraction-induced lipid composition in mouse skeletal muscle by matrix-assisted laser desorption/ionization imaging mass spectrometry. *Anal Bioanal Chem* **403**: 1863-1871

Grabielle-Madelmont C, Lesieur S, Ollivon M (2003) Characterization of loaded liposomes by size exclusion chromatography. *J Biochem Biophys Methods* **56**: 189-217

Greisheimer EM, Wiedeman MP (1972) SKELETAL MUSCLE. In *Physiology and Anatomy*, 9th edn. Washington: J.B. Lippincott Company

Grimm MO, Grosgen S, Riemenschneider M, Tanila H, Grimm HS, Hartmann T (2011) From brain to food: analysis of phosphatidylcholins, lyso-phosphatidylcholins and phosphatidylcholin-plasmalogens derivatives in Alzheimer's disease human post mortem brains and mice model via mass spectrometry. *J Chromatogr A* **1218**: 7713-7722

Guo T, Bond ND, Jou W, Gavrilova O, Portas J, McPherron AC (2012) Myostatin Inhibition Prevents Diabetes and Hyperphagia in a Mouse Model of Lipodystrophy. *Diabetes*

Guo T, Jou W, Chanturiya T, Portas J, Gavrilova O, McPherron AC (2009) Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. *PLoS One* **4**: e4937

Gustavsson J, Parpal S, Karlsson M, Ramsing C, Thorn H, Borg M, Lindroth M, Peterson KH, Magnusson KE, Stralfors P (1999) Localization of the insulin receptor in caveolae of adipocyte plasma membrane. *FASEB J* **13**: 1961-1971

Habegger KM, Penque BA, Sealls W, Tackett L, Bell LN, Blue EK, Gallagher PJ, Sturek M, Alloosh MA, Steinberg HO, Considine RV, Elmendorf JS (2012) Fat-induced membrane cholesterol accrual provokes cortical filamentous actin destabilisation and glucose transport dysfunction in skeletal muscle. *Diabetologia* **55**: 457-467

Hamelin M, Sayd T, Chambon C, Bouix J, Bibe B, Milenkovic D, Leveziel H, Georges M, Clop A, Marinova P, Laville E (2007) Differential expression of sarcoplasmic proteins in four heterogeneous ovine skeletal muscles. *Proteomics* **7**: 271-280

Hannun YA, Linaudic CM (1993) Sphingolipid breakdown products: anti-proliferative and tumor-suppressor lipids. *Biochim Biophys Acta* **1154**: 223-236

Hantschel C, Wagener A, Neuschl C, Teupser D, Brockmann GA (2011) Features of the metabolic syndrome in the Berlin Fat Mouse as a model for human obesity. *Obes Facts* **4**: 270-277

Haslam DW, James WP (2005) Obesity. *Lancet* **366**: 1197-1209

Hayashibe H, Asayama K, Nakane T, Uchida N, Kawada Y, Nakazawa S (1997) Increased plasma cholesteryl ester transfer activity in obese children. *Atherosclerosis* **129**: 53-58

<http://elchem.kaist.ac.kr/jhkwak/AnalChem/09/04/gif2.htm>.

<http://en.wikipedia.org/wiki/File:Cholesterol.svg>.

[http://en.wikipedia.org/wiki/File:Fat\\_triglyceride\\_shorthand\\_formula.PNG](http://en.wikipedia.org/wiki/File:Fat_triglyceride_shorthand_formula.PNG).

<http://en.wikipedia.org/wiki/File:Sarcomere.gif#file>.

<http://www.chemguide.co.uk/analysis/chromatography/hplc.html>.

<http://www.columbia.edu/cu/biology/courses/c2005/purves6/figure03-21.jpg>.

<http://www.cyberlipid.org/elsd/elsd0001.htm>.

<http://www.cyberlipid.org/glycer/glyc0002.htm>.

<http://www.nestgrp.com/pdf/colcare.pdf>.

<http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/lipoprot.htm>.

<http://www.s-cool.co.uk/a-level/biology/movement-and-support-in-animals/revise-it/muscles-and-movement>.

<https://www.msu.edu/~gallego7/MassSpect/Chromatography.htm>.

Hu J, Zhang Z, Shen WJ, Azhar S (2010) Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab (Lond)* **7**: 47

Iverson SJ, Lang SL, Cooper MH (2001) Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. *Lipids* **36**: 1283-1287

Jocken JW, Blaak EE (2008) Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. *Physiol Behav* **94**: 219-230

Joshi AS, Zhou J, Gohil VM, Chen S, Greenberg ML (2009) Cellular functions of cardiolipin in yeast. *Biochim Biophys Acta* **1793**: 212-218

Kanasaki K, Koya D (2011) Biology of obesity: lessons from animal models of obesity. *J Biomed Biotechnol* **2011**: 197636

Karst S, Cheng R, Schmitt AO, Yang H, de Villena FP, Palmer AA, Brockmann GA (2011) Genetic determinants for intramuscular fat content and water-holding capacity in mice selected for high muscle mass. *Mamm Genome* **22**: 530-543

Kelley DE, Goodpaster BH (2001) Skeletal muscle triglyceride. An aspect of regional adiposity and insulin resistance. *Diabetes Care* **24**: 933-941

Kent C, Carman GM (1999) Interactions among pathways for phosphatidylcholine metabolism, CTP synthesis and secretion through the Golgi apparatus. *Trends Biochem Sci* **24**: 146-150

Khaodhiar L, McCowen KC, Blackburn GL (1999) Obesity and its comorbid conditions. *Clin Cornerstone* **2**: 17-31

Khoo JC, Aquino AA, Steinberg D (1974) The mechanism of activation of hormone-sensitive lipase in human adipose tissue. *J Clin Invest* **53**: 1124-1131

Kiebish MA, Han X, Cheng H, Chuang JH, Seyfried TN (2008) Cardiolipin and electron transport chain abnormalities in mouse brain tumor mitochondria: lipidomic evidence supporting the Warburg theory of cancer. *J Lipid Res* **49**: 2545-2556

Kunji ER, Harding M, Butler PJ, Akamine P (2008) Determination of the molecular mass and dimensions of membrane proteins by size exclusion chromatography. *Methods* **46**: 62-72

Lean ME (2000) Pathophysiology of obesity. *Proc Nutr Soc* **59**: 331-336

Li L, Hossain MA, Sadat S, Hager L, Liu L, Tam L, Schroer S, Huogen L, Fantus IG, Connelly PW, Woo M, Ng DS (2011) Lecithin cholesterol acyltransferase null mice are protected from diet-induced obesity and insulin resistance in a gender-specific manner through multiple pathways. *J Biol Chem* **286**: 17809-17820

Licinio J, Caglayan S, Ozata M, Yildiz BO, de Miranda PB, O'Kirwan F, Whitby R, Liang L, Cohen P, Bhasin S, Krauss RM, Veldhuis JD, Wagner AJ, DePaoli AM, McCann SM, Wong ML (2004) Phenotypic effects of leptin replacement on morbid obesity, diabetes mellitus, hypogonadism, and behavior in leptin-deficient adults. *Proc Natl Acad Sci U S A* **101**: 4531-4536

Lottenberg AM, Afonso MD, Lavrador MS, Machado RM, Nakandakare ER (2012) The role of dietary fatty acids in the pathology of metabolic syndrome. *J Nutr Biochem*

Lutzke BS, Braugher JM (1990) An improved method for the identification and quantitation of biological lipids by HPLC using laser light-scattering detection. *J Lipid Res* **31**: 2127-2130

MacLean PS, Bower JF, Vadlamudi S, Osborne JN, Bradfield JF, Burden HW, Bensch WH, Kauffman RF, Barakat HA (2003) Cholesteryl ester transfer protein expression prevents diet-induced atherosclerotic lesions in male db/db mice. *Arterioscler Thromb Vasc Biol* **23**: 1412-1415

Magkos F, Mohammed BS, Mittendorfer B (2009) Plasma lipid transfer enzymes in non-diabetic lean and obese men and women. *Lipids* **44**: 459-464

Maraschiello C, Diaz I, Regueiro JAG (1996) Determination of Cholesterol in Fat and Muscle of Pig by HPLC and Capillary Gas Chromatography with Solvent Venting Injection. *J High Resol Chromatogr* **19**: 165-168

McMillin JB, Dowhan W (2002) Cardiolipin and apoptosis. *Biochim Biophys Acta* **1585**: 97-107

McPherron AC, Lee SJ (2002) Suppression of body fat accumulation in myostatin-deficient mice. *J Clin Invest* **109**: 595-601

Meyer CW, Wagener A, Rink N, Hantschel C, Heldmaier G, Klingenspor M, Brockmann GA (2009) High energy digestion efficiency and altered lipid metabolism contribute to obesity in BFMI mice. *Obesity (Silver Spring)* **17**: 1988-1993

Ng DS (2012) The role of lecithin:cholesterol acyltransferase in the modulation of cardiometabolic risks - A clinical update and emerging insights from animal models. *Biochim Biophys Acta* **1821**: 654-659

Olsson P, Holmback J, Herslof B (2012) Separation of lipid classes by HPLC on a cyanopropyl column. *Lipids* **47**: 93-99

Ono Y, Solomon MB, Evock-Clover CM, Steele NC, Maruyama K (1995) Effects of porcine somatotropin administration on porcine muscles located within different regions of the body. *J Anim Sci* **73**: 2282-2288

Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlien LH (1997) Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* **46**: 983-988

Parpal S, Karlsson M, Thorn H, Stralfors P (2001) Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. *J Biol Chem* **276**: 9670-9678

Perona JS, Ruiz-Gutierrez V (2005) Quantitative Lipid Composition of Iberian Pig Muscle and Adipose Tissue by HPLC. *Journal of Liquid Chromatography & Related Technologies* **28**: 2445 — 2457

Pike LJ (2003) Lipid rafts: bringing order to chaos. *J Lipid Res* **44**: 655-667

Rauch C, Loughna P (2005) C2C12 skeletal muscle cells exposure to phosphatidylcholine triggers IGF-1 like-responses. *Cell Physiol Biochem* **15**: 211-224

Reaven G, Abbasi F, McLaughlin T (2004) Obesity, insulin resistance, and cardiovascular disease. *Recent Prog Horm Res* **59**: 207-223

Reaven GM (2011) The metabolic syndrome: time to get off the merry-go-round? *J Intern Med* **269**: 127-136

Reed DR (2008) Animal models of gene-nutrient interactions. *Obesity (Silver Spring)* **16 Suppl 3**: S23-27

Riemens SC, van Tol A, Scheek LM, Dullaart RP (2001) Plasma cholesteryl ester transfer and hepatic lipase activity are related to high-density lipoprotein cholesterol in association with insulin resistance in type 2 diabetic and non-diabetic subjects. *Scand J Clin Lab Invest* **61**: 1-9

Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FG, Goodpaster BH, Ruderman NB, Kelley DE (2010) Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol Metab* **298**: E49-58

Rogner M. (1999) Size Exclusion Chromatography. In Kastner M (ed.), *Protein Liquid Chromatography*. Elsevier Science B.V., pp. 90-95.

Roos HP. (1999) Ion Exchange Chromatography. In Kastner M (ed.), *Protein Liquid Chromatography*. Elsevier Science B.V., pp. 5-9.

Saadeh M, Ferrante TC, Kane A, Shirihai O, Corkey BE, Deeney JT (2012) Reactive oxygen species stimulate insulin secretion in rat pancreatic islets: studies using mono-oleoyl-glycerol. *PLoS One* **7**: e30200

Schittmayer M, Birner-Gruenberger R (2009) Functional proteomics in lipid research: lipases, lipid droplets and lipoproteins. *J Proteomics* **72**: 1006-1018

Scott W, Stevens J, Binder-Macleod SA (2001) Human skeletal muscle fiber type classifications. *Phys Ther* **81**: 1810-1816

Shier D, Butler J, Lewis R (2010) Muscular System. In *Hole's Human Anatomy & Physiology*, 10th edn, 9, pp 280-300. New York: McGraw-Hill

Simons K, Ehehalt R (2002) Cholesterol, lipid rafts, and disease. *J Clin Invest* **110**: 597-603

Slack JM (1997) Growth control: action mouse. *Curr Biol* **7**: R467-469

Snyder LR, Kirkland JJ (1979) *Introduction to Modern Liquid Chromatography*, 2nd edn. New York: John Wiley & Sons

Speakman J, Hambly C, Mitchell S, Krol E (2008) The contribution of animal models to the study of obesity. *Lab Anim* **42**: 413-432

Stefanyk LE, Coverdale N, Roy BD, Peters SJ, LeBlanc PJ (2010) Skeletal muscle type comparison of subsarcolemmal mitochondrial membrane phospholipid fatty acid composition in rat. *J Membr Biol* **234**: 207-215

Stein DT (2004) Triglyceride in Muscle. In *Diabetes Mellitus: A Fundamental and Clinical Text*, LeRoith D (ed), 3rd edn. Philadelphia: Lippincott Williams & Wilkins

Stevens J, Couper D, Pankow J, Folsom AR, Duncan BB, Nieto FJ, Jones D, Tyroler HA (2001) Sensitivity and specificity of anthropometrics for the prediction of diabetes in a biracial cohort. *Obes Res* **9**: 696-705

Svenson KL, Von Smith R, Magnani PA, Suetin HR, Paigen B, Naggert JK, Li R, Churchill GA, Peters LL (2007) Multiple trait measurements in 43 inbred mouse strains capture the phenotypic diversity characteristic of human populations. *J Appl Physiol* **102**: 2369-2378

Tall AR (1993) Plasma cholesteryl ester transfer protein. *J Lipid Res* **34**: 1255-1274

Taschler U, Radner FP, Heier C, Schreiber R, Schweiger M, Schoiswohl G, Preiss-Landl K, Jaeger D, Reiter B, Koefeler HC, Wojciechowski J, Theussl C, Penninger JM, Lass A, Haemmerle G, Zechner R, Zimmermann R (2011) Monoglyceride lipase deficiency in mice impairs lipolysis and attenuates diet-induced insulin resistance. *J Biol Chem* **286**: 17467-17477

Toye AA, Lippiat JD, Proks P, Shimomura K, Bentley L, Hugill A, Mijat V, Goldsworthy M, Moir L, Haynes A, Quarterman J, Freeman HC, Ashcroft FM, Cox RD (2005) A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. *Diabetologia* **48**: 675-686

Vainio S, Heino S, Mansson JE, Fredman P, Kuismanen E, Vaarala O, Ikonen E (2002) Dynamic association of human insulin receptor with lipid rafts in cells lacking caveolae. *EMBO Rep* **3**: 95-100

Vander A, Sherman J, Luciano D (1998) Muscle. In *Human Physiology, The Mechanism of Body Function*, 7th edn, 11. New York: McGraw-Hill Companies, Inc.

Wagener A, Schmitt AO, Aksu S, Schlote W, Neuschl C, Brockmann GA (2006) Genetic, sex, and diet effects on body weight and obesity in the Berlin Fat Mouse Inbred lines. *Physiol Genomics* **27**: 264-270

Wang H, Eckel RH (2009) Lipoprotein lipase: from gene to obesity. *Am J Physiol Endocrinol Metab* **297**: E271-288

Wannamethee SG, Shaper AG (1999) Weight change and duration of overweight and obesity in the incidence of type 2 diabetes. *Diabetes Care* **22**: 1266-1272

WHO Report. (2008).

Yamakawa H, Higashino K, Ohara O (1996) Sequence-dependent DNA separation by anion-exchange high-performance liquid chromatography. *Anal Biochem* **240**: 242-250