DETERMINATION OF INBRED OBESE MOUSE ADIPOSE TISSUE LIPID PROFILES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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submitted by **TUĞÇE BAŞER** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Gülay Özcengiz _____
Head of Department, **Biology**

Prof. Dr. Feride Severcan _____
Supervisor, **Biological Sciences Dept., METU**

Examining Committee Members:

Doç. Dr. Ayşegül Çetin Gözen _____
Biological Sciences Dept., METU

Prof. Dr. Feride Severcan _____
Biological Sciences Dept., METU

Doç. Dr. Sreeparna Banerjee _____
Biological Sciences Dept., METU

Doç. Dr. Çağdaş Devrim Son _____
Biological Sciences Dept., METU

Prof. Dr. Belma Konuklugil _____
Faculty of Pharmacy, AU

Date: _____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Tuğçe Başer
Signature :

ABSTRACT

DETERMINATION OF INBRED OBESE MOUSE ADIPOSE TISSUE LIPID PROFILES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Başer, Tuğçe

M.Sc., Department of Biology

Supervisor: Prof. Dr. Feride Severcan

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Obesity becomes an important and serious health problem all over the world. Consumed too much calorie and less physical activity are the major issues in obesity. Obesity also causes some other important diseases such as hypertension, insulin resistance, dyslipidemia, type II diabetes and cardiovascular diseases.

The aim of this study is to identify and quantify lipid profiles of obese Berlin Fat Mouse Inbred (BFMI) which has high fat content. These mouse lines showed spontaneous obesity. In addition to obesity, BFMI 860 and 861 had insulin resistance. Also, BFMI 860 and 861 lines were used as animal model for cardiovascular diseases. High performance liquid chromatography (HPLC) was used to separate, identify and quantify the lipids of BFMI mouse. Evaporative light scattering detector (ELSD) was used as detector with HPLC.

Lipid extraction procedures were applied to BFMI. After that, HPLC were done with a gradient elution. Lipids that were identified and quantified were triglyceride (TG), diglyceride (DG), cholesterol (C), monoglyceride (MG) and phosphatidylcholin (PC). It is known that all of these lipids have role in cardiovascular diseases, insulin resistance and obesity. With respect to results, BFMI 860 and 861 mouse lines have more lipids especially TG, C, and MG than BFMI 852 and BFMI 856. Therefore, these lines are suitable for usage as animal models for obesity and type II diabetes.

Keywords: Lipid profiles, Obesity, HPLC, ELSD, BFMI

ÖZ

GENETİK OBEZ FARE ADİPOZ DOKULARI LİPİT PROFİLLERİNİN YÜKSEK PERFORMANSLI SIVI KROMOTOGRAFİSİ İLE TAYİNİ

Başer, Tuğçe

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Obezite, tüm dünyada önemli ve ciddi bir sağlık sorunu haline geldi. Çok fazla miktarda kalori almak ve az fiziksel aktivite yapmak obeziteye neden olan başlıca etken. Obezite aynı zamanda hipertansiyon, insulin direnci, dislipidemi, tip II diyabet ve kardiyovasküler hastalıklar gibi önemli başka hastalıklara da sebep oluyor.

Bu çalışmanın amacı yüksek yağ içeriğine sahip olan Berlin lipit inbred (BFMI) fare modelindeki yağ profillerini tanımlamak ve miktarlarını belirlemek. Bu fare modelinde yüksek derece obezite görülmektedir. Obeziteye ek olarak, BFMI 860 and 861 fare modellerinde insulin direnci görülmektedir. Aynı zamanda, bu modeller, kardiovasküler hastalıklar için de hayvan modeli olarak kullanılıyor. Yüksek performanslı likit kromatografisi (HPLC), BFMI fare modellerindeki lipitleri ayırmak, tanımlamak ve miktarlarını belirlemek için kullanıldı. Evaporatif ışık saçılımlı dedektör (ELSD), HPLC ile birlikte dedektör olarak kullanıldı.

Lipit ekstraksiyon prosedürü, BFMI fare hatları için uygulandı. Daha sonra, ekstraksiyonu yapılan lipitler iki solüsyonlu gradyan akışı ile HPLC metodu uygulandı. Trigliserit (TG), digliserit (DG), kolesterol (C), monogliserit (MG) ve fosfatidilkolin (PC), HPLC ile tanımlanan ve miktarları ölçülen lipitlerdir. Bu lipitlerin, kardiyovasküler hastalıklarda, insülin direncinde ve obezitede rölü olduğu biliniyor. Bu çalışmada, BFMI 860 ve 861 modellerinin BFMI 852 ve BFMI 856 modellerine göre daha fazla lipit, özellikle TG, C ve MG lipitlerini içerdiği saptanmıştır. Bu yüzden bu modellerin obezite çalışmaları için önemli ve kaydedeğer oldukları anlaşılmıştır.

Anahtar Kelimeler: Lipit profili, Obezite, HPLC, ELSD, BFMI

To my devoted family

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LIST OF ABBREVIATIONS

BFMI	Berlin Fat Mouse Inbred
C	Cholesterol
DG	Diglyceride
ELSD	Evaporative Light Scattering Detector
FFAs	Free Fatty Acids
GF	Gonadal fat
HPLC	High Performance Liquid Chromatography
IF	Inguinal fat
MG	Monoglyceride
PC	Phosphatidylcholin
SAT	Subcutaneous Adipose Tissue
TG	Triglyceride
VAT	Visceral Adipose Tissue

CHAPTER 1

INTRODUCTION

1.1. Adipose Tissue

Adipose tissue is a kind of connective tissue which is very loose. Mainly, adipocytes and fibroblasts are found in adipose tissue. Other constituents of adipose tissue are macrophages, stromal cells, monocytes and pre-adipocytes (Figure 1) (Geloen et al, 1989).

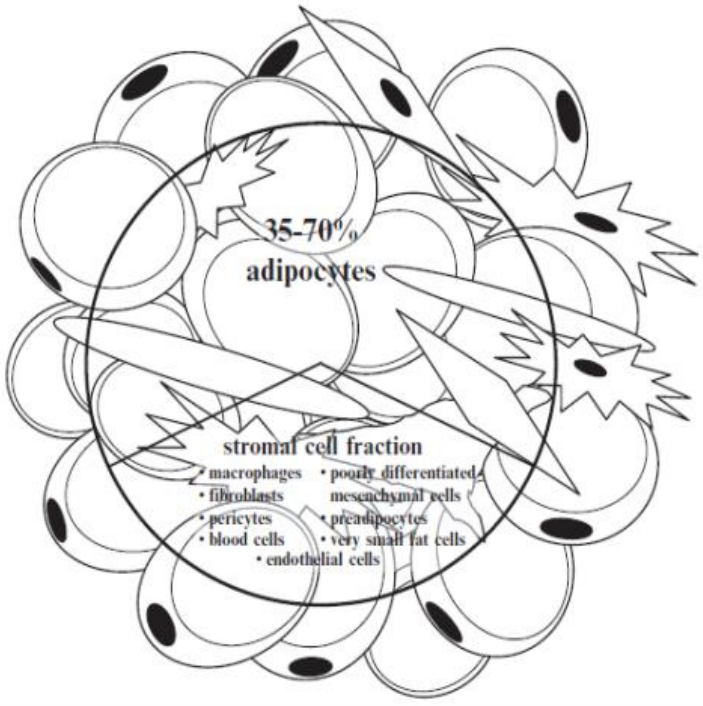


Figure 1. Different constituents of adipose tissue (Fruhbeck, 2008)

The main role of the adipose tissue is to store energy. Adipose tissue stores the energy in the form of lipid. Moreover, adipose tissue insulates the body and has role in thermogenesis (Rosen and Spiegelman, 2000).

1.1.1. Types of Adipose Tissue

Adipose tissue can be grouped into two groups according to its color. One is white adipose tissue (WAT). The other is brown adipose tissue (BAT). The difference between WAT and BAT is the histological composition. WAT contains white adipocytes. However, BAT contains brown adipocytes (Figure 2) (Cinti, 2006).

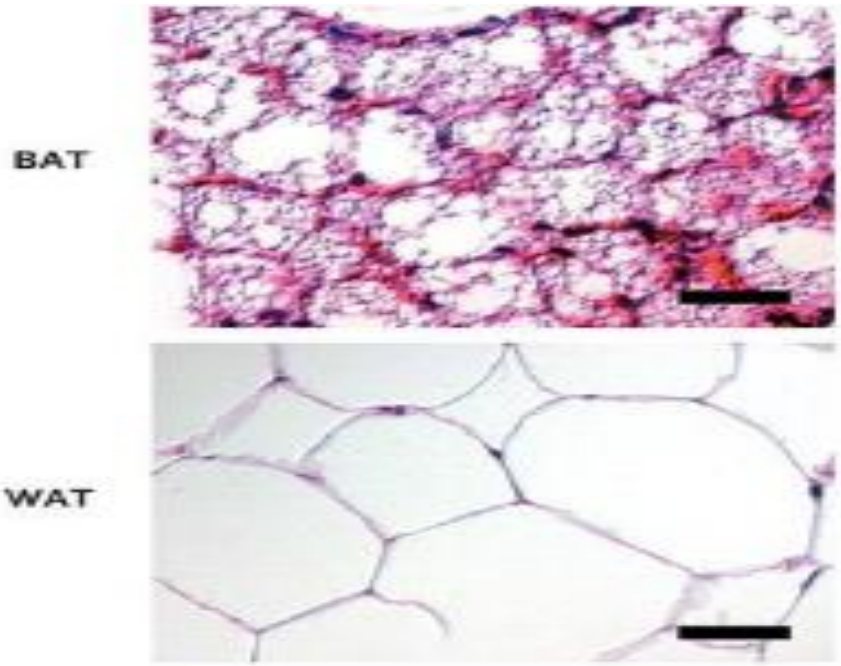


Figure 2. Different types of adipose tissue (Virtanen et al, 2009)

1.1.1.1. White Adipose Tissue

White adipose tissue stores highly energetic molecules. Therefore, WAT is the important energy storage site in mammals and birds (Trayhurn & Wood, 2004). In addition, WAT is responsible for secreting some factors which affect the metabolism for example leptin. One of the main function of the leptin is to affect eating function (Zhang et al, 1994). The accumulation of beta-3-adrenoceptors causes the transformation of white adipose tissue into brown adipose tissue phenotype. This is called transdifferentiaon. This event makes a significant contribution to treatment of diabetes and obesity in rats (Cinti, 2008).

1.1.1.2. Brown Adipose Tissue

Brown adipose tissue is found around the large blood vessels of thorax and neck mainly in newborns, hibernating mammals, migratory birds and rodents. The role of brown adipose tissue is different than white adipose tissue. Predominantly, BAT has role in thermogenesis. Furthermore, BAT preserves against diabetes and obesity (Cannon & Nedergaard, 2004; Rosen and Spiegelman, 2000; Klaus et al, 1991).

1.1.2. Adipocytes

The main constituent of adipose tissue is the adipocytes. The major role of adipocytes is to store energy as a fat. Adipocytes store fat in the form of triglycerides (TG). Moreover, adipocytes have role in thermogenesis, neuroendocrine function and feeding (Ahima, 2006).

The precursor of adipocytes are pre-adipocytes. Because adipocytes store energy as fat, they have role in satiety. Furthermore, fatty acids have many functions in the body. They are found in membrane and they can affect the fluidity of it. In addition, they have a role in channel and receptor function. In obesity, the over accumulation and storage of fatty acid are seen (Otto & Lane, 2005; Duplus et al, 2000; Garaulet et al, 2006).

Small adipocytes are sensitive to insulin, however, large adipocytes are resistant to insulin. When small adipocytes become larger, they procure hyperlipolytic feature. Therefore, insulin cannot affect large adipocytes. This causes the obesity (Freedland, 2004; Rajala & Scherer, 2003).

1.1.3. Types of Abdominal Adipocytes

Abdominal obesity is related to decrease pulmonary function. In obesity, especially the abdominal obesity, distrubition of regional fat has more significant role than distrubition of overall fat. Anatomically, there are two types of abdominal adipocytes. These are subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) (Park et al, 2011). In adults, the ratio SAT and VAT is different. The ratio of SAT is roughly 80%, however the ratio of VAT is approximately 10-20% of total body fat (Abate et al, 1995). Their metabolic function, insulin response, lipolytic activity and adipocytes types are different. For instance, the metabolic activity of VAT is higher than SAT. Excessive accumulation of VAT is closely associated to many diseases such as type 2 diabetes, insulin resistance and dyslipidemia (Park et al, 2011; Freedland, 2004).

1.1.3.1. Visceral Adipose Tissue (VAT)

Visceral adipose tissue (VAT) is intensively found in the abdominal viscera in body cavity underneath the abdominal muscles. VAT ratio is approximately 10-20% in men and in women, VAT ratio is 5-8%. The amount of VAT is affected and changed due to age (Freedland, 2004). There are many differences between VAT and SAT. the metabolic activity of VAT is higher than the metabolic activity of SAT. Furthermore, lipolytic activity of VAT is higher than the lipolytic activity of SAT. VAT also has a significant role in secretion of hormones which have role in some metabolic diseases, for example obesity (Ibrahim, 2010; Freedland, 2004; Wajchenberg, 2000).

1.1.3.2. Subcutaneous Adipose Tissue (SAT)

Subcutaneous adipose tissue (SAT) is found directly under the skin and upper side of the muscular system. In the body, the fat which are located in the lower part are constitute by subcutaneous adipocytes (Wajchenberg, 2000; Montague & O'Rahilly, 2000). SAT provides the insulation of heat in the body. In addition, SAT encloses inetrnal organs so it protects the internal organs. High caloric diet and physical inactivity cause the excess accumulation of subcutaneous fat. It acts as an buffer and stores excess glycerol and free fatty acids (FFAs) as triglycerides (Ibrahim, 2010; Frayn, 2002). This protects the non-adipose tissue from excess accumulation of FFAs and TGs. Therefore, organism can be protected from metabolic diseases such as type 2 diabetes. However, if there are too much lipid accumulation in subcutaneous adipocytes, they become functionless (Frayn, 2002; Freedland, 2004; Hajer et al, 2008).

1.1.4. SCAT and VAT Differences

1.1.4.1. Anatomical Differences

SAT is mainly found in abdominal wall, especially anterior and back part. The amount of SAT is very high in the body. Approximately, 80% of body fat is found in this area (Ibrahim, 2010; Wajchenberg, 2000). However, VAT ratio in body is 10-20% in men. The ratio VAT is less in women than the VAT ratio in men. In women, VAT ratio is 5-8%. In addition to gender, age also affect the VAT amount. It increases due to age in both women and men (Wajchenberg, 2000). Moreover, portal drainage of VAT and SAT are different because of their anatomical position. Venous drainage of VAT goes liver through portal vein but venous drainage of SAT goes systemic vein (Ibrahim, 2010).

1.1.4.2. Cellular Differences

There are many differences between SAT and VAT. Their adipocytes, lipolytic activity, endocrine function and insulin response are different. VAT adipocytes are large. They are more dysfunctional than SAT adipocytes. SAT adipocytes are smaller than VAT adipocytes. They are sensitive to insulin. They store free fatty acids and triglycerides much more than VAT adipocytes so this prevent excees accumulation of free fatty acids and triglycerides in non-adipose tissue. (Marin et al, 1992; Misra & Vikram, 2003). SAT has more correlation to BMI used in obesity diagnosis than VAT. In addition, both SAT adipocytes and VAT adipocytes have role in blood pressure regulation (Ibrahim, 2010).

1.2. Obesity

Overweight and especially obesity are major and important health problems all over the world. The number of obesity cases has become very serious. According to *World Health Organization* (WHO) 2013 report, because of obese or overweight, approximately 2.8 million people die in each year. Obesity, at past, is the serious problem of developing countries or high income countries, but now, it is also important and serious problems for low and middle income countries (WHO, 2013).

Obesity is defined that excess fat has accumulated in the body and this cause the negative effect on person's health. By using body mass index value (BMI), we consider the degree of obesity. BMI value can be found by a simple calculation. Person's weight is divided by the square of person's height (Figure 3) (Adams & Murphy, 2000).

$$BMI = \frac{\text{weight (lb)} * 703}{\text{height}^2\ (\text{in}^2)}$$

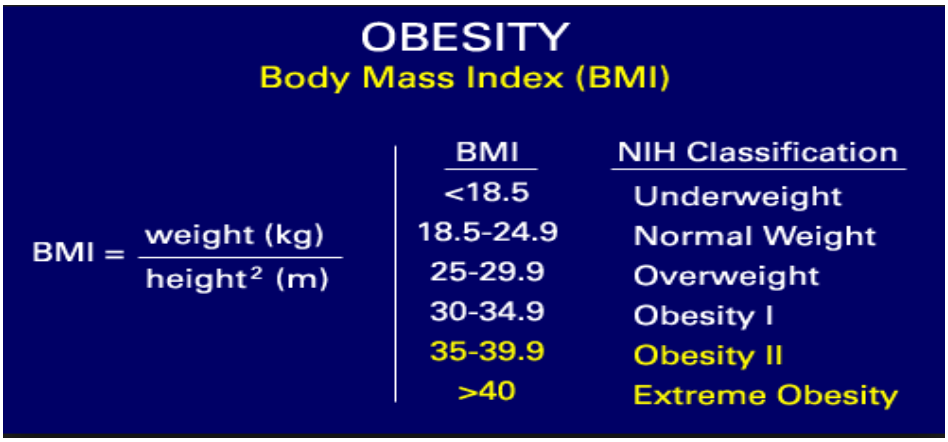
OR

$$BMI = \frac{\text{weight (kg)}}{\text{height}^2\ (\text{m}^2)} \quad \text{(metric)}$$

Figure 3. Calculation of BMI Value
(Retrieved from http://www.feelforfit.com/physiology/how-to-calculate-my-body-mass-index)

If BMI value is below 18.5, we consider that person is in underweight. If BMI value is between 18.5 and 24.9, we consider that person is in normal weight. If BMI value is between 25 and 29.9, we consider that person is overweight. If BMI value is between 30 and 34.9, we consider that person is obese. If BMI value is higher than 40, we consider that person is extreme obese (Table 1).

Table 1. Classification of BMI Value



(Retrieved from http://www.calculatebmi.co/&docid=scjJMtNnc1jZxM&imgurl)

High energy intake and less energy consumed such as lack of physical activity cause the obesity. In addition to energy imbalance, genetic tendency and some other external factors such as environmental factors are important factors in obesity (Khandelwal et al, 2010).

Leptin is an important hormone and marker for obesity. Fat cells produce leptin hormone. One of the main function of the leptin is to affect eating function. High level of leptin in the circulation system shows the leptin resistance. Researches show that leptin resitance is one of the reason of obesity (Martin et al, 2008; Hinuy et al,2006; Yang & Barouch, 2013).

Obesity causes some diseases such as hyperlipidemia, hypertension, insulin resistance, cardiovascular diseases and many others (Figure 4).

Figure 4. Diseases Related with Obesity (Gupta, 2011)

1.3. Gender and Obesity

Although the effect of gender in obesity has been carried out several times, it has not been determined exactly. Several studies have been done to understand the difference in distribution of body fat (Nguyen et al, 1996), metabolic rate (Henderson et al, 2008), energy consumption (Catala-Niell et al, 2008), physical activity and sex hormones (Rodriguez-Cuenca et al, 2002). These studies have demonstrated that males have higher risk to be obese than females (Erem et al, 2004; Hong et al, 2009). However, there are some reverse results in literature through nutritional strain and state of female and male aniamls (Kautzky &Willer, 2013; Justo et al, 2005). In addition, rate of energy consumption is higher in females than in males because thermogenic activity is higher in females (Valle et al, 2005).

1.4. BFMI: Obesity Mouse Models

Scientists use the animal models such as mouse to research and analyze the diseases and to find treatment. For obesity, there are several animal models that have been used so far such as Zucker rats, leptin deficient and Cav1 knockout mice and etc. (Speakman et al, 2007; Turban et al 2002; Zhang et al, 1994; Park et al, 2002; Razani et al, 2001).

In this study, Berlin fat mouse inbred (BFMI) is used as animal model to research the obesity. Although BFMI mouse are nourished with high fat diet, type II diabetes are not developed (Hantschel et al, 2011). BFMI mice have high fat content. In addition, there is no mutation in BFMI lines. Moreover, they can be affected by the severe obesity (Wagener et al, 2006).

1.5. Lipids

Lipids are group of hydrophobic naturally occurring compound. They contain very large and diverse group such as simple lipids, phospholipids, steroids like cholesterol, sphingolipids and waxes. The difference between lipids and other naturally occurring compound is that lipids can dissolve in nonpolar organic solvent such as chloroform, hydrocarbons, ethers and alcohols. They also cannot dissolve in water. Lipids have many important functions in the body. They are energy source of the body. They also join the membrane structure (Christie, 1987).

1.5.1. Triglyceride

Triglyceride (triacylglyceride or triacylglycerol) is a simple lipid. It has a glycerol that bound to three fatty acids (Figure 5 and 6). Triglyceride has role in metabolism. It can be synthesized by liver and fat cells. In addition, it can be stored in liver. High level of triglyceride is related to some important diseases such as, insulin resistance and obesity.

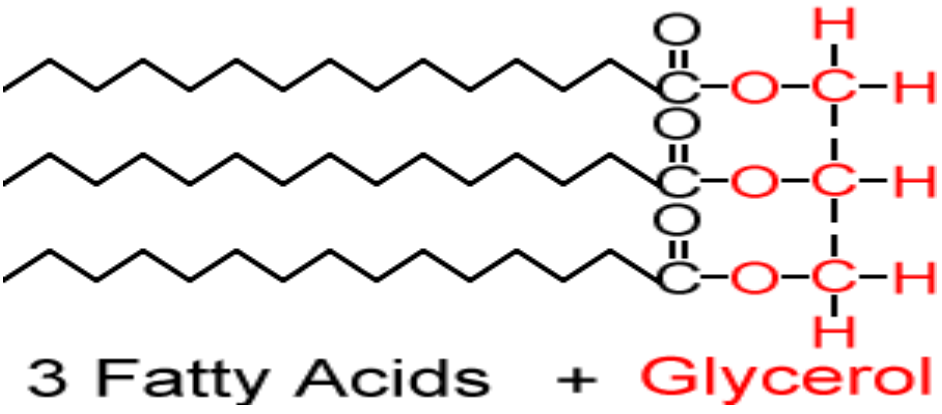


Figure 5. Structure of Triglyceride

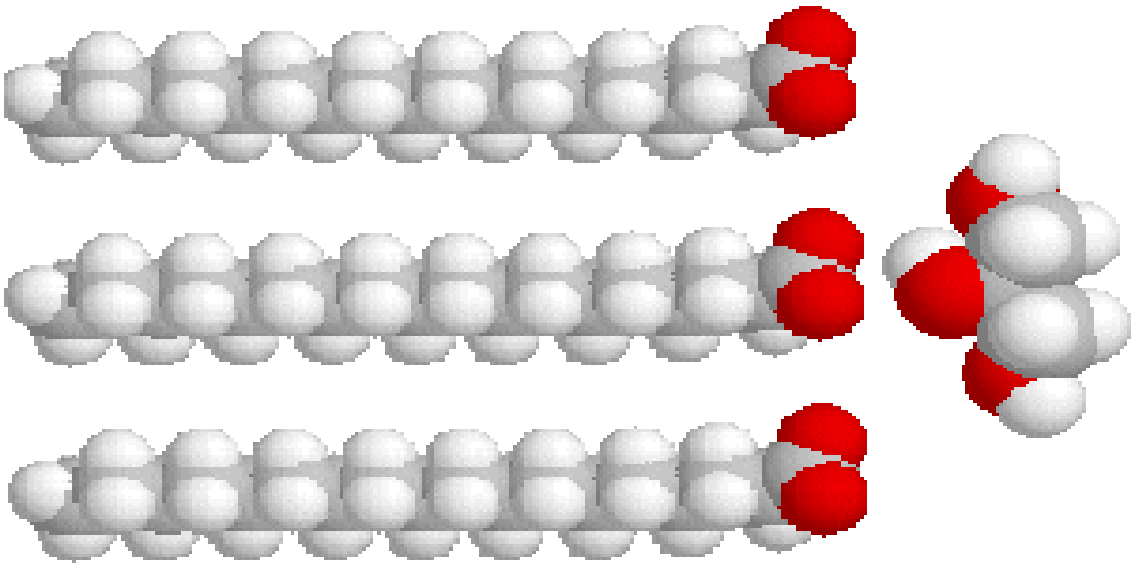


Figure 6. 3D Structure of Triglyceride
(Retrieved from http://bioweb.wku.edu/courses/BIOL115/Wyatt/Biochem/Lipid/triglyceride)

1.5.2. Diglyceride

Diglyceride or a diacyl-glycerol (DAG) has glycerol that bound to two fatty acids (Figure 7). It can be used as food additives, emulsifying agents and flour treatment. It can be found in chocolate products, ice cream and bakery products.

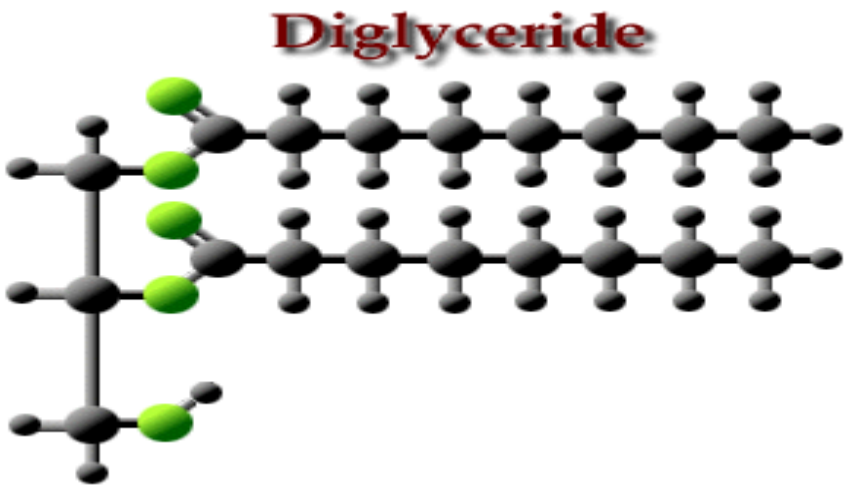


Figure 7. Structure of Diglyceride
(Retrieved from http://www.brooklyn.cuny.edu/bc/ahp/SDgraphics/PSgraphics/SD.PS.LG.Diglyceride)

1.5.3. Cholesterol

Cholesterol is an important member of lipid (Figure 8). It is one of the sterol lipid. Cholesterol can be synthesized in cell. One of the main roles of cholesterol is to have role in synthesizing steroid hormones (precursor of steroid hormones) such as estrogen (estradiol), androgen (testosterone), progesterone, aldesteron and cortisol. Moreover, it is found in the structure of cell membrane. In addition, it has role in membrane fluidity (Hu et al, 2010; Christie, 1987). The level of cholesterol is important. For example, increasing the cholesterol level causes some diseases such as atherosclerosis and insulin resistance (Pike, 2009; Gustavsson et al, 1999).

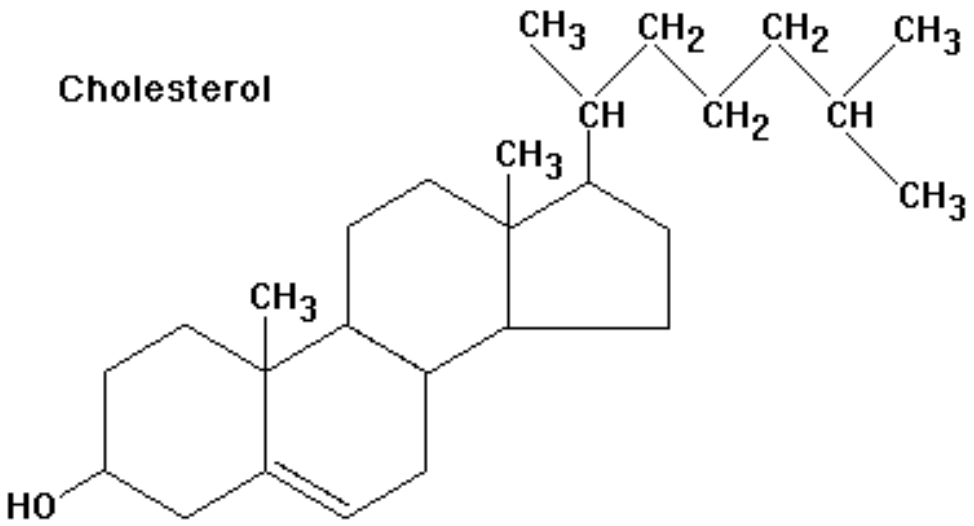


Figure 8. Structure of Cholesterol
(Retrieved from http://www.raw-milk-facts.com/images/Cholesterol)

1.5.4. Monoglyceride

Monoglyceride, also labeled as monoacyglycerol has glycerol that bound to one fatty acid chain (Figure 9). It is procured from animal fats or plant oil. Furthermore, it can be produced synthetically. In addition, it can be obtained by breaking down the triglyceride. It is added in the food product such as gum, baked goods and ice cream for emulsifying (Christie, 1987; Dinh et al, 2002).

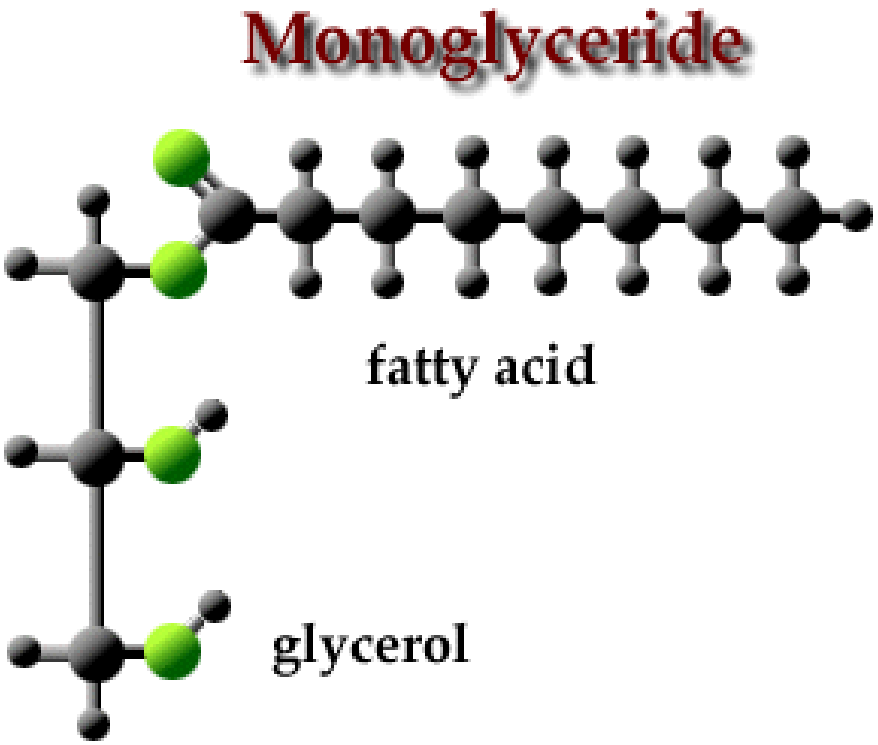


Figure 9. Structure of Monoglyceride
(Retrieved from http://www.brooklyn.cuny.edu/bc/ahp/SDgraphics/PSgraphics/SD.PS.LG. Monoglyceride)

1.5.5. Phosphatidylcholine

Phosphatidylcholine is one of the most abundant phospholipid in eukaryotes. Glycerol, phosphate group, choline head group and two fatty acid chain are found in the structure of phosphatidylcholine (Figure 10). It is one of the major components of cell membrane of plants and animals. It has role in membrane integrity and cell signaling. Moreover, it can be used to synthesize many molecules. It generally found in eggs, mustard, soybeans and sunflower (Gibellini & Smith, 2010; Pasenkiewicz-Gierul et al, 2000).

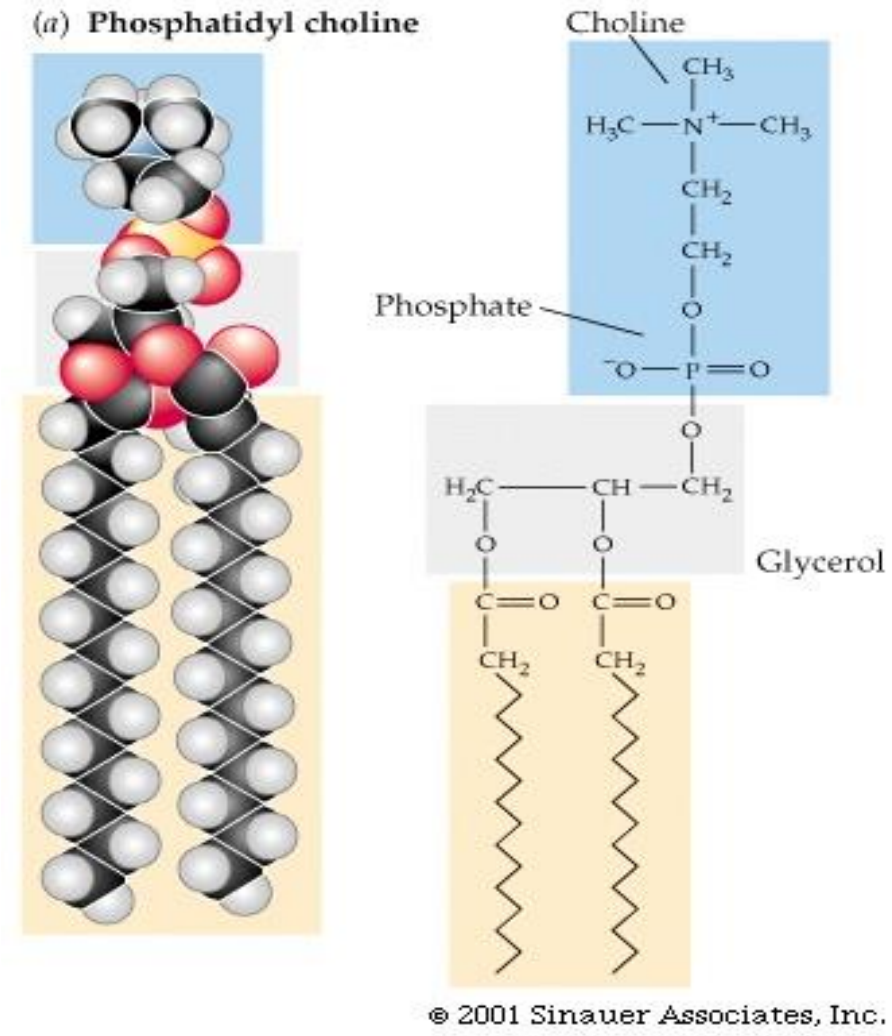


Figure 10. Structure of Phosphatidylcholine
(Retrieved from http://www.columbia.edu/cu/biology/courses/c2005/lectures/lec3_10.pdf)

1.6. Chromatography

Chromatography is an important physical method that separate and analyze complex mixtures. There are two phases and a complex mixture is scattered between them. One of the two phases is a stationary phase. Molecules of interest are adhered in stationary phase. The other phase is a mobile phase. Molecules of interest are dissolved in mobile phase and mobile phase supply the flow of mixture of complex through stationary bed (Homan & Anderson, 1998).

It is the first time that chromatography was developed and used in plant research to separate pigments in 1903 by Mikhail Tswett who is a Russian botanist (Ditz, 2012). After

that, chromatography experiment has been developed and many molecules, such as proteins, vitamins and lipids, can be analyzed by using chromatography techniques.

According to molecules that used in experiment, there are several chromatographic techniques. Some of them are paper, adsorption, gas, affinity, ion-exchange and high performance liquid chromatography.

1.6.1 High Performance Liquid Chromatography (HPLC)

High performance (or pressure) liquid chromatography (HPLC) is one of the chromatographic techniques used to separate the mixture component and then identify and quantify these components (Homan & Anderson, 1998; Moreau, 2006).

There are two phases in HPLC techniques. One is mobile phase and the other is stationary phase. Mobile phase flows with high pressure during stationary phase (Figure 11). When mixture flows during the stationary phase, components of mixture are resolved. In this process, resolution rate, interaction degree with components and stationary bed are very important.

There are several parameters that affect the HPLC analysis results such as peak area and retention time. Solvents which are used in mobile phase, HPLC and method, which used in experiment, parameters and HPLC detector used in experiment affect the retention time and peak area.

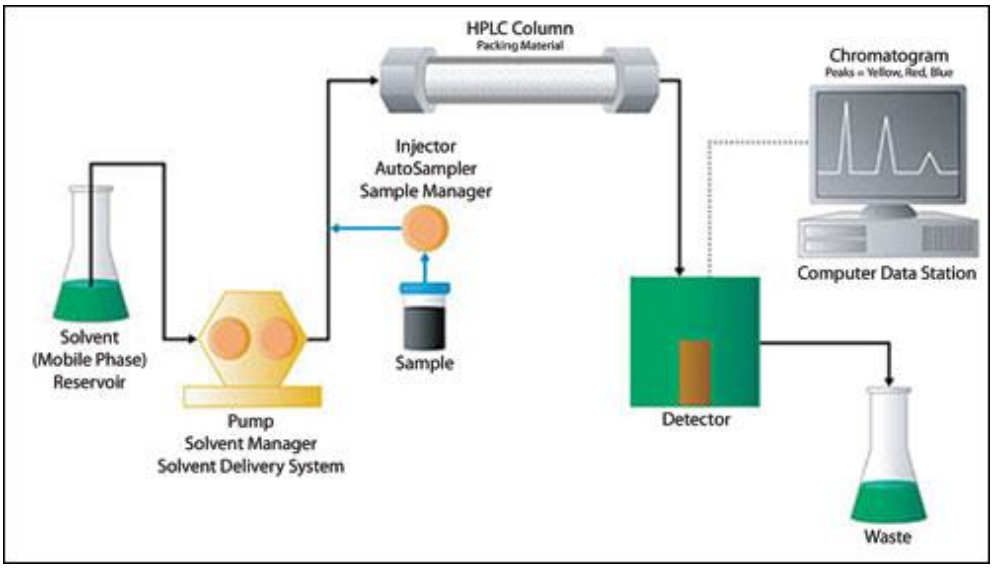


Figure 11. Parts of HPLC
(Retrieved from http://www.comsol.com/stories/waters_corp_hplc_systems)

Analyzing and detecting lipids are very important to analyze several diseases such as obesity, insulin resistance and type II diabetes. HPLC is useful tools to detect, separate and analyze the lipids (Seppanen-Laakso et al, 2001).

1.6.2. Evaporative Light Scattering (ELS) Detectors

ELSD detector is universal and useful detectors. After solvent or mobile phase evaporates, sample particles which are interested in experiment are measured by using light which scattered from sample particles.

ELSD does not need the sample optical properties for detection unlike UV detectors. Compounds which used in experiment must be less volatile than current solvent. It does not need the chromophores to detect sample particles. Therefore, lipids, polymers, saccharides, amino acids, surface active compounds, oil and small molecules can be detected by using ELSD. ELSD gives response to quantity so it is used to quantitate the sample particles which used in experiment. Gradient can be easily used with this detector. Some detectors such as refractive index (RI) detectors depend on temperature but ELSD does not. For evaporation and nebulization of solvent, gas generator is required. According to sample particles and solvent which is used in experiment, pressure air or nitrogen can be used (Rodriguez-Alcala & Fontecha, 2010; Torres et al, 2005; Nordback et al, 1998).

1.6.2.1. ELSD Working Principle

There are generally three steps in ELSD (Figure 12). The first step is nebulization step. Nebulization of mobile phase is occurred in here. Nebulized mobile phase turns to aerosol. Then, it goes into drift tube with pure nitrogen which is used as nebulizer gas in this work. Second step is evaporation. In drift tube which is heated, aerosol is evaporated completely. Remainders are just sample particles that used in study. The final step is detection. Sample particles go out the drift tube and go into light beam. In light beam, by using scattered light of laser diode or photomultiplier tube, detection is occurred (Christie, 1987).

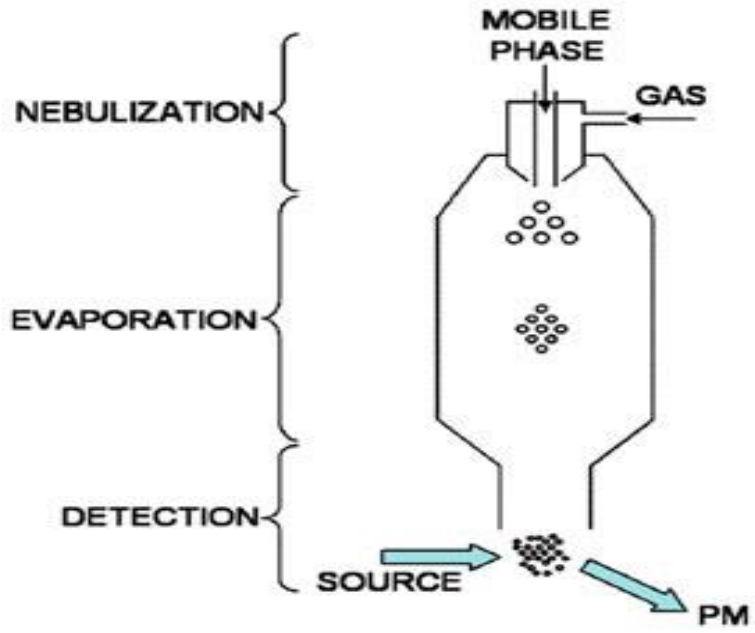


Figure 12. Running Steps of ELSD
(Retrieved from http://www.americanlaboratory.com/914-Application-Notes/19182-Optimizing-the-Sensitivity-of-an-Evaporative-Light-Scattering-Detector)

After detection, to analyze the data calibration curves are made from injected standards. To make calibration curve, different concentration of standards are injected. Quadratic equation is used for calibration curves.

$y = A + Bx + Cx^2$

In this equation, y represents the response of standard peak, x represents concentration or amount of standards. A, B and C are the coefficients.

To use the drawn calibration curve, coefficient of determination value (R^2) is important. It should be 0.99 or higher. If it is lower than this value, calibration curve is not used to calculate sample particles.

1.7. Aim of the Study

Aim of this study is to research obesity effect on the lipid profiles of the different adipose tissues from 10 week old Berlin Inbred (BFMI) mouse lines. BFMI lines demonstrate spontaneous obesity so these lines have contribution to investigation of spontaneous obesity with standard breeding diet (SBD). Spontaneous obesity arises in BFMI lines even they were fed standard breeding diet. In addition to obesity, they have insulin sensitivity. Since adipose tissue is important in metabolism and metabolic diseases such as obesity, type II diabetes and insulin resistance which affect the adipose tissue, lipid levels and profiles were analyzed by using HPLC. HPLC are used to determine lipid profiles of BFMI lines; BFMI 852, BFMI 856, BFMI 860 and BFMI 861. In addition, the difference of lipid profiles between two adipose tissues; visceral (VAT) or gonadal adipose tissue (GF) and subcutaneous (SAT) or inguinal adipose tissue (IF) which belong to different sex is investigated.

CHAPTER 2

MATERIALS AND METHODS

2.1. Animal Model

German Animal Welfare Authorities (approval no: G0171/10) approved whole experimental protocols for animal treatment. Animals that are found in BFMI lines were bought originally from random pet shops in Berlin, Germany.

Chosen animals had low protein content. In second selection, animals were selected according to high fat content and low body weight. In final selection, animals were selected according to high adiposity phenotype. By using these animals, after 58 generation BFMI lines (BFMI 852, BFMI 856, BFMI 860 and BFMI 861) were generated (Wagener et al, 2006).

Animals lived under conventional provision and controlled lighting with a 12:12h light/dark cycle at 22±°C and 65% humidity. All animals had ad libitum reach to food and water. When animals became 3 weeks old, they were fed with standard breeding diet (SBD) (V1534-000, ssniff R/M-H, Ssniff Spezialdiäten GmbH, Soest/Germany). It has 12.8 MJ/kg of metabolized energy. The major component of SBD is carbohydrate. The percentage of carbohydrates is 58%. In addition to carbohydrates, it contains 33% of proteins and 9% of fat (Hantschel et al., 2011).

2.2. Sample Collection

Gonadal fat (GF) and inguinal fat (IF) were taken from 10 weeks old mice. Then, dissected tissues were put into plastic tubes. Plastic tubes were snap-frozen in liquid nitrogen. After that, plastic tubes were stored at -80°C.

2.3. Methods

2.3.1. Lipid Extraction

Lipid extraction from BFMI adipose tissues were done by using Folch method (Folch et al, 1957). 100±5mg adipose tissues were taken from stock. Chloroform and methanol (2:1, v/v) were added (amount of chloroform/methanol was 20 ml for 1000 mg tissue). Then, tissues were homogenized by ULTRA-TURRAX T25 (IKA-Werke GmbH&CO, Staufen, Germany). The speed of ULTRA-TURRAX is 13000 1/minutes. Homogenized tissue was put into test tube and then it was shaked for 30 minutes, at 150 rpm and at room

temperature in orbital shaker. After that, filtration was done to get rid of non-homogenized particles. After filtration, 0.9% NaCl solution was added (amount of NaCl was 4 ml for 20 ml chloroform/methanol). Vortexing was done for 1 minute. Afterwards, centrifugation was performed at 5000 rpm and at room temperature for five minutes. Upper part of test tube was removed after centrifugation. Lower part of test tube was dried under gentle nitrogen gas and stored at - 20°C.

2.3.2. HPLC Studies

For HPLC studies, methods which tried according to literature were failed to separate lipids (Perona & Ruiz-Gutierrez, 2005; Descalzo et al, 2003). Therefore, Olsson method was rearranged and improved for better separation (Olsson et al, 2012; Restuccia et al, 2011).

HPLC analysis was performed by using Water Alliance e2695 Seperations Module and Waters 2424 ELS detector (Waters, Milford, USA). In addition, in analysis Agilent Polaris-Si (150 x 4.6 mm, 3µm particle size) column was used (Agilent Technologies, Santa Clara, USA). Before starting running, column was washed 2 hours with solvents which used in experiment to calibrate the column (http://www.nestgrp.com/pdf/colcare.pdf). To make condition, only solvents which used in method were run four times. Stroke volume was fit 50 µl. Column temperature was adjusted 22°C and sample temperature was 25°C. Binary gradient elution was used and the change of it was fit linear (Figure 13).

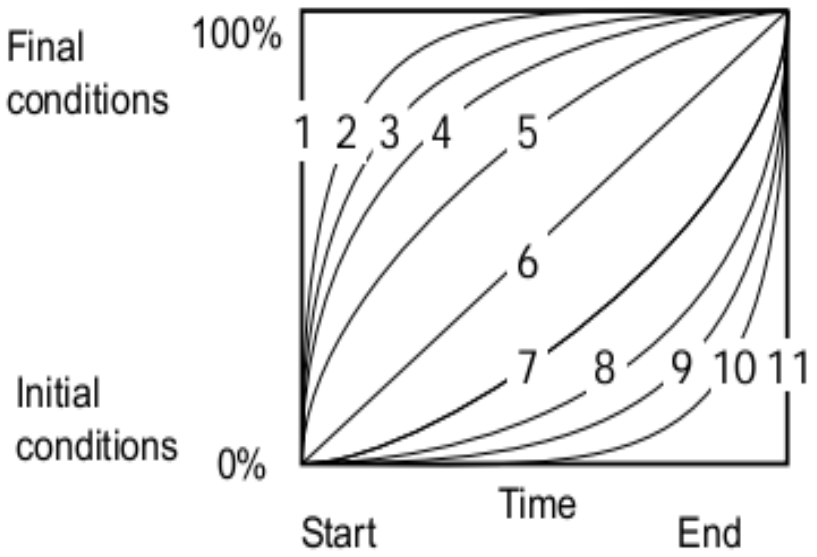


Figure 13. Visual Model of Gradient Curve (Waters e2695 Seperations Module Operator's Guide)

Binary gradient elution consisted of two solvent. Solvent A was n-Hexane. Solvent B composed of isopropanol (IPA), methanol (MeOH), acetic acid (AA) and triethlyamine (TEA) with the ratio 60:40:0.2:0.1 by weight. The gradient of solvents demonstrated in Table 2. First 3 minutes, 100% of solvent A flowed. Then, next 3 minutes solvent A became 80% and decreased 60% in 16 minutes. It decreased 0% in 18 minutes and increased 90% in 26 minutes. Then, solvent A became again 100% in 28 minutes (Olsson et al, 2012; Restuccia et al, 2011). In total, for one sample the time of run was 30 minutes. Flow rate of run is also different according to time. First 3 minutes, it was 1.20 ml/min. Next three minutes, flow rate became 1.00 ml/min. After that, flow rate became 0.80 ml/min last 24 minutes. Gas pressure was adjusted to 60 psi. Nebulizer mode was selected to cooling. Temperature of drift tube was adjusted to 70°C.

Table 2. Binary Gradient Table of Solvents

Time (min)	Flow (ml/min)	A (%)	B (%)	Curve
0	1.20	100.0	0.0	6
3.0	1.00	100.0	0.0	6
6.0	0.80	80.0	20.0	6
16.0	0.80	60.0	40.0	6
18.0	0.80	0.0	100.0	6
23.0	0.80	0.0	100.0	6
26.0	0.80	90.0	10.0	6
28.0	0.80	100.0	0.0	6

Samples and standards were dissolved in 2:1 chloroform/methanol mixture. For each sample and standard, two replicate tubes and for each tube three run were performed. So, in total, six run were performed for each sample and standard. The average results were taken from these replicates. Calibration and data analysis were performed by Empower 2 Chromatography Data Software (Waters, Milford, USA). Calibration curves were used for calibration. For calibration curves, five different molarity standards were prepared and run.

2.4. Statistical Analysis

Results were stated as SEM (mean±standard error of mean). First of all, ANOVA test was performed to all BFMI lines. And then, Tukey's test was performed to get the significant difference of one group to another. To say that results were significant, p value was used. If p value is equal or less than 0.05, results are statistically significant. Degree of p value is $p<0.05$*, $p<0.01$**, $p<0.001$***. Significance of results were shown with *, ¥, # and §, if results were statistically significance relative to BFMI 852, BFMI 856, BFMI 860 and BFMI 861, respectively.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. HPLC Standards

To define and quantify lipid profiles of BFMI lines, different concentrations of standards glyceryl trioleate (triglyceride, TG), 1,2-dioleoyl-sn-glycerol (diglyceride, DG), cholesterol (C), 1-oleyol-rac-glycerol (monoglyceride, MG), phosphatidylcholin (PC) were prepared and run. Retention times of each standard were determined. Table 3 demonstrates retention times of standards. Figures 14-18 show chromatograms of different concentration of each standard.

Table 3. Retention Times of Lipid Standards

Standards	**TG**	**DG**	**C**	**MG**	**PC**
Retention Time (min)	2.0786	3.1458	6.9653	8.9653	16.984

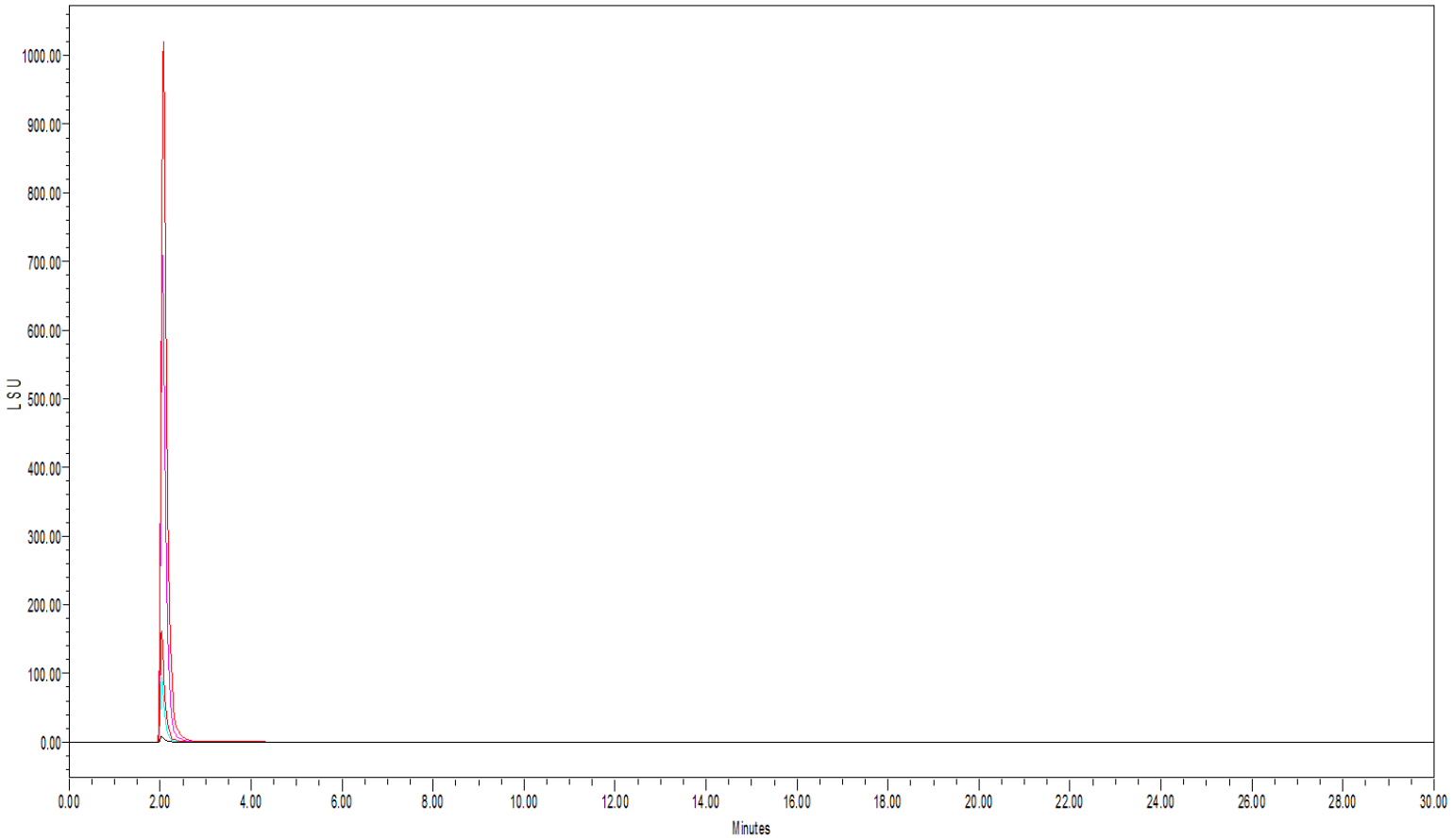


Figure 14. Chromatogram of the Glyceryl Trioleate (triglyceride, TG)

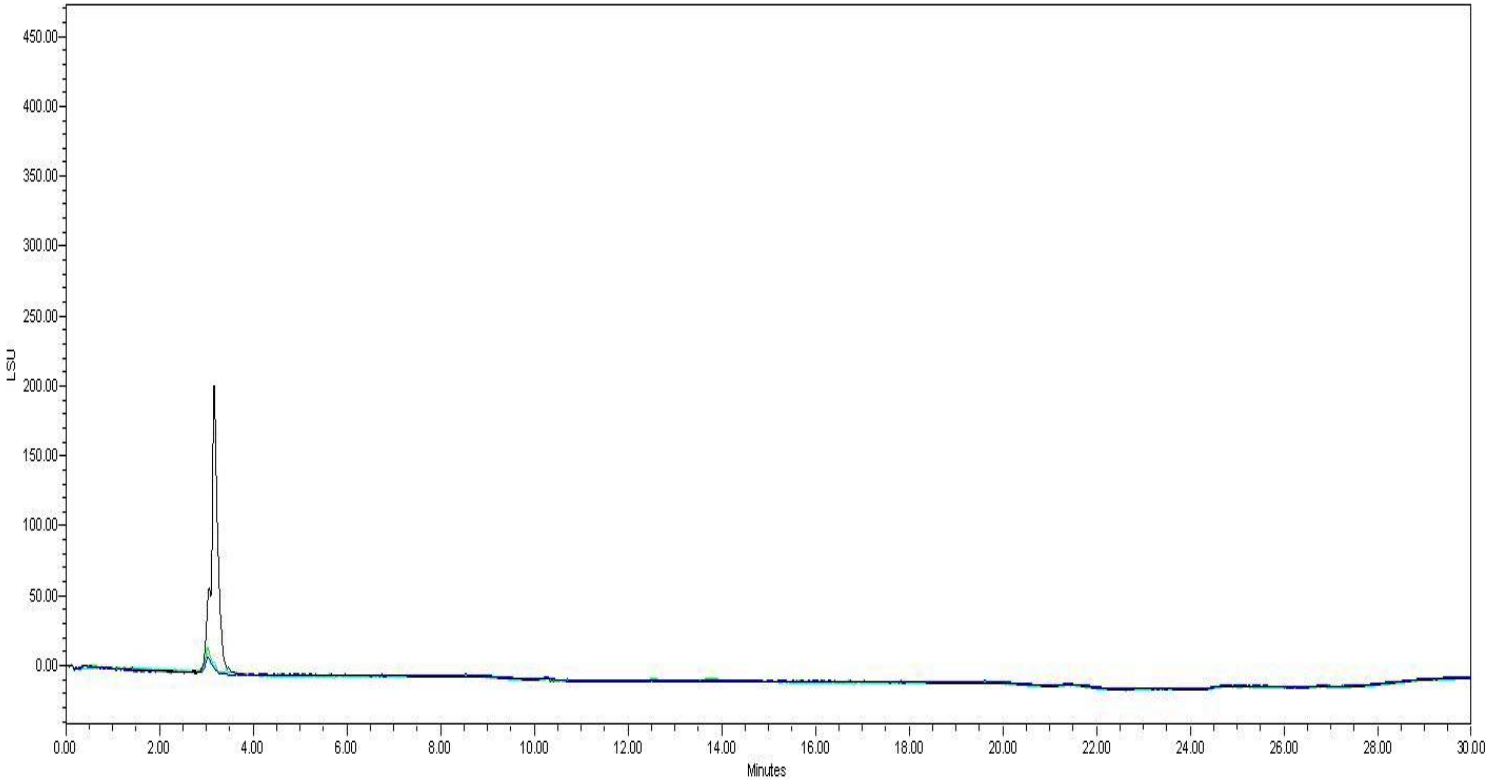


Figure 15. Chromatogram of the 1,2-dioleoyl-sn-glycerol (diglyceride, DG)

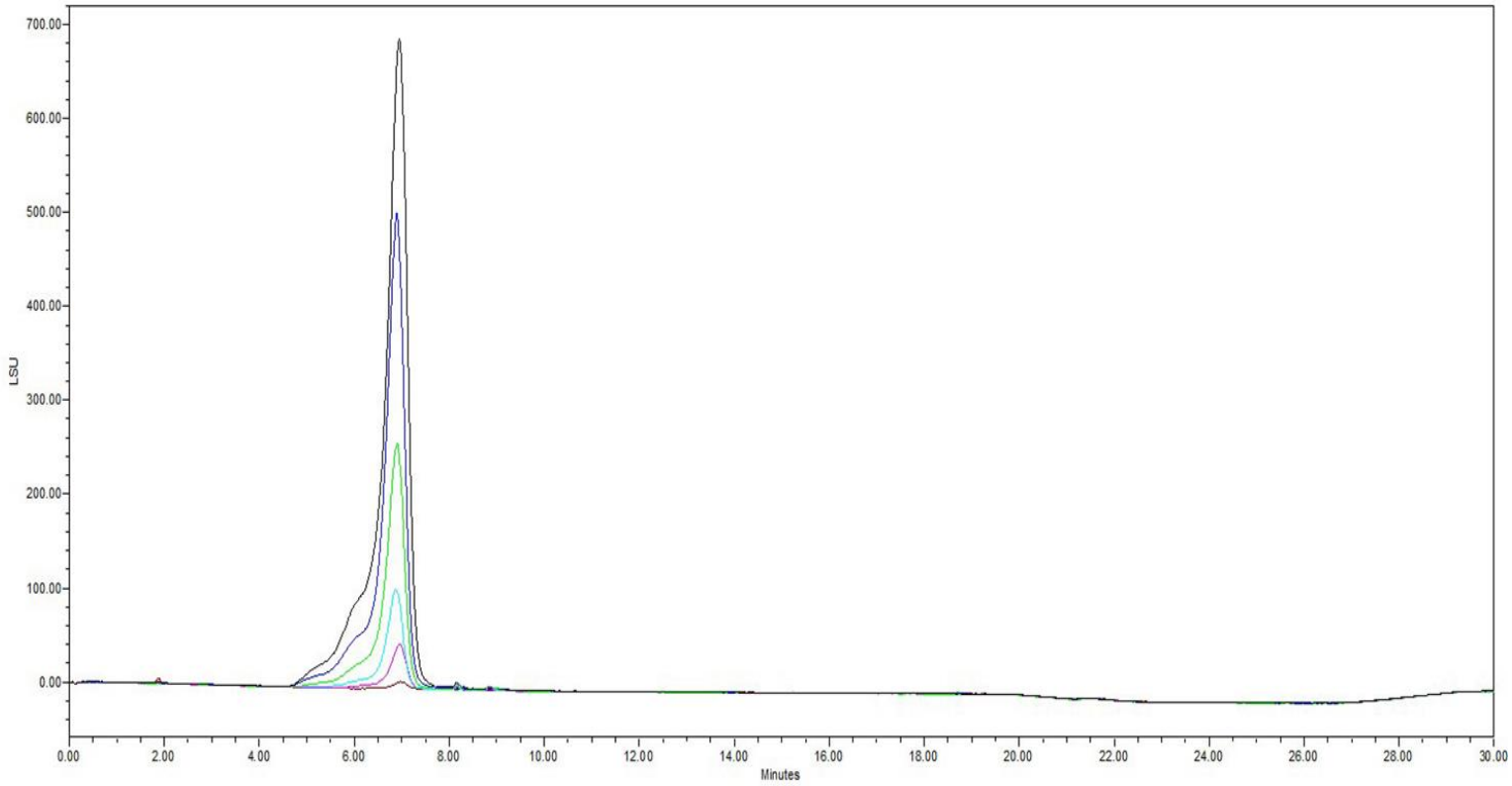


Figure 16. Chromatogram of the Cholesterol (C)

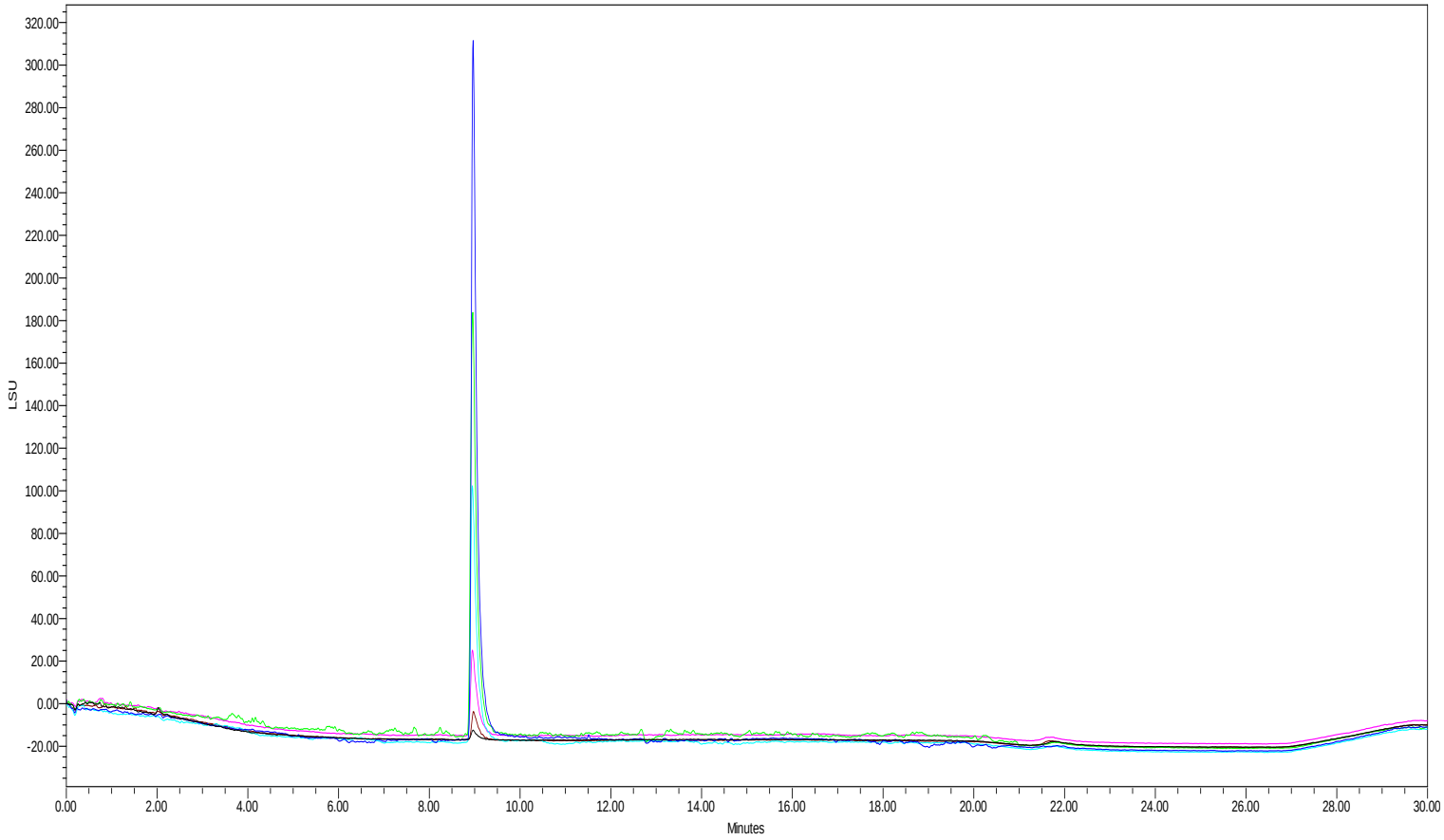


Figure 17. Chromatogram of the 1-oleyol-rac-glycerol (monoglyceride, MG)

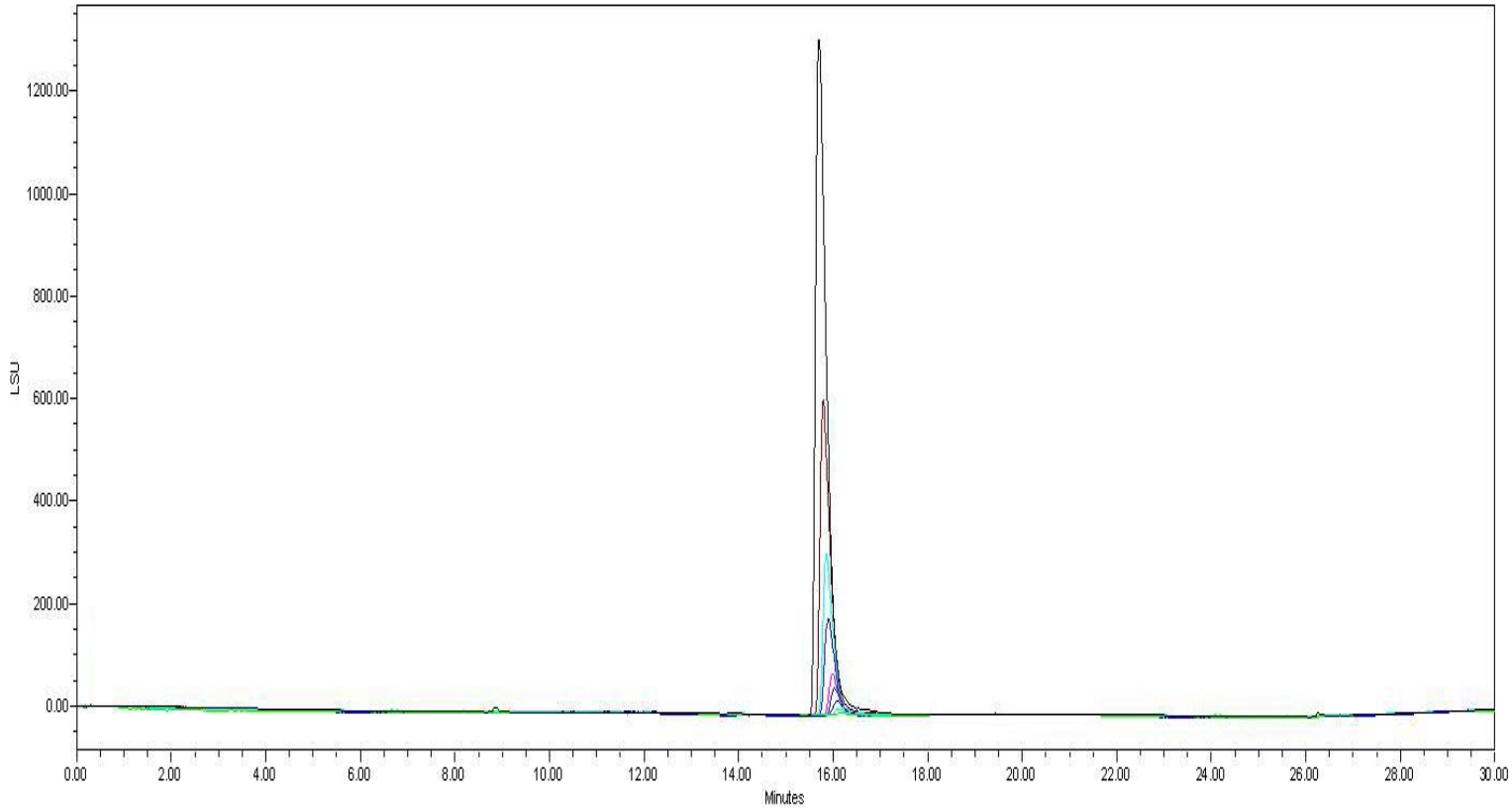


Figure 18. Chromatogram of the Phosphatidylcholin (PC)

3.2. Calibration Curves

Calibration curves of each standard were drawn to quantify BFMI lines lipids from different concentration of standards by using Waters Empower Pro 2 software. Calibration curves of each standard can be seen in Figure 19-23. R^2 of each calibration curve was higher than 0.99 which show the goodness of fit.

For TG calibration curve, five different standard concentrations were used. For DG curve, four different standard concentrations were used. In addition, for C and MG curves, six standard concentrations were used. Finally, for PC curve, nine standard concentrations were used. These numbers were chosen according to BFMI line samples.

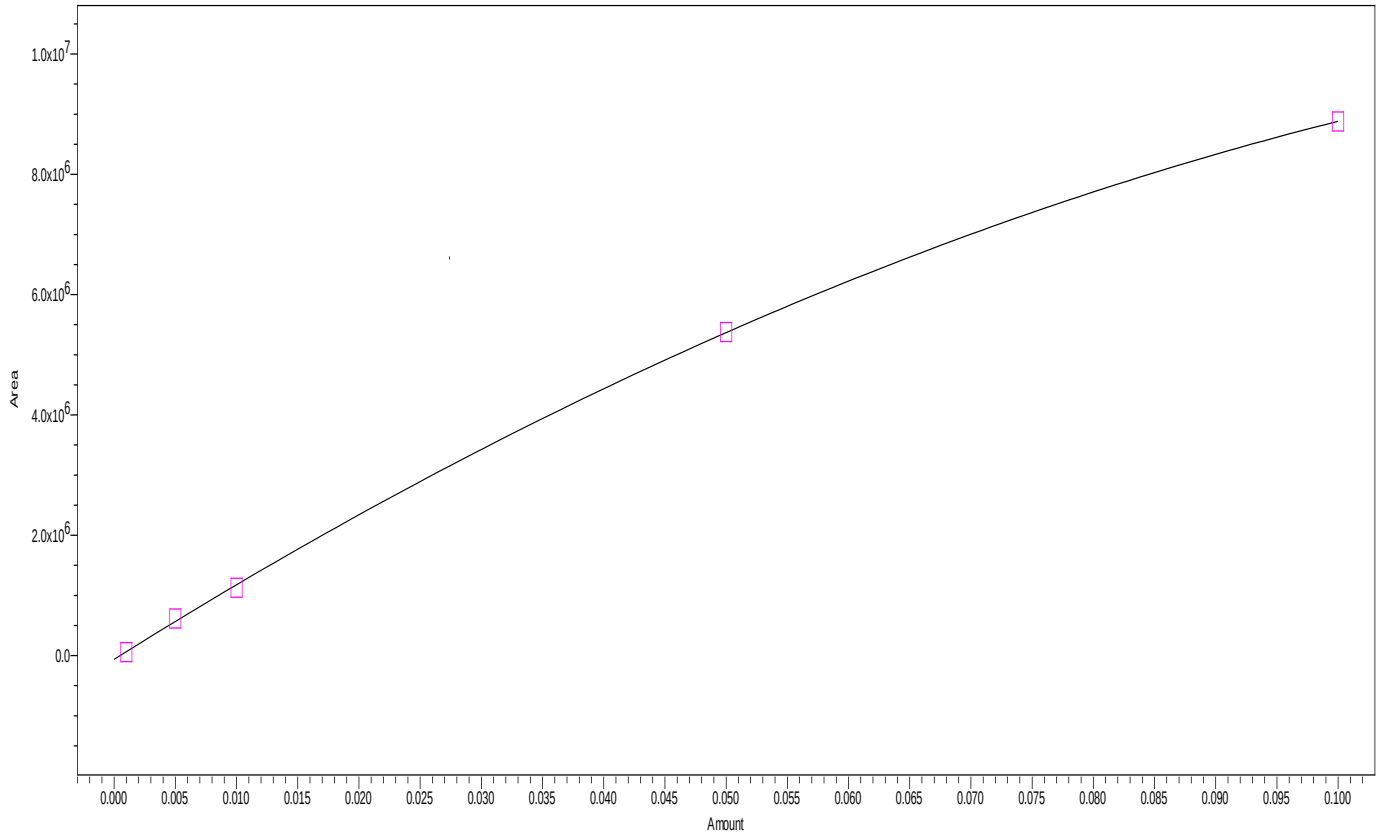


Figure 19. Calibration Curve of Glyceryl Trioleate (triglyceride, TG). Pink squares represent different concentrations of TG standard. R2 value of calibration curve is 0.999916.

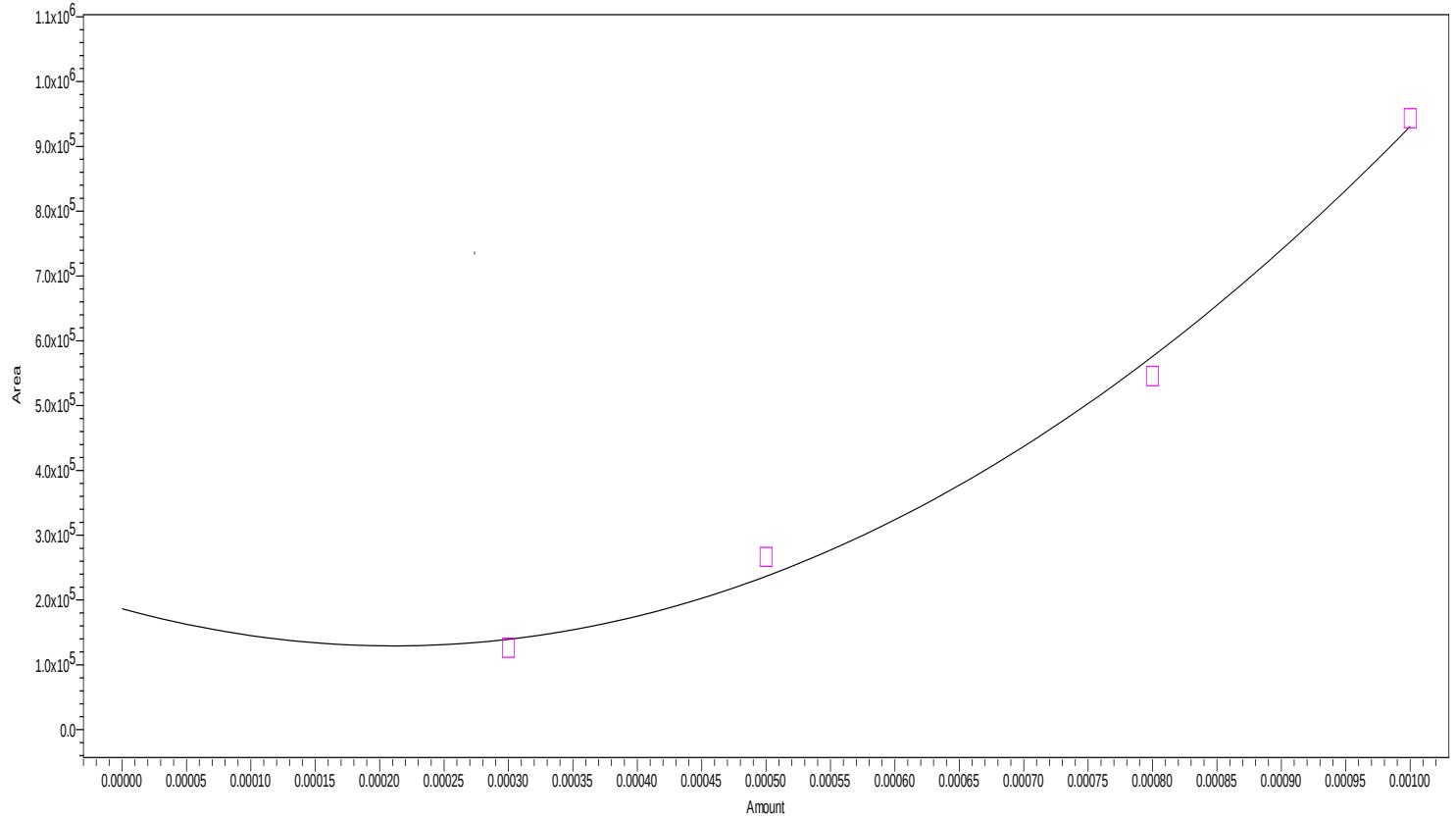


Figure 20. Calibration Curve of 1,2-dioleoyl-sn-glycerol (diglyceride, DG). Pink squares represent different concentrations of TG standard. R2 value of calibration curve is 0.994517.

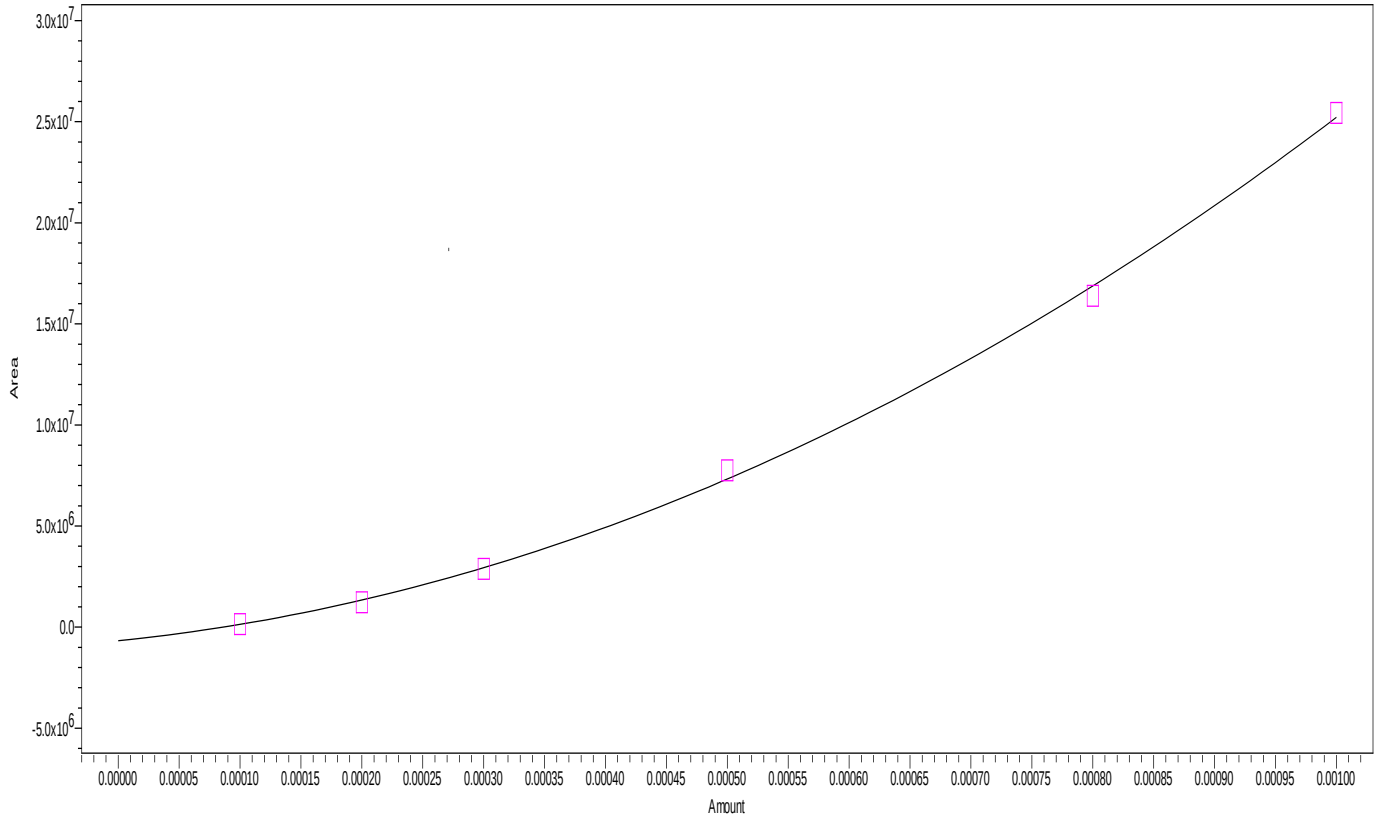


Figure 21. Calibration Curve of Cholesterol (C). Pink squares represent different concentrations of TG standard. R2 value of calibration curve is 0.999048.

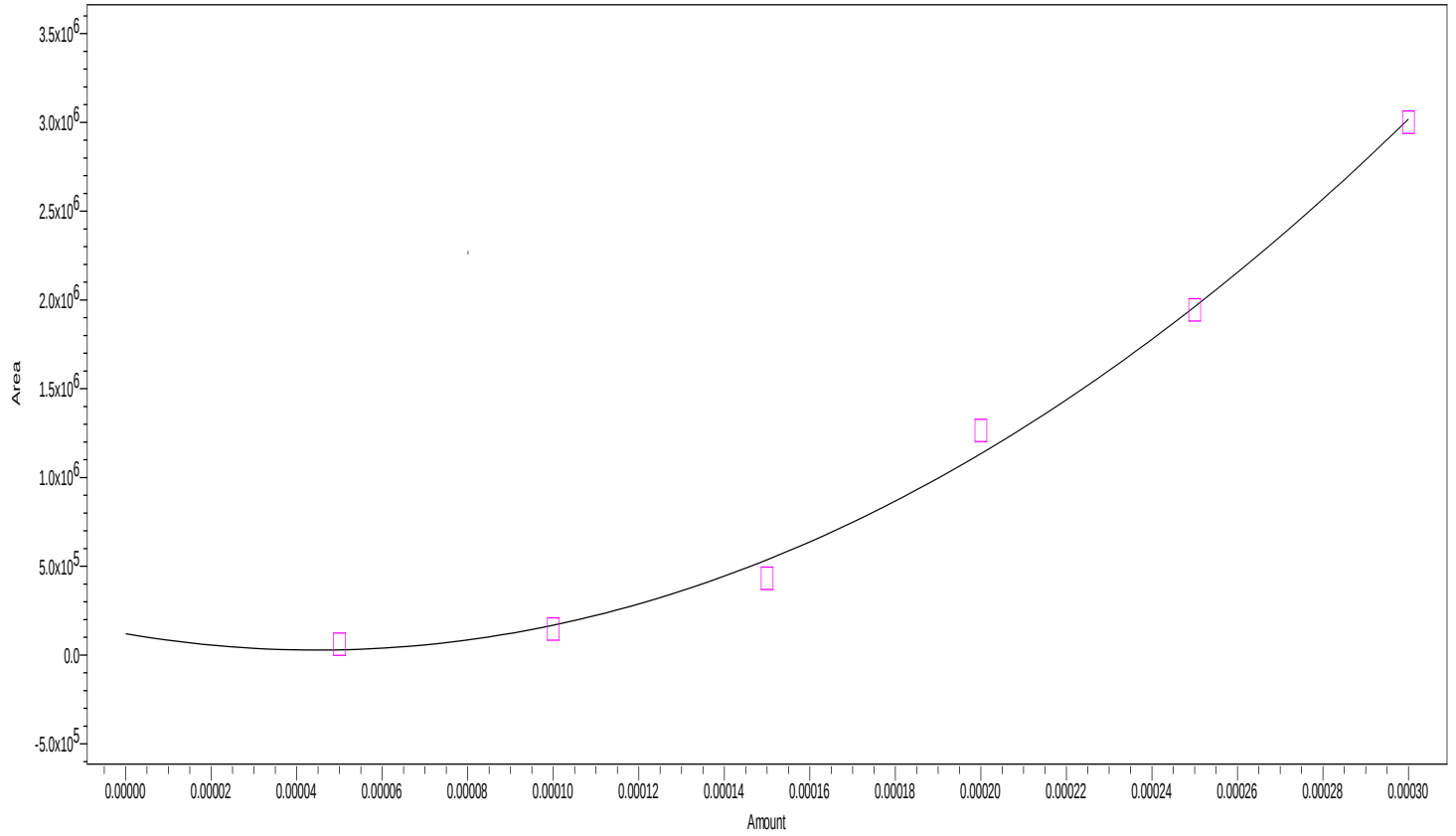


Figure 22. Calibration Curve of 1-oleyol-rac-glycerol (monoglyceride, MG). Pink squares represent different concentrations of TG standard. R2 value of calibration curve is 0.995599.

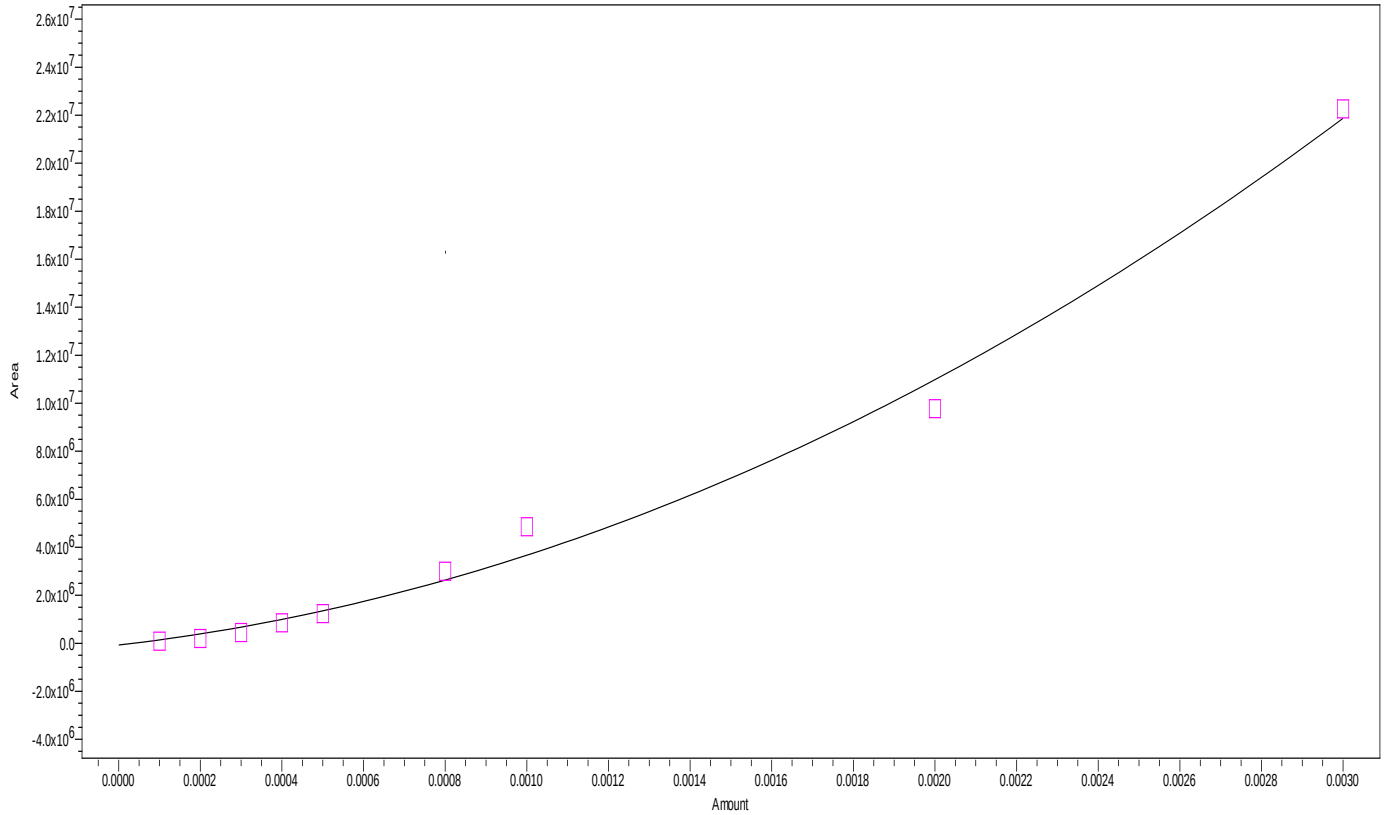


Figure 23. Calibration Curve of Phosphatidylcholin (PC). Pink squares represent different concentrations of TG standard. R2 value of calibration curve is 0.992231.

3.3. Lipid Profiles of BFMI Lines

Lipid profiles of BFMI 852, BFMI 856, BFMI 860 and BFMI 861 were separated, determined and quantified by using HPLC with ELS detector, as shown in Figure 24-43. Lipid profiles of BFMI lines were also separated as female, male and gonadal adipose tissue and inguinal adipose tissue. As shown in figures 24-35, gonadal adipose tissue had higher lipids than inguinal adipose tissue. Male and female had almost same lipid profiles in all BFMI lines, except BFMI 860. In BFMI 860 lines, in both inguinal and gonadal adipose tissue, female and male tissues had almost same lipid profile, as shown in figures 36-43. Figures and all data obtained from HPLC showed that BFMI 860 and BFMI 861 had better lipid profile, especially BFMI 861.

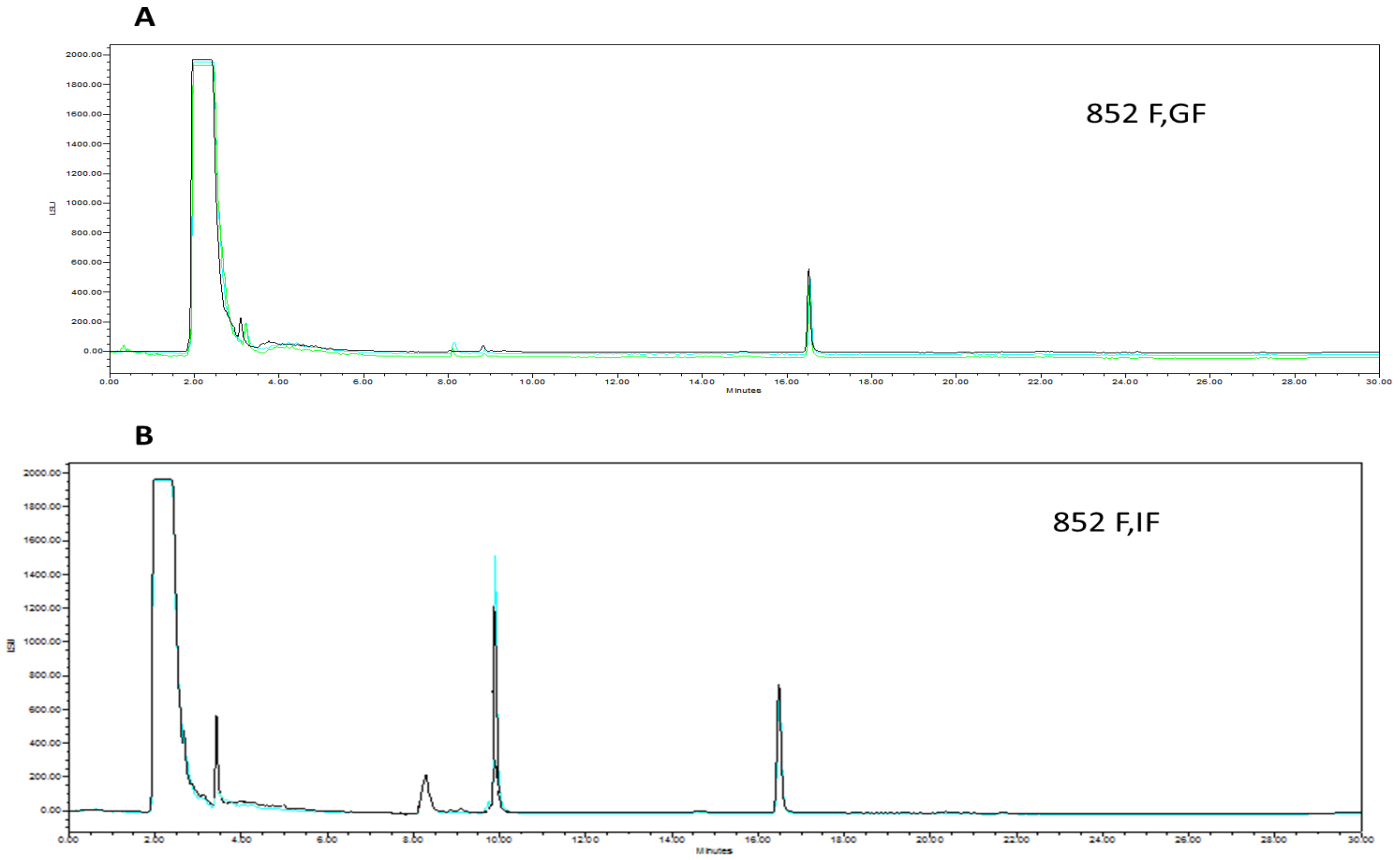


Figure 24. Chromatograms of Lipid Profile of (A) BFMI 852 Female, Gonadal Fat, (B) BFMI 852 Female, Inguinal Fat

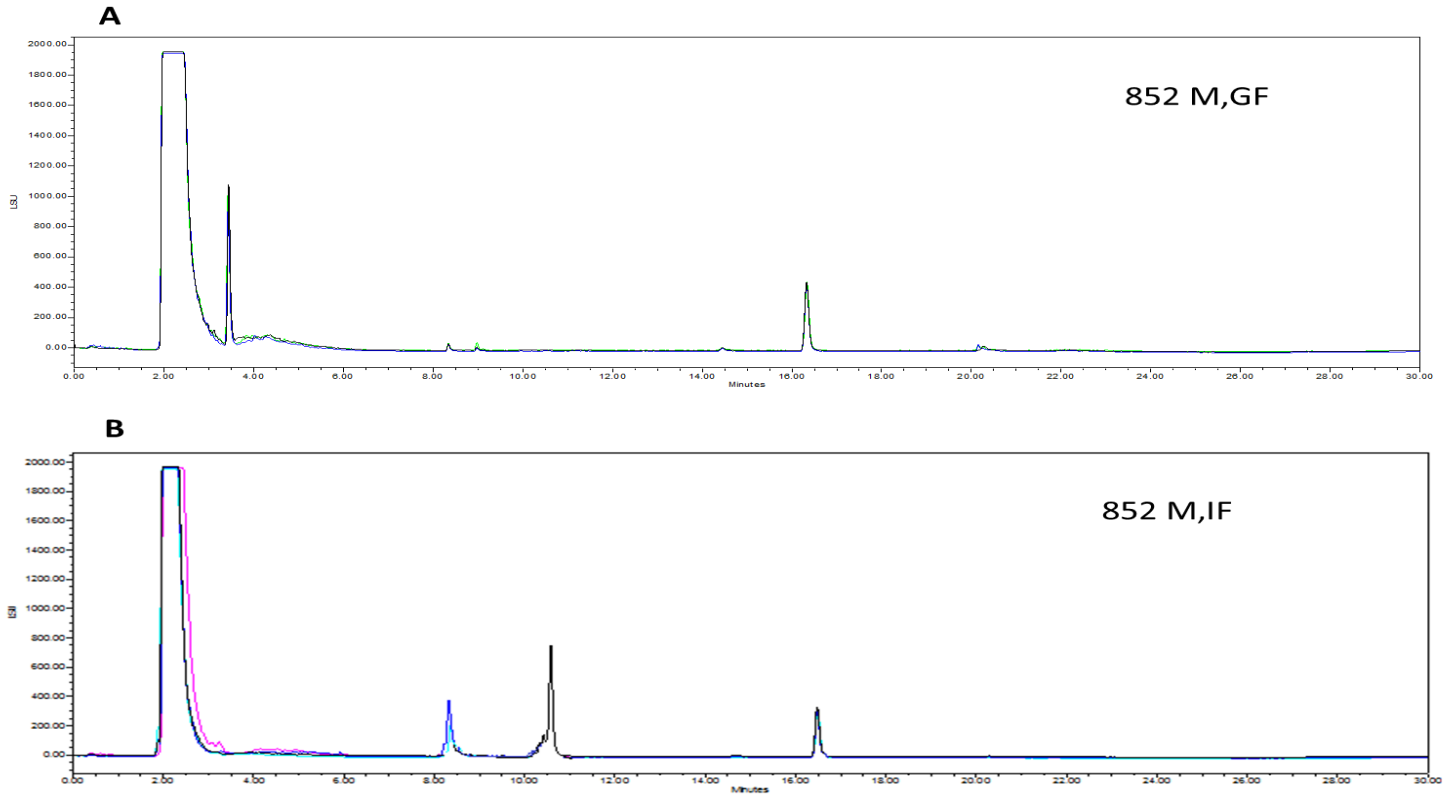


Figure 25. Chromatograms of Lipid Profile of (A) BFMI 852 Male, Gonadal Fat, (B) BFMI 852 Male, Inguinal Fat

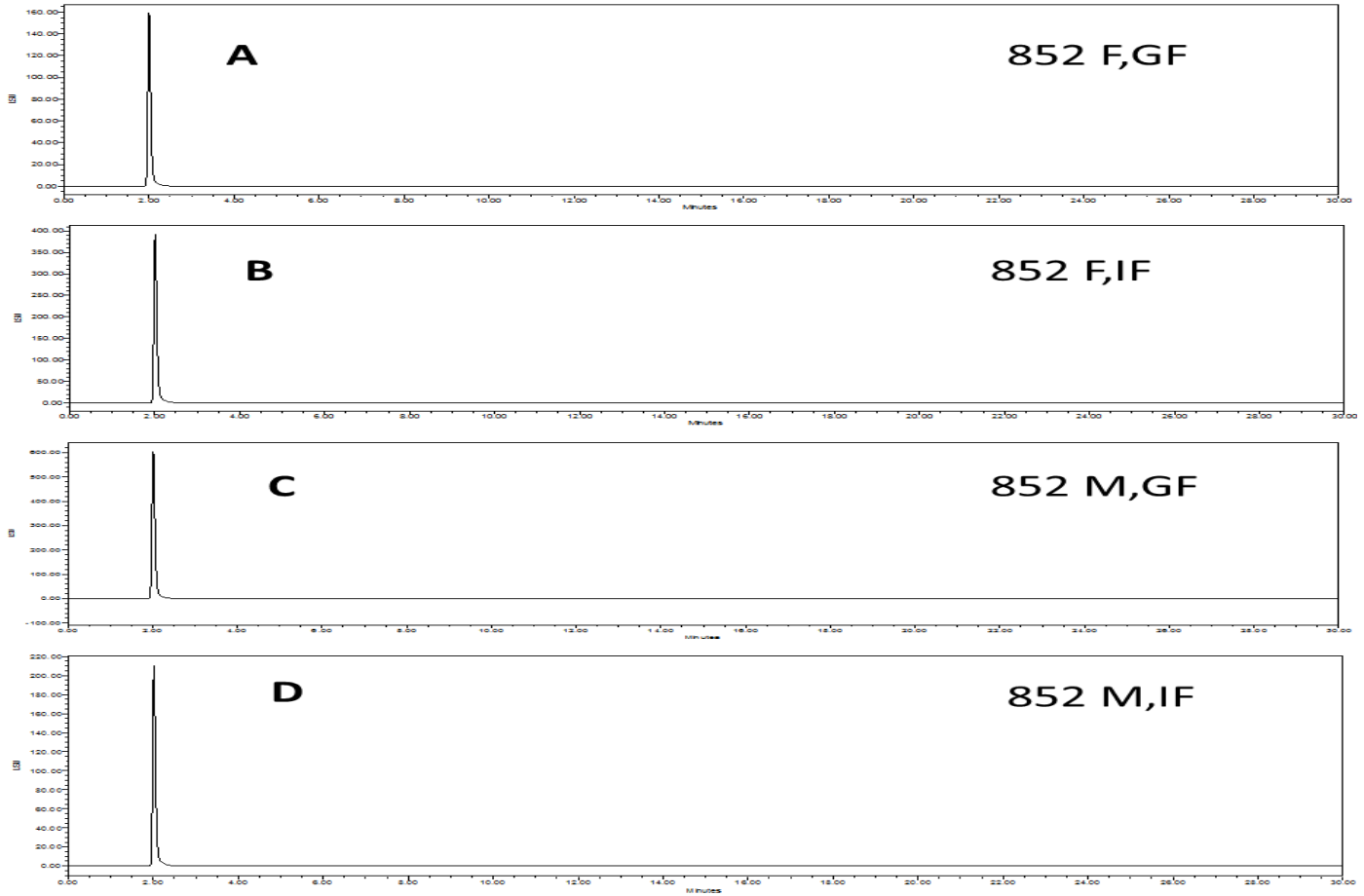


Figure 26. Chromatograms of TG of (A) 852 Female, Gonadal Fat (B) 852 Female, Inguinal Fat (C) 852 Male, Gonadal Fat and (D) 852 Male, Inguinal Fat

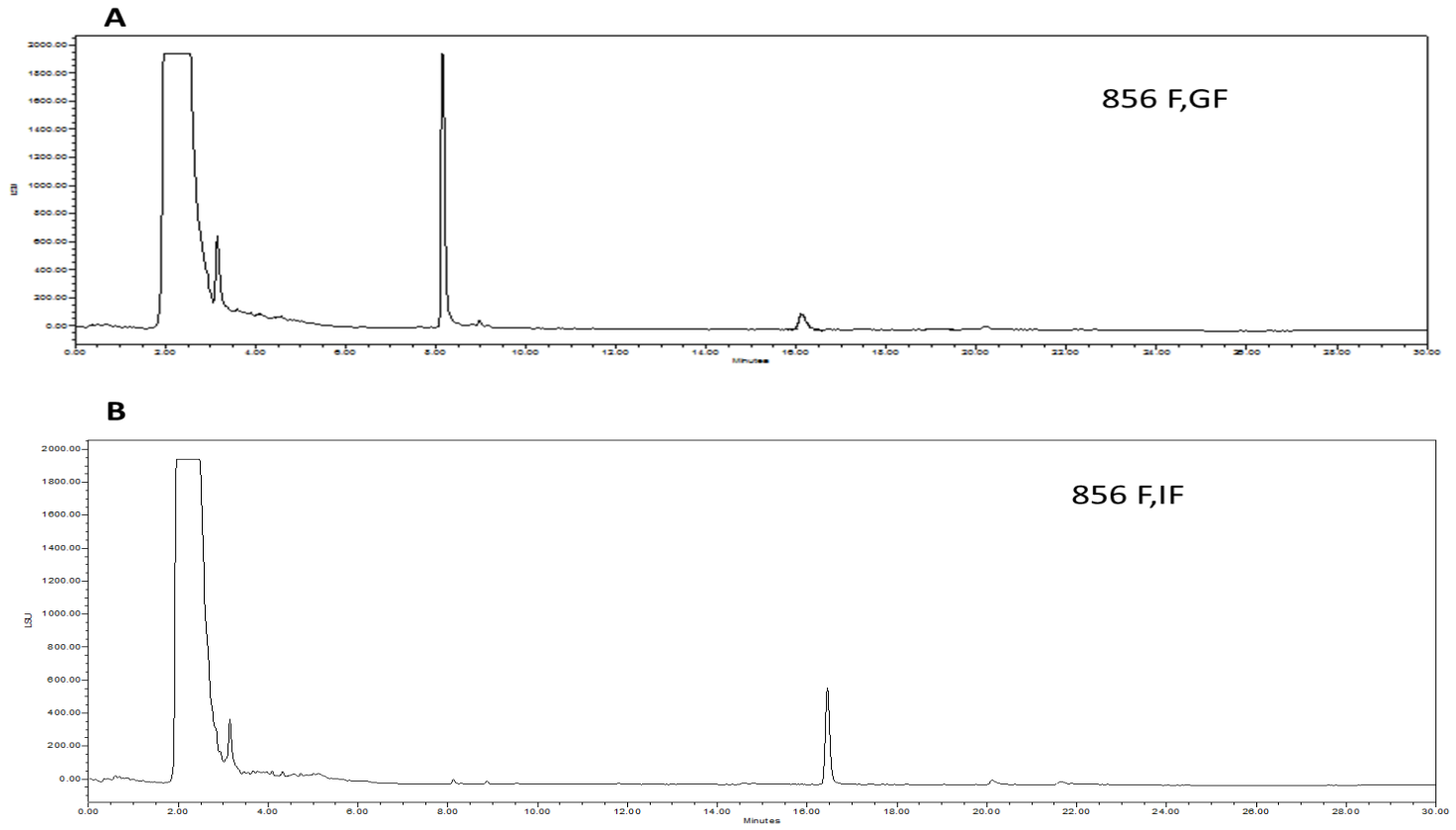


Figure 27. Chromatograms of Lipid Profile of (A) BFMI 856 Female, Gonadal Fat, (B) BFMI 856 Female, Inguinal Fat

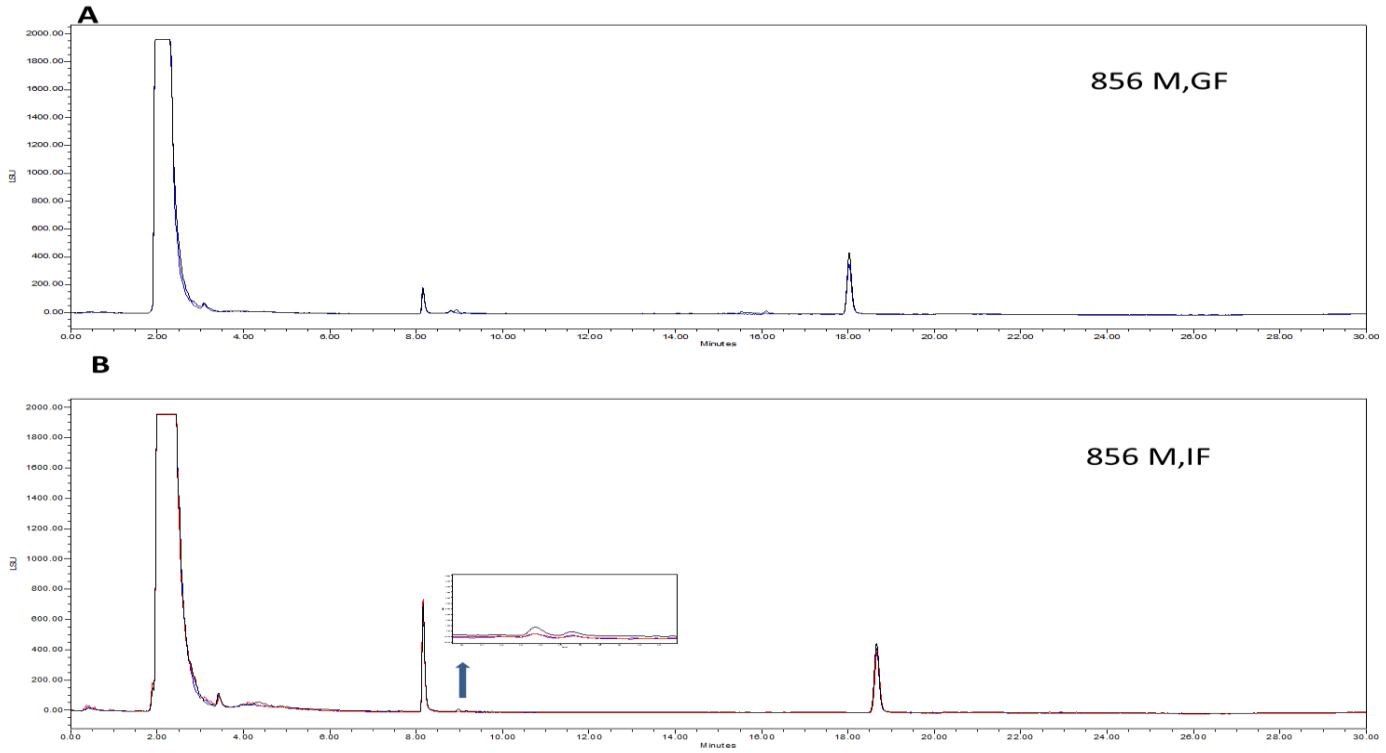


Figure 28. Chromatograms of Lipid Profile of (A) BFMI 856 Male, Gonadal Fat, (B) BFMI 856 Male, Inguinal Fat

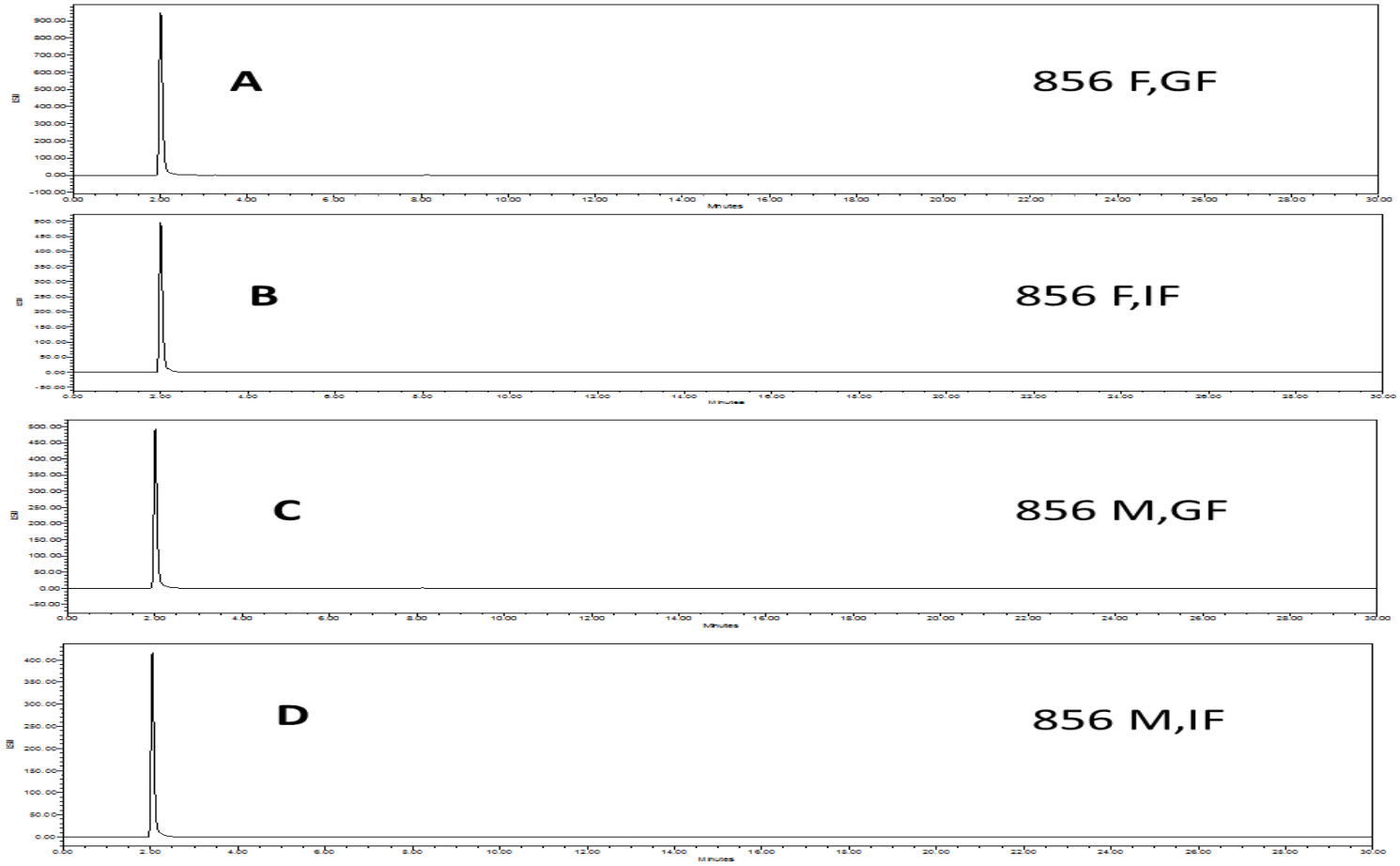


Figure 29. Chromatograms of TG of (A) 856 Female, Gonadal Fat (B) 856 Female, Inguinal Fat (C) 856 Male, Gonadal Fat and (D) 856 Male, Inguinal Fat

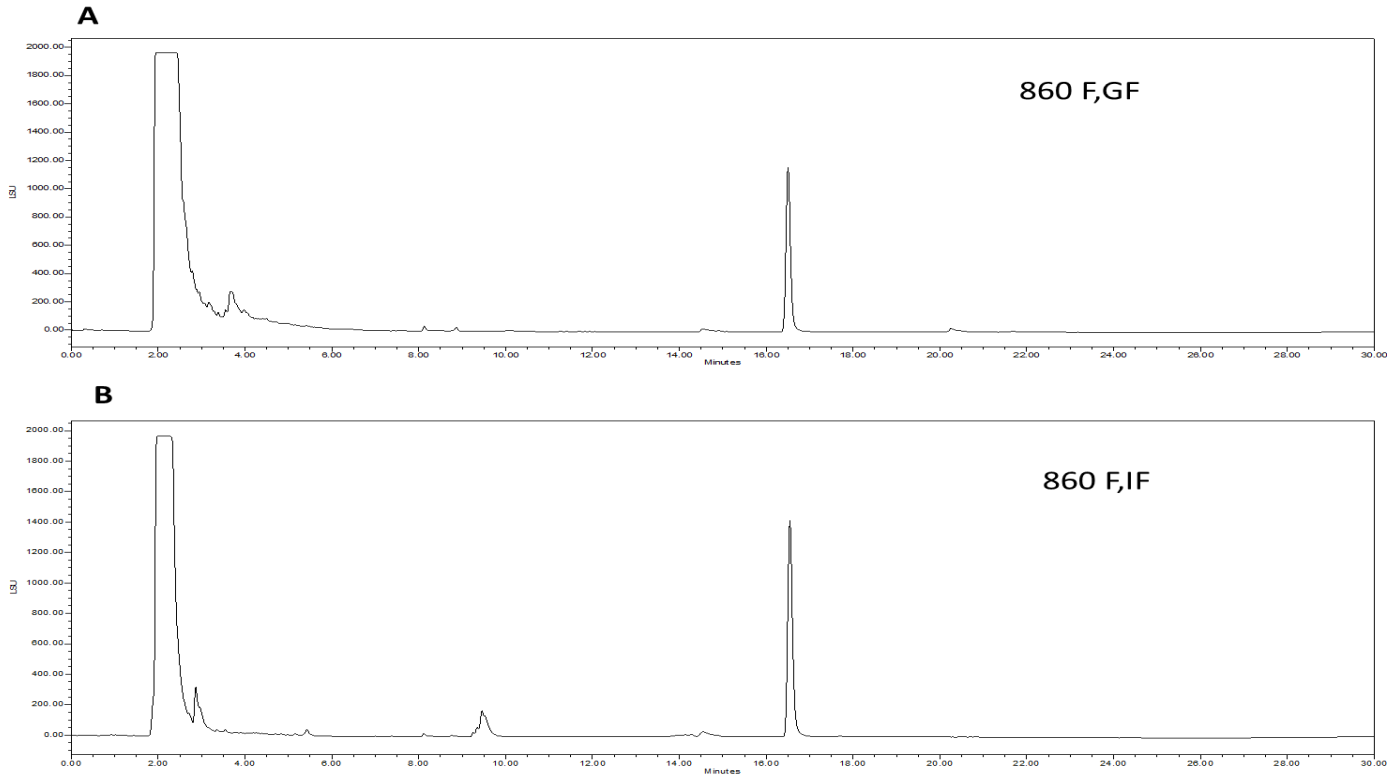


Figure 30. Chromatograms of Lipid Profile of (A) BFMI 860 Female, Gonadal Fat, (B) BFMI 860 Female, Inguinal Fat

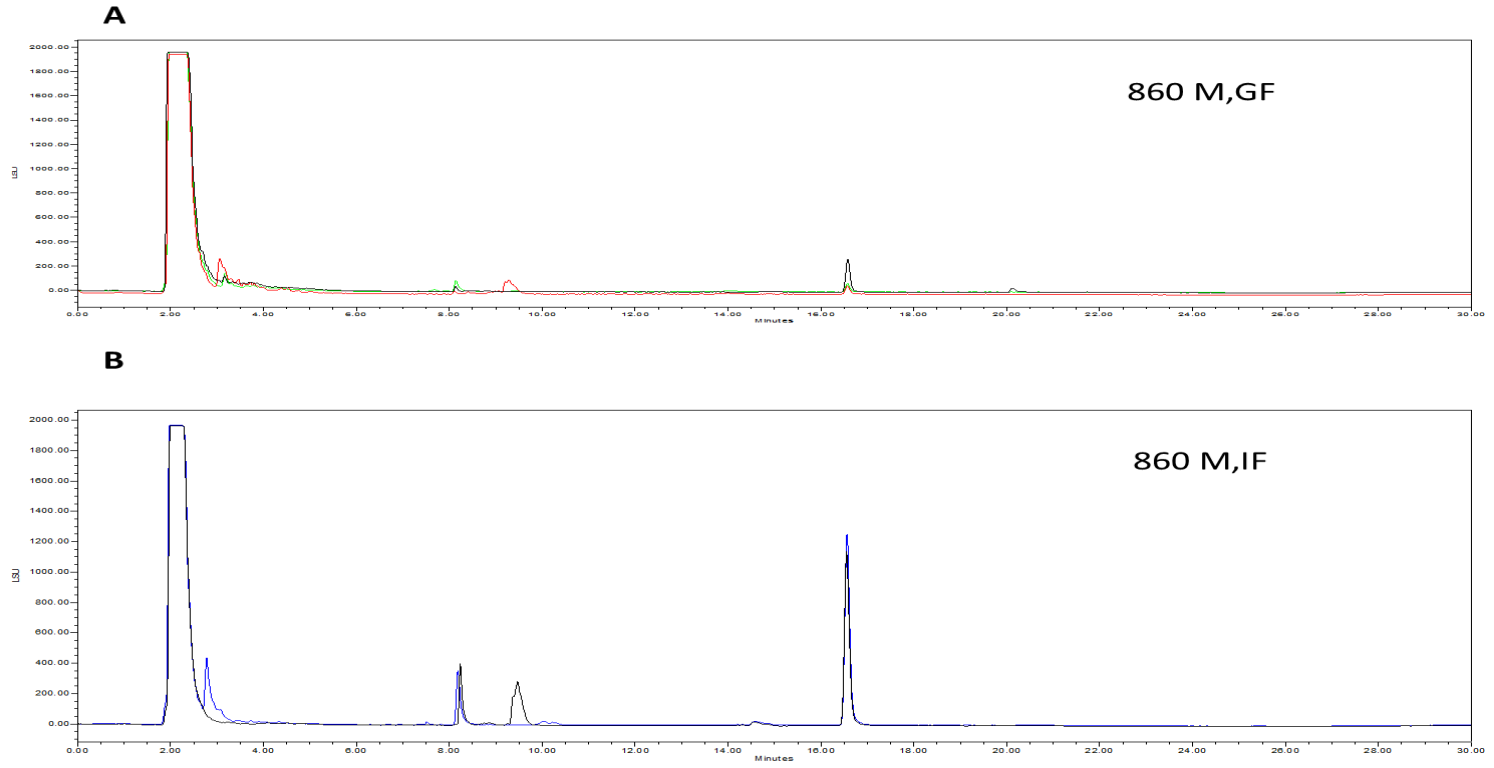


Figure 31. Chromatograms of Lipid Profile of (A) BFMI 860 Male, Gonadal Fat, (B) BFMI 860 Male, Inguinal Fat

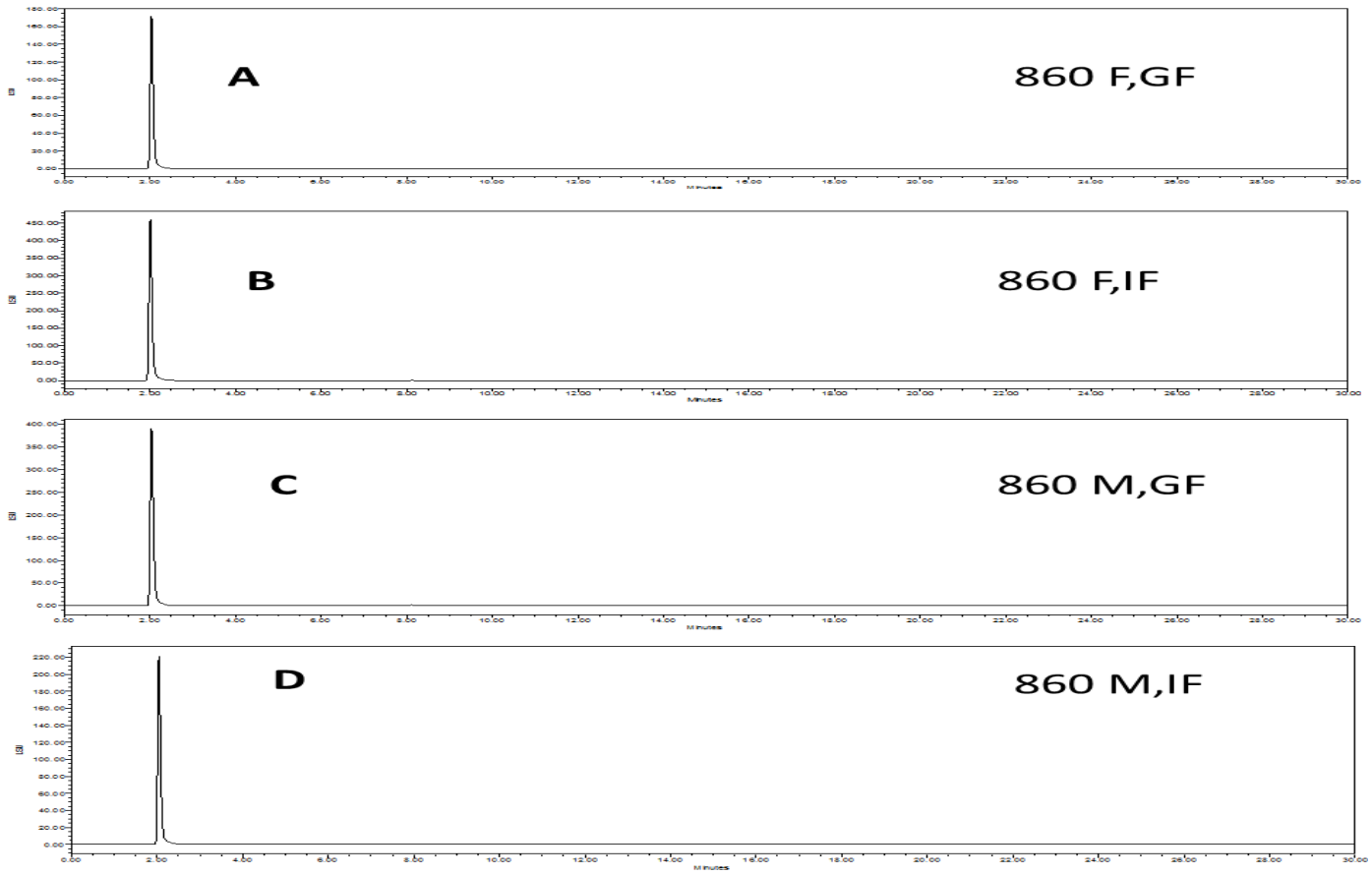


Figure 32. Chromatograms of TG of (A) 860 Female, Gonadal Fat (B) 860 Female, Inguinal Fat (C) 860 Male, Gonadal Fat and (D) 860 Male, Inguinal Fat

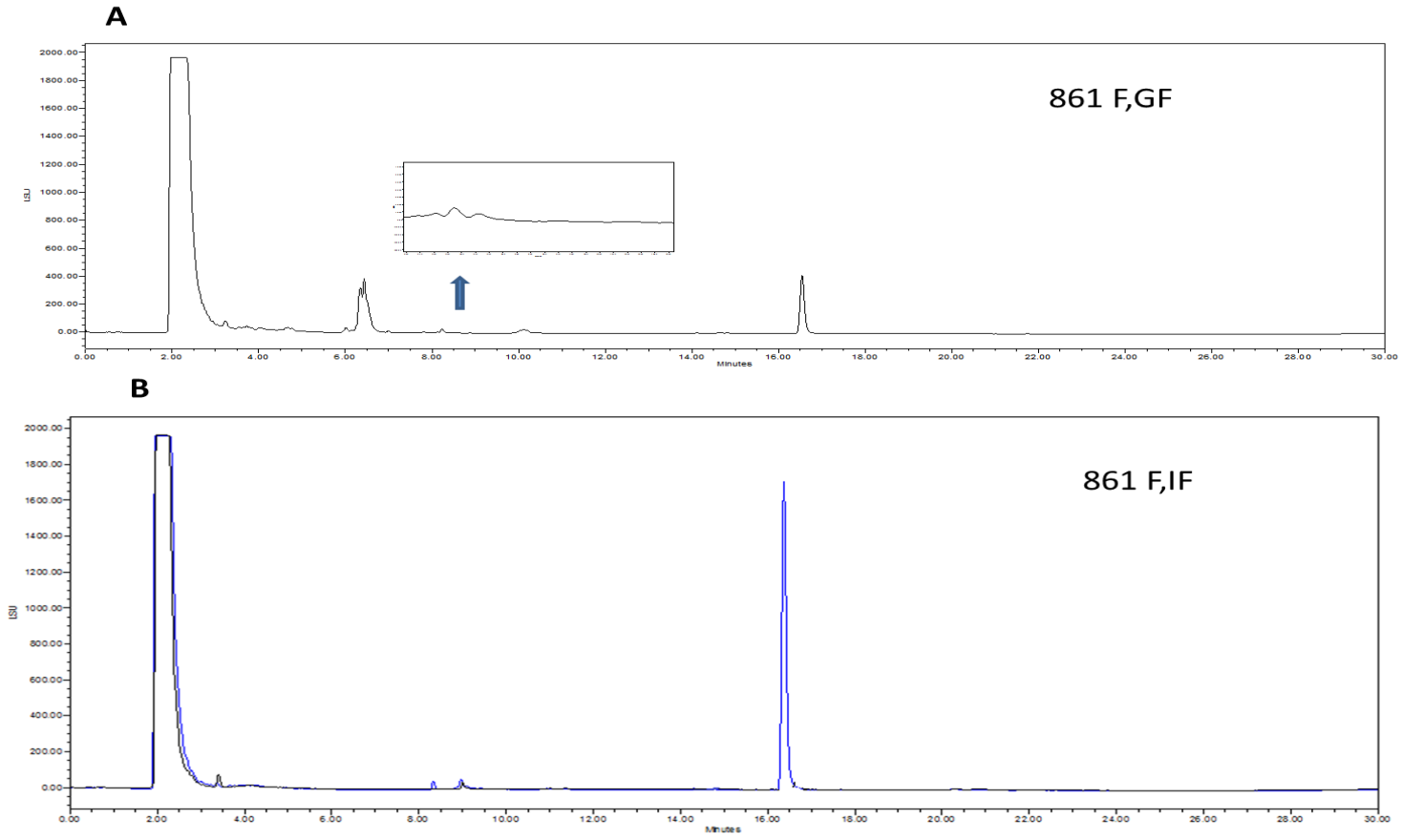


Figure 33. Chromatograms of Lipid Profile of (A) BFMI 861 Female, Gonadal Fat, (B) BFMI 861 Female, Inguinal Fat

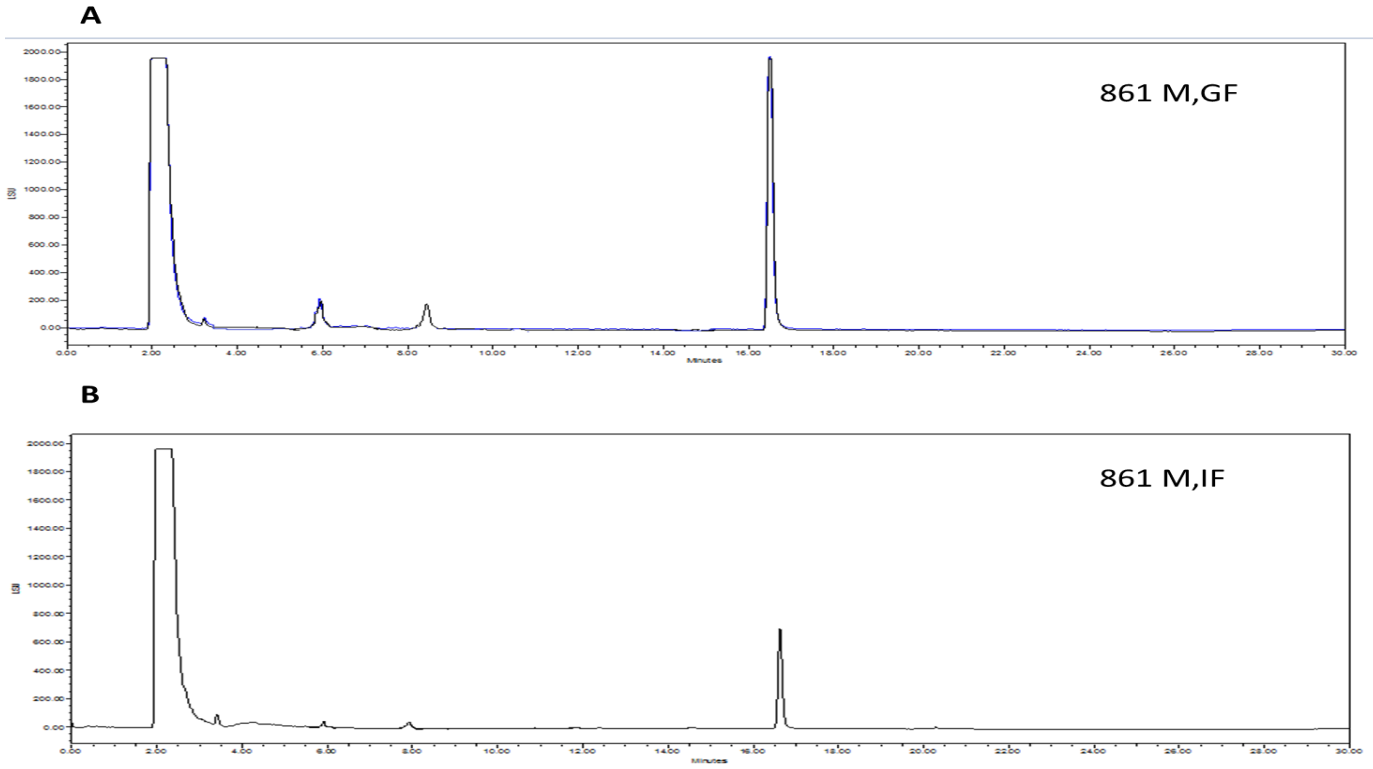


Figure 34. Chromatograms of Lipid Profile of (A) BFMI 861 Male, Gonadal Fat, (B) BFMI 861 Male, Inguinal Fat

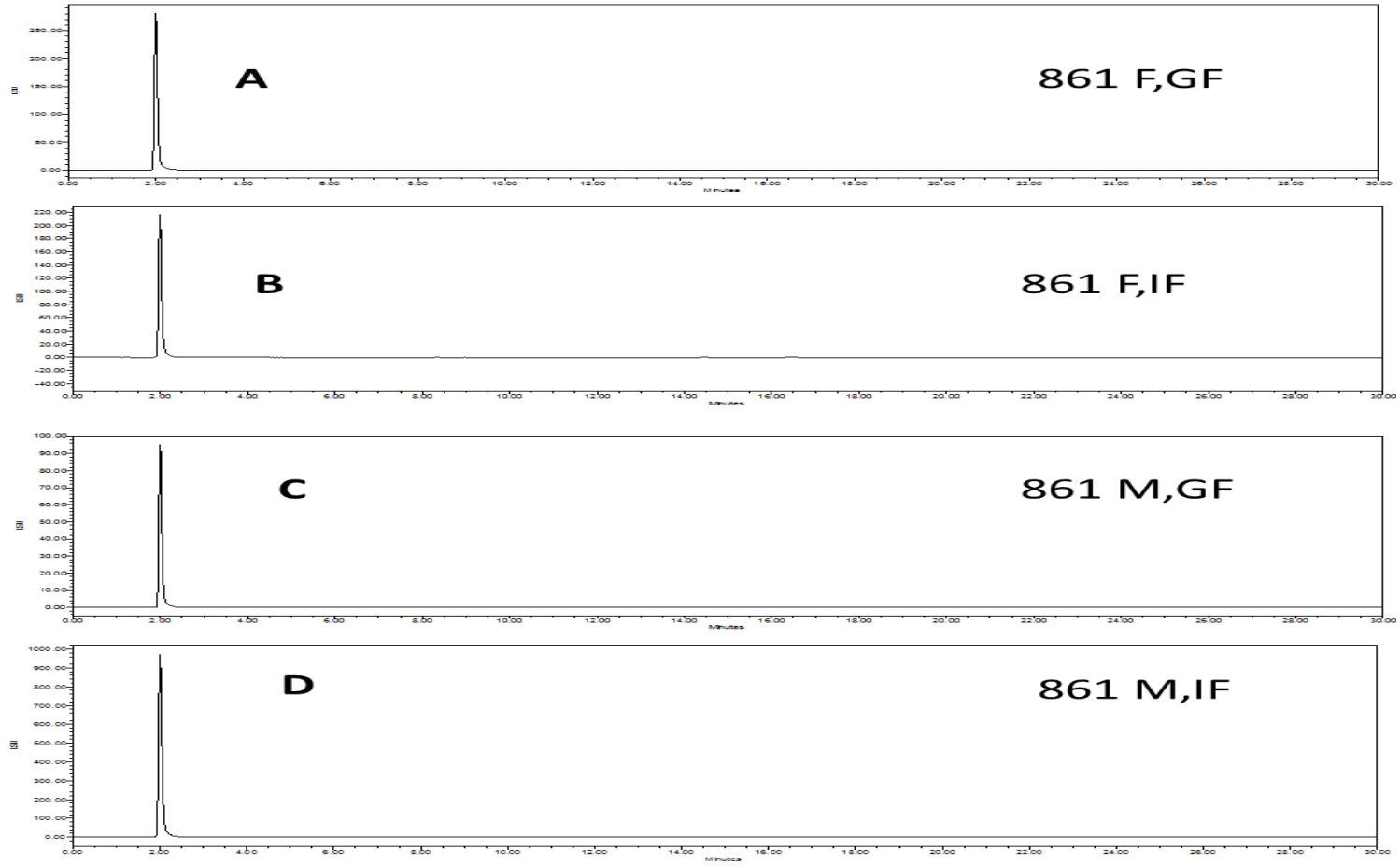


Figure 35. Chromatograms of TG of (A) 861 Female, Gonadal Fat (B) 861 Female, Inguinal Fat (C) 861 Male, Gonadal Fat and (D) 861 Male, Inguinal Fat

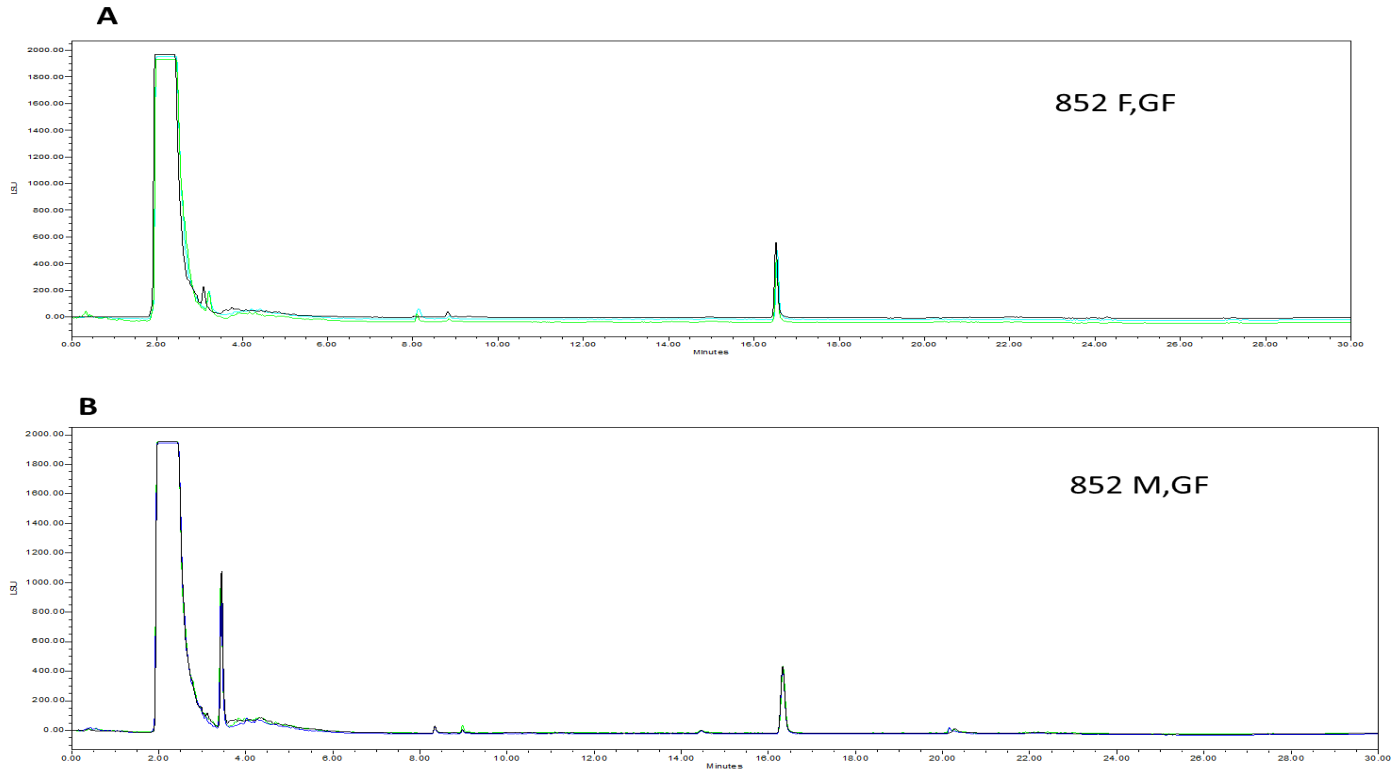


Figure 36. Chromatograms of Lipid Profile of (A) BFMI 852 Female, Gonadal Fat, (B) BFMI 852 Male, Gonadal Fat

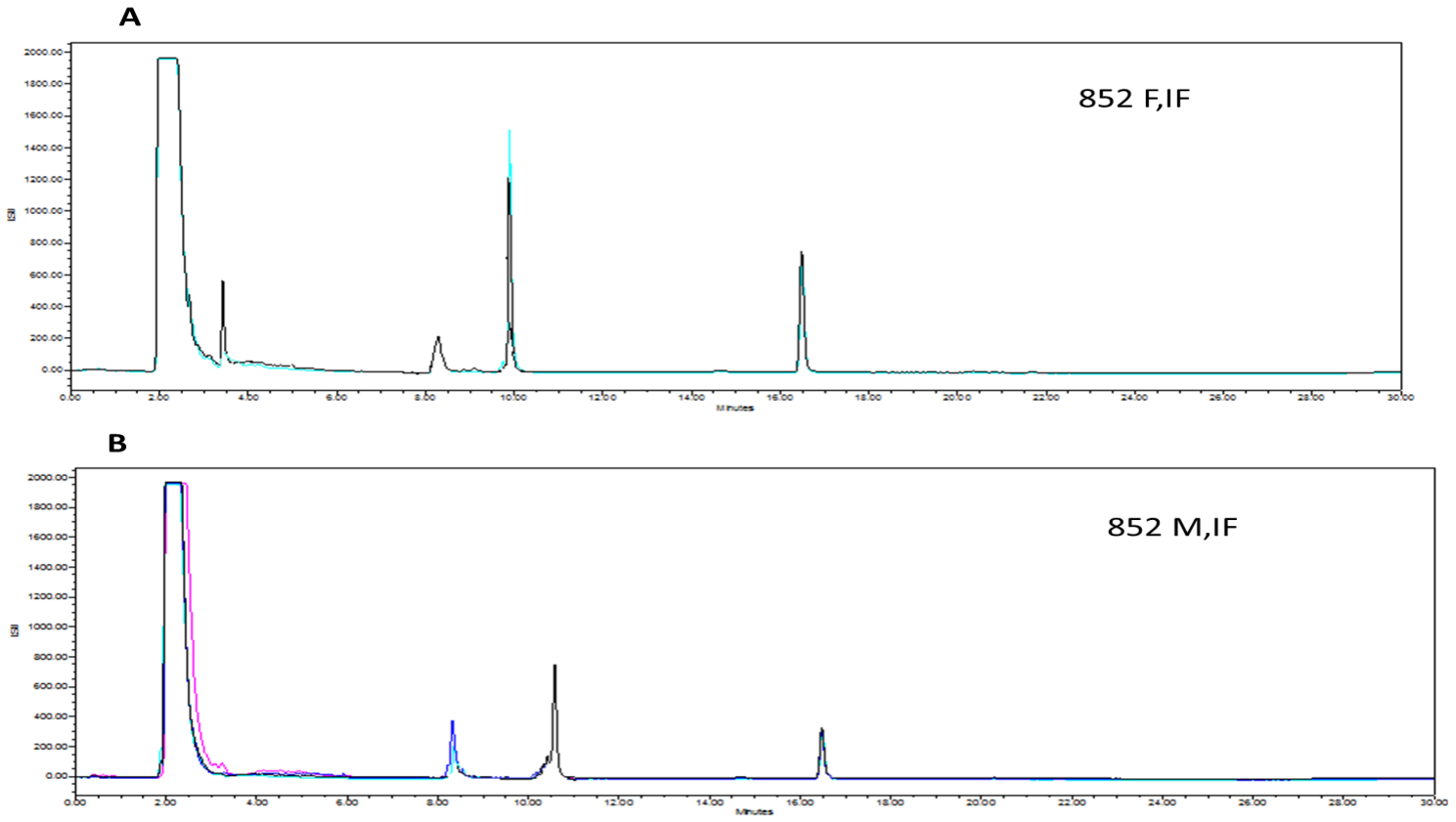


Figure 37. Chromatograms of Lipid Profile of (A) BFMI 852 Female, Inguinal Fat, (B) BFMI 852 Male, Inguinal Fat

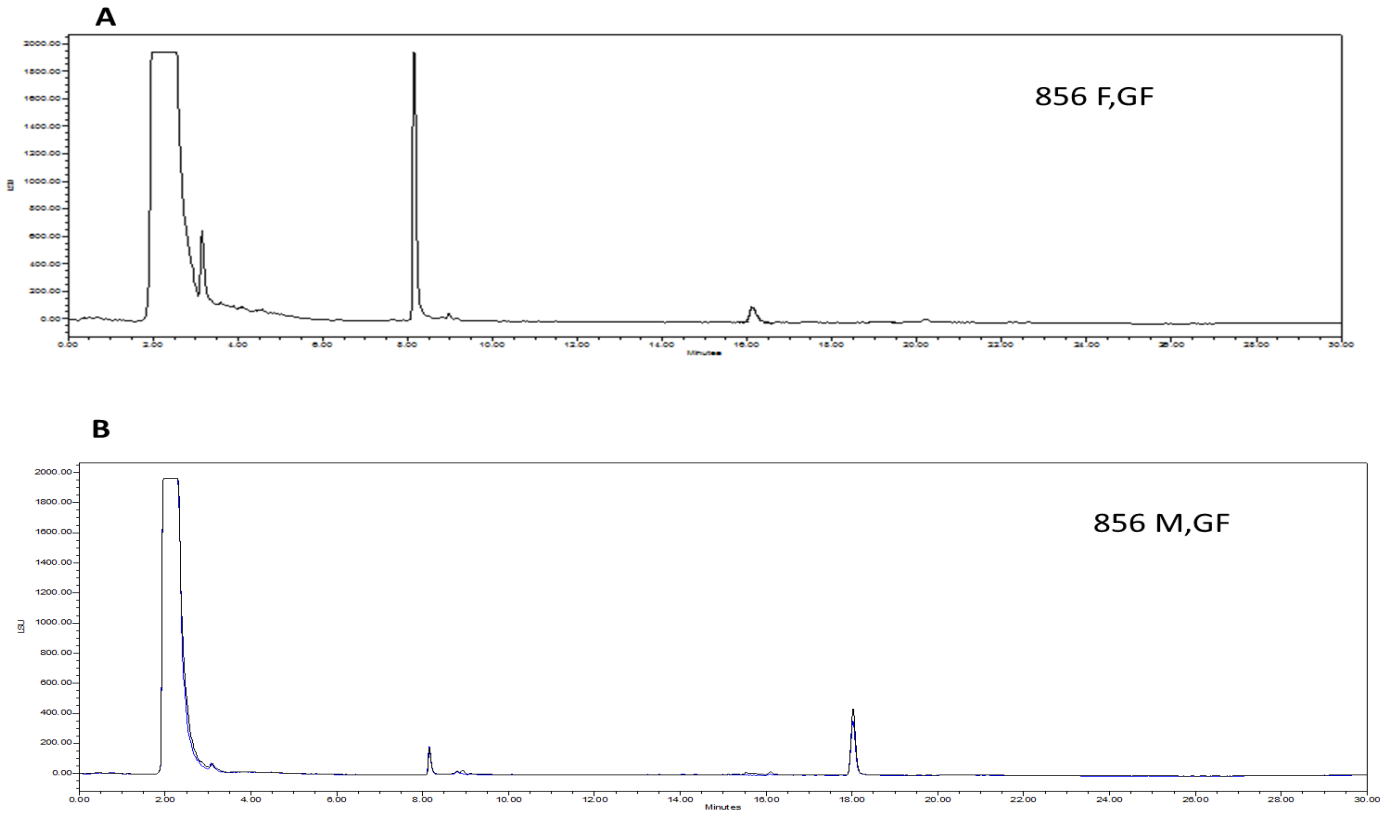


Figure 38. Chromatograms of Lipid Profile of (A) BFMI 856 Female, Gonadal Fat, (B) BFMI 856 Male, Gonadal Fat

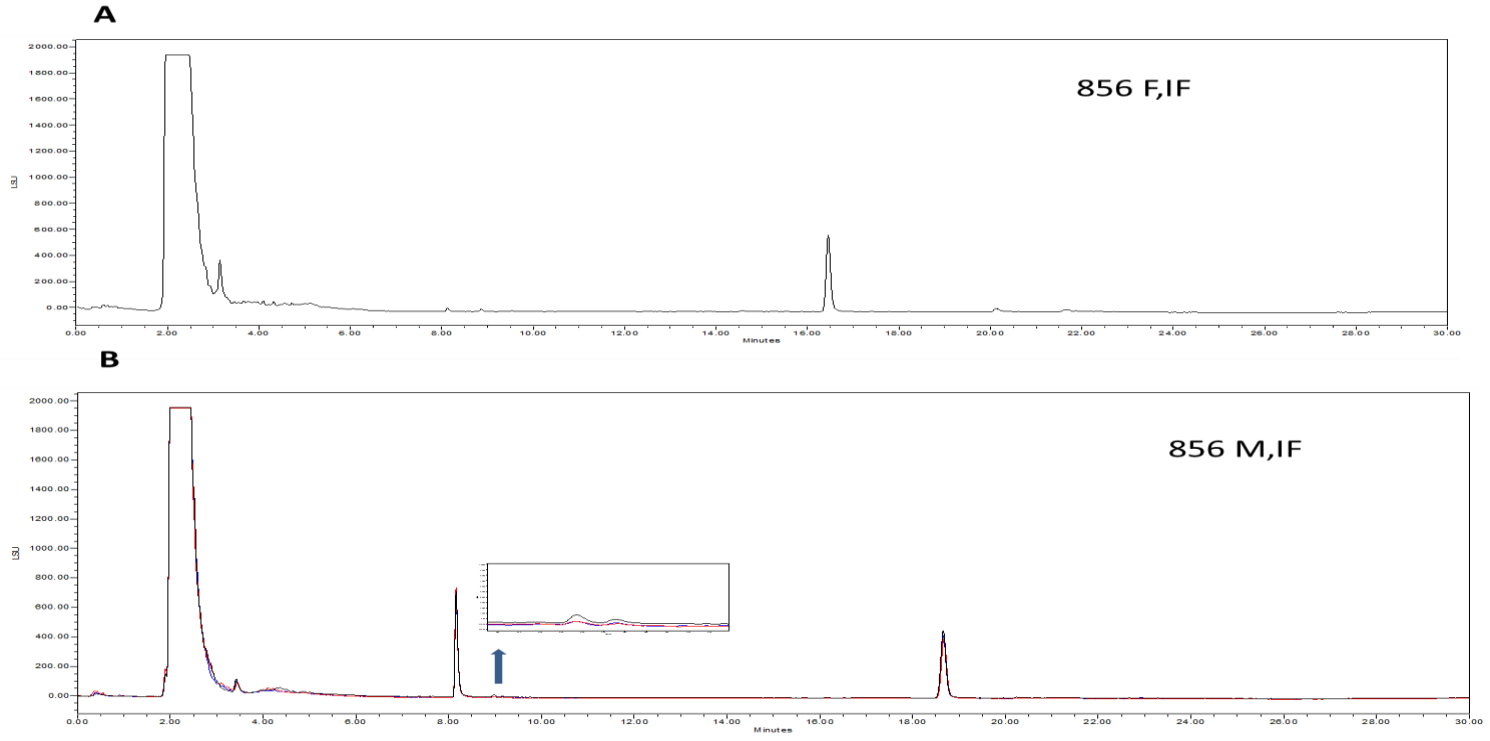


Figure 39. Chromatograms of Lipid Profile of (A) BFMI 856 Female, Inguinal Fat, (B) BFMI 856 Male, Inguinal Fat

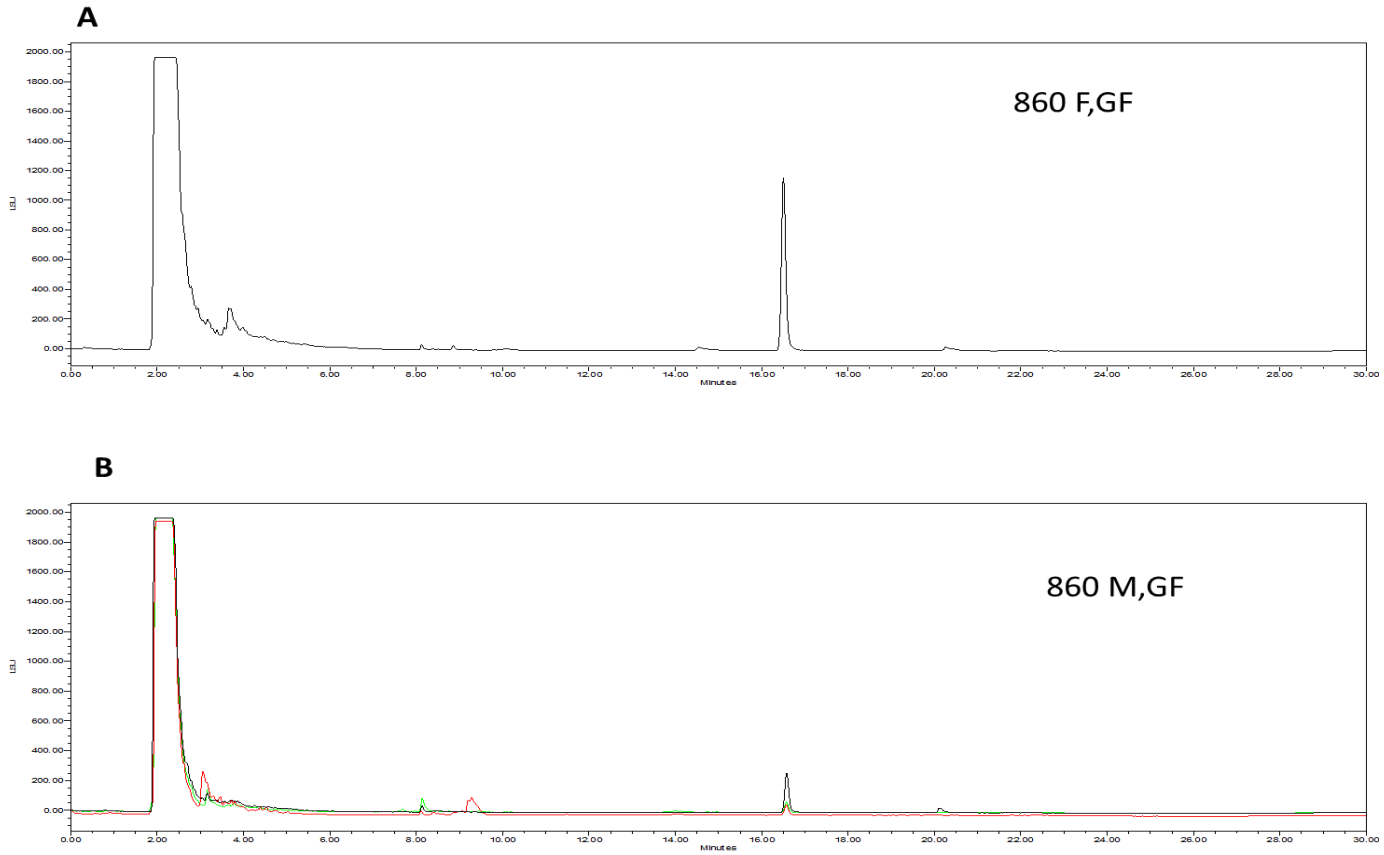


Figure 40. Chromatograms of Lipid Profile of (A) BFMI 860 Female, Gonadal Fat, (B) BFMI 860 Male, Gonadal Fat

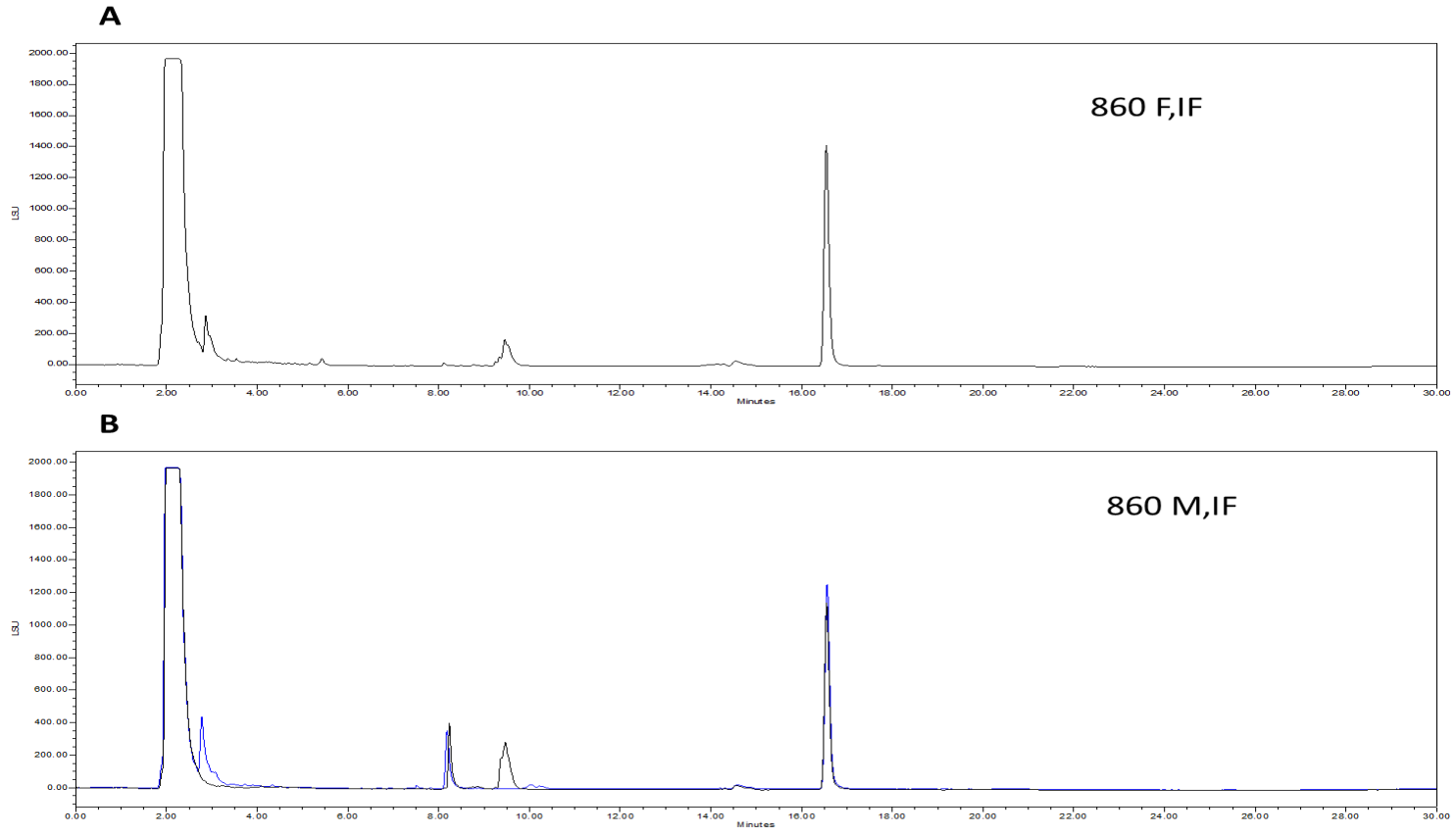


Figure 41. Chromatograms of Lipid Profile of (A) BFMI 860 Female, Inguinal Fat, (B) BFMI 860 Male, Inguinal Fat

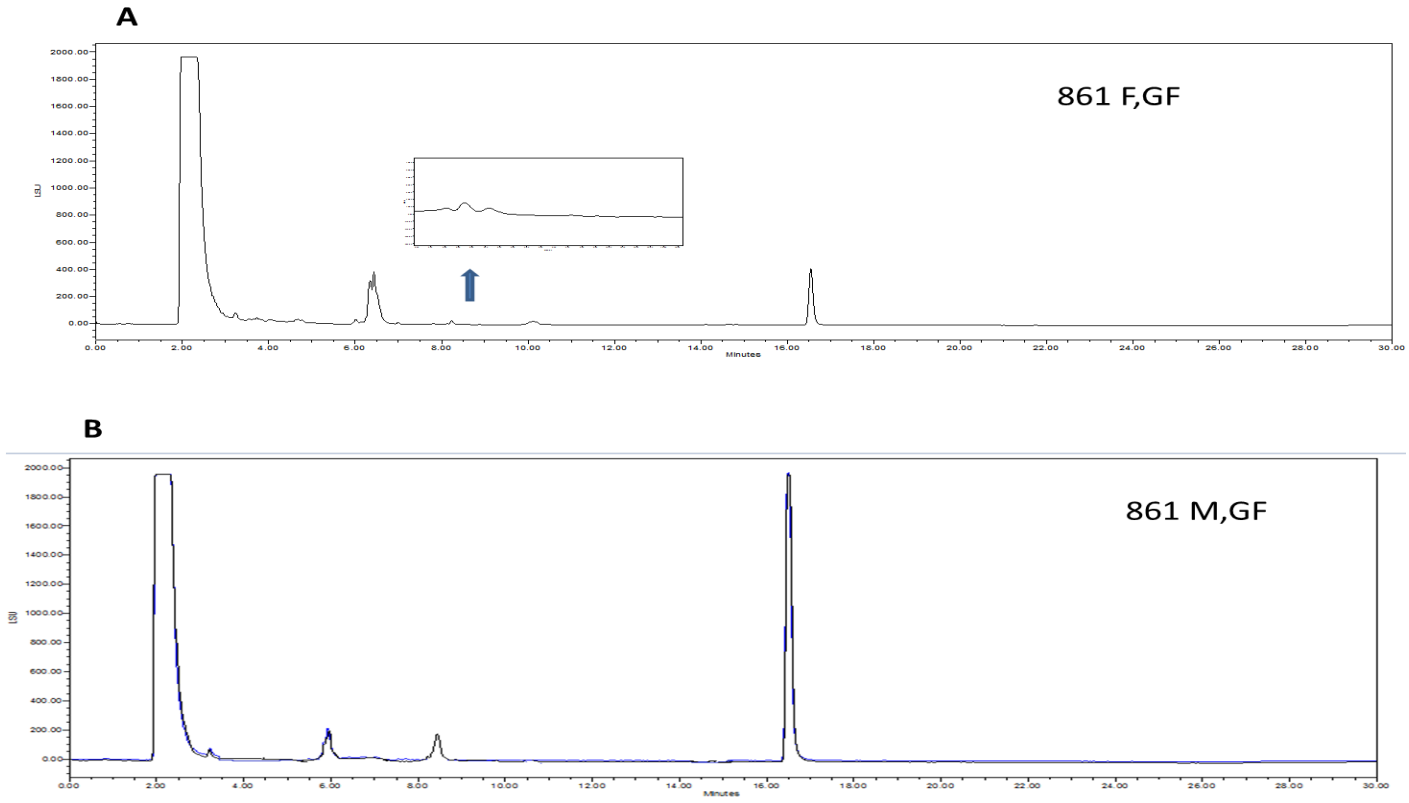


Figure 42. Chromatograms of Lipid Profile of (A) BFMI 861 Female, Gonadal Fat, (B) BFMI 861 Male, Gonadal Fat

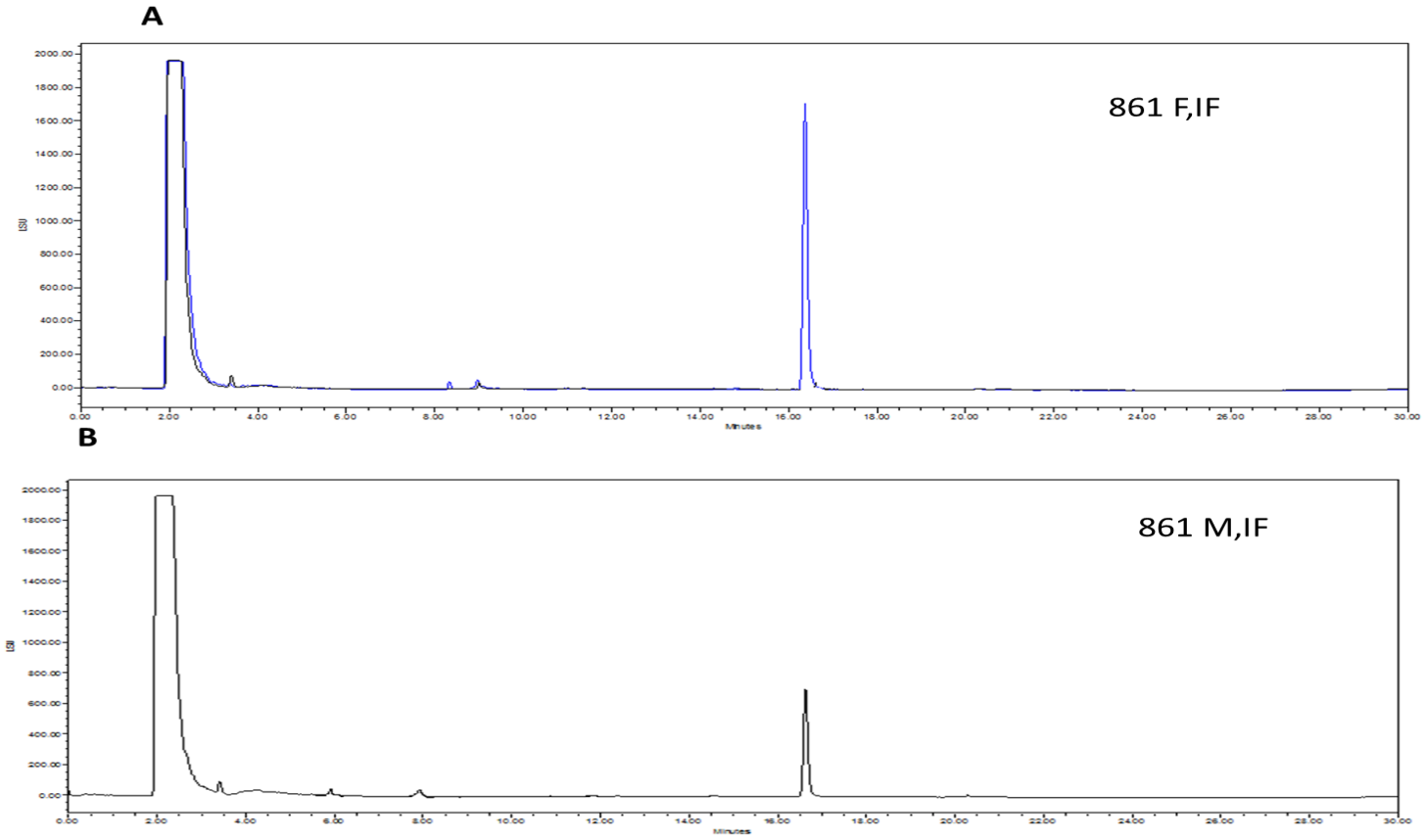


Figure 43. Chromatograms of Lipid Profile of (A) BFMI 861 Female, Inguinal Fat, (B) BFMI 861 Male, Inguinal Fat

3.4. Quantitative Results

Quantitative results of lipid concentrations were obtain by using Water Empower2 Pro Software. TG, DG, C, MG and PC lipids of each BFMI lines were statistically quantify.

TG concentrations of BFMI lines can be seen in Figures 44-45. According to results, BFMI 860 and 861 lines had higher TG concentration than BFMI 852 and BFMI 856. Only in male, inguinal tissue, also BFMI 856 showed higher concentration. In obese mouse, higher TG concentration is expected because concentration of simple lipids, particularly TG changes dramatically (St-Pierre et al, 2002; Karatayev et al, 2009; Christie, 1987). BFMI 860 and BFMI 861 had higher concentration. This shows that BFMI 860 and BFMI 86, especially BFMI 860 could have high level of insulin resistance. In fact, they were found that they are insulin resistant (Hantschel et al, 2011; Wagener et al, 2006).

Between BFMI lines, gonadal adipose tissue or visceral adipose tissue had higher TG concentration than inguinal adipose tissue. Visceral adiposity is closely related to some metabolic disorders such as obesity and insulin resistance (St-Pierre et al, 2002). Therefore, this is expected result.

Female tissues had almost same amount of TG as male tissue. Two exceptions were seen in BFMI 852 inguinal tissue and BFMI 856 gonadal tissue. In these tissues, female had higher TG concentration than male.

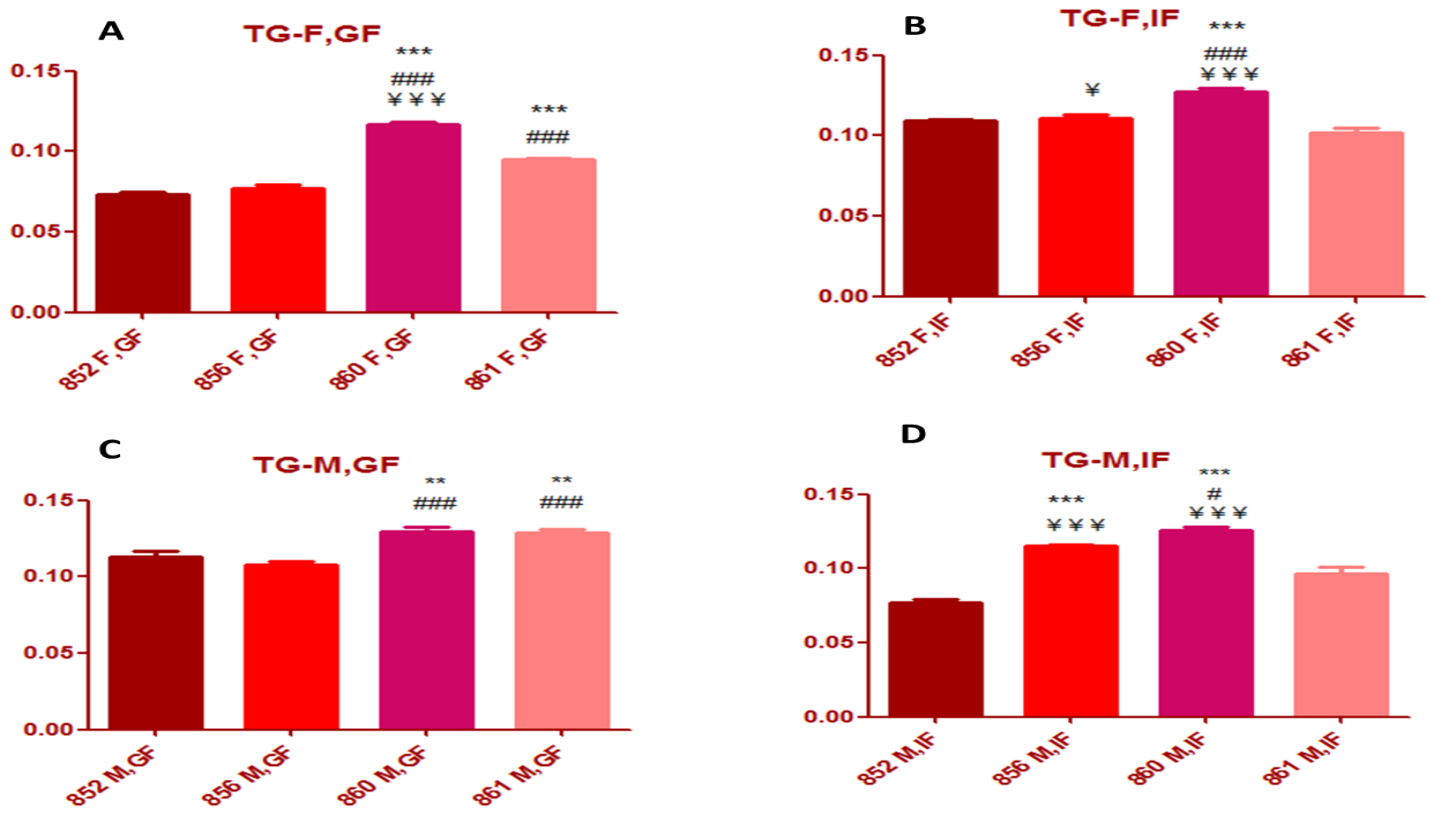


Figure 44. TG (mg/ml) concentrations between BFMI lines obtained from (A) Female, Gonadal Fat, (B) Female, Inguinal Fat, (C) Male, Gonadal Fat, and (D) Male, Inguinal Fat. (*, #, §, and ¥ indicated degree of significance of BFMI 852, BFMI 856, BFMI 860 and BFMI 861, respectively.)

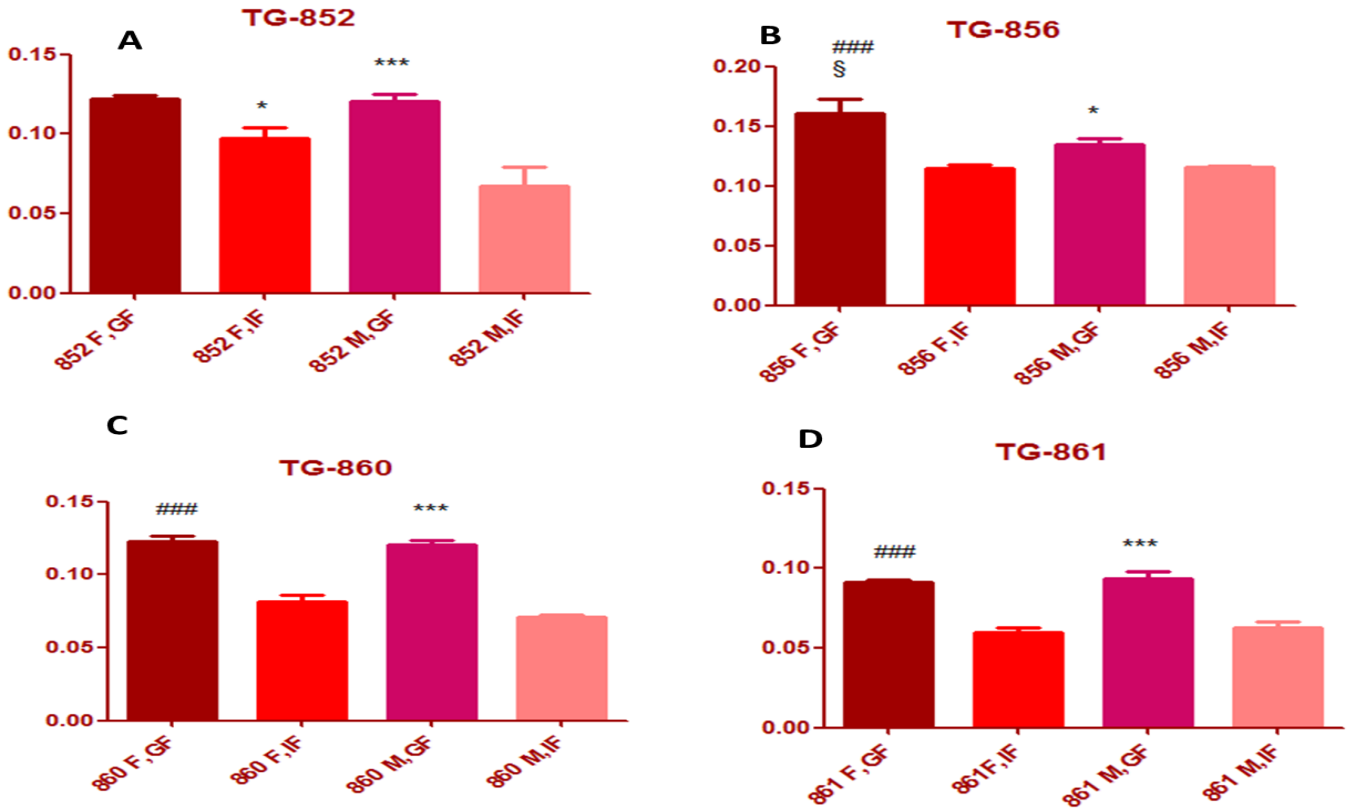


Figure 45. TG (mg/ml) concentrations between Female-Male and Gonadal-Inguinal obtained from (A) BFMI 852 (B) BFMI 856 (C) BFMI 860, and (D) BFMI 861. (¥, #, § and * indicated degree of significance of Female-Gonadal Fat, Female-Inguinal Fat, Male-Gonadal Fat and Male-Inguinal Fat, respectively).

Cholesterol is an important lipid. It is the most known steroid. One of the main roles of cholesterol is to have role in synthesizing steroid hormones (precursor of steroid hormones) such as estrogen (estradiol), androgen (testosterone), progesterone, aldesteron and cortisol. Moreover, it is found in the structure of cell membrane. In addition, it has a role in membrane fluidity (Duane, 1997; Hu et al, 2010; Christie, 1987). Insulin receptors of cell are located within lipids raft, name caveolae. If there are any problems such as ruin of this lipid raft, insulin receptors become dysfunction. Level of cholesterol is important. Increasing the cholesterol level causes some diseases such as atherosclerosis and insulin resistance (Pike, 2009; Gustavsson et al, 1999).

Cholesterol levels were higher in BFMI 860, BFMI 861and BFMI 856, especially BFMI 860. The least level of cholesterol were observed in BFMI 852 lines (Figures 46-47). High cholesterol concentration might indicate the decaying the insulin sensitivity. This indicates the increasing risk of obesity and type II diabetes.

According to results, gonadal fat had higher cholesterol levels than inguinal fat, especially in male tissues. Female and male tissues had almost same amount cholesterol, except BFMI 852.

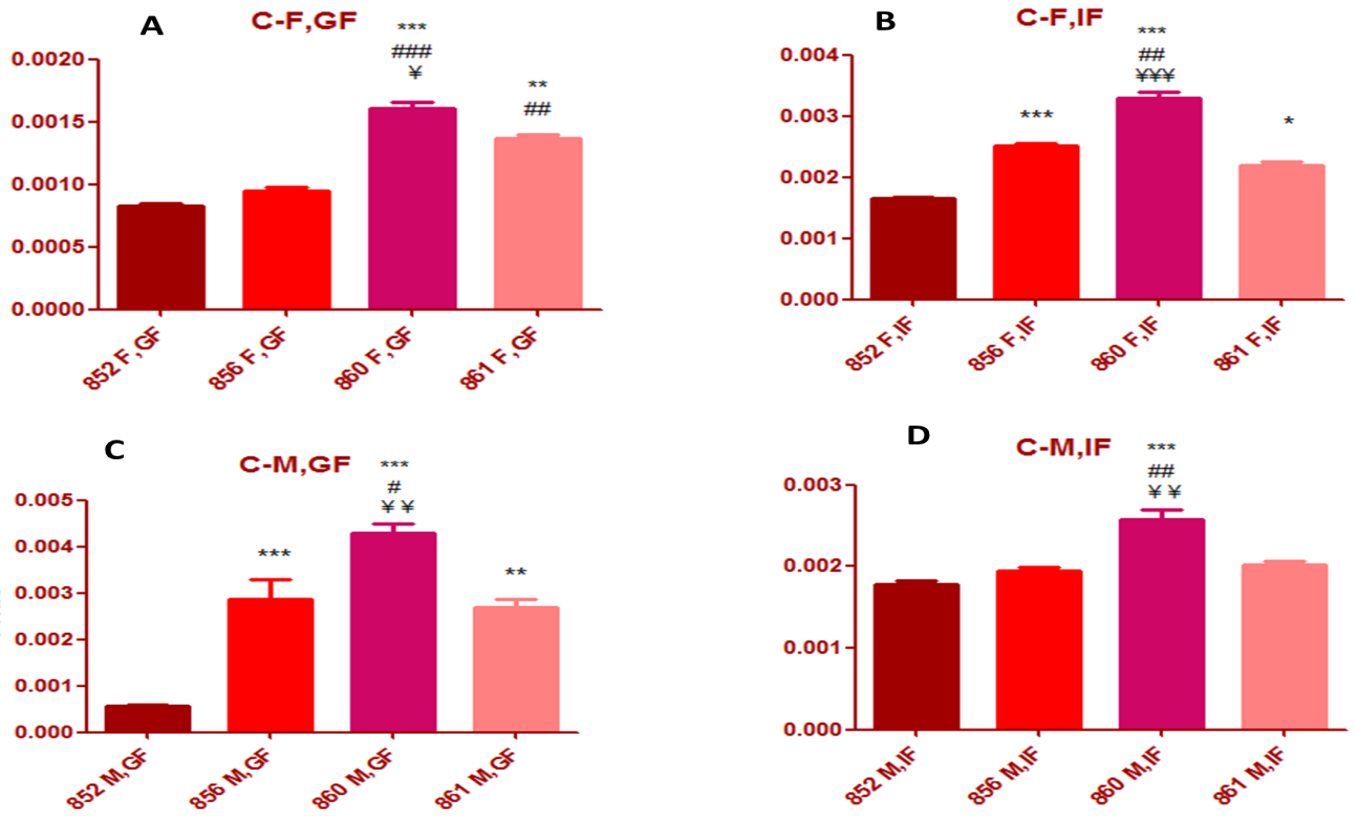


Figure 46. Cholesterol (mg/ml) concentrations between BFMI lines obtained from (A) Female, Gonadal Fat, (B) Female, Inguinal Fat, (C) Male, Gonadal Fat, and (D) Male, Inguinal Fat. (*, #, §, and ¥ indicated degree of significance of BFMI 852, BFMI 856, BFMI 860 and BFMI 861, respectively.)

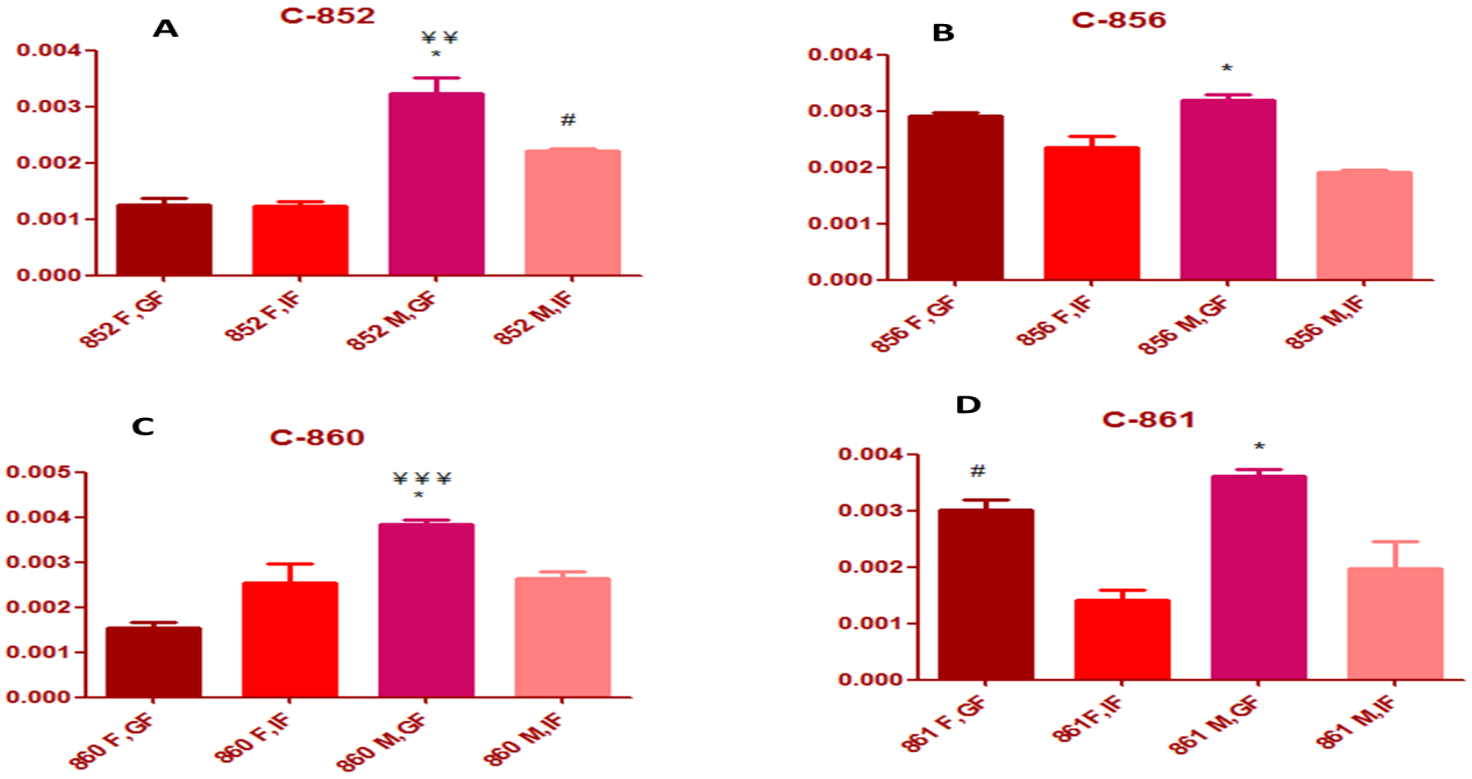


Figure 47. Cholesterol (mg/ml) concentrations between Female-Male and Gonadal-Inguinal obtained from (A) BFMI 852 (B) BFMI 856 (C) BFMI 860, and (D) BFMI 861. (¥, #, § and * indicated degree of significance of Female-Gonadal Fat, Female-Inguinal Fat, Male-Gonadal Fat and Male-Inguinal Fat, respectively.)

Diglyceride (DG) or a diacyl-glycerol (DAG) has glycerol that bound to two fatty acids. In lots of vegetable-based oil such as soybean and canola oil, there are few amount of diglyceride unlike triglyceride. The majority of vegetable-based oil is triglycerides. TG is quickly absorbed and then it is converted to body fat. However, DG is quickly metabolized and it is less converted to body fat (Anastasiou et al, 2009).

Figures 48-49 demonstrated statistical analysis of diglyceride. According to results, there was no significant change between BFMI lines. Also, there was no change between gonadal-inguinal and female-male, except BFMI 856 lines. In BFMI 856 lines, male tissues had a little bit higher DG than female only in inguinal fat. Moreover, in female tissues of BFMI 856, gonadal fat had a little bit higher DG than inguinal fat.

In literature, concentration of DG remains almost unchanged in obese mouse while TG levels increase (Anastasiou et al, 2009). Therefore, results are expected.

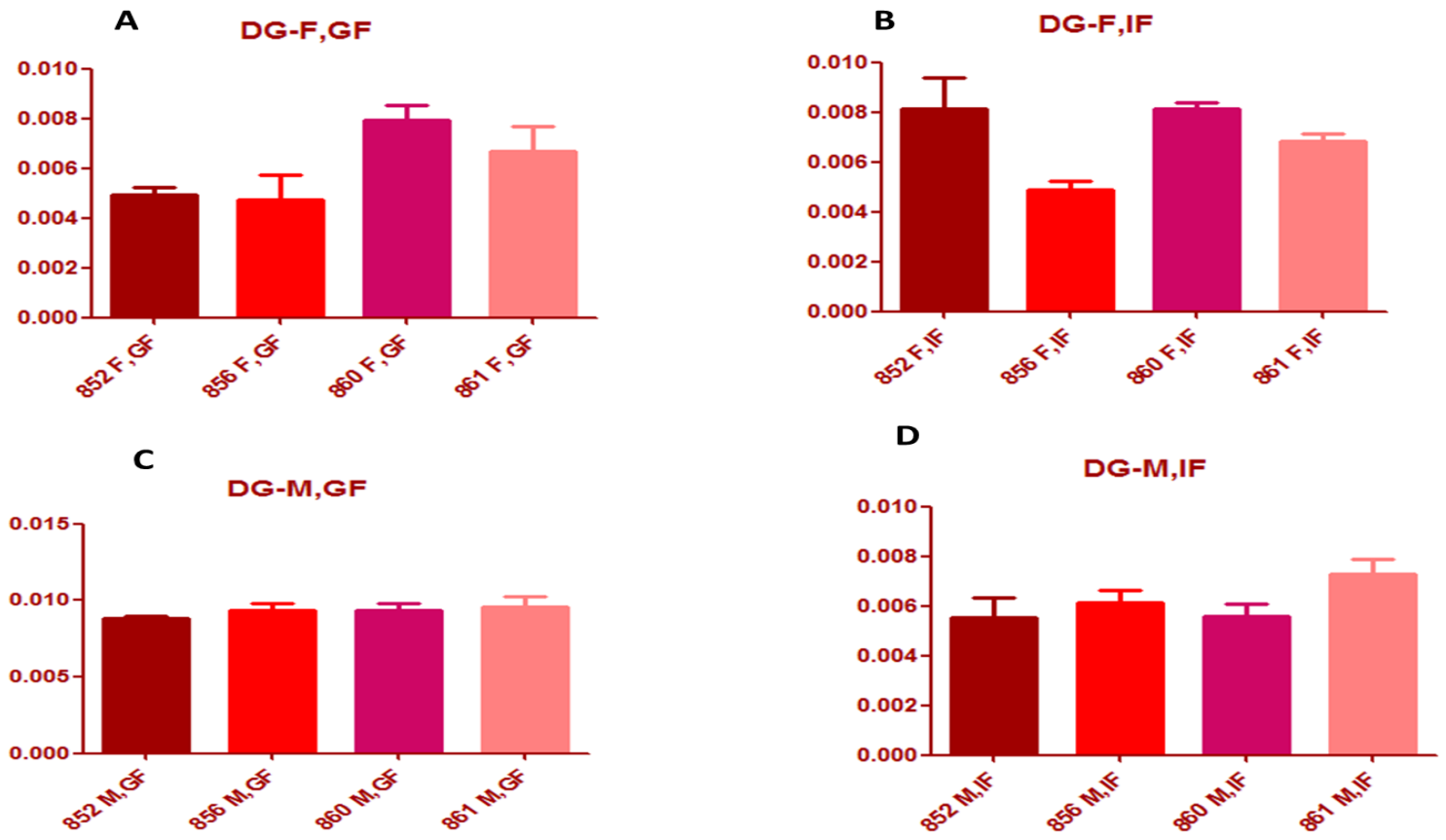


Figure 48. DG (mg/ml) concentrations between BFMI lines obtained from (A) Female, Gonadal Fat, (B) Female, Inguinal Fat, (C) Male, Gonadal Fat, and (D) Male, Inguinal Fat. (*, #, §, and ¥ indicated degree of significance of BFMI 852, BFMI 856, BFMI 860 and BFMI 861, respectively.)

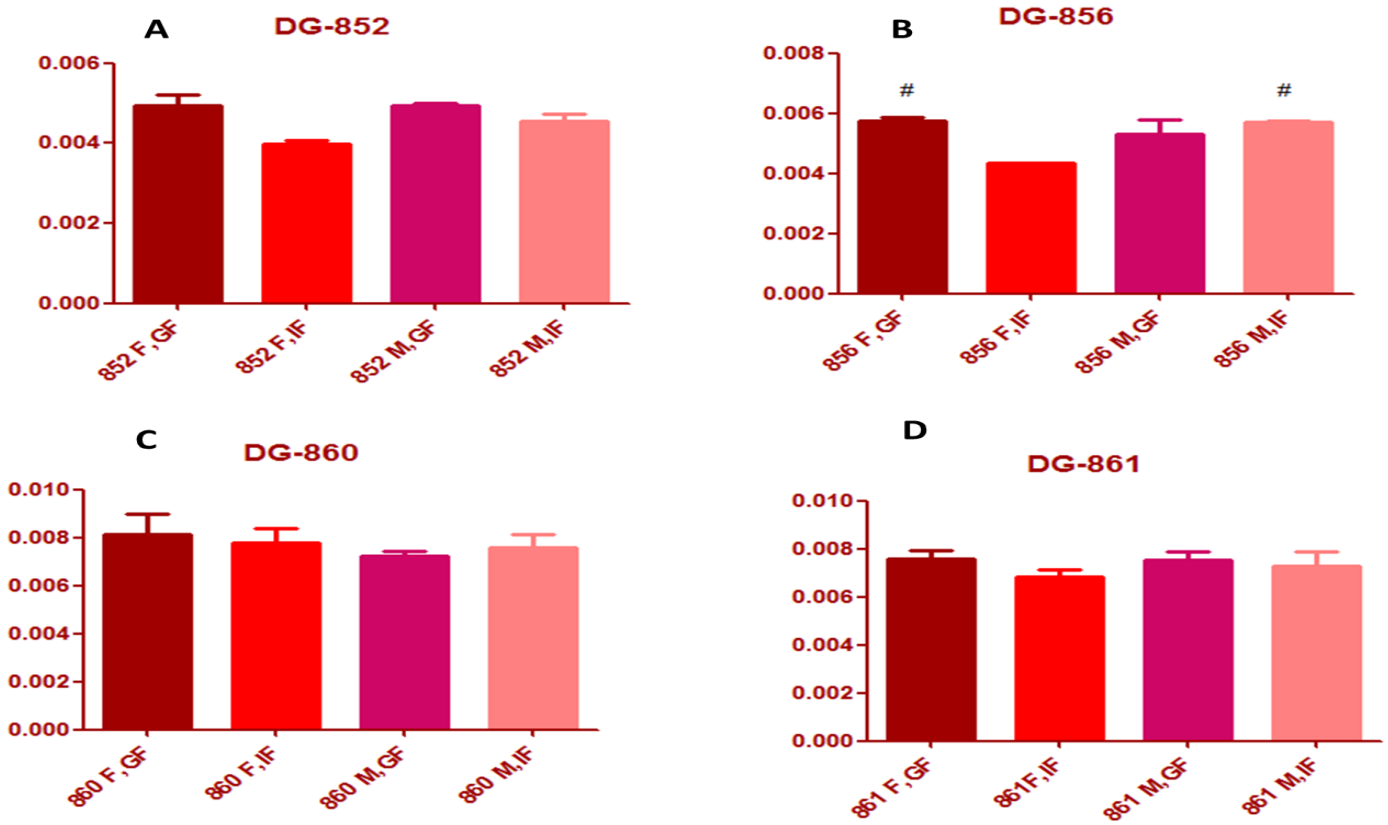


Figure 49. DG (mg/ml) concentrations between Female-Male and Gonadal-Inguinal obtained from (A) BFMI 852 (B) BFMI 856 (C) BFMI 860, and (D) BFMI 861. (¥, #, § and * indicated degree of significance of Female-Gonadal Fat, Female-Inguinal Fat, Male-Gonadal Fat and Male-Inguinal Fat, respectively.)

Monoglyceride, also labeled as monoacyglycerol has glycerol that bound to one fatty acid chain. It is procured from animal fats or plant oil. Furthermore, it can be produced synthetically. In addition, it can be obtained by breaking down the triglyceride (Christie, 1987; Dinh et al, 2002). Monoglycerides are broken down glycerol and fatty acid by monoglyceride lipase enzyme. Any deficiency on monoglyceride lipase enzyme has contribution to the insulin resistance. Therefore, monoglyceride and its lipase have effect on insulin resistance (Taschler et al, 2011).

Quantitative results of monoglycerides can be seen in Figures 50-51. Among all other BFMI lines BFMI 860 had the highest monoglyceride level. The reason might explain high level of TG in BFMI 860. In female and male tissue, there were no remarkable changes. In addition, gonadal and inguinal tissues had almost same amount of MG, except BFMI 856. In this tissue, gonadal had high MG than inguinal in male tissues.

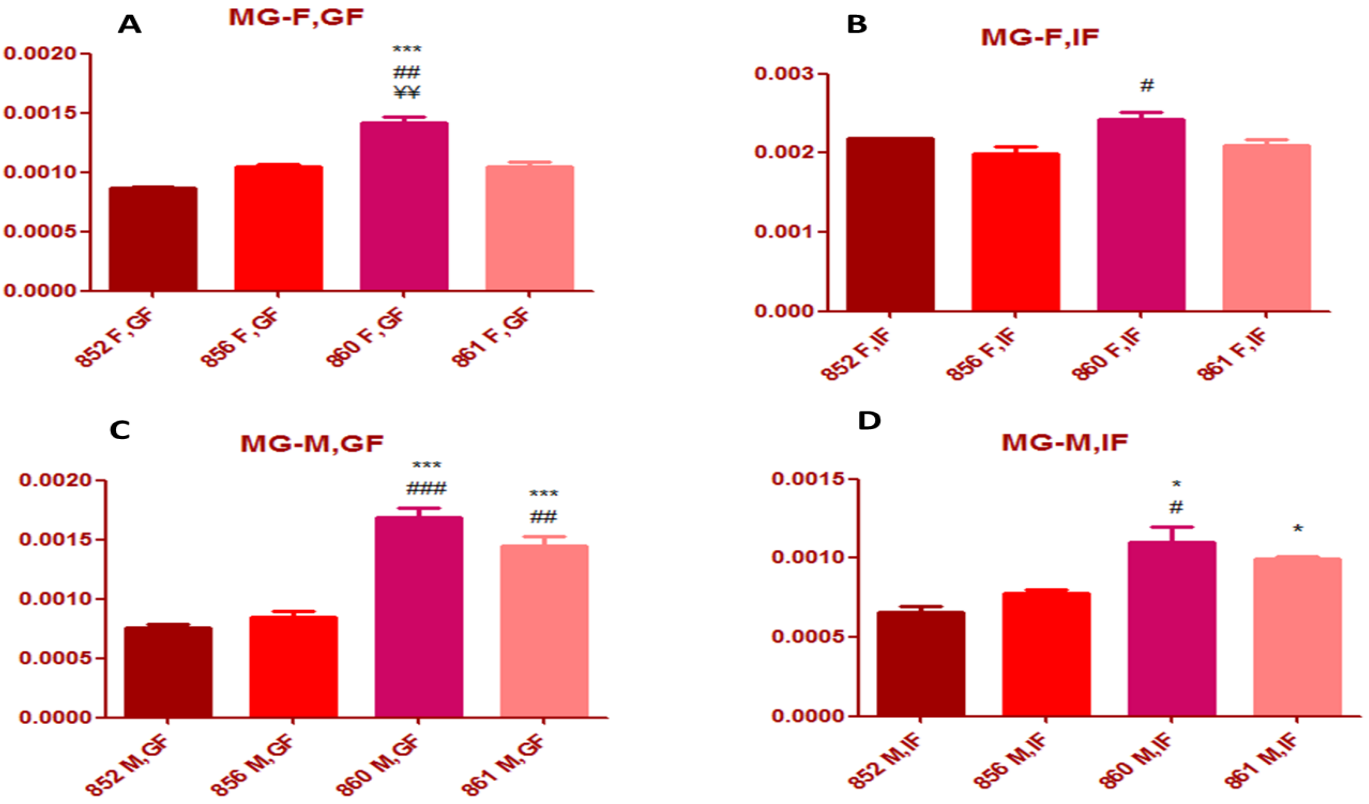


Figure 50. MG (mg/ml) concentrations between BFMI lines obtained from (A) Female, Gonadal Fat, (B) Female, Inguinal Fat, (C) Male, Gonadal Fat, and (D) Male, Inguinal Fat. (*, #, §, and ¥ indicated degree of significance of BFMI 852, BFMI 856, BFMI 860 and BFMI 861, respectively.)

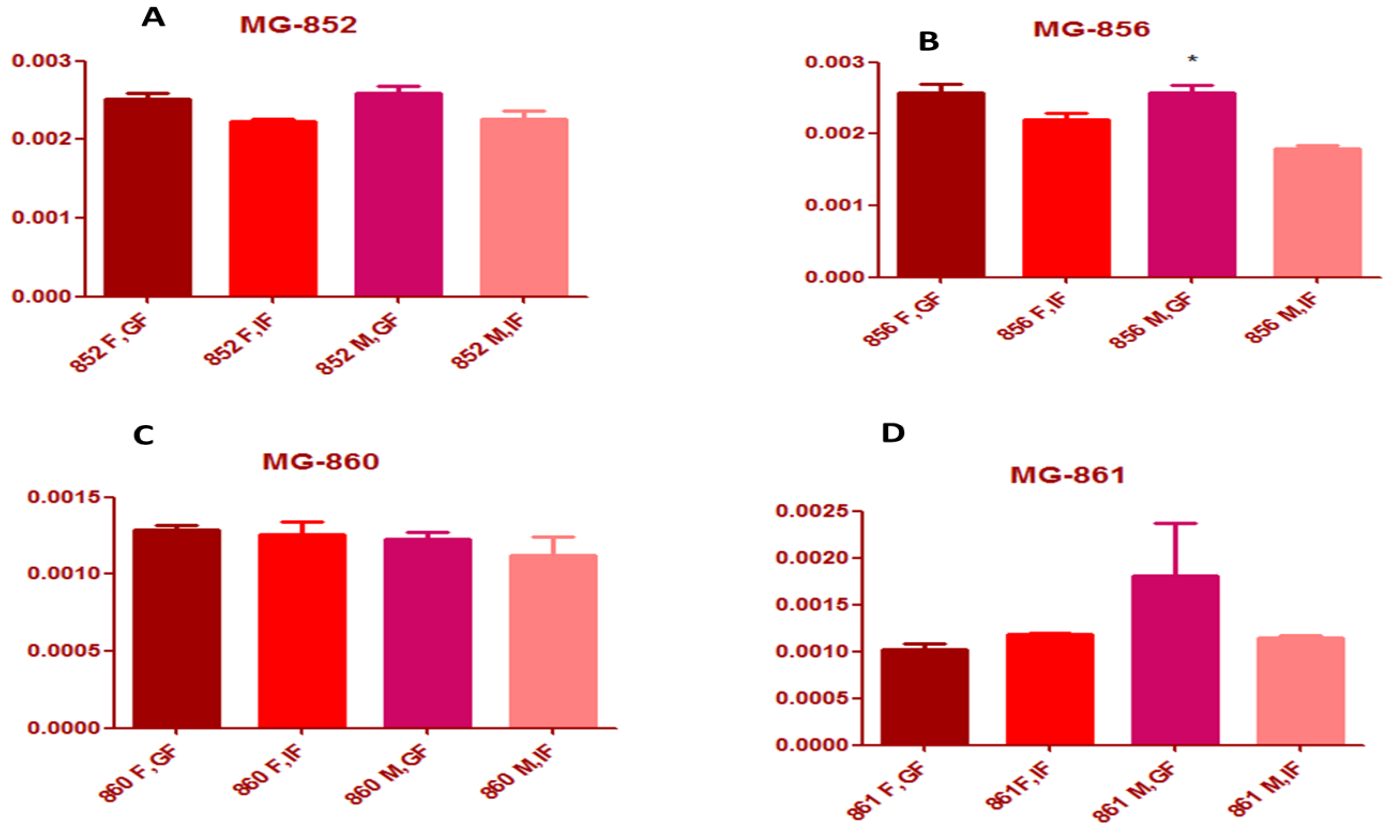


Figure 51. MG (mg/ml) concentrations between Female-Male and Gonadal-Inguinal obtained from (A) BFMI 852 (B) BFMI 856 (C) BFMI 860, and (D) BFMI 861. (¥, #, § and * indicated degree of significance of Female-Gonadal Fat, Female-Inguinal Fat, Male-Gonadal Fat and Male-Inguinal Fat, respectively.)

Phosphatidylcholine is one of the most abundant phospholipid in eukaryotes. Glycerol, phosphate group, choline head group and two fatty acid chain are found in the structure of phosphatidylcholine. It is one of the major components of cell membrane of plants and animals. It has role in membrane integrity and cell signaling. Moreover, it can be used to synthesize many molecules (Gibellini & Smith, 2010; Pasenkiewicz-Gierul et al, 2000).

Quantitative results of PC were seen in Figures 52-53. BFMI lines in female tissues had same PC content. However, in male tissues BFMI 860 and BFMI 861 had higher content because they had also insulin resistance and severe obesity. Gonadal and inguinal tissues had almost same PC content. Moreover, female and males tissues had almost same PC content.

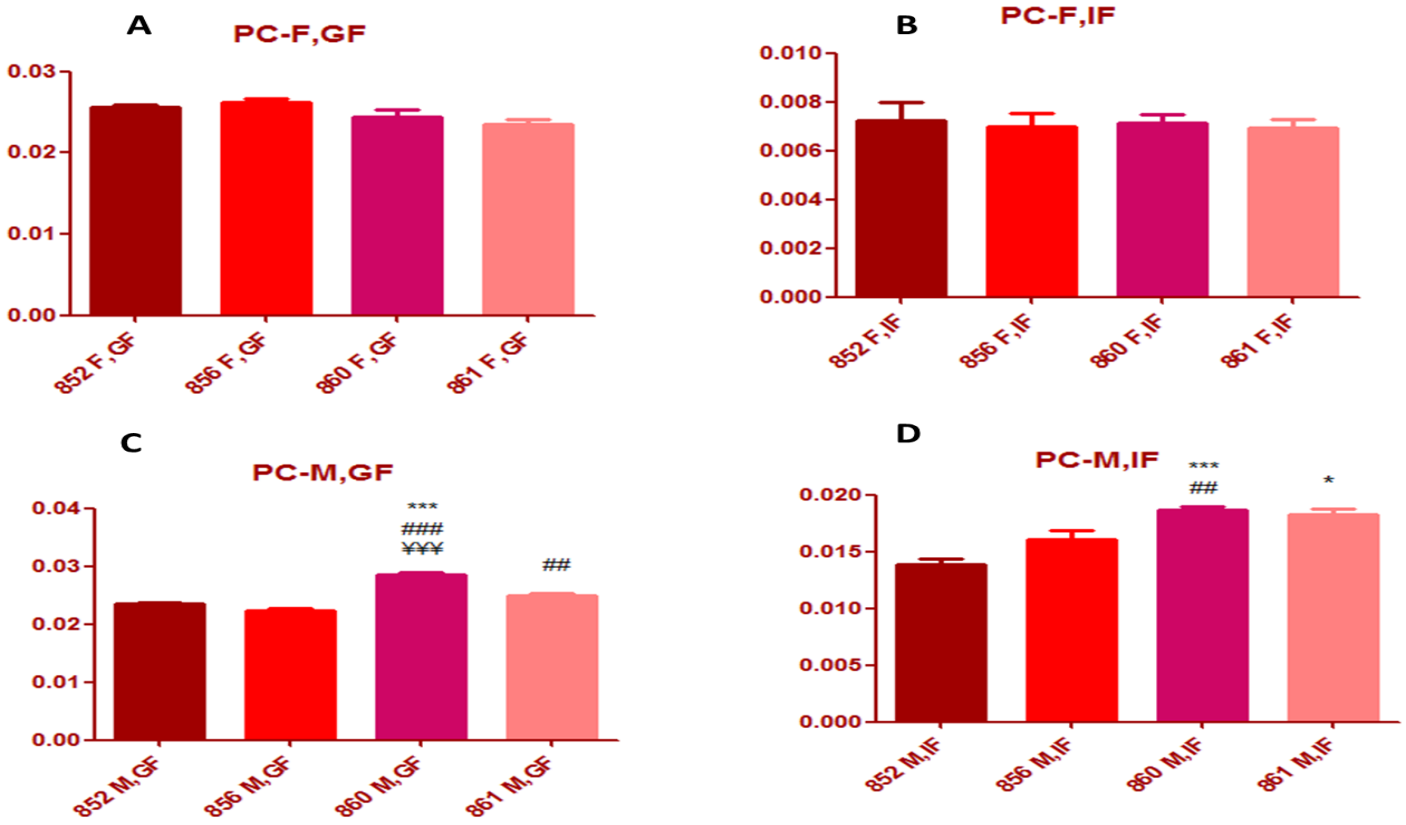


Figure 52. PC (mg/ml) concentrations between BFMI lines obtained from (A) Female, Gonadal Fat, (B) Female, Inguinal Fat, (C) Male, Gonadal Fat, and (D) Male, Inguinal Fat. (*, #, §, and ¥ indicated degree of significance of BFMI 852, BFMI 856, BFMI 860 and BFMI 861, respectively.)

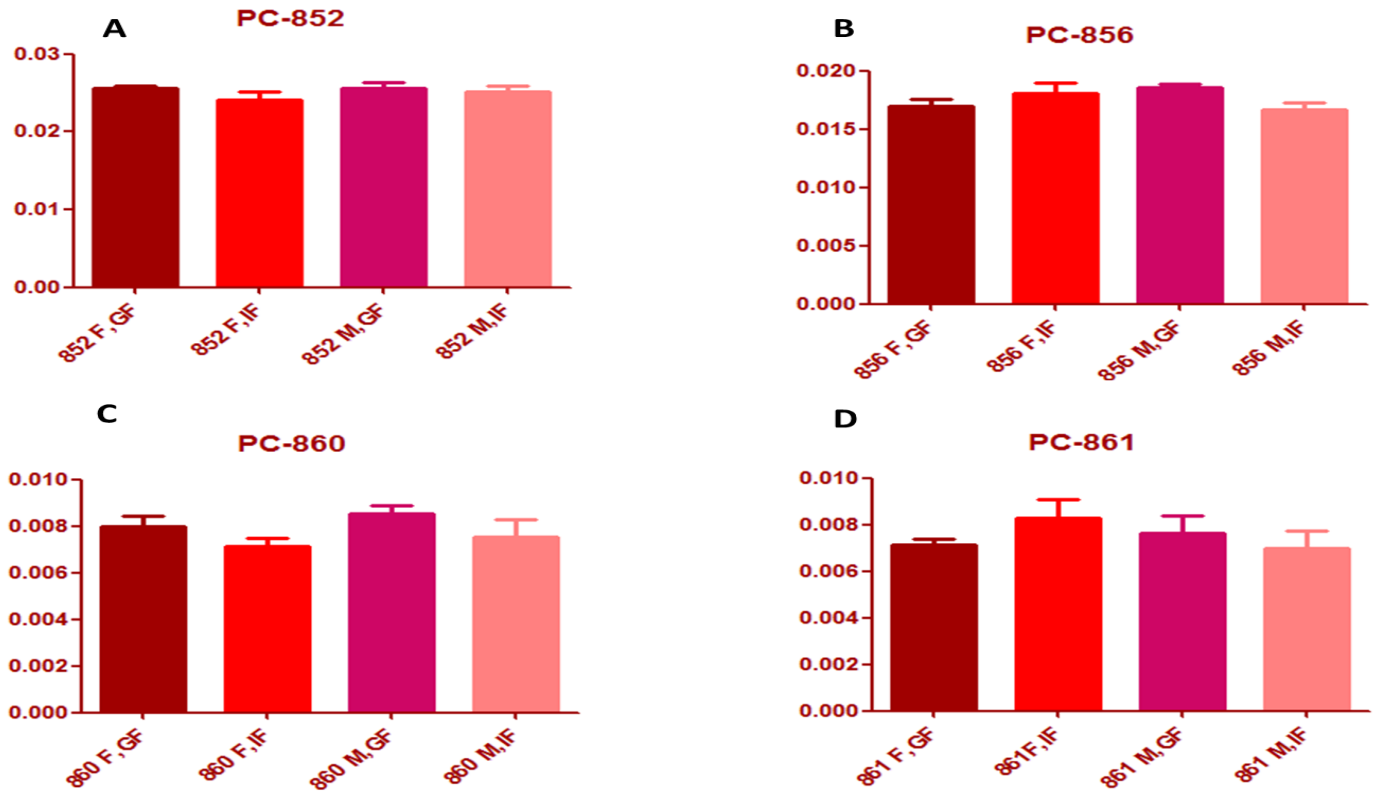


Figure 53. PC (mg/ml) concentrations between Female-Male and Gonadal-Inguinal obtained from (A) BFMI 852 (B) BFMI 856 (C) BFMI 860, and (D) BFMI 861. (¥, #, § and * indicated degree of significance of Female-Gonadal Fat, Female-Inguinal Fat, Male-Gonadal Fat and Male-Inguinal Fat, respectively.

Results which obtained from bar diagrams and German groups data (Appendix A) showed that BFMI 860 and BFMI 861 had higher lipid content except diglyceride (DG). Triglyceride (TG), cholesterol (C), monoglyceride (MG), and phosphatidylcholine (PC) were found higher amount in BFMI 860and 861. These results demonstrated that BFMI 860 and BFMI 861 had high level of obesity. Gonadal fat had higher lipids than inguinal fat in almost all BFMI lines. Female and male tissues had almost same amount lipids.

CHAPTER 4

CONCLUSION

In this study, quantification of obese mouse lipid profiles is performed by HPLC technique. Lipid profiles of BFMI lines (BFMI 852, BFMI 856, BFMI 860 and BFMI 861) were analyzed. Results showed that BFMI 860 and BFMI 861, particularly BFMI 860 had higher lipid content than other lines. In addition, gonadal or visceral adipose tissue had higher lipid content than inguinal adipose tissue. Differentiation between female and male tissue were different among lipid and BFMI lines. However, in general, female and male tissues had almost the same amount of lipid content.

Highest TG concentration was found in BFMI 860 and BFMI 861. This shows that BFMI 860 and BFMI 86, especially BFMI 860 could have high level of insulin resistance. Also, only in male tissues, BFMI 856 was found higher. Gonadal adipose tissue or visceral adipose tissue had higher TG content than inguinal adipose tissue. Female and male had same amount of TG, except BFMI 852-inguinal fat and BFMI 856-gonadal fat. In BFMI 852-inguinal fat and BFMI 856-gonadal fat, concentration of TG was higher in female tissues than in male tissues.

There were no changes in DG level in BFMI lines. All lines had same amount of DG. In addition, there were no differences between gonadal and inguinal adipose tissue, except BFMI 856 tissue. In BFMI 856 lines, in female tissues, DG concentration was higher in gonadal adipose tissue than inguinal adipose tissue. Moreover, there were no significant differences between female and male tissues, except BFMI 856. In BFMI 856, male had higher DG than female in inguinal adipose tissue.

Cholesterol levels were higher in BFMI 856, BFMI 860 and BFMI 861. The highest concentrations were observed in BFMI 860. The least concentration of cholesterol was found in BFMI 852. Gonadal fat had higher cholesterol concentration than inguinal fat. There were no significant changes in cholesterol level between female and male tissues, except BFMI 852 line. In BFMI 852 line, cholesterol was higher in male than female.

Monoglyceride concentrations were higher only in BFMI 860. It also had highest level of TG. Female and male tissue had same amount of TG content. In addition, gonadal fat and inguinal fat had same amount of monoglyceride level, except BFMI 856. In BFMI 856 line, gonadal fat was higher than inguinal fat in male tissue.

Phosphatidylcholine levels were observed the same amount among BFMI lines in female tissues. In male tissues, phosphatidylcholine levels showed differences unlike female tissues. Higher phosphatidyl levels were observed in BFMI 860 and BFMI 861 lines.

Gonadal and inguinal adipose tissues had same amount of phosphatidylcholine. In addition, there were differences in phosphatidylcholine between female and male tissues.

Results of this study were similar to the study done by the German group. Analysis of body weight, serum parameters (level of glucose, insulin, leptin, resistin, adiponectin, triglyceride (TG), cholesterol and non-esterified fatty acid (NEFA)) and intra-peritoneal insulin tolerance test for 10 weeks BFMI mouse were performed by German group. According to results, BFMI 860 had the highest body weight. Level of glucose, leptin, resistin, TG, cholesterol and NEFA were also higher in BFMI 860. High level of glucose, TG and NEFA demonstrated that insulin resistance might arise in BFMI 860. Furthermore, BFMI 860 and BFMI 861 lines gave late response to insulin. Results show that BFMI 860 have higher lipid content than other BFMI lines.

When results were evaluated, BFMI 860 and BFMI 861 had spontaneous and high level of obese. In addition, result showed that these lines might have insulin resistance. Therefore, these lines are suitable for usage as animal models for obesity and type II diabetes.

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APPENDIX A

GERMAN GROUP DATA

Analysis of body weight, serum parameters (level of glucose, insulin, leptin, resistin, adiponectin, triglyceride (TG), cholesterol and non-esterified fatty acid (NEFA)) and intra-peritoneal insulin tolerance test for 10 weeks BFMI mouse were performed by German group (Figure 54-55). According to results, BFMI 860 had the highest body weight. Level of glucose, leptin, resistin, TG, cholesterol and NEFA were also higher in BFMI 860. High level of glucose, TG and NEFA demonstrated that insulin resistance might arise in BFMI 860. Furthermore, BFMI 860 and BFMI 861 lines gave late response to insulin, as shown in figure 50B. Results show that BFMI 860 have higher lipid content than other BFMI lines.

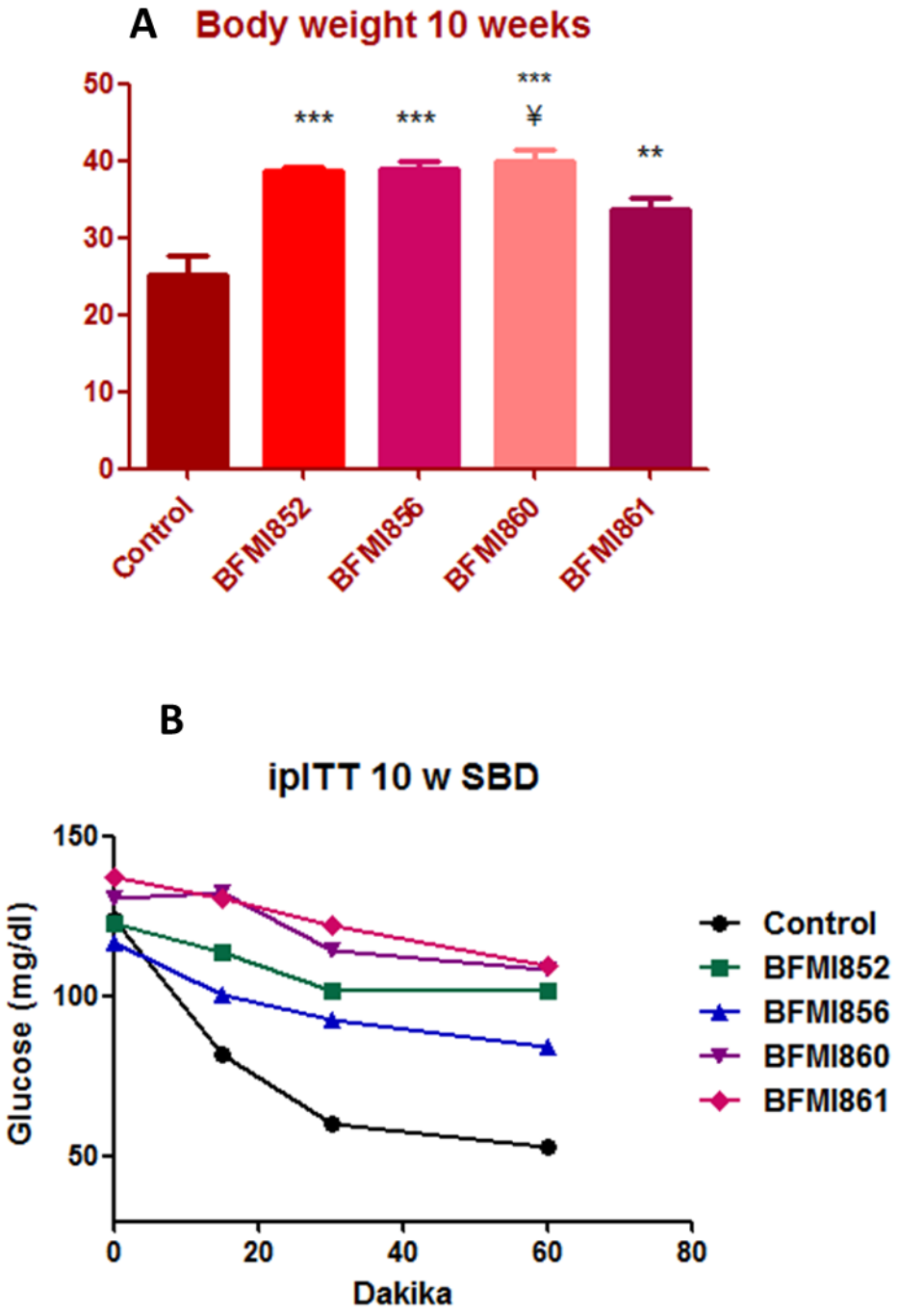

Figure A.1. Parameters of 10 weeks old BFMI lines which were fed with standard breeding diet (A) Body weight of control and BFMI lines (*,#,$,§, and ¥ indicated degree of significance of control, BFMI 852, BFMI 856, BFMI 860 and BFMI 861) (B) intra-peritoneal insulin tolerance test results of control and BFMI lines

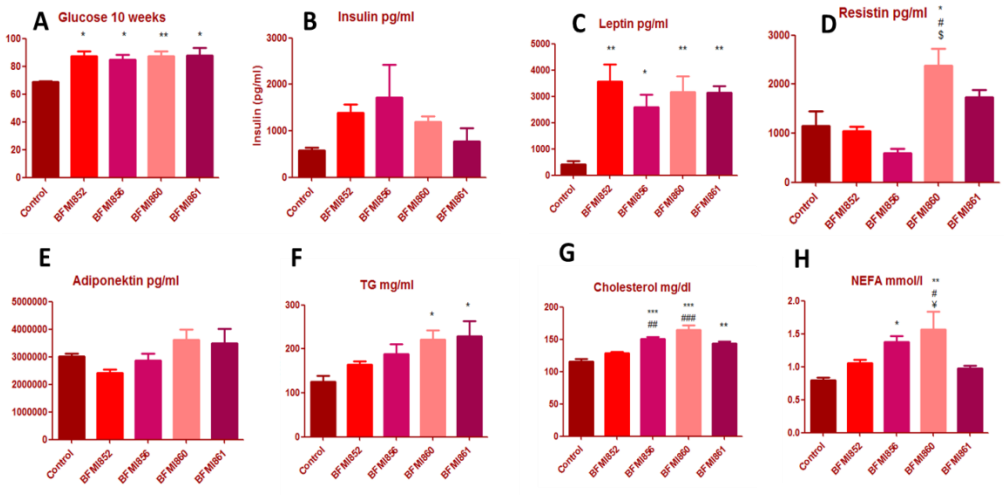

Figure A.2. Serum parameters of 10 weeks old control and BFMI Mouse lines (A) Glucose (B) İnsulin (C) Leptin (D) Resistin (E) Adiponektin (F) TG (G) Cholesterol (H) NEFA. (*,#,$,§, and ¥ indicated degree of significance of control, BFMI 852, BFMI 856, BFMI 860 and BFMI 861.