Quantitative analysis of fatty acids, cholesterol and oxidation products thereof in human breast adipose tissues

Doctoral Dissertation (thesis)

to obtain the degree of

“Doktor der Naturwissenschaften”

(Dr. rer. nat.)

awarded by the

Julius-Maximilians-Universität Würzburg

submitted by

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from Hamedan/Iran

Würzburg 2017

Submitted to the faculty of Chemistry and Pharmacy on:

_____

Evaluators of the written Dissertation (Thesis)

Supervisor - 1. Evaluator: _____

2. Evaluators: _____

Examiners of the Public Defense

1. Examiner: _____

2. Examiner: _____

3. Examiner: _____

Date of the Public Defense

_____

Doctoral Certificate awarded on

_____

To my Parents

This thesis is dedicated to my parents, who have provided me their unconditional love and support, and have always encouraged me to follow my dreams**.**

Acknowledgement

First I would like to express my gratitude to my supervisor professor Dr. Leane Lehmann who lets me work on very interesting topic. Her valuable suggestions, discussions and constructive criticism have contributed to the success of this work.

I'm also grateful to Dr. Harald Esch for his guidance, help, support and his valuable suggestions and comments during my work and also during writing this thesis.

I thank Daniela Pemp for her help in getting the GC-MS/MS analysis and her feedback during writing this thesis.

I would like to say thank to Dr. Carolin Kleider for her useful comments and feedback to this thesis.

I thank Annette Albrecht for her helps not only at the start of my PhD but also whenever I needed it.

I would like to say thank Anne Scheffler and Jan Fuchs for their valuable suggestion, they were always ready to listen when I needed to talk with them.

I would like to say thank to Dr. Katja Schmalbach for her help in statistic part of this research.

I thank Miriam Macziol and Benjamin Spielmann for their useful comments and feedback to this thesis.

I would like to thank the master student Lisa Bäuml for her work during her master thesis.

I would like to thank the other colleagues of the Prof.Lehmann working group (Dr.Susanne Futh, René Hauptstein, and Sabrina Jäger) for the good and always fair cooperation.

Last, but certainly not least, I must sincerely thank my lovely parents, my sister and my husband for their unconditional love, constant encouragement and fathomless support which I have gotten over the years, with special thanks to my father Dr. Alireza Mahdiani.

Contents

Abbreviations

α-epoxy-ChOL	5,6α-Epoxy-Cholesterol
β-epoxy-ChOL	5,6β-Epoxy-Cholesterol
5α-ChAN-3β-ol	5α-Cholestan-3β-ol
7-O-ChOL	7-Keto-Cholesterol
7α-HO-ChOL	7α-Hydroxy-Cholesterol
7β-HO-ChOL	7β-Hydroxy-Cholesterol
7α/β-OOH-ChOL	7 HydroperoxyChOL
AA	Arachidonic acid
ACC	Acetyl-CoA carboxylase
ALA	Alpha linolenic acid (18:3 n3)
BC	Breast cancer
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSTFA	N,O-Bis(trimethylsilyl)trifluoracetamide
ChOL	Cholesterol
CYP	Cytochrome P450
CYP7A1	Cytochrome P450 family 7 subfamily A member 1
D-ChOL	Deuterated ChOL-25,26,26,26,27,27,27-d7
DGF	Deutsche Gesellschaft für Fettwissenschaft
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid (22:6 n3)
EPA	Eicosapentaenoic acid (20:5 n3)
EtOH	Ethanol
FAME	Fatty acid methyl ester
FASN	Fatty acid synthase
FDA	Food and drug administration
FID	Flame ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GLA	Gamma-linolenic acid (18:3 n6)
HMG-CoA	3-Hydroxy-3-Methylglutaryl coenzyme A
IS	Internal Standard
ID	Inner diameter
LA	Linoleic acid (18:2 n6)
LCPUFA	Long chain polyunsaturated fatty acid
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MTBE	Methyl tert-butyl ether
MUFA	Monounsaturated fatty acid

OxyChOL(s)	Oxidation products of cholesterol
Oxy-D-ChOL(s)	Oxidation products of 6-fold deuterated cholesterol
PUFA	Polyunsaturated fatty acid
Q	Quadruple
ROS	Reactive Oxygen Species
RSD	Relative standard deviation
R_t	Retention time
SFA	Saturated fatty acid
SPE	Solid Phase Extraction
TFA	Trans fatty acid
TMSH	Trimethylsulfonium hydroxide

1. Introduction

Adipose tissue is commonly known as body fat and composed of adipocytes surrounded by a matrix of collagen fibers, blood vessels and immune cells. Adipose tissue is energy storage tissue and highly active metabolic and endocrine organ (Ailhaud et al., 1992; summarized in Galic et al., 2010). Its principal metabolic actions can be divided into: biosynthesis, incorporation and storage of triacylglycerols and liberation of free fatty acids and glycerol (Coelho et al., 2013). The cholesterol (ChOL) is stored in adipose tissue and used for the metabolism and production of steroid hormone by adipose tissue (Trayhurn and Beattie, 2001; summarized in Galic et al., 2010; Guerre-Millo, 2002). Moreover, adipose tissue produces hormones, including several cytokines and adipsin (Guerre-Millo, 2002). Adipose tissue plays a crucial role in the regulation of whole-body fatty acid homeostasis (summarized in Yu and Ginsberg, 2005). It is found all over the body. It presents under the skin (subcutaneous fat), packed around internal organs, between muscles, within bone marrow and in breast tissue (summarized in Tchkonia et al., 2010, Chapter 1.1).

1.1 Breast adipose tissue composition

The breast consists of glandular, fatty, and fibrous tissues positioned over the pectoral muscles of the chest wall. The mammary glands are surrounded by a layer of adipose tissue. This layer extends throughout the breast (Jesinger, 2014; summarized in Zhu and Nelson, 2013). Breast adipose tissue is a type of subcutaneous fat depot which consists of adipocytes, preadipocytes, endothelial cells, pericytes, monocytes, macrophages, and fibroblastic connective tissue (Bohler et al., 2010; Kershaw and Flier, 2004; Hovey et al., 1999). Breast adipose tissue is a source of lipids and their metabolites (Hovey and Aimo, 2010; Hovey et al., 1999). The development of mammary can be affected by fat content and fat type (summarized in Wang et al., 2010; Schedin and Hovey, 2010). Previously it has been shown that the development of normal mammary gland was prevented in the total absence of breast adipose tissue in transgenic mice (Couldrey et al., 2002). In addition, previous cell culture studies have shown that breast adipose tissue plays a role in metastatic progression of breast tumors (Wang et al., 2015; Manabe et al., 2003; Chamras et al., 1998).

The breast adipose tissue consists of major constituent including fatty acids (summarized in Lafontan, 2008) and ChOL (summarized in Yu et al., 2010). Oxidation products of ChOL (oxyChOL) present also in the adipose tissue. In this chapter, the breast adipose tissue components: fatty acids (Chapter 1.1.1), ChOL (Chapter 1.1.2) and oxyChOLs (1.1.3) are taken into account.

1.1.1 Fatty acids

The fatty acid composition of breast adipose tissue of breast cancer (BC) patients (Klein et al., 2000; Maillard et al., 2002; Chajes et al., 1992), breast adipose tissue of women without BC (Straka et al., 2015; Yee et al., 2010) and other biological adipose tissues such as gluteal (Mamalakis et al., 2009; Bagga et al., 1997; London et al., 1991) and abdominal (Boué et al., 2000) adipose tissues were investigated previously. The result indicated the fatty acid composition of subcutaneous adipose such as the gluteal, abdominal and breast were similar. The main fatty acids in different type of adipose tissue were oleic acid, palmitic acid, linoleic acid (LA), stearic acid, palmitoelic acid and myristic acid (Tjonneland et al., 1993; Maillard et al., 2002; Klein et al., 2000). Fatty acids are carboxylic acids which are categorized to saturate and unsaturated (Chapter 1.1.1.1). They play an important role in human health. There are several studies which demonstrated fatty acids associated with developing different diseases including BC (summarized in MacLennan and Ma, 2010; Makarem et al., 2013; Bhupathiraju and Tucker, 2011). Therefore, in chapter 1.1.1.2 the biological effect of fatty acids in adipose tissue is considered. Moreover, the source of fatty acids in breast adipose tissue is taken into account (Chapter 1.1.1.3).

1.1.1.1 Fatty acids structure and categorization

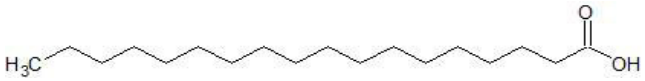
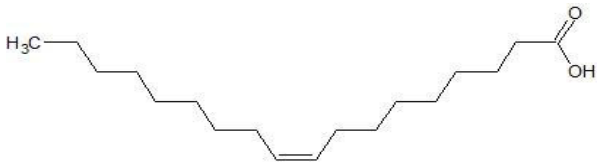
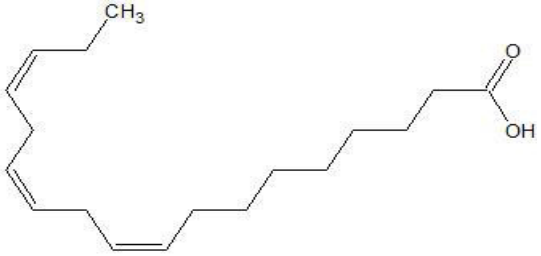
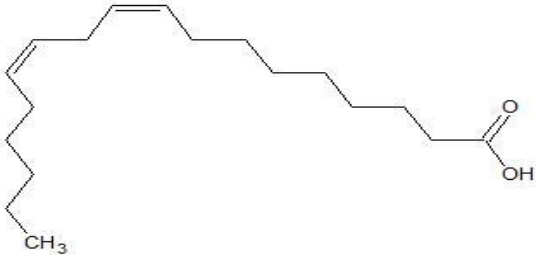
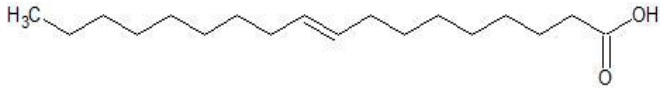
Fatty acids composed of a hydrocarbon chain of variable length, with a carboxyl group (COOH) at one end and a methyl (CH_3) group at the other end (Table 1). Fatty acids vary in carbon chain length and degree of unsaturation. The length of fatty acids is termed short, medium, long, and very long, having ≤ 6, 8 to 14, 16 to 22, or ≥ 22 carbons respectively (Rustan and Drevon, 2005). Fatty acids can be stored into the following categories: saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and trans fatty acid (TFA).

SFAs have no double bonds. MUFAs are characterised by having one double bond with the hydrogen atoms present on the same side of double bond. SFA divided to two groups: even numbered and odd-numbered SFA. Odd chain fatty acids are those that contain an odd number of carbon atoms in the structure (example pentadecanoic acid 15:0).

PUFAs have two or more double bonds generally separated by a single methylene group. Two main types of PUFAs are n3 and n6 fatty acids (Table 1). The first double bonds of n3 and n6 PUFA are located three and six carbons from the beginning of the carbon chain respectively (Table 1). They are a family of 18-24 carbon chain fatty acids with three or more methylene-interrupted double bonds.

TFAs are unsaturated fatty acids that contain at least one double bond in the trans configuration (Table 1).

Table 1 Chemical structure of fatty acids (modified according to MacLennan and Ma, 2010).

group	Example of fatty acids	Chemical structure
SFA	stearic acid (18:0)	
MUFA	oleic acid (18:1 9cis)	
n3 PUFA	α-linolenic acid (ALA, 18:3 n3)	
n6 PUFA	linoleic acid (LA, 18:2 n6)	
TFA	elaidic acid (18:1 9trans)	

1.1.1.2 Biological effect of fatty acids in adipose tissue

Fatty acids are required for membrane synthesis, modifications of proteins and carbohydrates, construction of various structural elements in cells and tissues and production of signalling compounds. Furthermore, there are several studies which demonstrated the type and content of dietary fat associated with developing different diseases including BC and heart diseases (summarized in MacLennan and Ma, 2010; Makarem et al., 2013; Bhupathiraju and Tucker, 2011). Mamalakis et al. (2009) investigated the gluteal fatty acid composition of BC patients and healthy control women. The result of conditional logistic regression analysis showed that gluteal adipose tissue myristic fatty acid was associated with an increase in BC risk. Furthermore, there is evidence that myristic acid may stimulate protein kinase C activation and

exert procancer effects (Kasahara and Kikkawa, 1995). The high protein kinase C activity has been found in malignant breast tumors (Ways et al., 1995).

There is some evidence which indicates oleic acids may play a role in BC. Indeed, it may reduce the BC risk by suppressing HER2/neu gene (summarized in MacLennan and Ma, 2010; Menendez et al., 2005). HER2/neu can promote metastasis in cells and decreases receptors of cell-surface HER2/neu (Menendez et al., 2005). Mamalakis et al. (2009) found out that a high level of MUFA and oleic acid can reduce the risk of BC.

A study on mice treated with MDA-MB-231 BC cells comparing diets of corn oil (1% ALA) with diets of canola oil (10% ALA) found, the tumor growth rate to be significantly reduced in canola-fed mice (Hardman et al., 2005). Cell culture work found that ALA strongly suppressed over-expression of HER2-positive mammary carcinomas (Menendez et al., 2006).

Previously published studies have shown that n6 PUFA stimulates the growth of human BC cell lines in vitro, whereas docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) inhibit growth (Rose et al., 1993; Rose et al., 1991). Reductions in tumor incidence have been observed in the estrogen receptor-negative MMTV-HER2/neu transgenic mice which fed 3% and 9% n3 PUFA diets (fish oil). Unlike the tumor-enhancing effects of diets containing elevated amounts of LA, diets enriched with EPA and DHA suppress tumor growth and metastasis in nude mice with human BC xenografts (Gonzalez et al., 1991).

TFAs may inhibit the conversion of essential fatty acids to long chain polyunsaturated fatty acids (LCPUFA), which are essential for tissue growth and development (Decsi and Koletzko, 1995; Larqué et al., 2000). TFAs compete with the essential fatty acids for the enzyme systems involved in these reactions (Larqué et al., 2000). Indeed, elaidic acid competes for desaturase enzyme sites and can reduce production of n3 and n6 PUFAs (Booyens et al., 1992). Furthermore, Szabó et al. (2007) studied the relation between TFAS and LCPUFAs in human milk. Significant negative correlation was found between relative level of elaidic acid and n3 and n6 PUFAs, which is essential for tissue growth and development.

1.1.1.3 The source of fatty acids in breast adipose tissue

Fatty acids can be taken up from the diet (Chapter 1.1.1.3.1) but can be also synthesized in body (Chapter 1.1.1.3.2).

1.1.1.3.1 Dietary source of fatty acid

The even numbered SFAs such as capric and lauric acid occur naturally in coconut oil and palm kernel oil (Table 2). Edible fats like coconut oil and milk fat contain a considerable amount of

myristic acid (Table 2). Palmitic acid is the long chain SFA which is found in high proportion in palm oil. SFAs (except odd-numbered) can be produced by the body as well.

Table 2 The dietary sources of SFA in human body.

Fatty acids	Common Name	Systematic Name	Diet	Ref
10:0	capric acid	decanoic acid	coconut oil, palm kernel oil	Deutsche Gesellschaft für Fettwissenschaft (DGF); Gunstone. (1996)
12:0	lauric acid	dodecanoic acid	coconut oil, palm kernel oil	DGF; Gunstone. (1996)
13:0	tridecylic acid	tridecanoic acid	milk	Beri et al. (2011); Medeiros et al. (2014)
14:0	myristic acid	tetradecanoic acid	coconut oil, dairy fat	DGF; Biong et al. (2006)
15:0	pentadecylic acid	pentadecanoic acid	milk	Biong et al. (2006); Brevik et al. (2005); Baylin et al. (2002)
16:0	palmitic acid	hexadecanoic acid	palm oil	DGF; London et al. (1991)
17:0	margaric acid	heptadecanoic acid	milk	Biong et al. (2006)
18:0	stearic acid	octadecanoic acid	cocoa butter, vegetable oils	DGF
20:0	arachidic acid	eicosanoic acid	cocoa butter, peanut oil	DGF; Gunstone. (1996)
21:0	heneicosylic acid	heneicosanoic acid	milk	Beri et al. (2011); Medeiros et al. (2014)
23:0	tricosylic acid	tricosanoic acid	cheese	Medeiros et al. (2014)

Odd chain fatty acids cannot be synthesised in humans. They originate from the diet. 15:0 and 17:0 are specific fatty acids of milk fat (Wolk et al., 1998). The amount of these fatty acids in adipose tissue may reflect the long-term dairy fat consumption. Also 13:0 and 21:0 are present in a low level in milk fat (Beri et al., 2011). 23:0 fatty acid is seen in cheese which is made from goat milk (Medeiros et al., 2014).

MUFAs

Plant sources such as canola oil, olive oil and sunflower oil are rich in MUFAs. Oleic acids accounts for about 92% of dietary MUFAs. The major sources for human food are olive oil (60 - 80%), almond oil, sunflower oil high oleic (Table 3). Dietary source of 14:1 fatty acid is milk fat. 16:1 is seen mainly in animal fats, particularly in fish and marine mammals (Table 3). Rapeseed oil is a rich source of 20:1.

Table 3 The dietary sources of MUFA in adipose tissue.

Fatty acids	Common Name	Systematic Name	Diet	Ref
14:1	myristoleic acid	cis-9-tetradecenoic acid	milk	Brevik et al. (2005)
16:1	palmitoleic acid	cis-9-hexadecenoic acid	beef tallow, marine oils	DGF, Gunstone. (1996); Jones et al. (1995).
18:1 9cis	oleic acid	cis-9-octadecenoic acid	olive oil, almond oil, sunflower oil high oleic	DGF, Gunstone. (1996)
18:1 11cis	vaccenic acid	cis-11-octadecenoic acid	most vegetable oils	DGF, Gunstone. (1996)
20:1 11cis	eicosenoic acid	cis-11-eicosenoic acid	rapeseed oil	DGF, Gunstone. (1996)

n3 and n6 PUFAs

There are PUFAs such as alpha linolenic acid (ALA, 18:3 n3) and Linoleic acid (LA, 18:2 n6) which cannot be synthesised in human body and the body must provide them from the diet. Therefore, they are called essential fatty acids (summarized in Liu and Ma, 2014).

ALA is essential fatty acid (n3). The adequate Intake of ALA was set at 1.1 g/d for women (summarized in Liu and Ma, 2014). To provide adequate intake of ALA, the consumption of oils rich in ALA such as flaxseed oil, canola and walnut oils are recommended (Table 4). DHA and EPA are two long chain n3 PUFAs that are present at high concentrations in fish oils and fatty fish. Examples of fish rich in DHA and EPA include salmon, cod, sardines, herring and anchovies.

The most important member of n6 PUFAs is LA. It is essential fatty acid and represents the basis of the n6 family. Safflower, corn, soybean, sunflower, cottonseed, peanut and rice bran oils are the sources of LA (Table 4).

Gamma-linolenic acid (GLA) is another n6 PUFA. The rich dietary sources of GLA are borage oil, blackcurrant seed oil and evening primrose oil (Table 4). Arachidonic acid (AA) is found naturally occurring in the diet, mostly through animal products.

Table 4 The dietary sources of PUFA (n6 and n3) in adipose tissue.

Fatty acids	Common Name	Systematic Name	Diet	Ref
18:2 n6	linoleic acid (LA)	cis-9,12-octadecadienoic acid	safflower oil, soybean oil, sunflower oil	DGF, Jones et al. (1995)
18:3 n6	gamma-linolenic acid (GLA)	cis-6,9,12-octadecatrienoic acid	borage, blackcurrant, evening primrose oil	DGF, Gunstone. (1996)
20:2 n6	eicosadienoic acid	cis-11,14-eicosadienoic acid	fish	Gunstone. (1996); Özogul et al. (2007)
20:3 n6	dihomo-gamma-linolenic acid (DGLA)	cis-8,11,14-eicosatrienoic acid	fish	Gunstone. (1996); Özogul et al. (2007)
20:4 n6	arachidonic acid (AA)	Cis-5,8,11,14-tetraenoic acid	meat, fish, poultry	DGF
18:3 n3	alpha-linolenic acid (ALA)	cis-9,12,15-octadecatrienoic acid	flaxseed oil, canola, walnut oil, soybean oil	DGF
20:3 n3	eicosatrienoic acid	cis-11,14,17-eicosatrienoic acid	fish	Özogul et al. (2007)
20:5 n3	eicosapentaenoic acid (EPA)	cis-5,8,11,14,17-eicosapentaenoic acid	fish	Gunstone. (1996); Baylin et al. (2002)
22:6 n3	docosahexaenoic acid (DHA)	cis-4,7,10,13,16,19-docosahexaenoic acid	fish	Gunstone. (1996); Baylin et al. (2002)

n6 to n3 ratios

Consumption of n3 and n6 fatty acids in the proper ratio is important. The n6 fatty acids compete with n3 fatty acids for use in the body (summarized in Lands, 2012) and therefore excessive intake of n6 PUFAs can inhibit n3 PUFAs. Moreover, a high n6/n3 ratio, involves in the pathogenesis of many diseases, including cardiovascular disease, BC, and inflammatory and autoimmune diseases, whereas increased levels of n3 PUFA (a lower n6/n3 ratio), can have suppressive effects (Yang et al., 2014; Patterson et al., 2012). Thus, the ratio of n6 to n3 fatty acids should be ideally between 1:1 and 4:1 (Simopoulos, 1999). Instead, most Americans consume these fatty acids at a ratio of n6:n3 between 10:1 and 20:1, consequently unable to get the benefits of n3 PUFAs (Simopoulos, 1999). Ratios of n6/n3 are 15:0 in United Kingdom and northern Europe (Sanders, 2000). This imbalance can cause by high consumption of processed foods and oils.

Experimental studies demonstrated that mammary tumors suppression is achieved with a diet balanced in n6/n3 (Cohen et al, 1993). Cohen et al. (1993) designed a study to investigate the effect of n6 to n3 PUFA ratio on N-nitrosomethylurea-induced rat mammary tumors. They

varied the proportion of n3 PUFA (provided as menhaden oil) to n6 PUFA (corn oil) in a high fat diet (23% per day). They found that tumor growth was inhibited only when equal parts of n6 and n3 PUFA were fed. In a rat model of chemically induced mammary tumors, a high dietary n3 to n6 PUFA ratio was found to decrease cellular proliferation by 60% and increase apoptosis in the mammary carcinomas compared to a low n3 to n6 PUFA ratio (Jiang et al., 2012).

TFAs

TFAs, produced by the method of partial hydrogenation of vegetable, have adverse effect on human health. In both animal and human studies, dietary TFAs are digested and absorbed in the same way as natural cis isomers. TFAs have been determined to incorporate into serum triglycerides, lipoproteins and adipose tissue (Vidgren et al., 1998; Aro et al., 1995).

Elaidic acid is the major TFA (the trans isomer of oleic acid) which derived from partially hydrogenated vegetable oils. Products such as crackers and cookies are also the rich source of this fatty acid.

Adipose tissue fatty acids as a biomarker of dietary fatty acids

The fatty acid composition of adipose tissue has been reported to reliably reflect dietary intake of the preceding two to three years period, whereas the fatty acid profile of serum or plasma phospholipids reflects medium-term intake of dietary fat (Summers et al., 2000; Tjonneland et al., 1993; Mamalakis et al., 2009). This is especially true for TFAs, the odd-chain SFAs (e.g. 15:0 and 17:0) and the essential PUFAs (both n3 and n6), which cannot be synthesized endogenously but are derived exclusively from dietary sources (London et al., 1991; Kohlmeier et al., 1997; Klein et al., 2000). Conversely, SFAs (except for odd-numbered fatty acids) and MUFAs are not expected to reflect intake.

Some studies have implemented biomarkers of fatty acid intake in adipose tissues. Biong et al. (2006) investigated the correlation of the fatty acids characteristic of dairy fat (15:0, 17:0) in gluteal adipose tissue. They observed the highest correlation between relative peak area percentages of 15:0 in gluteal adipose tissue and the estimated intakes of 15:0 (R-value = 0.55) and dairy fat (R-value = 0.55). Since 15:0 is odd-numbered fatty acids, it cannot be synthesised in human body. The presence of 15:0 in adipose tissue indicated its origin from diet. According to the Biong et al. (2006) study, the main dietary source of odd-numbered fatty acids is dairy fat.

The relation between habitual fish and marine n3 PUFA intake, and the fatty acid composition of gluteal adipose was studied by Marckmann et al. (1995) They demonstrated that the gluteal

adipose tissue DHA (relative peak area percentage) is the biomarker for long-term habitual dietary intake of fish and marine n3 PUFAs.

Moreover, dietary isomeric TFAs are reflected in the fatty acid composition of adipose tissue. Gluteal adipose tissue TFAs were determined by Aro et al. (1995). They concluded that hydrogenated vegetable fats are primarily dietary source of 18:1 trans fraction in adipose tissues.

Petrova et al. (2011) investigated the correlation between ALA and DHA availability and gluteal adipose tissue ALA and DHA in 50 men and 58 women. A strong positive correlation (R-value = 0.92) was found between ALA availability as a percent of total vegetable oils and adipose tissue ALA. Moreover, DHA associated strongly with fish (R-value = 0.88). Furthermore, gluteal adipose tissue ALA proportion was investigated in Costa Rican population. The subjects divided to 5 different quintiles. Adipose tissue ALA was 2-fold higher in the highest (0.75%) than in the lowest quintile (0.36%). In 70.4% of subjects were soybean oil users in the highest quintile (Baylin et al., 2003).

1.1.1.3.2 Fatty acids biosynthesis

SFAs (except odd numbered), MUFAs and PUFAs (except essential fatty acids) can also be synthesised in liver, white adipose tissue and the mammary glands (during lactation). In human, it seems that adipose tissue is not a major site of *de novo* fatty acid. Guo et al. (2000) measured the accumulation of *de novo* synthesized triglyceride fatty acids in human adipose tissue (gluteal and abdominal adipose tissue) *in vivo* using deuterated water and isotope ratio mass spectrometry. Rates of *de novo* fractional synthesis of triglyceride fatty acids were low (mean: 0.014%) for both lean and obese Caucasian women. They noted that the *de novo* synthesis of fatty acid seems to be highly variable in adipose tissue.

The major biosynthetic pathways for fatty acids involve up to 4 steps in human. These are 1) the *de novo* synthesis of saturated acids, 2) chain elongation, 3) delta 9-desaturation to produce monoenes and 4) further desaturation of LA and ALA.

***De novo* synthesis of SFAs**

The synthetic process involves alteration of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase (ACC). For the formation of saturated fatty acids of different carbon lengths, acetyl-CoA combines with a series of malonyl CoA molecules. The end product is palmitic acid (16:0). The *de novo* fatty-acid synthesis involves two key enzymes, ACC and fatty acid synthase (FASN). ACC carboxylates acetyl-CoA to form malonyl-CoA. The malonyl-CoA is further converted by FASN to long-chain fatty acids (Figure 1).

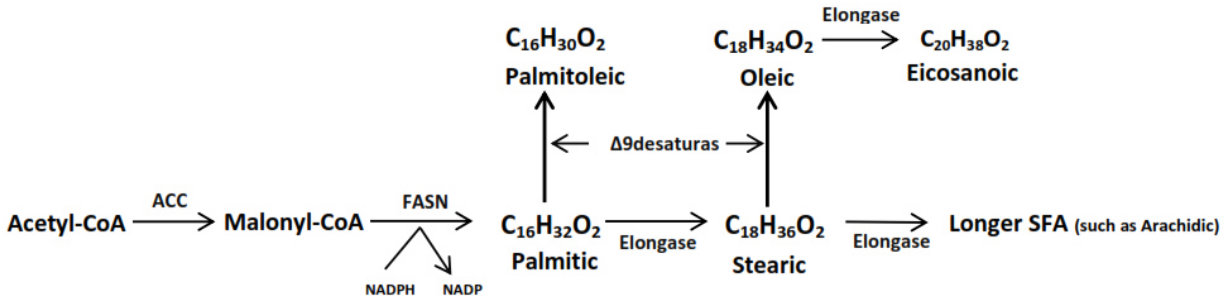


Figure 1 Schematic overview of fatty acids biosynthesis pathways (modified according to Ferré and Foufelle, 2007). The abbreviations used are the following: ACC = acetyl-CoA carboxylase; FASN: fatty acid synthase.

Chain elongation

The final product of *de novo* synthesis is mainly palmitic acid. By further addition of acetyl groups, palmitic acid can be elongated to stearic acid and even longer chain acids (Figure 1, summarized in Baenke et al., 2013).

Desaturation

A fatty acid desaturase removes two hydrogen atoms from a fatty acid and creating a carbon/carbon double bond. Four desaturases occur in humans: Δ9desaturase, Δ6desaturase, Δ5desaturase, and Δ4desaturase. A minimum chain length of 16 - 18 carbons is required. In the desaturation procedure of stearic acid and palmitic acid, the Δ9desaturase creates a double bond at the 9th position from the carboxyl end and in this way oleic acid and palmitoleic acid can be formed (Figure 1).

Metabolism of LA and ALA to long chain PUFA

Although fatty acids with double bonds at the Δ9 position can be formed in mammals, mammals cannot introduce additional double bonds between Δ10 and the methyl terminal end. Thus, fatty acids such as LA and ALA cannot be synthesized by mammals. ALA and LA are required for the synthesis of LCPUFA and eicosanoids. LA and ALA can be converted to n6 and n3 LCPUFA by a series of alternating desaturation and elongation reactions which needs Δ6- and Δ5-desaturases (Figure 2, Burdge and Cadler, 2005). In the first step, a double bond is inserted at the Δ6 position of LA and ALA by the action of Δ6 desaturase. After insertion of a double bond, the carbon chain is elongated by addition of two carbon units by elongase, which is followed by insertion of another double bond at the Δ5 position by Δ5-desaturase to form AA (20:4 n6) and EPA (20:5 n3), respectively (Burdge and Cadler, 2005). Conversions of LA and ALA to LCPUFAs are two independent pathways. However, same enzymes are used in both

pathways. Since LA is the predominant PUFA in human diet and intakes of ALA are generally low, levels of n3 LCPUFA derived from ALA tend to be lower than the n6 LCPUFA levels in cells and plasma. The relative levels of ALA in adipose tissue are usually around 0.5% (Mamalakis et al. 2009; Boué et al., 2000; Bagga et al., 1997). It seems that the low content of ALA is not sufficient to compete with LA for the Δ6 desaturase.

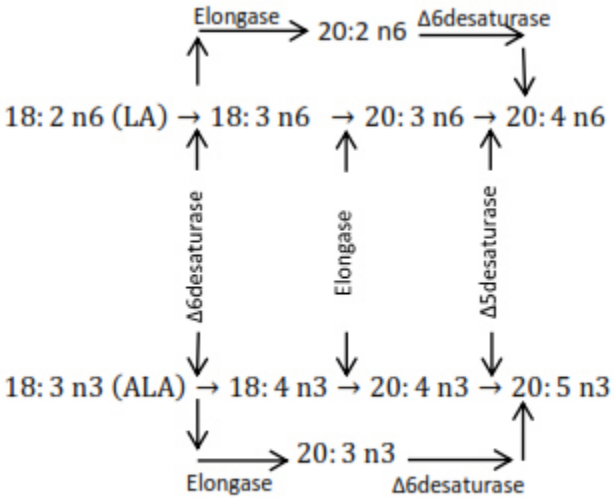


Figure 2 Metabolic pathways for the conversion of dietary LA and ALA to their long chain fatty acids (modified according to Lands, 2012).

1.1.2 ChOL

The amounts of ChOL have been studied in animals previously (Ariyoshi et al., 2002; Xu et al., 2011; Wang et al., 2012); but there is no information about the ChOL level in human breast adipose tissue. It was observed, that levels of ChOL may play a role in the regulation of tumor progression and can increase tumor formation in mouse (Llaverias et al., 2011). In following chapters ChOL structure (Chapter 1.1.2.1), biological effect of ChOL in adipose tissue (Chapter 1.1.2.2) and the source of ChOL in adipose tissue (Chapter 1.1.2.3) are taken into account.

1.1.2.1 ChOL structure

ChOL is a sterol because it is a steroid and alcohol at the same time (Figure 3). It is a solid alcohol of high molecular weight and like other steroids is a derivative of the cyclic hydrocarbon perhydrocyclopentanophenanthrene. It consists of four rings.

1.1.2.2 Biological effect of ChOL

ChOL is essential for some physiological functions. The functions of ChOL in body includes: insulate nerves, make cell membranes and is a precursor in production of steroid hormones (such as estrogen, testosterone, progesterone, aldosterone and cortisone), production of bile acid and vitamin D (Hongbao, 2004). It has an important role in health and disease. The previous findings suggested a role for ChOL in BC growth and development. A high-fat, high-ChOL diet enhanced the growth and metastasis of tumors in a mouse model of mammary cancer (Llaverias et al., 2011). In another research by Nelson et al. (2013), the impact of ChOL on breast tumor pathogenesis was considered. It was shown that, a high-ChOL diet (normal in fat) increased tumor growth in a mouse model of mammary cancer.

1.1.2.3 The source of ChOL

ChOL can be synthesized endogenously through *de novo* mechanism and also obtained from the diet (Chapter 1.1.2.3.1-2).

1.1.2.3.1 Dietary source of ChOL

Foods that are derived from animal are the rich source of ChOL (Alonso et al., 1995). These foods involve eggs, milk, dairy products, red meat (beef, pork), fish and sea food (Table 5). An average intake of ChOL from food is 300 mg per day. The intake of less than 300 mg/day of ChOL is recommended for healthy people (Ostlund, 2007). In a study by de Haan et al. (2014) high fat-high ChOL fed mice demonstrated increased adipose tissue ChOL content.

Table 5 The level of ChOL in different foods.

foods	egg	egg yolks	fish	fish oil	Beef	Pork	milk	butter
ChOL mg/100 g	372	1058	70	521	72	70	10	270

1.1.2.3.2 ChOL biosynthesis

Adipose tissue is the major source of free ChOL. However, the activity of it for ChOL synthesis is very low compared with other cell types (summarized in Yu et al., 2010). ChOL is synthesized from acetyl-coenzyme A. Enzymes including 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, HMG-CoA synthase, farnesyl diphosphate synthase and squalene synthase regulate synthesis of ChOL (Nes, 2011). The process of ChOL synthesis can be considered to be composed of following steps: conversion of acetyl-CoAs to HMG-CoA (through condensation between acetyl CoA and acetoacetyl-CoA), conversion of HMG-CoA to mevalonate, conversion of mevalonate to isopentenyl pyrophosphate (through two phosphorylation steps and one

decarboxylation step), condensation of three molecules of isopentenyl pyrophosphate to farnesyl pyrophosphate, synthesis of squalene from two molecules of farnesyl pyrophosphats, cyclization of squalene to lanosterol and series of changes, oxidations, removal or migration of methyl groups, lead to the production of ChOL (Figure 3).

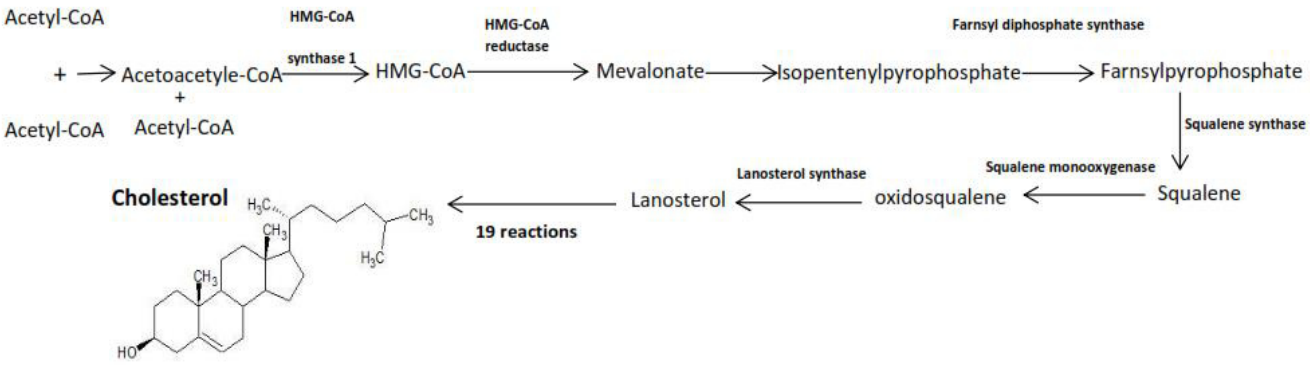


Figure 3 Schematic overview of ChOL biosynthesis pathways (modified according to Charlton-Menys and Durrington, 2007).

The HMG-CoA reductase is the rate-determining enzyme of the ChOL biosynthetic pathway and is regulated by supply of ChOL.

1.1.3 OxyChOLs

Due to the unsaturated structure of ChOL, it is susceptible to oxidation. The resulting oxidation products are called oxyChOLs. OxyChOLs are structurally identical to ChOL, but with one or more additional oxygen containing functional groups (such as alcohol, carbonyl or epoxide groups). To date, more than 80 various products of oxidation of ChOL were identified (Bösinger et al., 1993; Janoszka et al., 2003; summarized in Kulig et al., 2016). Up to now no study investigates the level of oxyChOLs in breast adipose tissue. Pathological effects of oxyChOLs have been considered. OxyChOL have been associated with various cancers including BC (Jusakul et al., 2011). In following chapters, the physiological function of oxyChOLs (Chapter 1.1.3.1) and source of oxyChOLs (Chapter 1.1.3.2) are taken into account.

1.1.3.1 Physiological function of oxyChOLs

OxyChOLs may involve in ChOL homeostasis by reduction of HMG-CoA reductase activity (Tamasawa et al., 1997). Furthermore, they may alter the membrane fluidity, permeability, stability, and activity of membrane-bound enzymes (summarized in Guardiola et al., 1996).

The oxyChOLs products of non-enzymatic (oxidative stress) are associated with several types of cancer, including cancers of the colon, lung and breast (Jusakul et al., 2011; Bösinger et al., 1993).

The pro-oxidative and pro-inflammatory effects of 7α/β-Hydroxy-Cholesterol (7α/β-HO-ChOL), 7-Keto-Cholesterol (7-O-ChOL), 5,6α/β-Epoxy-Cholesterol (α/β-epoxy-ChOL) were studied in U937 human promonocytic leukemia cells. 7β-HO-ChOL, 7-O-ChOL and β-epoxy-ChOL increased the production of single oxygen (Lemaire-Ewing et al., 2005). In addition to, oxyChOLs that were oxidized at C7 (7β-HO-ChOL and 7-O-ChOL) enhanced the production of free radicals *in vitro* study performed on U937 human monocytic cell line (Monier et al., 2003; Miguet-Alfonsi et al., 2002). Moreover, 7-O-ChOL caused oxidation of PUFAs and enhanced the production of reactive oxygen species (ROS) in U937 cells (Lizard et al., 1998). In another study, a diet containing 6% oxyChOLs (including 7-O-ChOL, 7β-HO-ChOL and α-epoxy-ChOL) given to rabbits provoked a 64% increase in total aortic ChOL compared to the pure ChOL control animals (Vine et al., 1998). Treatment with 7-O-ChOL, 7β-HO-ChOLs, epoxy-ChOLs has shown important oxidative processes, sometimes associated with a complex mode of cell death with characteristics of apoptosis, on various cell types *in vitro* (Lemaire-Ewing et al., 2005; Joffre et al., 2007; Roussi et al., 2007).

OxyChOLs may play role in inflammation process (summarized in Olkkonen et al., 2012). The expression and synthesis of inflammatory mediators, such as interleukin-18 receptor and monocyte chemotactic protein-1 were increased in the culture media of oxyChOL-treated human promonocytic leukemia cells U937 (Leonarduzzi et al., 2005). OxyChOL mixture contained: 7α-HO-ChOL (5%), 7β-HO-ChOL (10%), α-epoxy-ChOL (20%), β-epoxy-ChOL (20%), cholestan-3β,5α,6β-triol (9%), 7-O-ChOL (35%), and 25-hydroxycholesterol (1%, Leonarduzzi et al., 2005). Moreover, Soto-Rodríguez et al. (2012) suggested that oxyChOLs have an important pro-inflammatory effect. They observed an increase in circulating levels of plasma high-sensitivity C-reactive protein, tumor necrosis factor alpha, and resistin in rats which were fed with a diet contained oxyChOLs such as 7α/β-HO-ChOL, 7-O-ChOL and α/β-epoxy-ChOL.

1.1.3.2 Source of oxyChOLs

OxyChOLs may form in the human body through endogenous oxidation. Endogenous oxyChOLs are formed by enzymatic (Chapter 1.1.3.2.1) and non-enzymatic processes (Chapter 1.1.3.2.2) with the participation of ROS (Iuliano, 2011). Preferred oxidation states are the C7, and the double bond between C5 and C6. It mainly arises 7α/β-HO-ChOL, 7-O-ChOL and α/β-epoxy-ChOL (Plat et al., 2001; Apprich et al., 2004; summarized in Olkkonen et al., 2012; Orczewska-Dudek et al., 2012). OxyChOLs may also be derived from food (Chapter 1.1.3.2.3).

1.1.3.2.1 Enzymatic oxidation

The enzymatic oxidation of ChOL occurs in 7α-position of the sterol nucleus. The enzymes involved in the oxidation of ChOL are members of Cytochrome P450 (CYP). Monooxygenase, dehydrogenases, epoxidases, hydroxylases and oxidases are the most important CYPs. Cytochrome P450 family 7 subfamily A member 1 (CYP7A1) catalyzed the reaction of ChOL to 7α-HO-ChOL. The formation of 7α-HO-ChOL, by the enzyme 7α-hydroxylase is an initial step in the formation of bile acids (Figure 4, Björkhem et al., 2002; Micheletta and Luliano, 2006).

1.1.3.2.2 Non-enzymatic oxidation

Non-enzymatic oxidation occurs with ROS or other free radicals (including lipid peroxide radical, reactive nitrogen species). A free radical is any chemical specie that contains one or more unpaired electrons. Free radicals tend to accept electrons from other molecules and consequently they can change structure and/or function of these molecules. The damage produced by free radicals can be repaired by antioxidant defense system. Indeed, the antioxidant defense system maintains a delicate equilibrium known as oxidative balance. When this oxidative balance is broken, as a result of free radical production, oxidative stress happens (summarized in Thanan et al., 2014). Autoxidation is the main reaction in oxidative deterioration of ChOL, which can be initiated by abstraction of hydrogen, predominantly at the 7-position. The reaction is followed by the addition of an oxygen molecule, which leads to formation of 7 HydroperoxyChOL (7α/β-OOH-ChOL, Figure 4). The 7α/β-OOH-ChOL is reduced further to 7α/β-HO-ChOL. The 7α/β-OOH-ChOL can also undergo dehydration to form 7-O-ChOL (summarized in Otaegui-Arrazola et al., 2010; Murphy and Johnson, 2008). In addition, 7-O-ChOL can be formed through dehydrogenation of 7α/β-HO-ChOL in the presence of radicals and also through enzymatic pathway (7α-hydroxycholesterol dehydrogenase, Niki et al., 2005). Moreover, non-enzymatic oxidations happen at the double bond between position 5 and 6. In addition to the oxyChOLs initially formed by ROS, subsequent enzymatic metabolism can occur. For example, α/β-epoxy-ChOL is a substrate for epoxide hydrolase to yield the corresponding 3,5,6-triol-ChOL (summarized in Newman et al., 2005). The non-enzymatic oxidation generates 7α/β-HO-ChOL, 7-O-ChOL and α/β-epoxy-ChOL (Figure 4) which are the major non-enzymatic oxyChOLs present in most tissues (summarized in Otaegui-Arrazola et al., 2010).

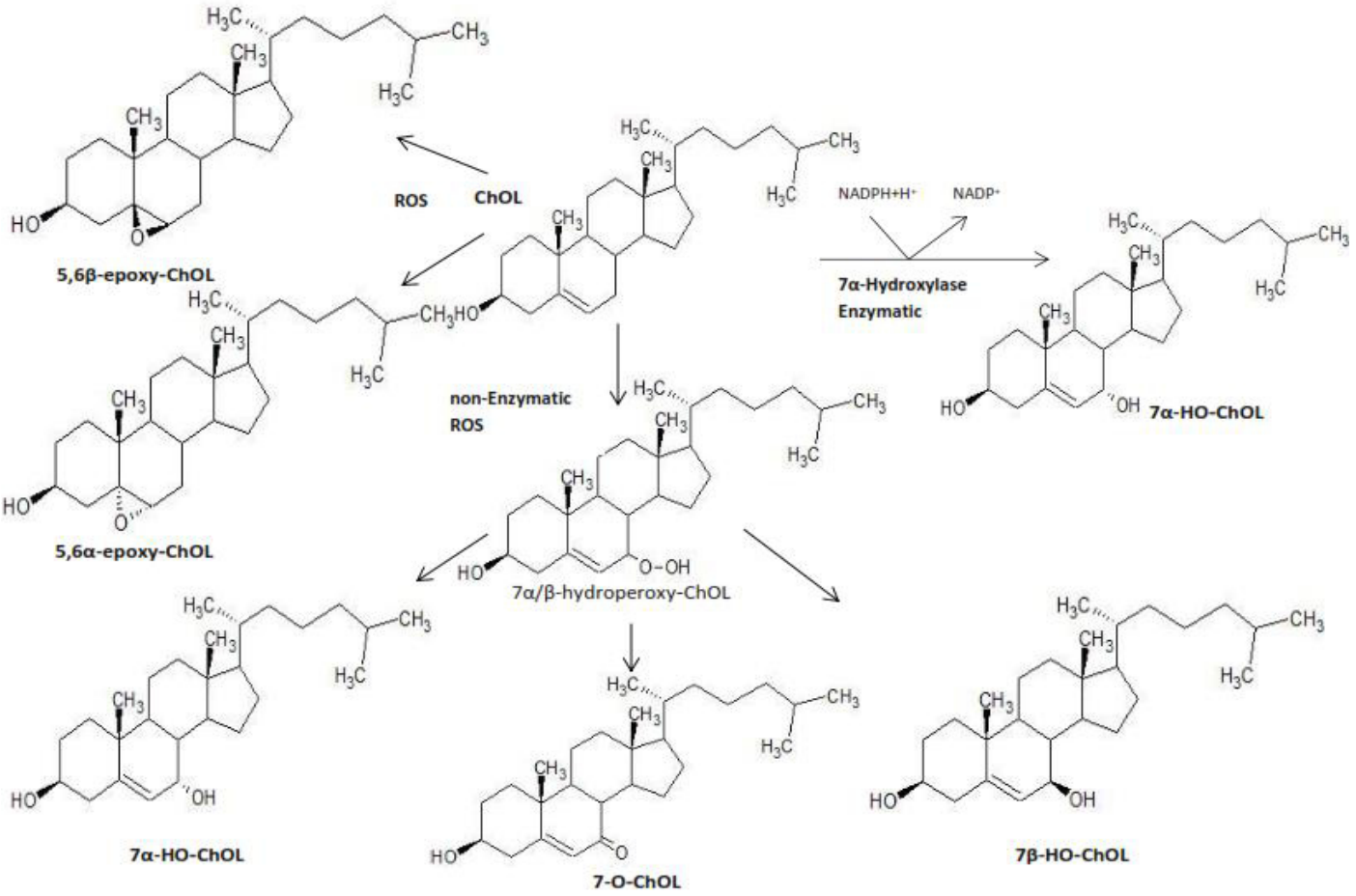


Figure 4 Endogenous biosynthesis (enzymatic and non-enzymatic) of oxyChOLs (modified according to Otaegui-Arrazola et al., 2010).

1.1.3.2.3 Dietary source of oxyChOLs

OxyChOLs can be formed in all types of foods and food ingredients that contain levels of ChOL such as eggs, milk, meat and sea foods (Table 5). They can be generated in minor level in fresh foods. However, the procedure of processing food (such as heat treatments) as well as storage condition (long periods subjected to sunlight and oxygen) may cause elevated levels of them (Schroepfer, 2000). Hence, the oxyChOLs may be taken from exogenous source as well. The most detected oxyChOLs in foods involves 7α/β-HO-ChOL, 7-O-ChOL and α/β-epoxy-ChOL. oxyChOL profiles in different food were not similar (Table 6).

The contribution of dietary oxyChOLs in the adipose tissue levels of these compounds is not clear. Meynier et al. (2002) examined the effect of high lipid diet enriched with oxyChOLs in myocardium of hamsters. No significant changes were observed in the level of oxyChOLs in

myocardium of hamsters. The most abundant oxyChOL was β-epoxy-ChOL in control hamsters, whereas α-epoxy-ChOL was the main oxyChOLs in high lipid diet. Staprans et al. (1998) investigated the influence of dietary oxyChOLs on the level of liver oxyChOLs in New Zealand white rabbits. Significantly more oxyChOL was found in livers of rabbits after consumption of the diet containing oxidized ChOL. There was an increase in 7β-HO-ChOL (0.16 ± 0.07 to 0.33 ± 0.02 μg/mg liver tissues), β-epoxy-ChOL (0.11 ± 0.04 to 0.22 ± 0.02 μg/mg liver tissue) and 7-O-ChOL (0.14 ± 0.06 to 0.29 ± 0.03 μg/mg liver tissue) in the liver after feeding the diet containing oxidation products of ChOL.

Table 6 The proportion of oxyChOLs (%) in egg, dairy, meat, fish and tallows (UHT* ultra-high temperature, nt* not tested, nd* not detected).

group	products	oxyChOLs (%)					Ranking	references
		β-epoxy	α-epoxy	7-O-	7α-HO	7β-HO		
egg product	spray-dried whole egg	47.8	10.9	9.8	12.3	19.1	β-epoxy-ChOL>7β-HO-ChOL>7α-HO-ChOL>α-epoxy-ChOL>7-O-ChOL	Lai et al. (1995)
	whole spray-dried egg powder	30.0	16.7	21.7	18.3	13.3	β-epoxy-ChOL>7-O-ChOL>7α-HO-ChOL>α-epoxy-ChOL>7β-HO-ChOL	Mazalli et al. (2007)
dairy product	UHT* milk (full cream)	nt*	5.1	nt*	nt	86.0	7β-HO-ChOL>α-epoxy-ChOL	Calderon-Santiago et al. (2012)
	whole milk powder (fresh)	nt*	60.0	40.0	nd*	nd*	α-epoxy-ChOL>7-O-ChOL	Angulo et al. (1997)
	bovine milk (raw)	14.6	10.3	27.0	29.2	19.9	7α-HO-ChOL>7-O-ChOL>7β-HO-ChOL>β-epoxy-ChOL>α-epoxy-ChOL	Pikul et al. (2013)
	bovine milk (pasturised)	13.8	10.3	23.9	28.1	23.9	7α-HO-ChOL>7-O-ChOL>7β-HO-ChOL>β-epoxy-ChOL>α-epoxy-ChOL	Pikul et al. (2013)
	bovine milk (UHT)	12.3	9.3	22.6	30.0	25.7	7α-HO-ChOL>7β-HO-ChOL>7-O-ChOL>β-epoxy-ChOL>α-epoxy-ChOL	Pikul et al. (2013)
meat	raw hamburger	29.1	14.5	18.2	12.7	25.5	β-epoxy-ChOL>7β-HO-ChOL>7-O-ChOL>α-epoxy-ChOL>7α-HO-ChOL	Larkeson et al. (2000)
	fried hamburger	15.9	11.1	12.7	27.0	33.3	7β-HO-ChOL>7α-HO-ChOL>β-epoxy-ChOL>7-O-ChOL>α-epoxy-ChOL	Larkeson et al. (2000)
	unirradiated Pork	38.4	26.1	35.6	nt*	nt*	β-epoxy-ChOL>7-Keto-ChOL>α-epoxy-ChOL	Hwang et al. (1993)
	irradiated Pork	34.5	18.1	47.4	nt*	nt*	7-O-ChOL>β-epoxy-ChOL>α-epoxy-ChOL	Hwang et al. (1993)
	beef (7days storage)	27.7	8.2	64.1	nt*	nt*	7-O-ChOL>β-epoxy-ChOL>α-epoxy-ChOL	Zubillaga et al. (1991)
	raw beef	31.2	12.3	32.9	9.7	10.0	7-O-ChOL>β-epoxy-ChOL>α-epoxy-ChOL>7β-HO-ChOL>7α-HO-ChOl	Pie et al. (1991)
	beef (cooked for 10 min)	22.3	9.4	36.0	9.9	11.4	7-O-ChOL>β-epoxy-ChOL>7β-HO-ChOL>7α-HO-ChOl>α-epoxy-ChOL	Pie et al. (1991)
	raw pork	15.6	9.8	41.1	8.5	12.5	7-O-ChOL>β-epoxy-ChOL>7β-HO-ChOL>α-epoxy-ChOL>7α-HO-ChOl	Pie et al. (1991)
	pork (cooked)	22.3	9.4	36.0	9.9	11.4	7-O-ChOL>β-epoxy-ChOL>7β-HO-ChOL>7α-HO-ChOl>α-epoxy-ChOL	Pie et al. (1991)
fish	raw shrimp	13.2	36.2	21.1	16.3	13.2	α-epoxy-ChOL>7-O-ChOL>7α-HO-ChOL)>β-epoxy-ChOL=7β-HO-ChOL	Hernández Becerra et al. (2014)
	boiled shrimp	11.3	36.1	17.5	20.1	14.9	α-epoxy-ChOL>7α-HO-ChOL>7-O-ChOL>7β-HO-ChOL>β-epoxy-ChOL	Hernández Becerra et al. (2014)
	fresh salmon roe	33.9	17.5	24.6	9.7	14.3	β-epoxy-ChOL>7-O-ChOL>α-epoxy-ChOL>7β-HO-ChOL>7α-HO-ChOL	Pickova et al. (2003)
	Norwegian herring fish oil	44.0	10.3	21.8	8.4	15.6	β-epoxy-ChOL>7-O-ChOL>7β-HO-ChOL>α-epoxy-ChOL>7α-HO-ChOL	Pickova et al. (2003)
tallow	natural tallow	77.9	<LOQ	<LOQ	<LOQ	22.1	β-epoxy-ChOL>7β-HO-ChOL	Verleyen et al. (2003)
	deodorised tallow	77.7	<LOQ	<LOQ	<LOQ	22.1	β-epoxy-ChOL>7β-HO-ChOL	Verleyen et al. (2003)

2. Objectives

Breast adipose tissue is involved in the development and function of the female breast by complex interactions with stromal tissue. However, there is little information about the quantitative lipid composition in breast adipose tissue, containing substances such as fatty acids, cholesterol and (aut)oxidation product of cholesterol. Fatty acids play an important role in human health, some studies suggested that specific types of fatty acids (such as n6 polyunsaturated fatty acids) may be involved in tumorigenesis, in contrast, n3 polyunsaturated fatty acids may have anticancer effects and (aut)oxidation products of cholesterol such as 7α-Hydroxy-Cholesterol, 7β-Hydroxy-Cholesterol, 7-Keto-Cholesterol, 5,6α-Epoxy-Cholesterol and 5,6β-Epoxy-Cholesterol may affect inflammation process. Moreover, there is little information about profile of fatty acids in breast adipose tissue of women without breast cancer and the absolute content of fatty acids, which are indicative for consumption of dairy products (pentadecanoic acid), processed fats (elaidic acid), vegetable oils such as flaxseed oil (linolenic acid) and fish (docosahexaenoic acid), have not been determined yet. Moreover, quantitative profiles of oxy-cholesterols and cholesterol have not been determined in human breast adipose tissues yet. Furthermore, it has not been determined if fatty acids, oxy-cholesterols and cholesterol are related with each other or if they are related to physiological parameters such as age or the body mass index. Hence, the aim of this work is to investigate the breast adipose tissue lipid composition regarding fatty acids, cholesterol and (aut)oxidation product of cholesterol.

Therefore the breast adipose tissues and personal information will be collected from women without breast cancer who undergo reduction mammoplasty for cosmetic reasons. To determine fatty acids in breast adipose tissues, a gas chromatography separation method with flame ionization detection will need to be optimized regarding resolution and separation of 37 relevant fatty acids (35 cis and 2 trans isomers of fatty acids). The relative proportions of fatty acids will be determined by integrating the peak at baseline and dividing the results by the total area for all fatty acids. Moreover, absolute levels of pentadecanoic, elaidic, α-linolenic and docosahexaenoic acids will be quantified for the first time. External calibration using an internal standard was used for quantification of fatty acids.

To quantify cholesterol in breast adipose tissue of human females an existing gas chromatography (flame ionization detector) method will be optimized and validated, and external calibration using an internal standard will be performed to calculate the absolute level of cholesterol. With regard to low levels of (aut)oxidation products of cholesterol in other biological tissues, a more sensitive analytical procedure will be required to identify and quantify these compounds. Thus, samples will be analyzed using gas chromatography coupled with triple

quadrupole mass spectrometry based on a multi-reaction monitoring method and the developed method will be validated. The absolute levels of oxy-cholesterols will be quantified using external calibration. With the obtained absolute levels, ratios of oxy-cholesterols levels to cholesterol levels and ratios of oxy-cholesterols levels to total oxy-cholesterol levels will be calculated.

Finally, appropriate statistical methods will be selected and applied to investigate the relation between the levels of fatty acids, cholesterol and oxy-cholesterol and to answer the question if physiological parameters such as age or body mass index are associated with levels of fatty acids, cholesterol and oxy-cholesterol.

3. Materials and methods

The following chapters provide an overview of the equipment such as gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS/MS, Chapter 3.1-2), laboratory consumables (Chapter 3.3), chemicals (Chapter 3.4), solutions (Chapter 3.5) and software (Chapter 3.6) which were used in this work. Furthermore, sampling process, developed and validated methods and the correlation analysis were discussed (Chapter 3.7).

3.1 Gas chromatography equipment

GC-FID 5890

Agilent 5890 GC Series II (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)

Agilent 7683 Autosampler (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)

Carbowax 20 M capillary fused silica, 30 m x 0.25 mm; film thickness: 0.25 µm; Column material: polyethylene glycol (Agilent technologies, CA, USA)

Supelco SP-2560 fused silica, 100 m x 0.25 mm; film thickness 0.20 µm, Column material: poly biscyanopropyl siloxane (Sigma-Aldrich, Munich, Germany)

GC-FID 6890

Agilent 6890 GC Series plus (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)
Agilent 7683 Autosampler (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)

Supelco SLB-5ms fused silica, 30 m x 0.25 mm; film thickness :0.25 µm; Column material: 5% Phenyl-, 95% Methylpolysiloxan (Supelco Analytical Bellefonte, PA, USA)

GC-MS/MS

Varian 450 GC (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)

Varian 300-MS/ triple quadrupole Mass Spectrometer (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)
Varian CP 8400 Autosampler (Agilent Technologies® Deutschland GmbH, Böblingen, Germany)

Supelco SLB-5ms fused silica, 30 m x 0.25 mm; film thickness: 0.25 µm; Column material: 5% Phenyl-, 95% Methylpolysiloxan (Supelco Analytical Bellefonte, PA, USA).

3.2 Laboratory equipment

Analytical balances
Mettler AE-240-S (Mettler Toledo GmbH, Gießen, Germany)
Mettler AB-204-S/PH (Mettler Toledo GmbH, Gießen, Germany)

Bio-freezers
Type 6483 (Part No.: 73537; GFL Laboratory Technology GmbH, Burgwedel, Germany)

Type UF 755G (Part No.: 991.7050.32; domestic medical systems, Luxemburg)

Centrifuges
Universal 16 (Hettich lab technology, Tuttlingen, Germany EBA 12 (Type: 1000; Hettich, Tuttlingen, Germany)

Drying oven
Memmert 800 (Memmert GmbH + Co. KG, Schwabach, Germany)

Evaporators
Büchi rotary evaporator (Model: R-200, Part No. Z626797, Sigma-aldrich, Steinheim, Germany)

Rotational vacuum concentrator (Model: RVC 2-25 CD plus, Part No.: 101225; Martin Christ Freeze Dryers GmbH, Osterode, Germany) with cold trap (Model: CT 04-50 SA, Part No.: 100505; Martin Christ freeze dryers GmbH, Osterode, Germany)

Magnetic stirrer
IKA® RCT basic (Part No.: 3810000; IKA laboratory technology, Staufen, Germany)

Pipettes
Eppendorf research
10-100 µl (Part No.: 3111 000.149, Eppendorf AG, Hamburg, Germany)
Multipipette® plus (Eppendorf AG, Hamburg, Germany)

Microman®
100-1000 µl (M1000; Part No.: F148506, Gilson, Middleton, Wisconsin, USA)
50-250 µl (M250; Part No.: F148505, Gilson, Middleton, Wisconsin, USA)
10-100 µl (M100; Part No.: F148504, Gilson, Middleton, Wisconsin, USA)

Solid phase extraction (SPE) chamber	Supelco Visiprep™ SPE Vacuum Manifold; 12-Port Model (Supelco Sigma Aldrich GmbH, Buchs, Switzerland)
Ultrasonic bath	Bandelin SONOREX SUPER RE 100 (BANDELIN electronic GmbH & Co. KG, Berlin, Germany)
Ultra microbalance	Mettler Toledo AT21 comparator (Mettler Toledo GmbH, Gießen, Germany)
Vortex	Vortex-Genie® 2 (Model: G560E, Part No.: 2-146860, Scientific industries, Scientific Industries™, New York, USA)

3.3 Laboratory consumables

Cannulas	100 Sterican®, one-time injection cannula, 0.90 x 40 mm, Gr. 1 (B. Braun Melsungen AG, Melsungen, Germany)
Centrifuge tubes	50 ml, 28 x 115 mm, conical base with screw cap (Part No.: 62.559.010, Sarstedt, Nümbrecht, Germany)
Cryos	1.8 ml, with screw cap (Part No.: 72.379, Sarstedt, Nümbrecht, Germany)
Liner	SSL-Liner, borosilicate, HP 78.5 x 6.3; 4mm inner diameter (ID) inactive.; Quartz wool (Part No.: 092019, Agilent Technologies Deutschland GmbH, Böblingen, Germany) SSL-liner, borosilicate, HP 78.5 x 6.3,4mm ID, taper, deactivate (Part No.: 5183-4691, CZT Klaus Trott Chromatographie, Kriftel, Germany)
Pasteur pipettes	Glass: length 230 mm (Part No.: 6355, facility for Chemicals and Materials, University of Würzburg, Germany)
Pipette tips	Tips for Microman® 1000 µl (Part No.: CP1000 F148560 Gilson, Middleton, Wisconsin, USA) 250 µl (Part No.: CP250 F148114, Gilson, Middleton, Wisconsin, USA) 100 µl (Part No.: CP100 F148414, Gilson, Middleton, Wisconsin, USA) Tips for Multi pipette Eppendorf®Combitips Biopur, 2.5 ml (Part No.: 5811, Webers, Oberhausen, Germany) Eppendorf®Combitips Biopur, 1.0 ml (Part No.: 5809, Webers, Oberhausen, Germany)

Septa	Septa BTO®, 9 mm, Center Guide® (Part No.: 170900142, CZT Klaus Trott chromatography, Kriftel, Germany)
SPE column	Discovery SPE DSC-Si Silica Tube 100 mg, 1 ml Volume (Part No.: 52653-U, Supelco, USA)
Glass culture tubes	GL 18, 100 x 16 mm with screw cap (Part No.: RG09, A. Hartenstein, Laborbedarf GmbH, Würzburg, Germany)
O-Ringe	non-stick Liner O-Ring (Part No.: 5188-5365, CTZ Klaus Trott Chromatographie, Kriftel, Germany)
Syringe	10 µl, SGE (Part No.: 002814, Agilent Technologies, Australia)
Vials	1.5 ml brown bottle (Part No.: 451101220, CZT Klaus Trott Chromatographie, Kriftel, Germany)
	Screw PP with seals , 8-425, NK/TEF, red, 1,3 mm (Part No.: 30 11 015 S1, CZT Klaus Trott Chromatographie, Kriftel, Germany)
	micro-inserts, conical base, 5 x 30 mm (Part No.: 501105031, CZT Klaus Trott chromatography, Kriftel, Germany)
	Feathers (Part No.: 501167011, CZT Klaus Trott chromatography, Kriftel, Germany)

3.4 Chemicals

Fatty acids standards

37 Component Fatty acid methyl ester (FAME)	Supelco™ 37 Component FAME mixture, 10 mg/ml in methylene chloride (Part No.: 47885-U, Supelco, PA, USA)
DHA	> 98%, 50 mg (Part No.: CAYM90310, VWR, Netherlands)
Elaidic acid	~ 98%, (Part No.: A14832.03, Alfa Aesar, Karlsruhe, Germany)
ALA	> 98%, 50 mg (Part No.: CAYM90210, VWR, Netherlands)
Myristic acid	≥ 98% (Part No.: 70082, Sigma-Aldrich, Steinheim, Germany)
Oleic acid	≥ 99% (Part No.: 7213.1, Carl Roth, GmbH)
Palmitic acid	≥ 99% (Part No.: P0500, Sigma-Aldrich, Steinheim, Germany)
Pentadecanoic acid	~ 99% (Part No.: P6125, Sigma-Aldrich)
Undecanoic acid	≥ 97.0% (Part No.: 94090, Fluka, Sigma-Aldrich, Steinheim, Germany)
Cis_Vaccenic acid	≥ 97% (Part No.: V0384, Sigma-Aldrich, USA)

ChOL and derivatives

5α-Cholestan-3β-ol	≥ 95% (Part No.: D6128, Sigma-Aldrich, Steinheim, Germany)

(5α-ChAN-3β-ol)	
ChOL	≥ 99% (Part No.: C8667, Sigma-Aldrich, Steinheim, Germany)
Deuterated ChOL-25,26,26,26,27,27,27-d7 (D-ChOL)	99%D (Part No.: D897, Medical Isotopes, Pelham, NH, USA)
α-epoxy-ChOL	≥ 80% (Part No.: C2773, Sigma-Aldrich, Steinheim, Germany)
β-epoxy-ChOL	≥ 98% (Part No.: C2648, Sigma-Aldrich, Steinheim, Germany)
7-O-ChOL	≥ 90% (Part No.: C2648, Sigma-Aldrich, Steinheim, Germany)
7α-HO-ChOL	10 nM in Ethanol (Part No.: C6420-000, Steraloids, USA)
7β-HO-ChOL	≥ 95%, 1 mM in EtOH (Part No.: H6891, Sigma-Aldrich, Steinheim, Germany)
Solvents and other chemicals	
Chloroform ($CHCl_3$)	stabilized with Ethanol, purity: 99.0-99.4% (Part No.: 24216, RiedeldeHaën, Seelze, Germany)
Dichloromethane (CH_2Cl_2)	stabilized with amylene, ≥ 99.9% (Part No.: 10010120, Fisher Scientific, Schwerte, Germany)
Butyl hydroxytoluene (BHT)	99% (Part No.: A16863, Alfa Aesar GmbH & Co KG, Karlsruhe, Germany)
Ethanol (EtOH)	≥ 99.8%, Absolut, GC-grade (Part No.: 24102, Sigma-Aldrich, Steinheim, Germany)
Ethyl acetate	≥ 99.5% (Part No.: CP42.2; Carl Roth GmbH + Co.KG, Karlsruhe, Germany)
n-Heptane	Chromasolv®, LC-MS-Grade, ≥ 99% (Part No.: 34999, Fluka, Sigma-Aldrich, Steinheim, Germany)
n-Hexane	Chromasolv®, LC-MS-grade, ≥ 97% (Part No.: 34986 Fluka, Sigma Aldrich, Steinheim, Germany)
Methanol (MeOH)	HPLC, ≥ 99.9% (Part No.: 12490.4700, Bernd Kraft, Duisburg, Germany)
Methyl-tert-butyl ether (MTBE)	anhydrous, 99.8% (Part No.: 306975, Sigma Aldrich, Steinheim, Germany)
N,O-Bis (trimethylsilyl) trifluoracetamide (BSTFA) + 1% Trimethylchlorsilan	99% (Part No.: 15238, Sigma Aldrich, Steinheim, Germany)
Potassium hydroxide	p.a. (Part No.: 2159, Facility for Chemicals and Materials, University of Würzburg, Germany)
2-Propanol	Rotipuran®, ≥ 99,8 %, p.a. (Part No.: 6752.1, Roth, Karlsruhe)

Sodium sulphate	anhydrous, p.a. (Part No.: 2019, Facility for Chemicals and Materials, University of Würzburg, Germany)
Sodium chloride (NaCl)	(Part No.: 2532, Facility for Chemicals and Materials, University of Würzburg, Germany)
Trimethylsulfonium hydroxide (TMSH)	0.25 M in MeOH (Part No.: 92732, Sigma Aldrich, Munich, Germany)
Toluene	Rotisolv® HPLC; ≥ 99,9% (Part No.: 7346.2, Roth, Karlsruhe, Germany)

3.5 Solution

The following chapters provide an information about the solution which were used for isolation of fatty acid from adipose tissue (Chapter 3.5.1), calibration solution for quantification of fatty acid (Chapter 3.5.2), solution for isolation of ChOL and oxyChOLs (Chapter 3.5.3) and calibration solutions for quantification of ChOL (Chapter 3.5.4) and oxyChOLs (Chapter 3.5.5).

3.5.1 Solutions for the isolation of fatty acids from adipose tissue

$CHCl_3$:MeOH	20 ml $CHCl_3$:MeOH (2:1; v:v) + 0.05 mg/ml undecanoic acid as an internal standard (IS)
NaCl solution (0.9%)	900 mg of NaCl dissolved in 100 ml of distilled water

3.5.2 Calibration solutions for the quantification of fatty acids

Solution of undecanoic acid as an internal standard

85.35 mg of undecanoic acid were dissolved in 500 ml of MeOH. From this stock solution, 6 ml were transferred by Eppendorf multi pipette to the 50 centrifuge tubes (50 ml) and then 14 ml $CHCl_3$ was added to each tube. The final amount of undecanoic was 1.0 mg (0.05 mg/ml) in each tube. All tubes were kept in -22˚C.

Stock solutions of fatty acids (calibration 1)

To create two different calibration lines (1 and 2), stock solutions were prepared.

Calibration 1: Three independent stock solutions (solutions 1, 2 and 3) of four fatty acids were produced. Around 16.0 mg of pentadecanoic acid and elaidic acid, 22.0 mg of ALA and 13.5 mg of DHA were dissolved in 50 ml MTBE.

Calibration 2: Three independent stock solutions (solution 1, 2 and 3) of four fatty acids were produced. 7.3 mg of pentadecanoic acid, 9.7 mg of elaidic acid, 17.0 mg of ALA and 6.0 mg of DHA were dissolved in 820 µl MTBE.

Preparation of calibration solutions of the fatty acid

Calibration 1:

Each calibration solution was prepared from the stock solutions. The calibration solutions were processed in the same procedure which was used for breast adipose tissues (Chapters 3.7.2-5). The final amount of IS was kept constant (0.016 µg on column). The final amounts of pentadecanoic acid in each calibration solution were 0.008, 0.028, 0.048 and 0.068 µg on column (oc). The final amounts of elaidic acid in each calibration solution were 0.008, 0.028 and 0.048 µg oc. For ALA the final amounts were 0.010, 0.038, 0.066 and 0.094 µg oc in calibration solutions 1 to 4 respectively. Final amounts of DHA in one point calibration were 0.006 and 0.024 µg oc in calibration solutions.

Calibration 2:

Each calibration solution was prepared from the stock solutions. The calibration solutions were processed in the same procedure which was used for breast adipose tissues (Chapters 3.7.2-5). The final amount of IS was kept constant (0.016 µg oc).The final amount of pentadecanoic acid in each calibration solution were 0.06, 0.15, 0.24 and 0.35 µg oc. The final amounts of elaidic acid in each calibration solution were 0.07, 0.20, 0.33 and 0.46 µg oc. For ALA the final amounts were 0.130, 0.360, 0.590 and 0.820 µg oc in calibration solution 1 to 4 respectively. For DHA the final amounts were 0.050, 0.130, 0.210 and 0.290 µg oc in calibration solutions 1 to 4 respectively.

3.5.3 Solutions for the isolation of ChOL and oxyChOL from adipose tissue

$CHCl_3$:MeOH	$CHCl_3$:MeOH (2:1; v:v) + 80 mg BHT
Eluent solution for oxyChOL fraction	To prepare the solution of 2-propanol:n-hexane (30%), 15 ml 2-propanol was mixed with 35 ml n-hexane.
Eluent solution for ChOL fraction	To prepare the solution of 2-propanol:n-hexane (0.5%), 400 µl 2-propanol was mixed with 80 ml n-hexane.
Ethanolic potassium hydroxide solution (2M)	2.8 g KOH dissolved in 25 ml EtOH.
NaCl solution (0.9%)	900 mg of NaCl dissolved in 100 ml of distilled water.

3.5.4 Calibration solutions for the quantification of ChOL

5α-ChAN-3β-ol solution as an IS

20.0 mg of 5α-ChAN-3β-ol was dissolved in 10.0 ml of EtOH (2.0 mg/ml). From this stock solution, a solution with concentration of 1.0 mg/ml was prepared by addition of 4.5 ml of EtOH to 5.5 ml of stock solution.

2.0 mg/ml // 5.5 ml + 4.5 ml EtOH (1.0 mg/ml)

ChOL stock solution

To create an external calibration of ChOL, three stock solutions with a concentration of 1.0 mg/ml were produced. 10 mg ChOL was dissolved in 10 ml of EtOH (three times independent).

Preparation of calibration solutions ChOL

Starting from ChOL stock solutions, 4 calibration solutions were prepared. The calibration solutions were processed in the same procedure which was used for breast adipose tissues (Chapters 3.7.6-7, Chapters 3.7.10-11). The final amounts of IS was kept constant (50.01 ng oc). The final amounts of ChOL in calibration solutions were 30.0, 50.0, 70.0 and 90.0 ng oc.

3.5.5 Solutions for the quantification of oxyChOLs

Oxy-D-ChOL as an IS

Oxy-D-ChOL was synthesized by Lisa Bäuml within her master thesis (2014). The IS mixture of Oxy-D-ChOLs was produced by thermal oxidation of D-ChOL. For this aim 500 µg D-ChOL were weighed in a glass culture tube and heated for 4 h at 160°C in a drying oven.

The residue was dissolved in 1 ml of toluene and applied onto SPE column to purifying the D-ChOL from oxy-D-ChOL fraction. After evaporation of solvents, the residue of oxy-D-ChOLs was dissolved in 1 ml EtOH. A further thermal oxidation was carried out on the residue of D-ChOL. The thermal oxidation of the D-ChOLs and the subsequent steps were repeated six times and in each step the SPE was done and after evaporating the solvent, the oxy-D-ChOLs were collected.

OxyChOLs stock solutions

To create two different calibration lines (1 and 2), stock solutions (three independent 1, 2 and 3) were prepared for 7α-HO-ChOL, 7β-HO-ChOL, 7-O-ChOL, α-epoxy-ChOL and β-epoxy-ChOL as follows:

7α-HO-ChOL

①90 µg 7α-HO-ChOL were dissolved in 260 µl EtOH (0.35 mg/ml)
0.35 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 100 µl + 780 EtOH
②138 µg 7α-HO-ChOL were dissolved in 355 µl EtOH (0.39 mg/ml)
0.39 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 100 µl + 780 EtOH
③240 µg 7α-HO-ChOL were dissolved in 560 µl EtOH (0.43 mg/ml)
0.43 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 100 µl + 780 EtOH

7β-HO-ChOL

①1 mM // 10 µl + 90 µl EtOH // 50 µl + 530 µl EtOH // 50 µl + 836 µl EtOH // 100 µl + 900 EtOH
②1 mM // 10 µl + 90 µl EtOH // 50 µl + 530 µl EtOH // 50 µl + 836 µl EtOH //100 µl + 900 EtOH
③1 mM // 10 µl + 90 µl // 50 µl + 530 µl EtOH // 50 µl + 836 µl EtOH // 100 µl + 900 EtOH

7-O-ChOL

①394 µg 7-O-ChOL were dissolved in 868 µl EtOH (0.45 mg/ml)
0.45 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 1030 µl EtOH // 40 µl + 764 EtOH
②278 µg 7-O-ChOL were dissolved in 638 µl EtOH (0.44 mg/ml)
0.44 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 1030 µl EtOH // 40 µl + 764 EtOH
③350 µg 7-O-ChOL were dissolved in 780 µl EtOH (0.45 mg/ml)
0.45 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 1030 µl EtOH //40 µl + 764 EtOH

α-epoxy-ChOL

①415 µg α-epoxy-ChOL were dissolved in 915 µl EtOH (0.45 mg/ml)
0.45 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 70 µl + 712 µl EtOH
②144 µg α-epoxy-ChOL were dissolved in 370 µl EtOH (0.39 mg/ml)
0.39 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 70 µl + 712 µl EtOH
③235 µg α-epoxy-ChOL were dissolved in 550 µl EtOH (0.43 mg/ml)
0.43 mg/ml // 50 µl + 530 µl EtOH // 50 µl + 530 µl EtOH // 70 µl + 712 µl EtOH

β-epoxy-ChOL

①644 µg β-epoxy-ChOL were dissolved in 1450 µl EtOH (0.44 mg/ml)
0.44 mg/ml // 50 µl + 580 µl EtOH // 50 µl + 580 µl EtOH // 70 µl + 712 µl EtOH
②382 µg β-epoxy-ChOL were dissolved in 845 µl EtOH (0.45 mg/ml)
0.45 mg/ml // 50 µl + 580 µl EtOH // 50 µl + 580 µl EtOH // 70 µl + 712 µl EtOH
③254 µg β-epoxy-ChOL were dissolved in 590 µl EtOH (0.43 mg/ml)

0.43 mg/ml // 50 µl + 580 µl EtOH // 50 µl + 580 µl EtOH // 70 µl + 712 µl EtOH

Preparing standard solution of calibration points with IS

To create an external calibration of oxyChOLs, three independent stock solutions (solutions 1, 2 and 3) were produced. The level of IS (oxy-D-ChOL) was kept constant (7α-HO-D-ChOL: 4.5, 7β-HO-D-ChOL: 6.0, 7-O-D-ChOL: 83.1, β-epoxy-D-ChOL: 448.3 and α-epoxy-D-ChOL: 279.0 pg oc) in all solutions.

The calibration solutions were processed in the same procedure which was used for breast adipose tissues (Chapter 3.7.6-9). In first calibration line the final level was 2 - 12 pg oc for 7α-HO-ChOL, 1.0 - 13.5 pg oc for 7β-HO-ChOL, 30 - 180 pg oc for 7-O-ChOL, 300 - 1300 pg oc for β-epoxy-ChOL and 50 - 625 pg oc for α-epoxy-ChOL. In second calibration line the final level was 0.57 - 1.67 pg oc for 7α-HO-ChOL, 0.18 - 0.67 pg oc for 7β-HO-ChOL, 1.59 - 16.26 pg oc for 7-O-ChOL.

3.6 Software

Software	Version, Provider
Agilent Chem Station	G1701DA, Agilent Technologies , Wilmington, USA
Agilent Chem Station	A.08.03 [847], Agilent Technologies , Santa Clara, USA
MS Data Review	6.9.2, Varian, California, USA
OriginPro	Version 9.0, OriginLab Corporation, Northampton, MA 01060,USA
R	Version 3.0.1, R Core Team (2013), R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/
Valoo	2.6, analytic-software, Leer, Germany, URL http:// www.analytik-software.de/

3.7 Methods

3.7.1 Preparation of breast adipose tissue samples

The breast adipose tissues and personal information were collected from women without BC who underwent reduction mammoplasty for cosmetic reasons. The ethics committee of the university of Würzburg (vote numbers: 74/10 and 168/10) approved the study.

Immediately after receipt the tissue samples were put in ice-cold Krebs buffer. For the determination of ChOL and oxyChOLs, 100 mg of human breast adipose tissue was frozen in

liquid nitrogen and homogenized portioned into cryos, and then it was kept in a freezer under -80˚C until the day of assay.

3.7.2 Extraction of fatty acids

Total lipid of breast adipose tissue was extracted from each sample by using $CHCl_3$:MeOH (2:1, v/v) solution mixture (Chapter 3.5.1). For each sample approximately 700 mg of breast adipose tissue was weighed and added to 20 ml of a $CHCl_3$:MeOH (2:1) solution containing 1 mg IS (Chapter 3.5.2). The solution was allowed to stand for one hour. Then 2 ml of MeOH (Chapter 3.1.4) and 2 ml of 0.9% NaCl solution (Chapter 3.5.1) was added to the mixture, shaken and centrifuged for 5 min at 4000 rpm with the centrifuge Universal 16 (Chapter 3.2). This washing step with MeOH and 0.9% NaCl was performed twice in total. Then the collected $CHCl_3$ layer (organic phase) was evaporated by rotary evaporator (Chapter 3.2). The extract residue was dissolved in 3 ml MTBE.

3.7.3 Preparing dilution

The residue of lipid extract (Chapter 3.7.2) was dissolved in 3000 µl MTBE. 50 µl of this solution was taken and diluted by 950 µl MTBE.

3.7.4 Derivatization of fatty acids

The fatty acids of samples (Chapter 3.7.3) and calibration solutions (Chapter 3.5.2) were converted to corresponding FAMEs by derivatization by 50 µl of TMSH. 1 µl of the derivatized solution was used for GC-FID analysis.

3000 µl extract// 50 µl + 950 µl MTBE + 50 µl TMSH// injection of 1 µl for fatty acids detection

3.7.5 Optimized GC/FID method for quantification of fatty acids

1 µl of analyte solution (Chapter 3.7.4) was injected manually at an injector temperature of 260°C. The flow of Helium as carrier was 1 ml/min equal to 111 kPa. The initial temperature of the oven was 140°C. It ramped to 230°C at a heating rate of 3°C/min, it was kept in 230°C for 5 min and then it was increased by 1°C per min to 240°C and hold in this temperature for 20 min. The temperature of the FID was 260°C. Detection was carried out at a hydrogen flow rate of 40 ml/min, an air flow rate of 450 ml/min and a nitrogen flow of 45 ml/min. This method was applied as a final method for quantification of fatty acids methyl ester in breast adipose tissue samples (Chapter 3.7.4) and calibration solutions (Chapter 3.5.2). To identify the fatty acids in 50 breast adipose tissue samples, the absolute retention time (R_t) and relative retention time (Appendix: Table 32) of fatty acids in samples were compared to those in Supelco^{TM} 37 (Chapter

3.4). For device control and integration of fatty acids peak area (Appendix: Table 33), the Agilent ChemStation software (G1701DA, Chapter 3.6) was used.

3.7.6 Preparation of adipose tissue for the determination of ChOL and oxyChOLs in human breast adipose tissue

In order to extract the fat, the frozen sample (Chapter 3.7.1) was transferred into 50 ml plastic centrifuge tube with 10 ml of a $CHCl_3$:MeOH + 80 mg BHT (2:1; v:v) solution (Chapter 3.5.3) containing 80 µl of 5α-ChAN-3β-ol (1.09 mg/ml, Chapter 3.5.4) or 100 µl of oxy-D-ChOLs (Chapter 3.5.5) as an IS for ChOL and oxyChOL respectively. The tube was shaken and left for one hour followed by washing steps (Chapter 3.7.2). The washed solution was transferred with plastic Pasteur pipette into a glass culture tube and the solvent was evaporated by means of rotational vacuum concentrator (Chapter 3.2). The dry residue was taken up in 5 ml of ethanolic potassium hydroxide in order to saponification overnight. The next day, the solution was transferred to a 50 ml plastic centrifuge tube. The evaporation vessel was rinsed with 10 ml of distilled water and then transferred into a centrifuge tube. After addition of 10 ml of CH_2Cl_2 (Chapter 3.3) in the tube, this was shaken and centrifuged for 5 min at 4000 rpm. The upper phase was removed with a plastic Pasteur pipette and discarded. The lower phase was mixed with 10 ml of distilled water, turned cautious (slowly rotated) and centrifuged for 5 min at 4000 rpm. The upper phase was removed and discarded again. The remaining solution in the centrifuge tube was treated with sodium sulphate until no more turbidity was observed. The upper phase was transferred with a glass Pasteur pipette into glass culture tubes and evaporated to dryness. The residue was dissolved in 1 ml of toluene.

3.7.7 Separation of ChOL and oxyChOLs by SPE

ChOL was separated from oxyChOLs by means of SPE extraction. The 100 mg silica column (Chapter 3.3) was first conditioned with 2 ml of n-hexane and then equilibrated with 1 ml of toluene. The residue, which was dissolved in 1 ml toluene, was loaded onto the cartridge and then washed with 2 ml of n-hexane. First, the ChOL fraction was eluted with 8 ml of 0.5% 2-propanol in n-hexane, then the oxyChOLs fraction eluted with 5 ml of 30% 2-propanol in n-hexane (Chapter 3.5.3). The solvents of both fractions were removed in an evaporator.

3.7.8 Derivatization of oxyChOLs

The derivatization of oxyChOLs was performed by adding 50 µl BSTFA/n-heptane (1:1) in the 10 ml glass culture tube containing the residue of oxyChOL after separation by SPE and evaporation of solvents (Chapter 3.7.7). The mixture was transferred with a glass Pasteur pipette into a 1.5 ml GC tube. After three hours at room temperature, the solution was diluted

(Chapter 3.7.9). Then it was transferred with a glass Pasteur pipette into a GC vial for GC analysis.

3.7.9 Dilution for detection of the oxyChOLs in human breast adipose tissue

The residue of oxyChOL fraction was dissolved in 50 µl BSTFA/n-heptane (1:1) (Chapter 3.7.8). This solution was referred to as "solution 0". For detecting of 7-O-ChOL, an aliquot of 10 µl of the "0 solution" was diluted by the addition of 40 µl of n-heptane. From this solution 10 µl were diluted in 30 µl n-heptane for the analysis of 7-HO-forms.

„Solution 0“// 2 µl Injection for detection of the epoxy-forms

// 10 µl + 40 µl n-Heptan // 1 µl Injection for detection of 7-O-ChOLs

// 10 µl + 30 µl n-Heptan // 1 µl Injection for detection of 7-HO-forms

3.7.10 Dilution for detection of the ChOL content

1750 µl EtOH were added to the residue of the ChOL fraction isolated by SPE (Chapter 3.7.7). To ChOL quantification by GC-FID 50 µl of this solution were evaporated and used for derivatization.

3.7.11 Derivatization of ChOL

50 µl of derivatization reagent BSTFA/n-heptane (1:1) were added to the ChOL residue (Chapter 3.7.10). After three hours at room temperature, the solution was transferred with a glass Pasteur pipette into a GC vial. 1 µl of the derivatized solution was used for GC-FID analysis.

3.7.12 Optimized GC-FID method for the quantification of ChOL

The GC-FID method for quantification of ChOL was optimized by Lisa Bäuml (2014). In an injection temperature of 250°C, 1 µl of the analyte solution (silylated ChOL, Chapter 3.7.11) was injected by autosampler (Chapter 3.1). Helium as a carrier gas was used at a flow of 1 ml/min and a gas pressure of 111 kPa. The oven temperature increased by 2 min at 200°C at a heating rate of 3.4°C/min to the final temperature of 300°C. This was held for 5 min. The total run time was 36.4 min. The temperature in the FID was 250°C. Detection was carried out at a hydrogen, air and nitrogen flow rate of 40, 450 and 45 ml/min respectively. Peak areas of ChOL and IS were determined using Agilent ChemStation software A.08.03 (Chapter 3.6). Forty nine breast adipose tissue samples were analyzed using this method.

3.7.13 Optimized GC-MS/MS method for the quantification of oxyChOL

In order to detection of oxyChOL, GC-MS/MS parameters including collision energies, dwell-time, collision gas pressure and temperatures of transfer line and ion source sensitivity were optimized for the highest sensitivity for quantification of the oxyChOL in human mammary adipose tissue. GC-MS/MS parameters were optimized by Lisa Bäuml (2014). The best sensitivity for quantification of the content of oxyChOL was achieved in the *multiple-reaction-monitoring mode* (MRM-Mode)

In the optimized method, the ionization of the substances was carried out by electron impact ionization at -70 eV. Argon was used as a collision gas in the quadrupole 2 (collision cell Q2). The collision gas pressure in the Q2 was 0.8 mTorr. The temperatures of the transfer line and ion source were 300°C, 270°C respectively. Helium as a carrier gas was used with a flow rate of 1 ml/min. The starting temperature of the column was 200°C with holding time of 2 minutes. With a heating rate of 4°C/min, the oven was heated up to the temperature of 315°C and this held for 5 min. This temperature program resulted in a total run time of 35.8 min. The injection volume of analyte solutions (Chapter 3.7.9) was 1 or 2 µl and was injected at an injection temperature of 250°C by means of auto sampler (Chapter 3.1) in splitless mode.

To capture the individual oxyChOL in fatty tissue of the breast, three different analytical methods were used. The α- and β-form of the epoxy-ChOL were analyzed together; likewise the two isomeric forms of 7-HO-ChOL (α, β) were analyzed together. 7-O-ChOL was measured with a separate method (Table 7). The Integration of the peaks was performed by the analysis software MS Data Review Varian (Chapter 3.6). Forty nine breast adipose tissue samples were analyzed using this method.

Table 7 Instrument parameters for detection of oxyChOLs using GC-MS/MS.

substance	R_t (min)	Mass transfer	collision energy (ev)	Dwell Time (s)	classification
7α-HO-D-ChOl	24.68	462 > 233	20	0.1	Quantifier
7β-HO-D-ChOl	26.64	462 >462	10	0.1	Qualifier
7β-HO-ChOl	26.73	456 > 233	20	0.1	Quantifier
7α-HO-ChOl	24.77	456 > 456	10	0.1	Qualifier
β-D-epoxy-ChOL	27.15	480 > 390	7.5	0.1	Quantifier
α-D-epoxy- ChOL	27.37	480 > 362	7.5	0.1	Qualifier
β-epoxy-ChOL	27.24	474 > 384	7.5	0.1	Quantifier
α-epoxy-ChOL	27.45	474 > 356	7.5	0.1	Qualifier
7-O-D-ChOL	29.46	478 > 461	15	0.1	Quantifier
		478 > 373	22.5	0.1	Qualifier
7-O-ChOL	29.54	472 > 455	15	0.1	Quantifier
		472 > 367	22.5	0.1	Qualifier

3.7.14 Validation procedure

The analytical method was validated with regard to the food and drug administration guideline (FDA, 2013) parameters: calibration curve using IS (Chapter 3.7.14.1), accuracy and precision (Chapter 3.7.14.2), limit of detection (LOD, Chapter 3.7.14.3) and limit of quantification (LOQ, Chapter 3.7.14.4).

3.7.14.1 Calibration curve using IS

The calibrations of analyte were performed 3 times independently, starting from weighing the compound. The calibration curve constructed by linear regression of the peak area ratio of analyte to IS and the corresponding analyte amount on the column. Linear relationship of amount and peak area ratios (analytes to IS) as well as homogeneity of variances of calibration points was verified using valoo 2.6 (Chapter 3.6) at the significance level of 5%.

3.7.14.2 Accuracy and precision

Accuracy and precision were determined by replicate analysis of samples containing known amounts of the analyte. It was carried out through addition and non-addition of oxyChOLs or ChOLs in samples contained low, middle and high levels of these components. The blank (solvent containing IS mixture), spiked and non-spiked samples of ChOL or oxyChOLs were prepared by the method which is described in Chapter 3.7.6-11 and then analyzed by GC-FID (Chapter 3.7.12) and GC-MS/MS (Chapter 3.7.13) respectively.

Accuracy

The accuracy was calculated by subtracting the original level from the corresponding spiked levels (Formula 1):

$$\text{Accuracy (\%)} = (C_r/C_a) \times 100 \quad (1)$$

C_r: subtracting the original level from the corresponding spiked levels.

C_a: spiked level.

Precision

Precision was measured as relative standard deviation (RSD) of the replicate measurements using formula 2.

$$\%\text{RSD} = \frac{\text{Standard deviation}}{\text{mean}} \times 100 \quad (2)$$

3.7.14.3 LOD

LOD is the smallest amount of analyte that is significantly different from the blank. Two times the area of the noise at the respective retention time was determined and the absolute level of it was calculated with the IS.

3.7.14.4 LOQ

LOQ is the smallest amount of analyte that we are able to quantify. The lowest standard point on the calibration curve should be accepted as the limit of quantification.

3.7.15 Correlation analysis

The correlation analysis was performed using R (Chapter 3.6). To find the association of fatty acids, ChOL and oxyChOLs the Spearman rank correlation coefficient was calculated. Spearman's rank is a non-parametric measure of the strength of association between two variables. Furthermore, the correlation analysis was performed between fatty acids, ChOL and oxyChOLs and age and body mass index (BMI).

4. Results and discussion

The breast adipose tissue composition of women without BC has not been determined yet. In order to investigate the breast adipose tissue composition, breast adipose tissues of women without BC were analyzed (Chapter 3.7). The breast adipose tissue fatty acids (Chapter 4.1), ChOL (Chapter 4.2) and oxyChOL (Chapter 4.3) were determined. Moreover, the statistical evaluation of fatty acids, ChOL and oxyChOL were performed (Chapter 4.4).

4.1 Fatty acids in breast adipose tissue

Fatty acids are the main compounds of triglycerides in adipose tissue. The composition of fatty acid in adipose tissue is of interest because fatty acids may play an important role in BC (Chapter 1.1.1.2) and it has been reported that the relative fatty acid composition of adipose tissue reflect fatty acid dietary intake of the preceding two to three years period (Chapter 1.1.1.3.1). Although, the fatty acid composition of breast adipose tissue of BC and other biological adipose tissues were investigated previously, there is little information about breast adipose tissue of women without BC (Straka et al., 2015; Yee et al., 2010). 16 fatty acids were reported by Straka et al. (2015) in breast adipose tissue. Furthermore, Yee et al. (2010) have found that there were 18 fatty acids present in breast adipose tissue of women without BC. To see if there are more fatty acids present in breast adipose tissue and to compare the result of this work with previous researches, the breast adipose tissue fatty acids compositions of 50 samples (without BC) were determined (Chapter 4.1.1). Furthermore, absolute levels of four fatty acids pentadecanoic acid, elaidic acid, ALA and DHA as reasoned in Chapter 4.1.2 were quantified.

4.1.1 Breast adipose tissue fatty acid composition

The fatty acids composition was determined in breast adipose tissues of women without BC. Fifty samples were processed and injected under optimized GC-FID method (Chapter 3.7.5). Twenty six fatty acids were identified. Among these 26 fatty acids, 11 fatty acids were SFAs, 5 fatty acids were MUFAs, 9 fatty acids were PUFAs and 1 was TFA in breast adipose tissues. To find the proportion of total SFA, MUFA and PUFA (Chapter 4.1.1.1) as well as individual proportion of fatty acid (Chapters 4.1.1.2-5) in breast adipose tissues and major fatty acids (Chapter 4.1.1.6) the relative peak areas of fatty acids were calculated. In addition to, the relative peak areas of breast adipose tissues were compared to the breast adipose tissues of other studies (Chapter 4.1.1.7) and other biological adipose tissues (Chapter 4.1.1.8).

4.1.1.1 Sum of SFA, MUFA and PUFA in breast adipose tissue

The proportion of SFAs, MUFAs, PUFAs and TFA were determined by integrating the peak at baseline (Appendix: Table 33) and dividing the results by the total area for all fatty acids. SFA, MUFA, and PUFA were calculated by summing all the SFAs (n=11), MUFAs (n=5), and PUFAs (n=9, Figure 5). TFA group included only elaidic acid (18:1 9trans). Saturated group involved capric (10:0), lauric (12:0), tridecanoic (13:0), myristic (14:0), pentadecanoic (15:0), palmitic (16:0), heptadecanoic (17:0), stearic (18:0), arachidic (20:0), heneicosanoic (21:0) and tricosanoic (23:0) acids (Chapter 4.1.1.2). In the breast adipose tissue of 50 women SFA ranged from 25.8 to 39.9% (median: 34.6%, Figure 5). Monounsaturated included myristoleic (14:1 9cis), palmitoleic (16:1 9cis), oleic (18:1 9cis), vaccenic (18:1 11cis) and cis-11-eicosenoic (20:1) acids (Chapter 4.1.1.3). MUFA were between 48.2 to 62.0% (median: 53.2%, Figure 5). The fatty acids in polyunsaturated group were LA, ALA, GLA (18:3 n6), eicosadienoic and eicosatrienoic n3 and n6, AA, EPA and DHA (Chapter 4.1.1.4). The PUFA relative peak areas of breast adipose tissues ranged from 8.9 to 15.8% (median: 12.1%, Figure 5).

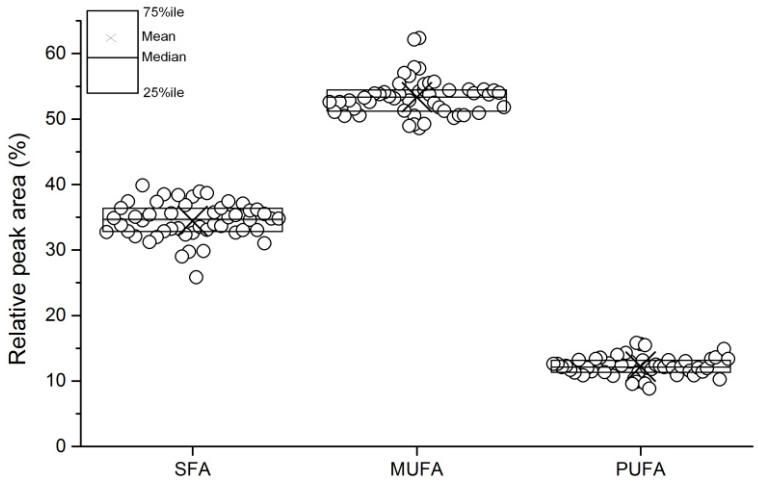


Figure 5 Sum of relative peak areas (%) of SFA, MUFA and PUFA in breast adipose tissues of 50 samples.

In the adipose tissue the largest proportion of fatty acids belonged to MUFA (mean: 53.0%), followed by SAF (mean: 34.4%) and PUFA (mean: 12.2%). Previously London et al. (1991) investigated the fatty acid composition of gluteal adipose tissue in postmenopausal American women. Their result is consistent with this work (relative peak areas of MUFA > SFA > PUFA). However, the mean peak areas of MUFA (54.49%) and PUFA (21.08%) were higher than MUFA and PUFA of breast adipose tissues.

4.1.1.2 Relative peak areas of SFAs

SFAs have no double bond in the carbon backbone (Chapter 1.1.1.1). In breast adipose tissues palmitic acid (16:0) devoted the highest proportion of SFA (Table 8). The relative peak area of this fatty acid ranged from 18.92 to 28.10% (median: 23.31%, Table 8). The second common SFA was stearic acid (18:0). The median proportion of stearic was 5.82% in breast adipose tissues (range: 2.75 - 7.97%). Myristic acid (14:0) ranged from 2.11 to 4.34% in samples (Table 8). The median proportions of capric (10:0) and lauric (12:0) acids were 0.05% (rang: 0.02 - 0.13%) and 0.55% (range: 0.21 - 1.35%) in breast adipose tissues respectively (Table 8). The median relative peak areas of odd numbered fatty acids were 0.02%, 0.32%, 0.31%, 0.22% and 0.38% for tridecanoic (13:0), pentadecanoic (15:0), heptadecanoic (17:0), heneicosanoic (21:0) and tricosanoic (23:0) acid respectively. These fatty acids cannot be synthesized in humans. They originate from diet (Chapter 1.1.1.3.1).

Arachidic acid (20:0) which is the long chain SFA accounted for 0.18% (median) in breast adipose tissue (Table 8). Short carbon chain SFAs such as 4:0, 6:0 and 8:0 were not observed in the analyzed samples.

All SFAs were detectable in 100% of samples (Table 8).

Table 8 The relative proportion of individual SFAs in breast adipose tissue of 50 samples. The presented data are the mean relative peak areas (%) of fatty acids from three injections by means of GC-FID (Chapter 3.7.5).

women	10:0	12:0	13:0	14:0	15:0	16:0	17:0	18:0	20:0	21:0	23:0
0	0.08	0.57	0.03	3.37	0.33	23.06	0.33	6.48	0.22	0.27	0.33
1	0.06	0.54	0.03	3.20	0.34	22.16	0.34	5.10	0.18	0.30	0.35
2	0.11	0.72	0.04	3.46	0.24	21.50	0.25	5.00	0.19	0.43	0.41
3	0.13	0.74	0.04	4.14	0.37	24.26	0.36	7.30	0.21	0.30	0.29
4	0.03	0.45	0.02	2.88	0.27	24.21	0.27	4.66	0.12	0.20	0.41
5	0.07	1.35	0.05	4.06	0.30	23.66	0.27	6.31	0.23	0.25	0.28
6	0.04	0.29	0.02	2.41	0.25	18.92	0.19	2.75	0.11	0.39	0.47
7	0.05	0.53	0.03	3.53	0.32	23.07	0.27	4.79	0.16	0.14	0.50
8	0.06	0.49	0.02	2.90	0.27	21.97	0.32	6.42	0.18	0.21	0.39
9	0.04	0.53	0.03	3.75	0.38	26.87	0.35	6.30	0.17	0.19	0.42
10	0.03	0.41	0.02	3.07	0.35	23.67	0.33	4.42	0.12	0.27	0.52
11	0.05	0.53	0.04	3.37	0.37	23.82	0.33	4.46	0.15	0.26	0.58
12	0.03	0.44	0.02	2.62	0.26	20.59	0.24	4.61	0.17	0.21	0.55
13	0.05	0.68	0.03	3.38	0.29	25.25	0.32	7.70	0.27	0.18	0.34
14	0.03	0.75	0.02	3.48	0.33	24.80	0.36	7.85	0.26	0.19	0.63
15	0.04	0.65	0.02	3.27	0.33	22.48	0.28	4.93	0.20	0.22	0.43

Table 8 (continued).

women	10:0	12:0	13:0	14:0	15:0	16:0	17:0	18:0	20:0	21:0	23:0
16	0.06	0.69	0.02	3.15	0.23	24.16	0.31	6.43	0.24	0.15	0.26
17	0.06	0.74	0.03	3.21	0.28	23.55	0.28	6.91	0.30	0.19	0.19
18	0.02	0.45	0.02	2.39	0.24	22.82	0.29	6.70	0.19	0.14	0.39
19	0.04	0.59	0.03	3.19	0.28	22.27	0.26	4.55	0.16	0.25	0.36
20	0.07	0.58	0.03	3.37	0.32	23.75	0.30	5.66	0.26	0.28	0.39
21	0.13	0.95	0.04	4.34	0.35	25.12	0.33	6.45	0.25	0.27	0.25
22	0.04	0.58	0.03	2.91	0.27	21.36	0.27	4.91	0.16	0.25	0.42
24	0.06	0.74	0.03	3.60	0.34	21.65	0.30	4.92	0.25	0.25	0.53
25	0.03	0.41	0.02	3.08	0.33	25.11	0.35	6.15	0.15	0.21	0.55
26	0.07	0.83	0.03	3.63	0.33	23.72	0.36	7.56	0.25	0.24	0.34
27	0.03	0.33	0.02	3.04	0.30	24.58	0.31	5.17	0.16	0.22	0.37
28	0.06	0.54	0.02	2.77	0.20	22.95	0.25	5.58	0.23	0.17	0.29
29	0.03	0.29	0.02	2.74	0.23	23.20	0.24	4.55	0.15	0.10	0.62
30	0.08	0.67	0.04	4.07	0.42	25.00	0.38	5.98	0.19	0.35	0.26
31	0.08	0.60	0.03	3.74	0.35	22.91	0.33	6.39	0.27	0.31	0.37
32	0.05	0.42	0.02	2.44	0.24	22.56	0.31	7.97	0.23	0.16	0.23
33	0.03	0.38	0.02	3.32	0.31	25.28	0.28	4.91	0.17	0.22	0.41
34	0.02	0.46	0.02	2.88	0.31	23.04	0.32	4.63	0.17	0.20	0.78
35	0.05	0.36	0.02	3.44	0.26	28.10	0.30	6.63	0.16	0.18	0.37
36	0.05	0.57	0.03	3.46	0.34	23.79	0.38	7.72	0.25	0.21	0.29
37	0.03	0.46	0.02	3.41	0.32	24.11	0.27	3.65	0.12	0.26	0.39
38	0.06	0.71	0.04	3.45	0.38	22.97	0.26	5.10	0.26	0.25	0.32
39	0.03	0.44	0.02	2.63	0.28	21.20	0.32	5.24	0.15	0.22	0.48
40	0.04	0.62	0.02	3.30	0.32	23.26	0.29	4.90	0.15	0.25	0.46
41	0.10	0.43	0.03	3.53	0.29	23.36	0.32	7.17	0.24	0.23	0.32
42	0.06	0.67	0.03	3.86	0.40	25.12	0.34	6.17	0.22	0.23	0.31
43	0.05	0.47	0.02	2.65	0.27	20.42	0.26	5.06	0.16	0.15	0.31
44	0.04	0.31	0.02	3.31	0.44	24.77	0.39	6.02	0.10	0.20	0.54
45	0.08	0.69	0.03	3.51	0.36	23.01	0.31	6.10	0.21	0.19	0.29
46	0.08	0.40	0.02	3.19	0.22	25.19	0.24	6.40	0.18	0.18	0.31
47	0.03	0.21	0.01	2.11	0.20	21.52	0.25	3.36	0.08	0.19	1.04
48	0.04	0.55	0.02	3.10	0.34	24.39	0.35	6.10	0.14	0.16	0.35
49	0.07	0.70	0.03	3.51	0.34	23.10	0.32	6.10	0.21	0.20	0.30
50	0.05	0.60	0.02	2.82	0.26	22.38	0.29	5.36	0.17	0.15	0.64
median	0.05	0.55	0.02	3.31	0.32	23.31	0.31	5.82	0.18	0.22	0.38
min	0.02	0.21	0.01	2.11	0.20	18.92	0.19	2.75	0.08	0.10	0.19
max	0.13	1.35	0.05	4.34	0.44	28.10	0.39	7.97	0.30	0.43	1.04

4.1.1.3 Relative peak areas of MUFAs

Monounsaturated are fatty acids with one double bond in the carbon chain (Chapter 1.1.1.1). Five MUFAs (myristoleic, palmitoleic, oleic, vaccenic acid and eicosenoic acid) were detected in 50 breast adipose tissues. Myristoleic acid ranged from 0.12% to 0.49% (median: 0.27%) in 50 samples (Table 9). Palmitoleic acid accounted for 3.55% (median, Table 9) in breast adipose tissues (rang: 1.87 - 7.58%). The main MUFA in breast adipose tissue was oleic acid that ranged from 41.12 to 50.25% (Table 9). The important diet sources of oleic acid are olive oil, almond oil, and sunflower oil high oleic (Table 3, Chapter 1.1.1.3.1). Also, desaturation of 18:0 can lead to the endogenous production of 18:1 9cis (Figure 1, Chapter 1.1.1.3.2).

Vaccenic acid (cis) is an n-7 acid produced by chain elongation of palmitoleic acid. It accounted for 2.66% in tissues of 50 women (median, Table 9). Moreover, eicosenoic acid (20:1) was present in the range of 0.18 - 1.14% (median: 0.84%, Table 9).

The result of previous studies indicated, the relative proportion of gluteal adipose tissue MUFAs (as a group) was poorly correlated with dietary intake (London et al., 1991; Tjonneland et al., 1993). MUFAs can be synthesized in body. Therefore the adipose tissue content of MUFA is not expected to reflect dietary intake of it (Chapter 1.1.1.3).

Table 9 The relative proportion of individual MUFAs in breast adipose tissue of 50 samples. The presented data are the mean relative peak areas (%) of fatty acids from three injections by means of GC-FID (Chapter 3.7.5).

Women	14:1 9cis	16:1 9cis	18:1 9cis	18:1 11cis	20:1 9cis
0	0.27	3.36	43.51	3.13	0.92
1	0.27	3.30	46.52	2.56	1.14
2	0.43	6.80	46.67	2.59	0.80
3	0.31	3.44	42.97	2.44	0.84
4	0.25	4.27	45.82	2.92	0.80
5	0.40	4.72	44.45	1.96	0.70
6	0.49	7.58	49.17	3.72	1.03
7	0.37	5.97	42.75	3.32	0.88
8	0.24	3.56	43.26	2.88	0.83
9	0.23	3.02	41.12	3.11	0.70
10	0.26	3.47	44.40	2.97	0.95
11	0.37	5.18	44.43	2.52	0.81
12	0.26	3.99	50.02	2.45	0.87
13	0.18	2.45	45.62	2.21	0.82
14	0.17	2.11	43.68	1.87	0.75
15	0.26	3.14	46.41	2.18	0.89
16	0.18	2.56	44.39	2.70	0.92
17	0.22	3.08	46.68	2.15	0.91
18	0.14	2.83	47.89	2.39	0.84
19	0.31	5.03	45.83	2.88	0.89
20	0.33	3.76	46.74	2.15	0.86
21	0.36	4.07	42.47	2.19	0.70
22	0.34	4.65	47.61	2.76	0.91
24	0.39	4.68	45.22	2.26	0.87
25	0.15	1.87	45.42	2.04	0.79
26	0.22	2.68	43.26	1.91	0.78
27	0.22	3.62	46.12	2.61	1.05
28	0.21	3.30	48.63	2.01	0.95
29	0.34	5.00	47.70	3.39	0.18
30	0.36	3.85	42.93	2.36	0.76
31	0.33	3.78	45.03	2.22	0.99
32	0.12	2.19	49.38	2.05	0.60
33	0.26	4.52	44.35	3.05	0.80
34	0.23	3.44	46.03	2.97	1.02
35	0.25	4.12	42.18	2.84	0.92
36	0.19	2.62	44.17	2.68	0.99
37	0.40	5.62	44.91	3.63	0.88
38	0.36	3.61	46.57	2.78	0.90

Table 9 (continued).

Women	14:1 9cis	16:1 9cis	18:1 9cis	18:1 11cis	20:1 9cis
39	0.21	3.53	47.12	3.20	1.00
40	0.27	3.84	46.42	2.98	0.77
41	0.28	4.55	44.67	3.13	0.89
42	0.25	2.83	44.15	2.35	0.61
43	0.16	2.22	48.33	2.73	0.73
44	0.16	2.20	43.04	2.64	0.67
45	0.22	2.98	45.17	3.01	0.79
46	0.28	5.78	43.69	3.22	0.79
47	0.32	7.08	50.25	3.63	0.65
48	0.16	1.95	45.49	2.42	0.84
49	0.22	2.98	45.17	3.02	0.79
50	0.17	2.89	45.33	2.24	0.87
median	0.26	3.55	45.28	2.66	0.84
min	0.12	1.87	41.12	1.87	0.18
max	0.49	7.58	50.25	3.72	1.14

4.1.1.4 Relative peak areas of PUFAs

The family of PUFA contains fatty acids with more than one double bond in structure (Chapter 1.1.1.1). There are two main types of polyunsaturated fats which are n3 and n6 fatty acids. In this research five n6 PUFAs and four n3 PUFAs were determined in 50 breast adipose tissues. The sum of n6 PUFAs and n3 PUFAs relative peak areas ranged from 8.36 to 15.03% and from 0.49 to 1.23% respectively (Table 10). The relative peak areas of n6 PUFAs (mean: 11.31%) were approximately 10 fold higher than n3 PUFAs (mean: 0.89%).

n6 PUFAs

The total n6 PUFAs (relative peak areas) reflected the sum of LA (18:2 n6), GLA (18:3 n6), eicosadienoic acid (20:2 n6); DGLA (20:3 n6) and AA (20:4 n6) in breast adipose tissues. The most important member of this group is LA, which is essential for the human body because it cannot be synthesized endogenously. It is found abundantly in vegetable oils especially safflower oil and sunflower oil (Chapter 1.1.1.3.1). The relative peak areas of this fatty acid were between 7.60 - 14.56% in breast adipose tissues (Table 10). The human body can convert LA to other types of n6 (Chapter 1.1.1.3.2). In the first step it can be changed to GLA by Δ6-desaturase (Chapter 1.1.1.3.2). The median relative peak area of GLA was 0.04% in breast adipose tissue of 50 women (Table 10). Moreover, DGLA can be formed through elongation of GLA (Chapter 1.1.1.3.2). The relative peak areas of DGLA varied between 0.14 and 0.54% in breast adipose tissues (Table 10). Another n6 PUFA is AA. In breast adipose tissues AA was not detectable in all samples (Table 10). The relative peak area percentages of this fatty acid in 10 samples were between 0.002 to 0.02%. Nelson et al. (1997) examined the influence of dietary intervention of AA on the level of this fatty acid in gluteal adipose tissue. No significant increase was observed in the adipose level of AA in intervention and control groups. However, the

significant changes were seen in the production of urinary eicosanoids. AA is the metabolic precursor of the biologically active eicosanoids, such as prostaglandins. They suggested that the metabolic pathway of dietary AA is changed into specific pathway by the physiological control mechanisms (for fatty acid homeostasis) and as a consequence the accumulation of AA is prevented in adipose tissue.

Eicosadienoic acid (20:2 n6) is a cis11-cis14-di-unsaturated fatty acid. The relative proportion of this fatty acid in breasts ranged from 0.13 to 0.31% (Table 10).

Table 10 The relative proportion of individual PUFAs in breast adipose tissue of 50 samples. The presented data are the mean relative peak areas (%) of fatty acids from three injections by means of GC-FID (Chapter 3.7.5, nd: not detected).

	n6						n3				
Women	18:2	18:3	20:2	20:3	20:4	total n6	18:3	20:3	20:5	22:6	total n3
0	12.00	0.04	0.29	0.18	nd	12.51	0.66	0.04	0.03	0.13	0.86
1	11.70	0.04	0.29	0.21	nd	12.24	0.76	0.04	0.03	0.09	0.92
2	8.78	0.04	0.20	0.19	nd	9.20	0.54	0.04	0.05	0.11	0.73
3	10.01	0.04	0.24	0.21	nd	10.50	0.68	0.04	0.03	0.12	0.87
4	10.53	0.04	0.24	0.29	nd	11.11	0.68	0.04	0.04	0.14	0.89
5	9.20	0.04	0.16	0.16	nd	9.55	0.63	0.03	0.04	0.09	0.79
6	10.25	0.04	0.27	0.29	0.01	10.85	0.64	0.04	0.07	0.20	0.95
7	11.30	0.05	0.20	0.29	nd	11.86	0.67	0.03	0.10	0.30	1.11
8	14.12	0.04	0.29	0.21	nd	14.70	0.68	0.04	0.04	0.17	0.94
9	11.30	0.05	0.22	0.24	nd	11.83	0.55	0.03	0.03	0.07	0.69
10	12.61	0.04	0.31	0.33	nd	13.29	0.62	0.05	0.07	0.26	0.99
11	10.81	0.05	0.23	0.34	nd	11.42	0.58	0.03	0.04	0.18	0.84
12	10.88	0.04	0.24	0.27	0.01	11.42	0.65	0.04	0.08	0.18	0.94
13	8.70	0.04	0.18	0.22	nd	9.17	0.50	0.03	0.03	0.12	0.69
14	10.85	0.07	0.18	0.50	nd	11.61	0.39	0.02	0.04	0.09	0.52
15	12.20	0.04	0.25	0.33	nd	12.82	0.84	0.05	0.08	0.21	1.17
16	11.53	0.04	0.21	0.14	0.01	11.93	1.07	0.04	0.03	0.09	1.23
17	9.47	0.04	0.20	0.21	nd	10.20	0.51	0.03	0.01	0.04	0.59
18	10.72	0.04	0.22	0.25	nd	11.23	0.53	0.04	0.04	0.11	0.72
19	11.14	0.04	0.22	0.23	nd	11.62	0.76	0.04	0.06	0.22	1.07
20	9.61	0.04	0.20	0.33	<0.01	10.17	0.53	0.03	0.03	0.14	0.72
21	10.00	0.04	0.16	0.17	0.02	10.36	0.84	0.03	0.03	0.09	0.98
22	10.64	0.04	0.21	0.19	0.01	11.09	0.88	0.04	0.07	0.15	1.13
24	12.00	0.07	0.18	0.24	nd	12.49	0.78	0.03	0.07	0.18	1.06
25	11.50	0.04	0.22	0.38	nd	12.14	0.60	0.03	0.06	0.19	0.88
26	12.15	0.04	0.21	0.16	nd	12.56	0.67	0.04	0.04	0.08	0.83
27	9.85	0.03	0.25	0.39	nd	10.53	0.63	0.05	0.08	0.25	1.01
28	10.14	0.04	0.22	0.24	nd	10.64	0.69	0.04	0.03	0.07	0.82
29	9.48	0.05	0.27	0.27	nd	10.07	0.37	0.03	0.08	0.31	0.79
30	10.58	0.03	0.20	0.19	0.01	11.00	0.84	0.03	0.03	0.10	1.00
31	10.72	0.04	0.21	0.17	0.01	11.13	0.64	0.03	0.04	0.15	0.86
32	9.89	0.04	0.14	0.18	nd	10.24	0.44	0.02	0.02	0.13	0.61
33	10.15	0.05	0.22	0.28	nd	10.69	0.61	0.03	0.03	0.08	0.75
34	11.35	0.05	0.31	0.45	nd	12.16	0.77	0.06	0.07	0.17	1.06
35	8.27	0.03	0.19	0.17	nd	8.67	0.68	0.05	0.06	0.13	0.92
36	10.57	0.03	0.23	0.18	nd	11.01	0.74	0.05	0.05	0.13	0.96
37	9.65	0.03	0.23	0.25	nd	10.16	0.89	0.05	0.06	0.11	1.11

Table 10 (continued).

	n6						n3				
Women	18:2	18:3	20:2	20:3	20:4	total n6	18:3	20:3	20:5	22:6	total n3
38	10.45	0.04	0.20	0.17	0.00	10.86	0.68	0.03	0.05	0.07	0.82
39	11.78	0.03	0.29	0.32	nd	12.43	0.82	0.06	0.07	0.25	1.18
40	10.26	0.05	0.21	0.30	nd	10.82	0.82	0.04	0.06	0.13	1.04
41	8.99	0.03	0.22	0.16	nd	9.40	0.67	0.04	0.02	0.13	0.86
42	11.03	0.04	0.13	0.24	nd	11.44	0.53	0.01	0.04	0.10	0.68
43	14.56	0.04	0.21	0.23	nd	15.03	0.63	0.03	0.03	0.10	0.79
44	13.11	0.03	0.22	0.36	nd	13.72	0.95	0.04	0.05	0.13	1.17
45	11.35	0.03	0.22	0.23	0.01	11.82	0.65	0.03	0.03	0.08	0.80
46	8.35	0.02	0.19	0.16	nd	8.73	0.70	0.04	0.02	0.08	0.84
47	7.60	0.06	0.16	0.54	nd	8.36	0.25	0.02	0.06	0.16	0.49
48	12.01	0.05	0.23	0.25	nd	12.53	0.75	0.03	0.03	0.05	0.86
49	11.35	0.03	0.21	0.22	nd	11.81	0.65	0.03	0.03	0.08	0.79
50	13.59	0.06	0.28	0.36	nd	14.29	0.72	0.04	0.09	0.33	1.18
median	10.72	0.04	0.22	0.24	0.01	11.20	0.67	0.04	0.04	0.13	0.87
min	7.60	0.02	0.13	0.14	<0.01	8.36	0.25	0.01	0.01	0.04	0.49
max	14.56	0.07	0.31	0.54	0.02	15.03	1.07	0.06	0.10	0.33	1.23

n3 PUFAs

Measured n3 PUFAs included ALA (18:3 n3), eicosatrienoic (20:3 n3), EPA (20:5 n3) and DHA (22:6 n3). ALA was the main n3 LCPUFA accounted median relative peak areas of 0.67% (range: 0.25 - 1.07%) in breast adipose tissue samples (Table 10). The body cannot produce this fatty acid (Chapter 1.1.1.3.2). The median proportions of EPA and DHA in breast adipose tissues were 0.04% (range: 0.01 - 0.10%) and 0.13% (range: 0.04 - 0.33%) respectively (Table 10). Another member of n3 fatty acids is eicosatrienoic (20:3 n3). The median relative peak areas of 20:3 n3 was 0.04% (range: 0.01 - 0.06%, Table 10).

Ratios of n6 to n3

The n6/n3 ratios in breast adipose of 50 women ranged from 9.2 to 22.1 (Figure 6). The n6/n3 ratios were obtained by dividing the total relative peak areas of n6 to total relative peak areas of n3. High consumption of plant oils rich in n6 PUFA and consumption of relatively low marine foods (as source of n3 PUFA) increases the n6/n3 ratio (Simopoulos, 1999). The average n6/n3 ratio of gluteal adipose tissue was 9.35 in healthy women (Mamalakis et al., 2009).

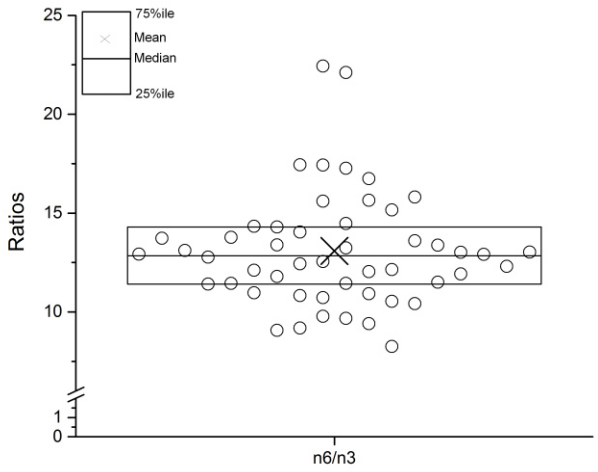


Figure 6 Ratios of n6 to n3 PUFAs in breast adipose tissues of 50 samples. The n6 PUFAs included LA, GLA, eicosadienoic acid, DGLA, AA and n3 PUFAs included ALA, eicosatrienoic, EPA and DHA.

4.1.1.5 TFA

In breast adipose tissues of 50 women the trans isomer of oleic acid was found. Elaidic acid (18:1 9trans) devoted 0.32% (median) of relative peak areas to itself (Figure 7). It was detectable in all samples. It ranged from 0.18 to 0.59%. Baylin et al. (2002) was reported the levels of elaidic acid in gluteal adipose tissue samples. The average proportion of elaidic acid was 0.63 ± 0.23%. Adipose tissue elaidic acid was positively correlated with eladic acid of dietary intake (Baylin et al., 2002). Margarine and baked product were mentioned as a major dietary source of TFA in their population (Baylin et al., 2002).

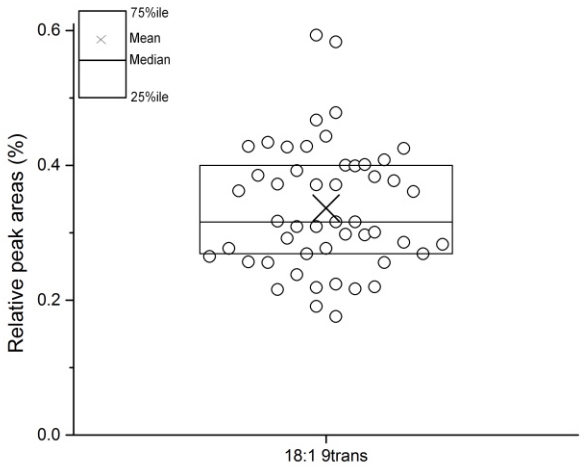


Figure 7 The relative peak area percentages of elaidic acid (18:1 9trans) in breast adipose tissue of 50 samples. The presented data are the mean relative peak areas (%) of fatty acids from three injections by means of GC-FID (Chapter 3.7.5).

4.1.1.6 Major fatty acids in breast adipose tissue

Among SFAs (Chapter 4.1.1.2), MUFAs (Chapter 4.1.1.3), PUFAs (Chapter 4.1.1.4) and TFA (Chapter 4.1.1.5), oleic acid with median of 45.3% (range: 41.1 - 50.2%) was the predominant fatty acid. The next most abundant fatty acids were palmitic acid 23.3% (median, range 18.9 - 28.1%), LA 10.7% (median, range: 7.6 - 14.6%), stearic acid 5.8% (median, range: 2.7 - 8.0%), palmitoleic acid 3.5% (median, range: 1.9-7.6%), myristic acid 3.3% (median, range: 2.1 - 4.3%) and vaccenic acid (18:1 11cis) 2.7% (median, range: 1.9-3.7%) respectively. These fatty acids accounted for about 95% average of relative peak area (range: 94.3 - 96.5%). Therefore they were assumed as major fatty acids and the rest of fatty acids were assumed as minor fatty acids in this work. The rest fatty acids devoted only about 5% (mean) of total peak area.

4.1.1.7 Comparison between breast adipose fatty acids of women

The relative proportions of breast adipose tissue fatty acids in women without BC were presented in Table 11. The fatty acid composition of breast adipose tissue of women without BC was investigated (Straka et al., 2015). The largest proportion of fatty acids devoted to oleic acid (mean: 43.2%), palmitic acid (mean: 20.9%), LA (mean: 18.6%), stearic acid (mean: 5.2%), palmitoleic acid (mean: 3.1%), myristic acid (mean: 2.6%) and vaccenic acid (mean: 2.4%). Yee et al. (2010) conducted a study in breast adipose tissue of women without BC. The major fatty acids were oleic acid (mean: 38.7%), palmitic acid (mean: 19.3%), LA (mean: 18.1%), stearic acid

(mean: 5.3%), palmitoleic acid (mean: 3.3%), myristic acid (mean: 3.1%) and vaccenic acid (mean: 2.3%).

Although in all tissues the major fatty acid was oleic acid, the proportion of it in this work was the highest in comparison to other studies. Likewise the proportion of palmitic and stearic acid were higher in this research than the others (Table 11). In all studies, palmitic acid devoted the highest proportion of SFAs. In studies which were conducted by Straka et al. (2015) and Yee et al. (2010), no proportion was reported for odd numbered fatty acids, while they were detected in this work. Likewise elaidic acid was detected in breast adipose tissue of women in this work, but it was not reported in others (Table 11). Furthermore, the average proportion of PUFAs (12.20%) in general and of LA (10.79%) and ALA (0.67%) were lower in this work than others. The proportion of DHA in this work was somehow similar to those observed in other studies (Table 11). The result of study by Straka et al. (2015) showed that dietary fish and n3 PUFA capsule supplements both increased EPA and DHA content of breast adipose tissue over 3 months. However, in this work there was no data from questionnaire. Moreover, a comparison of the proportion of GLA in this work and other studies showed lower levels of aforementioned fatty acid in this work versus others (Table 11). In addition to, AA was detected in 10 samples in this work and the average of this fatty acid was lower in comparison to other studies (Table 11).

The short carbon chain fatty acids like 4:0, 6:0 and 8:0 were not observed in breast adipose tissues.

Table 11 Comparison of the fatty acid distribution in breast adipose tissue of women. The data represented the mean relative proportion of fatty acid.

group	Fatty acid	Women without BC	Women without BC	Women without BC
SFA	10:0	0.05		
	12:0	0.56		
	13:0	0.03		
	14:0	3.24	2.58	3.14
	15:0	0.31		
	16:0	23.40	20.9	19.28
	17:0	0.30		
	18:0	5.71	5.19	5.27
	20:0	0.19		
	21:0	0.23		
	23:0	0.41		
	Total	34.43	28.67	27.69
MUFA	14:1	0.27		
	16:1 9cis	3.80	3.09	3.28
	18:1 9cis	45.46	43.22	36.68
	18:1 11cis	2.67	2.4	2.35
	20:1	0.83		
	Total	53.03	48.71	42.31
TFA	18:1 9trans	0.34		
PUFA	18:2 n6	10.79	18.58	18.12
	18:3 n6	0.04	0.11	0.29
	20:2 n6	0.22	0.26	0.38
	20:3 n6	0.26	0.29	2.53
	20:4 n6	0.01	0.45	0.67
	18:3 n3	0.67	0.93	1.07
	20:3 n3	0.04		
	20:5 n3	0.05	0.04	0.15
	22:4 n6		0.16	0.17
	22:5 n3		0.30	1.21
	22:6 n3	0.14	0.16	0.16
	Total	12.2	21.12	24.75
Number of subjects		50	12	12
References		Mahdiani dissertation	Straka et al. (2015)	Yee et al. (2010)

4.1.1.8 Comparison between breast adipose fatty acids and other adipose tissues

The fatty acid composition of subcutaneous adipose tissues from the gluteal and abdominal has been reported (Table 12). The fatty acid composition of adipose tissue observed in this work is consistent with the results of other studies showing that the major components of adipose tissue are oleic acid followed by palmitic acid and LA.

SFA values in breast tissue were higher than those found in other tissues. In addition to, odd numbered SFAs including 21:0 and 23:0 were detected in breast adipose tissue in this work and gluteal adipose tissue (London et al., 1991). However, no value was reported in other studies (Table 12). Moreover, the short carbon chain fatty acids like 4:0, 6:0 and 8:0 were not observed in gluteal, abdominal and breast adipose tissues.

18:1 11cis were present in gluteal (Mamalakis et al., 2009), abdominal (Boué et al., 2000) and breast adipose tissue, but no information was reported in gluteal tissues which were investigated by London et al. (1991) and Bagga et al. (1997). Furthermore, the proportion of 18:1 11cis in breast adipose tissue was higher than gluteal and abdominal tissues (Mamalakis et al., 2009; Boué et al., 2000).

The relative proportions of 14:1 5t, 16:1 9t, 18:2 tt, 18:2 ct, and 18:2 tc (TFAs) were determined in gluteal adipose tissues. However, no value was reported for elaidic acid (Table 15, Mamalakis et al., 2009). In this research elaidic acid was detected in 50 samples (Table 12).

The proportions of EPA and DHA in this work were somehow similar to those observed in gluteal (Mamalakis et al., 2009; London et al., 1991) and abdominal adipose tissue (Boué et al., 2000).

Table 12 Comparison of the fatty acid distribution in different types of adipose tissues. The data represented the mean relative proportion of fatty acid.

	Adipose tissue fatty acid (mean %)					
FA group	fatty acids	gluteal	breast	abdominal	gluteal	gluteal
SFA	10:0		0.05			
	12:0	0.12	0.56	0.50	0.10	0.18
	13:0		0.03			
	14:0	1.18	3.24	3.43	1.72	1.84
	15:0	0.17	0.31	0.37	0.3	
	16:0	15.63	23.40	22.15	19.06	18.13
	17:0	0.16	0.30	0.29	0.23	
	18:0	1.96	5.71	4.92	2.87	
	20:0	0.09	0.19	0.23	0.07	2.45
	21:0		0.23		0.01	
	23:0		0.41		0.05	
MUFA	14:1 9cis	0.14	0.27	0.36	0.29	0.28
	16:1 7cis	1.00		0.56		
	16:1 9cis	3.72	3.80	4.23	6.41	8.37
	18:1 9cis	59.25	45.46	39.69	41.52	49.36
	18:1 11cis	1.69	2.67	1.83		
	20:1 11cis		0.83	0.70	0.41	0.70
	22:1 n9			0.04	0.03	
Trans	14:1 5t	0.02				
	16:1 9t	0.03				
	18:1 9trans		0.34	0.27		
	18:2 tt	0.09			0.51	
	18:2 ct	0.12		0.13	0.54	
	18:2 tc	0.04		0.06	0.25	
PUFA	18:2 n6	9.83	10.79	14.35	17.23	17.24
	18:3 n6	0.04	0.04	0.05	0.11	
	20:2 n6	0.14	0.22	0.26	0.2	0.32
	20:3 n6	0.29	0.26	0.18	0.37	0.09
	20:4 n6	0.43	0.01	0.37	0.61	0.35
	22:2 n6				0.01	
	18:3 n3	0.57	0.67	0.43	0.77	0.61
	20:3 n3	0.06	0.04			
	20:5 n3	0.04	0.05	0.03	0.06	0
	22:3 n3	0.18				
	22:4 n6				0.20	
	22:5 n3	0.17		0.14		
	22:6 n3	0.17	0.14	0.14	0.19	0.06
Number of subjects		131	50	71	115	36
Ref		Mamalakis et al. (2009)	Mahdiani dissertation	Boué et al. (2000)	London et al. (1991)	Bagga et al. (1997)

4.1.2 Quantification of fatty acids in breast adipose tissue

Adipose tissue has been shown to reflect qualitative dietary exposure of essential fatty acids such as ALA, also n3 PUFAs such as DHA, odd-numbered SFAs (pentadecanoic acid) and TFAs (Chapter 1.1.1.3.1). However, no study has investigated the absolute level of these fatty acids in breast adipose tissue of woman without BC. These fatty acids are indicative for dairy products (pentadecanoic acid), processed fats (elaidic acid), vegetable oils such as flaxseed oil (ALA) and fish (DHA) consumption respectively (Chapter 1.1.1.3.1) and cannot be synthesised in human body. Therefore among 26 fatty acids which were identified in breast adipose tissues, the absolute level of pentadecanoic acid, elaidic, ALA and DHA were determined. These four fatty acids devoted to minor fatty acids group (Chapter 4.1.1.6). To quantify the absolute content of four fatty acid the external calibration using IS was performed (Chapter 4.1.2.1). The absolute level (mg/g) of pentadecanoic acid (Chapter 4.1.2.2), elaidic acid (Chapter 4.1.2.3), ALA (Chapter 4.1.2.4) and DHA (Chapter 4.1.2.5) were determined.

4.1.2.1 External calibration using IS

In this work, external calibration was applied to quantify the levels of pentadecanoic acid, elaidic, ALA and DHA in 50 adipose tissue samples. The external calibration method is suitable when many samples are to be measured with a similar matrix. To account for loss of analyte during sample preparation, the solutions of the external calibration containing IS (Chapter 4.1.2.1.1) were subjected to the same sample preparation procedure as the samples themselves.

4.1.2.1.1 IS

The IS is used to monitor and adjust instrument fluctuations. The IS is typically added to the sample prior at the first step, so that it undergoes the same extraction conditions as the sample (Wang et al., 2007; Zenkevich and Makarov, 2007).

IS should be a compound which is not present in the sample. Furthermore, a good gas chromatographic separation between IS and analyte is required (Laakso, 2005). Since undecanoic acid (11:0) was not present in breast adipose tissue (Chapter 4.1.1) and has similar structure to other fatty acids, it is an appropriate as an IS. Furthermore, undecanoic acid (11:0) with R_t of 15.4 min separated completely from pentadecanoic acid (R_t: 23.9 min), elaidic acid (R_t: 32.0 min), ALA (R_t: 38.0 min) and DHA (R_t: 56.7 min). For the quantitative analysis of pentadecanoic acid, elaidic acid, ALA and DHA, odd-chain fatty acids are a good choice as an IS. In this work undecanoic acid (11:0) was chosen as an IS.

4.1.2.1.2 Calibration curve

The calibration was carried out with known amounts of fatty acids (pentadecanoic acid, elaidic, ALA and DHA) mixed with a fixed quantity of IS (Chapter 3.5.2). In order to construct a calibration curve a minimum of 4 different calibration amounts of analyte were recommended which should span the range of amount of interest in analysis (European communities, 2002; Peters et al., 2007).

The four point calibration curve was constructed using pentadecanoic acid values ranging from 0.06 - 0.35 µg oc (Figure 8b), which covered the breast adipose tissue pentadecanoic levels between 0.07 to 0.30 µg oc (Appendix: Table 34). The calibration points were spaced equally over the range. Since the four point calibration curve depicted in Figure 8b did not cover the levels of pentadecanoic acid in 17 samples, another four point calibration curve was constructed (0.01 - 0.07 µg oc, Figure 8a). The pentadecanoic levels of 17 samples (range: 0.02 to 0.06 µg oc, Appendix: Table 34) were in the range of the calibration curves shown in Figure 8a.

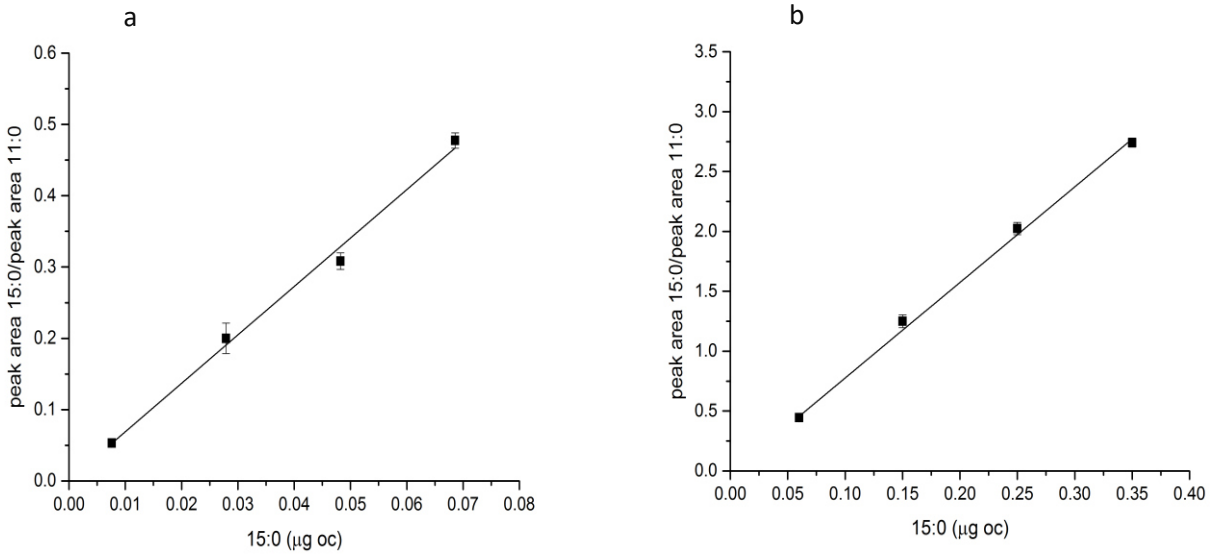


Figure 8 Calibration lines for quantification of pentadecanoic acid (averages and standards deviation of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of fatty acid to IS and the corresponding fatty acid amount on the column.)

The four point calibration curve was constructed using elaidic acid values ranging from 0.07 to 0.46 µg oc (Figure 9b), which was used to calculate levels of 39 samples (range: 0.07 to 0.43 µg oc Appendix: Table 35). Since this calibration curve did not cover the amount of 11 samples

(range: 0.02 - 0.04 µg oc, Appendix: Table 35), another calibration curve (Figure 9a) was generated for elaidic acid, which covered amounts of 0.01 to 0.05 µg oc.

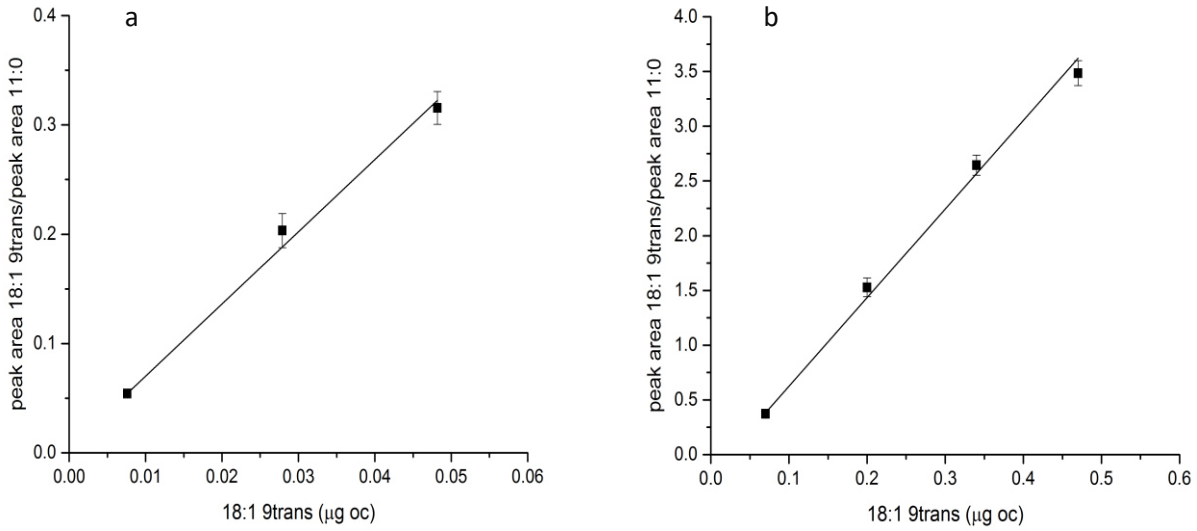


Figure 9 Calibration lines for quantification of elaidic acid (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of fatty acid to IS and the corresponding fatty acid amount on the column).

The four point calibration curve was constructed using ALA values ranging from 0.13 - 0.82 µg oc (Figure 10b), which was used to calculate levels of 40 samples (range: 0.13 - 0.71 µg oc Appendix: Table 36). Since this calibration curve did not cover the amount of 10 samples (range: 0.05 - 0.09 µg oc, Appendix: Table 36), another calibration curve (Figure 10a) was constructed for ALA, which covered amounts of 0.01 - 0.09 µg oc.

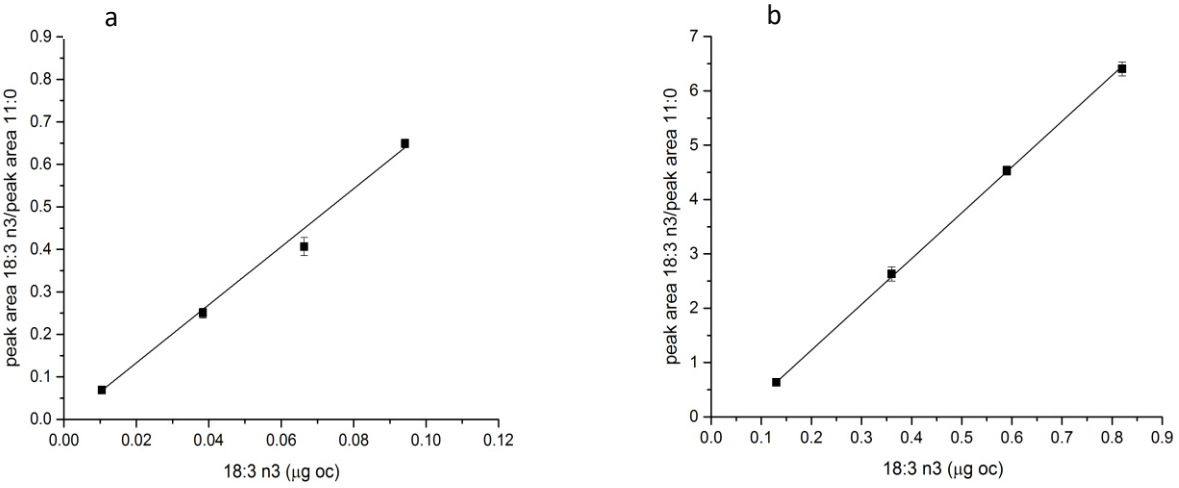


Figure 10 Calibration lines for quantification of ALA (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was

determined by linear regression of the peak area ratio of fatty acid to IS and the corresponding fatty acid amount on the column).

The calibration curve of DHA (22:6 n3) covered the DHA amount between 0.05 - 0.29 µg oc (Figure 11). The DHA level of 37 samples (range: 0.05 - 0.23 µg oc, Appendix: Table 37) were determined using Figure 11. The DHA levels of 2 samples (0.32 and 0.92 µg oc) were calculated using one calibration point. The last point of Figure 11 was used for this aim.

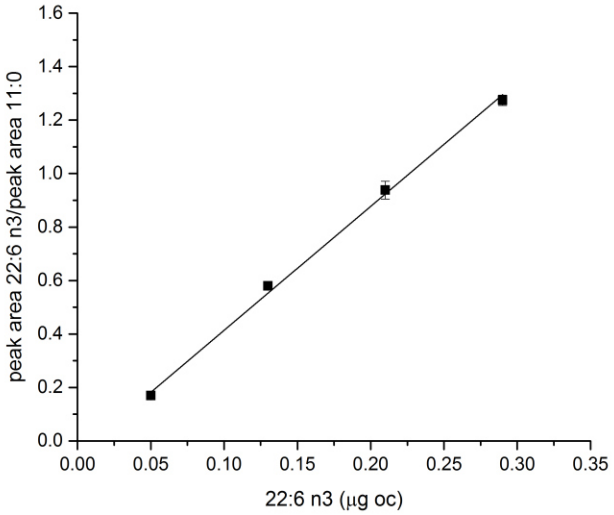


Figure 11 Calibration lines for quantification of DHA (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of fatty acid to IS and the corresponding fatty acid amount on the column).

Moreover, the DHA levels of 9 samples were calculated using one point calibration point (0.02 µg oc, DHA peak area/IS peak area: 0.09). For one point calibration, the ratio of calibration point should be 75% to 150% of the sample ratio. Among these 9 samples (Appendix: Table 37) the DHA peak area/IS ratios of one sample was 0.05. The ratio of calibration point was 159.3% of this sample ratio.

Another one point calibration (0.01 µg oc, DHA peak area/IS peak area: 0.02) was used to calculate the amount of DHA in 2 sample (0.01 µg oc, Appendix: Table 37).

Linearity and variance homogeneity (Chapter 3.7.14.1) of the all calibration curves (Figures 8a,b; 9a,b; 10a,b; 11) were confirmed (significance level 5%, Appendix: Table 57).

4.1.2.2 Absolute level of pentadecanoic acid (15:0)

The absolute content of pentadecanoic acid has not been determined in human breast adipose tissue of women yet. The absolute levels of pentadecanoic acid ranged from 0.08 to 1.31 mg/g (median: 0.37 mg/g) in breast adipose tissues (Figure 12, Appendix: Table 34). Pentadecanoic acid was quantifiable in 100% of samples. Previously Wang et al. (2011) reported the 0.34 mg/g absolute level (mean) of pentadecanoic acid in mice liver, which is somehow similar to the result of breast adipose tissue (mean: 0.44 mg/g) .

The relative peak areas (%) of pentadecanoic acid (in gluteal adipose tissues) have been shown to correlate with dairy fat intake (Chapter 1.1.1.3.1). There is no information about absolute level. The level of pentadecanoic acid in breast adipose tissue may reflect the consumption of dairy fat. However, in this work there was no dietary data from questionnaires to confirm this issue.

4.1.2.3 Absolute level of elaidic acid (18:1 9trans)

Absolute levels of elaidic acid were between 0.09 to 1.92 mg/g (median: 0.50 mg/g) in 50 samples (Figure 12, Appendix: Table 35). Clifton et al. (2004) determined the level of TFAs in abdominal subcutaneous fat. The level of elaidic acid was 0.63 ± 0.40 g/100 g fatty acids.

4.1.2.4 Absolute level of ALA (18:3 n3)

Up to now, no study has determined the absolute level of ALA in breast adipose tissue of women without BC. The absolute levels of ALA ranged from 0.10 to 3.05 mg/g (median: 0.88 mg/g) in breast adipose tissues of 50 women (Figure 12, Appendix: Table 36). Previously Bagga et al. (1997) reported the level of 4.06 mg/g (mean) in breast adipose tissue of women with BC.

Moreover, the absolute level of ALA was quantified by Wang et al. (2011) in mice liver. The average level of 2.76 mg/g was reported which is higher than the average ALA level in breast adipose tissues (1.04 mg/g).

4.1.2.5 Absolute level of DHA (22:6 n3)

The DHA (22:6 n3) levels were between 0.04 - 1.80 mg/g (median: 0.31 mg/g, Figure 12) and it was present in all samples (Appendix: Table 37). The mean absolute level of DHA (7.36 mg/g, Wang et al., 2011) in mice liver was higher than DHA level of breast adipose tissue in women (mean: 0.39 mg/g). DHA content of abdominal adipose tissue of female mice was 0.26 mg/g (mean, Ahn et al., 2010). The breast adipose tissue DHA level was slightly greater than abdominal adipose tissue of female mice. The mean level of DHA in breast adipose tissue of women with BC cancer was 0.48 mg/g (Bagga et al., 1997).

Fish is a unique rich source of n3 PUFA (EPA and DHA). Previously, Straka et al. (2015) found the significant increase of DHA level ($p < 0.01$) in breast adipose tissue of women who had dietary intervention with fish (4 servings of canned wild salmon ± pouched albacore tuna per week).

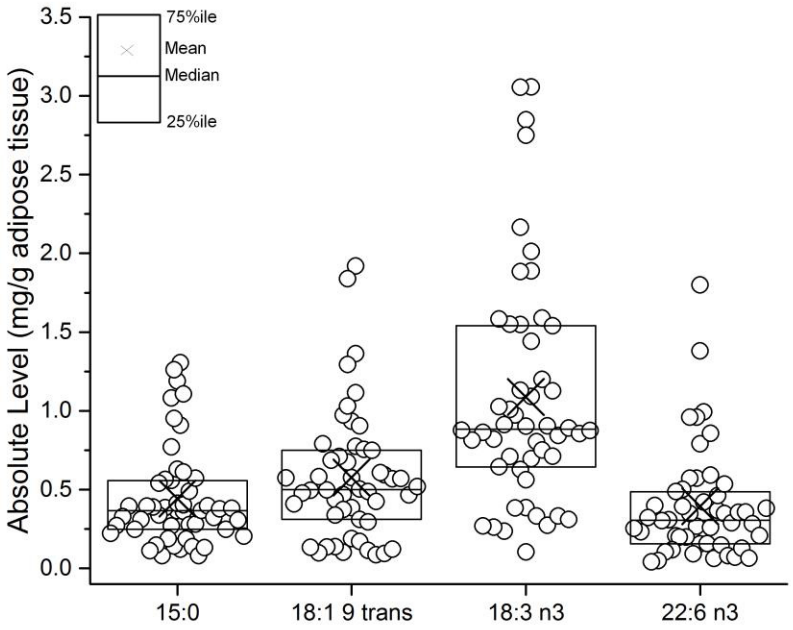


Figure 12 Absolute levels (mg/g) of four fatty acids in breast adipose tissues of 50 samples. The absolute level of fatty acid was determined by external calibrations (Chapter 4.1.2.1)

4.1.3 Discussion

The first purpose of this work was to determine the fatty acid composition of breast adipose tissues in women without BC. 26 fatty acids were found in samples. In this work more fatty acids were detected in comparison to previous studies (Straka et al., 2015; Yee et al., 2010). Furthermore, odd-numbered fatty acids (13:0, 15:0, 17:0, 21:0 and 23:0) as well as TFA (elaidic acid) were found in breast adipose tissues. However, they were not reported by previous studies. Among the 26 fatty acids, oleic acid, palmitic, LA, stearic acid, palmitoleic acid, myristic acid and vaccenic acid were the major fatty acids respectively which were consistent with expectation. In this work AA was detected in 10 samples which may indicate this fatty acid did not accumulate in all tissues. Moreover, for the first time the absolute levels of four fatty acids (pentadecanoic, elaidic acids, ALA and DHA) were quantified in breast adipose tissues of women without BC.

4.2 ChOL in breast adipose tissue

ChOL plays an important role in human health (Chapter 1.1.2.2). It is stored mainly in fatty tissue and constitutes more than 95% of sterols in adipose tissue. Although the levels of ChOL in animal tissues have been studied previously, there is little information about the ChOL level in human adipose tissue (Garcia-Cruset et al., 2001; Iuliano et al., 2003). Moreover, according to current information, no study has been considered the level of ChOL in breast adipose tissue. The ChOL can be oxidized and may be transformed through enzymatic and non-enzymatic oxidation into oxyChOLs. To compute the ratio of oxyChOLs to ChOLs, quantification of ChOL is necessary. In order to quantify the level of ChOL, a validated method is required. Thus in this study the validation of method has been specified to see if the quantification method of ChOL is accurate (Chapter 4.2.1) and the ChOL levels in breast adipose tissues were determined (Chapter 4.2.2).

4.2.1 Validation of the method

The validation of the method was performed according to the FDA guidelines (FDA, 2013). The results from method validation can be used to judge the quality, reliability and consistency of analytical results. In this work the method validation for quantification of ChOL was based on the following criteria: external calibration using IS (Chapter 4.2.1.1), accuracy (Chapter 4.2.1.2) and precision (Chapter 4.2.1.3).

4.2.1.1 External calibration using IS

There are various methods of quantitative analysis such as single point external calibration, multiple point external calibration, internal calibration and standard addition. The most common method is a multiple-point external calibration (Garcia-Cruset et al., 2001). It allows analyzing a series of samples using a single calibration curve. This is an important advantage when there are many samples to analyze. The fixed amount of IS (Chapter 4.2.1.1.1) was added to all samples and calibration solutions. The external calibration of ChOL was performed during the master thesis of Lisa Bäuml (2014).

4.2.1.1.1 IS

The IS is used to monitor and adjust instrument fluctuations. The IS is typically added to the sample prior at the first step, so that it undergoes the same extraction conditions as the sample (Wang et al., 2007; Zenkevich and Makarov, 2007).

IS should be a compound which is not present in the sample. Furthermore, a good gas chromatographic separation between IS and analyte is required (Laakso, 2005). Since 5α-ChAN-

3β-ol was not present in breast adipose tissue and has similar structure to ChOL, it is an appropriate IS. Furthermore, 5α-ChAN-3β-ol with a retention time of 30.4 min separated completely from ChOL (R_t: 30.2 min, Appendix: Figures 31-36). Thus, for the quantitative analysis of ChOL, 5α-ChAN-3β-ol was chosen as an IS.

4.2.1.1.2 Calibration curve

In order to construct a calibration curve a minimum of 4 different calibration levels of analyte were recommended which should span the range of amount of interest in analysis (European communities, 2002; Peters et al., 2007). Therefore, the four point calibration curve was constructed using ChOL values ranging from 30.0 - 90.0 ng oc (Figure 13), which covered the breast adipose tissue ChOL levels between 44.8 to 90.3 ng oc (Appendix: Table 39). The calibration points were spaced equally over the range. The ChOL calibration curve was constructed by Lisa Bäuml within her master thesis (2014).

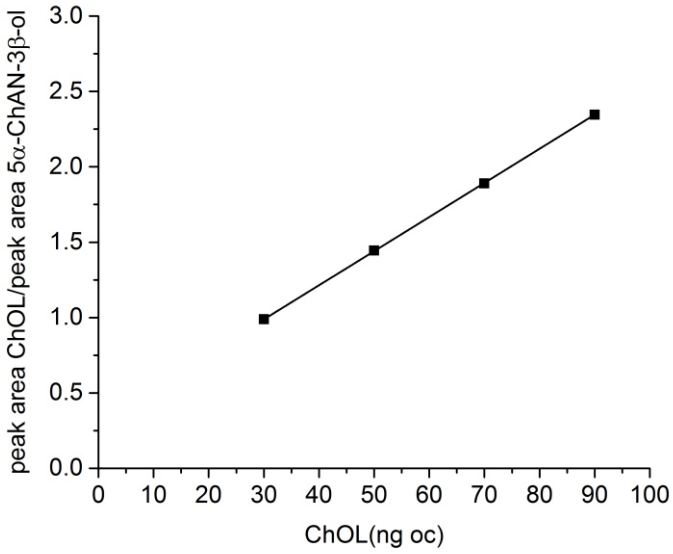


Figure 13 Calibration lines for quantification of ChOL (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of ChOL to IS and the corresponding ChOL amount on the column).

Linearity and variance homogeneity (Chapter 3.7.14.1) of the calibration line (Figure 13) were confirmed (significance level 5%, Appendix Table: 57).

4.2.1.2 Accuracy and Precision

In order to validate the analytical method, the accuracy and precision of the method were specified (FDA, 2013). Accuracy defines the degree of closeness of the determined value to the nominal or known true value under prescribed conditions and using precision, the closeness of agreement among a series of measurements (obtained from multiple sampling of the same homogenous sample under the prescribed conditions) can be determined. To determine the accuracy and precision of the method, adipose tissue samples which contained low (0.85 mg/g), medium (1.08 mg/g) and high (1.26 mg/g) levels of ChOL were chosen. The procedure was performed using both non-spiked and ChOL-spiked samples. For each level three spiked samples, one non-spiked and one blank (solvent contained IS mixture) were prepared. To reach the final amount of 1.18, 1.35, and 1.49 mg/g in spiked samples, the amount of 0.025 mg ChOL was added to 100 mg of the tissues with low, medium and high levels of ChOL. All samples were analysed with GC-FID (Chapter 3.7.12).

Accuracy

The accuracies of quantification of ChOL in three independent spiked samples with low, medium and high levels were 99.1 ± 10.1%, 87.0 ± 11.2%, and 103.4 ± 4.6% (Appendix: Table 38). According to the FDA guidelines, the mean value of accuracy should be within 15% of the nominal value. The accuracies of ChOL in low, medium and high ChOL contained samples were in the accepted range.

Precision

The mean, standard deviation and RSD were calculated with regards to ChOL content of three independent spiked samples. RSD was used to assess the precision. According to the FDA guidelines (FDA, 2013) the precision should not exceed 15% of the coefficient of variation.

The relative deviations of 2.15, 2.08, and 0.78% complied with the acceptance limit of 15% for precision (%RSD).

4.2.2 ChOL content of human breast adipose tissues

The amount of ChOL can be determined based on the linear equation of the calibration (Figure 13, Chapter 4.2.1.1.2). The ChOL was present in all adipose tissues (Appendix: Table 39). The ChOL levels were between 0.73 and 1.50 mg/g (median: 1.08 mg/g, Figure 14).

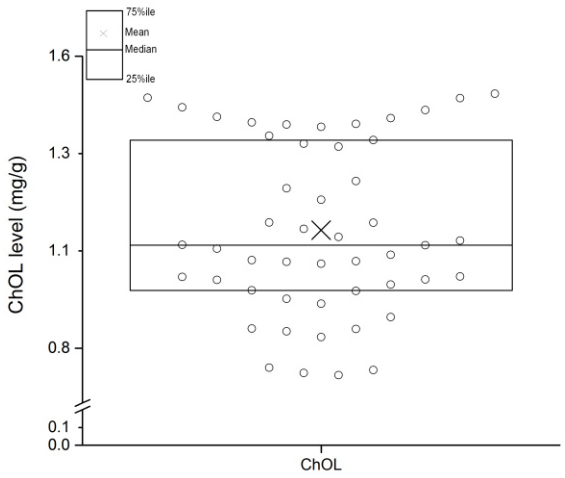


Figure 14 Absolute levels of ChOL (mg/g adipose tissue) in 49 breast adipose tissue. The absolute level of ChOL was determined by multiple external calibrations (Chapter 4.2.1).

4.2.2.1 Comparison of ChOLs in other tissues and human breast adipose tissues

According to the current knowledge, no study has investigated the levels of ChOLs in breast adipose tissue up to now. There are some researches which considered the ChOL in human artery, liver and animals tissues (Table 13).

Garcia-Cruset et al. (2001) determined the levels of ChOL in normal human artery (Table 13). The average level of ChOL was 3.71 ± 1.54 mg/g in normal human artery (Garcia-Cruset et al., 2001), which is higher than average level of ChOL in human breast adipose tissues. Moreover, levels of ChOL were 0.85 mg/g and 2.45 mg/g in normal human artery and liver tissue respectively (Iuliano et al., 2003). Furthermore, levels of ChOL were 12.40 mg/g and 2.10 mg/g in brain and liver tissues of rat (Xu et al., 2011). The ChOL content of pancreas and heart were 3.90 mg/g and 1.50 mg/g in rats (Wang et al., 2012).

In the study by Vaya et al. (2001), the content of ChOL was verified in human non-lesion aortic samples (Table 13). According to their data, the average level of ChOL was 4.50 mg/g in human non-lesion aortic samples. Ariyoshi et al. (2002) determined the level of ChOL in Male Wistar liver of rats (mean: 1.66 mg/g).

Table 13 Comparison of ChOLs absolute levels in different biological tissues.

Sample	unit	ChOL mean	reference
Breast adipose tissues	mg/g	1.12	Mahdiani dissertation
Human artery	mg/g	3.71	Garcia-Cruset et al. (2001)
Human artery	mg/g	0.85	Iuliano et al. (2003)
Human liver	mg/g	2.45	Iuliano et al. (2003)
Human non-lesion aortic tissue	mg/g	4.50	Vaya et al. (2001)
Liver (rat)	mg/g	1.66	Ariyoshi et al. (2002)
Myocardium (hamsters)	mg/g	0.80	Meynier et al. (2002)
Pancreas (rat)	mg/g	3.90	Wang et al. (2012)
Heart (rat)	mg/g	1.50	Wang et al. (2012)
Brain (rat)	mg/g	12.40	Xu et al. (2011)
Liver (rat)	mg/g	2.10	Xu et al. (2011)

4.3 OxyChOL in breast adipose tissues

ChOL has an unsaturated structure; therefore it is susceptible to oxidation processes (Chapter 1.1.3). The common forms of oxidized ChOL are 7α/β-HO-ChOL, 7-O-ChOL, α/β-epoxy-ChOLs (Chapter 1.1.3). Aforementioned OxyChOLs are able to enhance the production of free radicals including ROS (Lizard et al., 1998; Lemaire-Ewing et al., 2005; Gramajo et al., 2010) which are known to damage DNA (Cooke et al., 2003; Dizdaroglu and Jaruga, 2012; Jusakul et al., 2011). Although, levels of oxyChOLs in animal tissue have been studied previously, there is no information about their level in human adipose tissue. OxyChOLs are present in mammalian tissues at very low levels (130 - 200 ng/g in mice liver, summarized in Olkkonen et al., 2012). Thus for the detection of oxyChOLs in human adipose tissue, a sensitive analysis method is necessary. In this work oxyChOLs in breast adipose tissues were analyzed using GC-MS/MS (Chapter 3.7.13). To ensure that the developed method meets the requirement of analytical method, the validation of method was checked (Chapter 4.3.1). Moreover, the absolute levels of oxyChOLs (7α/β-HO-ChOLs, 7-O-ChOL, α/β-epoxy-ChOLs, Chapter 4.3.2) as well as relative (Chapter 4.3.3) levels of oxyChOLs and the ranking of oxyChOLs (Chapter 4.3.4) in breast adipose tissues were determined.

4.3.1 Validation of the method

Validating methods includes performing all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix is reliable and reproducible for the intended use. The validation of the method was performed according to the FDA guidelines (FDA, 2013). The quantification method of oxyChOLs (7α/β-HO-

ChOLs, 7-O-ChOL, 7α/β-epoxy-ChOLs) was validated in terms of external calibration using IS (Chapter 4.3.1.1), accuracy and precision (Chapter 4.3.1.2), LOD and LOQ (Chapter 4.3.1.3).

4.3.1.1 External calibration using IS

The quantification of oxyChOL was performed using external calibration method. To take into account the loss of analyte during sample preparation, the calibration solutions subjected to the same sample preparation. The fixed amount of IS (Chapter 4.3.1.1.1) was added to all samples and calibration solutions.

4.3.1.1.1 IS

The IS is used to monitor and adjust instrument fluctuations. Therefore use of IS is necessary. An appropriate IS mixture is required for quantifying oxyChOLs. The IS should have the similar chemical structure and the similar chromatographic behaviour during detection (Wang et al., 2007). Deuterated substances have nearly identical physical and chemical characteristics such as its non-deuterated analogs (Stokvis et al., 2005; Wang et al., 2007). A thermally oxidized D-ChOL standard mixture was synthesised and used as an IS (Chapter 3.5.5). For each oxyChOL the corresponding deuterated substance was produced.

After analysis with GC-MS/MS (Chapter 3.7.13) the following R_t was determined for non deuterated oxyChOLs: 27.22, 27.43, 29.51, 24.70, 26.64 min for β-epoxy-ChOL, α-epoxy-ChOL, 7-O-ChOL, 7α-HO-ChOL and 7β-HO-ChOL respectively. The R_t for oxy-D-ChOLs were 27.14 min (β-epoxy-D-ChOL), 27.34 min (α-epoxy-D-ChOL), 29.42 min (7-O-D-ChOL), 24.60 min (7α-HO-D-ChOL) and 26.54 min (7β-HO-D-ChOL). All oxyChOLs and oxy-D-ChOLs were separated in the samples (Appendix: Figures 37-57).

4.3.1.1.2 Calibration curve

In order to construct a calibration curve a minimum of 5 or 6 different calibration levels of analyte were recommended which should span the range of level of interest in analysis (ICH, 2005; FDA, 2013). Therefore, the six point calibration curve was constructed using α-epoxy-ChOL values ranging from 50.0 - 625.0 pg oc (Figure 15), which covered the breast adipose tissue α-epoxy-ChOL levels between 41.4 to 538.1 pg oc (Appendix: Table 41). The calibration points equally spaced over the range. The data related to this calibration curve was taken from master thesis of Lisa Bäuml (2014).

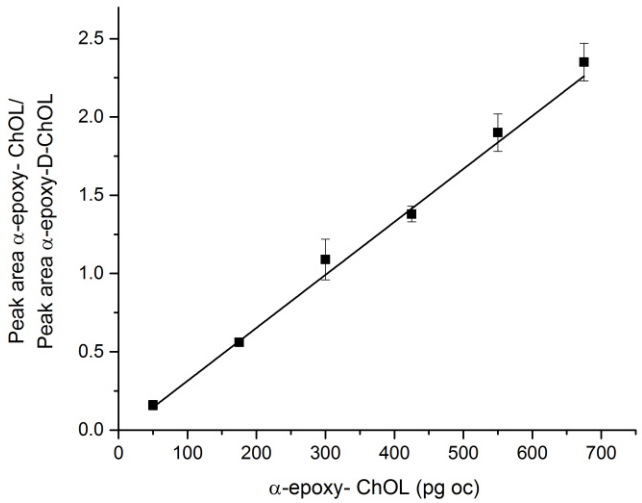


Figure 15 Calibration lines for quantification of α-epoxy-ChOL (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of oxyChOL to IS and the corresponding oxyChOL amount on the column).

According to the ICH and FDA suggestion, five point calibration curve was constructed using β-epoxy-ChOL values ranging from 500 - 1300 pg oc (Figure 16). This calibration curve was used to calculate the β-epoxy-ChOL levels of 30 samples (Appendix: Table 40). Levels of 18 samples ranging from 107.9 to 446.6 pg oc were determined using one calibration point depicted in Figure 16 (300 pg oc).

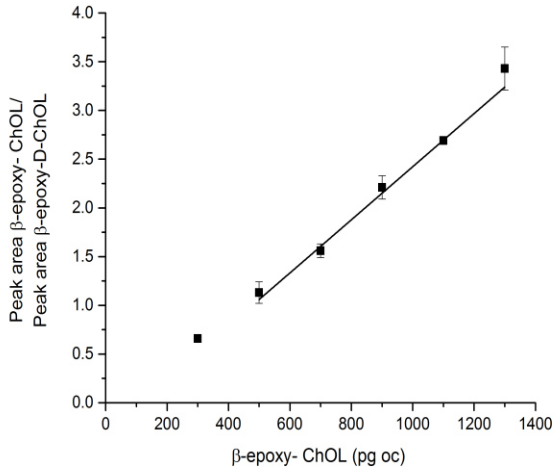


Figure 16 Calibration lines for quantification of β-epoxy-ChOL (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of oxyChOL to IS and the corresponding oxyChOL amount on the column).

The data related to this calibration curve (Figure 16) was taken from master thesis of Lisa Bäuml (2014).

The level of one sample (2684.0 pg oc) was not in the range of the calibration curve shown in Figure 16, therefore the level of this sample was quantified using one point calibration (calibration point: 3136 pg oc).

The six point calibration curve was constructed using 7-O-ChOL values ranging from 30.0 - 180.0 pg oc (Figure 17b), which was used to calculate levels of 11 samples (range: 28.0 - 110.3 pg oc, Appendix: Table 42). Since this calibration curve did not cover the amount of all samples, another six point calibration curve (Figure 17a) was generated for 7-O-ChOL, which covered amounts of 1.6 to 16.3 pg oc. The calibration curve depicted in Figure 17a was used to calculate 7-O-ChOL amounts of 38 samples (range: 4.0 - 17.5 pg oc, Appendix: Table 42). It should be noted the data related to calibration curve depicted in Figure 17b was taken from master thesis of Lisa Bäuml (2014).

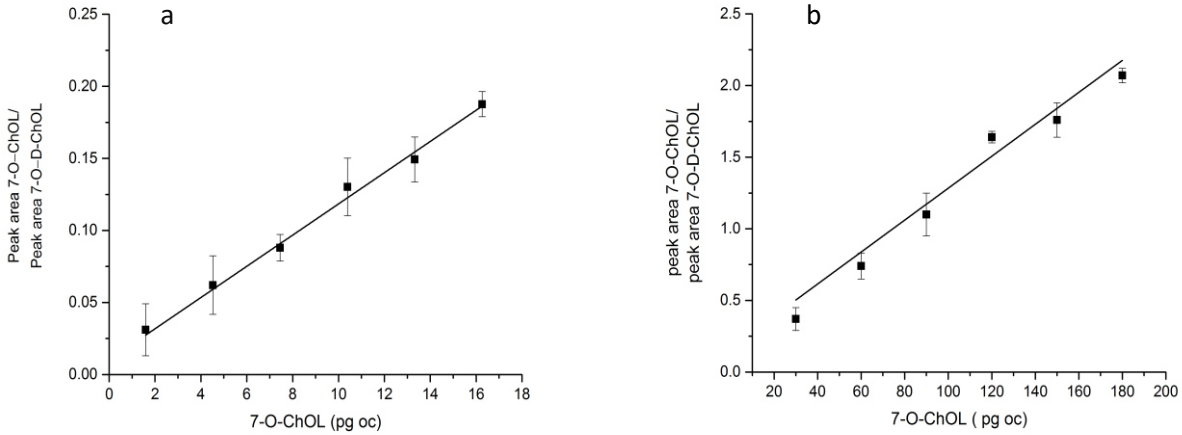


Figure 17 Calibration lines for quantification of 7-O-ChOL (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of oxyChOL to IS and the corresponding oxyChOL amount on the column).

The six point calibration curve was constructed using 7α-HO-ChOL values ranging from 2.0 - 12.0 pg oc (Figure 18b), which was used to calculate levels of 8 samples (Appendix: Table 43). The data related to this calibration curve (Figure 18b) was taken from master thesis of Lisa Bäuml (2014). Since this calibration curve did not cover the amount of 41 samples, another calibration curve (Figure 18a) was generated for 7α-HO-ChOL, which covered amounts of 0.57 to 1.67 pg oc. There are different recommendations about the number of calibration point. Peters et al. (2007) recommended four or five levels. Moreover, according to the commission of

the European communities, (2002) construction of four point calibration curve is acceptable for quantification. Thus in calibration curve depicted in 18a four points calibration curve was used to calculate the 7α-HO-ChOL content of 41 samples (Appendix: Table 43).

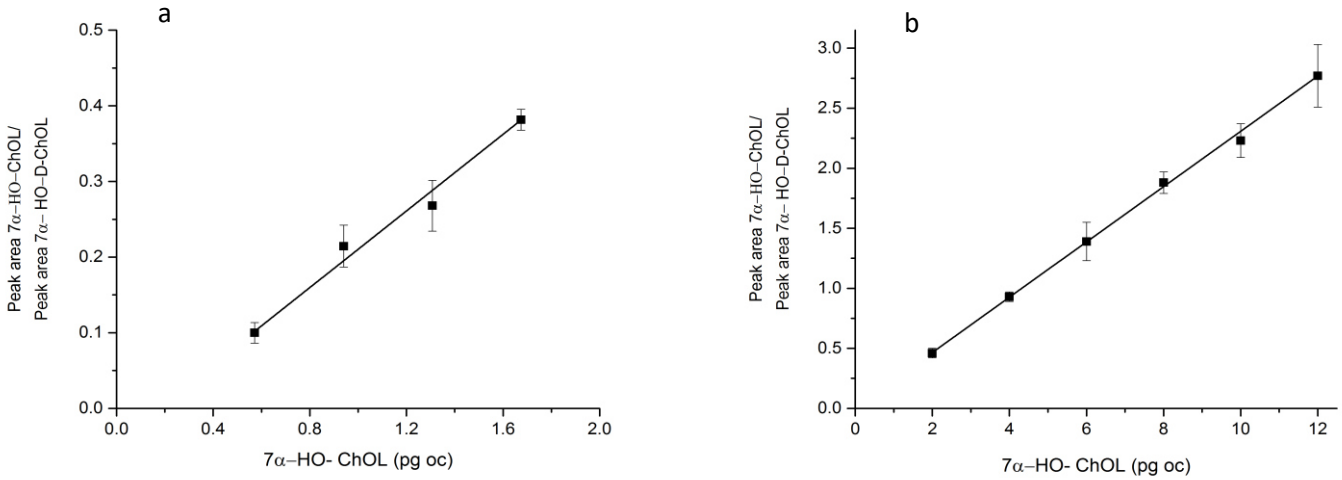


Figure 18 Calibration lines for quantification of 7α-HO-ChOL (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of oxyChOL to IS and the corresponding oxyChOL amount on the column).

Two calibration curves were created for 7β-HO-ChOL, which ranged from 0.18 to 0.67 pg oc (Figure 19a) and from 1 to 13.5 pg oc (Figure 19b). The amounts of 7β-HO-ChOL in 11 samples were in the range of the calibration curves depicted in Figure 19b (range: 0.99 - 5.70 pg oc; Appendix: Table 44). The data related to this calibration curve (Figure 19b) was taken from master thesis of Lisa Bäuml (2014). Since the six point calibration curve depicted in Figure 19b did not cover the low levels of 7β-HO-ChOL, another calibration curve was constructed (Figure 19a). The 7β-HO-ChOL levels of 38 samples (Appendix: Table 44) were in the range of the calibration curves shown in Figure 19a.

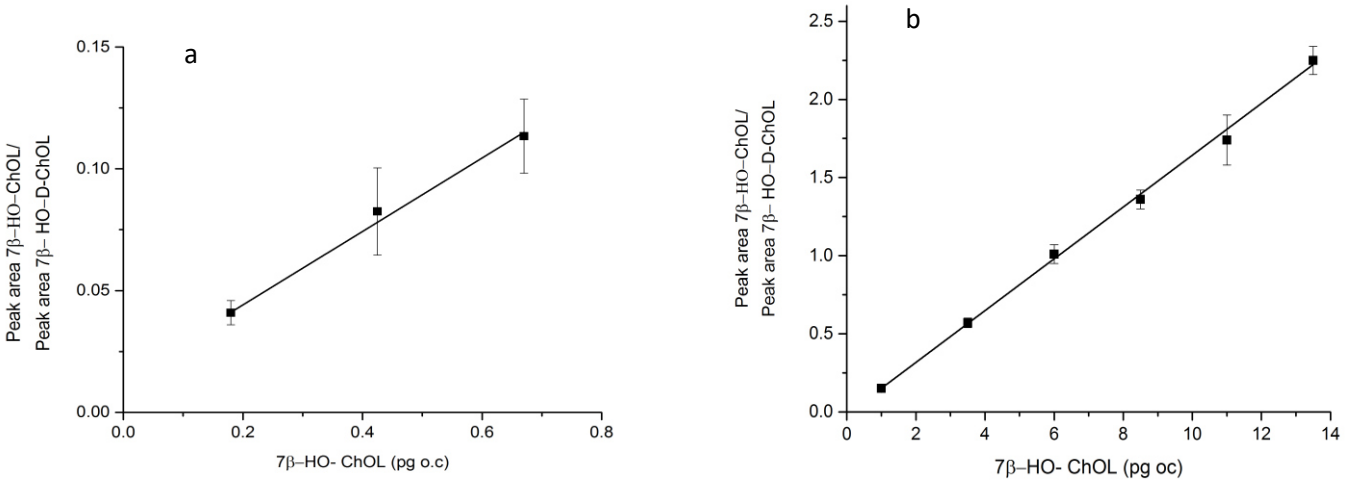


Figure 19 Calibration lines for quantification of 7β-HO-ChOL (averages and standard deviations of three independent experiments, starting from weighing of compounds. The calibration line was determined by linear regression of the peak area ratio of oxyChOL to IS and the corresponding oxyChOL amount on the column).

Linearity and variance homogeneity (Chapter 3.7.14.1) of the calibration lines (Figures 16; 17; 18a,b; 19a,b) were confirmed (significance level 5%, Appendix: Table 57).

4.3.1.2 Accuracy and precision

In order to validate the analytical method, the accuracy and precision of the method were specified according to the FDA guidelines (FDA, 2013). To determine the accuracy and precision of the method, adipose tissue samples were spiked (Table 14) with β-epoxy-ChOL, α-epoxy-ChOL, 7-O-ChOL, 7α-HO-ChOL, 7β-HO-ChOL to achieve the range of low, medium and high level of aforementioned oxyChOLs in breast adipose tissues. For each level (low, medium and high) 3 samples were spiked. Furthermore, one non-spiked sample and one blank (solvent contained IS mixture) were prepared. All samples were analysed with GC-MS/MS (Chapter 3.7.13).

Table 14 The initial and final levels of oxyChOLs in breast adipose tissue samples.

oxyChOL	initial level (ng/g)	spiked amount (ng)	Final level (ng/g) average of 3 analysis
β-epoxy-ChOL	32.2	85.0	117.9
	80.5	85.0	171.9
	152.6	85.0	228.9
α-epoxy-ChOL	12.7	25.0	37.9
	19.7	30.0	49.2
	46.8	25.0	71.3
7-O-ChOL	9.6	6.0	16.0
	17.4	4.5	21.4
	28.1	3.8	31.7
7α-HO-ChOL	4.0	4.0	7.3
	6.7	5.5	12.5
	12.2	4.0	16.1
7β-HO-ChOL	1.1	2.2	3.2
	1.7	5.0	7.5
	6.5	4.0	10.8

Accuracy

According to the FDA guidelines, the mean value of accuracy should be within 15% of the nominal value. The accuracies of quantification of 7α-HO-ChOL in three independent spiked samples with low, medium and high levels were 81.6 ± 12.4%, 106.8 ± 7.3%, and 97.5 ± 12.8% (Table 15). The accuracy should not deviate by more than 20% of the nominal value at LLOQ (FDA, 2013). The accuracy of 7α-HO-ChOL in low concentrated sample was in the accepted range for LOQ (FDA, 2013).

Accuracies of 7β-HO-ChOL were 95.3 ± 10.7%, 115.7 ± 12.6%, and 107.6 ± 5.9%, in low, medium and high 7β-HO-ChOL contained samples (Table 15). As well, the accuracies for the low, medium and high 7-O-ChOL content samples involved 104.9 ± 16.0%, 90.3 ± 13.4%, and 92.8 ± 12.6% (Table 15).

The accuracies of β-epoxy-ChOL ranged 89.7 ± 12.7%, to 107.6 ± 6.3% (table 15). Accuracies of α-epoxy-ChOL were 100.9 ± 14.3%, 98.6 ± 6.6%, and 98.0 ± 14.6% in low, medium and high samples respectively. Accuracies of 7β-HO-ChOL, 7-O-ChOL, β-epoxy-ChOL and α-epoxy-ChOL complied with the FDA acceptance limit.

Table 15 Accuracy data, averages and standard deviations of three independent experiments.

β-epoxy-ChOL			α-epoxy-ChOL		
sample	accuracy (%)	average accuracy (%)	sample	accuracy (%)	average accuracy (%)
Low-1	101.67		Low-1	114.26	
Low-2	108.87	100.89 ± 8.39	Low-2	102.81	100.91 ± 14.39
Low-3	92.13		Low-3	85.67	
Medium-1	102.54		Medium-1	91.72	
Medium-2	105.51	107.56 ± 6.30	Medium-2	99.18	98.60 ± 6.61
Medium-3	114.64		Medium-3	104.91	
High-1	101.54		High-1	107.66	
High-2	91.20	89.68 ± 12.69	High-2	105.19	98.02 ± 14.62
High-3	76.29		High-3	81.19	
7β-HO-ChOL			7α-HO-ChOL		
sample	accuracy (%)	average accuracy (%)	sample	accuracy (%)	average accuracy (%)
Low -1	107.68		Low-1	77.38	
Low-2	89.97	95.33 ± 10.72	Low-2	71.87	81.63 ± 12.43
Low-3	88.34		Low-3	95.63	
Medium-1	101.24		Medium-1	98.80	
Medium-2	124.36	115.70 ± 12.60	Medium-2	113.03	106.81 ± 7.28
Medium-3	121.51		Medium-3	108.62	
High-1	100.81		High-1	84.20	
High-2	111.99	107.63 ± 5.98	High-2	109.83	97.47 ± 12.84
High-3	110.10		High-3	98.40	

7-O-ChOL		
sample	accuracy (%)	average accuracy (%)
Low-1	105.95	
Low-2	120.47	104.95 ± 16.04
Low-3	88.42	
Medium-1	98.36	
Medium-2	74.92	90.34 ± 13.36
Medium-3	97.76	
High-1	99.58	
High-2	100.55	92.79 ± 12.60
High-3	78.25	

Precision

The mean, standard deviation and RSD were calculated using oxyChOLs content of three independent spiked samples (low, medium and high oxyChOL contain samples). The RSD was used to assess the precision (Chapter 3.7.14.2). According to the FDA guidelines (FDA, 2013) the precision should not exceed 15% of the coefficient of variation.

The precisions of 7α-HO-ChOL were 6.8, 3.2 and 3.2% (Table 16) for low, medium and high contained samples, respectively. As well, the precisions of 7.4, 8.4 and 2.2% were obtained for 7β-HO-ChOL in low, medium and high contained samples, respectively (Table 16). The precisions of 7-O-ChOL were 6.1, 2.8 and 1.5% in low, medium and high concentrated samples, respectively (Table 16).

The precisions of β-epoxy-ChOL were 6.1, 3.1 and 4.7% low, medium and high concentrated samples, respectively (Table 16). Furthermore, the precisions of 9.5, 4.0 and 5.1% were acquired for α-epoxy-ChOL (Table 16). The precisions were in accordance with FDA guidelines (FDA, 2013).

Table 16 The Precision of oxyChOLs quantification in breast adipose tissues of women.

	precision (RSD%)		
oxyChOLs	low	medium	high
β-epoxy-ChOL	6.05	3.12	4.72
α-epoxy-ChOL	9.48	4.03	5.13
7-O-ChOL	6.11	2.81	1.51
7α-HO-ChOL	6.79	3.19	3.20
7β-HO-ChOL	7.43	8.44	2.21

4.3.1.3 LOD and LOQ

LOD can be defined as the smallest amount of analyte that can be reliably detected by the instrumental method (Chapter 3.7.14.3). LODs were 3.89 ng/g for β-epoxy-ChOL, 1.99 ng/g for α-epoxy-ChOL, 0.01 ng/g for 7-O-ChOL, 0.41 ng/g for 7α-HO-ChOL and 0.13 ng/g for 7β-HO-ChOL. The absolute levels of all oxyChOLs were above the determined LODs (Table 17).

LOQ is the lowest amount of oxyChOLs which can be exactly quantitated (Chapter 3.7.14.4). The lowest points of oxyChOLs in calibration lines were assumed as LOQs (Table 17). It should be noted the 7-O-ChOLs levels (in all samples) were above LOQ. The level of α-epoxy-ChOL in one sample was below determined LOQ. The levels of 7α-HO-ChOL were below LOQ in 8% of samples (4 samples). The 7β-HO-ChOL levels of 5 samples were below LOQ. The levels of 9 samples were below LOQ in the case of β-epoxy-ChOL.

Table 17 Limit of detection and quantification of oxyChOLs.

	7β-HO-ChOL	7α-HO-ChOL	7-O-ChOL	α-epoxy-ChOL	β-epoxy-ChOL
LOD (ng/g)	0.13	0.41	0.01	1.99	3.89
LOQ (ng/g)	1.80	5.01	3.60	12.50	66.09

4.3.2 Absolute Level of oxyChOLs in breast adipose tissues

The absolute level of oxyChOLs was quantified by external calibration (Chapter 4.3.1). All oxyChOLs were present in all adipose tissues (Appendix: Tables 40-44). The most abundant oxyChOL was β-epoxy-ChOL (median: 147.2 ng/g tissue, range: 25.7 - 624.2 ng/g), followed by α-epoxy-ChOL (median: 34.6 ng/g tissue, range: 9.9 - 124.7 ng/g), 7-O-ChOL (median: 19.1 ng/g

tissue, range: 7.9 - 220.6 ng/g), 7α-HO-ChOL (median: 10.2 ng/g tissue, range: 3.8 - 111.3 ng/g), 7β-HO-ChOL (3.5 ng/g, range: 1.0 - 45.6 ng/g) respectively (Figure 20). The total oxyChOLs ranged from 52.1 to 760.2 ng/g.

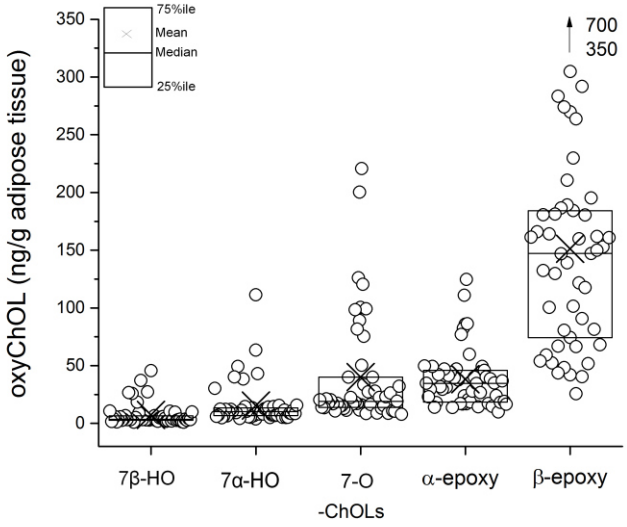


Figure 20 The absolute levels of oxyChOLs (ng/g adipose tissue) in 49 breast adipose tissue. The absolute level of oxyChOLs was determined by multiple external calibrations (Chapter 4.3.1).

According to the current knowledge, no study has investigated the levels of oxyChOLs in human adipose tissue yet. Therefore comparing the result of oxyChOL in breast adipose tissue with other human adipose tissues is not possible. There are some researches which considered the oxyChOLs in human artery, lesion and animals tissues (Chapter 4.3.2.1).

4.3.2.1 Comparison of oxyChOLs in other tissues and human breast adipose tissues

In this chapter the absolute levels of oxyChOLs in breast adipose tissues were compared with other tissues (Table 18). It should be noted, in previous studies, the level of oxyChOLs were reported as mean values. Therefore comparison between the result of this research and others limited to the mean absolute level of oxyChOLs. Moreover, the experimental conditions in previous studies were not exactly similar to the present work.

Garcia-Cruset et al. (2001) determined the levels of oxyChOLs (7α/β-HO-ChOLs, 7-O-ChOL and α/β-epoxy-ChOLs) in normal human artery (Table 18). The highest level belonged to α-epoxy-ChOL in artery tissues (Table 18). The mean human artery oxyChOLs were higher than breast adipose tissues (Table 18). Furthermore, the most abundant oxyChOL of human coronary lesions was β-epoxy-ChOL which is similar in this work. α-epoxy-ChOL, 7-O-ChOL and 7β-HO-ChOL were also present in human coronary lesions (Table 18, Vaya et al., 2001). The 7α-HO-

ChOL was reported as not detected in human coronary lesions. However, it was present in breast adipose tissues (Table 18).

The autoxidation profiles of ChOL in aorta and liver samples of apolipoprotein E-deficient mice were determined (Ando et al., 2002). The major oxyChOL was β-epoxy-ChOL followed by α-epoxy-ChOL > 7-O-ChOL > 7α-HO-ChOL > 7β-HO-ChOL in aorta samples (Table 18). The oxyChOLs composition in aorta samples appeared to be similar to the breast adipose tissue but not identical. 7-O-ChOL was the most abundant species in the liver tissues of mice, whereas in breast adipose tissue β-epoxy-ChOL was the main oxyChOL. Furthermore, in comparison with breast adipose tissues, aorta and liver samples of apolipoprotein E-deficient mice contained higher levels of oxyChOLs (Table 18).

In the study by Wooten et al. (2014), the content of oxyChOLs was verified in mice liver and adipose tissue (Table 18). According to their data, the highest level belonged to β-epoxy-ChOL followed by α-epoxy-ChOL > 7-O-ChOL > 7α-HO-ChOL > 7β-HO-ChOL in liver tissues, which is in accordance with the oxyChOLs ranking in breast adipose tissue (Chapter 4.3.2). The highest level of oxyChOLs belonged to 7-O-ChOL in adipose tissue of mice (Table 18). The result of this research indicated that the levels of oxyChOLs were lower in human adipose tissue than mice adipose tissue (Table 18).

Meynier et al. (2002) determined the level of oxyChOLs (7β-HO-ChOL, 7α-HO-ChOL, α-epoxy-ChOL, β-epoxy-ChOL and 7-O-ChOL) in myocardium of hamsters. The most abundant oxyChOL was β-epoxy-ChOL in samples, which is the same in breast adipose tissues. 7β-HO-ChOL was the second main oxyChOL in myocardium. However, it was the least oxyChOLs in human breast adipose tissues (Table 18). The least absolute value belonged to 7α-HO-ChOL in myocardium of hamsters.

In another research, 7β-HO-ChOL, 7α-HO-ChOL and α-epoxy-ChOL were found in aortic tissue of New Zealand White rabbits, whereas β-epoxy-ChOL and 7-O-ChOL were not present in aortic tissue (Hodis et al., 1992). In breast adipose tissue all aforementioned oxyChOLs were found. The result of breast adipose tissues demonstrated that the distribution of oxyChOLs differs in human adipose tissue and aortic tissue of rabbit.

In addition Helmschrodt et al. (2013) investigated the level of oxyChOLs in healthy human plasma and carotid plaque. The most abundant oxyChOL was 7-O-ChOL (63%) followed by 7α/β-HO-ChOL (13%) > β-epoxy-ChOL (12%) > α-epoxy-ChOL (11%). In breast adipose tissues the most abundant was β-epoxy-ChOL, whereas in artery 7-O-ChOL was the main oxyChOL. According to their result the distribution of oxyChOLs in carotid plaque were different from breast adipose tissues.

Table 18 Comparison of oxyChOLs absolute levels in different biological tissues (np*: not present in the sample).

Sample	unit	β-epoxy-ChOL mean	α-epoxy-ChOL mean	7-O-ChOL mean	7α-HO-ChOL mean	7β-HO-ChOL mean	reference
Breast adipose tissues	ng/g	151	38	31	15	7	Mahdiani dissertation
Myocardium (hamster)	ng/g	78000	46000	24000	21000	50000	Meynier et al. (2002)
Aorta (mice)	ng/g	3570	1730	1690	1090	930	Ando et al. (2002)
Liver (mice)	ng/g	150	160	200	190	130	Ando et al. (2002)
Liver (mice)	ng/g protein	12300	7800	7200	5400	3200	Wooten et al. (2014)
Adipose tissue (mice)	ng/g protein	49700	33700	62700	36900	35100	Wooten et al. (2014)
Human artery	ng/g	690	990	490	910	510	Garcia-Cruset et al. (2001)
Human coronary lesion	ng/g	8200000	3500000	400000	np*	2400000	Vaya et al. (2001)

4.3.3 Relative Level

The relative level of each oxyChOL to ChOL was also calculated for each woman (Appendix: Table 46). Median relative levels were 0.0001, 0.00003, 0.00002, 0.00001 and 0.000003 for β-epoxy-ChOL, α-epoxy-ChOL, 7-O-ChOL, 7α-HO-ChOL and 7β-HO-ChOL respectively (Figure 21).

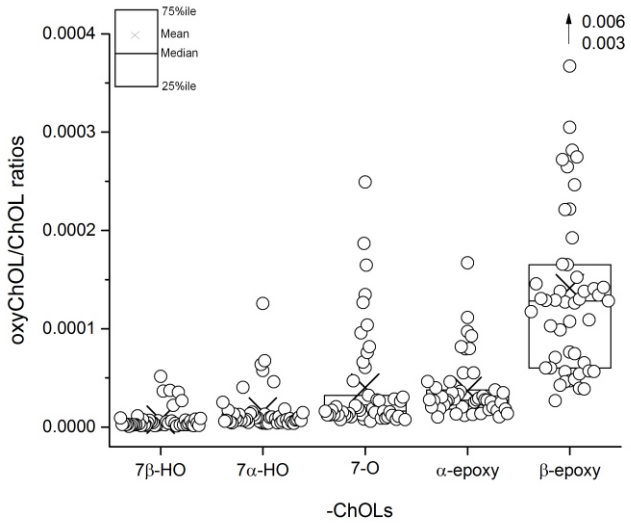


Figure 21 The relative levels of oxyChOLs to ChOL in 49 breast adipose tissues.

The relative level of each oxyChOL to total oxyChOL was also calculated for each woman (Appendix: Table 45). The most abundant ratio was devoted to β-epoxy-ChOL/total oxyChOL (median: 0.65, range: 0.28 - 0.82), followed by α-epoxy-ChOL/total oxyChOL (median: 0.16, range: 0.07 - 0.25), 7-O-ChOL/total oxyChOL (median: 0.11, range: 0.04 - 0.53), 7α-HO-

ChOL/total oxyChOL (median: 0.05, range: 0.02 - 0.15), 7β-HO-ChOL/total oxyChOL (0.02, range: 0.01 - 0.08) respectively (Appendix: Table 45).

4.3.4 OxyChOLs ranking in individual sample

According to the median levels of oxyChOLs the most abundant oxyChOL was β-epoxy followed by α-epoxy-ChOL, 7-O-ChOL, 7α-HO-ChOL and 7β-HO-ChOL respectively (Chapter 4.3.2-3). However, the oxyChOLs ranked in different way in some samples. The levels of oxyChOls (both relative and absolute) in individual samples demonstrated that in 96% of samples (47 tissues), β-epoxy was the most abundant oxyChOL. In two samples 7-O-ChOL was the main oxyChOL and α-epoxy-ChOL has the second place in 71% of samples (35 tissues). The least oxyChOL in all samples was 7β-HO-ChOL (Appendix: Table 47).

The oxyChOLs ranking was as follows:

1- in 71% of samples: β-epoxy-ChOL > α-epoxy-ChOL > 7-O-ChOL > 7α-HO-ChOL > 7β-HO-ChOL

2- in 22% of samples: β-epoxy-ChOL > 7-O-ChOL > α-epoxy-ChOL > 7α-HO-ChOL > 7β-HO-ChOL

3- in 1 sample: 7-O-ChOL > β-epoxy-ChOL > α-epoxy-ChOL > 7α-HO-ChOL > 7β-HO-ChOL

4- in 1 sample: 7-O-ChOL > β-epoxy-ChOL > 7α-HO-ChOL > α-epoxy-ChOL > 7β-HO-ChOL

5- in 1 sample: β-epoxy-ChOL > 7-O-ChOL > 7α-HO-ChOL > α-epoxy-ChOL > 7β-HO-ChOL

4.3.5 Discussion

For the first time, the validated method allows a determination of oxyChOLs (α/β-epoxy-ChOL, 7-O-ChOL and 7α/β-HO-ChOL) by GC-MS/MS in breast adipose tissues of women. The method is validated in aspects of the FDA and ICH guidelines for harmonisation and contains data about accuracy, precision; LOD and LOQ. Accuracy data between 81.6% and 115.7% and precision below 10.0% indicated the high precision and accuracy of the method. Using GC-MS/MS validated method, oxyChOLs (α/β-epoxy-ChOL, 7-O-ChOL and 7α/β-HO-ChOL) were determined in 49 samples. All oxyChOLs were present 100% of samples. In 96% of samples (47 tissues), β-epoxy was the most abundant oxyChOL. In two samples 7-O-ChOL was the main oxyChOL and α-epoxy-ChOL has the second place in 71% of samples (35 tissues). The least oxyChOL in all samples was 7β-HO-ChOL. The present study showed that the ranking of oxyChOLs (absolute and relative levels) in human breast adipose tissues were similar to mice liver. However, it differed to other tissues such as Human artery.

4.4 Statistical evaluation of fatty acids, ChOL and oxyChOLs

The breast adipose tissue composition of fatty acids (Chapter 4.1), ChOL (Chapter 4.2) and oxyChOLs (Chapter 4.3) were determined. Up to now no study has investigated the association between aforementioned components in breast adipose tissue. Hence, the aim was to find correlations between fatty acids, ChOL and oxyChOLs in breast adipose tissue. The statistical relationships between ChOLs and oxyChOLs (Chapter 4.4.1), ChOL and fatty acids (Chapter 4.4.2), oxyChOLs and fatty acids (chapter 4.4.3), between fatty acids (Chapter 4.4.4) and between oxyChOLs (Chapter 4.4.5) were established (Chapter 3.7.15).

Another objective of this study was to determine whether the fatty acid composition of breast adipose tissue as well as ChOL and oxyChOLs are associated with age and BMI. Previously Tjonneland et al. (1993) evaluated the correlation between age, BMI and fatty acids in gluteal adipose tissues. They reported neither age nor BMI associated with fatty acids. However, Bolton-Smith et al. (1997) reported an age related decline of LA and an increase in DHA in subcutaneous adipose tissue taken from the outer upper arm. No research has examined the association between the age or BMI and ChOL levels in breast adipose tissue. No data in breast adipose tissue concerning the correlation of oxyChOLs and age and BMI have been published up to now. Hence, the association between fatty acids, ChOL, oxyChOLs and age and BMI was checked (Chapters 4.4.6-7).

4.4.1 Correlation analysis between ChOL and oxyChOLs

To see the relation between tissue levels of ChOL and oxyChOLs, rank-based Spearman correlation test was carried out. Significant negative correlations were observed between absolute levels of ChOL and 7α-HO-ChOL, 7β-HO-ChOL and 7-O-ChOL levels (absolute and relative) in breast adipose tissues. The levels of epoxy-ChOLs, as well as sum of oxyChOLs did not correlate with ChOL (Table 19).

Table 19 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and absolute level of ChOL in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	-0.29	0.04	-0.30	0.05
7β-OH-ChOL	-0.32	0.02	-0.31	0.03
7-O-ChOL	-0.29	0.04	-0.36	0.01
α-epoxy-ChOL	-0.04	0.78	-0.04	0.84
β-epoxy-ChOL	0.06	0.66	0.04	0.81
Total oxyChOLs	-0.10	0.48	-0.07	0.65

Indeed oxyChOL may have inhibitory activity on ChOL homeostasis. HMG-CoA reductase is the rate-determining enzyme of the ChOL biosynthetic pathway. The activity of this enzyme is reduced by oxyChOLs (Tamasawa et al., 1997). A significant negative correlation was observed between sum of 7β-HO-ChOL and 7-O-ChOL and HMG-CoA reductase activity in rat liver (Tamasawa et al., 1997). Moreover, 7β-HO-ChOL, 7-O-ChOL and 7α-HO-ChOL decreased ChOL biosynthesis as well as ChOL level in the lesioned brain cortex of Wistar rats (Bochelen et al., 1995). It can be assumed 7-O-ChOL, 7β-HO-ChOL, and 7α-HO-ChOL can reduce ChOL synthesis in body.

4.4.2 Correlation analysis between ChOL and fatty acids

A significant negative association were found between absolute levels of ChOL and absolute levels of pentadecanoic acid (Table 20). The relative proportion of pentadecanoic acid did not correlate with ChOL (Appendix: Table 48). There are some studies which investigated the relation between fatty acids and ChOL content of the blood plasma. However, no data concerning breast adipose tissue has been published until now. Grela et al. (2014) investigated the relation between relative level of fatty acids and ChOL content in tissue samples (back fat, liver and heart) of 60 pigs. They found no significant correlation between SFA and ChOL. No information is available about the influence of pentadecanoic acid (biomarker of dairy product) on ChOL level.

Absolute levels of ALA were inversely correlated with levels of ChOL (Table 20). However, relative proportions of ALA did not associate with ChOL content of adipose tissue (Appendix: Table 48). Fukumitsu et al. (2013) demonstrated that ALA significantly reduced the mRNA expressions of sterol regulated element binding protein and consequently inhibited biosynthesis of ChOL in 3T3-L1 mouse cells. In addition to, negative significant correlation was observed between ChOL and elaidic acid absolute levels (Table 20). Study by Brouwer et al. (2010) summarized that TFAs have a ChOL-raising effect. Vendel Nielsen et al. (2013) investigated the hepatic response of human HepG2 cell to elaidic acid. They found that elaidic acid up regulates the proteins which are responsible for ChOL synthesis, esterification and hepatic import/export of ChOL. Moreover, Shao and Ford (2014) indicated elaidic acid treatment increased *de novo* synthesis of ChOL in HuH-7 cells.

Table 20 Spearman correlation results of ChOL and fatty acids absolute levels were obtained with data of 49 breast adipose tissue samples.

	R-value	p-value
15:0&ChOL	-0.40	0.01
18:1 9trans&ChOL	-0.41	0.01
18:3 n3&ChOL	-0.39	0.01

4.4.3 Correlation analysis between oxyChOL and fatty acids

The statistical relationship between four fatty acids (elaidic acid, ALA, pentadecanoic acid and DHA) and oxyChOLs were tested.

Correlation between elaidic acid and oxyChOLs:

A significant positive correlation was observed between absolute levels of elaidic acid (18:1 9 trans) and absolute levels of each oxyChOLs (except β-epoxy-ChOL), as well as total oxyChOLs. Moreover, relative levels (oxyChOLs to ChOL) of all oxyChOLs were positively correlated with absolute levels of elaidic acid (Table 21). TFAs induced the lipid peroxidation and oxidative stress in livers of rats which were fed with oxidized edible oil and margarine for four weeks compared with the control group (Dhibi et al., 2011). The result suggested that feeding margarine with higher TFA level may represent a direct source of oxidative stress. OxyChOLs can be formed through non-enzymatic oxidation (oxidative stress, Chapter 1.1.3.2.2). Therefore it can be assumed the elaidic acid may increase the formation of oxyChOLs indirectly through induction of oxidative stress.

Table 21 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and absolute levels of elaidic acid in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.41	0.01	0.47	<0.01
7β-HO-ChOL	0.41	<0.01	0.47	<0.01
7-O-ChOL	0.32	0.03	0.38	0.01
α-epoxy-ChOL	0.32	0.03	0.39	0.01
β-epoxy-ChOL	0.21	0.15	0.34	0.02
Total oxyChOLs	0.35	0.02	0.40	<0.01

In addition to, relative peak area percentages of elaidic acid were positively associated with the absolute and relative levels of 7β-HO-ChOL, β-epoxy-ChOL and total levels of oxyChOLs (Table 22).

Table 22 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and relative peak area percentages of elaidic acid in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.20	0.17	0.21	0.14
7β-HO-ChOL	0.32	0.02	0.30	0.03
7-O-ChOL	0.14	0.33	0.24	0.33
α-epoxy-ChOL	0.27	0.10	0.24	0.09
β-epoxy-ChOL	0.37	0.01	0.31	0.03
Total oxyChOLs	0.34	0.02	0.30	0.04

Correlation between ALA and oxyChOLs:

The positive correlation was found between 7α/β-HO-ChOL (absolute and relative levels) and absolute levels of ALA in breast adipose tissues (Table 23). There is some evidence which demonstrated that 7β-HO-ChOL inhibited the conversion of ALA to LCPUFA in cultured cells, which caused the higher level of ALA (Risé et al., 2004). Furthermore, absolute levels of ALA were positively associated with relative levels of α-epoxy-ChOL (Table 23). On the other hand Individual oxyChOL as well as total oxyChOLs levels (absolute and relative) did not correlate with relative peak area percentages of ALA (Appendix: Table 49). It was expected to obtain similar correlation result for both absolute and relative peak area percentages of ALA. The Spearman rank correlation measures the statistical relationship between ranked variables. The ranking of absolute levels of ALA and peak area percentages of ALA in 49 samples were not similar, which may cause the unexpected different correlation result in the case of ALA. Up to now no study has investigated the correlation between fatty acids and oxyChOLs in adipose tissue; therefore it is difficult to interpret the correlation result.

Table 23 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and absolute levels of ALA in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.26	0.09	0.33	0.03
7β-HO-ChOL	0.29	0.05	0.35	0.02
7-O-ChOL	0.16	0.27	0.22	0.13
α-epoxy-ChOL	0.20	0.18	0.28	0.05
β-epoxy-ChOL	0.05	0.75	0.22	0.13
Total oxyChOLs	0.17	0.23	0.24	0.09

Correlation between pentadecanoic acid and oxyChOLs:

Absolute levels of petadecanoic acid were positively correlated with levels (absolute and relative) of oxyChOLs (Table 24). It should be noted, the association of pentadecanoic acid (absolute levels) was weak with relative levels of β-epoxy-ChOL and there was no correlation with absolute levels of β-epoxy-ChOL and pentadecanoic acid (Table 24). Relative peak area percentages of pentadecanoic acid were not associated with oxyChOLs (Appendix: Table 50).

It was expected to obtain similar correlation result for both absolute and relative peak area percentages of pentadecanoic acid. The ranking of absolute levels of pentadecanoic acid and peak area percentages of it were not similar in 49 samples, which may cause the unexpected different correlation result in the case of pentadecanoic acid. No data in breast adipose tissue concerning the correlation of fatty acids and oxyChOLs have been published until now. Thus the interpretation of result is difficult.

Table 24 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and absolute levels of pentadecanoic acid in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.39	0.01	0.38	<0.01
7β-HO-ChOL	0.37	0.01	0.31	<0.01
7-O-ChOL	0.30	0.05	0.33	0.02
α-epoxy-ChOL	0.29	0.05	0.27	0.02
β-epoxy-ChOL	0.14	0.35	0.27	0.06
Total oxyChOLs	0.29	0.05	0.35	0.02

Correlation between DHA and oxyChOLs:

It should be noted the relative proportions and absolute levels of DHA were not associated with relative (oxyChOL to ChOL) and absolute levels of oxyChOL (Appendix: Tables 51, 52).

4.4.4 Correlation analysis between fatty acids

In order to investigate the correlation between 26 fatty acids which were found in breast adipose tissues, the mean relative peak area percentages of fatty acids (Chapter 4.4.4.1) as well as the absolute levels (Chapter 4.4.4.2) of four fatty acids (pentadecanoic acid, elaidic acid, ALA and DHA) were considered statistically.

4.4.4.1 Correlation between peak area percentages of fatty acids

The strong positive correlations (Table 25) were observed between proportions of capric (10:0) with lauric acid (12:0), myristic (14:0), stearic (18:0) and arachidic (20:0) acids. Likewise, proportions of lauric acid were positively associated with myristic, stearic and arachidic acids. Moreover, myristic acid was positively associated with palmitic and stearic acids (Table 25). Palmitic acid was positively correlated with stearic acid. Positive relation was found between stearic and arachidic acid. The observed positive correlation between these SFAs can be explained by the *de novo* biosynthesis of them in human body (Chapter 1.1.1.3.2). These SFAs are formed via chain elongation.

It is noticeable that pentadecanoic acid showed high positive correlation with heptadecanoic acid (R-value = 0.69, p-value < 0.01). The high positive correlation may hold because the food resources of both are the same. Some studies have suggested proportions of 15:0 and 17:0 acid in human adipose tissue reflect dairy fat consumption (Baylin et al., 2002; Biong et al., 2006; Brevik et al., 2005). In this work there is no food questionnaire to confirm this issue. These fatty acids cannot be synthesized in human body (Chapter 1.1.1.3.2).

On the other hand strong negative correlation was seen between myristic acid (14:0) and oleic acid (18:1 9cis). Myristic acid is produced via *de novo* system in body then it can be converted to palmitic and palmitic to stearic, and finally stearic acid can be desaturated to oleic (Chapter 1.1.1.3.2). Similar result obtained for palmitic (16:0) and oleic acid. The correlation between Stearic (18:0) and oleic acid was also negative (Table 25). The synthesis of oleic acid via desaturation of stearic acid may explain the negative correlation between these fatty acids.

Table 25 Statistical correlation (Spearman) between even numbered SFAs and MUFAs in human adipose breast tissue (presented data are R-values, p-value < 0.05, *p-value > 0.1).

	10:0	12:0	14:0	16:0	18:0	20:0	14:1 n9	16:1 n9	18:1 cis 9
10:0	—	0.57	0.63	*0.09	0.45	0.59	0.29	*0.09	-0.36
12:0	0.57	—	0.65	*0.04	0.37	0.68	*0.13	*-0.22	*-0.23
14:0	0.63	0.65	—	0.47	0.37	0.49	0.35	*0.02	-0.71
16:0	*0.09	*0.04	0.47	—	0.41	*0.09	*-0.14	*-0.23	-0.65
18:0	0.45	0.37	0.37	0.41	—	0.66	-0.49	-0.55	-0.39
20:0	0.59	0.68	0.49	*0.09	0.66	—	*-0.07	*-0.23	*-0.15
14:1 n9	0.29	*0.13	0.35	*-0.14	-0.49	*-0.07	—	0.82	*-0.07
16:1 n9	*0.09	*-0.22	*0.02	*-0.23	-0.55	*-0.23	0.82	—	*0.01
18:1 9cis	-0.36	*-0.23	-0.71	-0.65	-0.39	*-0.15	*-0.07	*0.01	—

Myristoleic acid was highly correlated with palmitoleic acid (Table 25). Myristoleic acid and palmitoleic acid are MUFAs and biosynthesized from myristic acid and palmitic acid respectively.

Strong correlation was observed between DHA and EPA (Table 26). These fatty acids belong to n3 PUFAs and they are available in high amount in fishes and fish oils (Table 4, Chapter 1.1.1.3.1). Previously, the strong association between dietary intake and adipose tissue content of these fatty acids was reported (Straka et al., 2015; Marckmann et al., 1995). However, in this work there is no food questionnaire to confirm this issue. Furthermore, a positive correlation was observed between peak area percentages of ALA (18:3 n3) and eicosatrienoic acid (20:3 n3, Table 26), which may cause by the metabolism of ALA to n3 PUFAs (Chapter 1.1.1.3.2). Moreover, peak area percentages of 20:3 n3 were positively correlated with DHA and EPA (Table 26). The rich source of 20:3 n3, EPA and DHA are fishes (Table 4, Chapter 1.1.1.3.1).

Eicosadienoic acid (20:2 n6) and DGLA (20:3 n6) were positively associated with each other (Table 26). These fatty acids are n6 LCPUFA and derived from LA in human body (Figure 2, Chapter 1.1.1.3.2). They are also present in fishes (Table 4, Chapter 1.1.1.3.1). GLA (18:3 n6) was positively correlated with DGLA. In human body GLA can be converted to DGLA. They both derived from LA (Figure 2, Chapter 1.1.1.3.2). Peak area percentages of LA were positively related with DGLA (Table 26) but not with GLA.

The positive correlation was also found between LA and ALA which are both essential fatty acids (Table 26).

Table 26 Statistical correlation (Spearman) between PUFAs in human adipose breast tissue (presented data are R-values, p-value ≤ 0.05, *p-value ≥ 0.1, **p-value = 0.07).

	18:2 n6	18:3 n6	18:3 n3	20:2 n6	20:3 n6	20:3 n3	20:5 n3	22:6 n3
18:2 n6	—	*0.15	0.34	0.44	**0.26	*0.11	*0.24	*0.16
18:3 n6	*0.15	—	-0.29	*-0.02	0.41	-0.34	0.31	*0.16
18:3 n3	0.34	-0.29	—	0.28	*-0.17	0.53	*0.14	*0.03
20:2 n6	0.44	*-0.02	0.28	—	0.44	0.67	0.39	0.45
20:3 n6	**0.26	0.41	*-0.17	0.44	—	*0.05	0.51	0.51
20:3 n3	*0.11	-0.34	0.53	0.67	*0.05	—	0.34	0.33
20:5 n3	*0.24	0.31	*0.14	0.39	0.51	0.34	—	0.75
22:6 n3	*0.16	*0.16	*0.03	0.45	0.51	0.33	0.75	—

In this work no association (Appendix: Table 53) was found between elaidic acid and LCPUFAs (18:3 n6, 20:2 n6, 20:3 n6, 20:3 n3, 20:5 n3 and 22:6 n3). Mosley et al. (2005) studied the relation between TFAs and LCPUFAs in women milk. They found no correlation between relative levels of 18:1 trans and n3 and n6 PUFAs, which is in lie with the finding of this research.

4.4.4.2 Correlation between absolute levels of four fatty acids

Absolute levels of pentadecanoic acid were positively associated with absolute levels of ALA, DHA and elaidic acid (Table 27). Furthermore, the positive correlation was observed between absolute levels of elaidic acid and ALA and DHA (Table 27). The ALA absolute levels were positively associated with DHA absolute levels (Table 27).

Table 27 Statistical correlation (Spearman) between individual fatty acids (absolute levels) in human adipose breast tissue (presented data are R-values, p-value < 0.0001).

	15:0	18:1 9 trans	18:3 n3	22:6 n3
15:0	—	0.92	0.84	0.70
18:1 9trans	0.92	—	0.79	0.68
18:3 n3	0.84	0.79	—	0.81
22:6 n3	0.70	0.68	0.81	—

4.4.5 Correlation between breast adipose tissue oxyChOLs

A strong significant association was observed between absolute and relative levels of individual oxyChOLs and total level of oxyChOLs (Table 28). Moreover, 7-O-ChOL and 7-HO-ChOLs correlated strongly with each other. They were positively associated with epoxy-ChOLs. Furthermore, strong positive correlation was observed between epoxy-ChOLs (Table 28).

Table 28 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs in human adipose breast tissue (presented data are R-value, p-values < 0.00001).

	Absolute level						Relative level					
oxyChOL	7α-HO	7β-HO	7-O	α-epoxy	β-epoxy	Total oxy	7α-HO	7β-HO	7-O	α-epoxy	β-epoxy	Total oxy
7α-HO-	—	0.93	0.81	0.72	0.69	0.85	—	0.92	0.80	0.71	0.70	0.83
7β-HO-	0.93	—	0.82	0.69	0.64	0.82	0.92	—	0.82	0.71	0.69	0.84
7-O-	0.81	0.82	—	0.60	0.59	0.78	0.80	0.82	—	0.62	0.63	0.80
α-epoxy-	0.72	0.69	0.60	—	0.88	0.91	0.71	0.71	0.62	—	0.93	0.91
β-epoxy-	0.69	0.64	0.59	0.88	—	0.94	0.70	0.69	0.63	0.93	—	0.94
Total oxyChOLs	0.85	0.82	0.78	0.91	0.94	—	0.83	0.84	0.80	0.91	0.94	—

4.4.6 Correlation between age and fatty acids, ChOL and oxyChOLs

To investigate the association of breast adipose tissue fatty acids (Chapter 4.4.6.1), ChOL (Chapter 4.4.6.2) and oxyChOLs (Chapter 4.4.6.3) with age, correlation analysis was done.

4.4.6.1 Fatty acids and age

The significant positive correlation was seen between relative proportions of EPA, DHA and the age of women. The slight positive correlation was also found between total n3 PUFAs and age (Table 29). Ogura et al. (2010) reported positive correlation between age and n3 fatty acid (sum up 18:3 n3, 20:5 n3, 22:5 n3 and DHA) in human abdominal subcutaneous adipose. The relative proportion of EPA and DHA in adipose tissue has been reported to increase with increasing age (Walker et al., 2014; Bolton-Smith et al., 1997). Straka et al. (2015) found no significant correlation between the relative level of DHA and age in breast adipose tissue.

Relative proportions of 18:2 n6 were positively associated with age. Since, LA is an essential fatty acid, this positive link can cause by higher intake of 18:2 n6 in elderlies or by decreases in the production of n6 PUFAs from LA. Moreover, the total n6 PUFAs were positively associated with age (Table 29).

In contrast, the proportions of palmitic, stearic, arachidic acids as well as total SFA were negatively correlated with age (Table 29). The reduction of SFA with age can be due to changes in the activity of enzymes, which they cooperate in the fatty acid synthesis. The activities of ACC, FASN were lower in old rats than younger ones (Nogalska et al., 2003). Furthermore, dietary habits may vary with the age.

Table 29 Statistical correlation (Spearman) between fatty acids (peak area percentages) and age in human adipose breast tissue.

	R-value	p-value
16:0	-0.53	< 0.01
18:0	-0.31	0.03
20:0	-0.29	0.04
Total SFA	-0.47	< 0.01
20:5 n3	0.53	< 0.01
22:6 n3	0.54	< 0.01
n3 PUFAs	0.26	0.06
18:2 n6	0.35	0.01
n6 PUFAs	0.36	0.01

No correlation was found between absolute level of pentadecanoic acid, ALA, elaidic acid and age. The weak positive associated was seen between absolute level of DHA and age (Table 30).

Table 30 Statistical correlation (Spearman) between fatty acids (absolute level) and age in human adipose breast tissue.

	R-value	p-value
15:0	-0.12	0.25
18:1 9trans	-0.08	0.40
18:3 n3	0.00	1.00
22:6 n3	0.26	0.07

4.4.6.2 ChOL and age

No correlation was observed between the ChOL content of breast adipose tissues and age (R-value = -0.02, p-value = 0.88). Smiljanic et al. (2013) studied the influence of age on ChOL metabolism in two brain regions, (the cortex and hippocampus), as well as liver of male Wistar rats. According to their result ChOL amounts were constant by aging in brain and liver. Moreover, reduced ChOL synthesis in the aging human hippocampus has been reported, while the amount of ChOL was unchanged (Thelena et al., 2006).

4.4.6.3 OxyChOL and age

No correlation was observed between the oxyChOL content (absolute and relative) of the breast adipose tissues and age. Till now, no study has investigated the relation between oxyChOLs and age in tissues (Appendix: Table 54).

4.4.7 Correlation between BMI and fatty acids, ChOL and oxyChOLs

BMI is a person's weight in kilograms divided by the square of height in meters. A high BMI can be an indicator of high body fatness. The World Health Organisation (WHO, 1998) has classified BMI rang in four classes including underweight range (less than 18.5), normal or healthy weight range (18.5 - 24.5), overweight (25.0 - 29.9) and the obese range (30.0 or higher).

In this research the BMI of women ranged from 19.6 to 45.6. Among the 49 women, sixteen women were in normal weight range. Twenty-seven of them had BMI between 25 and 29.9 and 7 of the women were obese. To investigate the association of breast adipose tissue fatty acids (Chapter 4.4.7.1), ChOL (Chapter 4.4.7.2) and oxyChOLs (Chapter 4.4.7.3) with BMI, correlation analysis was done using Spearman rank correlation.

4.4.7.1 BMI and breast adipose tissue fatty acids

Spearman correlation analysis revealed the significant negative correlation between SFAs (10:0, 12:0, 13:0, 14:0, 18:0, 20:0 and total SFA) and BMI (Table 31). Furthermore, peak area percentages of elaidic acid were inversely correlated with BMI (R-value = 0.33, p-value = 0.02).

A negative association was also found between relative levels of total 18:1 trans and BMI in gluteal adipose tissue (Smit et al., 2010).

Table 31 Statistical correlation (Spearman) between fatty acids (peak area percentages) and BMI in human adipose breast tissue.

	R-value	p-value
10:0	-0.52	<0.01
12:0	-0.49	<0.01
13:0	-0.55	<0.01
14:0	-0.52	<0.01
18:0	-0.27	0.06
20:0	-0.57	<0.01
Total SFA	-0.29	0.05
14:1	-0.28	0.05
18:1 9cis	0.32	0.02
18:1 9tranc	-0.33	0.02
20:2	0.27	0.06
20:3 n6	0.34	0.02

The positive significant correlation was seen between proportion of oleic acid (18:1 9cis) and BMI (Table 31).

No correlation was observed between the BMI and proportions of n3 PUFAs (Appendix: Table 55), whereas positive correlation was found between DGLA (20:3 n6) and BMI. A weak association was also observed between eicosadienoic acid (20:2) and BMI (Table 31). In study by Straka et al. (2015), levels of EPA and DHA in breast adipose tissue were positively associated with a higher BMI.

No correlation was seen between the absolute levels of pentadecanoic acid (R value: -0.12, p value: 0.41), elaidic acid (R-value = -0.21, p-value = 0.15), ALA (R-value = -0.11, p-value = 0.44), DHA (R-value = -0.08, p-value = 0.58) and BMI.

4.4.7.2 BMI and ChOL

In this work no significant correlation was seen between BMI and ChOL level in breast adipose tissue of women (R-value = -0.01, p-value = 0.97). Previously, the positive association was observed between serum ChOL level and BMI in men but not in women (Schröder et al, 2003), it should be noted no study investigated the association between BMI and ChOL in breast adipose tissue yet.

4.4.7.3 BMI and oxyChOL

In this research no correlation was found between oxyChOLs (absolute and relative levels) and BMI (Appendix: Table 56). No data in breast adipose tissue concerning the correlation of oxyChOLs and BMI have been published until now. Tremblay-Franco et al. (2015) investigated the effect of obesity on plasma oxyChOLs levels in women. No correlation was found in plasma oxyChOLs levels (including 7-HO-ChOL, 7-O-ChOL and β-epoxy-ChOL) between healthy control and obese women.

5. Summary

The aim of the present work was to determine the breast adipose tissue composition regarding fatty acids, cholesterol and (aut)oxidation products of cholesterol in women without breast cancer and to identify associated variables. Thus the necessary methods were optimized and validated where required and the breast adipose tissues of women without breast cancer were collected and analyzed.

The gas chromatography with flame ionization detection was optimized for detection and separation of 37 relevant fatty acids. Fifty breast adipose tissues were analyzed using the optimized method. 26 fatty acids were detected in breast adipose tissues. The median proportion of saturated (sum of 11 fatty acids), monounsaturated (sum of 5 fatty acids), polyunsaturated (sum of 9 fatty acids) and one trans fatty acid were 34.6%, 53.2%, 12.1% and 0.3% respectively. Moreover, absolute levels of pentadecanoic acid (median: 0.37 mg/g, range: 0.08 - 1.31 mg/g), elaidic acid (median: 0.50 mg/g, range: 0.09 - 1.92 mg/g), linolenic acid (median: 0.88 mg/g, range: 0.10 - 3.06 mg/g) and docosahexaenoic acid (median: 0.31 mg/g, range: 0.04 - 1.80 mg/g) were determined in breast adipose tissues for the first time. These four fatty acids are indicative for consumption of dairy products, processed fats, vegetable oils such as flax seed oil and fish respectively.

Furthermore, for the investigation of cholesterol in breast adipose tissues a gas chromatography was optimized and validated. The accuracies of the method in three independent spiked samples with low, medium and high levels of cholesterol were 99.1 ± 10.1%, 87.0 ± 11.2%, and 103.4 ± 4.6% with precisions of 2.1, 2.1, and 0.8% respectively. Using external calibration with internal standard cholesterol was quantified in samples (median: 1.1 mg/g, range: 0.7 - 1.5 mg/g).

In order to detect (aut)oxidation products of cholesterol, gas chromatography coupled triple quadrupole mass spectrometry was optimized and validated. The accuracy was between 81.6% and 115.7% and precisions for low, medium and high oxy-cholesterols levels were below 10.0%. The quantitative determination of (aut)oxidation products of cholesterol was established using external calibration with an internal standard. The most abundant oxy-cholesterol was 5,6β-Epoxy- (median: 147.2 ng/g, range: 25.7 – 624.2 ng/g), followed by 5,6α-Epoxy- (median: 34.6 ng/g, range: 9.9 – 124.7 ng/g), 7-Keto- (median: 19.1 ng/g, range: 7.9 – 220.6 ng/g), 7α-Hydroxy- (median: 10.2 ng/g, range: 3.8 – 111.3 ng/g) and 7β-Hydroxy-Cholesterol (median: 3.5 ng/g, range: 1.0 – 45.6 ng/g) respectively. Median oxy-cholesterol/cholesterol ratios ranged from 0.0001 (5,6β-Epoxy-Cholesterol) to 0.000003 (7β-Hydroxy-Cholesterol).

Finally the associations between fatty acids, cholesterol and oxy-cholesterol were investigated using Spearman's rank correlation. Absolute levels of elaidic acid were positively correlated with levels of linolenic and docosahexaenoic acid ($R = 0.79, 0.68$, $p < 0.01$). Absolute levels of linolenic acid were positively associated with levels of docosahexaenoic acid ($R = 0.81$, $p < 0.01$). Moreover, relative proportions of saturated fatty acids capric, myristic, palmitic and stearic acid were negatively correlated with oleic acid ($R = -0.36, -0.71, -0.65, -0.39$, $p < 0.05$). Tissue levels of cholesterol were not correlated with levels of 5,6α/β-Epoxy-Cholesterols but were negatively associated with that of 7α-Hydroxy-, 7β-Hydroxy- and 7-Keto-Cholesterol ($R = -0.29, -0.32, -0.29$ $p = 0.04, 0.02, 0.04$). Levels of 7-Keto- and 7-Hydoxy-Cholesterol were strongly correlated with each other ($R = 0.81, 0.91$, $p < 0.01$) and, weaker, with 5,6α/β-Epoxy-Cholesterols ($R = 0.60$-0.70, $p < 0.01$). 5,6α/β-Epoxy-Cholesterols were associated positively with each other ($R = 0.90$, $P < 0.01$). Total oxy-cholesterol, 7β-Hydroxy-Cholesterol, and 5,6β-Epoxy-Cholesterol levels were correlated with relative proportions of elaidic acid ($R = 0.30, 0.30$, and 0.31 respectively, $p = 0.04, 0.03, 0.03$, respectively), whereas no correlation was observed between levels of oxy-cholesterols and relative proportion of pentadecanoic acid, linolenic acid and docosahexaenoic acid.

Furthermore, Spearman's rank correlation was performed to investigate the relationship of fatty acids, cholesterol and oxy-cholesterol with age and body mass index. The relative proportions of total saturated fatty acids were negatively correlated with age ($R = -0.47$, $p < 0.01$) and body mass index ($R = -0.29$, $p = 0.05$). A positive significant correlation was observed between proportions of oleic acid and body mass index ($R = 0.32$, $p = 0.02$). There was no correlation between levels of cholesterol and body mass index or age. Likewise, no correlations of oxy-cholesterol levels with age or body mass index were observed.

In sum, in this work the quantification methods of cholesterol and oxy-cholesterol were validated. The validation data met the criteria according to the FDA guideline. Using the validated methods the absolute levels of cholesterol and oxy-cholesterols were determined in breast adipose tissue of human females for the first time.

6. Zusammenfassung

Brust-Fettgewebe ist in der Entwicklung und Funktion der weiblichen Brust durch komplexe Interaktionen mit Stroma beteiligt. Allerdings gibt es nur wenige Informationen über die quantitative Lipid-Zusammensetzung in Brust-Fettgewebe in Hinblick auf Substanzen wie Fettsäuren, Cholesterin und (Aut)Oxidationsprodukt von Cholesterin. Fettsäuren spielen eine wichtige Rolle in der menschlichen Gesundheit. Einige Studien deuten darauf hin, dass spezifische Arten von ungesättigten Fettsäuren (wie Omega-6-Fettsäuren) an der Tumorentstehung beteiligt sein können und im Gegensatz dazu können Omega-3-Fettsäuren präventive Wirkungen haben. Weiterhin können (Aut)Oxidationsprodukte von Cholesterin wie 7α-Hydroxy-Cholesterin, 7β-Hydroxy-Cholesterin, 7-Keto-Cholesterin, 5,6α-Epoxy-Cholesterin und 5,6β-Epoxy-Cholesterin Entzündungsprozesse beeinflussen. Darüber hinaus gibt es in Brust-Fettgewebe von Frauen ohne Brustkrebs wenig Informationen über das Profil von Fettsäuren, und der absolute Gehalt an Fettsäuren, das charakteristisch beeinflusst werden durch den Verzehr von bestimmten Lebensmitteln, wie Milchprodukten (Pentadecansäure), künstlich modifizierten Fetten (Elaidinsäure), Pflanzenölen wie Leinsamenöl (Linolensäure) und Fisch (Docosahexaensäure), noch nicht bestimmt wurden. Außerdem wurden quantitative Profile von Oxy-Cholesterinen und Cholesterin in Brust-Fettgewebe noch nicht bestimmt. Weiterhin ist nicht bekannt, ob Fettsäuren, Oxy-Cholesterinen und Cholesterin zum einen miteinander und zum anderen mit physiologischen Parametern wie dem Alter oder dem Body-Mass-Index assoziiert sind. Daher war das Ziel der vorliegenden Arbeit, das Brust-Fettgewebe von Frauen ohne Brustkrebs, die sich aus rein Kosmetischen Gründen einer Brustverkleinerung (Reduktions-Mammaplastik) unterzogen haben, zu sammeln um die Zusammensetzung in Bezug auf Fettsäuren, Cholesterin und (Aut)Oxidationsprodukte von Cholesterin zu untersuchen und hierzu entsprechende Methoden zu optimieren und validieren. Anschließend sollte mit geeigneten statistischen Methoden geprüft werden, ob ein Zusammenhang zwischen Fettsäuren, Cholesterin und Oxy-Cholesterinen untereinander und mit physiologischen Faktoren wie dem Alter und dem Body-Mass-Index besteht.

Für den Nachweis und die Trennung von 37 relevanten Fettsäuren wurde eine Methode mit gaschromatographischer Trennung und Flammenionisationsdetektion optimiert. Mit dieser optimierten Methode wurden 50 Brust-Fettgewebe analysiert. Der mittlere Fettsäureanteil (Median) von gesättigten (insgesamt 11 Fettsäuren), einfach ungesättigten (insgesamt 5 Fettsäuren), mehrfach ungesättigten Fettsäuren (insgesamt 9 Fettsäuren) und einer trans-Fettsäure lag bei 34,6%, 53,2%, 12,1% und 0,3%. Darüber hinaus wurde zum ersten Mal der absolute Gehalt von Pentadecansäure (Median: 0,37 mg/g, Bereich: 0,08 - 1,31 mg/g), Elaidinsäure (Median: 0,50 mg/g, Bereich: 0,09 - 1,92 mg/g), Linolensäure (Median: 0,88 mg/g, Bereich: 0,10 - 3,06 mg/g) und Docosahexaensäure (Median: 0,31 mg/g, Bereich: 0,04 - 1,81

mg/g) in Brust-Fettgeweben bestimmt. Pentadecansäure, Elaidinsäure, Linolensäure and Docosahexaensäure sind bezeichnend für den Verzehr von Milchprodukten, Künstlich modifizierte Fette, Pflanzenöle wie Leinsamenöl und Fische.

Weiterhin wurde nach gaschromatographischer Trennung mit anschließender Flammenionisationsdetektion der absolute Gehalt an Cholesterin in Brust-Fettgewebe von Frauen bestimmt. Die gaschromatographische Trennung wurde optimiert und die Methode validiert. Die Richtigkeit der Bestimmung wurde in drei unabhängigen Proben für einen niedrigen, mittleren und hohen Cholesteringehalt bestimmt und lag bei 99,1 ± 10,1%, 87,0 ± 11,2% und 103,4 ± 4,6% mit der Präzision von 2,1, 2,1 bzw. 0,8%. Der absolute Gehalt an Cholesterin, der mit externer Kalibrierung mittels eines internen Standards bestimmt wurde, lag zwischen 0,7 und 1,5 mg/g (Median: 1,1 mg/g).

Zur Erfassung von Oxy-Cholesterinen in den Brustfettgeweben wurde eine gaschromatographische Trennung mit anschließender Detektion mittels eines Triple-Quadrupol-Massenspektrometers im Multiple Reaction Monitoring Modus optimiert und validiert. Die Richtigkeiten der Bestimmung lagen zwischen 81,6% und 115,7% und die Präzision lagen unter 10,0%. Die quantitative Bestimmung von (Aut)Oxidationsprodukten von Cholesterin wurde mittels externer Kalibrierung mit internem Standard durchgeführt. Das am häufigsten vorkommende Oxy-Cholesterin war 5,6β-Epoxy (Median: 147,2 ng/g, Bereich: 25,7 – 624,2 ng/g), gefolgt von 5,6α-Epoxy (Median: 34,6 ng/g, Bereich: 9,9 – 124,7 ng/g), 7-Keto (Median: 19,1 ng/g, Bereich: 7,9 – 220,6 ng/g), 7α-Hydroxy (Median: 10,2 ng/g, Bereich: 3,8 – 111,3 ng/g) und 7β-Hydroxy (Median: 3,5 ng/g, Bereich: 1,0 – 45,6 ng/g). Der Median des Oxy-Cholesterin/Cholesterin-Verhältnisses reichte von 0,0001 (5,6β-Epoxy-Cholesterin) bis 0,000003 (7β-Hydroxy-Cholesterin).

Schließlich wurden die Korrelation zwischen Fettsäuren, Cholesterin und Oxy-Cholesterinen mithilfe der Spearman-Korrelation untersucht. Die absolute Gehalte an Elaidinsäure korrelierte positiv mit den Gehalten an Linolensäure und Docosahexaensäure ($R = 0,79, 0,68$, $p < 0,01$). Die Gehalte an Linolensäure waren positiv mit denen von Docosahexaensäure assoziiert ($R = 0,81$, $p < 0,01$). Darüber hinaus korrelierten die relative Anteile an gesättigten Fettsäuren Caprin-, Myristin-, Palmitin- und Stearinsäure negativ mit denen von Ölsäure ($R = -0,36, -0,71, -0,65, -0,39$, $p < 0,05$). Die Gehalte an Cholesterin korrelierten nicht mit denen von 5,6α/β-Epoxy-Cholesterin und waren negativ assoziiert mit denen von 7α-Hydroxy-, 7β-Hydroxy- und 7-Keto-Cholesterin ($R = -0,29, -0,32, -0,29$, $p = 0,04, 0,02, 0,04$). Die Gehalte von 7-Keto- und 7-Hydoxy-Cholesterin korrelierten stark miteinander ($R = 0,81, 0,91$, $p < 0,01$) und eine schwächere Korrelation mit denen von 5,6α/β-Epoxy-Cholesterin ($R = 0,60\text{-}0,70$, $p < 0,01$) wurde beobachtet. 5,6α/β-Epoxy-Cholesterin waren positiv miteinander assoziiert ($R = 0,90$, $P < 0,01$). Der Gesamt-Oxy-Cholesteringehalt, 7β-Hydroxy-Cholesterin und 5,6β-Epoxy-Cholesterin

korrelierten mit dem relativen Fettsäureanteil von Elaidinsäure (R = 0,30, 0,30 bzw. 0,31 p = 0,04, 0,03, 0,03). Dagegen wurde keine Korrelation zwischen den Gehalten von Oxy-cholesterinen und dem Anteil an Pentadecansäure, Linolensäure und Docosahexaensäure beobachtet.

Darüber hinaus wurde die Spearman-Korrelation durchgeführt, um einen möglichen Zusammenhang von Fettsäuren, Cholesterin und Oxy-Cholesterinen mit dem Alter und dem Body-Mass-Index zu untersuchen. Die Fettsäureanteile der gesättigten Fettsäuren korrelierten negativ mit dem Alter (R = -0,47, p < 0,01) und dem Body-Mass-Index (R = -0,29, p = 0,05). Es wurde eine signifikant positive Korrelation zwischen dem Anteil an Ölsäure und dem Body-Mass-Index (R = 0,32, p = 0,02) beobachtet. Es gab keine Korrelation zwischen den Gehalten von Cholesterin und dem Body-Mass-Index oder dem Alter. Ebenso wurden keine Korrelationen von Oxy-Cholesterinen mit dem Alter oder Body-Mass-Index beobachtet.

Zusammengefasst wurden die Quantifizierungsmethoden für Cholesterin und Oxy-Cholesterine validiert. Die Validierungsdaten entsprechen den Kriterien nach der FDA-Richtlinie. Mit den validierten Methoden wurden die absoluten Werte von Cholesterin und Oxy-Cholesterinen erstmals in Brust-Fettgewebe von Frauen bestimmt.

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Appendix

Table 32 R_t and relative R_t of fatty acids.

fatty acids	R_t (min)	relative R_t
10:0	13.49	0.90
11:0 (IS)	15.06	
12:0	16.93	1.12
13:0	19.01	1.26
14:0	21.27	1.41
14:1	23.28	1.55
15:0	23.60	1.57
16:0	25.98	1.72
16:1	27.68	1.84
17:0	28.28	1.88
18:0	30.63	2.03
18:1 9tranc	31.68	2.10
18:1 9cis	32.17	2.14
18:1 11cis	32.19	2.15
18:2 n6	34.43	2.29
20:0	35.20	2.34
18:3 n6	36.07	2.39
20:1	36.59	2.43
18:3 n3	36.93	2.45
21:0	37.28	2.47
20:2	38.68	2.57
20:3 n6	40.28	2.67
20:3 n3	41.14	2.73
20:4 n6	41.41	2.75
23:0	41.54	2.76
20:5 n3	44.20	2.93
22:6 n3	50.60	3.36

Table 33 peak areas (counts) of fatty acids (mean of three injections) in 50 breast adipose tissues (nd: not detected).

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10:0	278.4	126.0	299.0	256.8	243.5	268.4	228.7	292.0	197.4	135.5	128.8	195.4	202.3	202.8	121.2	315.7
12:0	1929.9	1173.0	1969.9	1472.5	3276.7	4923.4	1636.0	3050.0	1705.7	1842.2	1638.3	2011.4	3308.2	2644.3	3035.0	5401.1
13:0	113.6	68.4	104.4	85.1	165.2	166.3	131.4	170.2	81.2	94.4	96.6	138.2	163.4	101.5	79.4	204.7
14:0	11430.3	6956.7	9533.1	8210.0	20952.7	14846.1	13603.5	20331.3	10029.5	13114.8	12196.5	12746.7	19883.0	13200.3	14322.8	27218.8
14:1	922.1	593.6	1181.4	618.0	1855.4	1470.8	2744.2	2149.0	821.0	818.5	1040.8	1413.9	1946.4	713.2	676.0	2188.7
15:0	1104.2	746.8	664.9	732.4	1955.7	1102.2	1426.8	1841.0	935.4	1309.7	1400.6	1394.5	1940.1	1141.4	1374.1	2717.1
16:0	78203.7	48416.9	59303.2	47877.3	176278.9	86890.9	106938.1	132733.8	76091.8	93842.8	94015.8	89938.8	156204.4	98481.5	102915.4	187071.9
16:1	11395.8	7210.3	18614.5	6804.8	31110.7	17320.6	42835.3	34321.4	12334.2	10594.9	13789.9	19568.1	30281.5	9553.1	8740.7	26082.2
17:0	1119.4	729.2	692.2	724.6	1981.2	1005.0	1069.2	1534.7	1103.0	1209.8	1306.0	1251.8	1839.4	1259.2	1473.8	2298.0
18:0	21995.3	11195.3	13688.6	14349.5	33947.7	23208.9	15554.8	27544.4	22255.7	21967.3	17548.8	16826.5	34970.0	30014.2	32667.1	41080.9
18:1 9trans	1242.6	965.9	1171.7	922.2	2883.9	2158.5	2082.1	2135.1	1368.4	1088.7	1700.0	1766.2	2329.0	1520.6	2311.1	2313.3
18:1 9cis	147447.2	101970.4	127859.7	84745.5	333598.4	163457.2	277910.1	245913.4	149990.5	143462.6	176430.8	167715.9	379769.5	177935.8	181757.2	386157.4
18:1 11cis	10630.7	5607.9	7084.7	4812.1	21252.2	7218.1	21046.7	19128.1	10012.8	10875.6	11798.7	9497.8	18678.7	8596.6	7793.2	18105.5
18:2	40676.1	25651.5	24049.9	19767.7	76685.2	33834.8	57932.6	64985.6	48963.8	39416.1	50090.9	40799.5	82620.6	33938.2	45120.8	101523.3
20:0	732.7	397.6	528.3	420.0	878.8	859.0	648.5	912.3	611.0	579.8	488.9	548.8	1276.6	1055.1	1097.8	1693.5
18:3 n6	120.5	76.6	106.1	72.1	292.7	144.1	247.6	287.0	154.1	183.7	158.4	168.6	288.9	174.4	283.8	310.8
20:1	3129.8	2493.6	2199.5	1651.9	5815.0	2573.6	5807.6	5034.6	2891.7	2427.0	3791.1	3054.7	6605.3	3196.8	3134.2	7396.1
18:3 n3	2256.6	1666.7	1478.1	1344.9	4943.0	2317.8	3618.8	3879.5	2376.5	1914.1	2461.3	2203.3	4934.3	1942.9	1600.6	6976.0
21:0	920.0	655.0	1178.8	592.1	1482.0	933.7	2181.0	813.8	710.6	646.4	1092.3	978.6	1618.7	713.9	769.8	1812.5
20:2	984.2	633.8	546.5	472.9	1734.6	573.4	1511.0	1172.9	1009.9	758.2	1233.4	870.3	1783.8	709.7	769.9	2049.6
20:3 n6	627.5	464.6	507.2	409.8	2142.8	567.3	1632.7	1657.0	731.1	825.7	1312.8	1277.7	2033.5	867.8	2070.8	2771.1
20:3 n3	144.8	94.7	102.2	86.9	317.2	93.3	241.7	194.4	149.6	114.1	197.7	117.6	279.1	131.7	68.5	373.9
20:4 n6	nd	nd	nd	nd	nd	nd	33.6	nd	nd	nd	nd	nd	47.8	nd	nd	nd
23:0	1134.1	778.6	1131.6	570.4	3001.7	1046.1	2652.9	2902.4	1353.7	1480.4	2084.7	2202.5	4177.6	1328.8	2644.4	3618.4
20:5 n3	115.0	60.7	126.5	55.7	261.6	134.5	372.1	576.0	151.2	112.9	264.3	165.6	608.1	130.7	152.6	668.0
22:6 n3	431.2	200.4	300.9	237.6	982.9	343.5	1126.0	1701.6	581.8	240.0	1017.9	684.8	1336.6	464.2	350.1	1745.1

Table 33 (continued).

	16	17	18	19	20	21	22	24	25	26	27	28	29	30	31	32	33
10:0	252.5	329.8	107.1	161.4	294.6	458.9	343.7	314.9	138.7	228.2	190.0	202.8	164.9	687.9	843.0	416.9	174.7
12:0	2718.3	4383.3	1926.5	2411.0	2294.1	3283.2	4526.2	4087.6	1643.3	2697.3	1824.6	1958.8	1504.5	5544.0	6293.2	3761.5	2015.2
13:0	90.9	170.8	70.5	106.4	126.9	150.2	221.1	183.3	78.2	103.7	113.2	79.7	96.1	306.6	341.9	149.5	103.7
14:0	12562.5	19192.1	10241.5	13012.5	13377.9	15074.9	22841.7	19941.5	12342.4	11821.4	16565.9	10043.0	14214.0	33888.5	38933.6	21990.6	17545.2
14:1	717.2	1295.8	597.8	1246.8	1294.4	1264.1	2679.3	2171.0	604.0	727.5	1185.9	756.7	1769.4	3004.9	3418.8	1122.5	1378.6
15:0	913.0	1666.8	1025.1	1155.6	1261.4	1210.9	2110.1	1876.9	1334.7	1073.4	1642.9	734.3	1185.0	3528.2	3630.3	2153.8	1647.4
16:0	96467.7	140646.4	97965.4	90933.0	94118.7	87219.4	167673.9	119738.3	100838.4	77419.1	134065.3	83556.5	120282.5	208227.0	238551.5	203400.6	133695.0
16:1	10215.4	18389.7	12157.4	20521.6	14884.8	14122.2	36515.3	25893.2	7506.7	8746.0	19758.0	12007.3	25966.3	32079.1	39369.7	19733.0	23915.5
17:0	1246.3	1674.4	1260.7	1048.0	1187.7	1132.0	2165.2	1639.3	1419.3	1171.9	1685.3	907.8	1259.5	3145.7	3460.1	2837.0	1471.6
18:0	25720.8	41279.7	28783.1	18605.3	22403.2	22403.3	38575.7	27133.1	24700.9	24751.4	28199.0	20324.1	23618.2	49824.2	66533.5	71986.5	25977.8
18:1 9trans	1573.0	2580.7	1368.1	1561.8	1073.9	1295.2	2335.9	2068.2	1245.2	1326.3	1573.8	1409.3	1840.1	2469.5	2646.8	1595.0	1347.9
18:1 9cis	177602.8	278821.3	205633.9	187171.2	185178.5	147452.1	373915.1	249688.4	182536.4	141424.9	251587.2	177251.4	247441.2	357611.2	469229.2	445356.8	234477.4
18:1 11cis	10809.3	12829.8	10258.9	11787.8	8508.1	7616.0	21614.7	12459.0	8164.6	6224.6	14231.5	7330.7	17572.8	19613.2	23161.1	18476.8	16108.6
18:2	46126.9	58292.3	46005.8	45498.6	38074.2	34697.8	83549.7	66347.1	46212.6	39748.1	53756.7	36964.0	49146.7	88129.8	111663.2	89211.8	53673.3
20:0	958.9	1812.4	796.8	646.6	1018.1	875.4	1293.4	1385.0	611.3	834.0	898.7	828.5	795.7	1606.2	2839.8	2046.4	908.5
18:3 n6	140.2	222.8	178.9	150.9	143.3	136.3	352.4	404.6	175.8	124.9	184.6	152.2	255.6	259.5	401.5	350.0	262.4
20:1	3685.0	5458.4	3611.7	3634.0	3415.1	2426.9	7116.9	4771.9	3181.4	2559.0	5726.9	3480.4	916.3	6369.1	10329.7	5450.4	4220.7
18:3 n3	4289.1	3024.9	2254.9	3117.0	2100.9	2898.2	6875.6	4303.5	2422.7	2199.3	3460.9	2509.4	1892.9	6968.4	6681.0	3967.0	3216.1
21:0	1812.5	1141.8	620.0	973.9	1101.0	926.9	1985.0	1411.2	854.7	787.1	1209.1	601.9	601.9	2934.7	3269.0	1476.0	1160.9
20:2	848.7	1182.5	946.0	885.2	773.9	556.9	1666.0	974.7	892.2	687.1	1334.6	785.9	1385.4	1621.5	2148.2	1215.3	1140.0
20:3 n6	567.6	1235.6	1066.3	935.4	1324.7	573.8	1508.9	1309.8	1525.7	511.7	2150.9	862.8	1405.6	1578.8	1761.1	1603.4	1469.0
20:3 n3	178.3	175.7	160.4	145.4	100.4	109.0	330.0	159.6	132.6	112.9	255.9	137.1	173.4	285.2	344.3	149.9	174.9
20:4 n6	46.8	nd	nd	nd	17.6	58.3	35.9	nd	nd	nd	nd	nd	nd	69.1	86.0	nd	nd
23:0	1045.4	1145.1	1673.6	1476.8	1524.5	873.5	3339.4	2941.2	2209.3	1097.1	2045.7	1068.8	3191.4	2154.6	3901.5	2051.9	2161.8
20:5 n3	113.1	77.5	182.3	236.1	105.5	95.9	527.4	384.0	220.2	121.5	448.9	92.6	390.0	240.2	388.5	210.8	154.2
22:6 n3	346.4	220.1	474.4	886.2	563.5	311.7	1172.2	993.7	770.4	270.9	1345.0	239.7	1626.0	860.8	1568.9	1192.7	410.6

Table 33 (continued).

	34	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**
10:0	173.5	160.5	188.7	170.3	323.6	148.6	298.9	1032.1	380.2	287.8	316.7	309.6	469.5	190.9	267.1	283.7	530.6
12:0	3569.3	1069.7	2015.6	2356.8	4196.8	1996.2	4447.6	4329.7	4586.6	2797.7	2404.3	2743.9	2355.5	1396.5	3849.4	2784.4	7068.6
13:0	166.1	65.7	91.6	128.3	207.5	79.6	165.8	285.2	212.3	112.2	165.0	115.7	129.2	87.3	155.8	115.7	141.5
14:0	22415.9	10558.5	12213.5	17461.3	20333.7	12199.0	23548.0	35602.0	26447.6	15730.8	26175.8	14014.2	18721.2	13969.4	22163.0	14014.2	31591.9
14:1	1786.1	740.4	654.6	2044.4	2088.0	976.8	1929.3	2826.4	1716.2	930.9	1254.9	857.9	1620.8	2147.6	1126.3	861.1	1889.0
15:0	2451.0	763.7	1185.9	1634.3	2254.4	1309.8	2295.0	2880.1	2758.6	1573.6	3473.6	1422.8	1275.8	1346.3	2452.4	1402.3	2763.0
16:0	179347.7	87966.2	84163.3	123415.0	135204.2	99639.9	165877.6	233885.8	171831.9	121397.7	195042.7	91911.5	148004.0	142197.7	176688.9	91917.9	227711.8
16:1	26791.0	12887.8	9270.7	28768.4	21226.7	16562.1	2066.0	45554.0	19390.9	13182.3	17418.8	11885.4	33936.7	46763.0	14095.8	11885.4	29716.6
17:0	2535.0	953.7	1364.1	1383.6	1540.5	1530.1	2103.7	3152.9	2346.6	1564.7	3098.3	1256.5	1429.2	1638.9	2527.6	1259.6	2714.6
18:0	36061.7	21118.4	27435.9	18658.2	29996.7	24742.5	34925.4	71416.8	42160.6	30065.8	47093.4	24385.2	37584.4	22158.3	44410.5	24385.2	50001.3
18:1 9trans	2008.3	696.3	1001.4	1402.1	1773.5	1496.2	1562.2	2159.5	1838.1	1126.7	1814.4	1707.0	1552.8	1438.6	1571.5	1697.5	2940.3
18:1 9cis	358396.1	134124.0	156170.3	229865.9	274025.8	222885.6	331076.1	445173.0	301839.5	287678.5	345211.4	180486.9	256670.0	331760.4	331102.5	180493.3	421640.9
18:1 11cis	22888.1	8944.4	9504.1	18567.2	16423.9	15054.0	21272.2	31206.1	16021.2	16054.5	20271.0	12075.2	18914.9	23926.4	17609.7	12081.6	21229.9
18:2	88354.9	26355.5	37380.6	49400.6	61483.8	55713.2	73205.3	89587.9	75378.2	86583.0	105057.6	45340.5	49076.9	50200.1	87416.2	45350.0	126074.1
20:0	1315.4	525.1	903.7	635.1	1538.5	722.7	1041.7	2420.0	1529.3	944.5	814.5	856.5	1056.9	514.9	996.4	856.5	1469.4
18:3 n6	394.9	107.9	108.5	166.7	230.8	149.8	341.6	246.0	282.8	220.2	228.5	132.1	118.0	385.3	358.3	132.1	559.9
20:1	7919.6	3001.8	3488.1	4493.6	5292.0	4734.2	5516.7	8827.7	4178.4	4367.0	5374.1	3157.3	4653.2	4300.5	6098.0	3154.1	7135.2
18:3 n3	5977.0	2171.7	2600.8	4558.0	4020.4	3873.9	5847.1	6705.7	3612.7	3756.2	7678.3	2615.9	4139.0	1670.4	5446.5	2600.0	6795.4
21:0	1565.5	579.4	745.8	1308.8	1501.5	1073.1	1783.5	2251.7	1579.4	903.4	1606.7	776.8	1060.9	1241.9	1183.9	796.0	2512.6
20:2	2416.0	632.9	810.1	1157.6	1178.7	1389.3	1527.5	2177.5	884.6	1247.7	1796.8	869.1	1132.2	1081.8	1645.7	849.0	2557.8
20:3 n6	3515.8	548.7	641.6	1267.4	1003.9	1521.7	2115.2	1628.6	1637.9	1382.3	2908.3	900.5	964.2	3562.1	1792.5	885.5	3203.6
20:3 n3	435.1	148.0	170.1	241.2	161.2	261.0	252.3	368.1	95.5	161.0	308.5	118.7	221.3	113.5	221.9	128.3	348.0
20:4 n6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	47.8	nd	nd	nd	nd	nd
23:0	6038.0	1206.8	1014.0	1976.5	1878.3	2285.8	3310.0	3138.5	2136.3	1821.0	4390.0	1169.3	1804.1	6868.4	2545.0	1182.2	5714.5
20:5 n3	503.1	199.2	165.8	290.1	280.6	310.3	441.4	208.6	259.3	172.5	407.7	128.8	129.9	383.1	200.0	130.1	643.3
22:6 n3	1316.8	425.5	462.3	571.3	382.9	1182.4	903.1	1260.9	696.8	584.5	1058.6	329.1	452.1	1043.6	372.9	328.4	1121.9

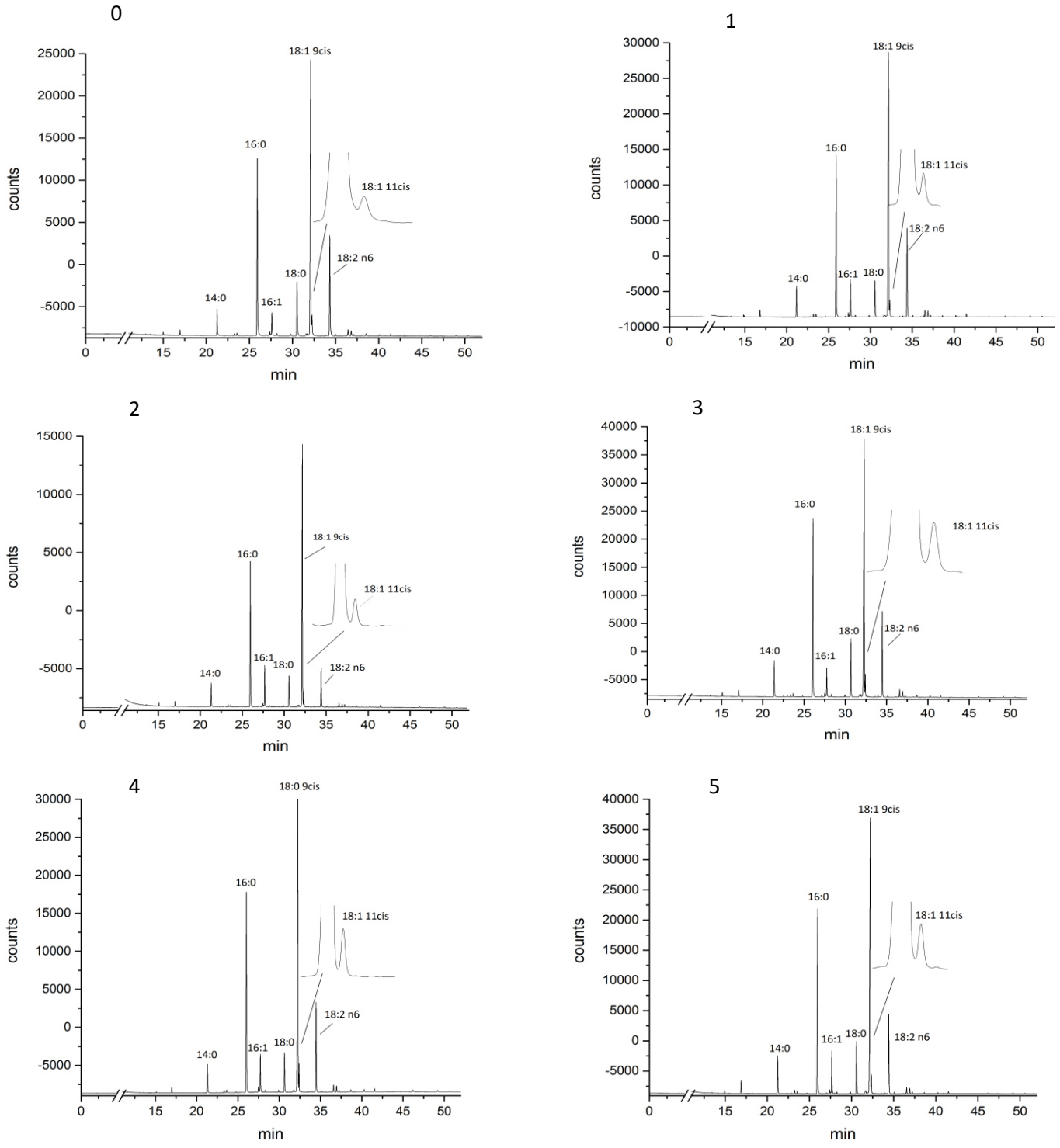

Figure 22 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 0, 1, 2, 3, 4 and 5.

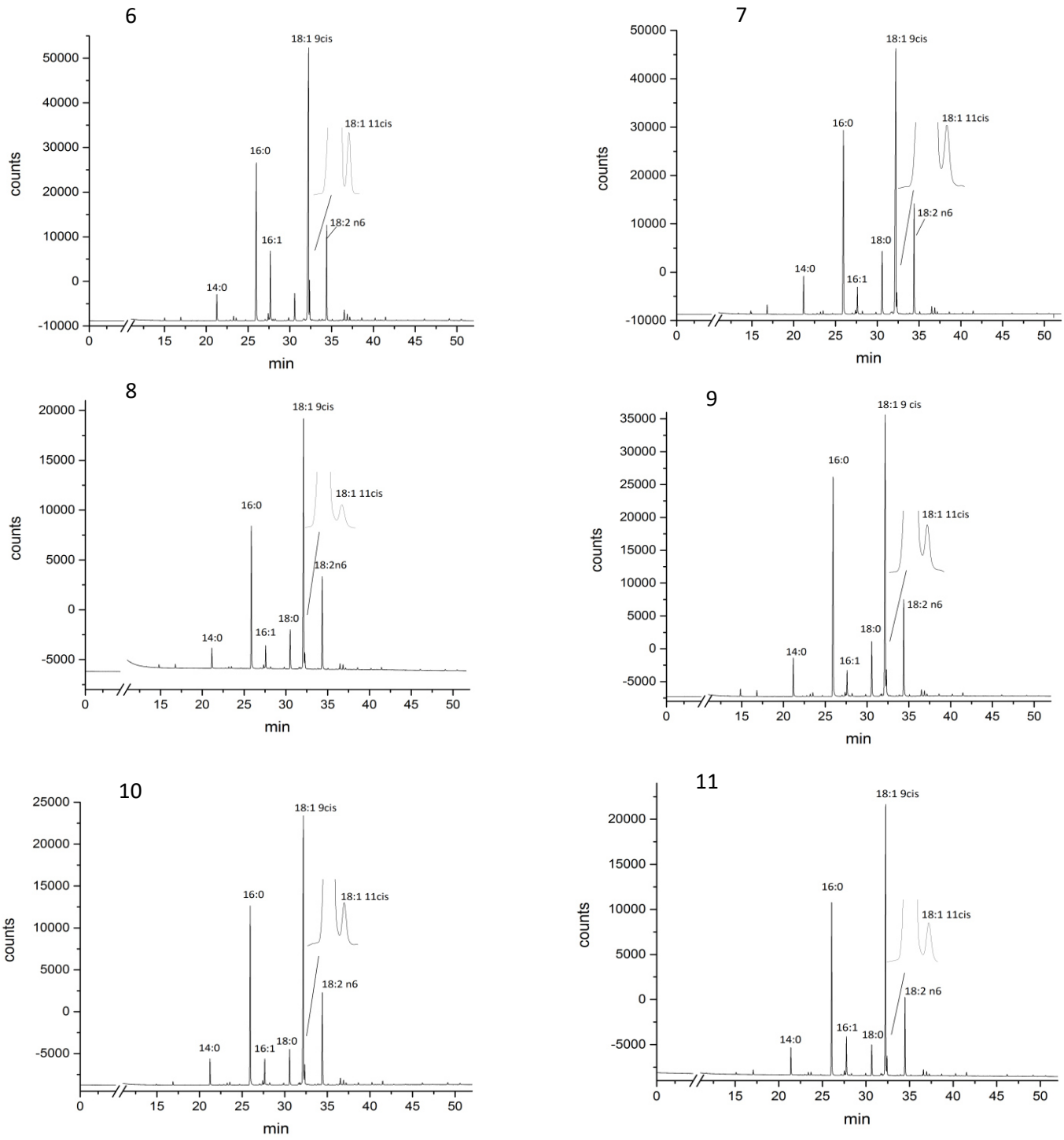

Figure 23 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 6, 7, 8, 9, 10 and 11.

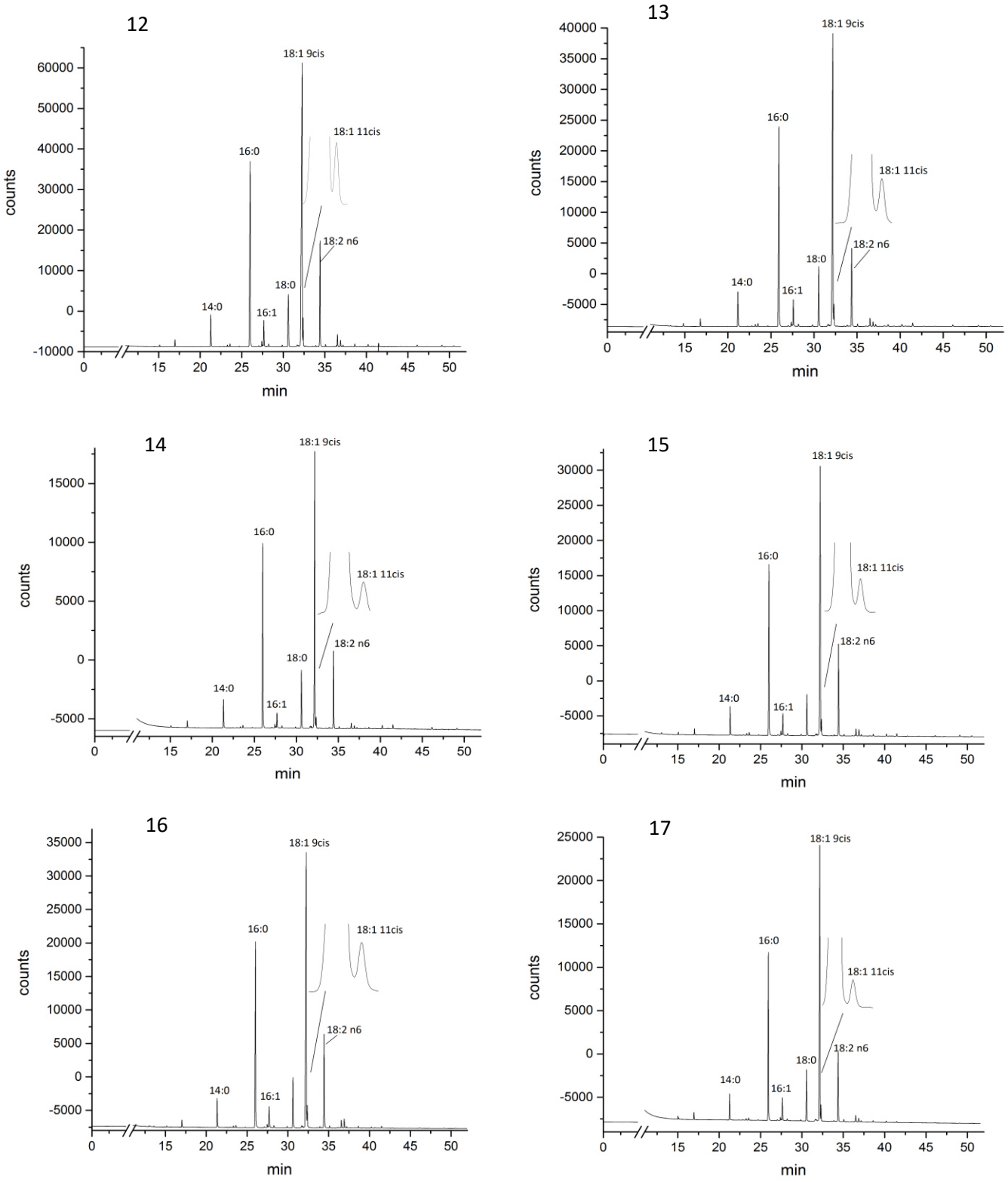

Figure 24 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 12, 13, 14, 15, 16 and 17.

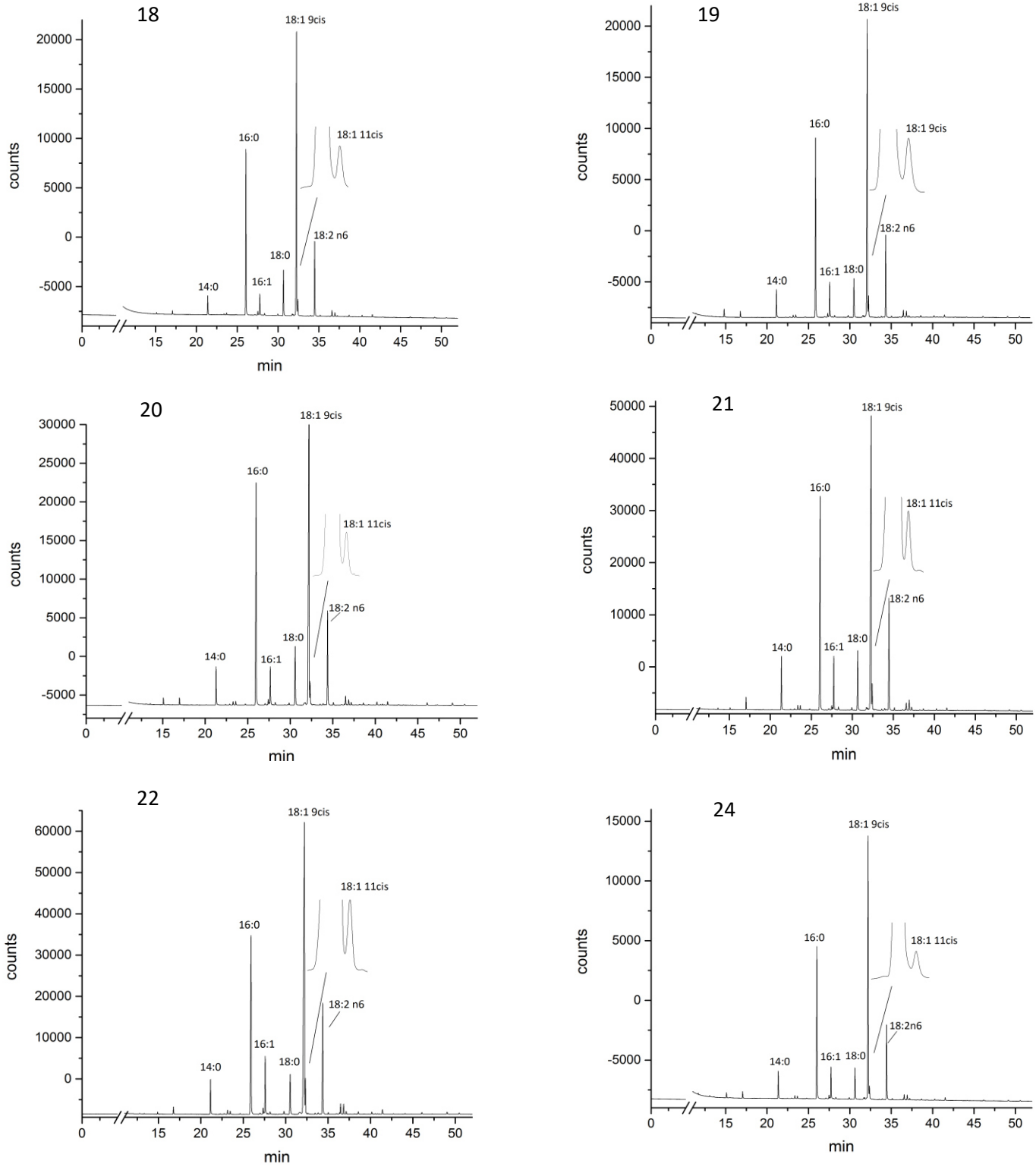

Figure 25 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 18, 19, 20, 21, 22 and 24.

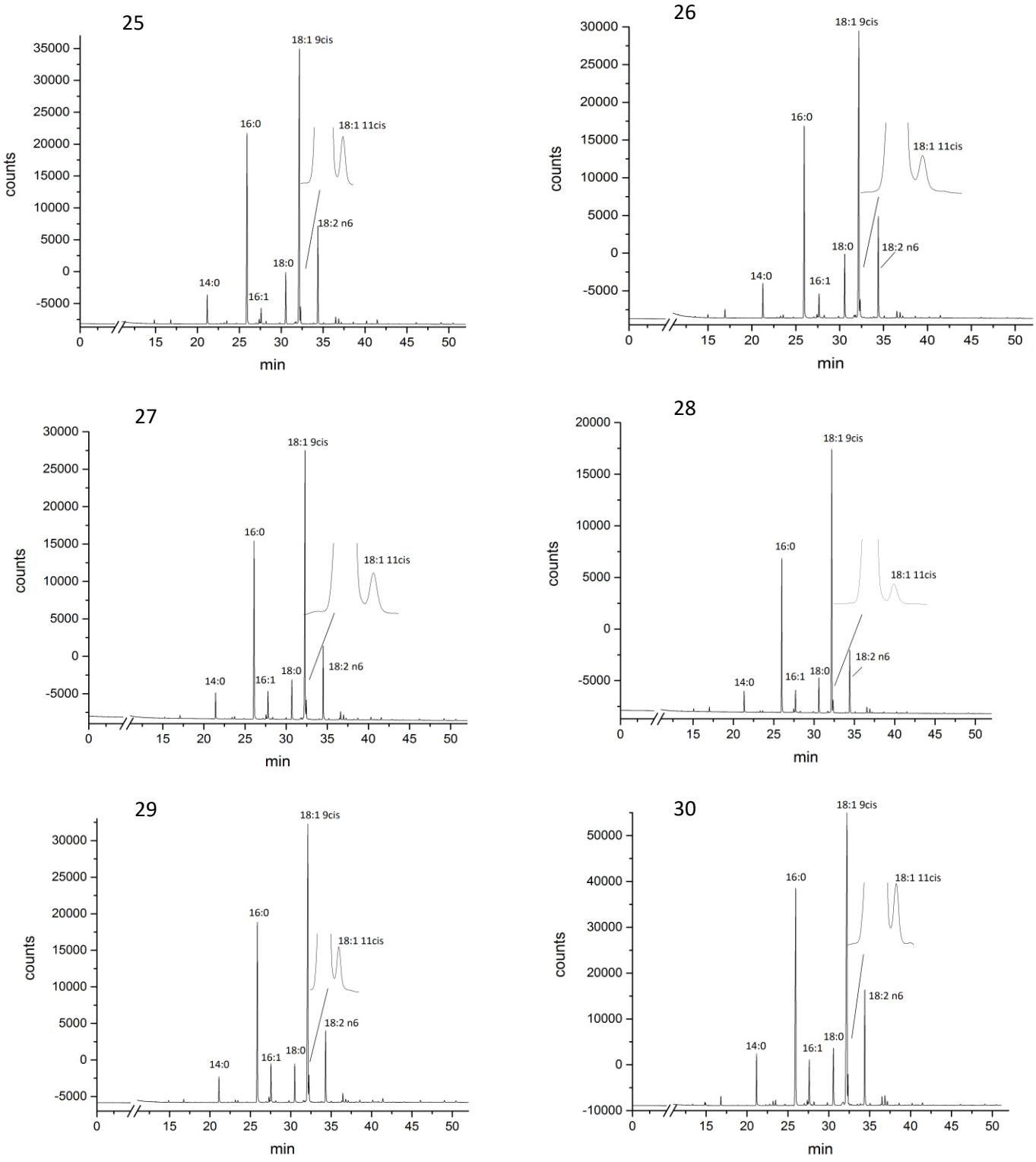

Figure 26 GC-FID chromatogram of fatty acids in breast adipose tissue of samples 25, 26, 27, 28, 29 and 30.

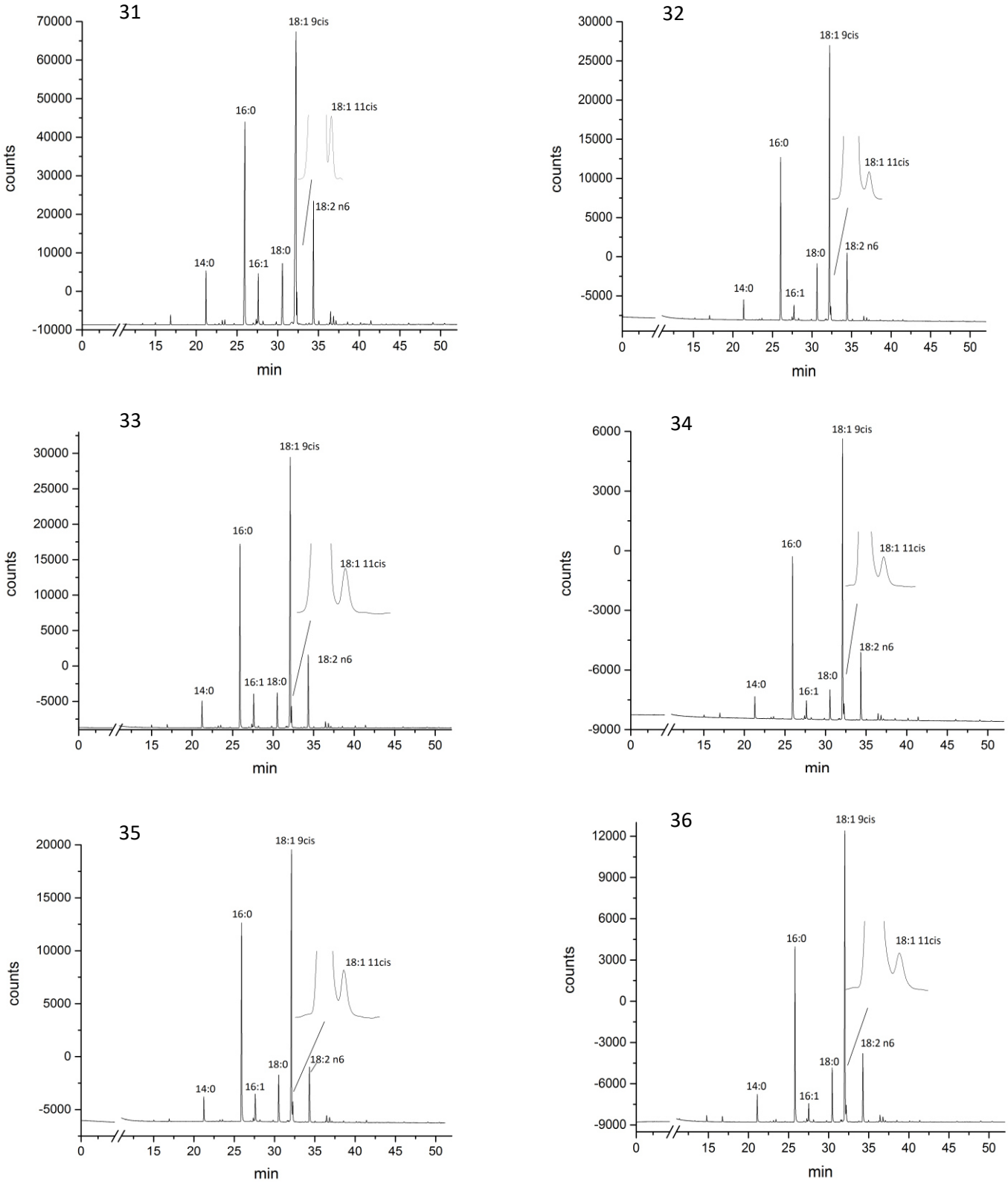

Figure 27 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 31, 32, 33, 34, 35 and 36.

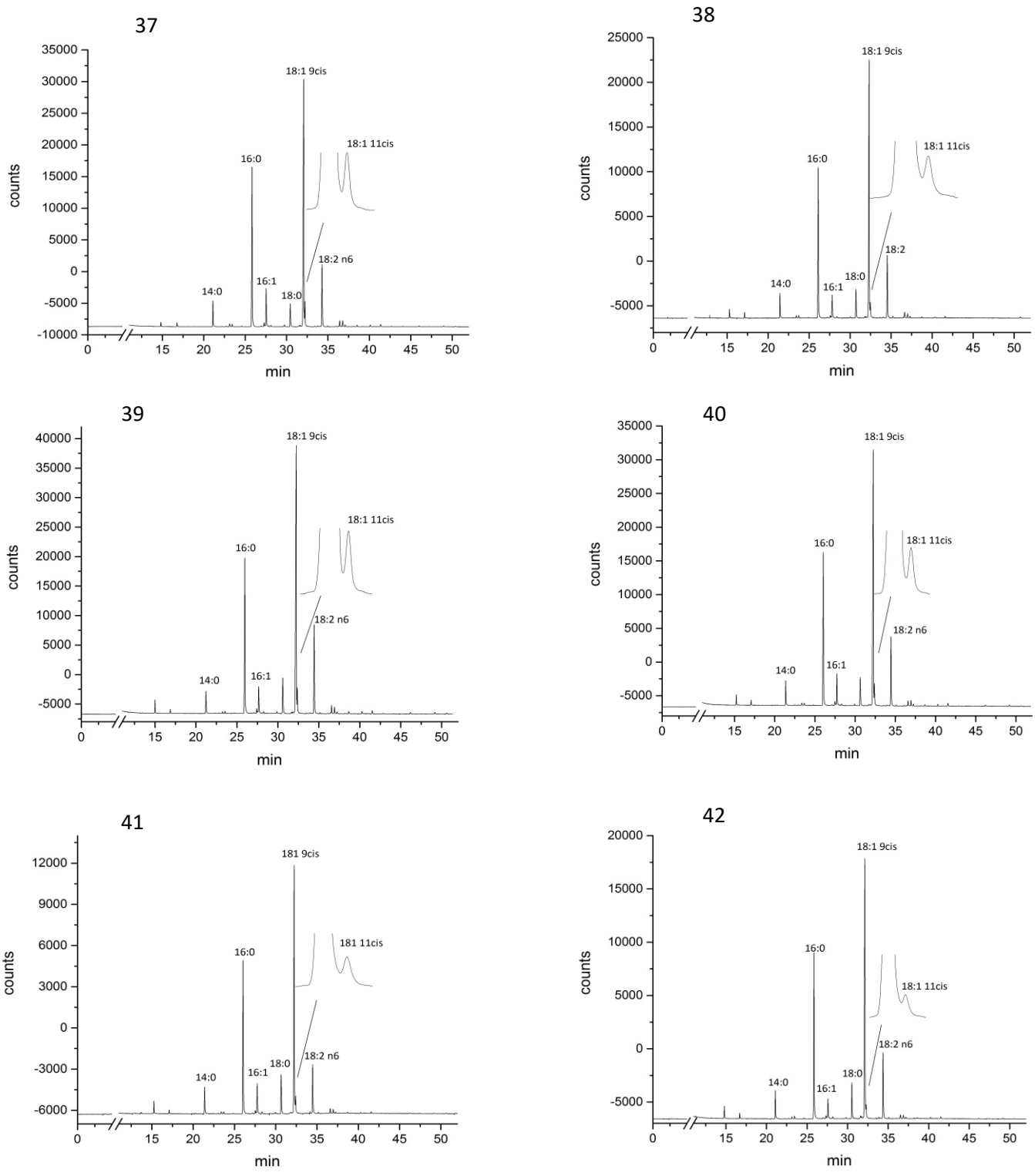

Figure 28 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 37, 38, 39, 40, 41 and 42.

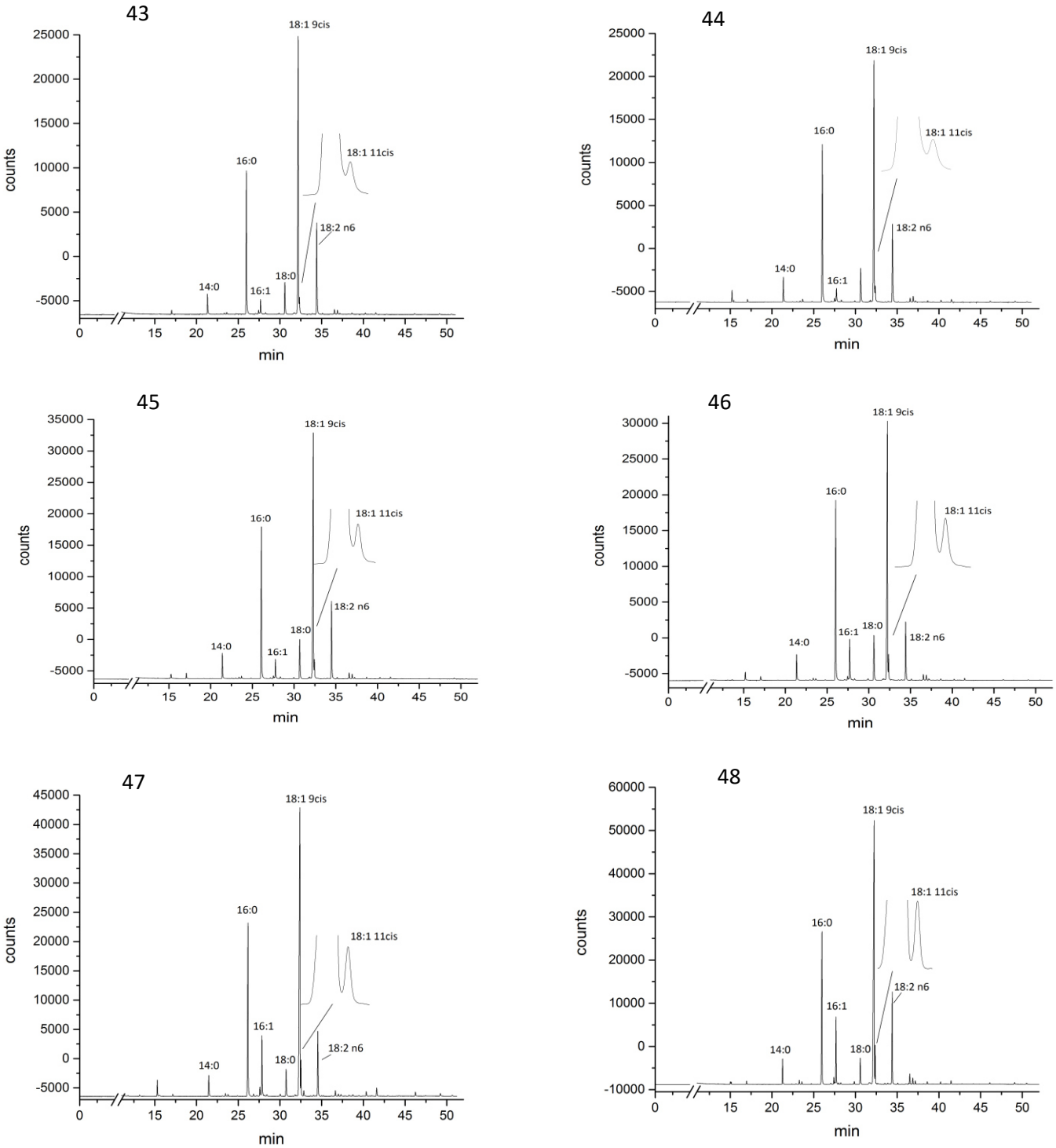

Figure 29 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 43, 44, 45, 46, 47 and 48.

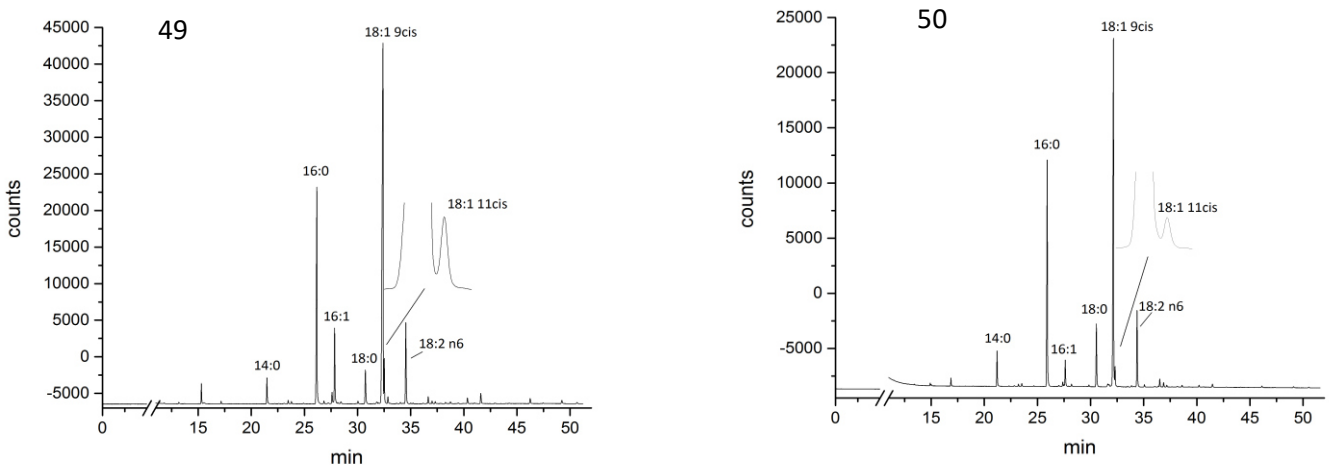

Figure 30 GC-FID chromatograms of fatty acids in breast adipose tissue of samples 49 and 50.

Table 34 Peak areas (counts) of pentadecanoic acid and undecanoic acid (IS) and ratios of pentadecanoic acid to IS. The pentadecanoic content calculated according to the calibration curve (µg oc) and the final level (mg/g adipose tissue) of pentadecanoic acid calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	Peak area 15:0	Peak area 11:0 (IS)	ratio	µg oc	mg/g
0	0.55	1156.57	2404.35	0.48	0.07	0.37
1	0.67	1341.37	576.59	2.33	0.29	1.31
2	0.70	502.02	1173.56	0.43	0.06	0.27
3	0.70	2291.11	3530.54	0.65	0.08	0.36
4	0.70	1275.08	581.30	2.19	0.28	1.19
5	0.70	1513.72	2158.33	0.70	0.09	0.39
6	0.70	1471.92	3376.07	0.44	0.06	0.28
7	0.70	2369.59	3912.94	0.61	0.08	0.34
8	0.70	978.54	1949.48	0.50	0.07	0.28
9	0.54	2058.08	3913.95	0.53	0.07	0.39
10	0.70	2089.39	1036.28	2.02	0.25	1.08
11	0.70	1635.31	2176.48	0.75	0.10	0.41
12	0.70	2836.78	2783.61	1.02	0.13	0.56
13	0.70	1426.16	1992.85	0.72	0.09	0.40
14	0.70	864.93	1297.25	0.67	0.09	0.37
15	0.70	2196.78	3129.26	0.70	0.09	0.40
16	0.70	950.72	1328.44	0.72	0.09	0.40
17	0.70	1107.67	2006.91	0.55	0.07	0.31
18	0.70	1085.27	1865.57	0.58	0.08	0.32
19	0.70	1170.65	2971.76	0.39	0.06	0.25
20	0.70	3418.99	2964.48	1.15	0.15	0.63
21	0.70	2505.59	2424.43	1.03	0.13	0.56
22	0.70	2867.94	1717.81	1.67	0.21	0.91
24	0.70	1110.42	1601.45	0.69	0.09	0.38
25	0.70	1774.22	2481.13	0.72	0.09	0.40
26	0.70	1182.22	1354.73	0.87	0.11	0.49
27	0.70	1542.83	659.32	2.34	0.29	1.26
28	0.70	680.34	1758.45	0.39	0.06	0.25
29	0.70	1089.10	1873.41	0.58	0.08	0.33
30	0.70	1588.78	1154.47	1.38	0.18	0.77
31	0.70	3479.03	1680.97	2.07	0.26	1.11
32	0.70	2316.16	1347.42	1.72	0.22	0.95
33	0.70	1218.22	1749.07	0.70	0.09	0.38
34	0.70	1700.58	1707.29	1.00	0.13	0.54
35	0.70	513.72	495.22	1.04	0.13	0.57
36	0.70	1616.04	3746.80	0.43	0.06	0.27
37	0.70	1722.92	3126.68	0.55	0.07	0.31
38	0.70	2389.29	7159.55	0.33	0.05	0.22
39	0.70	1602.26	8293.57	0.19	0.03	0.12
40	0.70	1279.04	4004.97	0.32	0.05	0.20
41	0.70	2108.29	9482.56	0.22	0.03	0.14
42	0.70	1654.21	5394.17	0.31	0.04	0.19
43	0.70	1095.62	978.16	1.12	0.14	0.61
44	0.70	2490.44	8412.18	0.30	0.04	0.19
45	0.70	1647.74	7510.42	0.22	0.03	0.14
46	0.70	1093.06	8597.50	0.13	0.02	0.08
47	0.71	1488.46	11248.15	0.13	0.02	0.08
48	0.70	3281.47	14019.16	0.23	0.03	0.15
49	0.70	1537.74	7410.42	0.21	0.03	0.13
50	0.70	3033.77	16920.08	0.18	0.03	0.11

Table 35 Peak areas (counts) of elaidic acid and undecanoic acid (IS) and ratios of elaidic acid to IS. The elaidic content calculated according to the calibration curve (µg oc) and the final level (mg/g adipose tissue) of elaidic acid calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	Peak area 18:1 9t	Peak area 11:0 (IS)	ratio	µg oc	mg/g
0	0.55	1521.64	2404.35	0.63	0.11	0.58
1	0.67	1913.17	576.59	3.32	0.43	1.92
2	0.70	821.94	1173.56	0.70	0.11	0.47
3	0.70	2666.25	3530.54	0.76	0.12	0.50
4	0.70	1913.94	581.30	3.29	0.43	1.84
5	0.70	3426.11	2158.33	1.59	0.22	0.93
6	0.70	2126.55	3376.07	0.63	0.10	0.44
7	0.70	2887.40	3912.94	0.74	0.11	0.49
8	0.70	1494.51	1949.48	0.77	0.12	0.50
9	0.54	1647.81	3913.95	0.42	0.08	0.42
10	0.70	2574.73	1036.28	2.48	0.32	1.36
11	0.70	2002.76	2176.48	0.92	0.14	0.58
12	0.70	3548.41	2783.61	1.27	0.18	0.77
13	0.70	2083.15	1992.85	1.05	0.16	0.67
14	0.70	1219.16	1297.25	0.94	0.14	0.59
15	0.70	2179.00	3129.26	0.70	0.12	0.50
16	0.70	1654.13	1328.44	1.25	0.18	0.75
17	0.70	1764.86	2006.91	0.88	0.13	0.57
18	0.70	1361.42	1865.57	0.73	0.11	0.48
19	0.70	1606.61	2971.76	0.54	0.09	0.39
20	0.70	3438.40	2964.48	1.16	0.17	0.71
21	0.70	2998.70	2424.43	1.24	0.18	0.75
22	0.70	3310.49	1717.81	1.93	0.26	1.12
24	0.70	1458.43	1601.45	0.91	0.13	0.57
25	0.70	1443.98	2481.13	0.58	0.10	0.41
26	0.70	1500.06	1354.73	1.11	0.16	0.69
27	0.70	1494.31	659.32	2.27	0.30	1.29
28	0.70	1179.63	1758.45	0.67	0.11	0.47
29	0.70	1766.31	1873.41	0.94	0.14	0.61
30	0.70	1458.58	1154.47	1.26	0.18	0.79
31	0.70	2787.23	1680.97	1.66	0.23	0.97
32	0.70	2008.52	1347.42	1.49	0.20	0.90
33	0.70	908.86	1749.07	0.52	0.09	0.37
34	0.70	1574.57	1707.29	0.92	0.13	0.57
35	0.70	847.08	495.22	1.71	0.24	1.03
36	0.70	1508.78	3746.80	0.40	0.07	0.31
37	0.70	1416.12	3126.68	0.45	0.08	0.34
38	0.70	2002.74	7159.55	0.28	0.04	0.19
39	0.70	1337.98	8293.57	0.16	0.02	0.10
40	0.70	1054.57	4004.97	0.26	0.04	0.17
41	0.70	1992.16	9482.58	0.21	0.03	0.14
42	0.70	1993.94	5394.17	0.37	0.07	0.30
43	0.70	784.33	978.16	0.80	0.12	0.52
44	0.70	1496.29	8412.18	0.18	0.03	0.11
45	0.70	1606.47	7510.42	0.21	0.03	0.13
46	0.70	1191.24	8597.50	0.14	0.02	0.09
47	0.71	1760.60	11248.15	0.16	0.02	0.10
48	0.70	2100.80	14019.16	0.15	0.02	0.09
49	0.70	1401.47	7410.42	0.19	0.03	0.13
50	0.70	3236.46	16920.08	0.19	0.03	0.12

Table 36 Peak areas (counts) of ALA and undecanoic acid (IS) and ratios of ALA acid to IS. The ALA content calculated according to the calibration curve (µg oc) and the final level (mg/g adipose tissue) of ALA calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	Peak area 18:3 n3	Peak area 11:0 (IS)	ratio	µg oc	mg/g
0	0.55	2761.27	2404.35	1.15	0.20	1.09
1	0.67	2857.80	576.59	4.96	0.63	2.85
2	0.70	1070.90	1173.56	0.91	0.16	0.70
3	0.70	4622.59	3530.54	1.31	0.21	0.90
4	0.70	3227.10	581.30	5.55	0.71	3.06
5	0.70	3170.25	2158.35	1.47	0.23	0.97
6	0.70	3741.36	3376.07	1.11	0.19	0.80
7	0.70	5217.27	3912.94	1.33	0.21	0.91
8	0.70	2581.43	1949.48	1.32	0.21	0.91
9	0.54	3028.55	3913.95	0.78	0.15	0.82
10	0.70	3684.00	1036.28	3.56	0.47	2.01
11	0.70	2629.77	2176.48	1.21	0.20	0.84
12	0.70	6631.51	2783.61	2.38	0.34	1.44
13	0.70	2433.30	1992.85	1.22	0.20	0.86
14	0.70	1015.07	1297.25	0.78	0.15	0.63
15	0.70	5485.63	3129.26	1.75	0.26	1.13
16	0.70	4328.13	1328.44	3.26	0.44	1.89
17	0.70	2037.68	2006.91	1.02	0.18	0.75
18	0.70	2441.13	1865.57	1.31	0.21	0.89
19	0.70	3380.93	2971.76	1.14	0.19	0.81
20	0.70	5666.36	2964.48	1.91	0.28	1.20
21	0.70	6292.09	2424.43	2.60	0.36	1.55
22	0.70	9526.14	1717.81	5.55	0.71	3.05
24	0.70	2491.24	1601.45	1.56	0.24	1.01
25	0.70	3034.17	2481.13	1.22	0.20	0.85
26	0.70	2329.77	1354.73	1.72	0.26	1.13
27	0.70	3283.95	659.32	4.98	0.64	2.75
28	0.70	2208.64	1758.45	1.26	0.20	0.88
29	0.70	1737.82	1873.41	0.93	0.17	0.71
30	0.70	3046.76	1154.47	2.64	0.37	1.59
31	0.70	6478.35	1680.97	3.85	0.51	2.16
32	0.70	4289.23	1347.42	3.18	0.44	1.88
33	0.70	2234.39	1749.07	1.28	0.20	0.87
34	0.70	4536.50	1707.29	2.66	0.36	1.55
35	0.70	1293.29	495.22	2.61	0.36	1.54
36	0.70	3540.52	3746.80	0.94	0.17	0.71
37	0.70	4890.17	3126.68	1.56	0.24	1.03
38	0.70	4177.12	7159.55	0.58	0.09	0.39
39	0.70	4991.20	8293.57	0.60	0.09	0.38
40	0.70	3193.37	4004.97	0.80	0.15	0.64
41	0.70	4968.95	9482.56	0.52	0.08	0.33
42	0.70	2076.64	5394.17	0.38	0.06	0.24
43	0.70	2604.94	978.16	2.66	0.37	1.58
44	0.70	5453.96	8412.18	0.65	0.13	0.56
45	0.70	3240.04	7510.42	0.43	0.06	0.27
46	0.70	3532.56	8597.50	0.41	0.06	0.26
47	0.71	1873.06	11248.15	0.17	0.02	0.10
48	0.70	7402.60	14019.16	0.53	0.08	0.33
49	0.70	3130.04	7410.42	0.42	0.06	0.27
50	0.70	8391.89	16920.08	0.50	0.07	0.31

Table 37 Peak areas (counts) of DHA and undecanoic acid (IS) and ratios of DHA acid to IS. The DHA content calculated according to the calibration curve (µg oc) and the final level (mg/g adipose tissue) of DHA calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	Peak area 22:6 n3	Peak area 11:0 (IS)	ratio	µg oc	mg/g
0	0.55	690.74	2404.35	0.29	0.08	0.42
1	0.67	316.15	576.59	0.55	0.13	0.57
2	0.70	260.31	1173.56	0.22	0.06	0.25
3	0.70	838.93	3530.54	0.24	0.06	0.27
4	0.70	596.47	581.30	1.03	0.23	0.99
5	0.70	503.15	2158.33	0.23	0.06	0.26
6	0.70	1124.22	3376.07	0.33	0.08	0.35
7	0.70	2310.66	3912.94	0.59	0.14	0.59
8	0.70	683.09	1949.48	0.35	0.09	0.37
9	0.54	388.02	3913.95	0.10	0.03	0.16
10	0.70	1488.47	1036.28	1.44	0.32	1.38
11	0.70	836.68	2176.48	0.38	0.09	0.39
12	0.70	1585.94	2783.61	0.57	0.13	0.57
13	0.70	645.01	1992.85	0.32	0.08	0.35
14	0.70	224.75	1297.25	0.17	0.05	0.21
15	0.70	1412.18	3129.26	0.45	0.11	0.46
16	0.70	350.59	1328.44	0.26	0.07	0.29
17	0.70	156.38	2006.91	0.08	0.02	0.09
18	0.70	535.12	1865.57	0.29	0.07	0.31
19	0.70	991.95	2971.76	0.33	0.08	0.36
20	0.70	1463.28	2964.48	0.49	0.12	0.50
21	0.70	676.92	2424.43	0.28	0.07	0.30
22	0.70	1698.04	1717.81	0.99	0.22	0.96
24	0.70	549.99	1601.45	0.34	0.08	0.36
25	0.70	947.10	2481.13	0.38	0.09	0.40
26	0.70	342.53	1354.73	0.25	0.07	0.28
27	0.70	1263.87	659.32	1.92	0.42	1.80
28	0.70	234.39	1758.45	0.13	0.04	0.16
29	0.70	1491.84	1873.41	0.80	0.19	0.79
30	0.70	340.50	1154.47	0.29	0.08	0.32
31	0.70	1505.49	1680.97	0.90	0.20	0.86
32	0.70	1307.05	1347.42	0.97	0.22	0.96
33	0.70	290.73	1749.07	0.17	0.05	0.20
34	0.70	933.58	1707.29	0.55	0.12	0.53
35	0.70	240.37	495.22	0.49	0.11	0.49
36	0.70	660.11	3746.80	0.18	0.05	0.21
37	0.70	630.54	3126.68	0.20	0.05	0.23
38	0.70	371.78	7159.55	0.05	0.01	0.06
39	0.70	1353.05	8293.57	0.16	0.05	0.20
40	0.70	500.37	4004.97	0.12	0.03	0.15
41	0.70	952.95	9482.56	0.10	0.03	0.12
42	0.70	371.00	5394.17	0.07	0.02	0.08
43	0.70	354.60	978.16	0.36	0.09	0.38
44	0.70	747.40	8412.18	0.09	0.02	0.10
45	0.70	456.78	7510.42	0.06	0.02	0.07
46	0.70	365.66	8597.50	0.04	0.01	0.05
47	0.71	1261.58	11248.15	0.11	0.03	0.13
48	0.70	493.44	14019.16	0.04	0.01	0.04
49	0.70	445.78	7410.42	0.06	0.02	0.07
50	0.70	3808.14	16920.08	0.23	0.06	0.25

Table 38 Accuracies of ChOL quantification method in breast adipose tissue samples.

Women	Accuracy (%)	Accuracy Average (%)	Standard deviation
Low-1	98.0	99.1	10.1
Low-2	109.7		
Low-3	89.6		
Medium-1	99.9	87.0	11.2
Medium-2	79.5		
Medium-3	81.7		
High-1	101.52	103.4	4.6
High-2	99.96		
High-3	108.63		

Table 39 Peak areas (counts) of ChOL and 5α-ChAN-3β-ol (IS) and ratios of ChOL to IS. The ChOL content calculated according to the calibration curve (µg oc) and the final level (mg/g adipose tissue) of ChOL calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	peak area ChOL	peak area IS	ratio ChOL/ IS	content oc (µg)	ChOL content (mg/g)
1	0.13	34,751,693	19,976,782	1.74	63.22	0.89
2	0.11	20,581,939	12,517,716	1.64	59.00	0.97
3	0.11	28,150,057	12,457,530	2.26	86.20	1.43
4	0.11	20,927,856	15,495,003	1.35	46.03	0.75
5	0.11	30,467,277	17,407,105	1.75	63.69	1.04
6	0.11	36,357,089	16,793,879	2.16	82.01	1.36
7	0.11	34,072,003	14,545,053	2.34	89.86	1.41
8	0.11	13,655,671	7,444,675	1.83	67.40	1.08
9	0.11	28,343,813	12,224,498	2.32	88.80	1.45
10	0.11	25,750,211	16,598,993	1.55	54.90	0.85
11	0.12	37,058,311	18,235,837	2.03	76.14	1.09
12	0.11	31,125,357	19,070,934	1.63	58.47	1.00
13	0.11	34,522,084	19,377,817	1.78	65.07	1.03
14	0.11	27,354,465	11,745,228	2.33	89.26	1.46
15	0.11	16,189,664	10,954,762	1.48	51.65	0.85
16	0.10	27,431,132	19,278,248	1.42	49.22	0.83
17	0.10	18,001,105	10,773,853	1.67	60.18	1.04
18	0.10	17,036,768	8,075,381	2.11	79.57	1.35
19	0.10	20,845,604	11,938,467	1.75	63.50	1.08
20	0.11	25,667,874	15,092,403	1.70	61.50	0.99
21	0.10	28,537,969	18,317,169	1.56	55.19	0.94
22	0.11	26,979,514	20,191,121	1.34	45.39	0.73
24	0.10	33,781,643	15,428,332	2.19	83.10	1.41
25	0.10	33,690,824	15,326,245	2.20	83.48	1.41
26	0.10	35,525,330	15,356,291	2.31	88.57	1.49
27	0.11	32,738,640	18,999,191	1.72	62.49	1.00
28	0.10	30,206,458	16,349,229	1.85	67.99	1.14
29	0.11	28,089,929	12,029,224	2.34	89.53	1.49
30	0.11	16,136,841	10,926,481	1.48	51.61	0.85
31	0.11	18,191,754	11,225,464	1.62	57.96	0.96
32	0.10	30,314,614	16,546,429	1.83	67.30	1.14
33	0.11	52,956,583	22,499,535	2.35	90.35	1.50
34	0.11	22,740,033	9,687,211	2.35	90.07	1.42
35	0.11	16,951,894	12,618,963	1.34	46.92	0.74
36	0.11	28,060,084	12,788,688	2.19	83.30	1.38
37	0.10	31,735,958	20,455,351	1.55	54.90	0.92
38	0.10	16,153,009	9,887,729	1.63	58.53	0.99
39	0.11	41,213,711	17,531,288	2.35	90.22	1.43
40	0.12	23,906,259	10,157,856	2.35	90.34	1.37
41	0.11	8,143,870	5,056,696	1.61	57.51	0.96
42	0.11	23,620,370	11,710,802	2.02	75.47	1.24
43	0.11	20,907,298	15,808,193	1.32	44.79	0.73
44	0.10	19,534,299	11,185,143	1.75	63.52	1.10
45	0.10	24,023,881	14,220,496	1.69	61.00	1.06
46	0.10	31,197,146	17,240,064	1.81	66.31	1.13
47	0.11	19,953,167	11,121,927	1.79	65.62	1.04
48	0.11	20,736,691	10,266,378	2.02	75.60	1.26
49	0.10	12,895,281	6,686,214	1.93	71.57	1.21
50	0.10	21,344,927	12,322,128	1.73	62.89	1.07

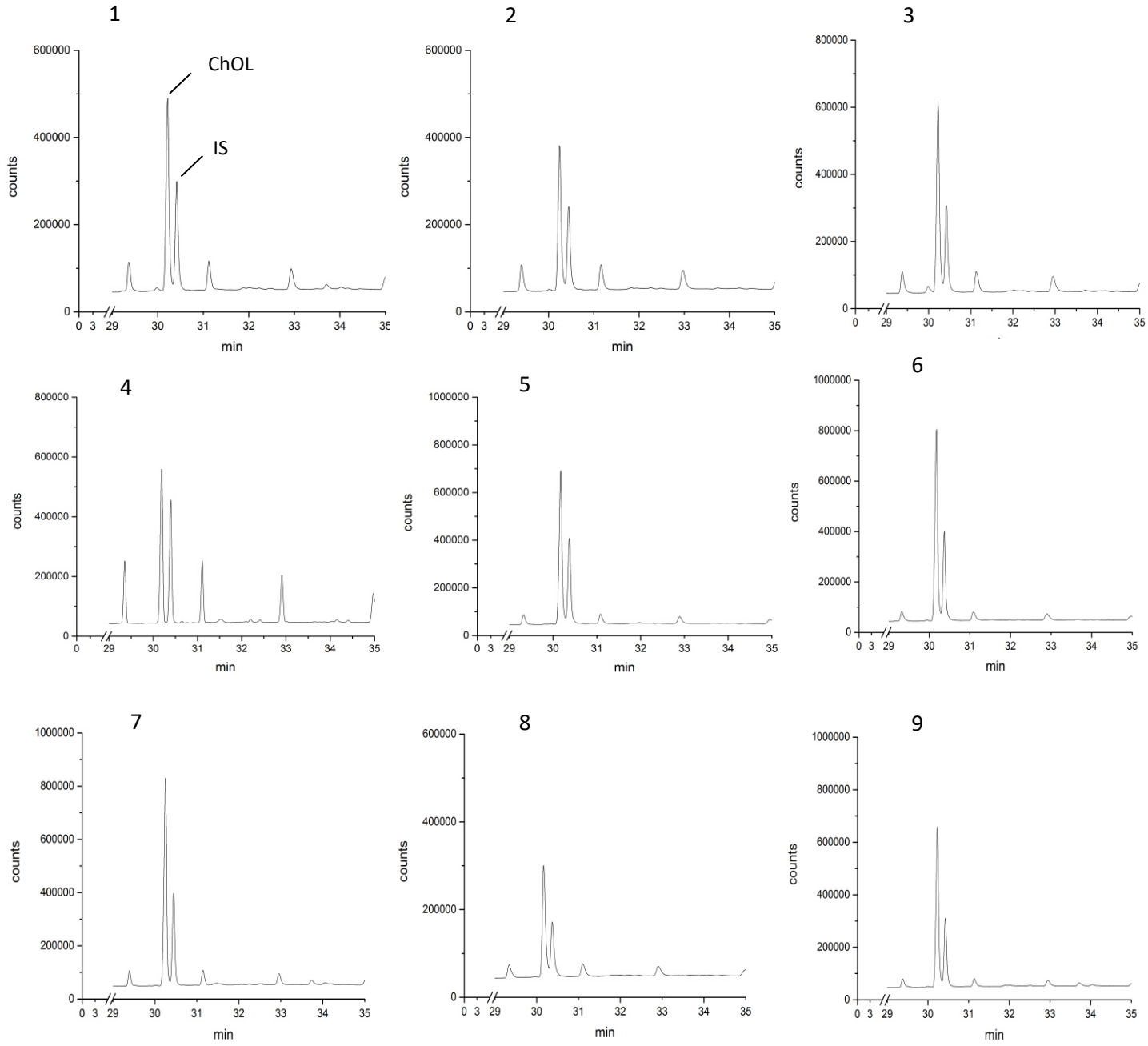

Figure 31 GC-FID chromatograms of ChOL and 5α-ChAN-3β-ol (IS) in breast adipose tissue of samples 1, 2, 3, 4, 5, 6, 7, 8 and 9. ChOL R_t: 30.23 min; 5α-ChAN-3β-ol (IS) R_t: 30.44 min.

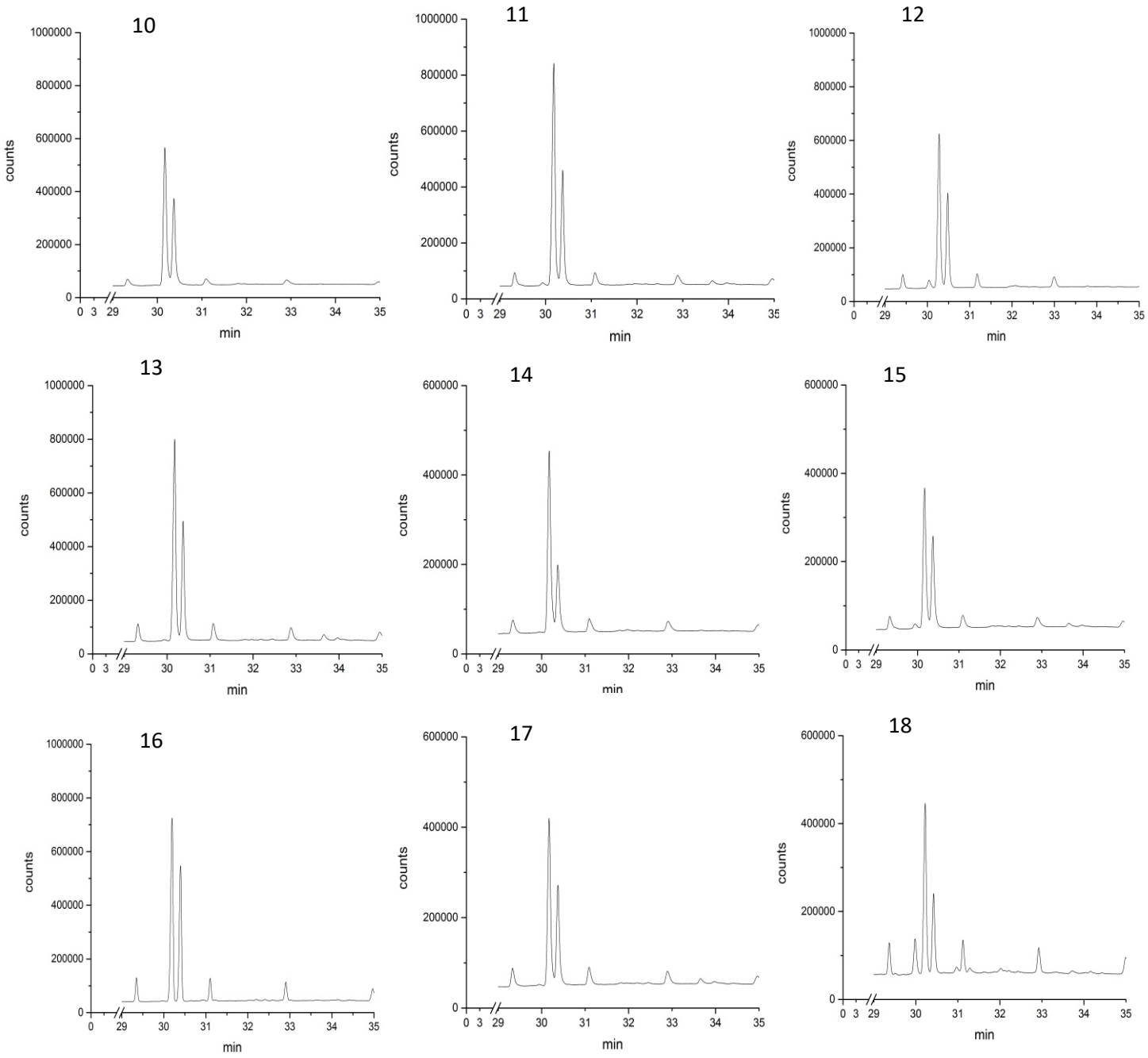

Figure 32 GC-FID chromatograms of ChOL and 5α-ChAN-3β-ol (IS) in breast adipose tissue of samples 10, 11, 12, 13, 14, 15, 16, 17 and 18. ChOL R_t: 30.23 min; 5α-ChAN-3β-ol (IS) R_t: 30.44 min.

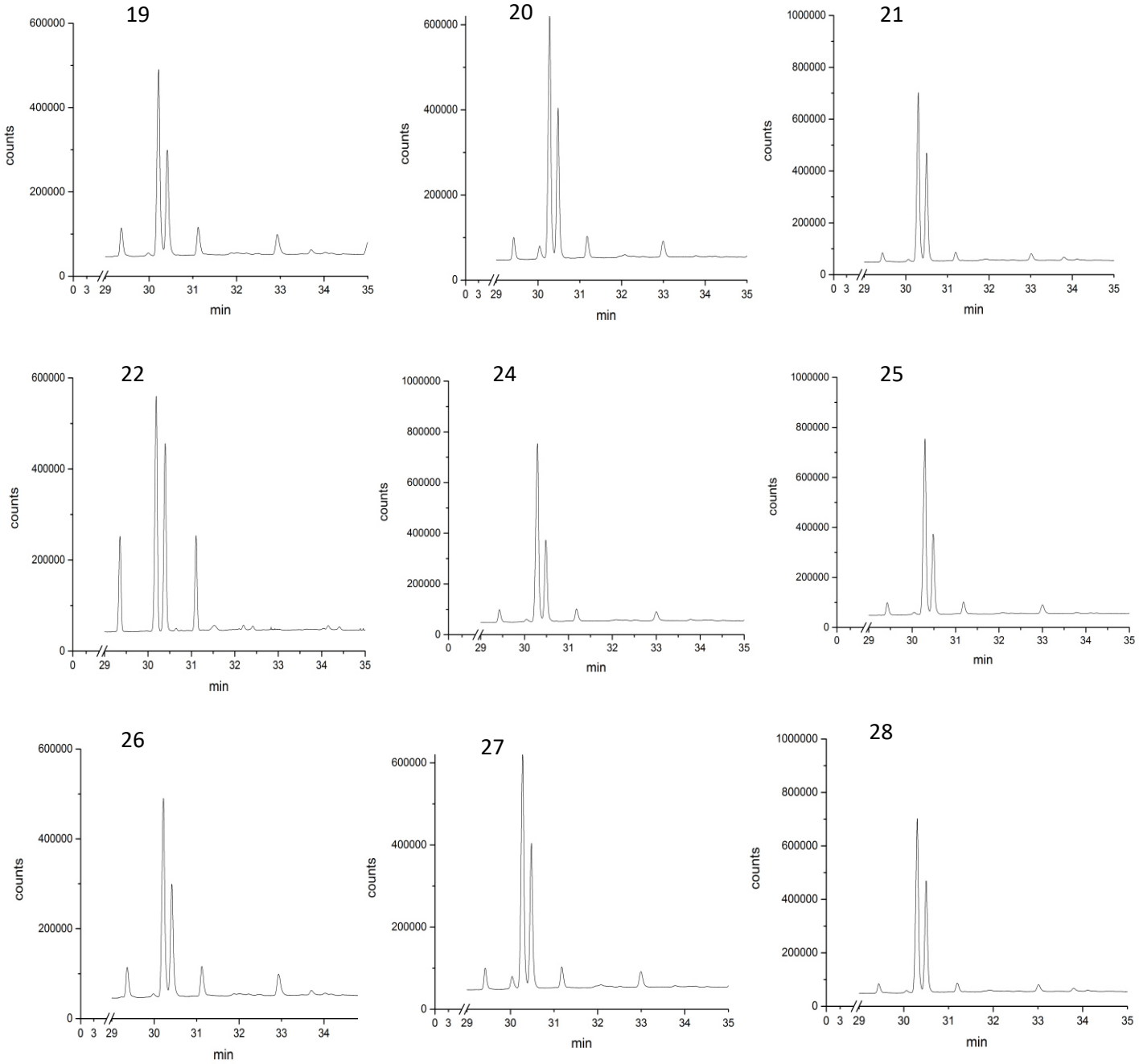

Figure 33 GC-FID chromatograms of ChOL and 5α-ChAN-3β-ol (IS) in breast adipose tissue of samples 19, 20, 21, 22, 24, 25, 26, 27 and 28. ChOL R_t: 30.23 min; 5α-ChAN-3β-ol (IS) R_t: 30.44 min.

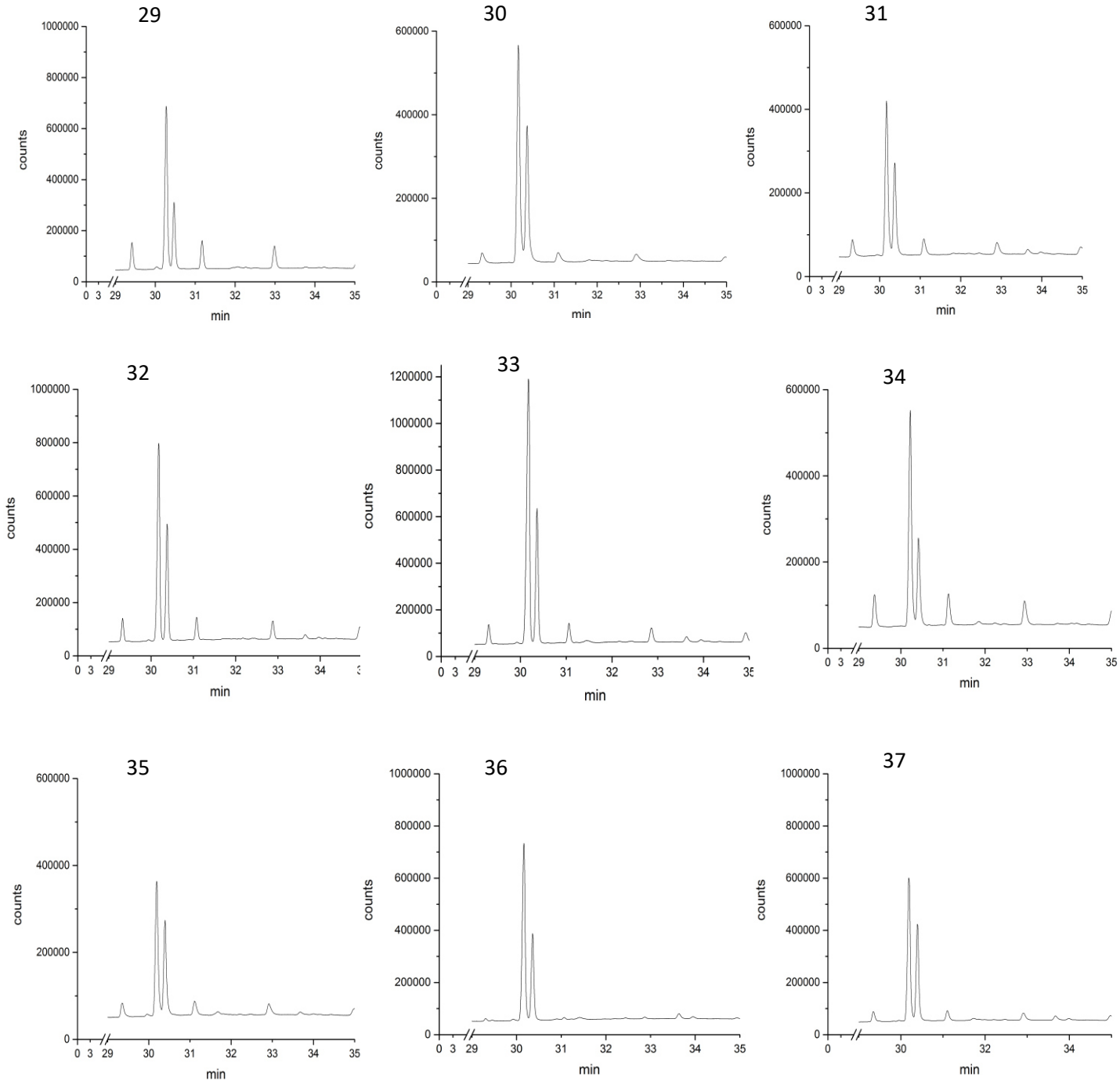

Figure 34 GC-FID chromatograms of ChOL and 5α-ChAN-3β-ol (IS) in breast adipose tissue of samples 29, 30, 31, 32, 33, 34, 35, 36 and 37. ChOL R_t: 30.23 min; 5α-ChAN-3β-ol (IS) R_t: 30.44 min.

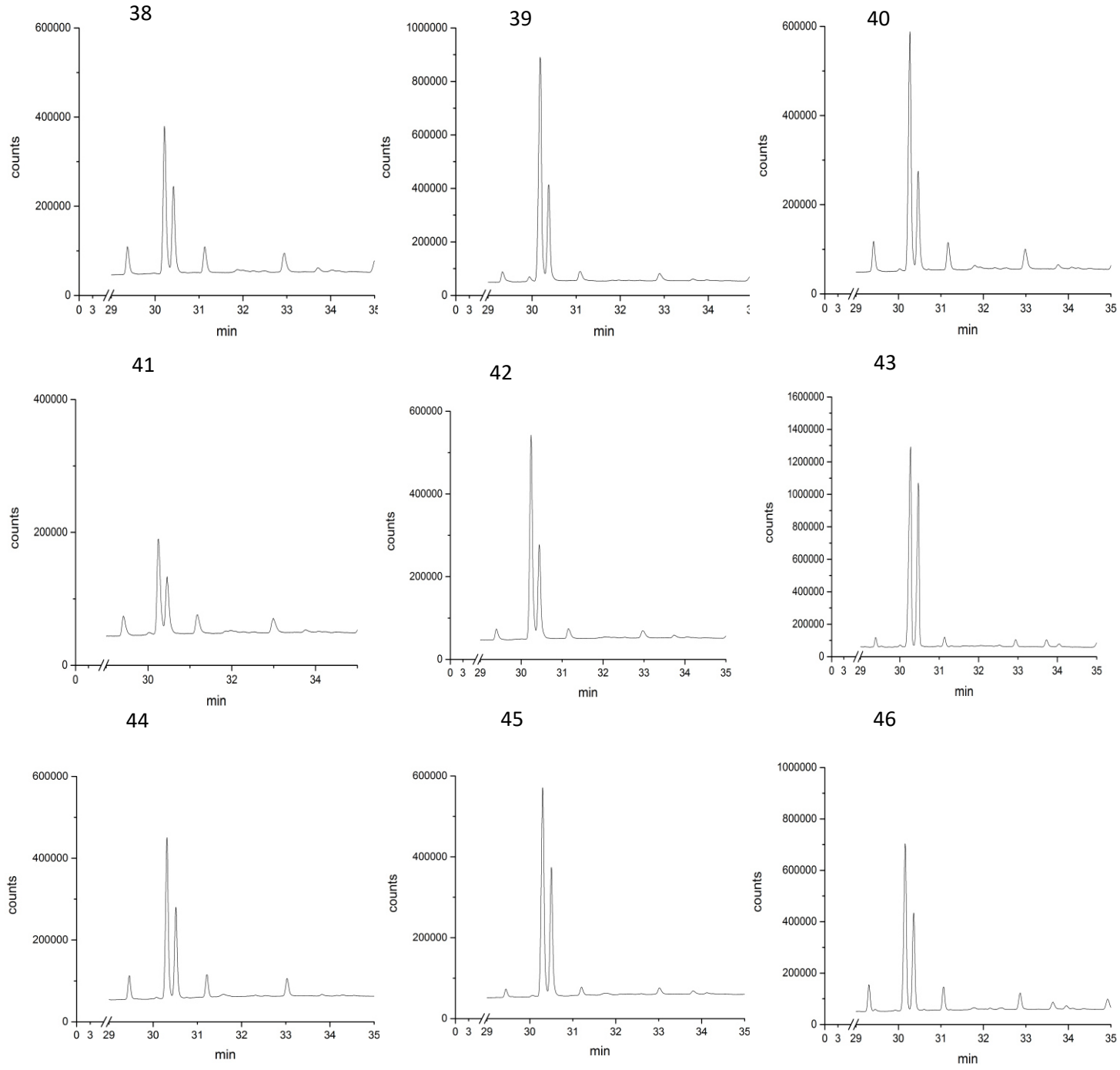

Figure 35 GC-FID chromatograms of ChOL and 5α-ChAN-3β-ol (IS) in breast adipose tissue of samples 38, 39, 40, 41, 42, 43, 44, 45 and 46. ChOL R_t: 30.23 min; 5α-ChAN-3β-ol (IS) R_t: 30.44 min.

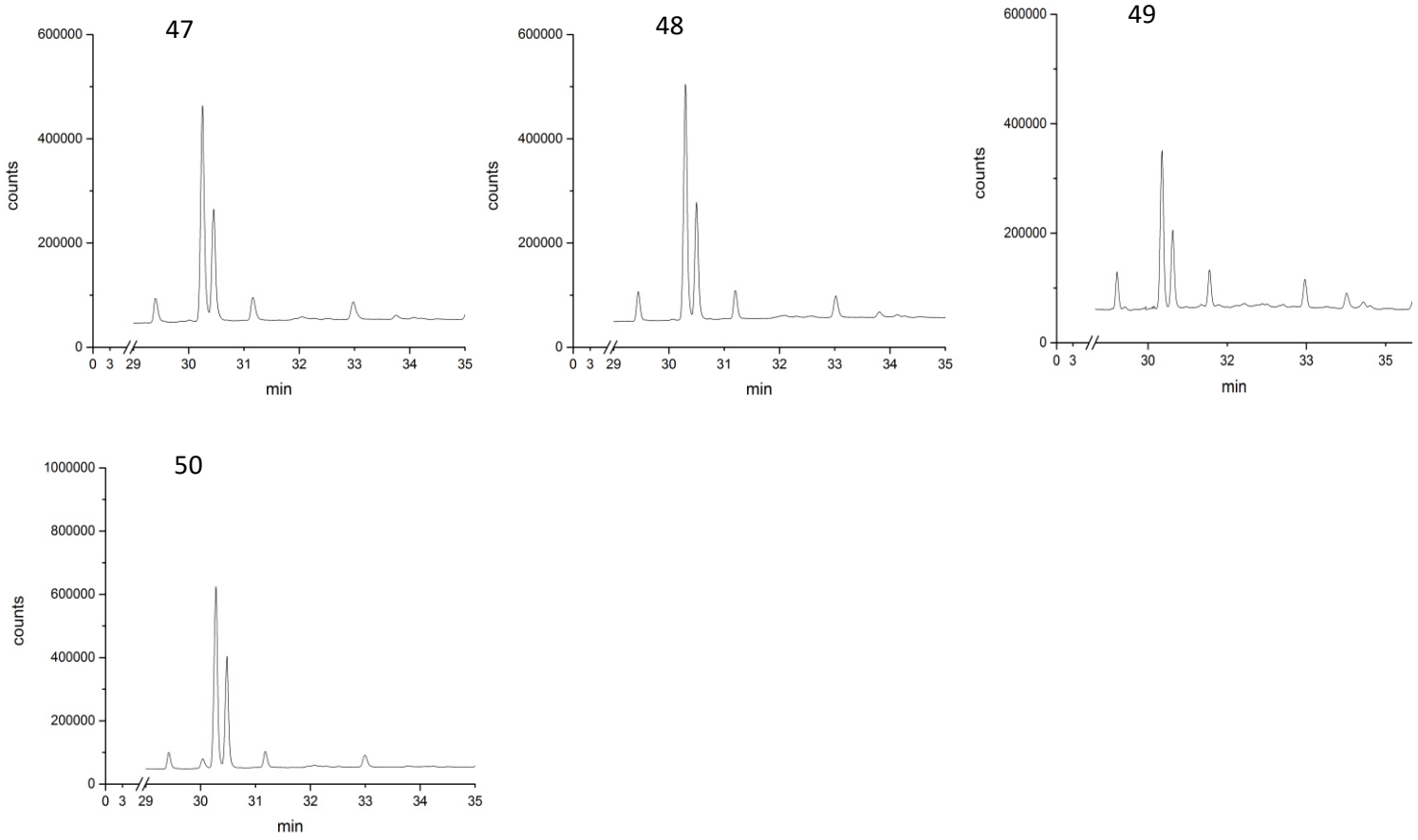

Figure 36 GC-FID chromatograms of ChOL and 5α-ChAN-3β-ol (IS) in breast adipose tissue of samples 47, 48, 49 and 50. ChOL R_t: 30.23 min; 5α-ChAN-3β-ol (IS) R_t: 30.44 min.

Table 40 Peak areas (counts) of β-epoxy-ChOL and β-epoxy-D-ChOL (IS) and ratios of β-epoxy-ChOL to IS. The β-epoxy-ChOL content calculated according to the calibration curve (pg oc) and the final level (ng/g adipose tissue) of β-epoxy-ChOL calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	β-epoxy-ChOL peak area	β-epoxy-D-ChOL peak area	peak area β-epoxy/peak area IS	content oc (pg)	β-epoxy-ChOL (ng/g)
1	0.13	3,490,000	999,000	3.49	1393.04	269.87
2	0.11	1,196,000	1,726,000	0.69	314.97	74.29
3	0.11	907,495	1,216,000	0.75	339.23	80.61
4	0.11	4,700,000	1,560,000	3.01	1228.01	274.07
5	0.11	8,114,000	1,606,000	5.05	2684.05	624.20
6	0.10	1,223,000	1,299,000	0.94	427.95	101.41
7	0.11	698,751	1,074,000	0.65	295.73	66.43
8	0.11	1,833,000	552,453	3.32	1328.65	304.74
9	0.11	689,672	1,097,000	0.63	285.77	66.77
10	0.11	933,656	1,030,000	0.91	412.03	90.76
11	0.12	1,639,000	1,063,000	1.54	677.21	139.11
12	0.10	3,520,000	1,290,000	2.73	1112.52	263.79
13	0.11	1,293,000	416,298	3.11	1250.90	283.26
14	0.11	1,703,000	897,538	1.90	807.62	184.22
15	0.11	1,013,000	538,880	1.88	801.17	189.13
16	0.10	1,730,000	1,140,000	1.52	668.29	159.81
17	0.10	1,102,000	492,962	2.24	931.61	229.69
18	0.10	3,381,000	1,889,000	1.79	768.16	186.45
19	0.10	249,405	527,140	0.47	215.06	52.40
20	0.11	371,110	923,100	0.40	182.74	41.95
21	0.10	1,013,000	950,838	1.07	502.44	121.60
22	0.11	1,520,000	815,000	1.87	795.74	180.44
24	0.10	1,348,000	774,627	1.74	749.95	181.32
25	0.10	1,146,000	604,573	1.90	806.94	195.29
26	0.10	1,080,000	789,939	1.37	613.14	146.97
27	0.11	1,230,000	730,000	1.68	729.68	164.17
28	0.10	1,026,000	996,974	1.03	489.14	117.58
29	0.11	1,022,000	656,334	1.56	682.81	161.80
30	0.10	2,465,000	2,111,000	1.17	539.97	129.80
31	0.10	592,808	1,264,000	0.47	213.18	51.74
32	0.10	709,366	526,038	1.35	606.29	147.16
33	0.11	1,600,000	754,305	2.12	889.69	210.63
34	0.11	494,318	503,157	0.98	446.56	100.49
35	0.11	222,628	486,583	0.46	207.97	48.28
36	0.11	679,692	383,157	1.77	762.33	180.65
37	0.10	940,015	656,613	1.43	636.77	152.78
38	0.10	317,319	586,301	0.54	246.01	59.25
39	0.11	306,024	377,921	0.81	368.07	81.51
40	0.12	332,749	608,742	0.55	248.46	53.83
41	0.11	82,444	347,174	0.24	107.94	25.70
42	0.11	563,725	346,446	1.63	708.50	166.00
43	0.10	105,359	272,569	0.39	175.70	43.66
44	0.10	461,307	315,885	1.46	647.32	160.86
45	0.10	606,052	449,506	1.35	606.20	149.90
46	0.10	575,730	487,204	1.18	545.11	132.31
47	0.11	174,452	446,112	0.39	177.75	40.29
48	0.11	507,083	328,162	1.55	678.44	161.23
49	0.10	1,747,000	2,807,000	0.62	282.90	68.13
50	0.13	1,785,000	564,128	3.16	1272.25	291.80

Table 41 Peak areas (counts) of α-epoxy-ChOL and α-epoxy-D-ChOL (IS) and ratios of α-epoxy-ChOL to IS. The α-epoxy-ChOL content calculated according to the calibration curve (pg oc) and the final level (ng/g adipose tissue) of α-epoxy-ChOL calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	α-epoxy-ChOL peak area	α-epoxy-D-ChOL peak area	peak area α-epoxy/peak area IS	content oc (pg)	α-epoxy-ChOL (ng/g)
1	0.13	1,620,000	1,100,000	1.47	429.39	85.88
2	0.11	642,051	2,948,000	0.22	71.55	16.88
3	0.11	549,291	1,746,000	0.31	100.16	23.80
4	0.11	2,450,000	1,310,000	1.87	538.31	124.73
5	0.11	3,028,000	2,574,000	1.18	354.80	82.51
6	0.10	541,706	2,015,000	0.27	86.63	20.53
7	0.11	368,219	1,618,000	0.23	74.44	16.72
8	0.11	1,055,000	843,917	1.25	376.59	86.37
9	0.11	498,608	2,049,000	0.24	79.10	18.48
10	0.11	360,769	1,507,000	0.24	77.93	17.17
11	0.12	770,475	1,772,000	0.43	135.67	27.87
12	0.10	1,670,000	1,060,000	1.58	455.74	110.94
13	0.11	416,298	776,863	0.54	165.54	37.49
14	0.11	839,706	1,474,000	0.57	175.53	40.04
15	0.11	560,044	862,919	0.65	198.97	46.97
16	0.10	788,000	718,000	1.10	319.24	76.96
17	0.10	443,827	986,060	0.45	140.19	34.56
18	0.10	1,567,000	2,982,000	0.53	162.47	39.43
19	0.10	164,075	947,348	0.17	58.37	14.22
20	0.11	274,746	1,559,000	0.18	59.27	13.61
21	0.10	608,849	1,755,000	0.35	109.71	26.55
22	0.11	627,000	705,000	0.89	259.86	59.88
24	0.10	742,744	1,249,000	0.59	182.91	44.22
25	0.10	788,335	1,185,000	0.67	203.77	49.32
26	0.10	627,774	1,122,000	0.56	172.52	41.35
27	0.11	532,000	777,000	0.68	201.55	45.85
28	0.10	675,558	1,634,000	0.41	129.36	31.10
29	0.11	580,432	1,057,000	0.55	169.46	40.16
30	0.10	1,642,000	3,909,000	0.64	195.69	47.04
31	0.10	439,840	2,950,000	0.22	71.69	17.07
32	0.10	368,690	952,823	0.39	121.53	29.50
33	0.11	715,560	1,355,000	0.53	163.24	38.65
34	0.11	360,646	795,244	0.45	141.20	31.77
35	0.11	139,244	735,667	0.19	63.12	14.65
36	0.11	360,277	622,752	0.58	178.14	42.21
37	0.10	608,621	1,303,000	0.47	145.22	34.84
38	0.10	212,215	933,188	0.23	74.39	17.92
39	0.11	233,081	680,689	0.34	108.38	24.00
40	0.12	164,387	854,496	0.19	64.04	13.87
41	0.11	64,994	560,841	0.12	41.44	9.87
42	0.11	446,632	649,123	0.69	210.51	49.32
43	0.10	95,458	426,535	0.22	73.33	18.22
44	0.10	334,502	887,303	0.38	118.59	29.47
45	0.10	402,060	838,315	0.48	148.91	36.82
46	0.10	258,218	872,562	0.30	94.64	22.97
47	0.11	167,023	644,053	0.26	83.82	19.00
48	0.11	261,665	555,114	0.47	146.48	34.81
49	0.10	933,591	4,464,000.	0.21	68.99	16.61
50	0.13	781,216	1,102,000	0.71	216.67	49.69

Table 42 Peak areas (counts) of 7-O-ChOL and 7-O-D-ChOL (IS) and ratios of 7-O-ChOL to IS. The 7-O-ChOL content calculated according to the calibration curve (pg oc) and the final level (ng/g adipose tissue) of 7-O-ChOL calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	7-O-ChOL peak area	7-O-D-ChOL peak area	Peak area 7-O-/peak area IS	content oc (pg)	7-O-ChOL (ng/g)
1	0.13	1,580,000	1,130,000	1.40	110.31	220.62
2	0.11	146,892	1,760,000	0.08	6.77	15.97
3	0.11	77,429	1,236,000	0.06	4.85	11.53
4	0.11	1,780,000	2,730,000	0.65	43.37	100.48
5	0.11	320,216	1,932,000	0.17	14.37	33.42
6	0.10	141,556	1,601,000	0.09	7.23	17.13
7	0.11	58,474	1,158,000	0.05	3.73	8.38
8	0.11	73,615	757,774	0.10	8.04	18.43
9	0.11	291,008	2,139,000	0.14	11.63	27.17
10	0.11	155,050	1,679,000	0.09	7.59	16.73
11	0.12	212,016	2,213,000	0.10	7.91	16.25
12	0.10	1,790,000	2,400,000	0.75	51.78	126.05
13	0.11	83,644	715,143	0.12	9.87	22.34
14	0.11	194,127	970,432	0.20	17.54	40.00
15	0.11	104,934	1,040,000	0.10	8.38	19.79
16	0.10	795,000	1,980,000	0.40	20.89	50.37
17	0.10	97,390	736,326	0.13	11.28	27.81
18	0.10	689,246	1,192,000	0.58	36.75	89.19
19	0.10	145,137	2,242,000	0.06	4.61	11.23
20	0.11	76,693	1,484,000	0.05	3.84	8.81
21	0.10	98,023	1,453,000	0.07	5.30	12.82
22	0.11	902,000	1,200,000	0.75	52.31	120.52
24	0.10	151,878	1,380,000	0.11	9.23	22.31
25	0.10	268,499	3,426,000	0.08	6.30	15.25
26	0.10	144,974	2,083,000	0.07	5.49	13.17
27	0.11	704,000	1,310,000	0.54	33.08	75.26
28	0.10	97,670	1,317,000	0.07	5.91	14.22
29	0.11	73,529	921,780	0.08	6.43	15.24
30	0.10	586,516	1,072,000	0.55	33.96	81.62
31	0.10	299,536	472,990	0.63	41.69	99.25
32	0.10	105,192	1,201,000	0.09	7.15	17.36
33	0.11	124,121	1,077,000	0.12	9.71	22.98
34	0.11	157,733	2,551,000	0.06	4.78	10.75
35	0.11	40,001	787,221	0.05	3.76	8.72
36	0.11	59,697	678,791	0.09	7.19	17.03
37	0.10	122,937	956,181	0.13	10.94	26.24
38	0.10	107,731	1,063,000	0.10	8.42	20.29
39	0.11	83,609	1,314,000	0.06	4.94	10.94
40	0.12	117,335	1,214,000	0.10	7.99	17.31
41	0.11	51,294	891,049	0.06	4.38	10.43
42	0.11	162,249	1,504,000	0.11	9.03	21.15
43	0.10	52,200	559,665	0.09	7.68	19.08
44	0.10	153,502	2,220,000	0.07	5.45	13.55
45	0.10	58,276	386,309	0.15	13.00	32.14
46	0.10	59,016	818,603	0.07	5.72	13.89
47	0.11	31,078	647,020	0.05	3.50	7.94
48	0.11	48,112	468,382	0.10	8.55	20.32
49	0.10	452,299	724,760	0.62	40.86	98.41
50	0.11	446,595	391,232	1.14	87.28	200.18

Table 43 Peak areas (counts) of 7α-HO-ChOL and 7α-HO-D-ChOL (IS) and ratios of 7α-HO-ChOL to IS. The 7α-HO-ChOL content calculated according to the calibration curve (pg oc) and the final level (ng/g adipose tissue) of 7α-HO-ChOL calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	7α-HO-ChOL peak area	7α-D-HO-ChOL peak area	peak area 7α-HO /peak area IS	content oc (pg)	7α-HO-ChOL (ng/g)
1	0.13	4,590,000	1,700,000	2.70	11.71	111.29
2	0.11	28,966	494,026	0.06	0.40	3.78
3	0.11	83,331	613,258	0.14	0.71	6.71
4	0.11	2,720,000	2,540,000	1.07	4.63	42.92
5	0.11	471,020	1,462,000	0.32	1.44	13.41
6	0.10	190,357	1,095,000	0.17	0.86	8.11
7	0.11	257,472	2,452,000	0.11	0.58	5.25
8	0.11	108,849	453,975	0.24	1.12	10.24
9	0.11	148,314	1,322,000	0.11	0.61	5.72
10	0.11	279,505	1,479,000	0.19	0.92	8.07
11	0.12	568,178	2,008,000	0.28	1.29	10.57
12	0.10	3,370,000	2,240,000	1.50	6.51	63.43
13	0.11	159,745	515,991	0.31	1.39	12.61
14	0.11	440,904	1,181,000	0.37	1.60	14.61
15	0.11	183,820	608,961	0.30	1.36	12.86
16	0.10	1,940,000	2,110,000	0.92	3.97	38.32
17	0.10	281,358	887,049	0.32	1.42	14.02
18	0.10	304,396	1,204,000	0.25	1.17	11.34
19	0.10	182,999	2,082,000	0.09	0.52	5.04
20	0.11	224,593	3,097,000	0.07	0.46	4.19
21	0.10	353,220	2,448,000	0.14	0.74	7.16
22	0.11	1,720,000	1,390,000	1.24	5.35	49.35
24	0.10	826,302	2,439,000	0.34	1.51	14.58
25	0.10	392,425	1,993,000	0.20	0.95	9.17
26	0.10	202,858	1,477,000	0.14	0.71	6.83
27	0.11	1,940,000	1,900,000	1.02	4.41	40.17
28	0.10	312,846	2,160,000	0.14	0.74	7.13
29	0.11	390,178	1,662,000	0.23	1.10	10.39
30	0.10	197,868	542,297	0.36	1.61	15.49
31	0.10	114,948	409,771	0.28	1.28	12.16
32	0.10	183,221	1,012,000	0.18	0.88	8.59
33	0.11	294,088	1,277,000	0.23	1.08	10.22
34	0.11	185,364	1,910,000	0.10	0.55	5.23
35	0.11	128,134	953,323	0.13	0.70	6.50
36	0.11	172,627	650,656	0.27	1.22	11.54
37	0.10	494,539	1,778,000	0.28	1.27	12.17
38	0.10	179,622	2,076,000	0.09	0.51	4.92
39	0.11	271,884	2,024,000	0.13	0.70	5.94
40	0.12	368,604	3,344,000	0.11	0.60	5.24
41	0.11	263,384	3,027,000	0.09	0.51	4.89
42	0.11	308,283	1,571,000	0.20	0.94	8.85
43	0.10	160,580	590,766	0.27	1.24	12.36
44	0.10	150,456	873,550	0.17	0.85	8.45
45	0.10	91,937	660,733	0.14	0.72	7.11
46	0.10	202,175	795,675	0.25	1.17	11.39
47	0.11	190,633	1,495,000	0.13	0.67	6.10
48	0.11	124,099	691,388	0.18	0.88	8.35
49	0.10	402,483	552,525	0.73	3.14	30.29
50	0.13	195,652	488,769	0.40	1.72	15.77

Table 44 Peak areas (counts) of 7β-HO-ChOL and 7β-HO-D-ChOL (IS) and ratios of 7β-HO-ChOL to IS. The 7β-HO-ChOL content calculated according to the calibration curve (pg oc) and the final level (ng/g adipose tissue) of 7β-HO-ChOL calculated with regards to the dilution and initial weight of each sample.

women	weight (g)	7β-HO-ChOL peak area	7β-HO-D-ChOL peak area	peak area7β-HO/peak area IS	content oc (pg)	7β-HO-ChOL (ng/g)
1	0.13	5,450,000	5,860,000	0.93	5.70	45.61
2	0.11	141,843	2,382,000	0.06	0.30	2.85
3	0.11	184,821	2,677,000	0.07	0.37	3.47
4	0.11	4,330,000	9,150,000	0.47	2.95	27.31
5	0.11	931,092	7,670,000	0.12	0.71	6.63
6	0.10	387,115	6,285,000	0.06	0.32	2.99
7	0.11	281,275	5,221,000	0.05	0.26	2.38
8	0.11	259,808	2,369,000	0.11	0.63	5.82
9	0.11	159,387	3,629,000	0.04	0.20	1.86
10	0.11	552,062	7,869,000	0.07	0.37	3.28
11	0.12	1,061,000	10,560,000	0.10	0.57	4.71
12	0.10	4,970,000	8,040,000	0.62	3.82	37.20
13	0.11	272,421	2,592,000	0.11	0.60	5.47
14	0.11	637,252	4,287,000	0.15	0.99	9.02
15	0.11	384,296	3,568,000	0.11	0.62	5.87
16	0.10	2,090,000	7,000,000	0.30	1.89	18.25
17	0.10	336,579	4,227,000	0.08	0.44	4.29
18	0.10	485,306	5,367,000	0.09	0.51	4.92
19	0.10	192,954	4,593,000	0.04	0.19	1.81
20	0.11	258,970	8,651,000	0.03	0.11	0.97
21	0.10	435,779	8,974,000	0.05	0.23	2.22
22	0.11	2,130,000	4,730,000	0.45	2.81	25.88
24	0.10	1,342,000	8,212,000	0.16	1.08	10.42
25	0.10	450,675	7,226,000	0.06	0.32	3.11
26	0.10	260,263	5,717,000	0.05	0.21	2.01
27	0.11	2,470,000	5,240,000	0.47	2.94	26.71
28	0.10	572,534	7,713,000	0.07	0.40	3.84
29	0.11	412,338	6,316,000	0.07	0.34	3.23
30	0.10	379,024	2,532,000	0.15	1.00	9.57
31	0.10	233,502	1,956,000	0.12	0.70	6.66
32	0.10	301,900	5,454,000	0.06	0.27	2.67
33	0.11	436,170	6,204,000	0.07	0.37	3.54
34	0.11	295,317	4,370,000	0.07	0.36	3.20
35	0.11	277,840	5,007,000	0.06	0.28	2.56
36	0.11	244,289	3,493,000	0.07	0.37	3.52
37	0.10	596,985	5,918,000	0.10	0.58	5.53
38	0.10	145,907	4,272,000	0.03	0.13	1.29
39	0.11	617,919	10,520,000	0.06	0.30	2.52
40	0.12	209,461	6,836,000	0.03	0.11	0.96
41	0.11	242,093	7,144,000	0.03	0.13	1.26
42	0.11	244,881	3,485,000	0.07	0.37	3.50
43	0.10	281,666	2,784,000	0.10	0.58	5.75
44	0.10	151,447	2,801,000	0.05	0.27	2.64
45	0.10	185,758	2,426,000	0.08	0.42	4.11
46	0.10	271,736	3,537,000	0.08	0.42	4.05
47	0.11	128,939	3,205,000	0.04	0.17	1.58
48	0.11	179,227	2,749,000	0.07	0.34	3.23
49	0.10	509,285	3,046,000	0.17	1.10	10.60
50	0.13	381,755	2,336,000	0.16	1.08	9.89

Table 45 Relative levels of oxyChOLs (ratios of oxyChOLs to total oxyChOLs).

women	7α-HO-ChOL/total	7β-HO-ChOL/total	7-O-ChOL/total	α-epoxy-ChOL/total	β-epoxy-ChOL/total
1	0.15	0.06	0.30	0.12	0.37
2	0.03	0.03	0.14	0.15	0.65
3	0.05	0.03	0.09	0.19	0.64
4	0.08	0.05	0.18	0.22	0.48
5	0.02	0.01	0.04	0.11	0.82
6	0.05	0.02	0.11	0.14	0.68
7	0.05	0.02	0.08	0.17	0.67
8	0.02	0.01	0.04	0.20	0.72
9	0.05	0.02	0.23	0.15	0.56
10	0.06	0.02	0.12	0.13	0.67
11	0.05	0.02	0.08	0.14	0.70
12	0.11	0.06	0.21	0.18	0.44
13	0.03	0.02	0.06	0.10	0.78
14	0.05	0.03	0.14	0.14	0.64
15	0.04	0.02	0.07	0.16	0.64
16	0.11	0.05	0.15	0.22	0.46
17	0.05	0.01	0.09	0.11	0.74
18	0.03	0.01	0.27	0.12	0.56
19	0.06	0.02	0.13	0.17	0.62
20	0.06	0.01	0.13	0.20	0.60
21	0.04	0.01	0.08	0.16	0.71
22	0.11	0.06	0.28	0.14	0.41
24	0.05	0.04	0.08	0.16	0.66
25	0.03	0.01	0.06	0.18	0.72
26	0.03	0.01	0.06	0.20	0.70
27	0.11	0.08	0.21	0.13	0.47
28	0.04	0.02	0.08	0.18	0.68
29	0.05	0.01	0.07	0.17	0.70
30	0.05	0.03	0.29	0.17	0.46
31	0.07	0.04	0.53	0.09	0.28
32	0.04	0.01	0.08	0.14	0.72
33	0.04	0.01	0.08	0.14	0.74
34	0.03	0.02	0.07	0.21	0.66
35	0.08	0.03	0.11	0.18	0.60
36	0.05	0.01	0.07	0.17	0.71
37	0.05	0.02	0.11	0.15	0.66
38	0.05	0.01	0.20	0.17	0.57
39	0.05	0.02	0.09	0.19	0.65
40	0.06	0.01	0.19	0.15	0.59
41	0.09	0.02	0.20	0.19	0.49
42	0.04	0.01	0.09	0.20	0.67
43	0.12	0.06	0.19	0.18	0.44
44	0.04	0.01	0.06	0.14	0.75
45	0.03	0.02	0.14	0.16	0.65
46	0.06	0.02	0.08	0.12	0.72
47	0.08	0.02	0.11	0.25	0.54
48	0.04	0.01	0.09	0.15	0.71
49	0.14	0.05	0.44	0.07	0.30
50	0.03	0.02	0.35	0.09	0.51
median	0.05	0.02	0.11	0.16	0.65
min	0.02	0.01	0.04	0.07	0.28
max	0.15	0.08	0.53	0.25	0.82

Table 46 Relative levels of oxyChOLs (ratios of oxyChOLs to ChOL).

women	7α-HO-ChOL/ChOL	7β-HO-ChOL/ChOL	7-O-ChOL/ChOL	α-epoxy-ChOL/ChOL	β-epoxy-ChOL/ChOL	Total oxyChOL/ChOL
1	1.26E-04	5.15E-05	2.49E-04	9.70E-05	3.05E-04	8.29E-04
2	3.88E-06	2.93E-06	1.64E-05	1.73E-05	7.63E-05	1.17E-04
3	4.68E-06	2.42E-06	8.04E-06	1.66E-05	5.62E-05	8.80E-05
4	5.75E-05	3.66E-05	1.35E-04	1.67E-04	3.67E-04	7.63E-04
5	1.29E-05	6.39E-06	3.22E-05	7.96E-05	6.02E-04	7.33E-04
6	5.96E-06	2.20E-06	1.26E-05	1.51E-05	7.45E-05	1.10E-04
7	3.71E-06	1.68E-06	5.93E-06	1.18E-05	4.70E-05	7.02E-05
8	9.46E-06	5.38E-06	1.70E-05	7.98E-05	2.82E-04	3.93E-04
9	3.94E-06	1.28E-06	1.87E-05	1.27E-05	4.60E-05	8.26E-05
10	9.53E-06	3.88E-06	1.98E-05	2.03E-05	1.07E-04	1.61E-04
11	9.66E-06	4.31E-06	1.48E-05	2.55E-05	1.27E-04	1.81E-04
12	6.37E-05	3.73E-05	1.27E-04	1.11E-04	2.65E-04	6.04E-04
13	1.22E-05	5.31E-06	2.17E-05	3.63E-05	2.75E-04	3.50E-04
14	1.00E-05	6.18E-06	2.74E-05	2.74E-05	1.26E-04	1.97E-04
15	1.51E-05	6.88E-06	2.32E-05	5.50E-05	2.22E-04	3.45E-04
16	4.61E-05	2.20E-05	6.06E-05	9.26E-05	1.92E-04	4.14E-04
17	1.35E-05	4.14E-06	2.68E-05	3.33E-05	2.21E-04	2.99E-04
18	8.39E-06	3.64E-06	6.60E-05	2.92E-05	1.38E-04	2.45E-04
19	4.65E-06	1.67E-06	1.04E-05	1.31E-05	4.84E-05	7.82E-05
20	4.24E-06	9.85E-07	8.92E-06	1.38E-05	4.24E-05	7.04E-05
21	7.65E-06	2.38E-06	1.37E-05	2.84E-05	1.30E-04	1.82E-04
22	6.74E-05	3.54E-05	1.65E-04	8.18E-05	2.46E-04	5.96E-04
24	1.04E-05	7.41E-06	1.59E-05	3.14E-05	1.29E-04	1.94E-04
25	6.48E-06	2.20E-06	1.08E-05	3.49E-05	1.38E-04	1.92E-04
26	4.59E-06	1.35E-06	8.86E-06	2.78E-05	9.89E-05	1.42E-04
27	4.04E-05	2.68E-05	7.56E-05	4.61E-05	1.65E-04	3.54E-04
28	6.23E-06	3.36E-06	1.24E-05	2.72E-05	1.03E-04	1.52E-04
29	7.00E-06	2.17E-06	1.03E-05	2.70E-05	1.09E-04	1.55E-04
30	1.82E-05	1.12E-05	9.57E-05	5.52E-05	1.52E-04	3.32E-04
31	1.27E-05	6.96E-06	1.04E-04	1.78E-05	5.41E-05	1.95E-04
32	7.51E-06	2.33E-06	1.52E-05	2.58E-05	1.29E-04	1.80E-04
33	6.82E-06	2.36E-06	1.54E-05	2.58E-05	1.41E-04	1.91E-04
34	3.69E-06	2.26E-06	7.57E-06	2.24E-05	7.08E-05	1.07E-04
35	8.79E-06	3.46E-06	1.18E-05	1.98E-05	6.52E-05	1.09E-04
36	8.35E-06	2.55E-06	1.23E-05	3.05E-05	1.31E-04	1.85E-04
37	1.32E-05	6.00E-06	2.85E-05	3.78E-05	1.66E-04	2.51E-04
38	4.99E-06	1.31E-06	2.06E-05	1.82E-05	6.00E-05	1.05E-04
39	4.15E-06	1.76E-06	7.65E-06	1.68E-05	5.70E-05	8.73E-05
40	3.83E-06	7.00E-07	1.26E-05	1.01E-05	3.93E-05	6.66E-05
41	5.10E-06	1.31E-06	1.09E-05	1.03E-05	2.68E-05	5.44E-05
42	7.15E-06	2.83E-06	1.71E-05	3.98E-05	1.34E-04	2.01E-04
43	1.70E-05	7.91E-06	2.63E-05	2.51E-05	6.01E-05	1.36E-04
44	7.64E-06	2.39E-06	1.23E-05	2.67E-05	1.46E-04	1.95E-04
45	6.74E-06	3.89E-06	3.04E-05	3.49E-05	1.42E-04	2.18E-04
46	1.01E-05	3.59E-06	1.23E-05	2.04E-05	1.17E-04	1.64E-04
47	5.86E-06	1.52E-06	7.62E-06	1.82E-05	3.87E-05	7.19E-05
48	6.64E-06	2.57E-06	1.62E-05	2.77E-05	1.28E-04	1.81E-04
49	2.51E-05	8.79E-06	8.16E-05	1.38E-05	5.65E-05	1.86E-04
50	1.47E-05	9.22E-06	1.87E-04	4.63E-05	2.72E-04	5.29E-04
median	8.35E-06	3.46E-06	1.64E-05	2.72E-05	1.28E-04	1.85E-04
min	3.69E-06	7.00E-07	5.93E-06	1.01E-05	2.68E-05	5.44E-05
max	1.26E-04	5.15E-05	2.49E-04	1.67E-04	6.02E-04	8.29E-04

Table 47 The ranking of oxyChOLs (absolute and relative levels) in breast adipose tissue samples.

women	Ranking
1	β-epoxy>7-O>7α-HO>α-epoxy>7β-HO
2	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
3	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
4	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
5	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
6	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
7	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
8	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
9	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
10	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
11	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
12	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
13	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
14	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
15	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
16	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
17	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
18	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
19	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
20	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
21	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
22	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
24	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
25	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
26	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
27	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
28	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
29	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
30	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
31	7-O>β-epoxy>α-epoxy>7α-HO>7β-HO
32	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
33	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
34	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
35	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
36	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
37	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
38	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
39	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
40	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
41	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
42	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
43	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO
44	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
45	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
46	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
47	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
48	β-epoxy>α-epoxy>7-O>7α-HO>7β-HO
49	7-O>β-epoxy>7α-HO>α-epoxy>7β-HO
50	β-epoxy>7-O>α-epoxy>7α-HO>7β-HO

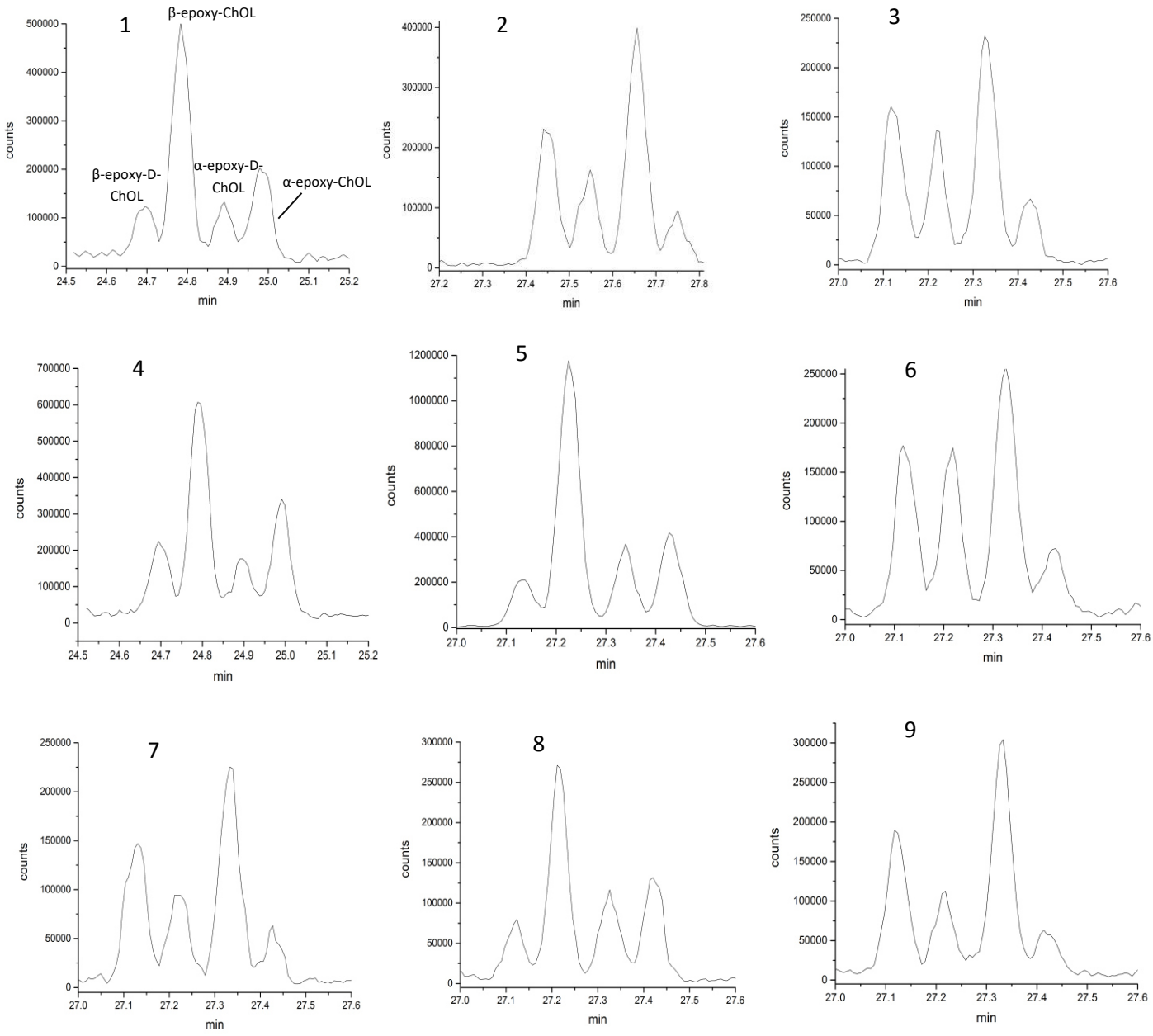

Figure 37 GC-MS/MS chromatograms of β-epoxy-ChOL, β-epoxy-D-ChOL (IS), α-epoxy-ChOL and α-epoxy-D-ChOL (IS) in breast adipose tissue of samples 1, 2, 3, 4, 5, 6, 7, 8 and 9. β-epoxy-ChOL R_t: 27.22, β-epoxy-D-ChOL (IS) R_t: 27.14, α-epoxy-ChOL R_t: 27.43 min; α-epoxy-D-ChOL (IS) R_t: 27.34 min.

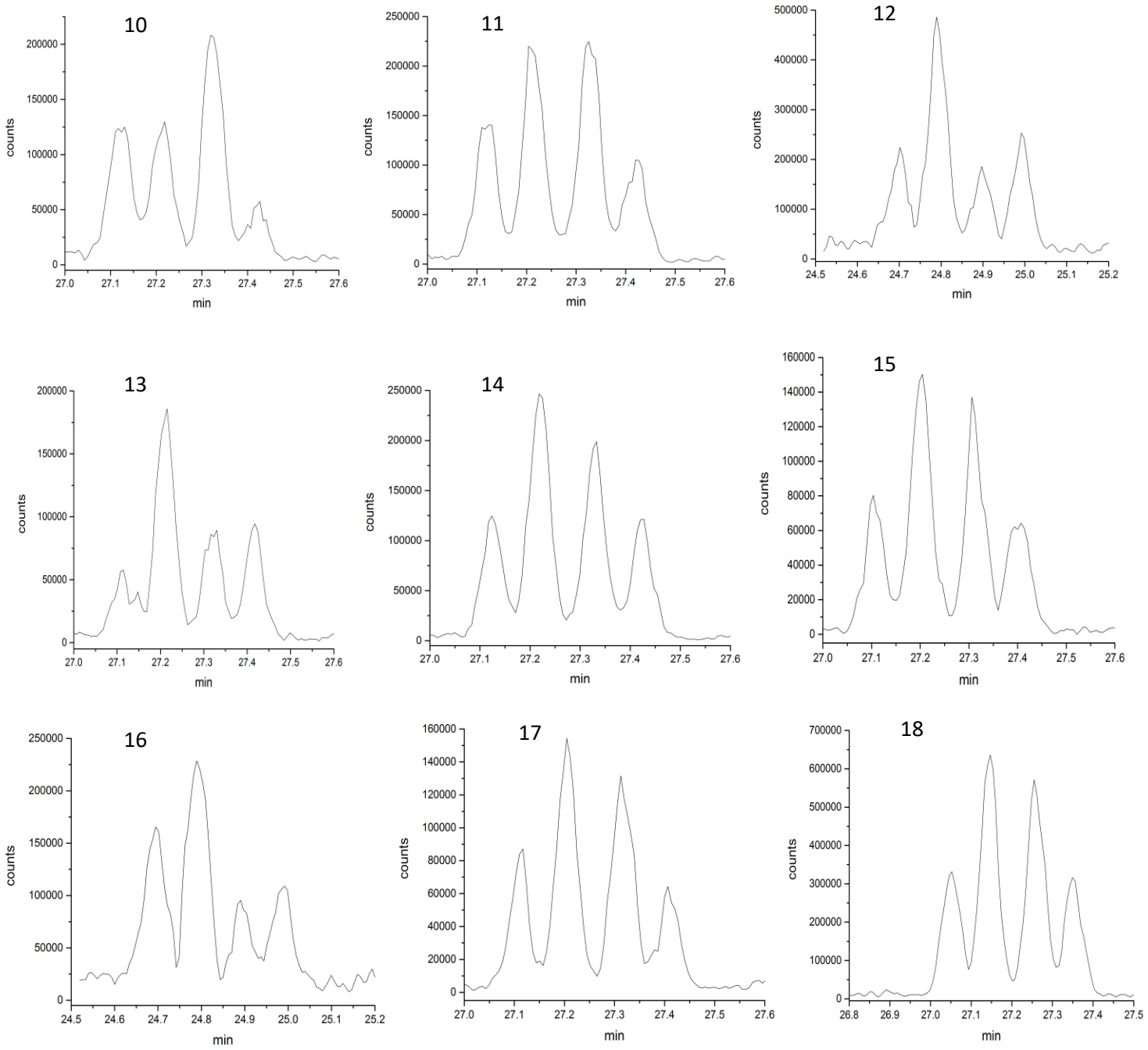

Figure 38 GC-MS/MS chromatograms of β-epoxy-ChOL, β-epoxy-D-ChOL (IS), α-epoxy-ChOL and α-epoxy-D-ChOL (IS) in breast adipose tissue of samples 10, 11, 12, 13, 14, 15, 16, 17 and 18. β-epoxy-ChOL R_t: 27.22, β-epoxy-D-ChOL (IS) R_t: 27.14, α-epoxy-ChOL R_t: 27.43 min; α-epoxy-D-ChOL (IS) R_t: 27.34 min.

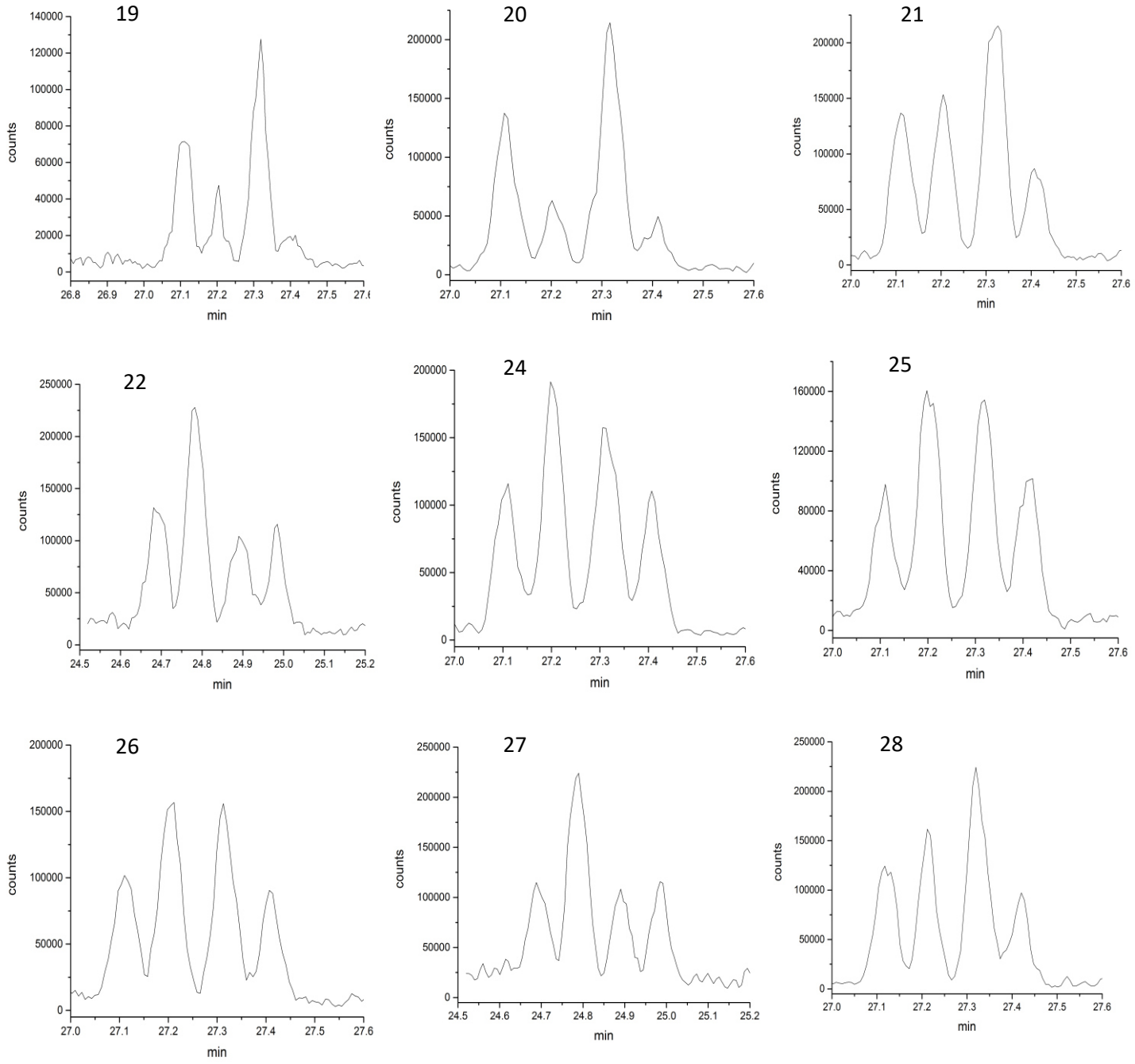

Figure 39 GC-MS/MS chromatograms of β-epoxy-ChOL, β-epoxy-D-ChOL (IS), α-epoxy-ChOL and α-epoxy-D-ChOL (IS) in breast adipose tissue of samples 19, 20, 21, 22, 24, 25, 26, 27 and 28. β-epoxy-ChOL R_t: 27.22, β-epoxy-D-ChOL (IS) R_t: 27.14, α-epoxy-ChOL R_t: 27.43 min; α-epoxy-D-ChOL (IS) R_t: 27.34 min.

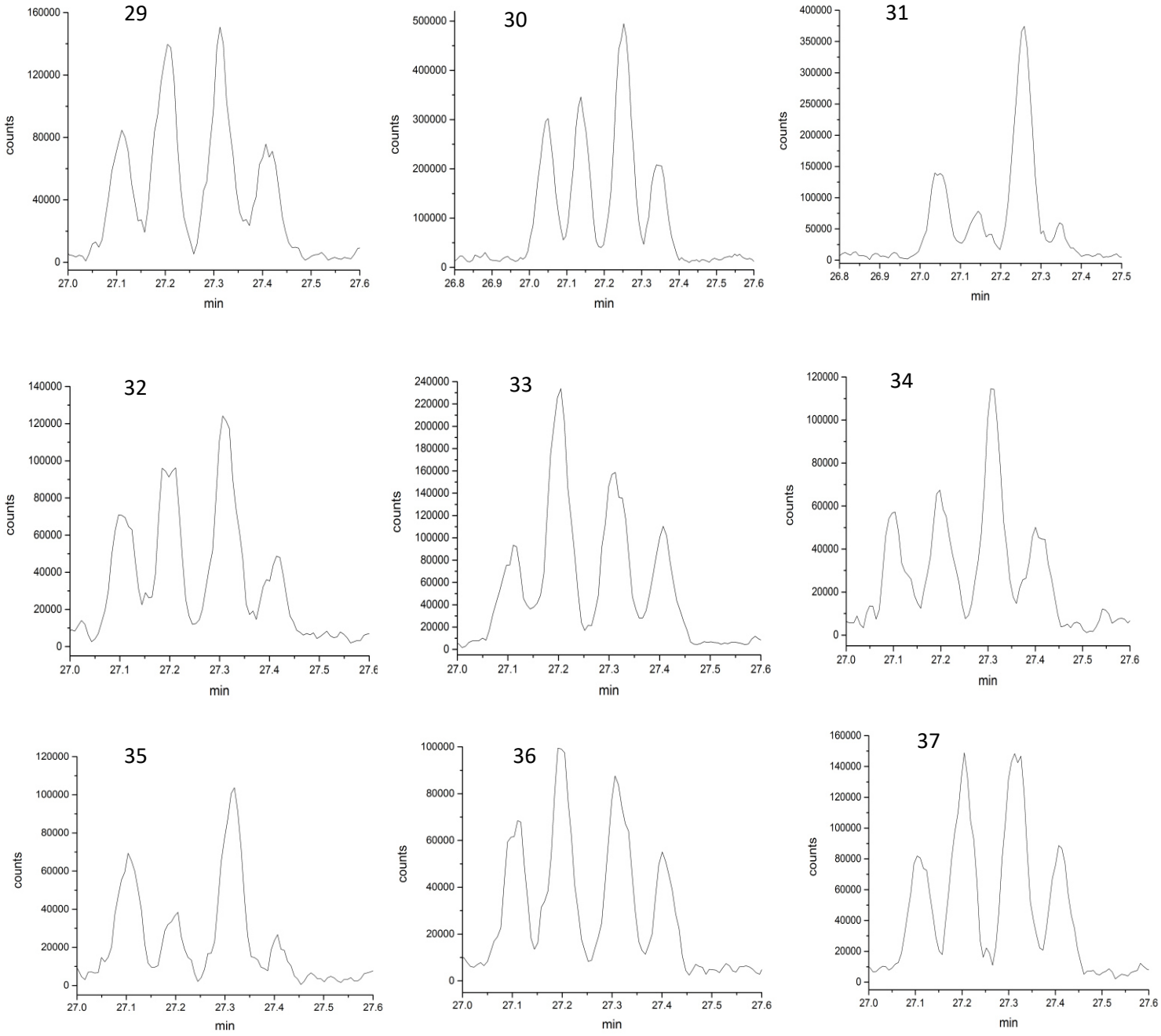

Figure 40 GC-MS/MS chromatograms of β-epoxy-ChOL, β-epoxy-D-ChOL (IS), α-epoxy-ChOL and α-epoxy-D-ChOL (IS) in breast adipose tissue of samples 29, 30, 31, 32, 33, 34, 35, 36 and 37. β-epoxy-ChOL R_t: 27.22, β-epoxy-D-ChOL (IS) R_t: 27.14, α-epoxy-ChOL R_t: 27.43 min; α-epoxy-D-ChOL (IS) R_t: 27.34 min.

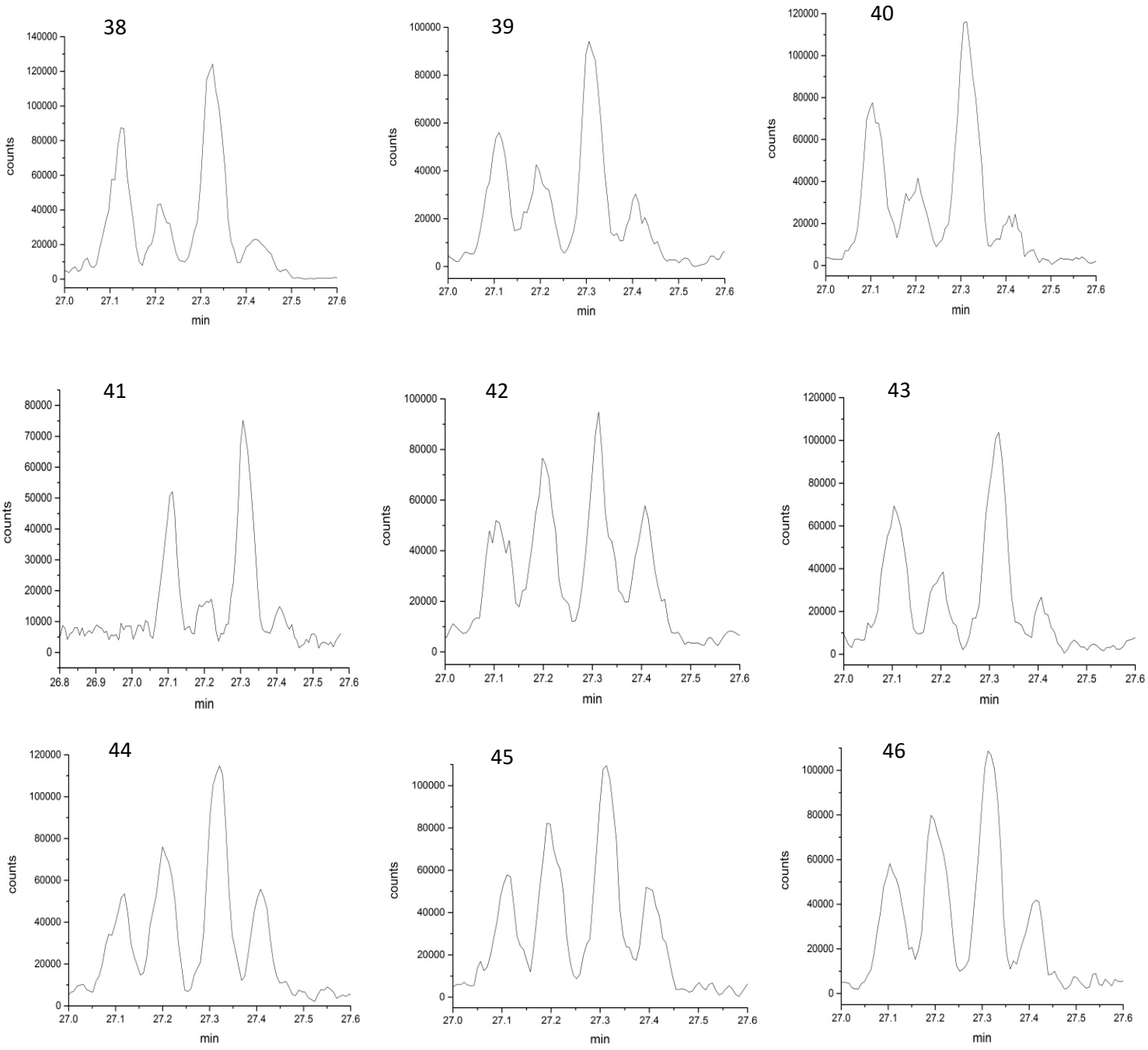

Figure 41 GC-MS/MS chromatograms of β-epoxy-ChOL, β-epoxy-D-ChOL (IS), α-epoxy-ChOL and α-epoxy-D-ChOL (IS) in breast adipose tissue of samples 38, 39, 40, 41, 42, 43, 44, 45 and 46. β-epoxy-ChOL R_t: 27.22, β-epoxy-D-ChOL (IS) R_t: 27.14, α-epoxy-ChOL R_t: 27.43 min; α-epoxy-D-ChOL (IS) R_t: 27.34 min.

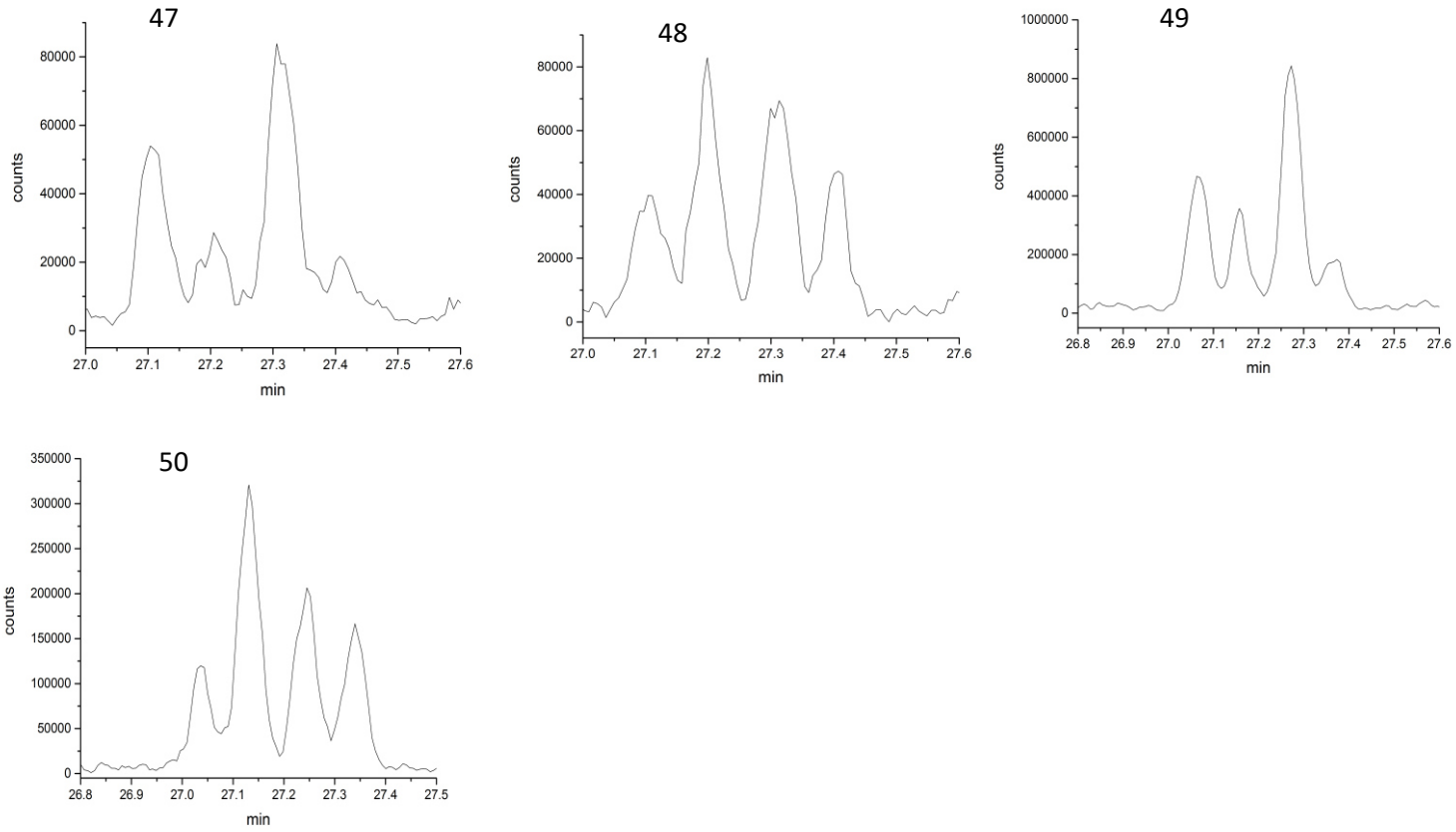

Figure 42 GC-MS/MS chromatograms of β-epoxy-ChOL, β-epoxy-D-ChOL (IS), α-epoxy-ChOL and α-epoxy-D-ChOL (IS) in breast adipose tissue of samples 47, 48, 49 and 50. β-epoxy-ChOL R_t: 27.22, β-epoxy-D-ChOL (IS) R_t: 27.14, α-epoxy-ChOL R_t: 27.43 min; α-epoxy-D-ChOL (IS) R_t: 27.34 min.

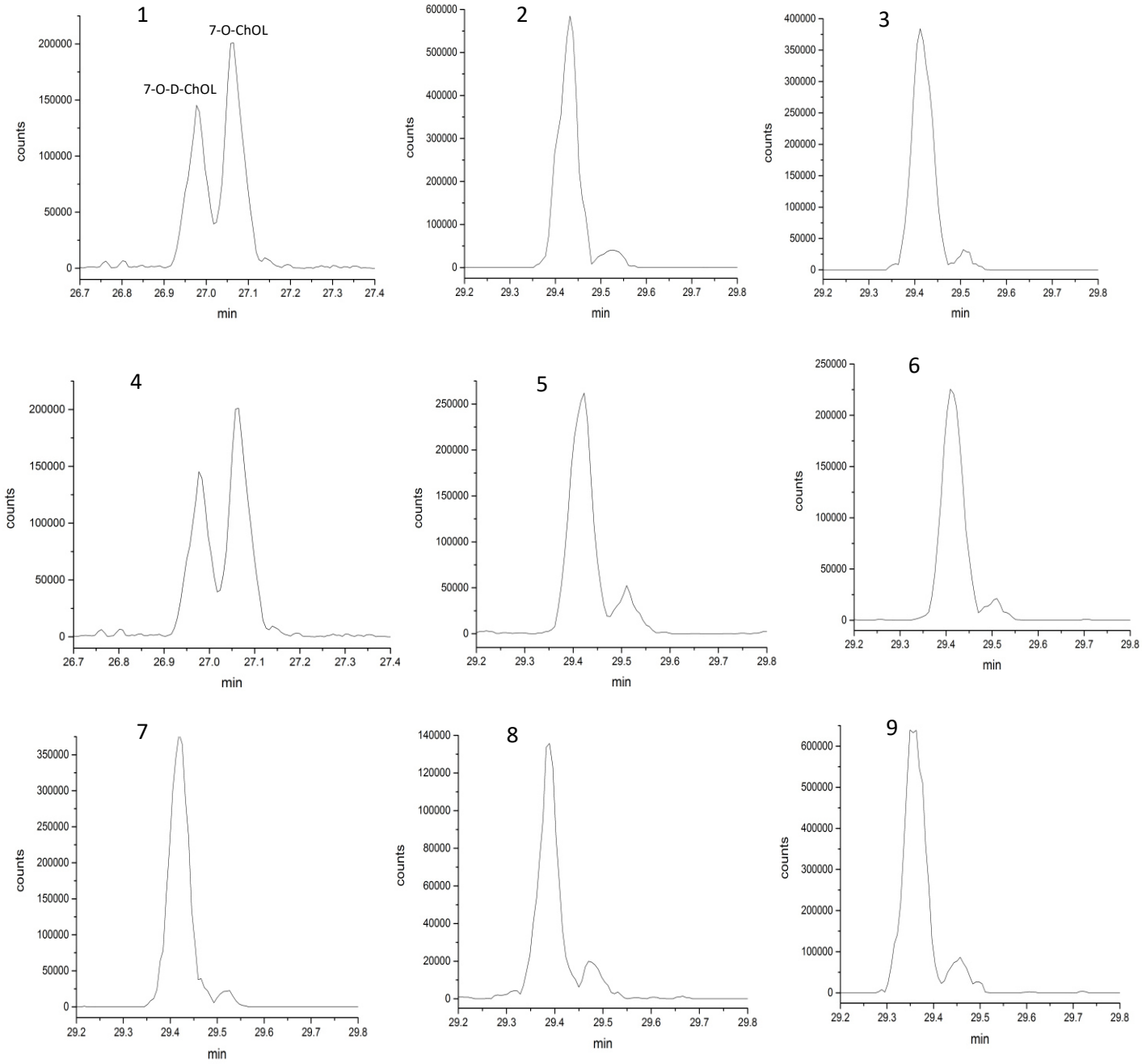

Figure 43 GC-MS/MS chromatograms of 7-O-ChOL, 7-O-D-ChOL (IS) in breast adipose tissue of samples 1, 2, 3, 4, 5, 6, 7, 8 and 9. 7-O-ChOL R_t: 29.51, 7-O-D-ChOL (IS) R_t: 29.42.

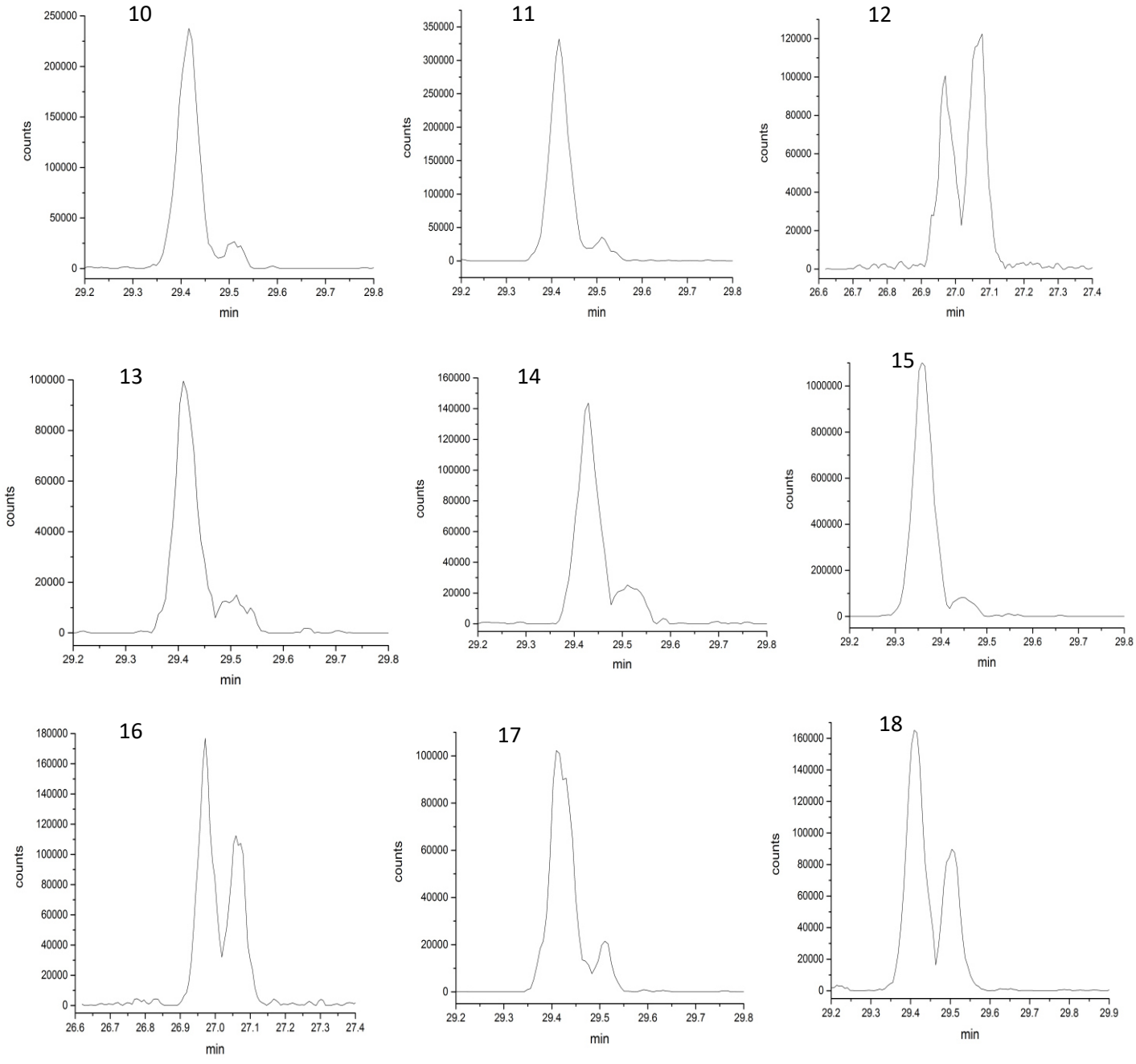

Figure 44 GC-MS/MS chromatograms of 7-O-ChOL, 7-O-D-ChOL (IS) in breast adipose tissue of samples 10, 11, 12, 13, 14, 15, 16, 17 and 18. 7-O-ChOL R_t: 29.51, 7-O-D-ChOL (IS) R_t: 29.42.

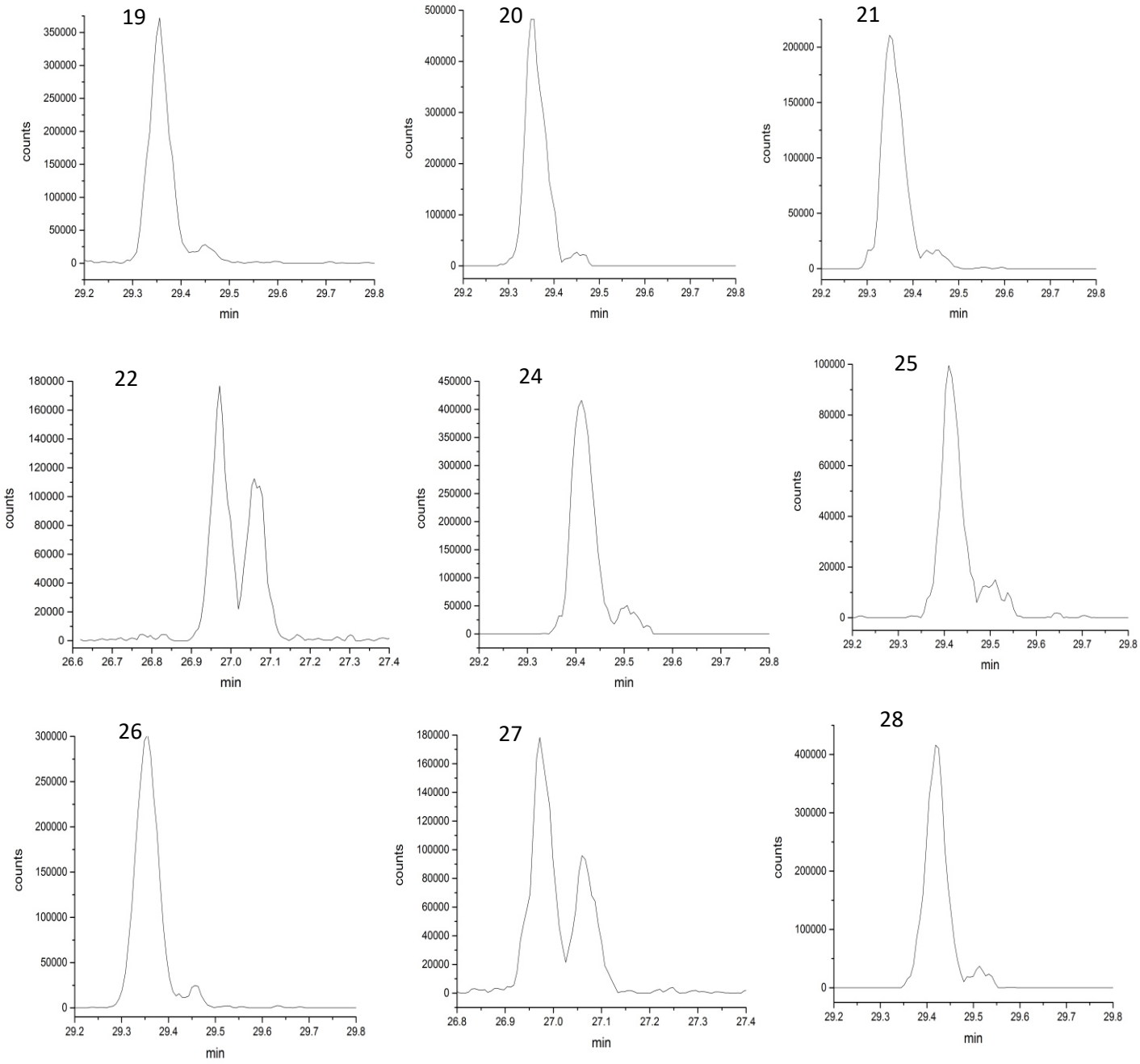

Figure 45 GC-MS/MS chromatograms of 7-O-ChOL, 7-O-D-ChOL (IS) in breast adipose tissue of samples 19, 20, 21, 22, 24, 25, 26, 27 and 28. 7-O-ChOL R_t: 29.51, 7-O-D-ChOL (IS) R_t: 29.42.

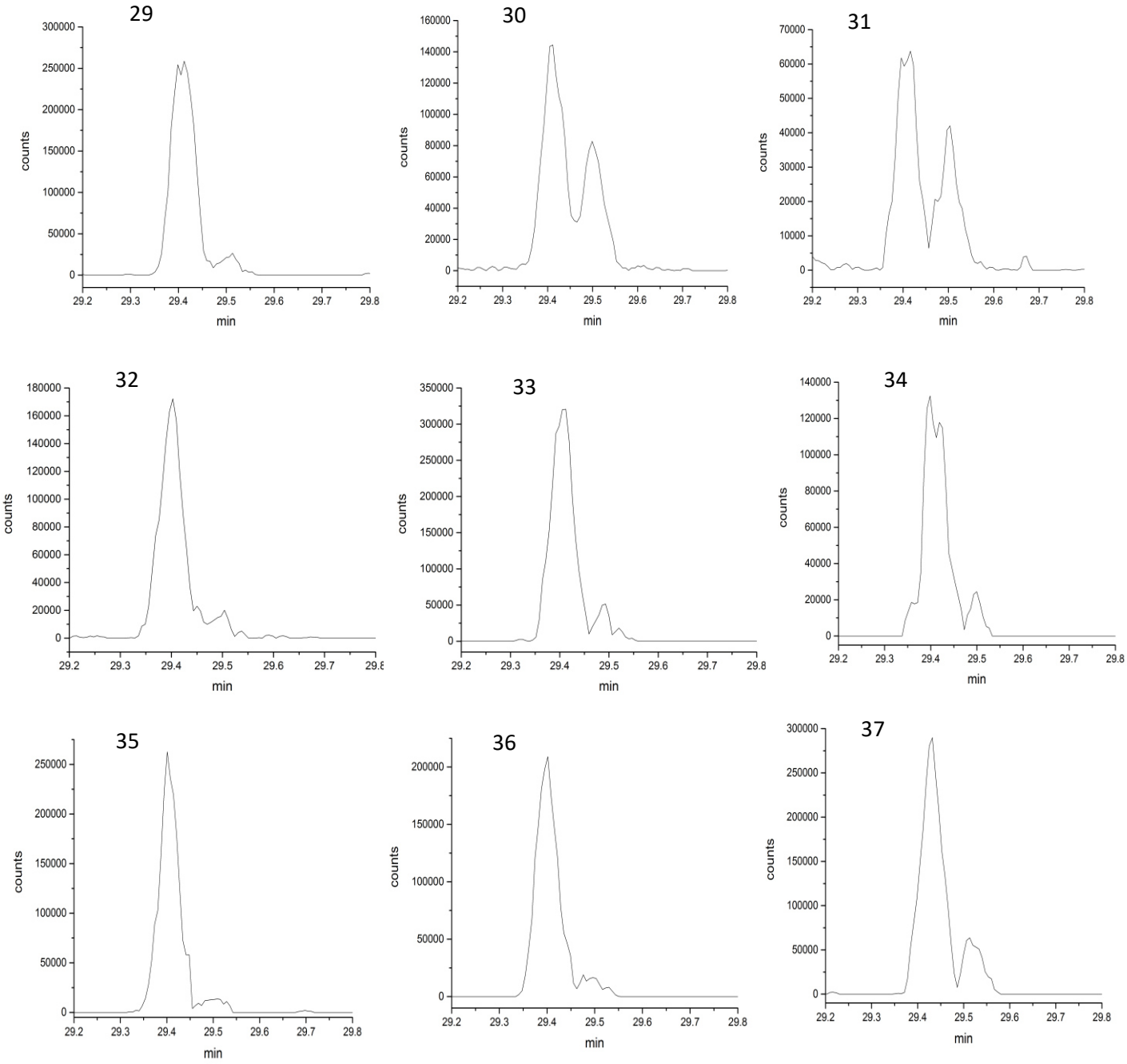

Figure 46 GC-MS/MS chromatograms of 7-O-ChOL, 7-O-D-ChOL (IS) in breast adipose tissue of samples 29, 30, 31, 32, 33, 34, 35, 36 and 37. 7-O-ChOL R_t: 29.51, 7-O-D-ChOL (IS) R_t: 29.42.

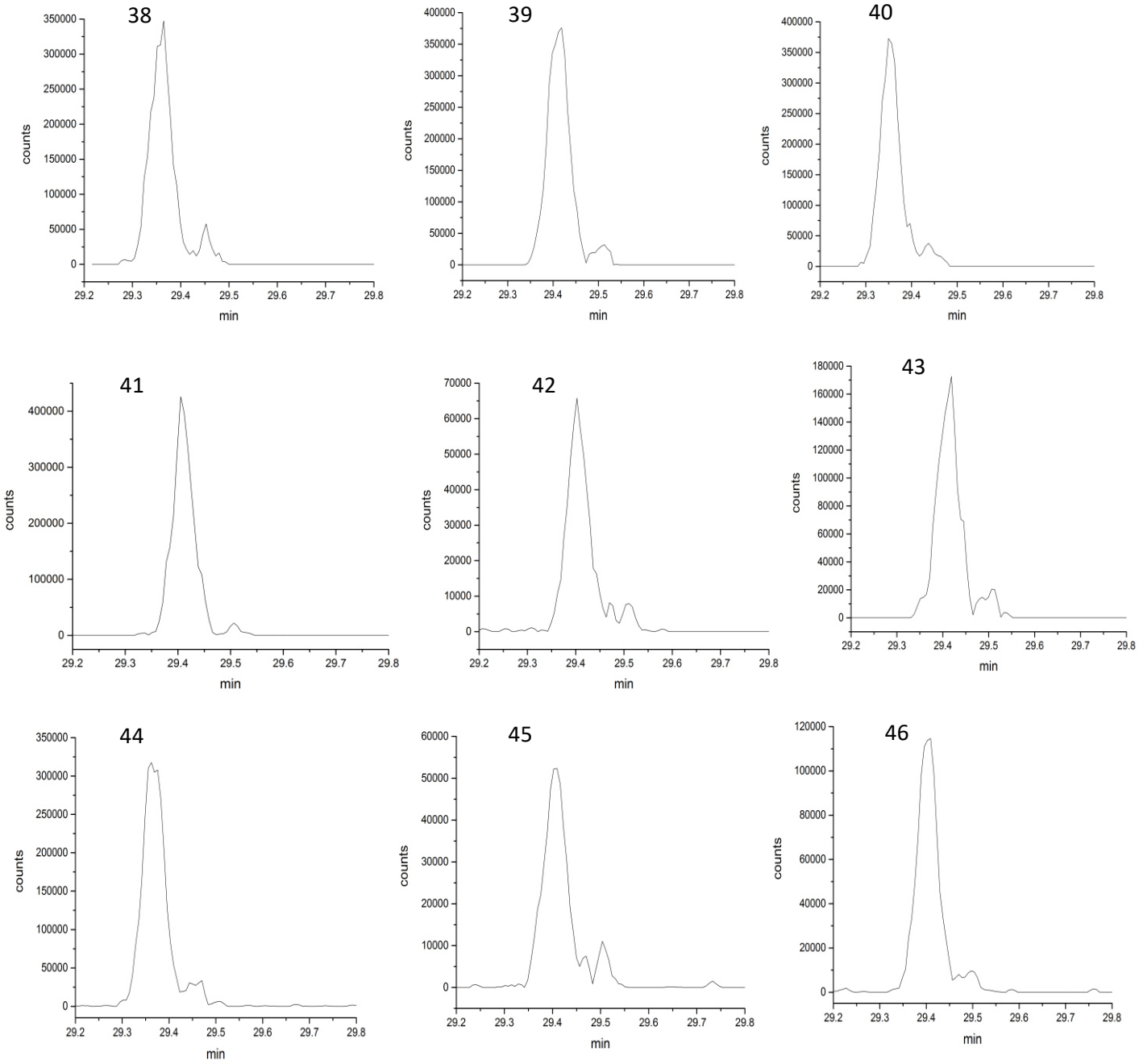

Figure 47 GC-MS/MS chromatograms of 7-O-ChOL, 7-O-D-ChOL (IS) in breast adipose tissue of samples 38, 39, 40, 41, 42, 43, 44, 45 and 46. 7-O-ChOL R_t: 29.51, 7-O-D-ChOL (IS) R_t: 29.42.

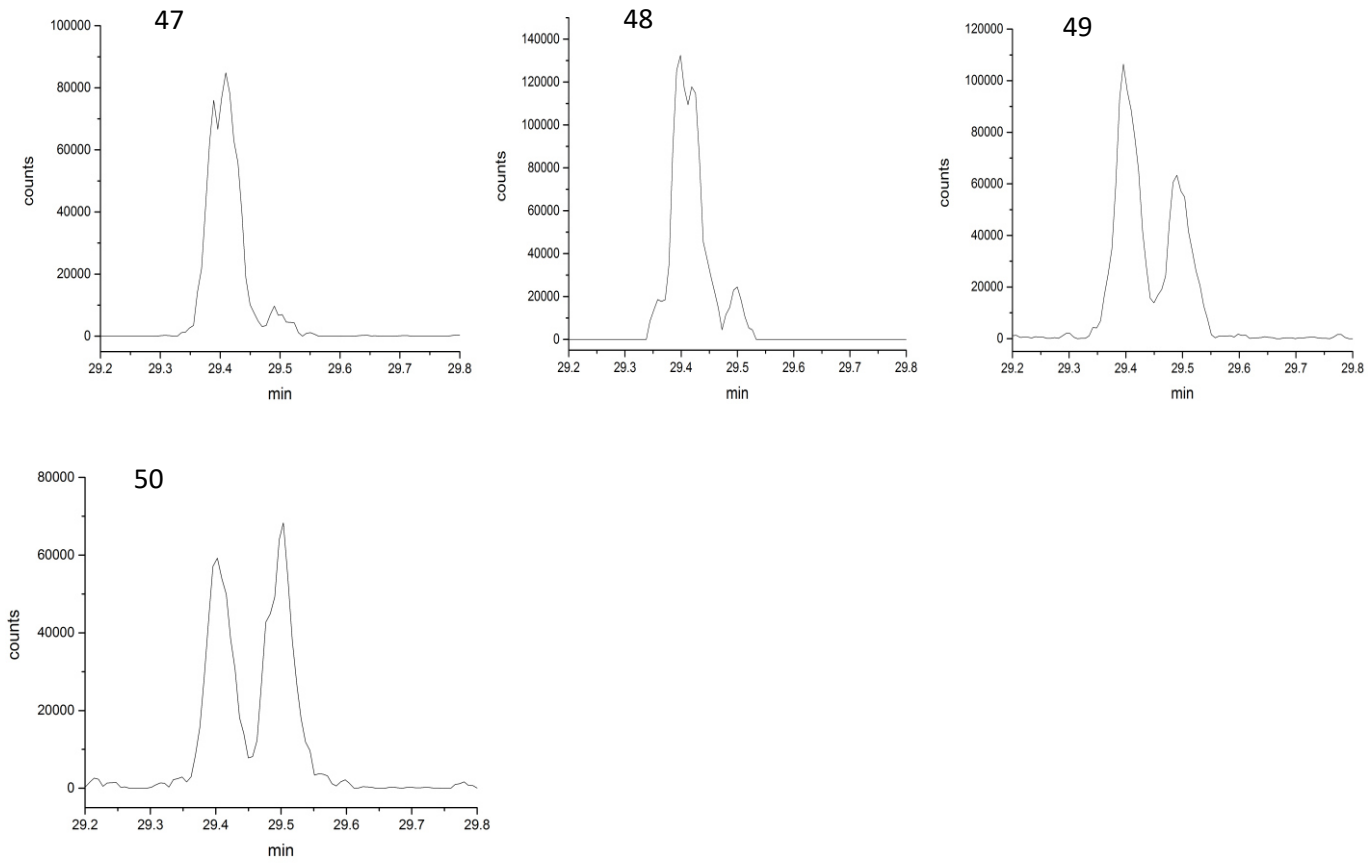

Figure 48 GC-MS/MS chromatograms of 7-O-ChOL, 7-O-D-ChOL (IS) in breast adipose tissue of samples 47, 48, 49 and 50. 7-O-ChOL R_t: 29.51, 7-O-D-ChOL (IS) R_t: 29.42.

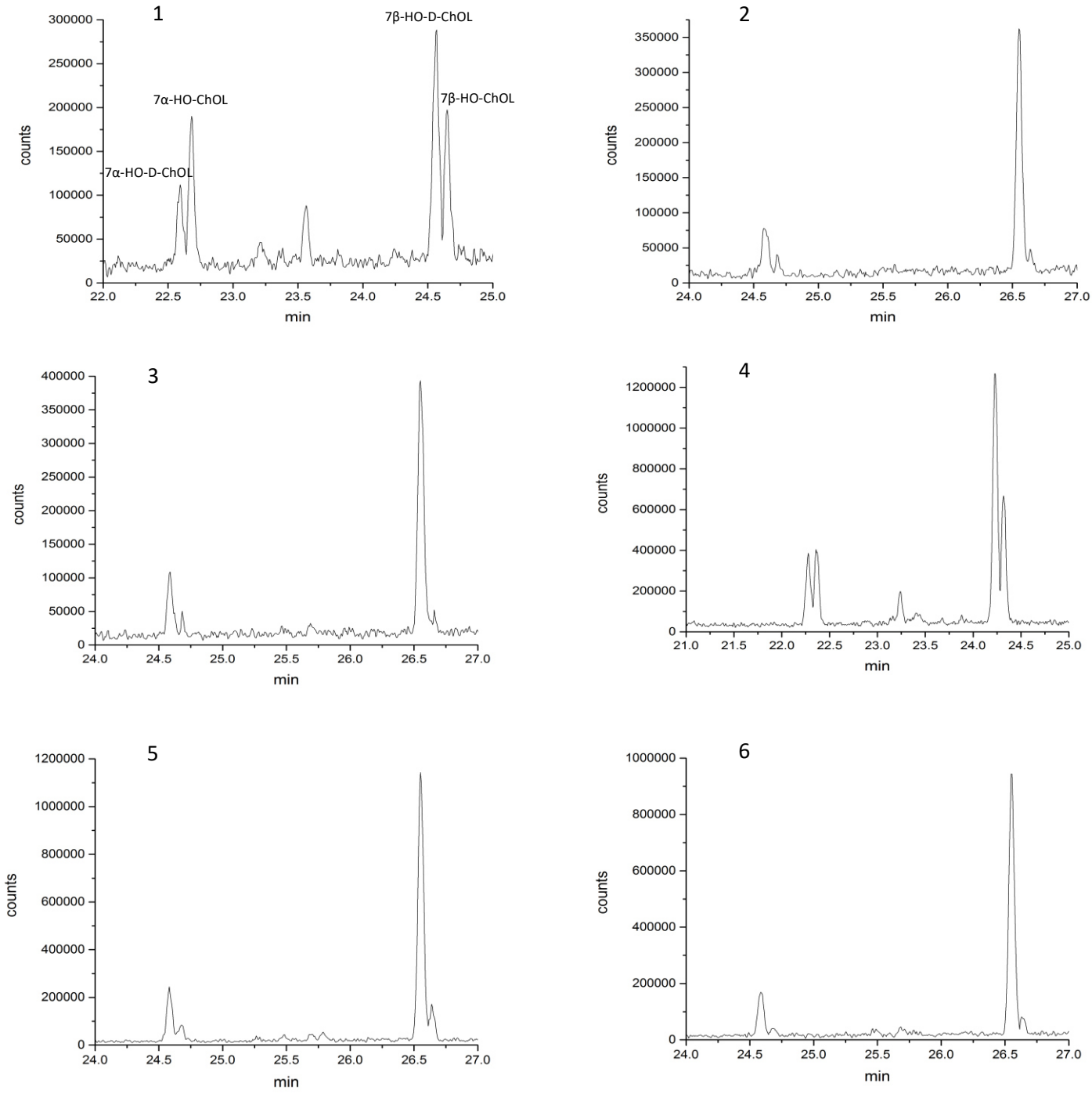

Figure 49 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 1, 2, 3, 4, 5 and 6. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

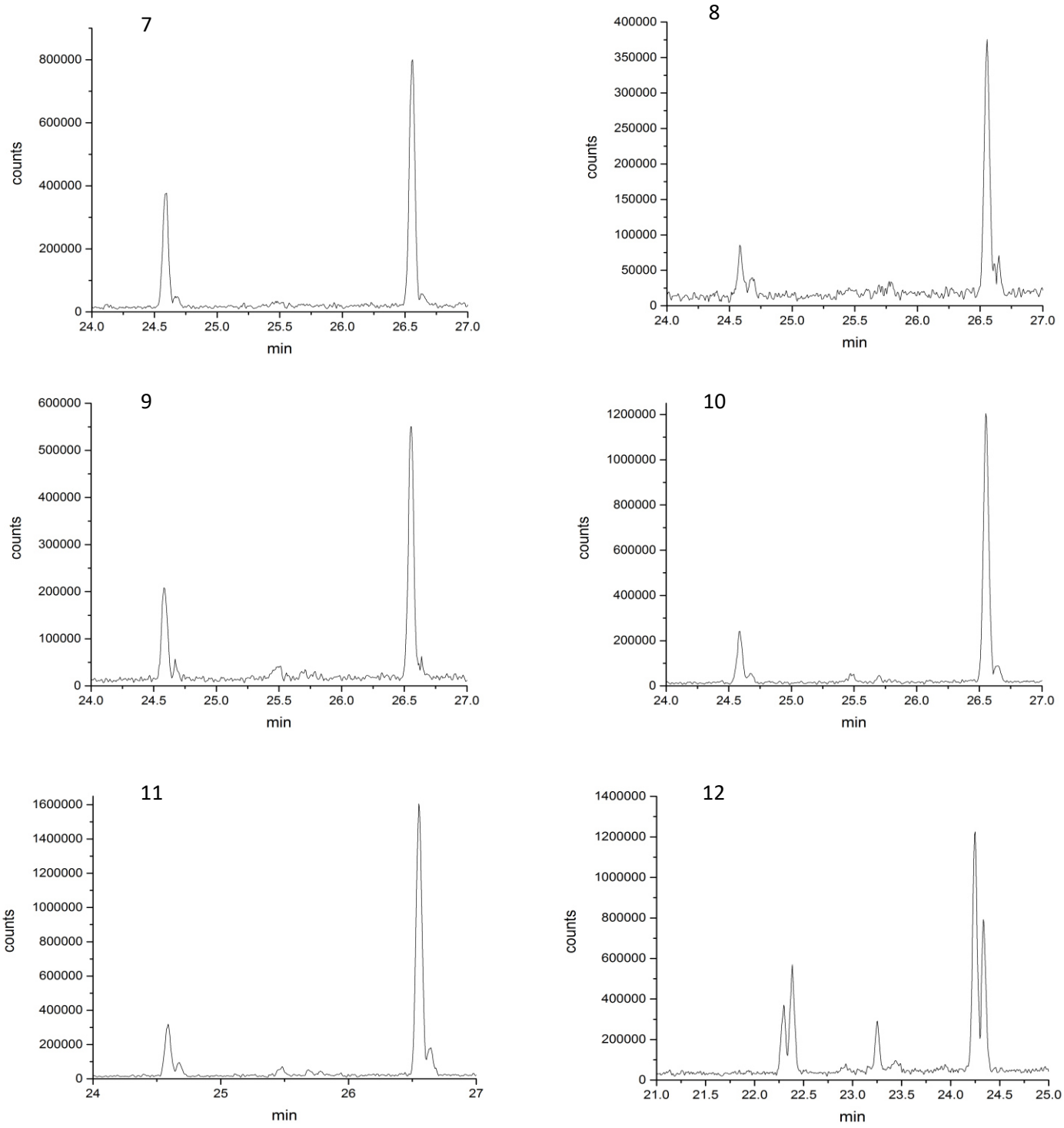

Figure 50 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 7, 8, 9, 10, 11 and 12. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

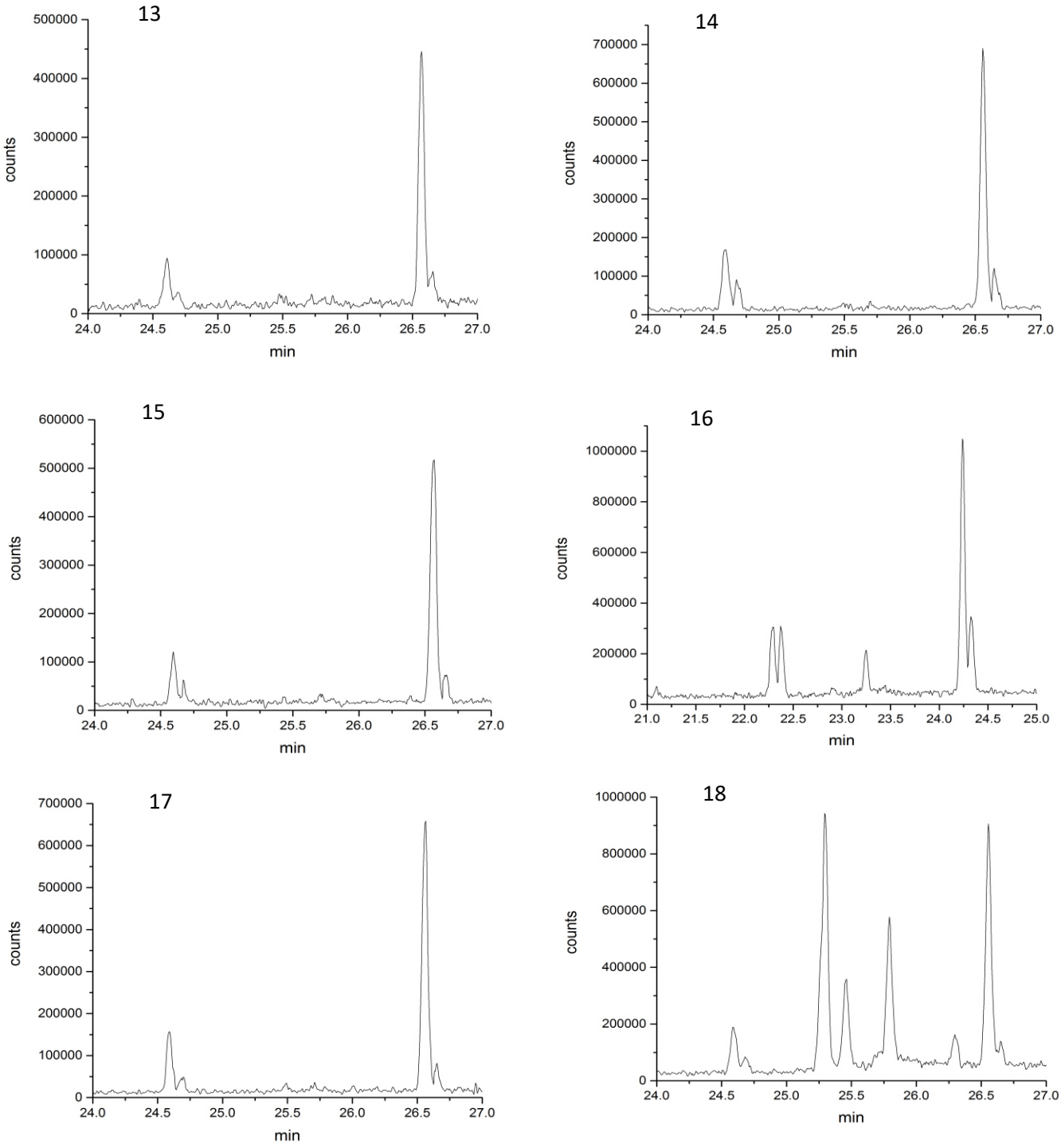

Figure 51 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 13, 14, 15, 16, 17 and 18. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

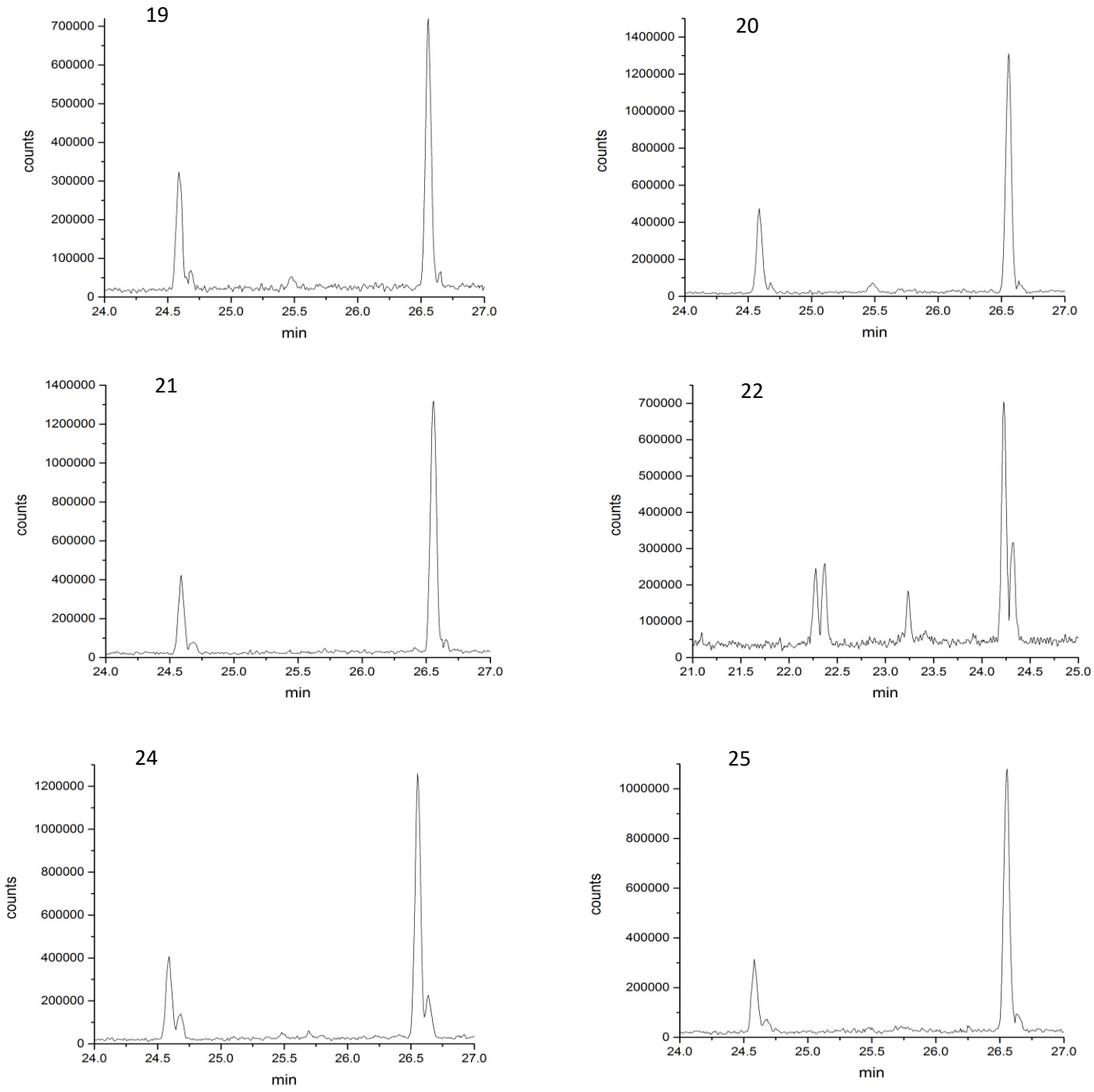

Figure 52 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 19, 20, 21, 22, 24 and 25. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

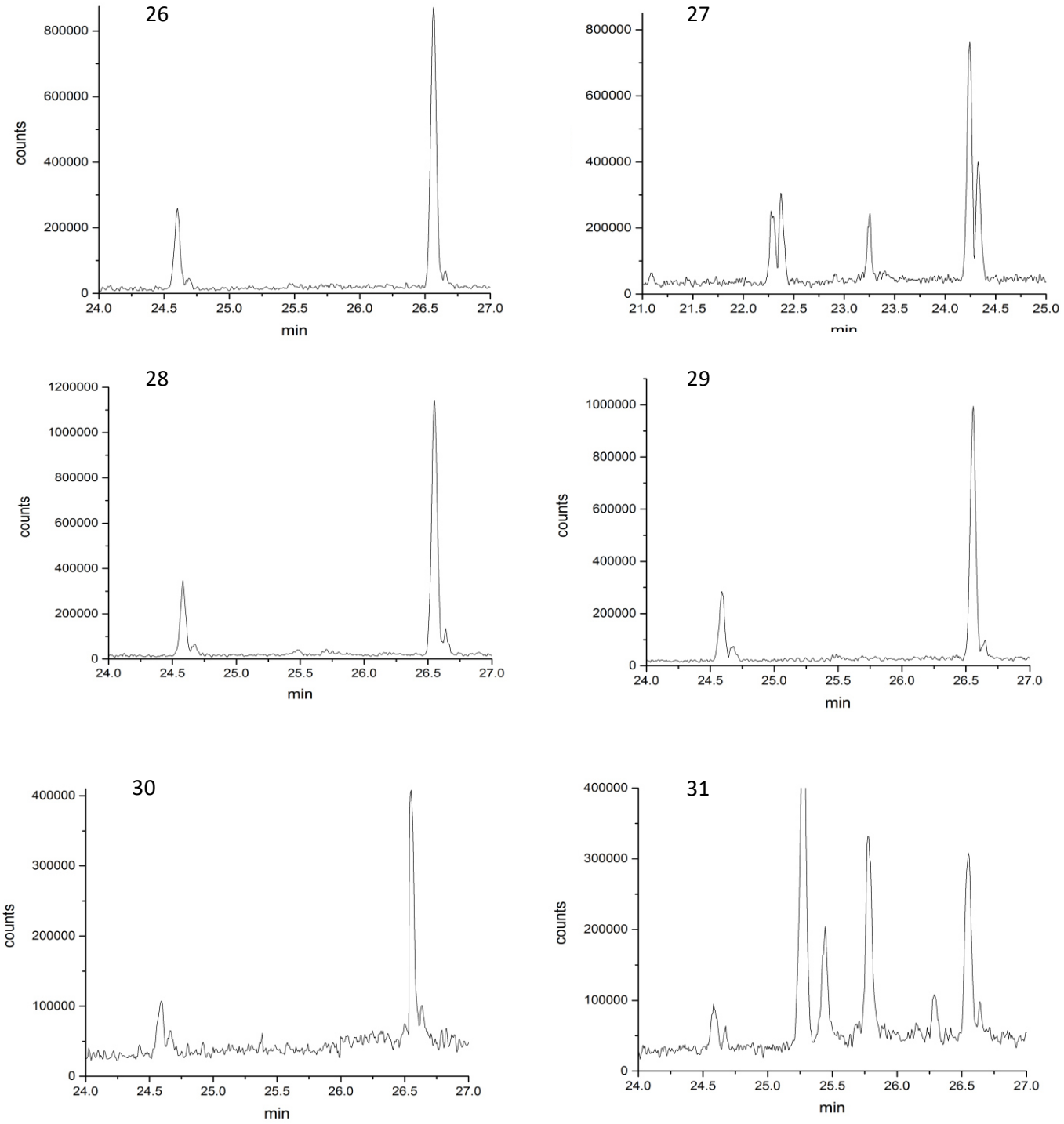

Figure 53 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 26, 27, 28, 29, 30 and 31. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

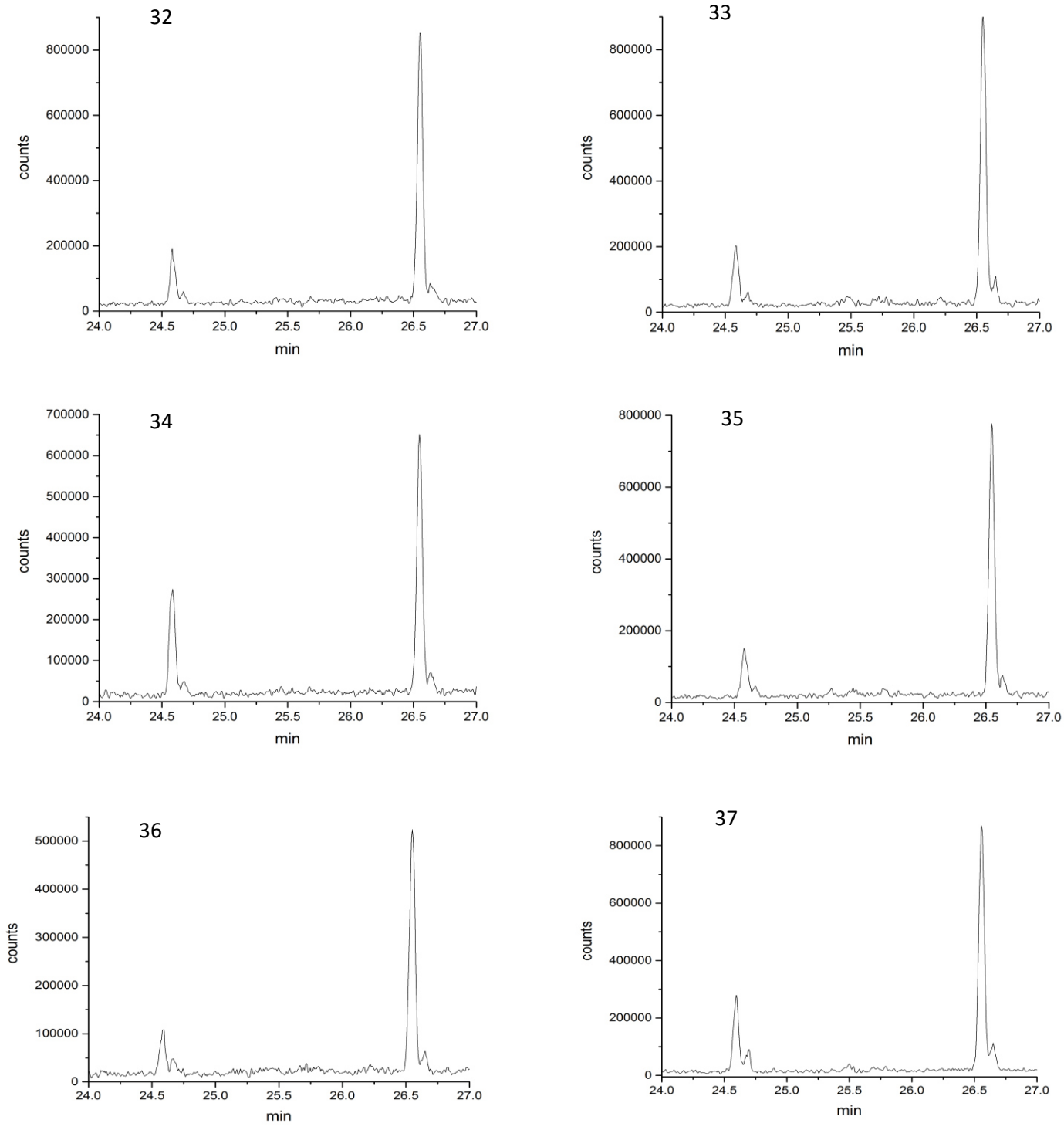

Figure 54 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 32, 33, 34, 35, 36, 37. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

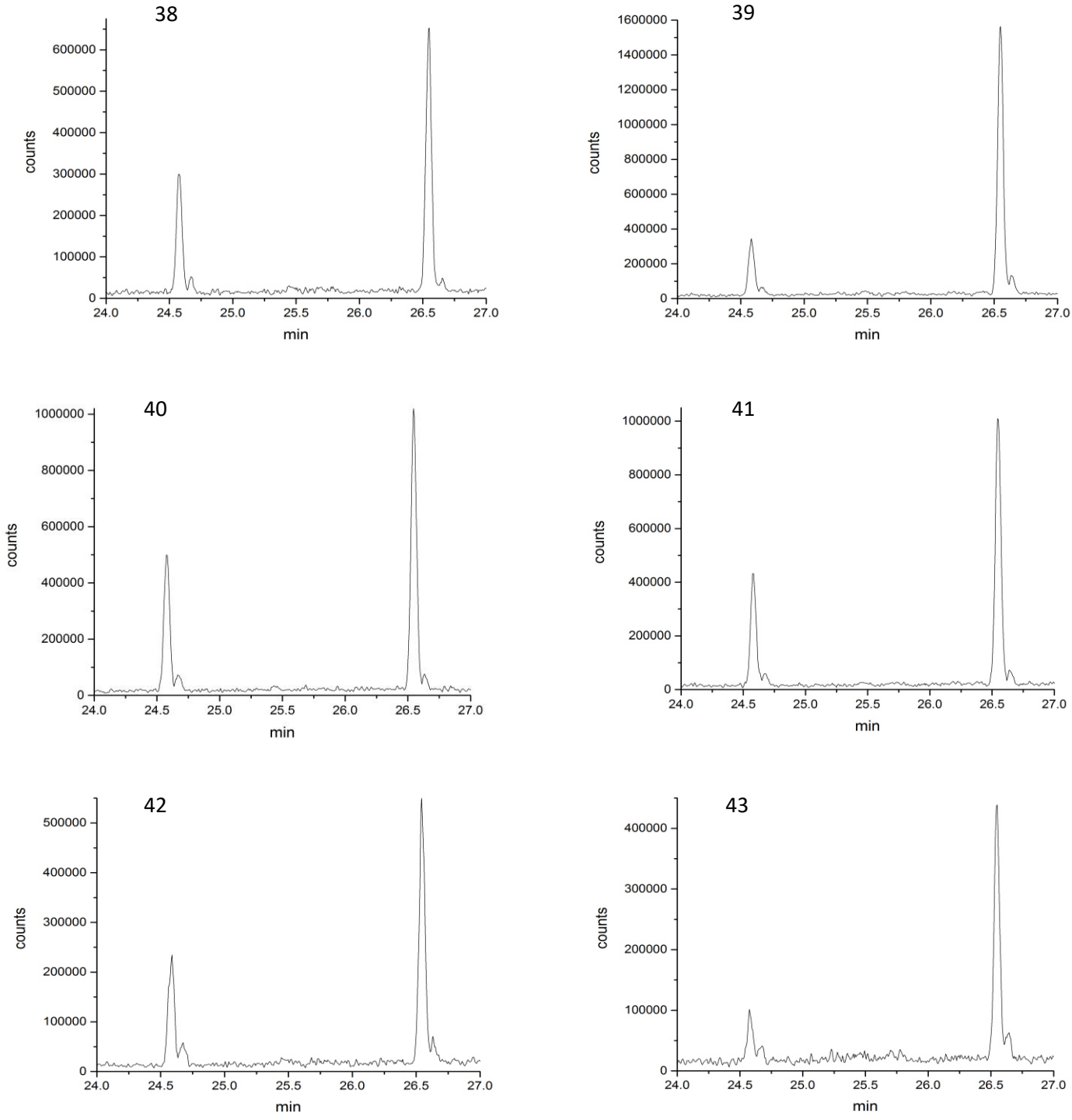

Figure 55 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 38, 39, 40, 41, 42, 43. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

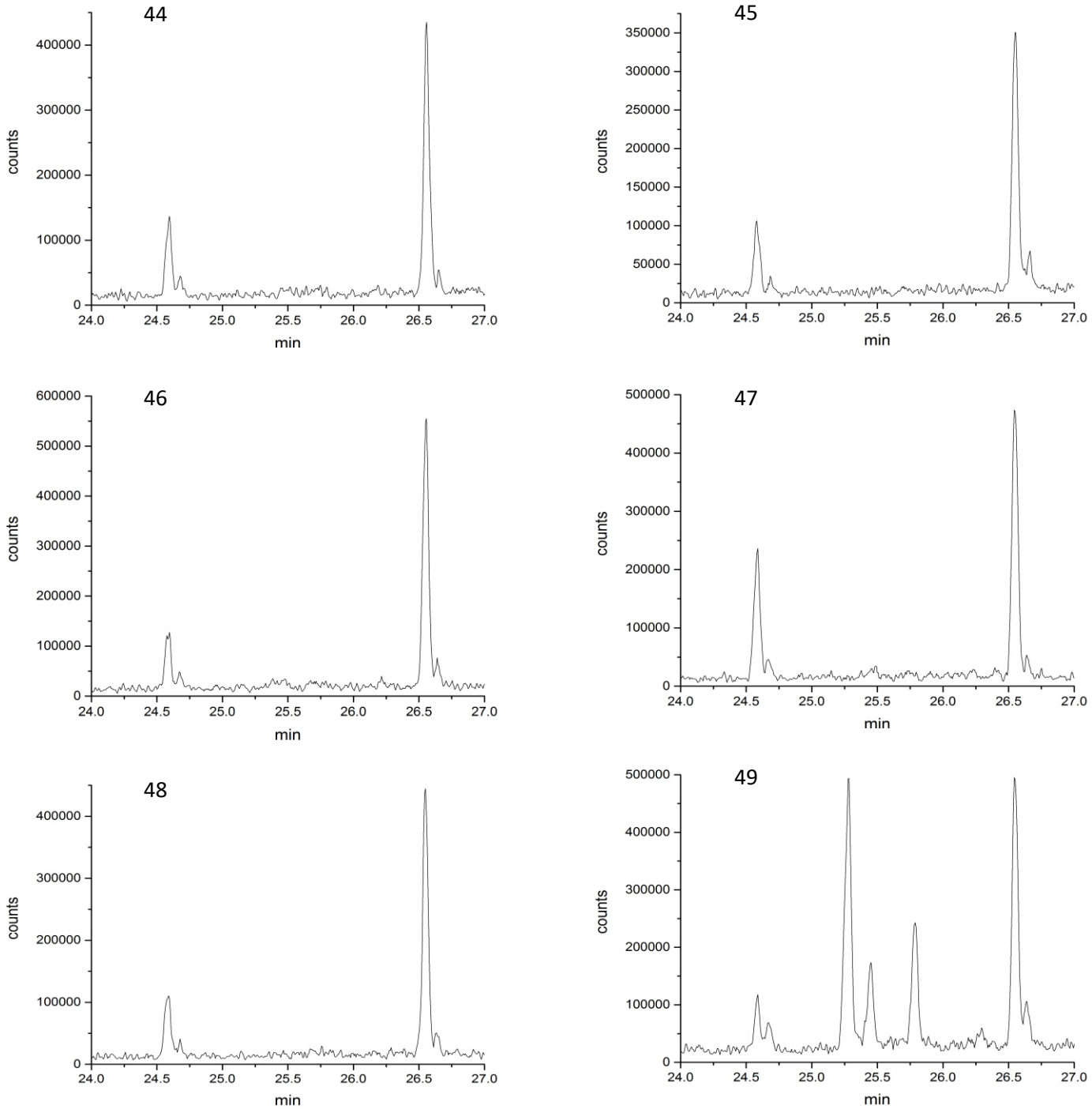

Figure 56 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 44, 45, 46, 47,48, 49, 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

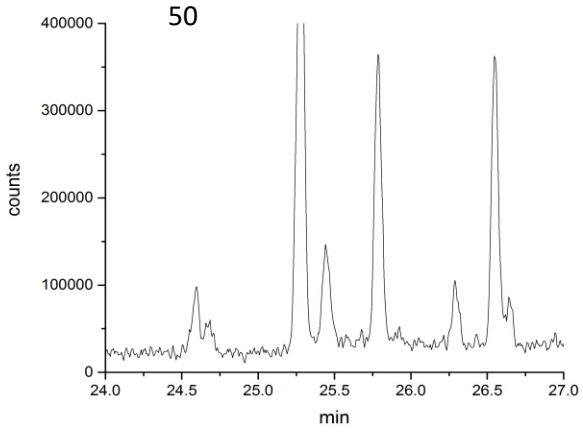

Figure 57 GC-MS/MS chromatograms of 7α-HO-ChOL, 7α-HO-D-ChOL (IS), 7β-HO-ChOL and 7β-HO-D-ChOL in breast adipose tissue of samples 50. 7α-HO-ChOL R_t: 24.70, 7α-HO-D-ChOL (IS) R_t: 24.60, 7β-HO-ChOL R_t: 26.64, 7β-HO-D-ChOL (IS) R_t: 26.54.

Table 48 Spearman correlation results of ChOL and fatty acids relative peak area percentages was obtained with data of 49 breast adipose tissue samples.

	R-value	p-value
15:0&ChOL	0.11	0.45
18:1 9trans&ChOL	0.08	0.57
18:3 n3&ChOL	-0.22	0.12

Table 49 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and relative peak area percentages of ALA in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.08	0.58	0.13	0.38
7β-HO-ChOL	0.11	0.45	0.17	0.24
7-O-ChOL	0.04	0.79	0.06	0.67
α-epoxy-ChOL	0.13	0.38	0.18	0.22
β-epoxy-ChOL	-0.02	0.86	0.16	0.28
Total oxyChOLs	0.05	0.71	0.11	0.43

Table 50 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and relative peak area percentages of pentadecanoic acid in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	-0.09	0.55	-0.08	0.57
7β-HO-ChOL	-0.02	0.88	-0.06	0.64
7-O-ChOL	0.08	0.57	0.06	0.67
α-epoxy-ChOL	-0.08	0.60	-0.08	0.57
β-epoxy-ChOL	-0.09	0.53	-0.07	0.63
Total oxyChOLs	-0.06	0.68	-0.01	0.93

Table 51 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and relative peak area percentages of DHA in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.07	0.64	0.00	0.98
7β-HO-ChOL	0.04	0.79	-0.01	0.96
7-O-ChOL	-0.12	0.41	-0.16	0.26
α-epoxy-ChOL	0.16	0.27	0.08	0.57
β-epoxy-ChOL	0.11	0.44	0.05	0.72
Total oxyChOLs	0.09	0.56	0.04	0.77

Table 52 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and absolute levels of DHA in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.16	0.27	0.18	0.20
7β-HO-ChOL	0.14	0.33	0.20	0.16
7-O-ChOL	0.01	0.97	0.03	0.83
α-epoxy-ChOL	0.13	0.37	0.14	0.33
β-epoxy-ChOL	0.04	0.76	0.10	0.49
Total oxyChOLs	0.07	0.60	0.11	0.44

Table 53 Statistical correlation (Spearman) between elaidic acid and LCPUFAs in human adipose breast tissue (presented data are R-values, p-value).

	18:3 n6	20:2 n6	20:3 n6	20:3 n3	20:5 n3	22:6 n3
R value	0.05	0.08	-0.12	0.03	-0.06	-0.11
P value	0.75	0.56	0.40	0.85	0.68	0.45

Table 54 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and age in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.02	0.91	0.02	0.88
7β-HO-ChOL	-0.03	0.83	0.15	0.31
7-O-ChOL	-0.10	0.48	-0.07	0.64
α-epoxy-ChOL	-0.10	0.51	-0.12	0.42
β-epoxy-ChOL	-0.15	0.29	-0.16	0.27
Total oxyChOLs	-0.13	0.36	-0.08	0.59

Table 55 Statistical correlation (Spearman) between n3 PUFAs (peak area percentages) and BMI in human adipose breast tissue.

	R-value	p-value
18:3 n3	0.12	0.41
20:3 n3	0.20	0.17
20:5 n3	0.22	0.12
22:6 n3	0.05	0.71

Table 56 Statistical correlation (Spearman) between absolute and relative levels (oxyChOL/ChOL) of individual and total oxyChOLs and BMI in human adipose breast tissue.

	Absolute level		Relative level	
	R-value	p-value	R-value	p-value
7α-HO-ChOL	0.09	0.50	0.08	0.58
7β-HO-ChOL	-0.11	0.42	0.07	0.63
7-O-ChOL	0.04	0.78	0.03	0.83
α-epoxy-ChOL	0.07	0.65	0.08	0.59
β-epoxy-ChOL	0.05	0.71	0.11	0.45
Total oxyChOLs	0.04	0.79	0.04	0.78

Table 57 Variance homogeneity (p-value) of calibration curves at significance level of 5%.

group	substance	p value		comments
		calibration curve 1	Calibration curve 2	
Fatty acids	15:0	0.97	1.00	variance homogen
	18:0 9trans	1.00	0.98	variance homogen
	18:3 n3	0.99	1.00	variance homogen
	22:6 n3	1.00		variance homogen
ChOL	ChOL	0.99	-	variance homogen
oxyChOLs	7α-HO-ChOL	0.91	0.95	variance homogen
	7β-HO-ChOL	0.90	0.93	variance homogen
	7-O-ChOL	0.99	0.60	variance homogen
	α-epoxy-ChOL	0.97	-	variance homogen
	β-epoxy-ChOL	0.92	-	variance homogen