GAS CHROMATOGRAPHY-MASS SPECTROMETRY PROFILING OF FATTY ACIDS IN BREAST MILK FROM NURSING MOTHERS IN BUNGOMA COUNTY, KENYA

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis is my original work and has not been submitted or presented for the award of any degree in this or any other university, either in part or as whole.

Signature ……. Date……..

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RECOMMENDATION

This thesis has been prepared with our guidance and assistance; it has been submitted with our approval as the University supervisors.

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DEDICATION

This thesis is dedicated to my parents, the late Mr and Mrs Solomon Kiprop Cheptoo who tirelessly ensured that we went to school. Their intuition to believe in the value of education and insist not just in words but also in deeds is a rare love and insight I will carry with me every step of my life to accomplish the vision they always desired. I also dedicate this thesis with deepest appreciation to my husband Isaac for his love and unwavering support that was always my inspiration during the many months of hard work.

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ABSTRACT

Human breast milk is universally recognized as the optimal food for term infants. Fatty acids play important roles in biological systems and the newborn's fatty acid requirements are wholly met by the mothers' breast milk. Although there have been many studies on fatty acid composition of human milk from Western countries and Africa, there is little information about that in the Kenyan population. This study was conducted to characterize and analyze quantitatively the fatty acid profile of breast milk at 2 stages of lactation from nursing mothers in Bungoma County, Kenya. Breast milk samples were obtained at the fourth and ninth month postpartum from nursing mothers who were participating in a Cohort for Vitamin A (COVA) study. The samples were collected by manual expression and stored at –20 ^{0}C until analysis. The fat was extracted from milk and methylated using the Association of Official Analytical Chemists International (AOAC) Official methods. The separation, identification and quantification of the fatty acid methyl esters was performed by gas chromatography coupled with mass spectrometry (GC-MS). Fatty acids were identified based on retention times and mass spectral data. These were compared with corresponding standards and a library of known mass spectra. The results showed that the major fatty acids of breast milk fat at the 4th month of lactation were oleic acid (66.6%), followed by linoleic acid (51.3%) and palmitoleic acid (28.9%). However, at the 9th month, the major component was linoleic acid (58.1%), followed by oleic acid (30.9%) and stearic acid (23.1%). The caprylic acid content was the least (< 4%) among the fatty acids in both stages of lactation. The fatty acid composition was expressed as weight percentage of all fatty acids detected with C8–C18 chain length. More than 80% of the total fatty acids were composed of C18:1n-9 (oleic acid) and C18:2n-6 (linoleic acid). The percentage average of the total saturated fatty acids at 4 months (16.62%) was significantly lower ($p < 0.05$) than the average percent of the total unsaturated FAs (48.93%). Similarly, at the 9th month the percentage average of the total saturated fatty acids (15.18%) was significantly lower ($p < 0.05$) than the average percent of the total unsaturated FAs (31.05%). The fatty acid composition was significantly different between the two lactation/ nursing periods. This study has shown that breast milk fat is an excellent source of the essential fatty acid omega-6 (linoleic acid) for infants in Bungoma County.

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ABBREVIATIONS

AA	–	Arachidonic acid
AOCS	–	American Oil Chemists Society
AOAC	–	Association of Official Analytical Chemists International
BM	–	Breast milk
COVA	–	Cohort for vitamin A
DHA	–	Docosahexaenoic acid
EPA	–	Eicosapentaenoic acid
EFA	–	Essential fatty acid
FA	–	Fatty acid
FAMEs	–	Fatty acid methyl esters
FAO	–	Food and Agriculture Organization of the United Nations
GC-MS	–	Gas chromatography- mass spectrometry
LA	–	Linoleic acid
LCPUFA	–	Long chain polyunsaturated fatty acid
MeOH	–	Methanol
NaOH	–	Sodium hydroxide
I*cipe*	–	International Centre of Insect Physiology and Ecology
SIM	–	Selected ion monitoring
SFA	–	Saturated fatty acids
SASHA	–	The Sweet potato Action for Security and Health in Africa
TAGs	–	Triacylglycerols
TIC	–	Total ion chromatogram
WHO	–	World Health Organization
ω	–	Omega

CHAPTER ONE

INTRODUCTION

1.1 Background information

Human breast milk is considered the optimal form of nourishment for infants during the first 6 months of life and provides essential fatty acids in early human life. Fat is a critical component of breast milk, providing energy and importantly, nutrients key to the development of the central nervous system, which cannot be synthesized *de novo* by the infant (Lauritzen et al., 2001). Principal among these are the long-chain polyunsaturated fatty acids (LCPUFAs), docosahexaenoic acid (DHA) (omega (ω) 3) and arachidonic acid (AA) (ω-6), which are components of infant formulas in developed countries. The fatty acid composition of milk is of considerable interest due to their nutritional and functional properties (Gomes et al., 2010). Fatty acids in breast milk may originate either from the recent dietary fatty acid intake and dietary carbohydrates, fatty acids released from maternal adipose tissue or further metabolism of dietary fatty acids in the maternal liver (Innis, 2007).

Beyond specific dietary fatty acid (FA), total maternal dietary fat intake alters the concentration of selected breast milk FA profile. For example, levels of lauric acid (C12:0) are relatively low in the milk of women who consume moderate- to high-fat diets. However, in women consuming low-fat diets, mammary gland FA synthesis are elevated, which increases the level of milk lauric acid (Jensen, 1999). A considerable number of observational studies that link higher habitual maternal DHA intake to some aspect of higher or more mature infant or child development are considered relevant in relation to the functional importance of LCPUFA transfer (Kannass et al., 2009; Mendez et al., 2009).

One of the qualities of BM is the fat content. Milk-fat serves as a source of energy, carrier of fat-soluble vitamins and also provides the essential fatty acids such as linoleic, which is required for the development and growth of central nervous system or tissue (Gomes et al., 2010). The levels of some FAs in human milk, such as DHA (22:6 ω3) are clearly dependent on dietary intake. Recently, the role of LCPUFAs has drawn special attention because of the potential source of anatomic and functional development of the central nervous system in early life (Agostoni, 2005; Agostoni, 2008). Human milk contains a large quantity of fatty acids that prevent and suppress autoimmune disease. It is shown that in newborns they have an important

role in the prevention of asthma, type I and type II diabetes, metabolic syndrome x and cardiovascular disease (Uauy et al., 2000). The fat content and fatty acid composition of human milk is variable. Milk's fatty acid composition is influenced by certain factors vis-à-vis diet, duration of pregnancy, maternal parity, stage of lactation, etc (Mitoulas et al., 2002). Maternal diet appears to be the most important variable determining milk's fatty acid composition (Lo´pez-Lo´pez et al., 2002).

Recent works have reported differences between the total fatty acid composition of human breast milk (Martin et al., 1993; Lo´pez-Lo´pez et al., 2002) and its TG composition (Martin et al., 1993; Lubetzky et al., 2012) at different stages of lactation. Fatty acid profile comparisons in human milk sampled from the same mothers at 2 different stages of lactation and the relation between trans isomeric and long-chain polyunsaturated fatty acids (LCPUFAs) in human milk at the sixth month of lactation has also been reported (Szabo et al., 2010). Whereas the component fatty acids of human milk are well documented in most countries in the developed world, reports describing the fatty acid profile for nursing mothers from Kenyan population are scanty. Therefore, the present study was designed to estimate the FA profile of breast milk of nursing mothers who were participating in a Cohort study of the impact of an integrated agriculture, nutrition and health intervention on the Vitamin A and health status of mothers and their infants from pregnancy through 9 months postpartum in Bungoma County, Kenya. The fatty acids were characterized and quantitated at the 4^{th} and 9^{th} month of lactation.

1.2 Problem statement

Fatty acids play important roles in biological systems. The newborns fatty acid requirements are met by the breast milk from the mother. Micronutrients and macronutrients deficiencies contribute to the greatest percentage of global childhood disability-adjusted life years. Although there have been many studies on fatty acid composition of human milk from Western countries and Africa, there is little information about that in the Kenyan population. Human milk is a dynamic system whose fat composition is influenced by factors such as maternal diet, duration of pregnancy or stage of nursing. Despite the importance of breast milk fatty acids, there is little information on the fatty acid profile of nursing mothers in Bungoma County, Kenya. It was also noted that no recent studies that focused on the fatty acid profile of human milk from East African populations have so far been reported.

1.3 Objectives

1.3.1 General objective

To study the profile of fatty acids in breast milk of nursing mothers using GC-MS at the fourth and ninth month postpartum in Bungoma County, Kenya

1.3.2 Specific objectives

1. To characterize the breast milk fatty acids from nursing mothers at the fourth month and ninth month postpartum
2. To quantitate the breast milk fatty acids from nursing mothers at the fourth month and ninth month postpartum

1.4 Hypothesis

The hypotheses tested were:

1. The profiles of fatty acids in breast milk of nursing mothers are not different at fourth and ninth month postpartum

2. There is no significant difference in concentrations of the fatty acids of breast milk from nursing mothers at both the fourth and ninth month postpartum

1.5 Justification

Breast milk is considered the ideal food for full-term infants. It is a very complex mixture of nutrients and non-nutritional factors. Breast milk-fat serves as a source of energy, carrier of fat-soluble vitamins and also provides the essential fatty acids needed for the development and growth of central nervous system (Gomes et al., 2010). Examination of milk- lipid concentration and composition is of interest because milk fat provides the major fraction of calories in human milk (Lauritzen et al., 2001). Whereas the fatty acid composition of milk is of considerable interest due to its nutritional and functional properties, it was of particular interest to quantify the content of the fatty acids in breast milk since it is the only dietary intake of a newborn. Fats and fatty acids should be considered as key nutrients that affect early growth and development and nutrition-related chronic disease later in life. Due to the important role of fatty acids in human milk assessing their qualities and quantities is essential. It helps to improve the nutrition and

health of pregnant, lactating women and their children. Although there was data from mature human milk reported for many countries that represented a wide range of geographic locales and cultures there was scanty data about milk from lactating women in Bungoma County, Kenya. Data from this work would really assist in quest for reduction in infant mortality and health in the country if well utilized at a policy level.

CHAPTER TWO
LITERATURE REVIEW

2.1 Dietary fat

Dietary fats provide the medium for the absorption of fat-soluble vitamins; are a primary contributor to the palatability of food; and are crucial to proper development and survival during the early stages of life-embryonic development and early growth after birth through infancy and childhood (Agostoni et al., 2005; Innis, 2007). Thus, the role of essential fatty acids during pregnancy and lactation is highlighted, and the role of long-chain ω-3 fatty acids as structural components for the development of the brain and central nervous system is now accepted (FAO, 2010). Fatty acids should be considered as key nutrients that affect early growth and development and nutrition-related chronic disease later in life. For example, specific ω-3 and ω-6 fatty acids are essential nutrients and also, as part of the overall fat supply may affect the prevalence and severity of cardiovascular disease, diabetes, cancer and age-related functional decline (FAO, 2010).

2.1.1 Fatty acids in human milk

Fatty acids represent 30–35% of total energy intake in many industrial countries and the most important dietary sources of fatty acids are vegetable oils, dairy products, meat products, grain and fatty fish or fish oils (Wiley, 2005). The main components of milk fat are fatty acids. These are esterified mainly in the form of triacylglycerols (TGs), which account for 98% of milk fat (Giovannini et al., 1995). Human breast milk provides all the dietary essential fatty acids, linoleic acid (LA: 18:2n-6), an omega 6 fatty acid and α-linolenic acid (18:3n-3), an omega 3 fatty acid. The ω-3 long chain polyunsaturated fatty acid, docosahexaenoic acid (DHA 22:6n-3), as well as the ω-6 arachidonic acid (AA 20:4n-6) support the growth and development of the breast-fed infant (Innis, 2007).

Mar´ın et al. (2005) examined the LCPUFA composition of milk of mothers living in La Plata in Argentina and analyzed the relationship between the fatty acid composition and the composition of the foods consumed by the mother. It was established that in case of overweight mothers the amount of the polyunsaturated fatty acids increased significantly and also the C18:2ω6/total ω-6 ratio was significantly higher compared to the ones with normal body weight (0.96%; 0.89%). No significant difference was found between the groups regarding the ω-3 fatty

acids, but the ratio between the ω-6 and ω-3 fatty acids was significantly higher for the overweight mothers (Mar´ın et al., 2005).

Another study that examined the essential fatty acid content of milk, of Chinese and Swedish mothers, on the effect of food on the composition of milk showed that Chinese mothers consumed mainly rice, steamed bean, noodles, Chinese cabbage and pork. Food of Swedish mothers consisted of bread, potato, pastry, milk, sour milk and cheese. The linoleic acid intake of the Chinese mothers was 14.06 g/day and that of the Swedish mothers was 9.91 g/day. The linoleic acid/ α -linolenic acid ratio was significantly higher for the Chinese than for the Swedish. It was established that the linoleic acid concentration in the milk of the Chinese mothers was significantly higher (22.69%) than that of the Swedish mothers (10.93%); whereas concentration of α -linolenic acid in the milk of the Chinese mothers was lower (1.19%) than in that of the Swedish mothers (1.60%). It was also established that the linoleic acid/ α -linolenic acid ratio was much higher in the milk of the Chinese mothers (22.97) than in that of the Swedish ones (7.50) and also the arachidonic acid/docosahexaenoic acid ratio (3.14; 1.56) was higher. Docosahexaenoic acid content of the food had a positive effect on the milk composition of both the Chinese and Swedish mother (Xiang et al., 2005).

2.1.2 Long chain polyunsaturated fatty acids and human milk lipids

Long-chain polyunsaturated fatty acids (LCPUFA) are bioactive compounds that include docosahexaenoic acid (DHA) and arachidonic acid (AA), that are necessary in optimizing growth and development of infant's neural functions (Agostoni et al., 2005). Human milk does contain both AA and DHA, and their quantitative levels are kept within a homogenous range. Their levels in western populations seem to be quite stable from colostrums through 12 months of life (Marangoni et al., 2002). Most LCPUFA are supplied as triacylglycerols, representing 98-99% of human milk fats, but a relatively high fraction is represented in the limited phospholipid fraction, that is 1-2% of human milk fats. This distribution may have functional consequences, since it has been speculated that fatty acids from the phospholipid fraction could be driven directly to target tissues and membranes (Agostoni et al., 2005; 2008).

Human milk DHA content may be influenced by maternal dietary intakes more than AA content. Women who consume fish and other foods containing high levels of omega 3 LCPUFAs have relatively high milk DHA levels compared with milk from women who consume diets low in these components (Innis and Kings, 1999). According to Koletzko et al. (2001) the different

LCPUFAs occur in considerable amount in the mother's milk. They analyzed the fatty acid composition of the ripe milk from industrial countries and found that the total ω-6 LCPUFA ranged between 0.83–1.40%, while the total ω-3 LCPUFA between 0.27–0.48% as percentage of the total fatty acids (Koletzko et al., 2001). Breast milk contains LCPUFAs, but the content also depends on fatty acid intakes and stores during pregnancy (Martin et al., 1993; Dunstan et al., 2007).

2.1.3 Omega 3 and omega 6 fatty acids ratio

The ratio between dietary ω-6 and ω-3 LCPUFAs (ω6: ω3 fatty acid ratio) is also of possible importance because endogenous synthesis of ω6 and ω3 LC-PUFAs competes for the same elongases and desaturases. During the past few decades, the ω6: ω3 fatty acid ratio has increased in Western diets, which could have some consequences for child neurodevelopment (Simopoulos, 2011). Previous studies have shown that ω 6 and ω 3 fatty acids have different effects on lipid metabolism, particularly with regard to plasma triacylglycerol, low density lipoprotein (LDL), high density lipoprotein (HDL) and lipoprotein subclasses (Harris, 1997; Sanders et al., 1997). Furthermore, PUFA status is affected by the ratio of ω-6 to ω-3 fatty acids of the habitual diet due to metabolic competition between them and may also be determined by dietary intake of energy, total fats and antioxidants.

2.2 Maternal dietary lipids and their effect on the composition of milk fat

The lipid fraction in breast milk in terms of its macronutrients is crucial in fulfilling a newborn's nutritional needs since 50% of dietary calories are supplied to the newborn infant as fat (Giovannini et al., 1995). However, only recently has interest been directed towards the possibility that maternal lipid nutrition may adversely effect, as well as promote, the growth and development of the breastfed infant. The effects of maternal dietary fat composition on the ω-6 and ω-3 fatty acid composition of human milk fatty acids are clearly shown by: changes in breast milk fatty acids over the last 50 years, comparison of fatty acids among women following vegan or mixed diets and the results of controlled interventions to alter maternal dietary fat intake (Innis, 2007). There is a strong association between the linoleic acid (LA) content of human milk and the LA levels in the plasma PUFA and phospholipids in the recipient breast-fed infant (Innis and King, 1999).

Dietary differences between different countries or between regions of the same country have been reported to affect the composition of breast milk. Diet also affects levels of fat soluble components of human milk, such as carotenoids, vitamin A and vitamin E (Jirapinyo et al., 2008). Earlier estimates of fat content in breast-milk from well-nourished communities of the United States of America and Britain gave an average of 0.045-0.048 g/ml (Macy and Kelly, 1981). However, results from poorly fed women in developing countries showed considerable variation and could sometimes be as low as 0.01 g/ml (Atkinson et al., 1980). This shows a drastic reduction in the energy content of milk, which may result in protein energy malnutrition (PEM) in the infants (Atkinson et al., 1980).

2.2.1 Benefits of breast milk

Evidence for promoting child survival demonstrates that breastfeeding saves lives and that exclusive breastfeeding protects against common childhood diseases such as diarrhoea and acute respiratory infections. Support for breastfeeding will be essential to achieve Kenya's targets for the Millennium Development Goals (MDGs) and child survival (GoK, 2007). Recent data indicates that exclusive breastfeeding is the most effective preventive intervention for ensuring child survival. It is estimated to prevent 13 per cent of all deaths of children under five years old (Jones et al., 2003). The introduction of appropriate complementary foods (after 6 months of age) is also a critical issue for child survival, and could save 6 percent of all under-five deaths (Jones et al., 2003).

Some studies have found striking results pertaining to the relative advantages that breastfeeding can confer on child neurodevelopment (Oddy and Kendall, 2003; Vohr and Poindexter, 2006; Kramer et al., 2008). Breastfeeding has previously been associated with improvements across neurodevelopmental domains for low birth weight babies in comparison with not breastfeeding at all (Vohr and Poindexter, 2006). Breastfeeding is promoted as beneficial to both the mother and newborn and exclusive breastfeeding for at least six months is recommended (World Health Organization, 2003). Although there are many reasons to encourage breastfeeding, its benefits for cognitive ability, intelligence and academic achievement have received increasing scientific scrutiny (Horwood and Fergusson, 1998; Silva and Mehta, 2006; Kramer et al., 2008).

Human milk is a complex living, biological fluid. It contains just the right amounts of nutrients, in the right proportions for a newborn (Oddy, 2002). It is processed gently through the baby's digestive system so that these important nutrients are easily absorbed. Breast milk's features include special factors and hormones that contribute to the optimal health, growth and development of infants (Gillman, 2002; Oddy, 2002).

Breast milk provides the only source of ω-6 and ω-3 fatty acids to support the growth and development of the breastfed infant during breast-feeding and for much of early infancy (Innis, 2007). Most infant formulae do not contain the full range of fatty acids found in human milk and are particularly low in the long chain PUFAs. In the brain DHA is enriched in synaptic terminal membranes and has diverse roles in brain growth and function and in protecting against oxidative stress (Innis, 2007).

A study which looked at the fatty acid composition of the brain tissues of infants dying unexpectedly compared breast-fed to formula-fed infants and found that those who had been breast fed had a higher mean concentration of DHA than those who had not (Farquharson, 1992). Several studies have indicated that human milk's protective qualities last well into adulthood. Adults who were breastfed as infants have a decreased risk of developing: Ulcerative colitis and Crohn's disease, diabetes, heart disease and obesity, multiple sclerosis, breast Cancer etc. (Hanson and Korotkonva, 2002; Gillman, 2002; Gartner et al., 2005; Innis, 2007).

2.2.2 Human milk as a model to define acceptable intakes (AI) for fats and fatty acids

Human milk is the preferred infant food; the current recommendations are that term infants be exclusively breastfed for the first 6 months of life (FAO, 2004). Mature human milk (after the first 2-3 weeks of life) provides a fat energy ratio (FER) of 50%. Human milk is a source of LA, ALA, DHA, AA, and other LCPUFA. The level of AA is relatively constant on a worldwide basis while the level of DHA is more variable and depends on maternal diet and lifestyle. Population means for AA in human milk range between 0.3-0.7 weight % of total fatty acids (Marangoni et al., 2002; Smith et al., 2002; Yuhas et al., 2006) while mean values for DHA range from 0.2–1.0% FA (Yuhas et al., 2006). Gibson et al. (1997) reported a dose-dependent response between maternal DHA consumption and DHA levels in human milk, although human milk DHA levels above 0.8% FA did little to increase the plasma or red blood cell DHA content of the infants studied. The content of human milk EFA and LCPUFA can serve to define acceptable intake values, taking into consideration the factors of expected volume of intake, the

fat content of human milk and the range of compositions measured in different regions of the world where children grow well and develop normally (FAO, 2010).

During prenatal life, lipids are mostly needed for structural and regulatory functions. After birth, the infant is challenged by changes in the lipid sources. The response to these changes can be altered by changes in the diet composition. For example, a newborn infant's response to human milk is considered to be ideal, therefore baby formulas try to mimic that response (Van Biervliet et al., 1992). Studies have shown that fatty acid accumulation in the brain increases as the pregnancy progresses (Innis and King, 1999; Innis, 2007). It reaches the maximum level of accumulation toward the end of the pregnancy. But there are differences in the rate of accumulation of these fatty acids. For example, DHA levels in the cerebrum increase as the percentage of the total fatty acid, whereas AA levels decrease during the last trimester (Martinez, 1992).

Studies have also shown that dietary deficiency in alpha-linolenic acid (18:3 n-3) during brain development results in impaired cognitive, visual, and motor skill development (Innis, 1992; Innis, 2007). Some of the problems caused by a deficiency in linoleic acid (18:2 n-6) are poor growth, skin lesions, loss of muscle tone, impaired water balance, and increased susceptibility to infections in infants. These problems disappear after providing an adequate diet that includes linoleic acid (Innis, 1992).

2.3 Lipid extraction

Several procedures for total lipid extraction (Soxhlet, Bligh & Dyer, Rosse-Gottlieb Mojonnier) have been described in the literature (Manirakiza et al., 2001); however there is no unanimous opinion on the most convenient technique. The purpose of extraction is to separate lipids from other constituents of the sample. Solvent extraction is one of the most widely used techniques to extract lipids (Sahena, 2009). Simple extraction procedures using non-polar organic solvents can be used for triacyglycerols-rich samples. Quantitative recovery of the complex lipid mixture from animal tissues is most conveniently achieved using procedures that employ a mixture of polar solvents such as the chloroform-methanol (Folch, 1957; FAO, 2010). In a study that evaluated the FA profiles of a large number of mature (postpartum day 30) human milk samples from each of nine countries, lipid was extracted from the milk using a modified Roese Gottlieb mixed ethers extraction (Yuhas et al., 2006). Solvent extraction methods have

been evaluated for the GC analysis of fatty acids from different food products (Seppänen-Laakso et al., 2002). For analysis of foods for fatty acid information, the AOAC Official method is recommended (AOAC, 2005).

2.3.1 Derivatization of Fatty Acids to Methyl Esters

GC-MS can be used to analyze fatty acids as fatty acid methyl esters. The primary reasons to analyze fatty acids as fatty acid methyl esters (FAMEs) include: in their free, underivatized form, fatty acids may be difficult to analyze, because these highly polar compounds tend to form hydrogen bonds, leading to adsorption issues. Reducing their polarity makes them more amenable for analysis. To distinguish between the very slight differences exhibited by unsaturated fatty acids, the polar carboxyl functional groups must first be neutralized. This then allows column chemistry to perform separations by degree of unsaturation, position of unsaturation and even the cis vs. trans configuration of unsaturation (AOCS, 2005).

The fatty acid composition is determined as the methyl esters of fatty acids by gas-liquid chromatography coupled with mass spectrometry (Christie, 2011). Methyl esters offer excellent stability and provide quick and quantitative samples for GC analysis (Christie, 2011; Brondz, 2002). Esterification is best done in the presence of an acidic catalyst (such as boron trifluoride). The catalyst protonates the oxygen atom of the carboxyl group making the acid much more reactive. Nucleophilic attack by an alcohol then yields an ester with the loss of water. The catalyst is removed with the water. The alcohol used determines the alkyl chain length of the resulting esters; the use of methanol will result in the formation of methyl esters whereas the use of ethanol will result in ethyl esters (Delmonte et al., 2011). Boron trifluoride (BF_3) in methanol or *n*-Butanol has a general formula F_3B: $HO–C_nH_{2n+1}$ where (n = 1 or 4) (Zenkevich, 2009).

This reagent is convenient and inexpensive method for preparing esters. It is most commonly used to form methyl (butyl) ester by reacting it with acids. The general equation for the formation of alkyl esters is shown in **Figure 1a** while that for the acid catalyzed esterification process in **Figure 1b**.

a. F_3B: $HO–CnH_{2n+1} + RCOOH \rightarrow RCOOC_nH_{2n+1}$

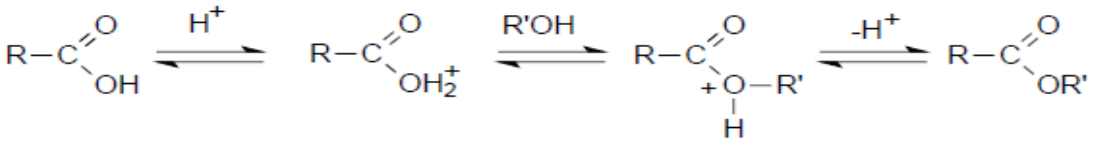
b 

Figure 1. Acid- catalyzed esterification of fatty acids (Christie, 2011).

2.4 Analytical methods

There are various methods for identifying the fatty acid composition of human milk. The technique most commonly used for fatty acids analysis is the gas chromatography coupled with mass spectrometry (GC-MS). With the coupling of MS methods to GC, much has been accomplished in the area of qualitative characterization of FAME mixtures. Since GC–MS provides spectrometric information on separated compounds, it provides a means of analyte selectivity; thus, detection with MS also represents a potentially powerful tool for quantitative analysis of FAME, especially in the presence of a convoluted biochemical background (Dodds et al., 2005).

Not all organic compounds are suitable for direct GC-MS analysis due to their involatile nature. Many important biological compounds, such as fatty acids, flavonoids, alkaloids, carbohydrates and amino acids are polar, and have limited volatility (Yayli et al., 2001). In GC-MS analysis, the components in a sample are eluted according to retention times by gas chromatograph and separated based on mass to charge ratio of the ions by the mass spectrometer (Hoffmann and Stroobant, 2001).

2.4.1 Gas Chromatography Mass Spectrometry (GC-MS)

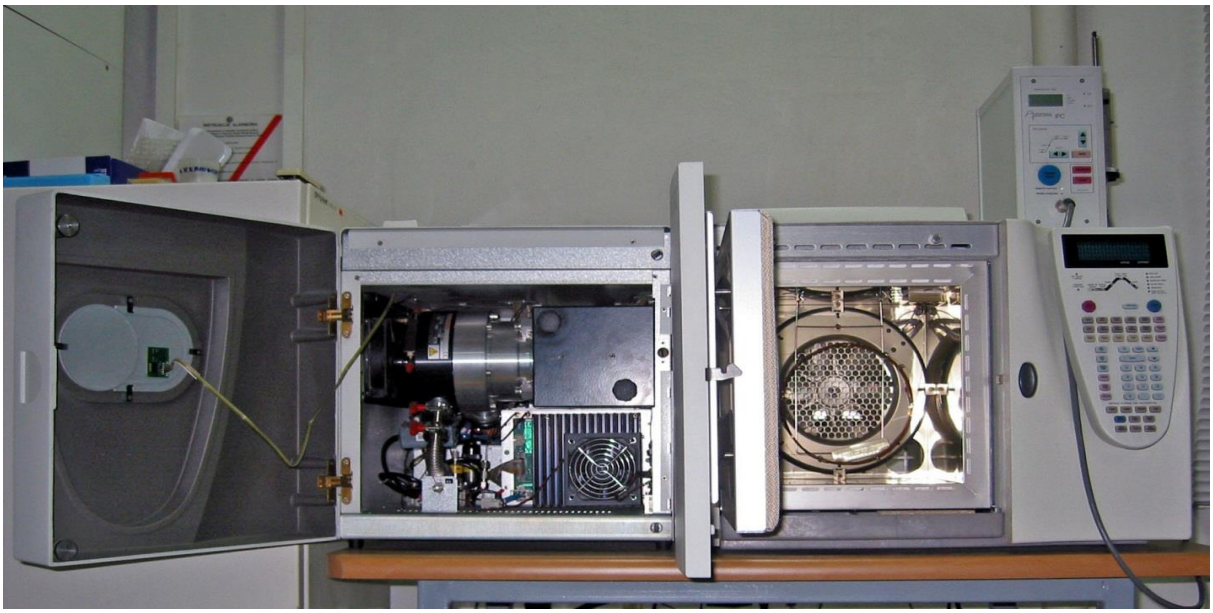
Present-day gas chromatographic methods with high-quality capillary columns allow sensitive and reproducible fatty acid analyses, as well as the characterization of complex mixtures of geometric isomers when combined with other chromatographic separations and spectroscopic identification (Seppänen-Laakso et al., 2002). The GC-MS is a gas chromatographic (GC) technique interfaced with mass selective detector. This is the method where peak identification problem, faced by GC flame ionization detector (FID) is solved. The GC-MS is a powerful tool in identifying peaks (Christie, 2003). In GC-MS, fatty acids are ionized, forming charged fragments with certain mass. Fatty acids are identified based on mass

to charge ratio, and compared with a library of known mass spectra which is stored on computer database. Usually fatty acids are analyzed by GC-MS as fatty acids methyl ester (FAME) (Christie, 1998; Christie, 2003).

2.4.2 Instrumentation

In GC-MS, a sample is injected into the GC column in the oven through an injection port. The temperature of the injector is high enough to vaporize all the components, so that the sample becomes a gas mixture. The mobile phase which is an inert gas (helium, nitrogen and hydrogen) carries the sample through the GC column (**Figure 2**). Due to the differences in the volatilities, the components are separated. The more volatile the component is, the sooner it is eluted. Polar stationary phases allow separation according to unsaturation and carbon chain length, thus they are commonly used for the analysis of FAMEs (Christie, 1998; Ruiz-Rodriguez et al., 2010).

The gas mixture is then introduced into the mass spectrometer. The first component of a mass spectrometer is an ion source (Ruiz-Rodriguez et al., 2010). The relative abundances of the ion fragments that are produced are a function of the energy of ionizing electrons and the temperature at which the ions are produced (Karasek and Clement, 1988). In a quadrupole mass analyzer, both a direct current (DC) voltage and a radio frequency (RF) voltage are applied to each of the four parallel rods. By adjusting the DC/RF ratio, the ions are filtered by their m/z ratios. Only the fragments having mass to charge ratios (m/z) within the selected range can pass the mass filter and reach the detector. The detector counts the number of ions hit it at each m/z. This information is collected by the computer and a chromatogram is generated using a gas chromatograph interfaced with a mass spectrometer (**Figure 3**) (Hoffmann and Stroobant, 2001; Ruiz-Rodriguez et al., 2010).

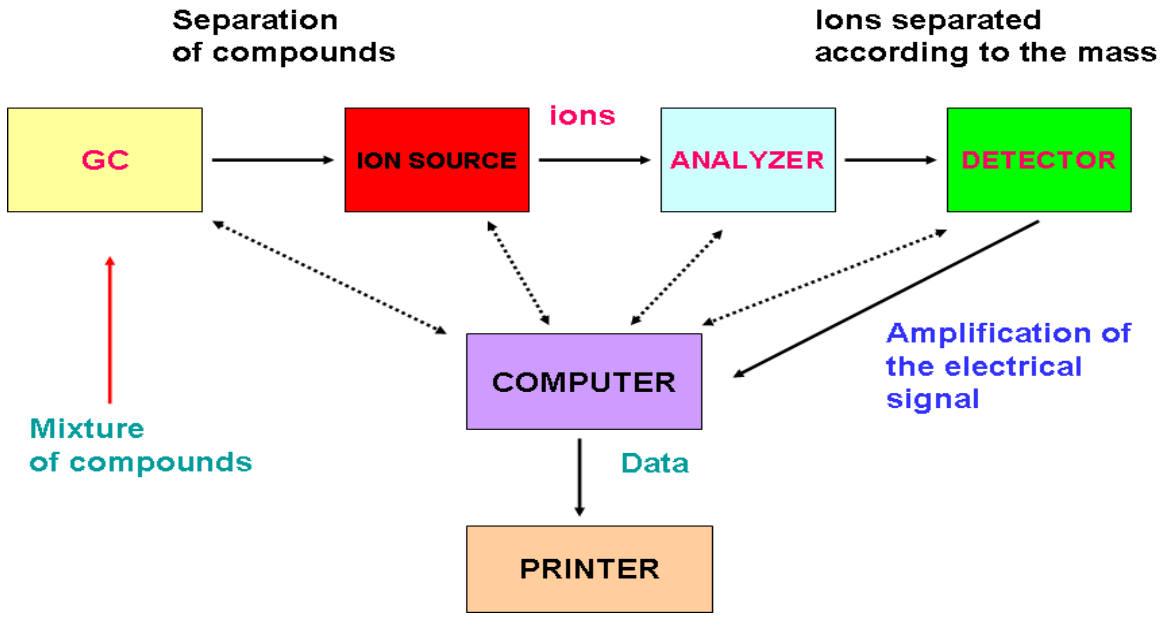


Source: http://www.specmetcrime.com/benchtop_gcms_introduction.htm . Accessed 2014 October 10

Figure 2. Bench top Gas Chromatograph Interfaced with a Mass Spectrometer: the insides of the GC-MS, with the column of the gas chromatograph in the oven on the right

2.5 Identification of the components

The identification of the components is achieved by using reference spectra. For a specific compound, at specific experimental conditions, the fragmentation pattern and retention time are the characteristics of the compound. By comparing the results against reference libraries, the identity of the compound can be determined (Hoffmann and Stroobant, 2001; Seppänen-Laakso et al., 2002).



Source: Ruiz-Rodriguez et al. (2010)

Figure 3. Schematic illustration of a GC/MS analytical System

2.6 Quantitative analysis

Quantification of FAME by GC with FID has been effectively performed for some time, whereas detection with MS has been used chiefly for qualitative analysis of FAME (Doods et al., 2005). The quantitative analysis of GC-MS technique can be conducted either in total ion chromatogram (TIC) or selected ion monitoring (SIM) mode. In the TIC scan mode, the mass spectrometer scans all the m/z in the selected range (for example, 50-400 m/z) repetitively and sums up the abundance of all the m/z over the whole range, and gives a TIC.

In the SIM mode, the mass spectrometer only monitors a limited number of m/z and records their abundances. Compared to TIC mode, SIM gives a much higher sensitivity and selectivity. Thus, SIM mode is usually applied to detect the substances with low concentrations (Krahmer et al., 1999). In addition to providing several acceptable options for quantification of FAME, GC–MS offers two powerful advantages over FID: the ability to confirm the identity of analytes based on spectral information in addition to retention time, and the ability to separate peaks from a noisy background or co-eluting peaks if unique ions are available (Doods et al., 2005).

2.7 Statistical tests

Prior to conducting analyses, the distribution of the data should be examined for departures from normality, such as skewness or outliers. If the data are normally distributed, and other assumptions are met, parametric tests are the most powerful. If the data are non-normal but other criteria are met, non-parametric statistics provide valid analyses (Zimmerman, 1998).

The Wilcoxon signed-rank test is a non-parametric statistical hypothesis test used when comparing two related samples, matched samples, or repeated measurements on a single sample to assess whether their population means ranks differ. It can be used as an alternative to the paired Student's t-test, *t*-test for matched pairs, or the *t*-test for dependent samples when the population cannot be assumed to be normally distributed. The Kruskal-Wallis test is a nonparametric (distribution free) test, and is used when the assumptions of analysis of variance (ANOVA) are not met. They both assess for significant differences on a continuous dependent variable by a grouping independent variable (with three or more groups). In the ANOVA, it is assumed that distribution of each group is normally distributed and there is approximately equal variance on the scores for each group (Corder and Foreman, 2009*;* McDonald, 2014).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Subjects

The study subjects were healthy nursing mothers (age 17 to 40 years) who were participating in a Cohort study of the impact of an integrated agriculture, nutrition and health intervention on the Vitamin A and health status of mothers and their infants from pregnancy to nine months postpartum in Bungoma County, Kenya. The subjects were enrolled at mid pregnancy (10-24 weeks) and followed with their infants through 9 months postpartum, first antenatal care visit, full term, health infant, exclusive breastfeeding for more than 9 months and residing in the catchment area were the inclusion criteria. Participation was voluntary, and informed consent was obtained from the participants. Recruitment was based on a proportion to population sampling procedure with the number of women recruited per facility specified. The study was approved by the Kenya Medical Research Institute (KEMRI) and Emory Internal Review Board (EIRB). Milk was collected by manual expression between 9:30am and 3:30pm by the mothers in a health facility and the breast milk (BM) samples were processed and kept at -20 ^{0}C prior to delivery and analysis at Egerton University Chemistry laboratory.

3.2 Sample preparation

Lipid fraction was extracted from the breast milk using the Association of Official Analytical Chemists International (AOAC) Official Method 989.05 with modifications (AOAC, 2005). The frozen milk sample was thawed for 30mins in a water bath heated to 37 ^{o}C then vortexed for 30seconds. 1ml of the milk was measured into a 50ml round bottomed flask and refluxed for 30 min after addition of 1ml of 3 N hydrochloric acid. The sample was cooled to about 20 ºC. To this sample, 1 ml of NH_4OH was added to neutralize the acidic sample and dissolve the casein followed by addition of 1 drop of phenolphthalein indicator to help sharpen visual appearance of the interface between the ether and aqueous layers during extraction. To the neutralized sample, 1ml of 95% ethanol was added (to prevent possible gel formation) and the flask vigorously shaken for 1 min while releasing built- up pressure by loosening stopper as necessary. 2.5 ml of diethyl ether were added and sample shaken for 1 min to dissolve the lipids. To the extract, 2.5 ml petroleum ether were added and the mixture shaken for 2 min to separate water and dissolve more non polar lipids. All the reagents used were of analytical grade.

The mixture was transferred to a separating funnel and left to stand for 5 min, and the upper ether layer was decanted into a weighed round bottomed flask. The solvent was evaporated on a hot plate in a hood at 100 ºC. The flask was dried to a constant weight in an oven at 100 ºC ± 1 ºC and the percent fat calculated as follows:

The % fat = 100 × {[wt dish + fat) – (wt of dish)] – (average wt blank residue)}/wt sample

Note: For blank determination, 1ml of distilled water was used instead of milk sample

Fat was extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using boron trifluoride (BF_3) in methanol (AOAC International, 2007).

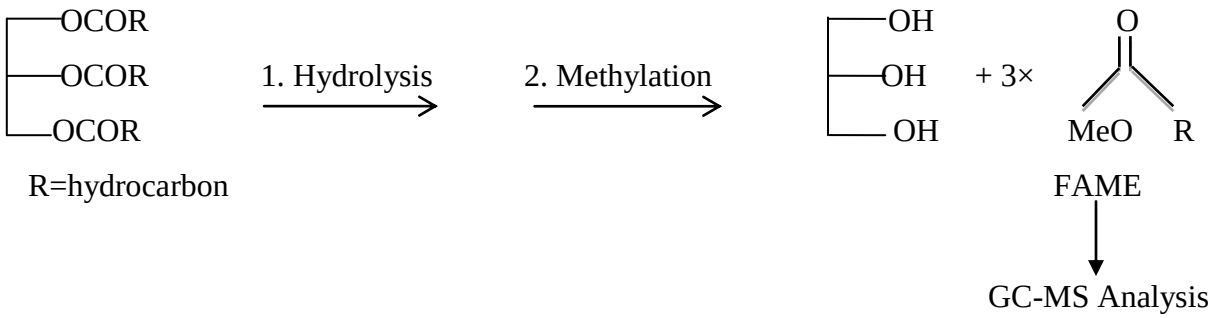


Figure 4. Schematic representation of the analytical process

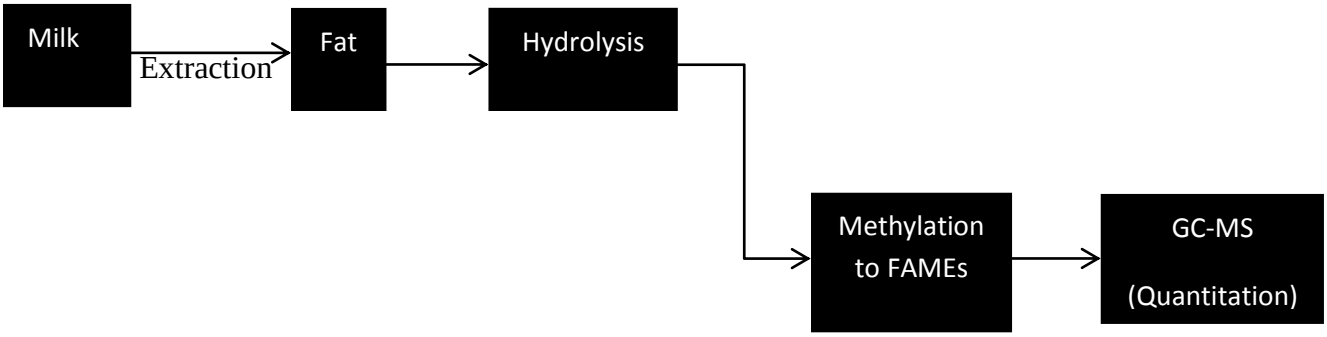


Figure 5. Flow diagram for the analysis of breast milk

3.3 Preparation of fatty acid methyl esters (Methylation)

Fatty acids were derivatized following the Official Methods of the American Oil Chemists' Society (AOCS, 2005). Lipids (1 mg) were suspended in 2 ml hexane prior to derivatization. 5mls of 0.5N sodium hydroxide (NaOH) in anhydrous methanol (MeOH) was added with a few boiling chips into the flask containing the lipids. Water cooled condenser was attached and refluxed on a heating plate for 1hr. 5mls of BF_3 - Methanol (10% w/w) was added through the top of the condenser then refluxed for 30 min. 5mls of hexane were then added and refluxed for 1 min longer. While still attached to the condenser, the round bottom flask was raised above the heating plate and allowed to cool for 15 min. To the cooled extract, 10 ml of saturated sodium chloride (NaCl) solution was added to the flask and stoppered after disconnecting the condenser. The contents were shaken vigorously for 2 min and left to stand for 10 min followed by additional 3 ml of saturated sodium chloride (NaCl) to float the hexane layer. The hexane layer containing the methyl esters was transferred into a test tube with a small scoop of anhydrous sodium sulfate to dry then caped. The dried sample was transferred into a vial and kept at -4 ^{o}C to await GC- MS analysis.

3.3.1 Preparation of sample for GC-MS analysis

To the sample in the vial, 1ml hexane was added to the sample then vortexed for 30 seconds. The sample was dried by passing through anhydrous sodium sulfate ($NaSO_4$) placed in a glass Pasteur pipette with a glass wool. The dried sample was transferred into Teflon caped sample vials from which 10µl was diluted by adding 990µl hexane then vortexed for 30seconds. 200µl of the mixture was pipetted into a clean Teflon capped sample vial with an insert. The vial was placed into an auto sampler where 1µl was injected into GC-MS.

3.3.2 Gas Chromatography-Mass Spectrometry analysis

The FAMEs were analyzed by splitless injection using an Agilent technologies-7890 gas chromatograph coupled to a 5975C inert XL EI/CI mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA). The GC was equipped with a carbowax HP-20M column (25 m × 0.20 mm ID × 0.20 µm film thickness, Agilent, Palo Alto, California, USA), with helium as the carrier gas at a flow rate of 1.2 ml/min. The oven temperature was held at 35 °C for 5 min, then programmed to increase at 10 °C/min to 220 °C and maintained at this temperature for 10 min.

3.3.3 Standard preparation

Stock solution was prepared by dissolving 10mg/ml of the commercial FAMEs mixed standards into100 ml of methylene chloride. Suitable volume of the stock solution of the FAMEs mixture was used for preparation of known concentrations (working standards).

3.4 Identification and quantitation of FAMEs

Fatty acid methyl esters were identified by comparison of retention times of the FAMEs from human milk samples with retention times of commercial FAMEs standards and with library data (Adams2.L, Chemecol.L and NIST05a.L). The amount of triacylglycerol in the different human milk samples was quantified based on peak areas. Through use of reference standards, verification and quantitation was possible.

3.5 Statistical analysis

Results are expressed as mean ± standard error (SEM). For statistical analysis the R software (R version 3.1.1) (R Development Core Team, 2011) was used. The Wilcoxon signed rank test was used to detect the difference between the fatty acid composition of human milk samples at the fourth month and the ninth month of lactation. The Wilcoxon test was also used to evaluate the difference between the individual fatty acids and between samples at both stages of lactation. The Kruskal-Wallis was used to test for the difference between the various fatty acid groups identified.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 FAME identification and quantification

In this study, the fatty acid methyl esters (FAMEs) of breast milk were characterized, a total of 70 samples were analyzed for fatty acid profile. In order to identify the hydrolyzed fatty acids, a capillary gas chromatographic-mass spectrometric method was employed for profiling total fatty acids methyl esters. Total run time of the FAMEs was 34 minutes. The FAMEs were identified in each sample by comparing the retention times of the human milk samples with retention times of the commercial standards and with those of known FAMEs using a comprehensive library databank. Concentrations of the identified fatty acids were calculated from the peak areas of corresponding FAME mixed standard using standard calibration curves. The FAMEs concentrations were converted to triacylglycerols (TAGs) using the conversion factor indicated in the Fatty Acid Conversion Table (Appendix V) (Nielsen, 2010).

4.2 Fatty Acids Analysis

Human milk fatty acids are the result of dietary intake, mobilization from fat depots and endogenous synthesis by the mammary gland which are capable of synthesizing saturated fatty acid (SFA), primarily 10 to 14 carbons in length. Fatty acids of chain lengths >16 carbons are not synthesized in the mammary gland and must be obtained either from the diet or mobilized from fat depots (VanderJagt et al., 2000). In the current study, the major fatty acids identified and quantitated are shown in **Table 1**. In the table, the esters are listed in order of elution on a capillary column depending on their relative affinities.

In the present study we compared fat contents and fatty acid composition of breast milk (BM) samples collected both at the fourth month and at the ninth month of lactation from the same lactating women participating in a large birth cohort study. The fat content in the current study ranged from 0.010- 0.065g/ml showing a slightly less average as compared to the US and Britain studies (0.045-0.048 g/ml) (Macy and Kelly, 1981). The conversion factors (**Table 1**) obtained from the Fatty Acid Conversion Table, were used to calculate the total fat in mg/ml of breast milk. The difference between our data and the others can be attributed to the difference in maternal diet as earlier reported (Jensen, 1999; Innis, 2007; Jirapinyo et al., 2008).

Table 1: Fatty acid methyl esters identified and quantified at 4 and 9 months postpartum in a cohort of 35 rural Kenyan women

Methyl ester IUPAC name	FA Common name	Retention time (min)	No. of carbons	No. of double bonds	Conversion factor
Octanoic	Caprylic	10.58	8	0	0.9915
Decanoic	Capric	14.35	10	0	0.9928
Dodecanoic	Lauric	16.97	12	0	0.9937
Tetradecanoic	Myristic	19.17	14	0	0.9945
Hexadecanoic	Palmitic	21.15	16	0	0.9950
9-hexadecenoic	Palmitoleic	21.36	16	1	0.9950
Octadecenoic	Stearic	22.96	18	0	0.9955
9-octadecenoic	Oleic	23.11	18	1	0.9955
9, 12 octadecadienoic	Linoleic	23.48	18	2	0.9954

ALA, LCPUFAs and Short chain (C4-C7) fatty acids were trace and not detected in most of the samples. The conversion factors were obtained from the Fatty acid conversion table, appendix V (Nielsen, 2010).

The distribution and variation of fatty acids during the fourth month and ninth month postpartum are shown in **Table 2**. Although, both stages of lactation were found to contain identical profile of the fatty acids in the breast milk, the relative proportion was however significantly (P<0.05) different. The fat content of milk samples was significantly higher at the 9th month than at the 4th month of lactation. More than 80% of the saturated fatty acids were composed of lauric acid (C12:0), myristic acid (C14:0), palmitic (C16:0), stearic (C18:0) at the 4th month, this results agree with that of Taiwanese study (Wu et al., 2010) that reported more than 80% of the total fatty acids composing of C12:0, C14:0, C16:0 and C18:0 among the saturated fatty acids. Oleic acid (C18:1n-9) was the most abundant unsaturated fatty acid (66.59%) at the 4th month while linoleic acid (C18:2n-6) was abundant unsaturated fatty acid (58.08%) at the 9th month of lactation (**Table 2**).

The major saturated fatty acid C18:0 constantly accounted for more than 20% of total fatty acids. For the unsaturated fatty acids, the proportion of C18:2n-6 increased significantly

while the monounsaturated fatty acids palmitoleic (C16:1n-7) and C18:1n-9 decreased significantly between the 4th and 9th month of lactation.

Table 2: Fat content and percentage composition of the fatty acids in human milk of 35 Kenyan rural women investigated both at the 4th month and the 9th month of lactation

	4th month		**9th month**	
Fatty acid (FA)	**Mean ± SEM**	**n**	**Mean ± SEM**	**n**
Fat content (mg/ml)	0.38 ± 0.04	35	1.21 ± 0.003	35
Fatty acid composition (% w/w)				
Saturated fatty acids				
C4-C7	ND		ND	
C8:0	3.52 ± 2.17	20	3.82 ± 0.88	20
C10:0	6.16 ± 1.79	35	9.05 ± 4.98	35
C12:0	18.35 ± 6.65	35	19.70 ± 8.33	35
C14:0	26.14 ± 9.62	35	18.77 ± 8.71	35
C16:0	19.04 ± 6.03	35	16.53 ± 6.56	35
C18:0	26.47 ± 6.13	35	23.14 ± 5.02	35
Unsaturated fatty acids				
C16:1n-7	28.88 ± 23.49	22	4.28 ± 0.88	22
C18:1n-9	66.59 ± 26.48	35	30.86 ± 7.74	35
C18:2n-6	51.32 ± 13.59	35	58.08 ± 16.81	35
Total saturated (Average)	16.62 ± 4.00		15.18 ± 2.97	
Total unsaturated (Average)	48.93 ± 10.95		31.05 ± 15.51	
Linolenic (C18:3n-3)	ND		ND	

Values are presented as mean ± standard error (SEM). Proportion of each fatty acid measured in all the samples is significantly different (p <0.0001) at both lactation stages using Wilcoxon signed test. Where: ND (not detectable), n (20 and 22) for C8:0 and C16:1n-7 respectively, are only for those samples that registered more than trace amounts.

There was significant difference between the two groups (4^{th} month and 9^{th} month) in terms of fatty acids (FA) percentages for all FA using Wilcoxon test. Wilcoxon signed analysis revealed a significant difference in percentage weight of individual fatty acid at the fourth month and at the ninth month of lactation (V = 45, *p*-value < 0.05). The percentage average of the total unsaturated fatty acids (48.93% and 31.05%) was significantly higher ($p < 0.05$) than the average percent of the total saturated FAs (16.62% and 15.18%) at the 4^{th} and 9^{th} month of lactation respectively (**Table 2**). Wilcoxon tests also showed significant difference in percentages of individual fatty acids among the subjects at both lactation stages ($p< 0.0001$).

Kruskal test revealed a significant difference in percentage weight between the component fatty acids at 4^{th} month (Kruskal-Wallis chi-squared = 77.1655, degrees of freedom (df) = 8, *p*-value < 0.0001) and at 9^{th} month (Kruskal-Wallis chi-squared = 114.7254, df = 8, *p*-value < 0.0001). The percentage weight of the individual fatty acids between the subjects were significantly different at both months (Kruskal-wallis chi-squared = 79.6161, df = 34, *p*-value < 0.0001). Two fatty acids, C8:0 and C16:1n-7, were only quantitated in 20 and 22 samples respectively since they were trace in all other samples. It is interesting to note that the unsaturated FAs were in high concentration as compared to saturated FAs and were different between women, perhaps suggesting the dependence of these FAs on immediate diet as opposed to body depots (Del Prado et al., 2000).

4.2.1 Saturated fatty acids (SFA) in human milk

There was a significant increase for C8:0-C12:0 fatty acids between the subjects at the two lactation groups, which was consistent with similar findings reported from Spain and Taiwan (Barbas and Herrera, 1998; Wu et al., 2010). These researchers observed an increased proportion of middle-chain fatty acids (MCFAs) compatible with the character of breast milk, presumably as the needs of infants' changed. These changes in milk composition may indicate variations in mammary gland biosynthetic capacity. The importance of a high percentage of lauric acid (C12) and myristic (C14) in the diet of human milk-fed infants may not be restricted to cholesterol metabolism and cardiovascular protection (Lubetzky et al., 2012). Indeed, lauric acid, metabolized into monolaurin, has been shown to play multiple roles as an antibacterial and antiviral agent (Carpo et al., 2007). Infants fed with human milk are notably protected against a multitude of infectious diseases, and it is possible that one of the many agents involved is indeed lauric acid (Lubetzky et al., 2012).

The SFA mean found in breast milk at the 4th (16%) and (15%) at the 9th month fell way below the European Range (39.0 – 51.3%),(Koletzko et al., 1992) and the Spanish (Granada) mature milk (40.66%) (Lo´pez-Lo´pez et al., 2002). The current study showed that stearic acid (C18:0) was the predominant fatty acid that accounted more than 20% of the total fatty acids than palmitic (19% and 16%) at 4th and 9th month, respectively. Lubetzky et al. (2012) also reported similar results where stearic acid (15%) was the predominant SFA both at short and long lactation periods. The results from the current study are however different from results of several studies, where the major saturated fatty acid, palmitic acid (C16:0), constantly accounted for 20% of total fatty acids (Lo´pez-Lo´pez et al., 2002; Sala-Vila et al., 2005; Szabo et al., 2010; Wu et al., 2010).

Studies on the fatty acid composition of human milk from mothers in a variety of geographical locations have been reported. It is well documented that the fatty acid composition of human milk is affected by dietary habits (Lammi-Keefe and Jensen 1984; Lo´pez-Lo´pez et al., 2002). Dietary differences between different countries or between regions of the same country have been reported to affect the composition of human milk (Golfetto et al., 2002; Innis 2007; Jirapinyo et al., 2008). For instance, previous studies have shown that Asian or African milk contains more saturated fatty acid than that of Western countries (Muskiet et al., 1987; Koletzko et al., 1992; Presa-Owens et al., 1996). Although Bungoma County, Kenya is geographically located in Africa, our results did not show typical characteristics for a population with a relatively high consumption of carbohydrate and low consumption of fat, as was expected according to the previous studies. In fact, the proportion of saturated fatty acids in the analyzed human milk samples from Bungoma County was only 16%, which is lower than that in Asian or West African countries.

4.2.2 Monounsaturated fatty acids (MUFA) in human milk

Oleic acid (C18:1 n-9), the major unsaturated fatty acid, and palmitoleic acid (C16:1n-7) decreased significantly at the 9th month, this agrees with similar findings reported from different geographic areas (Pago-Gunsam et al., 1999; Serra et al., 1997; Lo´pez-Lo´pez et al., 2002). Although oleic acid is not an essential fatty acid, it is very important because, in addition to the usual functions of fatty acids (source of energy and structural components), it reduces the melting point of triacylglycerides, thus providing the fluidity required for the formation, transport and metabolism of milk fat globules (Jensen, 1999). Similar MUFA percentages were

reported in a German study (Genzel-Boroviczeny et al., 1997), where the percentage of oleic acid was 32.16% and Spanish study (38.83%) (Lo´pez-Lo´pez et al., 2002). However, our study showed high mean value at fourth month (66.59%) and low at ninth month (30.86%) of lactation. The oleic acid mean values were also comparable to the mean value in mature milk of the European Range (ER) (34.42 – 44.90%) (Koletzko et al., 1992).

4.2.3 Polyunsaturated fatty acids (PUFA) in human milk

Linoleic acid (LA) was the most abundant fatty acid at both lactation stages. The LA mean values at both months (51.32 % and 58.08 %) was very high than the ER (6.9 –16.4) and Taiwanese mean value (23.62%) (Wu et al., 2010). The fact that the concentrations of alpha linolenic acid (ALA), arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were undetectable in all the samples studied was surprising. The differences in unsaturated composition between the mothers at both months may also reflect differences in body fat composition and parity-related mobilization of fat and FA stores (Butte and Hopkinson, 1998; Lassek and Gaulin, 2006). Indeed, generally reported concentrations of DHA in human milk range between 0.27 and 0.48 and are reported to be little influenced by maternal diet (Koletzko and Rodriguez, 1999). It must also be noted that Shehadeh et al. (2006) in a study of human milk during prolonged lactation also found undetectable values of DHA in the majority of samples. Similarly, Lubertzky et al. (2012) in another study found that four fatty acids from the long chain polyunsaturated group were undetectable in a large percentage of samples and fell below the limit of detection of the method they used in their study.

One of the important functions of long chain polyunsaturated fatty acids (LCPUFAs) is as a fundamental source of central nervous system development in early life. They are found abundantly in the prefrontal cortex and are associated with the development of retinal cells (Agostoni, 2008). Recent studies have even found improvement in psychological disorders such as attention-deficit/hyperactivity disorder, dyspraxia, and autism after dietary supplementation with LCPUFAs (Belkind-Gerson et al., 2008). Consequently, particular note has been taken in the content of essential fatty acids in human milk. The content of LA in the current study was in the range of 51-58%; this is higher than that found (< 20%) in the milk of Spanish, German, Australia, Tanzania, Mexico, Canada and American women (Muskiet et al., 1987; Yuhas, et al., 2006). Presa-Owens et al. (1996) reported an LA content of 12.3% in 40 Spanish women and

Koletzko et al. (1992) reported 10.6% for German women. These results suggest that Kenyan women from the analyzed group ingest a considerable amount of LA in their diet.

The increase in mono and di-unsaturated fatty acids in this study may be due to a reduction in the endogenous production of saturated fatty acids. The unsaturated fatty acids (C18:1 and C18:2) that were found to be predominant in the milk samples at both lactation periods may be a reflection of the dietary fat of the lactating mothers as earlier reported (Insull et al., 1979; Lassek and Gaulin, 2006). An increase in polyenoic acids (unsaturated fatty acids) of breast milk due to substitution of corn oil for lard in the maternal diet had earlier been reported (Innis and kings, 1999; Lawuyi et al., 2004). The higher content of LA, as shown by the profiles, is of advantage since they have been implicated in the infants' nervous system development (Golfetto et al., 2002; Lawuyi et al., 2004).

Levels of LA in the breast milk from the present study were among the highest reported in the extensive literature characterizing breast-milk fatty acid composition hence of concern with regard to omega 3 fatty acids. Calder (2006) reported that the high level of LA in Western diets has led to concern, particularly in relation to the concurrent low intake of n-3 fatty acids and the increasing prevalence of many inflammatory and immune disorders. The major portion of PUFA in milk, LA and AA, originate primarily from maternal fat stores, influenced by long-term dietary intake (Innis, 2007; Brenna et al., 2009). LCPUFA (more than 20 carbon chains in length) are also synthesized from their respective precursors, LA and ALA, although conversion of AA, EPA and DHA is low (Brenna et al., 2009). DHA conversion may also be diminished by competitive inhibition from LA and *trans* FAs (TFAs) (Brenna et al., 2009; Gibson et al., 2011). Smit et al. (2003) conducted an extensive evaluation of breast milk FA concentrations compared with regulatory standards and found that the levels of specific fatty acid (LA, ALA, AA, DHA, lauric acid, and myristic acid) from the milk of numerous mothers fell outside of the regulatory guidelines. Indeed, several studies investigated the fatty acid composition of mature BM at different points of lactation and reported increasing levels of the essential fatty acids but decreasing values of the most important LCPUFAs, AA, and DHA (Luukkainen et al., 1994; Agostoni et al., 2001; Mojska et al., 2003).

Figure 6 and **7** shows representatives of total ion chromatograms of FAME mixed standard and breast milk sample respectively obtained from the GCMS instrument. Other selected TICs are presented in appendices **I- III**. FAMEs of the analyzed breast milk samples

were identified by comparing the retention times of the milk samples with retention times of corresponding fame standards and mass spectra. Each compound has a unique or near unique mass spectrum that can be compared with a mass spectral database and thus identified. The mass spectrum for a representative FAME (9-Octadecenoic acid) is presented in f**igure 8**. The mass spectra of the other fames quantitated are presented in appendices (**IVa- IVh**).

Breast milk (BM) is remarkable for its variability, and ranges of intakes of milk constituents are comparable with normal patterns of infant growth and development. Lipids are by far the most variable constituents in human milk with both long-term maternal nutrition states and daily intake capable of exerting an influence (Picciano, 1998). The FA composition varied among the 35 mothers in the present study and between the two stages of lactation.

The FA composition of milk determined in the current study was similar to that previously reported (Marangoni et al., 2000). In contrast to the study of Marangoni et al. (2000), we observed that the FA composition did vary between the two lactation periods, most fatty acids decreasing in proportion at the 9^{th} month. Alpha-linolenic acid (ALA) and other LCPUFAs were trace in our study indicating that the mothers were not consuming omega-3 fatty acids hence of health concern, considering these FAs are necessary in optimizing growth and development of infant's neural functions. Despite living in close physical proximity the nursing mothers showed distinct cultural difference in dietary intake. The mothers were on ad libitum diets. These differences were reflected in the difference in the fatty acid composition of breast milk samples.

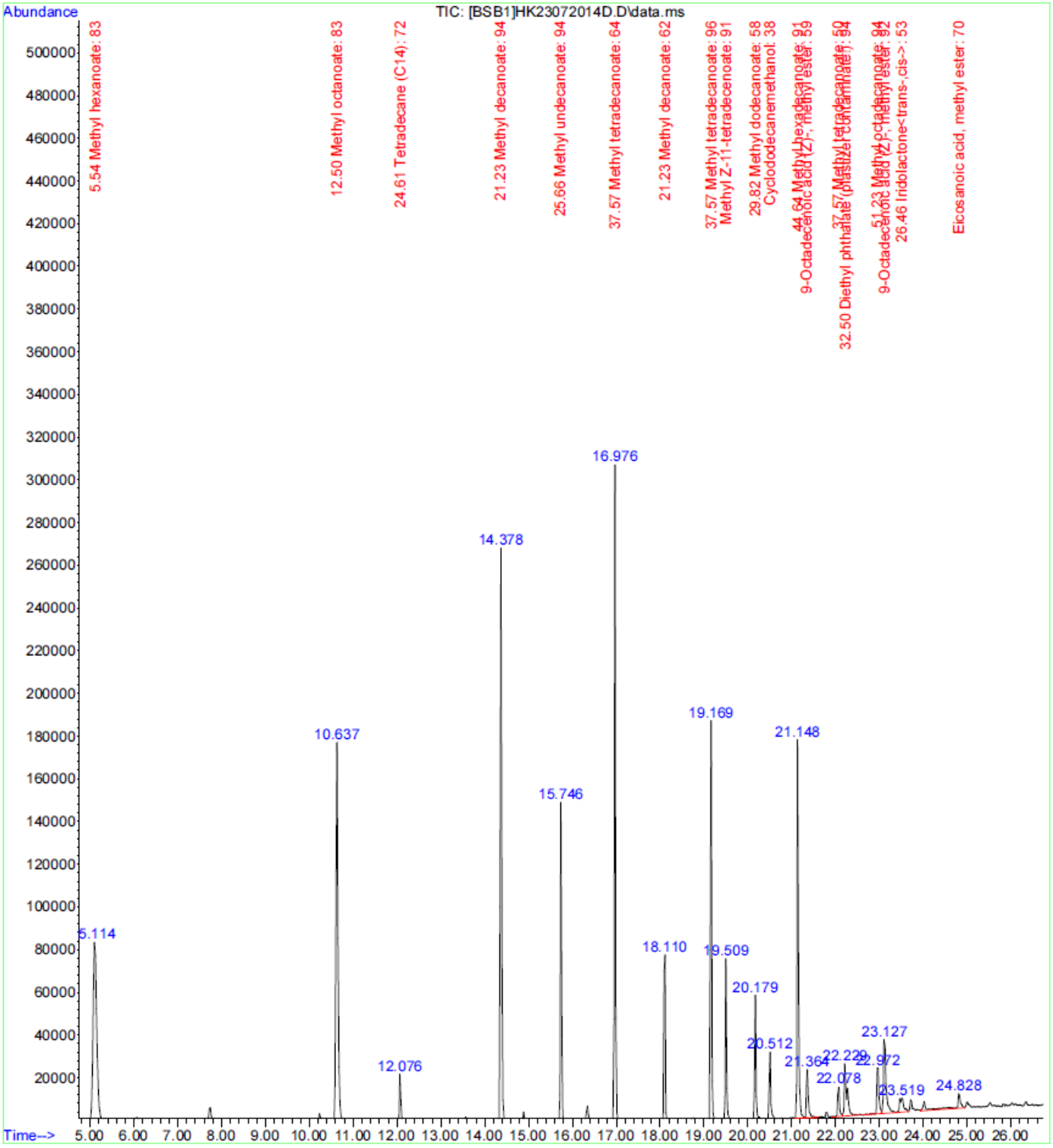


Figure 6. Representative of a total ion chromatogram of the FAME mixed standard

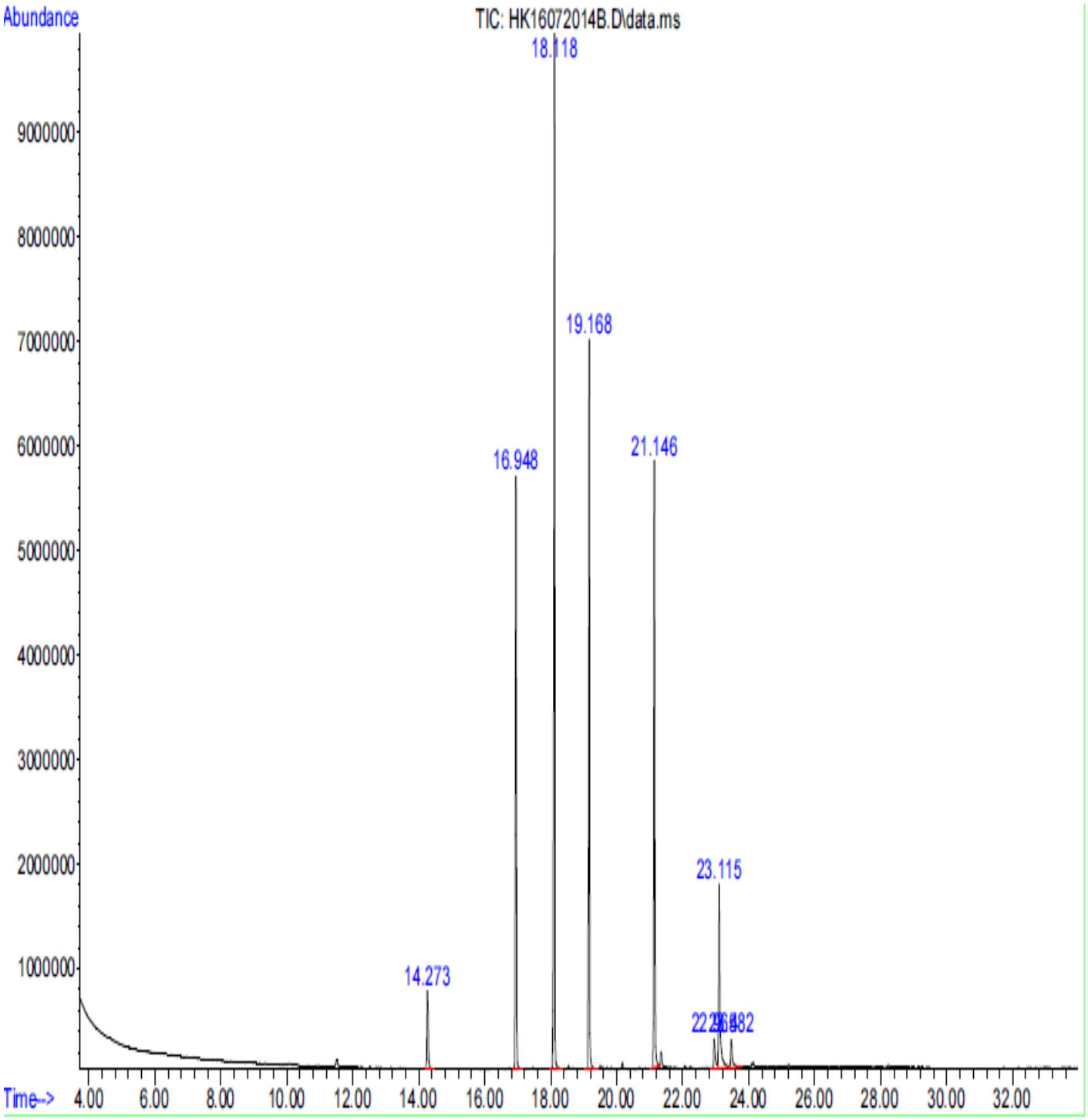
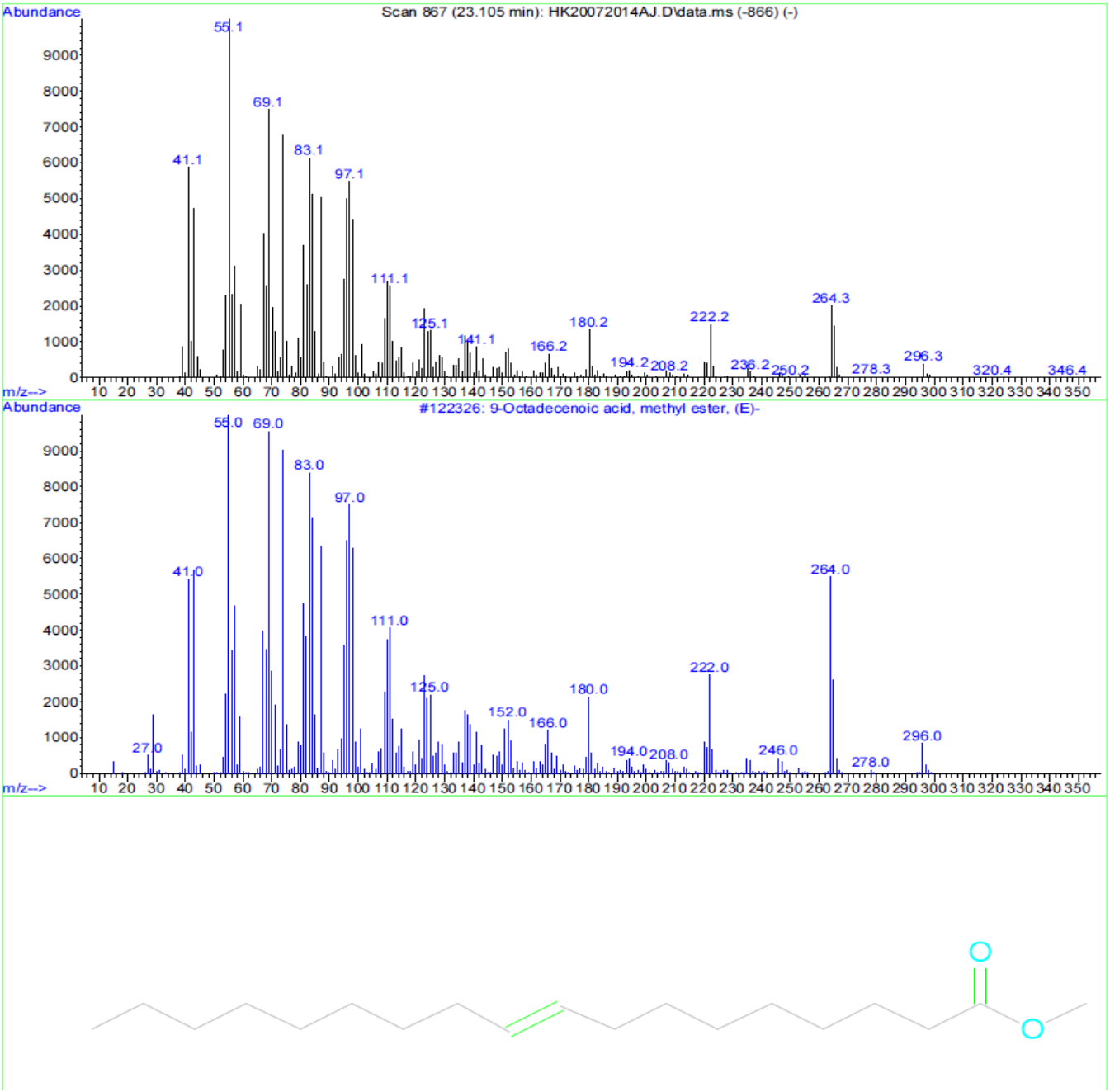


Figure 7. A representative of total ion chromatogram of the FAMEs of breast milk



9-Octadecenoic acid, methyl ester-E

Figure 8. Representative mass spectrum of FAME obtained from the GC-MS instrument library at *icipe* (Adams2.L, Chemecol.L and NIST05a.L

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study determined the FA profile of a total of 70 human milk samples from Bungoma County, Kenya.

1. Stearic (C18), Myristic (C14), palmitic (C16), lauric (C12), oleic (C18:1) and linoleic (18:2) acids were the predominant fatty acids found at both 4^{th} month and 9^{th} month postpartum BM samples. Among the saturated fatty acids, stearic acid (C18:0) was the predominant fatty acid that accounted more than 20% of the total fatty acids while oleic acid constituted the primary monounsaturated FA in all samples. These results indicate that the breast milk samples analyzed have identical profile but differ in their concentrations
2. The data suggest that dietary intake of food sources of n-3 LCPUFA is low and possibly deficient in Western region of Kenya, and that biochemical indices of maternal DHA status and breast milk DHA content are very low or undetectable compared to the international literature. Inadequate LCPUFA status of women during the reproductive cycle might have negative consequences for the developing infant as well as for the mother

5.2 Recommendations

This study is among few in literature to determine FA profile of breast milk in Kenyan populations hence from the results the following recommendations are suggested for further research:

1. The data from the current study indicate inadequate omega (ω) 3 fatty acid status among the analyzed group of Kenyan women during pregnancy and lactation hence there is need for intervention to increase consumption of foods rich in ω-3
2. Although the breast milk samples analyzed have identical profile there was no consistent pattern in FA variation between the two periods. It is therefore, recommended that this variation together with the variation in total fat content be accounted for. It will be of particular interest to profile the fatty acids over a considerable period of lactation (several months or a year) for better comparison

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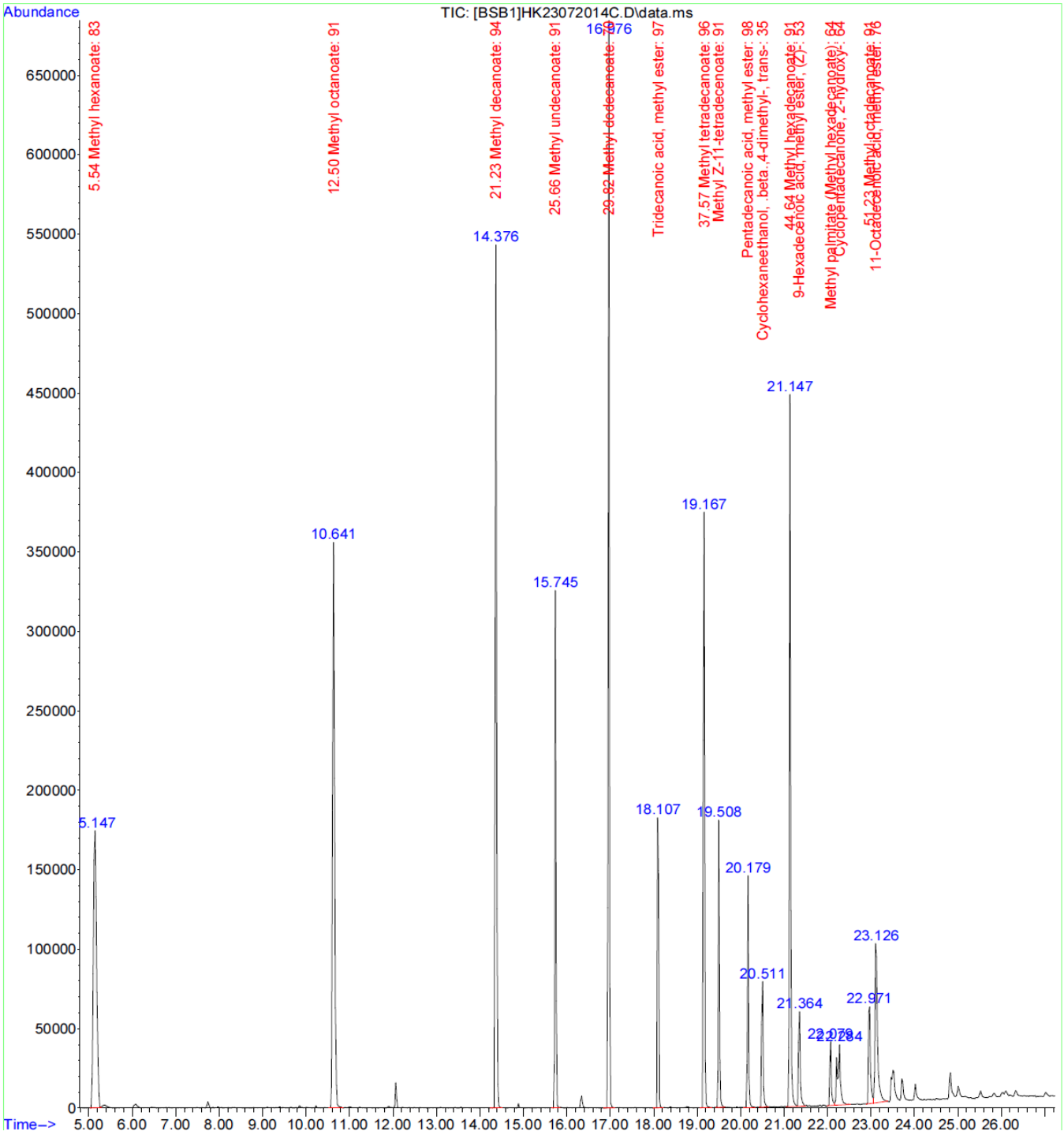
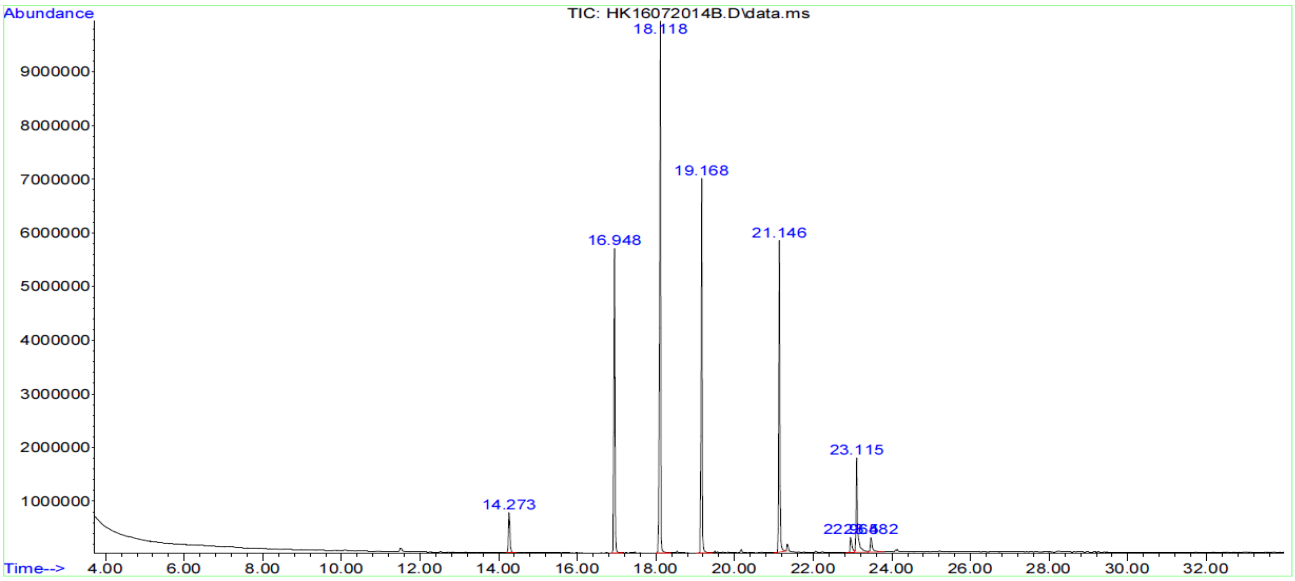
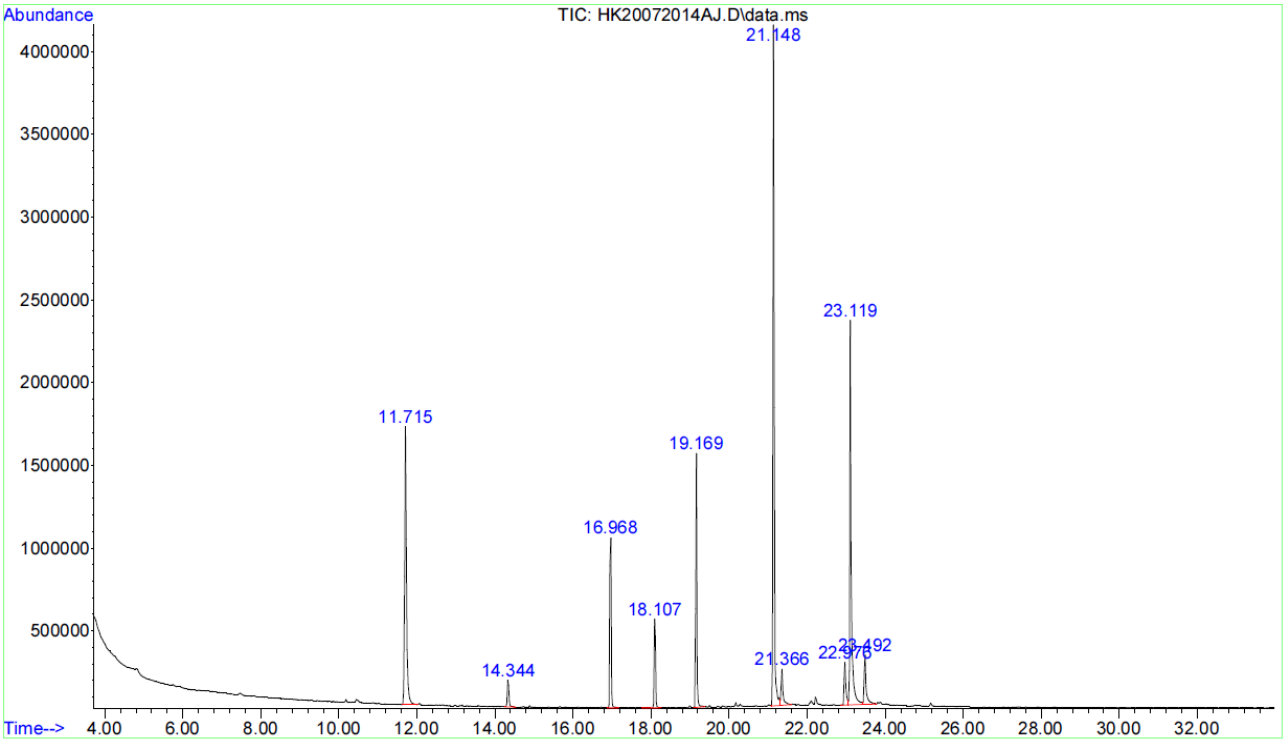
APPENDICES



Appendix I: A representative of a total ion chromatogram for the fame mix standard.

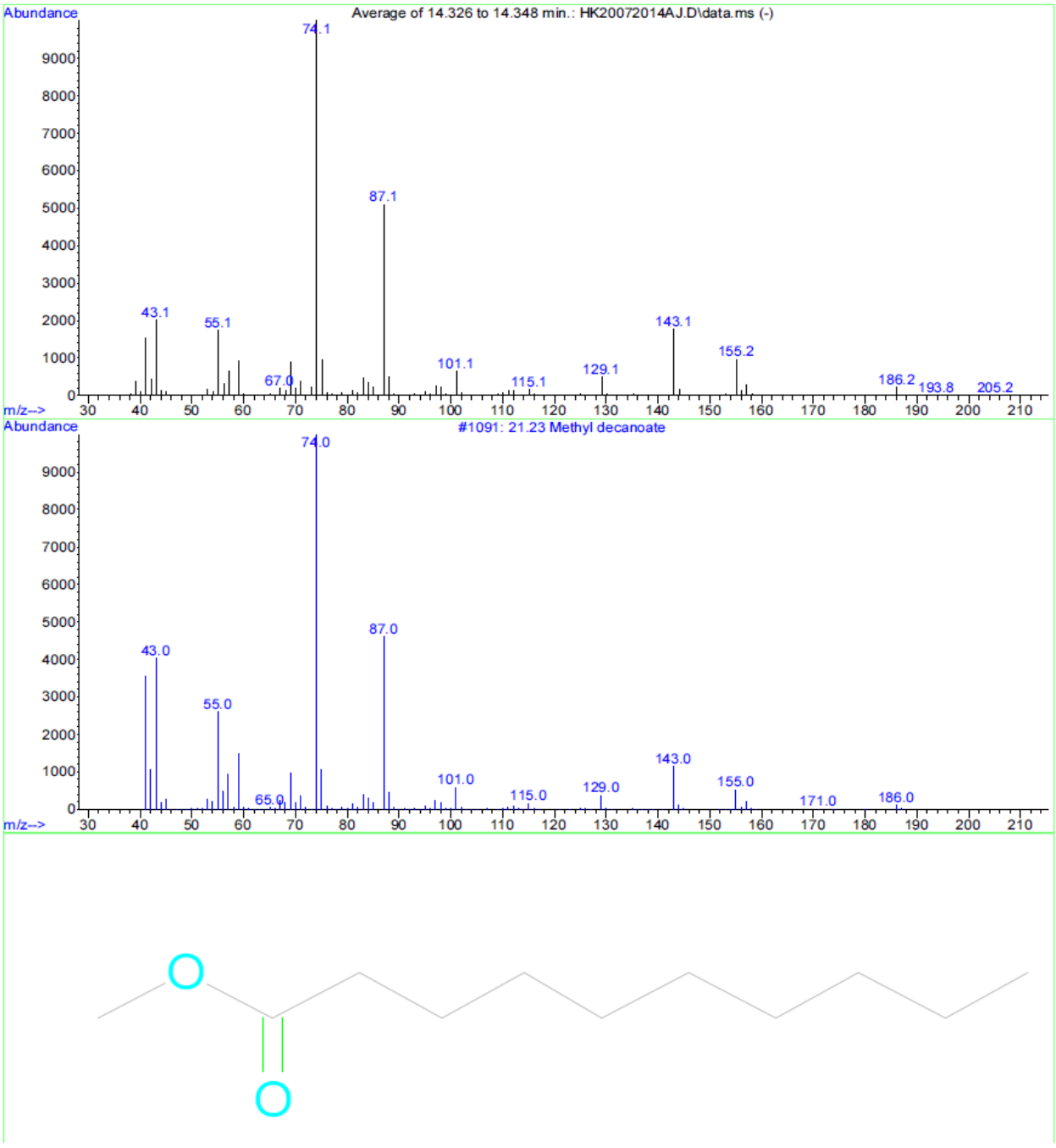


Appendix II: A representative of total ion chromatogram for a selected 4th month BM sample

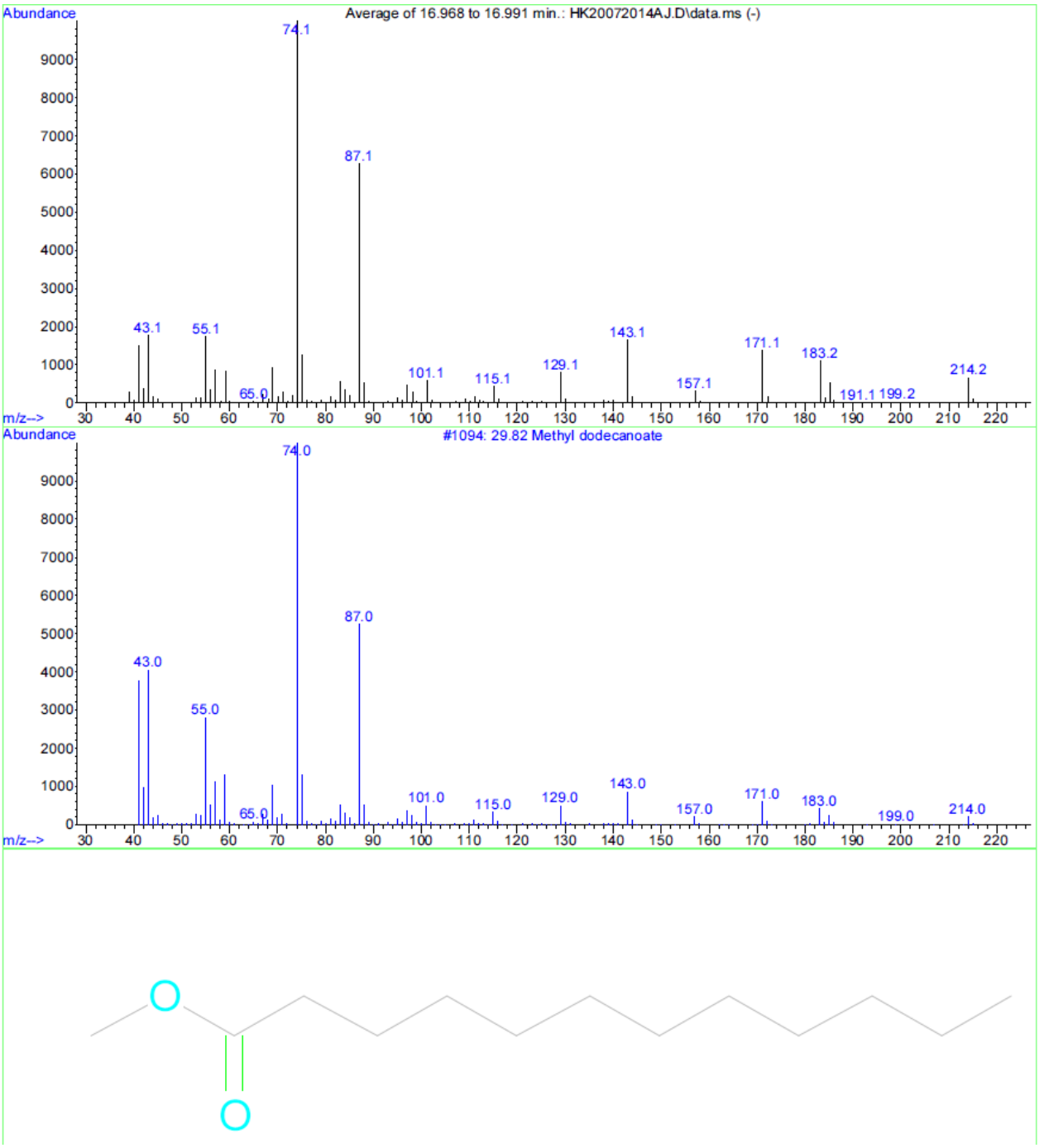


Appendix III: A representative of total ion chromatogram for a selected 9th month BM sample

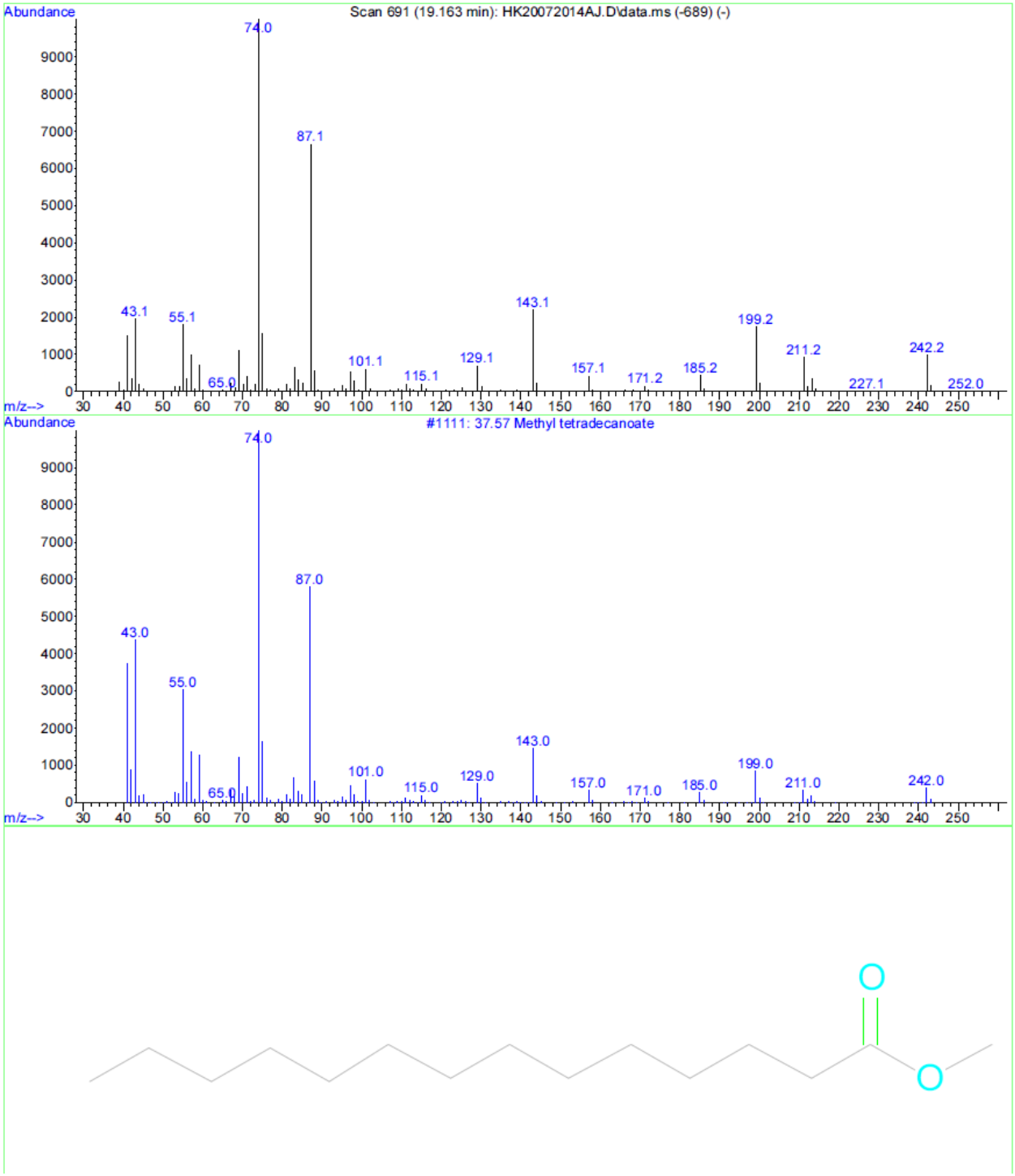
Appendix IVa- IVh shows mass spectra of the major FAMEs quantitated from the GC-MS instrument library at *icipe* (Adams2.L, Chemecol.L and NIST05a.L).



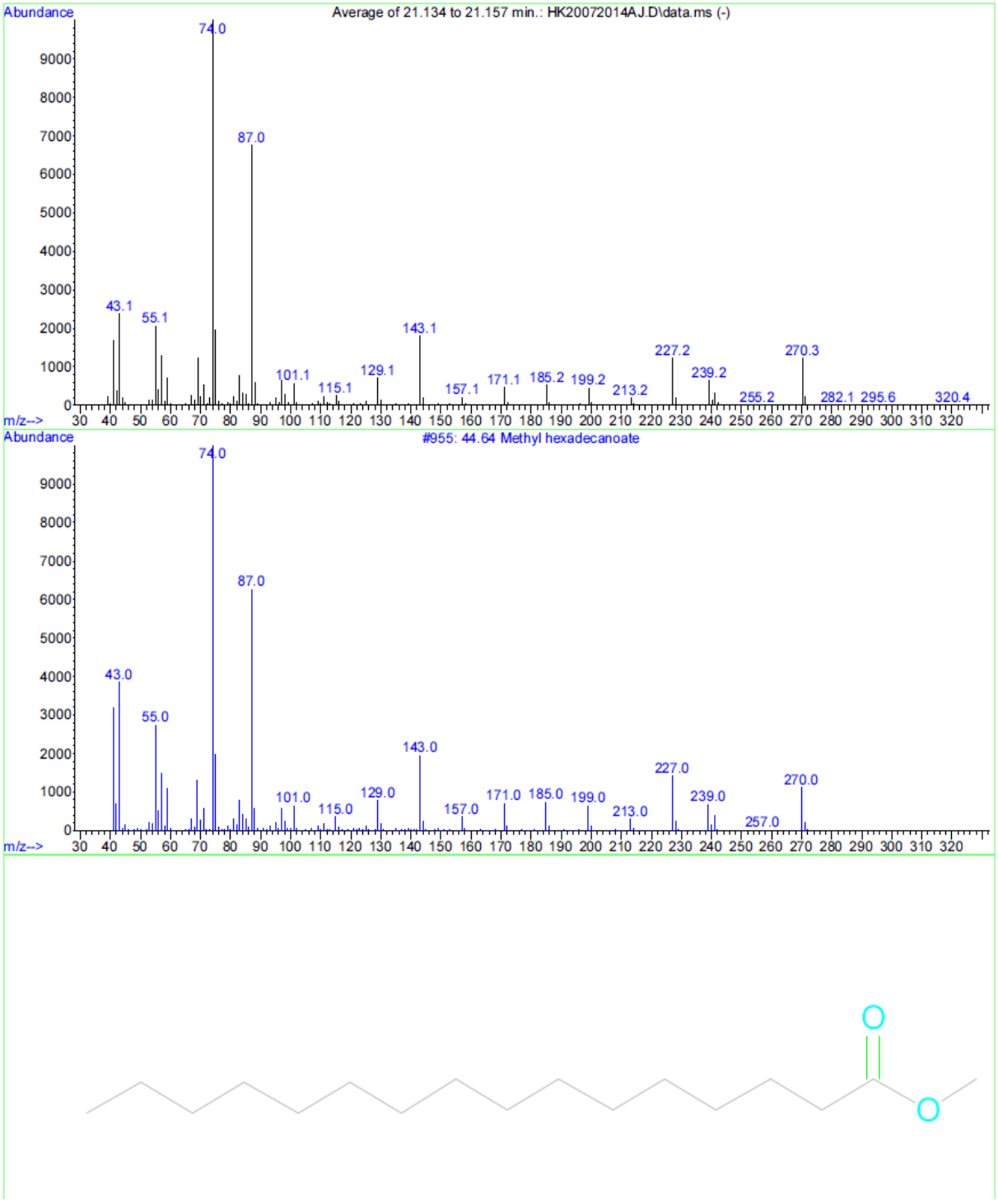
Appendix IVa. Methyl decanoate (C10:0)



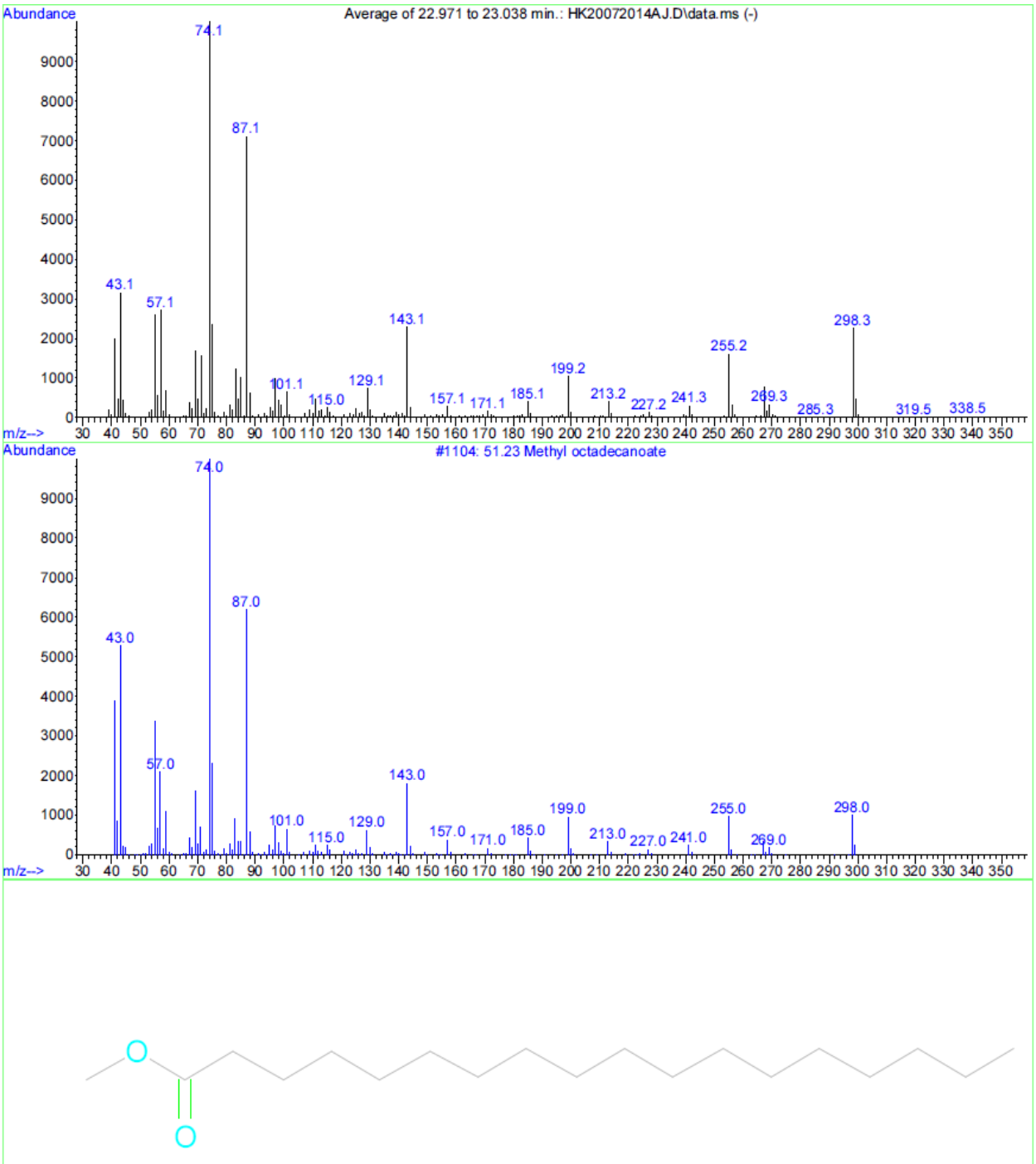
Appendix IVb. Methyl dodecanoate (C12:0)



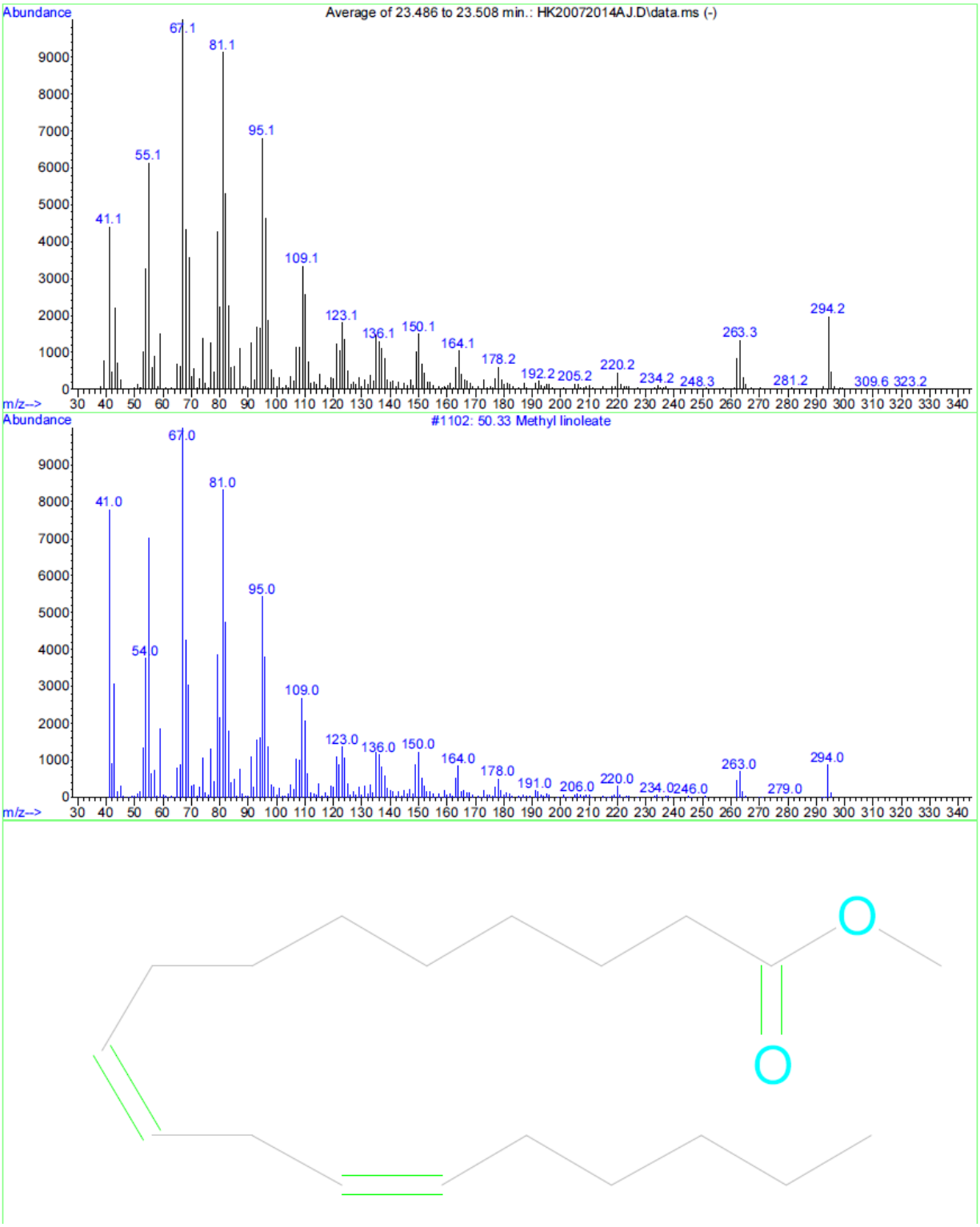
Appendix IVc. Methyl tetradecanoate (C14:0)



Appendix IVd. Methyl hexadecanoate (C16:0)

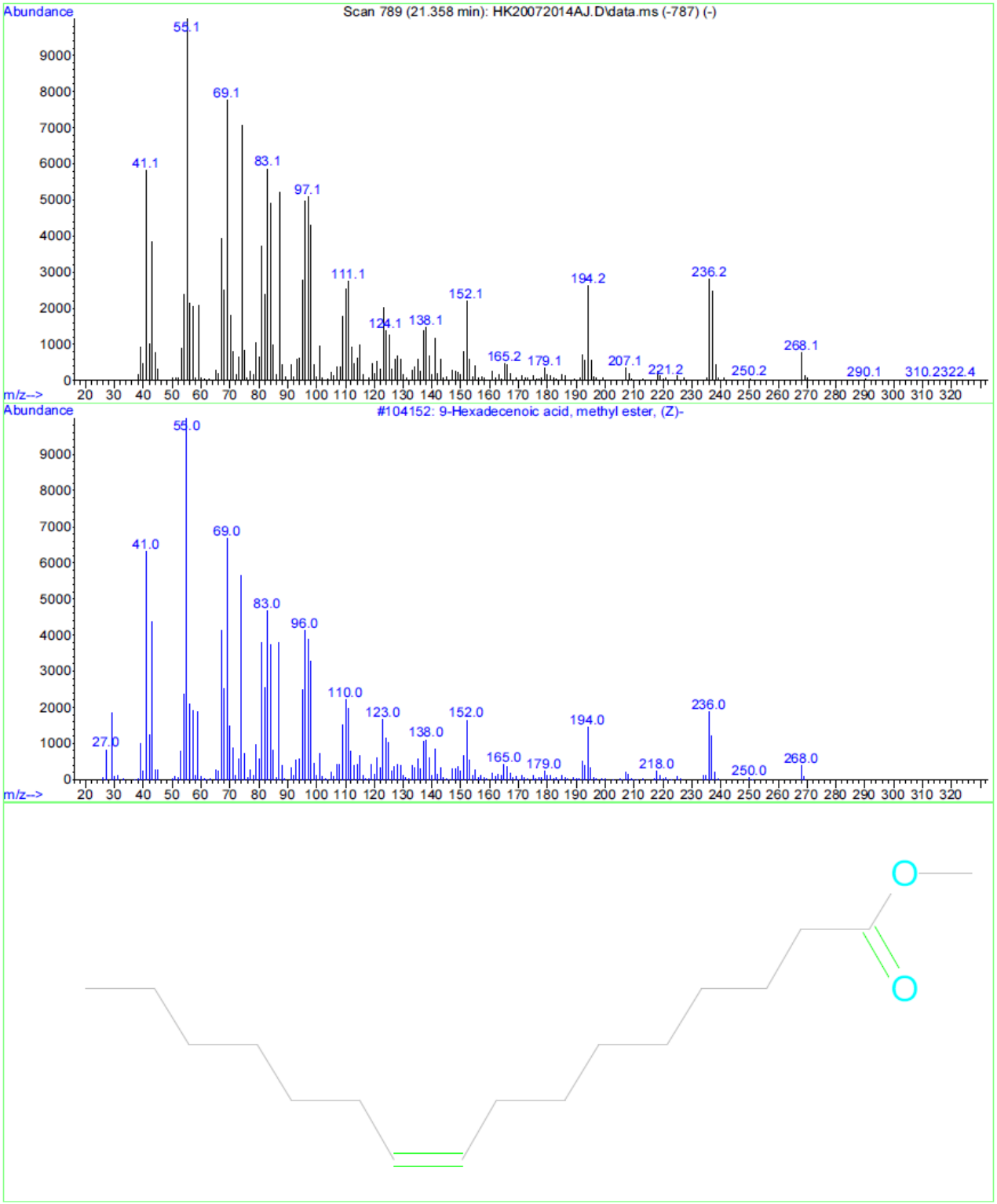


Appendix IVe. Methyl octadecanoate (C18:0)

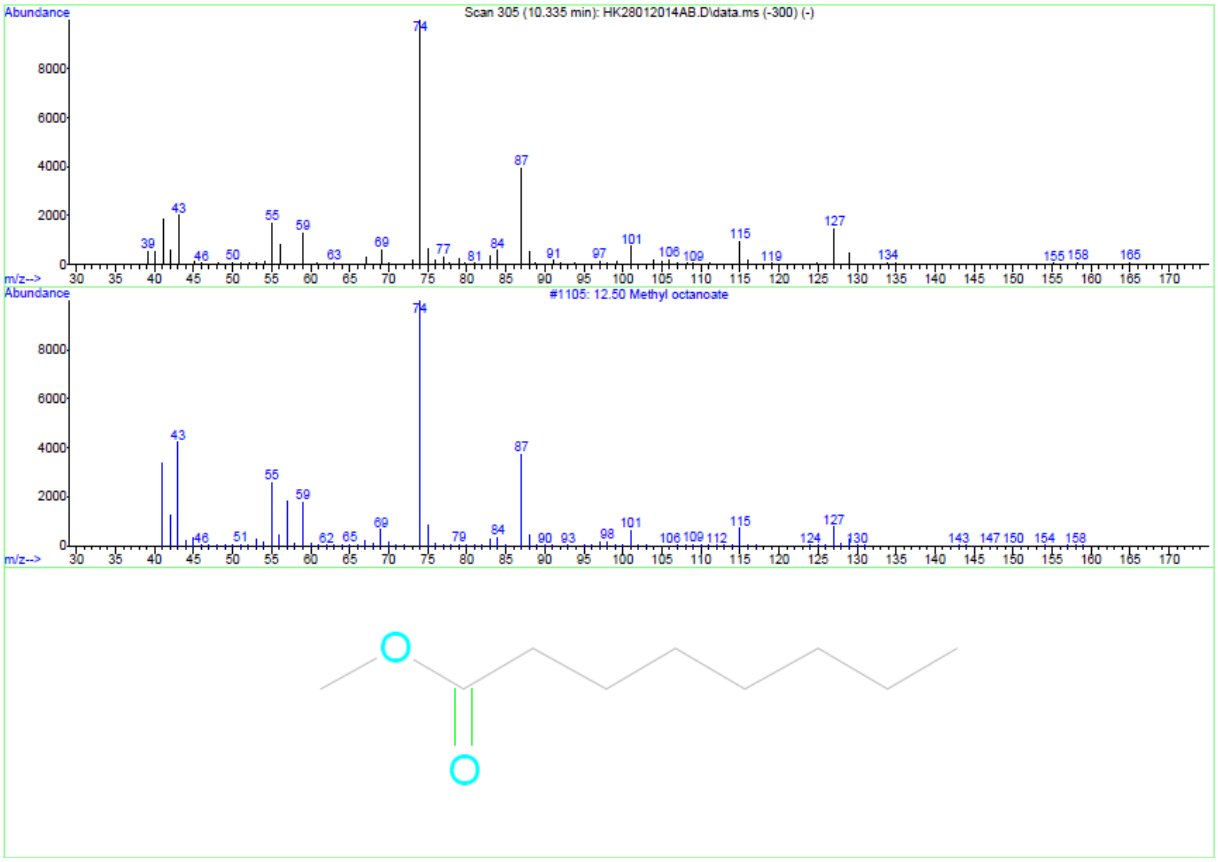


Appendix IVf. Methyl linoleate (C18:2n-6)

Library Searched : C:\Database\NIST05a.L
Quality : 99
ID : 9-Hexadecenoic acid, methyl ester, (Z)-

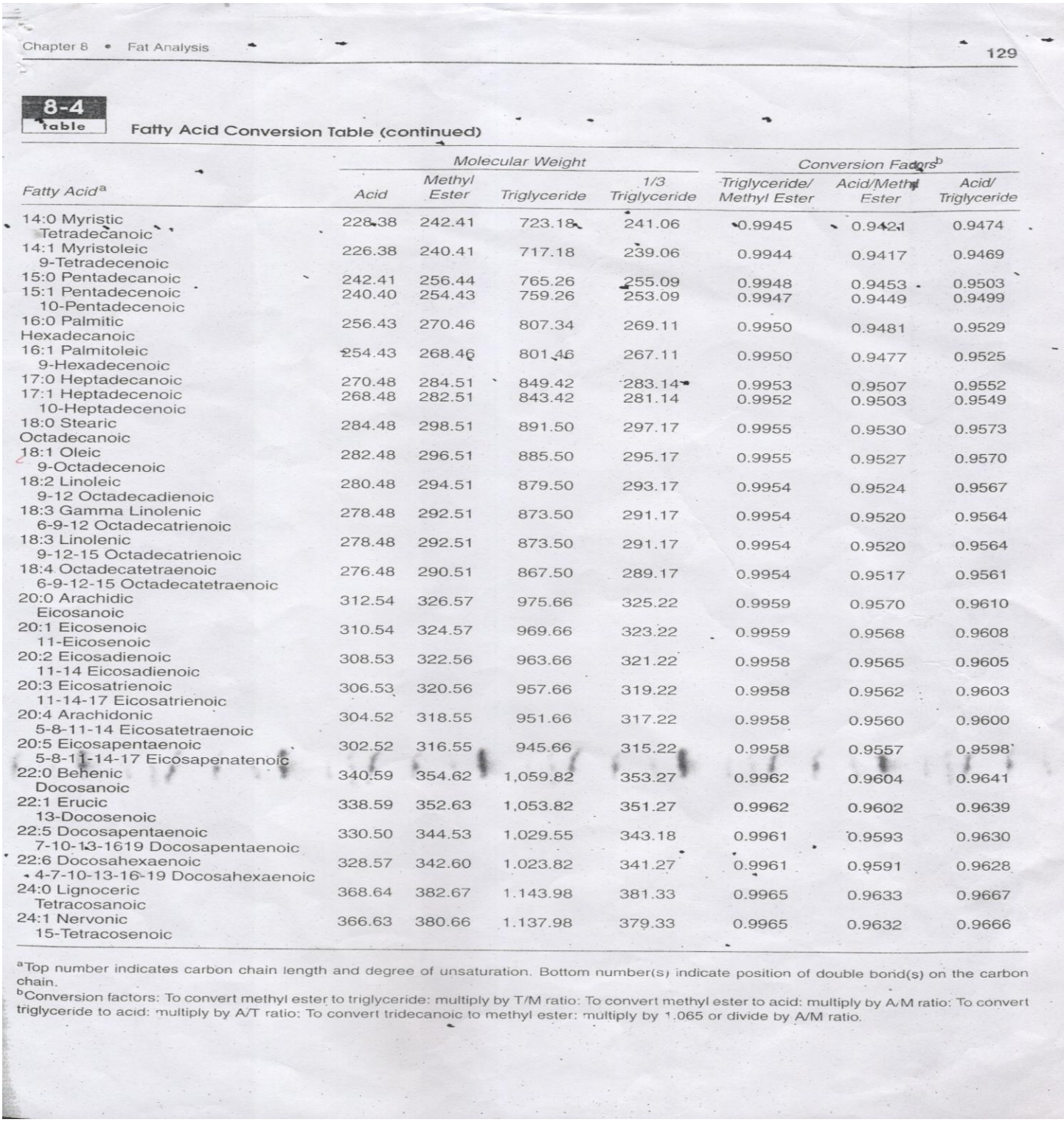


Appendix IVg. 9-Hexadecenoic acid, methyl ester- Z (C16:1n-7)



Appendix IVh. Methyl octanoate (C8:0)

Appendix V. Fatty Acid Conversion Table



8-4 table Fatty Acid Conversion Table (continued)

Fatty Acid[a]	Molecular Weight				Conversion Factors[b]		
	Acid	Methyl Ester	Triglyceride	1/3 Triglyceride	Triglyceride/ Methyl Ester	Acid/Methyl Ester	Acid/ Triglyceride
14:0 Myristic Tetradecanoic	228.38	242.41	723.18	241.06	0.9945	0.9421	0.9474
14:1 Myristoleic 9-Tetradecenoic	226.38	240.41	717.18	239.06	0.9944	0.9417	0.9469
15:0 Pentadecanoic	242.41	256.44	765.26	255.09	0.9948	0.9453	0.9503
15:1 Pentadecenoic 10-Pentadecenoic	240.40	254.43	759.26	253.09	0.9947	0.9449	0.9499
16:0 Palmitic Hexadecanoic	256.43	270.46	807.34	269.11	0.9950	0.9481	0.9529
16:1 Palmitoleic 9-Hexadecenoic	254.43	268.46	801.46	267.11	0.9950	0.9477	0.9525
17:0 Heptadecanoic	270.48	284.51	849.42	283.14	0.9953	0.9507	0.9552
17:1 Heptadecenoic 10-Heptadecenoic	268.48	282.51	843.42	281.14	0.9952	0.9503	0.9549
18:0 Stearic Octadecanoic	284.48	298.51	891.50	297.17	0.9955	0.9530	0.9573
18:1 Oleic 9-Octadecenoic	282.48	296.51	885.50	295.17	0.9955	0.9527	0.9570
18:2 Linoleic 9-12 Octadecadienoic	280.48	294.51	879.50	293.17	0.9954	0.9524	0.9567
18:3 Gamma Linolenic 6-9-12 Octadecatrienoic	278.48	292.51	873.50	291.17	0.9954	0.9520	0.9564
18:3 Linolenic 9-12-15 Octadecatrienoic	278.48	292.51	873.50	291.17	0.9954	0.9520	0.9564
18:4 Octadecatetraenoic 6-9-12-15 Octadecatetraenoic	276.48	290.51	867.50	289.17	0.9954	0.9517	0.9561
20:0 Arachidic Eicosanoic	312.54	326.57	975.66	325.22	0.9959	0.9570	0.9610
20:1 Eicosenoic 11-Eicosenoic	310.54	324.57	969.66	323.22	0.9959	0.9568	0.9608
20:2 Eicosadienoic 11-14 Eicosadienoic	308.53	322.56	963.66	321.22	0.9958	0.9565	0.9605
20:3 Eicosatrienoic 11-14-17 Eicosatrienoic	306.53	320.56	957.66	319.22	0.9958	0.9562	0.9603
20:4 Arachidonic 5-8-11-14 Eicosatetraenoic	304.52	318.55	951.66	317.22	0.9958	0.9560	0.9600
20:5 Eicosapentaenoic 5-8-11-14-17 Eicosapenatenoic	302.52	316.55	945.66	315.22	0.9958	0.9557	0.9598
22:0 Behenic Docosanoic	340.59	354.62	1,059.82	353.27	0.9962	0.9604	0.9641
22:1 Erucic 13-Docosenoic	338.59	352.63	1,053.82	351.27	0.9962	0.9602	0.9639
22:5 Docosapentaenoic 7-10-13-1619 Docosapentaenoic	330.50	344.53	1,029.55	343.18	0.9961	0.9593	0.9630
22:6 Docosahexaenoic 4-7-10-13-16-19 Docosahexaenoic	328.57	342.60	1.023.82	341.27	0.9961	0.9591	0.9628
24:0 Lignoceric Tetracosanoic	368.64	382.67	1.143.98	381.33	0.9965	0.9633	0.9667
24:1 Nervonic 15-Tetracosenoic	366.63	380.66	1.137.98	379.33	0.9965	0.9632	0.9666

[a]Top number indicates carbon chain length and degree of unsaturation. Bottom number(s) indicate position of double bond(s) on the carbon chain.

[b]Conversion factors: To convert methyl ester to triglyceride: multiply by T/M ratio: To convert methyl ester to acid: multiply by A/M ratio: To convert triglyceride to acid: multiply by A/T ratio: To convert tridecanoic to methyl ester: multiply by 1.065 or divide by A/M ratio.