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Optimalization of synthesis of FAME, FAEE, FAPE and FABE for analysis with GC-FID and GC-Orbitrap

Optimalisering av syntese av FAME, FAEE, FAPE og FABE for analyse med GC-FID og GC-Orbitrap

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Master's thesis

Optimalization of synthesis of FAME, FAEE, FAPE and FABE for analysis with GC-FID and GC-Orbitrap

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Linda Hummervoll

Abstract

Fatty acids are an important part of our diet and is mainly consumed in the form of fat. The most widely used technique for analysis of FA composition is by GC-FID and GC-MS. To analyse a FA profile, it is necessary to convert them to non-polar derivatives, such as methyl esters. One common problem with these analyses is the under reporting of short chain FAs. A method to prevent this is lengthening the carbon chain with derivatization forming ethyl, propyl, or butyl esters. The aim of this study was to develop a method for making different alkyl esters of fatty acid for analysis with a GC-MS instrument.

In this thesis five different DAGs/TAGs were transesterified with methanol, ethanol, propanol, and butanol, using C19 FAME as an internal standard. The shortest TAG C4:0 had the largest increase in yield from FAME to FABE with 16 precent. The least improved were C18:0 with an 8 precent increased. A mixture of fourteen different FFA were esterified with methanol, ethanol, propanol, and butanol, using C19 FAME as an internal standard. The short and medium long carbon chains improved significantly, while there was only a small increase for the longer and the unsaturated chains.

The yields of *cis* and *trans* isomers of C18-unsaturated FAMEs are also calculated and compared. The results showed a decrease in yield for the *cis* isomer with each added double bond, while the *trans* isomer kept an even yield. Mass spectra of all alkyl esters were obtained, and five of them are discussed. In the end, it was not possible to conclude that FAEE, FAPE and FABE are able to replace FAME yet. While the method used worked, it can still be improved.

Sammendrag

Fettsyrer er en viktig del av kostholdet vårt og inntas hovedsakelig i form av fett. Den mest brukte teknikken for analyse av fettsyre-sammensetning er ved GC-FID og GC-MS. For å analysere en FA-profil er det nødvendig å konvertere dem til ikke-polare derivater, for eksempel metylestere. Et vanlig problem med disse analysene er underrapportering av kortkjedede FA-er. En metode for å forhindre dette er å forlenge karbonkjeden med derivatisering som danner etyl-, propyl- eller butylestere. Målet med denne studien var å utvikle en metode for å lage forskjellige alkylestere av fettsyrer for analyse med et GC-MS instrument.

I denne oppgaven ble fem forskjellige DAG-er/TAG-er transesterifisert med metanol, etanol, propanol og butanol, ved å bruke C19 FAME som en intern standard. Den korteste TAG C4:0 hadde den største økningen i utbytte fra FAME til FABE med 16 prosent. De minst forbedrede var C18:0 med en økning på 8 prosent. En blanding av fjorten forskjellige FFA ble forestret med metanol, etanol, propanol og butanol, ved å bruke C19 FAME som en intern standard. De korte og middels lange karbonkjedene ble betydelig bedre, mens det kun var en liten økning for de lengre og de umettede kjedene.

Utbyttet av *cis*- og *trans*-isomerer av C18-umettede FAME-er blir også beregnet og sammenlignet. Resultatene viste en reduksjon i utbytte for *cis*-isomeren med hver tilsatt dobbeltbinding, mens *trans*-isomeren holdt et jevnt utbytte. Massespektre av alle alkylesterene ble oppnådd, og fem av dem er diskutert. Til slutt var det ikke mulig å konkludere med at FAEE, FAPE og FABE er i stand til å erstatte FAME ennå. Selv om metoden som ble brukt fungerte, kan den fortsatt forbedres.

Selected abbreviations

DAG	Diacylglycerol
EI	Electron ionization
FA	Fatty acid
FABE	Fatty acid butyl ester
FAEE	Fatty acid ethyl ester
FAME	Fatty acid methyl ester
FAPE	Fatty acid propyl ester
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography
GLC	Gas-liquid chromatography
GSC	Gas-solid chromatography
IS	Internal standard
m/z	Mass/charge
MAG	Monoacylglycerol
MS	Mass spectrometry
SCOT	Support-coated open tubular
TAG	Triacylglycerol
TCD	Thermal conductivity detector
WCOT	Wall-coated open tubular

Contents

1 Introduction

Fatty acids (FAs) are an important part of our diet, mainly consumed in the form of fat (Thompson 2020). The most common FAs in animal tissue and in plants are monocarboxylic acids with an even number of carbons in a straight chain. The term “essential FAs” is used about fatty acids which have a vital importance for biological functions. These cannot be synthesized in human tissue. Therefore, they must be included in our diet. One example of an essential acid is Linoleic acid (C18:2, 9z, 12z) (Calder 2004).

The most widely used techniques for measuring FA composition is by GC-FID and GC-MS (Zhiqian Liu 2018). To analyse FA by gas chromatography, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters. FAs are important components of certain fats and oils, such as milk fats and coconut oil. However, quantitative recovery of esters from these FAs can be difficult because of their high volatility and partial solubility in water. It is well known that short chain FA methyl esters (FAMEs) are highly volatile and thus can get under reported. (Christie 1989). Therefore, derivatization to ethyl, propyl or butyl esters have been used as an alternative. (Hallmann, van Aarssen, and Grice 2008) But a major disadvantage with using higher molecular weight alcohols is the lack of commercially available standards, while the availability of methyl esters is very broad (Liu 2018).

In this thesis we will study the use of FA ethyl esters (FAEEs), FA propyl esters (FAPEs) and FA butyl esters (FABEs) as a replacement for FAME. The aim of this study was to develop a method for making different alkyl esters of fatty acid for analysing with a GC-MS instrument and to study the difference in fragmentation between them.

2 Theory

2.1 Lipids

Lipids are a diverse group of naturally occurring compounds which have many key biological functions, such as serving as energy storage, participating in signalling pathways and as structural components of cell membranes. Lipids can be defined as any group of organic compounds that are insoluble in water, but soluble in organic solvents. This chemical feature is present in many organic molecules such as fatty acids, sterols, terpenes, phospholipids and more (Fahy et al. 2011). Lipids can be divided into simple and complex groups. Simple lipids are those who are hydrolysed into one or two products, such as free fatty acids, sterols and acylglycerols. Complex lipids are those who are hydrolysed into three or more products, such as glycerophospholipids (Christie 1989).

2.1.1 Fatty acids

Fatty acids are composed of a hydrocarbon chain with one terminal carboxyl group (COOH) (Thompson 2020). They are amphilic, which means they have one hydrophilic part (the carboxyl group) and one hydrophobic part (the hydrocarbon chain). How soluble they are depends on the hydrocarbon chain length (Calder 2004). Fatty acids that contain only single bonds are called saturated. They are most commonly straight with an even number of carbons, with 12-22 carbons being the most abundant. The carbon chains can also be branched. Unsaturated fatty acids contain one or more double bonds, with *cis* or *trans* configuration (Fig. 1). The most common configuration of unsaturated fatty acids in nature is the *cis* isomer (Rustan and Drevon 2001). Even though fatty acids usually contain more than 12 carbons; several short chained fatty acids are biochemically important. For example, butyric acid (C4:0) and caproic acid (C6:0) which are found in milk and dairy products (Thompson 2020).

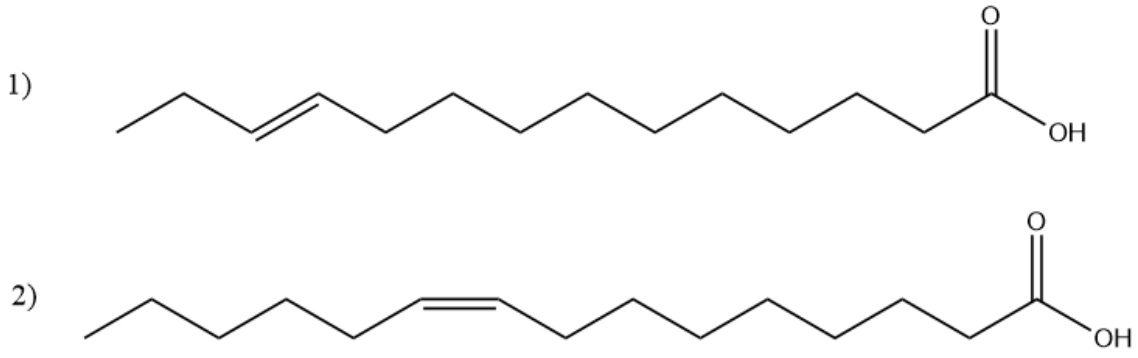


Figure 1: 1) C14:1n3 trans fatty acid, 11E-tetradecenoic acid 2) C14:1n6 cis fatty acid 9Z-tetradecenoic acid.

Many fatty acids have old trivial names along with their systematic names, the systematic names are based on the number of carbons they contain and the position and configuration of their double bonds. Two numbers are used to indicate the length of the chain and the number of double bonds, e.g. C14:1 indicates an unbranched C14 fatty acid with one double bond (Fig. 1) (Ratnayake 2009). Double bonds can be designated by using the "n" configuration. The position of the double bond can be denoted on the form (n-x), where n is the chain-length of the fatty acid and x is the number of carbon atoms from the double bond in the terminal region of molecule. Thus *cis*-9 tetradecenoic acid can be denoted C14:1n-6.

The geometry of the double bond is designated with *cis/trans* or *E/Z* nomenclature systems. The terms *cis/trans* are used to describe the position of atoms connected to the double bonded atoms. If the atoms lie on the same side, they're *cis*, are they on opposite sides they're *trans*. The *E/Z* nomenclature is used to describe isomers by using sequence ordering rules, like how it is done with the *R/S* system. The sequence rule-preferred group or atom attached to one of the double bonded carbons are compared to the other group or atom attached to the other double bonded carbon. If the preferred groups or atoms are on different sides of the reference plane, it is the *E* configuration. If they are on the same side as the reference plane, it is *Z* configuration (Akoh 2008).

2.1.2 Acylglycerols

Acylglycerols are the most predominant component in oils and fats. It consists of a glycerol that is esterified with one, two or three fatty acids, and are named as monoacylglycerols (MAGs), diacylglycerols (DAGs) or triacylglycerols (TAGs), respectively (Akoh, 2008). A TAG where all the three fatty acids are the same, is called a simple triglyceride, for example tristearin. Almost all naturally occurring triglycerides contains more than one type of fatty acid. These are called mixed triglycerides (Thompson, 2020).

Fatty acids can be esterified on the primary or the secondary hydroxyl groups of glycerol. Even though glycerol has no chiral centre, it becomes chiral if different FAs are esterified to the primary hydroxyl and we can get two possible configurations.

To differentiate the configurations of position isomers, we use terminology. The most common is the "*sn*" convention. In the numbering that describes the hydroxyl groups on the glycerol backbone in Fisher projection, sn1, sn2, and sn3 designations are used for the top, middle and bottom hydroxyl groups, Fig. 2 (Devle 2013).

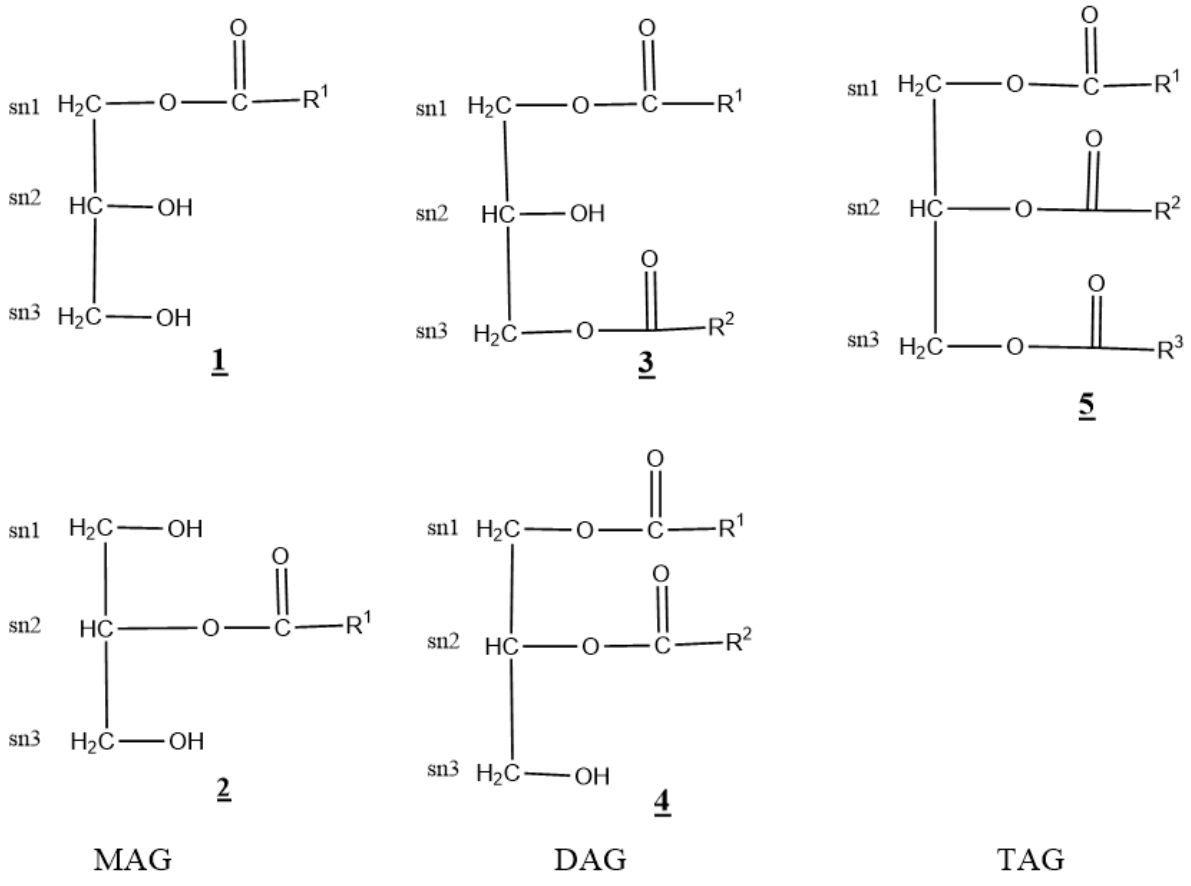
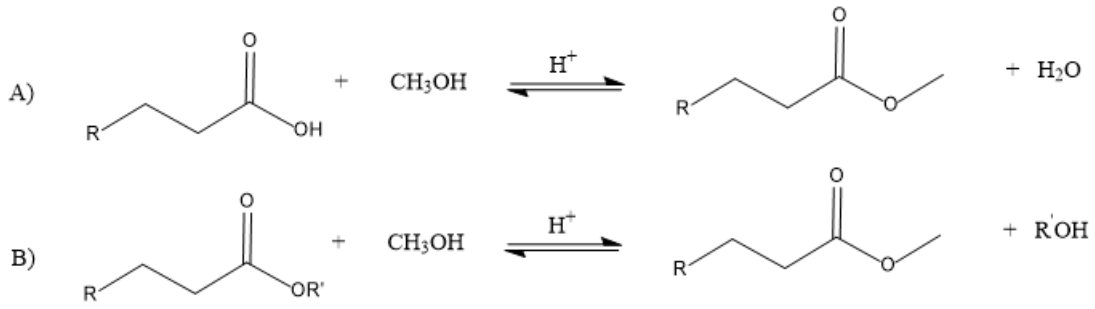


*Figure 2: **1** Examples of MAGs in sn1 position **2** and in sn2 position **3** DAG in sn1/sn3 position **4** and in sn1/sn2 position **5** TAG.*

2.2 Esterification of fatty acids

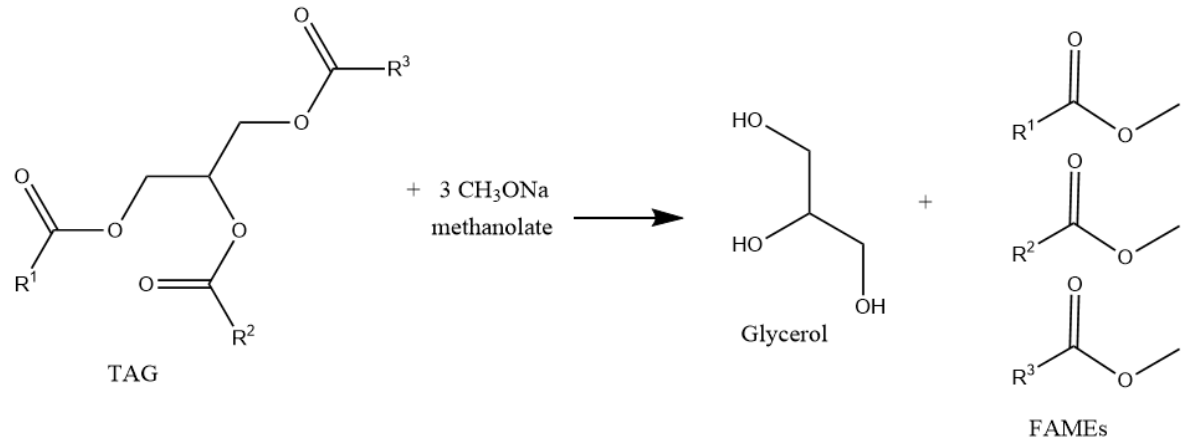
To analyse the fatty acid profiles of lipids, it is necessary to prepare non-polar, volatile, and thermally stable derivatives that are suitable for GC analysis. Preparation of methyl ester derivatives is by far the most common chemical reaction performed by lipid analysts (Christie 2012; Miller 2005). The most used derivatization methods for GC is transesterification of lipid bound FAs and esterification of FFA to FAMEs (Gutnikov 1995).

One method of esterification is acid-catalysed esterification and transesterification (Scheme 1). The FFAs are heated with a large excess of anhydrous methanol in the presence of an acidic catalyst. (Christie 2012) We can use BF_3, HCl or H_2SO_4 as catalysts, but 14 % BF_3 in methanol solution is a method that is often used (Gutnikov 1995). If water is present in the reaction, it may prevent the reaction from occurring (Christie 2012). This is because of the Le Châtelier's principle, water will push the reaction to the left, thus hindering the product formation.



Scheme 1) Examples of acid catalysed reactions to form FAMEs, A) FFAs are esterified with anhydrous methanol in the presence of an acidic catalyst (H^+) B) Esterified-FA are re-esterified into FAMES.

Base-catalysed transesterification proceeds faster than those in acid media and occur at room temperature and does not degrade FAs or isomerize the double bonds. The most used basic catalyst is methanolic solutions of sodium or potassium hydroxide or methoxide (Gutnikov 1995). The sodium methanolate reacts with the carbonyl carbon followed with elimination of water (Scheme 2) (Christie 2012).



Scheme 2) The TAG in excess amount of methanolate reacts and forms a glycerol and three FAMEs.

2.3 Use of internal standard

An internal standard (IS) is often used for quantitative analysis in chromatography. A known amount of standard is added to the sample in similar concentration as the analyte before any sample preparations. The standard that is chosen cannot be a component in the sample. It needs to be available in pure form and chemically similar to the analytes of interest. It cannot react with any of the sample's components and should elute near the peaks of interest but still be well resolved form them (Miller, 2005).

Quantitative detection using MS is often complicated because of the effect of the matrix components. When an analyte is introduced into the ion source it will compete with the other compounds that are introduced simultaneously, for ionization. Matrix components can decrease the analyte signal, called ion suppression and the degree of ion suppression depends on the chemical structure of the analyte. This means that if an analyte and IS are not sufficiently similar in structure, the ratio of analyte and IS detector response may vary, thus compromising the quantitation. To avoid this stable isotopically labelled (SIL) analogues of the analyte can be used. SIL internal standard are compounds in which several atoms in the analyte are replaced by their stable isotopes, ex. ^{2}H, ^{13}C, ^{18}O and ^{15}N. Labelling with three to eight ^{2}H or ^{13}C atoms is the most common. Since a compound and its SIL analogue will in theory co-elute, it is key that the mass difference between them is minimum three mass units, so that they get separated in the mass analyser (Stokvis, 2005).

2.4 Instrumentation

2.4.1 Gas chromatography

Gas chromatography (GC) has been the premier technique for the separation and analysis of volatile compounds since the 1950's. Before the GC, the only way to separate volatile compounds was by distillation, where compounds are separated based on boiling point and vapor pressure. A GC adopt similar principles, but there are more variables, such as the nature of the stationary phase, which gives it more versatility.

A GC uses gas as a mobile phase to carry the analyte through the column. The three most used carrier gases are N_2, He, and H_2. The gas used must be pure and chosen based on their inertness. Helium is a popular gas because of its high efficiency at fast flow rates, but it is expensive and limited in supply. Therefore, hydrogen is increasing in popularity, but since this gas carries an explosion risk, extra safety precautions is required.

There are two types of gas chromatography, gas-solid chromatography (GSC) and gas-liquid chromatography (GLC) (Miller 2005). GSC is a type of chromatography where the same material act as both the stationary phase and the support. In this type of chromatography, analytes are retained by their adsorption to the surface of the support. The support is an inorganic material such as alumina or silica (Nader Rifai 2018). The other and more common technique, GLC is a type of chromatography where the liquid stationary phase is immobilized on the capillary tubing walls or adsorbed onto a solid inert packing (Anonymous 2020; Miller 2005).

A GC contains an injector, a column placed in an oven, a detector, and a carrier gas supply (Fig 3).

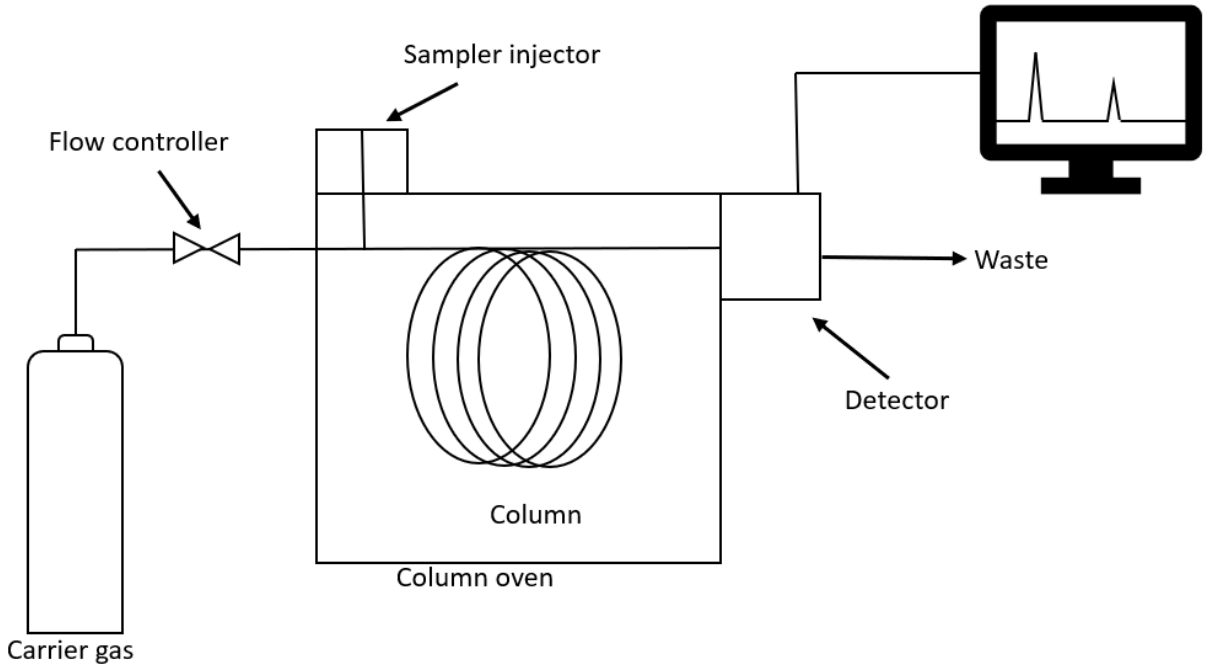


Figure 3: An illustration of a GC system.

One common type of injector is a split/splitless injector. Like the name suggest, the split/splitless injector can perform two types of injection, split injection and splitless injection. In a split injection, the sample is injected into a heated injection port with a syringe. The sample is vaporized rapidly and because of an open valve, only 0.1-10% of the vapor enters the column. The rest is flushed out with a large volume of carrier gas. The injection volume is normally 1 µL. The split ratio can vary from 1:1 to 1:1000. A higher split means a smaller amount of sample is applied on to the column. In a splitless injection, the valve is closed, and 100% of the sample is introduced onto the column (Miller 2005).

Open tubular column, also known as capillary columns, comes in two forms, wall-coated open tubular (WCOT) column and support-coated open tubular (SCOT) column. In a WCOT column, the walls are coated with a thin layer of the stationary phase. While in a SCOT column the walls are first coated with a thin layer of adsorbent solid that are treated with liquid stationary phase. The SCOT columns can hold a greater volume of stationary phase compared to the WCOT, but the WCOT are still preferred because it has greater column efficiencies (Anonymous 2020).

Many different detectors can be used with GC. This is discussed in chapter 2.4.2. (Miller 2005).

2.4.2 Detectors

The two most popular detectors for GC is the thermal conductivity detector (TCD) and the flame ionization detector (FID) (Miller 2005). The flame ionization detector is a commonly used detector for GC (Fig 4) (Nader Rifai 2018). The FID is a small oxygen-hydrogen flame where the sample is burned, creating ions, that are detected and turned into a signal (Miller 2005). As the carrier gas is leaving the column, it is mixed with hydrogen gas and the eluting compounds are burned by the flame. When the compounds are burned, ions are produced. These ions are detected by an electrode above the flame. The magnitude of the current that is generated is turned into a signal that are used for the detection and quantification. The advantages of a FID are its simplicity, versatility, robustness, and reliability. Also, this detector gives little to no signal for common carrier gases (e.g. He, Ar, N_2) or typical contaminates in these gases (e.g. O_2 and H_2O) (Nader Rifai 2018).

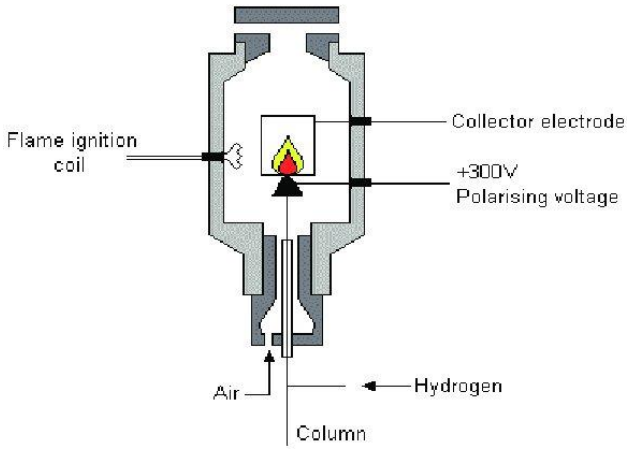


Figure 4: An illustration of a FID (Prasad 2012).

2.4.3 Mass spectrometry

Mass spectrometry (MS) is a powerful tool in analytical chemistry. The basic principle of MS is to generate ions from analytes, to separate them by their mass to charge ratio (*m/z*) and to detect them quantitatively and qualitatively by their unique *m/z*. A mass spectrometer consists of an ion source, an analyser and a detector, which operates under high vacuum conditions (Fig 5) (Gross 2006).

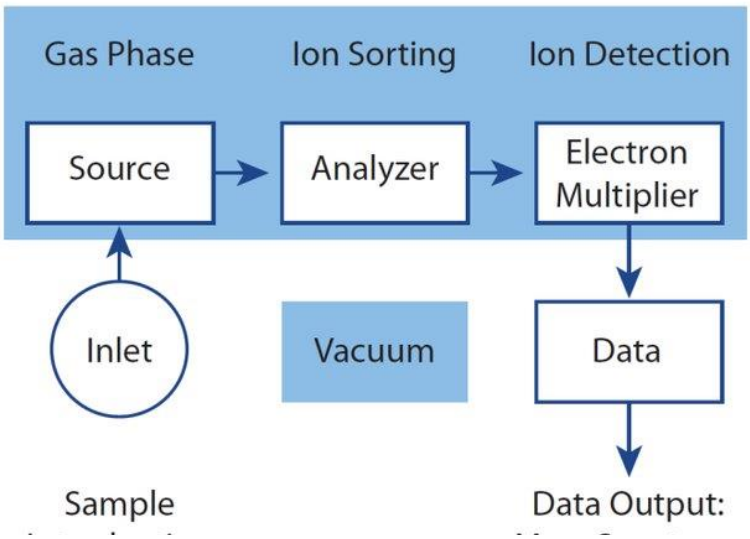


Figure 5: An illustration of a MS system (Vandenbroucke 2015).

In the ion sources, the sample is ionized prior to analysis. There are a variety of ionization techniques used. One of these techniques is called electron ionization (EI). EI works well for many gas-phase molecules and induces extensive fragmentation so that the molecular ion is not always observed. As you can see in Figure 6, the EI source consists of a heated source block in vacuum. The filament gives off energetic electrons (70 eV), that travel towards the electron trap, and on their way collide with the sample gas molecules. The ionized molecules are repelled by a repeller, that has a slight positive charge, towards the exit and into the analyser.

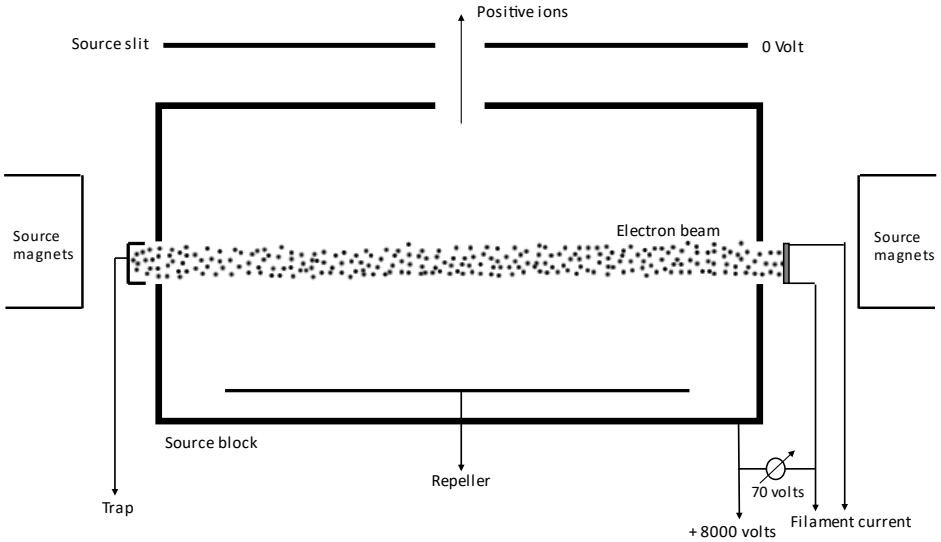


Figure 6: An illustration of an EI ion source.

In the mass analyser, the gas-phase ions are separated based on their m/z values. Since there are a great variety of sources, several types of mass analysers have been developed. All mass analysers use dynamic or static electric and magnetic fields, either alone or combined. Each analyser has its advantages and its limitations. Some examples of mass analysers are quadrupole, ion trap, time-of-flight analyser and orbitrap. The latter is described in chapter 2.4.4 (Hoffmann 2007).

2.4.4 Orbitrap

The orbitrap is one of the newest mass analysers, but we can find roots traced back to the 1920's, when Kingdon proposed the principle of orbital trapping. This mass analyser is an extremely powerful ion trap instrument, with a design that allows the system to act as both a mass analyser and a detector. The orbitrap consists of three electrodes, two outer that form a barrel-like enclosure and one in the centre (Hecht et al. 2019; Anonymous 2019). Since ions have different mass and charge, they oscillate at different frequencies, and when these frequencies are measured, we can obtain the mass spectra of the ions using image current detection (Anonymous 2019).

A key feature that separates the orbitrap from other mass analysers is the C-trap (Fig 7). The C-traps job is to make the ions kinetic energy match the voltage on the centre electrode. This is because one need the ions to have a stable trajectory. With a too high energy, the ions will crash into the outer electrodes, and if it's too low they will hit the centre electrode. The C-trap also introduces the ions to the orbitrap in relatively tight time windows so that ions of any given *m/z* will oscillate along the z-axis in narrow bands. This provides the optimal signal to noise for the image current detection method (Anonymous 2022).

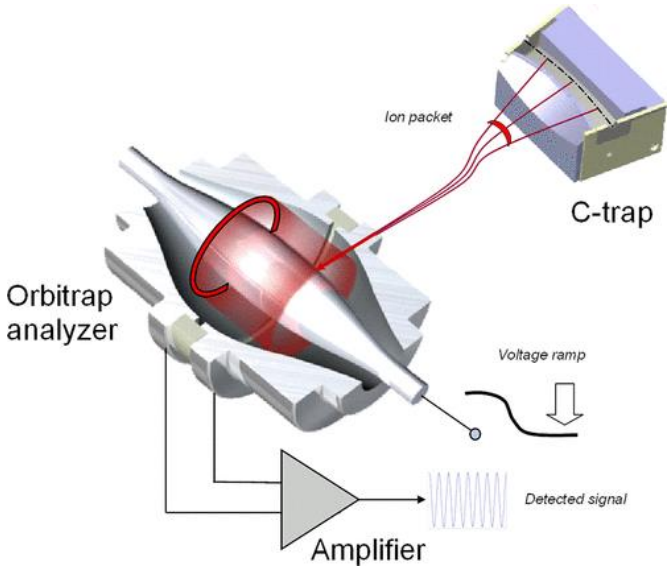
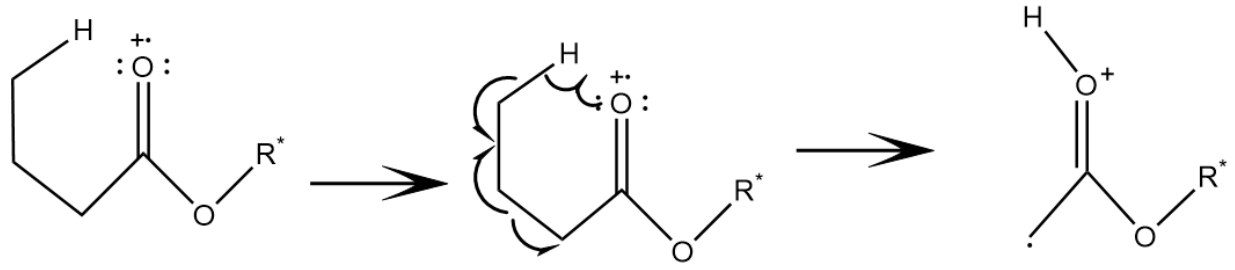


Figure 7: An illustration of an orbitrap (Scientific 2012).

2.4.5 Fragmentation

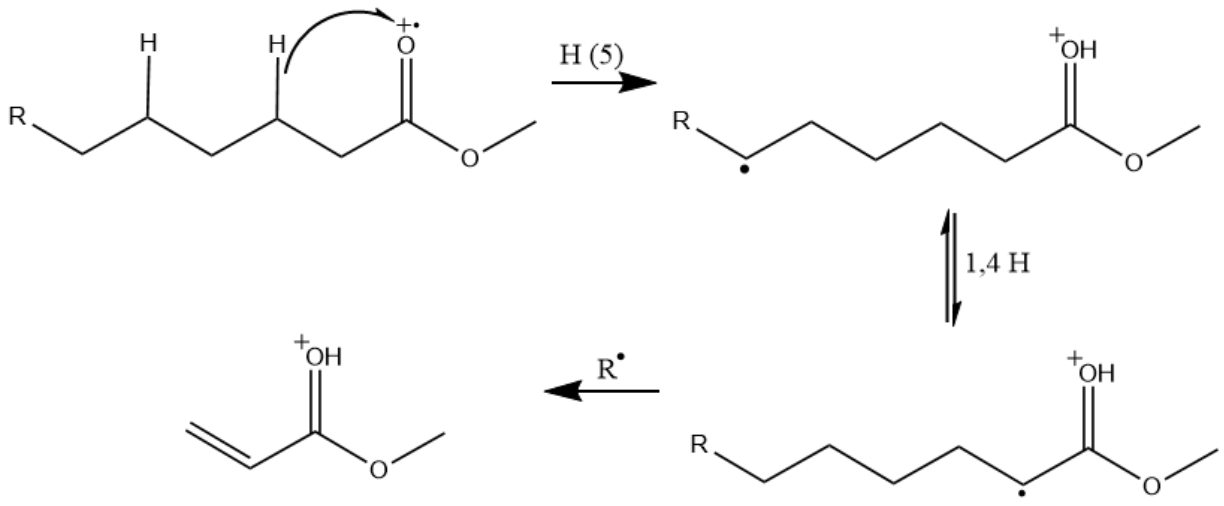
Methyl esters are by far the most used derivatives for fatty acid analysis. The methyl esters of straight-chain FAs have a characteristic spectrum. The spectra are relatively simple as the main ions tend to occur 14 amu apart, and not in clusters as with unsaturated esters.

A central fragmentation mechanism, especially for fatty acids, is the McLafferty rearrangement (Scheme 3). The McLafferty rearranegement is important for the identification of most ester derivatives of FAs. For a FAME, the ion at m/z = 74 indicates that a McLafferty rearrangement has occurred (Christie 2021).



Scheme 3) McLafferty rearrangement.

Scheme 4 describes another rearrangement often seen in fatty acids.



Scheme 4) H5 proton migration.

3 Materials and Methods

3.1 Chemicals

The chemicals, their quality and the supplier used in this study are listed in Table 3.1. Additionally, the GC-vials and Pasteur pipettes used were delivered by VWR Chemicals.

Table 3.1 List of chemicals used

Chemicals	Quality	Supplier
n-Heptane	AnalaR NORMAPUR	VWR Chemicals, part of AVANTOR, Radnor, PA, USA
Boron trifluoride-methanol solution, 14 %		Sigma-Aldrich, now Merck KPGaA, Darmstadt, Germany
Boron trifluoride-ethanol, 10 %		Sigma-Aldrich, now Merck KPGaA, Darmstadt, Germany
Boron trifluoride propanol complex, 14%		Sigma-Aldrich, now Merck KPGaA, Darmstadt, Germany
Boron trifluoride – 1-butanol solution, 10%		Sigma-Aldrich, now Merck KPGaA, Darmstadt, Germany
Ethanol absolute	AnalaR NORMAPUR	VWR Chemicals, part of AVANTOR, Radnor, PA, USA
Chloroform	HiPerSolv CHROMANORM	VWR Chemicals, part of AVANTOR, Radnor, PA, USA
1-propanol	HPLC grade	Rathburn Chemicals Ltd Walkerburn Scotland
Methanol	HiPerSolv CHROMANORM	VWR Chemicals, part of AVANTOR, Radnor, PA, USA
Butan-1-ol	GPR RECTAPUR	VWR Chemicals, part of AVANTOR, Radnor, PA, USA
Sodium (s)	Purum	Merck, Darmstadt, Germany

3.2 Standards

All standards used in this thesis are listed in Table 3.2

Nonadecanoic acid methyl ester, C19:0, (Larodan AB, Solna, Sweden) was used as internal standard, in order to quantify the FAs and the acylglycerols. Chloroform was used to dissolve 10.0 mg IS in a 10.0 mL volumetric flask, concentration was 1.0 mg/mL. The dissolved IS were stored in a freezer at -20 °C prior to esterification/transesterification.

The Mixture FA 61 was ordered from Larodan AB, Solna, Sweden and was containing 14 different FFA, Table 3.3. It was used to esterify into FAMEs, FAEEs, FAPEs and FABEs. The mixture was dissolved in chloroform to a total concentration of 10.0 mg/mL and stored in a freezer at -20 °C prior to esterification.

Three additional FAs were used, these where 9(E)-Octadecenoic acid (25 mg in 10 mL, 2.5 mg/mL), 9(E),12(E)-Octadecadienoic acid (100mg in 10 mL, 10 mg/mL) and 9(E),11(E),13(E)-Octadecatrienoic acid (10 mg in 10 mL, 1 mg/mL). These were stored at -20 °C prior to esterification.

Five acylglycerols was used for transesterification. 10 mg of Tristearin (TAG C18:0), Trilaurin (TAG C12:0), Dicaprin (DAG C10:0), Dicaprylin (DAG C8:0) and Tributyrin (TAG C4:0) were dissolved in 20 mL chloroform (0.5 mg/mL) and stored at -20 °C prior to transesterification.

All samples were diluted with heptane to 0.1 mg/mL prior to analysis.

Table 3.2 List of all standards used in this thesis.

Trivial Name	Number of C	IUPAC Name	Supplier
Mixture FA 61			Larodan AB, Sweden
Methyl nonadecanoate	C19:0	Methyl nonadecanoate	Larodan AB, Sweden
Eladic acid	C18:1 n-9	9E-Octadecenoic acid	Larodan AB, Sweden
Linoelaidic acid	C18:2 n-6	9E,12E-Octadecadienoic acid	Larodan AB, Sweden
β-eleostaric acid	C18:3 n-3	9E,11E,13E-Octadecatrienoic acid	Larodan AB, Sweden
Tristearin	C18:0	2,3-di(octadecanoyloxy)propyl octadecanoate	Larodan AB, Sweden
Trilaurin	C12:0	2,3-di(dodecanoyloxy)propyl dodecanoate	Larodan AB, Sweden
Dicaprin	C10:0	2-Hydroxy-1,3-propanediyl didecanoate	Larodan AB, Sweden
Dicaprylin	C8:0	1,2-Dioctanoylglycerol	Larodan AB, Sweden
Tributyrin	C4:0	2,3-di(butanoyloxy)propyl butanoate	Larodan AB, Sweden

Table 3.3 Concentration of free fatty acids in mix FA 61.

Trivial Name	Number of C*	IUPAC Name	FA concentration [mg/L]
Butyric acid	C4:0	Butanoic acid	0.4
Caproic acid	C6:0	Hexanoic acid	0.2
Caprylic acid	C8:0	Octanoic acid	0.1
Capric acid	C10:0	Decanoic acid	0.3
Lauric acid	C12:0	Dodecanoic acid	0.4
Myristic acid	C14:0	Tetradecanoic acid	1.0
Myristoleic acid	C14:1 n-5	9Z-tetradecenoic acid	0.2
Palmitic acid	C16:0	Hexadecanoic acid	2.51
Palmitoleic acid	C16:1 n-7	9Z-hexadecenoic acid	0.5
Octadecanoic acid	C18:0	Octadecanoic acid	1.0
Oleic acid	C18: 1n-9	9Z-octadecenoic acid	2.5
Linoleic acid	C18:2 n-6	9Z,12Z-octadecadienoic acid	0.29
Linolenic acid	C18:3 n-3	9Z,12Z,15Z-octadecatrienoic acid	0.4
Arachidic acid	C20:0	Eicosanoic acid	0.2

3.3 Derivatization

3.3.1 Esterification of free fatty acids

1.0 mL of dissolved FFA mix (10 mg/mL) and 1.0 mL dissolved IS (1 mg/mL) was transferred to a screw-capped plastic tube using a Hamilton syringe. The chloroform was evaporated using a jet of N_2 gas. The lipids were redissolved in 1 mL boron trifluoride 10-14% in alcohol solution, methanol, ethanol, propanol, or butanol. The sample was placed in a water bath at 70 °C for 5, 10, 15 and 20 minutes, respectively. After cooling the tube with running water, 2 mL n-heptane was added, and the sample was shaken with a vortex mixer for 2 minutes. 1 mL of saturated NaCl in water was added. The top layer was transferred to a GC vial and kept in a freezer until analysed by GC-FID. For analysis 6.0 µL sample and 294.0 µL n-heptane was transferred to a GC vial with glass insert. Total concentration of the alkyl esters was 0.1 mg/mL.

3.3.2 Transesterification of glycolipids

1 mL of each TAG/DAG standard and 1 mL IS in chloroform was transferred to a screw-capped plastic tube using a Hamilton syringe. The chloroform was evaporated using N_2 gas. The lipids were redissolved in 2 mL n-heptane. 1 mL of sodium alkoxide was prepared by dissolving 5 mg metallic sodium in 1 mL methanol, ethanol, 1-propanol, and 1-butanol. This solution was added to the tube. The sample was placed horizontally on a shaker platform with a frequency of 350 rpm for 30, 60, 90 and 120 minutes, respectively. 1 mL of saturated NaCl in water was added, and the sample was shaken with a vortex mixer for 2 minutes. The top layer was transferred to a GC vial and kept in a freezer until analysed by GC-FID. For analysis 20 µL sample and 280 µL n-heptane was transferred to a GC vial with glass insert. Total concentration of the alkyl esters was 0.1 mg/mL.

3.3.3 Instruments

Two different GC-instruments were used in this thesis, GC-FID and GC-MS. The GC-FID instrument was used primarily for quantification when optimization of the derivatization conditions was established. GC-MS was used to get information of the retention times for optimum separation and to get the mass spectra for qualitative purposes.

3.3.3.1 Gas chromatography flame ionization detector

The GC-FID used was a Thermo GC 1310, coupled with an FID. The liquid autoinjector was a Thermo AI 1310. The software used to acquire and process the data was Chromeleon™ Chromatography Data System, version 7.2.10.

3.3.3.2 Gas chromatography mass spectrometry

The GC-MS instrument used was a GC-Orbitrap where the instrument was a GC Trace 1310 coupled with an ExactiveTM GC Orbitrap GCMS, Thermo ScientificTM, Waltham, MA. The software used for instrument control was ExcaliburTM, version 4.5. Acquired data were processed using Tracefinder 5.1, Thermo ScientificTM, Waltham, MA.

4 Results and discussion

In this study five different TAGs/DAGs were transesterified and 17 different FFAs were esterified. The purpose of this was to evaluate how the yield would variate between the different alkyl esters. Mass spectra for the esters are also compared and discussed. No similar research is found in literature to our knowledge.

4.1 Transesterification of DAGs and TAGs

Five TAGs/DAGs were transesterified with methanol, ethanol, propanol, and butanol. We expected all the FAMEs to have a yield around 90-100 %. As shown on Figure 8, this did not happen. Only C18:0 gave a yield within the expected range. We expected an increase in yield from FAME to FABE for FAs with a short to medium carbon chain, and a decrease in the difference between FAME and FABE with an increase in carbon chain length. The reason for this is because a longer carbon chain is less volatile.

All compounds follow the expected pattern to some degree. The only exception is the ethyl ester of TAG C4:0, and the butyl ester of TAG C18:0. This might be due to errors made during sample preparations.

From FAME to FABE for C4:0 we have an 16 % increase in yield, C8:0 increses 14 %, C10:0 increases 11 % and C12:0 increases 6%. For C18:0, FAPE gave the highest yield at 105 %, and we had an increase from FAME at 8 %.

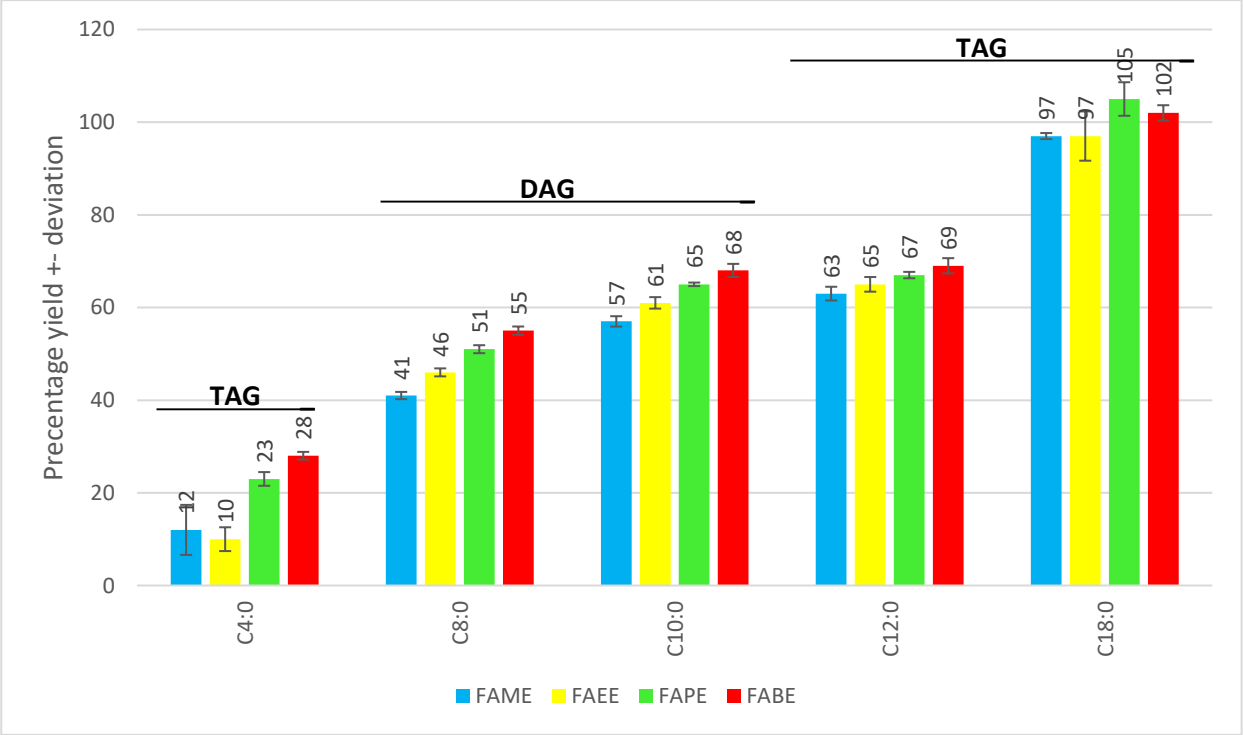


Figure 8: Yields of DAGs and TAGs transesterified into fatty acids methyl esters (FAMEs), fatty acid ethyl esters (FAEEs), fatty acid propyl esters (FAPEs), and fatty acid butyl esters (FABEs).

4.2 Esterification of free fatty acids

14 free fatty acids were esterified with methanol, ethanol, propanol, and butanol. Just like with the transesterified TAGs/DAGs, we expected the FAMEs to have yields between 90-100 %.

Most of the compounds shows the expectant pattern of FAME giving the lowest yield and increasing giving FABE the highest. Also, the difference between FAME and FABE decreasing with longer carbon chains. There are some exceptions. For the short chain and medium chain fatty acids (C4:0 – C12:0) the FAPE is not at the expectet level. For the carbon chains longer than 12, FAPE is were we expected, but not for the unsaturated medium fatty acids.

The one that really stands out is C20:0. The reason to why this one differs so much compared to the rest, may be in the sample preparation.

Even though the use of FABE shows promising results, there were some challenges. Butanol is both immiscible in water and heptane, which means we did not get a sufficient phase separation during the sample preparation. This may have effected the results.

The short FAs C4:0 and C6:0 was the most improved in yield from FAME to FABE, with a 16 % and 13 % increase, respectively. While the least improved FAs were the saturated and unsaturated C14, with a 4 % increase for both. The polyunsaturated C18 FAs also had 4 % increase for both.

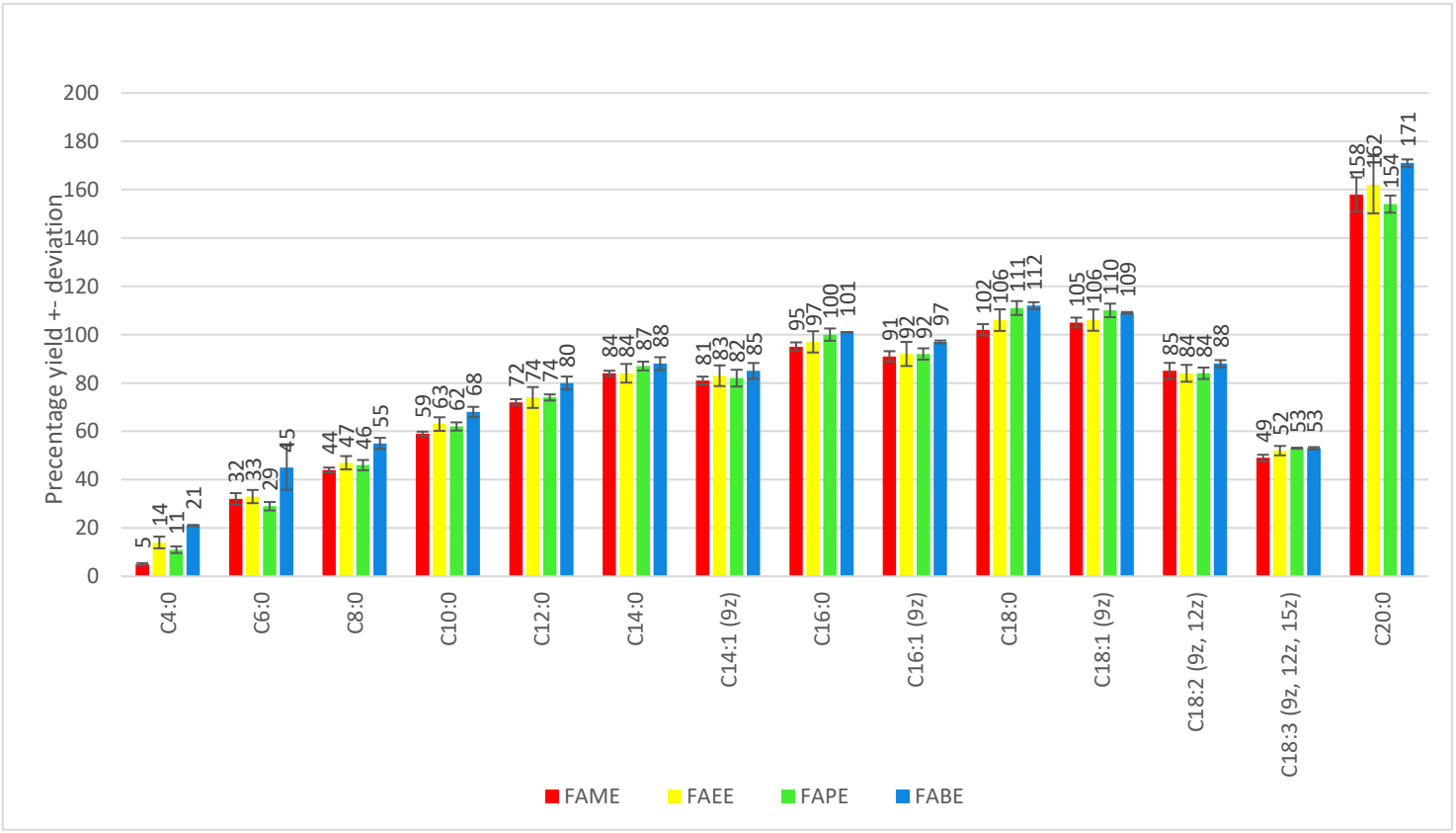


Figure 9: Yield of 14 FFA esterified into fatty acids methyl esters (FAMEs), fatty acid ethyl esters (FAEEs), fatty acid propyl esters (FAPEs), and fatty acid butyl esters (FABEs).

4.3 Esterification of unsaturated C18, *cis* versus *trans*

The FA mixture purchased for this thesis only contained *cis* unsaturated fatty acids, therefore separate *trans* FAs were bought to be compared. When esterified, the *trans* FAs gave a stable yield compared to the *cis* FAs where the yield is decreasing with the degree of unsaturation. From *cis* C18:1 to *cis* C18:3 there was a 55 % decrease in yield. While for *trans* C18:1 to *trans* C18:3 there was only a 8 % decrease. A reason for this may be the isomers shapes. While the *trans* FAMEs are straight no matter how unsaturated they are, the *cis* FAMEs gets more and more bent with increased degree of unsaturation. This may explain why the *trans* isomers keep an even yield, while the *cis* yield decreases with each new bend. In a littrature search, no journal found confirmed this.

We were only able to look at the *trans* FAMEs, not the other alkyl esters.The diffrent C18:2 esters and the esterified IS coeluted, and we were not able to find a suitable GC temperature for separation.

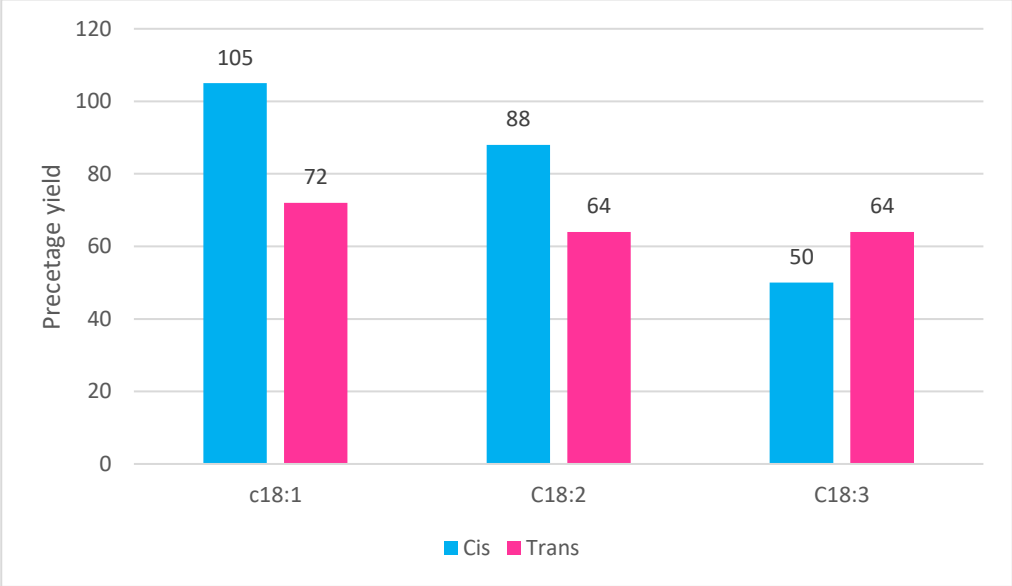


Figure 10: Comparison of yields of unsaturated C18 in cis configuration and unsaturated C18 in trans configuration.

4.4 Variations of internal standard yield

The same IS (C19:0 methyl ester) was used for all both derivatization techniques, for the esterification of FFA and for the transesterification of acylglycerols. When the IS was esterified with other alcohols than methanol, a portion internal standard remained as methyl ester. With ethanol we got the lowest amout esterified, with 53 % esterifying into FAEE and 47 % remaining as FAME. While propanol and butanol esterified the same amount, with 67 % esterifying into FAPE and FABE, and 33 % remaining as FAME. In the transesterified samples, all of the internal was 100 % esterified for each alcohol. A reason for this may be the temperature in which the esterified samples were prepared. A higher temperature during sample preparation might have increased the amount of IS esterified. Another possible reason can be the reaction time as this is a variable that was very different for to the two reactions types.

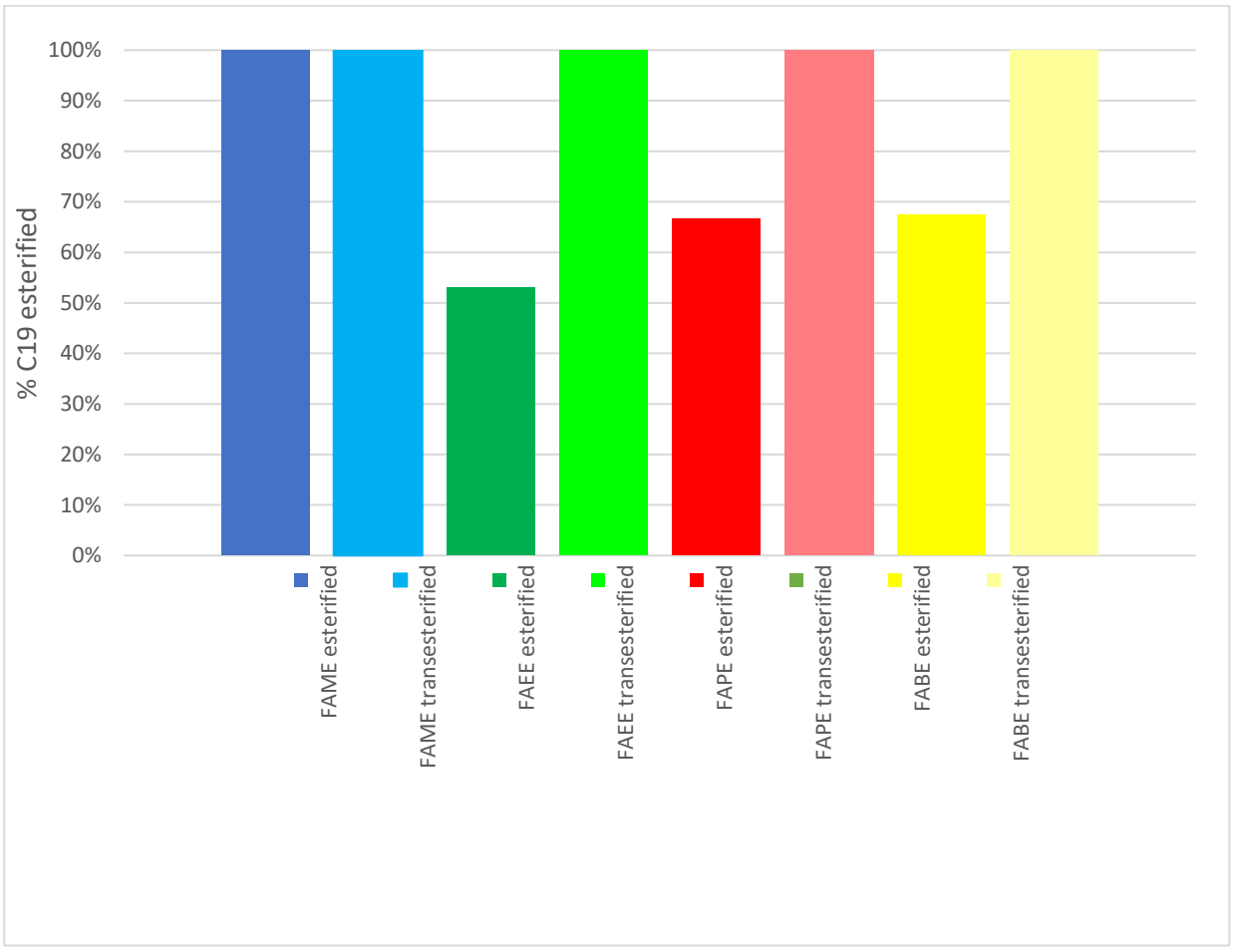


Figure 11: Comparison of yield of esterified and transesterified internal standard.

4.5 Fragmentation

All element compositions in the spectra are suggestions from the MS instruments, and are not always correct. The identification of the compounds are performed by comparing retention times with standards and search in NIST17 (National institute of standards and technology, Gaithersburg, MS, USA) mass spectral library. As the sample can contain contamination, the correct spectra is not always found. To determine the correct element composition, isotope marking or MS/MS studies could be used. All fragments draw in the figures are assumptions. The scan range used for acquiring the data was *m/z* 50 – 500 with a resolution set to 60'000. The electron energy were 70 eV and the ion source temperature was set to 250 °C.

4.5.1 Fragmentation; short chain fatty acid alkyl ester (C4:0)

It was expected to see similar fragmentation between all four esters, and there is a repeating pattern. The *m/z* = 71 fragment, which is the loss of the added alkyl group, is present in all spectres, but with varying intensity. It is lowest for FAME and highest for FAEE. Both FAME and FAEE has the McLafferty fragment as their main peak at *m/z* = 74 and *m/z* = 88, respectively. For FAPE and FABE, McLafferty can happen on both sides of the ketone but we don't see them in these spectra. FAPE and FABE both have the same main peak at *m/z* = 89.

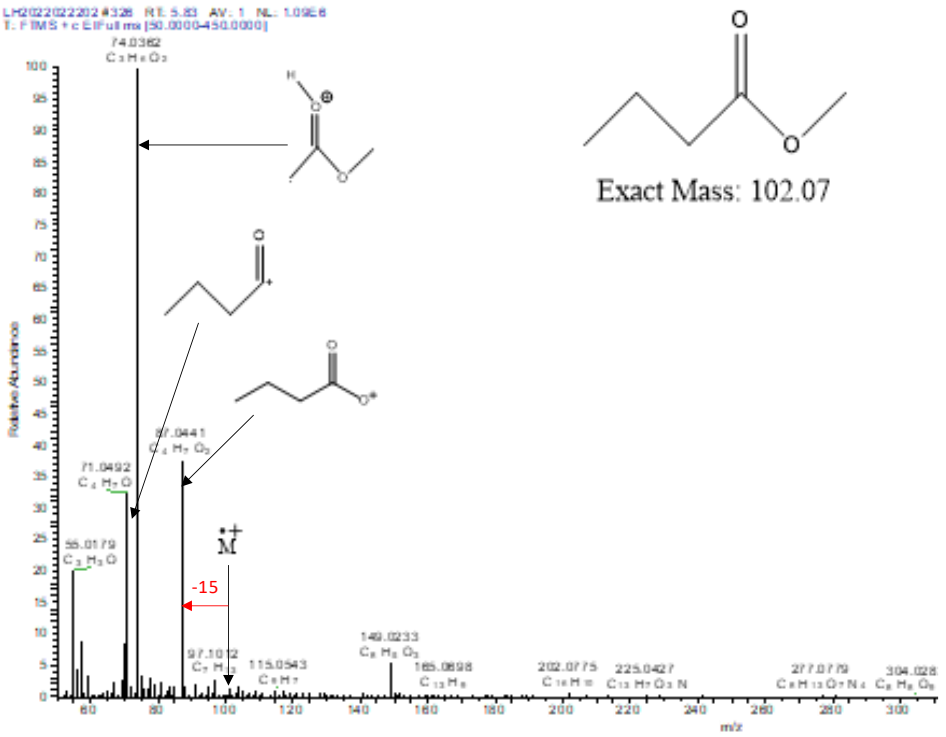


Figure 12: Fragmentation of C4:0 FAME.

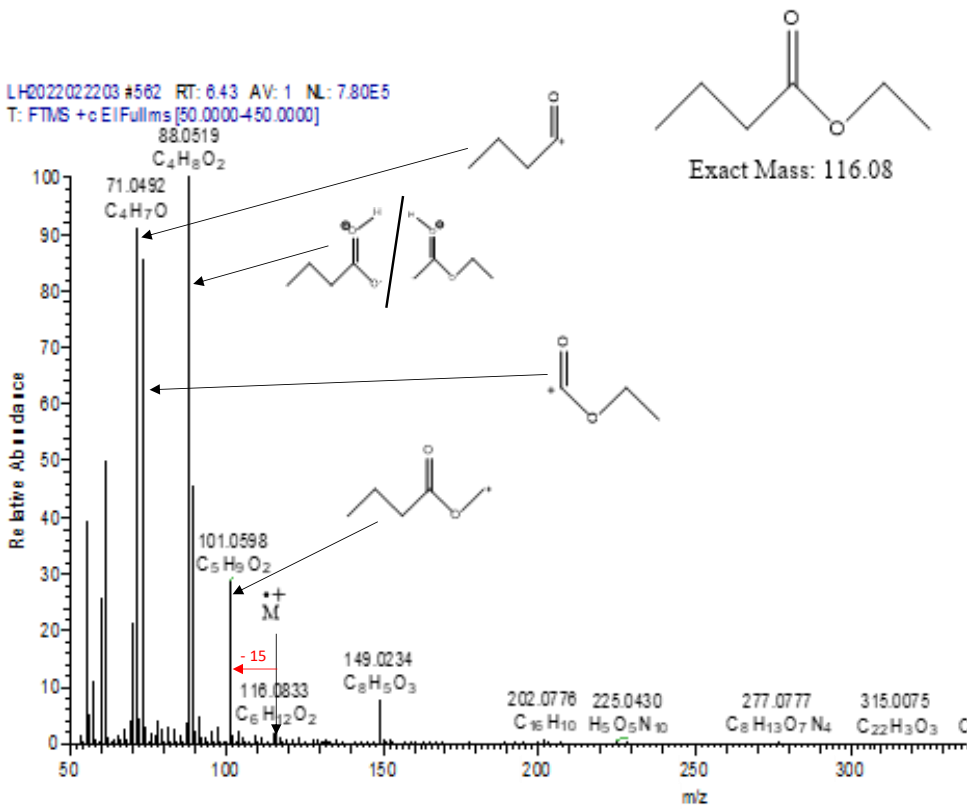


Figure 13: Fragmentation of C4:0 FAEE.

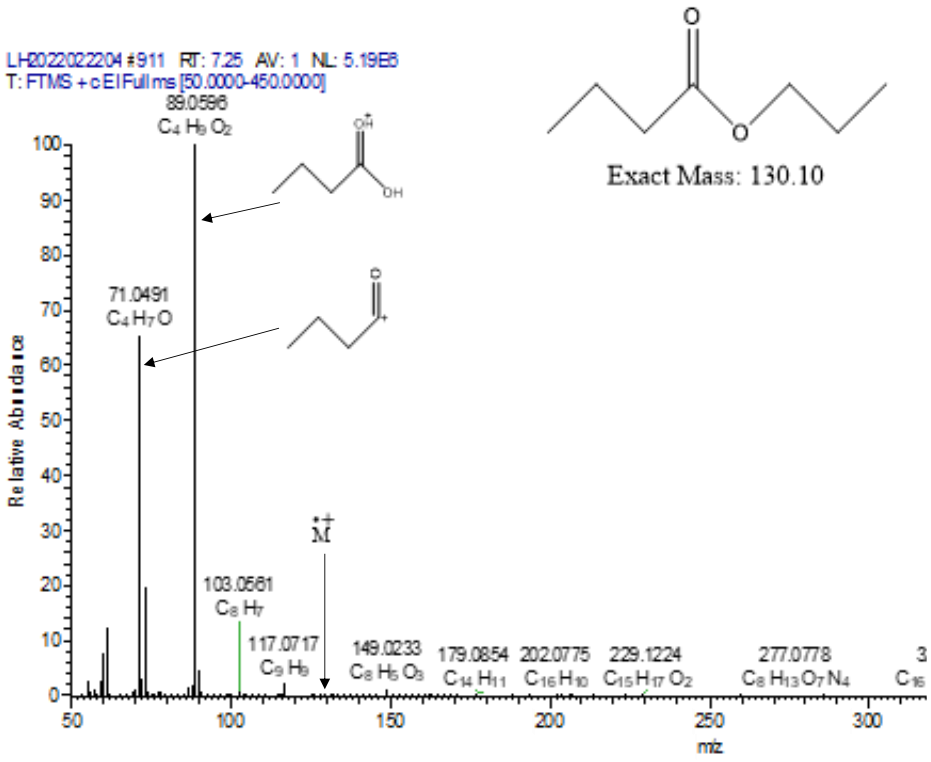


Figure 14: Fragmentation of C4:0 FAPE.

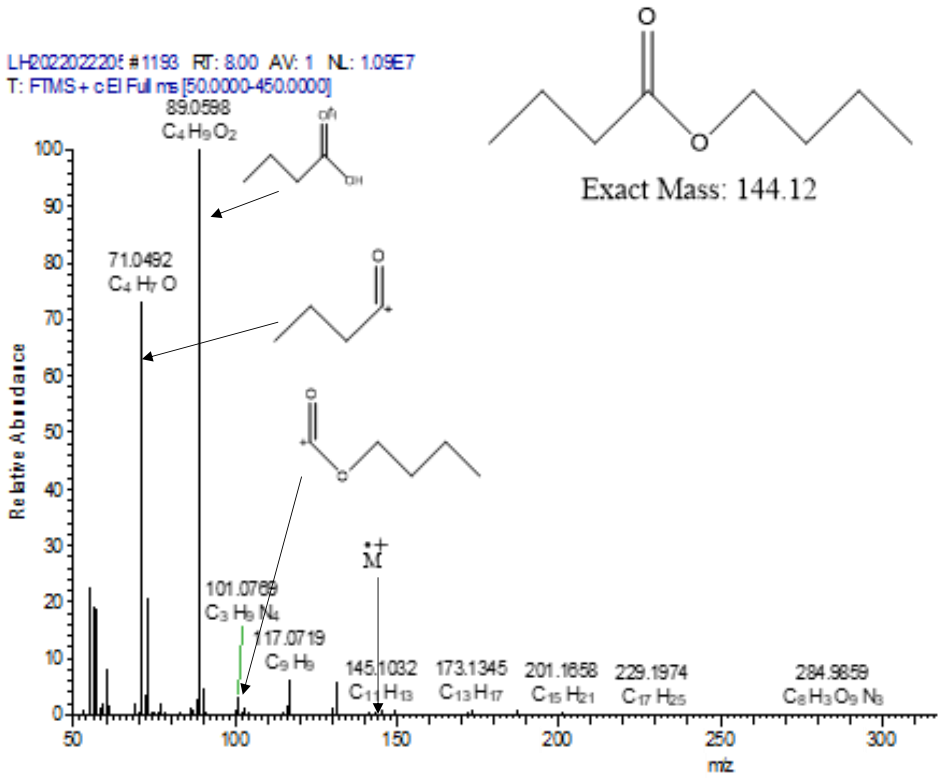


Figure 15: Fragmentation of C4:0 FABE.

4.5.2 Fragmentation; medium chain fatty acid alkyl ester (C10:0)

Just like the short chain FAME, the medium chain FAMEs main peak is the McLafferty fragment at *m/z* = 74. We also see the Mclafferty fragment in FAEE at *m/z* = 88, but it is not the main peak. Main peak for FAEE is *m/z* = 73 which is the loss of the carbon chain. In FAPE and FABE we have the same main peak at *m/z* = 173. We don't see the McLafferty fragment even though it might be present. We also see a lot more fragmentation for propyl and butyl, compared to methyl and ethyl.

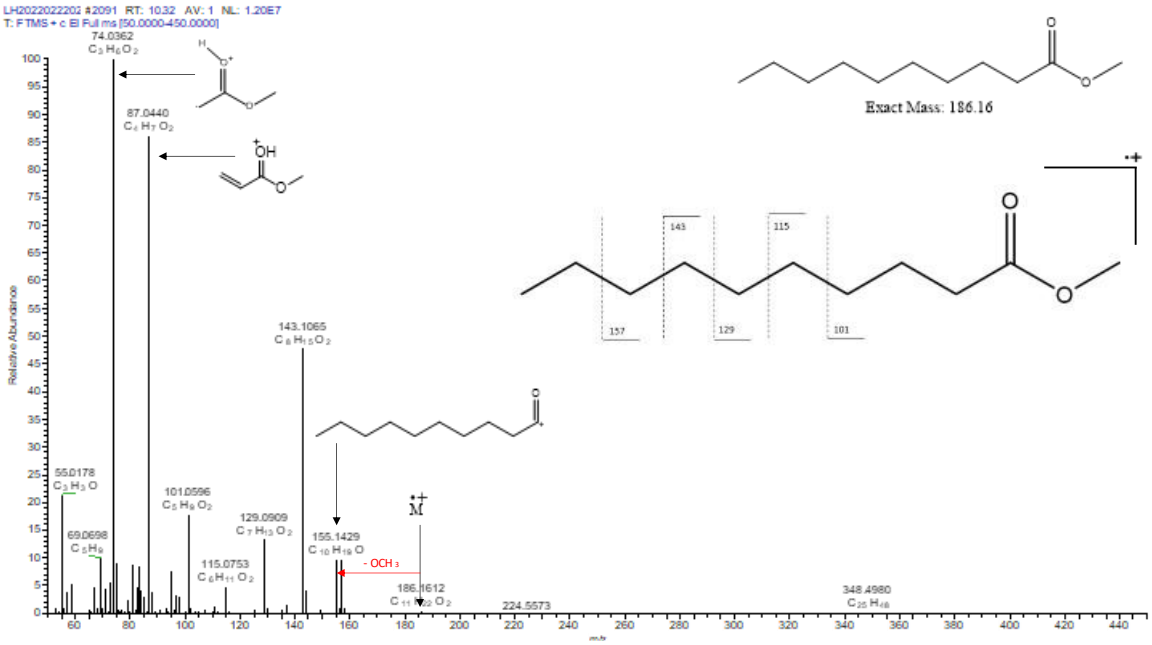


Figure 16: Fragmentation of C10:0 FAME.

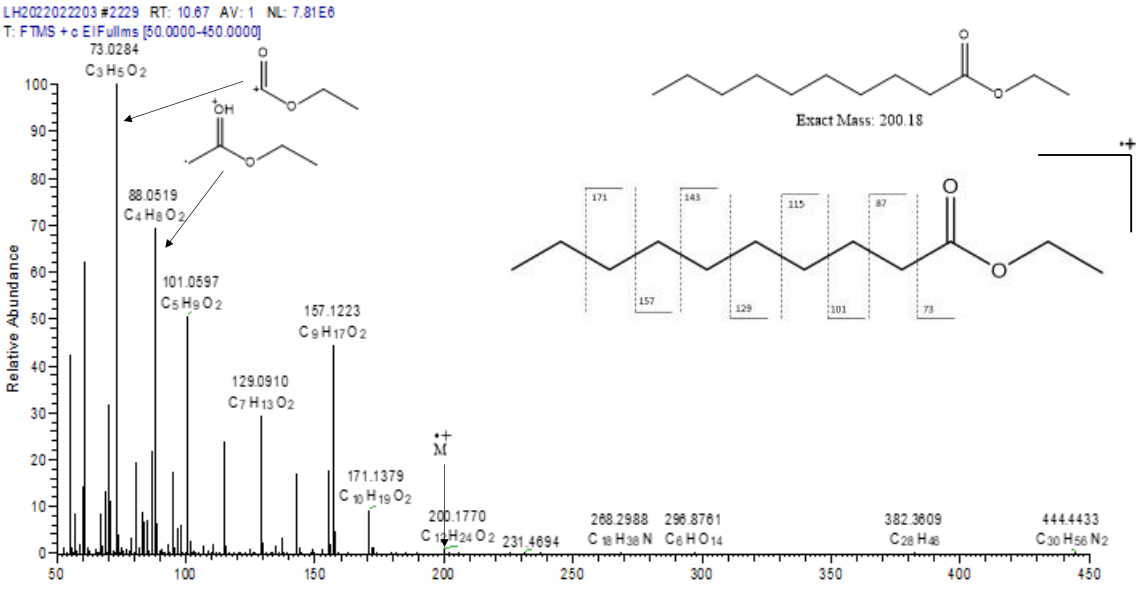


Figure 17: Fragmentation of C10:0 FAEE.

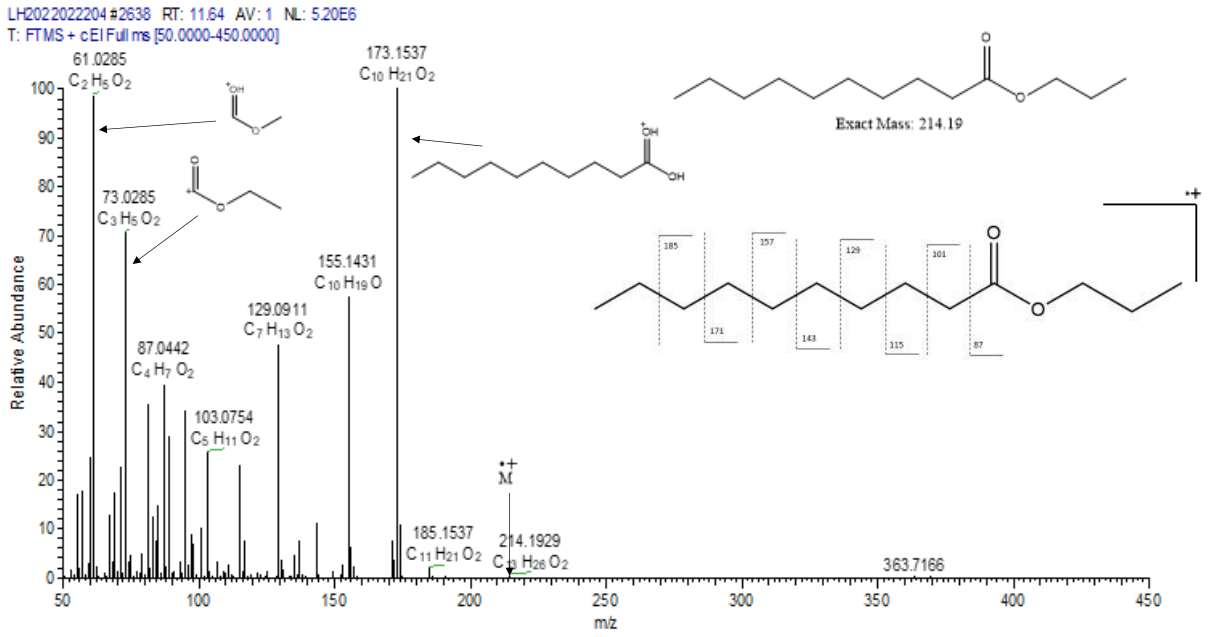


Figure 18: Fragmentation of C10:0 FAPE.

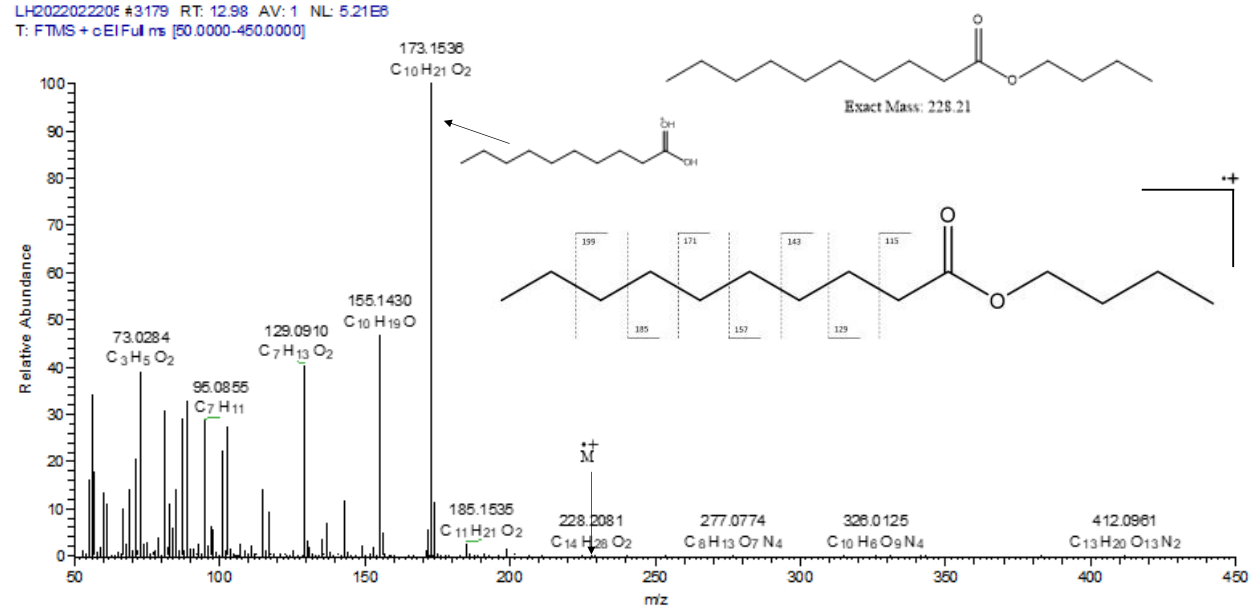


Figure 19: Fragmentation of C10:0 FABE.

4.5.3 Fragmentation; long chain fatty acid alkyl ester (C18:0)

The main peak for the long chained FAME is *m/z* = 87, the fragmentation reaction for this is described in scheme 4. For FAEE the main peak is *m/z* = 73, which is loss of the carbon chain. This signal is found in all the spectra, but with varying intensity. It is the main peak for FABE and barely present in the FAME spectra. We also see the same fatty acid fragmentation pattern in all spectra. The fatty acid fragmentation follow this series, $[(CH_2)_nCOOCH_3]^+$, with the most common fragments being *m/z* = 143, 199 and 255 (Mjøs 2004). All of these are present.

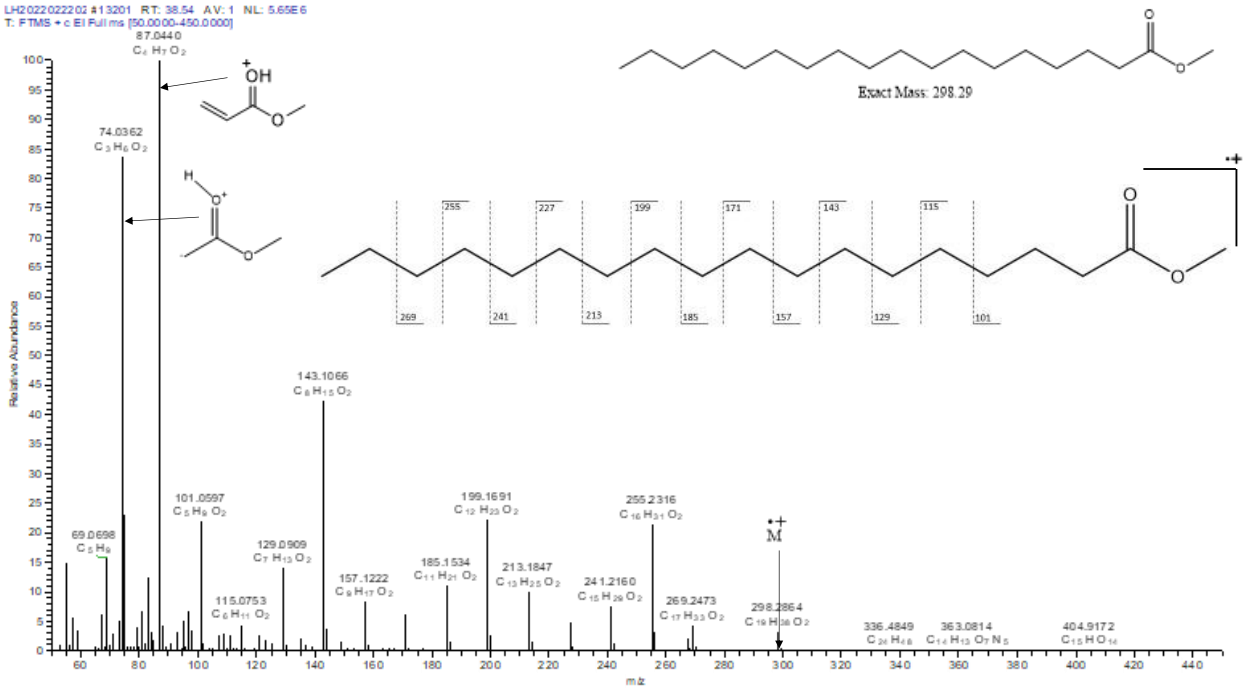


Figure 20: Fragmentation of C18:0 FAME.

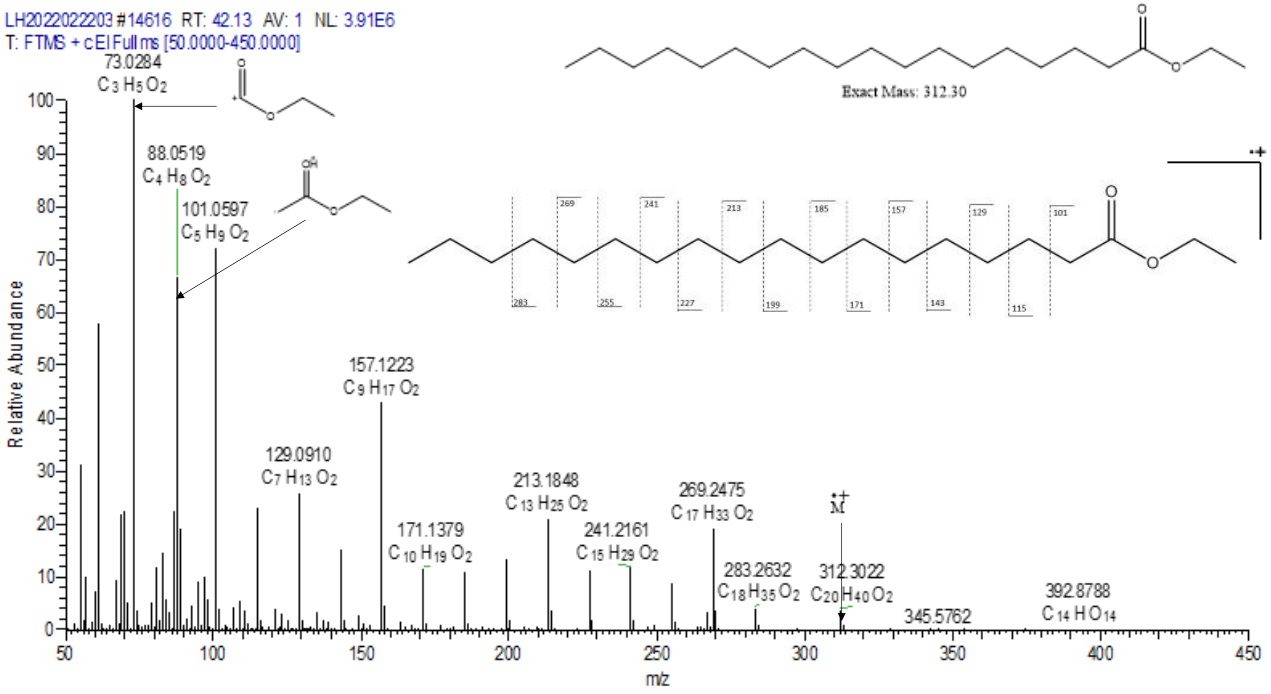


Figure 21: Fragmentation of C10:0 FAEE.

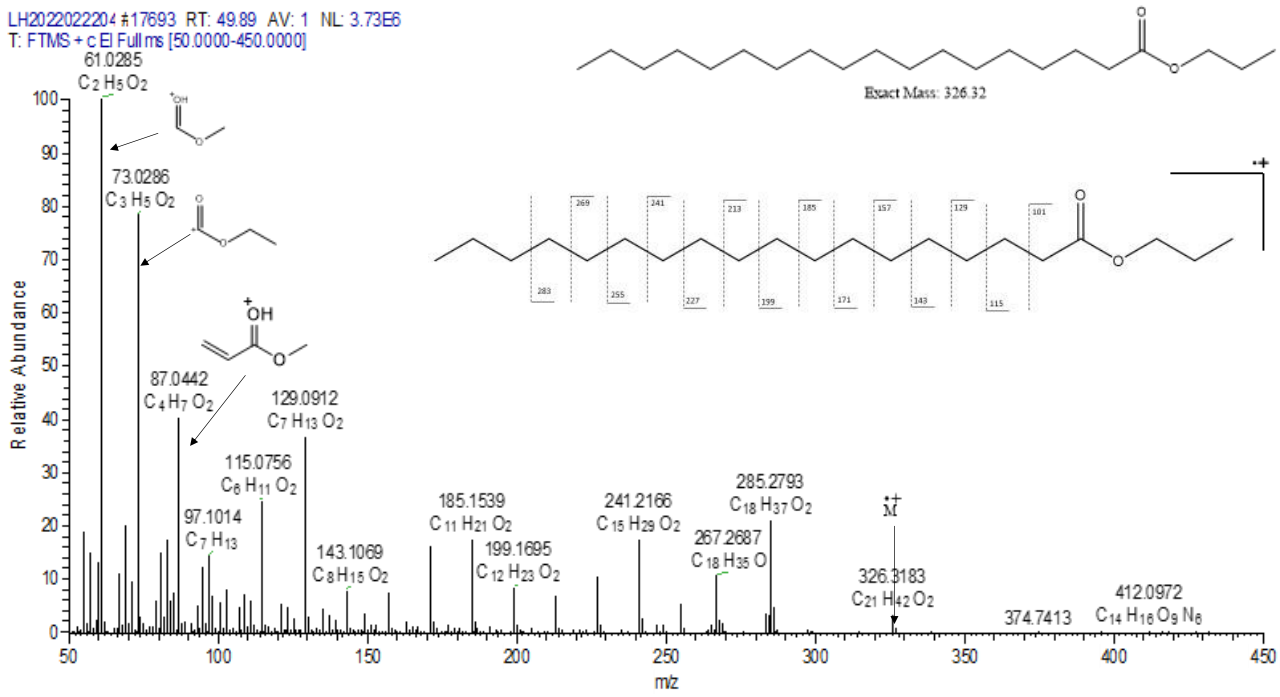


Figure 22: Fragmentation of C18:0 FAPE.

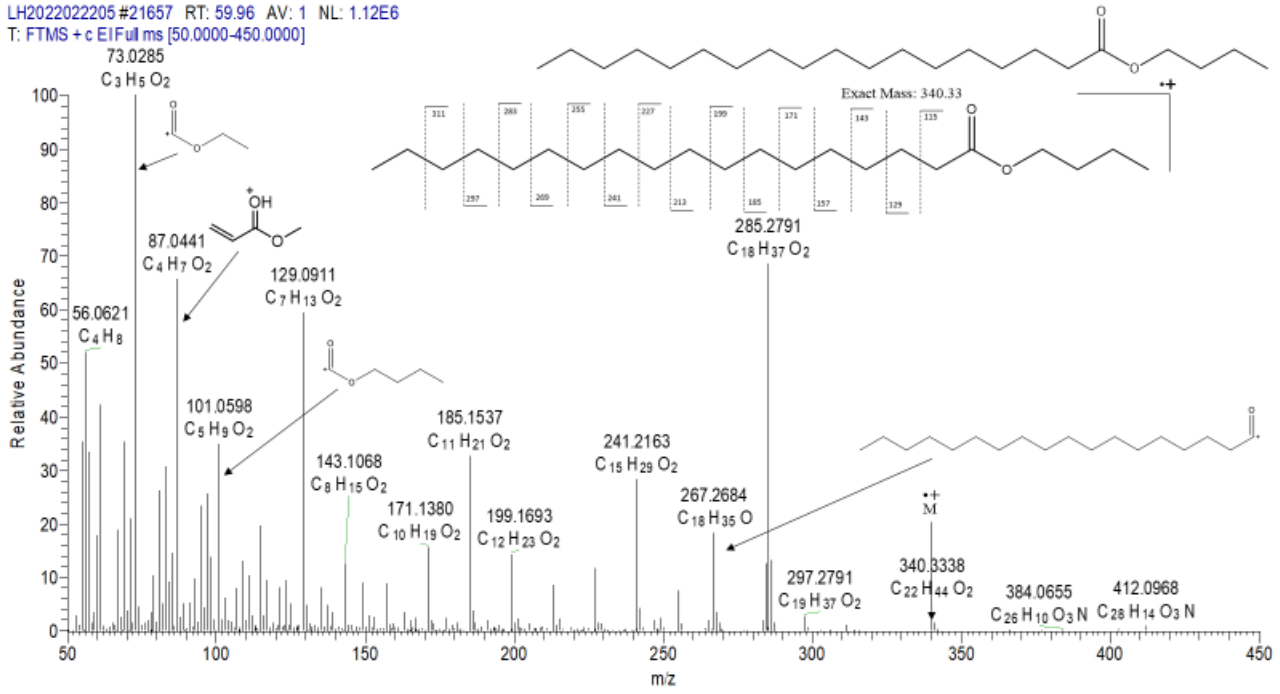


Figure 23: Fragmentation of C18:0 FABE.

4.5.4 Fragmentation; unsaturated long chain fatty acid alkyl ester (C18:1)

All the different alkyl esters have the same main peak at *m/z* = 81. Another dominate peak were the loss of the added alkyl group, *m/z* = 265, especially for FAPE and FABE. There is a lot of fragmentation for all the spectra, and many peaks are found in all four, so there is not much to differentiate.

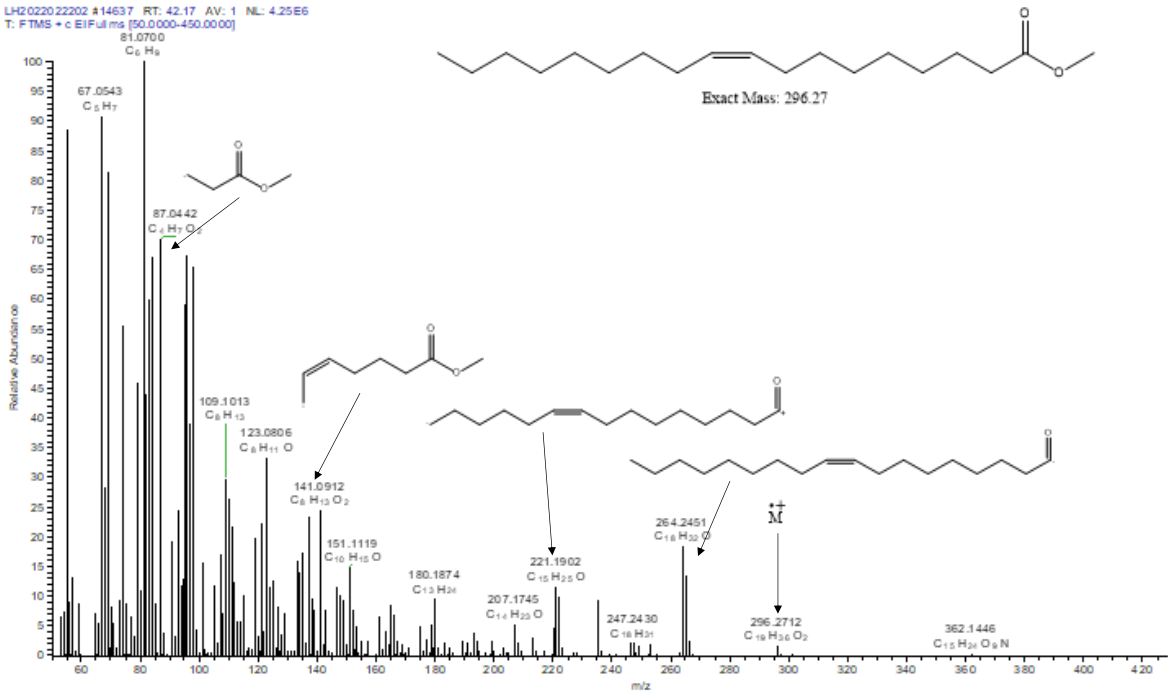


Figure 24: Fragmentation of C18:1 FAME.

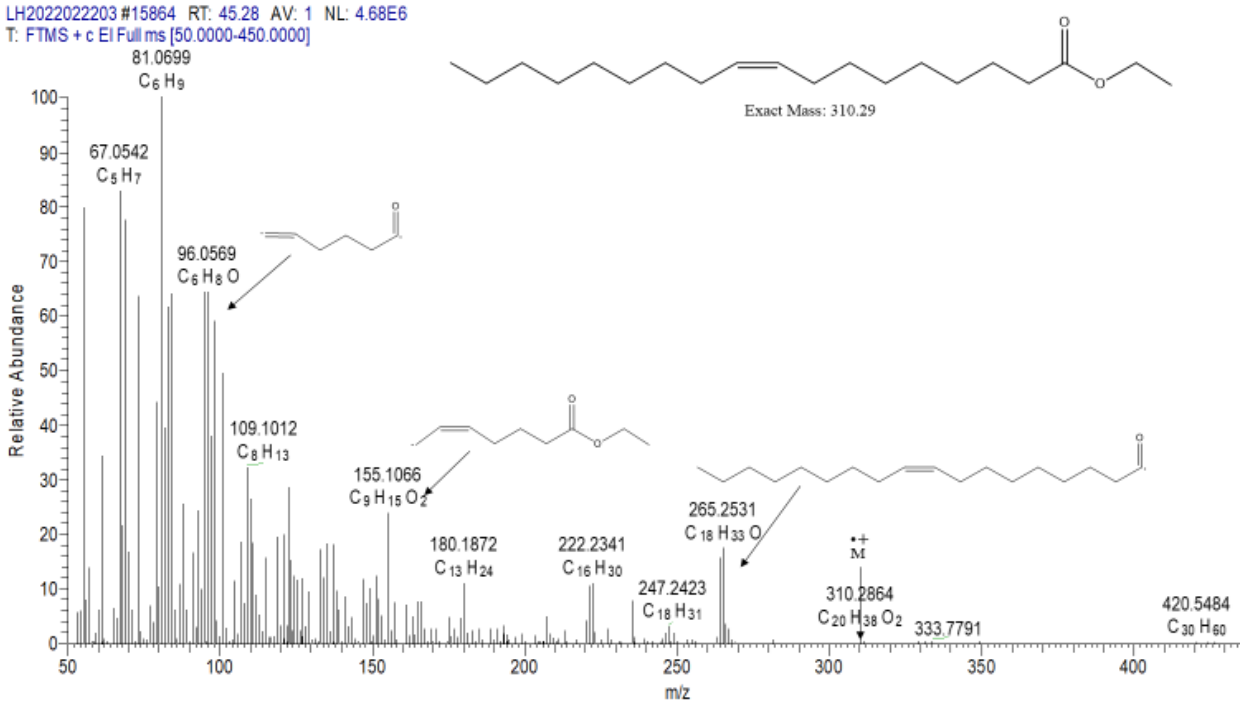


Figure 25: Fragmentation of C18:1 FAEE.

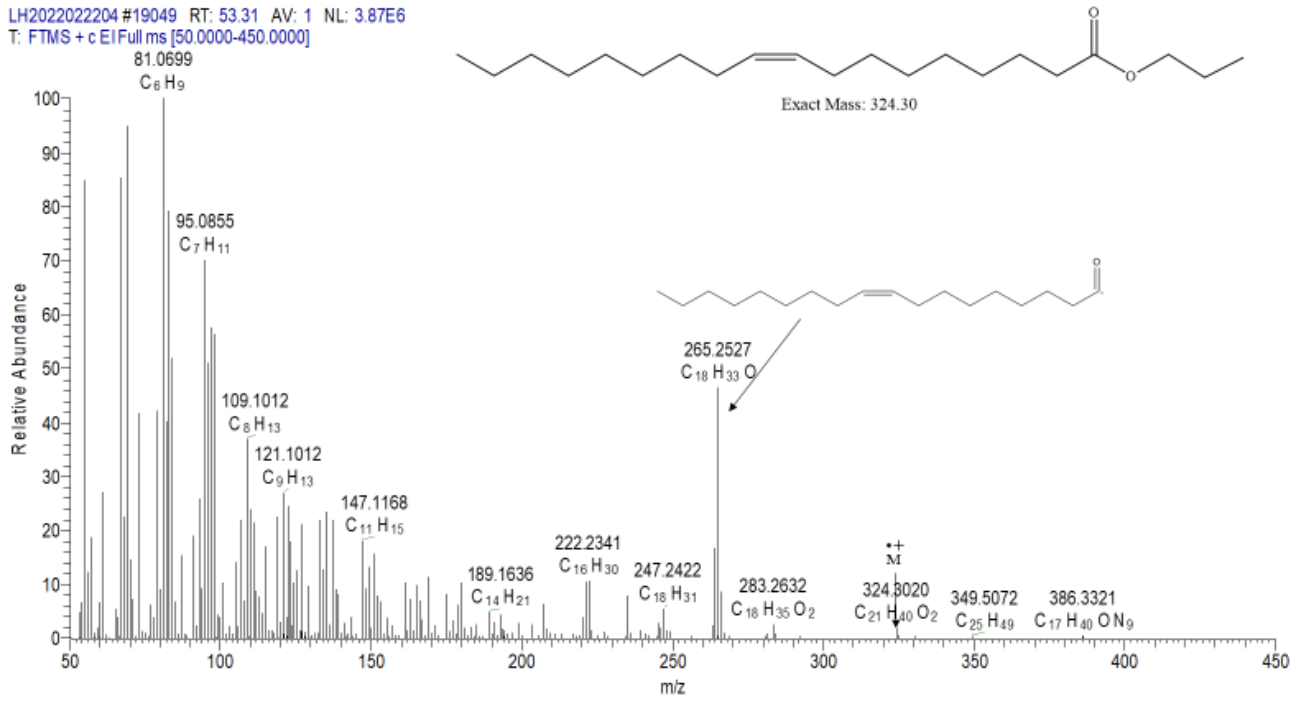


Figure 26: Fragmentation of C18:1 FAPE.

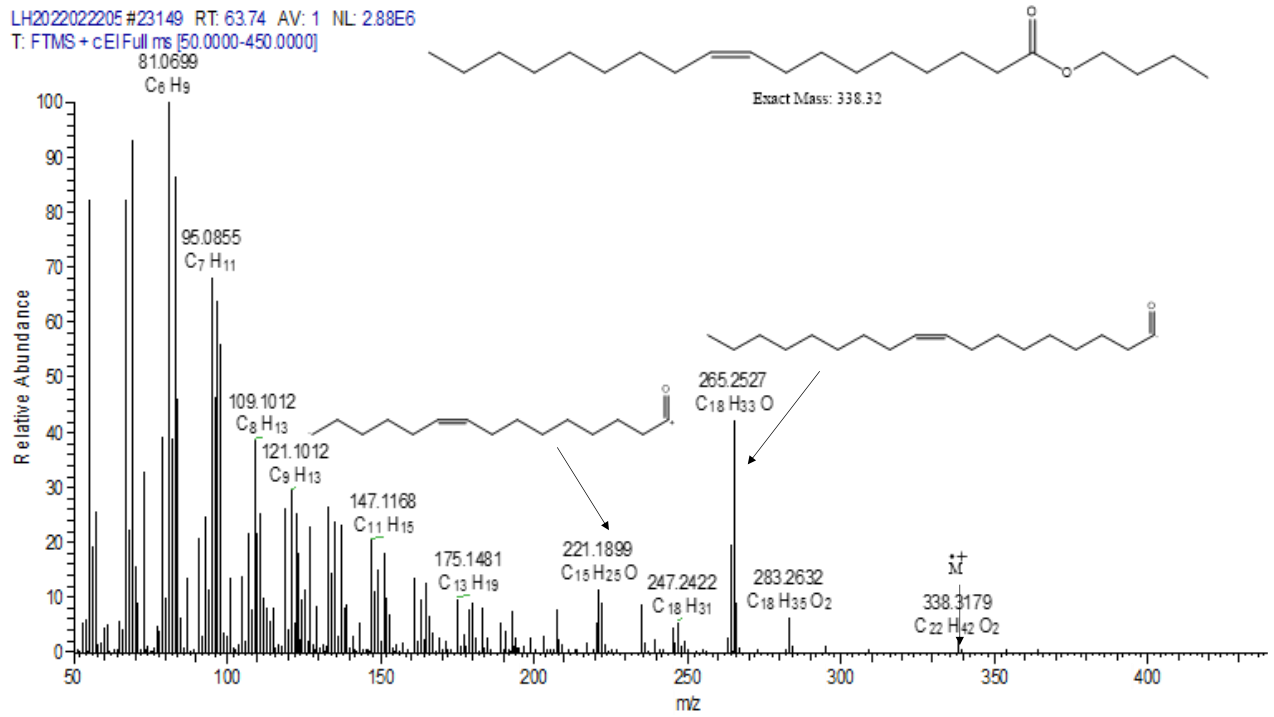


Figure 27: Fragmentation of C18:1 FABE.

4.5.5 Fragmentation; poly unsaturated long chain fatty acid alkyl ester (C18:3)

In the polyunsaturated spectra, there are very little fragmentation compared to all the other spectra. Though three peaks stand out, and are all present in all four, *m/z* = 67, 79 and 93. We see no peaks where the alkyl group can differentiate between the esters.

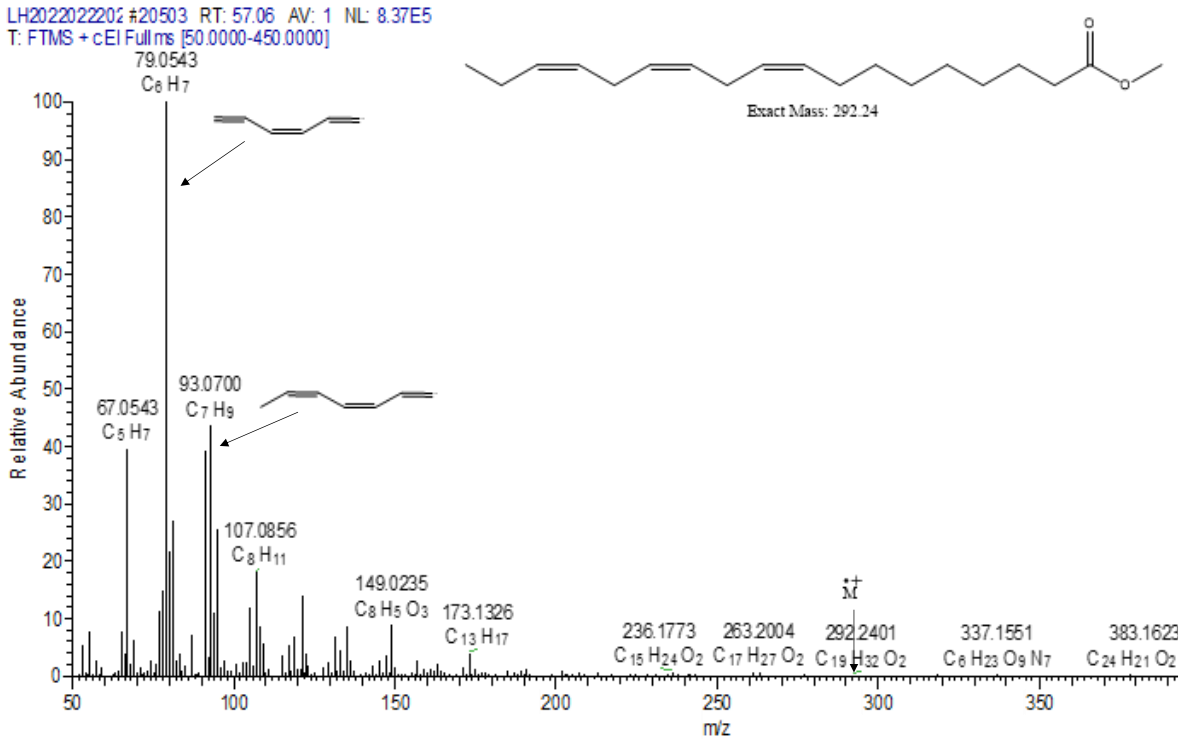


Figure 28: Fragmentation of C18:3 FAME.

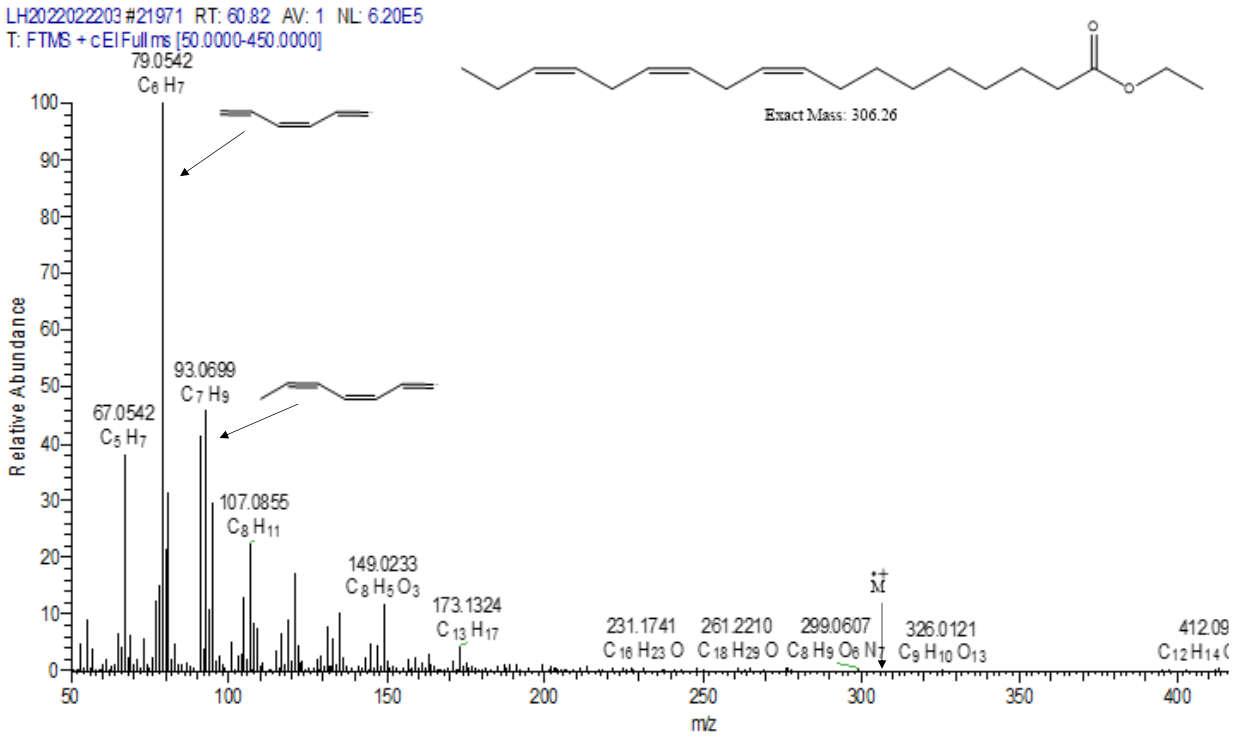


Figure 29: Fragmentation of C18:3 FAEE.

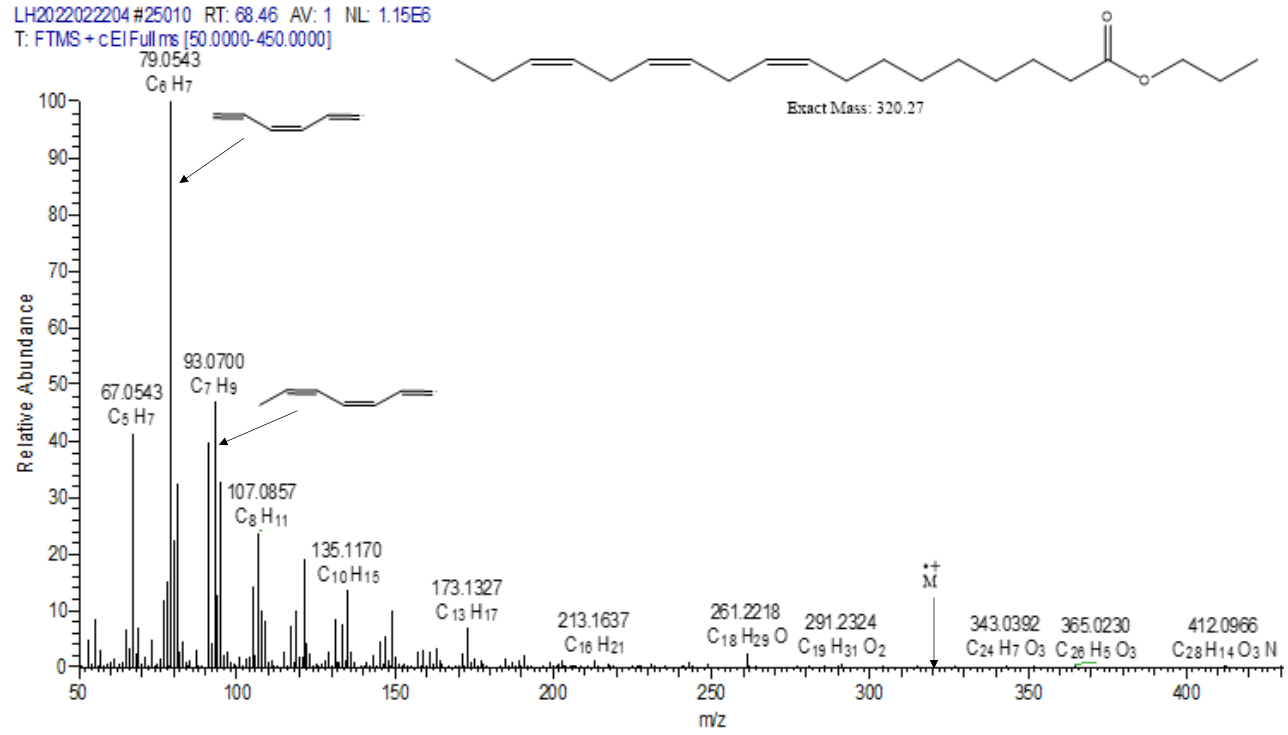


Figure 30: Fragmentation of C18:3 FAPE.

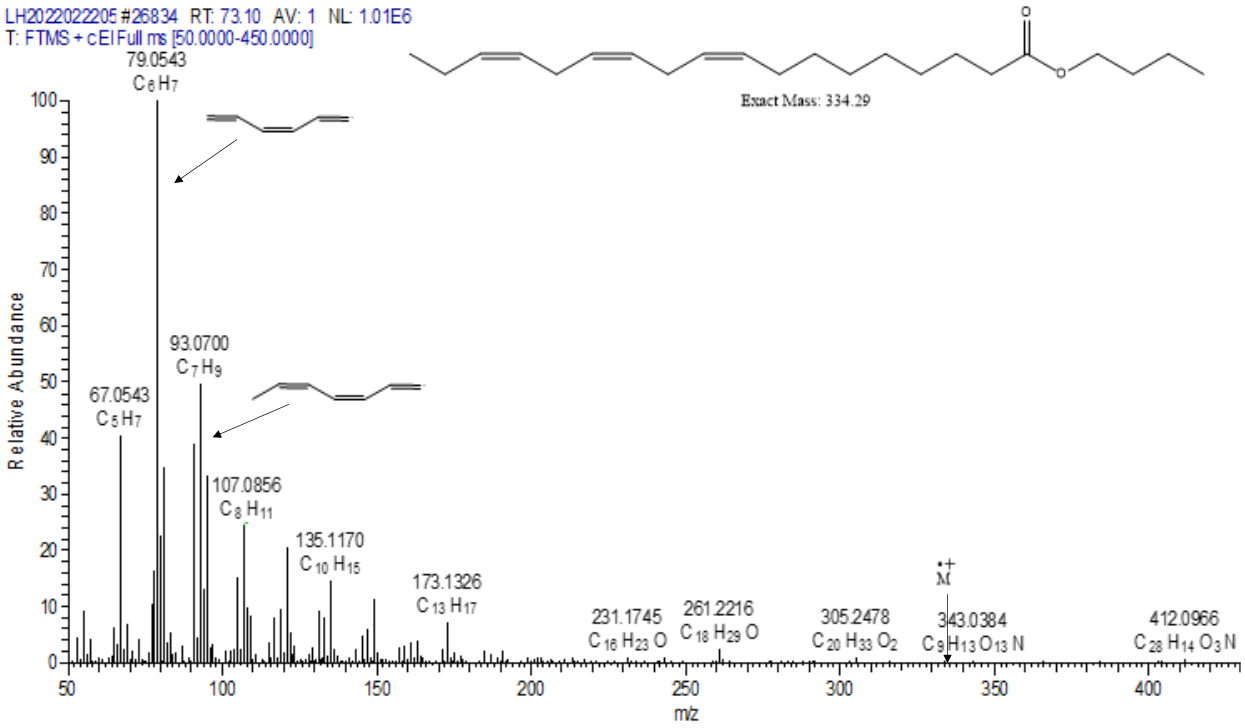


Figure 31: Fragmentation of C18:3 FABE.

5 Conclusion

In conclusion the use of other alkylesters are promising, but there is definitely a need for more testing and improvement on the method. For the transesterified TAGs and DAGs there were a significant increase in yield for the shorter FAs, while the longer only showed a small improvement. For the esterified FFAs, the short to medium improved the most, as expected. The unsaturated FFAs showed the least improvement.

6 Future work

We could not conclude that FAEE, FAPE and FABE are able to replace FAME yet. While the method used worked, it can still be improved. Other temperatures and reaction times should be used for the esterification. Also since butanol was immiscible in both water and heptane, another solvent should be tried for making butyl esters, e.g. isobutanol. Also further work on the seperation of the *trans* C18:2 and the C19 FAEE, FAPE, and FABE should be conducted. Since there is a major lack of commercially available standards for FAEE, FAPE and FABE, the development of these would greatly benefit this research.

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8 Appendices

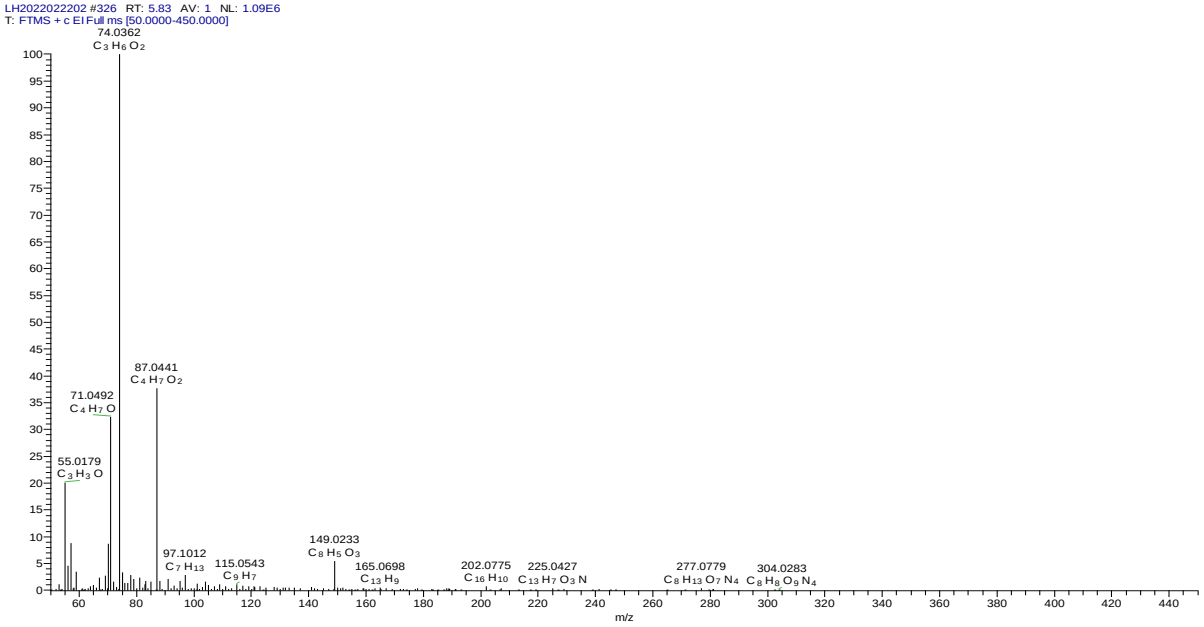
Appendix 1: MS spectra for all FAME

Appendix 2: MS spectra for all FAEE

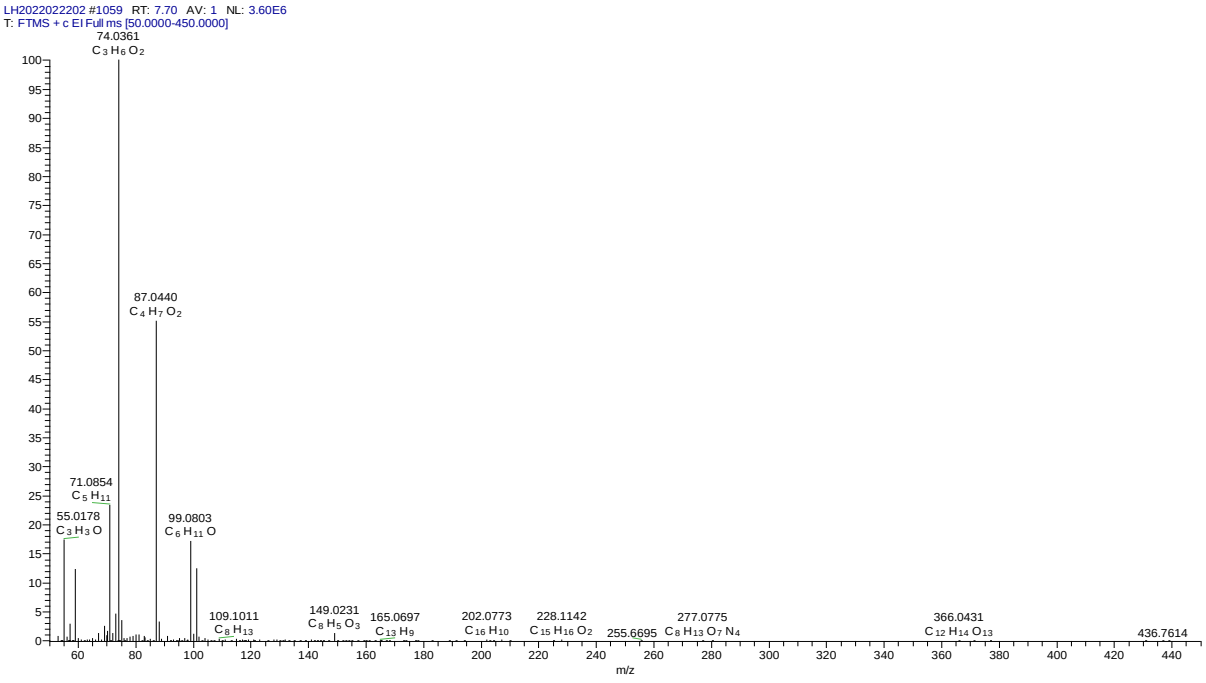
Appendix 3: MS spectra for all FAPE

Appendix 4: MS spectra for all FABE

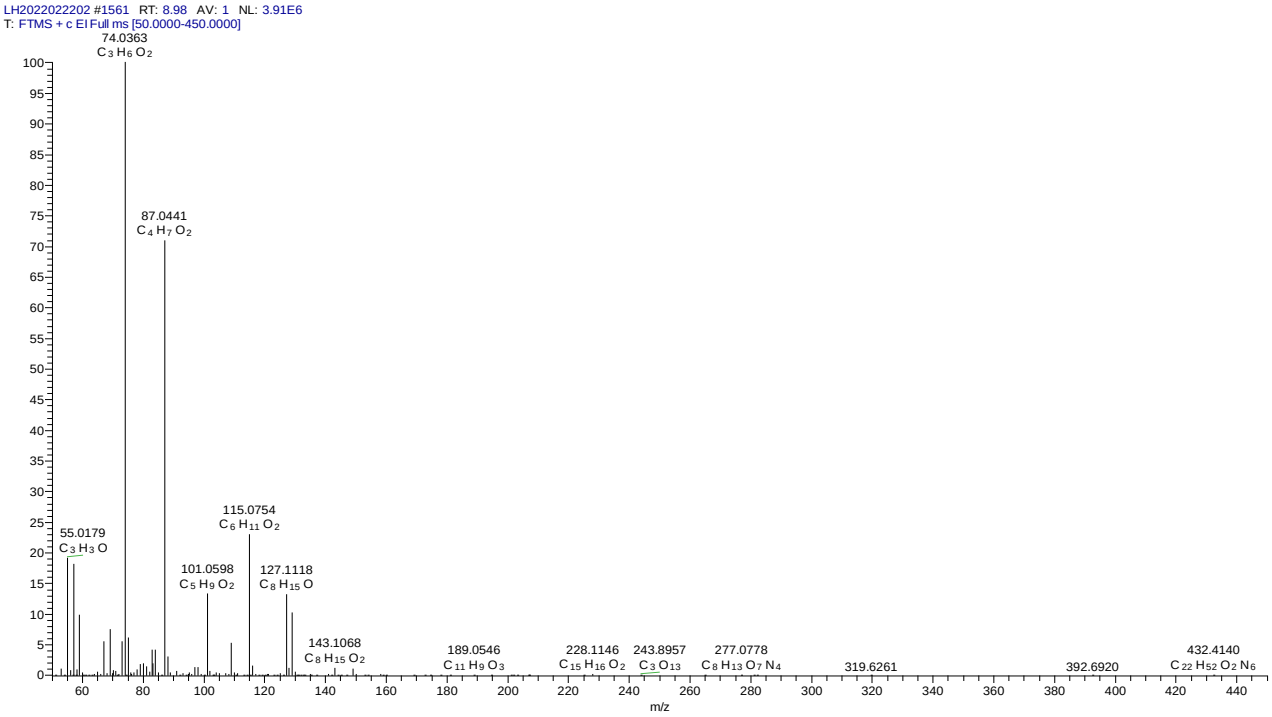
Appendix 1: MS spectra for all FAME



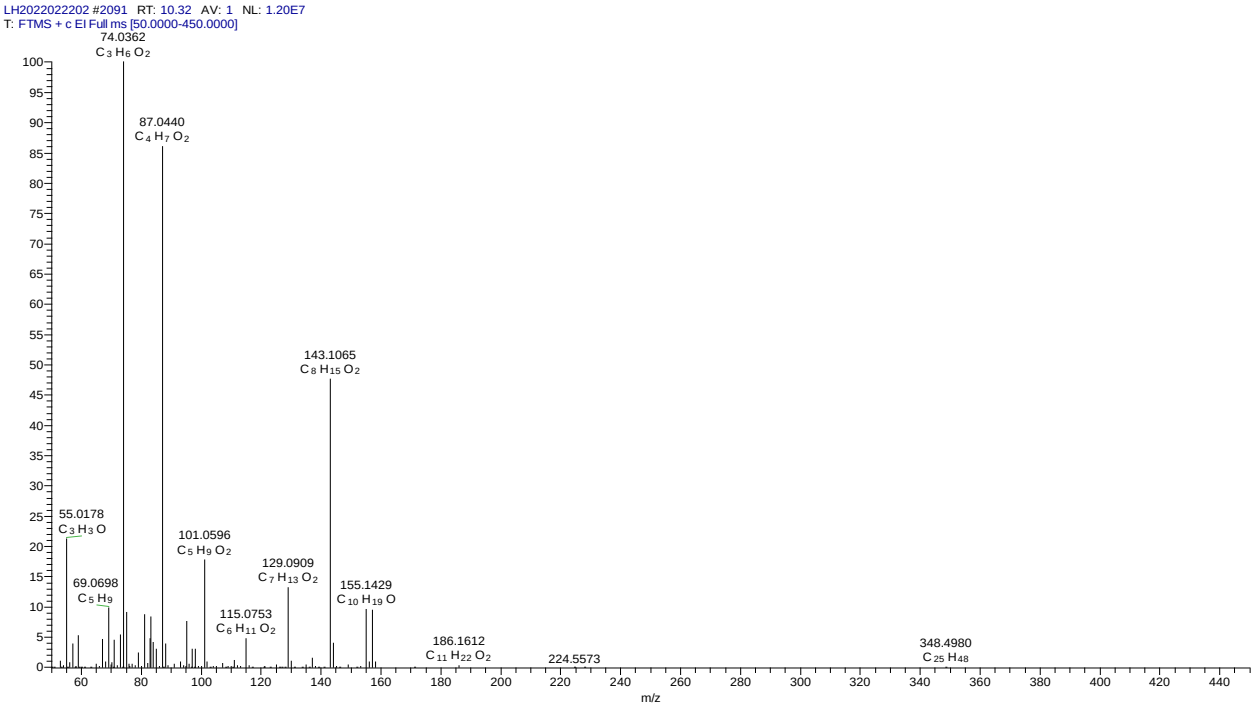
C4:0 MW: 102.13 $C_5H_{10}O_2$



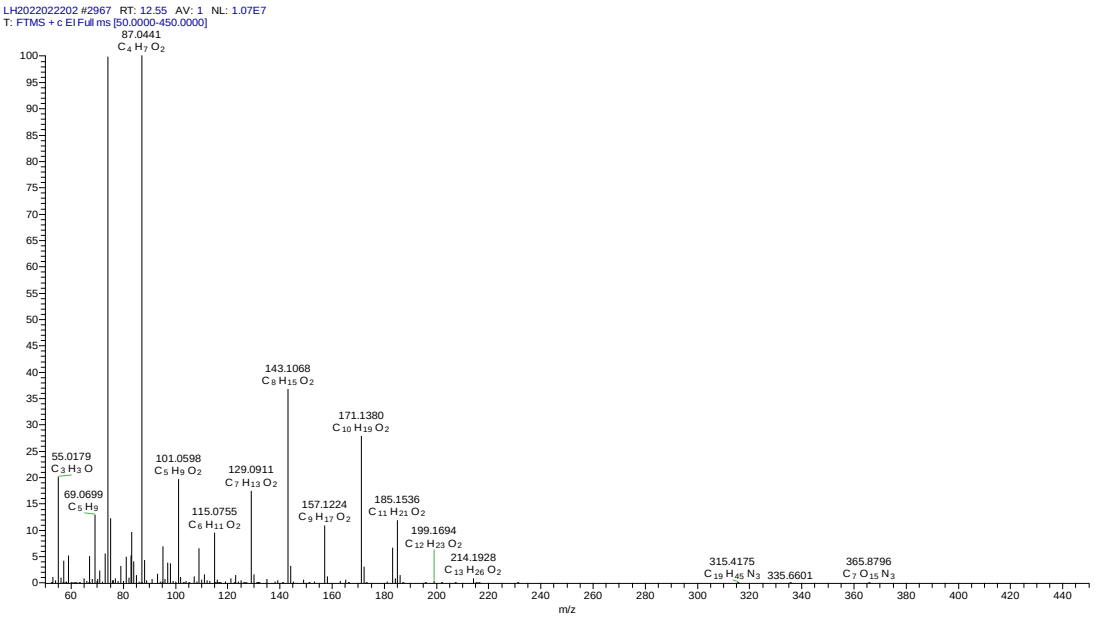
C:6 MW: 130.182 $C_7H_{14}O_2$



C8:0 MW: 158.234 $C_9H_{18}O_2$



C10:0 MW: 186.286 $C_{11}H_{22}O_2$



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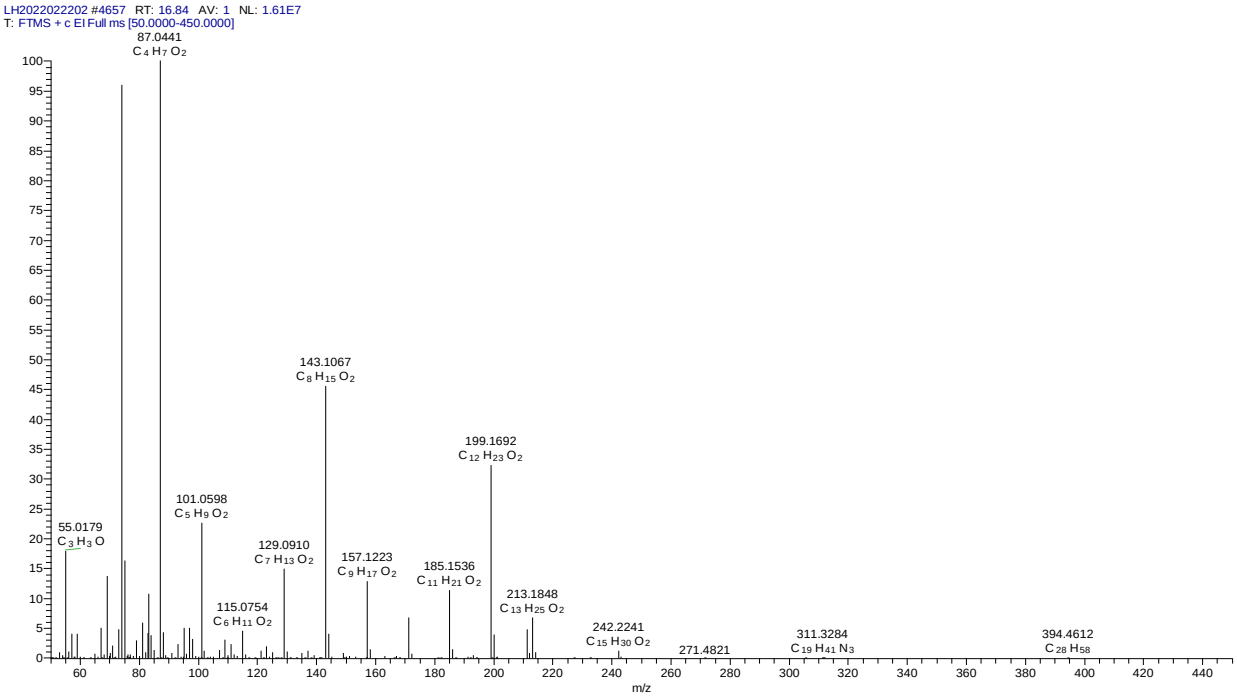
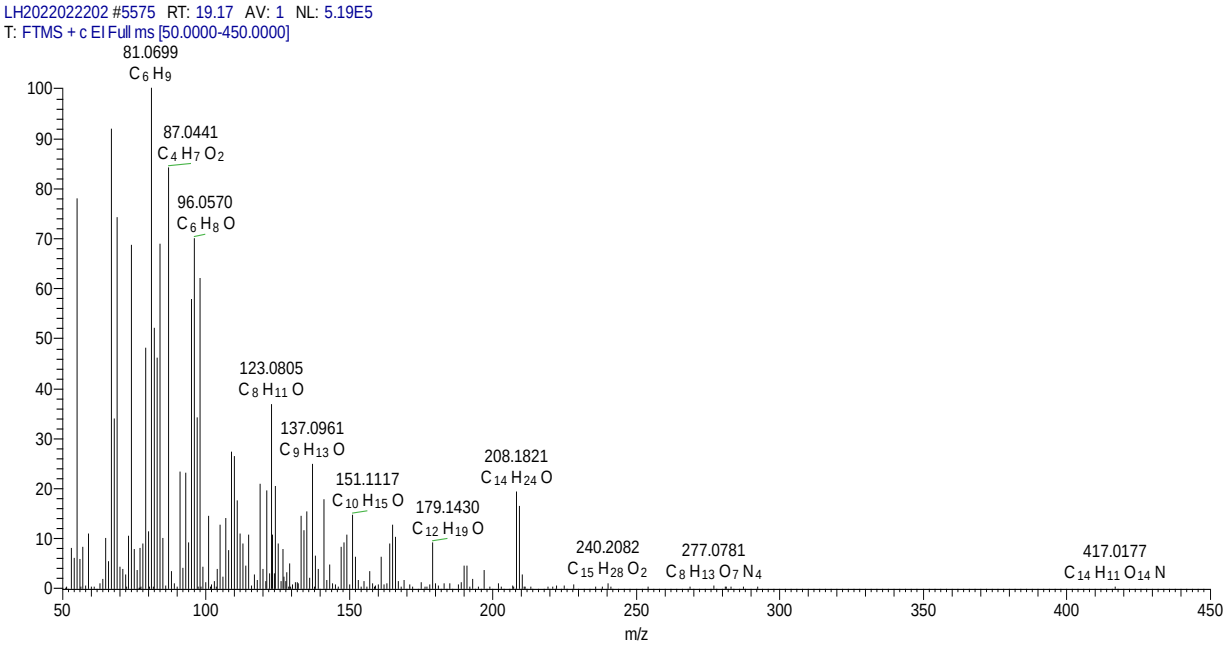
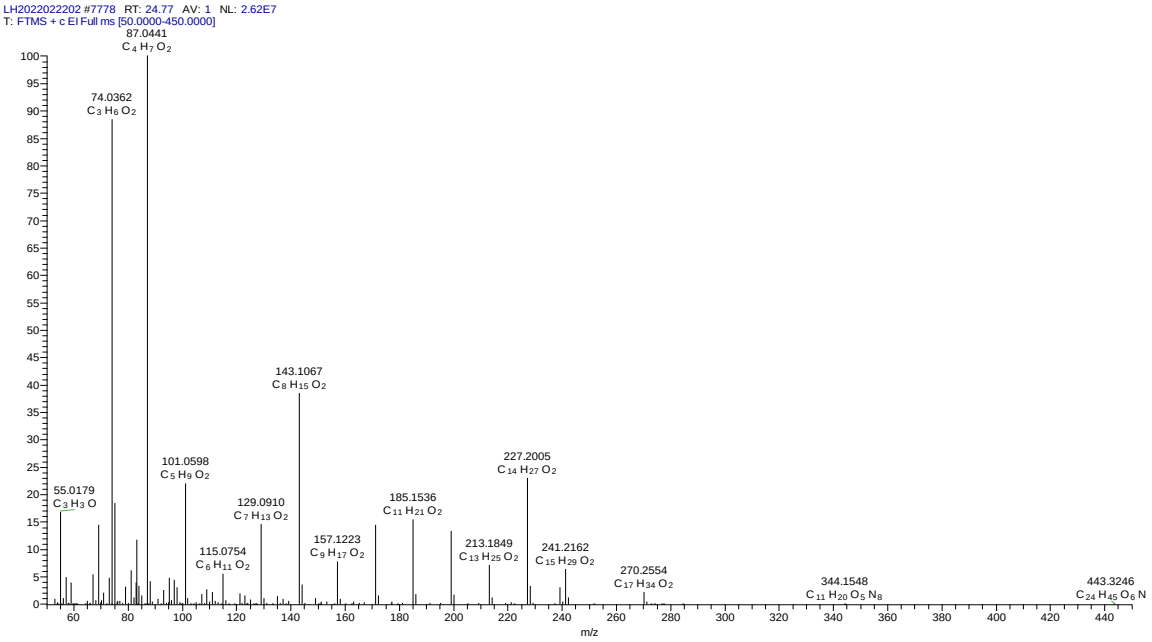


C14:0 MW: 242.39 $C_{15}H_{30}O_2$



C14:1 MW: 240.374 $C_{15}H_{28}O_2$



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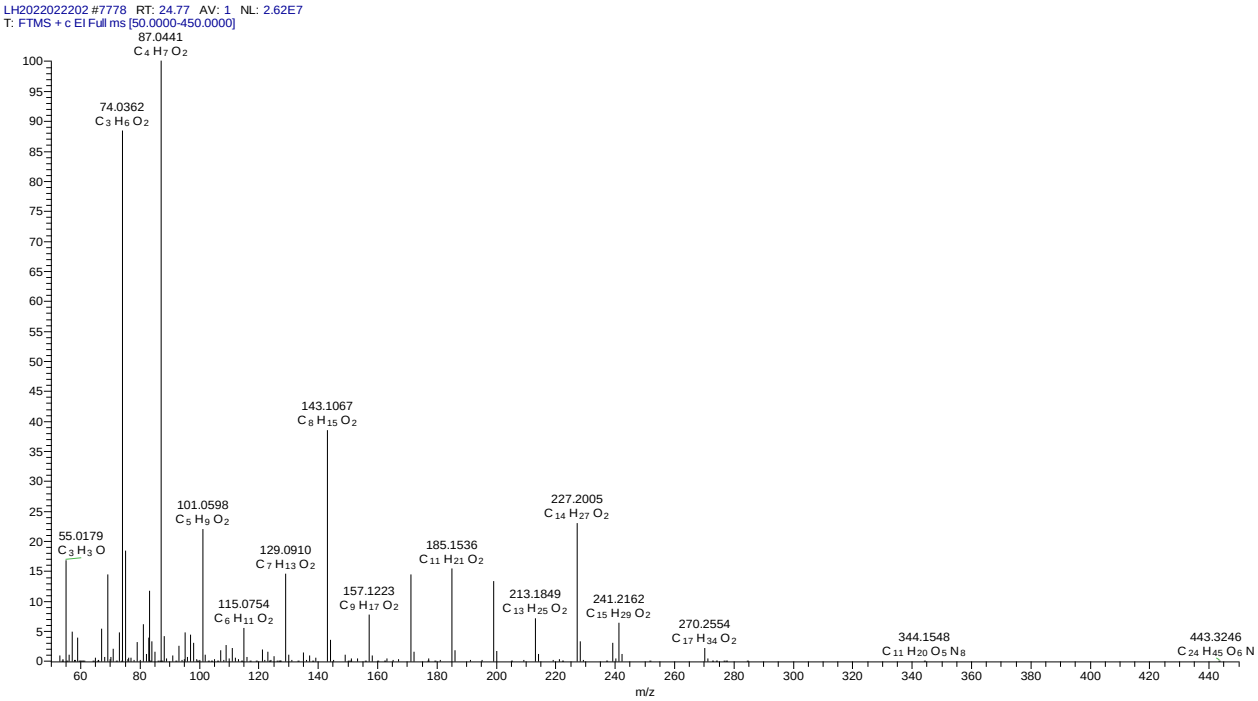

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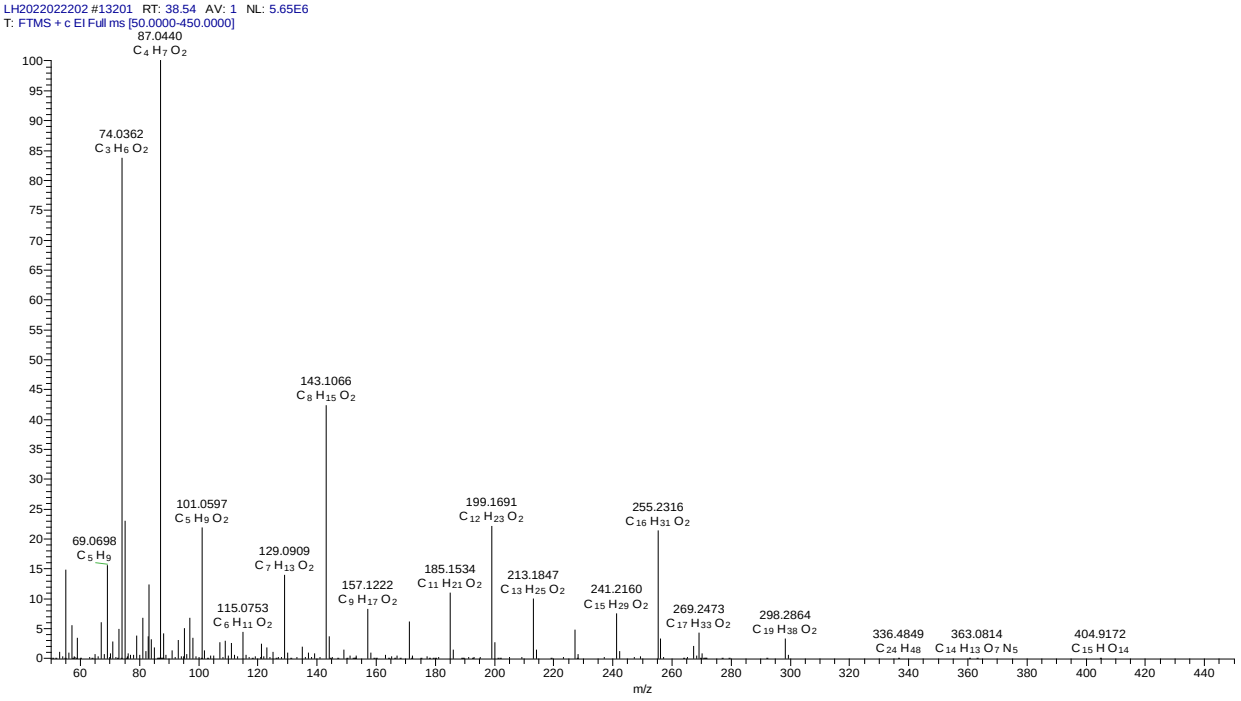
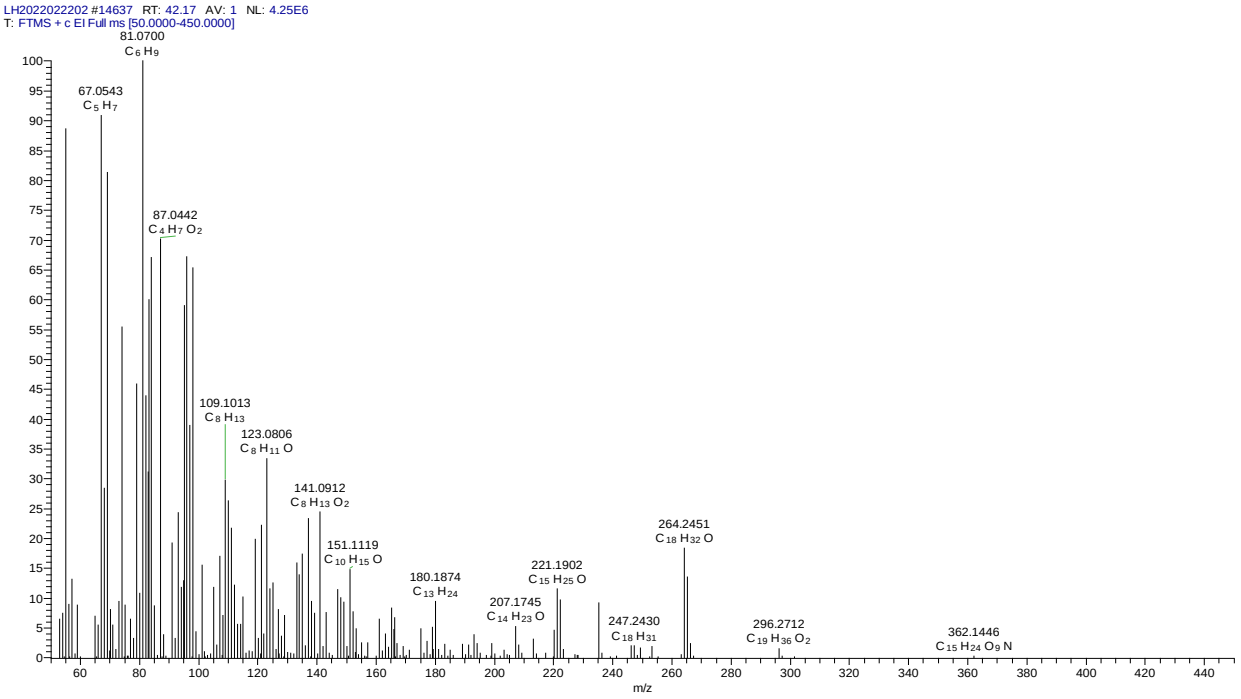
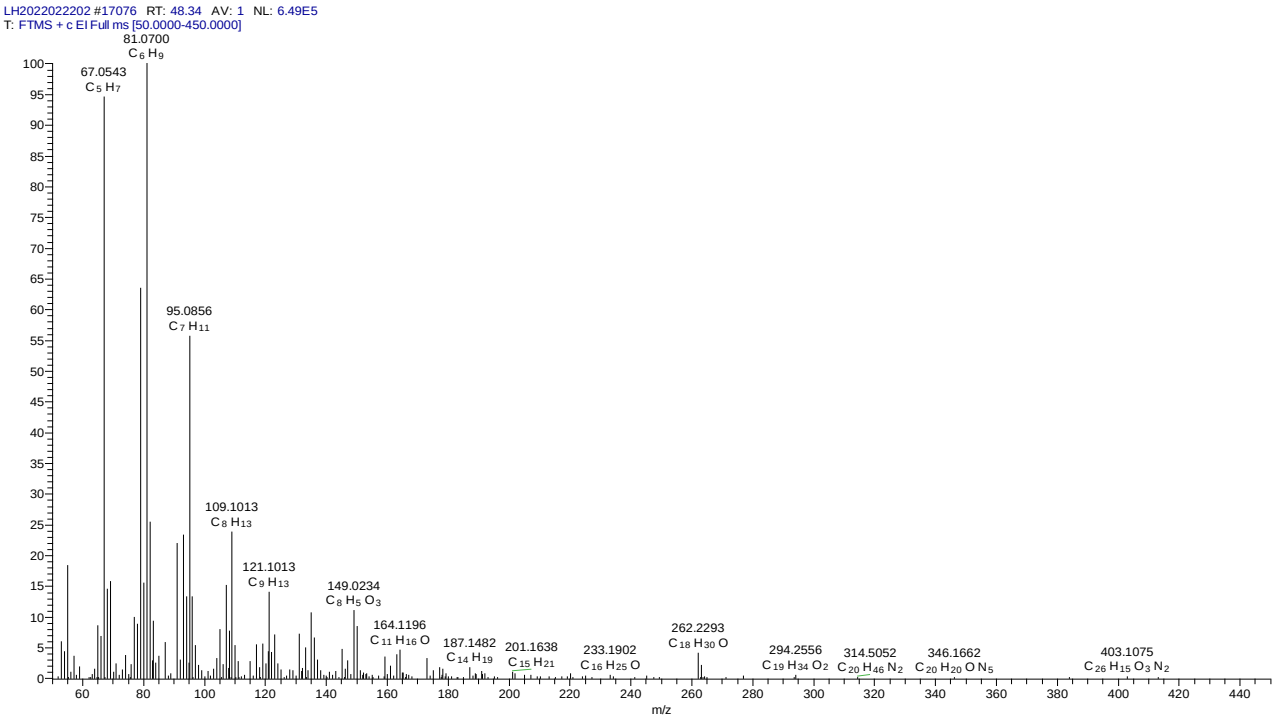


C18:0 MW: 298.494 $C_{19}H_{38}O_2$



C18:1 MW: 296.478 $C_{19}H_{36}O_2$



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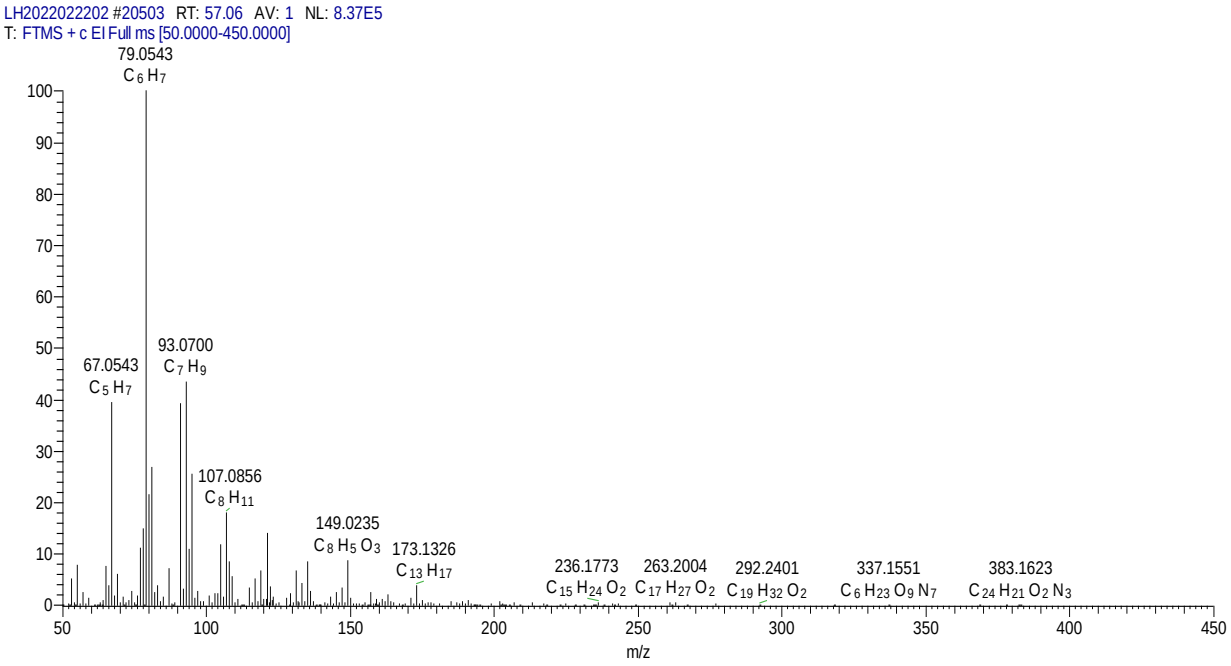

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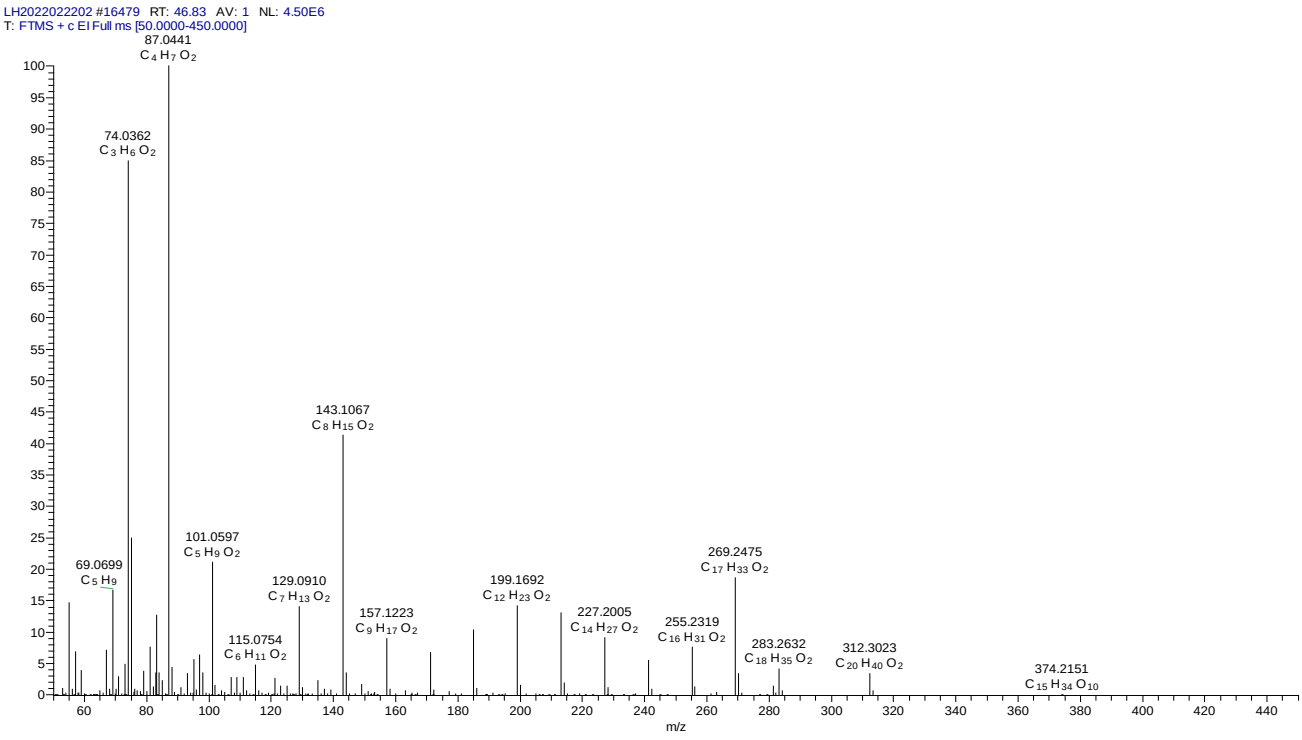

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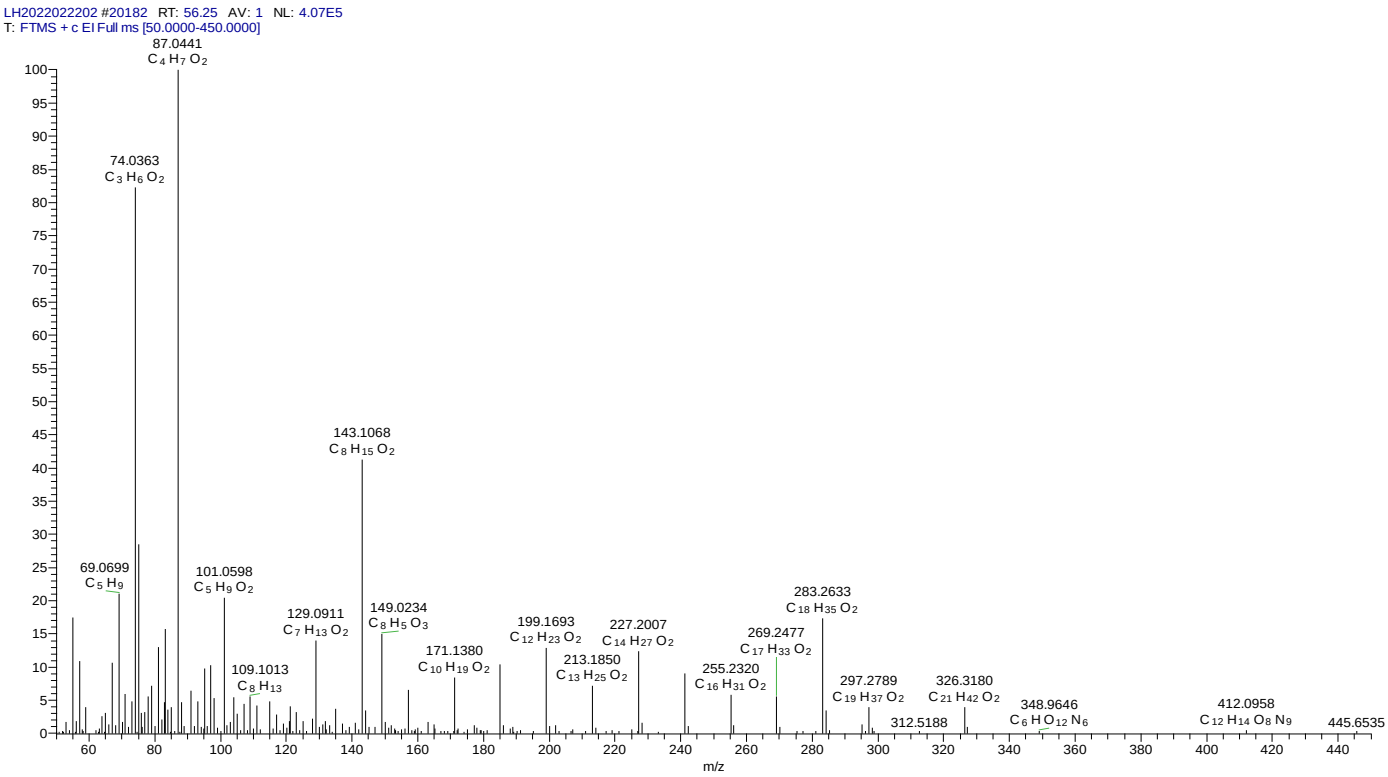
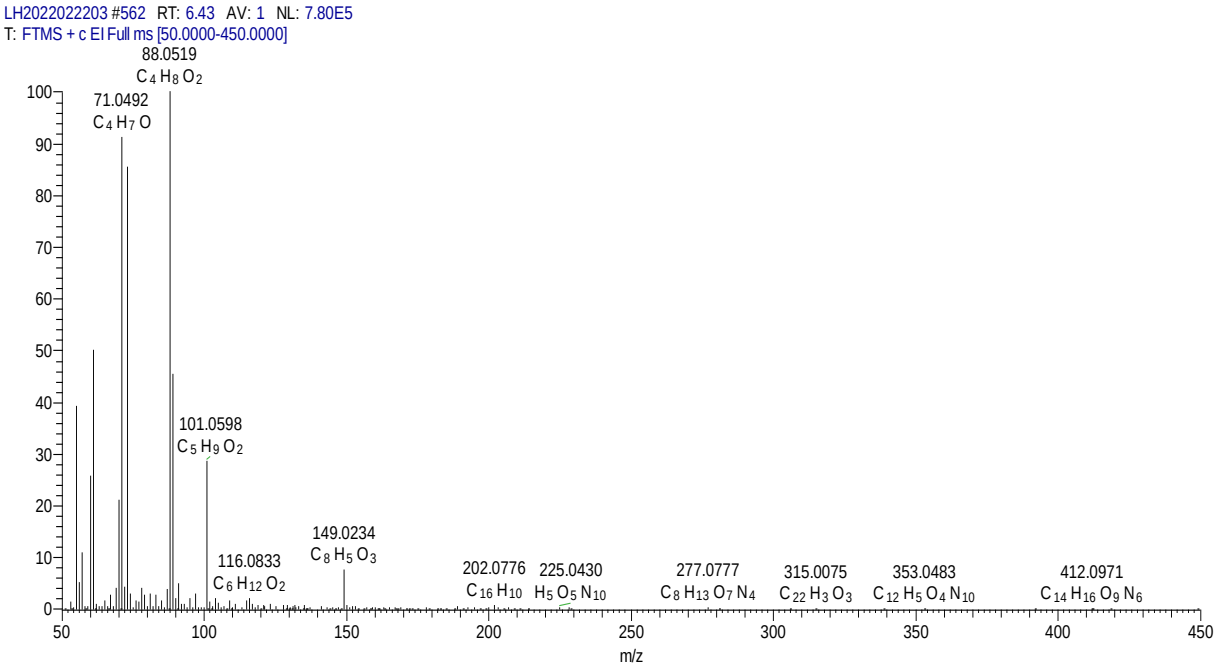


C20:0 MW: 326.546 $C_{21}H_{42}O_2$

Appendix 2: MS spectra for all FAEE



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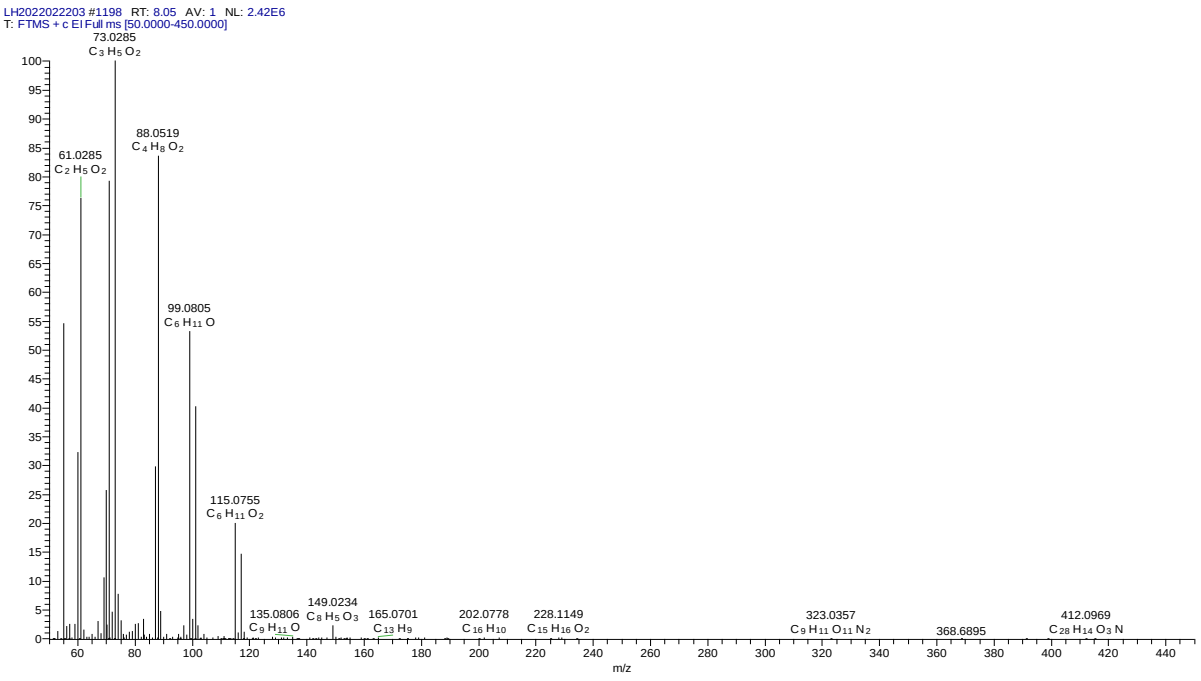

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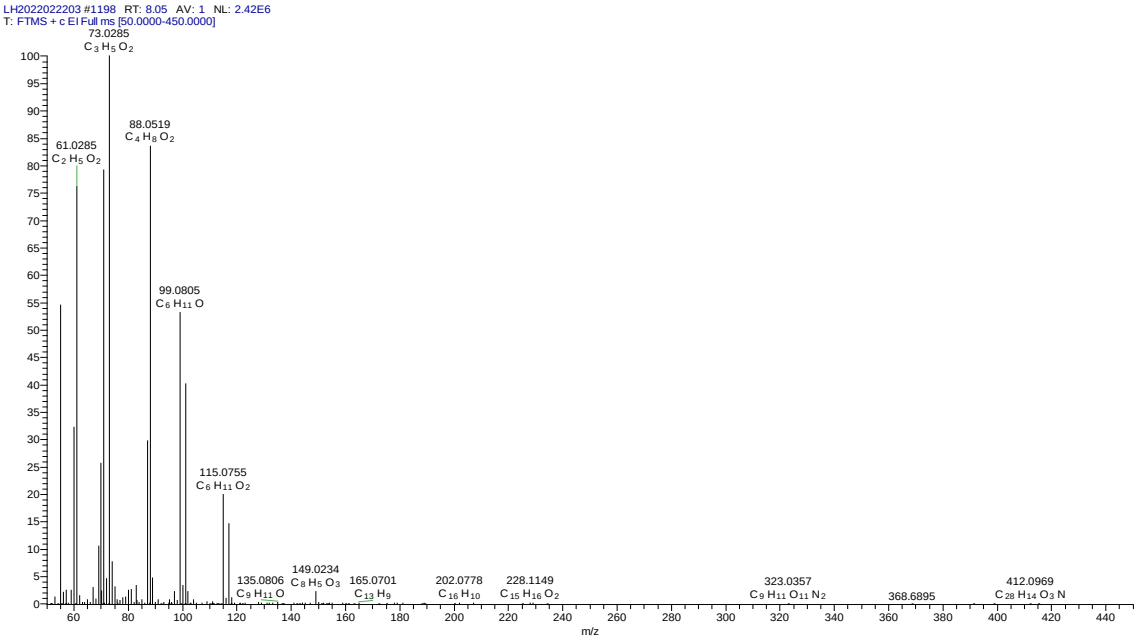

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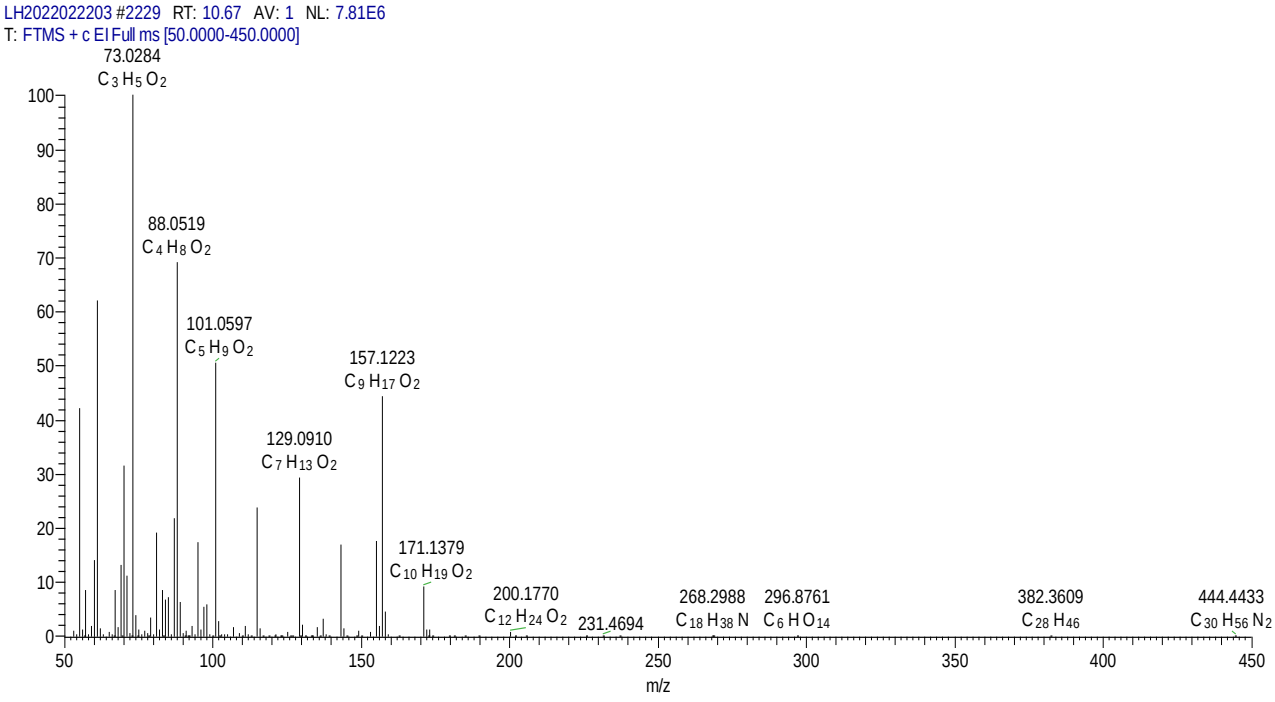

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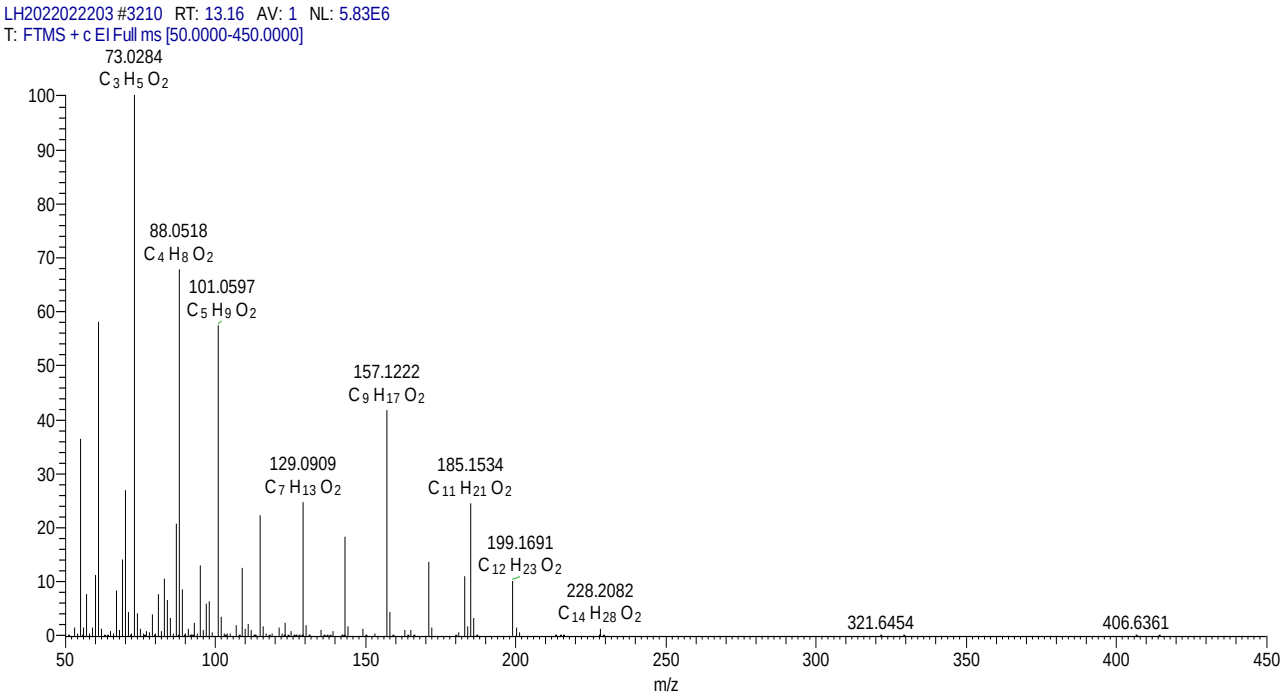

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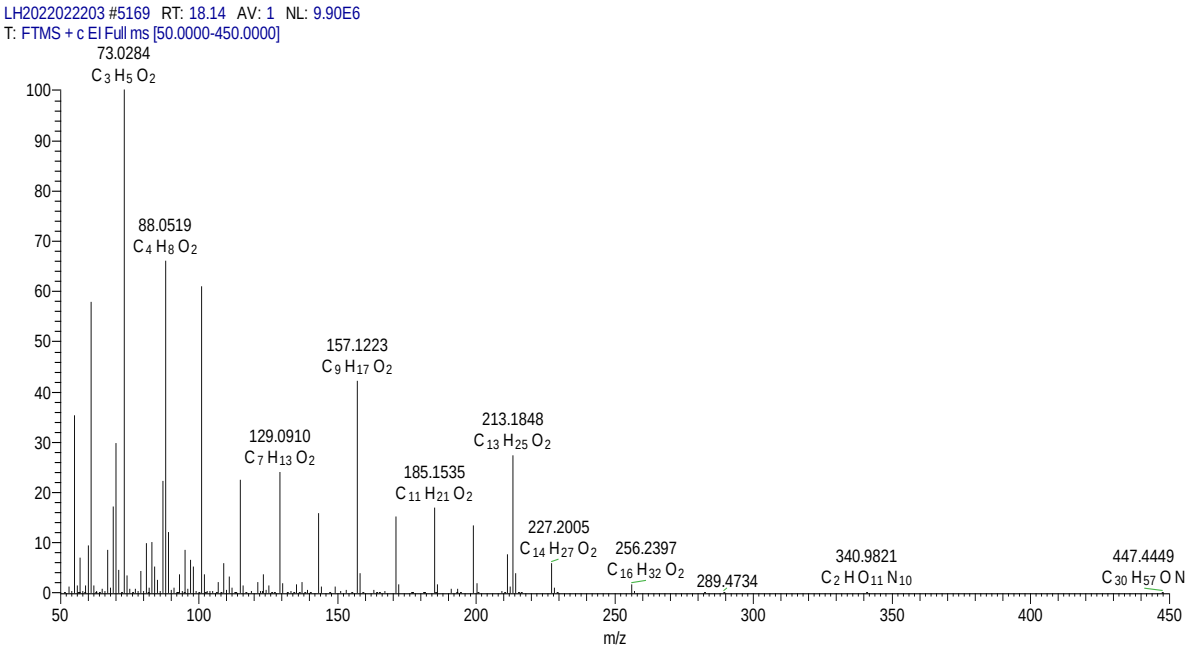

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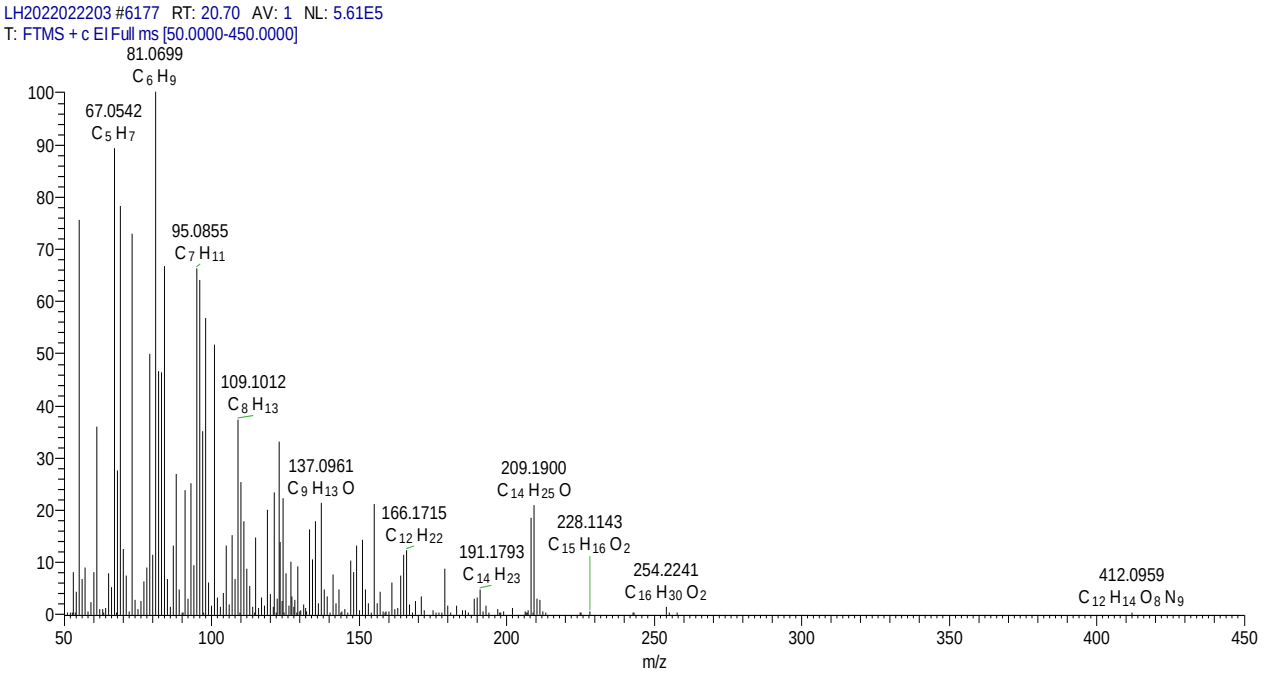

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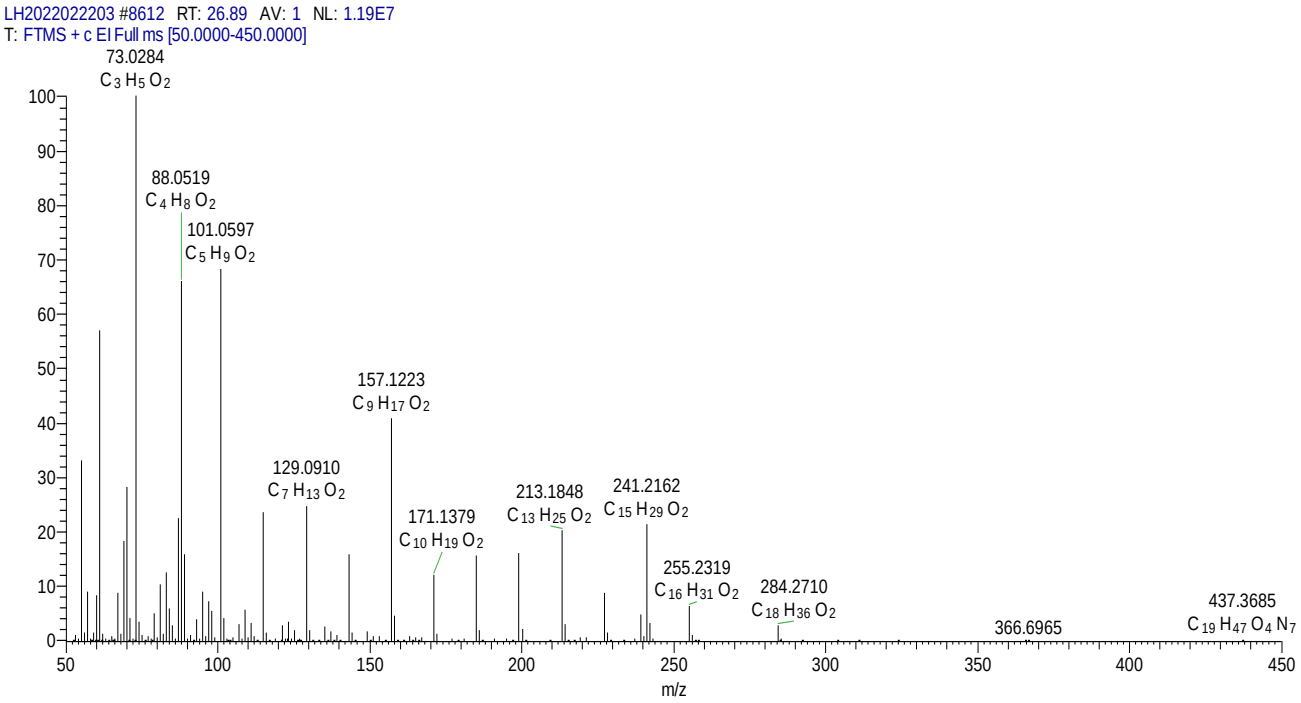

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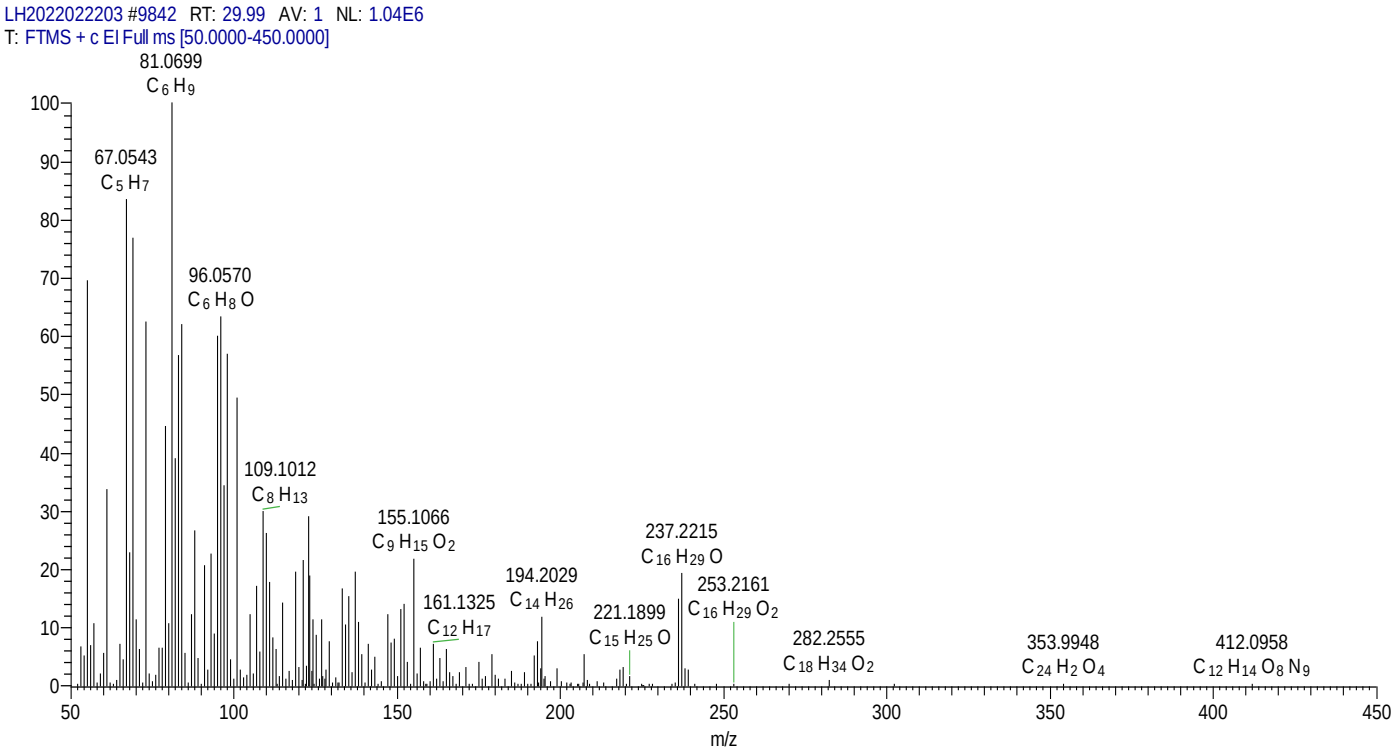

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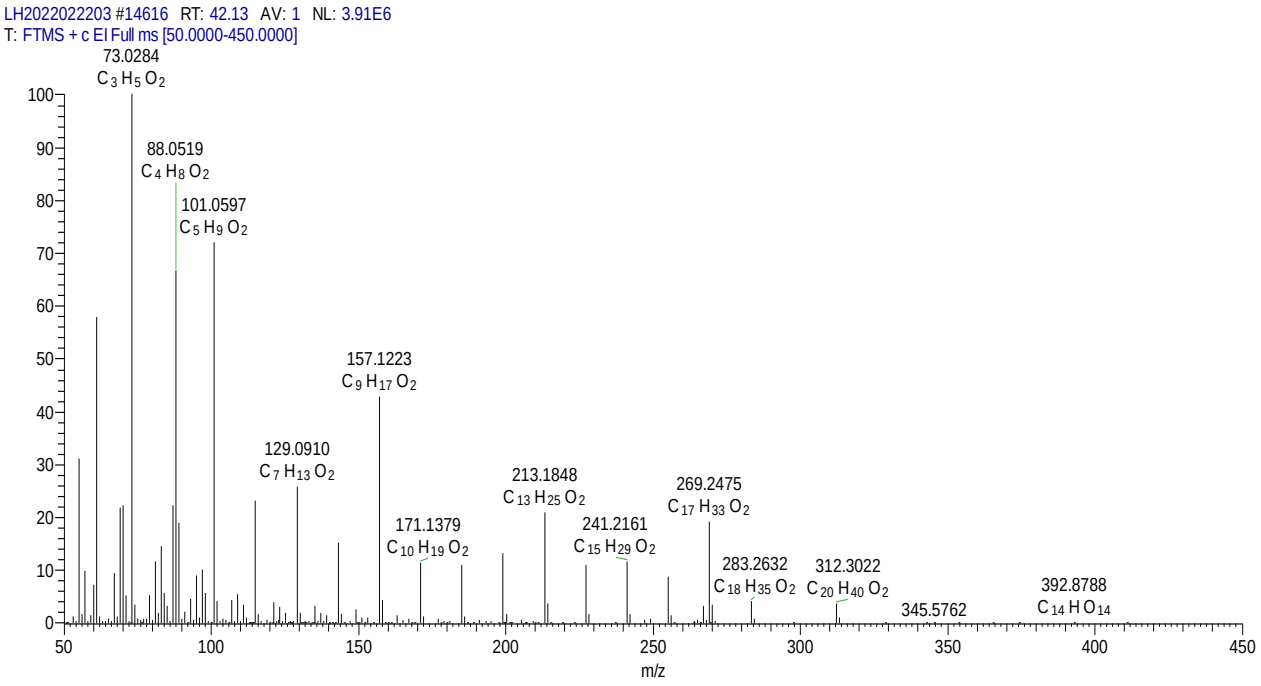
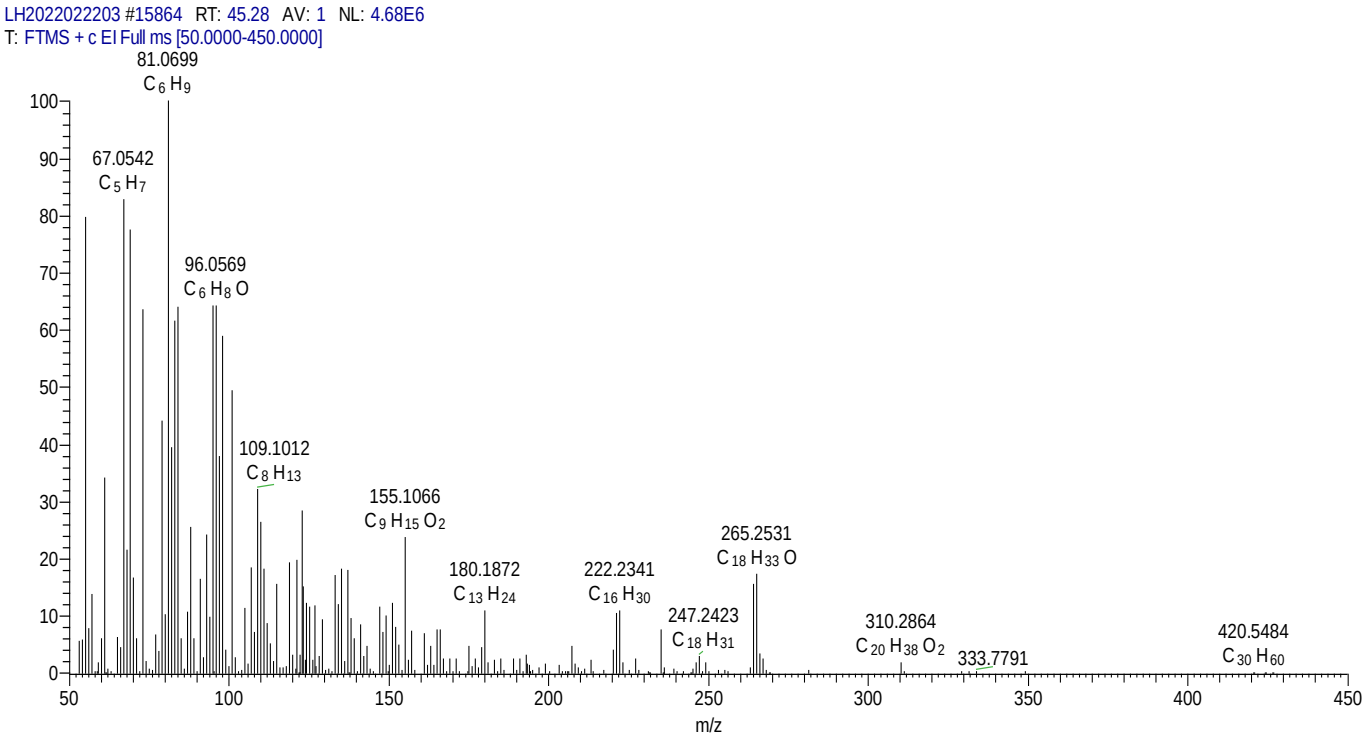


C18:0 MW: 312.52 $C_{20}H_{40}O_2$



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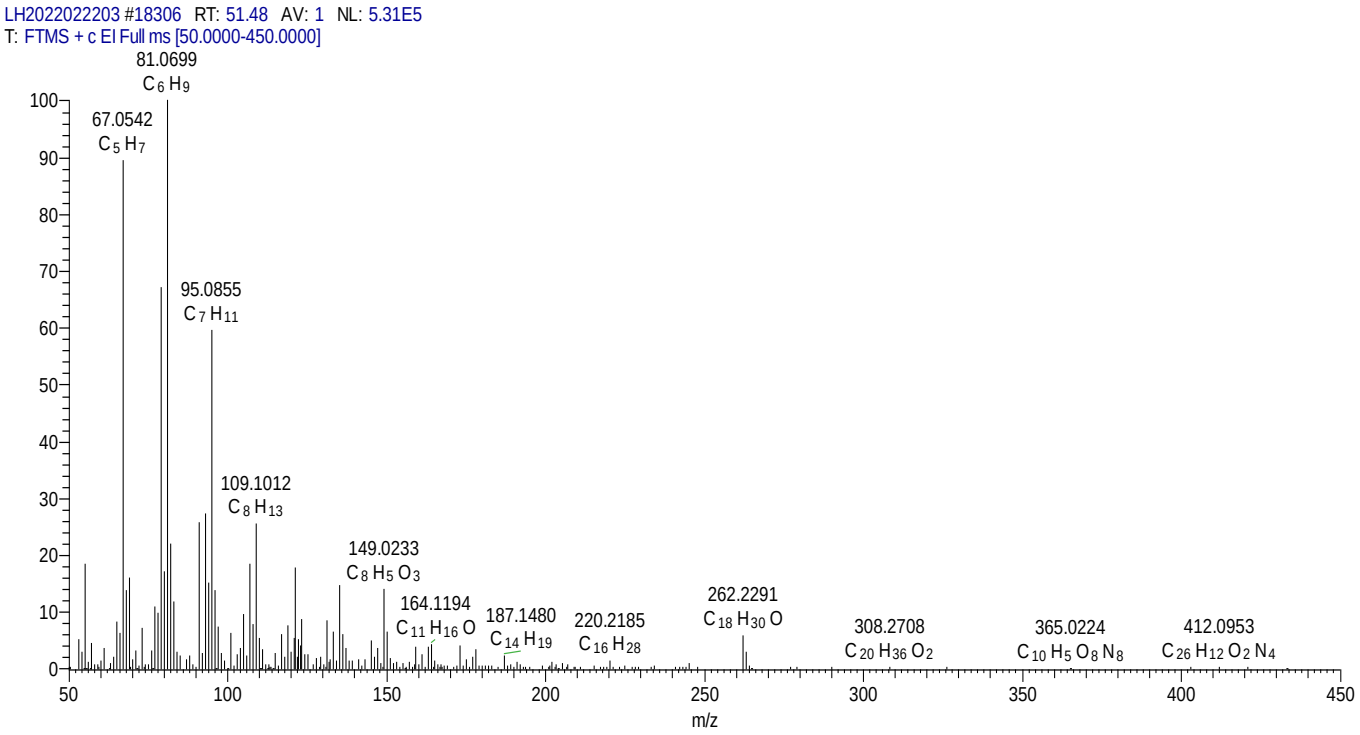

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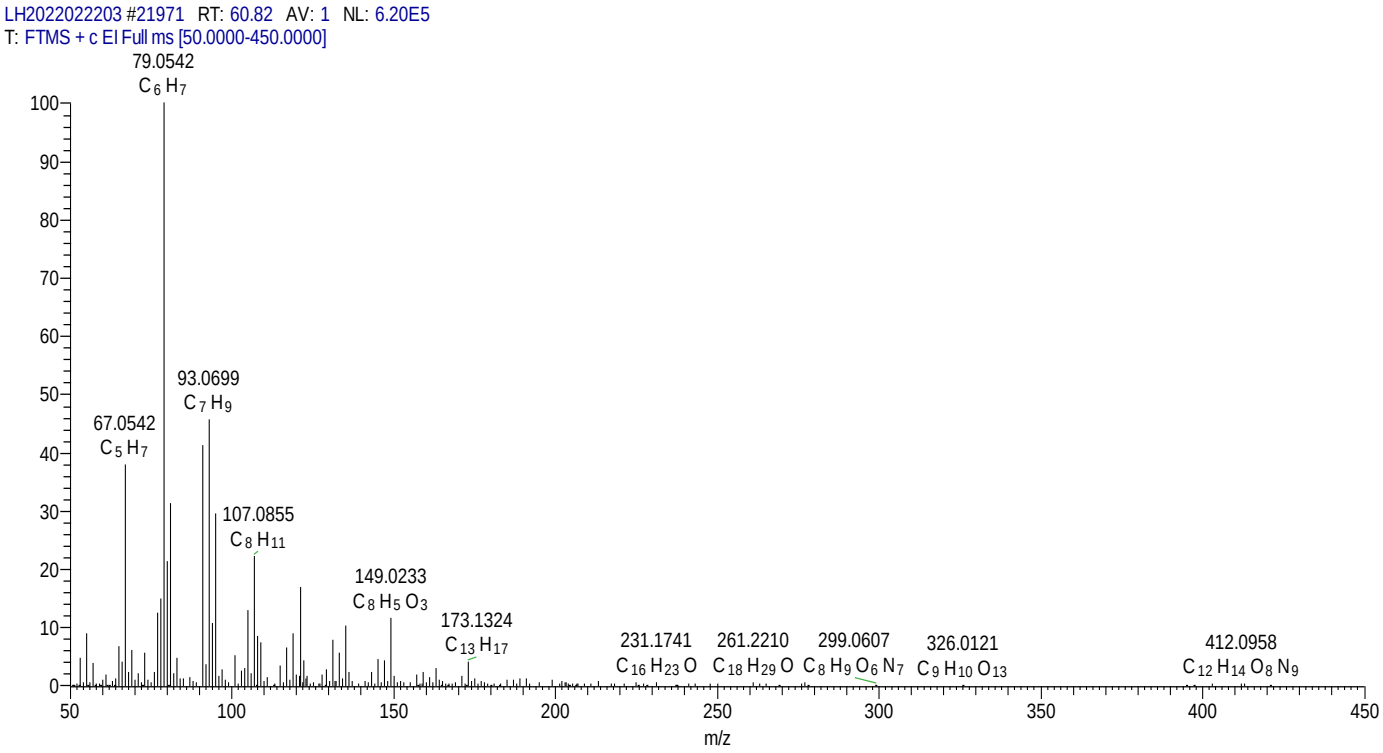

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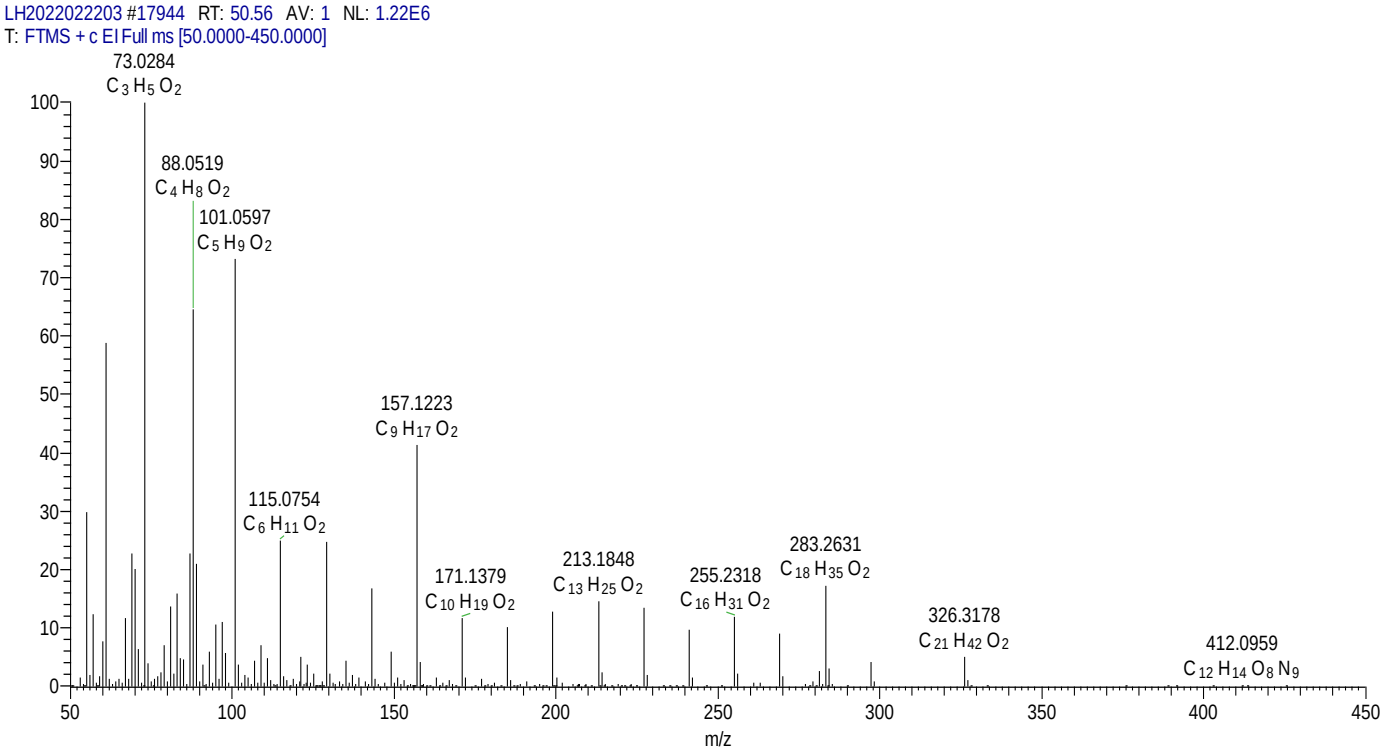
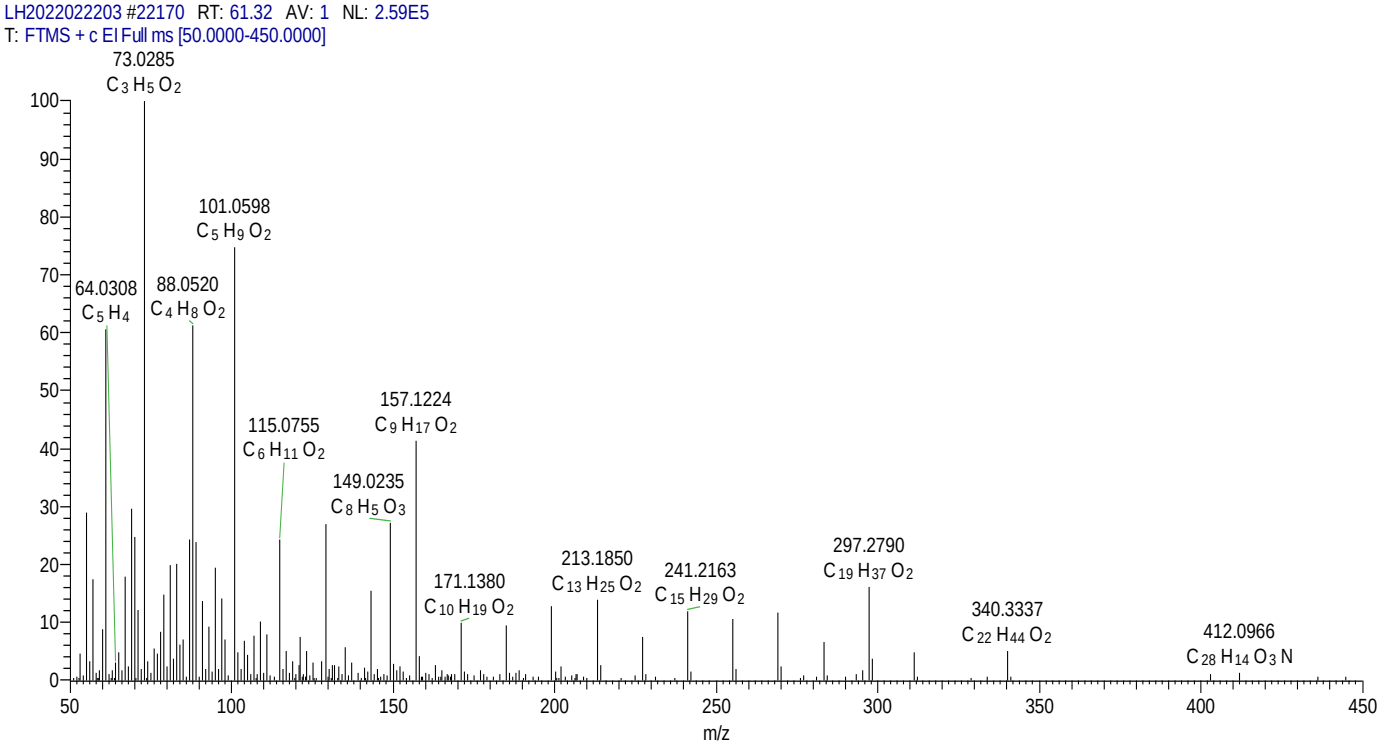
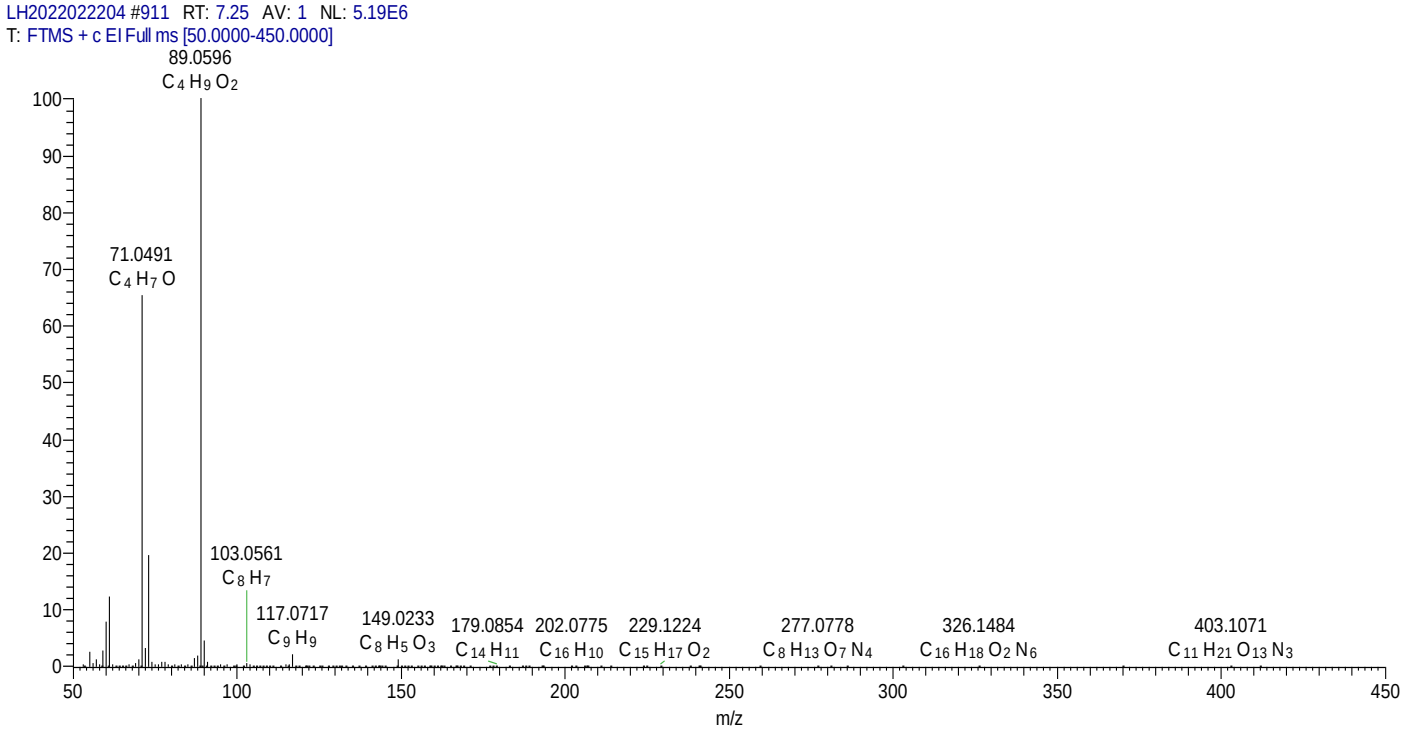


C19:0 MW: 326.546 $C_{21}H_{42}O_2$

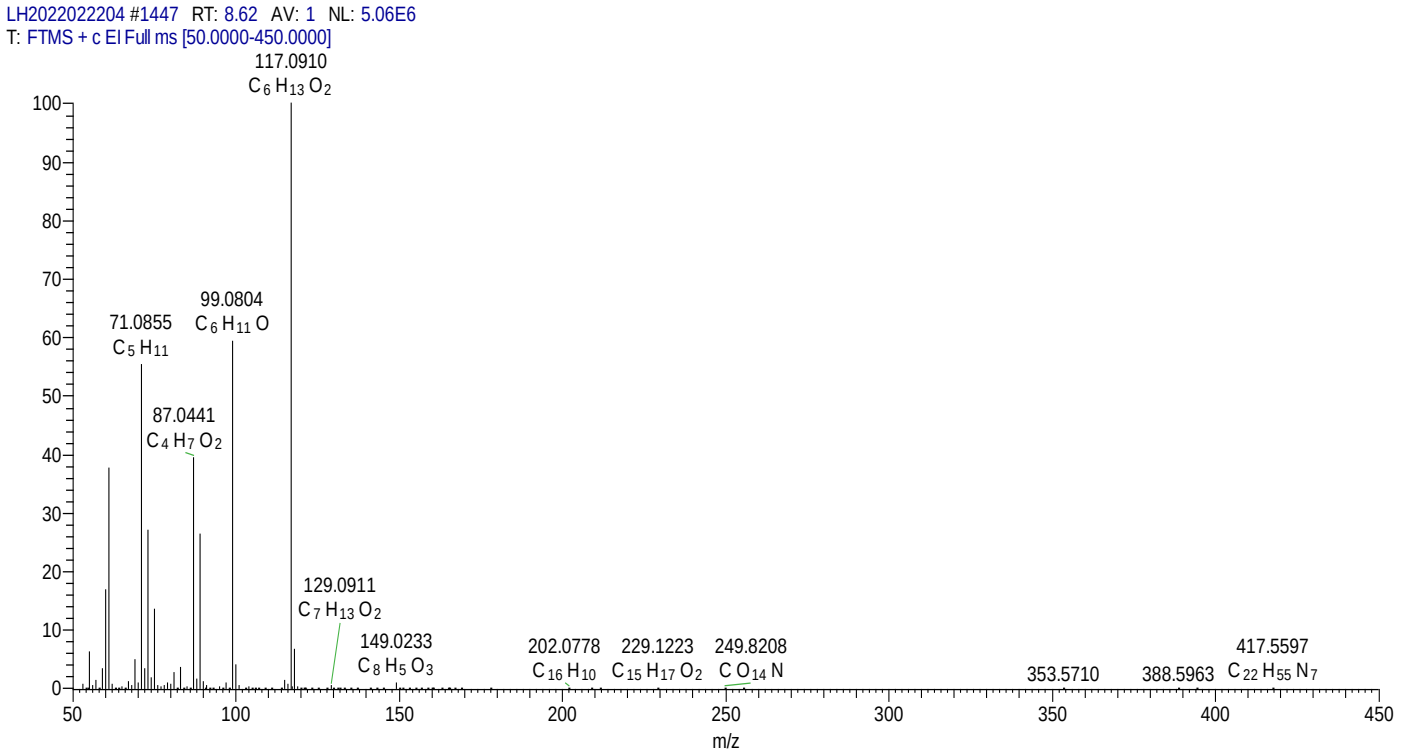


C20:0 MW: 340.572 $C_{22}H_{44}O_2$

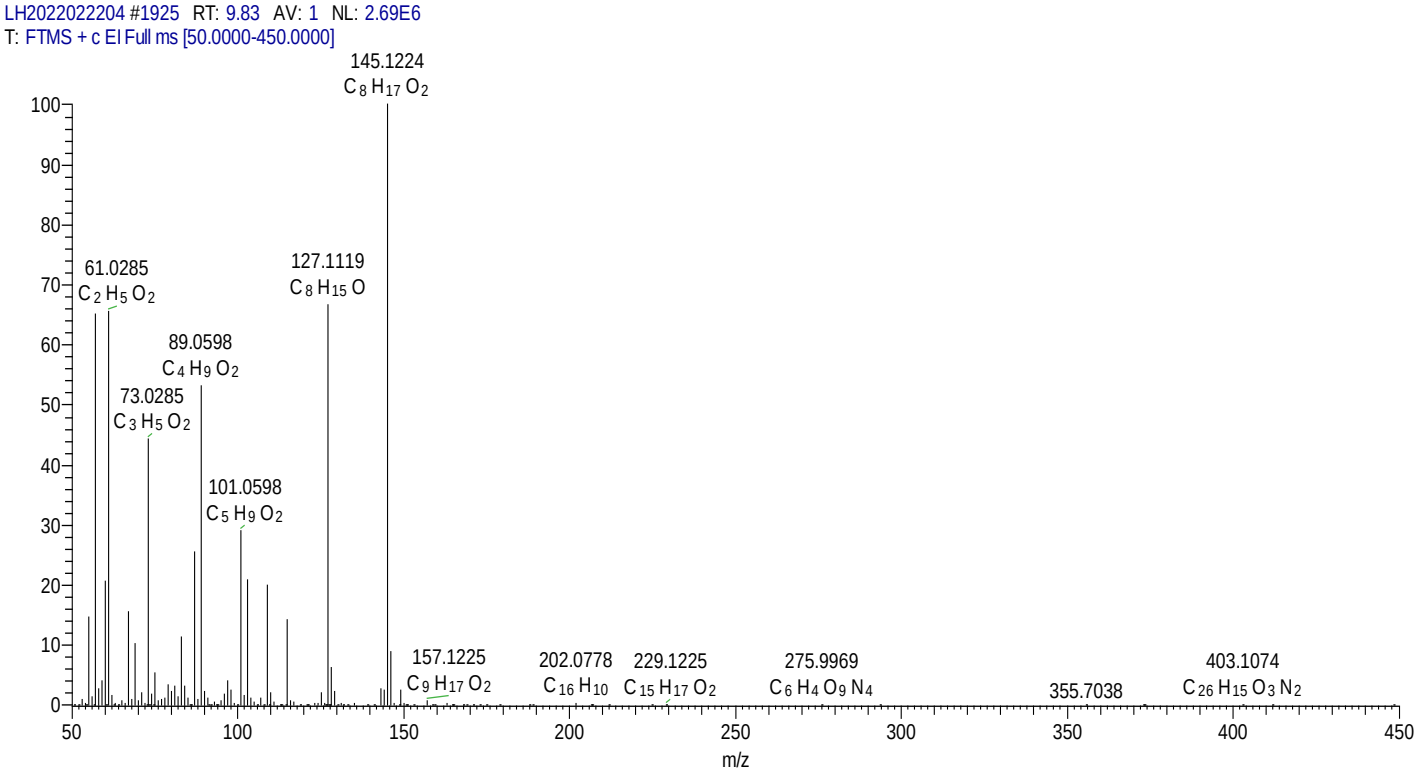
Appendix 3: MS spectra for all FAPE



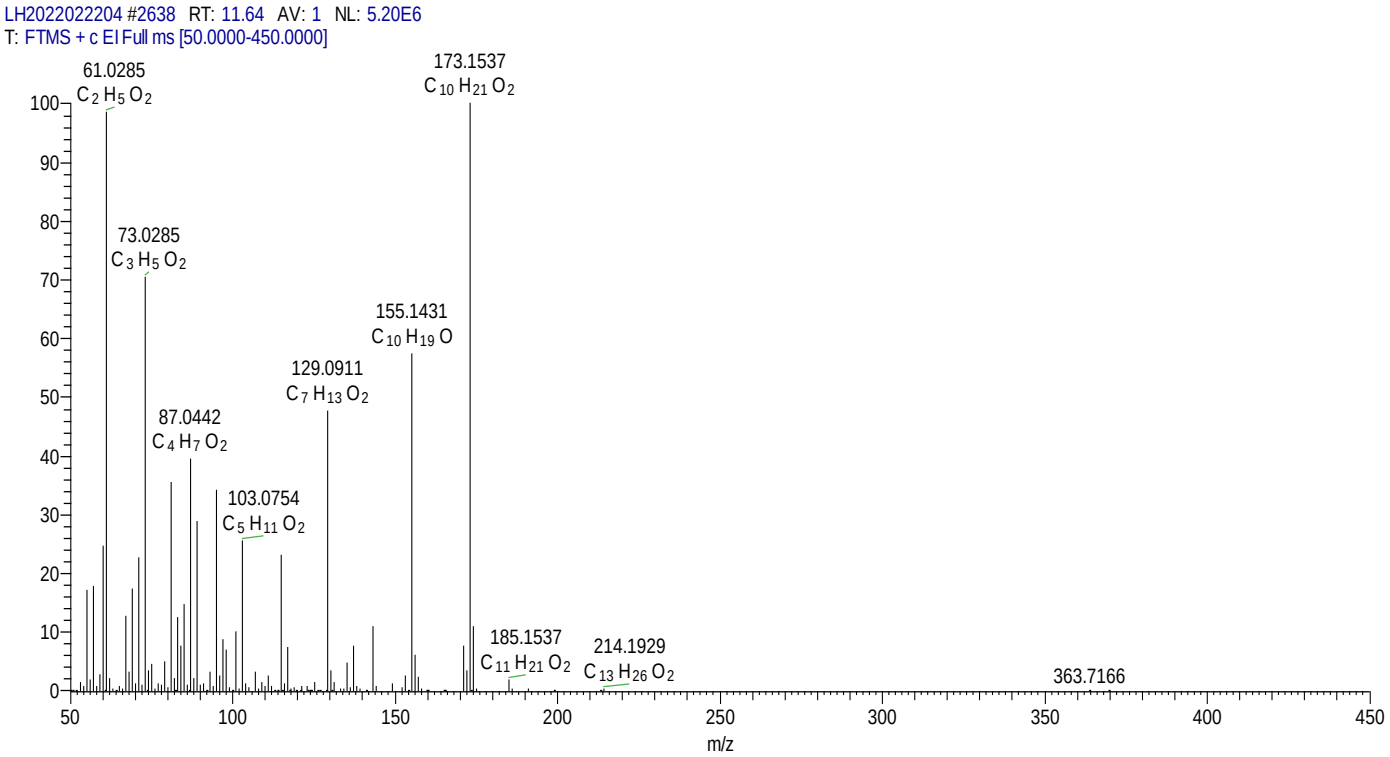
C4:0 MW: 130.182 $C_7H_{14}O_2$



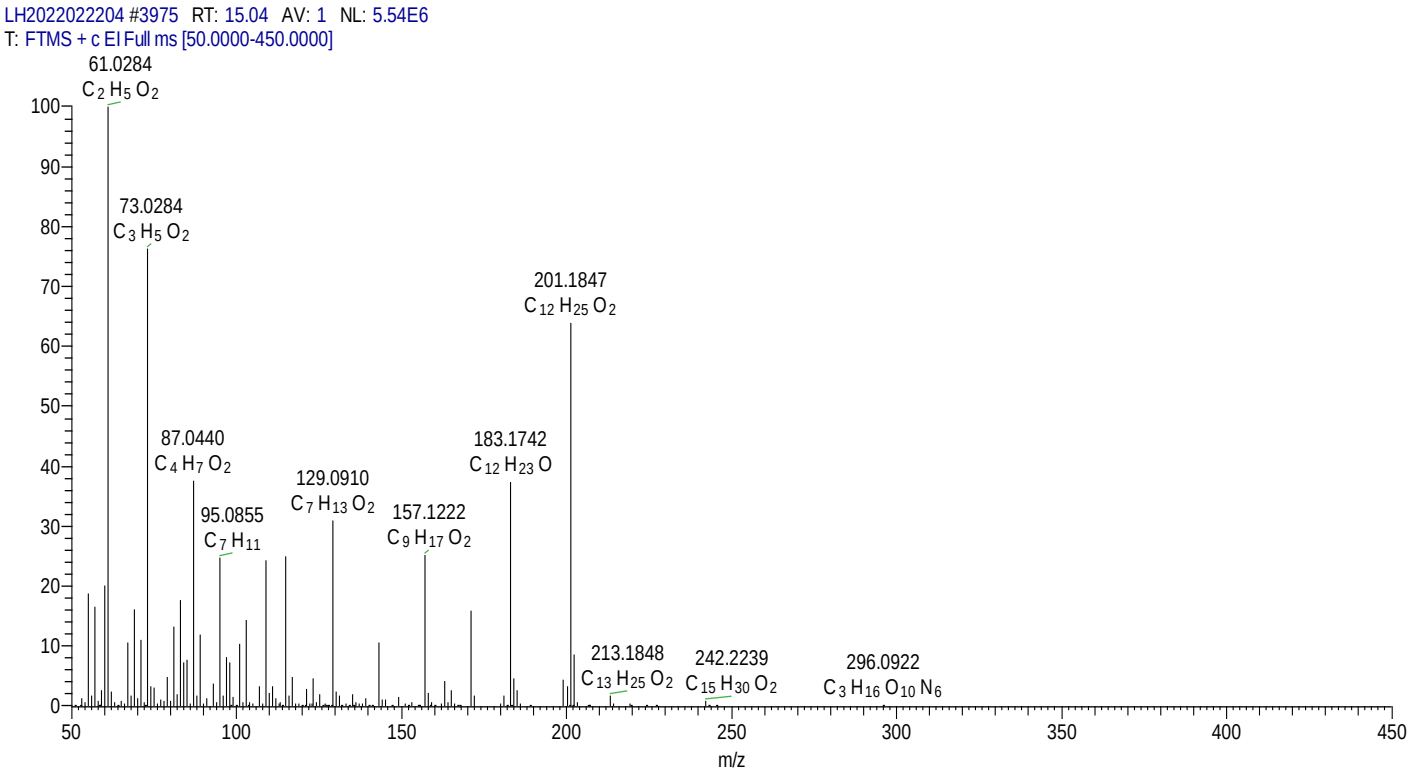
C6:0 MW: 158.234 $C_9H_{18}O_2$



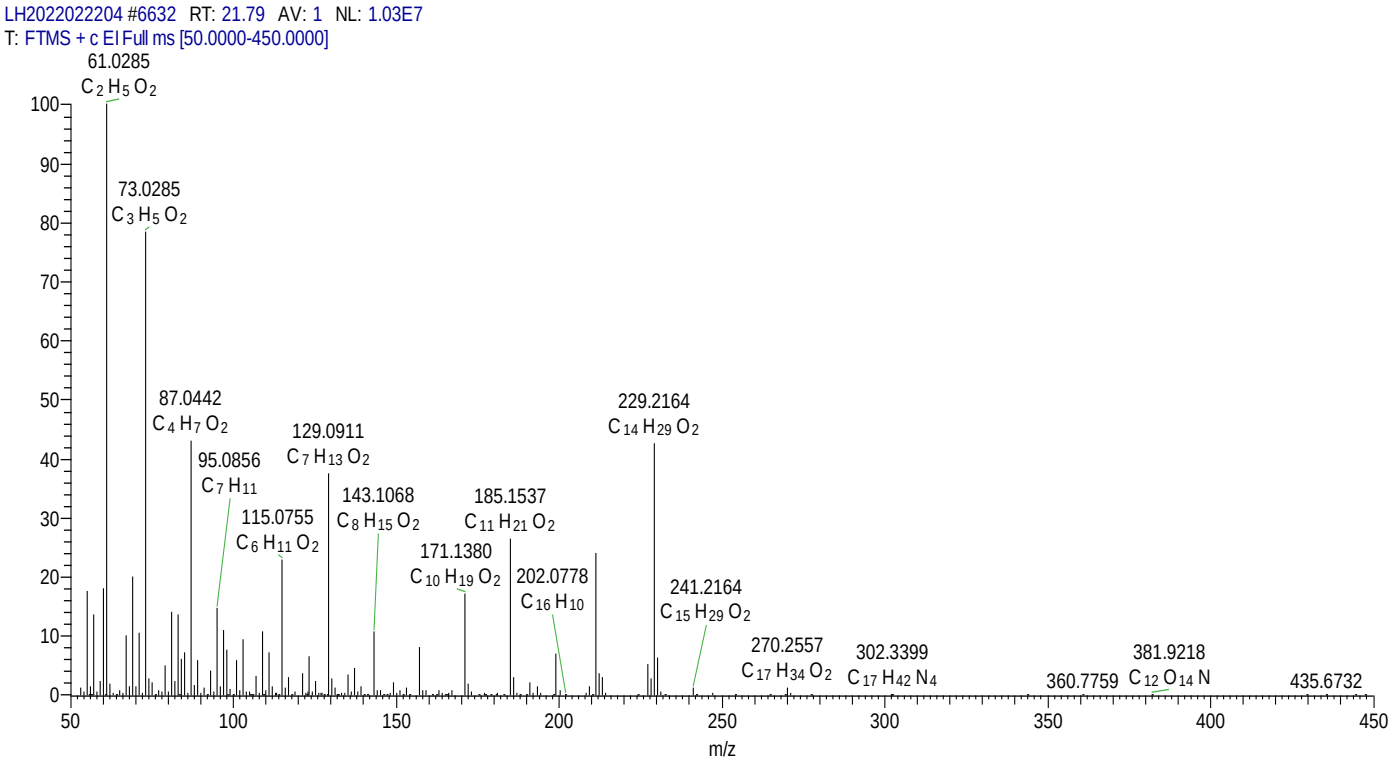
C8:0 MW: 186.286 $C_{11}H_{22}O_2$



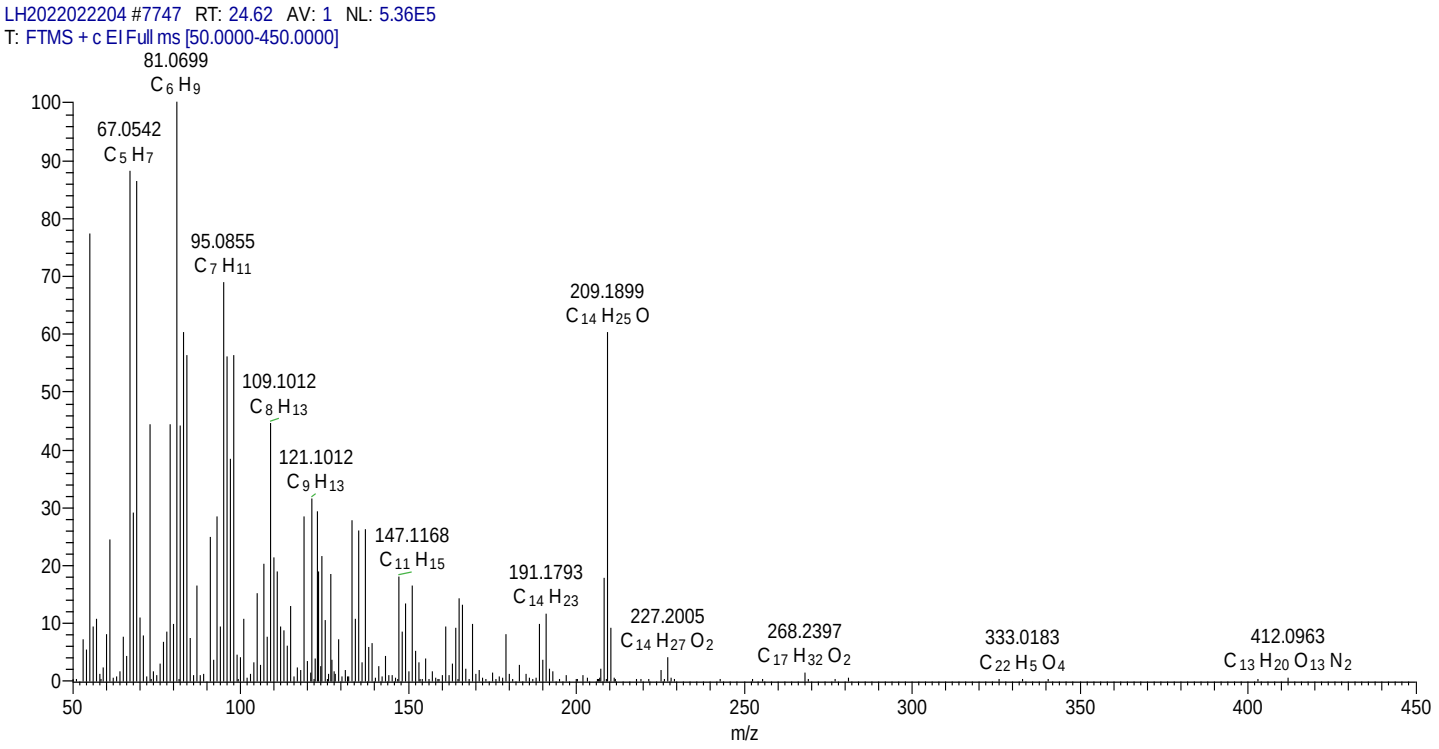
C10:0 MW: 214.338 $C_{13}H_{26}O_2$



C12:0 MW: 242.39 $C_{15}H_{30}O_2$



C14:0 MW: 270.442 $C_{17}H_{34}O_2$



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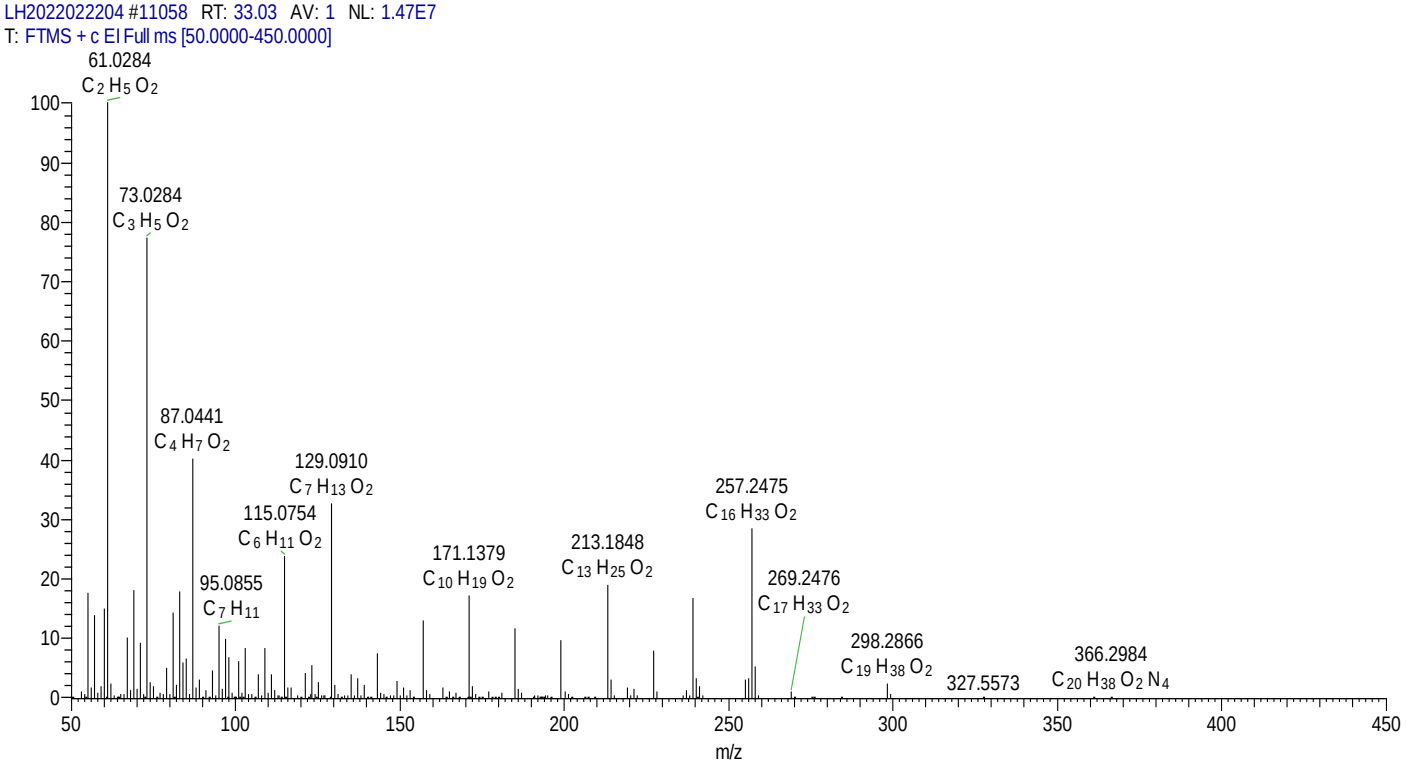
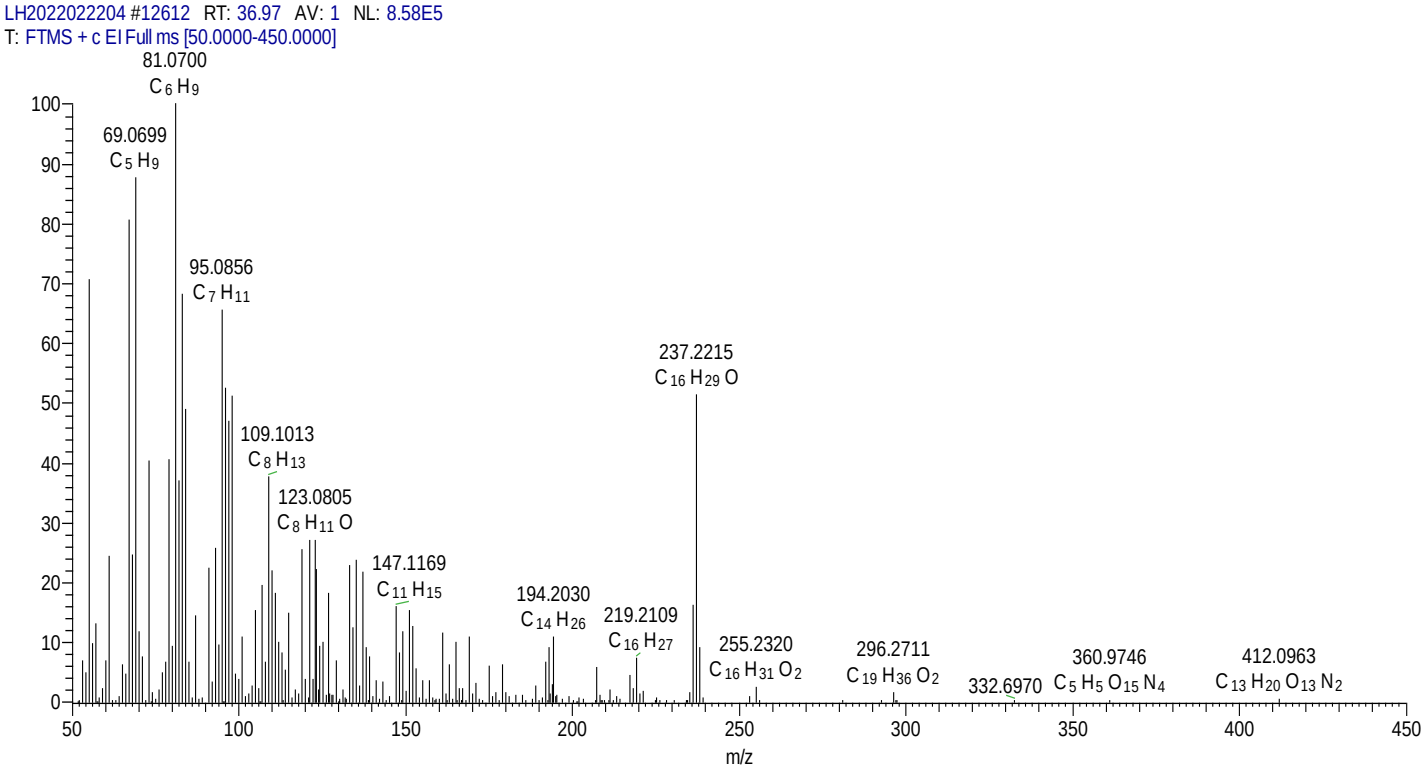


C16:0 MW: 298.494 $C_{19}H_{38}O_2$



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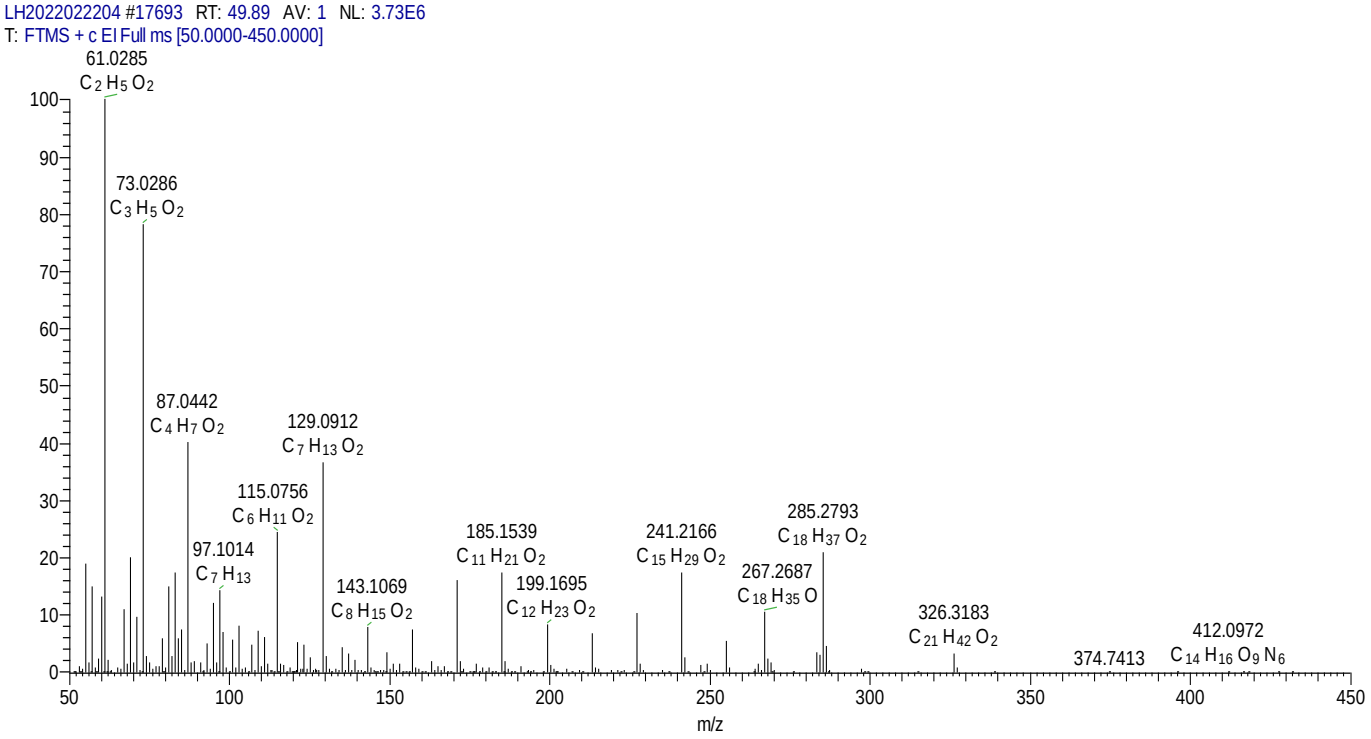
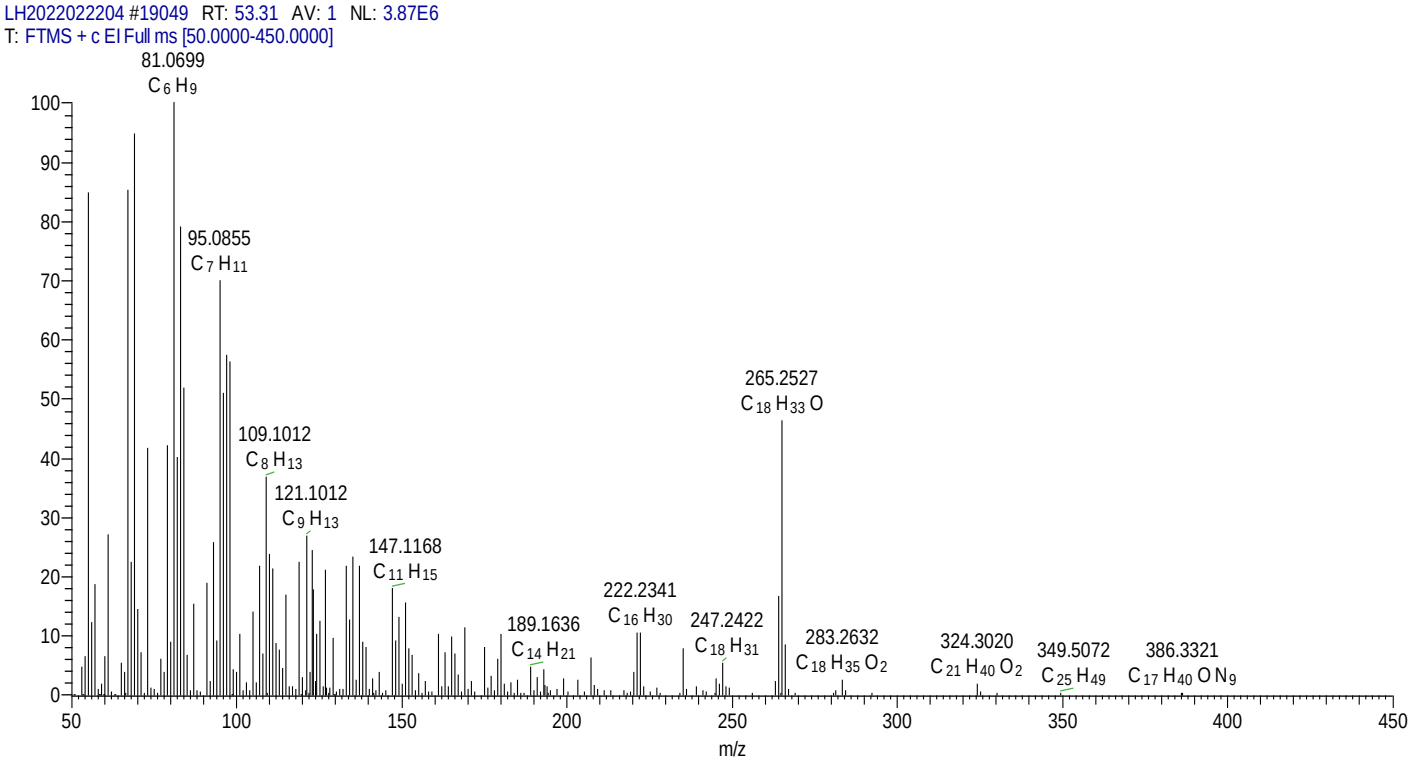
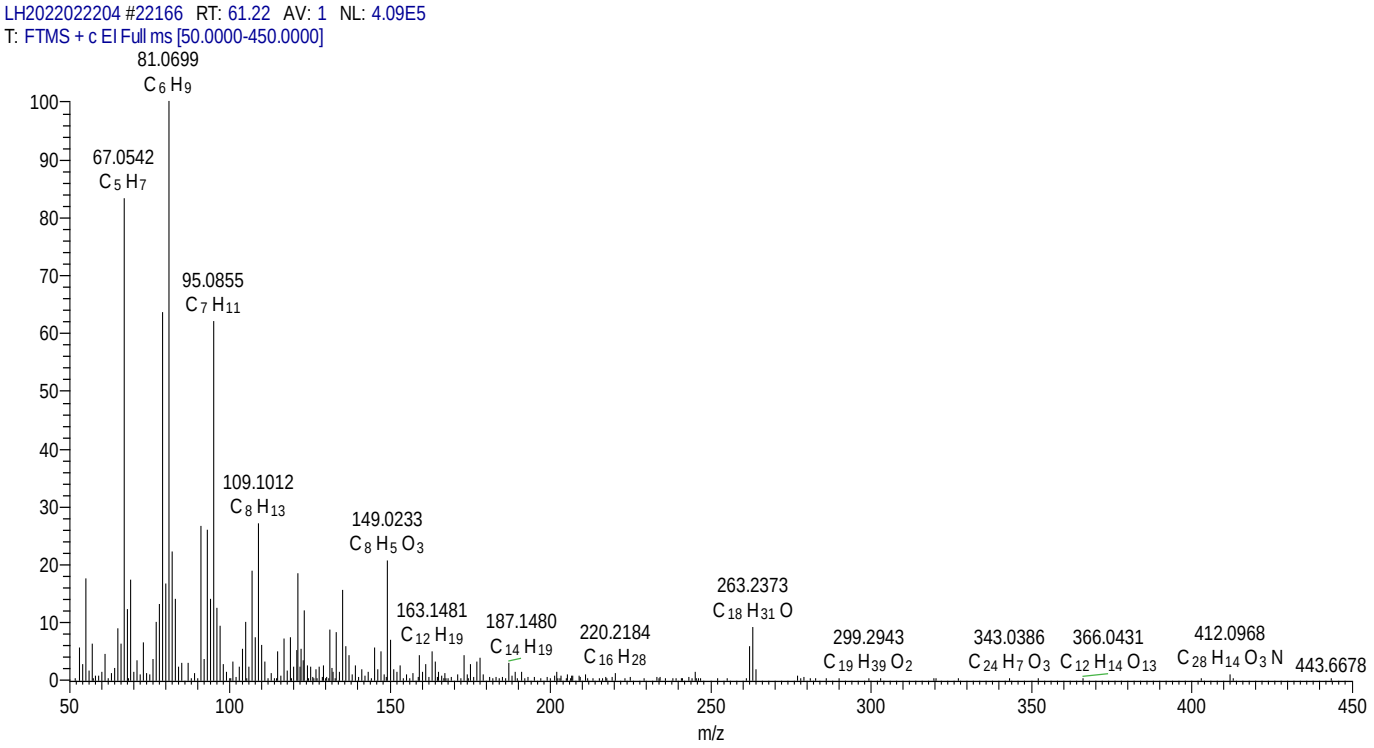


C18:0 MW: 326.546 $C_{21}H_{42}O_2$



C18:1 MW: 324.53 $C_{21}H_{40}O_2$



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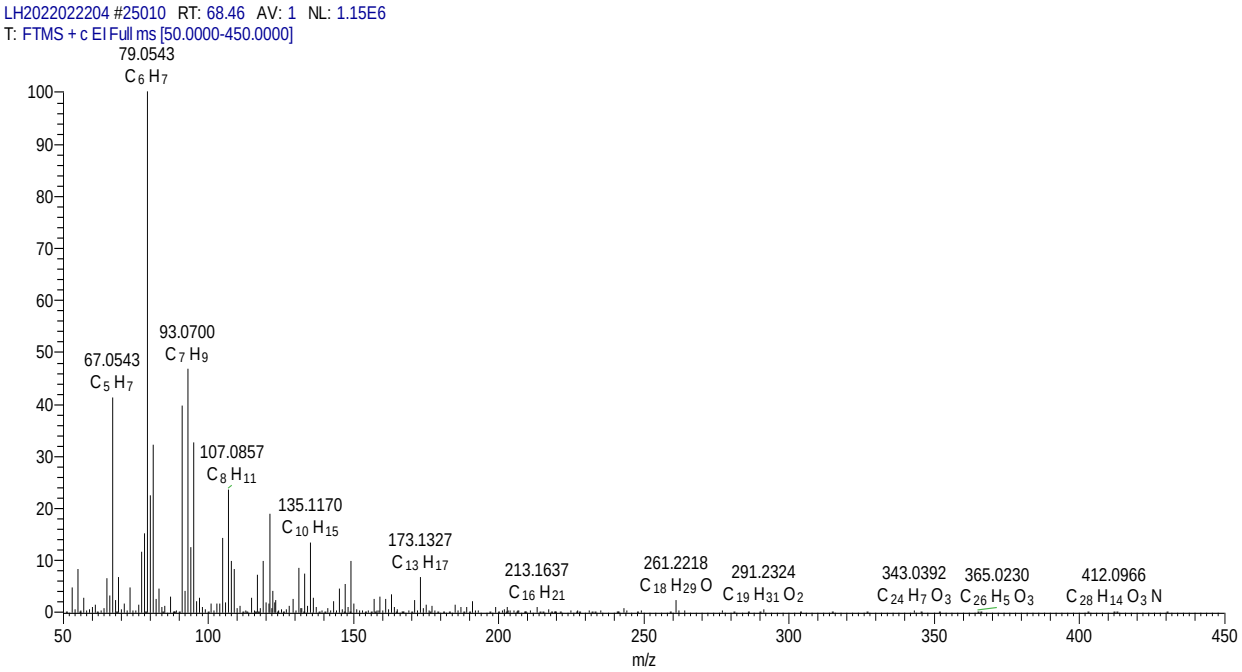

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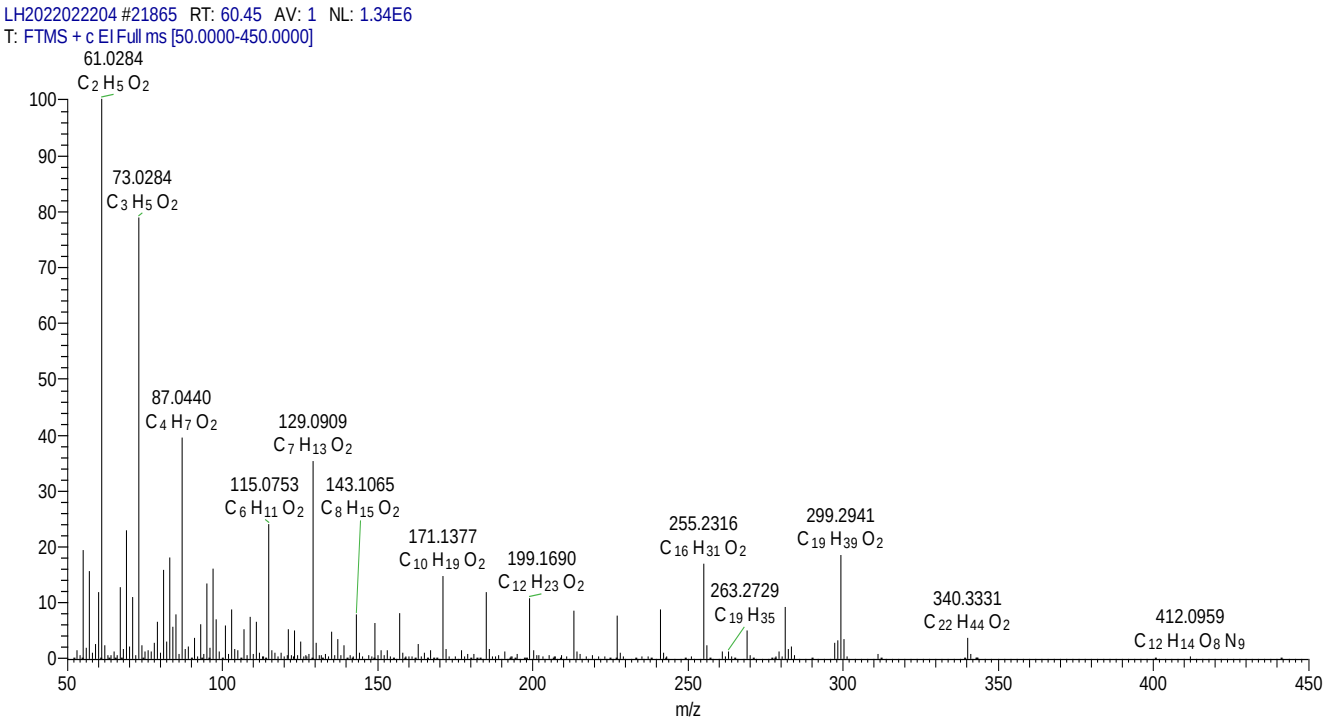
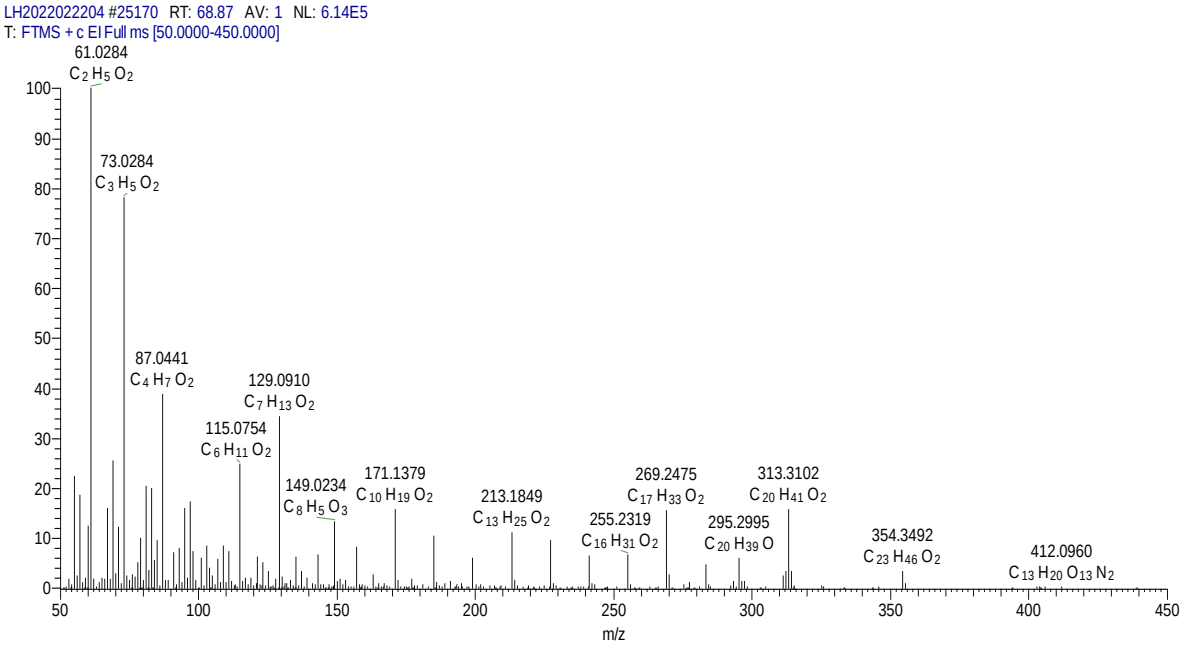
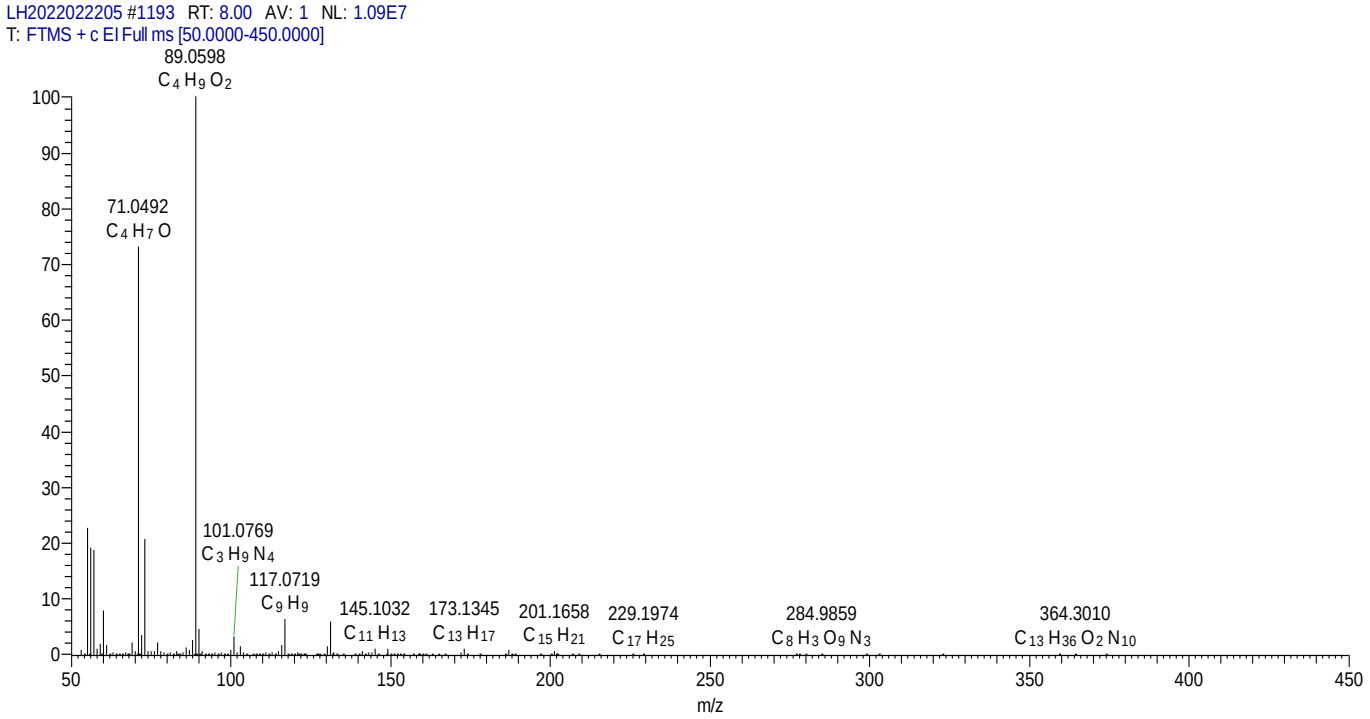


C19:0 MW: 340.572 $C_{22}H_{44}O_2$

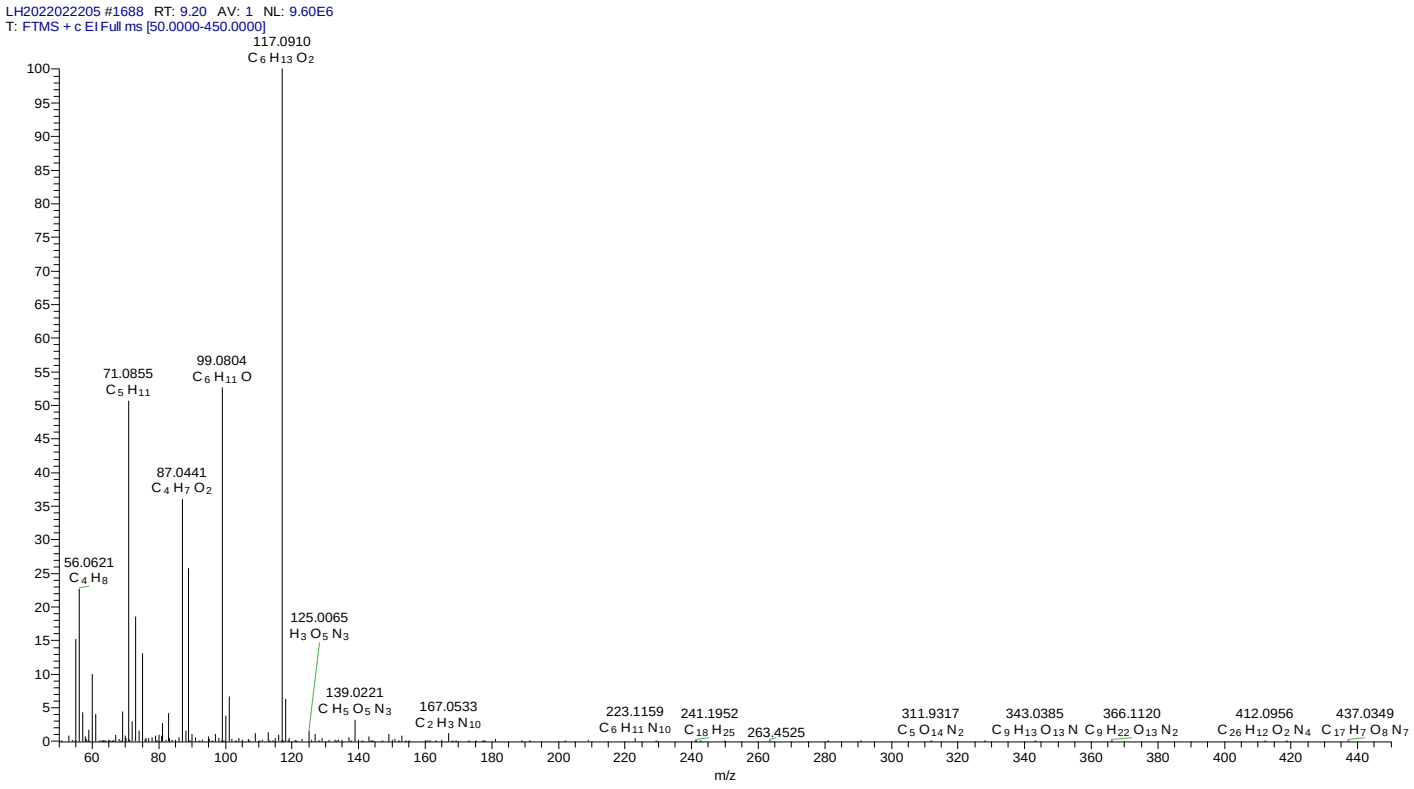


C20:0 MW: 354.598 $C_{23}H_{46}O_2$

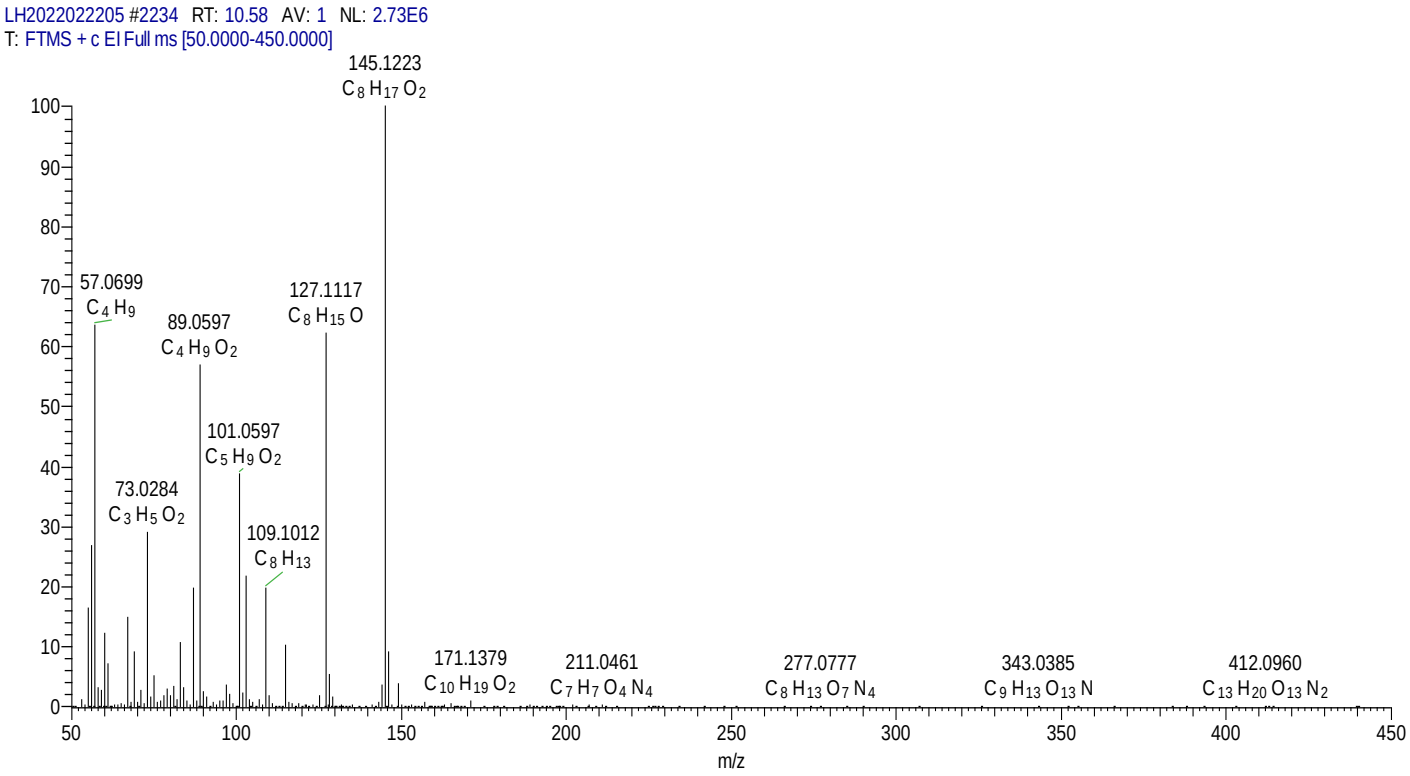
Appendix 4: MS spectra for all FABE



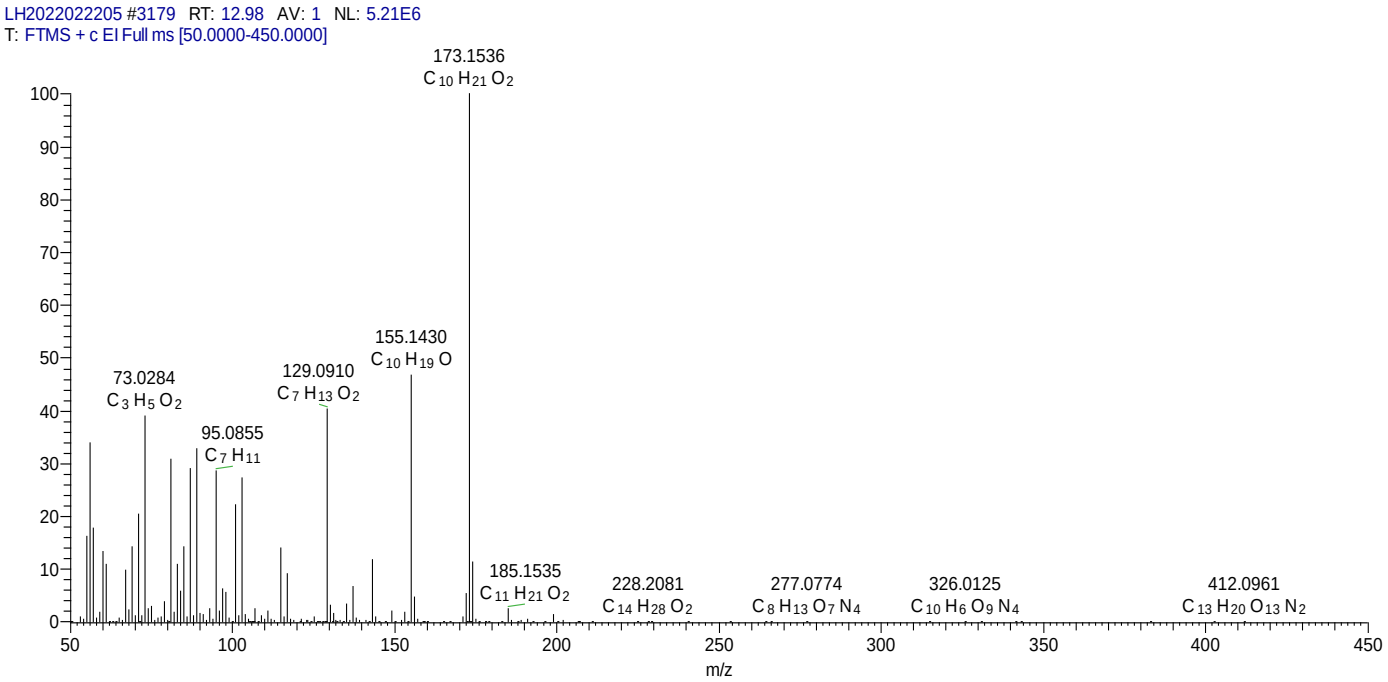
C4:0 MW: 144.206 $C_8H_{16}O_2$



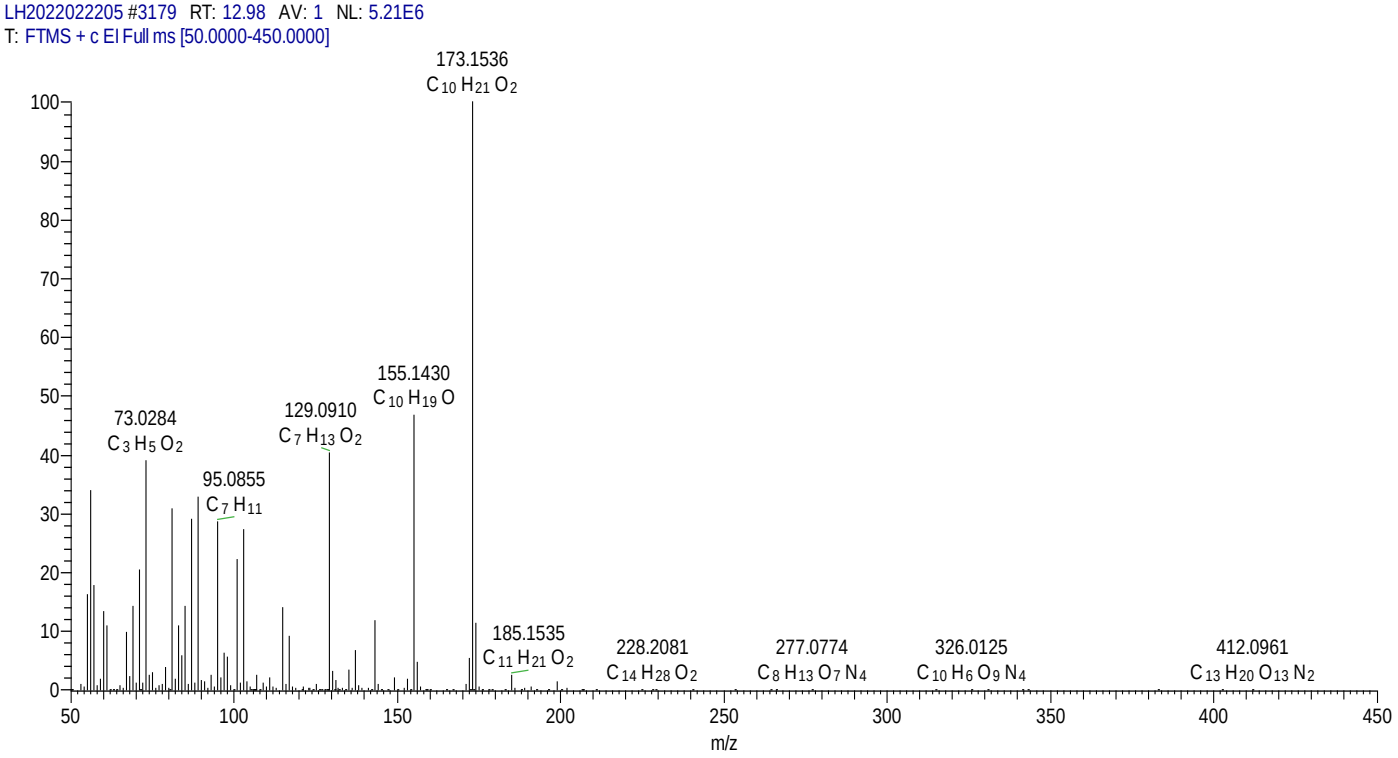
C6:0 MW: 172.26 $C_{10}H_{20}O_2$



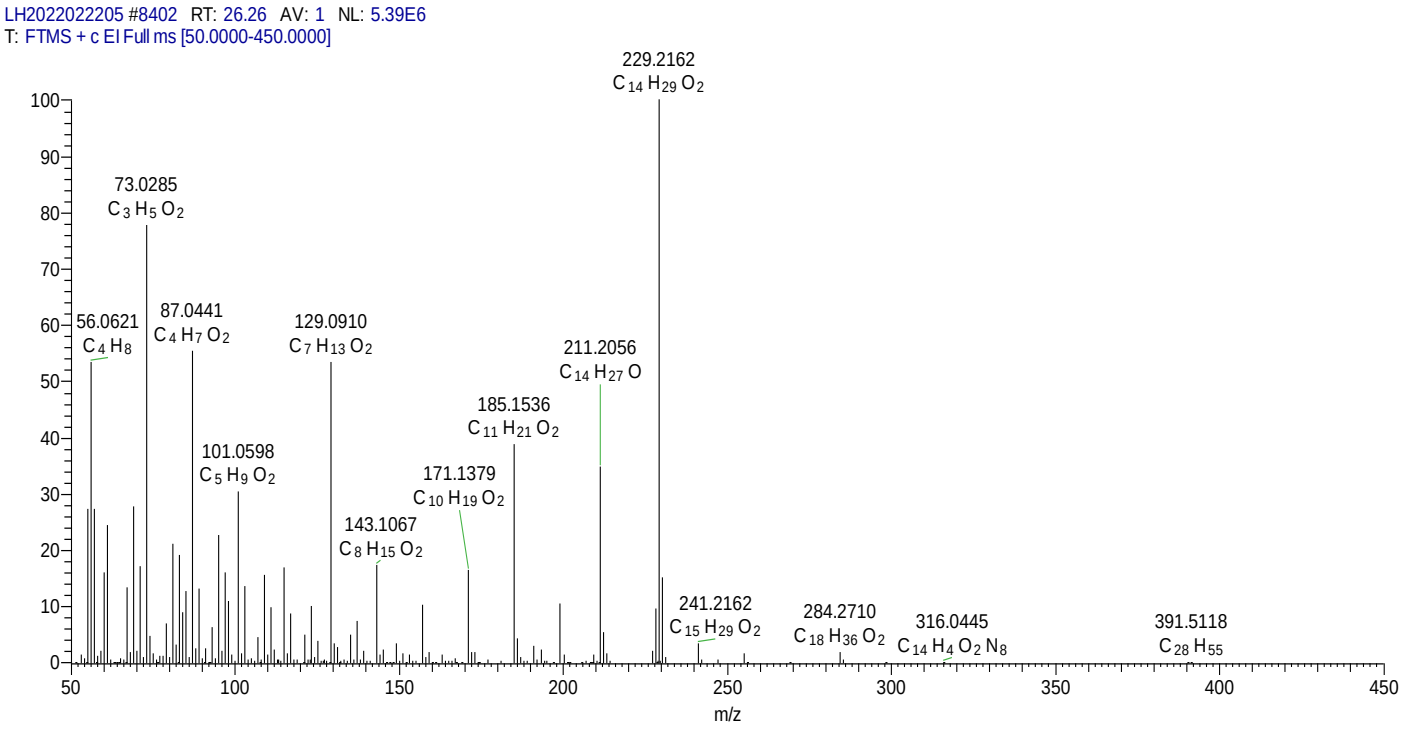
C8:0 MW: 200.312 $C_{12}H_{24}O_2$



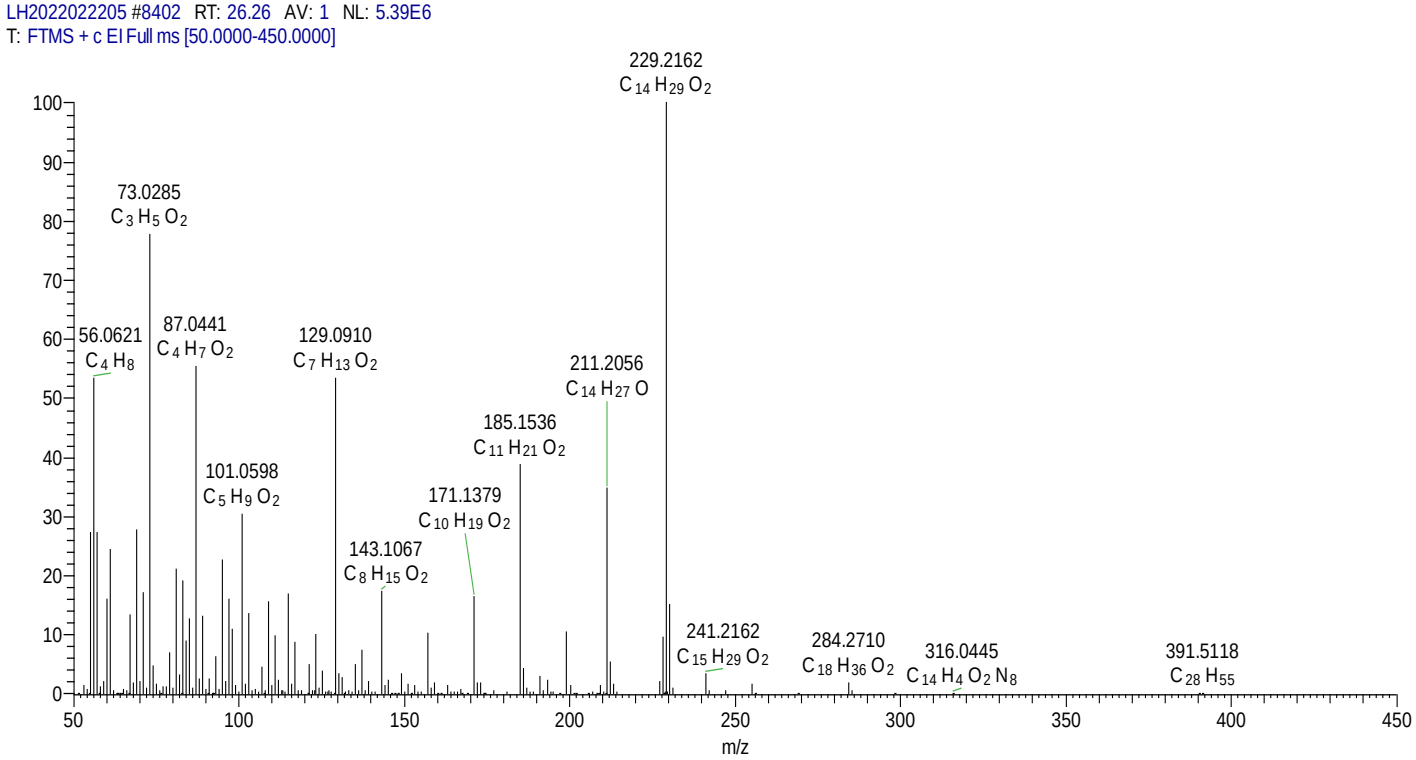
C10:0 MW: 228.364 $C_{14}H_{28}O_2$



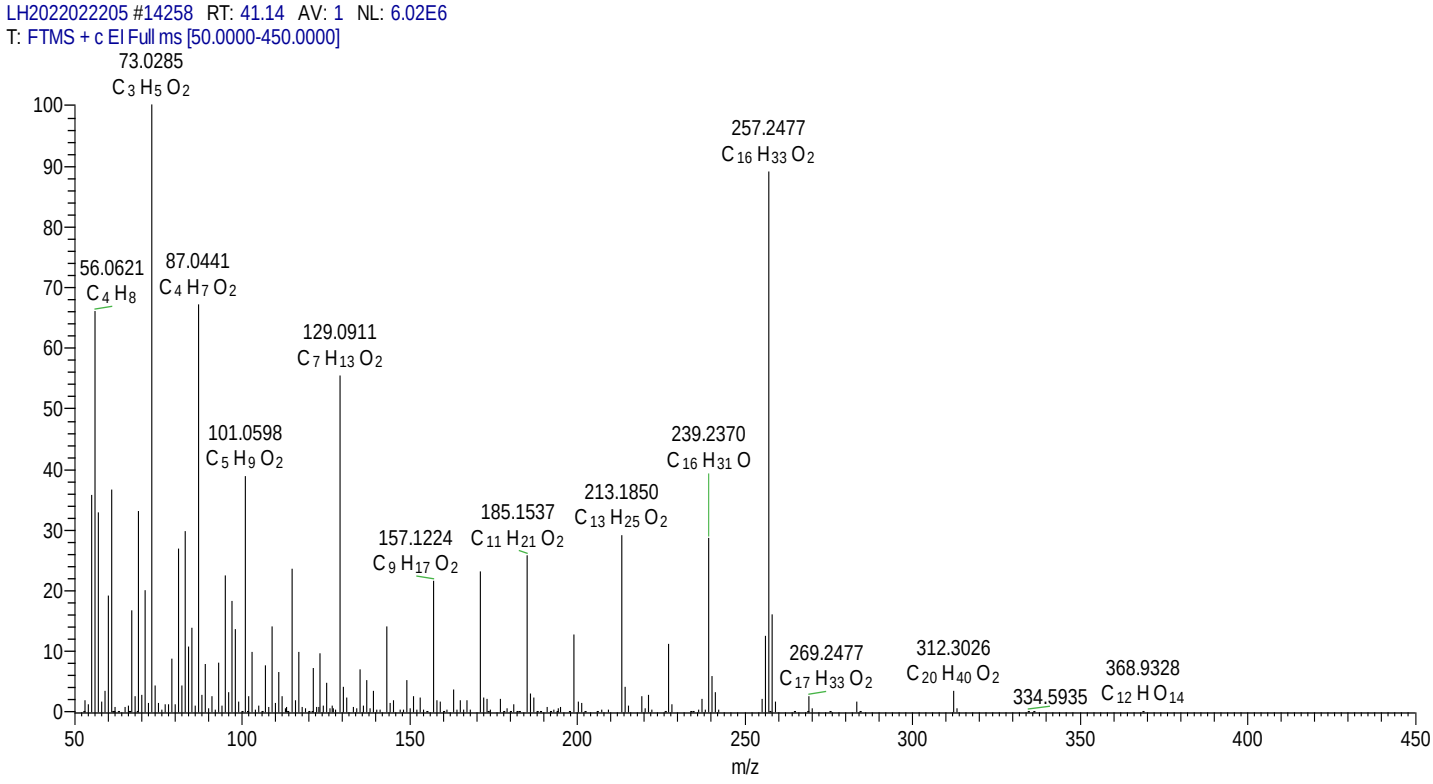
C12:0 MW: 256.416 $C_{16}H_{32}O_2$



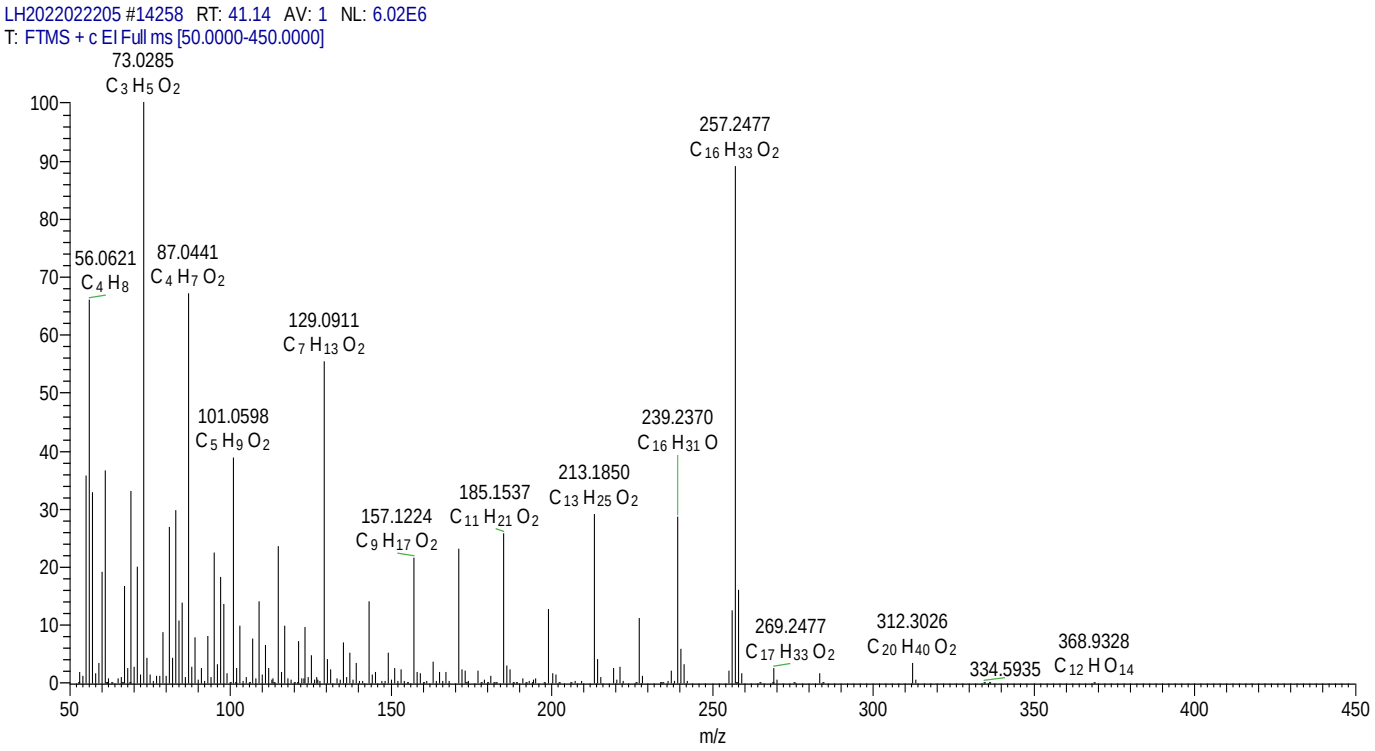
C14:0 MW: 284.468 $C_{18}H_{36}O_2$



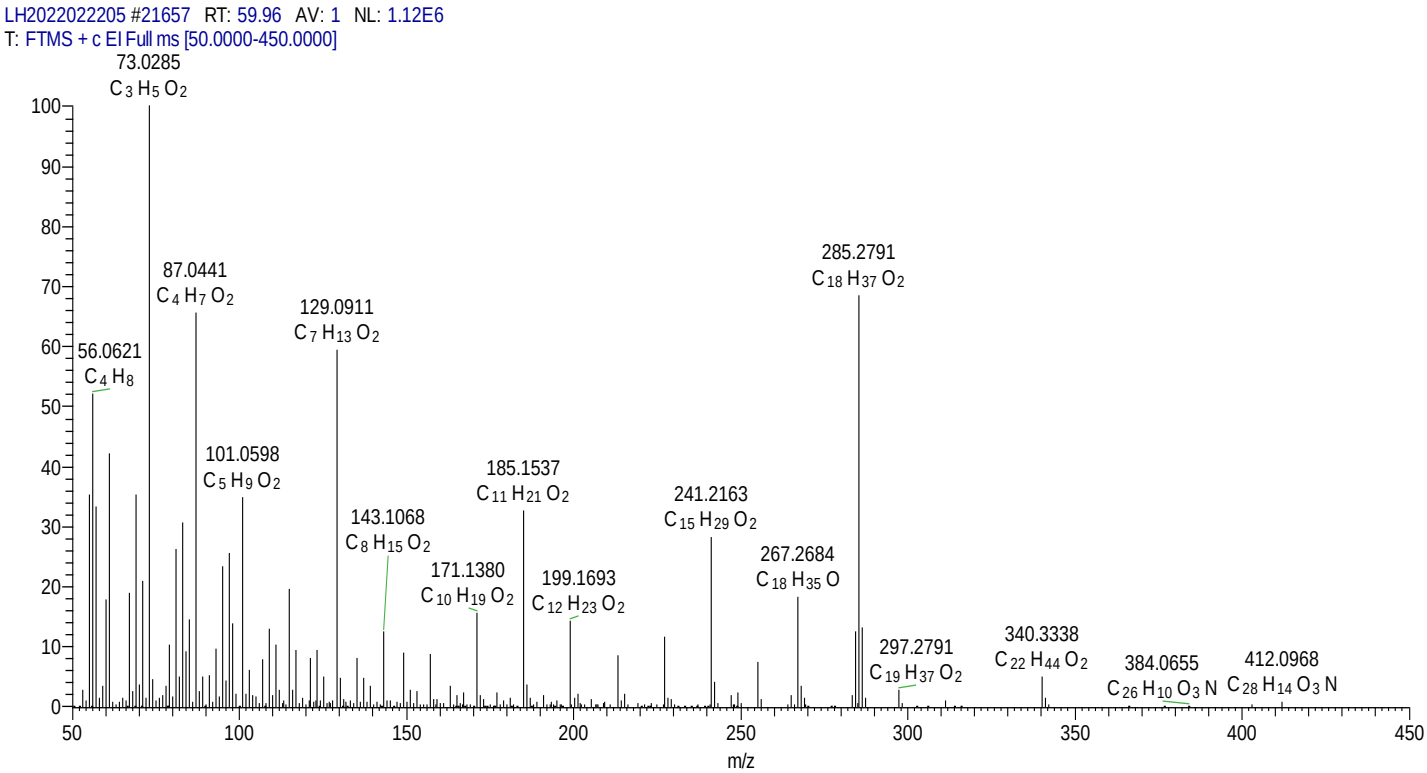
C14:1 MW: 282.452 $C_{18}H_{34}O_2$



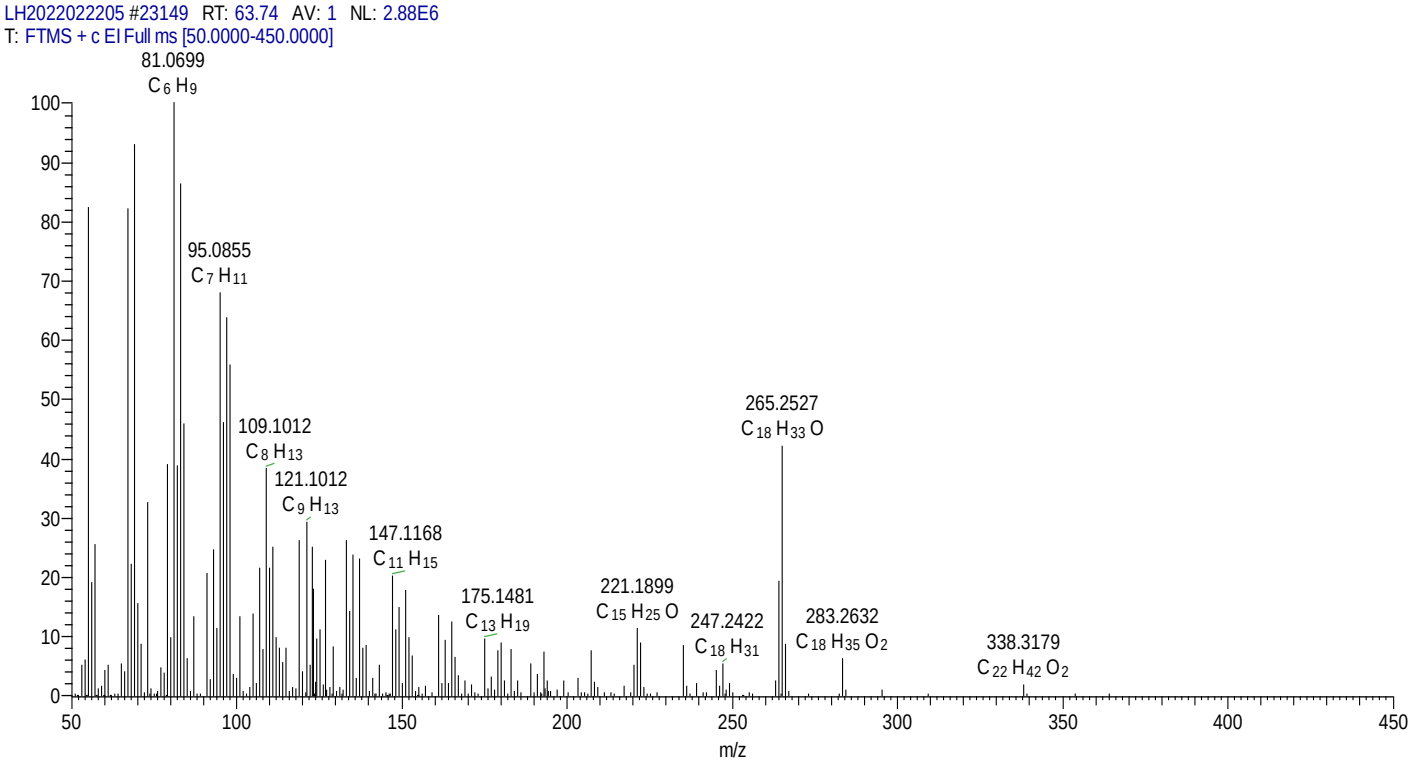
C16:0 MW: 312.52 $C_{20}H_{40}O_2$



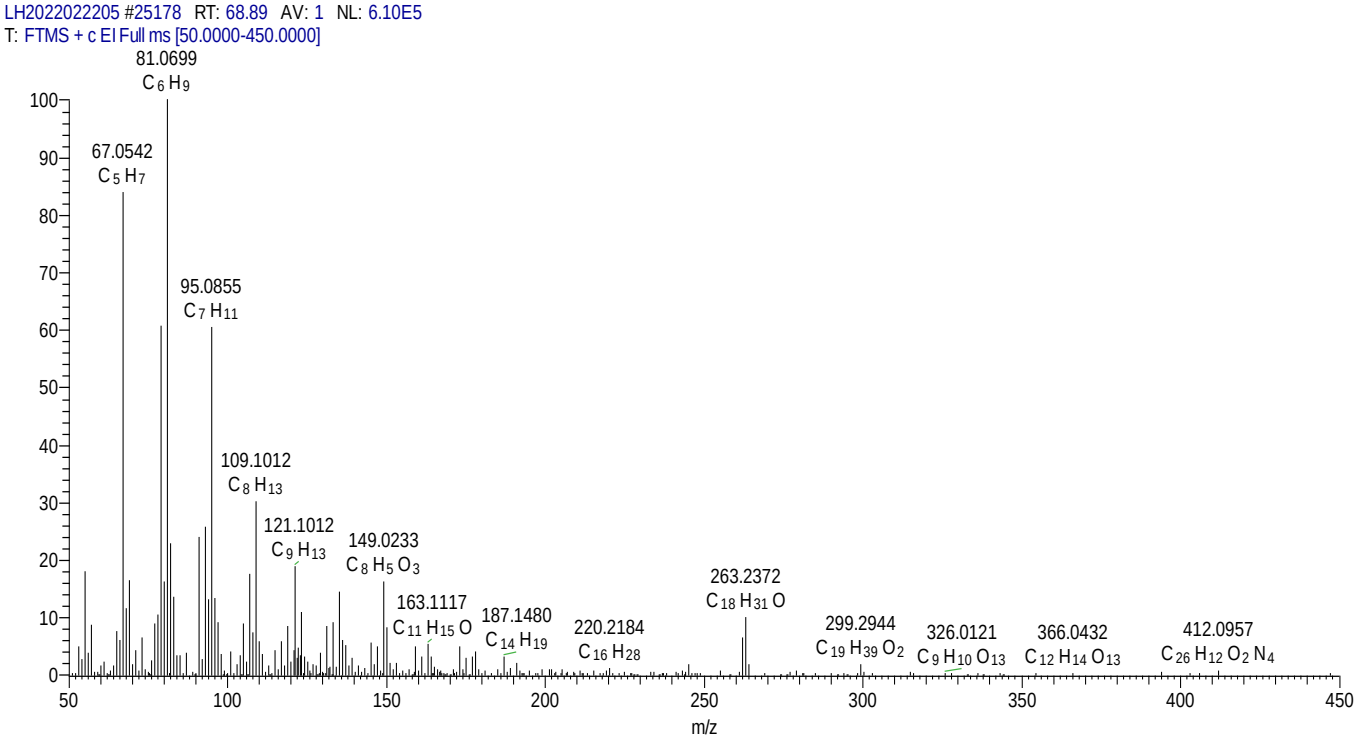
C16:1 MW: 310.504 $C_{20}H_{38}O_2$



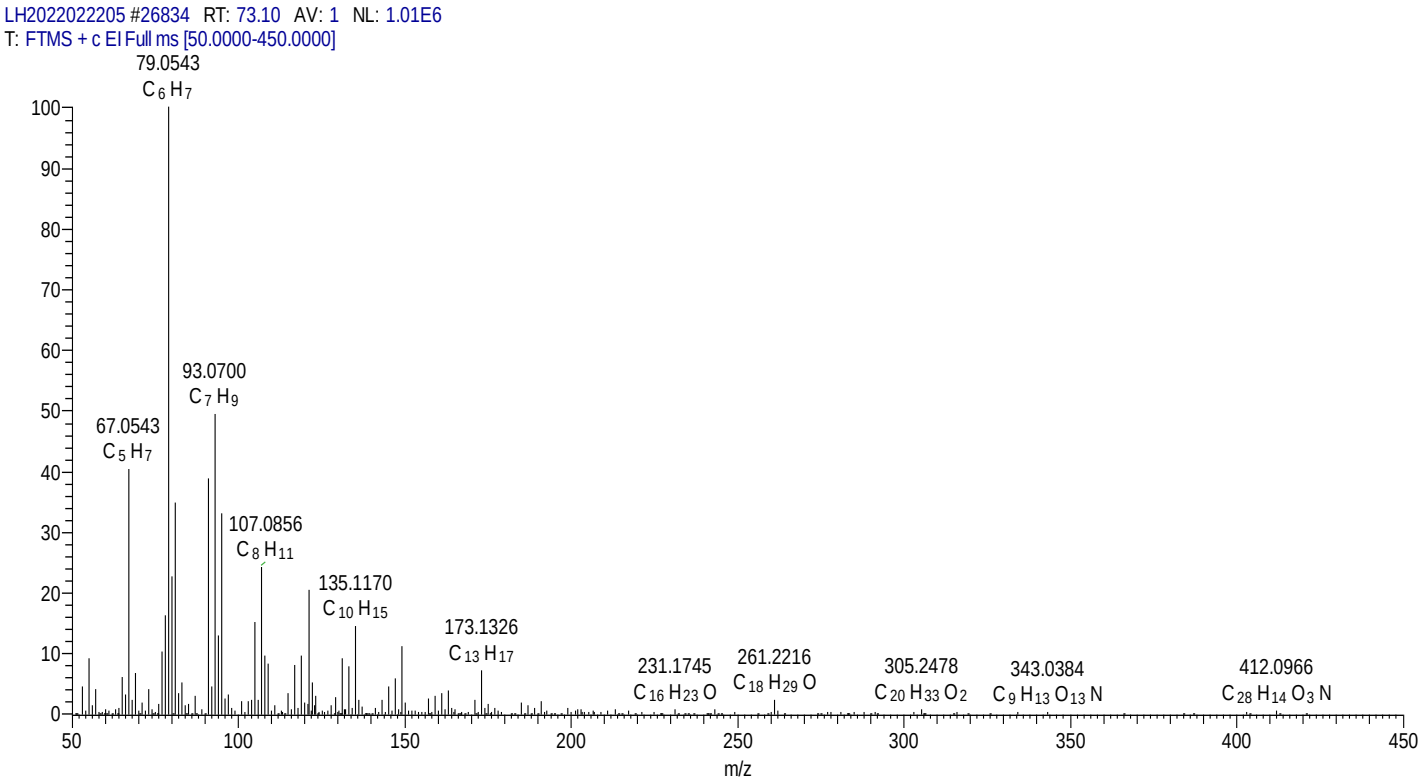
C18:0 MW: 340.572 $C_{22}H_{44}O_2$



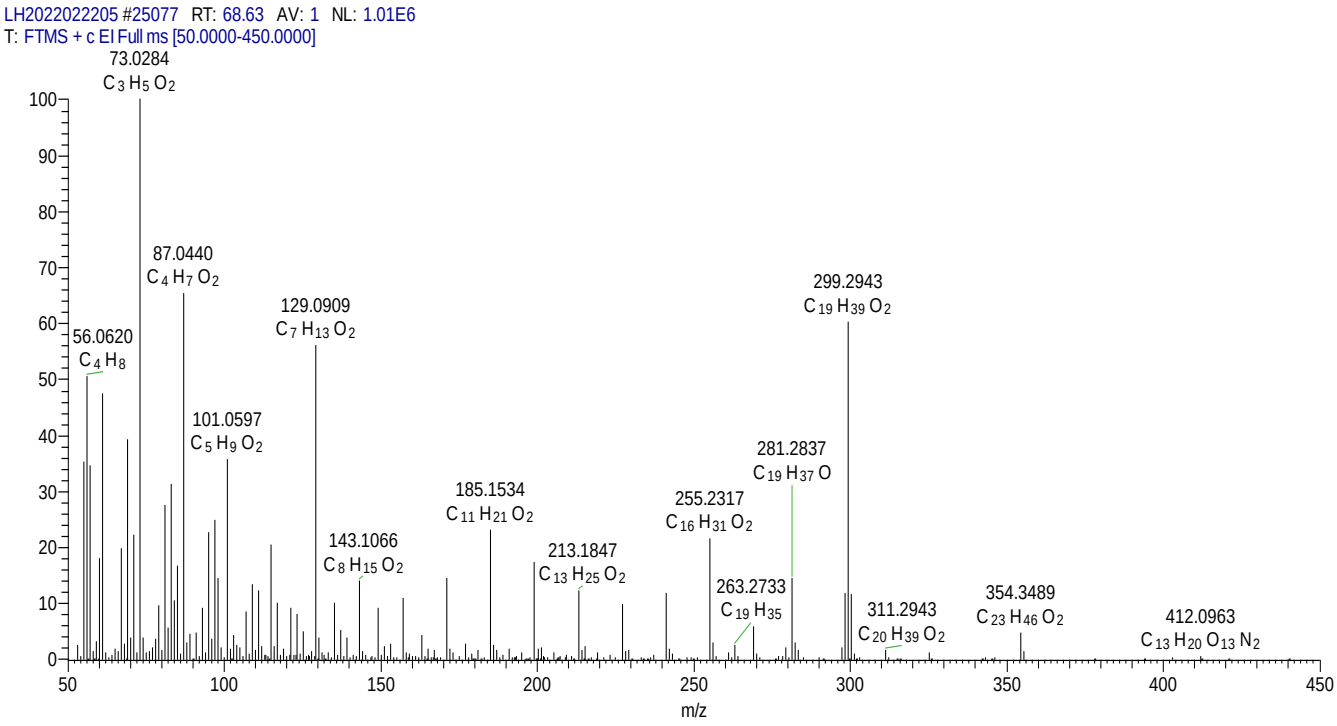
C18:1 MW: 338.556 $C_{22}H_{42}O_2$



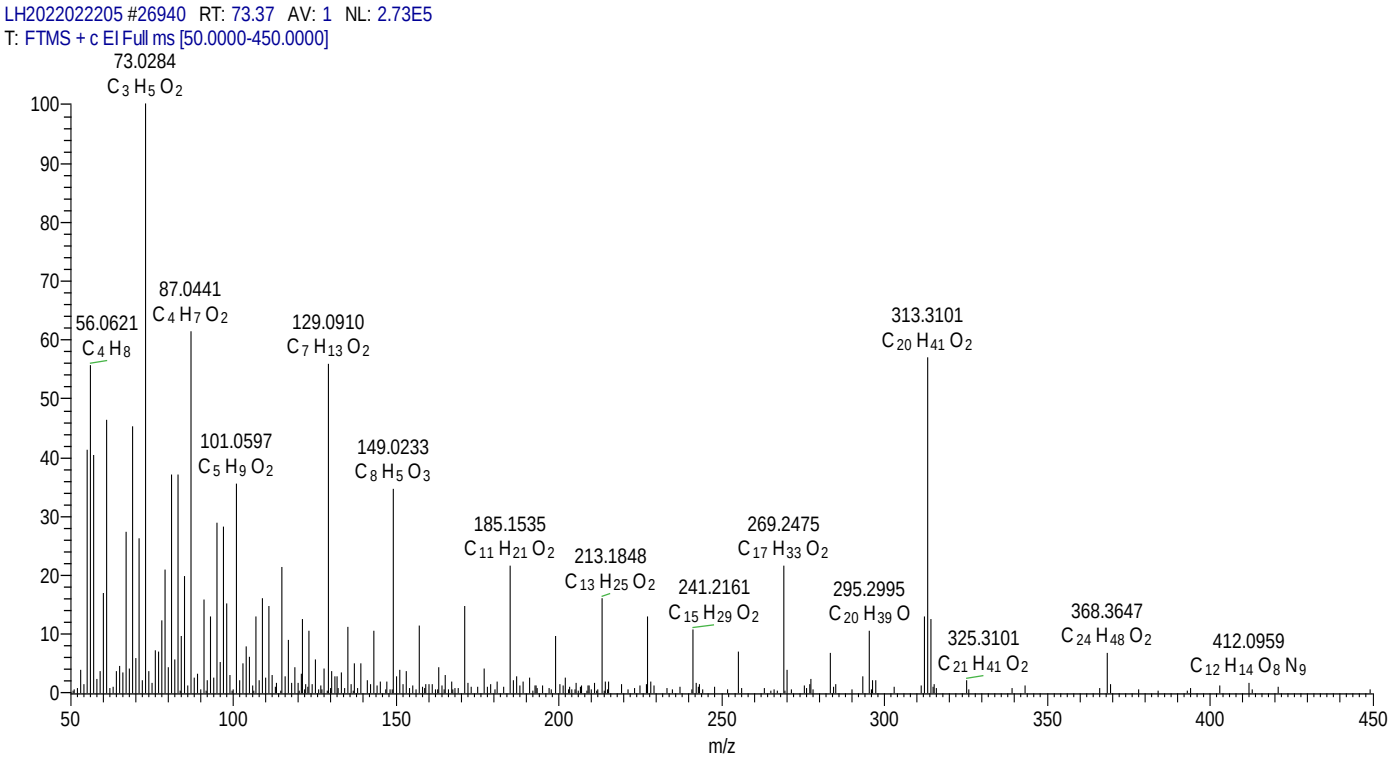
C18:2 MW: 336.54 $C_{22}H_{40}O_2$



C18:3 MW: 334.524 $C_{22}H_{38}O_2$



C19:0 MW: 354.598 $C_{23}H_{46}O_2$



C20:0 MW: 368.624 $C_{24}H_{48}O_2$

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