

**Development, Evaluation, and Application of a Gas
Chromatography – Atmospheric Pressure Chemical Ionization
Interface for Orbitrap Mass Spectrometers**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

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DEDICATION

To my family, friends, and colleagues who helped keep my grounded as well as lifted me up. You deserve as much credit as I do.

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ABSTRACT

Gas chromatography – [em dash] mass spectrometry (GC-MS) is a valuable tool used by numerous scientific fields including forensics, medicine, and petrochemicals. Until recently, many GC-MS instruments have relied on either electron ionization or chemical ionization sources with low resolution mass analyzers. With improving technology, Thermo Fisher Scientific launched the Q Exactive™ [trademark] GC hybrid quadrupole-Orbitrap MS, which utilizes these sources, while other companies also released GC-MS systems capable of exact mass measurements; however, these GC-MSs instead employ atmospheric pressure chemical ionization (APCI), which like other atmospheric pressure ionizations (API), provides “soft” ionization of analytes. Moreover, APCI extends the range of analytes that can be detected and identified compared to traditional ionization techniques. Thus, the combination of high-resolution mass spectrometry (HRMS) and APCI enables accurate yet versatile analyses. To utilize these advantages, an interface between a GC and a Thermo Scientific™ [trademark] Ion Max housing unit was designed. This simple, yet temporary interface enables the reversible conversion of a liquid chromatography system to a GC-MS. The GC-APCI interface was tested against a commercially available atmospheric pressure photoionization (APPI) interface for three types of analytes that span the breadth of typical GC analyses: fatty acid methyl esters (FAMES), polyaromatic hydrocarbons (PAHs), and saturated hydrocarbons analyses. The GC-APCI-Orbitrap was found to have similar or improved performance to the APPI interface and other reported methods. Additionally, it was found that the interface consistently produced unique ions of these analytes, which were also detected using the APPI source. Using a Q Exactive™ [trademark] Orbitrap, high-resolution and MS² [superscript 2] data were collected using standards of non-deuterated and select fully deuterated analytes. From these experiments, the data suggest unique, underreported mechanisms of GC-API due to in-source oxidation. Finally, the newly designed interface was used to characterize fatty acid composition of

subcutaneous adipose tissue from broiler chicks in support of studying polyunsaturated fatty acid (PUFA) pathways. Specifically, it was determined that expression of three genes significantly and positively correlated with seven fatty acids during embryonic and post hatch development. Together, these experiments demonstrate the utility and capability of a GC-APCI-Orbitrap as an alternative to traditional GC-MS.

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INTRODUCTION

Chemical Analyses

As time has passed, the fundamental understanding of many chemical systems has changed due in large part to advances in technology. With increased accuracy, better precision, and higher throughput, chemical analyses are now more effective at reporting qualitative and quantitative characteristics of complex mixtures. For instance, the field of metabolomics, which seeks to interpret biological systems through small molecule analyses of metabolism, has developed rapidly over the past two decades and its growth can be attributed to analytical innovations.¹ Specifically, the analytical techniques of nuclear magnetic resonance (NMR) and mass spectrometry (MS) have contributed to the working knowledge of the metabolic space. While NMR is directly quantitative, nondestructive, and requires minimal sample preparation, its sensitivity and selectivity is outclassed by MS when considering mixtures of varying chemical diversity.² By coupling MS to a separation technique, such as liquid chromatography (LC) or gas chromatography (GC), the ability to perform targeted and untargeted analyses is greatly refined. One significant result of both separation and detection improvements has been the discovery that identified analytes in metabolomics only make up approximately ten percent of mass spectrometric data.³ Continued advances in technological innovation will undoubtedly increase the percentage of known analytes in metabolomics as well as other analyses, and further aid in characterizing convoluted samples.

Mass Spectrometry

The history of MS can be traced back to the early twentieth century, when J.J. Thompson measured the mass-to-charge ratio (m/z) of electrons via cathode rays experiments and generated the first mass spectrum.⁴ Since its inception, mass spectrometry has undergone many changes and modifications; however, the core components of a mass spectrometer have remained the same. Every mass spectrometer requires an ionization source, a mass analyzer, and a detector to

generate m/z s of an analyte. Advances in the technology and design of these components have contributed to MS becoming one of the most valuable techniques for determining chemical identities.

Before a m/z can be determined using MS, analytes must first be converted to a suitable form, i.e. a gas-phase ion, in order to be manipulated and controlled by the MS. An analyte is transformed into an ion through the ionization source of the mass spectrometer. The type of ionization source employed often depends on the separation technique if any, the type of mass analyzer, as well as the analyte itself; nevertheless, sources can generally be sorted into two groups: “hard” ionization and “soft” ionization. A hard ionization technique will impart excess energy to an analyte, so as to destabilize and fragment the molecule. Charge is distributed amongst the fragments and the identities of the fragment ions as well as neutral losses can be used to elucidate the structure of the analyte. Additionally, the fragmentation pattern produced by the analyte can be used to identify the molecule when compared to a database. Soft ionization, in contrast, supplies just enough energy to ionize a molecule while keeping the structure intact, which allows for identification of the molecular ion peak, or mass of the molecule. Some of the most common ionization sources today include electron ionization (EI), chemical ionization (CI), inductively-coupled plasma (ICP), electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI). Although there are many additional ionization sources, including next generation sources being researched and developed, these sources adequately cover the range of compatible analytes, separation techniques, and mass analyzers.

Once the analyte is ionized, its path through the MS can be manipulated and controlled through various electric fields. It is important to note that mass analyzers and detectors are operated at high vacuum, so that atmospheric molecules do not interact with the charged species. Additionally, other optics can be utilized prior to the mass analyzer to focus the ions into a tighter path, or

compensate for irregular, kinetic distributions of ions generated by the source. Upon arriving at the mass analyzer, ions are sorted and separated by their m/z , usually via magnetic or electrostatic fields. It has become common practice to identify and label many MSs based on their mass analyzer. Some of the most common mass analyzers are time-of-flight (TOF), quadrupole (Q), linear and quadrupole ion traps (LIT)(QIT), Fourier transform – ion cyclotron resonance (FT-ICR), and orbitraps. One of the main distinctions between mass analyzers is their ability to resolve ions, which is a direct result of their operating principles. Resolution, which is defined as the capacity to differentiate between two adjacent masses, is of utmost importance when attempting to separate ions of similar mass. High-resolution MS (HRMS) is not only able to distinguish nominal mass, but also isotopic distribution as well due to exact mass measurements. FT-ICRs and orbitraps, with over one million and one hundred thousand resolution respectively, are the leading HRMSs in today's industry.

In addition to their unique capacity to separate analytes, FT-ICRs and orbitraps utilize distinct detectors to measure m/z . Specifically, HRMSs use image current, the process of generating a signal as ions pass near a metal surface and draw charge toward that surface, to register m/z . Other MSs, including TOF and Q mass analyzers, employ more traditional methods to detect m/z . Electron multipliers, which amplify the incoming signal from the m/z through several electrodes and dynodes, are the most common detector. Other detectors include Faraday cups and microchannel plates, though these are becoming increasingly rare.⁵ Regardless of which detector is incorporated into the MS, its function remains the same: to convert the energy of an ion into an electrical signal that can be measured and transferred to a computer for data processing.

Interpreting data acquired by a MS can be simplified with appropriate software and experimental knowledge. Depending on the type of ionization source as well as operational mode of the MS (full scan, parallel reaction monitoring (PRM), etc.), different ions can be expected to be produced and detected. For

instance, an ESI source is likely to generate intact, protonated species, $[M + H]^+$, whereas an EI source is more likely to generate $[M]^+$ of smaller, fragmented ions. Therefore, understanding experimental design is key to deciphering data. Total ion chromatograms (TICs) are summations of the intensities of all ions throughout the analysis and are particularly useful for identifying analytes of adequate signal to noise (S/N). Analytes will generate an ion species that is more intense than any background ion, which produces a peak, or bell-shaped curve. The profile of a particular m/z can be observed by plotting an extracted ion chromatograph and by measuring the area under the curve, quantitative comparisons can be made. Instrument manufacturers provide sufficient software to identify and calculate the areas of m/z ; however, there are several other programs and databases available to measure, calculate, and even identify m/z s.

Additional structural information, increased sensitivity, as well as verification of analyte identity are all possible using tandem MS ($MS^{n>1}$). Tandem MS relies on either two mass analyzers, such as the triple quadrupole (QqQ), or a trapping mass analyzer with the addition of collisional gas, such as a QIT, to fragment ions in space or time, respectively. There are several different modes by which data can be collected in a tandem MS experiment, such as PRM, MRM, as well as data dependent acquisitions, and the selected mode dictates whether ions are either selected or a range of ions are scanned for during each mass analysis. When designing a tandem MS experiment, it is important to consider if there is a sufficient amount of an analyte, i.e. concentration, to produce enough ions for MS^n analyses.

High Performance Liquid Chromatography

Although liquid chromatography – mass spectrometry (LC-MS) was developed after gas chromatography – mass spectrometry (GC-MS), it has become the industry standard for analyzing biologically relevant compounds. The development of LC-MS was initially slowed due to the technology of the time; ionization sources were incompatible with liquids.⁶ However, this limitation spurred

technological advancement and resulted in the development of ESI. The combination of LC and ESI proved to be ideal for analyzing peptides, proteins, and other molecules containing polar functional groups.

LC separates analytes based on individual hydrophilic or hydrophobic nature prior to ionization via the MS. By first separating analytes, there is less ionization suppression to interfere with MS analyses and higher degrees of selectivity. Each LC system is generally comprised of the following components: an injector/autosampler, a pump, solvents and mixing system, analytical column, and detector. Selection of solvents and analytical column is mediated by expected properties of the analytes within a mixture. Reverse-phase (RP) chromatography is the most common system, which uses a non-polar column and a large percentage of initially polar solvent, such as water. Analytes are injected using a syringe and switching valve to load the liquid sample to the head of the column. The pump supplies a constant flow of a solvent mixture that has been mixed and degassed prior to its interaction with the column. The solvent/column interaction creates hydrophilic and hydrophobic regions. Analytes spend time bound to the stationary phase based on surface area, pH of the system, and particle size. To elute analytes from the stationary phase, a higher percentage of less-polar solvent is applied over time through gradients. Analytes elute at different times, retention times (RTs), and travel in bands to the ionization source and subsequently detected by MS. There are many alternate types of liquid chromatography that are useful, such as hydrophilic interaction chromatography (HILIC), ion-exchange chromatography (IC), and size exclusion chromatography (SEC), but generally all rely on stationary phase/solvent/analyte interactions.

Gas Chromatography

GC was the logical choice as a separation technique to first be paired with MS. Analytes are already in the gas-phase and thus only need to be ionized. The delay in interfacing GC to MS, however, was due to slow scan speeds of mass

analyzers and the inability to maintain proper vacuum at the high flow rates of GC.⁷ These issues subsided with the advent of capillary columns, which separate analytes with slower flow rates, and faster mass analyzers. Since these developments, GC-MS has remained a crucial analytical tool for many fields. In particular, GC-MS remains the analytical tool of choice for fields that require analysis of non-polar compounds, such as petrochemicals. While biologically relevant molecules contain polar functional groups that may result in thermal lability or incompatibility with GC stationary phases, modifications such as derivatization, allow for fast and accurate analyses of these analytes.

GC-MS has remained relatively unchanged since its inception. Each GC-MS system uses a carrier gas, an injection port/autosampler, analytical column, an oven, and detector. Typical analyses consist of injecting a sample into the injection port that is kept at a constant temperature, usually well above the boiling point of the highest boiler in the sample mixture and close to the maximum allowable temperature of the analytical column. There are many different injection ports available to volatilize or in some cases cryogenically freeze the sample, but two of the most common are the split/splitless injector (SSL) and the programmable temperature vaporizer (PTV). A SSL injector can operate in split mode, where carrier gas is used to dilute the sample, or splitless mode depending on the desired experimental design. For instance, split mode is advantageous when a sample is particularly contaminated with involatile substances and concentrated analytes, whereas splitless mode is useful for trace analyses. The liner within the injection port focuses the volatilized analytes to the head of the column, and its design and shape are specific to each mode. Additionally, the amount of sample that can be properly injected is dictated by the size of the liner. PTV injectors are more multifaceted in that they can replicate SSL injections as well as other injection types. As their names suggest, PTVs can be programmed to change their temperature as injection occurs, which allows for selective volatilization of analytes and solvent prior to being separated on the column or large volumes of solvent to

be introduced while selectively discarding the solvent vapors. PTVs operating in these modes, however, tend to work best when the lowest boiling analyte of the mixture has a boiling point that is significantly greater than the solvent's boiling point.

Once analytes have condensed onto the column in a narrow band, temperature gradients and flow parameters separate analytes. Initial oven temperatures tend to be at or below the boiling point of the solvent and gradients are created to separate analytes based on boiling point. Analytes interact with the capillary column, usually an open tubular column with wall coated liquid stationary phase, and the parameters of the column affect its use. Besides the type of stationary phase, film thickness (d_f), inner diameter (i.d.), and length dictate maximum allowable temperature, bleed temperatures, selectivity, and theoretical number of plates. The inert carrier gas, often helium but nitrogen and hydrogen are common as well, is set to either constant flow or constant pressure throughout the analysis. The flow, temperature, and analytical column together dictate the retention and selectivity of the analytes while the injector greatly influences peak shape and sensitivity.

Outline of Dissertation

The work presented herein builds upon the fundamentals previously described through additional technological innovation. Specifically, the first chapter reports an interface that can be used to configure a LC-MS system to a GC-MS and back again. To test the analytical performance of the temporary GC-MS, common GC analyses that target analytes of varying polarity are reproduced and compared to other methods. Chapter two focuses on the unique ions detected during these analyses and the experiments designed to elucidate the structure as well as origin of these species. Finally, the third chapter demonstrates the real-world applicability of the designed interface by measuring fatty acids of *Gallus*

gallus from different tissues. GC-MS data are combined with targeted RNA sequencing to explore polyunsaturated fatty acid synthesis.

CHAPTER 1.
DESIGN AND EVALUATION OF A GAS CHROMATOGRAPH –
ATMOSPHERIC PRESSURE CHEMICAL IONIZATION
INTERFACE FOR AN EXACTIVE ORBITRAP MASS
SPECTROMETER

Preface

The first chapter of this dissertation presents the design and development of an interface capable of converting a UHPLC-MS to a GC-MS with minimal effort. The interface provides a means to use either LC or GC on the same HRMS and utilizes the Ion Max housing unit and APCI source of an Orbitrap MS. The idea for the project was conceived in early 2015, instrumentation was available late 2016/early 2017, and was fully optimized by early 2019. It was proposed that the fast scan speed of the Orbitrap mass analyzer could handle the high flow rates necessary for GC-MS analyses and that APCI would be amenable to ionizing analytes.

Consequently, the interface was designed with these principles in mind and an article detailing the process was published in the *Journal of the American Society for Mass Spectrometry*. After several iterations, a complete and reproducible setup is reported with minimal modifications. Specifically, the components of the interface, the implementation of contact closures, and the necessary software are presented and clearly defined for replication. It is highly encouraged that any laboratories, that would like to reproduce the interface and may require assistance, contact either of the authors.

Furthermore, the manuscript reports the optimization and evaluation of the interface and GC-MS system. Three common GC analyses are replicated including fatty acid methyl ester, polyaromatic hydrocarbon, and saturated hydrocarbon analyses. These analyses showcase the diverse sets of compounds that are typically difficult to analyze by LC but still capable of being ionized by APCI. Additionally, the GC-APCI interface was tested against a commercially available GC-APPI interface, and the results are reported within Chapter one. In total, sixty-six compounds were tested and evaluated. It was found that the GC-APCI-Orbitrap performed similarly or better than the GC-APPI-Orbitrap with respect to sensitivity, linearity of standard curves, and limits of quantitation. Moreover, literature provided

further context of these values by comparing the GC-APCI interface to other known methods, such as GC-FID.

By utilizing the GC-APCI interface, an analytical lab can greatly diversify its ability to qualify and quantitate additional analytes of interest for many fields of study.

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Contributions:

SRC and JBP designed the experiments and wrote the manuscript. JBP created the methods, ran the standards, and analyzed the data.

1.1. Abstract

Various separation and mass spectrometric (MS) techniques have furthered our ability to study complex mixtures, and the desire to measure every analyte in a system is of continual interest. For many complex mixtures, such as the total molecular content of a cell, it is becoming apparent that no one single separation technique or analysis is likely to achieve this goal. Therefore, having a variety of tools to measure the complexity of these mixtures is prudent. Orbitrap MSs are broadly used in systems biology studies due to their unique performance characteristics. However, GC-Orbitraps have only recently become available, and instruments that can use gas chromatography (GC) cannot use liquid chromatography (LC) and vice versa. This limits small molecule analyses, such as those that would be employed for metabolomics, lipidomics, or toxicological studies. Thus, a simple, temporary interface was designed for a GC and Thermo Scientific™ Ion Max housing unit. This interface enables either GC or LC separation to be used on the same MS, an Exactive™ Plus Orbitrap, and utilizes an atmospheric pressure chemical ionization (APCI) source. The GC-APCI interface was tested against a commercially available atmospheric pressure photoionization (APPI) interface for three types of analytes that span the breadth of typical GC analyses: fatty acid methyl esters (FAMES), polyaromatic hydrocarbons (PAHs), and saturated hydrocarbons. The GC-APCI-Orbitrap had similar or improved performance to the APPI and other reported methods in that it had a lower limit of quantitation, better signal to noise, and lower tendency to fragment analytes.

1.2. Introduction

As the ability to measure fully all analytes from complex mixtures has progressed, it has become apparent that two main factors still hinder such efforts: The chemical diversity of these mixtures is high, making it difficult to probe the full complexity of the samples with a single analysis technique; and the identity of many analytes is still undetermined. The obstacles facing analysis of complex mixtures have been highlighted with the growth of interest in metabolomics and lipidomics. Often, biological, environmental, and other samples contain large mixtures of compounds that can be similar in composition, which limits the utility of techniques like nuclear magnetic resonance (NMR) to measure all chemical species. For global analyses of mixtures, separation and detection techniques such as liquid chromatography and gas chromatography–mass spectrometry (LC-MS and GC-MS, respectively) are employed since these methods are better suited for such endeavors. However, many studies still focus on analyses using only one type of separation, even though use of both techniques would give a higher coverage of complex mixtures, such as the metabolome and lipidome. Although not addressed herein, the identification of unknown compounds is still difficult via MS. Recently, a common workflow to aid in the identification of unknowns by LCMS has been established,⁸ but GC-MS unknown identification has lagged behind. This discrepancy can be attributed to the frequent use of high-resolution mass spectrometry (HRMS) in LC-MS as well as the complexity of fragmentation generated by electron ionization (EI). Until recently, GC had not been routinely paired with HRMS as fast mass analyzers are required to fully utilize the high flow rates of GCs. In 2014, Peterson and coworkers published an article that detailed the coupling of a GC-MS (Thermo Scientific DSQ™ II) with an Orbitrap (Exactive™ Plus) as a possible tool for metabolomics analysis.⁹ Thermo Fisher Scientific™ followed with the introduction of a Q Exactive™ GC hybrid quadrupole-Orbitrap mass spectrometer system.¹⁰ These platforms pushed the boundaries of unknown identification, utilizing superior exact mass measurements; however, both still

relied on matching fragmentation data to accomplish these tasks. More recently, companies such as Waters Corporation, Bruker, and Agilent have also developed GC-APCI sources for traditional HRMS analyzers, such as the QTOF, as well as low energy EI sources in the push for softer ionization of GC amenable compounds.¹¹⁻¹³ Atmospheric pressure photoionization sources (APPI) have also been developed for GC-Orbitraps in order to provide softer, efficient ionization mechanisms for nonpolar systems.¹⁴ These sources rely on analytes that have ionization energies (IE) less than the energies of the photons from the source (direct APPI) or the addition of a doping agent (dopant-assisted APPI), such as toluene, to achieve ionization. The addition of doping agents is most practical when target analytes are already known as the doping agents cause higher background ions and can possibly react with the matrix. Thus, for a complex biological or environmental mixture, direct APPI is best suited for unknown identification. In 1973, Horning and coworkers reported atmospheric pressure chemical ionization (APCI),¹⁵ which has become an ionization technique of choice for LC-MS analyses of less polar molecules. Zuth and coworkers have also showed that an APCI-Orbitrap could be used for the detection of organic aerosols introduced through a unique apparatus, thus showing the utility of this instrument for the analysis of molecules introduced in the gas phase.¹⁶ Herein, an interface to allow coupling of a Trace 1300 GC system to a Thermo Scientific™ Ion Max housing is reported. This allows an APCI source to be used for GC-HRMS analyses with the unique capabilities of instruments such as the Orbitrap™ and does not preclude the use of an LC on the same system. This simple, inexpensive GC-MS interface was tested using three well-known, diverse types of analytes [fatty acid methyl esters (FAMES), polyaromatic hydrocarbons (PAHs), and saturated hydrocarbons] as they encompass the breadth of compounds that are routinely analyzed by GC-MS and are amenable to APCI. Moreover, the GCAPCI interface was benchmarked against a commercially available, aftermarket GC-APPI interface to evaluate and compare their analytical performance.

1.3. Experimental

1.3.1. Reagents and Materials

The 37 component FAME standard and the C8-C20 alkane standard solution were purchased from Sigma-Aldrich (St. Louis, MO). The 610 Calibration PAH Mix A standard was purchased from VWR International (Radnor, PA). HPLC-grade hexanes from Fischer Scientific (Hampton, NH) were used for preparations of all standards and syringe washes. Ultrahigh purity grade He was purchased from Airgas (Madison, WI). A stainless-steel sheet and silicon O-ring were purchased from McMaster-Carr (Elmhurst, IL). An 8-pin mini-din male to male cable was purchased from Thermo Fisher Scientific (Asheville, NC) and a 6-position, 3.81 mm Mini-COMBICON term block plug was included with the UPLC system.

1.3.2. Gas Chromatography – Mass Spectrometry

GC-MS experiments were conducted on a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a Triplus RSH autosampler and aux temperature/cryo module. The GC interfaces were coupled to an Exactive™Plus Orbitrap mass spectrometer (Thermo Fisher Scientific Waltham, MA). The commercially available GCAPPI interface, including the transfer line, was purchased from Gentech Scientific Inc. (Arcade, NY). Fatty Acid Methyl Ester Analysis

1.3.3. Fatty Acid Methyl Ester Analysis

FAMES were separated using a (FAMEWAX, 30 m × 0.25 mm i.d. × 0.25 µm df) polyethylene glycol column (Restek, Bellefonte, PA) and helium as the carrier gas. The FAME standard mix was serially diluted five times in hexanes to obtain concentrations ranging from approximately 5 nM to 400 µM, depending on the individual analyte. Triplicate, 2 µL injections of the standards were directed into a programmable temperature vaporizer (PTV) injection port operated in CT split

mode. Split flow was set to 5.1 mL/min (5:1 split ratio), and the temperature of the injection port was set to 240 °C. Analytes were separated on the column using a 1.7 mL/min He flow rate and a 26 min temperature program as follows: initial temperature 80 °C and hold for 5 min, 10.0 °C/min to 240 °C and hold at 240 °C for 5 additional minutes. The exact same standards were injected using both APCI and APPI sources. Analytes were transferred from the GC to the MS via the commercially available heated transfer line set to 240 °C. While using the APCI ionization source, sweep gas flow rate was set to 3 arbitrary units, aux gas flow rate to 8, and sheath gas flow rate set to 10. The discharge current was set to 10.00 μ A, ion transfer capillary temperature to 350 °C, S-lens RF level to 50.0, and vaporizer temperature set to 320 °C. The mass spectrometer was set to full scan from 50.0 to 700.0 m/z with a resolution of 140,000. Data was collected in positive mode, using 1 microscan, automated gain control (AGC) target of 3E6, and maximum inject time of 100 ms. APPI parameters were set as follows: sheath gas flow rate to 8 arbitrary units, aux gas flow rate to 0, sweep gas flow rate to 0, ion transfer capillary temperature to 280 °C, S-lens RF level of 50.0, vaporizer temperature to 30 °C, and ionization in APPI mode. The remaining parameters—scan range, polarity, microscans, AGC, and maximum inject time—were kept the same as the APCI source.

1.3.4. Polyaromatic Hydrocarbon Analysis

PAHs were separated on a (Select PAH, 15 m $\times$ 0.15 i.d. $\times$ 0.10 μ m df) capillary column (Agilent, Santa Clara, CA) using He as the carrier gas. The PAH standard mix was serially diluted seven times, resulting in approximately 0.1 nM to 800 μ M concentrations depending on the individual analyte. Triplicate, 2 μ L injections of the standards were directed into the PTV injection port operated in CT split mode. The injection port temperature was set to 315 °C with a split flow of 5.0 mL/min (5:1 split). Analytes were separated on the column using a 0.8-mL/min flow rate with the following 35.57-min temperature program: initial temperature of 100

°C and hold for 2.0 min, 7 °C/min to 230 °C and hold for 7 min, and 50 °C/min to 280 °C and hold for 7 min. The exact same standards were injected using both APCI and APPI sources. The settings for both were the same as those set for the FAME analyses. Analytes were transferred from the GC to the MS via the commercially available heated transfer line set to 280 °C.

1.3.5. Saturated Hydrocarbon Analysis

Alkanes were separated on a (TG1-MS, 30 m× 0.25 mm i.d., 0.25 µm df) capillary column (Thermo Fisher Scientific, Waltham, MA) using He as the carrier gas. The alkane standard mix was serially diluted six times in hexanes to obtain concentrations ranging from approximately 0.1 nM to 350 µM depending on the analyte. Triplicate, 2 µL injections of the standards were directed into the PTV injection port operated in CT split mode. The injection port was set to 330 °C with a split flow of 5.1 mL/min (5:1 split). Analytes were separated on the column using a 1.4 mL/min flow rate and the following 25 min temperature program: initial temperature of 55 °C and hold of 5.0 min and 15 °C/min to 280 °C and hold for 5 min. Each analysis required a new set of standards as the solutions evaporated noticeably. APCI and APPI settings were the same as those set for the FAME analysis. Analytes were transferred from the GC to MS via the commercially available heated transfer line set to 300 °C.

1.3.6. Data Analysis

Instrument control and data acquisition were supervised by Xcalibur software, Version 4.0.27.19. Data files generated by Xcalibur were of the .raw file extension, and these files were converted to .mzML format using msconvert¹⁷ and loaded into MAVEN¹⁸ (Metabolomic Analysis and Visualization Engine). In the case of $[M + H]^+$ ions, peaks were picked based on m/z calculated from chemical formula in positive mode using a ± 5 ppm mass window. Peaks picked based on the most abundant ion for each analyte were based on retention times of the $[M + H]^+$ ion and the exact m/z of the most abundant ion found in Xcalibur.

1.4. Results and Discussion

1.4.1. Interface Construction and Installation

The Ion Max housing is designed to allow both easy access and visibility, and the front of the unit contains a hinged, glass window that can be easily removed. Replacement of this window with a machined sheet of stainless-steel containing a hole for a transfer line provided a mechanism for the coupling of the GC to the MS (Figure 1.1). Earlier work by Lee and coworkers also used this aperture to introduce the eluent from a GC into an Orbitrap fitted with an APPI source, although they did not add additional modifications to the housing other than to remove the window.¹⁹

The opening of the stainless-steel piece was recessed, and a silicon O-ring was used to aid in the insertion and removal of the transfer line. A custom transfer line was built and used in the first iteration of the instrument (see Appendix Figure 1.8), although it was replaced with the transfer line purchased with the commercial APPI source as the latter was found to have superior temperature stability. This line was inserted to a depth of 8 cm through the stainless-steel insert of the Ion Max housing. The other end of the transfer line was inserted into the precut duct of the GC, and the temperature of the transfer line was controlled by the GC via a Quickmate connection (Figure 1.2).

Contact closures were used to synchronize data acquisition of the MS with the operation of the GC. These connections employed a male-to-male 8-pin mini din cable, and one end was connected through the generic handshake port on the back of the GC. Each wire at the opposite end of the 8-pin mini-din cable was separated and stripped since the Orbitrap had an otherwise incompatible 6-pin input configuration and required direct plug in with its existing contact closures. Using the pin configuration of the generic handshake port provided by the manufacturer, as well as measuring the resistance of each wire, it was determined that the wires corresponding to pins 4 and 6 were required to make the contact

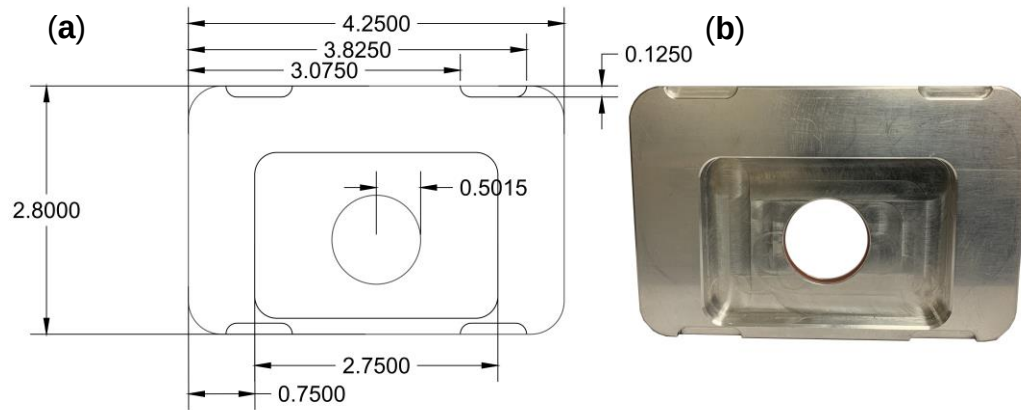


Figure 1.1. (a) Autocad rendering and (b) photograph of the fabricated stainless-steel insert that replaced the front window of the ion max housing. In (B), the orange, silicon O-ring can be seen recessed into the opening.

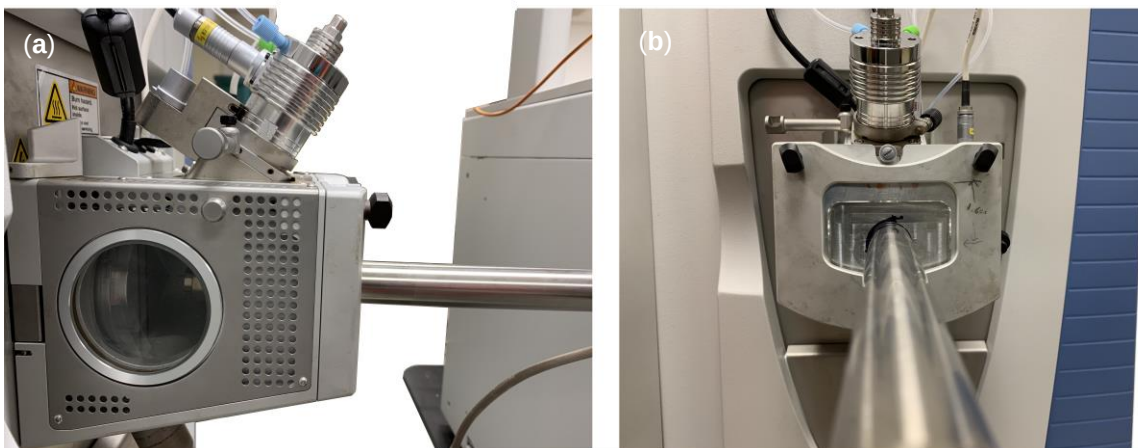


Figure 1.2. (a) Profile and (b) direct view of the completed APCI GC-Orbitrap interface. The insertion depth of the interface can be seen in (a) and the stainless-steel insert can be seen in (b).

closure. Pin 4 transmits the start run signal from the GC to the Orbitrap and Pin 6 transmits the end run signal from the GC to the Orbitrap. Through trial and error, the successful contact closure configuration consisted of connecting the wire from pin 4 to the positive digital input and the wire from pin 6 to the negative digital input using a Mini-COMBICON plug. GC devices software (Gentech Scientific Inc., Arcade, NY) was installed on the MS computer to allow for complete control of the autosampler, GC, and MS by ethernet connections. Additionally, it was necessary to change the software configuration of the GC, specifically “start of run out” to “low” and “end of run out” to “high.”

The first parameter to optimize was the placement of the transfer line in order to maximize the ionization for the APCI source. The configuration of the APCI-interface differed from that of either the APPI interface or of the normal source through introduction utilized by the Ion Max housing in that the eluent stream of the GC was pointed directly into the MS inlet rather than positioned at an angle. This was not deemed to be an issue since Thermo Scientific™ nano sources also utilize this geometry; therefore, it was postulated that the distance between the transfer line and the inlet was the more important parameter. It was noted that decreasing distance between the transfer line and the MS inlet had effects on both the number (observed as peak areas) and the composition (observed as m/z) of charged ions. When the transfer line was farther away from the corona discharge needle, there was a greater percentage of $[M + H]^+$ ions, but a decrease in peak area and tailing of the analyte peaks were observed. Conversely, when the transfer line was closer to the corona discharge needle, the percentage of $[M + H]^+$ ions was approximately equal to or less than the most abundant fragment ion for each analyte, and peak area increased by approximately two to threefold. Potential reasons for these changes in ion populations could be due to a variety of factors. A greater distance from the capillary column to the corona discharge would result in increased dispersion of analyte gas phase molecules and a higher probability of interacting with charged

atmospheric ion species. When considering molecular interactions at a closer distance, there is a higher probability that an analyte will be directly ionized by the corona discharge and be detected as a charged radical cation as observed in these experiments (Figure 1.3). Due to the increase in signal and better peak shape, the final operating configuration for the APCI utilized the minimum distance to the corona needle necessary to and all subsequent APCI analyses were performed at that distance.

1.4.2. FAME Analysis

In total, 36 of the 37 FAME standards were separated and quantitated using the chromatographic conditions discussed above (Figure 1.4). The only analytes that were not separated were trans-9-elaidic acid methyl ester and cis-9-oleic acid methyl ester. These isomers were not suited for the stationary phase, and the summation of the peak area from both compounds is listed as methyl oleate/elaidate. While the background generated by the APPI source was lower than that generated by the APCI source, the signal generated by the APCI source was more intense for each analyte and averaged one to two orders of magnitude larger. Comparing the performance of the APCI and APPI sources showed that the former performed as well as if not better than the latter in all cases. For the FAME analysis, the linear dynamic range for both ionization methods averaged four orders of magnitude. Correlation of determination (R^2) values were greater than 0.9936 for analytes ionized by the APCI source and 0.9627 for analytes ionized by APPI in all cases. When using a S/N of 3:1, the limits of quantitation (LOQs) for each analyte were the same for both ionization methods. However, some analytes had detectable peaks at a lower concentration than the LOQ by APCI; the signal did not reach the required S/N. The LOQ for the APCI source was also good with an average of 111 ± 80 nM and a range of 52.5 to 393 nM, which matched the values obtained by APPI for the analysis of FAMES. The APCI source had increased sensitivity and better R^2 values than the APPI source for most

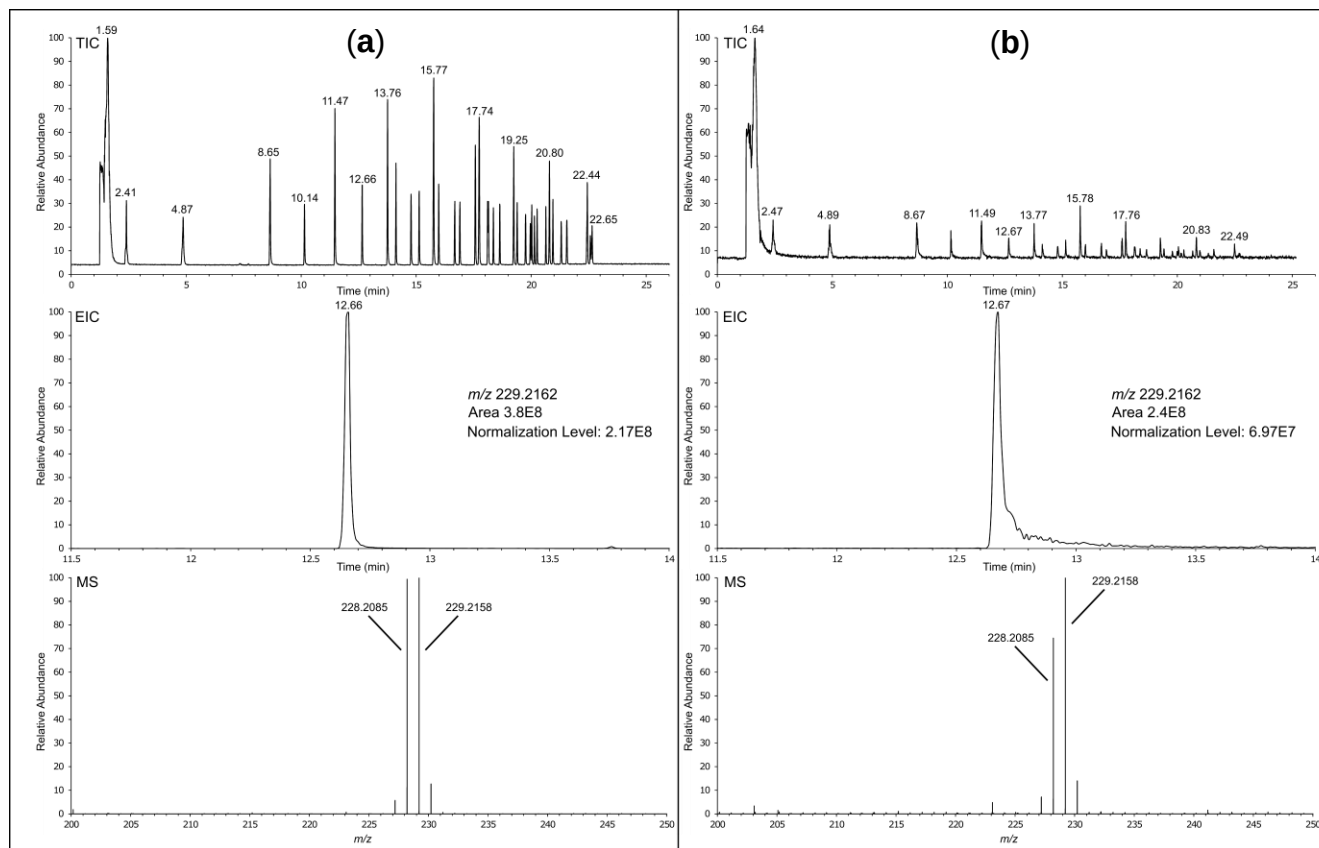


Figure 1.3. Side by side comparisons of TICs, EICs of m/z 229.2162, and MS from the transfer line being inserted to a depth of (a) 8 cm and (b) 4 cm. The difference in signal, peak shape, and ratio of $[M + H]^+$ to $M^{\bullet+}$ is dependent on the distance between the transfer line and corona discharge needle.

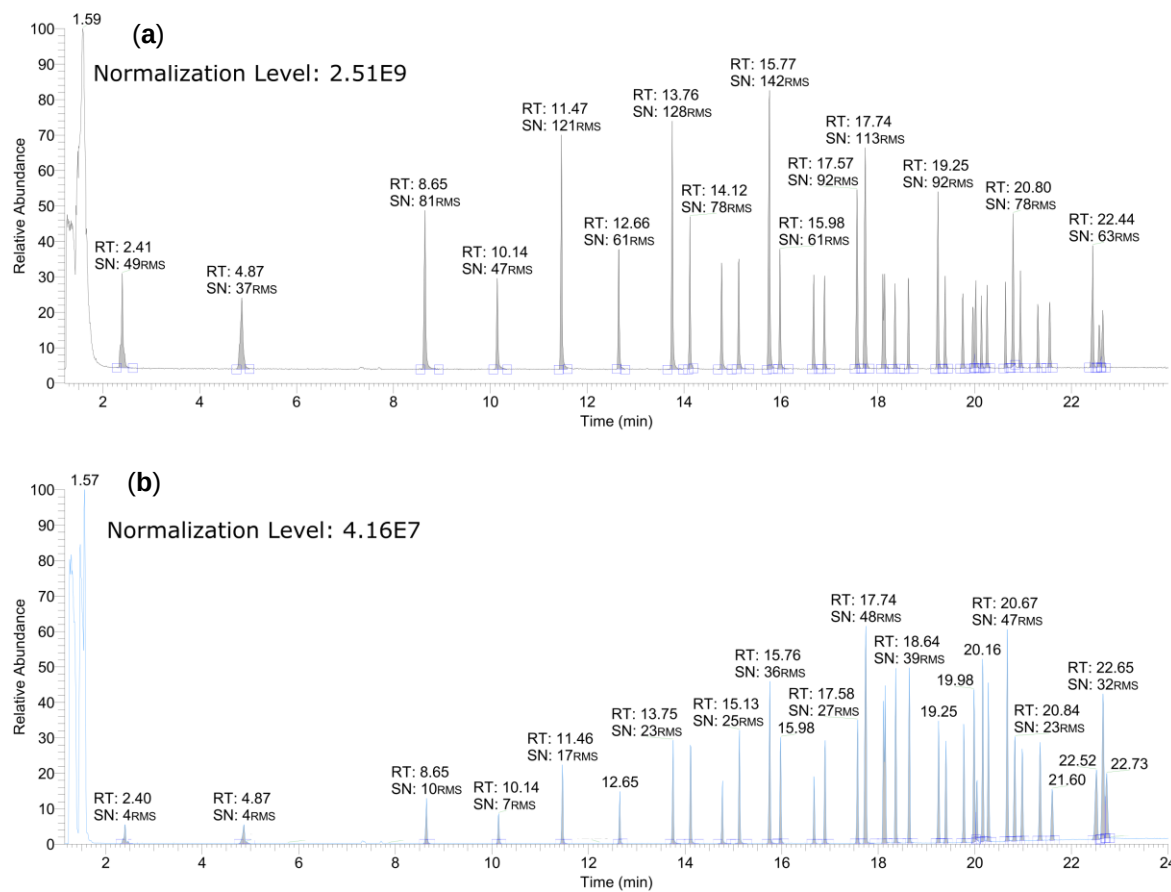


Figure 1.4. TICs of the 37 component FAME mixture ionized by (a) APCI and (b) APPI with selected S/N values.

compounds, although the detection of the lower molecular weight compounds was more sensitive using APPI. At low concentrations, the variability for the detection of the FAMES with APPI was of concern, and the apparent increase in sensitivity for this analysis is partially driven by fitting to the highly variable ion counts in this region (see Appendix Table 1.2). Detection of FAMES below the LOQ was not observed with APPI. The summation of these analytical data is presented in Figure 1.5.

Detection of FAMES by GC-MS is routine, and a rigorous study by Dodds and coworkers reported limits of detection (LODs) for a selection of FAMES based on flame ionization detection (FID) as well as quadrupole and trapping MS detection.²⁰ On average, Dodds and coworkers noted that the LOD for FAMES average 439 ± 103 , which compares favorably to the average LOQ observed in this study, and it is likely that the LODs for the reported GC-APCI interface would also be lower than this range since analyte was detected below the LOQ.

1.4.3. FAME Ionization

While Each FAME produced a number of ions and fragments, several reproducible trends for these analytes were noted (a complete table of the most abundant ions species generated by each ionization technique can be found in Appendix Table 1.9). In particular, the APCI source primarily generated m/z s indicative of $[M + H]^+$ ($M + 1.0072$ AMU) and $[M]^+\bullet$ ($M - 0.0005$ AMU) molecular ions as well as ions generated due to fragmentation of the FAMES with a reproducible loss of -32.0267 AMU ($[M - 32.0267]^+$) (Appendix Table 1.1) All but one of the 36 analytes had 40% or greater relative abundance of the $[M + H]^+$ species using the APCI source. The APPI source also generated m/z indicative of $[M + H]^+$ and $[M]^+\bullet$, although the number of FAMES whose base peak was the $[M + H]^+$ was lower than for APCI, with only 22 of the 36 analytes having 40% or greater relative abundance of this species. The number of major fragments for the APPI source was also greater than those for the APCI source, and a fragment with

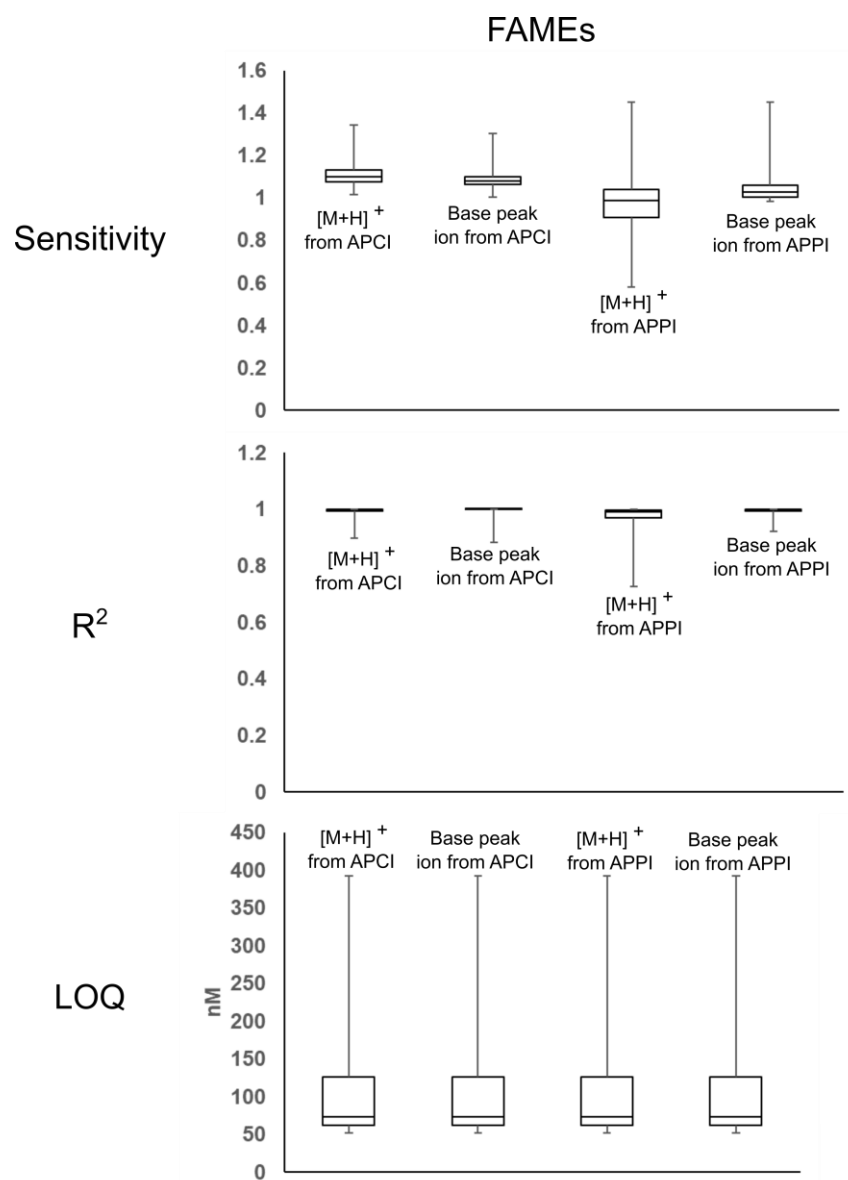


Figure 1.5. Box and whisker plots depicting sensitivity, R^2 , and LOQ values based on average values for all 36 analytes in the FAME analysis.

a loss of -45.0347 AMU ($[M - 45.0347]^+$) was observed along with the $[M - 32.0267]^+$ (Appendix Table 1.1).

1.4.4. PAH Analysis

The analysis of a mixture of 16 PAHs again showed that APCI performed favorably when compared to APPI. The linear dynamic range for the APCI source averaged four and a half orders of magnitude, with a range of 18.1 to 78.4 nM while that for the APPI source averaged four orders of magnitude with a range of 18.1 nM to 56.1 μ M. The dominate ionization mechanism for both APCI and APPI was loss of an electron yielding a radical cation; the sensitivity for this ion averaged 1.1153 with a range of 1.0802 to 1.2314 and 0.9819 with a range of 0.9114 to 1.0820 for APCI and APPI, respectively. The R^2 values were better than 0.9979 in all cases. While the average LOQs for both APCI and APPI were the same for the $[M]^+$ (35.0 nM), it was noted that APCI also produced a reasonable population of $[M + H]^+$ that could be detected with only a one order of magnitude loss in performance with an average LOQ of 291 nM. Detection of the $[M + H]^+$ via APPI was more difficult, with most LOQs being two orders of magnitude worse than those for the $[M]^+$ (6.0 μ M average). A summary of these data is presented in Figure 1.6. When both sensitivity and LOQ are taken into account, the APCI interface performs slightly better than the APPI interface. EPA Method 610 is widely utilized to detect PAHs and reports a GC-MS analysis. However, no analytical metrics for this technique are reported in the document. Alawi and coworkers have recently published a validated GC-MS method for detection of PAHs using a mass selective quadrupole detector in selective ion monitoring mode.²¹ They reported a dynamic range of 0.01 to 5.0 μ g/mL with LODs ranging from 0.0003 to 0.0089 μ g/mL, and the performance of the APCI interface reported herein is nearly identical. These data confirm that Orbitrap MSs operated in a full scan mode can perform as well as or better than quadrupole instruments using targeted analyses.

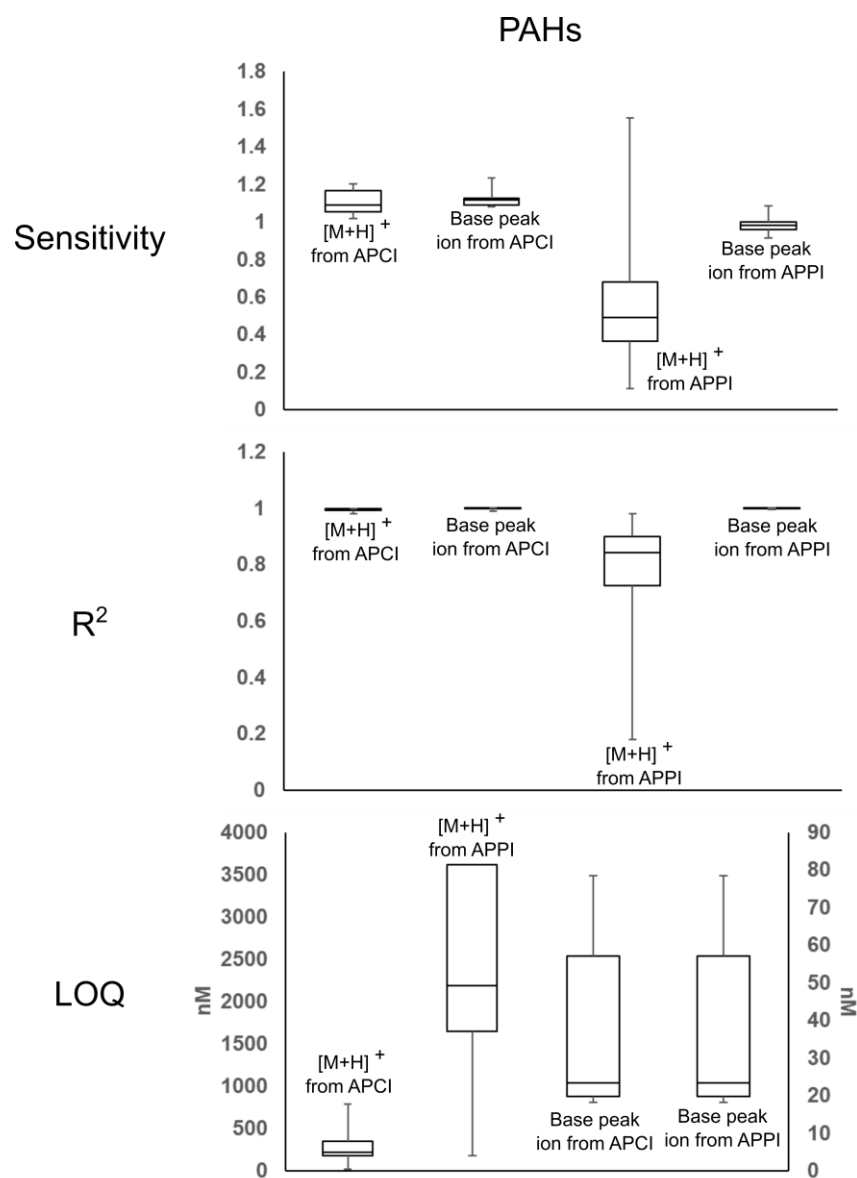


Figure 1.6. Box and whisker plots depicting sensitivity, R^2 , and LOQ values based on average values for all 16 analytes in the PAH analysis. In the plot depicting LOQs, $[M + H]^+$ values are assigned to the left axis and base peaks values are assigned to the right axis.

1.4.5. PAH Ionization

Ionization of the PAHs by both the APCI and APPI sources was rather routine with both generating m/z indicative of radical cations ($[M]^{+\bullet}$) as the most abundant ion with little to no fragmentation (Appendix Table 1.10). However, APCI was also able to generate $[M + H]^+$ ions, although with only an average relative abundance of $40 \pm 5\%$ (Appendix Table 1.10). It is of note that the mix of $[M]^{+\bullet}$ and $[M + H]^+$ did not lead to a significant loss in detection sensitivity for the APCI as this source led to an overall higher average population of ions for the PAHs.

1.4.6. Saturated Hydrocarbon Analysis

Though previous work, such as Marotta and Paradisi,²² has shown that ionization of saturated hydrocarbons is possible by APCI, such applications have been overlooked when evaluating the usefulness of this ionization method for the detection of nonpolar compounds.²³ Therefore, a mixture of 13 alkanes was analyzed using both the constructed GC-APCI and commercial GC-APPI systems. It was observed that both APCI and direct-APPI sources ionized alkanes, although with differing base peaks. The average sensitivity for the alkanes was 1.1592 ± 0.2537 with a range of 0.3393 to 1.3288 for the APCI source and 1.2207 ± 0.0683 with a range of 1.0709 to 1.2712 for the APPI source. If the sole outlier by the APCI source was to be excluded, the averages and standard deviations for both sources would be nearly identical. The average LOQs for the APCI and APPI source were both $2.2 \pm 0.6 \mu\text{M}$ with a range of 1.4 to $3.5 \mu\text{M}$. These LOQs (Figure 1.7) were not as good as for the other compound classes studied; however, both the APCI and

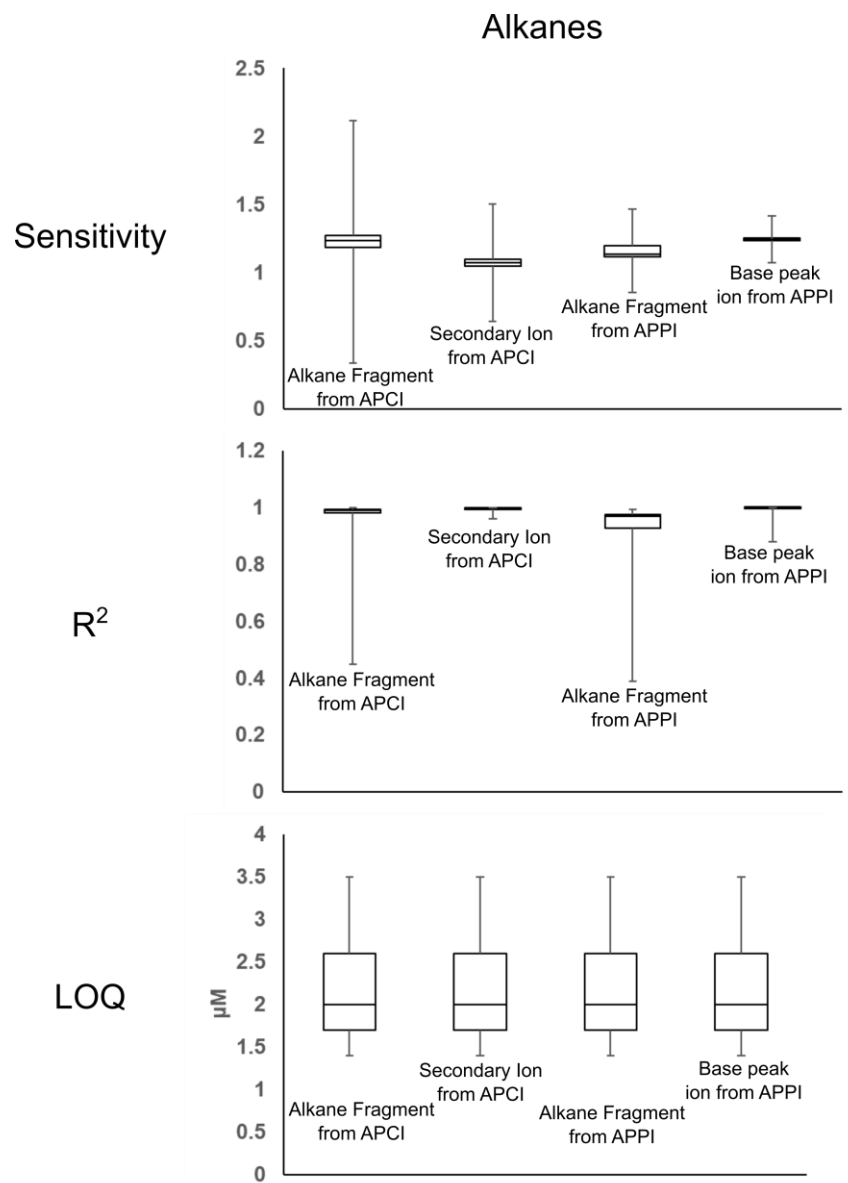


Figure 1.7. Box and whisker plots depicting sensitivity, R^2 , and LOQ values based on average values for all 13 analytes in the saturated hydrocarbons analysis.

APPI sources provided detection at levels comparable to other reported methods.²⁴

1.4.7. Saturated Hydrocarbon Ionization

Saturated hydrocarbons present a unique analytical challenge since soft, atmospheric pressure chemical ionization processes typically rely on protonation for positive mode, which is not amenable to these analytes. Although GC-APPI sources generate photons to be absorbed by analytes for direct ionization and are amenable to hydrocarbon analyses, EI remains the most common ionization source used for alkane analyses. Due to the generation of a mixture of positively charged fragments, neutral radicals, and radical cations; EI sources produce several characteristic m/z values: a low intensity $[M]^{+\bullet}$, a “ski-slope” pattern of small carbenium ions, and repeating peaks 14 m/z units apart.²⁵ In 1993, Bell and coworkers used an APCI source to ionize gas phase alkanes and reported $[M - 1]^+$ and $[M - 3]^+$ as characteristic ions, most likely generated via proton transfer pathways.²⁶ With these considerations, it was found that the GC-APCI ionized saturated hydrocarbons with a “ski-slope” pattern of small ions analogous to that of EI for each analyte (Appendix Figure 1.12). The ions closest in mass to the parent alkane were $[M - 1.0073]^+$ and $[M + 12.9707]^+$, and the latter species were on average $17.1 \pm 5.5\%$ of the base peak. The relative abundance of the $[M - 1.0073]^+$ ions increased and the relative abundance of the $[M + 12.9707]^+$ ions decreased as the analytes increased in molecular weight (Appendix Table 1.11). The most abundant “ski-slope” ion for all compounds has a mass of 57.0707 m/z and an average relative abundance of $96.6 \pm 7.1\%$ (Appendix Table 1.11). The $[M - 1.007276]^+$ ion could be detected for tetradecane and larger hydrocarbons, although with a very low relative abundance of no more than 3.3%. Overall, APCI produced characteristic alkane fragments similar to those observed with EI as well as unique species.

The APPI also produced ions with reproducible trends. The base peak for this ionization source was $[M + 28.9655]^+$, although $[M + 12.9707]^+$ species were also observed for each analyte with an average relative abundance of $23.9 \pm 2.2\%$ (Appendix Table 1.11). The relative abundances of the “ski-slope” ions also increased with increasing molecular weight of the analyte (Appendix Table 1.11). Finally, the most prominent ion ($69.0706\ m/z$) only had a maximum relative abundance of 10% for lighter compounds and this peak decreased as the molecular weight of the analyte increased. Although APPI provides unique adducts for saturated hydrocarbons, identifying the analytes from complex samples using mass alone could be challenging since the ions would be hard to distinguish from those arising from oxidized hydrocarbons.

1.4.8. Role of Ionization in Identifying Unknowns

The analysis of complex mixtures still remains a challenge, and MS has become an important tool for measuring the entire molecular content of many systems. One of the biggest challenges faced in these experiments is the identification of analytes, which is hindered by a lack of knowledge concerning their structure as well as issues with detecting unmodified, intact molecules due to factors such as in-source fragmentation, oxidation, and adduct formation.²³ When considering unknown chemical species in a complex mixture, another important facet is also the confidence to accurately predict how the unknown will be ionized. For example, a single analyte can often be ionized as the result of protonation, deprotonation, the loss of an electron, etc., and a mix of ionization types can be observed under more energetic ionization conditions. Beyond this, fragmentation and rearrangement of the ions can occur, and this phenomenon is much more prevalent under harder ionization conditions.²⁷ Current databases and computation resources that incorporate tools, such as Fiehn’s seven golden rules,²⁸ to help predict molecular formulae and/or structure require knowledge of predicted ionization mechanisms in order to determine the neutral mass of the

detected ions and often require the user to input the expected neutral mass for each compound. Therefore, it is critical to not only understand the ionization chemistry when dealing with mixtures of unknown compounds but also to use ionization sources that produce reasonable populations of intact ions. With these criteria in mind, the APCI source is well suited for such experiments as it reliably produced $[M + H]^+$ species for the majority of analytes with LOQs that are similar to or better than both the APPI source studied and other reported methods. Although saturated hydrocarbons posed a slightly more difficult analytical challenge due to the inability to reliably produce $[M + H]^+$ ions, molecular ion peaks were still produced by APCI. When they were not the base peak, the molecular ions still had 16–50% of the intensity as the most abundant ion whereas, the populations of these ions for analytes analyzed by the APPI source were < 1% that of the base peak. Further investigation into the identity and mechanistic formation of these $[M + 12.9707]^+$ and $[M + 28.9655]^+$ species is ongoing.

1.5. Conclusion

These data show that GC-APCI-MS offers an alternative to traditional GC-EI-MS, and that the interface of this source to an Orbitrap utilizing the Ion Max housing is inexpensive and requires only temporary and simple modification of the instrument. Therefore, both LC and GC can be used on the same MS with minimal down time between analyses. Further, the analytical performance of the GC-APCI source compares favorably with both EI and APPI sources, while also providing relatively soft ionization of the analytes. Compounds of varying polarity (FAMES, PAHs, and alkanes) that are routinely analyzed by GC-MS were studied, and as an underutilized technique in GC-MS analyses, APCI shows great potential for the detection of both known and unknown analytes.

1.6. Appendix

Preliminary interface

Reagents and Materials

Carbon dioxide compressed gas was of ultrahigh purity grade and purchased from Airgas (Madison, WI). Low thermal expansion alumina-silicate ceramic sheets and rod were purchased from McMaster-Carr (Elmhurst, IL). Ceramic fiber heater and AF thermocouple were purchased from Watlow (St. Louis, MO). X2 digital PID benchtop temperature controller and power plug adaptor were purchased from Briskheat corporation (Columbus, OH). A 1/8" union tee fitting and a 1/16" to 1/8" reducer fitting were purchased from Swagelok (Solon, OH).

Interface Assembly

Design for the preliminary interface was influenced and modified from previous work by Lee and coworkers.²⁹ Lee and coworkers proposed an interface utilizing the Ion Max housing unit packaged with the Thermo Fisher Scientific Exactive™ Plus orbitrap, which permitted the modified unit to be swapped among all instruments that use the same housing unit (i.e. Thermo Fisher Scientific's Exactive™ Plus Orbitraps, Q Exactive™ Orbitraps, and LTQ Velos Triple Quadrupole). Moreover, their design consisted of a nitrogen gas line running through the GC into a union tee fitting that connected with the GC capillary column. Nitrogen served as the sheath gas and was heated to the appropriate temperature as several feet of copper tubing were placed inside the GC and flowed into the heated transfer line. However, Lee and coworkers focused on APPI ionization using a LIT orbitrap.

The preliminary interface (Supplementary Figure 1) made use of the heated sheath gas line similar to that described by Lee and coworkers in conjunction with a constructed transfer line and modification of the Ion Max housing unit. In brief, a ¼" hole was drilled into a faux detector module shipped with the GC and copper tubing was plumbed through the hole of the module. Inside the GC, several feet of copper tubing were coiled at the base of the oven and the copper tubing was attached to the shaft of the union tee fitting via a ⅛" Swagelok fitting. A ⅛" outer diameter, 30 cm stainless-steel tube, held in place with a 1/8" graphite ferrule, was inserted into one side of the union tee fitting. On the other side of the union tee fitting was a ⅛" to 1/16" reducer fitting in which the GC column was fed through and a 0.4mm graphite ferrule was used to secure the column in place. The end of the column passed through the side of the union tee with the reducer fitting and into the ⅛" stainless-steel tubing. The stainless-steel tube, containing the GC capillary column, exited out the side of the GC and through the fabricated transfer line. The transfer line was created using a Watlow ceramic heater and two, reducing end caps machined from aluminum silicate. One end of the transfer line sat in the precut duct of the GC oven, while the other end was inserted into the fabricated stainless-steel insert for the Ion Max window. Due to the size of the ceramic heater, the transfer line could only be inserted to a depth of four cm into the Ion Max housing. The temperature of the transfer line was controlled by a X2 digital PID temperature controller via a J-type thermocouple inserted through the reducing endcap on the GC side. For analyses, the temperature was set to the maximum allowed by the column in each method as the temperature tended to drift over time. Data acquisition occurred via the contact closure described in the final interface design, except sequences used in this setup were replicated on both instrument computers running Xcalibur, that is the computer controlling the GC and the computer controlling the orbitrap, as well as changing the instruments in use on the MS computer to only the Exactive™ Plus orbitrap.

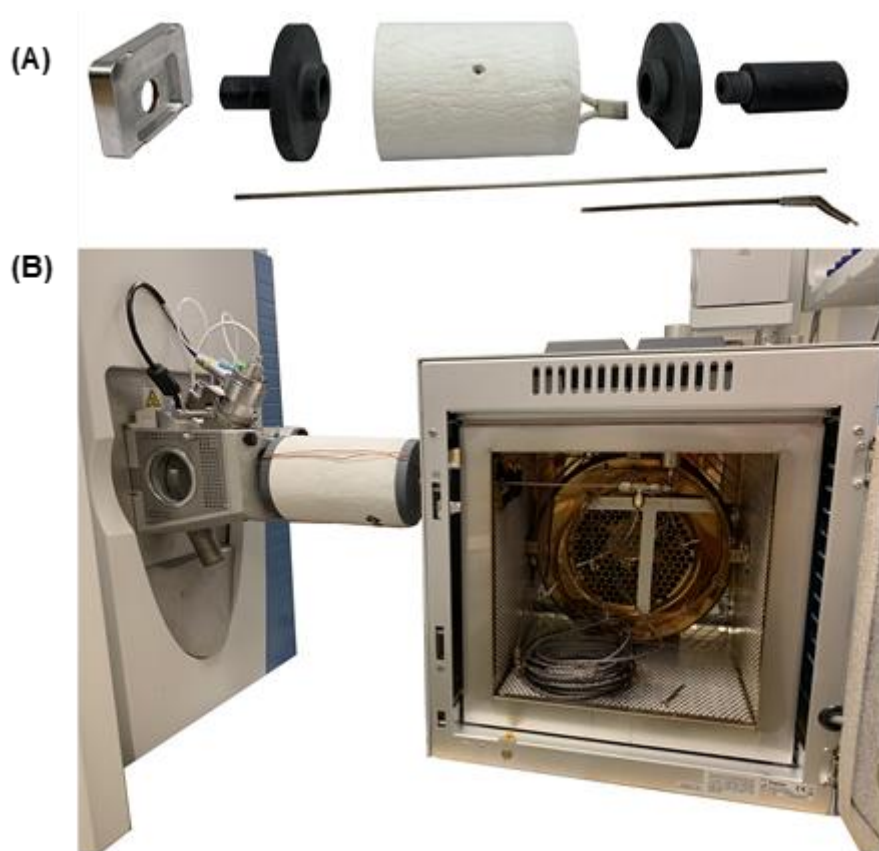


Figure 1.8. (a) Disassembled view of the transfer line and (b) full view of the preliminary interface. The nitrogen line coiled at the bottom of the GC oven was inserted through the bottom of the tee union and the GC capillary column was inserted into the right side of the tee union. The capillary column spanned from the tee union to the end of the transfer line.

Table 1.1. Different ions detected by APCI and APPI for FAME analysis.

Analyte	Retention Time Based on APPI (min)	Neutral Mass (AMU)	Most intense APCI ion (m/z)	Corresponding Neutral loss from Most Intense APCI ion (m/z)	Most intense APPI ion (m/z)	Corresponding Neutral loss from Most Intense APPI ion (AMU)
Methyl Butyrate	1.50	102.0681	103.0758	-1.0077	103.0758	N.A.
Methyl Hexanoate	2.40	130.0994	131.1067	-1.0073	131.1067	N.A.
Methyl Octanoate	4.85	158.1307	159.1379	-1.0072	159.1378	N.A.
Methyl Decanoate	8.64	186.1620	187.1692	-1.0072	187.1691	N.A.
Methyl Undecanoate	10.13	200.1776	200.1771	0.0005	201.1848	N.A.
Methyl Laurate	11.45	214.1933	215.2005	-1.0072	215.2005	N.A.
Methyl Tridecanoate	12.64	228.2089	229.2162	-1.0073	229.2159	N.A.
Methyl Myristate	13.74	242.2246	242.2238	0.0008	197.1899	45.0347
Methyl Myristoleate	14.11	240.2089	208.1822	32.0267	208.1822	32.0267
Methyl Pentadecanoate	14.77	256.2402	256.2396	0.0006	211.2055	45.0347
Methyl <i>Cis</i> -10-pentadecenoate	15.12	254.2246	222.1978	32.0268	222.1977	32.0269
Methyl Palmitate	15.74	270.2559	270.2552	0.0007	225.2212	45.0347
Methyl Palmitoleate	15.97	268.2402	236.2134	32.0268	236.2132	32.0270
Methyl Heptadecanoate	16.67	284.2715	284.2708	0.0007	239.2368	45.0347
<i>Cis</i> -10-Heptadecanoic Acid Methyl Ester	16.90	282.2559	250.2290	32.0269	250.2289	32.0270
Methyl Stearate	17.56	298.2872	298.2865	0.0007	253.2524	45.0348
Methyl Oleate/Elaidate	17.73	296.2715	264.2446	32.0269	264.2446	32.0269
Methyl Linolelaidate	18.10	294.2559	294.2553	0.0006	294.2551	0.0008
Methyl Linoleate	18.14	294.2559	294.2553	0.0006	294.2551	0.0008
Methyl Linolenate	18.36	292.2402	292.2396	0.0006	292.2393	0.0009
Methyl γ -linolenate	18.64	292.2402	292.2395	0.0007	292.2393	0.0009

Table 1.1. continued

Analyte	Retention Time Based on APPI (min)	Neutral Mass (AMU)	Most intense APCI ion (m/z)	Corresponding Neutral loss from Most Intense APCI ion (m/z)	Most intense APPI ion (m/z)	Corresponding Neutral loss from Most Intense APPI ion (AMU)
Methyl Arachidate	19.23	326.3185	326.3180	0.0005	281.2838	45.0347
Methyl <i>Cis</i> -11-Eicosenoate	19.39	324.3028	292.2760	32.0268	292.2759	32.0269
<i>Cis</i> -11,14-Eicosadienoic Acid Methyl Ester	19.77	322.2872	322.2867	0.0005	322.2865	0.0007
<i>Cis</i> -11,14,17-Eicosatrienoic Acid Methyl Ester	19.98	320.2715	320.2710	0.0005	320.2708	0.0007
Methyl Heneicosanoate	20.03	340.3341	340.3335	0.0006	295.2995	45.0346
<i>Cis</i> -5,8,11,14-Eicosatetraenoic acid methyl ester	20.16	318.2559	318.2553	0.0006	318.2552	0.0007
<i>Cis</i> -8,11,14-Eicosatrienoic Acid Methyl Ester	20.27	320.2715	320.2710	0.0005	320.2708	0.0007
<i>Cis</i> -5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	20.66	316.2402	316.2396	0.0006	316.2396	0.0006
Methyl Behenate	20.81	354.3498	354.3490	0.0008	309.3150	45.0348
Methyl Erucate	20.97	352.3341	320.3072	32.0269	320.3072	32.0269
<i>Cis</i> -13,16-Docosadienoic Acid Methyl Ester	21.34	350.3185	350.3178	0.0007	350.3176	0.0009
Methyl Tricosanoate	21.59	368.3654	368.3650	0.0004	323.3305	45.0349
Methyl Lignocerate	22.49	382.3811	382.3804	0.0007	337.3462	45.0349
<i>Cis</i> -4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	22.65	342.2559	342.2553	0.0006	342.2551	0.0008
Methyl Nervonate	22.71	380.3654	348.3386	32.0268	348.3384	32.027

Table 1.2. Analytical values generated by APCI for FAMES analysis

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APCI Ion (m/z)	Sensitivity of [M +H] ⁺ from APCI	Sensitivity of Most Abundant APCI Ion	APCI [M +H] ⁺ R ² Value	APCI Most Abundant Ion R ² Value	LOQ APCI [M+H] ⁺ (nM)	LOQ APCI Most Abundant ion (nM)
Methyl Butyrate	1.50	102.0681	103.0758	1.0711	1.0711	0.9984	0.9984	393.0	393.0
Methyl Hexanoate	2.40	130.0994	131.1067	1.0351	1.0351	0.9995	0.9995	308.0	308.0
Methyl Octanoate	4.85	158.1307	159.1379	1.0349	1.0349	0.9998	0.9998	253.0	253.0
Methyl Decanoate	8.64	186.1620	187.1692	1.0542	1.0542	0.9997	0.9997	215.0	215.0
Methyl Undecanoate	10.13	200.1776	200.1771	1.0811	1.0811	0.9990	0.9990	99.8	99.8
Methyl Laurate	11.45	214.1933	215.2005	1.0796	1.0796	0.9997	0.9970	187.0	187.0
Methyl Tridecanoate	12.64	228.2089	229.2162	1.0719	1.0719	0.9993	0.9993	88.0	88.0
Methyl Myristate	13.74	242.2246	242.2238	1.0957	1.0952	0.9996	0.9998	165.0	165.0
Methyl Myristoleate	14.11	240.2089	208.1822	1.0773	1.1275	0.9991	0.9990	83.6	83.6
Methyl Pentadecanoate	14.77	256.2402	256.2396	1.0763	1.1587	0.9975	0.9775	78.0	78.0
Methyl Cis-10-pentadecenoate	15.12	254.2246	222.1978	1.0472	1.1084	0.9969	0.9996	78.6	78.6
Methyl Palmitate	15.74	270.2559	270.2552	1.1355	1.0976	0.9990	0.9998	222.0	222.0
Methyl Palmitoleate	15.97	268.2402	236.2134	1.1005	1.1351	0.9991	0.9995	74.9	74.9
Methyl Heptadecanoate	16.67	284.2715	284.2708	1.1059	1.0974	0.9989	0.9992	70.7	70.7
Cis-10-Heptadecanoic Acid Methyl Ester	16.90	282.2559	250.2290	1.0832	1.1262	0.9991	0.9989	70.8	70.8
Methyl Stearate	17.56	298.2872	298.2865	1.3454	1.0805	0.8966	0.9998	134.0	134.0

Table 1.2. continued

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APCI Ion (m/z)	Sensitivity of [M +H] ⁺ from APCI	Sensitivity of Most Abundant APCI Ion	APCI [M +H] ⁺ R ² Value	APCI Most Abundant Ion R ² Value	LOQ APCI [M+H] ⁺ (nM)	LOQ APCI Most Abundant ion (nM)
Methyl Oleate/Elaidate	17.73	296.2715	264.2446	1.0948	1.3034	0.9995	0.8830	135.0	135.0
Methyl Linolelaidate	18.10	294.2559	294.2553	1.1345	1.0989	0.9939	0.9955	68.3	68.3
Methyl Linoleate	18.14	294.2559	294.2553	1.1983	1.1949	0.9924	0.9924	68.3	68.3
Methyl Linolenate	18.36	292.2402	292.2396	1.1145	1.0957	0.9961	0.9992	68.7	68.7
Methyl γ-linolenate	18.64	292.2402	292.2395	1.1205	1.1070	0.9974	0.9994	68.7	68.7
Methyl Arachidate	19.23	326.3185	326.3180	1.1220	1.0139	0.9936	0.9987	123.0	123.0
Methyl <i>Cis</i> -11-Eicosenoate	19.39	324.3028	292.2760	1.0479	1.0601	0.9954	0.9984	61.9	61.9
<i>Cis</i> -11,14-Eicosadienoic Acid Methyl Ester	19.77	322.2872	322.2867	1.1399	1.0663	0.9926	0.9993	62.0	62.0
<i>Cis</i> -11,14,17-Eicosatrienoic Acid Methyl Ester	19.98	320.2715	320.2710	1.1240	1.0772	0.9965	0.9998	62.4	62.4
Methyl Heneicosanoate	20.03	340.3341	340.3335	1.1175	1.0040	0.9903	0.9985	58.7	58.7
<i>Cis</i> -5,8,11,14-Eicosatetraenoic acid methyl ester	20.16	318.2559	318.2553	1.0985	1.0748	0.9963	0.9994	63.1	63.1
<i>Cis</i> -8,11,14-Eicosatrienoic Acid Methyl Ester	20.27	320.2715	320.2710	1.1345	1.0657	0.9965	0.9988	62.7	62.7
<i>Cis</i> -5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	20.66	316.2402	316.2396	1.1117	1.1117	0.9970	0.9990	63.5	63.5
Methyl Behenate	20.81	354.3498	354.3490	1.2111	1.0646	0.9934	0.9993	113.0	113.0

Table 1.2. continued

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APCI Ion (m/z)	Sensitivity of [M +H] ⁺ from APCI	Sensitivity of Most Abundant APCI Ion	APCI [M +H] ⁺ R ² Value	APCI Most Abundant Ion R ² Value	LOQ APCI [M+H] ⁺ (nM)	LOQ APCI Most Abundant ion (nM)
Methyl Erucate	20.97	352.3341	320.3072	1.0547	1.0415	0.9953	0.9981	57.0	57.0
<i>Cis</i>-13,16-Docosadienoic Acid Methyl Ester	21.34	350.3185	350.3178	1.1748	1.0368	0.9921	0.9986	57.0	57.0
Methyl Tricosanoate	21.59	368.3654	368.3650	1.2458	1.0810	0.9952	0.9992	54.3	54.3
Methyl Lignocerate	22.49	382.3811	382.3804	1.2589	1.0813	0.9915	0.9996	105.0	105.0
<i>Cis</i>-4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	22.65	342.2559	342.2553	1.0164	1.0042	0.9878	0.9947	58.7	58.7
Methyl Nervonate	22.71	380.3654	348.3386	1.0896	1.0819	0.9970	0.9992	52.5	52.5

Table 1.3. Analytical values generated by APPI for FAMES analysis

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APPI Ion (m/z)	Sensitivity of [M +H] ⁺ from APPI	Sensitivity of Most Abundant APPI Ion	APPI [M +H] ⁺ R ² Value	APPI Most Abundant Ion R ² Value	LOQ APPI [M+H] ⁺ (nM)	LOQ APPI Most Abundant ion (nM)
Methyl Butyrate	1.50	102.0681	103.0758	1.4523	1.4523	0.9829	0.9829	393.0	393.0
Methyl Hexanoate	2.40	130.0994	131.1067	1.0544	1.0544	0.9944	0.9944	308.0	308.0
Methyl Octanoate	4.85	158.1307	159.1378	1.1458	1.1458	0.9717	0.9717	253.0	253.0
Methyl Decanoate	8.64	186.1620	187.1691	0.9848	0.9848	0.9978	0.9978	215.0	215.0
Methyl Undecanoate	10.13	200.1776	201.1848	1.0174	1.0174	0.9966	0.9966	99.8	99.8
Methyl Laurate	11.45	214.1933	215.2005	0.9890	0.9890	0.9961	0.9961	187.0	187.0
Methyl Tridecanoate	12.64	228.2089	229.2159	1.0573	1.0573	0.9922	0.9222	88.0	88.0
Methyl Myristate	13.74	242.2246	197.1899	0.9904	1.0387	0.9990	0.9987	165.0	165.0
Methyl Myristoleate	14.11	240.2089	208.1822	1.0133	1.0773	0.9946	0.9965	83.6	83.6
Methyl Pentadecanoate	14.77	256.2402	211.2055	1.0877	1.1700	0.9829	0.9721	78.0	78.0
Methyl <i>Cis</i> -10-pentadecenoate	15.12	254.2246	222.1977	1.0491	1.0264	0.9902	0.9985	78.6	78.6
Methyl Palmitate	15.74	270.2559	225.2212	0.9946	1.0131	0.9988	0.9973	222.0	222.0
Methyl Palmitoleate	15.97	268.2402	236.2132	0.9927	1.0086	0.9993	0.9992	74.9	74.9
Methyl Heptadecanoate	16.67	284.2715	239.2368	1.0542	1.0840	0.9835	0.9943	70.7	70.7
<i>Cis</i> -10-Heptadecanoic Acid Methyl Ester	16.90	282.2559	250.2289	1.0376	1.0472	0.9936	0.9969	70.8	70.8
Methyl Stearate	17.56	298.2872	253.2524	0.9923	1.0186	0.9951	0.9984	134.0	134.0

Table 1.3. continued

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APPI Ion (m/z)	Sensitivity of [M +H] ⁺ from APPI	Sensitivity of Most Abundant APPI Ion	APPI [M +H] ⁺ R ² Value	APPI Most Abundant Ion R ² Value	LOQ APPI [M+H] ⁺ (nM)	LOQ APPI Most Abundant ion (nM)
Methyl Oleate/Elaidate	17.73	296.2715	264.2446	0.9573	0.9948	0.9988	0.9969	135.0	135.0
Methyl Linolelaidate	18.10	294.2559	294.2551	0.9173	1.0461	0.9674	0.9942	68.3	68.3
Methyl Linoleate	18.14	294.2559	294.2551	1.0318	1.0320	0.9696	0.9982	68.3	68.3
Methyl Linolenate	18.36	292.2402	292.2393	0.6627	0.9995	0.8200	0.9982	68.7	68.7
Methyl γ-linolenate	18.64	292.2402	292.2393	1.1409	1.0008	0.9947	0.9980	68.7	68.7
Methyl Arachidate	19.23	326.3185	281.2838	0.9471	1.0293	0.9957	0.9952	123.0	123.0
Methyl <i>Cis</i> -11-Eicosenoate	19.39	324.3028	292.2759	0.9177	1.0124	0.9934	0.9976	61.9	61.9
<i>Cis</i> -11,14-Eicosadienoic Acid Methyl Ester	19.77	322.2872	322.2865	0.9071	1.0411	0.9550	0.9963	62.0	62.0
<i>Cis</i> -11,14,17-Eicosatrienoic Acid Methyl Ester	19.98	320.2715	320.2708	0.6109	1.0013	0.8343	0.9978	62.4	62.4
Methyl Heneicosanoate	20.03	340.3341	295.2995	0.9788	1.1308	0.9883	0.9860	58.7	58.7
<i>Cis</i> -5,8,11,14-Eicosatetraenoic acid methyl ester	20.16	318.2559	318.2552	0.5793	0.9936	0.7276	0.9963	63.1	63.1
<i>Cis</i> -8,11,14-Eicosatrienoic Acid Methyl Ester	20.27	320.2715	320.2708	0.7226	0.9936	0.7726	0.9981	62.7	62.7
<i>Cis</i> -5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	20.66	316.2402	316.2396	0.7304	0.9840	0.8932	0.9970	63.5	63.5
Methyl Behenate	20.81	354.3498	309.3150	0.9105	1.0423	0.9958	0.9949	113.0	113.0

Table 1.3. continued

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APPI Ion (m/z)	Sensitivity of [M +H] ⁺ from APPI	Sensitivity of Most Abundant APPI Ion	APPI [M +H] ⁺ R ² Value	APPI Most Abundant Ion R ² Value	LOQ APPI [M+H] ⁺ (nM)	LOQ APPI Most Abundant ion (nM)
Methyl Erucate	20.97	352.3341	320.3072	0.8721	1.0094	0.9838	0.9972	57.0	57.0
<i>Cis</i> -13,16-Docosadienoic Acid Methyl Ester	21.34	350.3185	350.3176	0.8355	1.0625	0.9531	0.9963	57.0	57.0
Methyl Tricosanoate	21.59	368.3654	323.3305	1.0823	1.1008	0.9757	0.9907	54.3	54.3
Methyl Lignocerate	22.49	382.3811	337.3462	0.9572	1.1016	0.9932	0.9917	105.0	105.0
<i>Cis</i> -4,7,10,13,16,19-Docosahexaenoic Acid Methyl Ester	22.65	342.2559	342.2551	0.9336	1.0044	0.9936	0.9974	58.7	58.7
Methyl Nervonate	22.71	380.3654	348.3384	0.9014	1.0044	0.9827	0.9976	52.5	52.5

Table 1.4. Analytical values generated by APCI for PAHs analysis

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APCI Ion (m/z)	Sensitivity of [M +H] ⁺ from APCI	Sensitivity of Most Abundant APCI Ion	APCI [M +H] ⁺ R ² Value	APCI Most Abundant Ion R ² Value	LOQ APCI [M+H] ⁺ (nM)	LOQ APCI Most Abundant ion (nM)
Naphthalene	2.33	128.0626	128.0620	1.0391	1.0828	0.9992	0.9990	784	78.4
Acenaphthylene	6.37	152.0626	152.0619	1.0407	1.0884	0.9976	0.9997	65.7	65.7
Acenaphthene	6.79	154.0783	154.0775	1.0331	1.2314	0.9872	0.9903	650	65
Fluorene	8.40	166.0783	166.0776	1.0869	1.0802	0.9963	0.9996	602	60.2
Phenanthrene	12.02	178.0783	178.0775	1.0551	1.1182	0.9961	0.9988	281	28.1
Anthracene	12.10	178.0783	178.0775	1.0190	1.1140	0.9841	0.9993	561	56.1
Fluoranthene	16.02	202.0783	202.0776	1.0707	1.1097	0.9808	0.9950	247	24.7
Pyrene	16.92	202.0783	202.0776	1.0699	1.0846	0.9892	0.9995	247	24.7
Benzo(a)anthracene	21.12	228.0939	228.0932	1.0905	1.1409	0.9959	0.9955	219	21.9
Chrysene	21.41	228.0939	228.0930	1.1031	1.0895	0.9960	0.9992	219	21.9
Benzo(b)fluoranthene	27.40	252.0939	252.0930	1.1977	1.1257	0.9965	0.9983	19.8	19.8
Benzo(k)fluoranthene	27.60	252.0939	252.0932	1.2000	1.1147	0.9931	0.9990	19.9	19.9
Benzo(a)pyrene	28.93	252.0939	252.0930	1.1744	1.0968	0.9971	0.9989	198	19.8
Indeno(1,2,3-c,d)pyrene	32.01	276.0939	276.0931	1.1744	1.1267	0.9971	0.9966	181	18.1
Dibenz(a,h)anthracene	32.09	278.1096	278.1085	1.1591	1.1187	0.9964	0.9992	181	18.1
Benzo(g,h,i)perylene	33.20	276.0939	276.0933	1.1592	1.1225	0.9968	0.9983	182	18.2

Table 1.5. Analytical values generated by APPI for PAHs analysis

Analyte	Retention Time based on APPI (min)	[M] (m/z)	Most Abundant APPI Ion (m/z)	Sensitivity of [M +H] ⁺ from APPI	Sensitivity of Most Abundant APPI Ion	APPI [M +H] ⁺ R ² Value	APPI Most Abundant Ion R ² Value	LOQ APPI [M+H] ⁺ (nM)	LOQ APPI Most Abundant ion (nM)
Naphthalene	2.33	128.0626	128.0621	0.5430	1.0063	0.8964	0.9994	7840	78.4
Acenaphthylene	6.37	152.0626	152.0619	0.9171	0.9969	0.9750	0.9994	657	65.7
Acenaphthene	6.79	154.0783	154.0775	0.7113	1.0820	0.9804	0.9950	6500	65
Fluorene	8.40	166.0783	166.0775	0.3722	1.0026	0.8641	0.9994	6020	60.2
Phenanthrene	12.02	178.0783	178.0775	0.7857	0.9808	0.8904	0.9998	2810	28.1
Anthracene	12.10	178.0783	178.0775	1.5529	1.0002	0.8894	0.9991	56100	56.1
Fluoranthene	16.02	202.0783	202.0775	0.2011	0.9805	0.5282	0.9994	2470	24.7
Pyrene	16.92	202.0783	202.0775	0.4355	0.9733	0.8226	0.9995	2470	24.7
Benzo(a)anthracene	21.12	228.0939	228.0930	0.3047	0.9446	0.6862	0.9986	2190	21.9
Chrysene	21.41	228.0939	228.0930	0.1135	0.9114	0.1801	0.9962	2190	21.9
Benzo(b)fluoranthene	27.40	252.0939	252.0929	0.4452	0.9598	0.7360	0.9989	1980	19.8
Benzo(k)fluoranthene	27.60	252.0939	252.0929	0.4498	0.9598	0.7399	0.9989	1990	19.9
Benzo(a)pyrene	28.93	252.0939	252.0929	0.3457	0.9620	0.6925	0.9993	1980	19.8
Indeno(1,2,3-c,d)pyrene	32.01	276.0939	276.0928	0.6444	1.0196	0.9081	0.9971	181	18.1
Dibenz(a,h)anthracene	32.09	278.1096	278.1086	0.5348	0.9371	0.8198	0.9982	181	18.1
Benzo(g,h,i)perylene	33.20	276.0939	276.0928	0.6702	0.9948	0.9207	0.9989	182	18.2

Table 1.6. Analytical values generated by APCI for saturated hydrocarbons analysis.

Analyte	Retention Time based on APPI (min)	Most abundant alkane fragment [M-H] ⁺ (m/z)	Second Most Abundant APCI Ion (non-fragment) (m/z)	Sensitivity of Alkane Fragment from APCI	Sensitivity of Second Most Abundant APCI Ion	APCI Alkane Fragment R ² Value	APCI Second Most Abundant Ion R ² Value	LOQ APCI Alkane Fragment (μM)	LOQ APCI Second Most Abundant (μM)
Octane	3.21	57.0708	127.1119	0.3393	0.6412	0.4497	0.9617	3.5	3.5
Nonane	5.04	57.0707	141.1274	1.0989	1.0529	0.9827	0.9964	3.1	3.1
Decane	7.17	57.0708	155.1429	1.3288	1.0134	0.9895	0.9985	2.8	2.8
Undecane	8.78	57.0708	169.1586	1.1911	1.0450	0.8960	0.9970	2.6	2.6
Dodecane	10.06	57.0708	183.1743	1.2358	1.0469	0.9958	0.9988	2.3	2.3
Tridecane	11.10	57.0707	197.1900	1.2704	1.0485	0.9924	0.9990	2.2	2.2
Tetradecane	12.06	57.0708	211.2056	1.2274	1.0707	0.9974	0.9997	2.0	2.0
Pentadecane	12.91	57.0708	225.2212	1.2913	1.1810	0.9886	0.9838	1.9	1.9
Hexadecane	13.70	57.0708	239.2368	1.2434	1.0755	0.9787	0.9952	1.8	1.8
Heptadecane	14.43	71.0863	253.2524	1.2750	1.0995	0.9913	0.9981	1.7	1.7
Octadecane	15.12	71.0863	267.2682	1.1859	1.0872	0.9818	0.9982	1.6	1.6
Nonadecane	15.78	71.0863	281.2837	1.2322	1.1165	0.9931	0.9930	1.5	1.5
Icosane	16.41	71.0863	295.2993	1.1508	1.0991	0.9986	0.9988	1.4	1.4

Table 1.7. Analytical values generated by APPI for saturated hydrocarbons analysis continued.

Analyte	Retention Time based on APPI (min)	Most abundant alkane fragment [M-H] ⁺ (m/z)	Second Most Abundant APPI Ion (non-fragment) (m/z)	Sensitivity of Alkane Fragment from APPI	Sensitivity of Second Most Abundant APPI Ion	APPI Alkane Fragment R ² Value	APPI Second Most Abundant Ion R ² Value	LOQ APPI Alkane Fragment (μM)	LOQ APPI Second Most Abundant (μM)
Octane	3.21	69.0706	143.1066	1.1833	1.0709	0.3900	0.8790	3.5	3.5
Nonane	5.04	69.0706	157.1222	0.8540	1.0709	0.8930	0.8790	3.1	3.1
Decane	7.17	69.0706	171.1378	1.2004	1.2478	0.9185	0.9994	2.8	2.8
Undecane	8.78	69.0706	185.1535	1.1188	1.2660	0.9583	0.9975	2.6	2.6
Dodecane	10.06	69.0706	199.1691	1.1357	1.2676	0.9805	0.9989	2.3	2.3
Tridecane	11.10	69.0706	213.1848	1.4145	1.2434	0.9741	0.9979	2.2	2.2
Tetradecane	12.06	69.0706	227.2003	1.1361	1.2474	0.9265	0.9994	2.0	2.0
Pentadecane	12.91	69.0706	241.2160	1.2002	1.2507	0.9934	0.9992	1.9	1.9
Hexadecane	13.70	69.0706	255.2316	1.0895	1.2497	0.9922	0.9995	1.8	1.8
Heptadecane	14.43	69.0706	269.2472	1.1196	1.2712	0.9374	0.9997	1.7	1.7
Octadecane	15.12	69.0706	283.2628	1.1312	1.2380	0.9706	0.9997	1.6	1.6
Nonadecane	15.78	69.0706	297.2785	1.3081	1.2359	0.9705	0.9997	1.5	1.5
Icosane	16.41	69.0706	311.2941	1.1494	1.2099	0.9757	0.9997	1.4	1.4

Table 1.8. Different ions detected by APCI and APPI for saturated hydrocarbons analysis.

Analyte	Retention Time (min)	Neutral Mass (AMU)	Most intense APCI ion (m/z)	Corresponding Adduct from Second Most Intense APCI ion (m/z)	Most intense APPI (m/z)	Corresponding Adduct from Most Intense APPI ion (m/z)	Difference Between Each Adduct
Octane	3.21	114.1409	127.1119	12.9710	143.1066	28.9657	15.9947
Nonane	5.04	128.1565	141.1274	12.9709	157.1222	28.9657	15.9948
Decane	7.17	142.1722	155.1429	12.9707	171.1378	28.9656	15.9949
Undecane	8.78	156.1878	169.1586	12.9708	185.1535	28.9657	15.9949
Dodecane	10.06	170.2035	183.1743	12.9708	199.1691	28.9656	15.9948
Tridecane	11.10	184.2191	197.1900	12.9709	213.1848	28.9657	15.9948
Tetradecane	12.06	198.2348	211.2056	12.9708	227.2003	28.9655	15.9947
Pentadecane	12.91	212.2504	225.2212	12.9708	241.2160	28.9656	15.9948
Hexadecane	13.70	226.2661	239.2368	12.9707	255.2316	28.9655	15.9948
Heptadecane	14.43	240.2817	253.2524	12.9707	269.2472	28.9655	15.9948
Octadecane	15.12	254.2974	267.2682	12.9708	283.2628	28.9654	15.9946
Nonadecane	15.78	268.3130	281.2837	12.9707	297.2785	28.9655	15.9948
Icosane	16.41	282.3287	295.2993	12.9706	311.2941	28.9654	15.9948

Table 1.9. Most abundant ion species generated by APCI and APPI for FAMES analysis

Analyte	APCI				APPI			
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity
Methyl Butyrate	1.5	82.9456	100	[M+H] ⁺	1.55	103.0757	100	[M+H] ⁺
		84.9425	68.75					
		103.0757	9.21					
Methyl Hexanoate	2.4	131.1067	100	[M+H] ⁺	2.4	131.1067	100	[M+H] ⁺
Methyl Octanoate	4.87	159.1379	100	[M+H] ⁺	4.87	159.1378	100	[M+H] ⁺
Methyl Decanoate	8.64	187.1689	100	[M+H] ⁺	8.65	187.1691	100	[M+H] ⁺
		143.1066	67.87	[M-C ₇ H ₁₃] ⁺				
Methyl Undecanoate	10.14	201.1845	100	[M+H] ⁺	10.14	201.1848	100	[M+H] ⁺
		200.1771	53.95	[M] ^{+•}		155.1429	53.73	[M-C ₂ H ₅ O] ⁺
Methyl Laurate	11.47	215.2000	100	[M+H] ⁺	11.46	215.2005	100	[M+H] ⁺
		214.1928	84.26	[M] ^{+•}		169.1586	68.92	[M-C ₂ H ₅ O] ⁺
Methyl Tridecanoate	12.66	229.2154	100	[M+H] ⁺	12.65	183.1742	100	[M-C ₂ H ₅ O] ⁺
		228.2084	99.32	[M] ^{+•}		229.2160	96.06	[M+H] ⁺
Methyl Myristate	13.76	242.2240	100	[M] ^{+•}	13.76	197.1899	100	[M-C ₂ H ₅ O] ⁺
		243.2240	78.65	[M+H] ⁺		243.2317	79.92	[M+H] ⁺
Methyl Myristoleate	14.12	208.1822	100	[M-CH ₃ OH] ^{+•}	14.11	208.1820	100	[M-CH ₃ OH] ^{+•}
		241.2158	91.45	[M+H] ⁺		241.2159	37.51	[M+H] ⁺
		141.0910	60.34	[M-C ₇ H ₁₅] ⁺				
		209.1895	50.1	[M-CH ₃ O] ^{+•}				
		240.2083	91.45	[M] ^{+•}				
Methyl Pentadecanoate	14.78	256.2396	100	[M] ^{+•}	14.77	211.2055	100	[M-C ₂ H ₅ O] ⁺
		257.2461	66.02	[M+H] ⁺		257.2472	69.94	[M+H] ⁺

Table 1.9. continued

Analyte	APCI				APPI			
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity
Methyl <i>Cis</i> -10-pentadecenoate	15.14	222.1978	100	[M-CH ₃ OH] ⁺ •	15.13	222.1977	100	[M-CH ₃ OH] ⁺ •
		255.2313	87.67	[M+H] ⁺		255.2316	33.97	[M+H] ⁺
		141.0910	54.86	[M-C ₈ H ₁₇] ⁺				
		254.2239	47.34	[M] ⁺ •				
Methyl Palmitate	15.77	270.2552	100	[M] ⁺ •	15.76	225.2212	100	[M-C ₂ H ₅ O] ⁺
		271.2614	61.84	[M+H] ⁺		271.2629	63.67	[M+H] ⁺
Methyl Palmitoleate	15.98	236.2134	100	[M-CH ₃ OH] ⁺ •	15.98	236.2132	100	[M-CH ₃ OH] ⁺ •
		269.2469	90.43	[M+H] ⁺		269.2471	33.35	[M+H] ⁺
		268.2396	54.42	[M] ⁺ •				
		141.0910	51.31	[M-C ₉ H ₁₉] ⁺				
Methyl Heptadecanoate	16.69	284.2709	100	[M] ⁺ •	16.67	239.2367	100	[M-C ₂ H ₅ O] ⁺
		285.2770	56.99	[M+H] ⁺		285.2785	52.11	[M+H] ⁺
<i>Cis</i> -10-Heptadecanoic Acid Methyl Ester	16.91	250.2290	100	[M-CH ₃ OH] ⁺ •	16.9	250.2289	100	[M-CH ₃ OH] ⁺ •
		283.2624	83.87	[M+H] ⁺		283.2628	31.72	[M+H] ⁺
		282.2552	55.85	[M] ⁺ •				
		141.0910	44.28	[M-C ₁₀ H ₂₁] ⁺				
Methyl Stearate	17.57	298.2866	100	[M] ⁺ •	17.58	253.2524	100	[M-C ₂ H ₅ O] ⁺
		299.2924	53	[M+H] ⁺		299.2942	55.34	[M+H] ⁺
						281.2472	44.44	[M-CH ₅] ²⁺ •

Table 1.9. continued

Analyte	APCI				APPI			
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity
Methyl Oleate/Elaidate	17.74	264.2447	100	[M-CH ₃ OH] ^{•+}	17.74	264.2445	100	[M-CH ₃ OH] ^{•+}
		297.2780	76.01	[M+H] ⁺		297.2785	31.79	[M+H] ⁺
		296.2708	58.17	[M] ^{•+}				
Methyl Linolelaidate	18.11	294.2553	100	[M] ^{•+}	18.11	294.2547	100	[M] ^{•+}
		295.2612	51.23	[M+H] ⁺		293.2473	65.09	[M-H] ^{2•+}
						262.2289	54.01	[M-CH ₃ OH] ^{•+}
						295.2625	14.51	[M+H] ⁺
Methyl Linoleate	18.15	294.2553	100	[M] ^{•+}	18.14	294.2546	100	[M] ^{•+}
		295.2613	50.06	[M+H] ⁺		293.2473	78.74	[M-H] ^{2•+}
						262.2289	50.38	[M-CH ₃ OH] ^{•+}
						295.2624	14.49	[M+H] ⁺
Methyl Linolenate	18.36	292.2395	100	[M] ^{•+}	18.36	292.2393	100	[M] ^{•+}
		293.2457	58.55	[M+H] ⁺		293.2428	18.77	[M+H] ⁺
Methyl γ-linolenate	18.63	292.2397	100	[M] ^{•+}	18.64	292.2393	100	[M] ^{•+}
		293.2459	62.89	[M+H] ⁺		293.2428	18.39	[M+H] ⁺
Methyl Arachidate	19.25	326.3180	100	[M] ^{•+}	19.25	281.2836	100	[M-C ₂ H ₅ O] ⁺
		327.3235	49.96	[M+H] ⁺		327.3253	55.91	[M+H] ⁺
						309.2784	45.95	[M-CH ₅] ^{2•+}
Methyl <i>Cis</i> -11-Eicosenoate	19.4	292.2760	100	[M-CH ₃ OH] ^{•+}	19.4	292.2757	100	[M-CH ₃ OH] ^{•+}
		325.3092	61.7	[M+H] ⁺		325.3097	25.17	[M+H] ⁺
		324.3021	49.4	[M] ^{•+}				

Table 1.9. continued

Analyte	APCI				APPI			
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity
Cis-11,14-Eicosadienoic Acid Methyl Ester	19.76	322.2867	100	[M] ^{••}	19.77	322.2859	100	[M] ^{••}
		323.2921	45.16	[M+H] ⁺		323.2899	17.02	[M+H] ⁺
Cis-11,14,17-Eicosatrienoic Acid Methyl Ester	19.96	320.2709	100	[M] ^{••}	19.98	320.2707	100	[M] ^{••}
		321.2770	57.57	[M+H] ⁺		321.2745	20.82	[M+H] ⁺
Methyl Heneicosanoate	20.03	340.3335	100	[M] ^{••}	20.05	295.2994	100	[M-C ₂ H ₅ O] ⁺
		341.3390	49.56	[M+H] ⁺		341.3411	52.55	[M+H] ⁺
						323.2942	43.8	[M-CH ₅] ^{2•+}
Cis-5,8,11,14-Eicosatetraenoic acid methyl ester	20.14	318.2553	100	[M] ^{••}	20.16	318.2550	100	[M] ^{••}
		319.2616	68.45	[M+H] ⁺		319.2593	25.05	[M+H] ⁺
Cis-8,11,14-Eicosatrienoic Acid Methyl Ester	20.26	320.2710	100	[M] ^{••}	20.28	320.2707	100	[M] ^{••}
		321.2770	56.91	[M+H] ⁺		321.2745	20.64	[M+H] ⁺
Cis-5,8,11,14,17-Eicosapentaenoic Acid Methyl Ester	20.64	316.2397	100	[M] ^{••}	20.67	316.2394	100	[M] ^{••}
		317.2462	75.47	[M+H] ⁺		317.2440	25.42	[M+H] ⁺
Methyl Behenate	20.8	354.3492	100	[M] ^{••}	20.84	309.3148	100	[M-C ₂ H ₅ O] ⁺
		355.3545	49.46	[M+H] ⁺		355.3566	64.87	[M+H] ⁺
						337.3097	47.32	[M-CH ₅] ^{2•+}
Methyl Erucate	20.95	320.3073	100	[M-CH ₃ OH] ^{••}	20.98	320.3070	100	[M-CH ₃ OH] ^{••}
		353.3404	51.35	[M+H] ⁺		353.3410	27.33	[M+H] ⁺
		352.3334	42.34	[M] ^{••}				

Table 1.9. continued

Analyte	APCI				APPI			
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity
Cis-13,16- Docosadienoic Acid Methyl Ester	21.3	350.3179	100	[M] ^{+•}	21.36	350.3173	100	[M] ^{+•}
		351.3232	45.73	[M+H] ⁺		318.2915	49.37	[M-CH ₃ OH] ^{+•}
						349.3099	48.65	[M-H] ^{2•+}
						351.3251	16.19	[M+H] ⁺
Methyl Tricosanoate	21.56	368.3649	100	[M] ^{+•}	21.6	323.3306	100	[M-C ₂ H ₅ O] ⁺
		369.3702	49.94	[M+H] ⁺		369.3724	70.07	[M+H] ⁺
						351.3254	42.53	[M-CH ₅] ^{2•+}
Methyl Lignocerate	22.44	382.3806	100	[M] ^{+•}	22.52	337.3461	100	[M-C ₂ H ₅ O] ⁺
		383.3857	48.58	[M+H] ⁺		383.3880	76.22	[M+H] ⁺
						365.3411	44.19	[M-CH ₅] ^{2•+}
Cis- 4,7,10,13,16,19- Docosahexaenoic Acid Methyl Ester	22.57	342.2554	100	[M] ^{+•}	22.65	342.2551	100	[M] ^{+•}
		343.2618	77.56	[M+H] ⁺		343.2599	27.77	[M+H] ⁺
Methyl Nervonate	22.65	348.3387	100	[M-CH ₃ OH] ^{+•}	22.73	348.3385	100	[M-CH ₃ OH] ^{+•}
		381.3716	42.34	[M+H] ⁺		381.3724	26.57	[M+H] ⁺
		380.3647	36.55	[M] ^{+•}				
		349.3434	34.65	[M-CH ₃ O] ^{+•}				

Table 1.10. Most abundant ion species generated by APCI and APPI for PAHs analysis

Analyte	APCI				APPI			
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity
Naphthalene	2.4	128.0621	100	[M] ^{••}	2.33	128.0620	100	[M] ^{••}
		129.0692	30.33	[M+H] ⁺				
Acenaphthylene	6.37	152.0619	100	[M] ^{••}	6.37	152.0618	100	[M] ^{••}
		153.0687	38.88	[M+H] ⁺				
Acenaphthene	6.9	154.0776	100	[M] ^{••}	6.79	154.0774	100	[M] ^{••}
		155.0842	39.71	[M+H] ⁺				
Fluorene	8.4	166.0776	100	[M] ^{••}	8.4	166.0774	100	[M] ^{••}
		167.0841	50	[M+H] ⁺				
Phenanthrene	12.01	178.0776	100	[M] ^{••}	12.02	178.0774	100	[M] ^{••}
		179.0839	46.94	[M+H] ⁺				
Anthracene	12.17	178.0776	100	[M] ^{••}	12.1	178.0774	100	[M] ^{••}
		179.0839	41.41	[M+H] ⁺				
Fluoranthene	16.16	202.0775	100	[M] ^{••}	16.02	202.0774	100	[M] ^{••}
		203.0837	40.12	[M+H] ⁺				
Pyrene	17.07	202.0776	100	[M] ^{••}	16.92	202.0774	100	[M] ^{••}
		203.0838	40.72	[M+H] ⁺				
Benzo(a)anthracene	21.29	228.0931	100	[M] ^{••}	21.12	228.093	100	[M] ^{••}
		229.0991	42.74	[M+H] ⁺				
Chrysene	21.61	228.0931	100	[M] ^{••}	21.41	228.093	100	[M] ^{••}
		229.0991	48.26	[M+H] ⁺				
Benzo(b)fluoranthene	27.81	252.093	100	[M] ^{••}	27.4	252.093	100	[M] ^{••}
		253.099	48.94	[M+H] ⁺				

Table 1.10. continued

Analyte	RT (min)	APCI			RT (min)	APPI		
		Ions (<i>m/z</i>)	Relative Abundance	Probable Identity		Ions (<i>m/z</i>)	Relative Abundance	Probable Identity
Benzo(k)fluoranthene	27.97	252.0931	100	[M] ^{••}	27.6	252.093	100	[M] ^{••}
		253.0989	47.45	[M+H] ⁺				
Benzo(a)pyrene	29.04	252.0931	100	[M] ^{••}	28.93	252.093	100	[M] ^{••}
		253.0989	45.48	[M+H] ⁺				
Indeno(1,2,3-c,d)pyrene	32.17	276.0931	96.25	[M] ^{••}	32.01	276.0931	100	[M] ^{••}
		277.0989	35.16	[M+H] ⁺				
Dibenz(a,h)anthracene	32.21	278.1084	100	[M] ^{••}	32.09	278.1084	100	[M] ^{••}
		279.1144	43.98	[M+H] ⁺				
Benzo(g,h,i)perylene	33.36	276.0931	100	[M] ^{••}	33.2	276.093	100	[M] ^{••}
		277.0988	45.93	[M+H] ⁺				

Table 1.11. Most abundant ion species generated by APCI and APPI for saturated hydrocarbons analysis

Analyte	APCI				RT	APPI		
	RT (min)	Ions (m/z)	Relative Abundance	Probable Identity		Ions (m/z)	Relative Abundance	Probable Identity
Octane	3.21	57.0707	100	[C ₄ H ₉] ⁺	3.24	143.1066	100	[M-3H+2O] ⁺
		85.1017	89.4	[C ₆ H ₁₃] ⁺		125.0962	26.23	[M-5H+O] ⁺
		69.0706	80.27	[C ₅ H ₉] ⁺		127.1118	23.63	[M-3H+O] ⁺
		71.0862	55.91	[C ₅ H ₁₁] ⁺		57.0707	1.21	[C ₄ H ₉] ⁺
		127.1119	12.45	[M-3H+O] ⁺		69.0706	10.02	[C ₅ H ₉] ⁺
Nonane	5.04	57.0707	100	[C ₄ H ₉] ⁺	5.05	157.1222	100	[M-3H+2O] ⁺
		69.0706	43.78	[C ₅ H ₉] ⁺		139.1116	23.8	[M-5H+O] ⁺
		141.1274	12.81	[M-3H+O] ⁺		141.1272	22.02	[M-3H+O] ⁺
						69.07066	8.08	[C ₅ H ₉] ⁺
Decane	7.17	57.0707	100	[C ₄ H ₉] ⁺	7.19	171.1377	100	[M-3H+2O] ⁺
		69.0706	42.63	[C ₅ H ₉] ⁺		153.1272	22.14	[M-5H+O] ⁺
		71.0862	36.34	[C ₅ H ₁₁] ⁺		155.1428	21.21	[M-3H+O] ⁺
		155.143	16.24	[M-3H+O] ⁺		69.0706	5.51	[C ₅ H ₉] ⁺
Undecane	8.78	57.0707	100	[C ₄ H ₉] ⁺	8.8	185.1534	100	[M-3H+2O] ⁺
		71.0862	51.75	[C ₅ H ₁₁] ⁺		169.1585	19.87	[M-3H+O] ⁺
		69.0706	45.19	[C ₅ H ₉] ⁺		167.1428	16.7	[M-5H+O] ⁺
		169.1586	19.77	[M-3H+O] ⁺		69.0706	4.01	[C ₅ H ₉] ⁺

Table 1.11. continued

Analyte	APCI				APPI			
	RT (min)	Ions (<i>m/z</i>)	Relative Abundance	Probable Identity	RT	Ions (<i>m/z</i>)	Relative Abundance	Probable Identity
Dodecane	10.06	57.0707	100	[C ₄ H ₉] ⁺	10.06	199.169	100	[M-3H+2O] ⁺
		71.0862	64.6	[C ₅ H ₁₁] ⁺		183.1741	20.97	[M-3H+O] ⁺
		69.0706	46.72	[C ₅ H ₉] ⁺		181.1584	14.49	[M-5H+O] ⁺
		85.1017	36.27	[C ₆ H ₁₃] ⁺		69.0705	3.87	[C ₅ H ₉] ⁺
		183.1743	26.38	[M-3H+O] ⁺				
Tridecane	11.1	57.0707	100	[C ₄ H ₉] ⁺	11.13	213.1847	100	[M-3H+2O] ⁺
		71.0862	73.12	[C ₅ H ₁₁] ⁺		197.1898	23.34	[M-3H+O] ⁺
		85.1017	46.01	[C ₆ H ₁₃] ⁺		214.1880	14.25	
		69.0706	45.24	[C ₅ H ₉] ⁺		69.0706	3.21	[C ₅ H ₉] ⁺
		197.1899	14.85	[M-3H+O] ⁺				
		183.2104	0.07	[M-H] ⁺				
Tetradecane	12.06	57.0707	100	[C ₄ H ₉] ⁺	12.07	227.2003	100	[M-3H+2O] ⁺
		71.0862	83.41	[C ₅ H ₁₁] ⁺		211.2055	25.34	[M-3H+O] ⁺
		85.1017	55.72	[C ₆ H ₁₃] ⁺		69.0706	2.77	[C ₅ H ₉] ⁺
		69.0706	47.61	[C ₅ H ₉] ⁺				
		211.2056	27.78	[M-3H+O] ⁺				
		197.2264	0.08	[M-H] ⁺				

Table 1.11. continued

Analyte	APCI				APPI			
	RT (min)	Ions (<i>m/z</i>)	Relative Abundance	Probable Identity	RT	Ions (<i>m/z</i>)	Relative Abundance	Probable Identity
Pentadecane	12.91	57.0707	100	[C ₄ H ₉] ⁺	12.91	241.2159	100	[M-3H+2O] ⁺
		71.0862	90.99	[C ₅ H ₁₁] ⁺		225.221	26.14	[M-3H+O] ⁺
		85.1017	64.5	[C ₆ H ₁₃] ⁺		69.0706	2.39	[C ₅ H ₉] ⁺
		69.0706	52	[C ₅ H ₉] ⁺				
		67.055	42.23	[C ₅ H ₇] ⁺				
		225.2212	14.75	[M-3H+O] ⁺				
		211.2417	0.23	[M-H] ⁺				
Hexadecane	13.7	57.0707	100	[C ₄ H ₉] ⁺	13.71	255.2315	100	[M-3H+2O] ⁺
		71.0862	95	[C ₅ H ₁₁] ⁺		239.2366	26	[M-3H+O] ⁺
		85.1017	69.37	[C ₆ H ₁₃] ⁺		69.0706	2.11	[C ₅ H ₉] ⁺
		69.0706	50.04	[C ₅ H ₉] ⁺				
		239.2368	21.16	[M-3H+O] ⁺				
		225.2575	0.55	[M-H] ⁺				
Heptadecane	14.43	71.0862	100	[C ₅ H ₁₁] ⁺	14.44	269.2472	100	[M-3H+2O] ⁺
		57.0707	99.54	[C ₄ H ₉] ⁺		253.2523	25.47	[M-3H+O] ⁺
		85.1017	75.15	[C ₆ H ₁₃] ⁺		69.0706	1.99	[C ₅ H ₉] ⁺
		69.0706	47.54	[C ₅ H ₉] ⁺				
		253.2524	17.44	[M-3H+O] ⁺				
		239.2732	0.97	[M-H] ⁺				

Table 1.11. continued

Analyte	APCI				APPI			
	RT (min)	Ions (<i>m/z</i>)	Relative Abundance	Probable Identity	RT	Ions (<i>m/z</i>)	Relative Abundance	Probable Identity
Octadecane	15.12	71.0862	100	[C ₅ H ₁₁] ⁺	15.15	283.2627	100	[M-3H+2O] ⁺
		57.0707	93.23	[C ₄ H ₉] ⁺		267.2678	25.52	[M-3H+O] ⁺
		85.1017	76.01	[C ₆ H ₁₃] ⁺		69.0705	1.97	[C ₅ H ₉] ⁺
		69.0706	43.31	[C ₅ H ₉] ⁺				
		267.2681	16.48	[M-3H+O] ⁺				
		253.2888	1.74	[M-H] ⁺				
Nonadecane	15.78	71.0862	100	[C ₅ H ₁₁] ⁺	15.81	297.2784	100	[M-3H+2O] ⁺
		57.0707	84.78	[C ₄ H ₉] ⁺		281.2835	25.67	[M-3H+O] ⁺
		85.1017	75.97	[C ₆ H ₁₃] ⁺		69.0706	1.87	[C ₅ H ₉] ⁺
		69.0706	40.02	[C ₅ H ₉] ⁺				
		281.2838	13.59	[M-3H+O] ⁺				
		267.6044	2.65	[M-H] ⁺				
Icosane	16.41	71.0862	100	[C ₅ H ₁₁] ⁺	16.45	311.2939	100	[M-3H+2O] ⁺
		57.0707	78.21	[C ₄ H ₉] ⁺		295.2991	25.74	[M-3H+O] ⁺
		85.1017	77.36	[C ₆ H ₁₃] ⁺		69.0705	1.81	[C ₅ H ₉] ⁺
		295.2995	8.04	[M-3H+O] ⁺				
		281.3201	3.3	[M-H] ⁺				

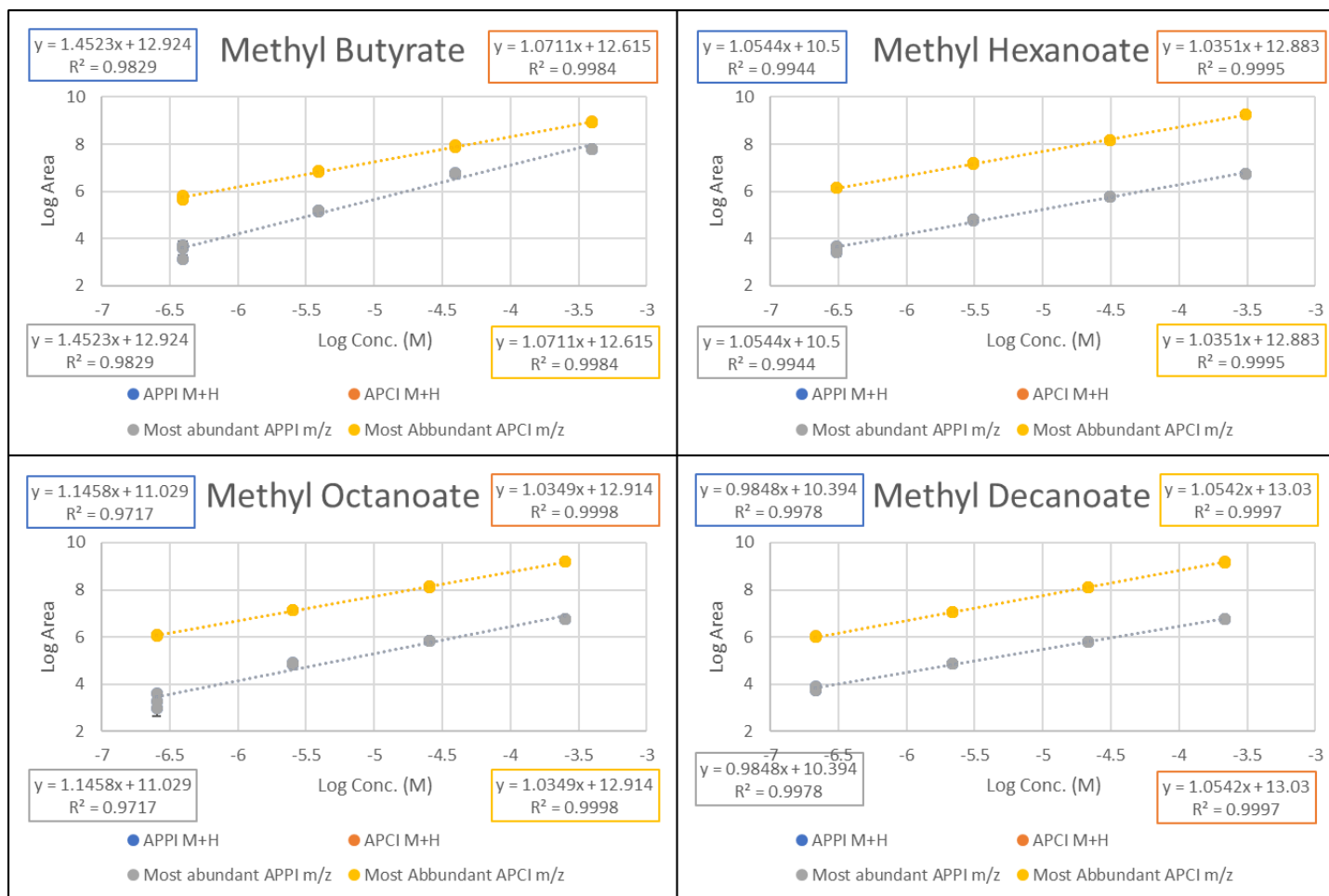


Figure 1.9. FAMES calibration curves for $[M + H]^+$ and most abundant ion (m/z) for each ionization technique. If there are only two curves, then the $[M + H]^+$ ion was the most abundant ion.

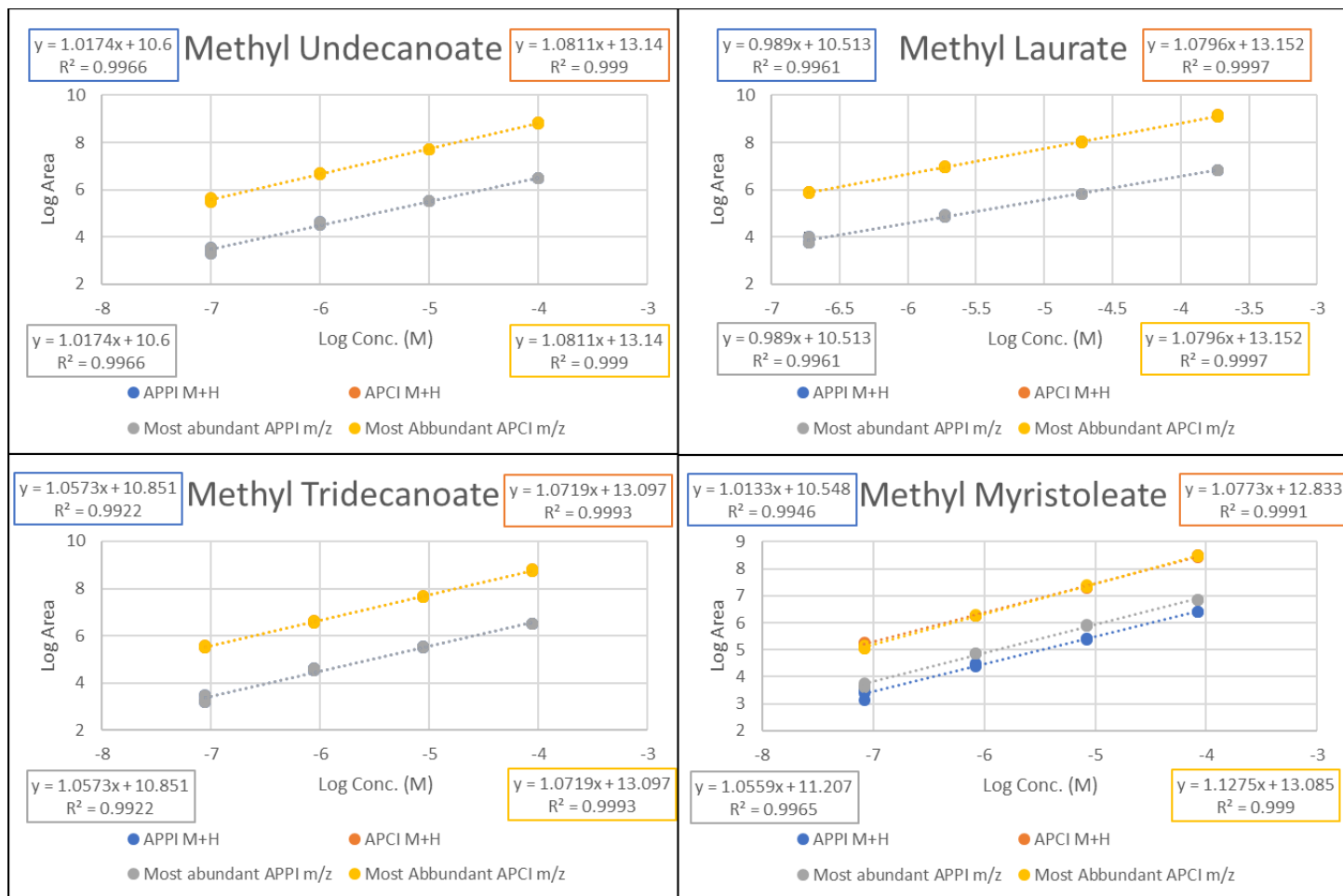


Figure 1.9. continued

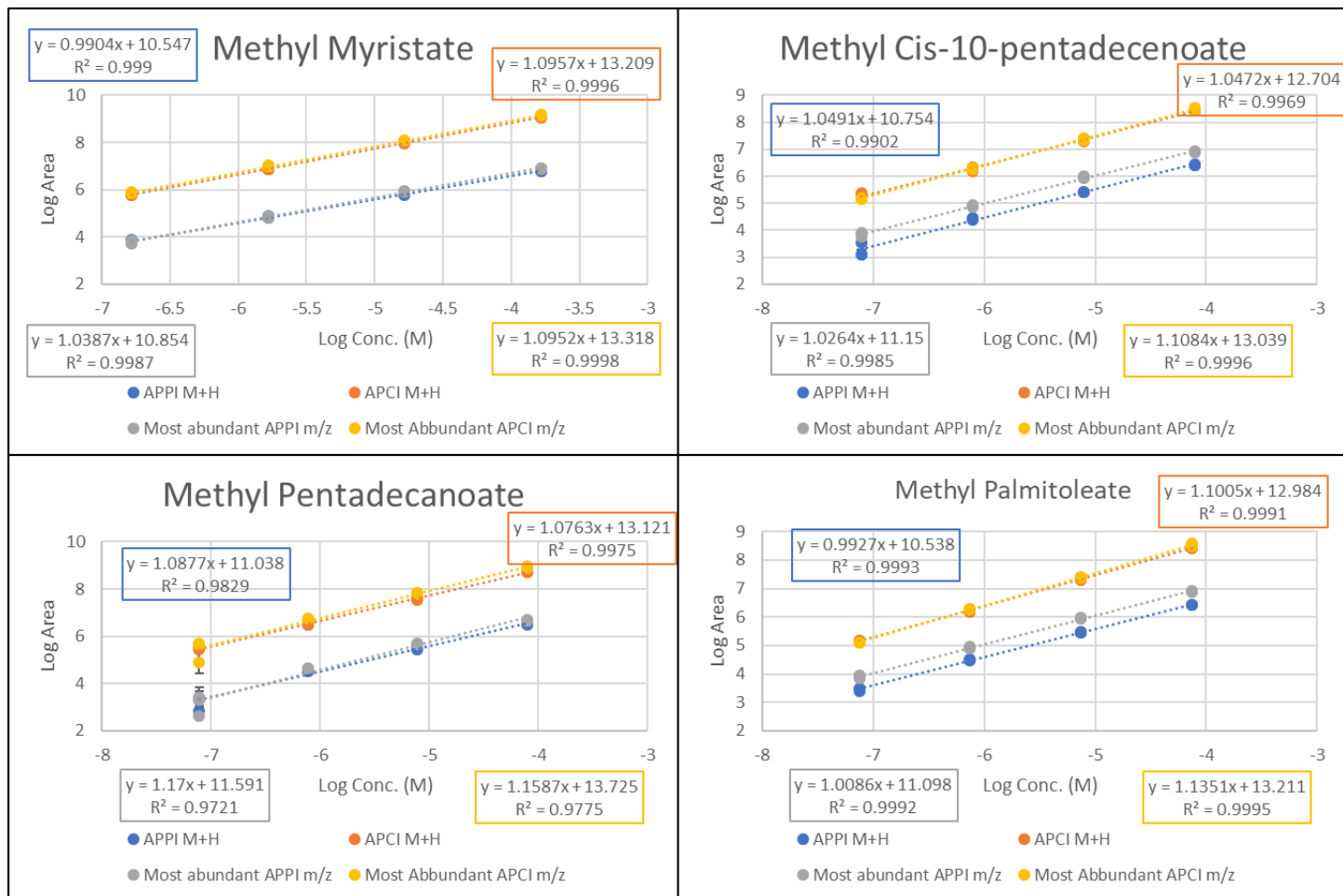


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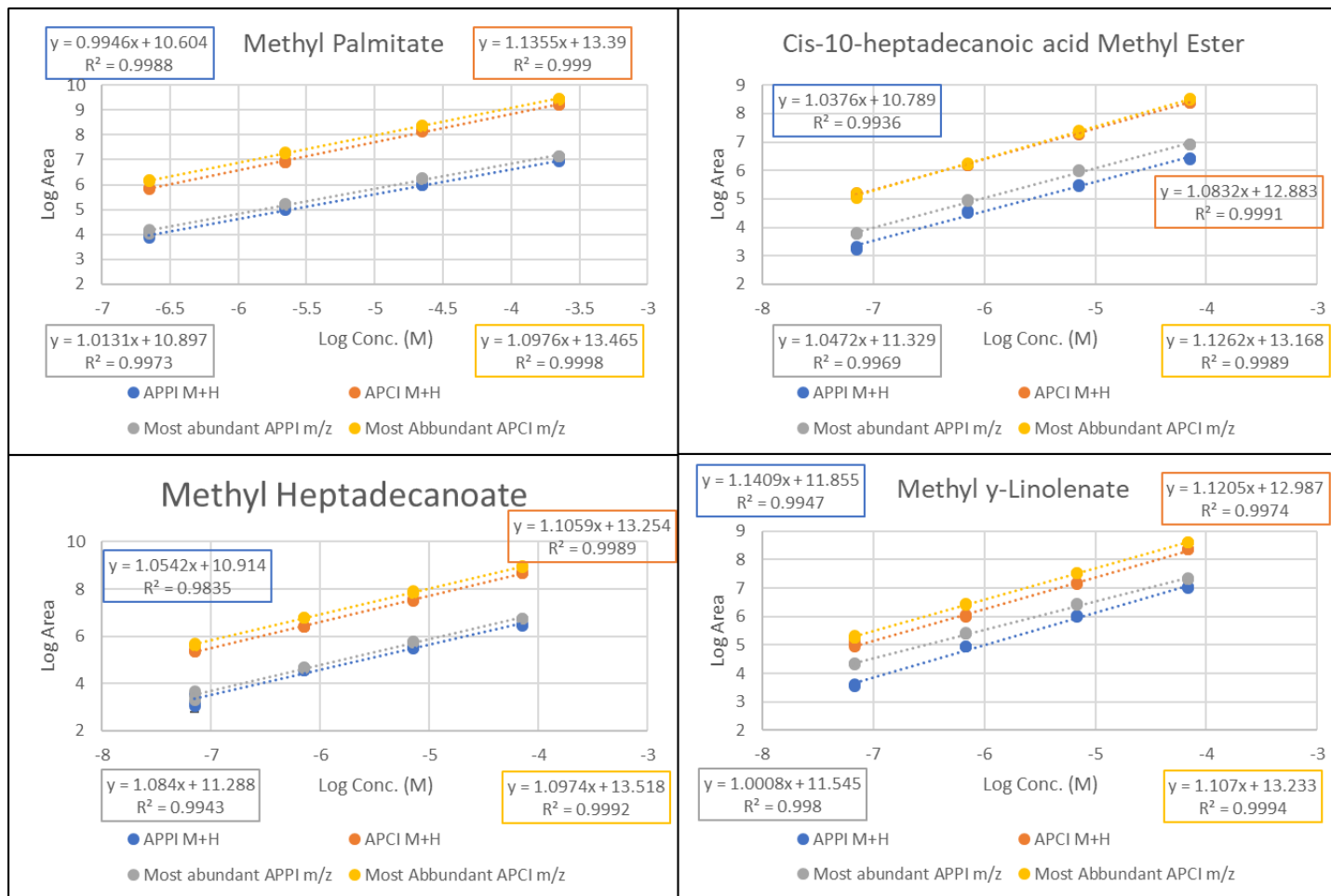


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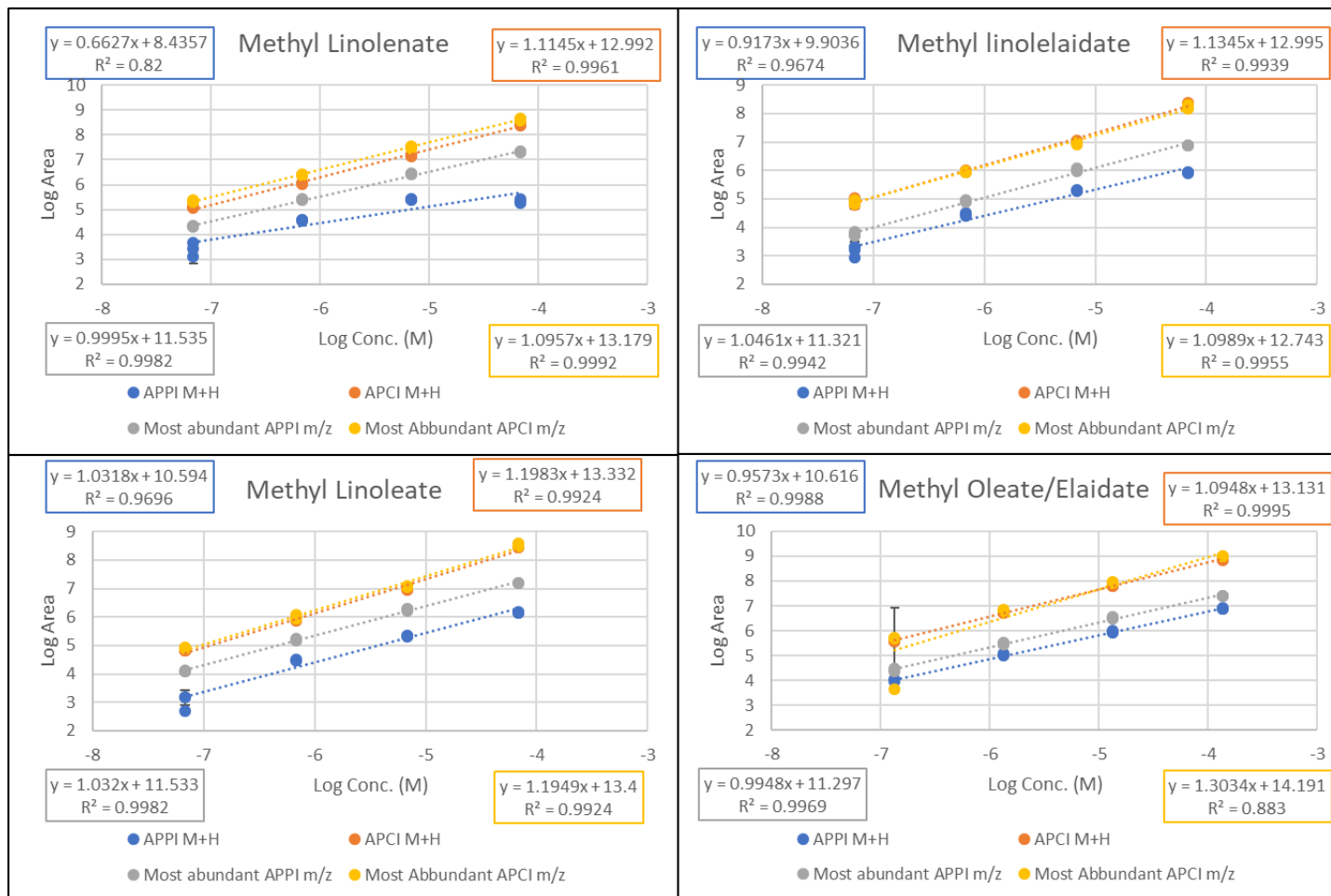


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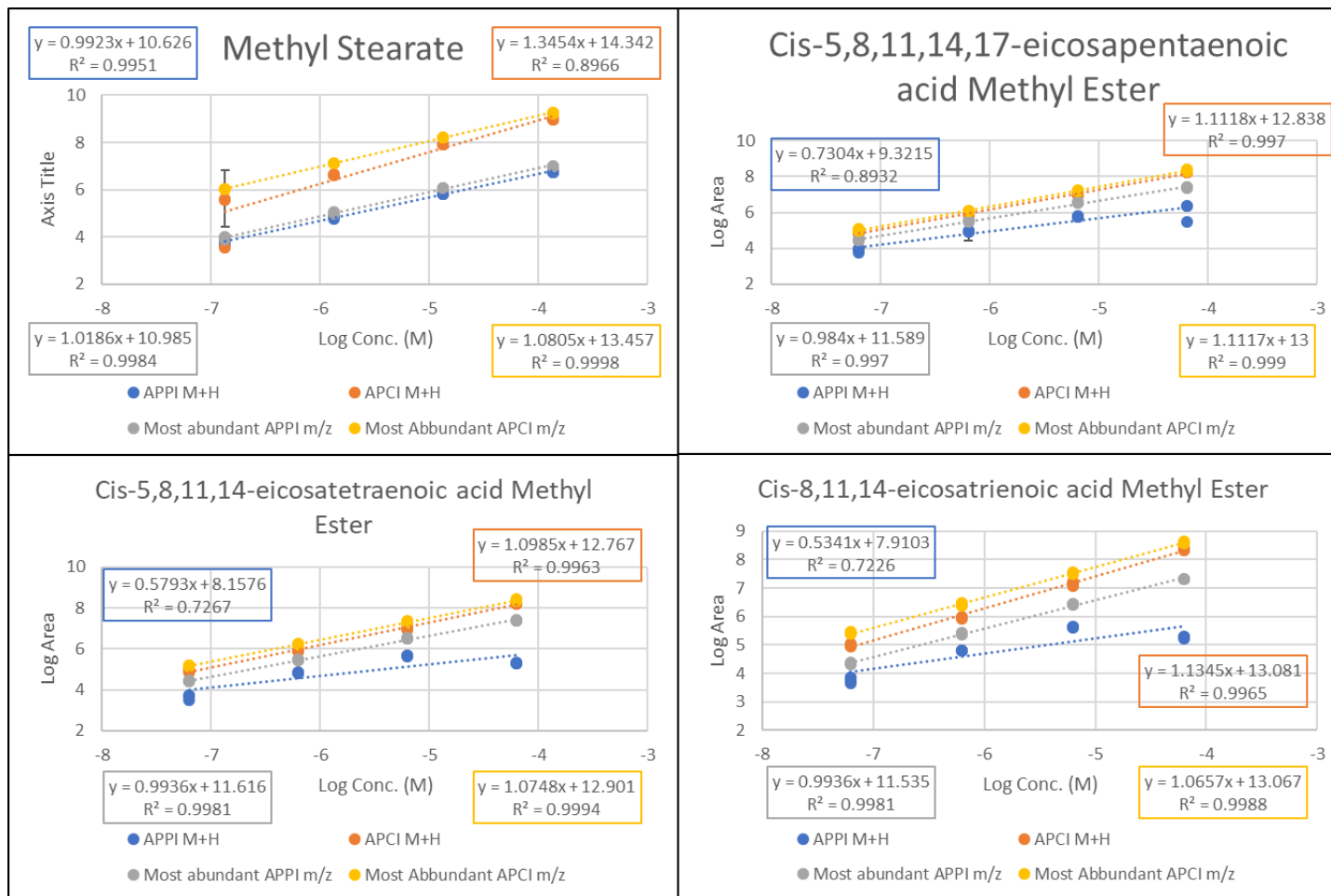


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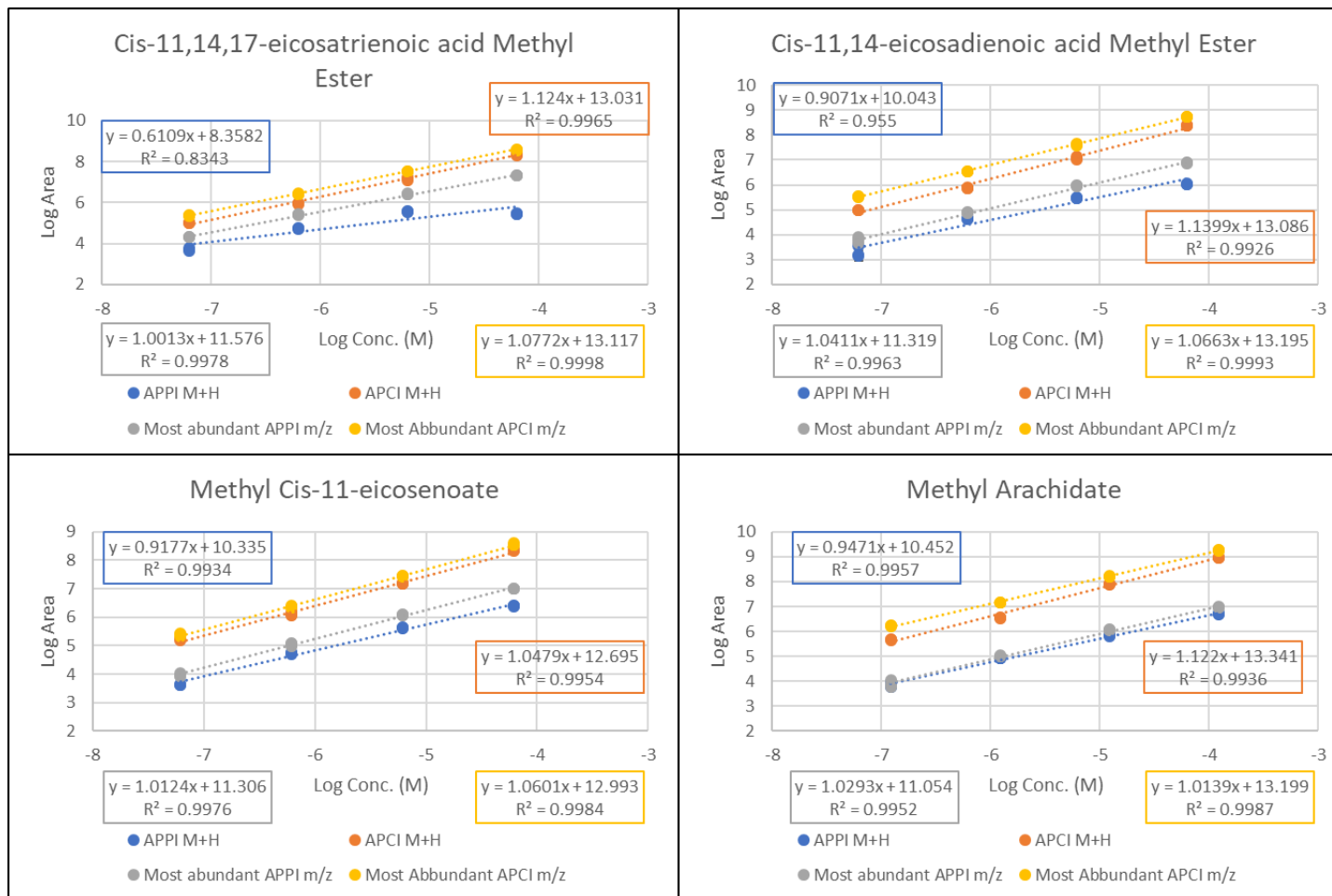


Figure 1.9. continued

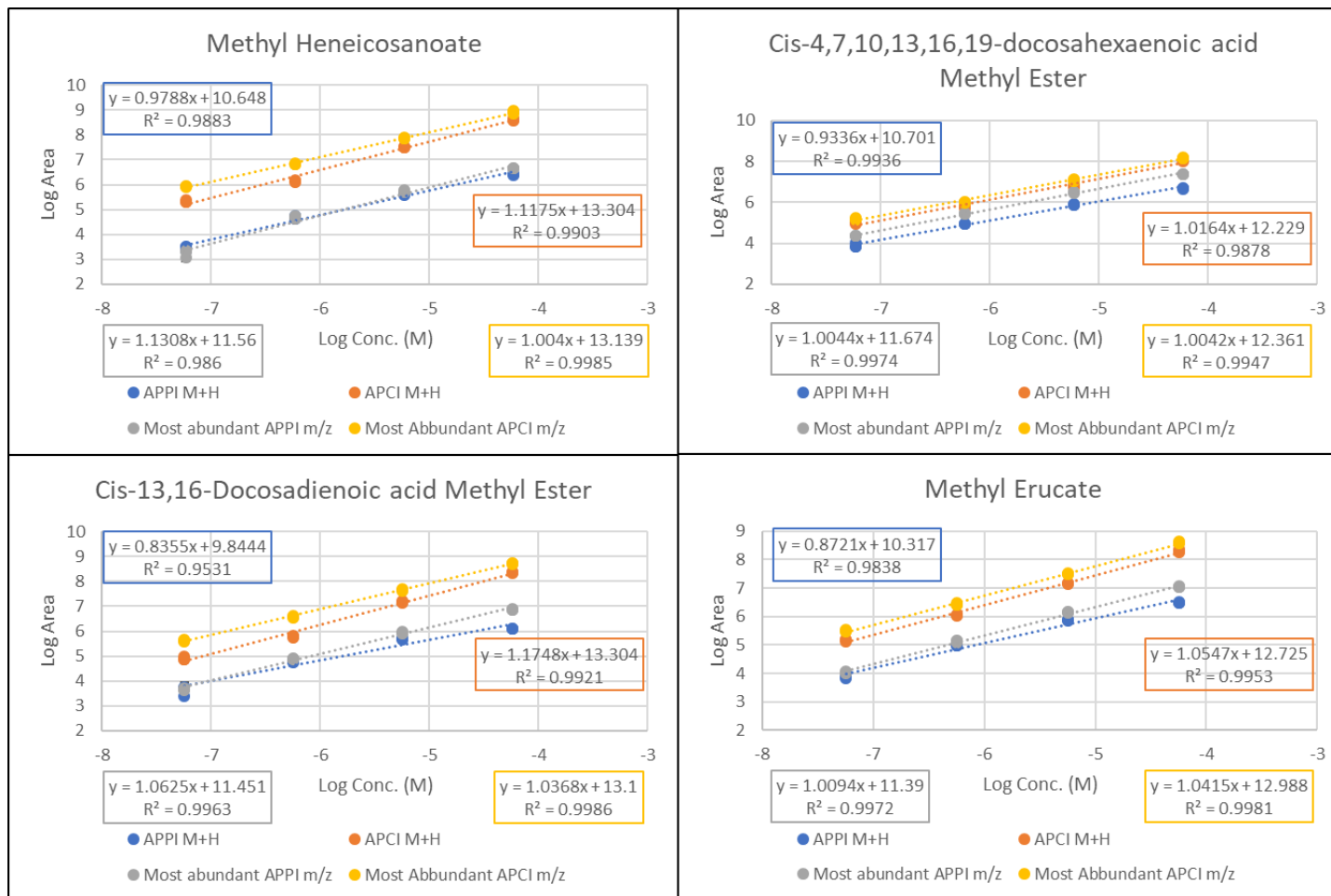


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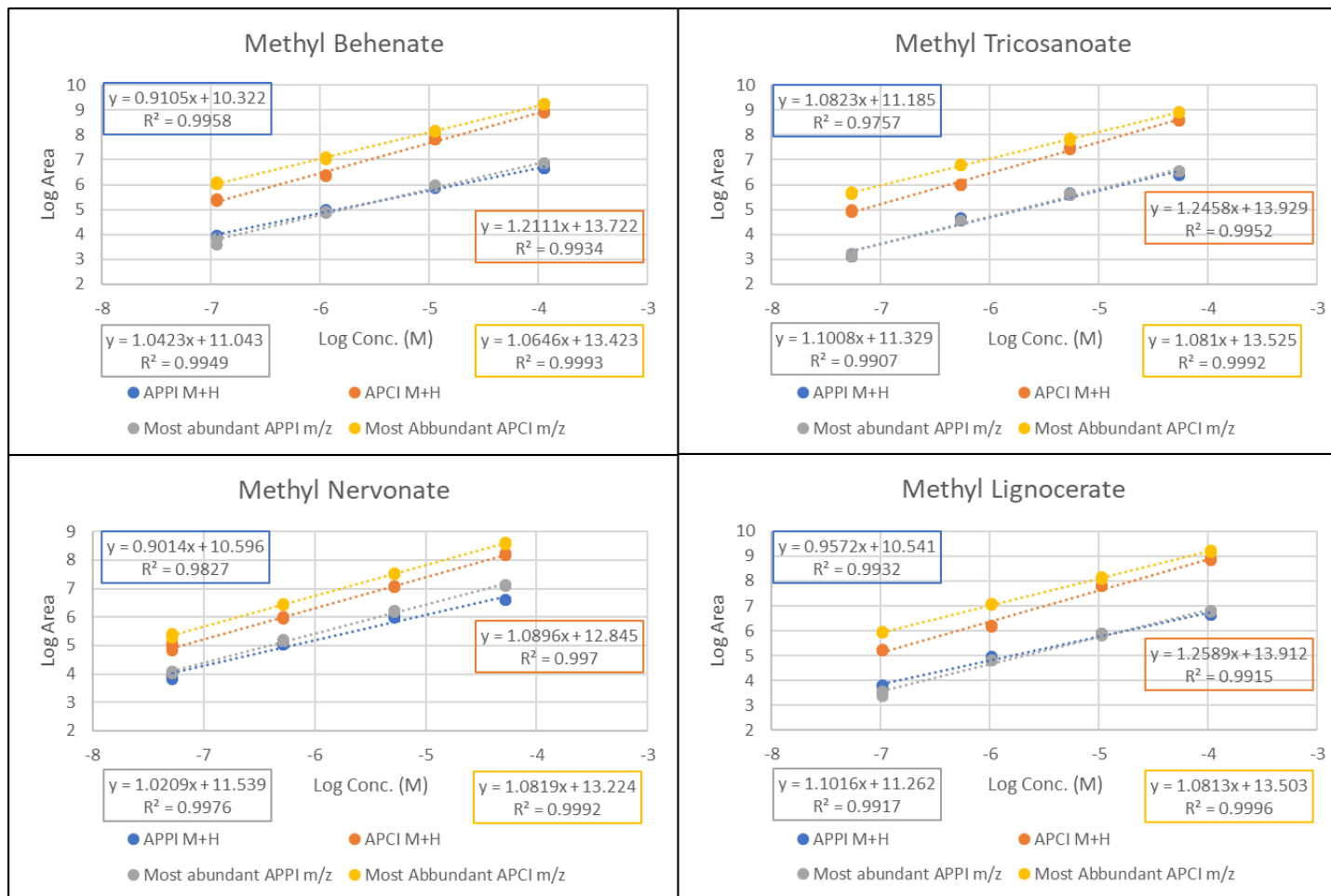


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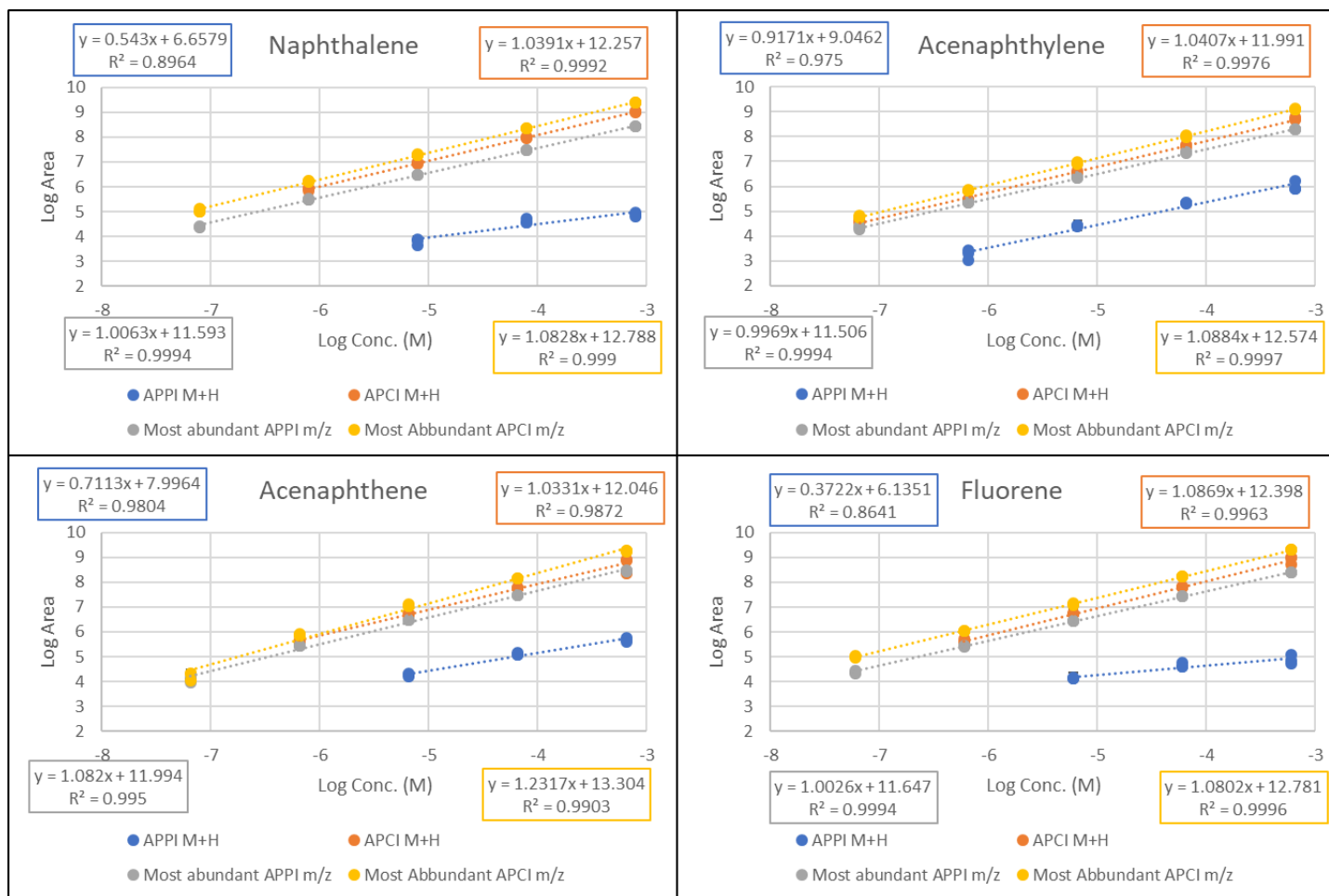


Figure 1.10. PAHs calibration curves for $[M + H]^+$ and most abundant ions (m/z) for each ionization technique

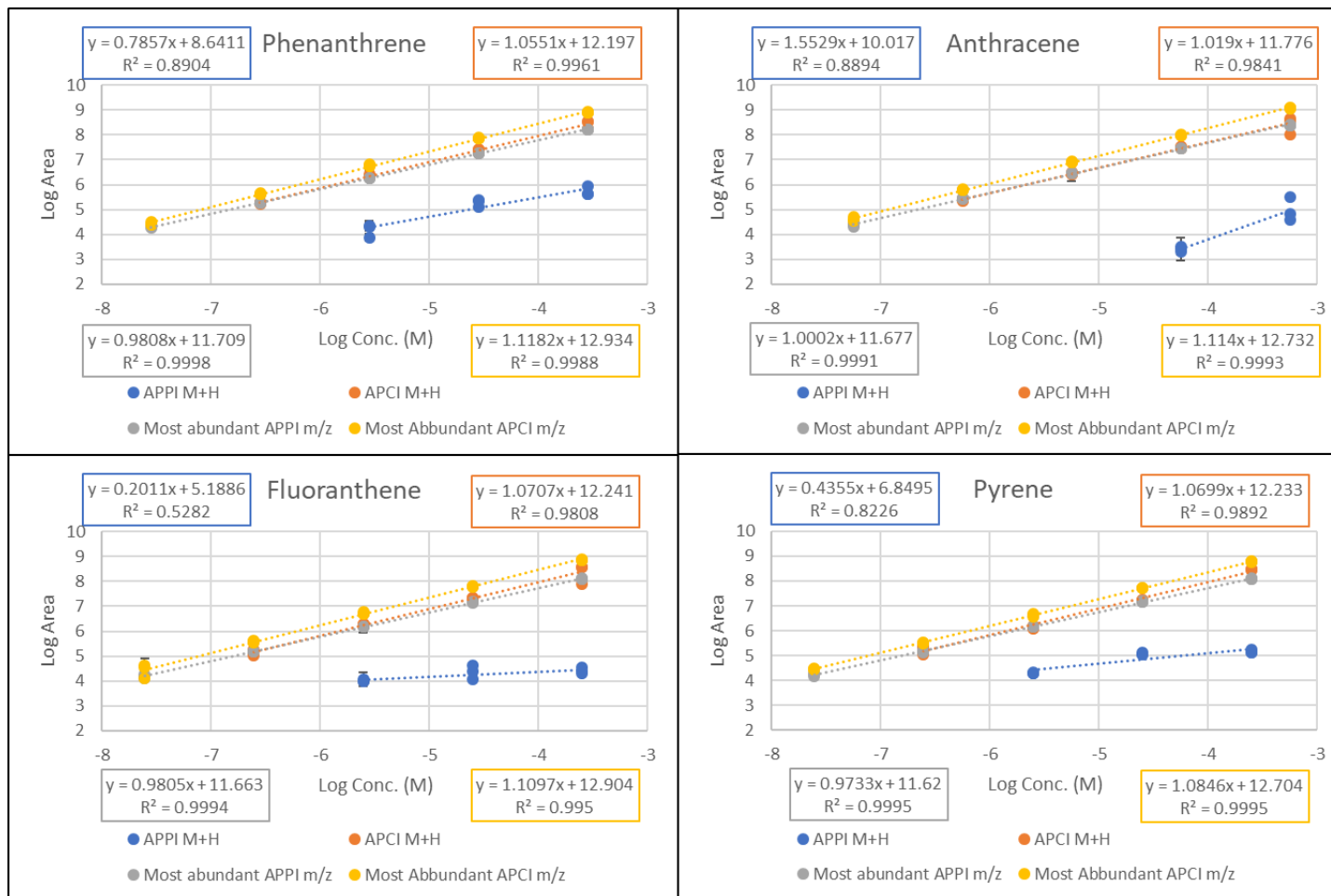


Figure 1.10. continued

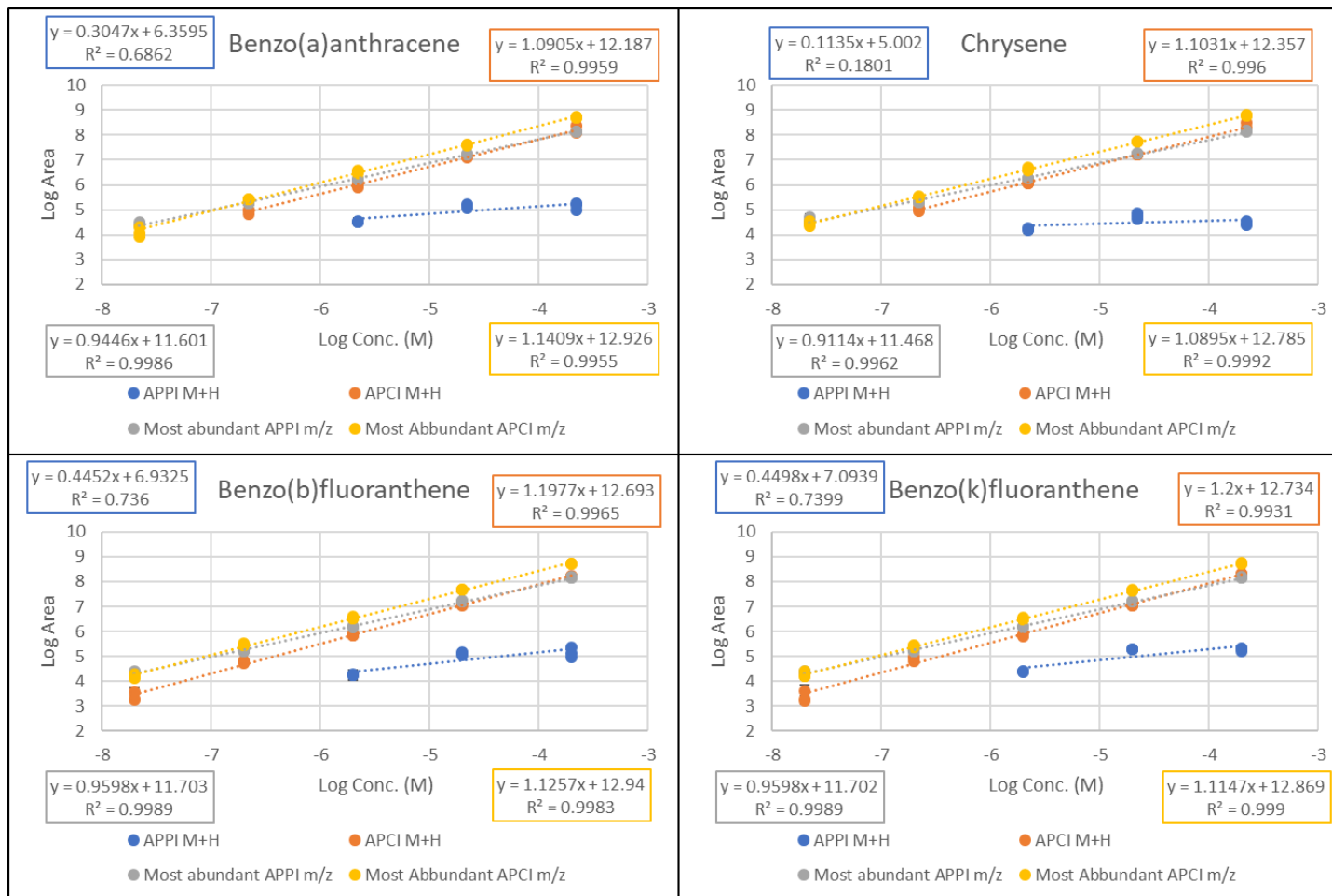


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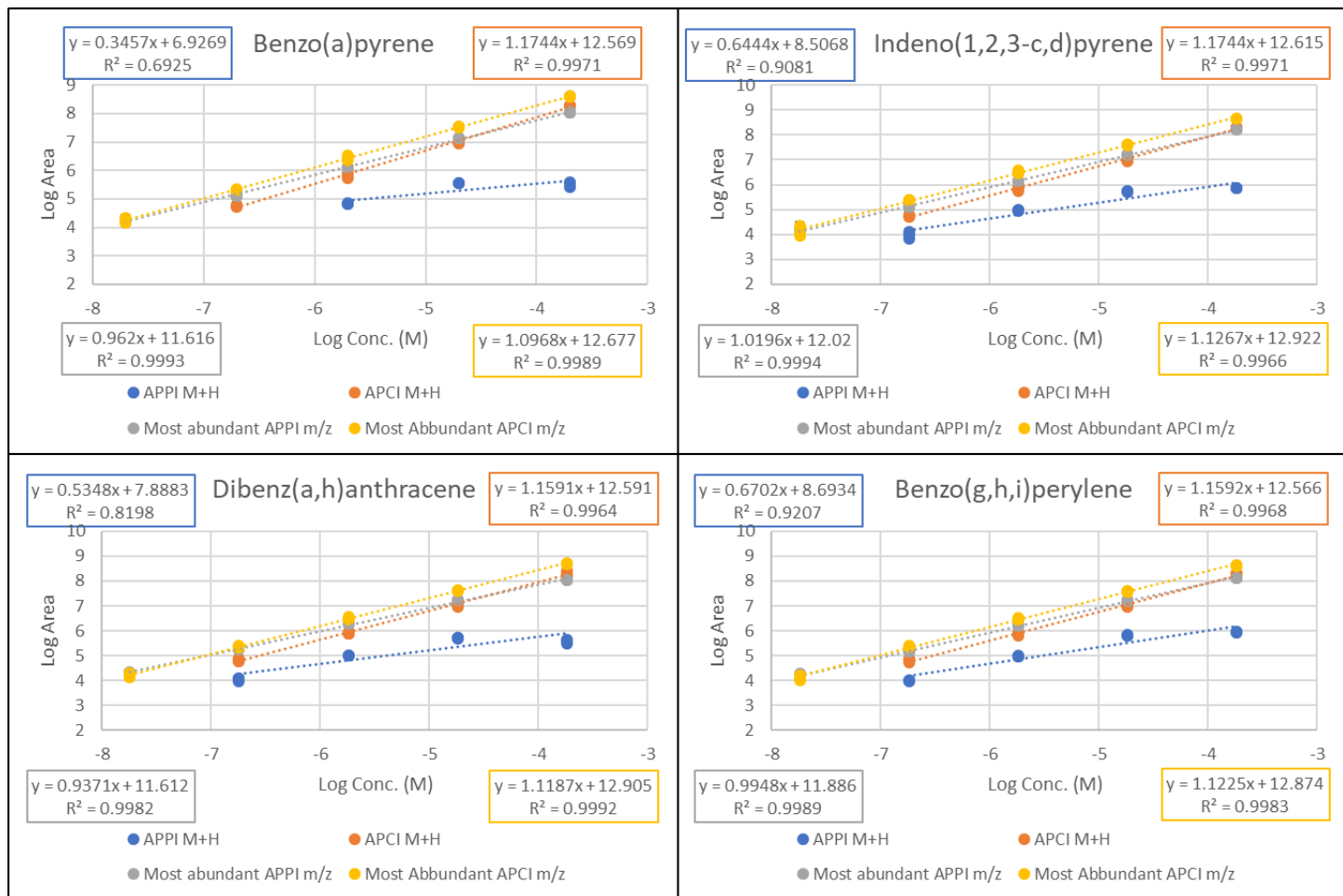


Figure 1.10. continued

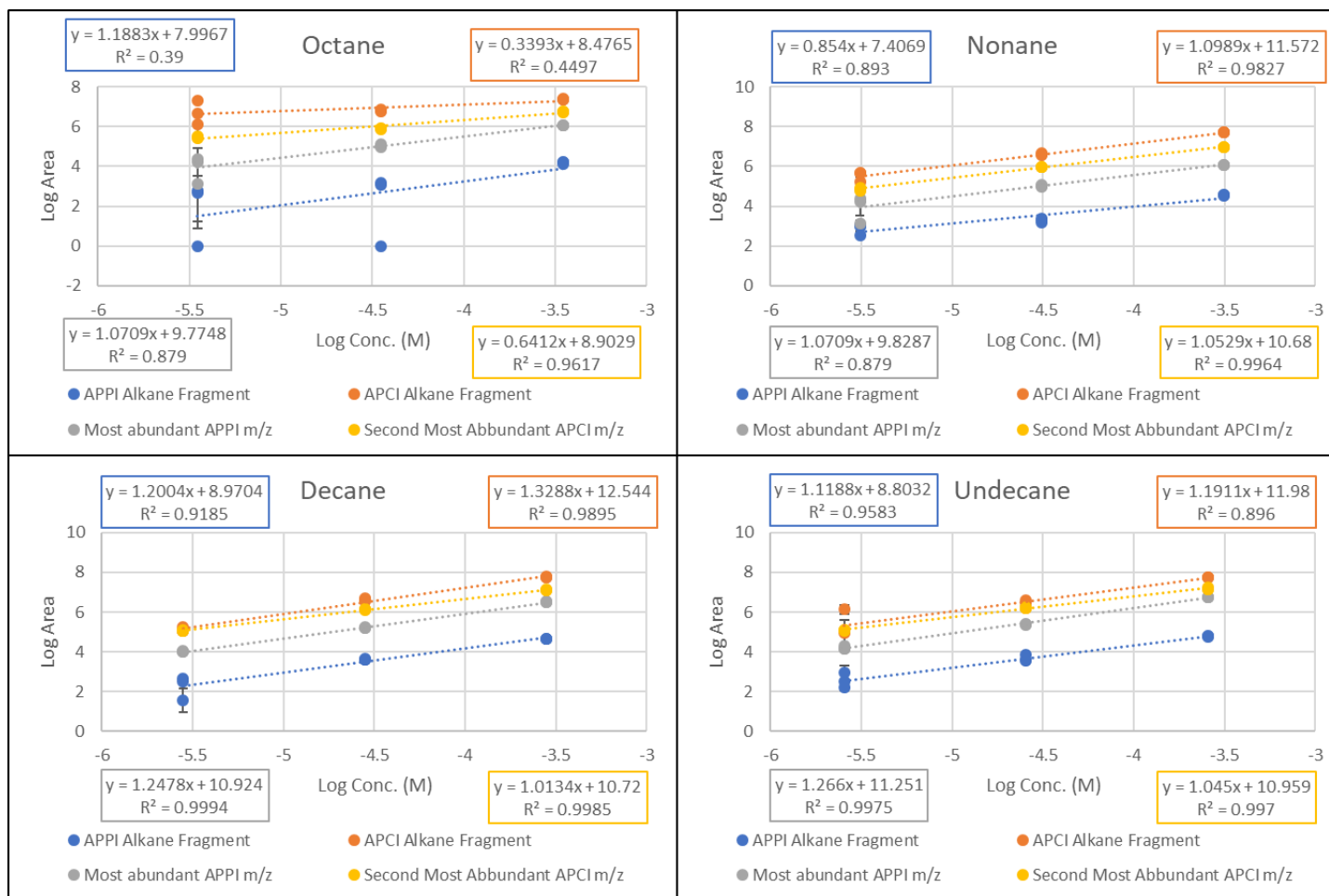


Figure 1.11. Saturated hydrocarbons calibration curves for the most abundant alkane fragment (m/z) and most abundant ion (non-fragment) (m/z) for each ionization technique

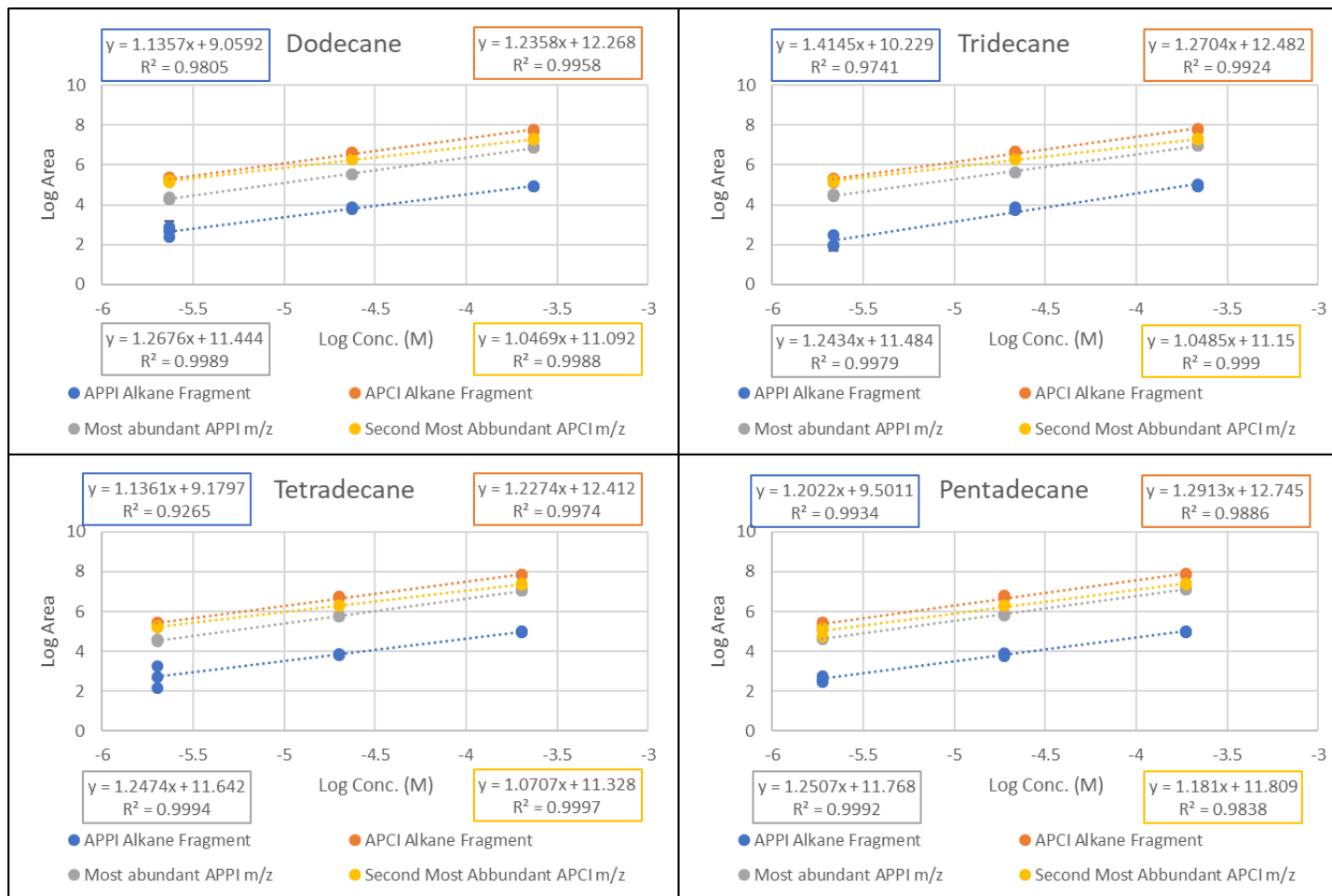


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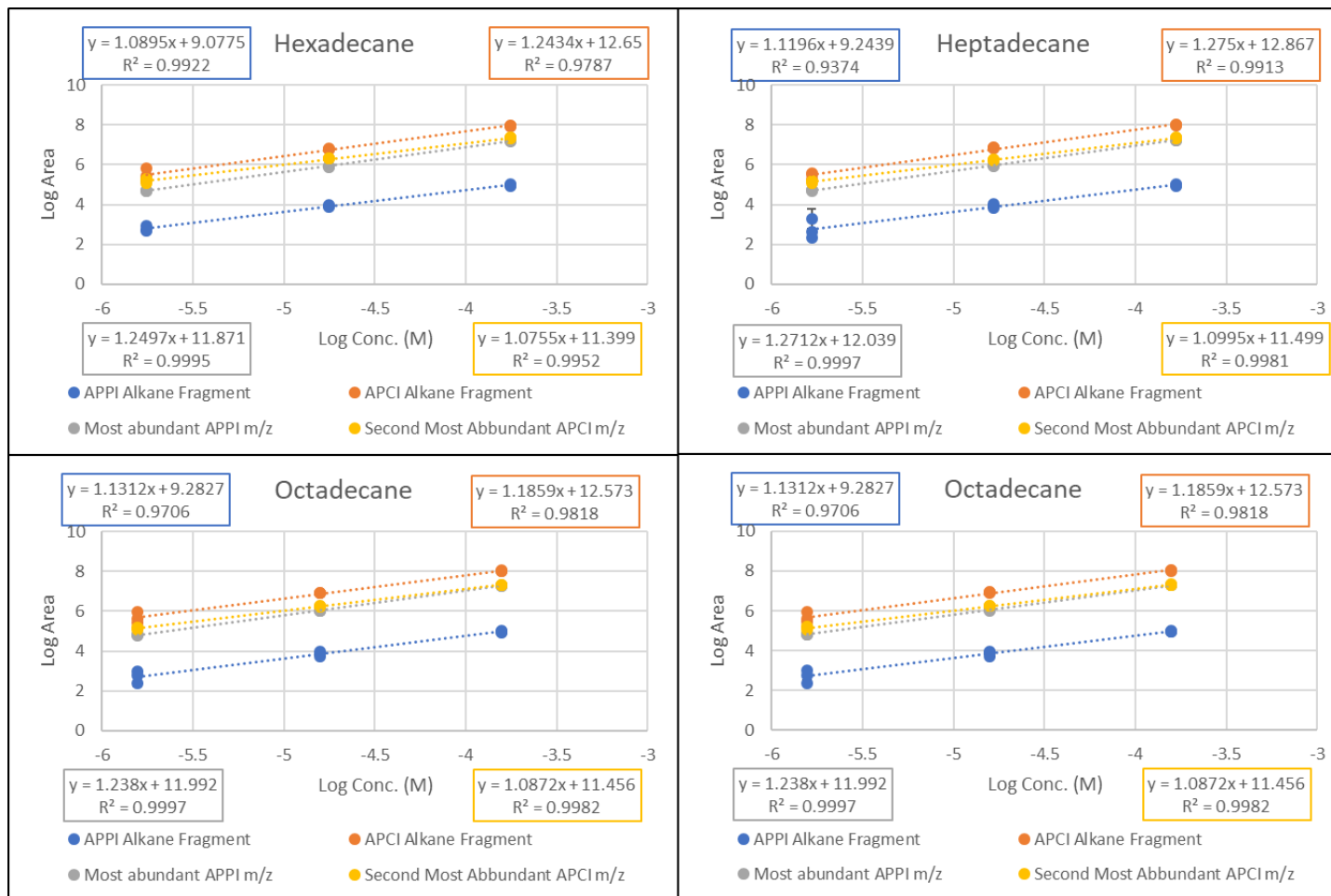


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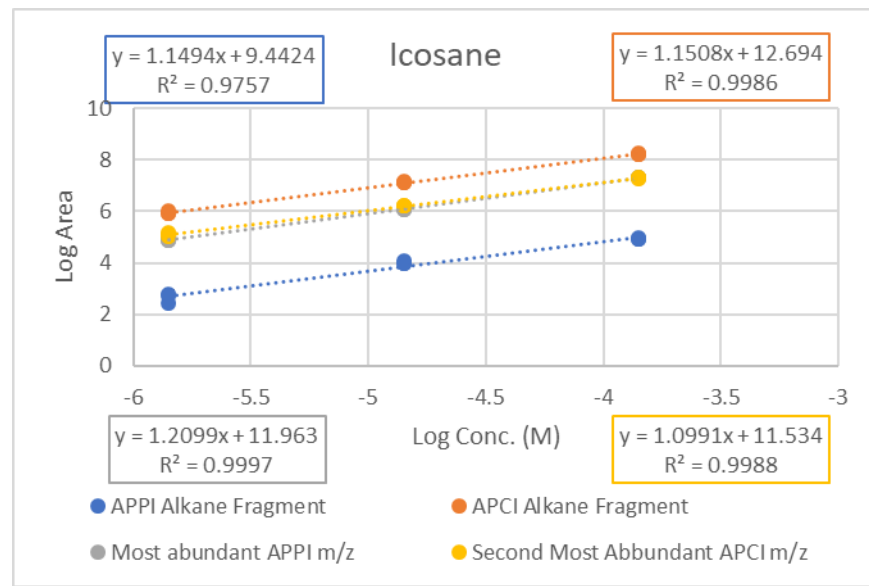


Figure 1.11. continued

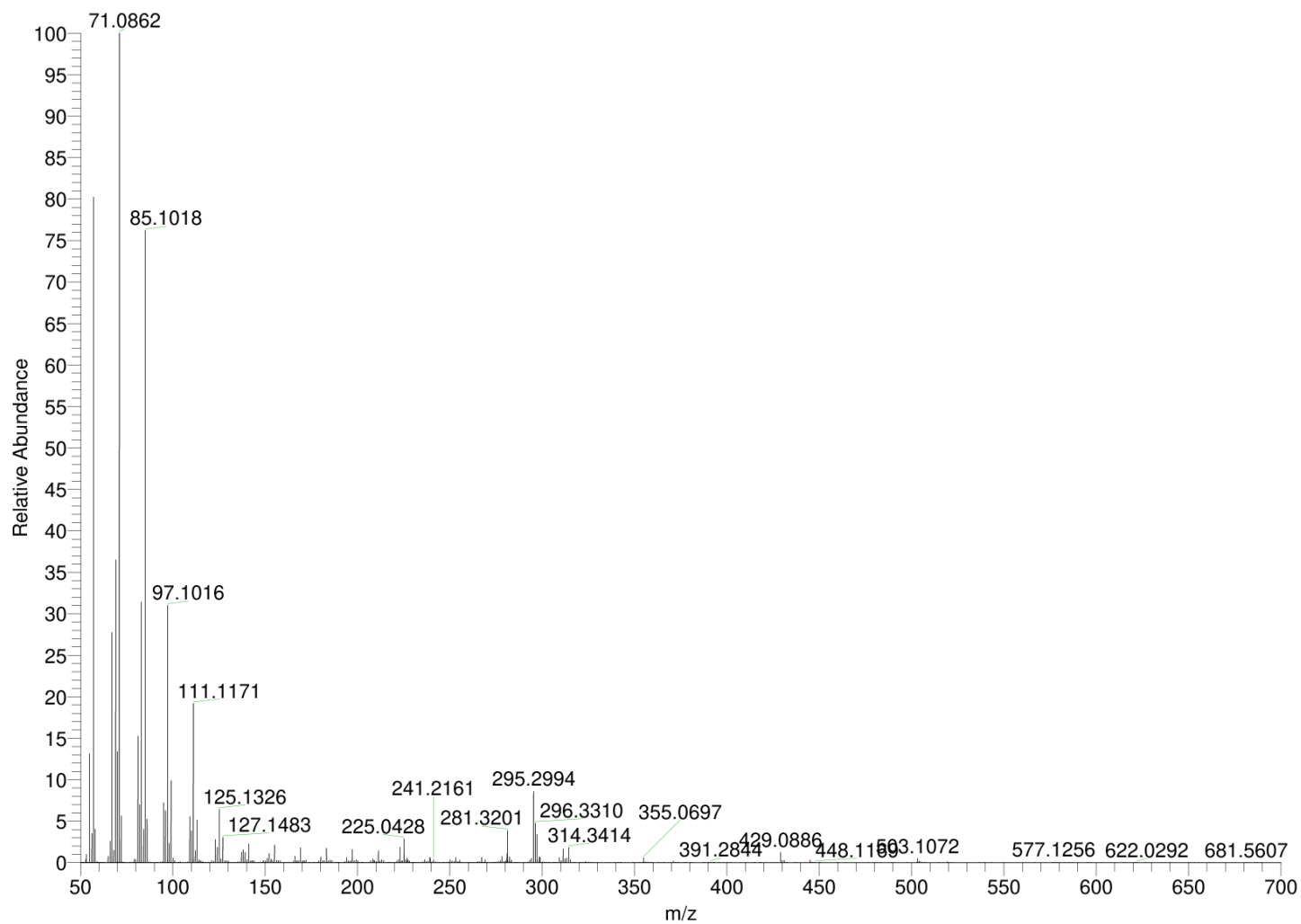


Figure 1.12. Full mass spectrum of eicosane ionized by APCI

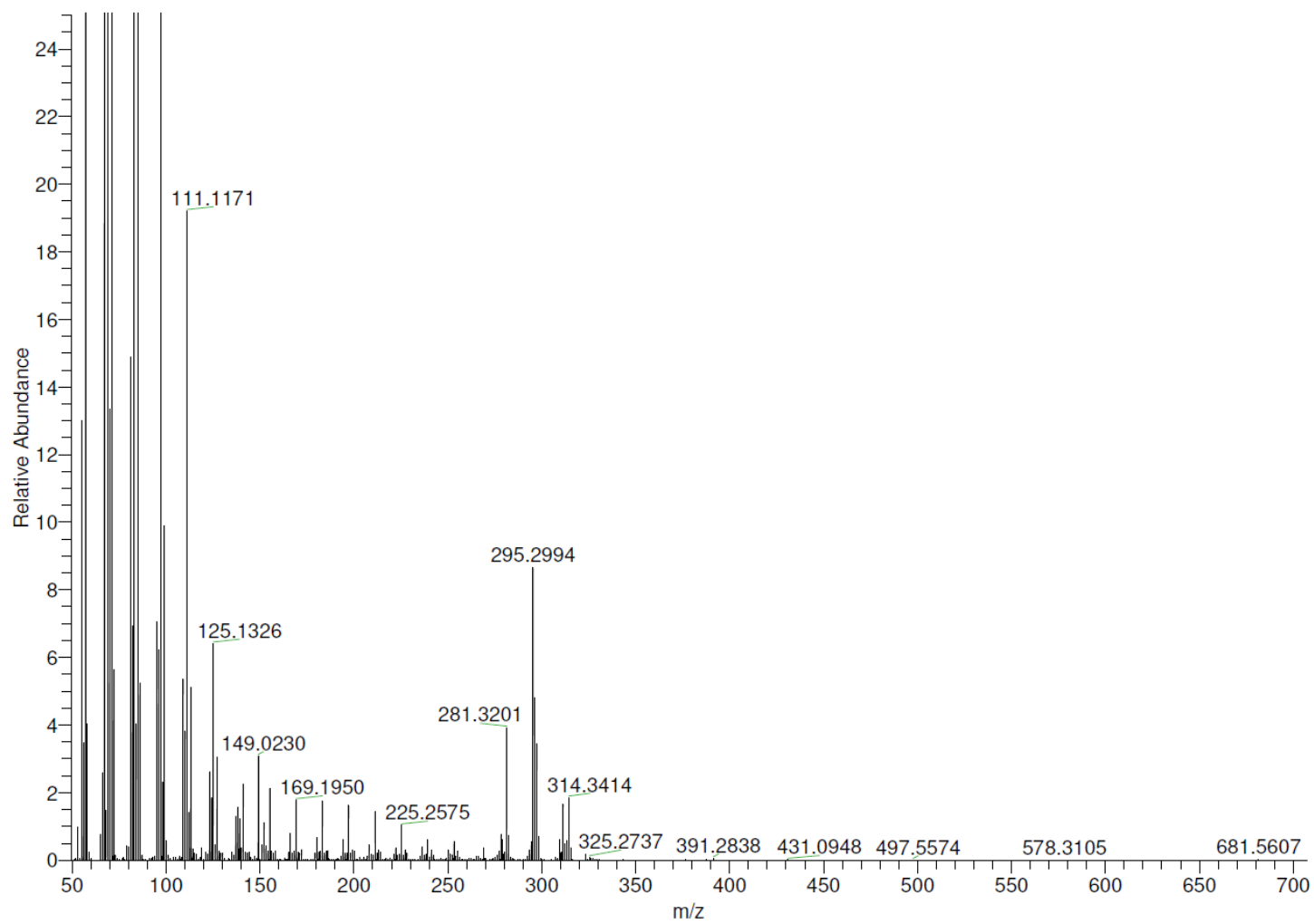


Figure 1.13. Zoomed in mass spectrum of eicosane ionized by APCI

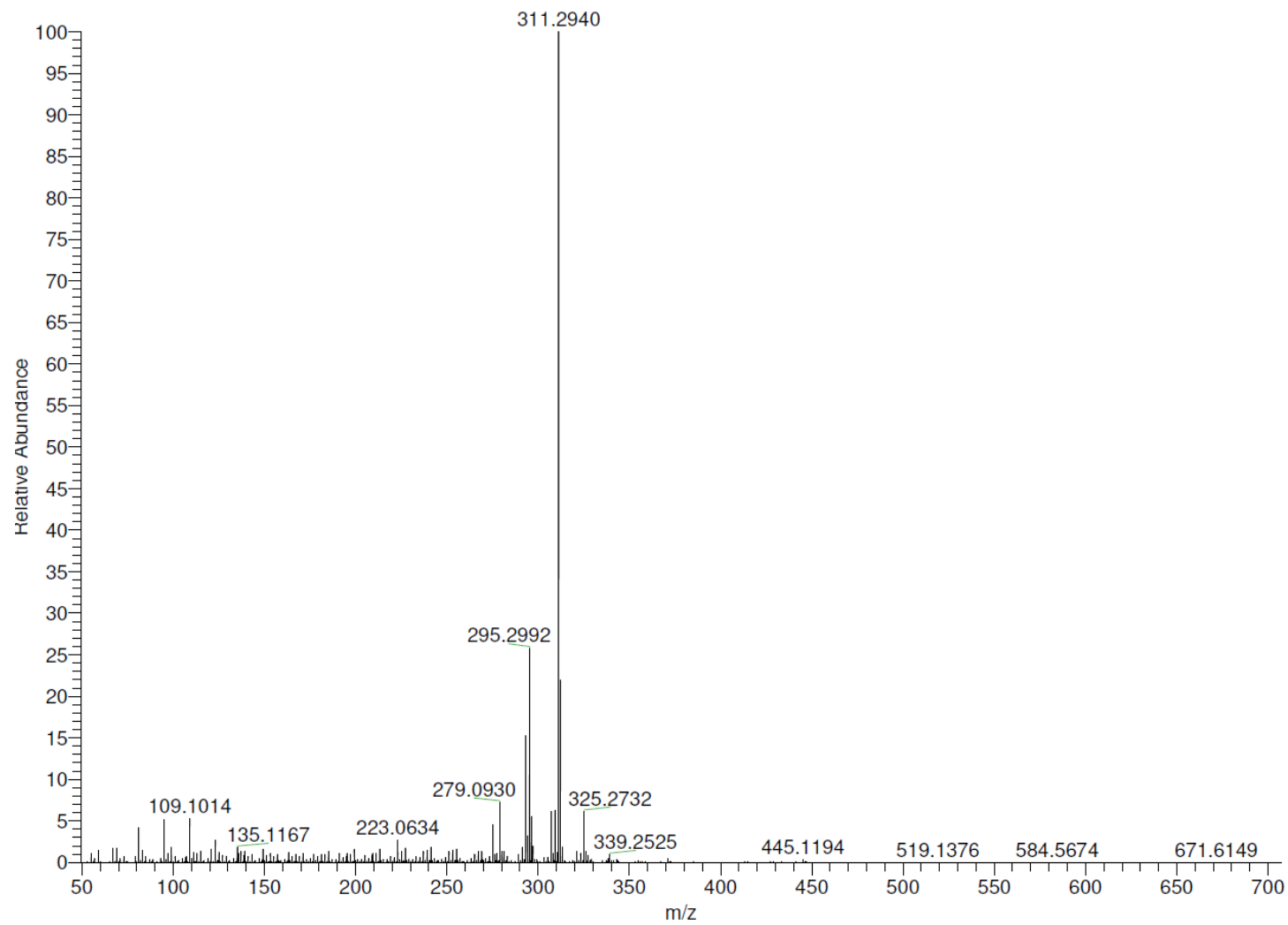


Figure 1.14. Mass spectrum of eicosane ionized by APPI

CHAPTER 2.
INVESTIGATION OF GAS CHROMATOGRAPHY –
ATMOSPHERIC PRESSURE IONIZATIONS OF ALKANES AND
FATTY ACID METHYL ESTERS

Preface

Chapter two focuses on findings from the first chapter and expands upon them. Specifically, unique ions generated from analytes were detected in large, relative abundances, which were at times the base peak, by both GC-APCI-Orbitrap as well as GC-APPI-Orbitrap. The ions detected are unique because they cannot be identified as simple adducts or fragmentations of the neutral mass. Rather, these ions are the result of ionization mechanisms specific to API sources or atmospheric conditions and have been underreported. Furthermore, these unique ions can hinder or aid in analyte identification depending on the sample mixture as well as the identities of the unique ion species. Thus, it is imperative to determine the identities and reasons for the creation of said unique species.

Fatty acid methyl esters and saturated hydrocarbons were the classes of compounds that generated the unique ions most significantly. Particularly, FAMES produced $[M - 32.0269]^+$ and $[M - 45.0347]^+$ ions whereas alkanes produced $[M + 12.9707]^+$ and $[M + 28.9655]^+$ ions. The elemental composition of these unique species can be narrowed down since they were generated from known, pure analytes, the Orbitrap was initially operated at 140,000 resolution, and the ionization occurred at atmospheric pressure. Ions generated from the alkanes were determined to have the formula $[M - 3H + O]^+$ and $[M - 3H + 2O]^+$ while ions from FAMES had formulas of $[M - CH_4O]^+$ and $[M - C_2H_5O]^+$. However, the potential structural compositions of these species could vary considerably and without knowing how the ions are generated, it would be difficult to characterize as well as to control them. Therefore, several experiments were designed to aid in structural elucidation of the characteristic ions, as well as to test the effects that source parameters and atmospheric conditions have on their generation.

To elucidate structures, a combination of isotopically labelled standards and parallel reaction monitoring were used. Select standards of FAMES and alkanes were fully deuterated and fragmented by a Q Exactive™ Plus Orbitrap. The substitution of deuteriums for hydrogens creates mass differences upon

fragmentation as well as ionization that can be measured due to the superior quality of the Orbitrap mass analyzer. Furthermore, the use of isotopically labeled standards is beneficial as the ionization efficiency will not change compared to the non-deuterated standards. Through MS² data as well as exact mass calculations, it was confirmed that oxygen was in fact a major component of these unique species.

Therefore, additional experiments were implemented to determine the source of the oxygen being incorporated into the standards. The distinct design of the GC-APCI interface allowed for the infusion of water during a GC analysis, which allowed for the volatilization and overall greater percentage of water available for ionization. Deuterium oxide was also infused by this method and the results of both these studies suggested that water was not a significant source of oxygen for incorporation into the analytes via routine analyses. However, testing the source parameters of both APCI and APPI yielded a key variable for API. Specifically, the flow rate of nitrogen gas and consequently the availability of atmospheric oxygen significantly changed the abundance of the unique species. This finding demonstrates the possibility of in-source oxidation for GC-API analyses.

Thus, this research has particular importance for the application of API for GC-MS analyses and the generation of new ion species. New API techniques being developed should take these findings into consideration.

A version of this chapter is expected to be part of a future publication by:
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Contributions:

JBP and SRC designed the experiment and wrote the manuscript. JBP conducted the experiments.

2.1. Abstract

Gas chromatography-mass spectrometry (GC-MS) is a valuable analytical tool that is used by numerous scientific fields including forensics, medicine, environmental monitoring, and petrochemicals. Until recently, many GC-MS instruments have relied on either electron ionization or chemical ionization sources. While these sources provide means to identify chemical species, their ability to do so can be limited by the quality of the mass analyzer, i.e. mass accuracy. In response to this need, Thermo Fisher Scientific launched the Q Exactive™ GC hybrid quadrupole-Orbitrap MS utilizing these sources, while other companies, such as Waters Corp, Bruker, and Agilent, have also released GC-MS systems capable of exact mass measurements; however, these GC-MSs employ a different ionization source: atmospheric pressure chemical ionization (APCI). APCI, like other atmospheric pressure ionizations (API), provides “soft” ionization of analytes which, when combined with high-resolution mass spectrometry, provides reasonable unknown identification by exact mass. Previously, an interface between a GC and Orbitrap MS, that allows users to easily switch between GC and liquid chromatography separations and utilizes APCI, was described. Using this GC-APCI interface, several analyses were conducted measuring analytes of decreasing polarities. Although the interface produced $[M + H]^+$ ions, it also generated unique, abundant species for some analyses. These species were also detected using an atmospheric pressure photoionization source. Specifically, $[M - 32.0267]^+$ and $[M - 45.0347]^+$ ions from fatty acid methyl esters (FAMES) as well as $[M + 12.9710]^+$ and $[M + 28.9657]^+$ ions from alkanes were generated consistently and significantly. When analyzing complex mixtures of unknown composition, the ability to accurately predict how known analytes will ionize is of the utmost importance. Herein, investigations undertaken to elucidate the ionization mechanisms of these compounds via APIs are presented. In particular, high-resolution and MS² data were collected using a Q Exactive™ Plus Orbitrap for fully deuterated analytes from both chemical classes. It was found that

alkanes were readily oxidized, and specific FAMES underwent a similar process following in-source fragmentation. From these experiments, the data suggest unique, underreported mechanisms of API and observations of in-source oxidation.

2.2. Introduction

Since its inception in the late 1950s, gas chromatography – mass spectrometry (GC-MS) has been a critical analytical tool for several diverse scientific fields including forensics, medicine, environmental monitoring and petrochemicals.⁷ Until relatively recently, GC-MS instruments have solely relied on electron ionization (EI) or chemical ionization (CI) methods to generate fragmented and intact ions, respectively, for analyses. While these ionization methods provide reliable data that can be matched to libraries or yield molecular peaks, there is a lack of exact mass data because these methods are often coupled to low-resolution mass analyzers.³⁰ Since exact mass data aid in identification and discernment of unknown analytes, it is substantially beneficial to obtain this information from high-resolution instrumentation.

In 2014, a high-resolution GC-MS instrument was assembled from a Thermo Scientific DSQ™ II GC-MS and an Exactive™ Plus Orbitrap by Peterson and coworkers.³¹ This instrumental design was one of the first GC-MS instruments capable of fragmentation as well as high-resolution data. Since then, Thermo Fischer Scientific™ has commercialized the instrument as the Q Exactive™ GC hybrid quadrupole-Orbitrap mass spectrometer system. Moreover, other companies have followed suit with the desire for exact mass data, though by utilizing an alternative ionization method. As first discussed by Horning and coworkers, atmospheric pressure chemical ionization (APCI) is capable of ionizing varying polarity analytes already in the gas-phase.¹⁵ Companies like Waters Corporation, Bruker, and Agilent have commercialized GC-APCI interfaces to couple to many of their new, high-resolution mass spectrometers. Additionally, atmospheric pressure photoionization (APPI) sources have also recently been implemented for GC-MS analyses with third-party interfaces available. GC-APPI sources utilize vacuum ultraviolet lamps, such as a 10.6 eV Krypton lamp, to ionize analytes with relatively “soft” or low energy processes.¹⁴ Together, APCI and APPI have provided alternative means to ionize analytes compared to traditional GC-

MS sources. Moreover, the incorporation of APCI and APPI sources into GC-MS analyses has demonstrated their utility, as it is increasingly recognized that these sources can be used with both GC and liquid chromatography (LC) analyses.

Previously, an interface between a GC and an Orbitrap MS was described. The interface enabled facile switching between GC and liquid chromatography, utilized an APCI source, and performed as well as or better than GC-MS analyses that used other sources, including EI and APPI. From comparison of the data generated by both GC-APCI-Orbitrap and GC-APPI-Orbitrap analyses, it was revealed that some analytes were not only detected as expected m/z s, including $[M + H]^+$ and $[M]^+\bullet$, but also as species that could not be assigned as simple adducts or rearrangements. Specifically, fatty acid methyl ester analyses consistently generated $[M - 32.0267]$ and $[M - 45.0347]$ ions while saturated hydrocarbon analyses consistently generated $[M + 12.9710]$ and $[M + 28.9657]$ ions. Although not always the most intense ions, these chemical species may provide additional insight, or fingerprints, when considering unknown identification through these sources. Moreover, it is particularly desirable to understand the potential mechanisms of these specific species as unknown identification workflows hinge on the ability to accurately predict how analytes will be ionized. To better understand the identity of these peculiar m/z s, several experiments were performed with naturally occurring and fully deuterated analytes from both chemical classes using a Q Exactive™ Orbitrap to generate high-resolution and MS² data. Additionally, environmental and instrumental parameters were tested to assess their effect on ion generation.

2.3. Experimental Section

2.3.1. Reagents and Materials

A 37 component FAME mixture and a C₈-C₂₀ alkane standard solution were purchased from Sigma-Aldrich (St. Louis, MO). Fully deuterated standards of select FAMES and alkanes were obtained from Cambridge Isotope Laboratories,

Inc. (Tewksbury, MA). Sample preparation and syringe washes were completed with HPLC-grade hexanes from Fisher Scientific (Hampton, NH). Ultrahigh purity grade helium was purchased from Airgas (Madison, WI).

2.3.2. Gas Chromatography – Mass Spectrometry

A Thermo Scientific Trace 1300 GC and a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap MS (Waltham, MA) were used for all GC-MS analyses. Two separate interfaces, an in-house APCI interface³² and a commercially available APPI interface from Gentech Scientific Inc. (Arcade, NY), were used to couple the instruments and generate ions. Control of the instrument and data analysis were performed using the Xcalibur software suite, Version 4.0.27.19.

2.3.3. Saturated Hydrocarbons

Alkanes were separated and detected using modified methods previously described.³² Briefly, a TG1-MS column (30 m x 0.25 mm i.d. x 0.25 µm dr) purchased from Thermo Fisher Scientific (Waltham, MA) and helium (1.4 mL/min) were used to separate analytes. Using a programmable temperature vaporizer (PTV) in CT split mode (330 °C, 7.0 mL/min), 2 µL injections of standards were volatilized. Analytes were separated on the column with the following temperature program: initial temperature of 55 °C held for 5 min; ramp of 15.0 °C/min to 280 °C; and final temperature of 280 °C held for 5 additional min. The transfer line from the GC to the MS was kept constant at 300 °C.

Initial settings for the APCI source were: sheath gas flow rate set to 10 arbitrary units, aux gas glow rate to 8, sweep gas flow rate to 3, discharge current set to 10.00 µA, ion transfer capillary temperature to 350 °C, S-lens RF to 50.0, and vaporizer temperature to 320 °C. MS settings were dependent on each analysis. In general, full scans were acquired in positive mode using a resolution of 140,000, AGC target of 3e6, maximum IT of 50 ms, scan range of 50-700 *m/z*, and estimated chromatographic peak width of 5s. Ions from the blanks were used to generate exclusion lists.

For parallel reaction monitoring (PRM) analyses, data was also acquired in positive mode using a resolution of 17,500, AGC target of 2e5, maximum IT of 50 ms, isolation window of 1.0 m/z , (N) CE/ stepped (N) CE of 20, 40, 60, default charge state of 1, MSX count of 1, and estimated chromatographic peak width of 5s. Analyte ions detected in full scan analyses were used to create inclusions lists and blanks were used to generate exclusion lists.

2.3.4. Fatty Acid Methyl Ester Analysis

Like alkanes, FAMES were separated and detected using modified methods previously described.³² Briefly, a FAMEWAX column (30 m x 0.25 mm i.d. x 0.25 μm d_f) purchased from Restek (Belefonte, PA) was used as the stationary phase and helium gas (1.7 mL/min) as the mobile phase to separate analytes. Using a programmable temperature vaporizer (PTV) in CT split mode (240 °C, 5.1 mL/min), 2 μL injections of standards were volatilized. Analytes were separated on the column with the following temperature program: initial temperature of 80 °C held for 5 min; ramp of 10.0 °C/min to 240 °C; and final temperature of 240 °C held for 5 additional min. The transfer line from the GC to the MS was kept constant at 245 °C. APCI and APPI settings were the same as those set for the alkanes analysis as well as MS settings.

2.4. Results and Discussion

2.4.1. Saturated Hydrocarbons

Three fully deuterated alkane standards were used to represent the range encompassed by the non-deuterated standard mixture. Specifically, *N*-decane (D₂₂, 99%), *N*-hexadecane (D₃₄, 99%), and *N*-eicosane (D₄₂, 98%) were analyzed individually, collectively, and in a 1:1 mixture of non-deuterated standards. Previously, non-deuterated *N*-decane, *N*-hexadecane, and *N*-eicosane standards were reported to have generated $[M + 12.9707]^+$ ions as well as $[M + 28.9655]^+$

ions with the intensity of each species dependent on the ionization type used.³² Table 2.1 lists characteristic ions unique to each analyte as well as their respective, relative abundances generated from the present experiments. From this table, a few simple observations can be made. Firstly, the unique ions of $[M + 12.9707]^+$ and $[M + 28.9655]^+$ for non-deuterated alkanes were detected once again in these sets of experiments. Moreover, similarly unique ions for each of the deuterated standards were also observed; though, in general the deuterated analytes produced $[M + 9.9512]^+$ and $[M + 25.9458]^+$ ions. Additionally, the deuterated versions of the analytes eluted earlier than their non-deuterated counterparts, which has been a well-documented phenomenon in gas chromatography.³³ Finally, one unique ion was preferentially observed depending on whether the APPI or APCI was used. Specifically, $[M + 12.9707]^+ / [M + 9.9512]^+$ ions were more abundant with the APCI source while $[M + 28.9655]^+ / [M + 25.9458]^+$ ions were more abundant with the APPI source.

Table 2.1. List of saturated hydrocarbon analytes and their characteristic ions produced by APCI and APPI. The (*) denotes that a contaminant was present in the deuterated standard with a mass similar to the unique ion.

Name	RT based on APPI (min)	Neutral Mass (<i>m/z</i>)	APPI Characteristic Ions (<i>m/z</i>)	Relative Abundance (%)	APCI Characteristic Ions (<i>m/z</i>)	Relative Abundance (%)
decane-D ₂₂	6.82	164.3102	190.2565	27.32	174.2624	100.00
			174.2617	21.70	189.2509	5.63
decane	7.07	142.1722	171.1373*	56.33	155.1431	100.00
			155.1424	16.50	157.1587	41.04
					171.1380	12.32
hexadecane-D ₃₄	13.43	260.4795	286.4253	79.24	270.4314	46.11
			273.4523	56.15	286.4253	2.66
			270.4304	51.33		
hexadecane	13.61	226.2661	255.2307	100.00	239.2370	100.00
			241.2515	61.21	255.2319	17.08
			239.2359	39.77		
eicosane-D ₄₂	16.15	324.5923	350.5377	100.00	334.5440	14.76
			355.0683	21.40	322.5773	10.68
			337.5648	66.68	353.5610	2.08
			334.5429	61.91		
eicosane	16.35	282.3287	311.2929	100.00	295.2995	89.57
			297.3138	55.50	311.2943	17.29
			295.2982	37.61		

To further elucidate some of these characteristic ions, MS² data were collected via parallel reaction monitoring (prm) methods. A full MS² spectrum of 295.2995 *m/z*, generated by APCI and associated with the analyte eicosane, is shown in Figure 2.1 as well as a zoomed spectrum around 85.1 *m/z*. From this example, three trends can be observed. First, ions indicative of oxidation to the alkane can be observed, including the more abundant series of larger, fragment ions (225.2207, 211.2055, 197.1898 *m/z*, etc.). Moreover, oxidation is present in smaller fragment masses but is not as obvious due to the abundance of many ions in this region. For example, in Figure 2.1 the oxidized, fragment ion 85.0655 *m/z* can only be observed by zooming in on the TIC. This mass is not readily apparent due to the greater abundance of the adjacent 85.1012 *m/z*, which is a saturated hydrocarbon fragment. In addition to the incorporation of oxygen, fragment ions indicating unsaturation of the parent ion are observed, specifically, alkene and diene fragments. Finally, alkane fragments that are positively charged are also present. Alkane, alkene, and diene fragments are characterized by a series of *m/z*s that differ by two AMU within the MS² spectrum. For instance, 67.0550, 69.0706, and 71.0863 *m/z* can be assigned as [C₅H₇]⁺, [C₅H₉]⁺, and [C₅H₁₁]⁺ or rather a diene, alkene, and alkane, respectively. Potential structures of some of the fragmented ions are shown within the spectra of Figure 2.1. These trends hold true for all alkane standards studied, including C₈-C₂₀.

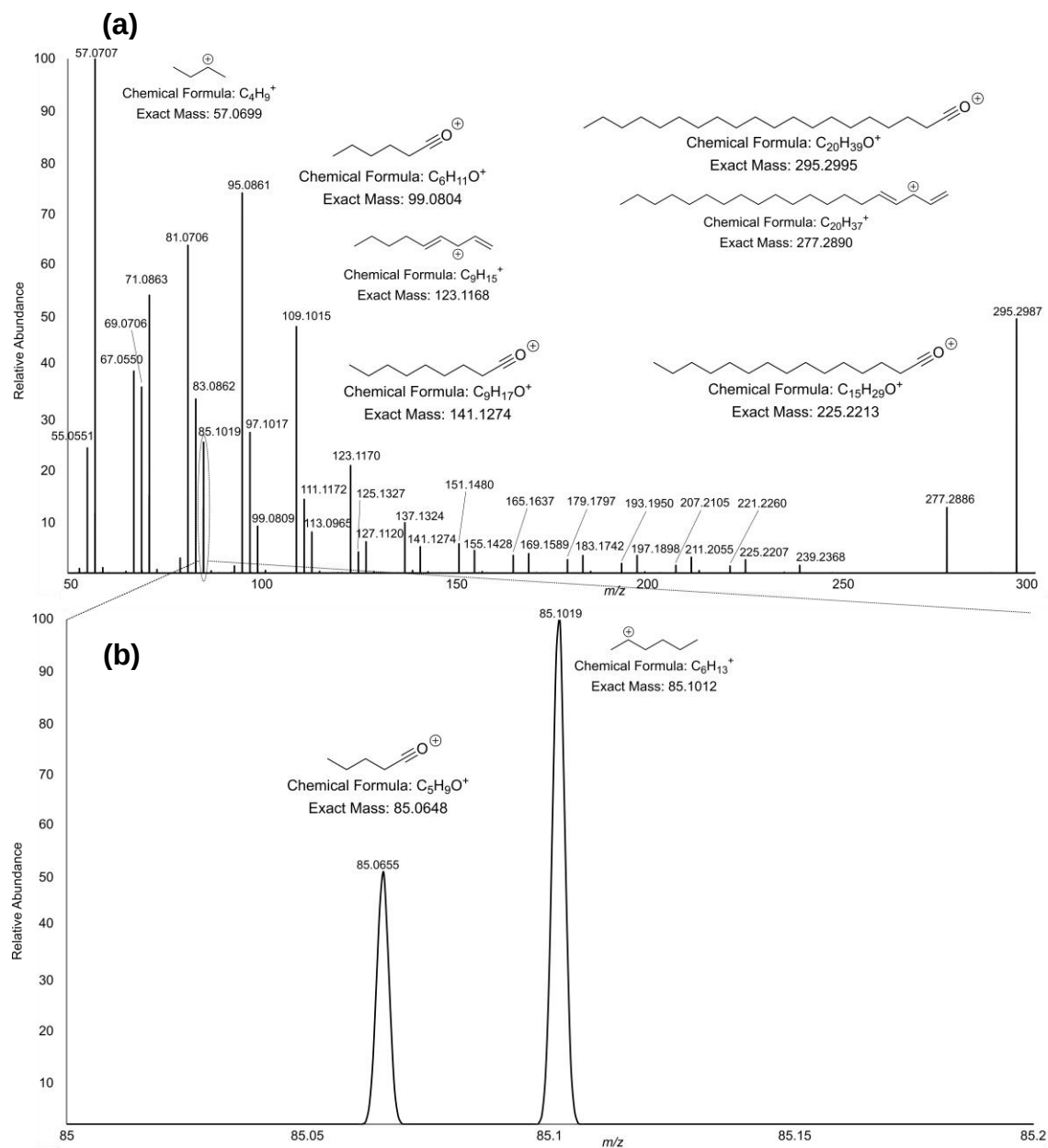


Figure 2.1. (a) Full MS² of *m/z* 295.2995 from the non-deuterated eicosane standard and (b) zoomed in spectra around *m/z* 85.1. Mass to charges indicative of protonated aldehydes, dienes, and alkenes can be assigned in (a) and resolution of similar masses can be observed in the zoomed spectrum (b). Additionally, potential structures of some fragment ions are depicted. Note that the exact location of the oxidation processes cannot be determined from these experiments and that multiple structures, including resonance structures, may still be possible.

While some of these non-deuterated structures can be rationalized solely with exact mass, further data was necessary to exclude other possibilities, including water clusters, protonated alcohols, and ketenes. The analysis of fully deuterated alkanes narrowed down these possibilities. Figure 2.2 shows the MS² data of m/z 334.5440 from eicosane-D₄₂, as well as another zoomed in spectrum produced by APCI. These spectra shared similar characteristics to those generated for the non-deuterated analyte, including fragment peaks indicative of dienes, alkenes, alkanes. Moreover, it was determined that moisture in the atmosphere was not being incorporated into the parent ion, either via water clusters or direct incorporation, as the mass shift of the fully deuterated ion did not correspond to these possibilities. For instance, eicosane has a neutral mass of 282.3287 AMU and a characteristic m/z of 295.2995. The m/z of 295.2995 could be indicative of several molecules as shown in Figure 2.3 including: the generation of an acylium ion; the unsaturation of the alkane to a diene, stabilized by water clusters or the addition of water; as well as the formation of a conjugated system consisting of a carbonyl and alkene similar to that of an enol. However, the structures that would result from the reaction of eicosane-D₄₂ and atmospheric water generate ions of varying m/z due to the additional hydrogens involved. Therefore, based on observed parent masses as well as fragment masses, it can be deduced that the formation of an acylium ion is most likely responsible for the characteristic ions of alkanes by APCI. This proposed structure is also equivalent to a terminal carbonyl that possesses a positive charge on the carbon. It is important to note that these unique ions and structures were observed for all fully deuterated alkanes, including decane-D₂₂, hexadecane-D₃₄, and eicosane-D₄₂. However, additional experimentation is still required to confirm the location of the oxidation site as well as whether the oxygen source is truly from moisture or atmospheric oxygen. The latter can be determined through the use of a controlled atmosphere with heavy oxygen, ¹⁸O, though at significant cost. Nonetheless, the

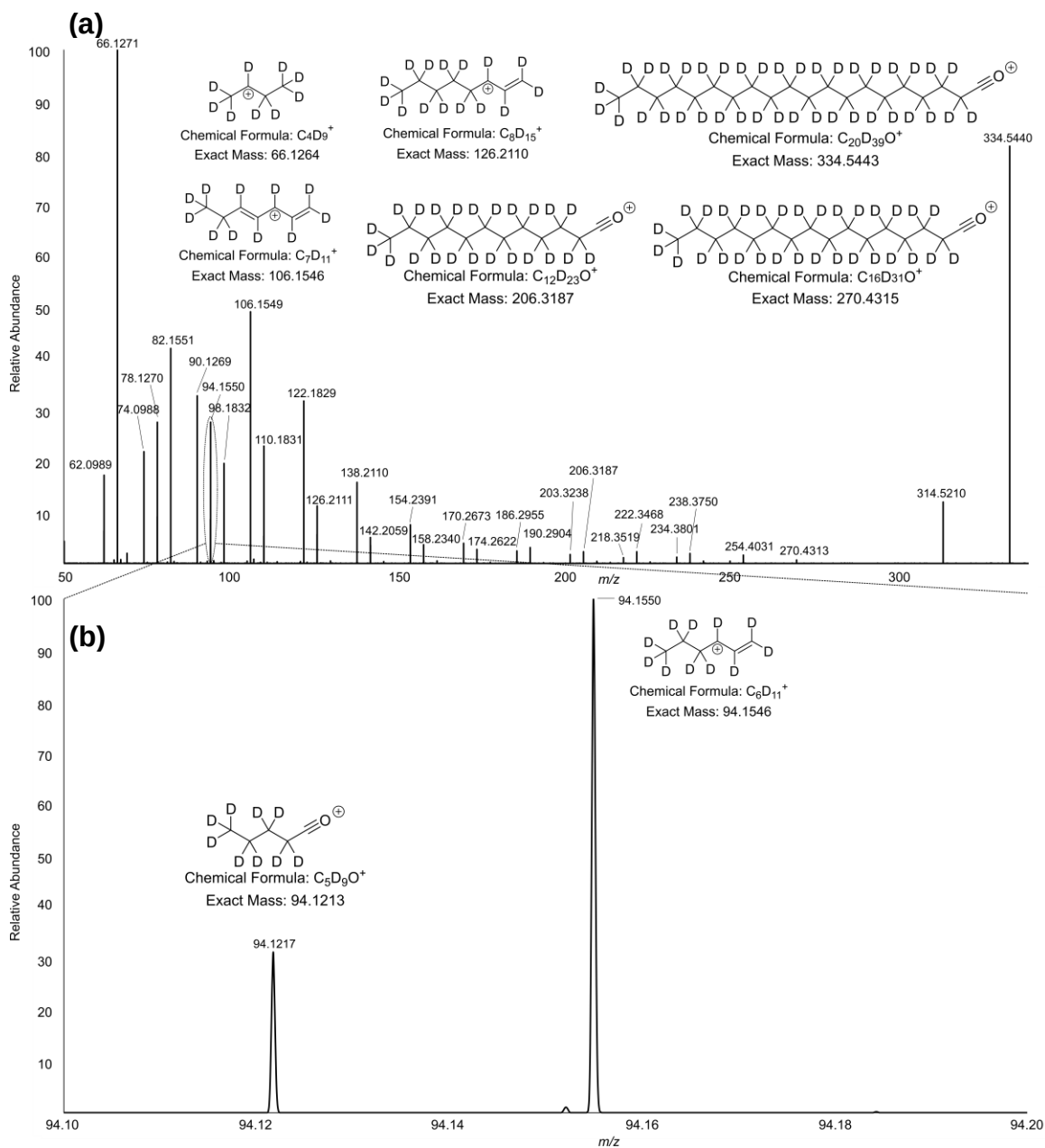
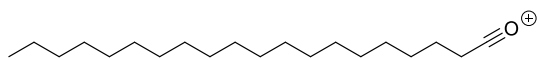
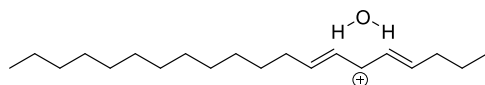


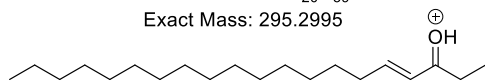
Figure 2.2. (a) Full MS² of m/z 334.5440 from eicosane-D₄₂ and (b) zoomed in spectra around m/z 94.15. Mass to charges indicative of deprotonated carbonyls, dienes, and alkenes can be assigned in (a) and resolution of similar masses can be observed in the zoomed spectrum (b). Additionally, potential structures of some fragment ions are depicted.

(a)

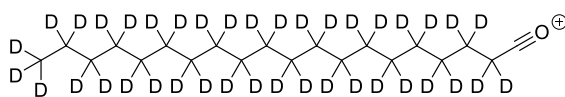
Chemical Formula: $C_{20}H_{39}O^+$
Exact Mass: 295.2995



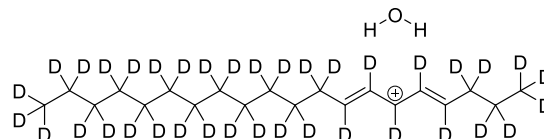
Chemical Formula: $C_{20}H_{39}O^+$
Exact Mass: 295.2995



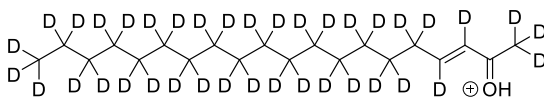
Chemical Formula: $C_{20}H_{39}O^+$
Exact Mass: 295.2995

(b)

Chemical Formula: $C_{20}D_{39}O^+$
Exact Mass: 334.5443



Chemical Formula: $C_{20}H_2D_{37}O^+$
Exact Mass: 332.53178



Chemical Formula: $C_{20}HD_{38}O^+$
Exact Mass: 333.5381

Figure 2.3. Possible structures of molecules associated with **(a)** eicosane and the analogous equivalents predicted for **(b)** eicosane- D_{42} . Due to the mass differences associated with possible structures from eicosane- D_{42} , the potential structure of the characteristic ion can be further narrowed down.

experiments discussed in the atmospheric water and gas experiments section have contributed additional support to the theory that atmospheric oxygen is indeed the source of the oxidized species.

Similarly, the $[M + 28.9655]^+ / [M + 25.9458]^+$ ions, which were most prominent by APPI but observed by both methods, were characterized by MS² spectra as well. Continuing the use of eicosane as an example, 311.2922 and 350.5353 *m/z* were observed for the non-deuterated and fully deuterated analytes, respectively. It is important to note that these sets of experiments were performed a week after initial calibration of the instrument, and thus mass accuracy had begun to drift. Nonetheless, consistent mass shifts enabled characterization of *m/z*s. Figure 2.4 displays the full MS² spectrum and potential structures of the fragment ions from the eicosane analytes. Similar to the spectra of the smaller adducts generated by APCI, a few trends were observed of the APPI generated spectra to help explain the larger 311.2922 and 350.5353 *m/z*s. In order to observe a mass even larger than the 295.2995 *m/z* of the non-deuterated analyte, there must be an additional adduct or modification to the ion. In this instance, an alcohol group substituted to the previously oxidized structure yields the correct exact mass, 311.2922 *m/z* and its proposed structure in Figure 2.4. This hypothesis is supported by the trend of fragment ions, which also indicate an ion that consists of a diol and point of unsaturation, such as 265.2519 and 269.2473 *m/z* and their respective proposed structures in Figure 2.4. Interestingly, the fully deuterated analytes not only supported this hypothesis with similar trends, but also indicated that a proton or deuterium was transferred to an oxygen to form the other alcohol in the process. For instance, 350.5353 *m/z* can only be calculated with oxygen, deuterium, and carbon. As previously stated, the predicted *m/z* is larger than expected, 350.5392, but this can be explained with the mass drift of the instrument as well as by the trend of fragments that follow suit. While similar to a McLafferty rearrangement, the neutral loss from the analytes does not appear to be an alkene;

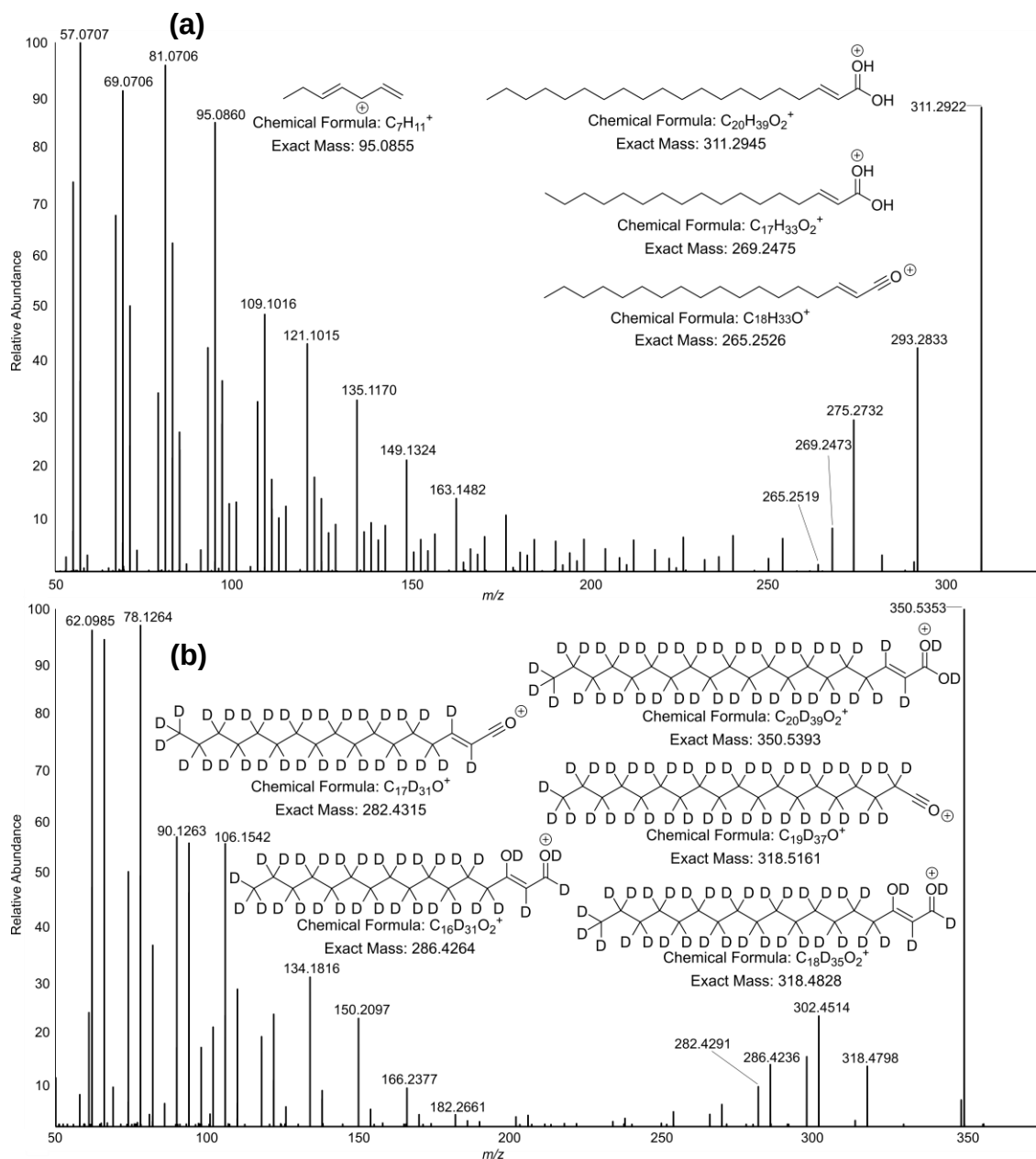


Figure 2.4. MS² of (a) eicosane and (b) eicosane-D₄₂ along with potential structures of fragment ions. Despite mass drift occurring towards the end of these experiments, the Orbitrap was still able to narrow down the potential structures of similar m/z, such as 318.4798 m/z in the eicosane-D₄₂ spectrum. The structure with the deuterated alcohol group and acylium ion is the more likely structure due after mass drift is considered.

however, this is nonetheless an insight into the ionization of such species. Further investigations into the amount of moisture in the atmosphere were taken in order to determine the source of the alcohol and carbonyl groups.

2.4.2. Atmospheric Water and Gas Experiments

To fully assess the effect of moisture in the atmosphere, the unique design of the APCI interface was utilized. Specifically, HPLC water or D₂O was infused at a rate of 0.3 mL/min through the heated APCI probe during the entirety of the run. The results of these experiments are expressed in Figure 2.5. The initial, characteristic ions 295.2995 and 311.2941 *m/z* as well as 334.5443 and 350.5392 *m/z* for eicosane and eicosane-D₄₂, respectively, continued to be the base peaks throughout each experiment. However, trials with the infusion of water resulted in larger relative abundances of other, adjacent ions. For instance, 297.3156 and 313.3105 *m/z* as well as 337.5663 and 352.5546 *m/z* were observed for eicosane and eicosane-D₄₂, respectively, with the addition of water while 298.3214 and 315.3226 *m/z* as well as 338.5723 and 354.5674 *m/z* were observed with the addition of D₂O. These *m/z* indicate that water or deuterium oxide is being substituted onto the alkane backbone, although in a different manner than previously deduced. If water had been the initial source of the oxidation, it would be expected that the relative abundances of the non-base peaks would decrease. Figure 2.6 shows potential structures of the characteristic ions during these experiments. For instance, it was previously determined that the structure of 295.2995 *m/z* most likely consisted of an acylium ion. However, the characteristic ion of the non-deuterated standard while water was being introduced was 297.3156 *m/z*. The structure of this *m/z* can be described as a protonated carbonyl, which is further supported by the deuterium oxide experiments. Therefore, it follows that water is being incorporated into these ions instead of from the previous source. This trend also holds true for the larger *m/z*, specifically the [M + 28.9655]⁺/[M + 25.9458]⁺ ions. For the fully deuterated analyte eicosane, 352.5546 and

354.5674 m/z can therefore be the result of a different alcohol addition to the previously oxidized structure. Thus, it is evident that the formation of the $[M + 28.9655]^+$ / $[M + 25.9458]^+$ ions is significantly different than the mechanism for the ions generated with water/deuterium oxide, since the former ions require a proton/deuterium transfer from a pseudo-McLafferty rearrangement while the latter ions would only require two alcohol additions.

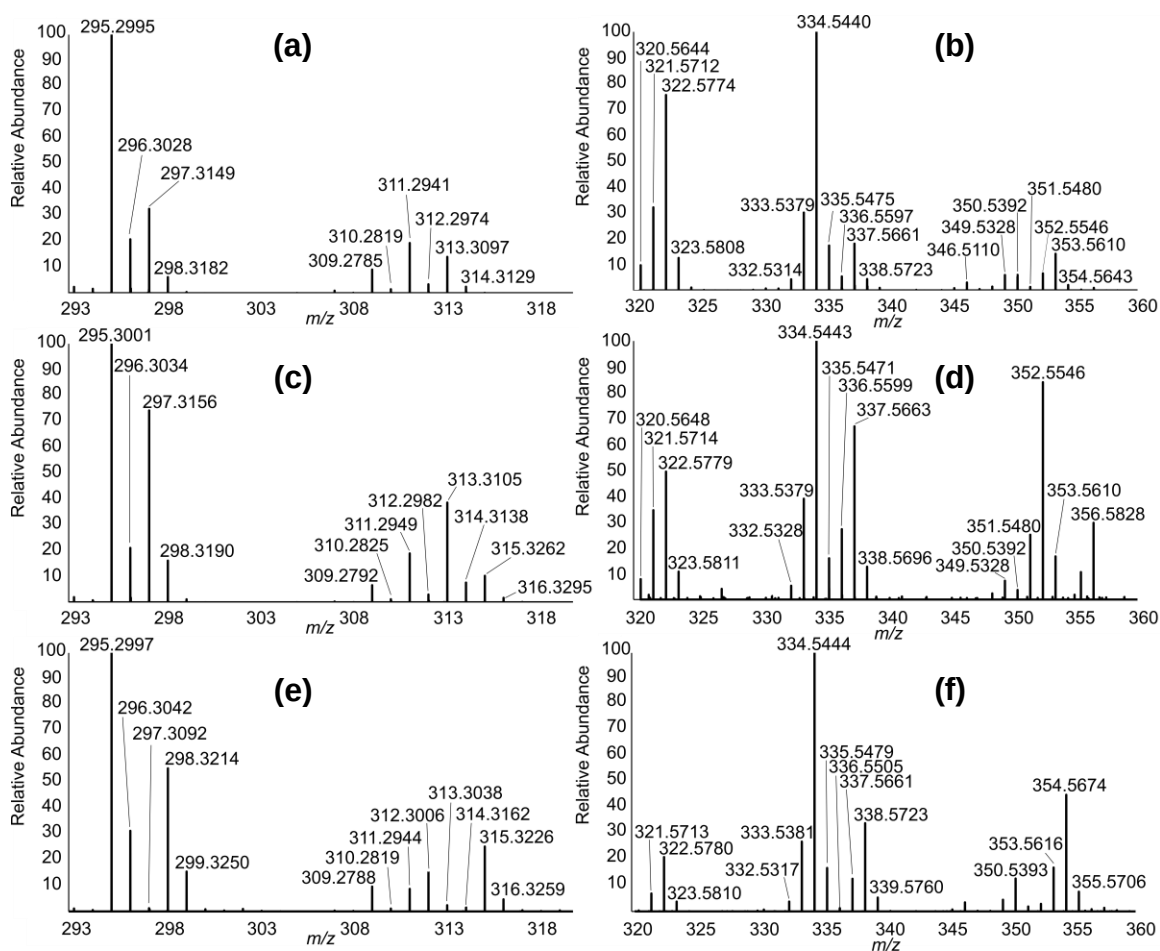


Figure 2.5. MS of (a) non-deuterated eicosane, (b) fully deuterated eicosane, (c) non-deuterated eicosane with water introduced into the atmosphere, (d) fully deuterated eicosane with water introduced into the atmosphere, (e) non-deuterated eicosane with deuterium oxide introduced into the atmosphere, and (f) fully-deuterated eicosane with deuterium oxide introduced into the atmosphere.

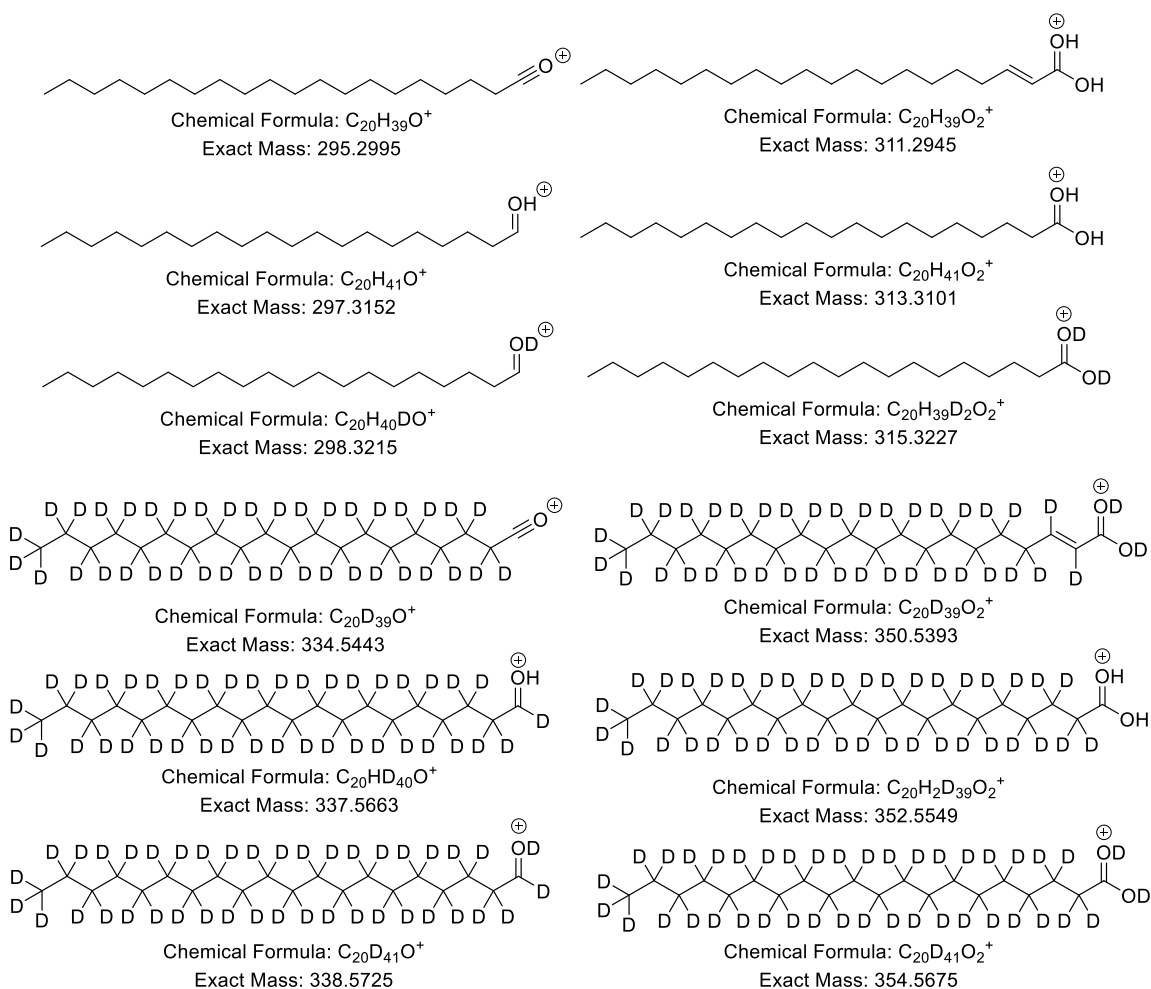


Figure 2.6. Potential structures of ion generated during the infusion of water or deuterium oxide. Note that the addition of the alcohol group, or groups, to the parent molecule can occur at any position based on available data.

Furthermore, the effect of changing source parameters was rigorously tested. Although changing the discharge current and S-lens RF level had no significant effect on the population of ions generated by either source, at least until settings were set so low that no signal was generated, it was determined that changing the gas settings significantly impacted the population of ions generated. Figure 2.7 shows an example of how a spectrum changes with these settings. As shown and described, the different settings of APCI and APPI generated differing abundances of the unique ion species. However, by changing the APCI gas settings to that of the APPI, the population of the unique ions shifts to those originally observed by APPI. However, the TIC generated by APCI with these settings was excessively noisy and features could only be detected using EICs. Therefore, the optimized settings of each source will continue to be used but with the knowledge that it is the gases that affect the abundance of each unique ion. Moreover, these experiments present indirect evidence that atmospheric oxygen is being incorporated into the analytes as the APPI settings involve less nitrogen than those of the APCI, which implies that a larger percentage of oxygen is available for ionization compared to APCI.

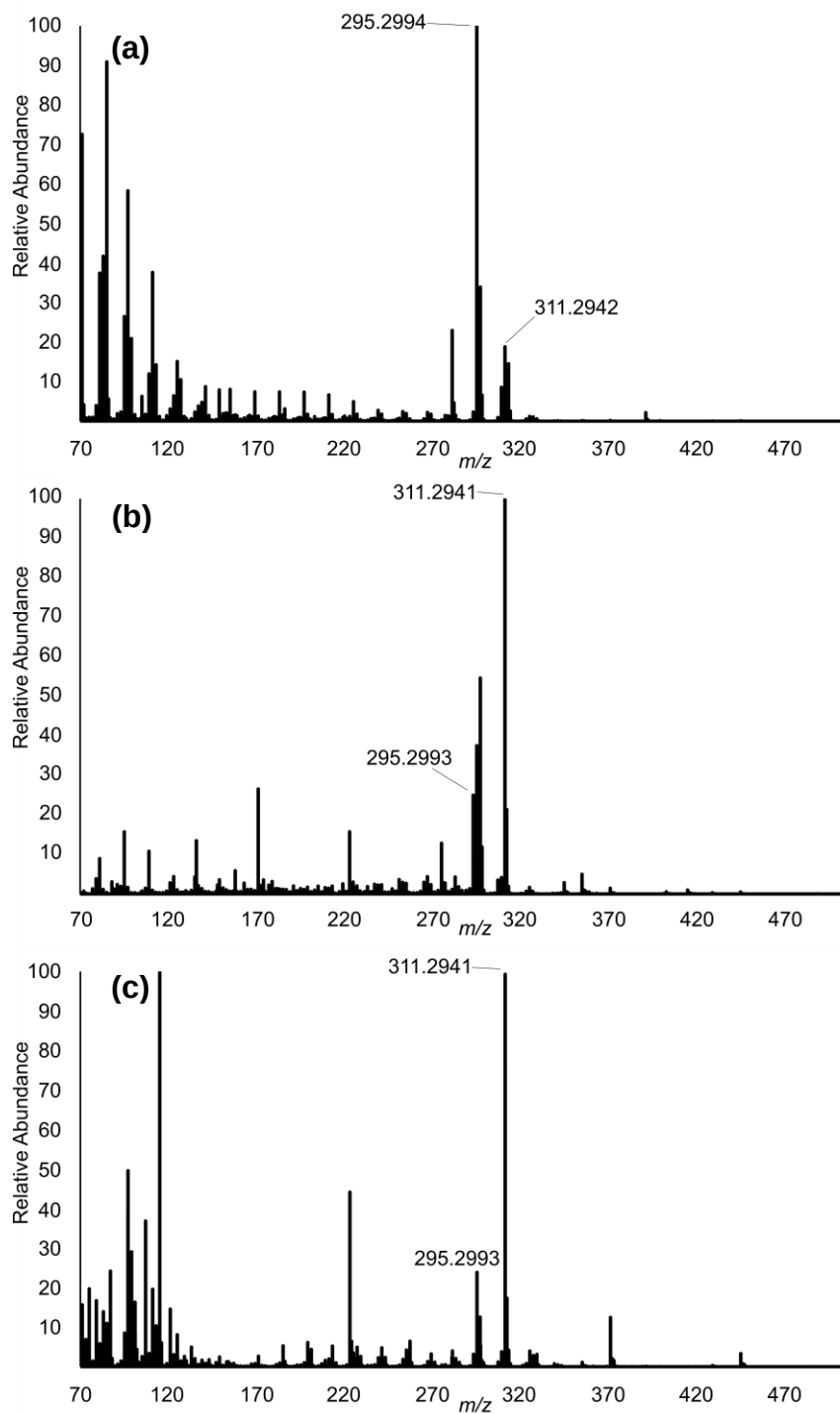


Figure 2.7. Full MS of the analyte eicosane generated by (a) APCI with its original settings, (b) APPI with its original settings, and (c) APCI with the same gas settings (sheath, aux, and sweep gas) as the APPI source.

2.4.3. Fatty Acid Methyl Ester Analysis

A mixture of four, fully deuterated FAMES were used to represent the various FAMES in a 37-component mix. Specifically, methyl palmitate (D_{31} , 98%), methyl stearate (D_{35} , 98%), methyl oleate (D_{33} , 98%), and methyl linoleate (D_{31} , 98%). This mixture of FAMES is excellent for analyses as there are different degrees of unsaturation yet similar masses. Previously, it was found that there were a few trends between ions detected, ionization source, and the FAME being analyzed. Methyl palmitate, methyl stearate, and methyl linoleate generated $[M]^+\bullet$ m/z s as base peaks when ionized by APCI while methyl oleate was represented by $[M - 32.0269]^+$ ions as the base peak. Alternatively, APPI generated $[M - 45.0347]^+$ m/z s as the base peaks for methyl palmitate and methyl stearate, $[M - 32.0269]^+$ ions as the base peak for methyl oleate, and $[M]^+\bullet$ as the base peak for methyl linoleate. Table 2.2 lists the total pertinent information on these compounds as well as their fully deuterated counterparts. While these experiments did not generate the same base peaks for each analyte as previously reported, the unique ions were still present. However, Table 2.2 does not accurately reflect the data necessary to elucidate chemical structures due to the complexity of the full spectra. Particularly, the spectrum of each analyte consisted of clusters of ions separated by 1.0062 m/z , which could potentially point to the exchange of deuteriums with hydrogens, unsaturation, or a combination of the two actions. Further complicating the spectra are the heteroatoms of the FAMES, which increase the potential for radical cations, as well as ^{13}C isotopic peaks. Thus, a closer look at the MS and MS² of each analyte is necessary.

Table 2.2. List of FAMES and their characteristic ions generated via either APPI or APCI.

Name	RT based on APPI (min)	Neutral Mass (<i>m/z</i>)	APPI Characteristic Ions (<i>m/z</i>)	Relative Abundance (%)	APCI Characteristic Ions (<i>m/z</i>)	Relative Abundance (%)
methyl palmitate-D ₃₁	15.39	301.4505	253.3965	100.00	301.4497	100.00
			301.4506	96.89	302.4558	71.78
			254.4027	91.03	269.4245	2.44
methyl palmitate	15.67	270.2559	271.2624	100.00	271.2620	100.00
			225.2208	92.68	270.2546	37.35
methyl stearate-D ₃₅	17.16	333.5069	285.4527	100.00	333.5058	100.00
			333.5069	85.77	332.4995	73.97
			286.4591	79.71	301.4807	2.79
methyl stearate	17.46	298.2872	253.2519	100.00	299.2930	100.00
			299.2936	85.89	298.2856	39.31
methyl oleate/elaidate-D ₃₃	17.35	329.4787	325.4504	100.00	329.4780	100.00
			324.4420	93.71	330.4841	77.43
			293.4248	53.87	295.4385	21.92
methyl oleate/elaidate	17.63	296.2715	295.2623	100.00	297.2776	100.00
			263.2363	67.88	264.2439	16.75
methyl linoleate-D ₃₁	17.76	325.4505	322.4296	100.00	325.4494	100.00
			321.4230	91.31	326.4556	75.83
			291.4109	31.62	293.4241	12.17
methyl linoleate	18.05	294.2559	293.2466	100.00	295.2620	100.00
			295.2622	90.40	294.2544	45.81
			263.2362	59.91		

Figure 2.8 shows MS of methyl linoleate and methyl linoleate-D₃₁ ionized by both APCI and APPI. From this example, it is apparent that it is possible to ionize each analyte by various means. For instance, methyl linoleate-D₃₁ ionized by APCI had a base peak represented by 325.4506 *m/z*. The structure of this ion is best characterized as a protonated form of the non-deuterated molecule with one deuterium replaced with a hydrogen. This hypothesis is supported by the presence of one peak 1.0060 *m/z* away (326.4566) and another subsequent peak 1.0035 *m/z* away (327.4601), which is indicative of a fully deuterated ¹³C isotope. The base peak of methyl linoleate-D₃₁ by APPI, however, is 323.4359 *m/z*, which most likely results from a positive charge along the non-polar backbone, stabilized by the two double bonds of the molecule. Adjacent ions 322.4293 and 321.4228 *m/z* are realized through substitutions of hydrogen for deuterium. Alternatively, the 325.4496 and 324.4430 *m/z* can be considered the result of the formation of a radical cation on the heteroatom and subsequently a substitution of hydrogen for deuterium. These ionization differences account for the small differences in mass between the spectra from either source, such as the 325.4506 and 325.4496 *m/z*.

Additionally, fragmentation to the molecules is dependent on the ionization source. For instance, APCI generated ions by partially cleaving the methyl ester, which could generate a triple bond between the previous carbonyl, as well as by putting a positive charge on the oxygen. The resulting ion, calculated as 294.4315 *m/z*, is indeed in the spectrum, observed as 294.4315 *m/z*, and the rest of the ions of this cluster can be attributed to deuteriums being replaced with hydrogens. For the APPI spectra, it can be concluded that a mixture of ions is responsible for the cluster observed. Specifically, the 292.4170 *m/z* would result from same molecule observed by APCI, though following deuterium abstraction could generate a radical cation with a structure similar to a ketene. Substitution of hydrogen for deuterium yields the two, smaller adjacent ions, 291.4107 and 290.4038 *m/z*, while replacing the radical with the original deuterium would yield the larger adjacent ion, 293.4244 *m/z*. Finally, the remaining smaller ions of the cluster can be explained by an

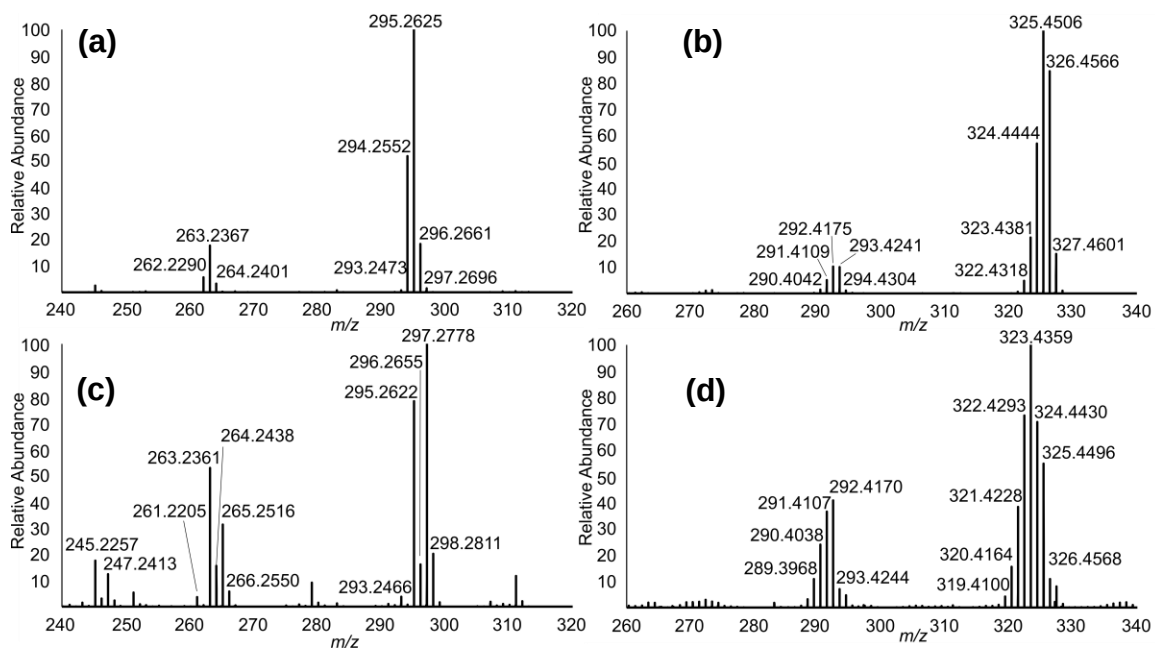


Figure 2.8. Selected MS of methyl linoleate by (a) APCI and (c) APPI as well as methyl linoleate- D_{31} by (b) APCI and (d) APPI.

additional point of unsaturation and hydrogen substitutions (289.3968 and 288.3906 m/z) on the proposed ketene structure. These unsaturations, replacement of deuterium with hydrogen, and partial loss of the ester group are also supported by the MS² data. However, similar to alkanes, the exact location of these modifications has not been determined. Additionally, other structures of varying stability and resonance are possible.

From these experiments, a conclusion can be made regarding the original $[M - 32.0267]^+$ species. In particular, this species must be the result of a ketene that is a radical cation. The proposed structure of the positively charged molecule is resonance stabilized due to the triple bond and radical, depicted in Figure 2.9. The observed spectra of the deuterated species support this conclusion as well as the original data, which shows multiple m/z due to the analyte either being a positively charged acylium ion, possessing a radical cation as a ketene, or possessing a ¹³C isotope as seen in Figure 2.10. Thus, the $[M - 32.0267]^+ / [M - 33.0335]^+$ ions for non-deuterated and deuterated analytes, respectively, are the result of in-source fragmentation.

Overall, the same trends initially observed for resulting ions from FAMES analyses with respect to structure generally hold true. Specifically, APCI continued to generate the unique ions $[M - 32.0267]^+ / [M - 33.0335]^+$, for non-deuterated and deuterated analytes respectively, though it did so for all FAME species studied. Conversely, APPI generated the characteristic $[M - 45.0347]^+$ and $[M - 47.0480]^+$ ions for non-deuterated and deuterated saturated FAMES, respectively. The formation of these ions appears to be driven by fragmentation at the carbon-carbonyl bond followed by oxidation to a new carbonyl, similar to the process observed for alkenes. Conversely, $[M - 32.0267]^+ / [M - 33.0335]^+$ ions and their isotopic clusters were the sole unique ions for unsaturated FAMES. Figure 2.9 demonstrates the potential for either species from methyl palmitate-D₃₁ as well as example spectra. While additional deuterated FAME standards of varying

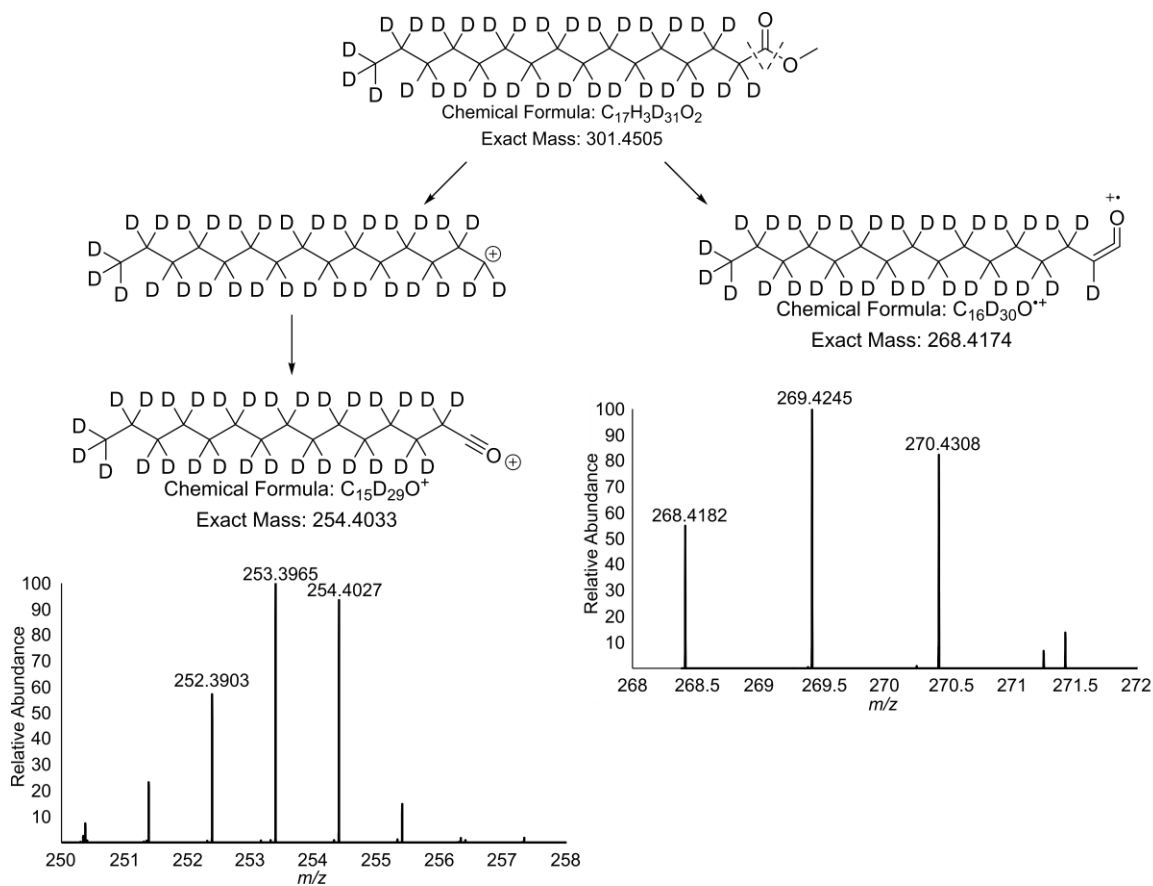


Figure 2.9. Methyl palmitate- D_{31} and possible structures of the unique ions produced by either ionization technique. 254.4027 m/z was produced by APPI and represents the $[M - 47.0480]^+$ ion while 268.4182 m/z was produced by APCI and represents the $[M - 33.0335]^+$ ion.

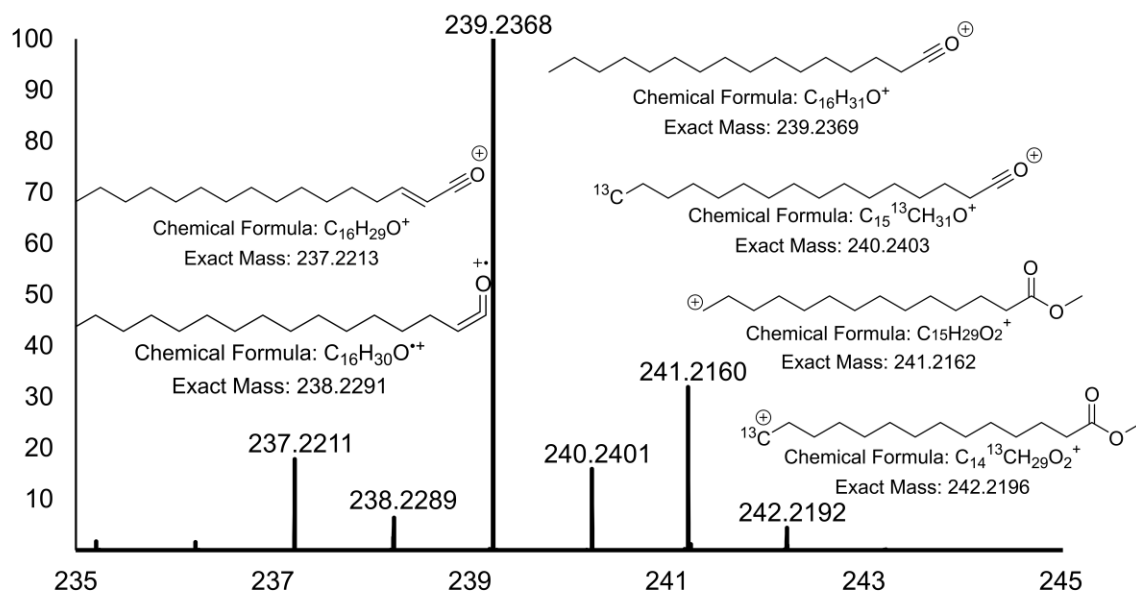


Figure 2.10. MS of methyl palmitate collected using a Thermo Fisher Scientific Exactive™ Orbitrap. The specific m/z s observed can be attributed to the various fragments of methyl palmitate and their chemical structure are depicted. Previously, the methyl palmitate fragment 239.2368 m/z was not reported as a significant ion and continues not to be, however, other FAMES and their respective fragments previously reported as $[M - 32.0267]^+$ would be share a similar structure to 238.2291 m/z reported here.³²

positionally different unsaturations would further strengthen these correlations, unique ions specific to molecular composition and ionization were observed.

2.5. Conclusion

Unique, characteristic ion species of saturated hydrocarbons and FAMES that have been previously underreported, are discussed. Through distinctive experimentation utilizing the GC-APCI interface, it was determined that atmospheric water had an insignificant role for the ionization mechanism of these species while the source gas settings shifted the population of ions significantly. Potential structures of these unique ions, generated by in-source oxidation and/or in-source fragmentation, are proposed based on isotopic and MS² analyses. Collectively, these data indicate a starting point for understanding the underlying mechanisms of APIs for GC, as well as provide considerable evidence to support future development and characterization of atmospheric pressure ionizations.

2.6. Appendix

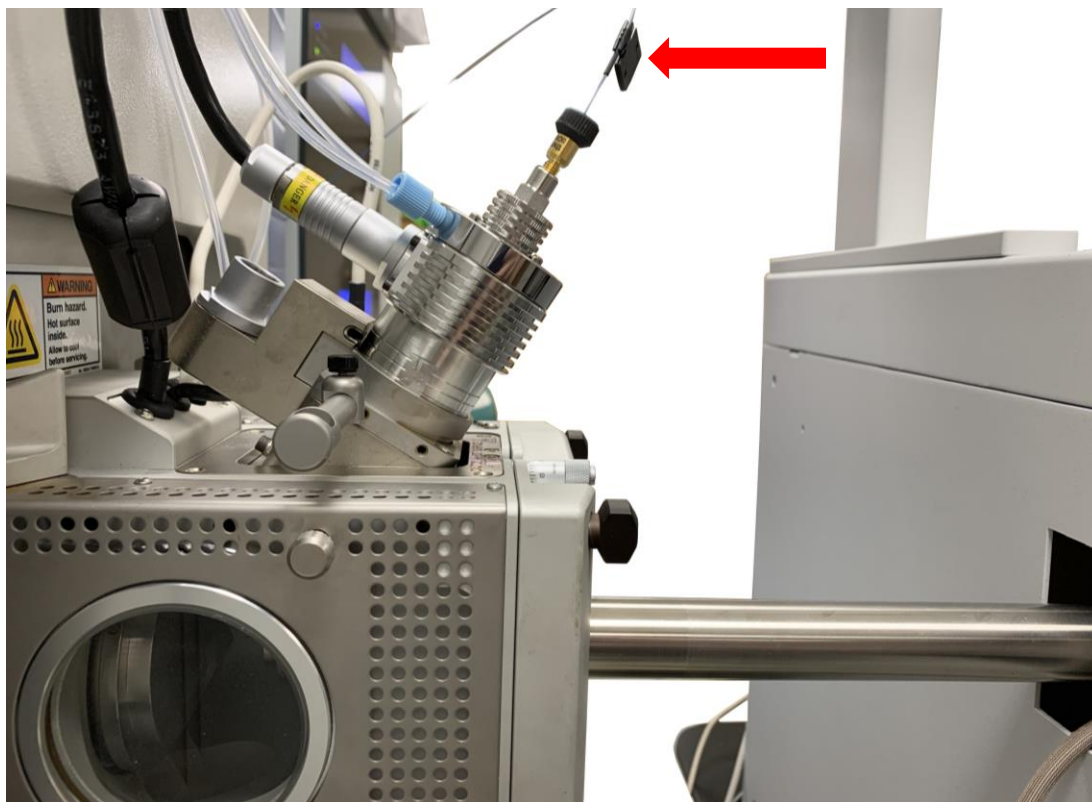


Figure 2.11. Picture of the GC-APCI interface with a Viper™ line attached to the APCI probe. Due to the unique setup of the interface, water can be infused and volatilized through the APCI probe as GC effluent is introduced into the Ion Max housing.

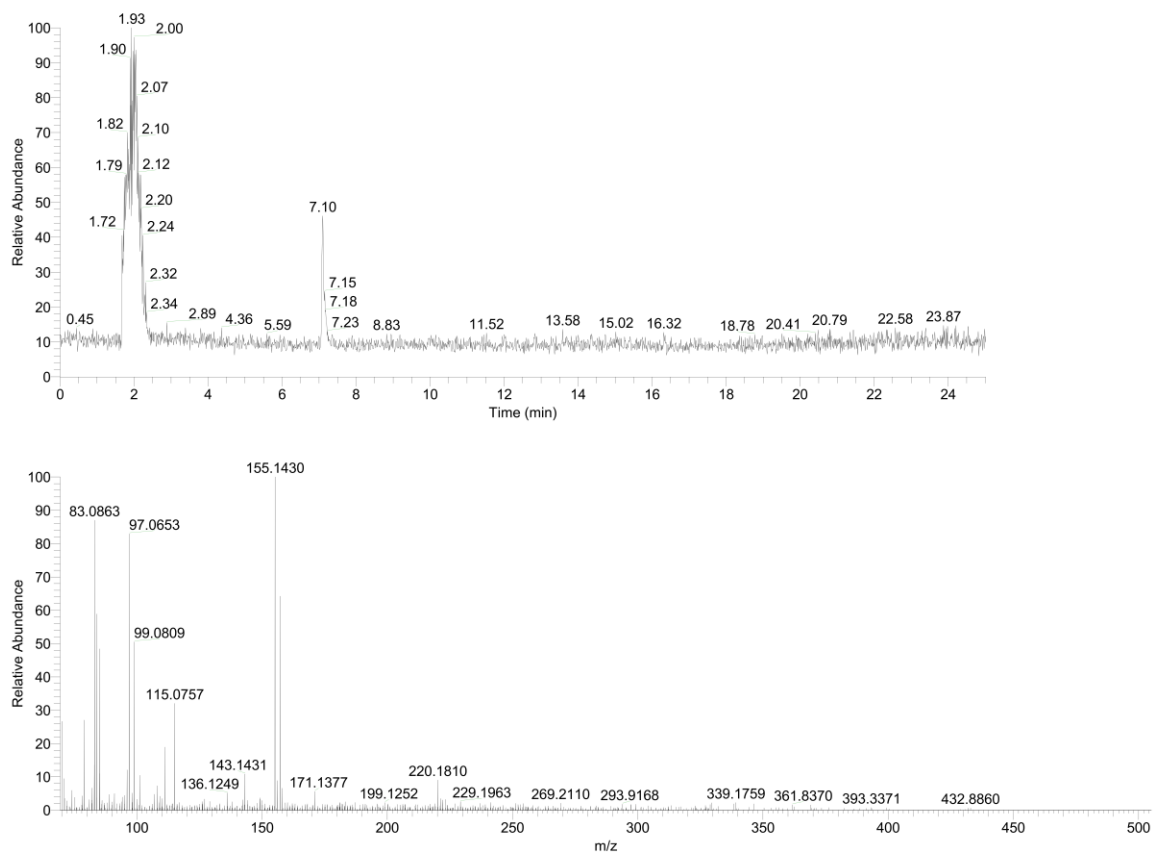


Figure 2.12. EIC (155.1430 m/z) and full MS of the analyte decane ionized using the APCI source.

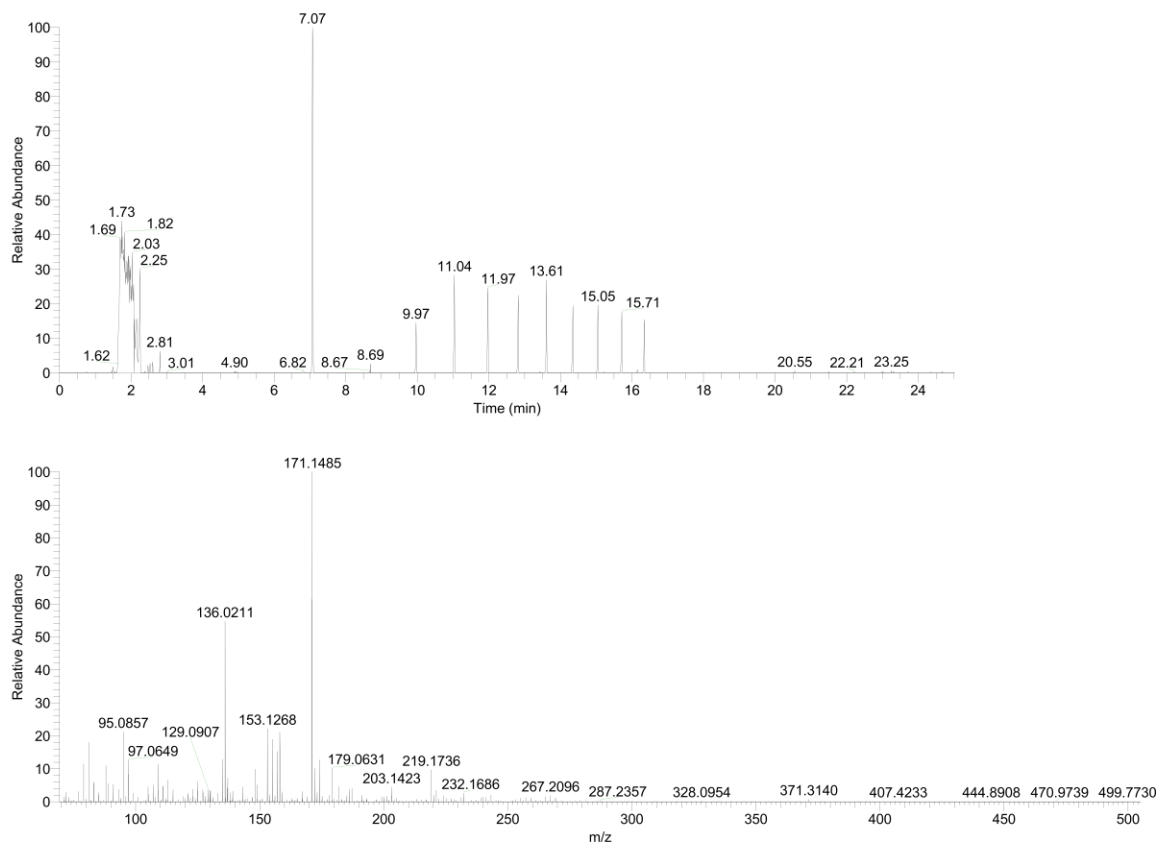


Figure 2.13. EIC (171.1485 m/z) and full MS of the analyte decane ionized using the APPI source.

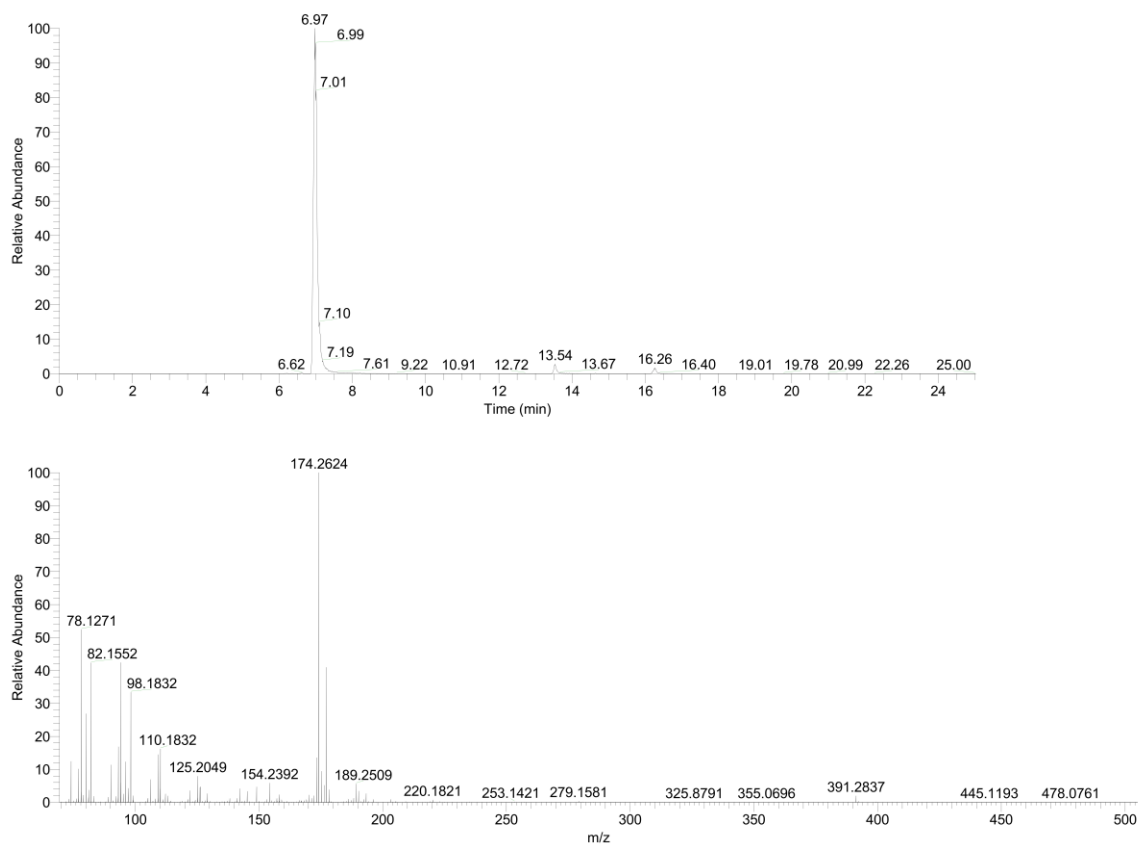


Figure 2.14. EIC (174.2624 m/z) and full MS of the analyte decane-D₂₂ ionized using the APCI source.

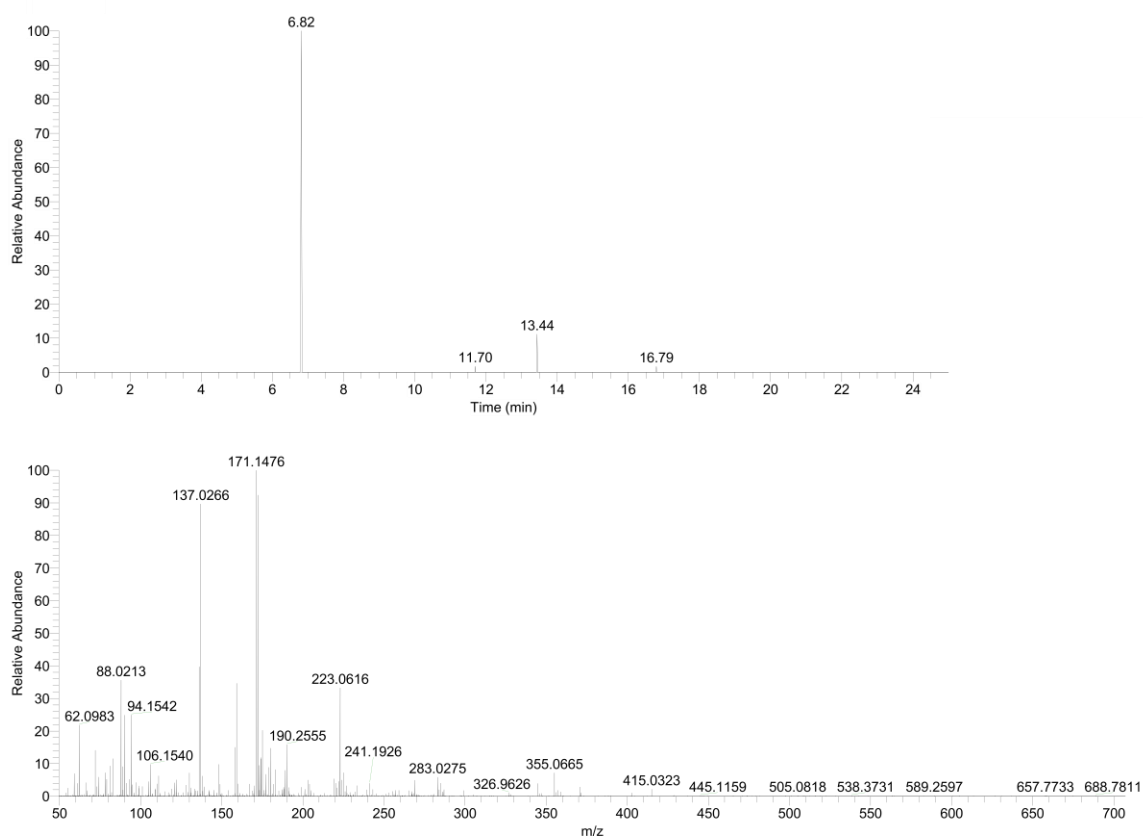


Figure 2.15. EIC (190.2555 m/z) and full MS of the analyte decane-D₂₂ ionized using the APPI source. The contaminant ion 171.1476 m/z is the base peak.

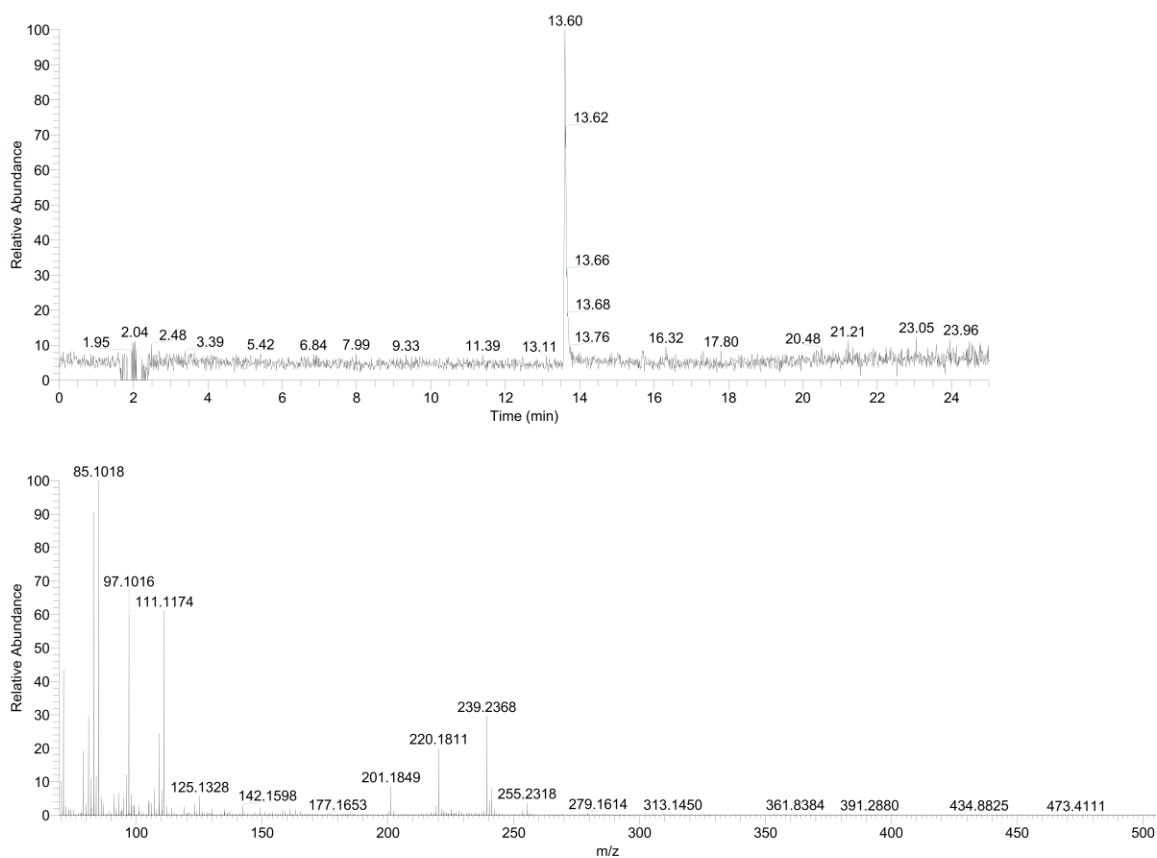


Figure 2.16. EIC (239.2368 m/z) and full MS of the analyte hexadecane ionized using the APCI source.

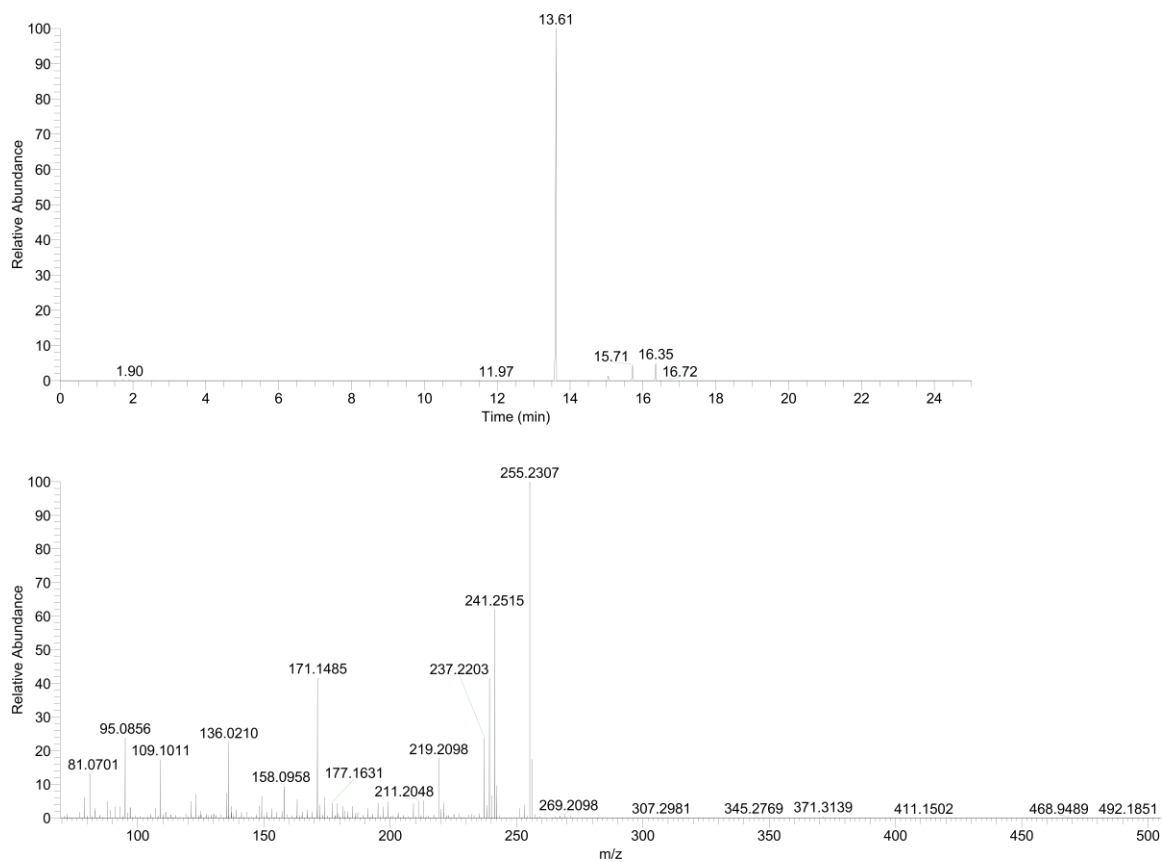


Figure 2.17. EIC (255.2307 m/z) and full MS of the analyte hexadecane ionized using the APPI source.

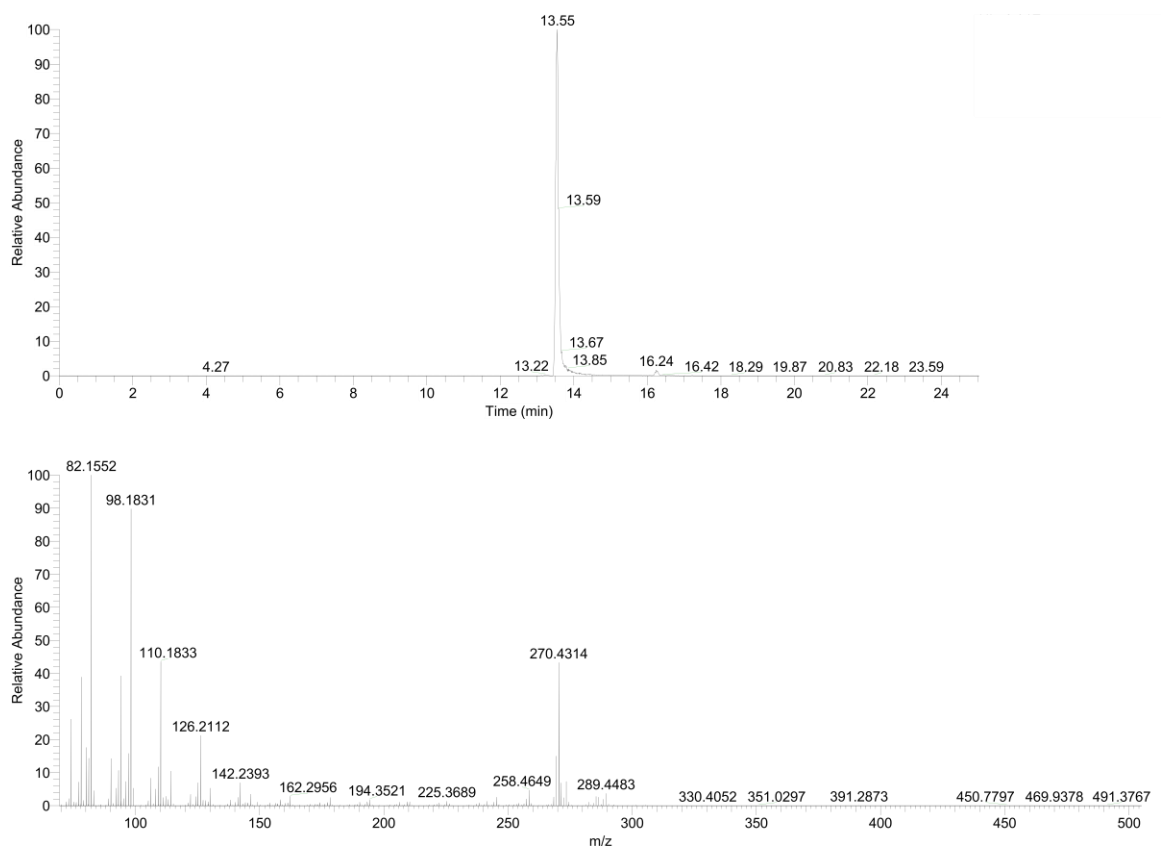


Figure 2.18. EIC (270.4314 m/z) and full MS of the analyte hexadecane- D_{34} ionized using the APCI source.

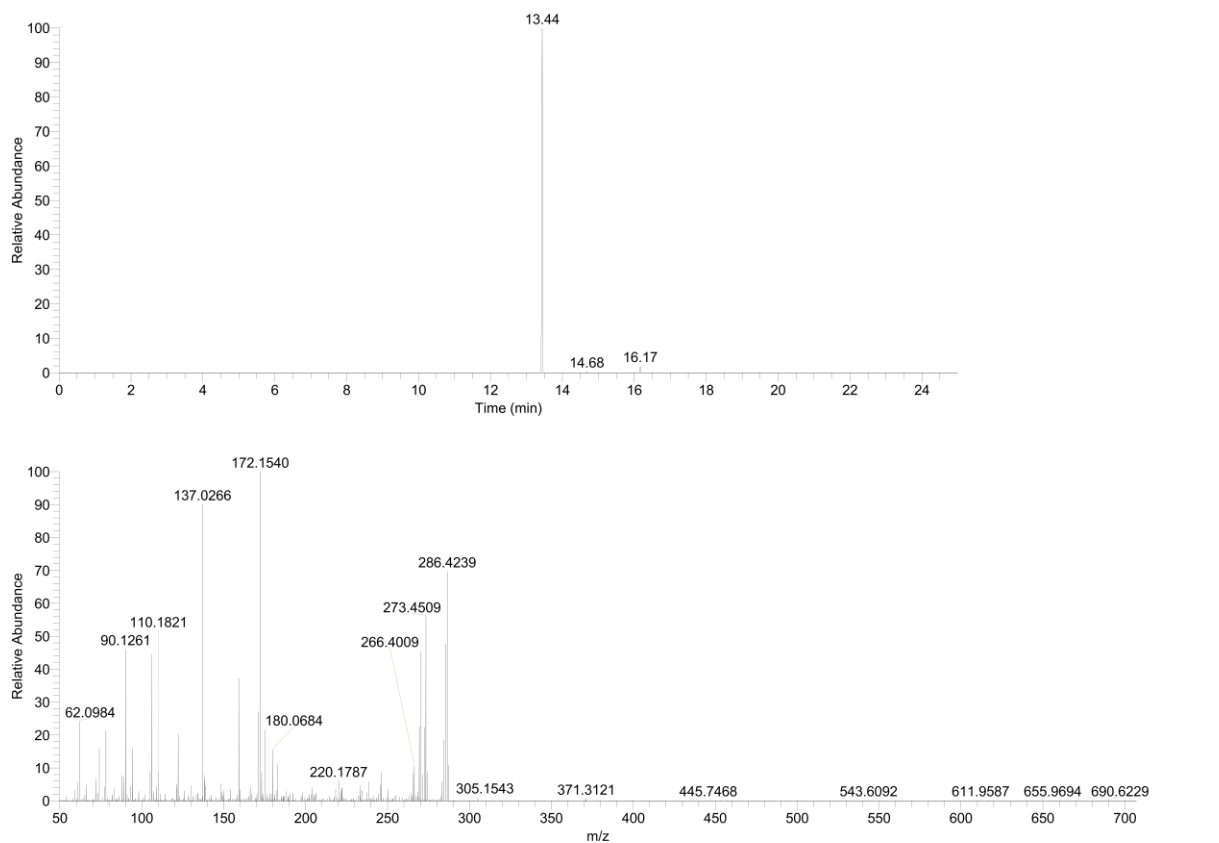


Figure 2.19. EIC (286.4239 m/z) and full MS of the analyte hexadecane- D_{34} ionized using the APCI source.

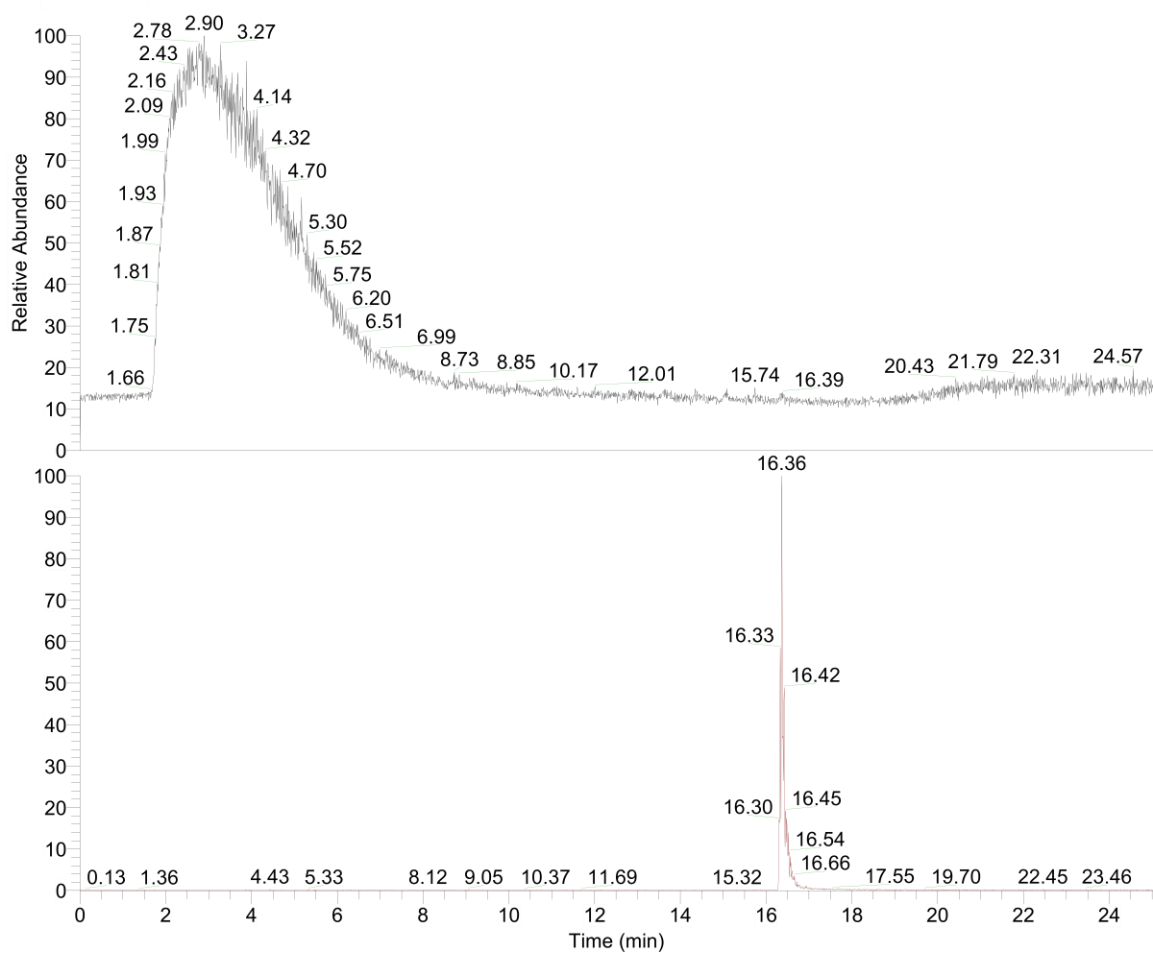


Figure 2.20. TIC and EIC (311.2929 m/z) of the C₈-C₂₀ mixed standard ionized by the APCI source with identical source gas settings to that of the APPI.

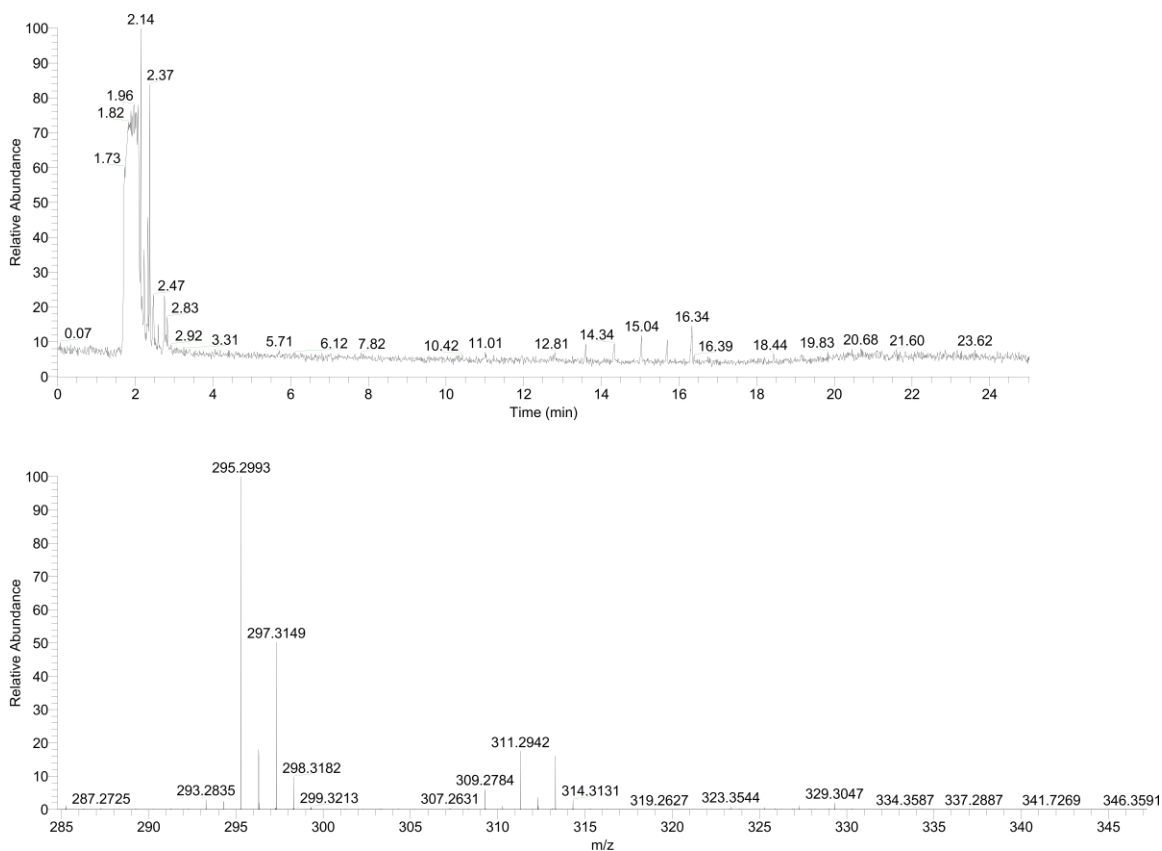


Figure 2.21. TIC of the C₈-C₂₀ mixed standard ionized by the APCI and zoomed MS of eicosane peak (RT 16.34). The discharge current was lowered to 5 μ A and the abundances of the unique ion species did not change.

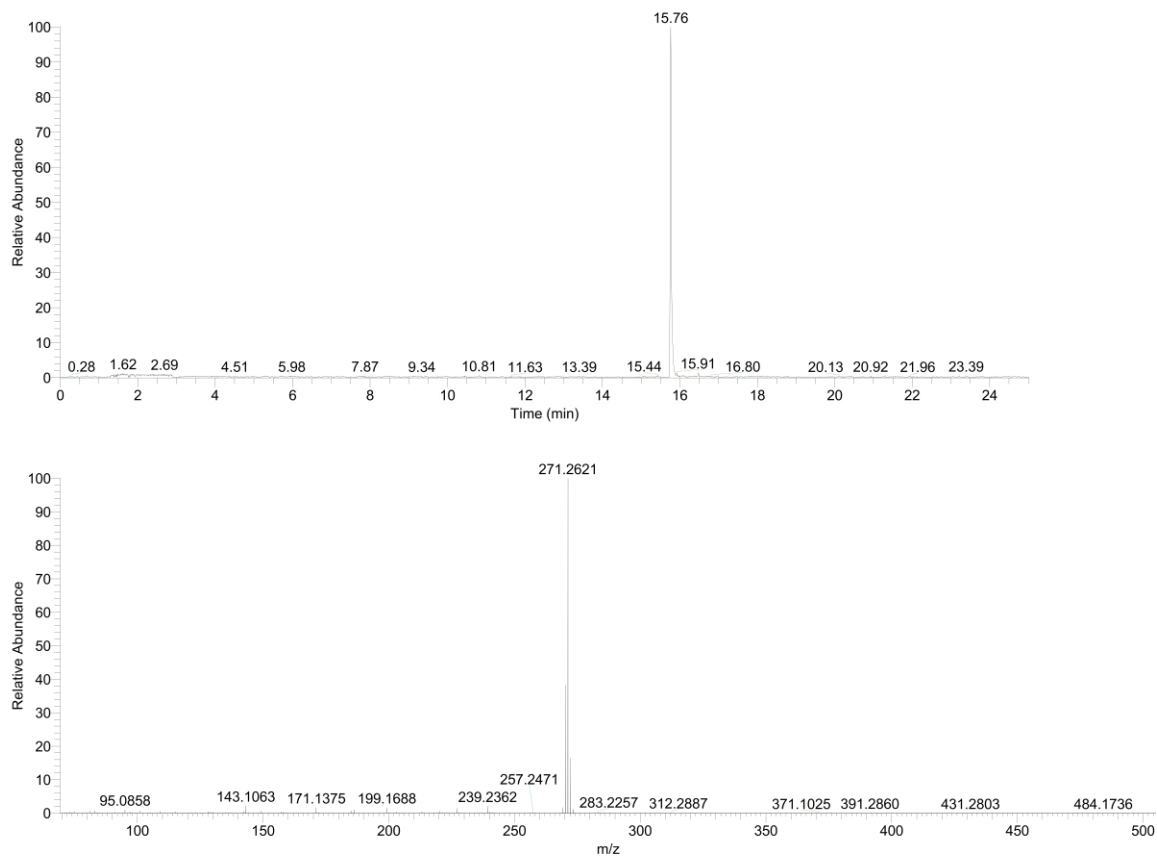


Figure 2.22. EIC (271.2621 m/z) and full MS of the analyte methyl palmitate ionized by the APCI source.

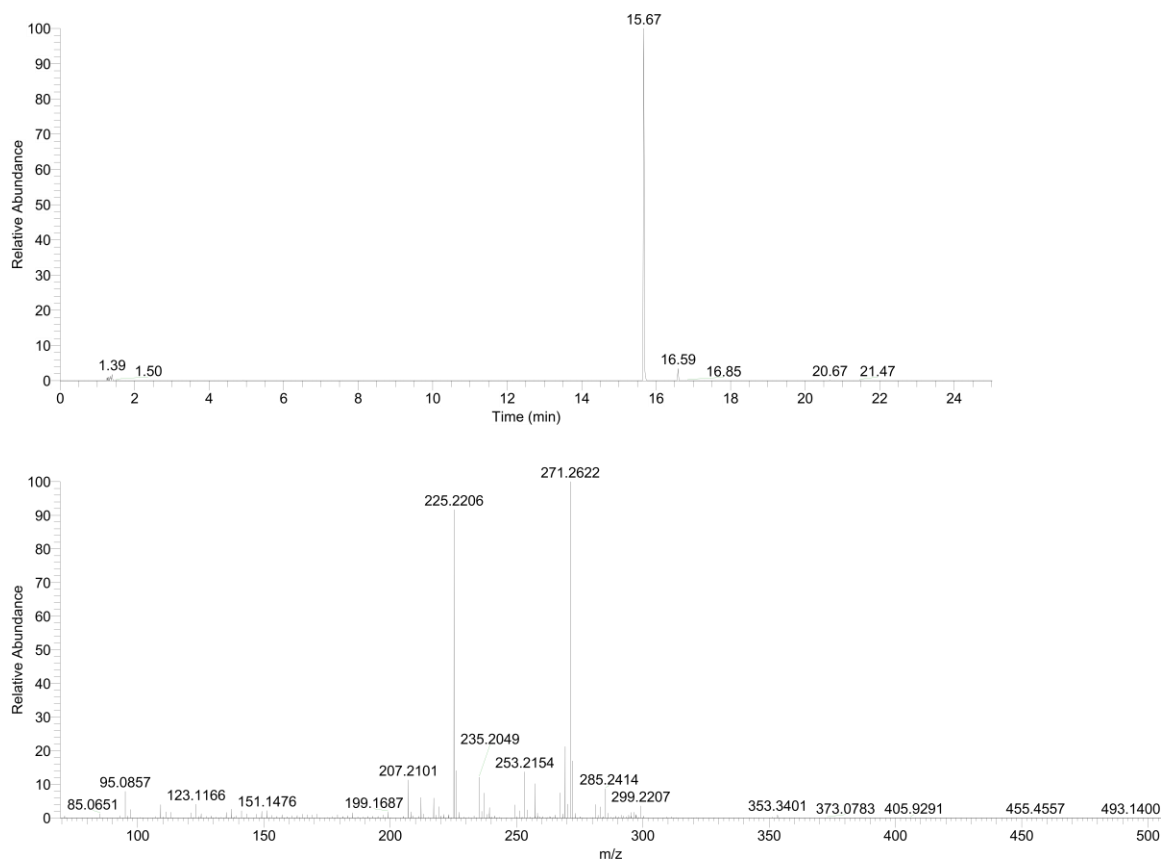


Figure 2.23. EIC (271.2621 m/z) and full MS of the analyte methyl palmitate ionized by the APPI source.

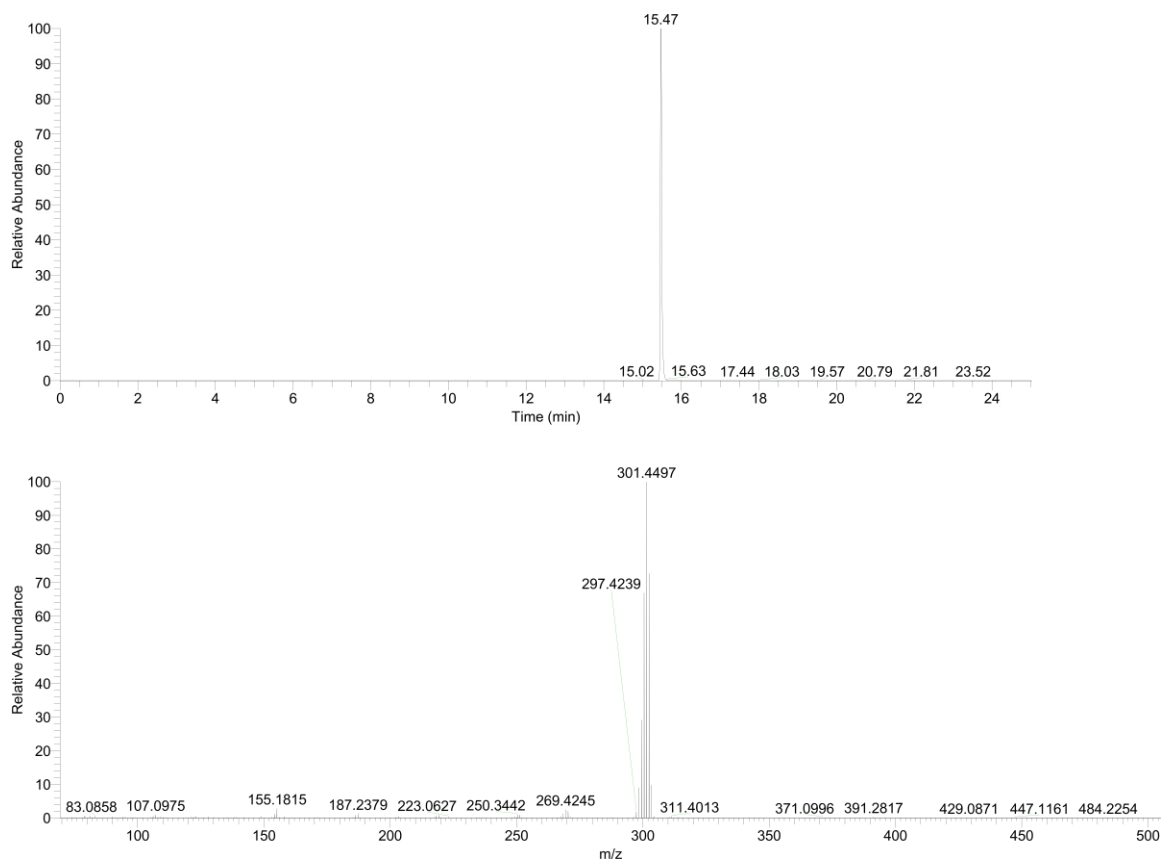


Figure 2.24. EIC (301.4497 m/z) and full MS of the analyte methyl palmitate- D_{31} ionized by the APCI source.

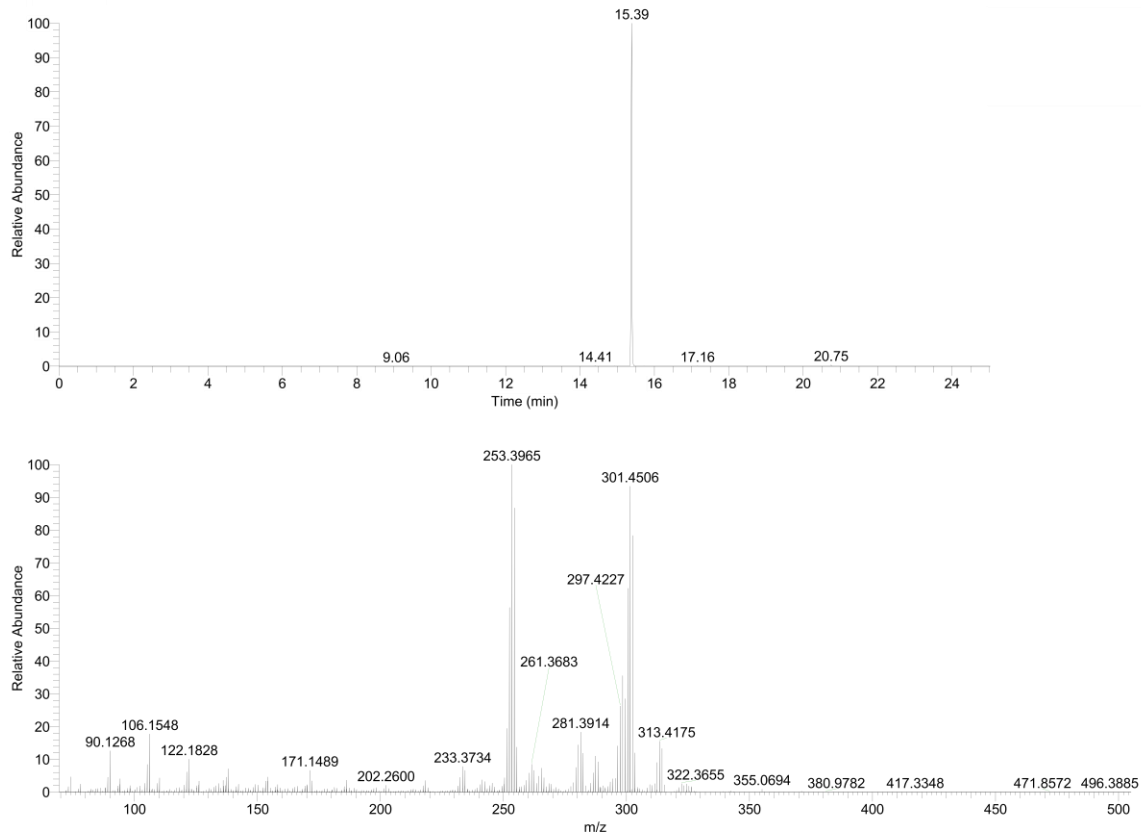


Figure 2.25. EIC (301.4497 m/z) and full MS of the analyte methyl palmitate- D_{31} ionized by the APPI source.

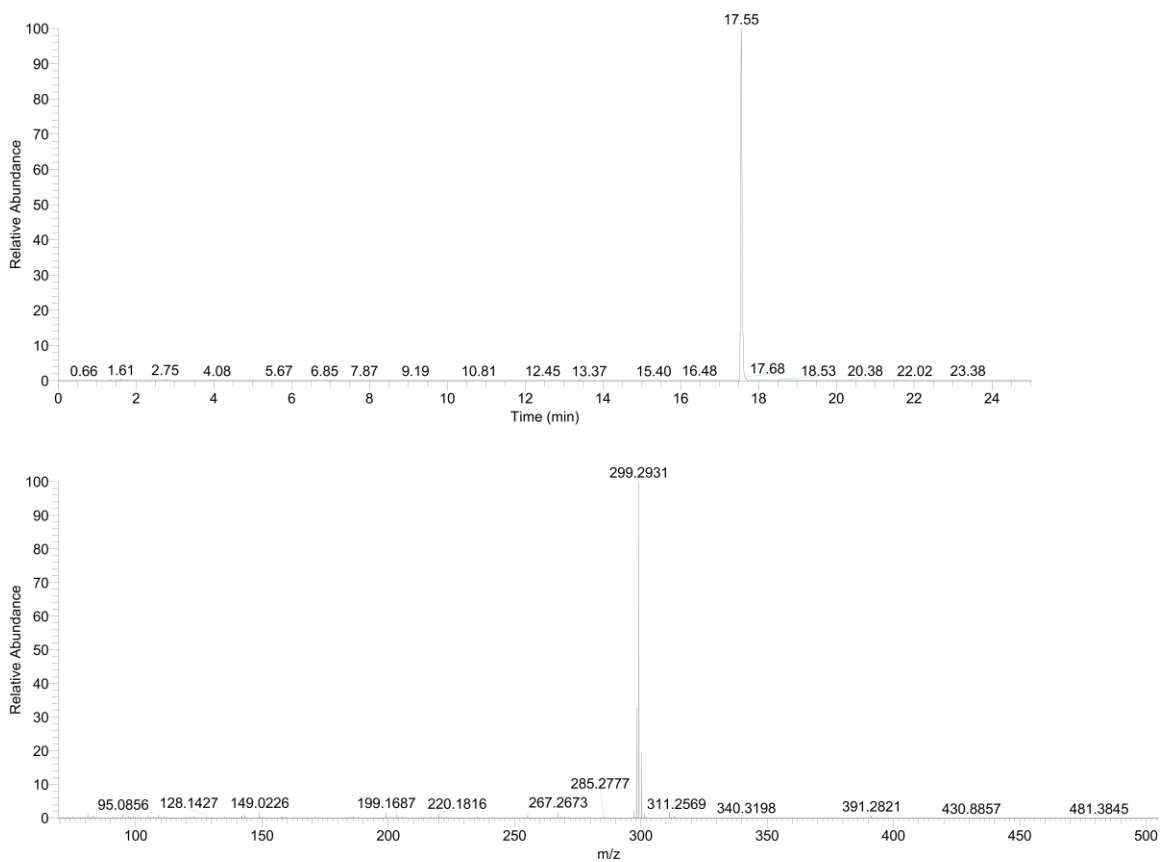


Figure 2.26. EIC (299.2931 m/z) and full MS of the analyte methyl stearate ionized by the APCI source.

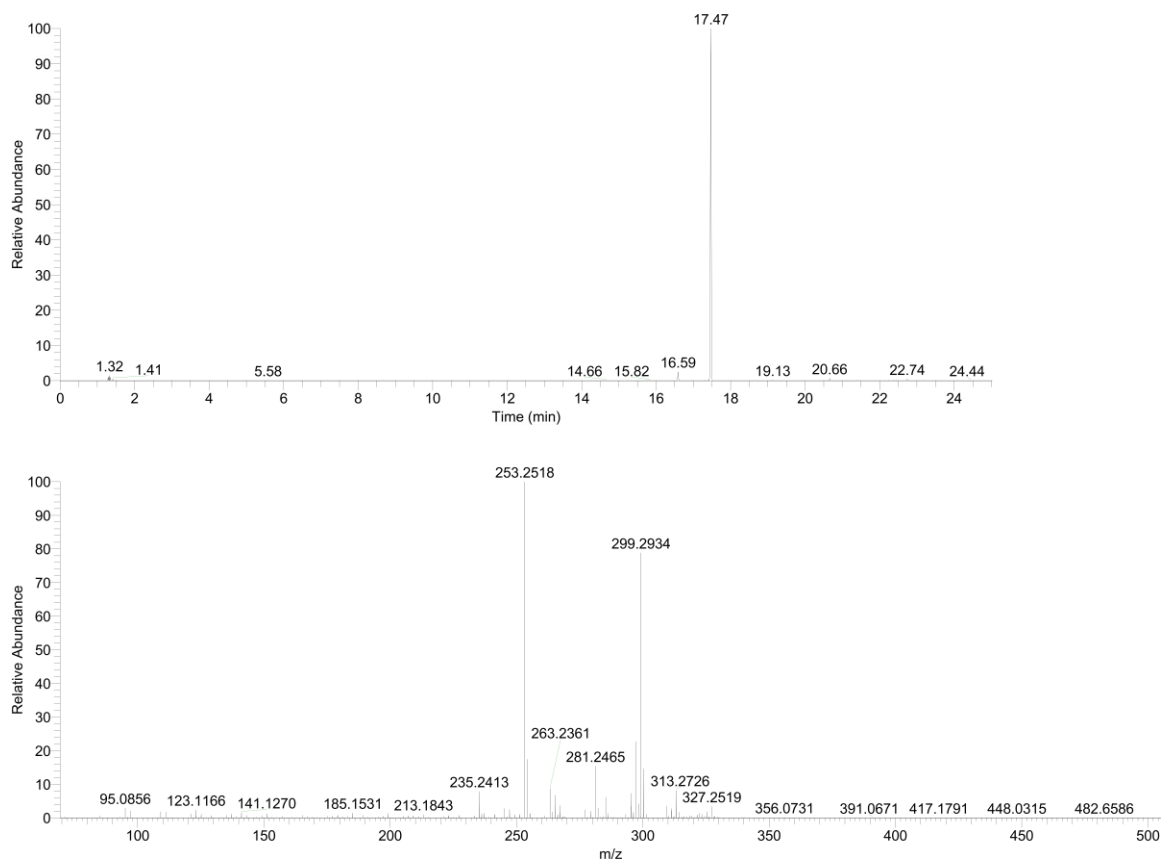


Figure 2.27. EIC (253.2518 m/z) and full MS of the analyte methyl stearate ionized by the APPI source.

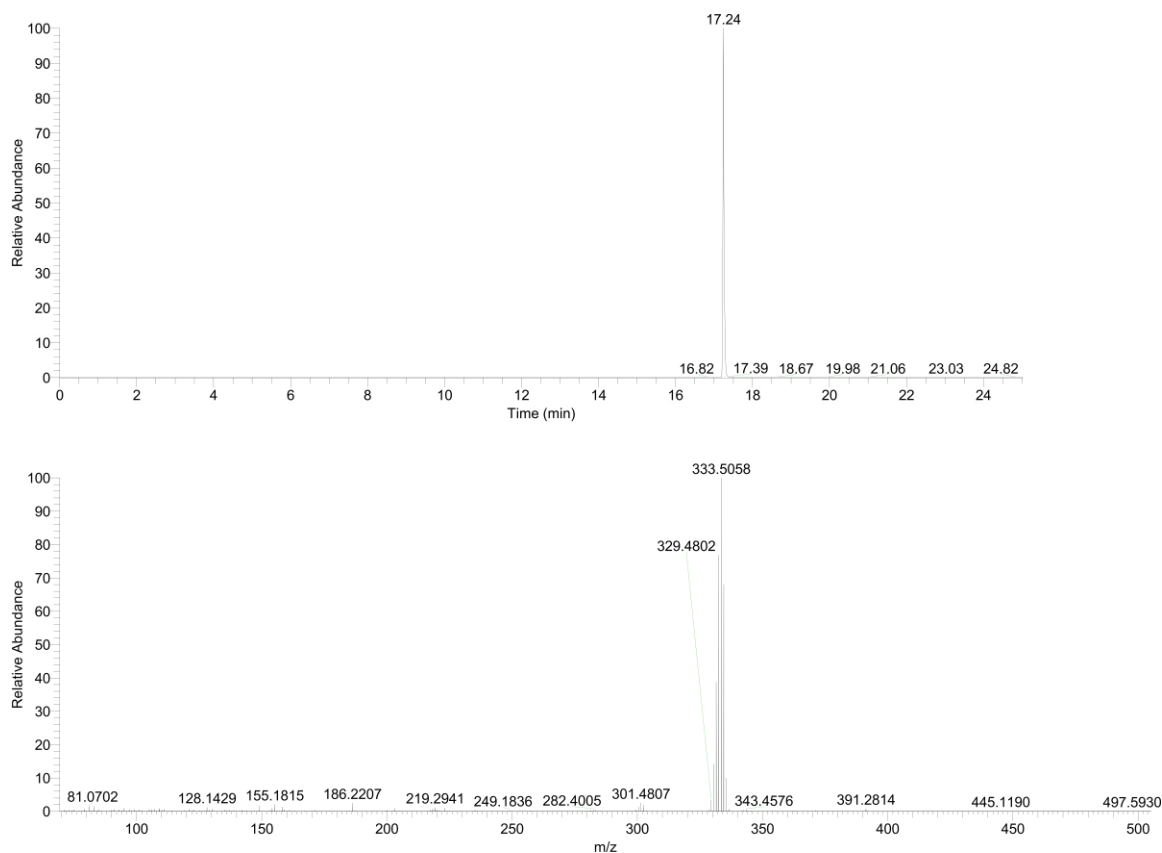


Figure 2.28. EIC (333.5058 m/z) and full MS of the analyte methyl stearate- D_{35} ionized by the APCI source.

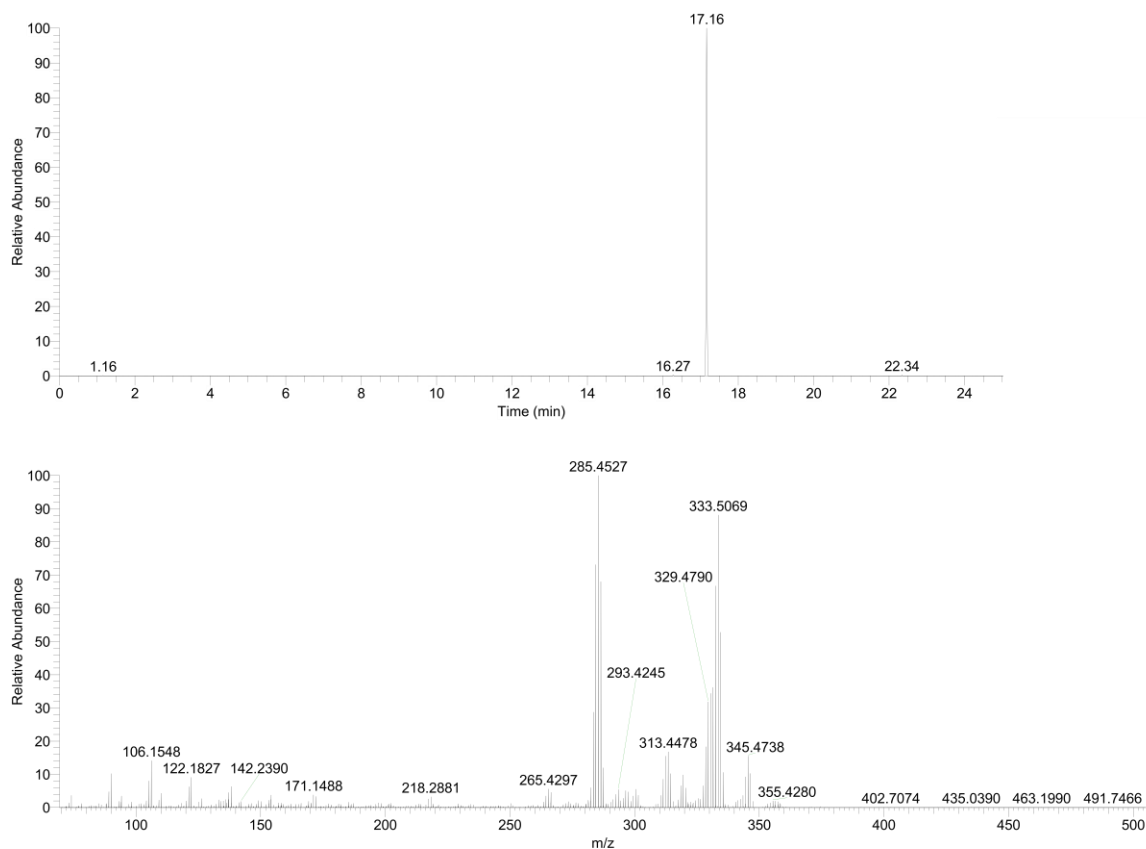


Figure 2.29. EIC (285.4527 m/z) and full MS of the analyte methyl stearate- D_{35} ionized by the APPI source.

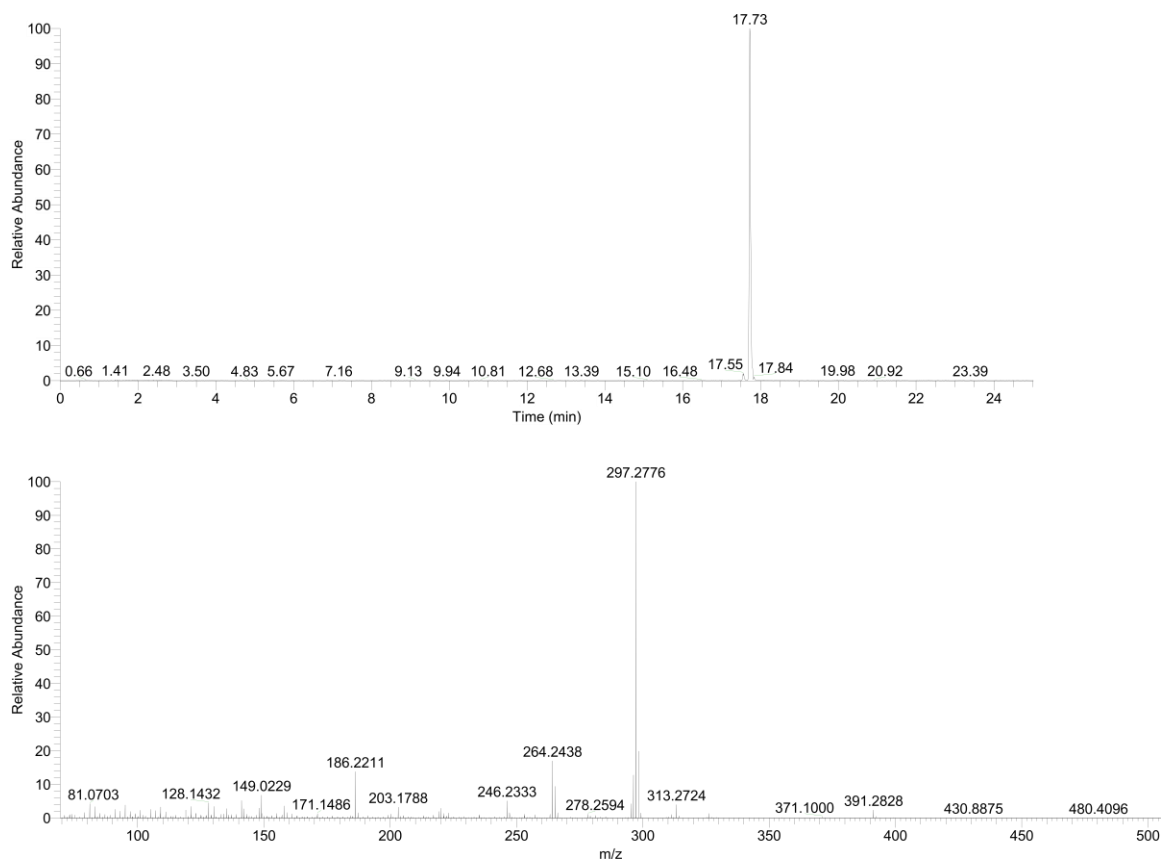


Figure 2.30. EIC (297.2776 m/z) and full MS of the analyte methyl oleate/elaidate ionized by the APCI source.

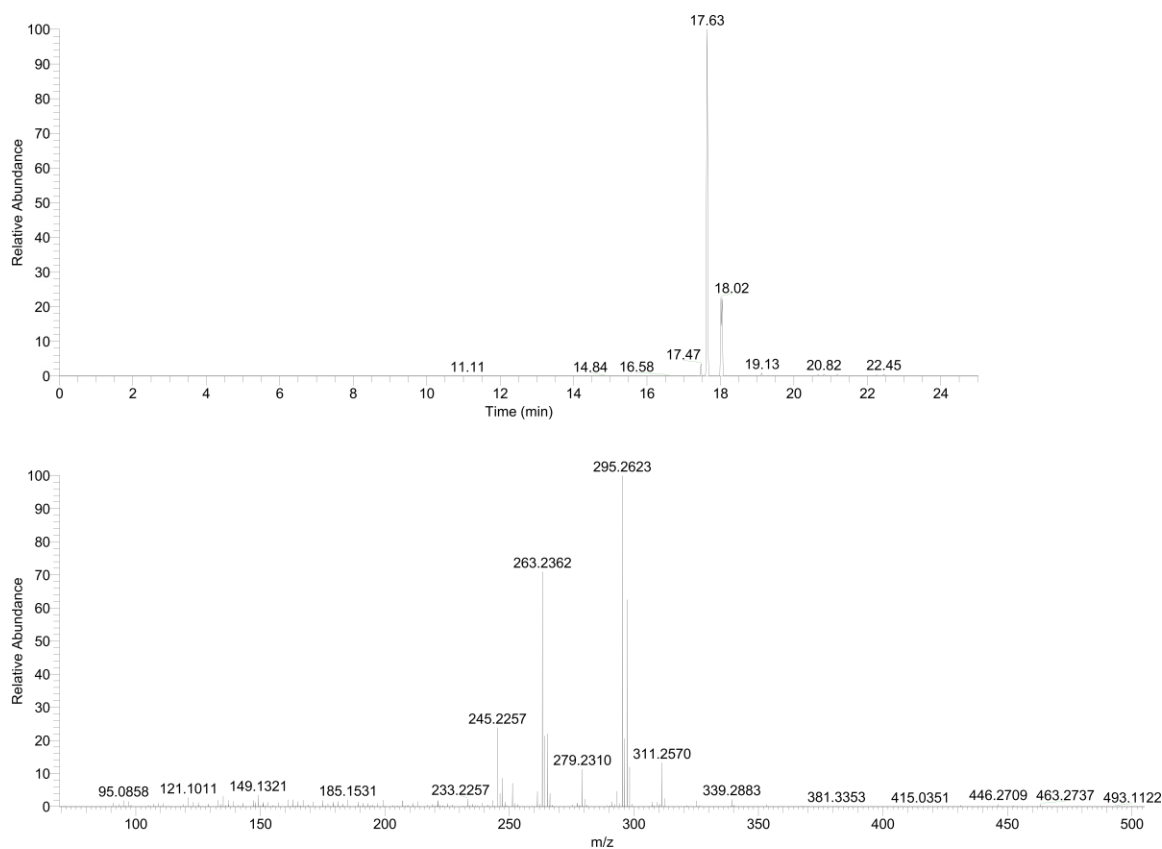


Figure 2.31. EIC (295.2623 m/z) and full MS of the analyte methyl oleate/elaidate ionized by the APPI source.

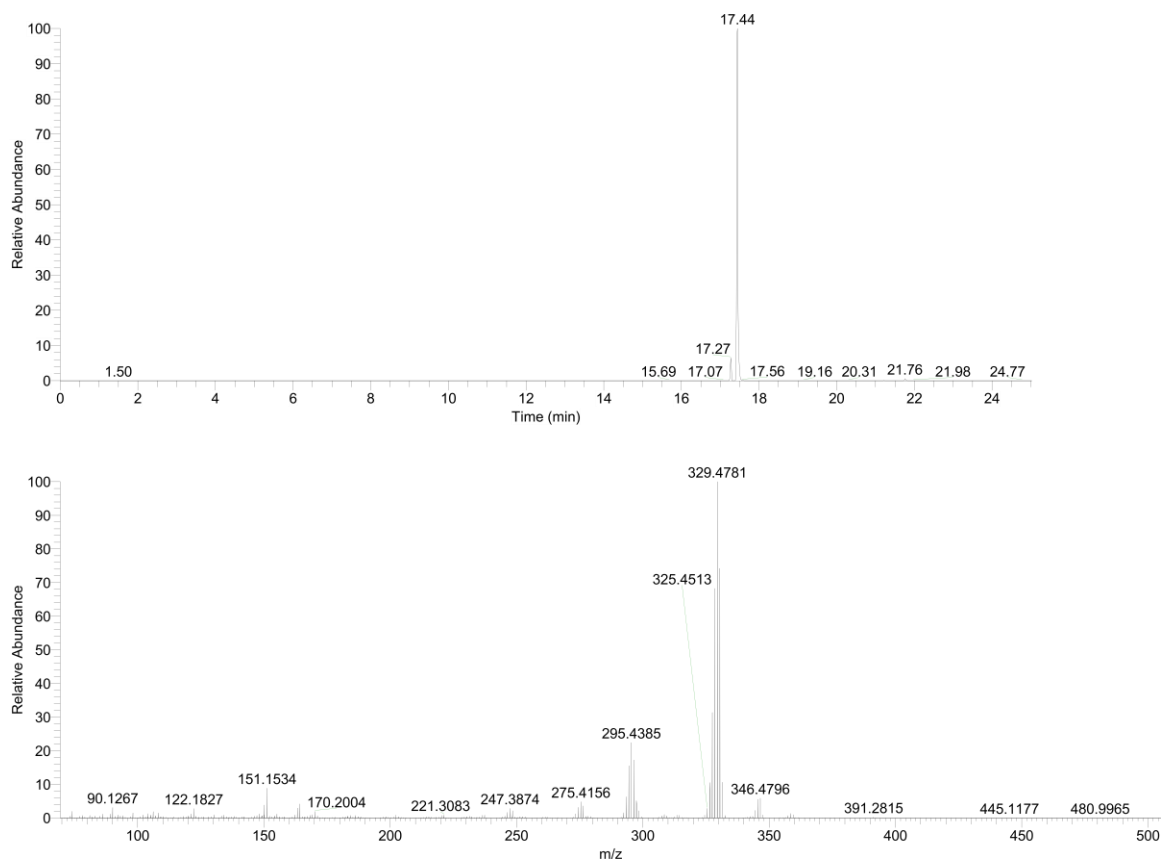


Figure 2.32. EIC (329.4781 m/z) and full MS of the analyte methyl oleate/elaidate- D_{33} ionized by the APCI source.

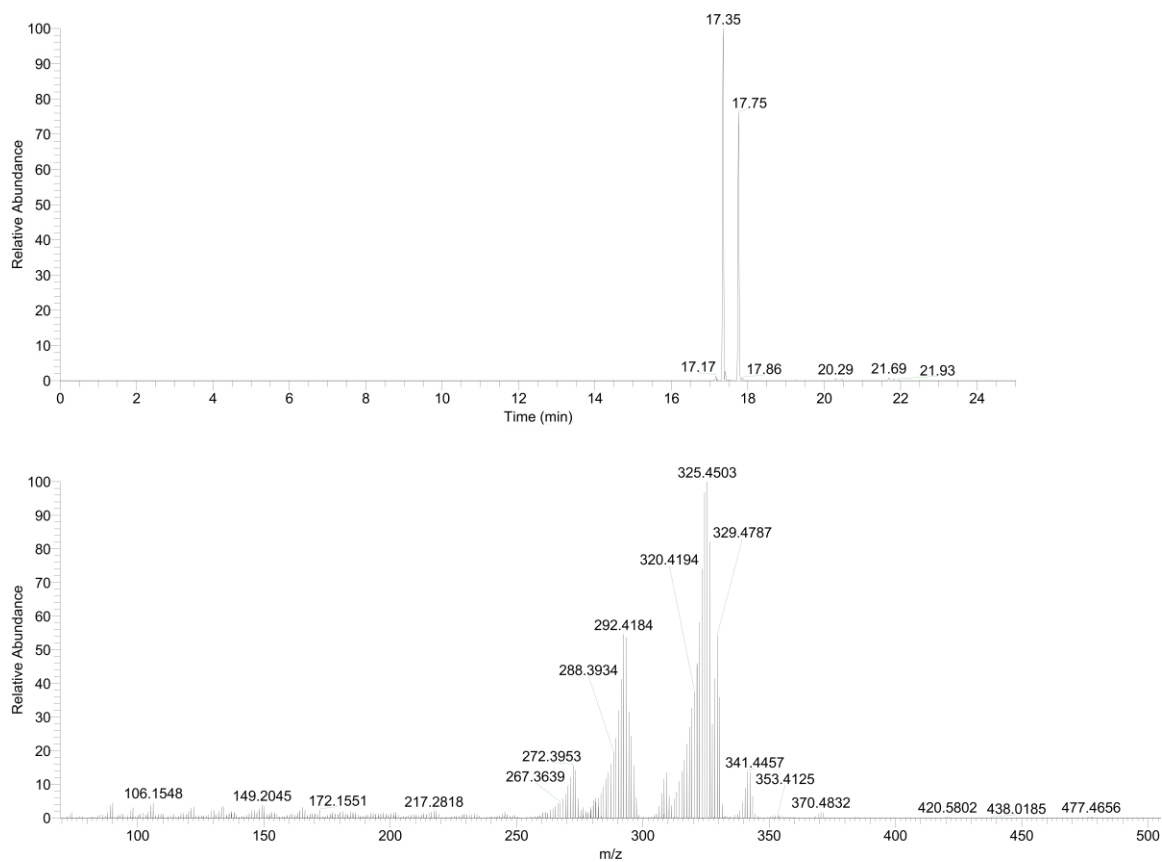


Figure 2.33. EIC (325.4503 m/z) and full MS of the analyte methyl oleate/elaidate- D_{33} ionized by the APPI source.

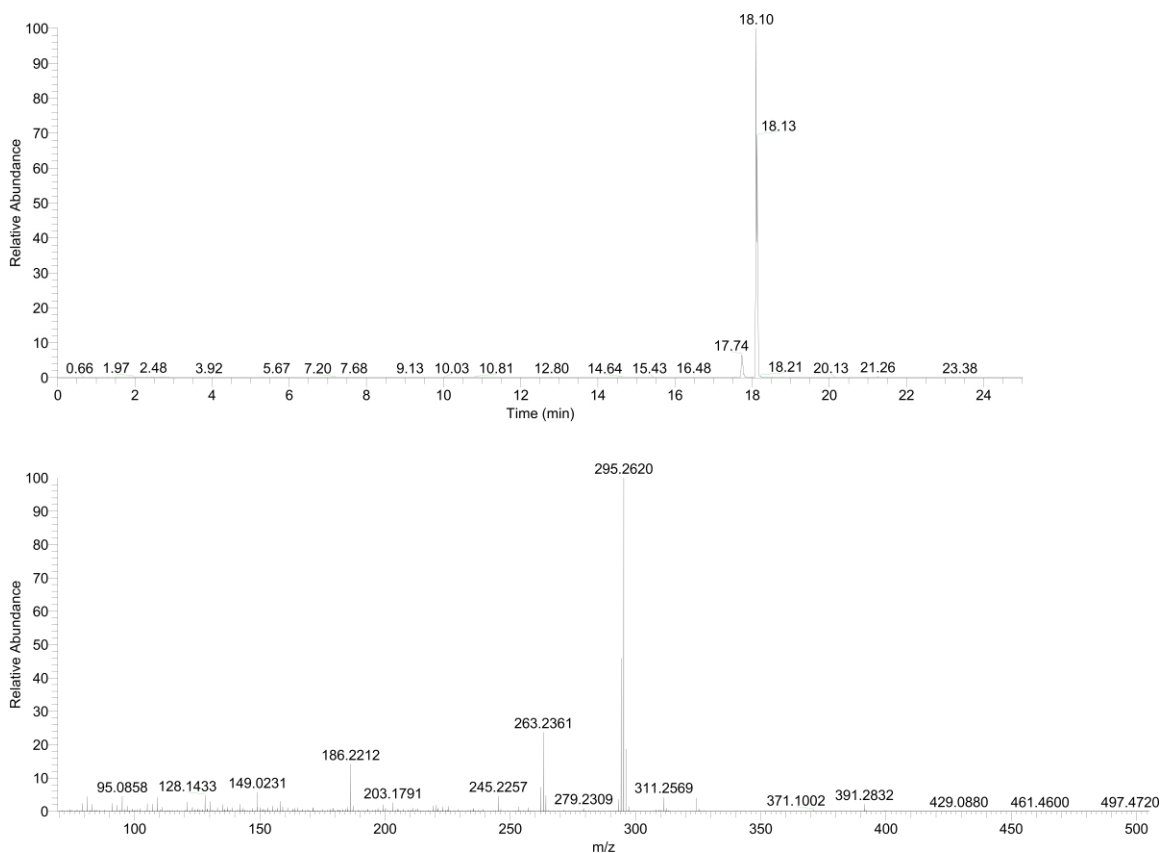


Figure 2.34. EIC (295.2620 m/z) and full MS of the analyte methyl linoleate ionized by the APCI source.

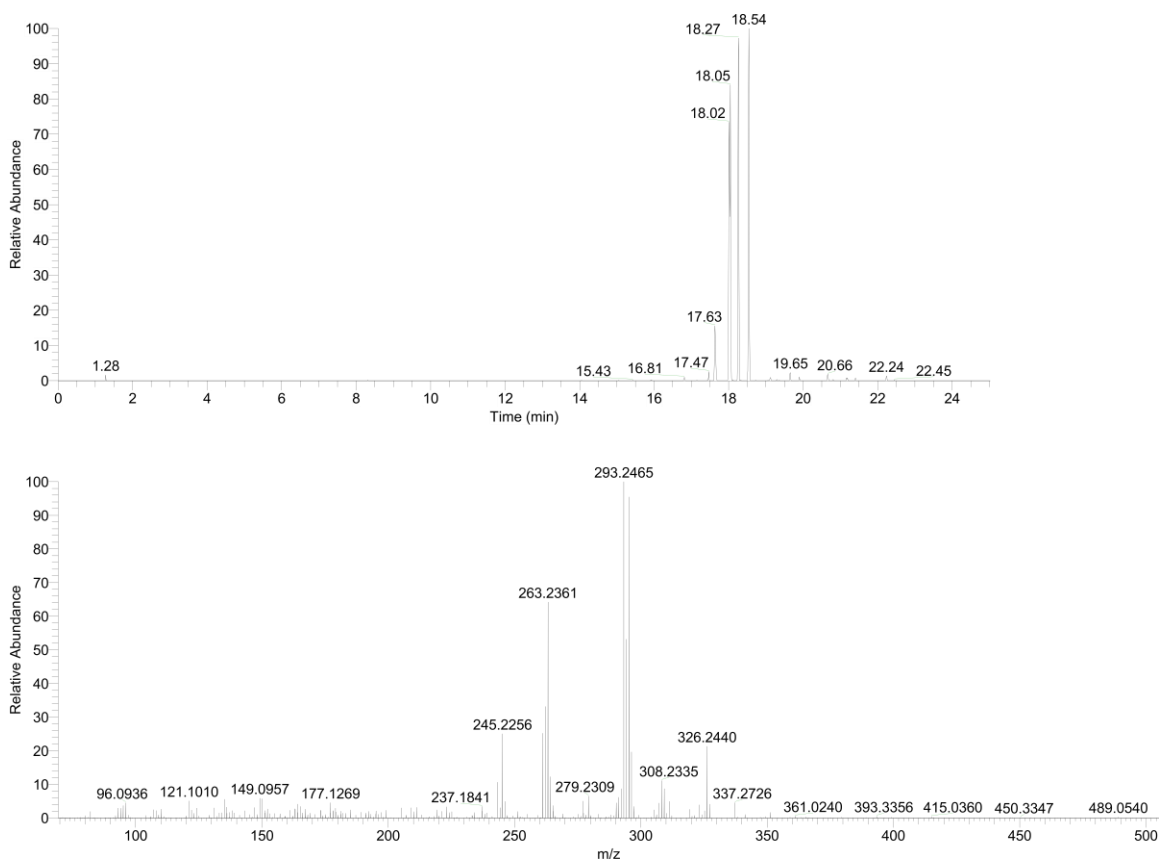


Figure 2.35. EIC (293.2465 m/z) and full MS of the analyte methyl linoleate ionized by the APPI source.

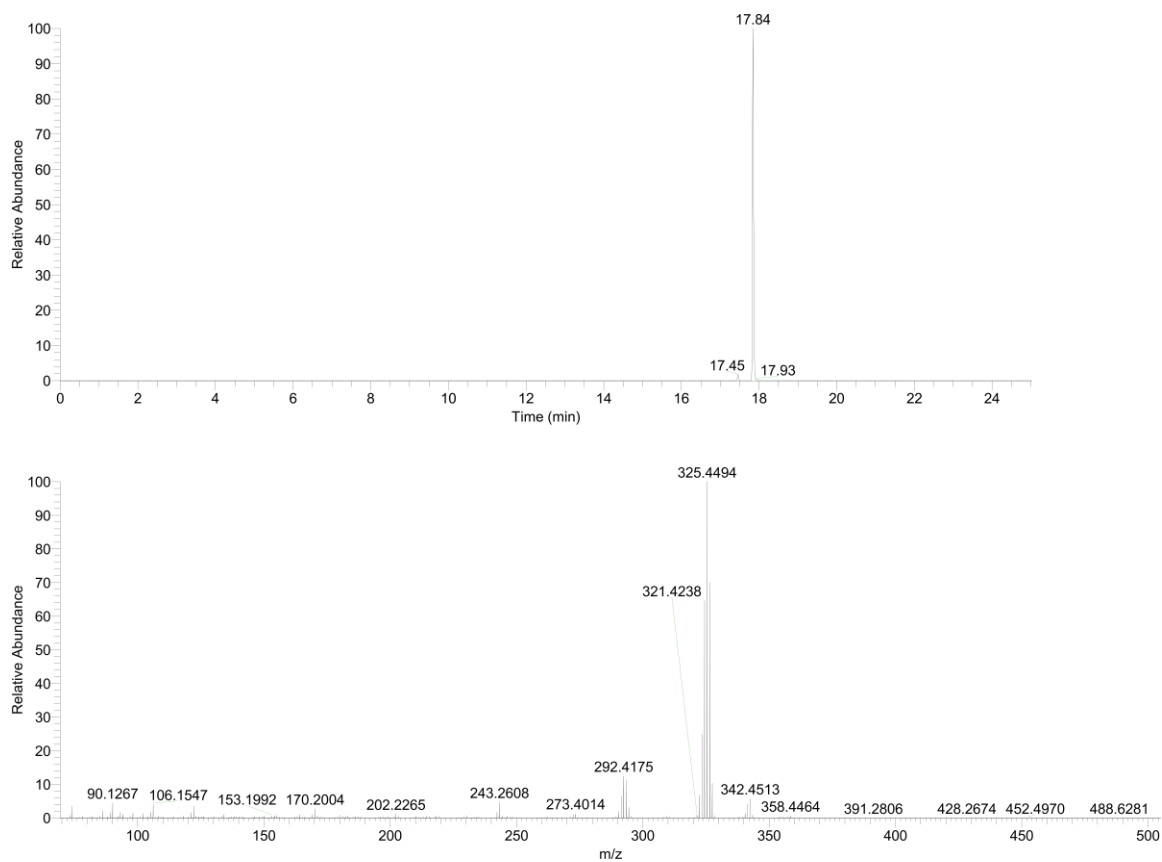


Figure 2.36. EIC (325.4494 m/z) and full MS of the analyte methyl linoleate- D_{31} ionized by the APCI source.

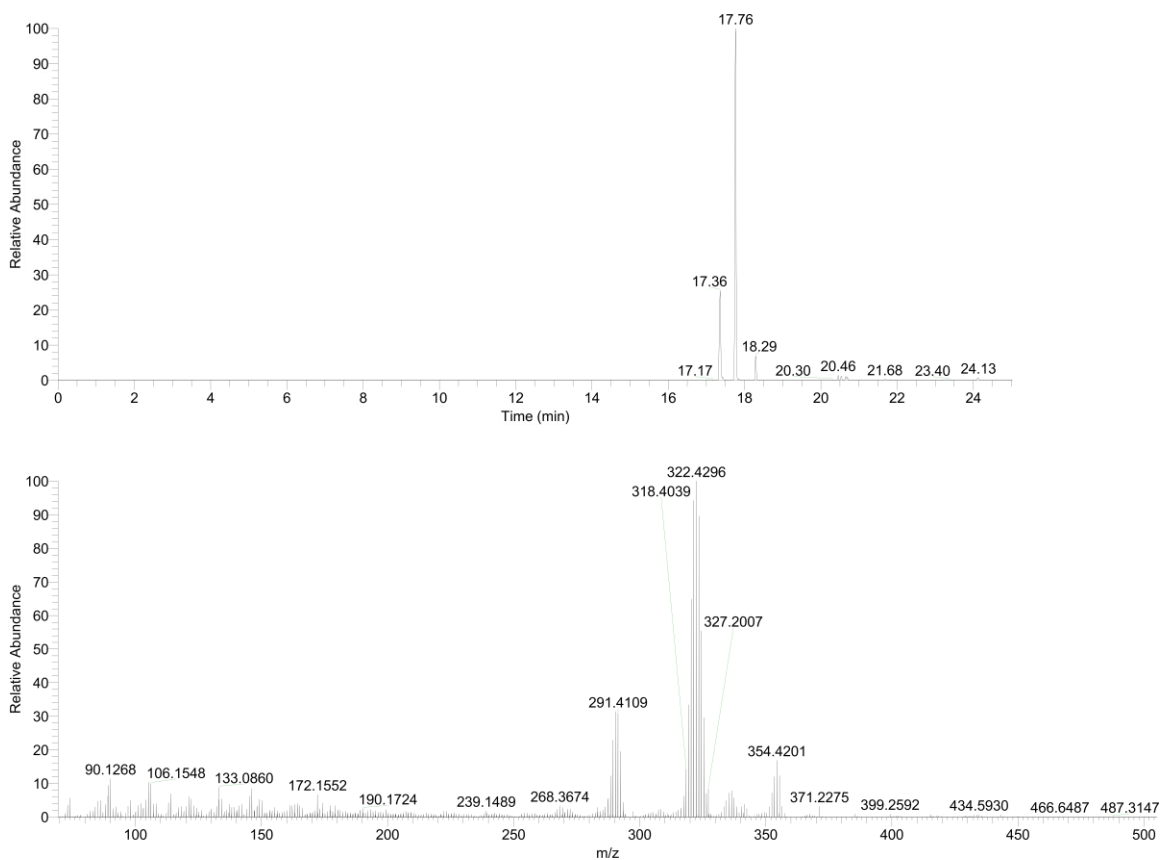


Figure 2.37. EIC (322.4296 m/z) and full MS of the analyte methyl linoleate-D₃₁ ionized by the APPI source.

CHAPTER 3.
DEVELOPMENTAL REGULATION OF THE GENES
CONTROLLING POLYUNSATURATED FATTY ACID
SYNTHESIS IN BROILER CHICKS

Preface

Chapter three of the dissertation describes the application of the GC-APCI interface to characterize a biological system. Particularly, a fatty acid methyl ester analysis was conducted in a collaborative effort to better characterize the synthesis of polyunsaturated fatty acids in broiler chickens. PUFAs are an important part of human health as they provide several benefits; however, both humans and mammals are unable to directly synthesize PUFAs. Yet, there is a clear distinction between humans and mammals as the latter can synthesize PUFAs more efficiently from the precursor alpha-linolenic acid. Additionally, an animal that has enriched production of omega-3 fatty acids has been shown to have an increased feed efficiency, which has further financial benefits. Therefore, understanding how to increase production of PUFAs within a common food source for human consumption would be especially advantageous.

Genes known to be associated with the synthesis of PUFAs, including *ELOVLs*, *FADS*, *SCDs*, and others, were quantitatively measured based on their expression during embryonic and post-hatch phases. These genes are not well characterized in *Gallus gallus* despite the fact that chickens demonstrate an improved ability to synthesize PUFAs from precursors. Additionally, there is evidence that some of these genes may be involved in the production of other important fatty acids. Measurements of fatty acid methyl esters by the GC-APCI-Orbitrap were correlated with the normalized gene expressions to investigate the PUFA pathway. It was found that three genes correlated positively and significantly with both each other and seven fatty acids throughout the development of the chickens. Additionally, these results provide further evidence that some genes have additional roles in fatty acid production and metabolism.

While the scope of this chapter focuses on metabolic profiling rather than technological innovation, it nevertheless serves as an example of the extended utility of the GC-APCI-Orbitrap. The simple modifications, previously described to

enable GC-MS analyses, facilitated the collaborative effort to be more efficient and direct in contrast to requiring the assistance of an external lab.

This article has been accepted for publication in British Poultry Science, published by Taylor & Francis.

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Contributions:

RM and SD isolated the RNA. HW and SD prepared the cDNA. RM analyzed the gene expression data. HW and JBP prepared fatty acid samples and analyzed the GC-MS data. KL performed the targeted RNAseq. BV, SRC, and KL designed the experiment. BV oversaw all data analyses. RM, KL, JBP, SRC, and BV helped write the manuscript.

3.1. Abstract

The objective of this study was to characterize regulation of the pathways that synthesize long chain polyunsaturated fatty acids (PUFA) in developing adipose depots of broiler embryos and chicks. Subcutaneous adipose depots were harvested from embryos E13, E15 and E17. Subcutaneous, abdominal and crop (neck) adipose, as well as liver, were collected at 7 and 14d post-hatch. Targeted RNA sequencing was used to quantify expression of six elongation of very long chain fatty acid (*ELOVL*) genes, two isoforms of stearoyl CoA desaturase (*SCD* and *SCD5*), and three fatty acid desaturases (*FADS1*, *FADS2*, and *FADS6*) in each depot and liver. Expression levels of marker genes for fatty acid oxidation and adipogenesis (peroxisome proliferator activated receptor gamma (*PPARG*)) were also quantified. Fatty acid composition of subcutaneous adipose was analyzed using gas chromatography – mass spectrometry (GC-MS). Genes in the PUFA synthetic pathway were differentially expressed across developmental ages and between depots. These include elongase and desaturase genes that have not been characterized in chicken. Correlation analyses identified subsets of co-regulated genes and fatty acids and highlighted relationships that may influence adipose metabolism and development. It was concluded that PUFA synthesis is an active and dynamically regulated pathway in developing adipose depots of the broiler chick. These data also highlight potential novel roles for specific elongase and desaturase genes in adipose growth and metabolism.

3.2. Introduction

Enriching poultry meat and eggs in long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) has the potential to alleviate a widespread dietary deficiency and improve global health. The LC n-3 PUFAs eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are associated with reduced risk of cardiovascular disease, obesity, neurological diseases, inflammation, and other major healthcare burdens.³⁴ However, despite these well-recognized health benefits, approximately 80% of the global population consumes less than the recommended amounts (250mg/day) of these critically important fatty acids.³⁵ Fish are the primary dietary source of EPA and DHA, but consumption of fish is relatively low in many countries due to cost and consumer preferences, and the sustainability of the global fish supply is in doubt.³⁶ Unlike fish, poultry is widely consumed and relatively affordable in most countries. The fatty acid profile of poultry meat and eggs can be readily enriched in EPA and DHA through diet. For example, replacing sunflower oil with fish oil in broiler diets increased muscle EPA and DHA content by approximately 40-fold.³⁷ However, this approach is not amenable to widespread use because of the shortage of fish. In addition, fish oil is prone to oxidation and rancidity, and consumer preference is affected by reported fishy taste of the resulting meat and eggs.

Docosahexaenoic acid and EPA are essential fatty acids because endogenous synthesis from their precursor alpha-linolenic acid (ALA; 18:3 n-3) is very low in humans and most vertebrates. However, chickens are thought to have an enhanced capacity to synthesize DHA from ALA, creating the possibility to enrich poultry meat in these fatty acids without the use of fish oil.³⁸ Synthesis of EPA and DHA from ALA requires progressive desaturation and elongation steps that are catalyzed by fatty acid desaturase (FADS) and elongation of very long chain fatty acid (ELOVL) enzymes. *In vitro* studies using a heterologous expression system in yeast demonstrated that the chicken isoforms of ELOVLs 2 and 5 have expanded substrate specificities that were predicted to increase the

capacity for DHA synthesis.³⁸ Consistent with this prediction, supplementing broiler diets with ALA has been shown to significantly increase tissue DHA levels.³⁹⁻⁴⁰ Both ALA and the C18 n-6 PUFA linoleic acid (LA; 18:2 n-6) are elongated by ELOVLs 2 and 5. Partial replacement of dietary LA with ALA for 28 days progressively increased breast muscle EPA and DHA by up to five-fold in broilers.⁴⁰ In addition, feed conversion ratio was significantly improved compared to soybean oil (LA)-only controls.⁴⁰ We and others have shown that enriching the broiler diet in LC n-3 PUFA also reduced fat accretion, which is a costly waste of feed.⁴¹ Other studies have demonstrated beneficial effects on immunity and bone strength with similar dietary manipulation.⁴²⁻⁴³ Therefore, increasing the LC n-3 PUFA content of poultry through dietary ALA may yield dual benefit for both consumers and producers.

The elongase and desaturase genes that are key to increasing the LC n-3 PUFA content of poultry products are not well-characterized. The *Gallus gallus* genome contains seven ELOVL (*ELOVL1-7*) genes, six of which (*ELOVL2-7*) are associated with PUFA synthesis based on what is known from other species.⁴⁴ Three fatty acid desaturase (*FADS1*, 2, and 6) genes have also been identified in chicken. Synthesis of DHA is attributed to the actions of *ELOVLs* 2 and 5, and of *FADS1* (delta-6 desaturase) and 2 (delta-5 desaturase).⁴⁵ However, other *ELOVL* and *FADS* enzymes may be involved in a tissue- or context-dependent manner. For example, *ELOVL7*, the most recently identified *ELOVL* gene, encodes an elongase with high specificity for ALA *in vitro*.⁴⁵ Although its function *in vivo* has not been defined, genetic variation at the *ELOVL7* locus has been associated with fatty acid composition and with feed efficiency in pigs.⁴⁶⁻⁴⁸ Likewise, a novel *FADS* gene (*FADS6*) may play an unappreciated role in DHA synthesis. Vertebrate *FADS6* has only been functionally characterized in pompano fish, in which it was shown to be a delta-4 desaturase with high specificity for direct synthesis of DHA from 22:5 n-3.⁴⁹ In addition to their roles in EPA and DHA synthesis, elongases and desaturases may synthesize other fatty acid species with specific metabolic

functions. For example, products of *ELOVL6* activity were recently shown to be necessary for thermogenic induction in brown adipose tissue in mice.⁵⁰

The objective of this study was to characterize the expression patterns of chicken elongase and desaturase genes and the associated impact on fatty acid levels in the developing broiler chick embryo and during the first two weeks of post-hatch growth. This period is one in which these enzymes are dynamically regulated as LC PUFA, particularly EPA and DHA, are heavily utilized to support growth and development of the embryo and young chick.⁵¹⁻⁵² It also represents a time of significant nutritional adaptation as chicks shift from lipid utilization *in ovo* to a diet that is high in carbohydrates and relatively low in fat after hatch. Gene expression was used as a proxy for elongase and desaturase enzyme activities, which are known to be regulated primarily at the transcriptional level in other species.⁵³⁻⁵⁴ Expression levels of six *ELOVL* (*ELOVLs* 2-7) and three *FADS* (*FADS1*, 2, and 6) genes were quantified in subcutaneous adipose tissue of the developing broiler chick embryo (days E13, 15, and 17) and in three adipose depots (subcutaneous, abdominal, and crop) and liver at days 7 and 14 after hatch. Two related desaturases (stearoyl-CoA desaturase (*SCD*) 1 and 5) that synthesize monounsaturated fatty acids (MUFA) were also profiled to associate the MUFA and PUFA synthesis pathways. We focused primarily on adipose tissue because of its role as a fatty acid reservoir for other tissues, and to represent adipocytes within muscle that determine the fatty acid quality of meat.⁵⁵ In addition, increasing synthesis of EPA and DHA within developing adipose tissue may be a means to reduce fat accretion in broilers and thus limit wasting of feed.⁴¹ Targeted RNAseq was utilized to efficiently profile expression of multiple genes across a relatively large set of tissue samples. In addition to components of the LC n-3 PUFA synthesis pathway, expression of genes that are markers for fatty acid synthesis (fatty acid synthase (*FASN*)), mitochondrial, and peroxisomal fatty acid oxidation (carnitine palmitoyl acyl transferase 1A (*CPT1A*) and acyl CoA oxidase 1 (*ACOX1*), respectively), and adipogenesis (peroxisomal proliferator activated receptor

gamma (*PPARG*)) were quantitated to associate specific elongase and desaturase genes with these pathways based on correlated gene expression. Fatty acid abundance in subcutaneous adipose samples was also measured to relate changes in *ELOVL* and *FADS* gene expression to tissue fatty acid profiles.

3.3. Experimental

3.3.1. Animals and Tissue Collection

All procedures were conducted under guidelines of the University of Tennessee Animal Care and Use Committee. Fertilized broiler eggs were acquired from a commercial hatchery in Chattanooga, TN and incubated using standard incubation methods. A subset of eggs was used to collect subcutaneous adipose from embryos prior to hatch at embryonic days 13, 15, and 17 (E13, E15, E17, respectively). Remaining eggs were hatched, brooded, housed in standard conditions, and fed a commercial chick starter diet until euthanized by CO₂ asphyxiation at 7 and 14 d of age. Adipose samples from the abdominal, subcutaneous (para-femoral) and crop depots and liver samples (ventral segment of the left lateral lobe) were collected and snap-frozen at -80 °C for subsequent RNA isolation. We use “crop fat” to describe the depot of fat that accumulates near the neck.

3.3.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted from tissues samples (~50-100 mg) using TRIzol (Invitrogen™; Carlsbad, CA) and quantitated spectrophotometrically by optical density at 260 nm using an Amersham Ultra Spec 1300 Pro Spectrophotometer (Amersham; Buckinghamshire, England). Quality was verified visually using gel electrophoresis. For both QPCR and targeted RNAseq, the iScript cDNA synthesis kit (Bio-Rad; Hercules, CA) was used to synthesize cDNA from ~500 ng of total RNA.

3.3.3. Targeted RNAseq

A targeted RNA sequencing approach was used to quantify expression levels of *Gallus gallus* fatty acid elongase and desaturase genes across embryonic and post-hatch adipose development. Primers were selected to produce amplicons that span exon-exon boundaries, using gene models extracted from Ensembl (ensembl.org) based on V5.0 of the chicken genome (*Gallus_gallus*-5.0/galGal5). The gene set included six fatty acid elongase (*ELOVL*) genes (*ELOVLs* 2-7), three fatty acid desaturase genes (*FADS1*, 2 and 6), and two stearoyl-CoA desaturase genes (*SCD1* and 5). Primers were also designed for peroxisome proliferator activated receptor gamma (*PPARG*), acyl-CoA oxidase 1 (*ACOX1*), and carnitine palmitoyltransferase 1 (*CPT1A*), which were included as markers of adipogenesis (*PPARG*) and fatty acid oxidation (*ACOX1* and *CPT1*). The following seven potential housekeeping genes were quantified for sample normalization: hypoxanthine guanine phosphoribosyl transferase (*HPRT*), peptidylprolyl isomerase A (*PPIA*), glucuronidase beta (*GUSB*), ornithine decarboxylase antizyme 1 (*OAZ1*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), actin beta (*ACTB*), and TATA-box binding protein (*TBP*). Primers for each gene of interest were designed using NCBI Primer Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>), restricting amplicons size to 90-100 bp and with amplicons spanning exon-exon junctions. For genes with more than one predicted transcript (NM or XM RefSeq transcript), a consensus partial transcript was used as a target for primer design. The fidelity of primer design for each gene was confirmed virtually using In-Silico PCR (genome.ucsc.edu) and empirically in PCR amplifications using chicken adipose cDNA as a template. The compatibility of primers with multiplexed amplification was then tested in a single multiplex mixture using control chicken cDNA, made from a pool of muscle, adipose, and liver RNA, as a template. The tests included amplification using an optimized Hi-Plex approach by Floodlight Genomics (Knoxville, Tennessee) as part of the Educational and Research Outreach

Program (EROP) and sequencing on an Illumina MiSeq device running a 2x150 configuration.⁵⁶ The resulting sequences were mapped to the original target sequences using CLC Genomics Workbench version 9.5.2 (Qiagen; Bethesda, MD) with default settings.

After validating the primer set, cDNAs from adipose tissue and liver at the ages collected were amplified in duplicate and sequenced using the same multiplex approach. Raw sequence reads were aligned to the chicken reference genome (Gallus_gallus-5.0) using STAR and counted using HtSeq.^{55, 57} Reads for each gene were normalized to the geometric mean of three housekeeping genes (*ACTB*, *YHAZ*, and *TBP*), which showed stable expression across all ages and tissue samples. Normalized reads for the two duplicates for each sample were averaged and used for statistical analyses.

3.3.4. QPCR

Primers were designed using the approach above to amplify and quantify *FADS1* expression using qPCR because the primers failed in the multiplex analysis. Quantification via qPCR was performed using SYBR Green Universal Master Mix and CFX96 Touch™ Real-Time PCR Detection System (BioRad; Hercules, CA). A standard curve was created from five serial dilutions of pooled cDNA and used to check efficiency of the *FADS1* primer pair prior to assays. Samples were run in duplicate and RNase-free water was used as a negative control.

3.3.5. Fatty Acid Analysis

Adipose samples were extracted for fatty acid profiling using a modified Folch method. Frozen tissue samples were pulverized in methanol/ chloroform (2:1, 600 µL) with a TissueLyser (Qiagen; Bethesda, MD) at 25hz for two minutes. Samples were sonicated for 15 minutes then chloroform/water (1:1, 200 µL of each) was added and samples were re-extracted. The organic phase was dried under a N₂ stream and resuspended in 750 µL methanol/chloroform (1:1). Acid

catalyzed esterification was carried out by adding 125 μL BF_3 – methanol and incubating at 90 $^\circ\text{C}$ for 90 minutes. Then 150 μL water and 300 μL hexane were added, followed by a one-minute vortex and centrifugation at 4 $^\circ\text{C}$. The organic fraction was dried under a N_2 stream and resuspended in 100 μL analytic grade hexane prior to GC-MS analysis.

GC-MS experiments were conducted using a Trace 1300 gas chromatograph (Thermo Fisher Scientific, Waltham, MA) coupled to an Exactive™ Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) using a custom interface equipped with an atmospheric pressure chemical ionization (APCI) source.³² The fatty acid methyl ester (FAMES) analysis and subsequent data processing were conducted as previously reported. Briefly, 2 μL injections performed in duplicate were directed into a programmable temperature vaporizer (PTV) injection port operated in CT split mode. Split flow was set to 5.1 mL/min (5:1 split ratio) and the temperature of the PTV was set to 240 $^\circ\text{C}$. Analytes were separated using a (FAMEWAX, 30 m x .25 mm i.d. x 0.25 μm di) polyethylene glycol column (Restek, Bellefonte, PA) and the carrier gas, helium, was set to constant flow at 1.7 mL/min. The oven temperature gradient consisted of the following: initial temperature 80 $^\circ\text{C}$ and hold for 5 min; 10 $^\circ\text{C}/\text{min}$ to 240 $^\circ\text{C}$; and hold at 240 $^\circ\text{C}$ for 5 additional min. Analytes were transferred from the GC to the MS via the transfer line set to 250 $^\circ\text{C}$. APCI and ion optics parameters were set to the following: sheath gas flow rate set to 10 arbitrary units, aux gas flow rate to 8, sweep gas 3, ion transfer capillary to 350 $^\circ\text{C}$, S-lens RF level to 50.0, and vaporizer temperature to 320 $^\circ\text{C}$. The mass spectrometer was set to full scan from 50.0 to 700.0 m/z with a resolution of 140,000, 1 microscan, automated gain control (AGC) target of 3E6, and maximum inject time of 100 ms. Control of the instrument, as well as generation of data files, was supervised by Xcalibur software, Version 4.0.27.19. The .RAW files generated by Xcalibur were converted to .mzML format using msconvert, a tool of ProteoWizard, and loaded into MAVEN (Metabolomic Analysis and Visualization Engine).^{18, 58} Known FAMES were picked based on

retention time from a standard as well as m/z calculated from chemical formula in positive mode using a ± 5 ppm mass window.

3.3.6. Statistical Analyses

Analysis of variance (ANOVA) was used to evaluate effects of age and tissue. Statistical analyses were performed in SAS 9.4 (SAS Institute Inc.; Cary, NC). Prior to statistical testing, assumptions of ANOVA including normality of residuals, equal variance, and independence of observations was checked using PROC UNIVARIATE diagnostics. As needed, natural log transformations were used for genes with non-normal distributions. Effects of embryonic-through post-hatch development on gene expression in SQ adipose tissue were analyzed using a single factor ANOVA. Differences between adipose depots and liver at 7 and 14d post-hatch were analyzed using a two-factor ANOVA (tissue and age) that included a term to detect age*tissue interactions, blocking on random variation between birds. For both ANOVA models, a significant F-test from the ANOVA was followed by least squares means (LSM) to detect significant pairwise differences between groups. Statistical significance for all tests was defined as $p \leq 0.05$. Gene expression and fatty acid data were inter-correlated using Spearman's rank correlation, executed in the language R, using Metaboanalyst.⁵⁹

3.4. Results

Expression levels of the six *ELOVL* genes varied widely during the window of embryonic adipose development (Table 3.1). The most abundant *ELOVL*, based on normalized read counts, was *ELOVL7* (Figure 3.1). *ELOVLS* 5 and 6 were expressed at levels that were approximately 4-fold lower than *ELOVL7*, based on average read counts across all embryonic SQ samples.

Table 3.1. Summary of ANOVA results comparing gene expression in SQ adipose tissue across development. P-value is from the one-way ANOVA for effect on age and age groups that do not share a letter differ significantly ($p < 0.05$) based on Fisher's LSD.

	<i>E13</i>	<i>E15</i>	<i>E17</i>	<i>7</i>	<i>14</i>	<i>p value</i>
<i>ELOVL2</i>	D	C	C	A	B	1.45E-7
<i>ELOVL3</i>	B	B	B	A	B	4.28E-7
<i>ELOVL4</i>	-	-	-	-	-	0.36
<i>ELOVL5</i>	B	B	C	A	B	1.85E-9
<i>ELOVL6</i>	-	-	-	-	-	0.10
<i>ELOVL7</i>	B	AB	AB	C	A	3.17E-6
<i>FADS1</i>	C	C	B	C	A	0.00014
<i>FADS2</i>	C	C	C	A	B	3.87E-9
<i>FADS6</i>	-	-	-	-	-	0.66
<i>SCD</i>	D	C	C	A	B	1.54E-8
<i>SCD5</i>	B	B	A	C	B	5.60E-12

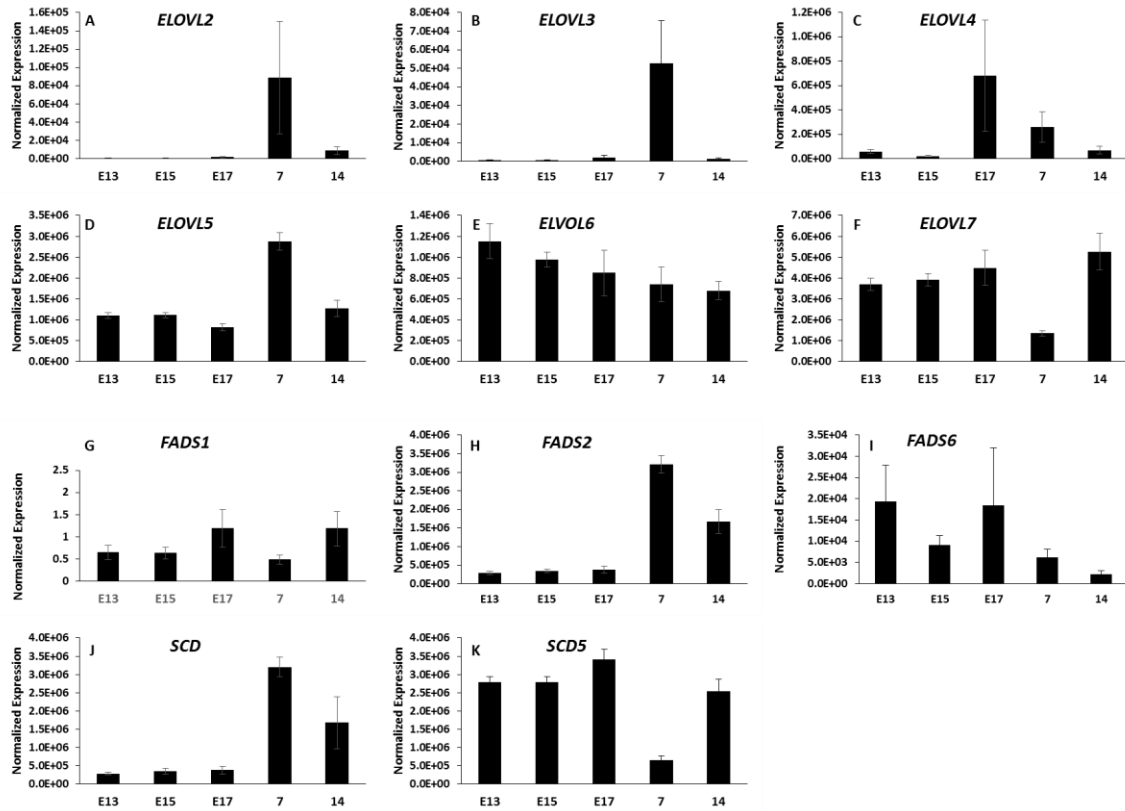


Figure 3.1. Developmental changes in expression of fatty acid elongase and desaturase genes in subcutaneous (SQ) adipose tissue. Values on the y-axes represent normalized total counts read from targeted RNA sequencing (A-F, H-K) or QPCR (I); n=5-7/age, mean $\pm$ std error. Note that the scale of the y-axes differs between genes due to differences in the magnitude of expression. Raw read counts from targeted RNAseq for each gene of interest were normalized to the geometric mean of three reference genes. Fatty acid desaturase 1 (*FADS1*) expression (I) was quantified by QPCR due to ambiguous results from targeted RNAseq. A-F, fatty acid elongases (*ELOVL*) 2-7, respectively; G-I, fatty acid desaturase (*FADS*) 1 (G), 2 (H) and 6 (I); J, stearoyl CoA desaturase (*SCD*), and K, *SCD5*. Data shown in graphs correspond to statistical analyses presented in Table 1.

ELOVL4 is the only other elongase gene that was detected in all embryonic SQ samples, albeit with read counts that were considerably lower (<20%) than for *ELOVLs* 5, 6, and 7. Expression of each of the *ELOVL* genes differed significantly (ANOVA p -value < 0.05) across embryonic and post-hatch SQ adipose development, and the temporal patterns of expression differed considerably between genes (Table 14). During the E13 – d14 window, both *ELOVLs* 2 and 5 were expressed at their highest levels at 7d, with relatively lower levels in the embryo and at d14. Expression for both genes at 7d differed significantly from all other ages (LSM p -value < 0.05). In contrast, *ELOVL7* was expressed at its lowest levels at 7d, with expression significantly lower than at both E17 and d14 ages (LSM p -value < 0.05). Expression of *ELOVL4* was relatively low in all SQ samples, with significantly higher levels in the embryo than at either age post-hatch. The least abundant elongase in terms of expression was *ELOVL3* (Figure 24), which was only detected (at least one read count) in all SQ samples with an age group of 7d.

Fatty acid desaturase 1 was expressed in a pattern similar to that of *ELOVL7*, with the lowest levels found at 7d, followed by a significant increase at 14d. In contrast, expression of *FADS2* followed a pattern similar to that of *ELOVLs* 5 and 2, which sharply and significantly (p <0.05) increased expression at 7d compared to both the embryonic ages and to 14d. Expression of *FADS6* in subcutaneous adipose was considerably lower than *FADS2* in both the embryo and after hatch. Levels declined significantly from E17 to d7, and further by d14. The two isoforms of *SCD* (*SCD1* and *SCD5*) exhibited inverse patterns of expression in developing subcutaneous fat. Expression of *SCD* paralleled that of *FADS2* and *ELOVLs* 2 and 5, with relatively low levels of expression in the embryo followed by a sharp increase at 7d. Conversely, *SCD5* was expressed at higher levels than *SCD1* in the embryo, with a significant decrease at 7d.

An ANOVA model was used to compare elongase and desaturase expression between the three adipose depots (subcutaneous, abdominal, and

supraclavicular) and between adipose and liver, to evaluate expression during the rapid growth and adaptation that occur during the first two weeks after hatch. Each of the five elongases (*ELOVLS* 2, 4, 5, 6 and 7) that were expressed at all ages showed a significant age- and tissue-dependent pattern of expression (ptissue*age <0.05; Table 3.2). *ELOVLS* 2, 5 and 6 were expressed at significantly higher levels in liver than in any of the three adipose depots at both 7d and 14d ($p < 0.05$). In contrast, *ELOVL7* was expressed at significantly higher levels in adipose tissue than in liver at both ages, except for in comparison to the subcutaneous depot at 7d. Within adipose tissue, effects of age varied by gene. *ELOVL5* (Figure 3.2) decreased significantly ($p < 0.05$) at 14d vs. 7d in both the abdominal and subcutaneous depots, while expression of *ELOVLS* 2 and 6 was not affected by age. Unlike the other elongases, *ELOVL7* expression significantly increased with age in both the abdominal and subcutaneous depots (but not in liver). Similar to *ELOVLS* 2, 5 and 6, *FADS2*, *FADS6* and *SCD* expression was significantly higher in liver than in adipose tissue after hatch (Table 3.2). Expression of *FADS2*, but not *FADS6* or *SCD*, showed a significant tissue* age interaction, increasing in liver but declining in subcutaneous adipose at 14d vs. 7d. In contrast to *SCD*, *SCD5* was expressed at significantly higher levels in adipose tissue than in liver after hatch.

Table 3.2. Summary of ANOVA results comparing gene expression across ages and tissues.

Tissue		AB		Crop		SQ		Liver	
Age (d)	ProbF	7	14	7	14	7	14	7	14
<i>ACOX1</i>	0.0012	C	BC	BC	B	D	B	B	A
<i>CPT1A</i>	1.08E-05	CD	CD	C	C	D	C	B	A
<i>ELOVL2</i>	0.0014	C	C	C	C	C	C	B	A
<i>ELOVL4</i>	0.017	B	B	B	B	A	B	B	B
<i>ELOVL5</i>	7.13E-06	C	D	D	CD	B	D	A	A
<i>ELOVL6</i>	0.00022	C	C	C	C	C	C	B	A
<i>ELOVL7</i>	0.012	B	A	AB	AB	C	AB	C	C
<i>FADS2</i>	1.96E-06	D	D	D	D	C	D	B	A
<i>FASN</i>	1.94E-10	D	E	E	E	C	E	B	A
<i>PPARA</i>	0.0042	CD	D	D	CD	C	CD	B	A
<i>PPARG</i>	0.030	B	A	AB	AB	C	B	D	D
<i>SCD5</i>	0.00022	B	AB	A	AB	D	B	CD	C
Tissue	ProbF	AB	Crop	SQ	Liver				
<i>FADS6</i>	4.18E-40	B	B	B	A				
<i>SCD</i>	2.41E-05	B	B	B	A				

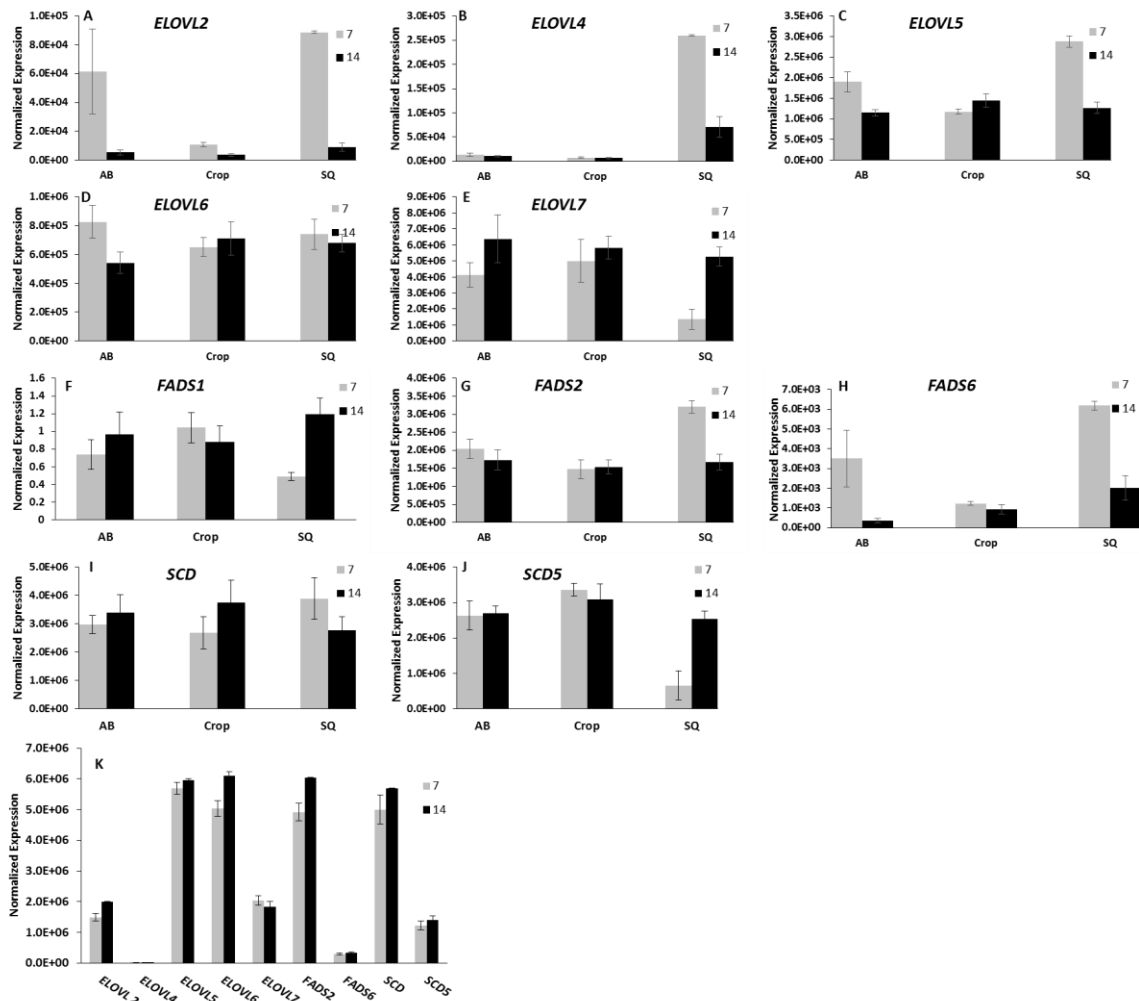


Figure 3.2. Comparison of elongase and desaturase gene expression between adipose depots and liver after hatch. Samples of three adipose depots (abdominal (AB), crop and SQ) and of liver were collected at 7 and 14 days of age after hatch. Values on the y-axes represent raw read counts from targeted RNAseq normalized to the geometric mean of three reference genes; $n = 5-7/\text{tissue}$ and age, mean $\pm$ std error. Note that the scale of the y-axes differs between genes due to differences in the magnitude of expression. A-E, fatty acid elongases (*ELOVL* 2, 4, 5, 6, and 7, respectively); F-H, fatty acid desaturase (*FADS*) 1 (F), 2 (G) and 6 (H); I, stearoyl CoA desaturase (*SCD*), and J, *SCD5*. Data for *ELOVL3* is not presented due to lack of consistent detection in post-hatch tissue samples other than SQ adipose from day 7. Expression values for liver are presented in a separate graph (K) due to differences in scale. Data shown in graphs correspond to statistical analyses presented in Table 2.

A panel of 27 fatty acids were measured using GC-MS in SQ adipose from E13 to d 14 (Table 3.3). As expected, both progression of embryonic development and the switch in nutrient use that comes with hatch and feeding correlated with significant changes in fatty acid abundance. Correlation analysis was used to identify subsets of genes and fatty acids that were co-expressed/ co-abundant across this period of development as a way to query potential functions of specific elongases and desaturases. Gene representing regulation of adipogenesis (PPARG) as well as fatty acid oxidation (*CPT1A*, *ACOX1*, *PPARA*), and synthesis (*FASN*) were included as markers of these pathways. These relationships are represented in a heat map (Figure 3.3). Expression levels of *ELOVL 2*, *FADS2* and *SCD* were significantly and positively inter-correlated with each other ($R^2 = 0.53 - 0.92$; $p < 0.05$) and with seven fatty acids across the E13 – d14 period of development. This set of fatty acids included oleic and its product eicosenoic (20:1), as well as linoleic, gamma-linolenic (18:3 n-6), and alpha-linolenic acids. Both *ELOVLs* 2 and 5 were significantly correlated with levels of EPA, but not DHA. *ELOVL7* was positively and significantly correlated with *SCD5* and with biomarkers of fatty acid oxidation (*ACOX1* and *CPT1A*) and adipogenesis (*PPARG*), and inversely correlated with *ELOVL2* and *ELOVL5*, but was not significantly related to abundance of any fatty acids that were measured. *ELOVL6* was positively correlated with several saturated fatty acids (e.g., palmitate, myristate) and with the n-3 PUFA stearidonic acid (18:4 n-3), while showing inverse relationships with *ELOVL2* and the series of fatty acids with which it was correlated. *ELOVL4* expression did not significantly correlate with any fatty acids.

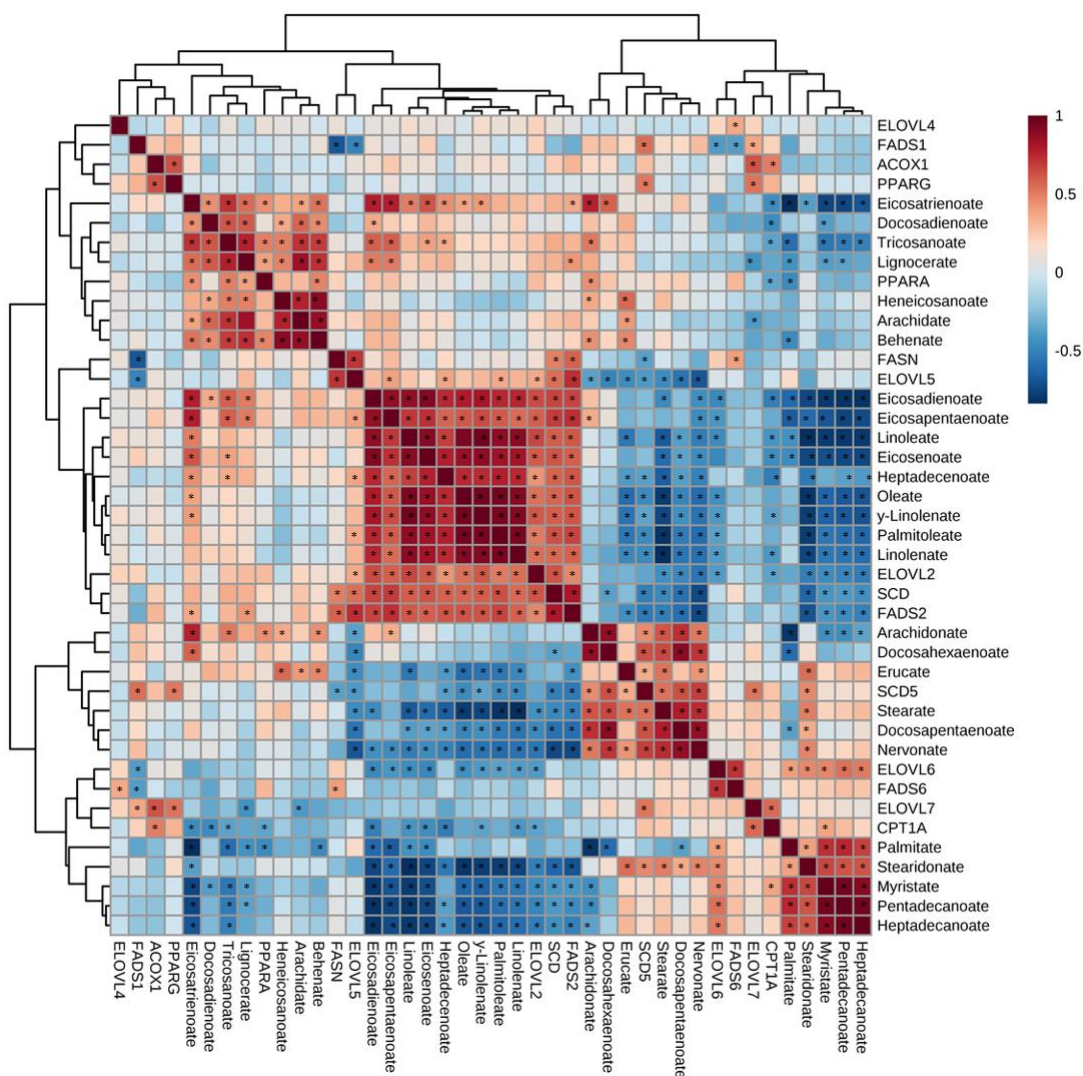


Figure 3.3. Heatmap illustrating gene and fatty acid correlations. Tissue fatty acids were profiled using GC-MS from the same SQ adipose samples from E13 – d14 that were used for gene expression. Relative abundance of each fatty acid in each sample was calculated as a percentage of total fatty acids measured. Fatty acid abundance and normalized gene expression values were correlated using Spearman's rank order in Metaboanalyst after normalizing expression data using logarithmic transformation. Asterisks indicate statistically significant pairwise correlations ($p \leq 0.05$).

3.5. Discussion

Enriching the developing broiler chick in LC n-3 PUFA presents a dual benefit opportunity for human health and for broiler chicken production.^{41, 60} Chickens are a feasible production animal in which to increase LC n-3 PUFA consumption in a sustainable manner because they can convert dietary ALA to EPA, DHA, and other VLC n-3 PUFA more efficiently than other livestock species. At the same time, increasing the supply of LC n-3 PUFA may directly benefit welfare by increasing bone strength, bolstering the immune system, and enhancing energy utilization. Adding sources of LC n-3 PUFA (e.g., flaxseed, fish oil, marine algae) to poultry diets has been shown to improve immune function, increase antioxidant status, anabolic response to insulin, bone mineral density and bone strength, and to reduce the incidence of bone fractures.^{42-43, 61-62} There is a lack of knowledge about how the elongase and desaturase genes that control LC n-3 PUFA synthesis are regulated during early growth and development, a period when chicks face significant metabolic adaptations and when PUFA metabolism is heightened. To address this gap in knowledge, we used targeted RNAseq to determine the developmental and depot-specific expression patterns of six *ELOVL* and five desaturase genes in broiler chick adipose tissue from E13 to 14d of age, and in three adipose depots and liver after hatch. Adipose tissue development begins in the embryo around E12 when fatty acids from the yolk begin to be mobilized and stored in subcutaneous adipocytes located in the parafeemoral area.⁶³ The age span (E13 – d14) that we profiled is a time of dynamic metabolic adaptation as the chick transitions from almost complete reliance on lipids *in ovo* to a carbohydrate-rich diet after hatch. Subcutaneous adipose tissue is a nexus for these adaptations, as it sequesters fatty acids from the yolk (E12-E19), mobilizes them during hatching, and then resumes fatty acid uptake and storage during post-hatch growth.^{51-52, 64} All of the elongase and desaturase genes that were expressed consistently across ages, with the exception of *ELOVL* 6, were differentially expressed in subcutaneous adipose tissue during this period of rapid development.

Elongases 2 and 5 catalyze the elongation steps in synthesis of VLC n-3 PUFA from the essential fatty acid ALA.⁴⁵ *ELOVL5* is widely considered to catalyze elongation of C18 and C20 PUFA, while *ELOVL2* elongates C20 and C22 PUFA substrates. However, the chicken isoform of *ELOVL5* has a unique ability, compared to *ELOVL5* in other species, to elongate DPA (22:5 n-3) to 24:5 n-3.³⁸ Using a heterologous expression system in yeast, chicken *ELOVL5* was shown to convert ~ 20% of DPA to 24:5 n-3, compared to low or undetectable conversion rates in mammals and fish. This broader substrate specificity has been proposed to underlie the enhanced capacity of chickens to synthesize DHA from ALA. We found that *ELOVL5* was expressed at relatively high levels in adipose tissue, including in SQ adipose of the embryo. Levels across adipose depots after hatch were within an order of magnitude of those in liver (normalized reads of ~ 1×10^6 vs. 6×10^6 , respectively). Therefore, adipose tissue of broiler chicks may also have an enhanced capacity to synthesize DHA if provided increased levels of ALA precursor in the diet due to the combined ability of chicken *ELOVLs* 2 and 5 to elongate DPA. Further studies are needed to determine if expression of *ELOVL5* in chick adipose tissue increases in response to dietary ALA, which has been demonstrated in liver of adult broilers.

Correlation analysis identified significant associations between specific *ELOVL* genes and fatty acid products. Although *ELOVL2* is known to use C20 and C22 substrates in other species, we found that expression of *ELOVL2* was significantly correlated with C18 substrates (e.g., oleic, linoleic and linolenic acids) and some of their products in chick SQ adipose tissue. While we acknowledge the limitations of correlation analyses, these relationships may reflect an expanded substrate use for *ELOVL2* in LC PUFA synthesis in chickens, at least in adipose tissue of developing chicks. In light of the unique activity of chicken *ELOVL5*, our association data may indicate that *ELOVL2* also has an expanded role in LC n-3 PUFA synthesis. If so, these two enzymes working together comprise a novel pathway for altering fatty acid composition in chick adipose tissue. It will be

important to determine if both are upregulated by dietary ALA in adipose tissue, which has been described in liver of adult broilers.⁶⁵

The most recently described *ELOVL* gene in vertebrates is *ELOVL7*. This elongase is highly conserved in mammals and avians, but little is known about its physiological role in any species. In the current study, *ELOVL7* was the most abundantly expressed elongase in adipose tissue, both in the embryo and after hatch. Expression levels were consistently higher in adipose tissue than in liver. Purified recombinant human *ELOVL7* enzyme was shown to have highest substrate specificity for ALA, suggesting it may catalyze an alternative initial step in DHA synthesis that begins with elongation rather than desaturation of ALA.⁶⁶ It is possible that this induction may represent a pathway to enhance ALA conversion, in addition to that yielded by the established pathway, when ALA levels are high. In fact, ALA was shown to increase *ELOVL7* expression in bovine mammary epithelial cells (bMECs) by strengthening binding of transcription factor Sp1 to a region in the proximal *ELOVL7* promoter.⁶⁷ The resultant increase in *ELOVL7* activity promoted lipid droplet accumulation in bMECs. In pigs, polymorphisms in *ELOVL7* are associated with variation in adipose and muscle fatty acid profiles, and with feed efficiency.^{48, 50} Collectively, these limited studies into function of *ELOVL7* suggest that its activity may yield downstream fatty acid species that play a regulatory role in metabolism and fat accretion. It is worth noting that in our dataset, *ELOVL7* was significantly correlated with markers of mitochondrial (*CPT1A*) and peroxisomal (*ACOX1*) beta-oxidation and adipogenesis (*PPARG*), with relatively little relation to expression of other elongases or to other fatty acids. Both *CPT1A* and *ACOX1*, which encode rate-limiting enzymes in fatty acid oxidation, are transcriptionally regulated by *PPARA*.⁶⁸ Like *PPARG*, *PPARA* is activated by binding of specific fatty acids and their metabolites. The intercorrelation of these genes with *ELOVL7* suggests that this novel elongase may contribute to synthesis of an endogenous ligand that contributes to transcriptional control of beta oxidation and adipogenesis. We have

previously reported that both of these pathways are associated with regulation of fat accretion in broilers, and are rapidly regulated by availability of free fatty acids in chick adipose tissue.⁶⁹⁻⁷⁰ Further characterization of *ELOVL7* and its products and regulation in broiler adipose tissue may yield new insight into ways to reduce fat accretion, a wasteful component of production. In future studies, we plan to expand our suite of measured FA species and FAME standards to screen for those that are induced in chick adipocytes with overexpression of *ELOVL7*.

The two desaturases (*FADS1* and 2) in the LC n-3 PUFA synthetic pathway play significant roles in determining the response to dietary fatty acids and tissue fat quality. *FADS1* (delta-5 desaturase) catalyzes the desaturation of 20:3 n-6 and 20:4 n-3 to arachidonic acid (20:4 n-6) and EPA, respectively.⁷¹ *FADS2* is responsible for multiple steps, including desaturations that are both upstream and downstream of the *FADS1*-catalyzed reactions.⁷² In humans, polymorphic variation in the *FADS* gene cluster underlies the shared genetic risk for multi-morbid traits such as obesity, inflammation and cancer, illustrating the physiological importance of these genes.⁷³ Higher expression of *FADS1* and 2 in chickens is associated with increased breast muscle EPA and DHA content and demonstrates the relationship between *FADS* expression and LC n-3 PUFA synthesis.⁷³ These two genes showed an inverse pattern of expression during adipose development, which is consistent with a tandem chromosomal arrangement (on Chr 5 in chicken) that has been shown to confer reciprocal regulation of *FADS1* and 2 in other species.⁷⁴⁻⁷⁵ The chicken genome also contains an additional *FADS* gene (*FADS6*) that resides on a different chromosome and is not part of the duplication events surrounding *FADS1* and 2. The *FADS6* gene is highly conserved across multiple taxa, but its role in the PUFA synthesis pathway is not defined. Recent functional characterization of *FADS6* in golden pompano (*Trachinatus ovatus*) demonstrated that it acts as a delta-4 desaturase that can directly convert 22:5 n-3 to DHA, bypassing the additional steps of elongation and peroxisomal beta-oxidation that are part of the DHA synthesis pathway defined by

Sprecher.^{49, 76} In our dataset, expression of *FADS6* was highest in embryonic adipose tissue. This additional route of DHA synthesis may serve to ensure the capacity of subcutaneous adipose in the embryo to adequately provide DHA to neural tissues.

Although stearoyl-CoA desaturase regulates MUFA rather than PUFA synthesis, we included the two *SCD genes* (*SCD* and *SCD5*) in the chicken genome in our panel because of this enzyme's critical role in regulating lipid composition and metabolism. Chickens, humans, swine and ruminants share a conserved isoform of *SCD* (*SCD5*) that is not found in rodents and that has not been well-characterized in any species.⁷⁷ Expression of *SCD* and *SCD5* were shown to be inversely correlated in bovine subcutaneous adipose, which is consistent with our results in the same depot from chicks.⁷⁸ Wu et al. suggested that different molecular pathways regulate expression of *SCD* and *SCD5*, and that they may contribute to MUFA synthesis in a context-dependent manner.⁷⁹ Overexpression of *SCD5* in neuronal cells altered the fatty acid content of specific phospholipid and cholesterol ester pools and modified cell proliferation and differentiation.⁸⁰ These effects were mediated through both canonical and non-canonical Wnt signaling pathways, which also regulate adipogenesis. In our dataset, *SCD5* expression was significantly correlated with levels of stearidonic acid (18:4 n-3), which has been shown to suppress adipocyte differentiation.⁸¹ In future studies it will be interesting to determine if chicken *SCD5* plays a role in regulation of fat accretion.

ELOVL3 was first identified in a screen for proteins induced by cold exposure in brown adipose tissue. It was originally named cold-inducible glycoprotein of 30 kDa (*Cig30*) because expression was strongly induced (~ 200-fold) in BAT of mice that were temporarily housed at 4 °C.⁸² *ELOVL3* encodes an elongase that regulates the synthesis of long chain (C20 and C22) saturated fatty acids.⁸²⁻⁸³ In mice this activity is necessary for brown adipocyte recruitment and hyper-induction of mitochondrial oxygen consumption during the initial adaptation

to cold exposure.⁸³ Expression of *ELOVL3* is induced by *PPARA* and by sympathetic stimulation, and levels correlate with beta-oxidation.⁸⁴ Because *ELOVL3* is associated with BAT, which is absent in avians, and it was not surprising that *ELOVL3* expression was not detected consistently in our adipose samples.⁸⁵ However, upon closer inspection, reads for *ELOVL3* were reliably measured in subcutaneous adipose samples, with a marked increase in reads at d7. Chicks at this age face a thermogenic challenge as they adapt to the transition from brooding to room temperatures. In our study, this coincides with the period during which lamps were removed as a source of supplemental heat. Therefore, it is interesting to speculate that the depot-and age-specific expression of *ELOVL3* at d7 may reflect a thermogenic mechanism that is similar to that in BAT. To our knowledge, such a mechanism has not previously been described in avians. To further explore the potential of a BAT-like thermogenic pathway in chick subcutaneous fat, we profiled expression of uncoupling protein 3 (*UCP3*) the avian homolog of the mammalian *UCP1* gene that characterizes BAT.⁸⁴ Like *ELOVL3*, expression of *UCP3* was primarily detected in d7 subcutaneous fat (data not shown). Taken together, these results suggest that mitochondrial uncoupling may play an unappreciated role in chicks' ability to adapt to ambient temperature after brooding. Further experiments are needed to explore this possibility.

Collectively, these data provide new insight into pathways that control fatty acid quality in broilers, specifically in adipose tissue during early growth and development. These findings are relevant to broiler production and to efforts to enhance the nutritional quality of poultry meat. At the same time, they are physiologically important for broilers because the products of these genes are bioactive lipids that regulate metabolism, growth and immunity. Adipose development in particular is a target of these products because of their roles in regulation of adipogenesis and fat accretion. Based on insight from genomes of humans and from other agricultural animals, genetic variants in these genes may play important roles in gene-by-environment interactions that influence production

traits in poultry. A better understanding of genes that control LC PUFA synthesis could therefore be important in selecting lines that are well-adapted to certain environments or that have improved fatty acid quality for consumers. Several limitations of our study should be acknowledged. Our profiling of elongase and desaturase genes relies exclusively on gene expression as a proxy for activity because these genes are accepted to be regulated at the transcriptional level. However other mechanisms of regulation (e.g., allosteric inhibition) may also exist. While correlation indicates associations between gene expression and fatty acid abundance, we recognize that causative relationships (e.g., by overexpressing specific enzymes and quantifying the effects on fatty acid profiles *in vitro*) are needed to definitively determine enzyme substrate use and products produced. By using targeted RNAseq, we may have missed novel or minor transcripts in our expression profiling that were not captured by our consensus amplicon reads. Finally, our study focused on early growth. Although this represents approximately one-third of the life of a market broiler and a dynamic period in adipose development, we may have missed insightful differences in gene expression that occur after d14. Nonetheless, our results provide new insight into the genes that regulate fatty acid quality in broilers. Ongoing efforts to determine how these genes and the corresponding tissue fatty acid profiles and performance are influenced by varying the ALA content of the diet.

3.6. Conclusion

In conclusion, we have demonstrated that the pathway that controls synthesis of LC n-3 PUFA is dynamically regulated during development of adipose tissue in broiler chicks. This period corresponds to an active time of adaptation to changes in nutrition, energetics, and growth. Our results suggest that this pathway and its products play important physiological roles in chick development, including the regulation of body composition. We have also increased functional annotation of the chicken genome by characterizing the expression patterns of novel elongase

and desaturase genes. Collectively, these data yield new insight that is relevant to both increasing the nutritional content of broiler products and to understanding the functional roles of LC n-3 PUFA in chick growth and development.

3.7. Appendix

Table 3.3. Relative abundance of each fatty acid in subcutaneous adipose tissue, expressed as a percentage of all fatty acids measured. Values represent averages within each age group (n =5-7/age) and identities are inferred from *m/z* and relative retention time.

Fatty acid	Numerical formula	Relative abundance (%) ¹				
		Age				
		E13	E15	E17	7	14
Myristate	C14:0	2.72	1.67	0.88	0.82	0.86
Pentadecanoate	C15:1	0.38	0.30	0.12	0.11	0.10
Palmitate	C16:0	43.03	37.25	23.76	31.28	31.12
Palmitoleate	C16:1	0.30	0.52	0.23	4.35	4.73
Heptadecanoate	C17:0	0.64	0.58	0.28	0.25	0.23
cis-10-Heptadecenoic acid	C17:1	0.01	0.01	0.01	0.08	0.07
Stearate	C18:0	38.86	41.66	47.12	18.94	20.15
Oleate	C18:1 n-9	6.79	8.71	8.58	24.26	27.63
Linoleate	C18:2 n-6	1.70	2.58	3.94	12.55	9.87
γ-linolenate	C18:3 n-6	0.01	0.01	0.02	0.17	0.10
Linolenate	C18:3 n-3	0.01	0.02	0.01	0.80	0.55
Stearidonate ²	C18:4 n-3	1.48	0.14	0.23	0.01	0.02
Arachidate	C20:0	0.25	0.23	0.25	0.40	0.44
cis-11-Eicosenoate	C20:1	0.06	0.10	0.11	0.29	0.31
cis-11-14-Eicosadienoic acid	C20:2 n-6	0.02	0.03	0.05	0.14	0.12
cis-11-14-17-Eicosatrienoic acid	C20:3 n-3	0.08	0.14	0.38	0.29	0.35
Heneicosanoate	C21:0	0.05	0.04	0.04	0.03	0.03
cis-5-8-11-14-Arachidonate	C20:4 n-6	2.05	3.89	8.85	2.75	3.02
cis-5-8-11-14-17-Eicosapentaenoate	C20:5 n-3	0.00	0.01	0.02	0.14	0.18
Behenate	C22:0	0.25	0.19	0.25	0.39	0.35
Erucate	C22:1	0.43	0.12	0.15	0.05	0.07
cis-13-16-Docosadienoate	C22:2 n-6	0.00	0.00	0.00	0.01	0.01
Tricosanoate	C23:0	0.01	0.01	0.03	0.05	0.05
Docosapentaenoate ²	C22:5 n-3	0.43	0.90	2.48	0.25	0.21
Lignocerate	C23:0	0.02	0.02	0.03	0.12	0.08
cis-4-7-10-13-16-19-docosahexaenoate	C22:6 n-3	0.23	0.52	1.36	0.18	0.21
Nervonate	C24:1	0.02	0.02	0.04	0.10	0.10

CONCLUSION

This work has demonstrated that a HRMS, initially intended for LC analyses, can have increased utility through simple modifications. Specifically, a UPLC – Orbitrap MS was temporarily converted for GC analyses via an interface, which allowed for additional coverage of chemically diverse species. These modifications cost significantly less than an independent GC-HRMS system while still delivering high quality data. The analytical performance, potential considerations for APIs, and application of the interface were described.

Chapter one reported the construction of the interface as well as the hardware and software necessary to allow for automation. The interface relies on the use of an APCI source, which provides desirable “soft” ionization of analytes. The specifications of the working GC-MS system, such as sensitivity, limit of quantitation, and correlation coefficients, were meticulously tested using a variety of chemical analyses. These analyses focused on typical GC analytes, including hydrocarbons, PAHs, as well as FAMES, and it was found that the GC-APCI interface performed similarly or better to a GC-APPI interface, in addition to other reported methods.

During the analyses of the first chapter, it was found that both the GC-APCI and GC-APPI interfaces consistently generated unique ions that could not be characterized as typical adducts or fragments. To investigate these species, the second chapter consisted of a series of experiments that were designed to help elucidate the potential structures and mechanisms of the generated ions. A Q Exactive™ Orbitrap and fully labeled, deuterated versions of select analytes were used to observe changes in mass caused by different possible ionizations. Additionally, source parameters and environmental factors were tested for their role in ionization. While the different chemical classes each had unique ionization mechanisms, it was concluded that both in-source oxidation and fragmentation by APCI and APPI were responsible for generating the unique species. Additionally,

these experiments provided important evidence for understanding and employing APIs for GC analyses.

Finally, the third chapter demonstrated the validity of the GC-APCI interface for real-world application using a collaborative study as an example. More accurately, GC-MS data generated via the interface was correlated with targeted RNA sequencing data to better understand PUFA biosynthesis in *Gallus gallus*. It was determined that the gene *ELOVL2* correlated significantly with C18 substrates, which has not been previously reported in other species and a role previously associated with *ELOVL5* solely, and that the relationship between *ELOVL2* and 5 must be investigated further.

Together, these chapters provide the means and insight of developing a GC interface for a HRMS. Through technological innovation, a highly selective and sensitive instrument can be made more robust without the requirements of additional MSs. By incorporating this straightforward, alternative interface, laboratories can increase their analytical coverage while still generating high quality data.

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VITA

Joshua B. Powers grew up in Clark, New Jersey and attended The College of New Jersey in the fall of 2009. It was at TCNJ that he committed to his passion of chemistry and received his Bachelor of Science in chemistry, with a minor in chemistry as well as concentration in forensics. While continuing to contemplate his next career steps, he took a position as a lab analyst at Accutest Laboratories in June 2013. Josh tested and analyzed environmental samples using dual GC ECD systems as part of the Semi-Volatile Organics Department. In April 2014, he took a new position at Product Safety Labs, where he utilized many analytical techniques, including mass spectrometry, for product studies. It was here that Josh realized that his desire for continued education with the behest and support of managers and former professors. He moved to Knoxville, Tennessee and began pursuing his chemistry Ph.D. in August 2015 at the University of Tennessee, Knoxville. Josh joined Dr. Shawn R. Campagna's group and was involved in a myriad of projects involving instrument modification, metabolomics studies, and method development. While at UTK, Josh also became an instrument operator for the department, running samples using the MALDI-TOF and ESI-TOF instruments, as well as a member of the Biological Small Molecule Mass Spectrometry Core. Following the completion of his Ph.D. program, Josh will begin a postdoctoral researcher position with North Carolina State University as part of a NIIMBL project that is focused on characterizing next generation biotherapeutics using mass spectrometry.