Development of a novel mass-selected internal positive chemical ionization quadrupole ion trap mass spectrometry technique for the quantitative analysis of isotopic polyunsaturated fatty acids

by

Hamid Izadi

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Kinesiology

Waterloo, Ontario, Canada, 2009

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

ABSTRACT

Analytical instrumentation for quantitative *in vivo* stable isotope metabolic studies has included gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Limitations of existing MS techniques include excessive parent ion fragmentation, time-consuming sample preparation, and complex instrument operating parameters. In this thesis, procedures for set up and implementation of four GC-MS techniques are described. The first three replicate existing GC-MS single quadrupole methods on an ion trap, and are electron ionization (EI), external methane positive chemical ionization (PCI), and methane negative chemical ionization (NCI). The fourth method is a novel GC-MS mass-selected ion trap internal isobutane positive chemical ionization technique. Four groups of rats were administered isotopic linoleic acid, and liver tissue was collected for labelled linoleic and n-6 polyunsaturated fatty acids (PUFA) metabolites analyses. Qualitative utility of EI was confirmed, and its quantitative limitations exposed. Labelled 18:2n-6 and n-6 PUFA metabolites were detected using external methane PCI, though limited due to significant fragmentation. Methane NCI also detected labelled 18:2n-6, as well as minimizing fragmentation. However, time-consuming sample preparation and non-linear responses were major limitations. Internal isobutane PCI was useful in detecting isotopic 18:2n-6 and n-6 PUFA metabolites. Fragmentation was reduced compared to EI and external methane PCI. Limitations include fragmentation of HUFAs such as EPA and DHA. The novel internal isobutane PCI is more sensitive than external methane PCI and NCI, produces highly linear responses, is simpler and less expensive to operate than C-IRMS, offers reliable instrument operation, and sample preparation time is minimal. Regular quantitative analyses of HUFAs such as EPA and DHA

may require further refinements such as using lower energy reagents than isobutane, including

acetonitrile and ammonia.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA	Arachidonic acid, 20:4n-6	GC	Gas chromatography
ALA	Alpha-linolenic acid, 18:3n-3	HUFA	Highly unsaturated fatty acid
BF_3	Boron trifluoride	LA	Linoleic acid, 18:2n-6
CI	Chemical ionization	LOD	Limit of detection
C-IRMS	Combustion isotope ratio mass spectrometry	MS	Mass spectrometry
		MUFA	Monounsaturated fatty acid
DHA	Docosahexaenoic acid, 22:6n-3	NCI	Negative chemical ionization
DPA	Docosapentaenoic acid, 22:5n-3 or 22:5n-6	PCI	Positive chemical ionization
		PFB	Pentafluorobenzyl
EI	Electron ionization	PUFA	Polyunsaturated fatty acid
EPA	Eicosapentaenoic acid	SFA	Saturated fatty acids
FAME	Fatty acid methyl ester	SIE	Selected ion extraction
FID	Flame ionization detector	U-^{13}C	Uniformly labelled carbon-13

CHAPTER 1

INTRODUCTION

Fatty acid isotopes are typically considered the gold standard technique for examining

fatty acid metabolism. Stable isotopes are typically used in human studies and chronic animal

feeding studies while radioisotopes are commonly used in cell culture and acute animal studies.

The main limitation associated with the use of fatty acid stable isotopes is the dependency on

advanced analytical techniques and instrumentation, mainly gas chromatography (GC) coupled

to mass spectrometry (MS) to detect, identify, and quantify the isotopes. GC-MS analyses of

fatty acid stable isotopes can be challenging due to low sensitivity and resolution of analytes,

complexity in equipment design, and standard curves that are non-linear. In the present thesis,

novel fatty acid stable isotope analytical techniques are developed using a Varian 4000 GC-MS

ion trap. The Varian 4000 GC-MS ion trap has the potential to measure fatty acid stable isotopes

with higher sensitivity than single quadrupole GC-MS instruments without the technical and

financial challenges of operating a highly sensitive GC combustion isotope-ratio MS (GC-C-

IRMS) instrument.

BIOCHEMICAL FOUNDATIONS

Metabolism of polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFA) affect key cellular and physiological processes in the

body, including cell signalling, and inflammation (Calder, 2008; Calder, 2007; Calder, 2006).

Linoleic acid (LA; 18:2n-6) and alpha-linolenic acid (ALA; 18:3n-3) are considered essential

fatty acids in mammals as they cannot be synthesized *de novo* and they are required in the diet to

1

prevent deficiencies (Pudelkewicz, Seufert, and Holman, 1968). In plants, algae and some bacteria, LA is synthesized by the action of Δ12 desaturase, which forms a double bond six positions from the methyl end of 16- and 18-carbon monounsaturated fatty acids. This linoleic acid product can be further desaturated by Δ15 desaturase, which forms a double bond, three positions from the methyl end forming ALA (Morimoto et al., 2005). Important dietary sources of LA include vegetable oils such as corn oil, and those of ALA include green leaves and some oils such as flaxseed and canola oil (Burdge and Calder, 2005). Both LA and ALA can be further metabolized by vertebrates to longer chain, highly unsaturated fatty acids (HUFA, ≥20 carbons, ≥3 carbon-carbon double bonds) by the actions of delta-6 and delta-5 desaturases and elongase enzymes in tissues (Igarashi et al., 2007; Burdge and Calder, 2005). The n-6 and n-3 precursor and metabolite fatty acids compete with each other for access to the same set of enzymes. The initial substrates, LA and ALA, undergo three rounds of desaturation and three rounds of elongation, followed in the end by β-oxidation to produce the final products 22:5n-6 and 22:6n-3 (Igarashi et al., 2007). These pathways are summarized below in **Figure 1.**

A key approach in the study of PUFA metabolism has been the *in vivo* administration of fatty acid stable isotopes (discussed further below) and their detection by mass spectrometry (discussed under "Methodological Foundations").

Fatty acid stable isotope studies

Isotopic fatty acids have the same chemical properties as their naturally occurring counterparts, but with a different mass. Common stable isotopes of fatty acids include carbon-13 (^{13}C) and deuterium (^{2}H). The ^{13}C isotope of the more abundant ^{12}C occurs in nature at a rate of about 1% of ^{12}C. The ^{13}C atom has one extra neutron, resulting in the one unit higher mass number (Rennie, 1999). Fatty acids with ^{13}C isotopes may contain any number of ^{13}C carbons,

n-6 PUFAs   n-3 PUFAs

(18:2n-6) → (18:3n-3)

Δ6 Desaturase

(18:3n-6) → (18:4n-3)

Elongase

(20:3n-6) → (20:4n-3)

Δ5 Desaturase

(20:4n-6) → (20:5n-3)

Elongase

(22:4n-6) → (22:5n-3)

Elongase

(24:4n-6) → (24:5n-3)

Δ6 Desaturase

(24:5n-6) → (24:6n-3)

β Oxidation

(22:5n-6) → (22:6n-3)
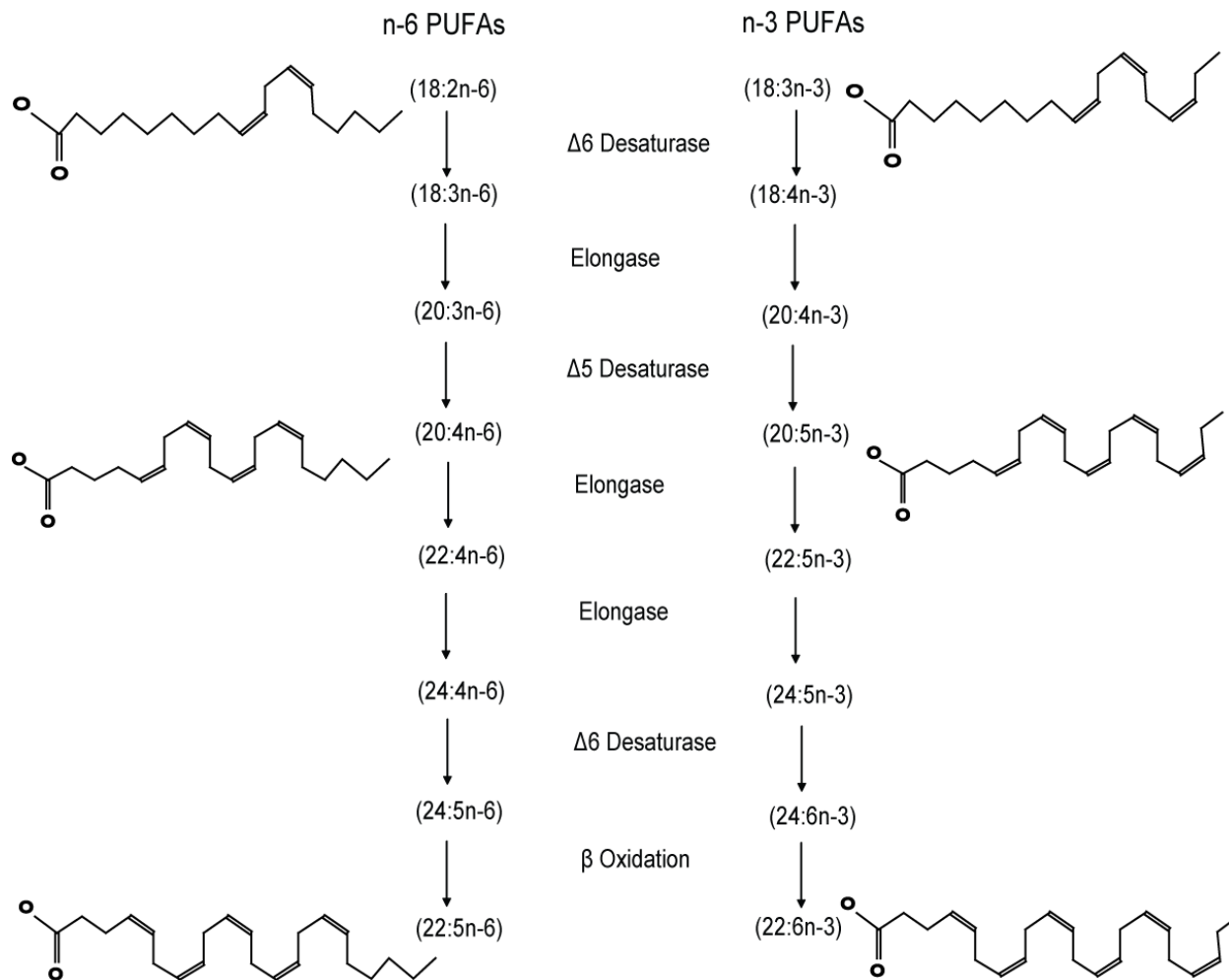
Figure 1. Metabolism of linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3). PUFA, polyunsaturated fatty acid.

up to uniform ^{13}C (U-^{13}C), where all carbons have the extra neutron. The ^{13}C and ^{12}C are chemically identical and therefore participate in the same chemical reactions at the same rate during metabolism (Rennie, 1999). Fatty acid metabolic processes such as desaturation, elongation, and beta oxidation can add carbon-carbon double bonds, and add or remove carbons. As a result, a uniformly labelled ^{13}C fatty acid will result in fatty acid metabolites such CO_2 also containing ^{13}C, allowing for a more in-depth tracer analysis. The ^2H isotope or deuterium is far rarer than ^{13}C. Deuterium-labelled fatty acids are not necessarily uniformly labelled, but the ^2H

label is present on all the hydrogen molecules associated with a specific carbon molecule. These isotopic fatty acids may contain any number of carbons that have been deuterated. Often the last two carbons of the methyl end (enzymatically inactive end) of the fatty acid are labelled resulting in a $^2H_5$ fatty acid stable isotope. Deuterated isotopes differ in gas chromatographic retention time from the more abundant parent molecule which can increase resolution (Lin and Salem, Jr., 2007; Pawlosky, Sprecher, and Salem, Jr., 1992). Fatty acid stable isotopes have been used in a variety of research applications in humans (Appendix A) and animals (Appendix B).

Complex matrices present in such tissues as liver, heart, and plasma significantly reduce isotopic signal detection. Isotopic fatty acid signal detection decreases with increasing plasma lipid concentrations and a plasma matrix effect has been demonstrated as being two-fold lower in $^2H_5$ 18:2n n-6 as compared with U-^{13}C 18:2 n-6 (Lin, Pawlosky, and Salem, Jr., 2005). Given the low dose of isotopic fatty acids in *in vivo* studies, tissue enrichment ranges from nmol/g levels in liver to fmol/g levels in muscle (Lin and Salem, Jr., 2007). Quantitative tracer studies therefore require MS limits of detection that are in the femtogram range, with high signal to noise ratios.

CHAPTER 2

METHODOLOGICAL FOUNDATIONS

Gas Chromatography (GC)

A GC separates and detects individual compounds from a complex mixture. The three

main components of a GC are the injector, the oven containing the chromatography column, and

the detector. Fatty acid methyl esters, dissolved in a non-polar solvent such as hexane, are first

vaporized in the injection port at temperatures nearing 300ºC. A carrier gas such as hydrogen or

helium then carries the gaseous fatty acids to the column as the mobile phase. The inside of the

column is coated with a liquid compound that is the stationary phase. In order to speed the

movement of the gaseous fatty acids through the column, it is necessary to raise the column oven

temperature in a systematic fashion up to temperatures nearing 250ºC. As the gaseous fatty acids

move through the column, they interact with the stationary phase. Factors relating to the

chemical makeup of the stationary phase, for example its polarity, as well as the molecular

weight and structure of the fatty acids determine the delay between the injection of the sample

and each fatty acid's column exit, also termed "retention time" (Christie, 1989). The longest

chain and most unsaturated fatty acids have the longest retention times. Separations using

conventional GC techniques typically lasted up to 120 minutes. Advances in GC techniques

have allowed this time to be reduced to as low as 8 minutes (Masood, Stark, and Salem, Jr.,

2005). Upon exiting the column, the sample is analyzed by a detector. For fatty acids, a flame

ionization detector (FID) is commonly used, which uses a hydrogen flame to combust the

hydrocarbon chain. This combustion produces ions, which contact an electrode, resulting in a

measurable electronic current that is converted into a response curve or "peak" for each fatty

acid. The area under the curve for each peak can be integrated to quantify the fatty acids. The

advantages of the FID include simple design, low cost and maintenance, high sensitivity, and linear responses (Christie, 1989). A standalone GC can be used to analyze individual fatty acids from a total lipid extract. The GC can also be coupled to a MS with the MS serving as the detector.

Mass Spectroscopy

Overview

The three main components of a MS are the ion source, the mass analyzer, and the detector. Various MS techniques differ based on the type, arrangement, and configuration of these three components (Downward, 2004).
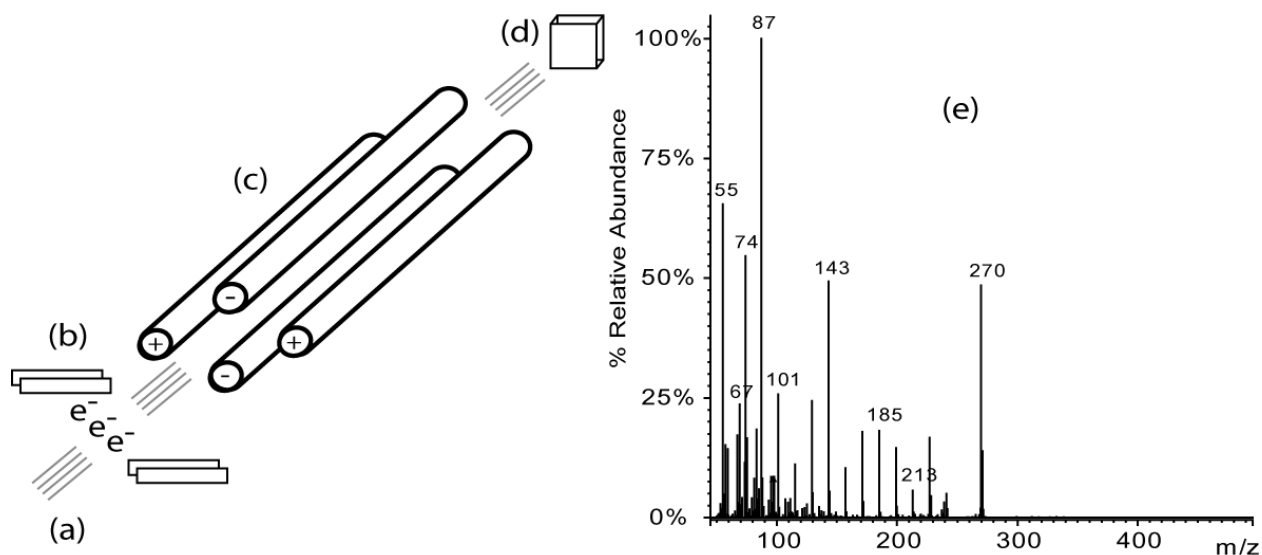
Figure 2. Schematic of a typical quadrupole mass spectrometer. Gaseous sample (a), is ionized by energetic electrons of the ion source (b), manipulated in the quadrupole mass analyzer (c), and amplified at the detector (d). The result is a mass spectrum (e), showing mass to charge ratios (m/z) on the x axis and the percent relative abundance of each ion on the y axis.

In GC-MS, analytes are first separated chromatographically by the GC. Analytes are then moved into the ion source of the MS and ionized. The resulting charged particles are manipulated in the mass analyzer based on their unique mass to charge ratios (m/z). Each

charged particle is then ejected from the mass analyzer toward the detector where the charge is

magnified manifold (Downward, 2004). This process is summarized above in **Figure 2.**

GC-MS hardware and techniques

Presently, GC-MS techniques used for fatty acid stable isotope analyses include single

quadrupoles and combustion isotope ratio mass spectrometry (C-IRMS). Ion trap mass analyzers

have occasionally been used for fatty acid analyses, but not for the detection and quantitation of

fatty acid stable isotopes.

Quadrupole mass analyzer

A quadrupole analyzer, also known as a "single quadrupole", is made up of two pairs of

rods that form a tunnel, into which sample ions are propelled. Ions are typically generated

through electron ionization (EI) and chemical ionization (CI). Direct current and radio frequency

currents are applied to alternating pairs of rods. As these voltages are increased, ions with

increasing instability are ejected from the tunnel and propelled toward the detector (Barker,

2000). The single quadrupole is considered a "molecular MS" technique because specific mass

and fragmentation spectrum of each compound is generated and can be used for sample

identification. Advances to quadrupole mass analysis have included the use of multiple

quadrupoles to enable several rounds of mass analysis, as for example in triple quadrupoles.

Combustion isotope-ratio MS or C-IRMS is another commonly used technique in lipid analysis.

Combustion isotope-ratio MS

In C-IRMS, samples are generally combusted or pyrolyzed completely, and the desired

elemental species is then detected by measuring the ratio between the labelled and the naturally

occurring isotopes of the compound. For example, in the case of ^{13}C labelled compounds, the

sample is first combusted to CO_2 gas in the presence of an oxidizing agent and then ionized. The

detector then measures the ratio between $^{13}CO_2$ and $^{12}CO_2$ (Godin, Fay, and Hopfgartner, 2007). Isotope ratio MS is the most sensitive means of measuring isotope ratios in tissues, and has therefore been an important tool in lipid metabolic studies. However, certain key shortcomings limit the application of C-IRMS to fatty acid stable isotope analysis. Firstly, in applications where ^{13}C compounds are analyzed, carbon components of the derivitization compound, as well as any other carbon contaminants will dilute the isotope pool, and will significantly affect the accuracy of isotopic ratios. It is therefore necessary to ensure that samples are extremely clean. Secondly, C-IRMS is element-specific, requiring the analyzer assembly to be changed over to allow analysis of other elemental isotopes. For example, if analysis is to be changed from ^{13}C to ^{15}N compounds, all combustion parameters have to be changed (Godin, Fay, and Hopfgartner, 2007). These include the possible replacement of the combustion chamber, the use of different oxidizing agents, and alterations to various other instrument analytical parameters. Thirdly, C-IRMS is far more expensive than more conventional MS techniques, and due to its complex operational requirements discussed above, it requires dedicated and highly trained technical personnel (Godin, Fay, and Hopfgartner, 2007). A quadrupole ion trap is a more recent MS technology that mitigates many of the above challenges.

Quadrupole ion trap

A modern quadrupole ion trap mass analyzer is represented schematically below in **Figure 3.** The ion trap is essentially made up of two "endcap" electrodes and one "ring" electrode. The endcap electrodes are hyperboloid discs, located at each end of the ion trap assembly, with the inside of their concave surfaces facing away from each other (March and Todd, 2005). At the centre of each endcap there is one aperture for the movement of ions.

Situated between the two endcaps is the ring electrode. The interior of the ring electrode is also hyperboloidal in geometry, and it is here where ions are trapped (March and Todd, 2005).
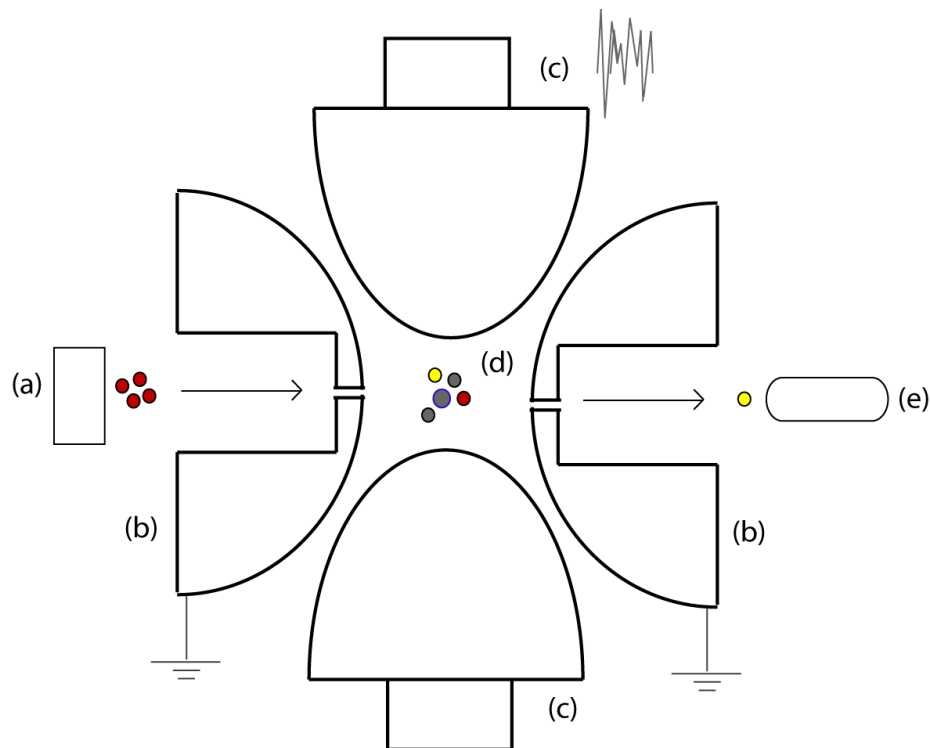
Figure 3. Schematic diagram of a ion trap in cross-section, showing the ion source (a); the two endcap electrodes (b) to which are applied DC voltages; the ring electrode (c) to which is applied DC and radio frequency voltages; trapped ions (d); and the detector (e).

Energetic ions from the ion source are first moved through the entrance endcap by the application of DC voltages of various strengths. A given radio frequency (RF) potential is then applied to the ring electrode to stabilize these initial ions. Gaseous sample molecules are then moved into the ring electrode cavity, where they are ionized by the trapped ions from the ion source. A different RF potential is then applied to stabilize the newly ionized sample molecules, and to eject any unwanted ions. Ions that remain in the trap follow a complex hyperboloidal trajectory that has been termed "flat boomerang" (March and Todd, 2005). Unwanted ions are

moved through the exit endcap by the application, once again, of various DC voltages. The

trapped ions can undergo successive series of reactions before they too are ejected. Ions that are

ejected are either discarded, or amplified by the detector (March and Todd, 2005).

External and internal ionization

The Varian 4000 ion trap is capable of operation in both external *and* internal ionization,

both of which were used in this thesis.

In external ionization, the ion source is situated outside of the ion trap. It is a self-

contained assembly, consisting of two filaments, two ionizing chambers, electron lenses, and a

heater. Each filament is made up of a rhenium ribbon placed in between a repeller plate and an

electron lens. As current moves through the filament, the heat that is generated causes the

emission of high energy electrons (thermionic emission). These electrons can then be either used

for the direct ionization of sample (electron ionization) or the ionization of a reagent gas

(chemical ionization). In external configuration, gaseous fatty acids enter the external source

where they are ionized by one of the two filaments. Depending on the mode of ionization, the

sample may enter the low pressure chamber (electron ionization) or the high pressure chamber

(chemical ionization). These two ionization techniques are described in detail in the next

section. Upon completion of ionization, sample ions are moved through an electron gate into the

trap, where they are manipulated and ejected toward the multiplier based on their m/z. External

ionization enables the ion trap to simulate ionization in single quadrupoles, thus allowing for

more flexibility and ionization options. For example, in external ionization, it is possible to use

negative chemical ionization, discussed later.

In internal ionization, the ion source assembly is located just outside of the entrance

endcap electrode, and energetic electrons are emitted directly into the ion trap. Similar to

external configuration, the ion source assembly in internal configuration is made up of two filaments and an electron gate. The gate enables movement of ions into the trap by reversing voltage from -120 to +120Vdc. The length of time the gate is open is variable and can be adjusted by the user to ensure varying degrees of sample ionization. Although internal ionization does not allow for negative chemical ionization it has several advantages over external ionization. The close proximity of the internal ion source assembly to the ion trap results in emitted electrons having shorter distances to travel, and sample molecules are already in the trap before ionization. As a result, the high energy electrons undergo fewer alterations to their energy states, and a lower concentration of sample may be used. These factors allow for more uniform electron ionization, enable more linear responses, and higher sensitivity. In addition, in external chemical ionization, the reagent gas must be ionized in the external ion assembly before moving to the trap. These reagent gas ions are often unstable with short half-lives and may have undergone significant changes by the time they enter the trap. Therefore, the issue of the proximity of the ion sources to the ion trap significantly affects both electron and chemical ionization. Internal chemical ionization also takes places at a lower pressure. This is because the neutral reagent gas in the trap is ionized over a longer period, therefore not requiring high pressures. This enables a softer ionization, reducing parent compound fragmentation. Finally, internal ionization requires a lower emission current, enabling longer filament life. These differences enable internal ionization to be more sensitive, have more linear responses, better resolution, and a more reliable long-term operating life.

Advances in ion trap MS

One of the advances of an ion trap is its ability to perform many rounds of MS analysis (MS^n) on the same set of ions, given that those ions are stored in the trap's cavity for variable

lengths of time. In quadrupole analyzers, ions continuously move through the mass analyzer, and therefore the number of MS analyses is restricted by the number of mass analyzers. For example, a single quadrupole can only perform a single round of mass analysis, whereas a triple quadrupole can perform two, with the middle quadrupole functioning as a dissociation chamber (Downward, 2004). Ion preparation techniques such as MS^n, combined with the short distance between the trap and the detector allow for very high sensitivity and resolution compared to conventional quadrupole analyzers where ions flow continuously from the ionization source to the detector (Downward, 2004; Barker, 2000). The ion trap MS is also a fairly resilient instrument that is less technically demanding than a C-IRMS. The Varian 4000 ion trap, in either internal or external ionization, is capable of electron ionization (EI) and chemical ionization (CI) of analytes as typically used in lipid and fatty acid research.

Electron ionization (EI)

In EI, an ionizing filament in the MS ion source generates a beam of electrons that are directed at a right angle against a stream of vaporized sample molecules (Downward, 2004). As the charge field of each electron nears or moves through that of each sample molecule, the latter emits an electron and becomes positively charged. The efficiency with which this process occurs depends in part on the energy of the bombarding electrons, as well as sample chemistry. Thus high energy electrons are able to ionize the sample more thoroughly. The low mass of the bombarding electron causes little change in a sample molecule's translational energy, however, the molecule's electron emission places it in an excited vibrational and rotational state. As the molecule returns to a lesser excited state it breaks into distinct fragments that form a highly specific spectrum (Barker, 2000). The degree of fragmentation is a function of the particular

structure of that sample molecule. For example, polyunsaturated fatty acids with unstable double bonds undergo more fragmentation as compared with saturated fatty acids. The specific molecular fragmentation spectrum can be compared against spectral libraries in order to conclusively identify the compound. The particular location, conformation, or other structural characteristics of any given sub-structure within a molecule can also affect the way that compound fragments. It is possible to use this concept to study the structure of the molecule. Electron ionization is therefore a powerful tool in qualitative applications especially in the identification of unknown compounds (Downward, 2004). However, an important limitation of EI in metabolic studies is that it causes near complete fragmentation of many compounds. As a result, little of the original molecule or parent ion remains, making quantitation difficult (Barker, 2000). Chemical ionization has important advantages over EI, and is better suited to quantitative determination of known analytes as in fatty acid stable isotope studies.

Chemical ionization (CI)

In CI, a reagent gas is bombarded with electrons, which results in the formation of positively charged reagent ions just as in EI. However, in CI, the positively charged reagent ions are further reacted with and transfer a proton to the sample, producing positively charged sample ions (Downward, 2004). It is possible to use a reagent that is ionized in such a way so as to release its proton with great difficulty and only to sample molecules with the highest proton affinity (Barker, 2000). Thus selectively increasing sample proton affinity and reagent protonation threshold raises the overall resolution of the spectra. Many different reagent systems have been used to significantly improve sensitivity and resolution of CI-MS. The choice of reagent system depends on analytical requirements such as sensitivity, resolution, and linear

quantitation (Barker, 2000). Common chemical ionization reagents include methane, hydrogen

gas, isobutane, methanol, and ammonia. Common CI reagents and recombination energies for

their reactant ions are listed below in **Table 1**.

Table 1. Comparison of the recombination energies of the reactant ions of common chemical
ionization reagents (Harrison, 1992).

CI Reagent	Reactant Ion(s)	Mass to charge ratio (m/z)	Recombination Energy (eV)
H_2	H_3^+	3	9.2
Methane	CH_5^+	17	7.9
	$C_2H_5^+$	29	8.3
	$C_3H_5^+$	41	?
Isobutane	$C_3H_5^+$	41	?
	$C_3H_7^+$	43	?
	$C_4H_9^+$	57	6.7
Methanol	$[(CH_3OH)H^+]$	33	5.7
Ammonia	$[(NH_3)H^+]$	18	4.8

A discussion of the potential utility of reagents with lower energies than isobutane is provided in
the Discussion section under "Future Directions".

One of the most common CI reagent gases is methane gas (CH_4). Reagents such as CH_4

and hydrogen gas are generally used when a sample contains an analyte with little background

contamination. Methane and hydrogen gas are not readily protonated, but when protonated, they

can easily release unstable protons to a broad class of compounds if present in the sample. Thus

more contaminants can be ionized and this high energy transfer of protons causes significant

levels of fragmentation that can reduce resolution and sensitivity (Harrison, 1992). Low energy

reagents such as ammonia can ionize specific compounds in complex samples containing many

classes of organic compounds. This is because positive ions of reagents such as ammonia only

relinquish their protons to compounds with higher proton affinity than background or contaminating compounds in the same sample (Harrison, 1992).

Electron bombardment of certain reagent gases can result in a large array of positively charged ions. These can be placed into three categories; low-mass and highly unstable primary ions, secondary ions that are formed as the result of the association of primary ions, and tertiary high-mass stable ions (Berberich et al., 1989). Low-mass and highly unstable primary ions quickly relinquish their positive charge to fatty acid methyl esters (FAMEs), causing significant fragmentation. Using parameters set for external methane CI, we determined the most abundant ion in this category to be CH_5^+ at a mass to charge (m/z) of 17, which makes up nearly 10% of all positively charged methane ions. Higher mass secondary ions result from the interaction of primary ions and neutral methane molecules, and relinquish their positive charges to the analyte with less energy, causing much less fragmentation of the parent compound (Berberich et al., 1989). We found the most abundant ion in this category to be $C_2H_5^+$ at an m/z 29, which makes up about 77% of all positively charged methane ions. High-mass tertiary ions are created as the result of complex interactions between combinations of the primary and secondary ions. These ions release their positive charges to the analyte with great difficulty, and only to the most nucleophilic compounds, therefore causing very little fragmentation (Berberich et al., 1989). We found the most abundant ion in this category to be $C_3H_5^+$ at an m/z of 41, which makes up about 4% of all positively charged methane ions.

Positive chemical ionization (PCI)

Chemical ionization using positively charged reagent ions is termed positive chemical ionization (PCI). Two important factors contribute to the higher sensitivity and resolution of PCI

as compared with EI; lower sample fragmentation, and higher selectivity in sample ionization. The low internal energy associated with the PCI protonation process results in a much lower excitation state for the ionized sample molecule. As a result, fragmentation in PCI is lower than EI (Harrison, 1992). The efficiency of the protonation process depends in part on the sample's proton affinity and the reagent's acidity, both of which can be manipulated. Thus it is possible, through certain sample preparation techniques, to increase the proton affinity of a desired set of compounds in a complex mixture so as to ensure that only those particular compounds are ionized by reagent ions (Harrison, 1992).

Fragmentation in PCI takes place through several complex processes that lead to the formation of certain distinct types of fragments. These fragments can be monitored to enhance the sensitivity of detection and accuracy of quantification. The most common of these fragmenting processes include small mass fragmentation, high mass fragmentation, hydride abstraction, and adduct formation (Berberich et al., 1989). Small mass fragments are created by repeated ionization of a larger fragment, and consequent rearrangement reactions that lead to a final, positively charged, low mass ion. Ionization in this case is not only from positively charged methane ions, but also residual charged particles and contaminants in the ion source and the trap (Berberich et al., 1989). The initial energy of the ionized sample molecule in part determines the extent to which it undergoes successive rounds of fragmentation. High mass fragments dissociate from the parent compound and then undergo another round of dissociation to produce distinct high mass ions. Hydride abstraction results from the parent compound having a reduction of its m/z by one mass unit, through the removal of a hydrogen atom (Harrison, 1992). High mass adducts form when the parent compound associates with a low-energy, ionizing methane ion. This adduct ion formation occurs more frequently for the highest

mass reagent ions, i.e., those with the least energy (Harrison, 1992). These processes are

summarized below in **Figure 4.**   Fragments that result from the high mass fragmentation,

hydride abstraction, and adduct formation can be monitored to enhance detection and accuracy of

quantification, discussed in Methods under "Selected ion extraction".
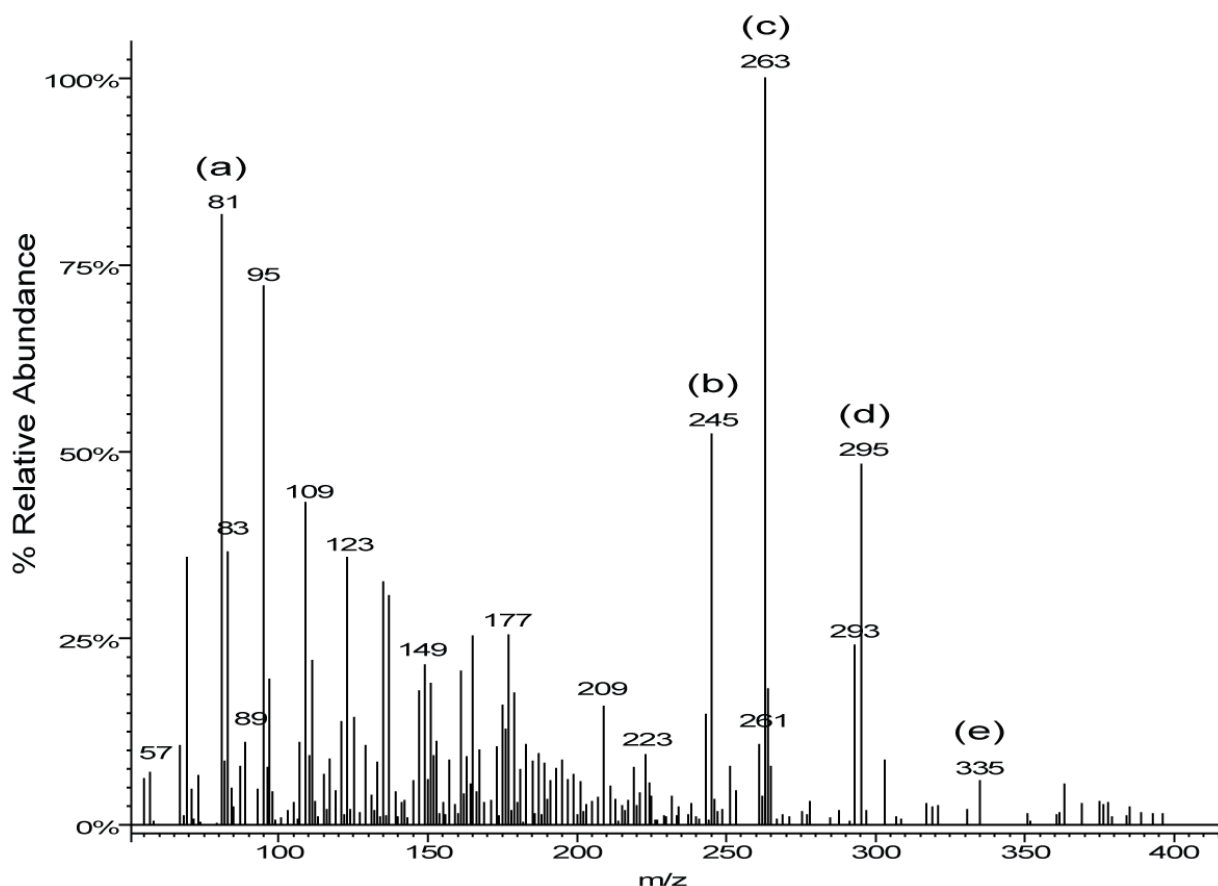
Figure 4. Low and high mass fragments of 18:2n-6 methyl ester standard in methane positive chemical ionization. Low mass fragments ranging up to m/z values of 230 (a). The MH-50 (loss of methanol and water) fragment in (b), MH-32 (loss of methanol) fragment in (c), the parent ion [MH] in (d), and the M+41 (C_3H_5 adduct) ion in (e).

Negative chemical ionization (NCI)

In negative CI, energetic electrons are first reacted with a reagent gas. This reaction

causes the electrons to impart much of their energy onto the reagent molecules, and in the

process become low energy or "thermal" electrons. These thermal electrons can then readily

attach to molecules with high electron affinity (Harrison, 1992). This technique is also known as "electron-capture" chemical ionization. Since this electron capture occurs with very little internal energy, there is even less fragmentation of the sample molecule as compared with PCI (Harrison, 1992). As well, sample preparation techniques allow for the ionization of very select groups of compounds, significantly reducing background ionization. These two factors give NCI higher sensitivity and resolution as compared with PCI (Harrison, 1992). Limits of detection for fatty acids have been reported at as low as 20fg/uL (Goto et al., 1987), and responses more than 2000 times higher than PCI (Pawlosky, Sprecher, and Salem, Jr., 1992). Less fragmentation also means more available sample, and therefore lower sample concentration requirements (Harrison, 1992). However, NCI is not without its limitations. These include complex and time-consuming sample preparation techniques, and high equipment maintenance demands. Fatty acid analysis with NCI requires preparation of pentafluorobenzyl (PFB) esters. The PFB group enables the fatty acid to be more electrophilic so as to better attract thermalized electrons. The entire PFB derivitization process requires more steps than methyl ester formation that is done in PCI. It also requires larger volumes of solvents such as hexane, and longer dry times under N_2. The electron capture process also requires the availability of a larger volume of thermal electrons. As a result, the filament emission current in NCI is more than 15 times higher than that of PCI. This results in shorter filament operating times which is not trivial as filament assemblies are expensive, and instrument shutdown results in significant post start-up diagnostics and tuning.

Mass-selection

Mass-selection allows for the selection of a single reagent ion, based on its mass, and then allows only that selected reagent ion to ionize the sample. Using this technique, the reagent

ion with the lowest energy can be used thereby minimizing the fragmentation of the parent

compound. Mass-selection techniques have been demonstrated in environmental contaminant

assessments for PCBs (Lausevic et al., 1995), and alkylbenzenes (Berberich et al., 1989) but not

for fatty acid analysis. Mass-selection was used in the development of the novel internal

isobutane technique for this thesis. Detailed scanning procedures for mass-selection are provided

in Methods under "Mass selection".

CHAPTER 3

RATIONALE & OBJECTIVES

RATIONALE

The Varian 4000 MS is equipped with a quadrupole ion trap, and is capable of EI and CI in internal and external mode as well as a hybrid internal-external CI mode. As such, the Varian 4000 ion trap offers significant flexibility and various analytical solutions. Although GC-MS ion traps have been used for fatty acid analyses, to date no published work exists documenting the use of a the Varian 4000 ion trap in quantitative fatty acid stable isotope analyses. In the present thesis proposal the use of this instrument for fatty acid stable isotope measures will be explored. *In vivo* fatty acid stable isotope analyses using GC-MS allow for in-depth investigations of the absorption, transport, and metabolism of dietary fatty acids. Existing GC-MS methods have been limited by low sensitivity, overly complex methodology, and non-linear responses. Therefore the novel quadrupole ion trap method may potentially enhance fatty acid stable isotope investigations. Initial efforts will be directed at duplicating existing single high pressure PCI and NCI quadrupole techniques using methane as the ionization reagent. This is equivalent to PCI and NCI with methane with the GC-MS ion trap in external mode. A novel low pressure, internal PCI with isobutane as reagent gas, GC-MS ion trap method will be developed. With this technique it is anticipated that limit of detection can be lowered to at least 500fg/uL, resolution will be significantly improved, quantitation will be linear, sample preparation will be simplified, and instrument maintenance demands will decrease. As well, this technique can potentially be a simpler, more robust substitution for GC-C-IRMS and NCI single quadrupole methods.

OBJECTIVES

The main objective of this thesis will be to develop a novel internal isobutane GC-PCI-MS technique that would overcome key limitations of existing single quadrupole quantitative GC-MS techniques. These limitations include excessive fragmentation of the parent ion and low sensitivity in GC-PCI-MS, non-linear responses and high instrument maintenance demands in GC-NCI-MS. In order to compare the novel technique to existing methods, it will be necessary to replicate existing single quadrupole quantitative methods on the ion trap. These techniques will be methane PCI and methane NCI. Both will be replicated using the ion trap's external mode. The *in vivo* quantitative performance of the novel technique to existing methods will be assessed by analyzing livers containing isotopic PUFA from rats orally dosed with isotopic 18:2n-6. The use of the three isotopes, U-^{13}C ethyl linoleate, U-^{13}C non-esterified (NEFA) linoleate, and $^{2}H_{5}$ ethyl linoleate will allow comparisons of the enrichment efficiency of each isotope. Fatty acid standards will also be analyzed in order to study the fragmenting behaviour of parent ions with each quantitative method. Finally, given the need for the qualitative analysis of fatty acids, a GC-EI-MS technique will be developed and a spectral database search program will be tested using fatty acid standards.

HYPOTHESIS

1. A novel internal isobutane positive chemical ionization GC-MS quadruopole ion trap method will be more sensitive than external methane PCI and external methane NCI GC-MS.

2. The novel technique will be suitable for measuring U-^{13}C and ^{2}H$_5$ labelled 18:2n-6 and 18-^{13}C and ^{2}H$_5$ n-6 PUFA metabolites in rat liver 8 hours after dosing with U-^{13}C and ^{2}H$_5$ 18:2n-6 by oral gavage.

CHAPTER 4

METHODS

Study design

The design intends to achieve one of the objectives outlined under "Objectives", and summarized below. More detailed discussions of each aspect of these phases are provided in the later sections of Methods.

Fatty acid analysis by GC-MS in EI mode was completed first. The GC oven temperature protocol was determined from previously published methods (Pawlosky, Sprecher, and Salem, Jr., 1992). Ionization parameters for EI were optimized by detecting and analyzing fatty acid methyl ester standards ranging from 10:0 to 24:1n-9. Spectral library searches were conducted to confirm functionality of this utility and proper EI operation.

Prior to development of further MS techniques, an animal study was conducted to obtain tissue samples that contained isotopic fatty acids as sample matrix is known to influence analytical conditions. Fasted Sprague-Dawley rats were dosed with isotopic 18:2n-6 by oral gavage, given access to food for 8 hours, then sacrificed. Livers were excised and rinsed in cold saline, total lipids were extracted and stored at -80°C. Fatty acid methyl esters were prepared for PCI GC-MS analysis. A separate batch of fatty acid PFB esters were prepared for NCI GC-MS analysis.

The ion trap MS was initially set up in the external ionization mode as this setting best emulates a single quadrupole GC-MS. There are existing single quadrupole GC-MS PCI and NCI methods for fatty acid stable isotope measurements in the literature (Pawlosky, Sprecher, and Salem, Jr., 1992). Adapting these single quadrupole methods to the ion trap would serve as

a method development starting point. GC-PCI-MS using methane as the reagent gas was developed from existing single quadrupole methods (Pawlosky, Sprecher, and Salem, Jr., 1992). To optimize ionization parameters and study parent ion fragmenting behaviour, fatty acid methyl ester standards ranging from 10:0 to 24:1n-9 were analyzed. Upon optimization, the analytical process was tested with the prepared liver fatty acid methyl ester samples with fatty acid stable isotopes. Selected ion monitoring (SIE) was used to detect isotopic 18:2n-6 and n-6 PUFA metabolites. After successfully duplicating GC-PCI-MS results, GC-NCI-MS methodology using methane was developed from methods previously published for GC-MS quadrupole instruments (Pawlosky, Sprecher, and Salem, Jr., 1992). Ionization parameters and gas pressure were altered and tested using fatty acid PFB ester standards (ranging from 10:0 to 24:1n-9). This was followed by analysis of fatty acid stable isotopes from experimental rat livers.

Finally, a novel internal ionization method using isobutane as the reagent gas was developed. This required a reconfiguration of the MS hardware from external to internal ionization and the connection of research grade isobutane to the ion trap. The fatty acid methyl ester standards were utilized to develop GC oven temperature and mass-selected ionization protocols followed by a check with the biological samples containing fatty acid stable isotopes (fatty acid methyl esters extracted from the liver of gavaged rats). Mass spectra were compared across all ionization modes, and quantification fatty acid stable isotopes were compared across the three CI modes.

Isotopes, standards and reagents

Ethyl linoleate U-^{13}C and ethyl linoleate 17, 17, 18, 18, 18-D5 were obtained from Cambridge Isotope Laboratories (Andover, MA) and isotope purities were determined by GC-FID were 95% for the former, and 91% for the latter. Non-esterified fatty acids (NEFA) of U-

[13]C linoleate were obtained from Spectra Isotopes and isotope purity determined by GC-FID was 81%. Heptadecanoic acid (17:0) was obtained from Nu-Chek Prep (Elysian, MN). Separate serial dilutions were made using fatty acid methyl esters and PFB esters of 18:2 n-6 for use in PCI and NCI, respectively. Heptadecanoate (10ug/mL) was added to each dilution of 18:2 n-6. Research grade methane and isobutane gases (99% purity) were purchased from Praxair (Danbury, CT).

Instrumentation

Gas Chromatography

Isotopic and standard fatty acid peaks were analyzed on a Varian 3800 GC coupled to a Varian 4000 MS with a quadrupole ion trap (Varian Canada Inc., Mississauga, ON). Fatty acid PFB esters and methyl esters in hexane were injected onto a DB-FFAP 30m x 0.25mm i.d. x 25um film thickness, capillary column (J&W Scientific from Agilent Technologies, Mississauga, ON) interfaced directly onto the ion source with helium as carrier gas. Oven temperature programming for the analysis of FAMEs and PFB esters are presented below in **Table 2.**

Table 2. Oven temperature protocol for fatty acid analyses.

Temp (C)	Rate (C/min)	Hold (min)	Total (min)
FAMEs			
50		1	1
130	30	0	3.67
175	10	0	8.17
230	5	9.5	28.67
240	50	25	53.87
PFB esters			
125		1	1
245	8	39	55

FAMEs, fatty acid methyl esters; PFB, pentafluorobenzyl

Mass spectrometry

In order to clear the ion trap of residual air and water post instrument maintenance, temperatures for the trap, the manifold, and the transfer line were maintained at 220°C, 100°C, and 280°C respectively for 24 hours, and were thereafter decreased to analysis temperatures of 175°C, 50°C, and 230°C. At analytical temperatures, the trap was allowed to stabilize for two hours. Afterwards the radio frequency coil was tuned to obtain a straight line in the spectral display window with a minimum ion count. The perfluorotributylamine (PFTBA) calibration gas was adjusted to show prominent peaks at m/z 69, 100, and 131, corresponding to CF_3^+, CF_4^+, and CF_5^+ respectively. Mass calibration and trap frequency calibration were re-set by auto tuning.

External electron ionization

Configuring the MS for external ionization requires removal of the analyzer assembly from the MS manifold and switching the ion source to external and pressing the heat shield to the forward position. In addition the transfer line was moved to the front position with a metallic tip (rather than the internal ionization polymeric tip), with the GC column extending 1mm past this tip. Electron ionization software parameters were set as follows: Mass defect, 100 mass units; uScan averaged, 3; automatic gain control, 20,000 counts; maximum ionization time, 65,000 usec; emission current, 25uAmp; tune, automatic. Automatic tune determined the optimal radio frequency storage level and ion time factors for ion mass ranges. The low-mass range was selected as 50 m/z to exclude air and water contaminants, and the high-mass range was 400 m/z to include the full range of fatty acid methyl esters.

External methane PCI

The external ion source and the position of the transfer line were similar to EI configuration. Methane reagent gas was connected to the MS through a 4mL/min restrictor tube

connected to a 1/8" gas supply line connected to a two-stage pressure regulator set at 20 psi on a methane dewar. The CI gas valve on the MS was adjusted so that the ion at m/z 17 (CH_5^+) is about 25% of that at 29 ($C_2H_5^+$) during a CI gas test. This operation produces a final pressure of 1 to 2 x 10^{-5} Torr of CH_4 gas in the ion trap. External PCI ionization parameters were as follows: Mass defect, 100 mass units; uScan averaged, 3; automatic gain control, 5000 counts; maximum ionization time, 25,000 usec; emission current, 25uAmp; tune, automatic. The low-mass range was selected as 50 m/z to exclude air and water contaminants, and the high-mass range was 400 m/z to include the full range of fatty acid methyl esters.

Methane NCI

Hardware configuration and gas settings in methane NCI were the same as those in methane PCI. Methane NCI ionization parameters were as follows: Mass defect, 100 mass units; uScan averaged, 3; automatic gain control, 5000 counts; maximum ionization time, 25,000 usec; emission current, 250uAmp; tune, automatic. The low-mass range was selected as 50 m/z to exclude air and water contaminants, and the high-mass range was 400 m/z to include the full range of fatty acid methyl esters.

Internal isobutane PCI

Ultra high purity isobutane gas cylinder was connected to the MS in place of methane. The outlet pressure on the isobutane cylinder was set to 20 psi. The MS was configured to internal ionization by removing the assembly and switching the ion source to internal and pressing the heat shield to the rear position. The transfer line was moved to the rear position with the polymeric tip and the GC column extended 7mm past the tip of the transfer line. The CI gas valve on the MS was adjusted so that the ion at m/z 43 ($C_3H_7^+$) and 57 ($C_4H_9^+$) are equal,

producing a final pressure of 1 to 2 x 10^{-5} Torr of isobutane in the trap. Ionization parameters are given in detail in the following section.

Mass selection

The scanning procedure for mass selection spans four stages. In stage one, isobutane gas is ionized by EI at 10uAmps, producing primary ions, which must be stored to allow them to further react with neutral isobutane molecules to form secondary and tertiary ions. In order to store the primary ions, the ejection amplitude voltage is set to store all ions above m/z 35. During the second stage, radio frequency ring voltage is increased to 15 volts to eject all but the isobutane ions, and to mass-select for a specific ion. Mass-selection is made possible by the application of a single-notch selected-ion storage waveform. The mass range for the notch and the amplitude of the ejection voltage are user-defined. For the isobutane ion $C_4H_9^+$ at m/z 57, low mass is set to 35 and high mass to 65. The reaction storage level is set to 46. At the third stage, the mass-selected reagent ion is allowed to react with the gaseous FAMEs for a maximum of 100msec. At the final stage, the ring RF voltage is increased to sequentially eject sample ions in the ascending order of mass to the detector, from a low m/z of 50 to a high of 500.

Selected ion extraction

Selected ion extraction (SIE) generates peaks that correspond to the abundance of one or more user-defined ion masses. This tool was used in all isotopic quantification work in this thesis.

Fatty acid stable isotopes labelled with ^{13}C co-elute chromatographically with endogenous unlabeled fatty acids. As a result, the total ion chromatographic peak cannot be integrated to quantify the isotope. The first step is to select for the most abundant ion of a FAME, by its mass to charge (m/z) value. In PCI however, any given FAME generally produces

more than one high mass fragment ions. Common ion m/z values that are used in SIE to quantify the respective isotopic FAMEs are listed (**Table 3**). When more than one m/z is used in SIE, the response is amplified, the shape of the chromatographic peak is clearer, and is thus simpler to quantify. This process becomes complicated when a highly unsaturated FAME completely fragments and leaves no parent compound intact. Methyl esters such as EPA and DHA leave no intact parent ion in methane PCI. As a result, SIE can only be used in restricted ionization environments, and only with those FAMEs that do not completely fragment. The quantification of 18:2n-6 using the Varian 4000 SIE utility is depicted below (**Figure 5**).

Table 3. Mass to charge (m/z) values of select isotopic FAMEs, ionized using methane PCI.

Isotopic FAME	Monitored m/z values
U-^{13}C 18:2n-6	**313**, 311, 281, 263
U-^{13}C 20:3n-6	**339**, 337, 307, 289
U-^{13}C 20:4n-6	**337**, 335, 305, 287
^{2}H$_5$ 18:2n-6	**284**, 282, 252, 234
^{2}H$_5$ 20:3n-6	**310**, 308, 278, 260
^{2}H$_5$ 20:4n-6	**308**, 306, 276, 258

Bolded numbers are parent ion m/z values; the rest are other high-mass fragment ions.
FAME, fatty acid methyl esters; PCI, positive chemical ionization; m/z, mass to charge ratio.
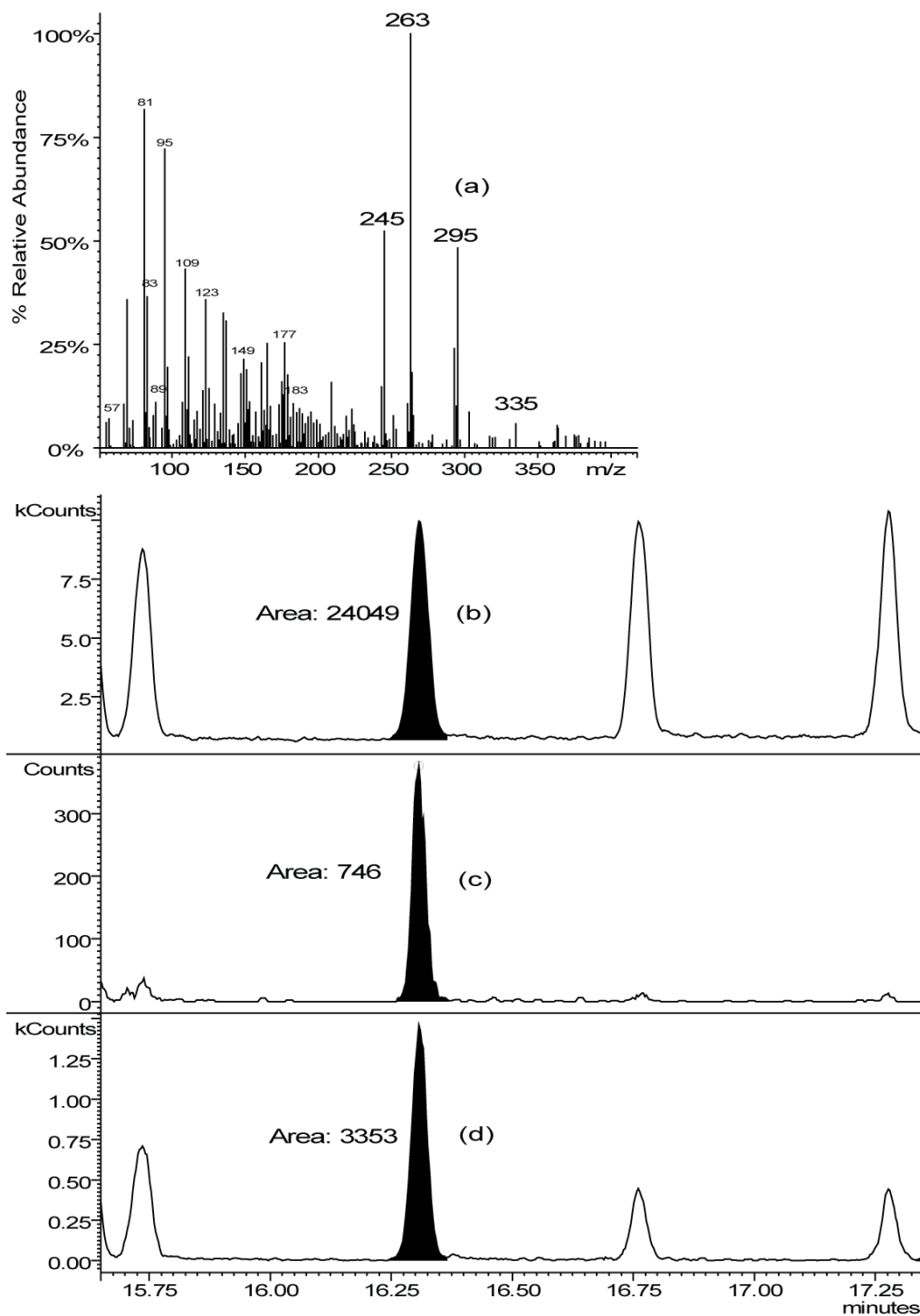
Figure 5. Selected ion extraction to quantify 18:2n-6 standard in external methane PCI. Spectrum in (a) shows high mass fragments at m/z 245 [MH-50] (loss of methanol and water), 263 [MH-32] (loss of methanol), 295 [MH] (the parent ion), 293 [M-H] (loss of hydrogen) and 335 [M+ 41] (C_3H_5 adduct). The total ion chromatograph of 18:2n-6 (b) is quantified using only the parent ion at (c) m/z 295 [MH], and later quantified using (d) m/z values 245, 263, 295, and 335.

Animals & diet

Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) were raised on Harlan Teklad 8640 rodent diet (**Table 4**). Upon reaching 110-120g of body weight, rats were fasted over night. Three treatment groups of 4 animals each were each dosed with 0.150mg/g of body weight of one of three stable isotopes; U-^{13}C 18:2n-6 ethyl esters, U-^{13}C 18:2n-6 NEFA, and ^{2}H$_5$ 18:2n-6 ethyl esters. The calculated dose took isotope impurity into account and dosing was by oral gavage, using 400uL of olive oil as vehicle. A fourth group of four animals received only the olive oil vehicle and served as the control group.

Table 4. Fatty acid composition of Harlan Teklad 8640 rodent diet.

Fatty Acid	Relative Composition (%)
14:0	0.5
16:0	14.7
18:0	4.6
Total saturates	20.1
18:1 n-9	20.7
Total monounsaturates	23.5
18:2 n-6	46.6
20:4 n-6	0.04
Total n-6 polyunsaturates	47
18:3 n-3	5.1
20:5 n-3	0.3
22:6 n-3	0.2
Total n-3 polyunsaturates	5.7

Tissue collection, homogenization, lipid extraction, and derivitization

Eight hours post gavage, the animals were anaesthetized using pentobarbital (250ug per 100g body weight) and sacrificed through exsanguination via cardiac puncture. Livers were removed, washed in cold 0.9% sodium chloride, and frozen immediately at -80°C. Two hundred milligrams of liver was homogenized in 2mL of methanol containing 50ug/mL of BHT and 10ug/mL of 17:0 as internal standard. Two millilitres of chloroform was added to the homogenates, followed by 1.5mL of 0.2M sodium phosphate (Folch, Lees, and Stanley, 1957). Total lipids in the bottom chloroform layer were removed, dried under N_2 gas, and trans-esterified using 14% methanolic boron trifluoride to form methyl esters for GC-EI-MS and GC-PCI-MS analysis (Morrison and Smith, 1964). For GC-NCI-MS, a portion of the TLE was saponified with 0.3M methanolic potassium hydroxide and the free fatty acids were derivitized using pentafluorobenzyl bromide (PFBBr) reagent [PFBBr, diisopropylamine in acetonitrile (1:100:1,000, v/v/v)] (Pawlosky, Sprecher, and Salem, Jr., 1992).

CHAPTER 5

RESULTS

Detection and identification of fatty acid standards by GC-EI-MS

Standard FAMEs ranging from 10:0 to 24:1n-9 were successfully detected in EI mode,

and identified using a National Institute of Standards and Technology (NIST) spectral search.

Spectral search

A search for the chromatographic peak corresponding to individual FAMEs such as

18:2n-6 yields a large number of potential matches. Each of these matches has a particular

degree of probability of being the correct match. Highly unsaturated FAMEs such as 22:6n-3

(DHA) yielded probabilities of up to 70%, whereas the least saturated FAMEs such as 16:0

(palmitic acid) yielded very low probabilities of as low as 3%. **Figure 6** below shows a segment

of a typical search result for 18:2n-6.
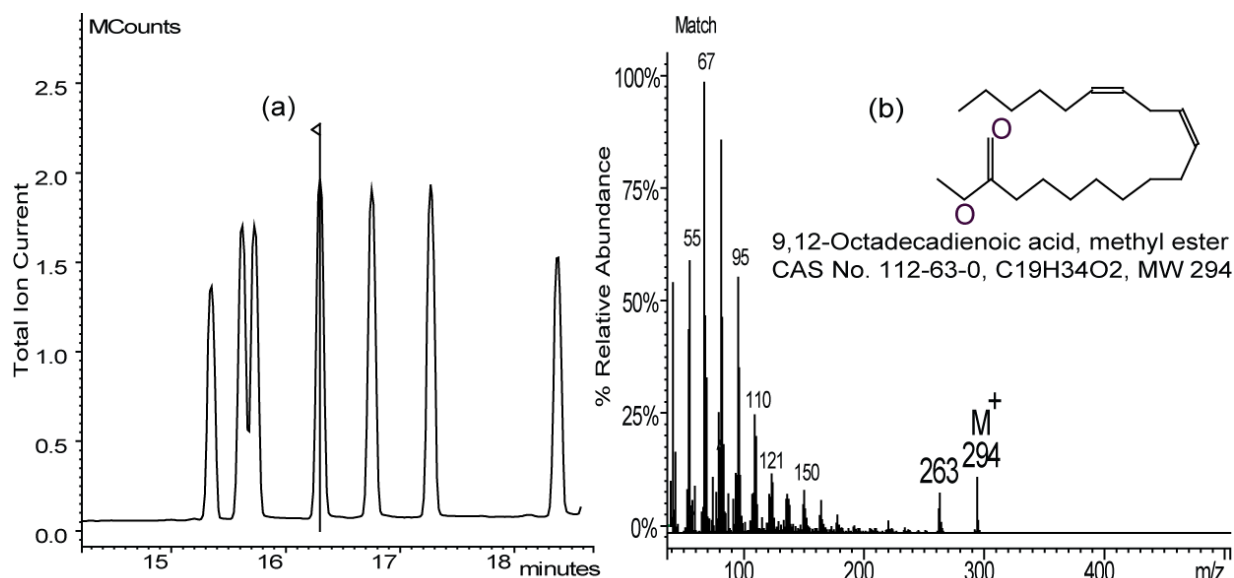
Figure 6. Search of electron ionization spectral library. A fatty acid methyl ester standard peak at 16.3 minutes (a) produces a spectrum that is positively identified as linoleic acid (b) with a probability of 15%, using the National Institute of Standards and Technology EI spectral library search.

Parent ion fragmentation

With EI, the majority of fragments are small mass, ranging from m/z of 50 to about 150. The most abundant ion, or the base peak, is mostly a small mass ion. Saturated FAMEs such as palmitic acid (16:0) show the highest yield in parent ion detection, and least amount of small mass fragments. Parent ions in these FAMEs make up nearly 50% of the base peak. Moderately unsaturated FAMEs such as linoleic acid (18:2n-6), fragment more and the parent ion makes up only about 5% of the base peak. Highly unsaturated FAMEs such as DHA (22:6n-3), show extremely high amounts of small mass fragments, and parent ions are absent (**Figure 7**).

Figure 7. External EI spectra of methyl ester standards of 16:0(a), 18:2n-6 (b), 20:4n-6 (c), and 22:6n-3 (d), showing diminished parent ion (M^+) detection with higher unsaturation. Note absence of 22:6n-3 parent ion.

Administration of fatty acid stable isotopes to rats by oral gavage

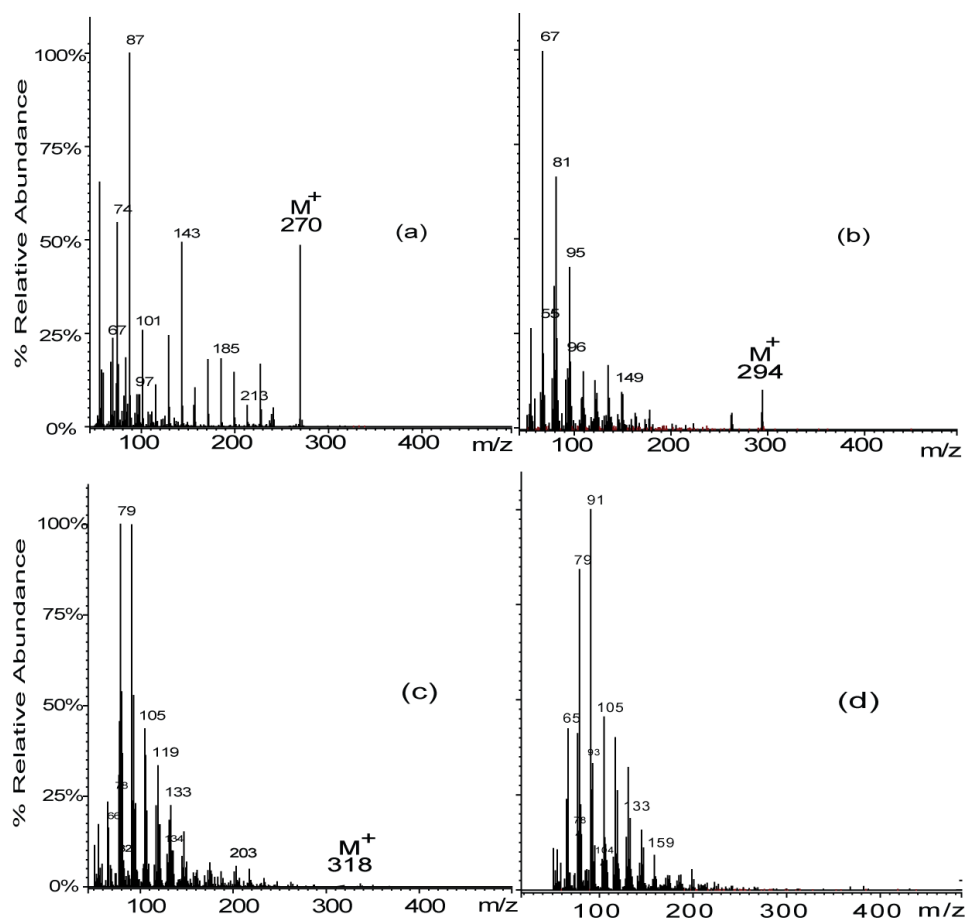Ethyl U-[13]C 18:2n-6, NEFA U-[13]C 18:2n-6, and ethyl $^2H_5$ 18:2n-6 were successfully

administered by oral gavage to rats. Mean body weight was 113g ($\pm$3.7), and mean liver weight

was 5.6g ($\pm$0.43). The concentration of the three isotopic forms of 18:2n-6 in liver varied by

analytical method in individual animals (**Table 5).**

Table 5. Variation in concentrations of the three isotopic forms of 18:2n-6, in internal isobutane
PCI, external methane PCI, and external methane NCI

Isotope group	Animal	Concentration of isotopic 18:2n-6 [nmol/g Liver]		
		Internal Isobutane PCI	External Methane PCI	External Methane NCI
NEFA U-[13]C 18:2n-6				
	1	129.30	397.02	29.51
	2	430.47	538.78	34.29
	3	267.36	397.53	31.29
	4	300.52	414.57	27.73
Ethyl $^2H_5$ 18:2n-6				
	1	137.36	119.02	276.58
	2	113.60	102.81	123.56
	3	118.18	103.47	166.34
	4	140.55	106.12	110.33
Ethyl U-[13]C 18:2n-6				
	1	379.93	NA	NA
	2	570.47	NA	NA
	3	449.09	NA	NA
	4	403.52	NA	NA

NEFA, Non-esterified fatty acids; NA, not analyzed

Development of an external methane PCI ion trap method for measuring fatty acid stable

isotopes

In vivo isotope measurement

Liver enrichment of ethyl U-[13]C 18:2n-6 surpassed that ethyl $^2H_5$ 18:2n-6 by about four

times. Neither 18-^{13}C nor $^2H_5$ 20:3n-6 was detectable. Only 18-[13]C 20:4n-6 was detected at a

concentration of 41nmol/g (**Figure 8**). Limits of detection for ethyl U-^{13}C 18:2n-6 in external

methane PCI was determined to be 300pg/uL.
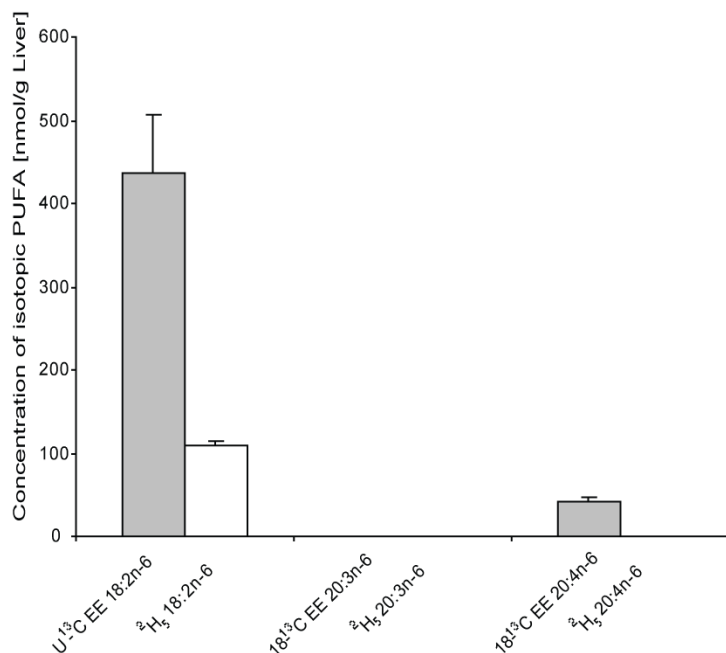
Figure 8. Concentrations of isotopic 18:2n-6, 20:3n-6, and
20:4n-6 in rat liver at 8 hours post gavage, using external methane PCI.

Parent ion detection

External methane PCI spectra of FAMEs show moderate fragmentation and more parent

ion detection than EI. The fragments were not entirely low-mass, but ranged from m/z of 50 to

500. Saturated FAMEs such as palmitic acid (16:0) show very high yields of parent ion, and few

low-mass fragments. In the case of 16:0, the parent ion is the base peak. Unsaturated FAMEs

such as linoleic acid (18:2n-6) fragment more, though producing prominent high mass fragment

peaks such as the (MH-32) at m/z 263 and (MH-52) at m/z 243. The parent ion at m/z 295 makes

up about 30% of the base peak. Highly unsaturated FAMEs such as arachidonic acid (20:4n-6)

fragment significantly more, with parent ions up to 4% of the base peak (**Figure 9**). However, in

the most unsaturated FAMEs such as DHA, parent ion is absent.
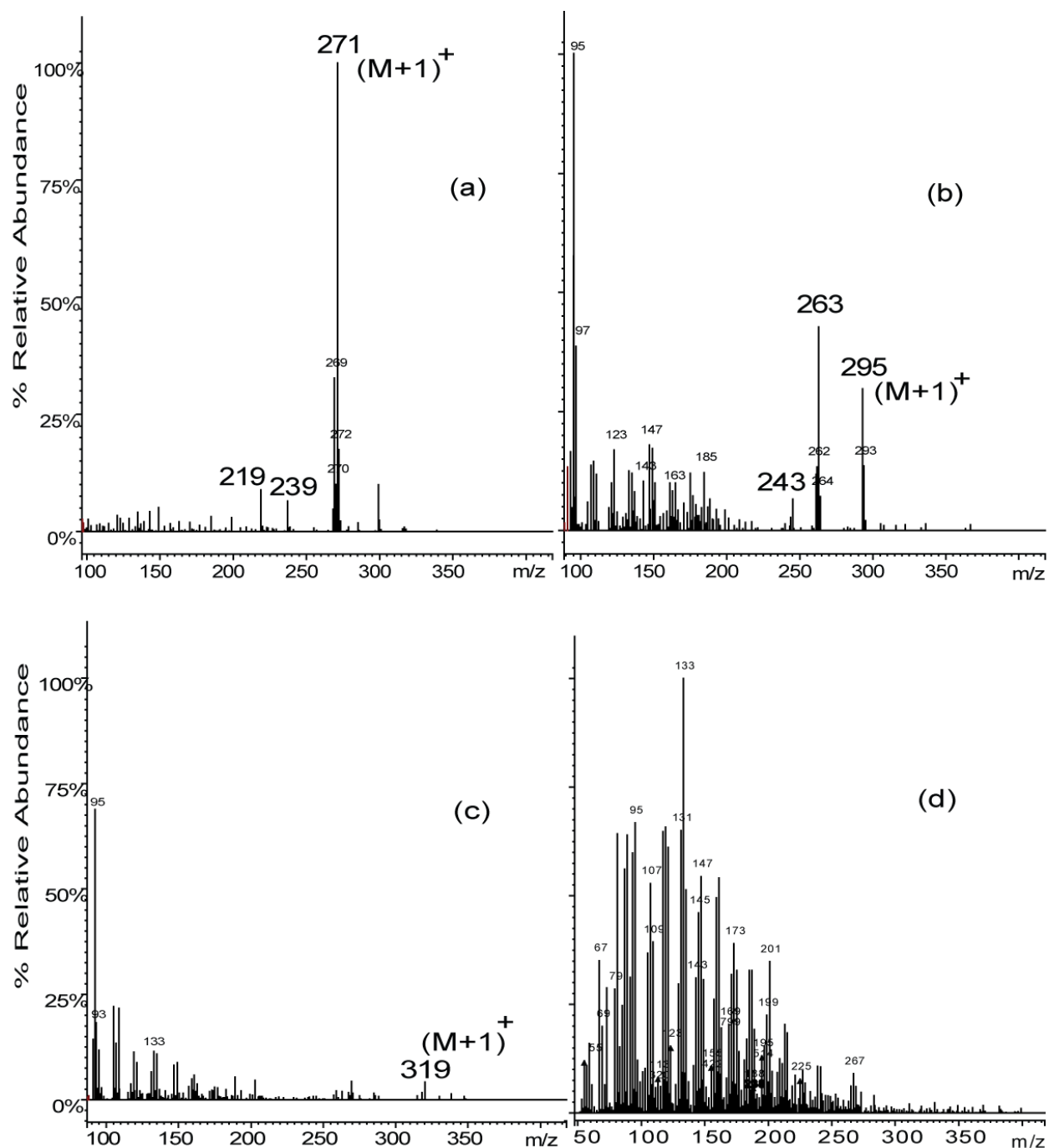
Figure 9. External methane PCI spectra of methyl ester standards of (a) 16:0, showing the parent ion at m/z 271 at 100% relative abundance, the MH-32 fragment at m/z 239, and the MH-52 fragment at m/z 219; (b) 18:2n-6, showing parent ion at m/z 295 at ~ 30% of base peak, MH-32 fragment at m/z 263, and MH-52 fragment at m/z 243; (c) 20:4n-6, showing parent ion at m/z 319 and at ~ 4% of base peak; (d) 22:6n-3, showing absence of parent ion and high mass fragments.

Development of an external methane NCI ion trap method for measuring fatty acid stable isotopes

In vivo isotope measurement

Liver enrichment of ethyl ^{2}H$_5$ 18:2n-6 surpassed that of ethyl U-^{13}C by about six times (188 vs. 30nmol/g). As well, ethyl ^{2}H$_5$ 18:2n-6 showed considerable variation among the four rats (Mean=188; ±79). The initial metabolite, 20:3n-6, was only detected in its ^{13}C form at a concentration of about 2nmol/g. Labelled 20:4n-6 was not detected (**Figure 10**). Limits of detection for ethyl U-^{13}C 18:2n-6 in methane NCI was determined to be 5pg/uL.
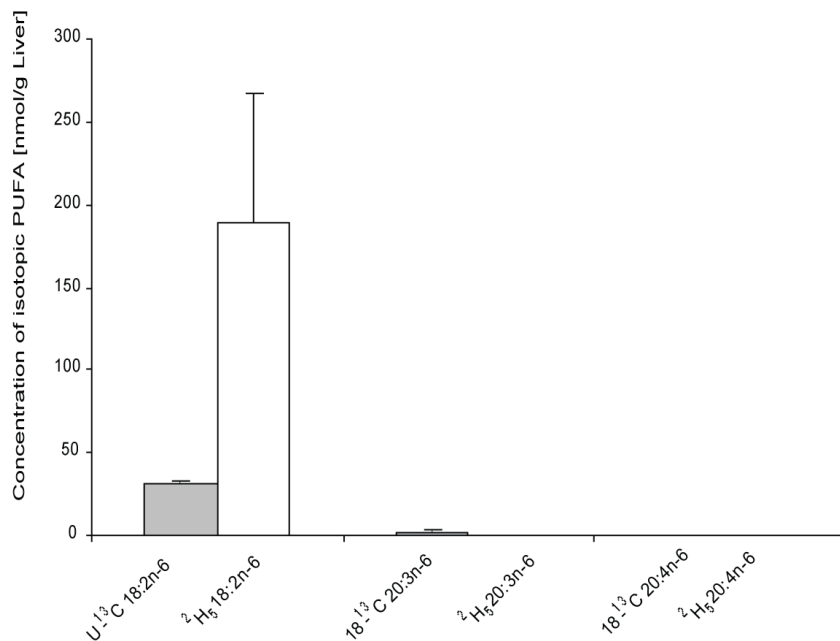
Figure 10. Concentrations of isotopic 18:2n-6, 18:3n-6, and 20:4n-6 in rat liver at 8 hours post gavage, using methane NCI.

Parent ion detection

Negative CI spectra of fatty acid PFB esters show almost no fragmentation of the parent ion (M-PFB). This trend is not dependent on the degree of saturation, as even the most unsaturated FAMEs such as DHA hardly fragment (**Figure 11**).
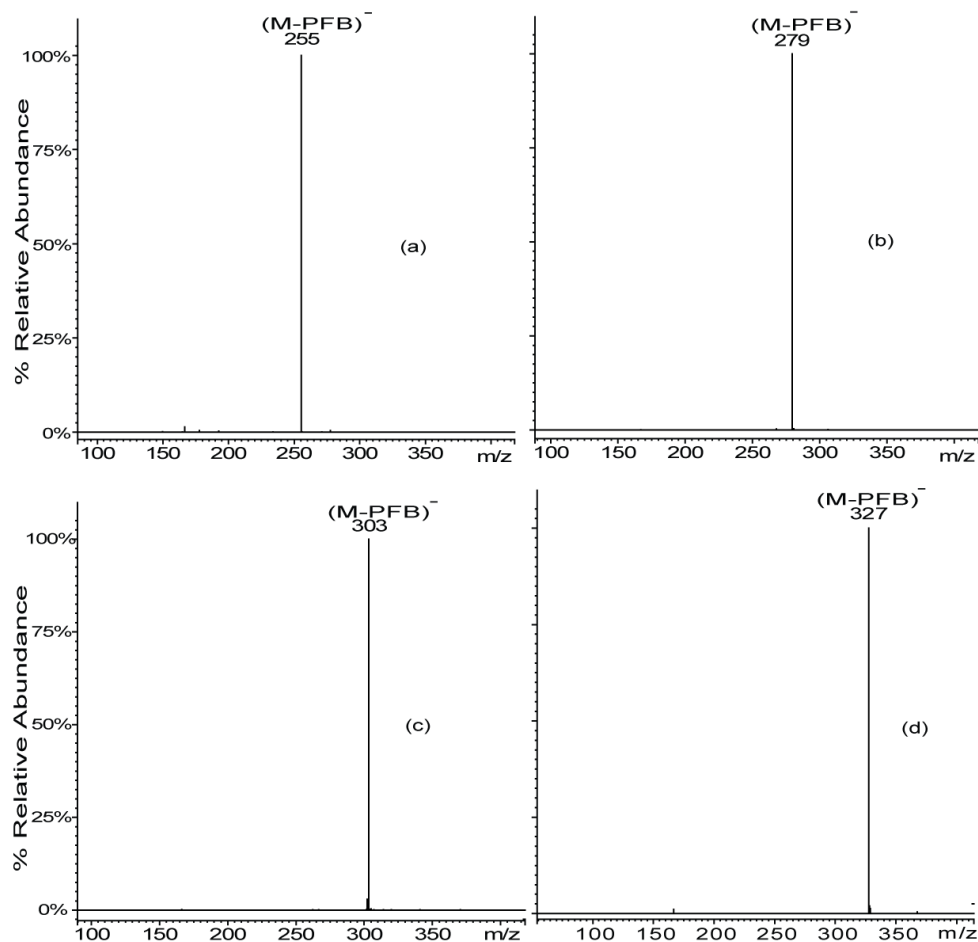
Figure 11. External methane NCI spectra of PFB ester standards of 16:0(a), with parent ion at m/z 255 as the base peak, 18:2n-6 (b) with parent ion at m/z 279 as the base peak, 20:4n-6 (c) with parent ion at m/z 303 as the base peak, and 22:6n-3 (d) with parent ion at m/z 327 as the base peak. Spectra show near complete parent ion yield and minimal fragmentation.

Development of an internal isobutane PCI ion trap method

In vivo isotope measurement

Liver enrichment of ethyl U-[13]C 18:2n-6 was nearly twice as high as the NEFA form, and about three times as high as ethyl $^2H_5$ 18:2n-6 (Mean = 450; 248; 158 nmol/g respectively). The amount of variation was very high in the U-^{13}C NEFA 18:2n-6 compared to either the ethyl U-^{13}C or ethyl $^2H_5$ (SD ± 176, ± 63, and ± 20, respectively) and thus the only statistical difference was between ethyl U-^{13}C and ethyl $^2H_5$ 18:2n-6. The concentrations of 18-[13]C 20:3n-6

metabolite of ethyl U-^{13}C 18:2n-6 were higher than either the NEFA ^{13}C or ethyl ^{2}H$_5$ forms

(Mean = 6.5, 1.9, and 3.9 nmol/g, respectively), but were only statistically different than 18-^{13}C

NEFA 20:3n-6. There was no difference between the concentrations of the two 18-^{13}C 20:4n-6

metabolites, which were 22 and 30 nmol/g (SD ± 6.8 and ± 6.2) for ethyl ester and NEFA forms

respectively. The ^{2}H$_5$ 20:4n-6 was not detected (**Figure 12**). Limits of detection for ethyl U-^{13}C

18:2n-6 in internal isobutane PCI was established as 500fg/uL.

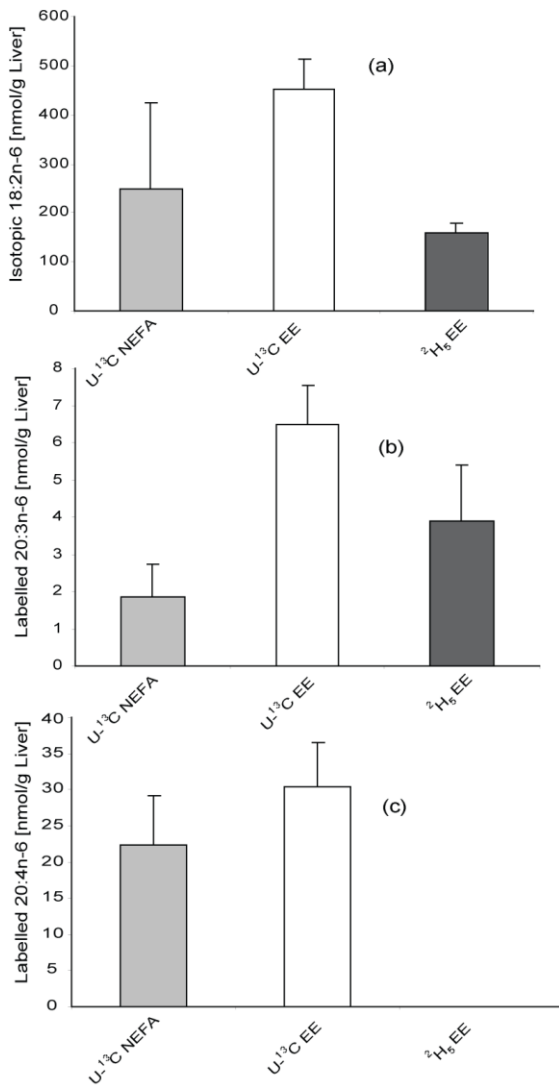**Figure 12.** Concentrations of isotopic 18:2n-6 (a) and labelled n-6 PUFA metabolites, 20:3n-6 (b), and 20:4n-6 (c) in rat liver at 8 hours post oral gavage using internal isobutane PCI. NEFA, Non-esterified fatty acids; EE, ethyl esters.

Parent ion detection

Internal isobutane PCI spectra of FAMEs show significantly less fragmentation than both EI and external methane PCI, and significantly more parent ion detection. Saturated FAMEs such as 16:0 are almost entirely made up of the parent ion. Moderately unsaturated FAMEs such as 18:2n-6 fragment more, though still producing parent ions that are as high as 60% of the base peak. In these spectra, a large proportion of the fragments are made up of only two high mass ions that cluster around the base peak. In the case of 18:2n-6 with a parent ion m/z of 295, the ions at m/z 263 (MH – 32) and m/z 245 (MH – 50) make up 35% of the total ion count. When combined with the parent ion, this number increases to 50% of the total ion count. Highly unsaturated FAMEs such as 20:4n-6 fragment, but produce parent ion base peaks, while DHA has the poorest yield in parent ion. However, unlike external methane PCI, the DHA parent ion is clearly detected, and makes up about 5% of the base peak (**Figure 13**).

A Comparison of external methane PCI and NCI (single quadrupole emulation) techniques to novel internal ion trap isobutane PCI method

Spectra for NCI is not included in this comparison as it produces virtually no fragmentation of the parent ion. In EI, the parent ion makes up about 4% of the base peak, and 0.5% of the total ion count. The base peak itself is a low-mass ion at m/z 67. In methane PCI, more high-mass fragments are produced at m/z 263 and 245, and these together make up about 15% of the base peak, and 5% of the total ion count. Similar to EI, the base peak in methane PCI is a low-mass ion. In internal isobutane PCI, the parent ion at m/z 295 makes up about 44% of the base peak. The base peak is the high-mass fragment at m/z 263 (MH-32). The parent ion

and the two high-mass fragments at m/z 263 and 245 make up about 30% of the total ion count
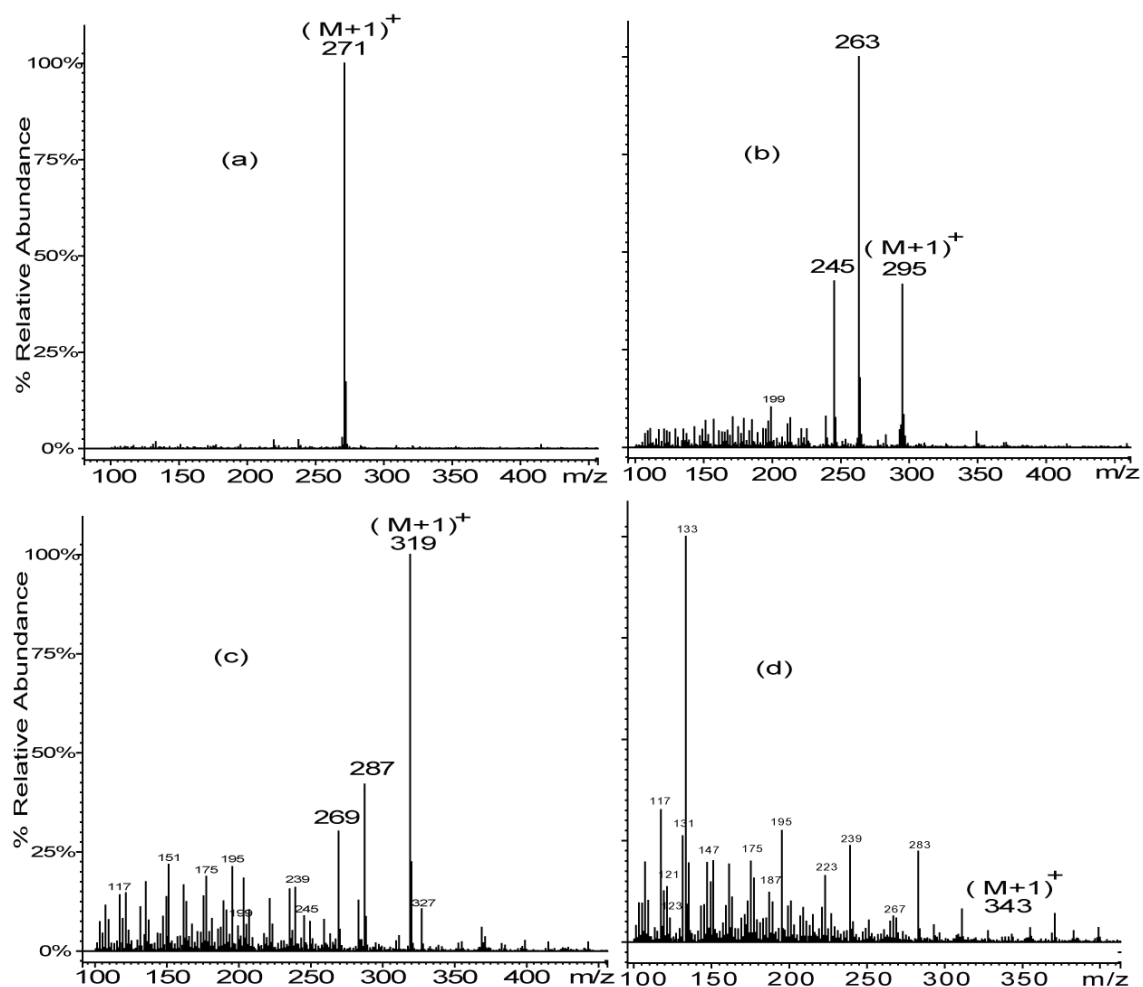
Figure 13. Internal isobutane PCI spectra of methyl ester standards of 16:0(a) showing complete yield of parent ion at m/z 271; 18:2n-6 (b) showing parent ion at m/z 295 and at ~60% of base peak, the MH-32 fragment at m/z 263, and the MH-50 fragment at m/z 245; 20:4n-6 (c) showing parent ion at m/z 319 as the base peak, the MH-32 fragment at m/z 287, and the MH-50 fragment at m/z 269; 22:6n-3 (d) showing parent ion at m/z 343 at ~ 5% of base peak.

for 18:2n-6 (**Figure 14**). The impact of the extent of fragmentation in each ionization mode on

parent ion quantification is illustrated in **Figure 15.**
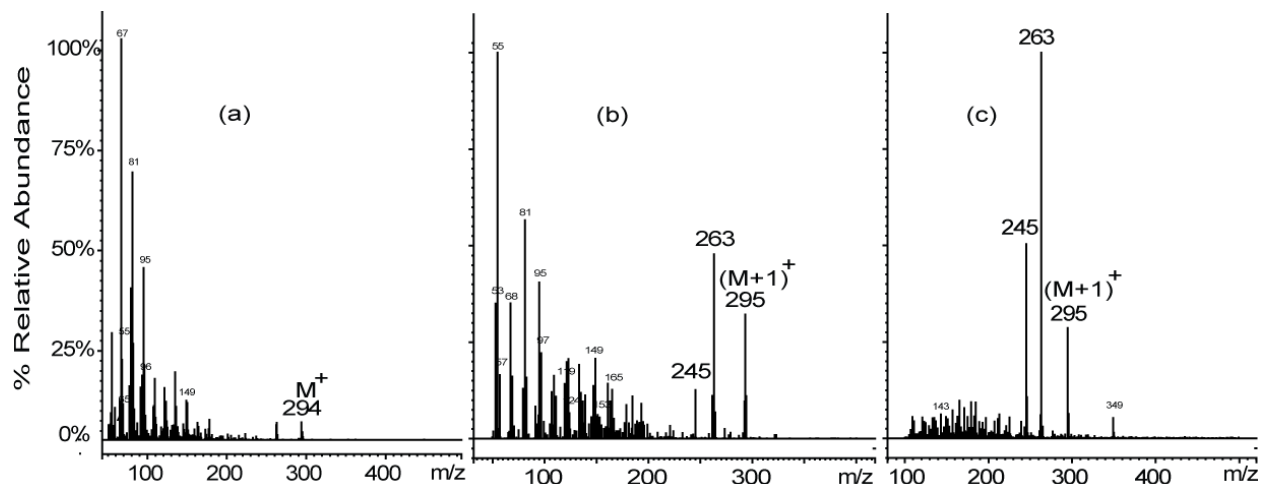
Figure 14. Extent of low-mass fragment formation in spectra of 18:2n-6 methyl ester standard in EI (a), external methane PCI (b), and internal isobutane PCI (c).
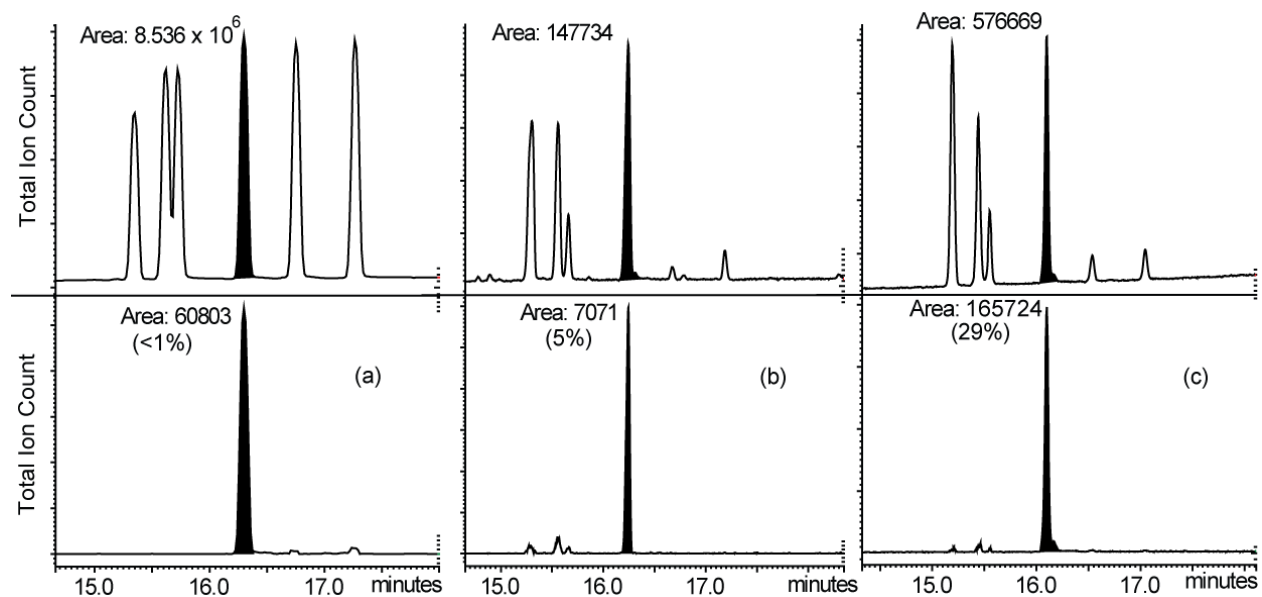
Figure 15. The effect of fragmentation on 18:2n-6 parent ion quantification. Peaks are extracted using SIE and the proportion of the extracted peak to the total ion peak is shown in EI (a), external methane PCI (b), and internal isobutane PCI (c).

CHAPTER 6

DISCUSSION

Comments on hypotheses

1. *The novel internal isobutane PCI technique will be more sensitive than methane NCI and external methane PCI.*

   The limit of detection for the new technique was established to be 500fg/uL for ethyl U-^{13}C 18:2n-6, which is ten times more sensitive than methane NCI (5pg/uL) and 1000 times more sensitive than external methane PCI (300pg/uL). This is likely an underestimate of the instrument's true capability, given that it was established in an operating environment that sacrifices sensitivity for resolution and reliable long-term operation.

2. *Labelled 18:2n-6 and n-6 PUFA metabolites will be detectable in rat liver 8 hours after oral gavage.*

   Labelled 18:2n-6 was detected in liver at 8 hours post oral dose in all three CI modes. Detection of ethyl ^{2}H$_5$ 18:2n-6 and its n-6 metabolites were lower than U-^{13}C isotopes in the two PCI modes, though this trend was reversed in NCI. Labelled n-6 metabolite concentrations, particularly 20:3n-6 differed across each CI mode. Specifically, labelled 20:3n-6 was not detectable in external methane PCI or NCI. These results suggest that the novel internal isobutane method is capable of detecting trace amounts of orally administered n-6 fatty acid stable isotopes and their metabolites in rat liver.

FAME analysis using EI

      Results indicate that FAMEs undergo significant fragmentation with EI, reducing parent ion detection in some unsaturated FAMEs to zero. For example, FAMEs such as 20:4n-6, 20:5n-3, and 22:6n-3, undergo complete fragmentation of the parent ion. Saturated FAMEs such as 16:0 and 18:0 are more stable and therefore fragment less; their parent ions make up about 50% of base peaks. FAME spectra indicate that most fragments are low-mass. This is because fragmentation occurs in a stepwise fashion, whereby primary higher mass fragments undergo further dissociation and complex rearrangement reactions to reach their most stable states (Berberich et al., 1989). Although all FAMEs were positively identified with the spectral library search utility, not all compounds were identified with the same degree of certainty. Saturated FAMEs that fragment less are identified with less certainty than the most unsaturated FAMEs. Extensive dissociation of a FAME produces a spectrum that is more diagnostic of its structure and can be better matched against a spectral library, providing more accurate results. Importantly, spectral searches are not restricted to FAMEs; all unidentified peaks can be searched against a library. It is thus possible to identify such contaminants as column bleed, and by-products of lipid oxidation.

      Despite the significant fragmentation in EI, it can still be used for limited quantitative applications because the total ion chromatographic peak of all unlabelled FAMEs can be integrated. This function essentially mimics GC-FID, and cannot be used in tracer metabolic studies. However, given the linear responses in EI and its high sensitivity, this quantification technique can be useful where a GC-MS is a standalone instrument. For example, limits of detection for saturated methyl esters can be 30% lower than GC-FID by using SIE in EI-MS, but GC-FID may be more sensitive for unsaturated FAMEs (Dodds et al., 2005). This is likely due

to the significant fragmentation of parent ions in unsaturated FAMEs. In the case of isotopic FAMEs, it is necessary to monitor the parent ion or certain diagnostic high-mass fragments, such as is done in SIE. This is because the chromatographic peaks of the labelled FAME co-elutes with that of the endogenous FAME. Highly unsaturated FAMEs almost completely fragment in EI and thus leave no parent ion intact, therefore SIE can be used only for those FAMEs that undergo the least fragmentation (**Figure 16**).
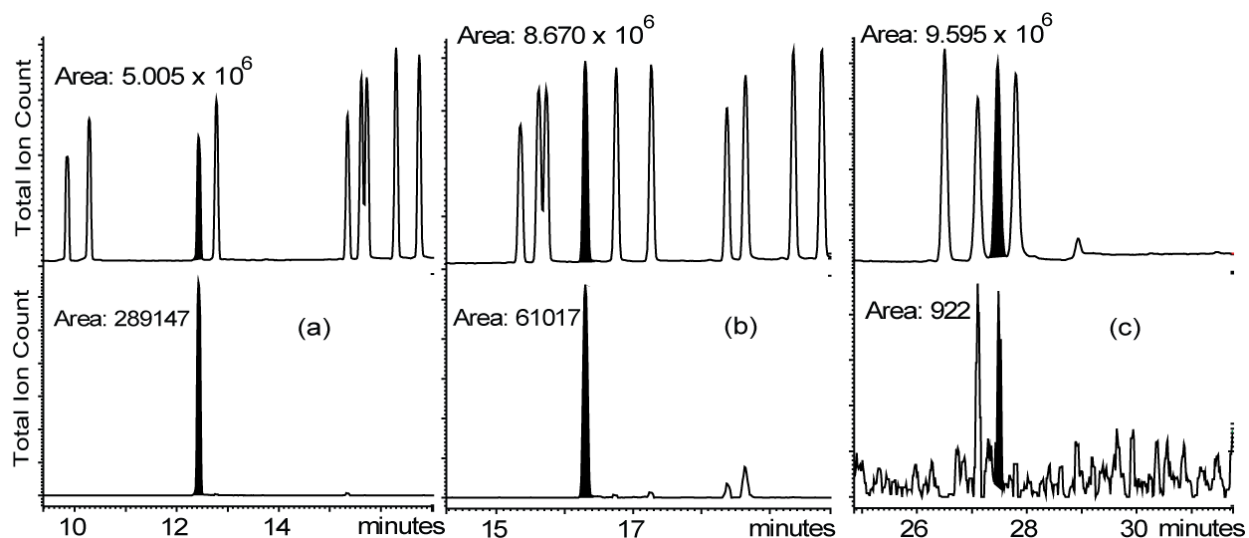
Figure 16. Challenge of quantification in EI-MS due to parent ion fragmentation. Integration of the total ion chromatographic (TIC) peak on the top row, and integration of the parent ion peak in the bottom row. The proportion of SIE area to TIC area is (a) 6% for 16:0, (b) 0.7% for 18:2n-6 and (c) 0.009% for 22:6n-3..

External methane CI

PCI vs. NCI

Methane NCI and PCI are still widely used and were replicated in this study to assess their advantages and expose their limitations in order to justify the development of the novel internal isobutane method. Methane PCI responses were linear across at least four orders of magnitude, which was similar to EI responses. As a result, it is possible to use a single

calibration curve for quantification of typical biological concentrations. In comparison to methane PCI, methane NCI produced little fragmentation in *any* FAME, allowing good extraction of the parent ion by SIE. These results confirm the reported low energy electron capture process by which fatty acid PFB esters are ionized (Harrison, 1992). Sensitivity of methane NCI was established to be 5pg/uL, which is about 60 times more sensitive than methane PCI. This result in part confirms previous work in which isotopic fatty acid analysis by methane PCI and NCI were compared for the first time, although they reported a greater difference in sensitivity between PCI and NCI with a limit of detection for NCI of 10fg, using a clean sample of a fatty acid standard (Pawlosky, Sprecher, and Salem, Jr., 1992).

The concentration of $^2H_5$ 18:2n-6 in NCI was roughly equal to that found using the other two CI methods (180nmol/g). However, the quantification of U-^{13}C 18:2n-6 in NCI proved problematic, primarily due to complications of producing a calibration curve as response rates in NCI are nonlinear. As a result, U-^{13}C 18:2n-6 enrichment using methane NCI analysis was nearly $1/6^{th}$ of that of methane PCI analysis. This issue is discussed in more detail below. In contrast, quantitation with methane PCI was simple and reproducible, but parent compound fragmentation limited its utility for analyzing highly unsaturated fatty acids such as 22:6n-3.

As yet there are no published methane PCI spectral libraries of FAMEs to which the spectra from this study can be compared. Compared to EI, methane PCI caused less parent ion fragmentation, allowing quantification by SIE for most FAMEs, including labelled 20:4n-6. Despite this, external methane PCI still caused considerable fragmentation of HUFAs, which limited quantification. For some FAMEs, the parent ion in SIE only makes up a small percentage of the base peaks. Therefore, if the concentration of the FAME in the sample is very low, it will not be possible to generate a peak in SIE. Also, similar to EI, HUFAs such as 22:6n-

3 undergo complete fragmentation in methane PCI, making quantification by SIE impossible. Therefore, in cases where isotope concentration is high and the FAMEs are not highly unsaturated, external methane PCI can be satisfactorily used for quantification. In all other applications, a more sensitive, less energetic CI technique is necessary.

NCI spectral results confirm that methane NCI is superior to methane PCI in parent ion detection and sensitivity. In fact, the single quadrupole NCI method (Pawlosky, Sprecher, and Salem, Jr., 1992) emulated in the present study has been extensively used in fatty acid metabolic investigations in rats (Lin, Pawlosky, and Salem, Jr., 2005; Lin and Salem, Jr., 2005a; Lin and Salem, Jr., 2007), fish (Bell, Dick, and Porter, 2003; Bell, Dick, and Porter, 2001), and humans (Pawlosky et al., 2006). However, three key disadvantages were discovered that limit the usefulness of methane NCI. Firstly, the PFB derivitization process for NCI is time consuming and complex. This preparation method used up five times more hexane, took nearly three hours longer, and required twice as many steps as compared with the FAME preparation process for PCI. Secondly, the present range of response linearity was only between 10-100pg in the ion trap as compared with 10-1000pg previously reported in the single quadrupole (Pawlosky, Sprecher, and Salem, Jr., 1992). This difference is not surprising given the previous reports of lower linearity of NCI in ion traps when compared to quadrupoles (Dodds et al., 2005). In order to compensate for the nonlinearity, individual calibration curves had to be built around the detected sample responses. Thirdly, methane NCI tended to significantly foul the ionizing filaments, requiring instrument shutdown after only about 1100 minutes of operation. This downtime was compounded by the fact that each time the instrument was re-started, a new calibration curve had to be produced to account for changed responses. These three factors combined to make methane NCI extremely time consuming, expensive, and overly complex.

Internal isobutane PCI

The objectives for development of the internal isobutane PCI technique was to minimize parent ion fragmentation, increase sensitivity, produce linear responses, and allow simple sample preparation. Fragmentation of all FAMEs was reduced compared to either EI or methane PCI. The methane and isobutane PCI methods did not differ much in the profiles of saturated FAMEs, but in isobutane PCI, all unsaturated FAMEs were significantly less fragmented, and *all* FAMEs showed some level of intact parent ion. As yet, no published work has documented the use of isobutane PCI for isotopic FAME analysis in a quadrupole ion trap. Moreover, mass-selection has never been used in any MS study examining fatty acids.

Unlike methane PCI, high-mass fragments formed by hydride abstraction and adduct formation were absent in isobutane PCI, suggesting these processes do not occur in lower energy ionization environments. This is the first time this ion behaviour has been reported. Fragment profiles of saturated FAMEs in isobutane PCI resembled those of NCI, suggesting this novel method produces an ideal ionization environment for these FAMEs. The spectra of MUFAs and some highly unsaturated PUFAs such as 20:4n-6 show that most fragments are high-mass. These correspond to the loss of methanol and water, and their occurrence is predictable. It is thus possible to use SIE to quantify nearly all FAMEs with this technique.

Using isobutane PCI, it was possible to detect the 18-^{13}C 20:3n-6 metabolite of U-^{13}C 18:2n-6 at 70pg/uL. These amounts were later re-calculated and expressed as nmol/g. Final limit of detection for U-^{13}C 18:2n-6 was determined to be 500fg/uL. This surpasses sensitivity attained in either of EI, methane PCI, or methane NCI. The lowest reported *in vivo* isotope concentration using a quadrupole NCI technique was approximately 0.25pmol of ^{2}H$_{5}$ 24:5n-6 per mL of plasma. Other investigators using the same NCI method have reported metabolite

concentrations ranging well above 1pmol/mL (Bell, Dick, and Porter, 2003; Bell, Dick, and Porter, 2001; Pawlosky et al., 2006; Lin and Salem, Jr., 2007). Therefore, if 1pmol/mL is the practical limit of detection for *in vivo* analyses of isotopic fatty acids, our limit of detection of 500fg/uL, which amounts to about 1.6pmol/mL, is comparable.

We have reason to believe the instrument has the capability to detect FAMEs at even smaller concentrations, perhaps in sub-pmol/mL level. However, steps were taken to optimize the technique not only for sensitivity, but also for resolution, and long filament service life. Presently, filament emission current is set to 10% of its permitted maximum with an estimated filament burnout time of 15,000 minutes, as compared with 1100 minutes in NCI. As well, this novel internal isobutane technique has highly linear responses coupled with short sample preparation times.

Labelled 18:2n-6 and n-6 PUFA metabolites

Labelled 18:2n-6 was detected in all three CI modes. In both PCI modes, U-^{13}C 18:2n-6 was detected at higher concentrations than ^{2}H$_{5}$ 18:2n-6, whereas in NCI, ^{2}H$_{5}$ 18:2n-6 was detected at a higher concentration. Concentration of ^{2}H$_{5}$ 18:2n-6 in all CI modes was roughly similar (~150nmol/g). As well, the only ^{2}H$_{5}$ n-6 metabolite detected was 20:3n-6 using isobutane PCI. The higher yield in U-^{13}C vs. ^{2}H$_{5}$ isotopes in PCI ion trap was not expected. Our NCI results tend to agree with the existing literature, when the decrease in linearity of U-^{13}C isotopes in the ion trap is considered. In studies using methane NCI analyses, deuterium-labelled fatty acids were detected at the same or even slightly higher concentrations as their ^{13}C-labelled counterparts in rat plasma homogenates (Lin, Pawlosky, and Salem, Jr., 2005), and ^{2}H$_{5}$ 18:2n-6 and its n-6 metabolites were detected at concentrations similar to our U-^{13}C isobutane PCI data (Lin and Salem, Jr., 2007). These results tend to indicate that orally delivered deuterated

isotopes are absorbed just as well as ^{13}C isotopes, and that in the quadrupole mass analyzer, the deuterated signal is not suppressed. The decreased quantitation of $^2H_5$ 18:2n-6 in PCI in the ion trap was not expected. As of yet, the behaviour of deuterated fatty acid isotopes in quadrupole ion traps has not been determined. Further work in this area may enable more clarity in explaining our findings.

In our study, the concentration of ethyl ester and NEFA forms of U-^{13}C 18:2n-6 and n-6 metabolites were similar using internal isobutane PCI (**Figure 12**). The NEFA form of the isotope is considerably less expensive, and given that no published work has to date assessed its enrichment efficiency in rats, it was included in this study. NEFAs may be absorbed more efficiently than fatty acid ethyl esters (elBoustani et al., 1987; Lawson and Hughes, 1988b; Chen et al., 1987). In the gut, pancreatic lipase hydrolyses ethyl esters to form NEFA fatty acids which can then be absorbed into lymph vessels. The impaired absorption of ethyl esters is thought to be the result of ethyl esters being poor substrates for pancreatic lipase and actively resisting hydrolysis (Lawson and Hughes, 1988a). However, the absorption of ethyl esters of EPA and DHA were reported to increase nearly three-fold when administered with a high fat meal (Lawson and Hughes, 1988a). This improvement is thought to be due to the upregulation of pancreatic lipase activity by the presence of high levels of fat in the gastrointestinal tract. In our study, each isotopic dose was administered with 400uL of olive oil, which represents a high fat meal, therefore allowing for a high enrichment of ethyl esters. These results suggest that the less expensive NEFA form of U-^{13}C 18:2n-6 is a viable alternative to the ethyl ester.

Liver concentrations of ethyl U-^{13}C 18:2n-6 in external methane PCI and internal isobutane PCI were 420 and 450 nmol/g respectively, and are slightly higher than previous reports of ~400nmol/g using methane NCI (Lin and Salem, Jr., 2007). In addition, U-^{13}C 18:3n-

6, 20:3n-6 and 20:4n-6 have been reported at 12, 1 and 40nmol/g at 24 hrs post-gavage (Lin and Salem, Jr., 2007). In the present study at 8 hours post gavage, 18:3n-6 was not detected, while 18-^{13}C labelled 20:3n-6 and 20:4n-6 were detected at 7 and 30 nmol/g respectively. These discrepancies are likely explained by the fact that our gavage dose was 1.4 times higher than the previous study. In addition the strain of rat used may contribute to the differences. Presently, Sprague-Dawley rats were utilized, while previous work also examined retinal function and used the non albino Long-Evans hooded rat (Lin and Salem, Jr., 2007).

Study limitations

Shortcomings in this study relate to derivitization solvents, quantification procedures, sub-optimal analytical MS parameters, and the inability to minimize fragmentation of parent ions in some HUFAs. The first limitation concerns the use of hexane as a solvent. Concentration of FAMEs due to hexane volatility necessitated separate FAME preparation for each different ionization mode, thus potentially introducing variability. Heptane was substituted, but this also is slightly volatile and only marginally improved results. A heavier hydrocarbon such as decane, though significantly more expensive, would have been a better solution. Secondly, labelled standards such as 18-^{13}C 20:3n-6, 20:4n-6, and ^{2}H$_5$ 20:3n-6 and 20:4n-6 were not available. Calibration curves for labelled 20:3n-6 and 20:4n-6 were made using unlabelled 20:4n-6, supported by previous work (Lin, Pawlosky, and Salem, Jr., 2005). Responses for 20:3n-6 are slightly different than that of 20:4n-6, and those of labelled FAMEs are different than unlabelled FAMEs. It is therefore likely that n-6 metabolite enrichments reported in this study are not entirely accurate. Thirdly, MS parameters were not set for maximum sensitivity, but set for regular reliable operation and long filament life, particularly in NCI. In NCI, filament operation time was about 1100 minutes, allowing analysis of an average of 55 samples before the need to

shut down the instrument for maintenance. This number would have been lower had the instrument been optimized for sensitivity. However, at every post-maintenance start-up, it is necessary to re-develop calibration curves. Therefore, filament operating times shorter than 1100 minutes would have been unfeasible. Finally, despite the markedly reduced FAME fragmentation under internal isobutane PCI, some HUFAs such as EPA and DHA still fragment significantly. The reason for this is that the ionization of these HUFAs requires much lower energies than can be sustained by more unsaturated FAMEs. Although these HUFAs can still be quantified by using high mass fragments rather than parent ions, further methodological developments that result in higher percentages of HUFA parent ions is desirable to improve sensitivity. Further work using n-3 PUFAs such as labelled 18:3n-3 needs to be completed.

Future directions

Further studies on ion preparation techniques such as selected ion storage to enhance sensitivity of existing methods and the use of other reagents to reduce HUFA fragmentation should be explored. Selected ion storage enables the multiplication and detection of only those ions that are defined by the user. When 18:2n-6 is ionized, a wide range of masses from 50 to 500 m/z is multiplied and detected. However, if only the parent ion and a few high-mass diagnostic ions are ejected toward the detector, electronic noise is reduced, thus allowing for a higher signal to noise ratio. In early experimentation, we have found this technique to nearly double the sensitivity of the current internal isobutane method. A second potential future project is experimentation with lower energy reagent gases. Despite high parent ion yields for virtually all fatty acids using the novel internal isobutane PCI technique, EPA and DHA significantly fragment. A lower energy CI reagent can reduce this fragmentation. Published acetonitrile PCI spectra of EPA and DHA show a high mass adduct ion at M+54 as the base peak, as well as a

small parent ion peak (Van Pelt and Brenna, 1999). These spectra suggest that fragmentation of EPA and DHA in acetonitrile PCI is significantly lower than that of our novel isobutane PCI method. However, both of these spectra also show a significant volume of small mass fragments, which may complicate quantitation in cases where trace amounts of n-3 HUFA metabolites are analyzed. Ammonia is a lower energy reagent than acetonitrile, and although utility in a quadrupole MS has been previously demonstrated (DeMark and Klein, 1981), as of yet no published spectra of HUFAs in ammonia PCI exists. Therefore, a potential alternative to isobutane PCI could be the development of a novel internal acetonitrile or ammonia PCI ion trap technique. However, given that these are liquid reagents, installation and maintenance may be more challenging that gaseous isobutane.

Conclusions

The objectives of this thesis were to initially replicate current qualitative and quantitative MS methodology, and then to develop a novel quantitative MS technique for *in vivo* fatty acid isotope analyses that mitigates the limitations of existing methods. These aims were achieved, and a novel mass-selected internal isobutane PCI technique has been developed. This method was tested and compared to external EI and external methane PCI and NCI techniques by examining fatty acids extracted from rats that had been dosed with various commercially available forms of 18:2n-6 fatty acid stable isotopes. The novel mass-selected internal isobutane technique confirmed our two hypotheses: The novel technique was more sensitive than both methane PCI and NCI; and isotopic 18:2n-6 and n-6 PUFA metabolites were detected in rat liver at 8 hours post dose. The internal isobutane method has a significant limitation in regards to the quantitation of n-3 HUFAs such as EPA and DHA as they remain highly fragmented after ionization. The present findings do provide the basis for further experimentation with lower

energy reagents such as ammonia. Overall, mass-selected internal isobutane PCI provides an analytical method for fatty acid stable isotope determinations that has more sensitivity and linearity and less instrument operating demands than methane NCI-MS, causes less fragmentation and is more sensitive than methane PCI-MS, and is simpler, less expensive, and more flexible than C-IRMS.

APPENDIX A

Human fatty acid stable isotope studies using GC-MS and GC-C-IRMS. List of abbreviations provided at end of table.

Author	Year	Subjects	Isotopic dose	Analysis	(Investigation) & main findings
(Rohwedder, Emken, and Wolf)	1985	1 subject, 9 separate blood samples	$^2H_{2^-}$, $^2H_{4^-}$, & $^2H_{6^-}$ 18:1n-9	PCI-MS	**(Analysis of deuterated blood lipids by CI-MS).** Used multiple isotopes. Near 85x increase in parent ion detection in PCI compared to EI for 18:1n-9. CI-MS more suitable for isotopic lipid analysis than EI-MS.
(Emken et al.)	1989	3 lactating females	$^2H_2$ 16:0, $^2H_6$ 18:1n-9, $^2H_4$ 18:2n-6	PCI-MS	**(Incorporation of labelled fatty acids into human milk and plasma).** Lower $^2H_4$ 18:2n-6 enrichment relative to $^2H_2$ 16:0 and $^2H_6$ 18:1n-9 in milk CE and PL compared to plasma CE and PL. This may be due to mammary tissue using fatty acids from chylomicrons and VLDL TG to synthesize PL, much like intestinal cells.
(Emken et al.)	1993	7 males	$^2H_2$ & $^2H_6$ 16:0, 18:0, & 18:1n-9 (i.e., 6 isotopes)	PCI-MS	**(Effect of 18:2n-6 on desaturation and uptake of 16:0 and 18:0).** Low absorption of isotopic 18:0 TG (33-58%), which increased (87-94%) when higher temperature was used in formulating the dose. *In vivo* desaturation of 16:0 and 18:0 demonstrated for the first time, and amounted to 3-5% and 6-14% respectively, which are lower than those reported for rats (33 & 53%) and mice (47 & 83%). Feeding 18:2n-6 did not affect desaturation, though reducing chain shortening, possibly via affecting β-oxidation.
(Emken et al.)	1998	6 males (ages 20-39)	$^2H_2$ 18:2n-6	PCI-MS	**(Effect of dietary 20:4n-6 on metabolism of 18:2n-6).** 8x increase in dietary 20:4n-6 did not significantly affect conversion of 18:2n-6 to n-6 metabolites. Conversion of 18:2n-6 to 20:4n-6 reaches its peak between 32-44 hours post feeding.
(Demmelmair et al.)	1998	6 females (age = 31)	U-^{13}C 18:2n-6	C-IRMS	**(Metabolism of U-^{13}C 18:2n-6 in lactating women).** More evidence regarding lower intake and milk content of 18:2n-6 in Europe vs. North America. Proportion of milk 20:4n-6 is reduced with longer lactation. About 13% of labelled 18:2n-6 recovered in milk until about 110 hours after dosing. Oxidation of labelled 18:2n-6 was higher than milk enrichment. No increased labelled 18:2n-6 enrichment with longer lactation. Authors conclude that 30% of milk 18:2n-6 is of dietary origin. Less than 3% of milk 20:4n-6 is from endogenous pools; though this is dependent on diet.
(Bosner et al.)	1999	32 males & 62 females (avg age = 41)	$^{13}C_{5^-}$ $^2H_{5^-}$, & $^2H_6$ cholesterol esters	NCI-MS	**(Extent and distribution of dietary cholesterol absorption).** Nearly half of administered cholesterol was absorbed, with no age or gender differences. Percent absorption was not correlated to plasma cholesterol

or LDL. No relationship between cholesterol absorption and fibre intake. Positive correlation between absorbed cholesterol and fasting plasma insulin, C-peptide, and glucagon.

(Emken et al.)	1999	6 males (ages 28-39)	$^2H_6$ 18:1n-9, $^2H_2$ 18:2n-6, $^2H_4$18:3n-3	PCI-MS	**(Effect of dietary DHA on uptake and desaturation of 18:1n-9, 18:2n-6, and 18:3n-3).** High DHA diet enhanced TG clearance, had no effect on serum cholesterol, and did not change proportions of 18:1, 18:2, and 18:3 in plasma PL and CE. High DHA diet lowered accumulation of n-6 metabolites to 380mg/d compared to 900mg/d for the low DHA diet.
(Salem, Jr. et al.)	1999	13 adult volunteers	$^2H_5$ 18:2n-6 & 18:3n-3	NCI-MS	**(*In vivo* conversion of 18:2n-6 to 20:4n-6 in adult humans).** Using more than 4 deuteria allows for better resolution and more accurate results, since the label does not overlap with endogenous ions. Maximal signal for labelled 20:4n-6 at 96h. Labelled EPA is maximal at 24h, and its DHA metabolite at 96h. Conversion of n-6 is much higher than n-3, confirming the inefficiency of 18:3n-3 conversion.
(Vermunt et al.)	2000	15 males & females (ages 21-66)	U-^{13}C 18:3n-3	IRMS	**(Effect of dietary 18:3n-3 on conversion and oxidation of labelled 18:3n-3).** First study to report on these two effects. Labelled EPA, but not DPA and DHA, was lower in the 18:3 dietary group. Slightly higher 18:3 oxidation in the 18:3 group, suggesting 18:3 intake does not inhibit 18:3 oxidation.
(Lagerstedt et al.)	2001	Adult & pediatric with various nutritional and metabolic disorders.	$^{13}C_4$ 8:0, $^2H_3$ 10:0-20:0, $^2H_4$ 22:0-26:0	NCI-MS	**(Quantification of plasma C6-C26 fatty acids for diagnosing nutritional and metabolic disorders).** GC-FID data has high false positive rate, since most fatty acid data is presented in percentages, which may be artificially reduced due to high intake of other fatty acids. Absolute quantitation is therefore necessary. Using this method, the authors successfully diagnosed essential fatty acid deficiency, disorders of fatty acid transport and mitochondrial oxidation, and peroxismal disorders.
(Pawlosky et al.)	2001	4 males & 4 females (avg age = 28)	$^2H_5$ 18:3n-3	NCI-MS	**(Compartmental analysis of 18:3n-3 metabolism in adult humans).** Subjects were subsisted on beef-based diet, hypothesized to increase 18:3n-3 conversion to n-3 PUFAs. 18:3n-3 was quickly removed from plasma, while other n-3 PUFAs remained for several hours. Only 0.2% of 18:3n-3 was converted to 20:5n-3, though 22:5n-3 and 22:6n-3 were converted more efficiently from their immediate precursors. Authors conclude that body n-3 stores contribute more to maintaining 22:6n-3 than dietary 18:3n-3.
(Burdge, Jones, and Wootton)	2002	6 males (avg age = 36)	U-^{13}C 18:3n-3	C-IRMS	**(Metabolism of 18:3n-3 in young men).** Labelled 18:3n-3 is detected in plasma TG for up to 24 hours. One third of isotopic 18:3n-3 dose was used as energy. No evidence of 18:3n-3 conversion to DHA in plasma; only EPA and DPA (22:5n-3). Men may require higher intake of DHA

(Burdge and Wootton)	2002	6 females (avg age = 28)	U-^{13}C 18:3n-3	C-IRMS	**(Conversion of 18:3n-3 to EPA, DPA and DHA in young women).** First study to assess 18:3n-3 metabolism in young women. Appearance of labelled 18:3n-3 in TG similar to men, though in CE, labelled 18:3n-3 was detected for up to 21 days, suggesting CE may be long-term storage for 18:3n-3, enabling constant supply for membranes. Higher 18:3n-3 conversion to EPA and DHA than men. Oxidation of 18:3n-3 was slightly lower than men (27% vs. 32%), possibly reflecting the lower metabolic rate in women.
(Emken et al.)	2002	3 Females (ages 23-41)	$^2H_2$18:2n-6, $^2H_8$ 18:1n-9, $^2H_6$ 9c, 11t- 18:2n-6, and$^2H_4$ 10t, 12c –18:2n-6.	PCI-MS	**(Effect of dietary CLA on metabolism of 18:1n-9, 18:2n-6, and CLA isomers in women).** No differences between CLA and non-CLA groups in plasma isotopic enrichment, suggesting CLA does not alter 18:1n-9 and 18:2n-6 metabolism. Absorption of the two ^{2}H CLA isomers was lower than 18:1n-9. Incorporation of ^{2}H CLA isomers into TG and CE was inhibited, but enhanced in phospholipid. More CLA isomers than 18:1n-9 were oxidized. Minimal elongation and desaturation of CLA isomers.
(Hernandez-Perez et al.)	2002	9 Males & 6 Females (avg age = 54)	^{13}C$_1$ 16:0 & 18:1n-9	EI-MS	**(Improved GC-MS method for analyzing 1-^{13}C plasma long chain fatty acids).** Authors initially used *tert*-butyldimethylsilyl (TBDMS) esters, which as standards performed well, but showed poor chromatographic resolution with plasma lipids. Trimethylsilyl (TMS) esters showed better chromatographic resolution and more precise MS measurements for 16:0 and 18:1. This method is not suitable for analyzing PUFA spectra.
(McCloy et al.)	2004	6 post-menopausal females	U-^{13}C 18:1n-9, 18:2n-6, 18:3n-3, and *trans*-18:1n-9	C-IRMS	**(Metabolism of 18-carbon 13C-unsaturated fatty acids in healthy women).** The four isotopes were mostly oxidized (46-71% of dose), followed by abdominal adipose storage (5-12% of dose), and plasma enrichment (3-8%). Plasma labelled n-3 and n-6 PUFAs amounted to less than 0.1% of dose.
(Emken et al.)	2004	4 Males	$^2H_2$ & $^2H_4$ 18:2n-6; $^2H_4$ & $^2H_6$ 16:0	PCI-MS	**(Effect of TG structure on the absorption and metabolism of 16:0 and 18:2n-6 by humans).** Labelled 16:0 and 18:2n-6 at *sn-1,3* and *sn-2* did not change position, and were well absorbed. After absorption, 16:0 at *sn-2* of chylomicron TG moved to *sn-1,3*, and 18:2n-6 at *sn-1,3* moved to *sn-2*, though the reverse of these two processes was not seen. No influence of TG *sn* position of 16:0 on 16:0,18:2n-6, and labelled n-6 metabolite enrichment of plasma TG and CE, but higher labelled 20:4n-6 and lower labelled 16:1n-9 in plasma PL.

(Pawlosky et al.)	2006	Newborns (8 Males & 2 Females)	U-^{13}C 18:2n-6 & ^{2}H$_5$ 20:3n-6	NCI-MS	**(Labelled n-6 metabolites in infants).** Turnover rate for 18:2n-6 in infants (1.8 - 6.6g/kg/d) higher than adults probably due to higher energy demands. 10% of 18:2n-6 was converted to metabolites, which is 5 times higher than in adults, and probably due to higher developmental demand for 20:4n-6. Daily 20:4n-6 intake requirement of nearly 4mg/kg/d to sustain plasma 20:4n-6 levels.
(Pawlosky, Hibbeln, and Salem, Jr.)	2007	10 males & 10 females (ages 20 – 44)	^{2}H$_5$ 18:3n-3	NCI-MS	**(Plasma n-3 essential fatty acids in male and female smokers and non-smokers).** Higher rate of conversion of 22:5n-3 to 22:6n-3 in women. Smoking had no effect on absorption of labelled 18:3n-3, though its turnover was 3 times higher, and no effect on conversion of 18:3n-3 to 20:5n-3, though it increased conversion of 20:5n-3 to 22:5n-3.
(Pawlosky et al.)	2009	5 control (avg age = 37) & 9 alcohol-dependent (avg age = 41)	^{2}H$_5$ 18:3n-3	NCI-MS	**(Plasma and liver n-3 essential fatty acids in alcohol-dependent (AD) men during withdrawal).** Higher 18:3n-3 conversion to and turnover of 20:5n-3 in AD subjects who smoked. AD subjects also had normal rates of production of 22:5n-3 and 22:6n-3.

GC, Gas chromatography; MS, Mass spectrometry; CI, Chemical ionization; EI, Electron ionization; PCI, Positive chemical ionization; NCI, Negative chemical ionization; CE, Cholesterol ester; PL, Phospholipid; TG, Triacylglycerol; C-IRMS, Combustion isotope-ratio mass spectrometry; CLA, Conjugated linoleic acid.

Animal fatty acid stable isotope studies using GC-MS or GC-C-IRMS.

Author(s)	Year	Animal model	Isotopic dose	Tissues	Analysis	(**Investigation**) & main findings
(Pawlosky, Sprecher, and Salem, Jr.)	1992	Rat (Sprague-Dawley)	$^2H_4$ 18:2n-6 & $^2H_5$ 18:3n-3	Liver	NCI & PCI – MS	(**Novel NCI-MS method for detection of labelled fatty acids**). Novel methane NCI was more than 2000 times more sensitive than methane PCI; limit of detection was 10fg/uL.
(Brossard et al.)	1994	Rat (Sprague-Dawley)	U -^{13}C 22:6n-3	Plasma, RBC	C-IRMS	(***In vivo* study of DHA metabolism using stable isotopes and GC-C-IRMS**). GC-C-IRMS reported as being more sensitive than conventional GC-MS, and some limitations are discussed. Microalgae reported useful for synthesizing isotopic PUFAs. Labelled DHA detected in VLDL and chylomicron fractions, and RBC PC.
(Bell, Dick, and Porter)	2003	Rainbow trout	$^2H_5$ 18:3n-3	Liver, stomach, ceca, hind gut	NCI-MS	(**Pyloric ceca as important sites of 22:6n-3 synthesis in rainbow trout**). Ceca (mid gut) accumulated 5 times more labelled DHA than liver. Blood labelled DHA closely matched liver data. At day 9 liver and ceca equalized in labelled DHA enrichment. Results suggest that ceca is the most important site of DHA synthesis in the trout.
(Bell and Dick)	2005	Rainbow trout	$^2H_5$ 18:3n-3	Liver, Cecal mucosa, brain	NCI-MS	(**Distribution of labelled 22:6n-3 in rainbow trout tissues**). $^2H_5$ -labelled DHA peaked within 2 days in cecal mucosa compared to 7 days in liver. Higher enrichment reported in PC, PE, TG than PE or PI; TG was relatively more important in liver; brain DHA enrichment higher in TG than phospholipids.
(Lin and Salem, Jr.)	2005	Rat (Sprague-Dawley)	$^2H_5$ 18:3n-3 & 20:3n-6, U-^{13}C 18:2n-6 & 20:5n-3	Plasma	NCI-MS	(***In vivo* conversion of 18-20-carbon essential fatty acids in rats using multiple isotopes**). Higher conversion of U-^{13}C 20:5n-3 to 22:6n-3 than $^2H_5$ -18:3n-3, and of $^2H_5$ -20:3n-6 to 20:4n-6 and 22:5n-6 than U-^{13}C 18:2n-6. Data shows conversion of the two EFAs 18:2n-6 and 18:3n-3 to longer chain metabolites is inefficient.
(Lin, Pawlosky, and Salem, Jr.)	2005	Rat (Sprague-Dawley)	$^2H_5$ 18:3n-3, 18:2n-6 & 20:3n-6; U-^{13}C 18:3n-3, 18:2n-6 & 20:5n-3	Plasma	NCI-MS	(**Simultaneous quantitative analysis of ^{13}C than $^2H_5$ in rat plasma**). Higher signal suppression of ^{13}C than $^2H_5$ isotopes by plasma. This phenomenon termed *matrix effect*, and thought to result from lower ionization due to analyte competition.
(Lin and Salem, Jr.)	2007	Rat (Long-Evans hooded)	$^2H_5$ 18:3n-3 & 18:2n-6	All tissues	NCI-MS	(**Whole-body distribution of deuterated 18:2n-6, 18:3n-3 and their n-6 and n-3 metabolites in rats**). Accumulation of 18:2n-6 and 18:3n-3 equalled ~ 20% of initial dose, mostly in

					first 100 hours; highest in adipose tissue, followed by muscle and skin. Liver showed highest enrichment of labelled 20:5n-3, 22:5n-3, and 22:6n-3 between 8 and 96 hours post-dose, and highest labelled 18:3n-6, 20:3n-6, and 20:4n-6 between 8 and 168 hours post dose.
(Gao et al.)	2008	Rat (Fisher344 - CDF)	U -^{13}C 18:3n-3	Plasma	NCI-MS	**(Whole-body synthesis-secretion rates of EPA, DPA, and DHA).** Isotope was infused. Labelled 18:3n-3 appeared in plasma by 30 minutes; its metabolites by 60.Synthesis rates for EPA, DPA and DHA were 8.4, 6.27, and 9.84 umol/day.

RBC, Red blood cells; PC, Phophatidylcholine; PE, Phosphatidylethanolamine; PI, Phophatidylinositol

References

Barker, J. (2000) Mass Spectrometry John Wiley & Sons, New York.

Bell, M. V. & Dick, J. R. (2005) Distribution of 22:6n-3 newly synthesized from 18:3n-3 into glycerolipid classes from tissues of rainbow trout (Oncorhynchus mykiss). Lipids 40: 703-708.

Bell, M. V., Dick, J. R., & Porter, A. E. (2001) Biosynthesis and tissue deposition of docosahexaenoic acid (22:6n-3) in rainbow trout (Oncorhynchus mykiss). Lipids 36: 1153-1159.

Bell, M. V., Dick, J. R., & Porter, A. E. (2003) Pyloric ceca are significant sites of newly synthesized 22:6n-3 in rainbow trout (Oncorhynchus mykiss). Lipids 38: 39-44.

Berberich, D. W., Hail, M. E., Johnson, J. V. et al. (1989) Mass-selection of reactant ions for chemical ionization in quadrupole ion trap and triple quadrupole mass spectrometer. International Journal of Mass Spectrometry and Ion Processes 94.

Bosner, M. S., Lange, L. G., Stenson, W. F. et al. (1999) Percent cholesterol absorption in normal women and men quantified with dual stable isotopic tracers and negative ion mass spectrometry. J.Lipid Res. 40: 302-308.

Brossard, N., Pachiaudi, C., Croset, M. et al. (1994) Stable isotope tracer and gas-chromatography combustion isotope ratio mass spectrometry to study the in vivo compartmental metabolism of docosahexaenoic acid. Anal.Biochem. 220: 192-199.

Burdge, G. C. & Calder, P. C. (2005) Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reprod.Nutr.Dev. 45: 581-597.

Burdge, G. C., Jones, A. E., & Wootton, S. A. (2002) Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. Br.J.Nutr. 88: 355-363.

Burdge, G. C. & Wootton, S. A. (2002) Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br.J.Nutr. 88: 411-420.

Calder, P. C. (2006) Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot.Essent.Fatty Acids 75: 197-202.

Calder, P. C. (2007) Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot.Essent.Fatty Acids 77: 327-335.

Calder, P. C. (2008) The relationship between the fatty acid composition of immune cells and their function. Prostaglandins Leukot.Essent.Fatty Acids 79: 101-108.

Chen, I. S., Hotta, S. S., Ikeda, I. et al. (1987) Digestion, absorption and effects on cholesterol absorption of menhaden oil, fish oil concentrate and corn oil by rats. J.Nutr. 117: 1676-1680.

Christie, W. W. (1989) Gas chromatography and lipids The Oily Press, Glasgow.

DeMark, B. R. & Klein, P. D. (1981) Ammonia gas: an improved reagent for chemical ionization mass spectrometry of bile acid methyl ester acetates. J.Lipid Res. 22: 166-171.

Demmelmair, H., Baumheuer, M., Koletzko, B. et al. (1998) Metabolism of U13C-labeled linoleic acid in lactating women. J.Lipid Res. 39: 1389-1396.

Dodds, E. D., McCoy, M. R., Rea, L. D. et al. (2005) Gas chromatographic quantification of fatty acid methyl esters: flame ionization detection vs. electron impact mass spectrometry. Lipids 40: 419-428.

Downward, K. (2004) Mass spectrometry: A foundation course The Royal Society of Chemistry, Cambridge.

elBoustani, S., Colette, C., Monnier, L. et al. (1987) Enteral absorption in man of eicosapentaenoic acid in different chemical forms. Lipids 22: 711-714.

Emken, E. A., Adlof, R. O., Duval, S. et al. (2002) Effect of dietary conjugated linoleic acid (CLA) on metabolism of isotope-labeled oleic, linoleic, and CLA isomers in women. Lipids 37: 741-750.

Emken, E. A., Adlof, R. O., Duval, S. M. et al. (1998) Effect of dietary arachidonic acid on metabolism of deuterated linoleic acid by adult male subjects. Lipids 33: 471-480.

Emken, E. A., Adlof, R. O., Duval, S. M. et al. (1999) Effect of dietary docosahexaenoic acid on desaturation and uptake in vivo of isotope-labeled oleic, linoleic, and linolenic acids by male subjects. Lipids 34: 785-791.

Emken, E. A., Adlof, R. O., Duval, S. M. et al. (2004) Effect of triacylglycerol structure on absorption and metabolism of isotope-labeled palmitic and linoleic acids by humans. Lipids 39: 1-9.

Emken, E. A., Adlof, R. O., Hachey, D. L. et al. (1989) Incorporation of deuterium-labeled fatty acids into human milk, plasma, and lipoprotein phospholipids and cholesteryl esters. J.Lipid Res. 30: 395-402.

Emken, E. A., Adlof, R. O., Rohwedder, W. K. et al. (1993) Influence of linoleic acid on desaturation and uptake of deuterium-labeled palmitic and stearic acids in humans. Biochim.Biophys.Acta 1170: 173-181.

Folch, J., Lees, M., & Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J.Biol.Chem. 226: 497-509.

Gao, F., Kiesewetter, D., Chang, L. et al. (2009) Whole-body synthesis-secretion rates of long-chain n-3 PUFAs from circulating unesterified alpha-linolenic acid in unanesthetized rats. J.Lipid Res. 50: 749-758.

Godin, J. P., Fay, L. B., & Hopfgartner, G. (2007) Liquid chromatography combined with mass spectrometry for 13C isotopic analysis in life science research. Mass Spectrom.Rev. 26: 751-774.

Goto, J., Watanabe, K., Miura, H. et al. (1987) Studies on steroids. CCXXVIII Trace analysis of bile acids by gas chromatography-mass spectrometry with negative ion chemical ionization detection. J.Chromatogr. 388: 379-387.

Harrison, A. (1992) Chemical Ionization Mass Spectrometry CRC, Boca Raton.

Hernandez-Perez, J. M., Cabre, E., Fluvia, L. et al. (2002) Improved method for gas chromatographic-mass spectrometric analysis of 1-(13)C-labeled long-chain fatty acids in plasma samples. Clin.Chem. 48: 906-912.

Igarashi, M., Ma, K., Chang, L. et al. (2007) Dietary n-3 PUFA deprivation for 15 weeks upregulates elongase and desaturase expression in rat liver but not brain. J.Lipid Res. 48: 2463-2470.

Lagerstedt, S. A., Hinrichs, D. R., Batt, S. M. et al. (2001) Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. Mol.Genet.Metab 73: 38-45.

Lausevic, M., Plomley, J., Jiang, X. et al. (1995) Analysis of polychlorinated biphenyls by quadrupole ion trap mass spectrometry: Part I - Chemical ionization studies of co-eluting congeners 77 and 110. Eur.Mass Spectrom 1: 149-159.

Lawson, L. D. & Hughes, B. G. (1988a) Absorption of eicosapentaenoic acid and docosahexaenoic acid from fish oil triacylglycerols or fish oil ethyl esters co-ingested with a high-fat meal. Biochem.Biophys.Res.Commun. 156: 960-963.

Lawson, L. D. & Hughes, B. G. (1988b) Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters. Biochem.Biophys.Res.Commun. 152: 328-335.

Lin, Y. H., Pawlosky, R. J., & Salem, N., Jr. (2005) Simultaneous quantitative determination of deuterium- and carbon-13-labeled essential fatty acids in rat plasma. J.Lipid Res. 46: 1974-1982.

Lin, Y. H. & Salem, N., Jr. (2005a) In vivo conversion of 18- and 20-C essential fatty acids in rats using the multiple simultaneous stable isotope method. J.Lipid Res. 46: 1962-1973.

Lin, Y. H. & Salem, N., Jr. (2005b) In vivo conversion of 18- and 20-C essential fatty acids in rats using the multiple simultaneous stable isotope method. J.Lipid Res. 46: 1962-1973.

Lin, Y. H. & Salem, N., Jr. (2007) Whole body distribution of deuterated linoleic and alpha-linolenic acids and their metabolites in the rat. J.Lipid Res. 48: 2709-2724.

March, R. E. & Todd, J. F. J. (2005) Quadrupole Ion Trap Mass Spectrometry John Wiley & Sons Inc., New Jersey.

Masood, A., Stark, K. D., & Salem, N., Jr. (2005) A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. J.Lipid Res. 46: 2299-2305.

McCloy, U., Ryan, M. A., Pencharz, P. B. et al. (2004) A comparison of the metabolism of eighteen-carbon 13C-unsaturated fatty acids in healthy women. J.Lipid Res. 45: 474-485.

Morimoto, K. C., Van Eenennaam, A. L., DePeters, E. J. et al. (2005) Endogenous production of n-3 and n-6 fatty acids in mammalian cells. J.Dairy Sci. 88: 1142-1146.

Morrison, W. R. & Smith, L. M. (1964) Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J.Lipid Res. 5: 600-608.

Pawlosky, R. J., Hibbeln, J. R., Herion, D. et al. (2009) Compartmental analysis of plasma and liver n-3 essential fatty acids in alcohol-dependent men during withdrawal. J.Lipid Res. 50: 154-161.

Pawlosky, R. J., Hibbeln, J. R., Novotny, J. A. et al. (2001) Physiological compartmental analysis of alpha-linolenic acid metabolism in adult humans. J.Lipid Res. 42: 1257-1265.

Pawlosky, R. J., Hibbeln, J. R., & Salem, N., Jr. (2007) Compartmental analyses of plasma n-3 essential fatty acids among male and female smokers and nonsmokers. J.Lipid Res. 48: 935-943.

Pawlosky, R. J., Lin, Y. H., Llanos, A. et al. (2006) Compartmental analyses of plasma 13C- and 2H-labeled n-6 fatty acids arising from oral administrations of 13C-U-18:2n-6 and 2H5-20:3n-6 in newborn infants. Pediatr.Res. 60: 327-333.

Pawlosky, R. J., Sprecher, H. W., & Salem, N., Jr. (1992) High sensitivity negative ion GC-MS method for detection of desaturated and chain-elongated products of deuterated linoleic and linolenic acids. J.Lipid Res. 33: 1711-1717.

Pudelkewicz, C., Seufert, J., & Holman, R. T. (1968) Requirements of the female rat for linoleic and linolenic acids. J.Nutr. 94: 138-146.

Rennie, M. J. (1999) An introduction to the use of tracers in nutrition and metabolism. Proc.Nutr.Soc. 58: 935-944.

Rohwedder, W. K., Emken, E. A., & Wolf, D. J. (1985) Analysis of deuterium labeled blood lipids by chemical ionization mass spectrometry. Lipids 20: 303-311.

Salem, N., Jr., Pawlosky, R. J., Wegher, B. et al. (1999) In vivo conveadultsrsion of linoleic acid to arachidonic acid in human adults. Prostaglandins, Leukotrienes, and essential fatty acids 60.

Van Pelt, C. K. & Brenna, J. T. (1999) Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters. Anal.Chem. 71: 1981-1989.

Vermunt, S. H., Mensink, R. P., Simonis, M. M. et al. (2000) Effects of dietary alpha-linolenic acid on the conversion and oxidation of 13C-alpha-linolenic acid. Lipids 35: 137-142.