

## Advances in Fatty Acid Analysis for Clinical Investigation and Diagnosis using GC/MS Methodology

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### Abstract

Fatty acids, clinically known as organic acids, play an important role in human health as essential nutrients and metabolites. This review outlines the classification of fatty acids, as well as demonstrates their significant role in clinical diagnosis as biomarkers for manifestations of disease progression. It is, therefore, crucial to present the latest methodology available for their clinical investigation. Additionally, this review presents several analytical methods, used for qualitative and quantitative fatty acid analyses; including, but not limited to, extraction, derivatization, and full mass spectroscopic analysis. Recent advances pertaining to GC/MS analysis of fatty acids, lipid extraction, and derivatization methodology in preparation for clinical investigation are also summarized.

**Keywords:** Biomarkers; Derivatization; Fatty Acid analysis; Gas Chromatography-Mass Spectrometry (GC/MS); Lipid Profile

**Abbreviations:** EFA: Essential Fatty Acid; FAME: Fatty Acid Methyl Ester; FFA: Free Fatty Acid; GC/MS: Gas Chromatography-Mass Spectrometry; TG: Triglyceride; PL: Phospholipid; CE: Cholesterol Ester; PUFA: Polyunsaturated Fatty Acid; SPE: Solid Phase Extraction

## Introduction

### Fatty Acids

Fatty acids are omnipresent in nature and have diverse functional roles, both free and involved in complex lipid structures [1]. Fatty acids can be classified by their chain length: short-chain (<6C), medium-chain (6C-12C), long-chain (13C-21C), and very long-chain (>22C). The most common chain length range for fatty acids is between 12C-22C; with most naturally occurring fatty acids containing an even number of carbon atoms [2]. As seen in Figure 1, they can also be characterized as saturated or unsaturated (with one or more double bonds present). Unsaturated fatty acids can be further classified into two possible configurations: *cis* (where H atoms of the vinylic carbons are on the same side of the double bond) and *trans* (where H atoms of the vinylic carbons are on opposite sides of the double bond) [3]. In addition to utilizing IUPAC nomenclature, fatty acids can be referred to by their common names (e.g., oleic acid); by numerical characterization (e.g., 18:0, which indicates an acid containing 18 carbons with no degree of unsaturation); and by the position of the double bond, when applicable. If designating a degree of unsaturation relative to the terminal methyl group,  $\omega$  or  $n$  is used; while  $\Delta$  indicates the position of the double bond relative to the carboxyl group [2]. Table 1 illustrates common fatty acids and their relative classifications.

### Lipid classification

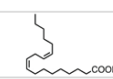


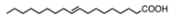
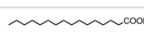
Lipids are water-insoluble molecules that have a wide variety of functions within the cell; and consequently, significant physiological importance. Lipids are classified into eight major categories: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [4,5]. Lipids fulfill three general functions: providing energy storage for cellular requirements, principally

as triglycerides (TG) and cholesteryl esters (CE) in lipid droplets [6-8]; forming the matrix of cellular lipid bilayers; and acting as first and second messengers in signal transduction and molecular recognition processes [8,9]. Fatty acids are major components required for the biosynthesis of more complex lipid structures such as triglycerides and phospholipids. Triglycerides, neutral energy storage molecules, are formed by the esterification of one glycerol molecule with three equivalents of fatty acid. Phospholipids are the main component of membrane bilayers, which provide a permeable barrier for cells and support for an array of proteins [10]. These predominantly non-polar lipid biomolecules require the assistance of various lipoprotein (lipid-protein assemblies) for transport in blood (Figure 2).

### Physiological relevance

Fatty acids play a significant role in physiology; and depending on the cellular process, lipids can both negatively and positively impact the performance and health of the human body. Conditions stemming

**Table 1:** Nomenclature styles and structure of various long-chain fatty acids.

COMMON	NAMES		ABBREVIATIONS		STRUCTURES
	IUPAC	$\Delta$ DESIGNATION	$n$ DESIGNATION		
linoleic acid	(9Z,12Z)-9,12-octadecenoic acid	18:2 $\Delta$ 9	18:2n-6		
stearic acid	octadecanoic acid	18:0	18:0		
oleic acid	(9Z)-octadecenoic acid	18:1 $\Delta$ 9	18:1 n-9		
elaidic acid	(9E)-octadecenoic acid	18:1 $\Delta$ 9	18:1n-9		
palmitic acid	hexadecanoic acid	16:0	16:0		

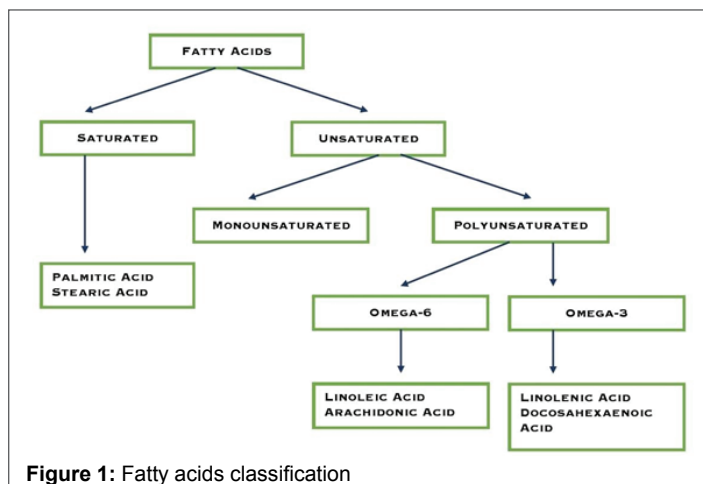


Figure 1: Fatty acids classification

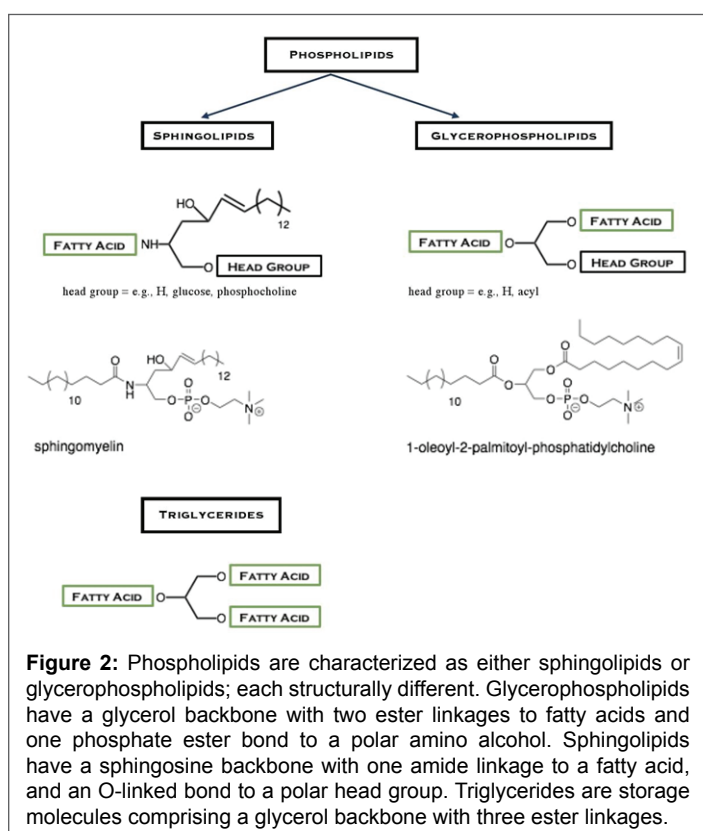


Figure 2: Phospholipids are characterized as either sphingolipids or glycerophospholipids; each structurally different. Glycerophospholipids have a glycerol backbone with two ester linkages to fatty acids and one phosphate ester bond to a polar amino alcohol. Sphingolipids have a sphingosine backbone with one amide linkage to a fatty acid, and an O-linked bond to a polar head group. Triglycerides are storage molecules comprising a glycerol backbone with three ester linkages.

from the negative impact of fatty acid function include: hypertension, diabetes [11], cancer [12], inflammation, sepsis, and cardiovascular disease [13,14]. However, fatty acids are utilized as therapeutic targets for drug development [15,16]. Furthermore, some fatty acids are crucial to the success of specific cellular processes and play a role in preventing disease. Polyunsaturated acids (PUFA), specifically n-6 and n-3, are collectively termed “essential fatty acids” (EFA). However, these acids are not biosynthesized by the human body and are, therefore, exclusively obtained from dietary sources [17,18]. Two prominent examples are linoleic acid (18:2n-6) [19], and docosahexaenoic acid (22:6n-3) [20]. Linoleic acid has *cis* double bonds at the 9 and 12 carbon positions, while conjugated linoleic acid is a mixture of various octadecadienoic acids with (9*cis*, 11*trans*) being the major isomer. Recently, the anti-carcinogenic properties of conjugated linoleic acid have been acknowledged, especially in the case of breast cancer prevention [19]. Docosahexaenoic acid has

compelling therapeutic implications as its inclusion in the diet is positively linked to the prevention of numerous pathologies [20]. Essential fatty acids play a major role in multiple processes, where the balance between dietary n-6 and n-3 fatty acids is critical.

Arachidonic acid exemplifies a metabolically active fatty acid; modified to create numerous lipid mediators such as eicosanoids, endocannabinoids, and lipoxins (Figure 3). The central involvement of lipid metabolism provides wide-reaching physiological consequences. During biosynthesis, fatty acids are liberated from a storage form and mobilized before utilization in cells. Eicosanoids [13,21,22] are a class of bioactive lipid mediators including prostaglandins and leukotrienes. These molecules have been implicated in the inflammatory responses present in diseases, such as arthritis and asthma.

### Fatty Acid analysis

Fatty acid analysis has progressed significantly over the past fifty years. During the last decade, numerous advances in extraction, derivatization, and analytical methodology were reported. The purpose of this review is to summarize recent literature; focusing on contributions regarding the analysis of medium and long-chain fatty acids (specific to most metabolites), using gas chromatography-mass spectrometry (GC/MS). Lipids prove to be a challenging field of study, mainly due to their structural complexity, which affects their subsequent analysis. Furthermore, the lack of internal standards for rare fatty acids hinders their use as clinical diagnostic tools. The novel approaches outlined here contribute to biomedical research, biomarker clinical diagnosis, and drug development.

### Extraction methodology

Due to their inherent hydrophobicity, lipids are soluble in organic solvents. As a result, most methods of isolation include a phase separation between immiscible solvents, with the lipid partitioning into the non-polar organic phase. The Folch et al. [23,24] and Bligh et al. [25] protocols are classic chloroform/methanol extraction methodologies (2:1 v/v and 1:2 v/v, respectively); with the latter approach best suited for extraction of samples low in lipid concentration (<1%). In both methods, the tissue is homogenized with solvent before the addition of water, which creates a biphasic system. The lower chloroform layer contains lipids from the sample, which is subsequently isolated; with the miscible methanol/water layer containing the non-lipid components of the sample. The advantage

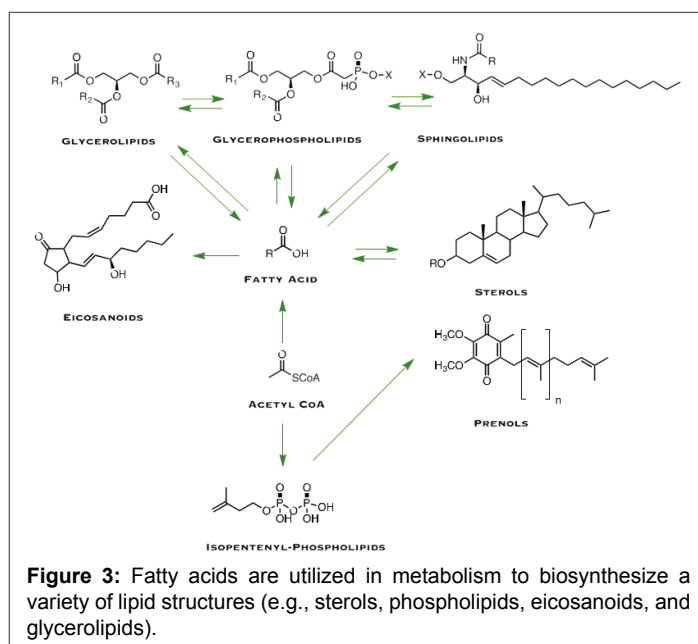


Figure 3: Fatty acids are utilized in metabolism to biosynthesize a variety of lipid structures (e.g., sterols, phospholipids, eicosanoids, and glycerolipids).

that the Bligh [25] method has over the Folch et al. [26] method is that significantly less solvent is required for sample extraction. Although frequently utilized, both approaches are found to be laborious and time-consuming. Additionally, a direct transesterification approach was employed by LePage et al. [26] using a 4:1 methanol/benzene solution with acetyl chloride. The main advantage of this methodology is the reduced number of sample preparation steps required for subsequent analysis. Matyash et al. [27] described a methodology utilizing milder and less toxic conditions for extraction, using methyl-*tert*-butyl ether (MTBE) in methanol. Upon using these solvent conditions, where lipids were extracted in the top layer, significant improvement of the lipid percent recovery was achieved.

The specific extraction technique is determined by the lipid class under investigation and its ultimate purpose. For example, blood samples are predominately composed of fatty acids and cholesterol; free fatty acids (FFA) represent a small percentage of the total fatty acid composition in biological samples, which require a tailored extraction scheme to isolate them for analysis [28,29].

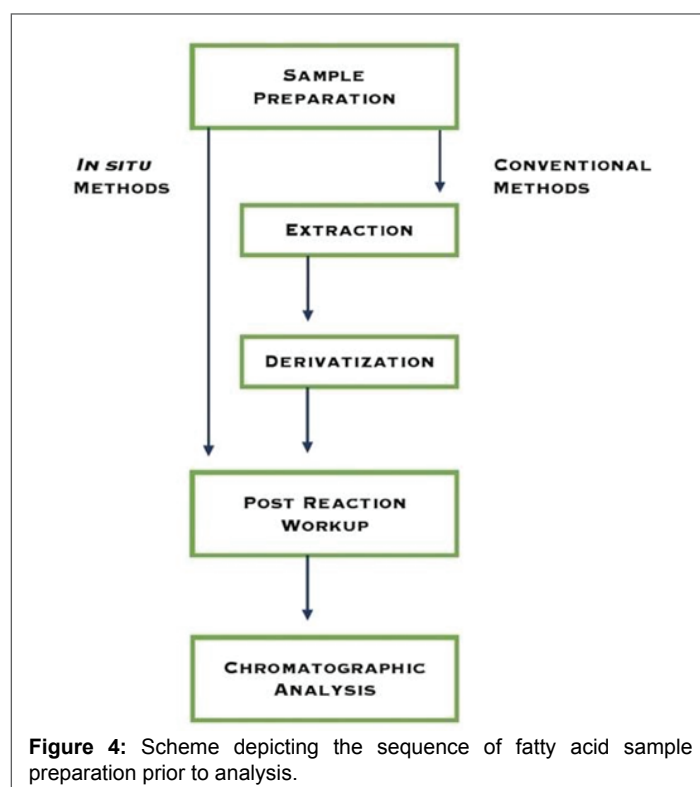
**Recent advances in lipid extraction methodology:** Lipid extraction is considered one of the most challenging, and thus critical, steps in quantitative analyses of biological samples. Method development for lipid extraction is continually optimized; providing wider-reaching applications. The current review summarizes the latest lipid extraction protocols with their applications.

**Extraction of triglycerides:** The extraction of medium-chain triglycerides (MCT) from virgin coconut oil was achieved in a 2009 study [30] using supercritical carbon dioxide (SC CO<sub>2</sub>). With this technique, >99% of the total oil from samples was extracted. This approach resulted in significantly higher yields than traditional extraction methodology using *n*-hexane. Researchers varied extraction parameters of temperature, pressure, and CO<sub>2</sub> consumption to achieve optimized conditions. The fatty acid composition was subsequently analyzed as FAME using gas chromatography-flame ionization detector (GC-FID). Results indicated that extraction yield and MCT content varied depending on the parameters selected.

**Extraction of phospholipids:** A simple and reproducible method was reported [31] for lipid extraction, specifically phospholipids and lysophospholipids, which was termed the “methanol method.” Methanol was added to the samples before vortexing and incubation on ice. After centrifugation, the supernatant was directly analyzed by MS. This method reproducibly indicated higher yields of phospholipids after extraction, with no formation of artifacts upon comparison with traditional methodologies [32].

**Extraction of Fatty acids:** For the preparation of fatty acid samples, a sequence of steps is generally followed as outlined in Figure 4. In order to characterize fatty acid profiles of lipids in a biological matrix, the fatty acids must first be obtained by either conventional methods of extraction and subsequent derivatization, or by simultaneous *in situ* extraction and derivatization [26,33]. Traditionally, solvent extraction is preceded by drying and digestion processes, in order to allow for solvent penetration and to liberate bound lipids, respectively [34]. Subsequent purification, hydrolysis, transesterification, and post-reaction work-up are performed to prepare the sample for GC/MS analysis. The *in situ* method of extraction and derivatization circumvents some of the limitations of traditional methodologies, such as time commitment, consumption of large volumes of solvent, large sample sizes, and potential contamination [33].

**Blood plasma:** Recent studies explored the best applications of various extraction methods by performing comparative analyses. The classical methods of extraction, as well as an alternative approach, which utilized microwave chemistry, were gauged for the preparation and analysis



**Figure 4:** Scheme depicting the sequence of fatty acid sample preparation prior to analysis.

of plasma samples. The Folch et al. [23] method was found to be the most suitable for fatty acid extraction and determination of relative concentrations of common fatty acids (e.g., linoleic, palmitic, oleic, stearic, and arachidonic acid) found in plasma [35]. Current reports indicate that the conventional Folch extraction and subsequent methylation was deemed more reliable for quantification of fatty acids from plasma. However, this method was more time consuming and utilized more resources, upon comparison with the LePage direct methods. The study also recommended that the choice of internal standards should be determined based on the nature of the targeted fatty acids. A recent study [36] compared details of the Matyash et al. [27] method of extraction with the classical Bligh and Dyer chloroform/methanol method on small plasma samples. No significant differences between these methods concerning recovered fatty acid concentrations were observed. However, the Matyash et al. [27] method led to a slightly higher concentration of recovered PUFA. When samples with high-fat content (>2%) were extracted, the classical Bligh and Dyer method worked better. The Folch et al. [23] method of extraction was utilized in an Alzheimer’s disease comparative study establishing fatty acid profiles for plasma and brain tissue samples [37]. Plasma lipids were extracted with chloroform/methanol (2:1); followed by saponification, methylation (BH<sub>3</sub>/CH<sub>3</sub>OH), and GC analysis. Analyses determined no significant difference between the fatty acid profiles of the two sample types.

**Tissue:** A rapid protocol with high-throughput applications was developed for the isolation of free fatty acids from tissue (and blood) [38]. Tissue samples, including adipose and liver, required a homogenization step as part of the extraction procedure. Adipose tissue, methanolic HCl, and internal standards (deuterated fatty acids) were briefly sonicated to achieve dispersion (however, liver tissue required homogenization prior to sonication). The tissue samples were then extracted with isooctane, and separated by centrifugation. The organic layer was removed and concentrated. The extracted fatty acids were subsequently analyzed using GC/MS in negative chemical ionization mode. To establish fatty acid profiles, and specifically evaluate the ratio of omega-6 to omega-3 fatty

acids in tissue samples, a simple procedure which combined extraction and methylation into a single step was described [39]. The homogenated tissues were treated with hexane,  $\text{BF}_3$  in methanol, and subjected to heating for 1 hour at 100 °C. Data indicated that recovery of long-chain fatty acids was significantly higher compared to traditional two-step methods. However, no difference in relative fatty acid composition between the methods was observed. This simplified method was more time efficient and decreased potential sample loss and contamination.

**Urine:** In order to simultaneously quantify short-chain fatty acids and branched-chain amino acids in fecal and urine samples, a novel derivatization method [40] was developed. After initial extraction, the samples were homogenized for 10 min and centrifuged for 20 min at 4 °C. The resulting supernatant was derivatized in one step using propyl chloroformate in a solution of water, propanol, and pyridine (8:3:2 v/v/v). After extraction, the samples were ultimately quantified using GC/MS. Furthermore, these conditions were optimized with fecal samples, but the procedure can be applied successfully to plasma and urine samples. A rapid methodology of this nature has numerous clinical applications; namely in obesity and metabolic disorder diagnosis.

## Analytical Methodology

### Derivatization of fatty acids

Fatty acid derivatization is performed prior to GC/MS analysis in order to elute less-polar, more-volatile analytes at practical temperatures without rearrangement or decomposition [41,42]. Modification of the functional groups by derivatization enables the analysis of compounds that otherwise would not be easily separated by GC. As outlined in Figure 4, there are generally two approaches to derivatization: either following hydrolysis of the fatty acids from the lipid structure, or direct *in situ* derivatization of the lipid structure [42]. Most reported derivatization procedures involve the conversion of the fatty acid moieties to corresponding esters through reversible esterification and transesterification mechanisms. Methodology varies considerably with respect to steps involved, solvents and reagents used, and experimental conditions [34]. Derivatization using trimethylchlorosilane (TMCS) is regarded as one of the simplest approaches. Analysis of resulting mass spectra reveals a characteristic ion fragment of M-117, which represents a loss of ( $\text{CO}_2\text{TMS}$ ) [43-45]. However, fatty acid methyl esters (FAME) are the most commonly utilized derivatives used for analysis [28, 46]. FAME is prepared more often than other fatty acid esters in biological tissues, because of the lower temperatures required to change their volatility during GC, improving subsequent separation [33].

Derivatization is typically carried out in the presence of a catalyst; characterized as either alkaline ( $\text{NaOCH}_3$ ,  $\text{KOH}$ , and  $\text{NaOH}$ ), or acidic ( $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ , and  $\text{BF}_3$ ). Acid-catalyzed derivatization reactions transesterify triglycerides and other complex lipids, in addition to free fatty acids, in the presence of methanol. However, base-catalyzed reactions are not able to esterify free fatty acids [42]. The majority of errors associated with derivatization (e.g., incomplete extraction, and unwanted saponification) occur during the post-production work-up, making this step crucial in obtaining accurate and reliable results [34]. To obtain optimal and accurate results during subsequent analysis, the researcher must select the ideal derivatization method based on the nature of the sample. Therefore, potential difficulties such as incomplete conversion, modification to the original fatty acid profile, formation of artifacts, and contamination can be reduced or eliminated [47,48].

## Recent Advances In Fatty Acid and Lipid Derivatization Methodology

### Fatty acid methyl esters (FAME)

FAME preparation using boron trifluoride and methanol is one of the most widely used derivatization approaches [49]. However, this methodology has several disadvantages due to the toxicity, volatility, cost, and limited shelf-life of  $\text{BF}_3$ . Moreover, possible formation of artifacts during synthesis is also a concern. These byproducts contribute to reduced selectivity and increased separation time. Therefore, employing a methodology that claims reduced reaction times is crucial in the reduction of byproducts [50,51]. Traditionally, synthesis of FAME is carried out in a hot water bath for extended periods of time with the use of catalysts. Microwave-assisted organic synthesis (MAOS) is a modern method where molecules of a sample absorb electromagnetic radiation; ultimately converting it to heat. The technique has significant advantages over conventional heating methods, such as reduced reaction times, decreased side reactions, increased percent recovery, and overall improved reproducibility (which are crucial factors for analyzing clinical samples). The utilization of microwaves in synthesis has gained popularity over the past few decades [49,52-54]; with microwave-assisted derivatization (MAD) for FAME synthesis being one specific application [51,55-58]. In 2013, Lin presented the direct transesterification of fatty acids from human serum samples using microwave irradiation [59]. The samples were extracted using a methanol/hexane/acetyl chloride solution based on LePage et al. [26] protocols. Fatty acid profiles obtained for the serum samples subjected to microwave-assisted FAME production (100 °C for 1 min in single mode or 125 °C for 5 minutes in multimode) were compared to profiles of samples where derivatives were obtained using traditional conductive heating (60 min at 100 °C). Fatty acid recoveries of the two sample groups were found to be quantitative or nearly quantitative. The one-step direct transesterification methodology was concluded to be accurate and comparable to the traditional LePage et al. [26] approach. Expedition of the derivatization process could prove beneficial for biotechnology applications where high-throughput analysis is paramount. A study of fatty acids in human plasma by Masood et al. [60] outlined two modifications of standard FAME analytical techniques, which would allow for the implementation of high-throughput analysis. The first modification related to the derivatization procedure, which was simplified to one step based on the LePage et al. [26] transesterification method. All required reagents were combined into a stock solution that was administered simultaneously; eliminating several laborious and time-intensive postreaction steps which characterize the traditional methodology. Due to the simplicity of this modified procedure, automatic robotic derivatization reactions were also explored. Results from the modified one-step method and the automated variation of one-step method were quantitatively similar to results obtained using the original LePage et al. [26] method, where differences for major fatty acids were minimal.

The efficiency of frequently-used protocols (e.g., trimethylsulfonium hydroxide (TMSH),  $\text{KOH}$ ,  $\text{BF}_3$  in hexane/methanol, methanolic hydrochloric acid, and  $\text{BF}_3$  with methanolic sodium hydroxide) for FAME derivatization was described [36]. This work concluded that the  $\text{HCl}$ -catalyzed derivation produced a complete transformation (>80%) for a variety of chain lengths and functionality of different fatty acid classes. Transesterification using TMSH lead to the insufficient derivation of PUFA, while the  $\text{KOH}$  procedure failed to derivatize FFA, and the  $\text{BF}_3$  in hexane/methanol failed to derivatize cholesterol esters. Methodologies for the analysis of hydroxy and cyclopropane-containing fatty acids, present in soil bacterium *Sinorhizobium meliloti*, were evaluated and optimized [61]. The three methodologies evaluated were: a) basic transesterification using  $\text{KOH}$  in methanol; b) acid-catalyzed

transmethylation using sulfuric acid and methanol, and c) base-catalyzed transmethylation using sodium methoxide in methanol. The study also introduced appropriate analytical standards to monitor recoveries and losses during the derivatization process. Data indicated that although all three methods yielded comparable quantities of fatty acids in general, significant concentration differences were observed in the recovery of 3-hydroxy and cyclopropane-containing fatty acids. Basic conditions (KOH in methanol) provided the best recovery of 3-hydroxy fatty acids, while the base-catalyzed transmethylation provided the best recovery for fatty acids containing cyclopropane. The derivatization strategy applied to phospholipids in this study is outlined in Figure 5.

### Trimethylsilyldiazomethane (TMSDM)

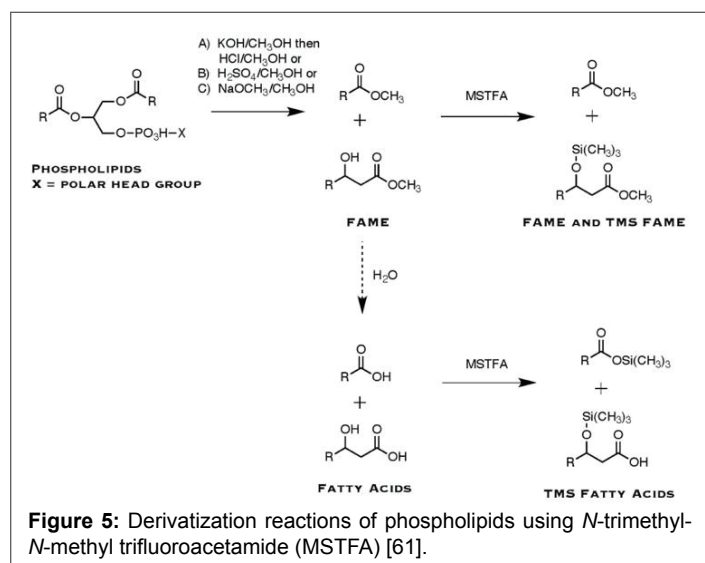
TMSDM is a commercially available methylating reagent, which has been used with methanol as a derivatizing agent to prepare FAME [42,62]. This reagent is commonly used as a replacement for diazomethane (DM) [47,63] since the use of DM is limited due to concerns regarding its safety, toxicity, and shelf-life [64]. Details regarding the mechanism of esterification of carboxylic acids were lacking until 2007 reports. Isotopic labeling technology was utilized to determine that TMSDM-catalyzed esterification of carboxylic acids proceeded through *in situ* liberation of diazomethane upon introduction of methanol [65]. TMSDM has been a common derivatizing agent. However, some disadvantages of TMSDM use have been recently reported, such as slower reaction rates [64,66] and lower yields [64] upon comparison with DM derivatization. Additionally, the presence of artifacts resulting from fatty acid trimethylsilylation has also been reported [48,63].

### Pentafluorobenzyl bromide (PFBB)

Pentafluorobenzyl bromide (PFBB) is a common derivatizing agent of fatty acids [67]. With this reagent, halo-based pentafluorobenzyl esters are generated. The use of PFBB for derivatization of fatty acids samples prior to analysis for profiling of complex lipids were reported [38,68].

### Chromatography

There is precedence for using gas chromatography to characterize fatty acid profiles in numerous studies for a variety of applications [3,46,69]. Generally, upon injection of the sample, the carrier gas advances the sample through the column containing the stationary phase, which is commonly made of polymer-bound silica or alumina. The separation of analytes of a sample is based on the degree of interaction with the stationary phase, as well as the difference in analyte boiling points.



The physical and chemical property differences of sample constituents contribute to distinctions in their retention times. Analyte identity may be confirmed solely based on retention times; however, MS may be required for more accurate identification purposes. Based on the nature of the sample, there are numerous parameters that can be altered to ensure proper method development (e.g., carrier gas, flow rate, column length and diameter, stationary phase, the temperature of the column, and injection technique). For GC analysis of fatty acids, a variety of columns with different properties are available. Polar columns are most frequently employed for analyses of fatty acids; with fused silica, polyesters, and cyanopropyl polysiloxane commonly used as stationary phases. Polar stationary phases allow for separation of fatty acids with different chain lengths, degrees of unsaturation (and their constitutional and geometrical isomers). Generally, the greater the length of the column, the better the separation of analytes in a sample. When the stationary phase has comparable polarity to the sample, increased resolution and separation will be exhibited, with a reduction in runtime [70].

Previous GC/MS methodologies have been reported for analysis of fatty acids [71]. Acid samples were derivatized with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and TMCS (99:1). The resulting TMS derivative was injected into a split/splitter injector set at 280 °C. The capillary column was 30 × 0.25 mm and 0.25 μm. The oven temperature was programmed to change from 80 to 150 °C at a rate of 30 °C per min (over 2.5 min), and then from 150 to 280 °C at a rate of 5 °C per min (over 30.0 min), and finally it was held at 280 °C for 10.0 min. The capillary column stationary solid phase is commonly made of silicon bound polymer, with a polar-activated surface and helium used as a mobile phase carrier gas; at a flow rate of 1.5 mL/min, with the electron ionization setting at 70 eV, and the heating source set at 230 °C [71]. Gas chromatographic analyses offer significant resolving power, high sensitivity, and reproducible results [72,73]. Additionally, lipid analysis can be carried out on derivatized or native fatty acid samples. However, recent reports [74,75] suggest that fatty acids with longer chain lengths require derivatization for optimal results. HPLC is an additional methodology, that is less utilized with regard to fatty acid analysis [9].

### Recent advances in fatty acid separation methodology

The sophisticated resolving power of gas chromatography has been harnessed for analysis of complex samples. In 2006, analyses for the determination of fatty acid profiling were reported; which provided data regarding the total conjugated linoleic acid content in various samples [41]. Separation of the closely related isomers was achieved using a cyanosilicone capillary column. Specific isomer identification was based largely on the comparison of retention times, as commercially available standards are limited.

Gas chromatography with FID or EI-MS detectors is the most often employed analytical approach used to study fatty acids. However, one-dimensional gas chromatography is not always the best option to elucidate fatty acid profiles from complex samples. Two-dimensional gas chromatography (GC/GC) is a relatively new technique able to successfully resolve complex mixtures of fatty acids due to significant separation power; offering an alternative approach that may circumvent some of the current limitations of FAME analysis. The GC/GC separation process is typically carried out on two columns with different polarities; with a conventional column configuration consisting of a nonpolar first phase and a polar second phase [76]. In 2011 [77] a comprehensive 2-D gas chromatographic analysis of derivatized polyunsaturated fatty acids in marine algae was performed. This study specifically assessed the utility of polar ionic liquid stationary phases in one-dimensional GC-MS and in two-dimensional chromatography (used as the second dimension). Additionally, polarity, sensitivity, and degree of column bleeding were compared with various traditional stationary phase compositions. Results

indicated that ionic liquid columns are more polar and bleed less than other polar columns resulting in better detectability. One-dimensional analysis using the ionic liquid column provided detailed fatty acid profiles, leading to the characterization of the algae samples. However, the high selectivity provided by the ionic liquid phase used in GC/GC, combined with mass spectral detection, made it possible to identify more compounds which were co-eluting in one-dimensional GC.

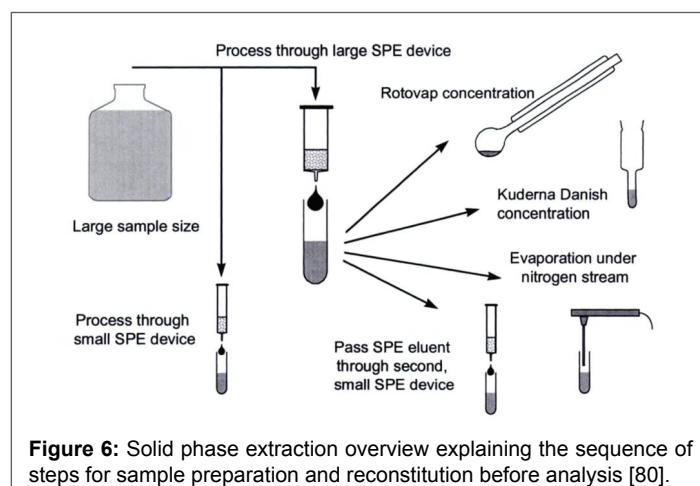
In an effort to diagnostically discern patients with diabetes mellitus (T2DM) from healthy individuals, metabolites in plasma were identified using 2-D gas chromatography/time of flight mass spectrometry (GC/GC-TOFMS) [78]. Using traditional one-dimensional gas chromatography in the analysis of complex metabolite samples can be problematic regarding resolution, due to peak overlap. In this case, two-dimensional gas chromatography coupled with a TOF-MS detector and peak analysis software led to exceptional analyte separation and biomarker identification for complex samples. Five metabolites including linoleic and palmitic acid were identified as potential biomarkers as their levels were elevated in patients with type 2 diabetes mellitus relative to healthy subjects. The methodology reported here has the potential to be used in clinical diagnosis, for several biological applications.

Manzano et al. [76] reported the separation and identification of FAME from complex samples, including linoleic and linolenic acid isomers. The utility of a two-dimensional gas chromatography system equipped with a capillary flow technology modulator partnered with FID detection was established. Method optimization including the exploration of different column combinations (both conventional and inverted phase sets) and column length of the second dimension was assessed to optimize results. Parameters such as oven temperature, modulation time, and column flow were also assessed. Greater FAME separation was observed when inverted phase column combinations were used. Furthermore, when the length of the second dimension (in inverted phase) was shorter, the resolution and analysis time were improved. The report also touts the separation of the eight isomers of linolenic acid for the first time in less than one hour.

The performance of two GC-FID methods for the determination of FFA in dairy products [79] was evaluated. Specifically, a direct on-column approach and a derivatization approach (where the FFA were derivatized in the injector) were assessed; with the latter method identified as more robust. This approach displayed more opportunity for routine use due to the automation potential of the derivatization step. The direct injection method displayed a lower detection and quantification level. Additionally, fatty acid absorption and column degradation were observed with the direct injection on-column method.

**Solid phase extraction:** In addition to gas chromatographic separation, there is also a pre-injection process for preparative separation to consider. Sample preparation is a crucial step in the analysis, as biological sample matrix can interfere with the analysis of the metabolites to be detected. Although there are many different separation methodologies available, solid phase extraction (SPE) chromatography remains one of the most useful and practical techniques for preparative separation of crude lipid mixtures. SPE, sometimes used in conjunction with other preparatory steps, acts to fractionate and subsequently concentrate complex mixtures to more manageable samples (Figure 6); which leads to more accurate analyses and lipid profiling. Depending on the nature of the sample, both the stationary phase and carrier gas can be modified. Columns are frequently purchased pre-packed, with a wide-range of variable specifications [9,80].

Recent publications report on the applications and advancements in solid phase extraction methodology. Solid phase micro-extraction (SPME) methods were applied for sample separation prior to analysis in a 2008 study. With SPME, a fiber coated with extracting phase was applied



**Figure 6:** Solid phase extraction overview explaining the sequence of steps for sample preparation and reconstitution before analysis [80].

to the sample, absorbing the analytes of interest. After extraction, the fiber was transferred to the injection port of the separating instrument, where analyte desorption occurred; and analysis subsequently took place. SPME is a simple and fast technique, not requiring the use of solvents. This protocol used a 2 mL sample, which was heated in contact with the absorbent fiber (divinyl benzene/carboxen/polydimethylsiloxane) to trap the volatile molecules. After subsequent desorption, the analysis was performed [81].

The process of analyzing urine samples generally requires the use of solid phase extraction methodology, to optimize the fatty acid concentration in samples. SPE methodology was utilized to explore new biomarkers, especially when investigating the secretion of fatty acids in urine samples for biomarker discovery studies [82]. Methanol was used first to activate the SPE cartridge before equilibration was performed with 2% acetic acid. Once the urine sample was loaded, the column was washed with a methanol/acetic acid solution; and the fatty acids were ultimately eluted with acetone. Another study [83] reported the characterization and quantification of nitroalkene metabolites, specifically 9-nitrooctadeca-9, 11-dienoic acid, and 12-nitro-octadeca-9, 11-dienoic acid. Before extraction, the urine was incubated with 10 mM Hg<sub>2</sub>Cl<sub>2</sub> for 30 min.

The effects of using various sorbents for SPE in the fatty acid analysis of extracted tissue were reported. Dehydrated silica, hydrated silica, and aminopropyl-bonded silica (NH<sub>2</sub>) were evaluated under diverse conditions. Results indicated that recovery of polar lipids was best when the hydrated silica matrix was used. Additionally, recovery of polar lipids improved by increasing the mass ratio of lipid to sorbent [84].

## Mass Spectrometry

Lipid characterization includes both quantitative and qualitative mass spectrometric analyses. Lipid profiling provides data relating to the composition and abundance of the lipids contained in a crude extract, which can be used to examine fluctuations over time [9,34]. A variety of different MS ionization sources (e.g., Electron Ionization (EI); Chemical Ionization (CI); Matrix-Assisted Laser Desorption Ionization (MALDI); Electrospray Ionization (ESI); and fast atom bombardment) are available, depending on the nature of the sample state (solid, liquid, or gas) [9]. EI and CI are generally used for the analysis of gases and volatile organic molecules, while MALDI and ESI can be used for the analysis of liquids and solids. Each ionization method is characterized by inherent advantages and disadvantages, depending on the specific application. There are also numerous mass analyzers, such as the triple quadrupole (QQQ), ion trap, and time-of-flight (TOF). In general, after injection, the sample is bombarded with a stream of electrons, resulting in the formation of

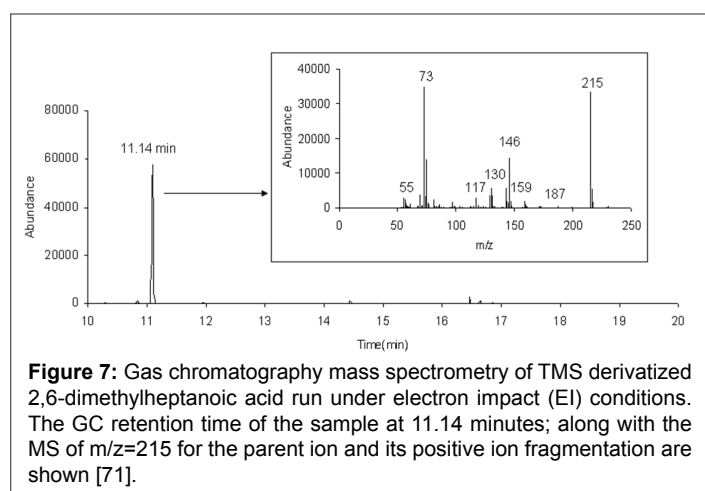
charged fragment ions. These fragments are then separated based on their mass to charge ratio ( $m/z$ ). After detection, the resulting spectra present the relative abundance of the detected ions as a function of their mass to charge ratio. Characteristic fragmentation patterns are observed for fatty acids, specifically FAME, with ions of  $m/z=74$  (McLafferty rearrangement ion from ester moiety),  $m/z=43$  (isopropyl cation), and  $m/z=41$  (propenyl cation) [85].

In addition to MS analyses, there are other modes of detection, such as flame ionization detection and thermal conductivity detection. FID detection has been widely used for forensic applications and in the investigative sciences. Its primary use is for analysis of hydrocarbons and is unable to generate ions for carbonyl-containing compounds [86,87]. GC separation with MS detection provides high chromatographic resolution with structural confirmation (Figure 7). This pairing generally leads to better sensitivity and selectivity over other detection modes [88]. GC/MS is highly recommended for clinical and organic acid analysis due to its capability to distinguish stereoisomers of different fatty acids, which would otherwise not be possible with other means of detection.

### Recent advances in Fatty Acids and Lipid MS methodology

Gas chromatography coupled to mass spectrometry is the most widely used analytical method for fatty acids. Its prevalence can be seen in the utilization in numerous current reports [89,90]. The effects of rosuvastatin treatment on the lipid profiles in plasma samples from healthy patients were reported [91]. Using ultra-performance liquid chromatography quadrupole time-of-flight (UPLC/Q-TOF) mass spectrometry, a simple and efficient method was established to detect and identify multiple classes of lipids. This method consisted of construction and implementation of a phosphatidylcholine retention time index, which aided in identification. Results of the study indicated that the concentration of detected lipids, namely sphingomyelin, triglyceride, phosphatidylinositol, and phosphatidylethanolamine was reduced significantly due to the administration of rosuvastatin (with plasma collection at peak drug levels). Although changes in several lipid levels were observed after drug treatment, this study could not ascertain the causal relationship.

A one vial method, with sample sizes reduced by 100-fold, was assessed in a base-catalyzed derivatization of phospholipids from *Sinorhizobium meliloti* samples [61]. A GC/MS vial with a 200  $\mu\text{L}$  insert was used for the derivatization and subsequent GC/MS analysis; additional instrumentation or modification was not required. Recent findings concluded micro-scale methodology to be reproducible; providing quantitative yields. This practical approach required minimal sample



manipulation and is amenable to a large sample number. Studies also indicated that reactions performed at higher temperatures were responsible for increased losses in product formation, most likely due to side reactions. Hydrolysis, which is typically a concern with reduced sample size, did not appear to influence the transformation.

In order to study bowel disorder pathologies, phosphatidylcholine was quantified using a novel, a highly-sensitive method developed [92]. Crude extract analyses were performed using nano-electrospray ionization tandem mass spectrometry with a triple quadrupole mass spectrometer. This method allowed for direct analyses of crude extracts and the quantification of small concentrations that most clinical techniques do not recognize due to their limitations.

Recent advances in mass spectrometry, specifically MALDI imaging mass spectrometry, have led to lipid analysis of tissue slices, and even single-cell samples [93-95]. This approach allowed for the circumnavigation of laborious lipid separation and extraction procedures, providing *in situ* analysis [96-98]. Furthermore, GC-MS was employed to identify and quantify fatty acids in seed extracts [99]; the relative percentages of monounsaturated, polyunsaturated, unsaturated, and saturated fatty acids were reported. The resulting data indicated  $\omega$ -linolenic acid, an omega-3 fatty acid, to have the highest concentration in the seed extracts. This fatty acid was present in all seeds analyzed except sesame; with the highest incidence in chia seeds and linseeds. Studies also indicated that the seeds contained greater concentrations of PUFA than saturated fatty acids.

Current efforts aimed to explore methodology in which the analysis time of fatty acids by GC/MS is reduced; providing the potential for a high-throughput investigation [100]. A most recent methodology using gas chromatography-tandem mass spectrometry (GC-MS/MS) with ammonia-induced chemical ionization was developed, and used to assess fatty acid profiles in red blood cells. A 9 minute total run time was observed with this method, as compared to analysis times between 40 and 60 minutes for conventional GC-MS/MS methodology. Furthermore, this methodology utilizing chemical ionization produces a higher yield of molecular ions relative to electron ionization fragmentation methodology, where typically only fragment ions, not molecular ions, are produced. This leads to increased compound specificity and thus, confidence in identification.

Isotope analysis methodology was developed in recent years to obtain accurate isotopic composition at abundance levels. Isotope ratio mass spectrometry (IRMS), when coupled to GC, allows for the sensitive detection and measurement of isotopic ratios in samples. This methodology offers reliable analysis and is a safe alternative for the use of radioactive tracers [101]. Most recent analysis of organic and fatty acids has employed IRMS to quantitate specific metabolites [70,102].

### Biomarkers

Biomarkers play an essential role in quantifying the presence and progress of various pathologies; in addition to evaluating potential therapeutic applications. Progress in this field of study is therefore imperative because it can aid in early disease diagnosis and prevention. For example, palmitoleic acid is regulated by the mechanistic target of rapamycin (mTOR) signaling; and inhibition of mTORC1 decreases palmitoleic acid levels in peripheral blood mononuclear cells (PBMC). Therefore, mTORC1 may regulate the level of palmitoleic acid by controlling its biosynthesis. The elevation of palmitoleic acid was used as an indicator of abnormal PBMC in polymyositis. The latest findings recommend this biomarker for early detection and therapy of polymyositis [103].

Based on evidence that FFA plays a role in cancer development, this lipid class was used as biomarkers to distinguish cancer from non-cancer status

in patients [104]. Serum samples from patients with adenocarcinoma and patients without known cancer were examined. Arachidonic acid and linoleic acid levels were observed to be elevated in patients with lung cancer compared to those without. Serum FFA and metabolites demonstrated good sensitivity and specificity for identification of adenocarcinoma of the lung. One advantage of FFA-based biomarkers is that they are stable; making them clinically practical with high predictive value. A 2017 study [105] identified aminomalonic acid and various metabolites as potential biomarkers of melanoma. Evidence of cancer formation, advancement, and treatment progress are potential applications of these reported molecular relationships. Lipid profiling was achieved using GC/MS and direct infusion-mass spectrometry (DI-MS). Metabolic profiles were used to distinguish between different cancer types in patients diagnosed with ovarian lesions [70]. Human tumor samples were analyzed by GC-TOF MS to provide signatures for patients diagnosed with invasive carcinoma and borderline tumors. Known primary metabolites, which included free fatty acids (e.g., nonadecanoic, stearic, heptadecanoic, and malic acid) were quantified. Approximately 40% were successfully identified using mass spectral comparison and retention times relative to reference compounds. Significant differences in metabolic profiles were found between tumor types after extensive statistical analysis. Specifically, the level of malic acid was elevated in patients suffering from invasive carcinoma, while the levels of stearic, heptadecanoic, and nonadecanoic acids increased in samples from patients diagnosed with borderline tumors. This methodology is a promising predictive model for high throughput tumor diagnosis; able to distinguish between 90% of tumor types. Experiments on blood plasma have validated the viability of this technique to quantify endogenous levels of free fatty acids and monoglycerides.

Current studies indicate consistent correlations between cardiovascular health and specific fatty acid levels, suggesting their relevance as biomarkers of heart health. A 2013 study [106] reported an inversely proportional relationship between cardiovascular disease and fatty acid levels. Phospholipid fatty acids including 15:0, 14:0, and *trans*-C16:1n-7 was investigated as possible biomarkers of dietary fat and incidence of cardiovascular disease. Data indicated that the 15:0 fatty acid was inversely associated with the incidence of cardiovascular disease, while no association was found with 14:0 and *trans*-C16:1n-7. Higher levels of 15:0 fatty acid was associated with lower blood pressure, lower triglycerides, and lower incidence of cardiovascular disease and coronary heart disease. Furthermore, in a similar study, the relationship between polyunsaturated n-3 fatty acids (specifically EPA and DHA), and markers of inflammation were investigated [107]. The blood samples were derivatized to FAME before assessment by capillary gas chromatography. Results indicated an inverse association between EPA and DHA levels and the inflammatory biomarkers C-reactive protein (CRP) and Interleukin-6 (IL-6). The preventative nature of fatty acids in the prevention of cardiovascular events by attenuation of systemic inflammation is suggested by the data, and may be employed as a biomarker.

Metabolic profiles for liver fibrosis samples were established in a 2017 report [90]. Biomarkers could prove to be significant in the treatment and ultimate prevention using targeted drug development. Carbon tetrachloride was used to induce liver damage in a sample of rats. GC/MS analysis was employed to establish metabolic profiles for both the model and control groups. Analysis of the data indicated that exposure to the organic solvent caused significant fluctuations in the profiles, affecting metabolic pathways. Seven metabolites from serum samples were targeted; and five from urine samples. Serum samples from the model group displayed increased levels of isoleucine, L-malic acid,  $\alpha$ -copper, and hippuric acid relative to the control group; while urine samples from the model group displayed increased levels of 2-hydroxybutyric acid, isoleucine, and corticoid relative to the control group. The authors

inferred that liver fibrosis is associated with the dysfunction of numerous metabolic pathways. Wang et al. [108] were able to distinguish between patients suffering from Alzheimer's disease (AD) and healthy controls using assessment of free fatty acid profiles results obtained with GC/MS and subsequent statistical analysis indicated that concentrations of five specific fatty acids varied between AD patients and those deemed healthy. Data analyses indicated drastically lower FFA levels in affected patients; with the most change observed with DHA. The results of this analysis demonstrate that FFA can be used to diagnose and determine the degree of disease progression in patients with AD. It was reported that fatty acids in urine were utilized as potential biomarkers for type 2 diabetes mellitus. The following fatty acids demonstrated the greatest utility as biomarkers: C16:0, C18:0, C18:2n-6, C20:5n-3, C22:6n-3 [82]. Fatty acid profiles of patients' urine samples were analyzed by UPLC/Q-TOF-MS. Recent developments focused on the urine metabolic profile, as sample collection was simple, non-evasive, and directly reflected the metabolic status of patients. Fatty acid biomarkers were used to distinguish between patients exhibiting pathology and those that were healthy.

## Summary

Fatty acids are recognized as powerful signaling compounds that are involved in many metabolic processes. Quantitative determination of fatty acids and exploration of fatty acid profiles have become essential for lipid analysis. The collective tools of gas chromatography and mass spectrometry represent a powerful strategy for the analysis of fatty acids in complex samples. This allows for the selective detection of several classes of fatty acids (small, medium, long, and very long-chains), and their counter isomers with great sensitivity. We hereby present a comprehensive review of methods for the analysis of free fatty acids and fatty acid composition of complex lipids in several biological matrixes. Our search horizon includes extraction, purification, detection, and finally, quantification. The analytical methodology focuses on GC/MS analyses and includes the most recent extraction and derivatization protocols, as well as latest detection techniques. The procedures outlined provide a baseline separation between saturated and unsaturated fatty acids of different chain lengths, as well as between isomers. Furthermore, we extended our review to other lipid-based signaling molecules, such as phospholipids and triglycerides.

Additionally, we compared different protocols for the derivatization of fatty acids, which are optimized to achieve acceptable quantitative results that can be validated for clinical diagnosis purposes. In general, transesterification methods, regardless of acidic or basic conditions, will be suitable for esterification of the tested sample. Recent procedures, especially where removal of the derivatizing reagent is not recommended, should be assessed more frequently for their long-term effect on the gas chromatography column efficiency. Optimization of spectrometer conditions for broad detection capacity and sensitivity, capable of measuring trace amounts of fatty acids in complex biological samples, is required.

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## Conflict of Interest

The authors contributed equally to this work and should be considered as co-first authors. The authors have declared no conflict of interest.



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