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Review Article

Gas chromatography-mass spectrometry-based analytical strategies for fatty acid analysis in biological samples

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ABSTRACT

Fatty acids play critical roles in biological systems. Imbalances in fatty acids are related to a variety of diseases, which makes the measurement of fatty acids in biological samples important. Many analytical strategies have been developed to investigate fatty acids in various biological samples. Due to the structural diversity of fatty acids, many factors need to be considered when developing analytical methods including extraction methods, derivatization methods, column selections, and internal standard selections. This review focused on gas chromatography-mass spectrometry (GC–MS)-based methods. We reviewed several commonly used fatty acid extraction approaches, including liquid–liquid extraction and solid-phase microextraction. Moreover, both acid and base derivatization methods and other specially designed methods were comprehensively reviewed, and their strengths and limitations were discussed. Having good separation efficiency is essential to building an accurate and reliable GC–MS platform for fatty acid analysis. We reviewed the separation performance of different columns and discussed the application of multidimensional GC for improving separations. The selection of internal standards was also discussed. In the final section, we introduced several biomedical studies that measured fatty acid levels in different sample matrices and provided hints on the relationships between fatty acid imbalances and diseases.

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1. Introduction

Fatty acids are essential molecules in biological systems and have several important biological functions, including being constituents of cell membranes and regulating the activity of enzymes and inflammatory processes. Studies have indicated that imbalances in fatty acids are associated with a wide variety of diseases, such as inflammation [1–3], cardiovascular disease [4–6], tumorigenesis [7–10] and Alzheimer's disease [11,12]. As fatty acids play critical roles in biological systems, many studies have analyzed fatty acids in various biological samples, such as plasma, skin, urine, and tissue samples, using a variety of analytical strategies [13–16].

Fatty acids are carboxylic acids with either saturated or unsaturated aliphatic chains. They can be divided into four groups, namely, short-chain fatty acids (<C6), medium-chain fatty acids (C6–C12), long-chain fatty acids (C13–C21) and very-long-chain fatty acids (≥C22), according to the chain length. In addition, fatty acids may contain different numbers of double bonds in their aliphatic chain at different positions, which results in large families of isomeric fatty acids, e.g., geometric isomers and structural isomers. In biological systems, although fatty acids can be present in their free fatty acid (FFA) forms, they most often exist in bound forms, such as cholesterol and phospholipids. The total fatty acids included FFAs and bound fatty acids. Analytical tools, including gas-chromatography mass spectrometry (GC–MS), gas-chromatography with flame ionization detection (GC-FID), and liquid-chromatography mass spectrometry (LC-MS), have been used to perform fatty acid analyses [10,17,18]. LC-MS methods for fatty acid analysis showed some disadvantages such as larger solvent consumption and lower selectivity [19,20]. Compared to GC-FID, GC–MS could provide more structural information [21,22]. Moreover, GC–MS has well-established databases for FA identification with higher efficiency and better selectivity compared to GC-FID. As a result, GC–MS is the most frequently used method for fatty acid analysis. This article reviewed the GC-MS-based methods used for fatty acid analysis in terms of their sample preparation methods, column selection, and recent applications in biomedical studies.

2. Sample preparation

2.1. *Extraction methods*

Generally, GC-MS-based analytical methods for fatty acid analysis included three steps: (1) extraction of the fatty acids from the sample matrix, (2) derivatization of the fatty acids, and (3) GC–MS analysis. There are various well-established extraction protocols, and generally, these extraction methods could be applied to different types of samples; however, to achieve the best performance for specific target analytes, some method optimization is required. Since many parameters such as instrumental settings would affect the method performance, we did not provide quantitative comparison for different extraction methods. In the following section, we briefly introduce the frequently used extraction methods.

2.1.1. *Liquid–liquid extraction*

Liquid–liquid extraction methods are frequently used in fatty acid analysis using different combinations of organic solvents. The most well-known liquid-based fatty acid extraction methods adopted in biomedical fields are those proposed by Folch et al. [23] and Bligh and Dyer [24]. Folch used a mixture of chloroform and methanol at a ratio of 2:1 (v/v) as the extraction solvent and a final volume of 20 times the volume of the tissue sample (1 g in 20 mL of solvent mixture). Then, water or a salt solution (e.g., 0.9% NaCl solution) was added to cause phase separation. The lower phase was then collected for fatty acid analysis. Folch extraction is considered the gold standard method for lipid extraction. The method described by Bligh and Dyer is also widely used in fatty acid analysis, and it was first developed as an extraction approach for determining the total lipid content in fish muscle. This method is usually applied to biological samples (e.g., tissue and blood) that contains ~80% water in the sample, and used chloroform/methanol/water for extraction to achieve a final ratio with chloroform/methanol/water 2:2:1.8. This method offers the advantage of low solvent consumption while still providing high recovery. However, using chloroform as part of the extraction solvent is a concern due to its high toxicity, making this method poorly suited for large-scale applications [25]. Therefore, other extraction solvents have been used to replace chloroform [24,26]. For example, one study proposed the use of methyl-*tert*-butyl ether (MTBE), and they claimed that this method provided faster and cleaner lipid extraction [27]. The overall recoveries achieved by MTBE method were 90–98%, which is similar to Folch method. The only one exception was phosphatidylinositol (PI) standard which showed higher recovery by MTBE. In addition, Hara and Radin have proposed a lipid extraction approach using the low toxicity solvents hexane and isopropanol [28]. This approach was applied to both plasma and erythrocytes samples, and was shown to provide higher extraction recovery for total FA compared to the Bligh and Dyer method, moreover, the sample preparation time of this method is comparatively shorter which made this method be more efficient [29,30]. Another chloroform free lipid extraction method, butanol:methanol (BUME) method, was also used for extracting FA. This method included an initial one-phase extraction with 300 μL butanol:methanol (3:1) mixture followed by a two-phase extraction with 300 μL heptane:ethyl acetate (3:1) and 300 μL 1% acetic acid [31]. Since BUME did not use chloroform for extraction, this method is more environment-friendly and also less toxic. If there is a specific requirement to analyze FFAs from biological samples, further isolation procedures (e.g., lipid fractionation) or a specific extraction approach may be necessary [32]. For example, Han et al. used both potassium hydroxide/methanol and hexane to separately extract esterified fatty acids and FFAs from plasma samples [33]. This approach could be used to determine both FFAs and esterified fatty acids with a small volume of samples. Alternatively, FFAs could be specifically isolated using a solid-phase extraction (SPE) approach (with aminopropyl-silica cartridges) or solid-phase microextraction (SPME), which are introduced in the following section [34]. Some studies have applied an additional saponification step after lipid extraction to separate FA from other lipids by

cleaving the ester bond between the fatty acid moiety and the glycerol part [35,36].

To improve extraction speed or reduce solvent consumption, some modified approaches have been proposed. Liu et al. used ultrasonic-assisted extraction (UAE) to extract fatty acids from tissue samples [37]. They evaluated their extraction procedure in the extraction of 16 FFAs from liver samples. They optimized the parameters of extraction time, extractant volume and ultrasound power level. The recoveries of this method ranged from 87 to 120%, and their results indicated that this method was comparable to conventional liquid–liquid extraction method but with the advantage of being more environmentally friendly due to the lower solvent consumption. The microwave-assisted extraction (MAE) approach has the advantages of being fast and robust and consuming a small amount of solvent. Several studies have used MAE to extract fatty acids from biological samples [38–40]. Costa et al. developed and validated an MAE method for lipid extraction from fish samples [40]. Their result showed that the contents of each fatty acid and the total lipid contents were similar between Folch and MAE method. They indicated that compared to the Folch method, MAE is a relatively fast and robust technique with lower solvent consumption. Although there were some concerns about the stability of the fatty acids during microwave treatment, their results indicated that there were no significant differences in the contents of any of the fatty acids between the Folch extraction method and MAE method.

2.1.2. *Solid-phase microextraction*

Solid-phase microextraction (SPME) has been used to extract FFAs since 1995 [41]. SPME is a fast, simple and solvent-free sample preparation approach. Fiorini et al. improved the efficacy of headspace SPME by using a salting out system, and this method could measure both short-chain and medium-chain fatty acids in the free form [42]. Sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) are commonly used salting-out reagents in SPME systems. Fiorini et al. used a combination of $(NH_4)_2SO_4/NaH_2PO_4$ as the salting-out reagent to improve the recovery for SPME. They used both biological samples (rat feces) and food samples (cheese and wine) to prove the applicability of the method and demonstrated the improvement in sensitivity.

2.2. *Derivatization methods*

Derivatization is usually necessary for fatty acid analysis by GC–MS, especially for fatty acids with carbon numbers larger than 10. Fatty acids are commonly derivatized to form fatty acid methyl esters (FAMEs), which are then detected by GC–MS. In this section, we introduced the methods frequently used for fatty acid derivatization. Generally, acid derivatization methods can be applied to total fatty acids (including FFA and esterified fatty acids); however, basic derivatization methods are limited to esterified fatty acids [43].

2.2.1. *Acid derivatization methods*

The commonly used acid derivatization reagents are hydrochloric acid (HCl), acetyl chloride (CH_3COCl), sulfuric acid (H_2SO_4), and boron trifluoride (BF_3). HCl derivatization is one of the most commonly used fatty acid analysis methods because of its operational simplicity [44,45]. In HCl derivatization, methanolic HCl is added to the dried lipid extract, and the solution is heated for a certain period. However, due to the solubility of certain lipids in methanolic HCl, the addition of a second solvent before the derivatization step may be necessary [44]. The acetyl chloride derivatization method was introduced in 1986 [46–48]. When using this method, acetyl chloride is added to the sample containing methanol, and the sample is generally heated at 95–100 °C for 60 min. After derivatization, the samples are neutralized, and the FAMEs are extracted with an organic solvent for further GC analysis. Some potential problems and safety issues may need to be considered when using the acetyl chloride derivatization method. For example, acetyl chloride derivatization is an exothermic reaction, causing the sample spill out of the vial, which could be dangerous. In addition, some polyunsaturated fatty acids (PUFAs) are relatively unstable at the high temperatures required for derivatization, which could lead to inaccurate quantification results [49]. The H_2SO_4 derivatization method has also been widely used for the analysis of fatty acids in biological samples [50,51]. The reaction procedure is similar to that of other derivatization methods. Because H_2SO_4 is a strong oxidizing agent, this method is not recommended for PUFA analysis [52]. The BF_3 derivatization method has been used for fatty acid analysis for the last several decades, and it is now widely used for derivatizing various biological samples [53–55]. This protocol has the advantage of a short reaction time, and previous studies have shown that after adding the BF_3-methanol reagent, the reaction could be completed within 10 min. When using the BF_3 derivatization method, the lipid extract is first dissolved in an organic solvent. Generally, the derivatization is performed by adding BF_3-methanol reagent (14%, w/v) and heating at 80–100 °C for 45–60 min. Finally, the FAMEs are extracted with an organic solvent and analyzed by GC. Although the BF_3 method provides efficient derivatization, its instability and the formation of artifacts have been subjects of concern in several studies [56–58]. To summarize, acidic derivatization approaches are commonly used for biological samples and have many advantages; however, the potential for altering the isomer distribution of the conjugated system remains a concern [57,59]. Stability evaluations for each fatty acid are suggested prior to the application of these techniques in biomedical analysis. Moreover, some of the artifact formation during acid derivatization could be reduced by avoiding using high reaction temperatures or amounts of derivatization reagent, or adding some dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) during the reaction [60–62].

In addition to being derivatized after lipid extraction, the HCl, acetyl chloride, H_2SO_4 and BF_3 derivatization methods could also be used for one-step extraction-derivatization approach [46,63–65]. Previous study has investigated the efficiencies of direct derivatization and conventional derivatization procedures, including the extraction of the lipids by the Folch method followed by derivatization. The results showed that similar FAME profiles were obtained from the two approaches. Moreover, a higher recovery of the total FAMEs was

achieved in the one-step approach [63]. This direct derivatization, which bypassed the extraction steps, is especially beneficial for large sample analyses in many clinical studies.

2.2.2. *Basic derivatization methods*

Basic derivatization methods offer the advantages of short derivatization times, no double bond isomerization issue, easy operation and uses less aggressive reagents, however, they are not suitable for derivatizing FFAs [57,59,66–68]. The sodium methoxide ($NaOCH_3$) derivatization method has been used in several studies [69,70]. Typically, 0.5 M $NaOCH_3$ in anhydrous methanol is added to the lipid extract, and the solution is reacted at 45 °C for 5 min. $NaHSO_4$ (15%) is then added to neutralize the mixture. Finally, the FAMEs are extracted with an organic solvent and analyzed by GC. Potassium hydroxide (KOH) can also be used in basic derivatization methods. The protocol is quite simple, and the reaction time is quite short [71]. When using KOH, methanolic KOH (2 mol/L) is added to the lipid extract, and the mixture is incubated at room temperature or heated to 50 °C for a few minutes for fatty acid derivatization. Then, sodium bisulfate is added, and the supernatant is collected and analyzed by GC [72].

2.2.3. *Other derivatization methods*

In addition to acid and basic derivatization methods, other derivatization strategies have also been proposed. Trimethylsulfonium hydroxide (TMSH) allows rapid derivatization in only one step without any further extraction and shows the ability to reduce the artifact compared to the acidic derivatization method [73,74]. This method has been used to investigate the fatty acid profiles of neutral lipids, FFAs and phospholipids in human plasma [75]. Due to the simplified protocol, this method was useful for large batch analysis, but the limit of the TMSH method is the insufficient derivatization efficiency for PUFA [74,76]. For studies specifically interested in free fatty acids, pentafluorobenzyl bromide (PFB–Br) is recommended. This method converts fatty acids into halogenated derivatives, which can be easily detected by negative chemical ionization (NCI) GC–MS [77]. The PFB-Br derivatization method was first introduced by Kawahara in 1968 and was specifically used for FFA analysis [78]. Briefly, a mixture containing PFB-Br and N,N-diisopropylethylamine (DIPEA) at a ratio of 1:1 is added to dried lipid extract. The derivatization is performed at room temperature for 15–30 min and produces the pentafluorobenzyl esters of fatty acids (PFB–FAs) [79].

Since numerous derivatization methods can be used for fatty acid analysis, it is better to understand the pros and cons of each method and to consider the limitations of the methods. In addition, the derivatization conditions can be optimized to meet the needs of a specific application. Ostermann et al. compared different fatty acid derivatization methods, including TMSH derivatization, BF_3 derivatization, HCl derivatization, KOH derivatization, combined NaOH + BF_3 derivatization, and direct TMSH derivatization, with plasma and tissue samples as well as fatty acid standards. The standards they used included saturated/unsaturated FFAs, phosphatidylcholine (PC), cholesterol ester (CE) and triacylglycerols (TG) [72]. Their results indicated that each method has its own limitations; for example, derivatization with KOH has good efficiency for the fatty acids in PC and TG but failed to derivatize the FFAs and fatty acids in CE. The results of their comparison using plasma samples suggested that MTBE/methanol extraction followed by HCl derivatization was good for all the tested lipid classes. Our group also compared different derivatization methods, including HCl derivatization, H_2SO_4 derivatization, BF_3 derivatization, acetyl chloride derivatization, and sodium methoxide derivatization, for the analysis of fatty acids in human plasm samples [80]. Our results showed that acetyl chloride derivatization has high derivatization efficiency and the lowest cost. We validated this method and then applied it to the investigation of potential breast cancer biomarkers in plasma samples. The results indicated that acetyl chloride derivatization provided the advantages of good accuracy and precision, which is important for clinical sample analysis [80]. In addition, we used a modified acetyl chloride derivatization method to achieve differential labeling by derivatization with unlabeled (D0) or deuterated (D3) methanol of pooled control and pooled test samples. This method allows the efficient and economical comparative analysis of fatty acids [81]. Selected fatty acid derivatization methods and their reaction conditions are listed in Table 1 [10,13,22,33,36,44,46,47,50,69,70,75,79,82–99].

3. GC column selection

A suitable column with good separation is essential for analyzing isomeric mixtures of fatty acids. Many columns have been demonstrated to be effective for separating fatty acids with different chain lengths, degrees of saturation, double bond locations, and *cis* or *trans* isomers. High-polarity columns such as HP-88 column (88% - cyanopropyl aryl-polysiloxane), DB-FFAP column (nitroterephthalic acid-modified polyethylene glycol) and SLB-IL series columns (ionic liquids) are commonly used for fatty acid analysis in biological samples [46,100,101]. Previous studies have indicated that ionic liquid (IL) columns provide better selectivity than wax or cyanopropylsiloxane columns for FAME mixtures. Moreover, IL columns can separate geometric and positional fatty acid isomers [102,103]. Zeng et al. characterized the FAME retention behaviors of various IL columns. They compared IL columns including SLB-IL59, SLB-IL60, SLB-IL61, SLB-76, SLB-82, SLB-100 and SLB-IL111 as well as a SLB-5ms. The total ion chromatograms of C18 to C24 obtained from the different columns are shown in Fig. 1, and the peak details are listed in Table 2 [104] (Fig. 1 and Table 2 were adapted from Ref. [104]). Several FAME geometric isomers, such as C18:2n6t and C18:2n6c, could not be separated by a nonpolar column (SLB-5ms), while better resolutions could be obtained on ionic series columns. In addition, imidazolium-based SLB-IL82, SLB-IL100, and SLB-IL111 columns provided better resolution of *cis* and *trans* isomers than phosphonium-based SLB-IL59, SLB-IL60, SLB-IL61 and SLB-IL76 columns, which is consistent with a previous report that IL columns with an imidazolium instead of a tripropylphosphonium moiety formed stronger interactions with polar compounds, resulting in better selectivity [104,105]. Weatherly et al. also compared several IL columns. For the *cis* and *trans* separation, they used C18:2 as a test standard, their result was similar to previous studies

Table 1 – Selected fatty acid derivatization methods and their reaction conditions.

Derivatization method	Sample type	Analyte	Reaction condition	Reference
Hydrochloride	blood	C14:0, C16:0,C16:1, C18:0, C18:1, C18:2 n-6, C18:3 n-3, C18:4 n-3, C20:1, C20:4 n-6, C20:4 n-3, C20:5 n-3, C22:1, C21:5 n-3, C22:5 n-3, C22:6 n-3	45 °C for 16 h	[44]
Hydrochloride	plasma	C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:4, C20:5, C22:5, C22:6	80 °C overnight	[82]
Hydrochloride	plasma	C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:4, C20:5, C22:0, C22:6, C24:0, C24:1	85 °C for 45 min	[83]
Acetylchloride	plasma	C14:0, C14:1, C16:0, C16:1 n-7, C18:0, C18:1 n-7, C18:1 n-9, C18:2 n-6, C18:3 n-6, C18:3 n-3, C20:0, C20:1 n-9, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:0, C22:4 n-6, C22:5 n-6, C22:5 n-3, C22:6 n-3, C24:0, C24: 1n9	100 °C for 1 h	[46]
Acetylchloride	plasma/fecal/bile	C12:0, C14:0, C14:1 (n-5), C15:0, C16:0,C16:1 (n-7), C17:0, C18:0, C18:1 (n-9), C18:2 (n-6), C18:3 (n-3), C20:0, C20:1 (n-9), C20:2 (n-6), C20:3 (n-6), C20:4 (n-6), C22:0, C22:1 (n-9), C22:4 (n-6), C22:5 (n-6), C22:6 (n-3), C24:0, C24:1 (n-9), C26:0	100 °C for 1 h	[47]
Acetylchloride	plasma/cell homogenate	C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1 (n−7), C17:0, C17:1, C18:0, C18:1 (n−7), C18:1 (n−9), C18:1 (n−9), C18:2 (n−6), C18:2 (n−6), C18:3 (n−6), C18:3 (n−3), C20:0, C20:1 (n−9), C20:2 (n−6), C20:3 (n−6), C20:3 (n−3), C20:4 (n−6), C20:4 (n−3), C20:5 (n−3), C21:0, C22:0, C22:1 (n−9), C22:2 (n−6), C22:4 (n−6), C22:5 (n−3), C22:6 (n−3), C23:0, C24:0, C24:1 (n−9), C25:0, C26:0, C28:0	95 °C for 1 h	[22]
Acetylchloride	plasma	FFA C12:0, C16:0, C16:1-c9, C17:0, C18:0, C18:1 (n-9), C18:2 (n-6), C18:3 (n-3), C20:0, C20:4 (n-6), C22:0, C24:0	overnight at 20 °C	[13]
Acetylchloride	plasma	C14:0, C15:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C20:3, C20:4, C22:6, C24:0, C24:1	100 °C for 1 h	[84]
Acetylchloride	dried blood spots	C8:0, C10:0, C10:1, C14:1,	45 min at room temperature.	[85]
Acetylchloride	plasma	C14:0, C16:0, C18:0, C20:0, C22:0, C24:0, C26:0, C14:1n-5, C16:1n-7, C18:1n-7, C18:1n-9, C20:1n-9, C22:1n-9, C24:1n-9, C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, C22:2n-6, C22:4n-6, C22:5n-6, C18:3n-3, C20:3n-3, C20:5n-3, C22:5n-3, C22:6n-3, C20:3n-9, C18:1n-9t, C18:2n-6t, C16:1n-7t, C16:1n-9, 9c11t-CLA	100 °C for 1 h	[86]
Sulfuric acid	plasma	C10:0, C12:0, C14:0, C14:1, C16:0, C16:1n-9, C18:0, C18:1 n-9, C18: 1n-11, C18:2 n-6, C18:3 n-3, C20:0, C20:1, C20:4 n-6, C20:5 n-3, C22:0, C22: 5 n-3, C22:6 n −3, C24:0	50 °C for 15 min	[50]
Sulfuric acid	serum	C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, γ-C18:3, C20:2, C20:4, C20:5, C22:5, C22:6, C24:0, C24:1	62 °C for 2 h	[87]
Sulfuric acid	plasma	FFA: C12:0, C14:0, C15:0, C16:0, C16:1n-9, C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2, C18:3n-3, C18:3n-6, C20:0, C20:3, C20:4, C20:5, C22:4, C22:5, C22:6, C24:0	70 °C for 30 min	[88]
Sulfuric acid	serum	C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, γ-C18:3, C18:3, C20:2, C20:4, C20:5, C22:5, C22:6, C24:0, C24:1	62 °C for 2 h	[89]
Sulfuric acid	serum	C12:0, C14:0, C16:0, C16:1n9 + C16:1n7, C17:0, C17:1n10, C18:0, C18:1n9t, C18:1n9c, C18:1n7, t9, t12 C18:2, c9, t12 C18:2, t9, c12 C18:2, c9, c12 C18:2, t9,t12, t15 C18:3, t9,t12, c15 C18:3 + t9,c12, t15 C18:3, c9,t12, t15 C18:3 + c9,c12, t15 C18:3, c9,t12, c15 C18:3, t9,c12, c15 C18:3, c9,c12, c15 C18:3, C20:0, C20:1n9, C20:2n6, C20:3n6, C22:0, C20:4n6, C22:2n6, C20:5n3, C22:4n6, C22:5n3, C26:0, C22:6n3	70 °C for 30 min	[90]
Sulfuric acid	plasma	C10:0, C12:0, C14:0, C16:1n-9, C16:0, C18:2, C18:1n-9, C18:1n-11, C18:0, C20:4, C20:5, C20:3, C20:2, C20:0, C22:6	70 °C for 30 min	[33]
Sulfuric acid	serum	C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2,γ-C −18:3, C18:3, C20:2, C20:4, C20:5, C22:4, C22:5, C22:6, C24:0, C24:1	62 °C for 2 h	[10]
Sulfuric acid	plasma	C14:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:3, C20:4, C21:0, C22:6, C23:0	50 °C for 18 h	[91]
Boron trifluoride	tissue	C16:0, C18:0, C18:1, C18:2, C20:4, C22:5, C22:6	90–110 °C for 1 h	[92]

Boron trifluoride	plasma	C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n9t, C18:1n9c, C18:2n6t, C18:2n6c, C18:3n6, C18:3n3, C20:0, C20:1, C20:2, C20:3n6, C20:4n6, C20:5n3, C22:0, C22:1n9, C22:4, C22:5n6, C22:5n3, C22:6n3, C23:0, C24:0, C24:1	75 °C for 45 min	[93]
Boron trifluoride	plasma	C14:0, C15:0, C16:0, C16:1n-7, C17:0, C18:0, C18:1n-9, C18:2n-6, C18:3n-3, C20:0, C20:1n-9, C20:2n-6, C20:3n-6, C20:4n-6, C20:5n-3, C22:0, C22:1n-9, C22:4n-6, C22:5n-3, C22:6n-3, C24:0, C24:1n-9	100 °C for 1 h	[94]
Boron trifluoride	plasma	C20:5n-3, C22:5n-3, C22:6n-3, C20:4n-6	100 °C for 10 min	[36]
Sodium methoxide	plasma	C14:0, C16:0, C16:1n-7, C17:0, C18:0, C18:1n-7, C18:1n-9, C18:2n-6, C18:3n-6, C18:3n-3, C20:0, C20:1n-9, C20:2n-6, C20:3n-6, C20:3n-9, C20:4n-6, C20:5n-3, C22:0, C22:4n-6, C22:5n-6, C22:5n-3, C22:6n-3, C24:0, C24:1n-9	room temperature for 3 min	[69]
Sodium methoxide	plasma	C14:0, C16:0, C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2n-6, C18:3n-6, C18:3n-3, C20:3n-6, C20:4n-6, C20:5n-3, C22:5n-3, C22:6n-3	45 °C for 5 min	[70]
Potassium hydroxide	blood	C14:0, C15:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n9c, C18:1n9t, C18:2n6, C18:3n6, C20:0, C20:1, C20:2, C20:3n3, C20:4n6, C22:6n3	50 °C for 20 min	[96]
Potassium hydroxide	plasma	C12:0, C14:0, C15:0, C16:0, C16:1n-9, C16:1n-7, C18:0, C18:1n-7, C18:1n-9, C18:2, C18:3n-3, C18:3n-6, C20:0, C20:3, C20:4, C20:5, C22:4, C22:5, C22:6, C24:0	room temperature for 10 min	[88]
Potassium hydroxide	serum	C12:0, C14:0, C16:0, C16:1n9 + C16:1n7, C17:0, C17:1n10, C18:0, C18:1n9t, C18:1n9c, C18:1n7, t9, t12 C18:2, c9, t12 C18:2, t9, c12 C18:2, c9, c12 C18:2, t9,t12, t15 C18:3, t9,t12, c15 C18:3 + t9,c12, t15 C18:3, c9,t12, t15 C18:3 + c9,c12, t15 C18:3, c9,t12, c15 C18:3, t9,c12, c15 C18:3, c9,c12, c15 C18:3, C20:0, C20:1n9, C20:2n6, C20:3n6, C22:0, C20:4n6, C22:2n6, C20:5n3, C22:4n6, C22:5n3, C26:0, C22:6n3	room temperature for 10 min	[90]
Trimethylsulfonium hydroxide	plasma	C8:0, C10:0, C12:0, C13:0, C14:0, C14:1, C15:0, C16:0, C16:1, C17:0, C18:0, C18:1, C18:2, C18:3, C20:1, C20:2, C20:3, C20:4, C20:5, C22:5, C22:6	room temperature for 2 min	[75]
Pentafluorobenzyl bromide	salivary	C12:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C19:0, C20:0, C20:3, C20:4, C22:0	room temperature for 30 min	[79]
Pentafluorobenzyl bromide	plasma/serum/red blood cells	C14:0, C14:1n-5, C16:0, C16:1n-7, C16:1n-7t, C18:0, C18:1n-7, C18:1n-7t, C18:1n-9, C18:1n-9t, C18:2n-6,9, C18:2n-6t,9t, C18:3n-3,6,9, C18:3n-6,9,12, C20:0, C20:1n-9, C20:2n-6,9, C20:3n-6,9,12, C20:4n-6,9,12,15, C20:5n-3,6,9,12,15, C22:0, C22:4n-6,9,12,15, C22:5n-6,9,12,15,18, C22:5n-3,6,9,12,15, C22:6n-3,6,9,12,15,18, C24:0, C24:1n9	room temperature for 15 min	[97]
Pentafluorobenzyl bromide.	plasma	C12:0, C14:0, C16:0, C16:1ω7, C16:1ω9, C18:0, C18:1ω7, C18:1ω9, C18:2ω6, C18:3ω3, C18:3ω6, C20:0, C20:3ω6, C20:3ω9, C20:4ω6, C20:5ω3, C22:1ω9, C22:4ω6, C22:5ω3, C22:5ω6, C22:6ω3, C24: 1ω9	room temperature for 30 min	[98]
Pentafluorobenzyl bromide.	plasma	C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3	60 °C for 12 min	[99]

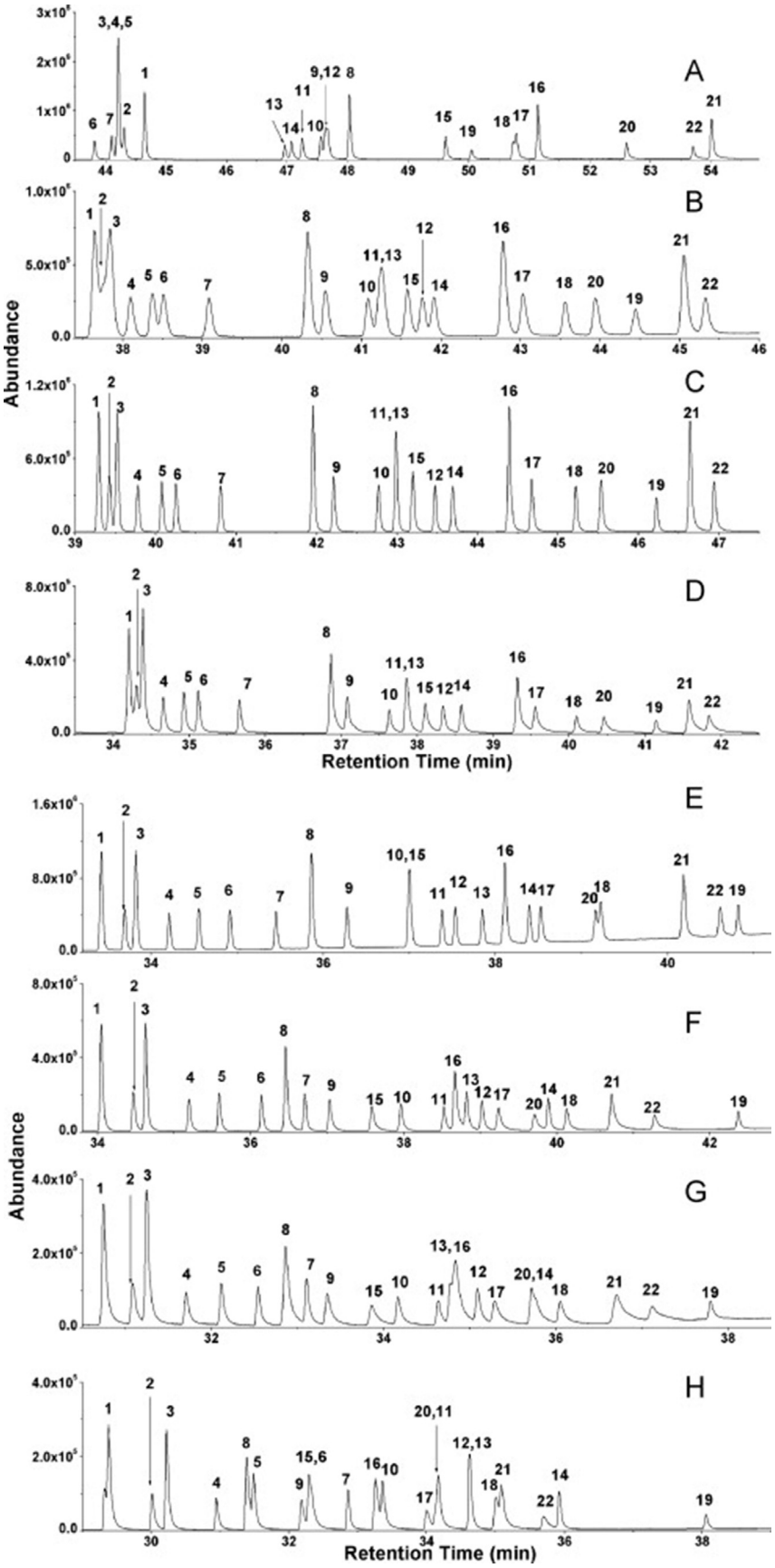

Fig. 1 – A comparison of the separation performances of different columns. Total ion chromatograms of the C18 to C24 region by using (A) 5 m s (B) IL59 (C) IL60 (D) IL61 (E) IL76 (F) IL82 (G) IL100, and (H) IL111 columns (This figure is reprinted from Ref. [104] with permission.)

Table 2 – List of the peaks illustrated in Fig. 1 (This table is reprinted from ref. [104] with permission.)

Peak no.	FAME compounds	Abbreviations
1	Stearic acid	C18:0
2	Elaidic acid	t9-C18:1
3	Oleic acid	c9-C18:1
4	Linolelaidic acid	t9,t12-C18:2
5	Linoleic acid	c9, c12-C18:2
6	γ-Linolenic acid	c6,c9,c12-C18:3
7	α-Linolenic acid	c9,c12,c15-C18:3
8	Arachidic acid	C20:0
9	cis-11-Eicosenoic acid	c11-C20:1
10	cis-11,14-Eicosadienoic acid	c11,c14-C20:2
11	cis-8,11,14-Eicosatrienoic acid	c8,c11,c14-C20:3
12	cis-11,14,17-Eicosatrienoic acid	c11,c14,c17-C20:3
13	Arachidonic acid (AA)	c5,c8,c11, c14-C20:4
14	cis-5,8,11,14,17-Eicosapentaenoic acid (EPA)	EPA
15	Heneicosanoic acid	C21:0
16	Behenic acid	C22:0
17	Erucic acid	c13-C22:1
18	cis-13,16-Docosadienoic acid	c13,c16-C22:2
19	cis-4,7,10,13,16,19-Docosahexaenoic (DHA)	DHA
20	Tricosanoic acid	C23:0
21	Lignoceric acid	C24:0
22	Nervonic acid	c15-C24:1

show that the *cis* and *trans* isomer of C18:2 could be baseline separated on SLB-IL 59 and SLB-IL60. Moreover, this study included SLB-IL 65 and SLB-IL 111 as well; in the four evaluated SLB-IL columns (SLB-IL 59, SLB-IL 60, SLB-IL 65, and SLB-IL 111) only SLB-IL 65 failed to separate the C18:2 isomer [106]. To summarize, high-polarity columns such as HP-88 column and DB-FFAP column are capable to separate fatty acids with different carbon chain length. Ionic liquid series columns (especially SLB-IL82, SLB-IL 110, SLB-IL111) are especially useful for separating fatty acid isomers.

3.1. *Multidimensional GC*

Multidimensional GC (MDGC) approaches, such as two-dimensional GC (GC×GC) and heart-cut MDGC, have recently attracted substantial interest. MDGC could provide greater resolving power and enhance peak capacity and sensitivity. The GC×GC technique has been used for determining fatty acid components in several types of samples, including cultured mammalian cells, animal tissue samples and lanolin [35,107,108]. Zeng et al. demonstrated an integrated GC system incorporating GC×GC and MDGC for analyzing fatty acids in fish oil and dairy milk fat samples [109]. They applied different IL columns using MDGC to increase the number of isomeric compounds identified. Compared to conventional 1D GC systems, this approach could provide more reliable data with a relatively shorter analysis time. Payeur et al. used GC×GC system to identify fatty acid composition in insulin secreting cells, and their results show that this system could largely increase the number of identified fatty acids [35]. To summarize, MDGC strategies have many advantages such as shorter analytical time and improved separation which could additional facilitate FA identification It is anticipated that these techniques would be beneficial for fatty acid profiling in complex biological samples.

4. Fatty acid internal standards

To acquire accurate and precise quantification results, internal standards are commonly used in biomedical analysis. Since fatty acids are diverse compounds, using stable isotope internal standards for each analyte is not cost effective and the relevant compounds may not even be commercial available. In previous studies, fatty acids with an odd number of carbons (such as C13:0, C17:0, C19:0 and C23:0) were frequently applied as the internal standards [37,48,76,110]. These fatty acids are not endogenous compounds and thus could be added during the sample preparation steps and used to correct potential variations in the experiments.

5. Fatty acid analysis in biological samples

Fatty acids play important roles in many biological systems. Many studies have analyzed the fatty acid levels in various biological samples, such as plasma, red blood cells, sweat, and saliva. In the following section, we discuss several previous studies that measured fatty acid levels in different types of sample matrices, and list several dysregulated fatty acids in different biological samples and the corresponding diseases in Table 3.

5.1. *Plasma samples*

Plasma is the most frequently used sample type in biomedical studies on fatty acids. Abdelmagid et al. analyzed 61 different kinds of fatty acids in a large cohort (n = 826), and their results provide foundational knowledge regarding a broad panel of circulating fatty acids, which may be helpful for further fatty acid-related biomedical studies [111]. Previous studies have

Table 3 – Selected examples of dysregulated fatty acids in biological samples and the associated diseases.

Disease	Dysregulated fatty acid	Sample	Reference
Breast cancer	C14:0, C16:0, C18:0, C18:2, C18:3 C20:5	serum	[10]
Breast cancer	C16:0, C18:0, C18:1n9c, C18:2n6, C20:0, C20:4n6, C22:0, C22:6n3, C24:0, C24:1n9	plasma	[80]
Breast cancer	C14:0, C17:0, C18:1, C20:0	serum	[113]
Breast cancer	C18:2w6, C18:1n-7	serum	[114]
Prostate cancer	long-chain ω-3 polyunsaturated fatty acids (20:5ω3; 22:5ω3; 22:6ω3)	plasma	[115]
Prostate cancer	C16:1n-7	blood	[116]
Colorectal cancer	PUFAs	plasma	[117]
Colorectal cancer	PUFAs, C18:3n3, C18:2n6	plasma	[118]
Lung cancer	FFA: C20:4n6, C18:2n6	serum	[8]
Lung cancer	FFA: C16:1, C18:3, C18:2, C18:1, C20:4, C22:6	serum	[119]
Pancreatic cancer	n-3 fatty acid	plasma	[120]
Multiple myeloma	saturated and n-6 polyunsaturated fatty acids	plasma	[121]
Normal aging and neurodegenerative diseases	C22:6n-3	blood	[122]
Alzheimer's disease/ Dementia/Cognitive impairment	C20:5n-3, C22:6n-3, total n-3 fatty acids	plasma	[11]
Alzheimer's disease	C14:0, C16:0, C18:1, C18:3, C22:6	serum	[87]
Alzheimer's disease/Mild cognitive impairment	FFA: oleic acid isomers and omega-6 fatty acids omega-3 fatty acids	plasma	[123]
Metabolic syndrome	C14:0, C16:0, C16:1n-7, C18:2n-6	plasma	[112]
Metabolic syndrome	C16:1n-7, C20:4n-6, C22:5n-6	plasma	[124]
Diabetes	mega-6 polyunsaturated fatty acids (n-6PUFA), omega-3 polyunsaturated fatty acid (n-3PUFA), C24:0	blood	[125]
Diabetes	C16:0, C18:0, C18:1n-9	plasma	[88]
Diabetes	C10:0, C14:0, C16:1n-9, C16:0, C18:2, C18:1, C18:0, C20:4, C20:5, C20:3, C20:2, C22:6 FFA: C10:0, C16:0, C18:2, C18:1, C18:3, C18:0, C20:4, C20:3, C20:2, C20:0, C22:6	plasma	[33]
Diabetes	FFA: C18:1, C18:2, C18:3	serum	[89]
Heart failure	FFA	plasma	[126]
Cirrhotic	monounsaturated FA, n-6 polyunsaturated FA, n-3 polyunsaturated FA,	plasma	[127]
Liver disease	the sums of nonessential/essential fatty acids,(n-7+n-9)/(n-3+n-6)	plasma	[128]
Nonalcoholic steatohepatitis	C20:4, C22:6	plasma	[129]
Ischemic stroke	palmitoleic acid, linoleic acid	plasma	[130]
Dengue fever	C14:0, C15:0, C16:1, C16:0, C18:3n6, C18:2n6, C18:1n9, C18:0, C20:4n6, C20:3n3, C20:2, C22:6n3	blood	[96]
Inflammatory	omega-3 fatty acid	red blood cell	[132]
Schizophrenia	PUFA	red blood cell	[138,139]
Schizophrenia	C22:5n3, C22:6n3 and C20:4n6	red blood cell	[134]
Sjogren's syndrome	FFA	saliva	[141]

discovered that fatty acid levels in plasma are closely related to many diseases, such as metabolic syndromes, several chronic diseases, Alzheimer's disease, and cancer. In Table 3, although various biological samples have been used to study the relationship between dysregulated fatty acids and the disease, plasma is still the most commonly used biological sample. Table 3 summarizes the dysregulated fatty acids in plasma/serum/blood samples and the corresponding diseases [8,10,11,33,80,87–89,96,112–130]. Jordi et al. showed that relative to healthy controls, higher levels of C14:0, C16:0, and C16:1n-7 and lower levels of C18:2n-6 were observed in people with metabolic syndromes [112]. Lv et al. indicated that the concentrations of C14:0, C16:0, C18:0, C18:2, C18:3 and C20:5 were significantly different between breast cancer patients and healthy controls [10].

5.2. *Red blood cell (RBC) samples*

Recently, many studies have investigated the fatty acid profiles of red blood cells [131–134]. Compared to plasma samples, the fatty acid compositions of red blood cell membranes could reflect longer-term (up to 2–3 month) dietary intake [135–137]. It has been found that the fatty acid profiles of red blood cells are related to inflammation and several mental diseases, such as schizophrenia and autism spectrum disorders. Fontes et al. observed modest inverse associations between the levels of omega-3 fatty acid in the RBCs and several inflammatory biomarkers [132]. Many studies have observed the depletion of polyunsaturated fatty acids (PUFA) in erythrocytes of schizophrenia patients [138,139]. Hoen et al. performed a meta-analysis on the relationship between the

PUFAs measured in erythrocyte cell membranes and schizophrenia and found that decreased levels of C22:5n3, C22:6n3 and C20:4n6 are associated with schizophrenia [134]. Bystrická et al. reviewed the GC-based analytical approaches for fatty acid analysis in human erythrocyte membranes [140]. This review summarized the analytical strategies for erythrocyte membranes, including erythrocyte membrane isolation, lipid extraction, fatty acid derivatization and GC analysis.

5.3. *Saliva samples*

The concentrations of FFAs in saliva are thought to be associated with several diseases, such as cystic fibrosis and Sjogren's syndrome [141]. Kulkarni et al. used the PFB-Br derivatization method to analyze salivary FFAs. They identified 16 FFAs in human saliva samples and mentioned that the fatty acids in the saliva included four major FFAs, C16:0, C18:2, C18:1, and C18:0 [79]. Moon et al. used N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) to derivatize fatty acids in relatively small volumes (100 μL) of saliva samples [142]. In their study, C12:0 and C14:0 were quantified in all samples, and C16:1, C18:0, C18:1, and C18:2n6 could be quantified in >40% of saliva samples. This optimized and validated method could be used to investigate the FFA levels in small volumes of saliva.

6. Conclusion

Fatty acids play important roles in many biological systems, and the dysregulation of fatty acids is associated with many diseases. Accurate and efficient analytical methods are essential for elucidating the mechanism of fatty acid dysregulation-associated diseases and advancing the use of these fatty acids as clinical therapeutic markers. This review has summarized the commonly used GC-MS-based analytical strategies for fatty acid analysis and their applications in analyzing biological samples. There is no perfect approach for all kinds of fatty acids and sample types. Not only the sample type but also the properties of the target analyte must be considered when developing analytical methods. More sophisticated analytical strategies for fatty acid analysis are anticipated to provide a more comprehensive understanding of the biological functions of these compounds and increase their clinical usage.

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