2 Chromatography–mass spectrometry based analysis of biologically active endogenous carboxylic acids

Based on:
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Accepted in Trends Analyt Chem.
Chapter 2

2.1 Abstract

Carboxylic acids, such as small carboxylic acids, fatty acids, eicosanoids and bile acids are involved in numerous biological processes. The mapping of the participating pathways potentially yields great insight into the physiological state of an organism. There is an urge for more sensitive, faster and easier to handle analysis techniques. These would enable advanced analysis and validation in larger clinical cohorts and hopefully deliver useful disease markers. The analysis of carboxylic acids is not only driven by the carboxylic acid group, but is largely dependent on chain length and other functional groups present. Several LC and GC strategies, with or without derivatisation have been developed in recent years. Here, we review the most recent trends in endogenous carboxylic acid analysis by chromatography–mass spectrometry. We critically evaluate sample preparation, derivatisation techniques, separation and mass spectrometric detection. Ultimately, the reader is guided to the most versatile, sensitive and facile analytical methods.

2.2 Introduction

One of the most interesting developments in the biomedical sciences of the post-genomic era is a “paradigm shift” in our understanding of the biological/regulatory activity of the chemical entities for, which such a function was disregarded. The compounds that traditionally were seen only as the constituents of energy metabolism (e.g. pyruvate, succinate, fatty acids) or emulsifiers of lipids (bile acids) are being more and more recognized as important immune-modulatory and/or signalling molecules. In the first approximation, all those compounds appear to be far too structurally divers for being reviewed together, but taking a broader view, one will realize that most of those “upcoming regulatory compounds” are endogenous
carboxylic acids (CAs). Their common functional features are the presence of a CA function, dictating many but not all of their physicochemical properties, and an alkyl part which can be decorated with different functional groups. One can distinguish four important subclasses of endogenous CAs, namely: (A) small CAs, crucial to aerobic respiration and energy metabolism\(^1\), (B) fatty acids (FAs), fundamental to energy storage, membrane formation and involved in numerous physiological processes such as inflammation\(^2\)-\(^4\), (C) eicosanoids and docosanoids, forming a class of highly important signalling molecules during several inflammatory and immunological events\(^5\), and (D) bile acids (BAs), being the main metabolites of endogenous cholesterol\(^6\) (see Figure 1 for their general structures). Recent advances in analytical techniques for the analysis of CAs are discussed here.

**Figure 1**
General structures of the four relevant classes of CAs discussed in this paper: small CAs, fatty acids, eicosanoids, and bile acids

All of these analytes reside in the body and can be quantitatively analysed in one of the body fluids or in cellular extracts. Their isolation and separation from matrix components is fundamental to their analysis. While sample preparation can be
distinctly different for the four CA subclasses, analyte separation is commonly performed by either gas chromatography (GC) or liquid chromatography (LC). Both separation techniques are nowadays frequently combined with mass spectrometric (MS) detection for highest selectivity and sensitivity in quantitative analysis. Both GC–MS and LC–MS feature common requirements owing to the functional groups present in the analytes of interest. Analysis of endogenous CAs is done with different aims, e.g., (I) qualitative profiling of CAs present, thus involving structure elucidation and resolving isomerism issues, (II) relative quantitation for comparison of physiological states and/or flux determination, involving the use of isotopologues, and (III) targeted (absolute) quantitative analysis. The aims of the study determine the analytical strategy chosen, but in all cases general issues in the analysis of CAs are important as well.

2.3 General aspects

All four analyte subclasses share the presence of a CA function, i.e., they are weak organic Brønsted-Lowry acids. However, other features shared by the CAs might also have significant effects on the analytical process and thus demand rather similar analytical solutions. Figure 2 gives a schematic overview of the most prominent functional groups that may be present in an endogenous CA.
Figure 2
Overview of relevant functional groups that may be present and influence the analysis of endogenous CAs
2.3.1 **GC–MS analysis**

Intrinsically, GC–MS analysis demands the evaporation of the analyte under investigation prior to separation and detection. With respect to CAs, several functional groups demand attention. Firstly, the CA function has to be derivatised for successful GC–MS analysis. From the various possible derivatisation strategies, silylation and esterification of the CA function are the most prominent ones. Other crucial functional groups (Figure 2) that have to be derivatised prior to GC–MS analysis are: (A) hydroxyl groups, which can either undergo silylation or ether formation, and (B) ketone groups, which are frequently converted into oximes, thereby blocking possible tautomerism during subsequent derivatisation as well as stabilizing particularly α- and β-keto CAs. After derivatisation, the CA derivatives can be analysed in routine GC–MS systems, involving common dimethylsiloxane-coated capillary columns and wide temperature gradients. Generally, analyte ionization is achieved by electron ionization (EI), although electron-capture negative ionization (ECNI) has been used as well.

Functional groups at the side chain in many cases lead to the formation of a stereo-centre. In these cases, either chiral derivatisation yielding diastereomers or the use of chiral GC columns form possible solutions. The degree of unsaturation may influence GC–MS analysis in different ways. Compounds with higher degrees of unsaturation are more prone to autoxidation. Fragmentation of highly unsaturated CAs in EI is mainly driven by double bonds, limiting the usefulness of the obtained spectra. The most important derivatisation strategies for CAs in GC–MS analysis are summarized in Table 1.
Table 1
Overview of important functional groups in endogenous CAs that require derivatisation in GC–MS and widely applied derivatisation strategies and reagents for this.

<table>
<thead>
<tr>
<th>Derivatisation reagent</th>
<th>Reaction</th>
<th>Functional group</th>
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<tbody>
<tr>
<td>N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA)</td>
<td>Trimethylsilylation</td>
<td>Carboxylic acid</td>
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<tr>
<td>Bis(trimethylsilyl)acetamide (BSA)</td>
<td>t-butylmethyldimethylation</td>
<td></td>
</tr>
<tr>
<td>N,O-Bis(trimethylsilyl) trifluoroacetamide (BSTFA)</td>
<td>Methyl ester formation</td>
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<tr>
<td>N-methyl-N-tert-butylimidysilyl trifluoroacetamide (MtBSTFA)</td>
<td>Esterification</td>
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<tr>
<td>Dimethylsulfoxonium hydroxide (TMSH)</td>
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<td></td>
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<tr>
<td>Methanol / inorganic acid</td>
<td></td>
<td></td>
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<tr>
<td>Methanol / Acyl chloride</td>
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<tr>
<td>Alcohol / BF₃</td>
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<tr>
<td>Pentafluorobenzyl bromide (PFBBr)</td>
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<tr>
<td>Picolinyl esters (3-Pyridylcarbinol esters)</td>
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<tr>
<td>4,4-dimethyloxazoline (DMOX)</td>
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<td>Diazomethane</td>
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<tr>
<td>Mosher’s acid chloride (S-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride)</td>
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<tr>
<td>Hydroxylamine</td>
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<tr>
<td>Methoxylamine</td>
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<tr>
<td>Oximation</td>
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</tbody>
</table>
2.3.2 LC–MS analysis

The most widely applied LC mode for CAs is ion-suppressed reversed-phase LC (RPLC), using methanol/water or acetonitrile/water gradients, frequently using acetic or formic acid as additives. In LC–MS, underivatised CAs must be analysed in negative-ion mode using electrospray ionization (ESI) or atmospheric-pressure chemical ionization (APCI). In some LC–MS instruments, negative ionization seems to be less efficient than positive ionization. Furthermore, most CA subclasses do not provide readily applicable intense fragment ions upon collision-induced dissociation (CID) to be applied in the selected-reaction monitoring (SRM). Quantitative analysis using LC–MS is mostly performed in SRM mode using tandem-quadrupole (TQ) or quadrupole–linear-ion-trap hybrid (QTRAP™, Q–LIT) instruments. The latter provides enhanced full-spectrum sensitivity in MS–MS as well. Underivatised CAs upon CID may primarily show the loss of CO\(_2\), i.e., [M-H-CO\(_2\)]\(^-\), and of H\(_2\)O from alcohol or ketone functions in the molecule, if present. Other fragmentation of CAs is largely influenced by functional groups, for instance double bonds, present in the alkyl part of the molecule. As a rule of thumb, it can be stated that the higher the degree of unsaturation and the higher the number of functional groups present in a CA, the easier is its fragmentation and the easier is the formation of fragments not related to the loss of CO\(_2\).

Although for LC–MS analysis of CAs derivatisation is not always necessary, there are a number of reasons to perform derivatisation of CAs also in LC–MS\(^{15,16}\). Derivatisation may facilitate the RPLC separation of CAs and may also direct fragmentation and yield characteristic other neutral losses, related to the derivatisation reagent, applicable in SRM.
Separation of chiral CAs for LC–MS analysis can be accomplished by either the use of chiral stationary phases, or chiral derivatisation. The usage of chiral stationary phases has long been associated with the use of normal-phase separation systems being less compatible with LC–MS. However, chiral RPLC separations can be achieved using for example amylose tris(3,5-dimethylphenylcarbamate) coated ChiralPak AD-RH columns\textsuperscript{12}. Chiral derivatisation for RPLC can be successful if the chiral centres are in close proximity, otherwise the resulting diastereomers might not be separable\textsuperscript{11}.

### 2.3.3 Sample pre-treatment

All subclasses of CAs may be analysed in body fluids, e.g., plasma, serum, urine, cerebrospinal fluid (CSF), while especially small CAs may also be analysed in cellular extracts. For GC–MS, analyte extraction to an organic solvent is required prior to derivatisation. This can be achieved by (ion-suppressed) liquid-liquid extraction (LLE), using solvents like ethyl acetate or \textit{n}-hexane. For LC–MS, protein precipitation is performed for blood-related samples, eventually followed by a sample clean-up step using LLE or solid-phase extraction (SPE), using RPLC or mixed-mode materials. Removal of endogenous phospholipids is important to reduce matrix effects in LC–MS. The combination of highly polar groups and hydrophobic alkyl chains may present challenges to analyte recovery in LLE or SPE. For urine analysis, either dilute-and-shoot procedures\textsuperscript{13} or clean-up using LLE or SPE is performed. In the analysis of small CAs in cellular extracts, quenching of the cellular metabolism is important, requiring specialized protocols\textsuperscript{14}. 
2.3.4 **Use of internal standards**

If absolute quantitation is to be achieved, the use of stable-isotope-labelled (SIL) internal standards (ISs) is crucial for both GC–MS and LC–MS analysis of CAs. Although D\textsubscript{n}-labelled ISs can be used, there is a risk of D/H-exchange during sample pre-treatment in acidic or alkaline media\textsuperscript{15}. Therefore, [\textsuperscript{13}C\textsubscript{n}]-labelled ISs are generally preferred. SIL-ISs can be produced by organic synthesis. In the analysis of cellular extracts, the use of mass isotopomer ratio analysis of uniformly-[\textsuperscript{13}C\textsubscript{n}]-labelled extracts (MIRACLE)\textsuperscript{16}, based on the biosynthesis of SIL-ISs in yeast-cell cultures grown on [\textsuperscript{13}C\textsubscript{6}]-glucose, is a powerful tool. Other approaches involve stable isotope coding by derivatisation\textsuperscript{17} or quantification by standard addition.

2.4 **Small carboxylic acids with less than 6 carbon atoms**

The subclass of small CAs consists of short-chain FAs (≤6 carbons in the aliphatic tail) and their hydroxylated and/or ketone containing analogues\textsuperscript{1,8}. Both mono-, di-, and tri-CAs are among this group. Prominent examples of this subclass are the intermediates of the Krebs’s or tricarboxylic acid (TCA) cycle and the important clinical markers D-lactic acid\textsuperscript{18} and methylmalonic acid\textsuperscript{8} (see Figure 3). Recent interest in small CAs has largely been boosted by translational research into metabolic phenomena such as the Warburg effect\textsuperscript{19} and autophagy\textsuperscript{1}. Therefore, the current interest in the analysis of small CAs is likely to grow. General pitfalls include: their low molecular weight, their high polarity, their limited stability, leading to challenges in their extraction from aqueous matrices and, for some, volatility issues\textsuperscript{1,20}. The importance of these issues depends on the analytical technique applied. There is a general stability problem with keto CAs\textsuperscript{21}, e.g., β-keto CAs very readily undergo decarboxylation, as a preferred six-membered transition state can be formed\textsuperscript{22}. 
2.4.1 GC–MS analysis of small carboxylic acids

Derivatisation is crucial for successful GC–MS analysis of small CAs. Esterification of small CAs with small alcohols is generally not successful due to the high volatility of such derivatives. Larger alcohols could be used, but generally require the use of rather harsh reaction conditions, involving catalysts like anhydrous sulfuric acid or...
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boron trifluoride\textsuperscript{23}. In this respect, silylation seems to be a better choice, as less volatile, higher molecular-weight derivatives are formed. As modern analytical strategies tend to move more and more towards comprehensive multi-component analysis, the derivatisation protocols to be used have to become increasingly generic\textsuperscript{24}. A frequently applied approach consists of a combination of oximation and silylation. Oximation using methoxylamine or hydroxylamine derivatises the ketone groups, if present, whereas trimethylsilylation or \textit{t}-butyldimethylsilylation\textsuperscript{24,25} modifies both the hydroxyl and the CA groups into trimethylsilyl (TMS) and \textit{t}-butyldimethylsilyl (tBDMS) derivatives, respectively. Typical reaction conditions comprise oximation in a solution of the corresponding alkoxylamine hydrochloride in pyridine at a concentration of typically 20 mg/mL at 30 °C for 90 min followed by silylation using MtBSTFA at 70 °C or MSTFA at 37 °C for 30 min\textsuperscript{25}.

In EI-MS, TMS derivatives yield abundant M–CH\textsubscript{3}● ions with \textit{m}/\textit{z} \textit{M}^{+}–15 as well as relatively abundant non-specific ions with \textit{m}/\textit{z} 73, due to (CH\textsubscript{3})\textsubscript{3}Si●, and \textit{m}/\textit{z} 75, due to (CH\textsubscript{3})\textsubscript{2}Si=OH●. The most prominent fragment of the tBDMS derivatives in EI-MS usually is M–(CH\textsubscript{3})\textsubscript{3}C● with \textit{m}/\textit{z} \textit{M}^{+}–57, together with some low-abundance fragments\textsuperscript{26}. Whereas the derivatisation of CAs using MtBSTFA is a straightforward reaction\textsuperscript{26}, the reaction of hydroxyl groups with MtBSTFA is less favourable and thus may lead to partial derivatisation and skewing of the results\textsuperscript{27}.

Prior to derivatisation, the (highly) hydrophilic small CAs should be extracted from the usually aqueous sample matrix into an appropriate organic solvent, e.g., diethyl ether, methanol or ethanol. Given the volatility of some CAs and the limited stability of the keto CAs, temperatures should be kept as low as possible throughout the sample pre-treatment procedure. Generic protocols have recently been described for extraction from cellular incubations and body fluids. Extraction of small
CAs, among other cellular metabolites, from mammalian cells involves quenching using liquid nitrogen and extraction using a methanol/chloroform mixture\textsuperscript{28}. For body fluids such as plasma or urine, quenching of the metabolic reactions is generally less of a concern. Protein precipitation with methanol\textsuperscript{29} has become the gold standard for wide-range analysis of low molecular-weight analytes, including small organic CAs, in plasma, eventually in combination with SPE if more targeted methods for a limited number of analytes are aimed at. For urine analysis, the sample pre-treatment usually comprises of an eventual urease step followed by freeze drying, reconstitution in an organic solvent, and derivatisation\textsuperscript{24}.

Metabolic fluxes in melanoma cell lines using oximation and tBDMS ester formation were recently investigated by Scott et al.\textsuperscript{30}. The effects of valproic acid in children were studied by urinary analysis of small CAs\textsuperscript{31}. Urine samples were directly oximated using hydroxylamine and sodium hydroxide, the analytes extracted by LLE and further derivatised by silylation using BSTFA, prior to GC-MS analysis.

### 2.4.2 LC–MS analysis of small carboxylic acids

LC seems to be the method-of-choice for the analysis of small CAs, being highly polar compounds. At first, no derivatisation seems to be required. In practice, the situation is somewhat more complicated. In RPLC, the small CAs generally show insufficient retention. Therefore, the use of ion-pairing agents like tetrabutylamine (TBA) have been proposed\textsuperscript{32}. However, this leads to substantial ionization suppression in ESI-MS and is detrimental to the equipment used\textsuperscript{33}. As an alternative, methods based on either hydrophilic interaction chromatography (HILIC)\textsuperscript{34} or anion-exchange chromatography (HPAEC)\textsuperscript{35} have been proposed. In the latter case, post-column electrolytic suppressors are required for the removal of high salt concentrations applied\textsuperscript{36}. Performance comparison of various column chemistries for
HILIC, e.g., aminopropyl, amide, cyano, diol, or silica\textsuperscript{37}, as well as their comparison with RPLC, for the analysis of small CAs has been reported\textsuperscript{34, 38}. Although HILIC with aminopropyl or diol columns appear to be most successful, it seems difficult to select an LC phase system especially directed at small CAs.

The indicated problems in both LC separation and MS detection lead to reconsidering pre-column derivatisation of the small CAs\textsuperscript{39}. The use of $N$-methyl-2-phenylethanamine (MPEA) after carbodiimide activation has been applied to TCA cycle intermediates\textsuperscript{20}. The analytes were derivatised with 3-(ethyliminomethyleneamino)-$N,N$-dimethyl-propan-1-amine hydrochloride salt (EDC) and MPEA at 60 °C for 45 min in 90% ACN. After dilution with water, the sample could be directly analysed by online SPE–LC–MS. Even though derivatisation might advance the analysis of small CAs, the instability of particularly the keto CAs might hamper successful analysis of these species.

For sample pre-treatments aimed at LC–MS, similar protocols are used as for GC–MS, involving freeze drying with urine, protein precipitation with plasma, eventually complemented by SPE\textsuperscript{24, 40}. In the analysis of cellular metabolites, combined quenching and extraction methods are needed\textsuperscript{41}.

A recent example identifying succinate as an inflammatory signal in innate immunity was reported by Tannahill et al.\textsuperscript{42}. The authors applied several LC–MS platforms with different HILIC separations; for succinate analysis the one based on a zwitter ionic (ZIC) HILIC column was used.
2.5 Fatty acids

FAs are mono-CAs with a long-chain aliphatic end. In mammals, straight-chain FAs with normally an even carbon number are observed, whereas in bacteria also branched alkyl chains and/or higher levels of odd carbon numbered FAs occur. One distinguishes short-chain (≤6 C atoms, i.e., the small CAs in this paper, Section 3), medium-chain (6–12 C atoms), long-chain (12–22 C atoms), and very-long-chain FAs (>22 C atoms). The aliphatic chain may contain several double bonds. FAs with a degree of unsaturation of two or higher are frequently called poly-unsaturated fatty acids (PUFAs). Each double bond may be either E or Z (trans or cis); a PUFA with three double bonds could theoretically form 8 E/Z-isomers. PUFAs formed biochemically usually show all-Z (all-cis) configurations. The ω(n)-nomenclature is applied to indicate the position of the first double bond relative to the aliphatic end rather than relative to the CA end (IUPAC). Besides double bonds, FAs might also contain ketone, hydroxyl, hydroperoxide, epoxide and other functional groups. Each of these functionalities put specific demands to the analytical strategies, which cannot be discussed in detail here. We focus on FAs, keto FAs, and mono-hydroxylated FAs, the latter being the biochemical precursors of certain eicosanoids and docosanoids. Hydroxyl groups usually lead to a stereo-centre in the FA side chain; biochemically formed hydroxylated FAs normally pose the S-configuration, whereas autoxidation products are racemic mixtures. The oxidative stability is a major concern in PUFA analysis. Until recently, FAs were primarily analysed by GC–MS, but currently also LC–MS methods are frequently reported.

Depending on the application, either free FA (f-FA) or total FA (t-FA) content is to be determined. f-FA determination requires an appropriate extraction method, e.g., using LLE with n-hexane, i-octane or a similar solvent, without affecting the FAs.
bound in triglycerides (phospholipids and other storage forms) or to, for instance, proteins. For t-FA determination, a saponification step must be performed, mostly under alkaline conditions. Care must be taken to avoid autoxidation and double-bond isomerization. Because of the risk of D/H-exchange, Dₙ-SIL-ISs can only be added after saponification\textsuperscript{15}. Saponification and extraction can be combined with esterification in a process called transesterification, which is carried out by acid-catalysed methylation usually by the use of methanol, hexane and acetylchloride\textsuperscript{44}, thus yielding fatty acid methyl esters (FAMEs), which can be analysed by GC–MS.

### 2.5.1 GC–MS analysis of fatty acids

LLE of f-FAs from a biological matrix yields the FAs in non-polar organic solvent. The samples can be subjected to derivatisation either directly or after drying under a stream of nitrogen or in a SpeedVac\textsuperscript{45}. Similar to small CAs, the most favourable derivatisation methods are esterification and silylation\textsuperscript{11}. The formation of FAMEs is the most prominent derivatisation strategy for GC–MS\textsuperscript{46}. While PFBBr and silyl-ester derivatives are frequently separated on standard phenyl-polysiloxane columns, cyanopropyl polysilphenylsiloxane columns have become the standard GC columns for FAME analysis. A recent application involves acetylchloride based transesterification incubating the samples overnight at room temperature, thereby overcoming acid induced E/Z isomerization, and the separation of positional and geometrical FAME isomers\textsuperscript{46}. However, FAMEs tend to provide excessive fragmentation in EI-MS with the ion with \(m/z\) 74, i.e., \(\text{CH}_2\text{C(OH)OCH}_3\)\textsuperscript{**}, resulting from a McLafferty rearrangement, being the most abundant ion 10. As the ion with \(m/z\) 74 is a class-specific and not a
compound-specific fragment, it cannot be used in isotopologue analysis (\(^{13}\)C-flux determination), as most of the molecular information is lost.

A number of alternative derivatisation strategies have been described\(^{47}\), including the formation of TMS or \(t\)BDMS derivatives\(^{26}\), picolinyl esters\(^{48}\), and DMOX\(^{49}\) derivatives. The latter can also be used for double-bond localization and branching analysis\(^{49}\). Derivatisation using PFBB\(\text{Br}\) enables the use of ECNI in GC–MS, which provides highly selective and mild ionization, i.e., dissociative electron capture to generate predominantly \([M–PFB]^–\) ions without much further fragmentation, thus facilitating isotopologue analysis\(^{50}\). Recently, an overview of the use of stable isotopes in studying lipid metabolism was published\(^{51}\). High-resolution GC is crucial for the differentiation of E/Z isomers\(^{46}\). Another important topic is the determination of double-bond positions, which can be achieved in different ways, e.g., specific derivatisation agents such as picolinyl esters or DMOX derivatives\(^{52}\), from careful interpretation of the fragmentation observed in EI mass spectra\(^{10}\), or by using covalent adduct chemical ionization tandem MS (CACI–MS–MS) using acetonitrile in ion-trap instruments\(^{53}\).

2.5.2 LC–MS analysis of fatty acids

The general interest in LC–MS and especially the introduction of ultra-high-performance LC (UHPLC) has boosted the developments in FA analysis by LC–MS rather than GC–MS\(^{54}\). Unless derivatisation is performed, FAs are analysed as \(M–H^+\) in negative-ion mode using ESI or APCI. Upon CID, little fragmentation is observed for saturated FAs, and minor losses of \(CO\_2\) for PUFAs\(^{55}\). Therefore, either SIM or SRM with the same \(m/z\) for both precursor and product ion, thus attempting to at least fragment possible co-eluting isobaric species\(^{56}\), is used. Post-column addition of Ba\(^{2+}\)
was reported, to generate [M–H+Ba]⁺-ions, which readily undergo charge-remote fragmentation of the alkyl chain, providing specific fragment ions for SRM⁵⁷.

In this way, f-FAs can be analysed in the low nM range, e.g., after MeOH protein precipitation for plasma⁵⁵. An interesting example is the analysis of 36 f-FAs in human plasma, using a calibration set of known FAs to enable identification and quantification of unknown f-FAs. The method made use of the above-described SRM procedure⁵⁶ and showed lower limits of quantification in the nM range with run times below 10 min⁵⁷. Transesterification procedures, applied to determine t-FA, yield FAMEs, which show poor ionization characteristics in ESI and APCI. Mostly, RPLC is used for the separation of FAs. Separation of FAs on Ag⁺-loaded columns provides enhanced resolution of FAs with different E/Z isomers and double-bond positions, eventually in combination with ozonolysis⁵⁸.

Like with small CAs, quantitative performance may be enhanced by derivatisation, either to enhance the ionization efficiency or to implement fragmentation characteristics for SRM. A 60,000-times improved sensitivity, compared to the analysis of underivatised FAs, has been claimed for N-(4-aminomethylphenyl)pyridinium (AMPP) derivatives of FAs, introducing a permanent charge⁵⁹. Other derivatisation strategies involve for instance trimethylaminoethyl (TMAE)⁶⁰, 2-bromo-1-methylpyridinium iodide (BMP)⁶¹, MPEA²⁰, and 4-(2-((4-bromophenethyl)dimethylammonio)ethoxy)benzenaminium dibromide (4-APC)⁶². Derivatisation techniques for FA analysis using LC–MS have recently been reviewed³⁹. Carbodiimide coupling using EDC in combination with AMMP derivatisation and stable isotope coding was applied in the analysis of t-FAs in human serum samples⁶¹. The derivatives were separated on a C₄-column using an acetonitrile/water gradient.
2.6 Eicosanoids

Phospholipases release mainly 20-carbon PUFAs from membrane-phospholipids. Eicosanoids are the enzymatic oxidation products of these PUFAs, generated by enzymes from the cyclooxygenase (COX), cytochrome P450 (CYP), and lipoxygenase (LOX) families\(^5\). Typical eicosanoids are arachidonic acid (FA 20:4) derived prostaglandins and leukotrienes. Isoprostanes are closely related eicosanoids, generated by non-enzymatic oxidation of FA 20:4\(^6\). Many eicosanoids mediate critical biological effects such as for example, chemotaxis, blood clotting or broncho-constriction. Particularly during inflammatory processes, prostaglandins and leukotrienes, derived from FA 20:4, are important in the initial phase\(^4\), whereas eicosapentaenoic acid derived mediators play a crucial role in the active resolution phase of inflammation\(^4\). In addition, the family of 22-carbon PUFA derived docosanoids comprise related highly active mediators\(^8\). The biological activity of the eicosanoids and related compounds strongly relies on stereo-, positional- and geometrical- isomerism\(^4\).

Artificial eicosanoids may be formed by oxidation of FA 20:4, which is present at high levels in human body fluids such as plasma, or by activation of platelets during venipuncture. To avoid errors in analysis, it is important to use ion chelators such as EDTA, to freeze samples immediately at -80 °C, and to consider the use of antioxidants such as butylated hydroxytoluene (BHT) and/or enzyme inhibitors such as indomethacin\(^43\).
2.6.1 GC–MS analysis of eicosanoids

Multistep derivatisation is required to achieve compatibility of eicosanoids with GC–MS analysis. The gold standard is a combination of trimethylsilylation of hydroxyl groups, oximation of the ketone groups (if necessary), and PFBBBr derivatisation of the CA group, thus enabling selective and sensitive analysis using ECNI in GC–MS. A protocol for the assessment of F$_2$-isoprostanes as markers of oxidative stress in vivo has been reported. Following PFBBBr ester formation, sample clean-up by thin-layer chromatography and silylation with BSTFA, analysis is performed by ECNI in GC–MS. Particularly for structural confirmation purposes, GC–MS with EI fragmentation after diazomethane derivatisation is still an important tool.

2.6.2 LC–MS analysis of eicosanoids

The sample pre-treatment protocol for GC–MS, involving a two (three)-step derivatisation, is obviously quite laborious, which explains why nowadays LC–MS analysis is frequently applied instead. LC–MS allows the analysis of underivatised compounds, greatly facilitating sample pre-treatment and minimizing possible analyte losses.

An important challenge in eicosanoid analysis is the resolution of the high number of possible stereo- and E/Z-isomers. Leukotriene B$_4$, for example, contains 4 double bonds and 2 stereo-centres and can thus theoretically exist as different isomers. The high separation efficiency achievable by the use of UHPLC with columns packed with small porous or solid-core particles (<2 μm) and excellent retention time stability are of utmost importance, especially because differentiation based on fragmentation in MS–MS is not always possible. As eluent systems in RP-separations MeOH/water, ACN/water as well as mixtures thereof have been
described. Given the impact of stereo- and E/Z isomerism on their biological activity, chiral separation of eicosanoids can be of considerable concern. As the elution order of enantiomers cannot be predicted, only comparison with standards or with published results obtained under identical conditions allows the deduction of absolute stereochemistry. Sample pre-treatments of plasma samples is mainly based on protein precipitation followed by sample clean-up using C<sub>18</sub>-SPE with or without the involvement of a hexane wash step. While sample preparation of blood derived samples is rather straightforward, the analysis of urinary samples does involve more tedious sample preparation protocols which is mainly due to the occurrence of strong matrix effects. A protocol using a mixed mode SPE (Oasis HLB) in combination with APCI LC–MS was described. Another protocol involves the use of a weak anion-exchange material. Compared to C<sub>18</sub> based SPE, very clean extracts were obtained by methanol elution of the eicosanoids; most matrix components remained on the SPE cartridge under these conditions.

Given the low endogenous levels of eicosanoids, ultimate sensitivity must be achieved using SRM in TQ or Q–LIT instruments. Upon CID, the presence of hydroxyl and ketone groups in the alkyl side-chain induces specific cleavages leading to analyte-specific fragment ions. Current trends in the implementation of high-resolution mass spectrometry (HRMS) in quantitative bio analysis may be beneficial in eicosanoid analysis, as (almost) co-eluting isobaric compounds can be resolved by HRMS. In this respect, combined ion-mobility spectrometry and MS (IMS–MS) should be explored as well.

LC–MS analysis of eicosanoids has recently been reviewed. Recent applications involving AMPP labelling of oxidized fatty acids and LC–MS using either a TQ instrument or an LTQ-Orbitrap mass spectrometer were reported. Mouse serum
samples were derivatised after SPE clean-up and analysed by a generic RPLC separation using an acetonitrile/water gradient.

2.7 Bile Acids

BAs, in particular cholic and chenodeoxycholic acids, are the major CYP-mediated catabolic-metabolites of cholesterol 6. Just recently, BAs have emerged as signalling molecules with systemic endocrine function76. Particularly in the context of metabolic diseases such as obesity or type-2 diabetes, BA signalling might possibly be exploited as novel therapeutic intervention strategy76, 77. As a result, analysis and profiling of BAs has recently received considerable attention. In this respect, a comprehensive sample pre-treatments protocol allowing the analysis of neutral, acidic and basic sterol-derivatives is needed78. All aspects of the analysis of BAs have been extensively reviewed recently79.

2.7.1 GC–MS analysis of bile acids

The subclass of BAs is not a favourable compound class for GC–MS. Apart from the CA and hydroxyl groups, which already require derivatisation, BAs may contain several other polar and labile conjugates with groups like sulfate, phosphate, amide and glucuronate, that are not readily derivatised towards GC–MS 8. Thus, BA analysis by GC–MS is limited to deconjugated compounds, which can be analysed as TMS/methyl ester derivatives. The fragmentation of BAs in EI can be highly useful and complementary in structure elucidation to product-ion mass spectra obtained by ESI-MS and CID79.
2.7.2 LC–MS analysis of bile acids

LC–MS can be readily used for the analysis of BAs as well as their conjugated analogues\textsuperscript{80, 81}. In all instances, sample pre-treatment is less complicated than for GC–MS. The presence of multiple isomeric BAs put high demands on efficient separation, especially because CID provides little compound-specific fragmentation. Reversed-phase UHPLC is generally applied\textsuperscript{81, 82}. For SRM in negative-ion mode, mostly group-specific product ions are applied, e.g., \(m/z\) 74 (\(\text{C}_2\text{H}_4\text{NO}_2^-\)) for glyco-BAs, \(m/z\) 80 (\(\text{SO}_3^-\)) for tauro-BAs, and \(m/z\) 97 (\(\text{HSO}_4^-\)) (or neutral loss of 80 Da, \(\text{SO}_3\)) for sulfate-conjugated BAs, whereas unconjugated BAs do not show significant fragmentation\textsuperscript{83}. As such, CID readily enables the identification of the conjugates, but provides little structural information on the BAs themselves\textsuperscript{80}.

An interesting recent study provided evidence that dietary fats can result in changes of the host BA composition, thus altering conditions for gut microbial assemblage perturbing immune homeostasis\textsuperscript{84}.

2.8 Conclusion and perspectives

Analytical strategies for the analysis of four subclasses of CAs by GC–MS and LC–MS are reviewed. Attention is paid to typical protocols for the analysis of these compound classes. The biological importance of the compounds discussed clarifies the analytical relevance. Despite the progress in the development of useful analytical protocols, many challenges remain to be (further) addressed, including differentiation between various isomeric species, both in terms of separation and MS–MS fragmentation, and low-level analysis for both absolute quantification and flux analysis. Significant progress in this respect may be expected in the years to come.
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