Analysis of Lipids: Triacylglycerols, Phospholipids, Fatty Acids, and Others

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1. INTRODUCTION

Lipids comprise large and disparate classes of molecules having very different overall polarities, but the one characteristic they typically have in common is a nonpolar hydrocarbon chain that is hydrophobic in nature. Most definitions of lipids are based on a combination of structural characteristics (i.e. hydrocarbon chains) and behavioral characteristics (i.e. insolubility in water). A typical definition comes from Merriam Webster [1]: "any of various substances that are soluble in nonpolar organic solvents (such as chloroform and ether), that are usually insoluble in water, that with proteins and carbohydrates constitute the principal structural components of living cells, and that include fats, waxes, phosphatides, cerebrosides, and related and derived compounds." This article will focus mainly on the classes of edible food lipids that contain linear (sequential) hydrocarbon chains with or without one or more double bonds (DBs) (i.e. sites of unsaturation). Sterols and related molecules are considered lipids, but these contain a four-ring backbone exemplified by cholesterol. But since these do not follow the

Bailey's Industrial Oil and Fat Products, *Seventh Edition*, edited by Fereidoon Shahidi.

DOI: 10.1002/047167849X.bio087

rules below that describe behavior based on chain length and degree of unsaturation, they will be mentioned only briefly in this article. This article will also focus on the most popular current method for lipid analysis, mass spectrometry (MS).

The characteristics of each class of lipid dictate the most appropriate tool and approach for analysis. For instance, phospholipids (PLs) are amphiphilic molecules that contain a polar head group attached to a (usually) glycerol backbone (except sphingolipids), plus two hydrophobic hydrocarbon chains (except lyso-PLs, which contain only one hydrocarbon chain) attached via carboxyl moieties derived from fatty acids (except plasmalogens and other ether-linked hydrocarbon chains). In many cases, PLs are zwitterionic, having a negative charge at the phosphate group and a cation at, for instance a quaternary amine group. Since many PLs are inherently charged, the polar head group makes them ideally amenable to soft ionization by electrospray ionization (ESI) coupled to MS. Since PLs always contain the phosphate moiety, but only some contain a complementary positively charged functional group, some classes of PLs are best analyzed in the negative-ion mode, while zwitterionic ones readily form positive ions by providing a proton to counteract the phosphate's negative charge. Thus, the charge state (+ versus –) is an important variable in selecting the right approach for PL analysis.

In contrast, triacylglycerols (TAGs) contain three fatty acyl chains derived from fatty acids (FAs), and are therefore large neutral molecules that are not easily directly ionized by electrospray ionization mass spectrometry (ESI-MS). In such cases, formation of adducts is necessary for ESI-MS analysis, which is accomplished by addition of an electrolyte to the flow stream, either in the solvents or supplied via a tee attached to the ionization source. In contrast, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are both readily able to ionize neutral molecules such as TAGs. Similarly, sterols are neutral molecules that are not very amenable to ESI-MS without derivatization, but are readily ionized by APCI-MS and APPI-MS. Thus, this article will outline the characteristics of each class of lipid, and how these affect the choice for the most appropriate tool for their analysis, including special steps and considerations that need to be taken into account to optimize the ionization efficiency for each class. However, emphasis will be given to neutral lipids (i.e. TAGs), which are the major components of many edible lipid mixtures.

This article will also focus on the different approaches necessary to derive the desired structural information from lipid molecules. As mentioned, TAGs and most PLs contain a three-carbon glycerol backbone with fatty acyl chains attached (these will also be referred to as FAs). In biological systems, these FAs are often not randomly distributed, but instead different types of FAs, depending on chain length and degree of unsaturation, exhibit preferences for stereospecific locations, dictated by enzymatic preferences during anabolism (structural synthesis). In a complementary manner, catabolizing enzymes also show stereospecific preferences for metabolism. Thus, it is important to know the locations of the FAs on the glycerol backbone to get an idea of the bioavailability of the FAs. These different positions are designated using the stereospecific numbering system (*sn*), with *sn*-1, *sn*-2, and *sn*-3 representing the three positions on the glycerol moiety. In PLs, the polar head group is assumed to be located at *sn*-3. During metabolism of TAGs, FAs at *sn*-1 and *sn*-3 are more readily available to enzymatic removal to form free FAs (FFAs), whereas the FA in the *sn*-2 position is generally retained, leaving a 2-monoacylglycerol (MAG). Interestingly, fragment formation during MS and MS/MS also exhibits preferences for stereospecific FAs, allowing stereospecific information to be derived from MS spectra.

Structural information that is desired from lipids includes, but is not limited to, the following: (i) lipid class (including PL class); (ii) molecular mass; (iii) carbon chain lengths of FAs;

(iv) number of DBs in FAs; (v) positions of DBs in hydrocarbon chains; (vi) stereospecific locations of FA chains on the backbone; and (vii) identities of different enantiomers, if present (*sn*-2 is a chiral center). Unfortunately, there is not a quick and easy approach to the determination of all of these structural characteristics. The more information that is required, the more difficult and time-consuming is the analytical approach to obtain that information. Thus, there are always trade-offs between analysis time and the amount of information provided.

This article is intended to serve as a guide for decision-making, by discussing the strengths and weaknesses and the benefits and shortcomings of the available options for detailed lipid analysis. The literature in the area of lipid analysis by MS has burgeoned in recent years to hundreds or thousands of articles on each topic. Many of the principles fundamental to lipid analysis in foods and edible oils were developed for analysis of lipids in biological samples, but are directly applicable to fats and oils. Thus, many of the references discussed below cite analyses of a wide range of sample types. Since any single review article quickly becomes outdated, this article will emphasize the first reports and primary works that laid the foundations for understanding the structural information provided for different classes by various techniques, which should be cited by later work, the history of developments in each area, references to review articles that help the reader be aware of the latest developments in the areas presented, and finally recent articles that point the way toward new technologies being adopted.

FA Abbreviations Used in TAGs and PLs: M – myristic acid, 14:0; P – palmitic acid, 16:0; S – stearic acid, 18:0; O – oleic acid, 18:1; L – linoleic, 18:2; Ln – linolenic, 18:3.

2. ANALYTICAL APPROACHES

There are numerous MS interfaces that have diminished in use (fast-atom bombardment, particle beam, thermospray, etc.) and many more that are still commercially available after years of use or that have been developed more recently (matrix-assisted laser desorption ionization (MALDI), direct analysis in real time (DART), desorption electrospray ionization (DESI), etc.). In fact, a recent review referred to more than 80 variations of just ambient ionization approaches [2]. Another recent review summarized the most common approaches currently in use [3], while another review focused on a subset of ambient techniques applied to metabolomics analysis [4]. One recent review focused specifically on the latest MS techniques applied to cooking oil analysis, with an emphasis on newly developed ionization methods [5]. The point is, there is no way to cover all possibilities for lipid analysis in one article. Therefore, this focuses primarily on analysis of intact molecules in the liquid phase, either using infusion via syringe pump without prior separation or using separation into either lipid classes or molecular species using high-performance liquid chromatography (HPLC) or ultrahigh-performance liquid chromatography (UHPLC) coupled to MS via atmospheric pressure ionization (API) techniques, because these are the methods most commonly in use for analysis of edible oils nowadays. Supporting this approach is a review of developments in the field of MS in the decade up to 2012 [6]. The two biggest developments in mass analyzers used for MS in the past decade are the development of the orbitrap ion trap developed by Makarov and coworkers [7], which allows facile high-resolution accurate-mass (HRAM) spectra to be acquired on a compact, affordable instrument, and the development of multiple commercial options for ion mobility mass spectrometry (IM-MS), which is discussed in a dedicated section below.

The biggest developments in the area of chromatography for edible oil analysis in recent years are the development of UHPLC and the increased adoption of comprehensive two-dimensional (2D) gas chromatography (GC) and LC techniques. Although 2D-TLC has been used for decades, especially for biological samples, the development of 2D-LC (or LC × LC) has been a more recent advance, facilitated especially by the introduction of UHPLC. UHPLC has allowed very short analyses, such that multiple UHPLC separations can be accomplished across the width of a conventional HPLC peak. In his recent thorough treatment of the subject, Stoll [8] describes the history, milestones, theoretical principles, and practical considerations associated with modern 2D-LC, as well as elsewhere providing a useful tutorial [9] and summary of recent advancements [10]. Dugo et al. [11] reviewed recent applications in 2D-LC, including separations of carotenoids and TAGs, and that same group has reviewed applications of 2D-LC to food analysis [12–15], as well as reviewing fundamental principles [16, 17]. Purcaro et al. [18] reviewed 2D-GC (or GC × GC) instrumentation, principles, and applications, with an emphasis on FA methyl esters (FAMEs), other lipid components, and volatiles in oils and fats. The principles and early applications of 2D-GC, starting in 1991, were summarized in the early review by Marriott and Shellie [19]. Users should not be hesitant to embrace these new technologies, because the complexity of lipids and the numerous structural aspects described above mean that no one single technique is adequate to provide all structural information desired. It is often necessary to combine complementary approaches into a single analysis to provide all of the desired information.

Although the emphasis here is on LC-API-MS, there is still great value in the information provided by the tried-and-true technique of GC for determination of the FA composition of a lipid mixture. The next section briefly discusses GC analysis, while the rest of the article is devoted to MS and LC-MS approaches. There are hundreds of valuable reviews that have addressed both specific and broad topics of lipid analysis over the years, including, to cite only a few, TAGs [20–28], PLs [29–31], FAs [32–36], and lipids in general [37–69]. One review worth special mention was targeted specifically at methods for DB localization [70].

But before any analysis is undertaken, it is common to perform an extraction to isolate lipids from nonlipid components that would not be compatible with the chromatography used for lipids, or which might cause ion suppression in the ionization source of the mass spectrometer. Only oil samples that have undergone oilseed processing (e.g. pressed/expelled and bleached, deodorized) are typically analyzed directly after dilution without the need for further extraction. Some samples that contain only nonpolar lipids can be extracted using, for example hexane or the Soxhlet approach. But for complex mixtures of polar and nonpolar lipids, a more universal approach is required. Of course, the types of extractions used prior to chemical analysis may be very different from those used for production of an edible product.

Despite decades of attempts to find better extraction methods than the very popular chloroform/methanol-based classic approaches of Folch et al. [71] and Bligh and Dyer [72], these approaches remain the "gold standard" techniques against which other total lipid extraction techniques continue to be measured. The literature is replete with comparisons of extraction techniques [73], but it appears that the most effective methods for complex lipid mixtures continue to be long-standing gold standard approaches. For subsets of lipids, such as PLs, targeted approaches can be successful, such as using methyl-*t*-butyl ether (MTBE) for PL extraction prior to lipidomic analysis [74]. Here, however, we will focus on total lipid analyses containing both polar and nonpolar lipids.

Before delving into the details of individual MS approaches, it is worth mentioning some general considerations that have arisen over the years. First, when listing masses, it is best to give masses to one decimal place in data from unit mass resolution instruments (quadrupoles, tandem sector quadrupoles, hyperbolic, and linear ion traps). This provides an indication of the accuracy of the calibration of the instrument (it is best to be accurate to ±0.1) and eliminates ambiguity between nominal masses and rounded masses. If nominal masses are used (integer formed from the decimal mass minus the mass defect), this should be stated explicitly. The least desirable format is simply rounding masses, because $[RCO]^+$, $[RCO + 74]^+$, other FA-related masses, and some $[DAG]^+$ fragments will be rounded down (e.g. $[PPo]^+$ at 549.488 (calculated) and $[LLn]^+$ at 597.488), while other $[DAG]^+$ fragments (e.g. $[PP]^+$ at 551.504 and $[LL]^+$ at 599.504), $[M+H]^+$, and adduct masses will be rounded up. Thus, some lower mass fragments would be rounded up ($[PP]^+$), while higher mass fragments are rounded down ($[LLn]^+$). Also, in early work by the authors, the designation $[M+1]^+$ or $[M+23]^+$ was sometimes used for the mass of the protonated molecule or sodium adduct, respectively, whereas $[M+H]^+$ or $[M+Na]^+$ should be used instead (or similarly $[M–1]^-$ used for negative-ion analysis of dinitrophenylhydrazine (DNPH) derivatives, etc., whereas $[M–H]^-$ is now preferred). One last point is that the term "molecular ion" should not be used for most API techniques, because this has a specific and limited meaning from the IUPAC [75]: "Ion formed by the removal of one or more electrons from a molecule to form a positive ion or the addition of one or more electrons to a molecule to form a negative ion." Thus, in the most common case where one electron is removed to form a positive ion, an odd-electron cation is formed, $M^+\cdot$, whereas in API techniques, even-electron species are usually formed by addition of a proton, H^+, or adduct ion, Na^+, K^+, NH_4^+, etc. In general, those undertaking analysis of lipids using the techniques in this article should be familiar with the IUPAC terms for MS referenced above, which are also readily available online [76].

2.1. Gas Chromatography (GC)

2.1.1. GC of FAMEs Since GC requires analytes to be volatile enough to be in the gas phase at a temperature that is compatible with the GC column being used, derivatization is required for GC analysis of FAs. By far the most common derivatives are FAMEs. Thorough discussions of FA derivatization options and procedures have been given by Christie [77] (available online at the American Oil Chemists' Society (AOCS) Lipid Library [78]) and in various reviews [32–35]. Generally, FAMEs are formed using either an acid-catalyzed approach or one that is base catalyzed. Acid-catalyzed approaches tend to be slower, but are applicable to both bound FA and free FA (FFA). They may not be appropriate for FA containing unusual functional groups, such as epoxides. Base-catalyzed approaches, on the other hand, may be faster, but do not apply to FFA. Those interested in a dedicated discussion of FA analysis should consult the reviews referenced earlier.

It should be noted that if a total lipid extract is derivatized, the FAMEs may arise from PLs, diacylglycerols (DAGs), TAGs, or FFAs. Thus, it will not be possible to differentiate the FA compositions of the different lipid classes without prior fractionation. In such cases, it may be necessary to collect LC fractions of the different classes and then derivatize them separately to obtain the separate FA compositions. In more classic approaches, thin-layer chromatography (TLC) is used to separate lipid classes prior to FA derivatization and analysis, and each spot is analyzed separately. In the cases of edible oils that contain almost exclusively TAGs and other neutral lipids (NLs), it is often adequate to analyze the oils without prior class separation.

In such cases, the amounts of DAGs are often only a small percentage relative to TAGs, and the assumption is made that the distribution of FAs is similar throughout.

It is valuable, and should be a standard procedure, to compare the FA composition determined by GC with flame ionization detection (FID) or MS detection to the FA composition that is calculated from the intact DAG and TAG composition determined by MS. Of course, the FID detector response is proportional to the number of carbons present (i.e. it is a weight proportional detector), so the weight percentage composition by GC-FID should be converted to a molar percentage composition by dividing the weight percentages by the accurate isotopic masses of the FAME molecular species and renormalizing to 100%. GC–MS provides a molar response (based on one (+) charge per molecule), so such conversion is not necessary. GC signal response factors (RFs) should be derived from analysis of a known FAME mixture, many of which are readily commercially available (e.g. Matreya, Nu-Chek Prep, Restek, and Supelco (now part of MilliporeSigma, formerly Sigma–Aldrich)). The FA composition calculated from the DAG + TAG composition is affected by differences in response during LC-MS due to the carbon chain length and degree of unsaturation, so RFs should be applied for LC-MS analysis, also. But since only a limited number of DAG and TAG standards are available, various approaches [79, 80] to determining RFs for TAGs analyzed by LC-MS are discussed below.

2.1.2. High-Temperature GC (HT-GC) of TAGs As mentioned, TAGs are large neutral molecules, which makes them nonvolatile at conventional GC temperatures. However, in the 1980s, new generations of highly durable columns [81] were developed that allowed separation of TAGs mainly by degree of unsaturation, without complete resolution of molecular species. Such columns are able to withstand temperatures of 360 °C or higher, enabling elution of intact TAGs. HT-GC columns have continued to advance, and a recent review discusses developments in this area over the past decade [82]. Christie has also discussed the topic of HT-GC in his early review, available online [83]. Other reviews have addressed this area, as well [21, 22, 25].

The elution characteristics of TAGs by HT-GC were succinctly summarized by Mares [21]: "The separation of TAG on the basis of the carbon number (CN) (total number of carbon atoms in all the acyl groups) can be achieved using a short capillary with a nonpolar stationary phase. Medium-length (about 10 m) nonpolar columns yield partial separations of unsaturated TAG. The unsaturation becomes important on nonpolar columns with high efficiency, but the separation is not based on the number of DBs, but rather on the number of unsaturated FAs in the TAG molecule. Similarly, when the TAG molecule contains FAs with very different chain lengths, a certain degree of separation can be observed on nonpolar columns. Columns with polarizable stationary phases can be employed to separate TAG also on the basis of the number of DBs and, in some cases, according to the FA composition."

The main complaints regarding HT-GC of TAGs are the potential for thermal decomposition, especially of polyunsaturated TAGs, and discrimination of short-chain versus longer chain TAGs, both of which may occur in a conventional heated split/splitless injector [25]. Therefore, use of an on-column injector was recommended.

2.2. Electrospray Ionization Mass Spectrometry (ESI-MS)

The thorough treatise by Cole [84], with foreword by 2002 Nobel Prize winner John B. Fenn, is a must-read volume for those interested in ESI-MS. The article by McEwen and Larsen [85]

describes the history of ESI, starting with experiments laying the foundation in 1917 by Zeleny, which were followed much later by the extensive work of Dole on polymer macromolecules in the 1960s and 1970s. But it was the work of Yamashita and Fenn [86] in 1984 that proved its value as an ionization source for MS, and that work was quickly followed up to produce an interface for liquid chromatography [87]. Niessen [88] has authored a comprehensive volume on a variety of ionization sources for coupling liquid chromatography and MS, which remains a valuable resource on the topic.

Early ESI sources were capable of ionization of only very low flow rates, in the microliter per minute range. The introduction of "pneumatically assisted" electrospray by Bruins et al. [89], which employs a coaxial nebulizing gas to aid in solvent evaporation, opened the way for commercially available LC–ESI-MS sources operating at atmospheric pressure using higher flow rates. The term "ionspray" was used to differentiate this approach from the original method, but this moniker is no longer used, since it has become the default approach for modern ESI-MS. Thus, the 1980s became the decade when ESI-MS went from a theoretical curiosity to a widely used analytical tool, giving rise to the "omics" era. The application of ESI-MS to lipids came a little later, described below.

2.2.1. Ion Mobility Mass Spectrometry While most principles of MS have been well established and used for lipid analysis for as much as two decades, the most recent development that is now blossoming into wider acceptance and usage is the use of ion mobility spectrometry (IMS) or IM-MS, which relies on one of the three approaches, according to May and McLean [90]: "(i) time dispersive, (ii) space dispersive, and (iii) ion confinement (trapping) and selective release." This article provides an excellent background and presentation of applicable theory, as do those of Lapthorn et al. [91], Kanu et al. [92], and others [93–95]. Several instrument manufacturers have made IM-MS instruments available based on these principles. As discussed by Hinz et al. [96], the various commercially available instruments are as follow: "(i) Drift-time ion mobility spectrometry (DT-IMS), developed by Agilent Technologies, (ii) Field-asymmetric ion mobility spectrometry/differential-mobility spectrometry (FAIMS/DMS) approach, provided by AB Sciex, (iii) Traveling-wave ion mobility spectrometry (TW-IMS), developed by Waters, and (iv) Trapped ion mobility spectrometry (TIMS), developed by Bruker." As explained by Paglia et al., "Currently, MS detection employs one of the three major IMS separation approaches: (i) drift-time IMS (DT-IMS); (ii) traveling-wave IMS (TW-IMS); and (iii) field-asymmetric IMS, also known as differential-mobility spectrometry. In DT-IMS, ions migrate through a buffer gas in the presence of an axial, linear electric-field gradient. In TW-IMS, a sequence of applied voltages generates a traveling wave that propels the ions through the buffer gas. Thus, both DT-IMS and TW-IMS allow all the ions to pass through the mobility cell. Field-asymmetric IMS, however, operates by varying the compensation voltage, filtering selected ions in a space-dispersive fashion."

While not strictly limited to use with ESI-MS, discussion of IM-MS is included in this section because ESI-MS is a soft ionization technique that forms intact protonated molecules or adduct ions with little or no fragmentation (unless energy is provided for nonspecific upfront, also known as nozzle-skimmer, collision-induced dissociation (CID)). Intact molecules are more suited for the differentiation of molecular species of lipids by IM-MS than molecular fragments.

Recent reviews have summarized the application of IM-MS to lipid analysis [95–98]. Such applications include a survey analysis of a broad range of lipids [99], a comparison of

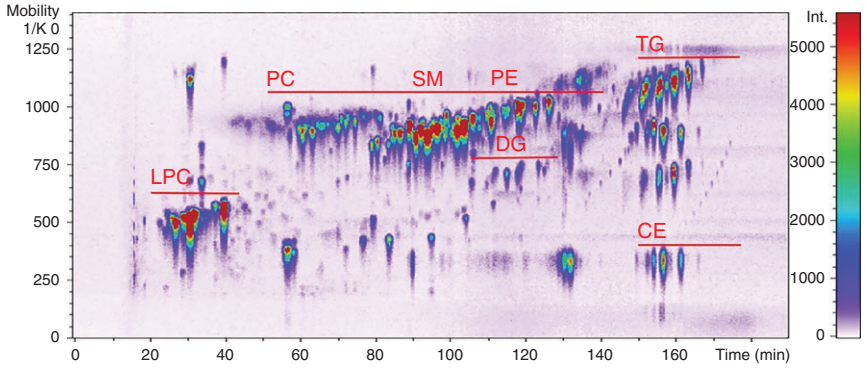

Figure 1. *LC–TIMS–MS separation of human plasma under optimized conditions acquired in positive ionization mode. Tentative regions are assigned to different classes of lipids. Source: Reprinted from Ref. [100] with permission. Copyright 2017 Elsevier, Inc.*

2D-LC to trapped-IM-MS for lipidomics [100] (pictured in Figure 1), the use of IM-MS for differentiation of lipid isomers [101–104], and many others. A search of "'ion mobility' AND lipids" yields nearly 200 references for this adolescent technology.

2.2.2. Without Liquid Chromatography

2.2.2.1. Neutral Lipids (NLs) The applications of ESI-MS to lipids appear to have started in the 1990s, with the report by Duffin et al. [105] on acylglycerols (AGs) using infusion without prior separation of standards and a mammalian cell culture sample, which were dissolved in chloroform/methanol (70:30). The trends reported therein hold true for analysis of AG NLs with or without online chromatographic separation, so it is worth citing several observations mentioned in that first report. AGs did not form abundant protonated molecules, $[M+H]^+$, by ESI-MS even when 2% formic acid was the electrolyte added to the solvent. On the other hand, adducts that provided abundant signal could readily be formed. Using either sodium acetate or ammonium acetate as electrolyte gave approximately equal sensitivities of sodium, $[M+Na]^+$, or ammonium, $[M+NH_4]^+$, adducts, respectively.

The response under ESI-MS conditions was substantially affected by the polarity of the molecules, with the most nonpolar molecules giving the least signal. MAGs gave more signal than DAGs, which gave more signal than TAGs (MAGs > DAGs > TAGs). Also, within a class of AGs, shorter FA chains gave more response than longer FA chains (short > long), and those with polyunsaturated FA chains produced more signal than saturated FAs (unsaturated > saturated). *This clearly points to the need for some form of response factors when using ESI-MS for MAGs, DAGs, and TAGs*. For individual molecular species, the response was approximately linear over four orders of magnitude.

For MS/MS, $[M+NH_4]^+$ adducts were preferred as precursors, as these produced abundant $[M+H]^+$ product ions at low collision energies (CEs). At medium CEs (e.g. 50 V) mass spectra (such as shown in Figure 2) produced predominantly DAG-like fragments, $[DAG]^+$, formed from loss of the various FA chains. At high CEs (e.g. 130 V), spectra rich in fragments were formed that included $[DAG]^+$ and also FA-related fragments $[RCO]^+$ and $[RCO + 74]^+$, as well as alkyl fragments $[CxHy]^+$ from fragmentation along the FA chain (the latter were not identified as such, but we now know that is what they are). $[M+Na]^+$ precursors, on the other hand, were sufficiently stable that they did not produce structurally informative MS/MS

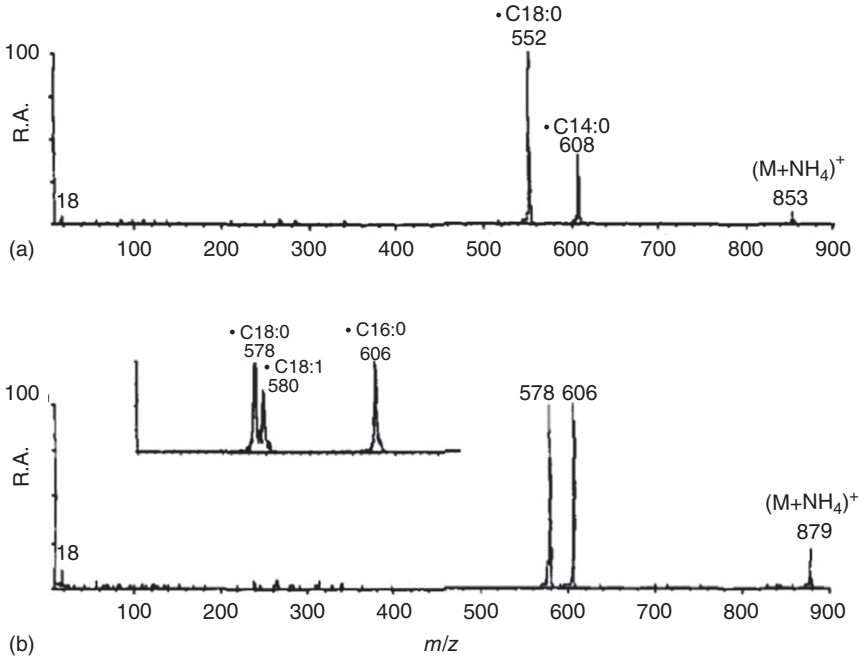

Figure 2. *Positive-ion MS/MS product ion spectra of two ammoniated triglycerides that contain mixed fatty acid components. (a) Spectrum results from 1,2-stearoyl-3-myristoylglycerol, and (b) the results from 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol. Both MS/MS spectra were acquired at 50-eV collision energy. Source: Reprinted from Ref. [105] with permission. Copyright 1991 American Chemical Society.*

spectra because they required very high CEs, making them less desirable as precursor ions. In that report [105], it was mentioned that the FA locations on the glycerol backbone (i.e. regioisomers) could not be determined. With hindsight gained from tabulated values [106–108] collected in the intervening years, we now know that some regioisomers of AAB/BAA TAG simply give ratios of $[AA]^+/[AB]^+$ that are close to the ratio of 1/2 (or 50%) that is statistically expected (e.g. OOP, OOS, and SSO by ESI-MS, and SSP, which is similar to the SSM in that report [105]), while the corresponding ABA regioisomer gives a ratio substantially less than 1/2. Therefore, standards of both regioisomers need to be obtained before firm conclusions can be drawn. Furthermore, reassessing the mass spectrum shown for POS in their Figure 5b (reproduced here as Figure 2) using insight gained since that time indicates that the $[DAG]^+$ fragment that is formed from loss of the FA chain in the *sn*-2 position (i.e. $[PS]^+$) had the lowest abundance of the three $[DAG]^+$ fragments ($[PO]^+$, $[OS]^+$, and $[PS]^+$), which is consistent with the later finding that loss of the FA in the *sn*-2 position is energetically disfavored (first shown for APCI-MS [110, 111] and later confirmed for LC-ESI-MS [112–117]). Thus, while most trends in that first report remain valid to this day, in hindsight it is no longer assumed that $[DAG]^+$ fragment ratios in MS/MS spectra of $[M+NH_4]^+$ adducts cannot be used to assign partial regioisomeric structures (i.e. the identity of the *sn*-2 FA). Other principles of structural elucidation of NLs by MS are given in Section 2.2.3, because many of the foundation works employed LC-MS, not infusion. Regardless of the details, ESI-MS with an appropriate electrolyte has proved itself to be a highly sensitive, valuable approach for TAG analysis.

Nowadays, analysis of NLs without prior separation is mostly done as part of an overall lipidomics approach, instead of analysis targeted just at TAGs, while TAG-specific analysis is more often done using LC-MS. There are over 3000 citations from a database search of "(lipidomics AND (triacylglycerol* OR triglyceride*))" and over 600 reviews for the same search at the time of this writing. The review by Murphy et al. [118] focused on MS of NLs, and serves as a good starting point for those undertaking NL analysis.

Shortly after the report of Duffin et al., Glenn et al. [119] used infusion to identify odd-chain (C15 and C17) TAGs in 3T3-L1 cells, and Geoffrey Haigh et al. [120] used infusion of a TLC fraction for ESI-MS analysis of (diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine) from the marine alga *Chlorella minutissima*, describing both positive- and negative-ion ESI-MS spectra (the negative-ion spectrum from the formate adduct). An early application of ESI-MS to food lipids by Kallio et al. [121] showed the use of negative-ion ESI-MS, with negative ions formed by ionization with ammonia, which produced structurally indicative fragments, and allowed regioisomers to be identified in primrose, borage, and black currant oils. This approach is used in other reports cited in the following paragraph.

In 1998 and 1999, two reports came out that remain foundation works for those interested in TAG analysis by ESI-MS. In contrast to the work by Duffin et al., who used low-energy CID, Cheng et al. [122] used high-energy collisionally activated dissociation (CAD, which is equivalent to CID, with the latter term more preferred nowadays according to the Murray Lab at http://mass-spec.lsu.edu/msterms/index.php/CAD_vs._CID) to determine not only the FA identities but also the identities of the FAs at the *sn*-2 positions and DB positions in the acyl chains. Descriptions of the $[RCO]^+$, $[RCO+74]^+$, and $[RCO+128]^+$ ions, and of course the $[DAG]^+$ fragments such as $[M+NH_4-RCOONH_4]^+$, are all still apropos to TAG analysis by MS/MS with a variety of API techniques. Similar to Duffin et al., Cheng et al. reported that the regioisomers could not be identified by ESI-MS/MS using $[N+NH_4]^+$ ions because the fragments from the different FAs were very similar in abundances. However, as with the note on hindsight above, the mass spectra of POS in Figure 3 (MS/MS of $[M+NH_4]^+$ on two different instruments) by Cheng et al. [122] do show the FA at *sn*-2 as the smallest of the $[DAG]^+$ fragments, even though the difference is not dramatic. The authors reported that $[M+metal]^+$ cations, where the metal was Li, Na, or Cs, produced a higher degree of fragmentation, which was better for DB localization. Smaller abundances of $[M+met.-RCOOmet]^+$ ($[DAG]^+$) ions were formed, so the *sn*-2 position could not be determined from those, but lower mass fragments formed from loss of two FAs, such as $[M-R_1COOH-R_3COOH-12]^+$, allowed identification of the acyl group at *sn*-2. While structural elucidation was relatively straightforward with known molecular species, the abundances of diagnostic ions were similar enough to make identification of unknowns more challenging. Only enantiomers could not be determined.

While the report above identified the carbon chain length, *sn*-2 FA chain positions, and DB locations, the mechanisms presented were proposed but not proven with deuterium-labeled studies. In fact, the situation is somewhat complex, since two different groups have reported different mechanisms using deuterium-labeled studies, and another group recently proposed another mechanism based on theoretical calculations. The first fragment mechanism backed up by a deuterium-labeled study came from Hsu and Turk [123] in 1999, who used MS/MS of deuterium-labeled $[M+Li]^+$ precursors on a triple-stage quadrupole instrument to precisely identify the mechanism of $[M+Li-RCOOLi]^+$ (=the DAG-like fragment, $[DAG]^+$) and $[M+Li-RCOOH]^+$ (=$[DAG+Li]^+$) fragment formation from deuterium-labeled TAGs. In both series of fragments, the smallest abundance resulted from loss of the FA at *sn*-2.

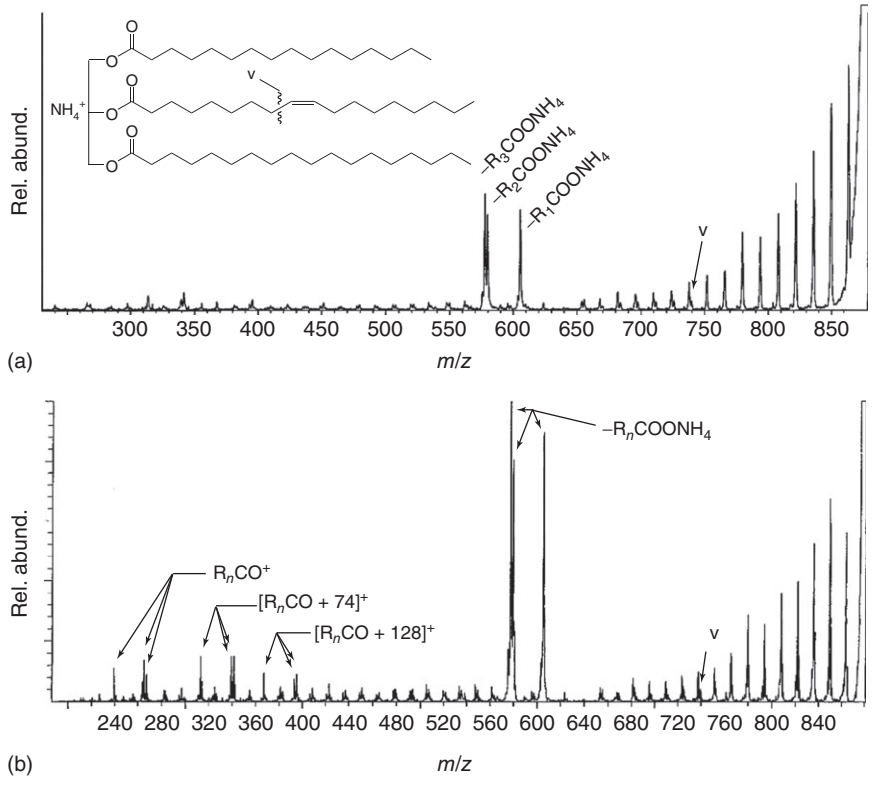

Figure 3. *CAD spectra of ESI-produced $[M+NH_4]^+$ ions of 1-palmitoyl-2-oleoyl-3-stearoyl-glycerol (16 : 0/18 : 1/18 : 0, MW = 860.8) obtained by different instruments: (a) an MS/MS spectrum from a four-sector mass spectrometer and (b) a linked-scan spectrum from a two-sector mass spectrometer. The peak corresponding to a vinyl cleavage is labeled with "v." Source: Reprinted from Ref. [122] with permission. Copyright 1998 American Chemical Society.*

Furthermore, compared to the high-energy CAD by Cheng et al. [122], the low-energy CAD MS/MS spectra of Hsu and Turk exhibited somewhat simpler spectra. Hsu and Turk's experiments seemed to convincingly show that the DAG-related fragments formed from $[M+Li]^+$ precursors include formation of α,β unsaturation. Also, $[M+Li]^+$ produced more structurally diagnostic ions than $[M+Na]^+$ precursors, so these are the preferred adducts for structural elucidation. Hsu and Turk [124] followed up that initial work using MS^n on a linear ion trap (LIT) instrument for localization of DB positions. However, the latter report did not include deuterium-labeled experiments for proof of the proposed mechanisms.

In 2005, McAnoy et al. [125] reported a different mechanism based on the use of deuterium-labeled TAGs as $[M+NH_4]^+$ adducts analyzed on a LIT instrument. They reported that under these conditions, an α-methylene deuterium was not lost. Furthermore, using deuterium labels on the glycerol backbone indicated that none of those deuteriums were involved, either. Using deuterium-labeled ND_4 as the ammonium source, they concluded that the mechanism for diacyl fragment formation involved two competing processes, representing $[(M+ND_4)-ND_3-RCOOD]^+$ (neutral loss mass shifted by 4 Da) and $[(M+ND_4)-ND_3-RCOOH]^+$ (neutral loss mass shifted by 3 Da). In MS^3 spectra, both

α-methylene protons were retained in $[RCO]^+$ acylium ions, and an additional α-methylene hydrogen atom was retained in the $[RCO + 74]^+$.

Thus, two different fragmentation mechanisms have been demonstrated by deuterium labeling experiments using either $[M+Li]^+$ or $[M+NH_4]^+$ adduct precursors. Until additional clarity is achieved, it is not unreasonable for those analyzing TAGs as $[M+Li]^+$ to cite the literature describing the former mechanism, and those analyzing $[M+NH_4]^+$ precursors to cite the latter mechanism. A recent theoretical consideration of fragmentation mechanisms [126] that used tripropionyl (C3) TAG as a model incorporated and confirmed the $[DAG]^+$ fragments with α,β unsaturation reported for MS/MS of the $[M+Li]^+$ by Hsu and Turk [123], but arrived at different conclusions regarding the fragments formed by MS^3. Grossert et al. proposed two lactones (γ- and δ-lactones having five- and six-membered rings, respectively) that fragmented during MS^3 along parallel pathways, one of which formed an α-lactone (three-membered ring). Thus, it appears that those engaging in regioisomer analysis of $[M+Li]^+$ adducts of TAGs should be aware of both the initial MS/MS mechanism of Hsu and Turk [123] that described the α,β unsaturation and the further description of lactones and the MS^3 mechanism of Grossert et al. [126].

Another important factor regarding neutral lipid analysis by ESI-MS is the degree to which the carbon chain length and degree of unsaturation affect the ionization efficiency and therefore the signal response. Han and Gross [127] reported quantification of TAGs in rat heart, which also applies to TAG analysis in foods, fats, and oils. The response under ESI-MS conditions for $[M+Li]^+$ adducts varied by as much as a factor of 2 based on the chain length (Figure 4) and degree of unsaturation, with polyunsaturated TAGs and those with shorter chain lengths exhibiting higher sensitivity. A polynomial equation was calculated from a set of standards, which was first order in degree of unsaturation and second order in acyl CN. Quantification was done relative to tri-17:1, using neutral-loss scanning of FA fragment masses. While the majority of biologically relevant TAGs contain less than eight sites of unsaturation, those with $\geq$12 dB required separate polyunsaturated internal standards, and those with $\geq$8 dB exhibited greater deviation from the algorithm. Using NL scans to form a 2D map, an iterative process of sequentially subtracting areas attributable to NL scans from the total areas from all NL scans for a particular TAG allowed quantification of total areas for each TAG. The main shortcoming of this approach is that multiple levels of standards of a wide range of TAGs need to be made and analyzed under the conditions of sample analysis. And since the signal depends on instrument-operating conditions, any time the conditions are changed, the standards need to be reanalyzed and the molar correction factors (MCFs) updated. The important point here is that ESI-MS data require RFs for accurate quantitation, even for percent relative quantification. While this report provides one option for MCFs for quantitative analysis of TAG molecular species, other approaches have also been reported, described below.

The report by Han and Gross [127] also raised another important issue that should not be ignored in quantitative analysis of TAGs, especially when no chromatographic separation is used. Because of the naturally occurring isotopes of carbon, hydrogen, and oxygen, every TAG produces a series of mass spectral peaks at $[M+adduct]^+$, $[M+adduct+1_{iso}]^+$, $[M+adduct+2_{iso}]^+$, $[M+adduct+3_{iso}]^+$, $[M+adduct+4_{iso}]^+$, etc. And since TAGs typically differ by degrees of unsaturation, with a degree of unsaturation being a difference of two hydrogens, the $[M+adduct+2_{iso}]^+$ isotopic variant of a monounsaturated TAG will have the same mass at unit resolution as the saturated TAG at two mass units higher, and so on. For instance, the calculated mass for the $[M+Li]^+$ of SSO is 895.8, and so it has an isotopic variant of $[M+adduct+2_{iso}]^+$ at 897.8, which would overlap the monoisotopic mass of the $[M+Li]^+$ of

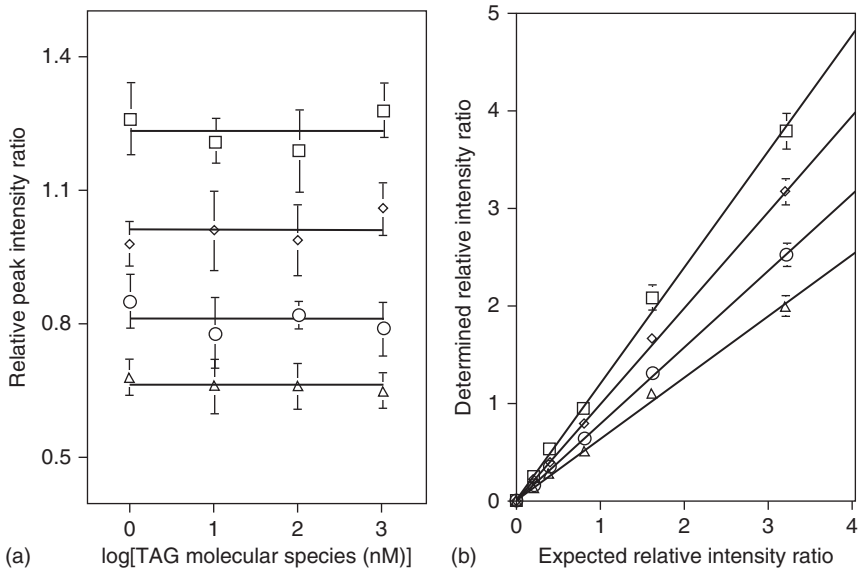

Figure 4. *Relationship of ESI/MS relative peak intensities with TAG molecular species concentration. In (a), mixtures of TAG molecular species containing identical molar ratios, but different concentrations, of individual TAG components were prepared as described under in Section 2. Positive-ion ESI mass spectra were acquired as described in the legend to Figure 1. The lithiated molecular ion peaks of each individual TAG molecular species were quantified relative to the internal standard (T17:1 TAG) after corrections for ^{13}C isotope effects. Experiments were performed over a three-order magnitude of concentration range (1–1000 nM) in mixtures containing T14:1 TAG (□), T16:1 (◇), T18:1 TAG (○), or T20:1 TAG (△). In (b), samples were prepared containing different molar ratios relative to the internal standard (T17:1), and the intensity of the molecular ion was quantified by ESI/MS after corrections for ^{13}C isotope effects. The ratios of molecular ion intensities of T14:1 TAG (□), T16:1 (◇), T18:1 TAG (○), or T20:1 TAG (△) with the molar ratio in the prepared solutions had coefficients (γ^2) > 0.99. The slope for each individual TAG molecular species was defined as the correction factor for the sensitivity effect relative to T17:1 TAG. Data are presented as means ± SEM from at least four separate sample preparations. Source: Reprinted from Ref. [127] with permission. Copyright 2001 Elsevier, Inc.*

SSS at 897.8. Therefore, to accurately quantify SSS, the isotopic contribution from SSO must be subtracted from the integrated area for *m/z* 897.9. This correction becomes especially vital when the lower mass TAG is present in a higher amount than the higher mass TAG, such that the $[M+adduct+2_{iso}]^+$ contribution can overwhelm the $[M+adduct]^+$ of the higher mass ion. Han and Gross gave two equations to calculate the isotopic contributions from ^{13}C, one for the CN difference between the analyte and the internal standard, and one for the overlapping of the $2 \times {}^{13}C$ isotopic variant with that of the TAG having a mass 2 Da higher. In this approximation, isotopic contributions from other atoms (i.e. oxygen and hydrogen) are ignored, since ^{13}C is the largest contributor to the isotopic variants. The important message here, though, is that isotope correction is necessary for accurate quantification of TAGs, especially when infusion is used without LC separation. When LC separation is used, the number of TAG molecular species that overlap and require isotopic correction is reduced. Isotope correction with LC separations coupled to API-MS using an alternative approach is discussed below.

With the groundwork laid by the abovementioned articles, other reports using infusion or LC with ESI-MS are further applications that, to one degree or another, confirm or support

the trends and characteristics described above. The same trends that were observed using infusion are usually observed using LC hyphenated to ESI-MS. In addition to the ability to often identify the *sn*-2 FA in ESI-MS/MS spectra, Byrdwell has repeatedly shown [128–132] that ESI-MS/MS with low-energy CID produces mass spectra that are very similar to full-scan APCI-MS mass spectra, including the dependence of the abundance of the protonated molecule (from the $[M+NH_4]^+$ precursor) on the degree of unsaturation. But since those reports all used LC with ESI-MS and APCI-MS, they are discussed further below.

While the capabilities of instruments continue to increase, with HRAM-MS now readily available, one of the greatest modern challenges is handling and interpreting the voluminous data that result from lipidomic analysis. Early software comprised mostly custom-made proprietary programs with limited applicability. The Fiehn Lab (University of California at Davis) maintains a compendium of software tools and databases at http://fiehnlab.ucdavis.edu/staff/kind/metabolomics/lipidanalysis. Nowadays, some of the major MS instrument manufacturers provide lipidomics software packages tailored to their data formats, including LipidSearch from Thermo Scientific and LipidView from Sciex. Others employ metabolomics software for lipidomics applications, such as Mass Profiler from Agilent and Progenesis QI from Waters, Inc. There are also a range of standalone lipidomics packages available, including free packages such as LipidXplorer (https://wiki.mpi-cbg.de/lipidx/Main_Page) and commercial products such as SimLipid. Some of these have their own proprietary databases, which limits the ability to be routinely updated with new lipid species. Other packages are able to access one or more of the online lipid databases (DBs), with perhaps the most notable being the LipidMAPS DB (http://www.lipidmaps.org/data/structure/index.php) and the LipidBank DB (http://www.lipidbank.jp/). Lipids are also included in general metabolomics databases such as METLIN at the Scripps Research Institute (https://metlin.scripps.edu) and the Human Metabolome DB (http://www.hmdb.ca/). While individual lipids can be manually searched in these DBs, this is not an efficient approach when hundreds to thousands of lipids are being identified, which is why lipidomics software with DB access integrated into it allows much greater productivity and higher throughput analyses. In addition to these resources, online tutorials and knowledge bases are very helpful, such as those at the AOCS Lipid Library (http://lipidlibrary.aocs.org), William W. Christie's LipidWeb (formerly LipidHome) (http://www.lipidhome.co.uk/), and the CyberLipid Center (http://www.cyberlipid.org/). One other very useful tool, especially for determining the differences between samples, is XCMS Online, also provided by the Scripps Research Institute (https://xcmsonline.scripps.edu). This portal allows users to submit MS files online, after which the system performs automated compound identification, statistical analysis, principal components analysis (PCA), fold-change analysis, and other tools. The point here is that the amount of data produced during modern lipidomic analysis is rapidly outpacing our ability to analyze the data, so software and automated tools are becoming crucial to handle data load and to extract the desired information. Of course, recent computing trends are pointing toward the use of artificial intelligence to aid in data analysis in the coming decade(s).

Many reviews of LC–ESI-MS for lipid analysis have been published, with the more recent ones more focused on lipidomics [69, 133], in which ESI-MS plays a key role, while others cast an even wider net to include Raman and NMR spectroscopies in addition to MS [66]. An especially thorough review and overview by Rustam and Reid [134] should be read by those interested in the field of lipidomics. An important review that highlights the importance of the proper selection and use of internal standards in lipidomics is also necessary reading [135]. A special issue of Rapid Communications in Mass Spectrometry recently had a selection of articles on lipidomics (http://onlinelibrary.wiley.com/journal/10.1002/

(ISSN)1097-0231/homepage/lipidomics_special_issue.htm). A valuable "virtual issue" of Analytical Chemistry covers numerous reports on lipidomics in that journal from 2008 to 2014 (https://pubs.acs.org/page/vi/2014/Lipidomics.html), while an earlier special issue of reviews on lipidomics appeared in Biochimica et Biophysica Acta (https://www.sciencedirect.com/science/journal/13881981/1811/11). Special issues of other journals focused on lipidomics include the journal Metabolites (http://www.mdpi.com/journal/metabolites/special_issues/lipidomics and http://www.mdpi.com/journal/metabolites/special_issues/Lipidomics_Volume_2). The Journal of Glycomics and Lipidomics had an issue on Emerging Techniques in Lipidomics (https://www.omicsonline.org/special-issues/emerging-techniques-in-lipidomics.html), and the European Journal of Lipid Science and Technology had a special issue on Lipidomic Methodologies (http://onlinelibrary.wiley.com/doi/10.1002/ejlt.v111:1/issuetoc). While not all of the numerous articles in these issues involve infusion without LC separation, they are included here for thoroughness. As with many MS approaches, those first developed for biological and clinical applications have been and are being adapted for analysis of foods [133], including lipids in edible fats and oils.

Han and Gross pioneered the use of "shotgun lipidomics" for ESI-MS using infusion without prior separation, using the principle of "intrasource separation" [136], of total lipid extracts [47, 137]. He has provided recent updated reviews and perspective on the burgeoning field [63, 64, 138]. These references provide an excellent record of applications of the technique across more than a decade and a half.

Recent applications (in 2018 and 2017) to food lipids of ESI-MS by infusion include PL analysis of edible whelk from the Red Sea [139], *N*,*N*-diethyl-1,2-ethanediamine (DEEA) derivatives of FFAs in a variety of edible oils [140], and analysis of EPA and DHA in TAGs and as ethyl esters in fish oil supplements [141]. A recent report on oxidation products of PLs showed that ESI-MS by infusion on a Qtrap quadrupole/linear ion trap (QqLIT) hybrid instrument was also well suited to the characterization of oxidized PLs [142]. The potential for identification of adulteration of olive oil with soybean oil was demonstrated using ESI-MS [143]. PLs in oysters were determined using a shotgun lipidomics approach to monitor enzymatic degradation of PLs under different storage conditions [144]. Infusion was used after HPLC fractionation and pooling of samples to analyze hydroxyl FAs in TAGs from Philippine wild edible mushrooms, as their lithium adducts [145]. Shotgun lipidomic analysis of both human and bovine milk TAGs and PLs was performed using (±)ESI-triple-TOF-MS with a Nanomate source [146]. Wheat PLs, FFAs, DAGs, and TAGs were analyzed using (±)ESI-QTrap-MS for assessment of the effect of nitrogen supply on the lipid composition. Unusual tetraacylglycerols in fractions collected from NARP–HPLC of *Lesquerella fendleri* oil have been analyzed by infusion using (+)ESI-MS of lithium adducts, with normal FAs (as opposed to hydroxyl FAs) found at the *sn*-2 position [147].

2.2.3. With Liquid Chromatography Before discussing LC–ESI-MS, it is worthwhile to mention separately the evolution of LC of lipids without necessarily being coupled to ESI-MS. Liquid chromatography applied to lipids falls into two primary categories: (i) class separation and (ii) molecular species separation. When a total lipid extract is analyzed, the class separation is often conducted using normal-phase HPLC on a silica column [148, 149], since the classes vary dramatically in polarity. Molecular species separations are often done using reversed-phase (RP) HPLC on C18 columns, often with nonaqueous (NA) solvents, for NARP–HPLC [22]. In such separations, molecules are separated by carbon chain length and degree of unsaturation, so in the case of PLs especially, molecular species from different

PL classes having similar CN and degree of unsaturation may coelute with other classes. For NLs, FAs typically elute first, followed by MAGs, DAGs, and TAGs, with little or no overlap between classes, but molecular species within each class may be separated. There is usually some partitioning of regioisomers within peaks, as described in the article and review article by Mottram et al. [150, 151]. DB isomers (e.g. TAGs with ω-3 vs ω-6 FAs) cannot usually be separated using NARP–HPLC, so silver-ion chromatography is used, the mechanism of which is based on coordination of DBs to column-immobilized silver ions, as recently thoroughly reviewed by Holčapek [152] in addition to previous expert reviews [153, 154], which are necessary reading on the topic. In such cases, α-Ln (ω-3) was retained longer than γ-Ln (ω-6) [110, 155], though this was solvent dependent [108]. Ag-ion chromatography also effectively differentiates TAGs with *cis* vs *trans* DBs, with the former retained more strongly than the latter. Ag-ion chromatography is also able to differentiate regioisomers, based on differences in spatial availability of the DBs, where unsaturated FAs at *sn*-2 are less available for coordination to silver ions than those in the *sn*-1 and *sn*-3 positions. Additional elution trends are discussed in detail in the reviews cited above. The disadvantage of Ag-ion HPLC for TAG analysis is that TAGs with similar degrees of unsaturation group in clusters, which presents a problem in complex natural mixtures, and saturated TAGs are unretained.

An important concept that analysts routinely encounter in HPLC of lipids is that of equivalent carbon number (ECN). The CN is simply the total number of carbons in the FA chains, for instance triolein has $3 \times 18 = 54$ carbons (the glycerol backbone is not included), so CN = 54. In its simplest form, the ECN = CN $- 2 \times$ #DBs, so, for instance triolein has a simple ECN of 48 ($=54 - 2 \times 3$). This is intended to better reflect the elution of lipids during chromatography, where TAGs elute from lowest to highest ECN by RP-HPLC [156], and from highest to lowest ECN by NP–HPLC [157]. However, the term partition number (PN) (originally integral PN [158]) had already been defined [159] as PN = CN $- 2 \times$ #DBs, and was widely used. To further complicate matters, some authors used additional terms to better fit the ECN to observed elution patterns [160, 161], as described in the IUPAC supplement to the seventh edition on standard methods for analysis of oils and fats [162], while yet others defined other, similar terms (e.g. theoretical carbon number) for the same reason [156, 163]. Therefore, to avoid confusion, the original term PN is the least ambiguous. While the term ECN is most commonly used nowadays as interchangeable with PN, it may also include additional terms to better reflect effects of unsaturation [162], or be empirically determined [160], etc. Therefore, some caution and awareness of the factors above is necessary when using ECN.

As seen from some of the older references earlier, lipid separations go back to the origins of liquid chromatography, and have been applied with many types of mass spectrometries over the decades. Thus, the separation process is more mature than is analysis by API-MS techniques, and much of that work is now being translated into methods for UHPLC. Almost 9400 articles and 200 reviews show up in a database search of HPLC for lipid or TAG analysis. Since chromatography instruments and columns have changed substantially over the years, the focus here is on more recent reports on LC-MS of lipids. Representative reviews of chromatographic aspects of analysis of FAs [34, 164–166], TAGs [24–27, 167–169], carotenoids [170], and overviews of lipid analysis [171, 172] and lipidomics [50, 66, 173], including 2D-LC [174], provide starting points for further investigation into the HPLC and UHPLC techniques in recent use. Owing to space limitations, PLs are not discussed in depth, so reviews [175–178] of food and dairy PLs serve as a starting point.

Early reports using ESI-MS for TAGs showed that it is sometimes beneficial to chemically derivatize TAGs and related molecules to provide a readily ionizable charge site. An early example of this was the formation of dinitrophenylurethane (DNPU) derivatives by Myher et al. [179], who formed these derivatives to be able to separate regioisomers by chiral HPLC of TAGs in fractions collected from TLC and analyze them by negative-ion HPLC. DNPU derivatives were then used for chiral chromatography (CC)/ESI-MS of phosphatidylglycerols (PGs) [180], and were later used for analysis of 1-*O*-alkylglycerols [181] and for algal digalactosyldiacylglycerols (DGDGs) [182]. Thus, DNPU derivatization followed by chiral LC–ESI-MS remains a useful tool for definitive determination of both regioisomers (e.g. *sn*-1,2 vs *sn*-2,3) and enantiomers (*R* vs *S* configuration at *sn*-2), the latter of which are especially difficult to differentiate by LC-MS.

Early applications of ESI-MS applied to NLs revealed that it is particularly amenable to analysis of oxidation products. ESI-MS was applied to multiple types of oxidation products that were prepared by three different approaches by Sjovall et al. [183], and DNPH derivatives were used for analysis of the core aldehydes, while underivatized hydroperoxides were observed directly as their $[M+Na]^+$ adducts, with partial separation of regioisomers. Sjovall et al. [184–186] published a series of reports in the early 2000s that used *t*-butyl hydroperoxide (TBHP) to produce TAG oxidation products (TAGOX), followed by their analysis using RP–LC–ESI-MS. The TAGOX formed abundant ammonium adducts, $[M+NH_4]^+$, and when upfront (nonspecific) collision energy was provided, they produced structurally significant $[M+NH_4-RCOONH_4]^+$ (=$[DAG]^+$) fragments for identification of the FA chains. ESI-MS proved highly valuable for structural elucidation of TAGOX, although factors such as TBHP that bridged FA chains and other issues complicated the interpretation of data. Early reports on the analysis of TAGOX by ESI-MS have been reviewed previously [107, 128, 187, 188], and coverage of the topic has been updated in a recent review by Zeb [169].

At the same time that TAGOX were being studied by the methods mentioned earlier, ESI-MS was being applied to conventional TAG analysis by others, such as Schuyl et al. [189], who employed silver-ion (argentation) chromatography coupled to ESI-MS on two different instruments. Among the points worth mentioning are that the sensitivity was dramatically solvent and flow rate dependent. Using dichloromethane (DCM):methanol (MeOH) as an infusion solvent, $[N+NH_4]^+$ ions gave a very good S/N. But when the solvent was changed to the toluene–hexane used for Ag-ion chromatography, the S/N dropped by half, and it fell further when the flow rate of infusion increased. Therefore, the authors switched to using $[M+Na]^+$ as adducts because these were less susceptible to solvent and flow rate effects. Of course, for those who use DCM and MeOH in their LC solvents (e.g. in nonaqueous reversed-phase (NARP) HPLC), $[M+NH_4]^+$ adducts provide a very sensitive and robust alternative to $[M+Na]^+$ adducts. Results for interesterified palm oil by HPLC–ESI-MS were compared to those by HPLC-FID and found to be in fair agreement ($\leq$5%).

Only the most recent applications of HPLC– and UHPLC–ESI-MS to food and edible lipid analysis are included here, owing to the large volume of applications, which could easily constitute a separate article. These provide an indication of which methods have evolved to most prominent current usage. A report on the PLs of yellow croaker roe used RP–ULPC–(±)ESI-QTOF-MS [190] (note that UPLC is a trademarked term by Waters, Inc., and should only be used to describe data from those systems; UHPLC is the generic widely applicable term). PLs of buffalo and bovine milk were analyzed by hydrophilic interaction liquid chromatography (HILIC) UPLC–(−)QTOF-MS [191]. TAGS in whale oil and archeological samples used RP-HPLC–(+)ESI-QTOF-MS [192]. Rapeseed extracts were

analyzed using HILIC–UHPLC–ESI-MS for PLs and RP–UHPLC–ESI-MS for TAGs and DAGs [193]. A lipid class determination of gonads (also known as "roe") from three types of sea urchins was first done by TLC-FID, followed by analysis of the PLs by HPLC-(+ → –) ESI-MS on a QqLIT hybrid instrument, and also by ^{31}P NMR, which is an ideal tool for PL class analysis [194]. The same research group used HILIC–ESI-QqLIT for analysis of PLs and also TAGs in hexane/ethanol extracts of six species of clams, again with ^{31}P NMR for PLs [195]. PLs in methanol/MTBE extracts of the same six clam species had previously been analyzed using the same HILIC–ESI-QqLIT approach [196]. PLs in six kinds of nuts were reported using HILIC–(±)ESI-IT-TOF, showing the class separations and partial molecular species separations within classes. Seasonal variations in the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) molecular species in mussels were reported from HILIC coupled to a HRAM quadrupole-orbitrap hybrid instrument (a QExactive) using ESI [197]. Very long chain FAs (VLCFAs) were reported in PLs in brewer's yeast using NP-HPLC coupled to a hybrid LIT-Orbitrap instrument (LTQ Oritrap Velos) in (−)ESI mode [198]. The first report of the use of UHPLC–ESI-MS for the Ag-ion separation of TAGs in African mobola plum kernel oil by degree of unsaturation and DB type (*cis* vs *trans*) was recently reported, as it was used as the second dimension in a comprehensive 2D-LC separation, in the so-called LC1MS2 × LC2MS2 = LC2MS4 experiments [199]. Oxidized lipids (TAGs, MAGs, DAGs, and PLs) in wheat seed samples under different storage conditions were analyzed from $[M+NH_4]^+$ and $[M+H]^+$ precursors using UHPLC–(+)ESI-MS/MS [200]. Similarly, LC-MS was included among the multiple techniques reviewed for analysis of minor TAGOX, including epoxides alcohols, and ketones [201]. Fractions collected from silica column chromatography followed by infusion were used for lipid class analysis of European salmon roe using (+)ESI-MS on a QTrap hybrid system, which was then followed by HPLC analysis of TAGs as $[M+Na]^+$ using a QTOF-MS/MS instrument [200]. Changes in acetogenin, TAG, and DAG compositions in avocado seeds during growing and ripening were assessed using RP-HPLC–(+)ESI-TOF-MS of multiple adducts with lipidomic analysis [202]. The molecular species of lipids (ceramides (Cer), PLs, DAGs, and TAGs) in goat milk, soymilk, and bovine milk were analyzed using RP–UHPLC–(±)ESI-qOrbitrap-MS (QExacitve) [203]. The lipids of yellow head catfish were determined in response to including fucoidan (a fucose-containing sulfated polysaccharide) in their diet, using UPLC–ESI-Q-TOF-MS [204]. HILIC–ESI-MS/MS of egg yolk was reported, with an emphasis on PE and PC molecular species containing PUFAs, using multiple reaction monitoring (MRM) transitions [205]. Similarly, PL molecular species in duck, hen, and quail eggs were determined using HILIC–UPLC–(±)ESI-QTOF-MS [206]. The products of drying oils (i.e. fresh linseed oil) and model and historical oil-based paints, which included TAGs and their hydrolysis products (MAGs, DAGs, and FFAs) and their oxidation products, have been analyzed using RP-HPLC–(±)ESI-QTOF-MS [207].

2.2.4. Triacylglycerol Regioisomers by ESI-MS Regioisomer determination was fundamental to the early development of LC–ESI-MS analysis of TAGs, and has been an area of ongoing interest since then. Therefore, this section presents work covering more than just the most recent two years that the previous sections encompassed. Insights gained from the first reports of ESI-MS for structural elucidation were mentioned above, and early works on characterization of TAG positional isomers by ESI-MS have been reviewed previously [44, 107]. Kalo reviewed regioisomer analysis of TAGs in 2013 [208]. Holčapek reviewed methods for TAG regioisomer analysis in his recent article on Ag-ion LC-MS [152]. In addition to the

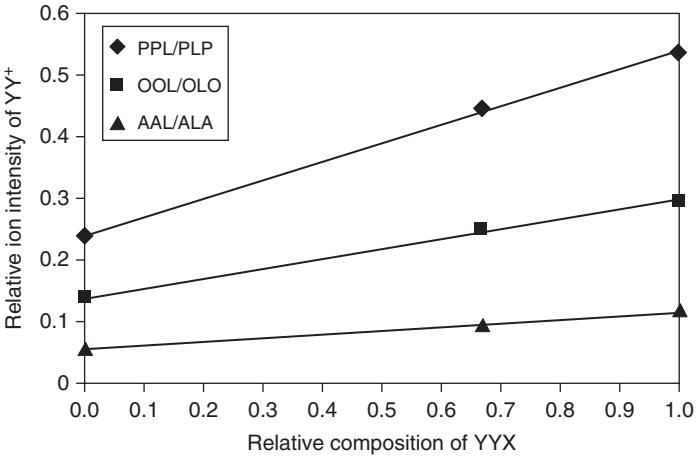

Figure 5. *Calibration plots for the PPL/PLP, OOL/OLO, and AAL/ALA positional isomer systems. These are representative examples of the calibration plots obtained in this work, and provide some indication of the precision possible in performing positional analysis using these plots. The letter "P" represents the palmitate ($C_{16:0}$), the letter "O" represents the oleate ($C_{18:1(c-9)}$), the letter "A" represents the arachidonate ($C_{20:4(cccc-5,8,11,14)}$), and the letter "L" represents the linoleate ($C_{18:2(cc-9,12)}$). Source: Reprinted from Ref. [109] with permission. Copyright 2017 John Wiley & Sons, Inc.*

IM-MS approaches referenced above for lipid isomer analysis [101, 102, 104], a recent review addressed other approaches for structural characterization of isomeric lipids [103]. Although enzymatic approaches remain as very useful tools for regioisomer determination [209], this article focuses on LC-MS methods for their analysis. While most approaches mentioned here employed LC coupled to MS, reports that utilized infusion are included in this section, as are some applications to biological samples, since these may be directly applicable to edible fats and oils. Most reports of IM-MS for regioisomer separation are mentioned above, in the section devoted to that topic.

Recently, Kallio et al. [210] used RP-UPLC–ESI-MS of lithium adducts for quantification of TAG regioisomers in human milk samples. They used a proprietary algorithm written using *GNU Octave* (having originally been implemented in MATLAB) to solve solutions using sequential quadratic programming (SQP). With this approach, they showed calibration curves for several TAG standards, and reported the identities of hundreds of TAG regioisomers in milk from Finnish and Chinese women. Another recent report [109] demonstrated the approach to using calibration lines from ESI-MS, which had previously been demonstrated using APCI-MS [211, 212], using infusion into a simple ESI-IT-MS instrument to construct calibration curves for calculation of the composition of YYX TAGS in regioisomeric mixtures using relative ion intensities for 42 YYX/YXY TAG pairs. Typical calibrations for three TAG molecular species are shown in Figure 5. Byrdwell recently demonstrated the use of Ag-ion UHPLC–ESI-MS (and APPI-MS) as the second dimension in a 2D-LC separation to produce substantial resolution of regioisomers, based on the Ag-ion separation principles reviewed by Holčapek [152] and others [154]. Interestingly, Sala et al. used differential-mobility spectrometry (DMS) coupled to infusion ESI-QTrap-MS of silver adducts (not Ag-ion LC) to effect DMS separation of four TAG regioisomer pairs, as standards and then in porcine adipose tissue [213]; the $[M+NH_4]^+$ and $[M+Na]^+$ adducts showed no separation by DMS, but

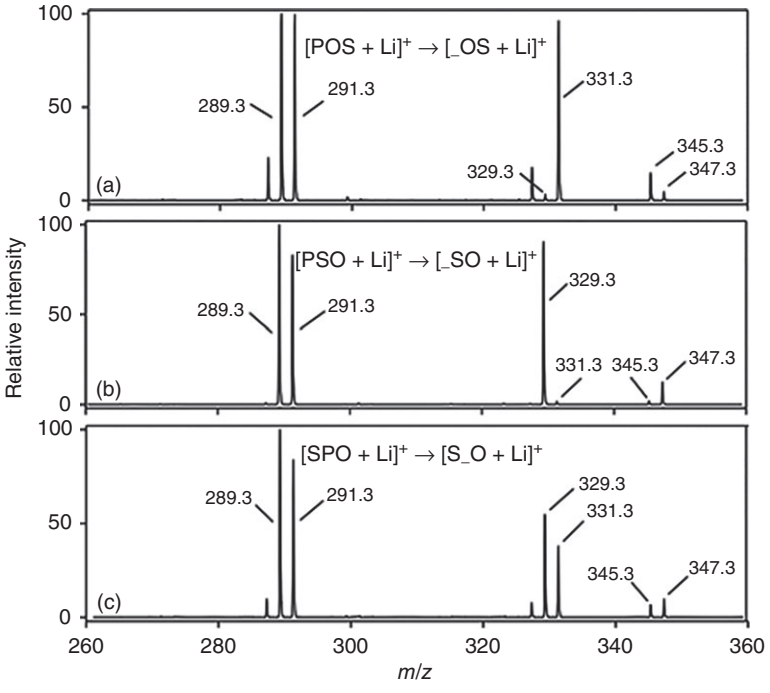

Figure 6. *MS^3 spectra of lithium adducts of (a) POS, (b) PSO, and (c) SPO. In each case, the first-generation precursor ion is* m/z *867.8, and the second-generation precursor ion,* m/z *611.6, has lost a neutral P acid side chain. Source: Reprinted from Ref. [216] with permission. Copyright 2015 John Wiley & Sons, Inc.*

$[M+Ag]^+$ ions did. Lin and Chen identified regioisomers of tetraacylglycerol $[M+Li]^+$ ions from lesquerella oil by infusion of NARP-HPLC fractions into an ESI-MS instrument [147], as described earlier. In 2016, they had analyzed the regioisomers of 72 lesquerella TAGs using the same approach [214]. The regioisomer assignments were made based on MS^3 using the mechanism developed from the model of Grossert et al. [126]. In 2015, Byrdwell [215] compared the regioisomer identification approach used for APCI-MS based on tabulated values [108] to ESI-MS and APPI-MS using NARP–HPLC–(APCI + ESI + APPI)-MS analysis of soybean oil in a dietary supplement. Regioisomer assignments agreed well for many, but not all TAGs, and the agreement was better between APPI-MS and APCI-MS than ESI-MS where TAGs with PUFAs showed more disagreement. Ramaley et al. [216] used MS^3 with infusion on a QTrap instrument for (+)ESI-QqLITMS of $[M+Li]^+$ adducts, based on the precedent described earlier [123, 124, 126]. They analyzed PPO/POP and POS/PSO/SPO isomer sets as standards, and then analyzed those in natural and spiked fish oils. Figure 6 shows typical MS^3 mass spectra of the three TAG regioisomers POS, PSO, and SPO. In 2014, Li et al. [217] used infusion into (+)ESI-QTOF-MS, with MS/MS of $[M+Li]^+$ precursors, for the determination of TAG regioisomers in the marine diatom *Thalassiosira pseudonana*. They used the presence of $[M+Li\text{-}R_{1/3}COOH\text{-}R_2CH=CHCOOH]^+$ fragments in MS/MS spectra to identify the components at *sn*-2. In 2013, the group of Ramaley et al. reported two applications of their approach of using (+)ESI-QqLITMS of $[M+Li]^+$ adducts, one using infusion [218], and another using NARP–HPLC–ESI-MS of TAGs in fish oils [219]. For the fish oils, they

used five pairs of TAG regioisomer standards containing EPA or DHA, and then quantified those in fish oils. In their other report they demonstrated, based on those same five standard pairs, that calibration curves by MS^3 of $[M+Li]^+$ ions should be considered inherently nonlinear and fit to second-order polynomials. Nagy et al. used an LTQ Orbitrap XL instrument for RP-HPLC–(+)ESI-LIT-orbitrap-MS of interesterified fat, beef tallow, pork lard, and butter fat samples using $[M+NH_4]^+$ ions for quantification and precursor ion selection and $[M+Na]^+$ ions as qualifying ions to reduce false positives. They used data-dependent scans from an inclusion list of 28 000 potential TAGs (up to 3 DBs), HRAM masses to distinguish monoisotopic TAGs from isotopic variants of closely related TAGs, and estimated the regioisomer compositions based on tabulated values [108]. One last older article should be mentioned [220], since it demonstrated the use of calibration lines formed from mixtures of OOS/OSO, OOP/OPO, and PPO/POP, to quantify regioisomers of these TAGs in rapeseed and soybean oils by NARP–HPLC–ESI-MS. This was an approach that had been well demonstrated for APCI-MS analysis, discussed earlier.

Kalo reviewed [208] the area of analysis of TAG regioisomers in 2013, so no further reports are mentioned here. The above references make it appear that lithium adducts, $[M+Li]^+$, have become the favored tool for identification of regioisomers, especially by MS^3, and are also commonly used for quantification of intact TAG molecular species. If there is one disadvantage to the use of lithium adducts, it is that the $[M+Li]^+$ precursors produce both $[M+Li-RCOOLi]^+$ and $[M+Li-RCOOH]^+$ fragments for loss of each FA by MS/MS, giving twice as many ions as MS/MS of $[M+NH_4]^+$ precursors, which can complicate data interpretation. Nevertheless, it seems that this approach is approaching becoming the norm for ESI-MS analysis of TAGs.

2.3. Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS)

The early reports of APCI-MS for TAG analysis have been reviewed multiple times [26, 44, 106, 107, 221, 222]. Lipid analysis by SFC–APCI-MS seems to have started with Tyrefors et al. [223] in 1993, who reported a custom-built APCI source attached to a Sciex API III instrument for SFC–APCI-MS of lipid standards (C15:0 FA and FAME, trilaurin (tri-12:0), cholesterol, and cholesterylpalmitate). However, since the makeup gas was sparged through water, the saturated TAG trilaurin produced a protonated molecule base peak and large $[M+H_2O]^+$ adduct (as did other lipid classes), which are now known to be atypical. The era of HPLC–APCI-MS started in 1995 with the publications of Byrdwell et al. [224–226], who used a commercially available APCI-MS instrument coupled to NARP–HPLC for qualitative analysis of TAG standards [224] and seed oils [225, 226]. Figure 7 shows typical APCI-MS mass spectra of two soybean oils TAGs [226]. The main upshot of those reports was that the amount of protonated molecule, $[M+H]^+$, versus diacylglycerol-like fragment, $[M+H-RCOOH]^+ = [M-RCOO]^+$ or $[DAG]^+$, increased with increasing unsaturation, and decreased with decreasing unsaturation, to the point that saturated TAGs gave little or no $[M+H]^+$. It was obvious from this dependence of degree of unsaturation that RFs would be necessary for accurate quantification of TAGs based on APCI-MS. But the $[DAG]^+$ fragments readily allowed the FA chains on TAGs to be identified for qualitative analysis of TAG molecular species. In 1996, Byrdwell et al. [79, 227] reported a comparison of several approaches to quantification of TAGs. Linear calibration lines of TAG standards were constructed using an internal standard method with d_{12}-PPP I.S., but given the limited number of TAG standards available and the large number of molecular species in normal samples, it was deemed impractical to construct calibration lines

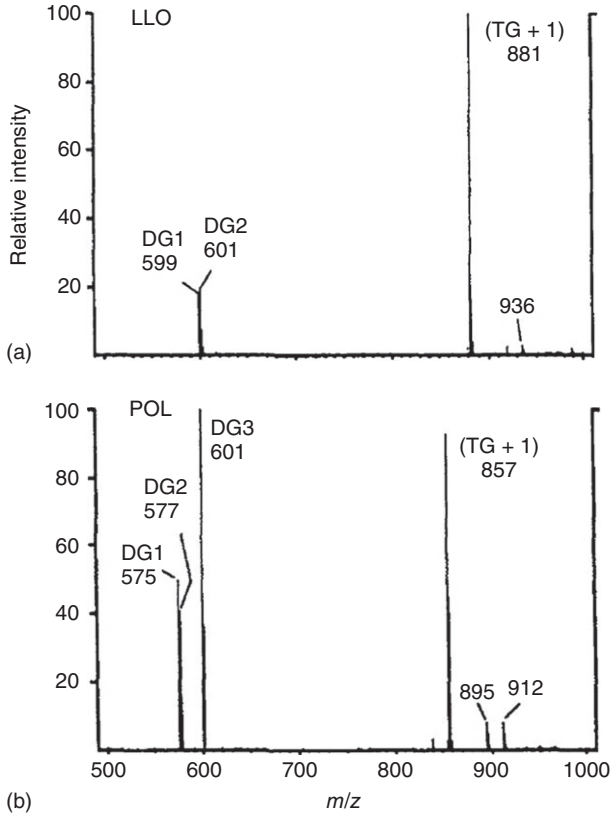

Figure 7. *(a) Mass spectrum of oleoyl–dilinoleoyl–glycerol (LOL/OLL/LOO). TG + 1 is the protonated molecule. DG1 and DG2 are the two characteristic diacylglycerol fragments for the triacylglycerol. (b) Mass spectrum of palmitoyl–oleoyl–linoleoyl–glycerol (POL/**OLP**/LPO/LOP/**PLO**/OPL). TG + 1 is the protonated molecular ion. DG1, DG2, and DG3 are the three diacylglycerol fragments for the triacylglycerol. Abbreviations as in Figure 1. The second TAG is now recognized as OLP/PLO due to $[PO]^+$ at* m/z *577.5 being the smallest. Source: Reprinted from Ref. [226] with permission. Copyright 1995 John Wiley & Sons, Inc.*

for all TAGs or to try to quantify all TAGs based on a few standards. Another approach was comparison to a TAG mixture of known composition, synthesized from FAs and glycerol. The approach to quantification that was the most successful was that in which the FA composition was calculated from the TAG composition, and that was compared to the FA composition from calibrated FAME analysis (converted from weight% to mol%) to produce RFs for each FA, which were then multiplied together to get TAG RFs. When the TAG RFs were applied to the TAG composition and then the FA composition was calculated from the RF-adjusted TAG composition, the agreement to the calibrated FA composition from GC-FID was excellent, and was better than the agreement of the FAs from the TAG composition found from an LC-FID detector. Furthermore, the method required no synthesis of a TAG mixture or preparation of numerous TAG standards at multiple concentration levels. This approach has now been used numerous times, and continues to consistently give better results (i.e. agreement of the FA composition from TAG to that by GC-FID) than other approaches that have been reported.

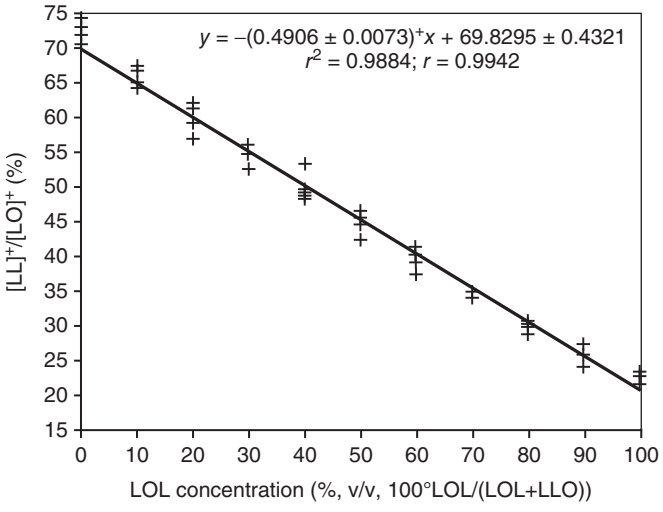

Figure 8. *Ratio of the $[LL]^+$ and $[LO]^+$ fragment ions (%) in mixtures of LOL and LLO at various percentages (calibration curve). Source: Reprinted from Ref. [211] with permission. Copyright 2003 John Wiley & Sons, Inc.*

The next milestones in APCI-MS analysis of TAGs came in 1996 with the recognition that APCI-MS could be used for discrimination of regioisomers, specifically the component in the *sn*-2 position. Laakso and Voutilainen used Ag-ion HPLC–APCI-MS [110] to identify TAGs in cloudberry seed oil, evening primrose oil, borage oil, alpine currant seed oil, and black currant seed oil. Using OOP and OPO standards as examples, they reported that the $[M–RCO_2]^+$ fragment formed loss of the palmitic acid FA at the *sn*-2 position was less than that formed when the palmitic acid was in the *sn*-1 or *sn*-3 position. Because of the sample complexity, the regioisomers in the seed oils were not determined. On the basis of the Ag-ion chromatography and not APCI-MS, TAGs with γ-Ln (ω-6 18:3) eluted before TAGs with α-Ln (ω-3 18:3), all else being equal. Also, TAGs with unsaturation in the outer positions (*sn*-1,3) were retained longer than when the unsaturation was located in the *sn*-2 position (with the exception of OOP versus OPO). Mottram and Evershed [111] used mixtures of SSO/SOS and PPO/POP to also demonstrate that the ratio of the $[AA]^+/[AB]^+$ in generic ABA/AAB/BAA TAGs was smaller when "B" was in *sn*-2. They further demonstrated for the first time that for an ABC TAG (three different FAs), the $[M–RCO_2]^+$ fragment from loss of the "B" FA was the smallest of the $[DAG]^+$ fragments.

Recognition of these trends was used to identify the predominant regioisomer in standards or plant oils [150], but applications to quantify the relative amounts of isomers in complex mixtures required further work. In 2003, Jakab et al. [211] reported the analysis of mixtures of LOL/LLO regioisomers in known amounts, which produced linear calibration lines (such as shown in Figure 8) based on the ratio of $[LL]^+/[LO]^+$ versus the %LOL. Given that there is one way to get an $[LL]^+$ fragment (only if it has both "L" FAs), and two possible ways to get an $[LO]^+$ fragment (one or the other "L" FAs with the "O" FA), the statistically expected ratio of $[LL]^+/[LO]^+$ should always be 1/2 or 0.5. But given the fact that loss of the *sn*-2 is energetically disfavored [110, 111], the $[LL]^+/[LO]^+$ was <0.5 for LOL and >0.5 for LLO. But the exact ratio required calibration from mixtures of known regioisomer composition.

In 2004, Fauconnot et al. developed a similar approach, in which seven TAG regioisomer pairs were analyzed, and equations for calibration lines were calculated from the values of $100 \times [AA]^+/([AA]^+ + [AB]^+)$ (in this case, the statistically expected value would be 1/3, or 0.333, since $[AB]^+$ should still be $2 \times [AA]^+$). This approach was used to identify those seven regioisomers in palm oil, cocoa butter, and in beef, pork, and chicken fats. Construction of calibration lines from a series of mixtures of known composition for multiple TAG regioisomer pairs still remains the most accurate means of regioisomer quantification by APCI-MS. However, making and analyzing multiple sets of mixtures is labor-intensive and time-consuming, and only a few regioisomer pairs are commercially available.

Because of the linearity of the plots in both the reports, Byrdwell simplified the approach to use only two pure regioisomers in his 2005 review [107] and article [228]. The simple equations were presented to determine the %ABA or %AAB from the $[AA]^+/[AB]^+$ ratios for the two pure regioisomers, and any $[AA]^+/[AB]^+$ observed from a real sample. It was also shown why the $[AA]^+/[AB]^+$ ratio of Jakab et al. [211] is simpler and preferred over the value of 100 x $[AA]^+/([AA]^+ + [AB]^+)$ [212]. The ratios of fragments from numerous reports of both APCI-MS and ESI-MS in the literature were tabulated to show the similarities across multiple users of similar instruments, though one instrument was an outlier, producing ratios far from statistically expected values [107, 228]. As mentioned earlier, some TAGs produce $[AA]^+/[AB]^+$ ratios close to the statistically expected value of 0.5 for the AAB regioisomer, while the ratio from the ABA isomer is usually much lower. On the other hand, some other AAB TAGs produce ratios higher than the statistically expected value. Therefore*, looking at one ABA/AAB/BAA regioisomer alone is not sufficient to draw conclusions about regioisomer identities*. However, owing to fairly consistent behavior, comparison to tabulated values can be useful. Also, the tabulated values [107] indicated that ratios obtained by ESI-MS/MS (or ESI-MS with upfront CID) were similar to those by APCI-MS. The most thorough and useful tabulation of fragment abundances from TAG regioisomers is that produced by Holčapek et al. [108], who compared raw abundances for synthesized regioisomers analyzed using Ag-ion HPLC–APCI-MS on five different mass spectrometers.

Two major trends described earlier for APCI-MS of TAGs can be summarized as: (i) the dependence of the relative amounts of $[M+H]^+$ versus $[DAG]^+$ on the degree of unsaturation and (ii) the dependence of the ratio of $[DAG]^+$ fragments on the identity of the FA at the *sn*-2 position. The first trend has been characterized by Byrdwell using the $[MH]^+/\Sigma[DAG]^+$ ratio versus the number of sites of unsaturation, and the relationship has a sigmoid appearance [228, 229]. The second trend has been characterized by Jakab et al. and by Byrdwell using the $[AA]^+/[AB]^+$ ratio for ABA/AAB/BAA TAGs [211, 228, 229] and the $[AC]^+/([AB]^+ + [BC]^+)$ ratio for ABC TAGs [228, 229]. Until recently, no trends were reported regarding *sn*-1,2 or *sn*-2,3 $[DAG]^+$ fragments based on APCI-MS data. Differentiation of TAGs based on differences in *sn*-1,2 and *sn*-2,3 requires chiral chromatography [230, 231]. However, using the $[AB]^+/[BC]^+$ ratios [228, 229] calculated from the tabulated abundances reported by Holčapek et al. [108], Byrdwell reported that the factors that determine the ratio of the $[AB]^+$ and $[BC]^+$ fragments are the degree of unsaturation and the grouping of that unsaturation (whether next to each other or separated).

A different approach to TAG regioisomer determination was reported by Leskinen et al. [232], who reported NARP–UHPLC coupled to negative-ion APCI-QqQMS using ammonia as the nebulizing gas. On the basis of the formation of $[(M-H)-RCOOH-100]^-$ ions, they constructed five-point calibration lines, such as those shown in Figure 9, from the fragment ratio

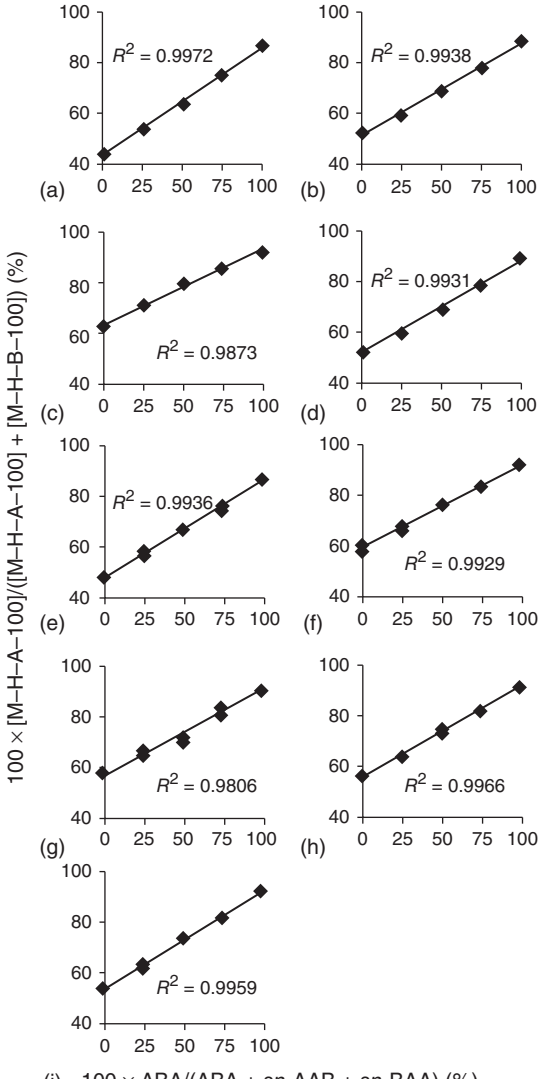

Figure 9. *The calibration curves obtained by UHPLC/ammonia NIAPCI-MS/MS. The ratio of product ions [M−H−A−100]⁻ and [M−H−B−100]⁻ is determined in the mixtures of ABA and* sn-*AAB* + sn-*BAA. (a) Ala/L/L,* y = *0.4275*x + *43.518; (b) Gla/L/L,* y = *0.3677*x + *51.061; (c) L/L/O,* y = *0.2952*x + *64.032; (d) L/O/O,* y = *0.375*x + *51.899; (e) P/O/O,* y = *0.3849*x + *47.563; (f) P/P/O,* y = *0.335*x + *59.475; (g) Po/Po/V,* y = *0.3388*x + *56.892; (h) Po/Po/O,* y = *0.3614*x + *56.054, and (i) C/O/O,* y = *0.3912*x + *53.114. Four parallel analyses were conducted from each regioisomer mixture. A and B denote different fatty acids. Source: Reprinted from Ref. [232] with permission. Copyright 2010 John Wiley & Sons, Inc.*

values $100 \times [(M-H)-R_ACOOH-100]^-/([(M-H)-R_ACOOH-100]^- + [(M-H)-R_BCOOH-100]^-)\%$ versus $100 \times ABA/(ABA + sn\text{-}AAB + sn\text{-}BAA)\%$ for nine TAG regioisomer pairs. Then, subsets of the nine TAGs were quantified in plant oils: black currant, rapeseed, sunflower seed, palm, and sea buckthorn pulp oils. The authors state that their approach "… is a clear

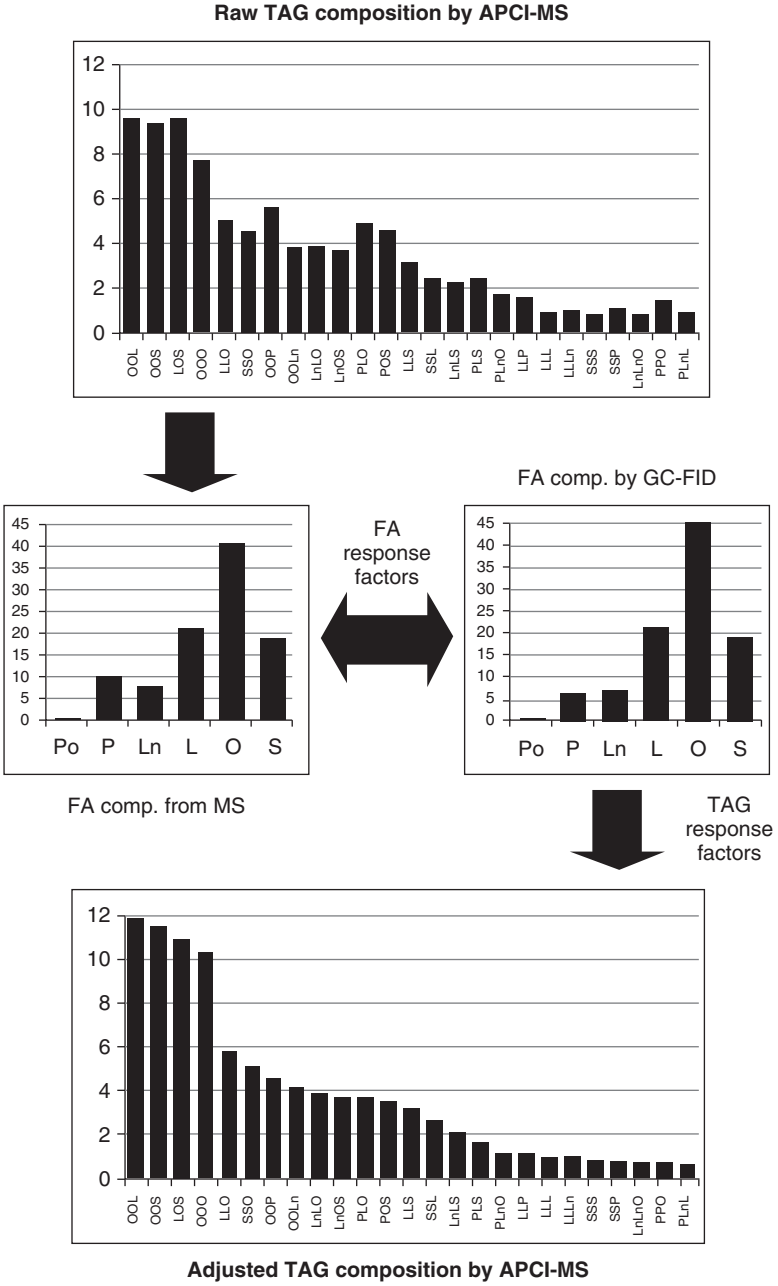

Figure 10. *Process for producing response factors from FA composition calculated from the TAG composition compared to that determined by GC-FID, producing TAG response factors, and producing a response-factor-adjusted TAG composition. Source: Figure reproduced from Ref. [236], without copyright restrictions.*

improvement to the methods earlier applied." Thus, their approach represents another option for the TAG analyst to consider.

With the general characteristics of APCI-MS spectra of TAGs outlined earlier, other reports address issues such as quantification of TAGs, coupling to different types of chromatography, and applications to a wide range of samples. Řezanka and coworkers have published multiple reports [233–235] and several reviews [26, 230, 231] describing the chiral separation of enantiomeric TAGs. While their recent CC applications were directed toward biological samples, the methods are entirely transferable to food and edible oil analysis. Although CC is one of the few approaches to differentiation of TAG enantiomers, its primary shortcoming is that the CC-HPLC chromatographic separations tend to be very lengthy (as much as 200 min or more).

A number of approaches for quantification of TAGs have been reported. The method of Byrdwell et al. [79] (pictured in Figure 10), which uses RFs from comparison of the FA composition calculated from the TAGs to that from calibrated GC-FID mentioned before, has been described in greater detail several times [107, 236, 237] and applied to a variety of samples including genetically modified canola oils [227], margarine base stocks [236], rice bran oil [131], olive oil [132], and others. Furthermore, it has been demonstrated to apply equally well to TAG analysis by NARP–HPLC–ESI-MS [131, 215] and NARP–HPLC–APPI-MS [131, 132, 215]. A recent improvement to the method, which applies to all API-MS techniques, has been to include the $1 \times {}^{13}C$ isotopic peaks for the $[M+H]^+$ and $[DAG]^+$ fragments to increase the signal without loss of specificity (since TAGs differ by sites of unsaturation, 2 Da). There are two variations of the method of Byrdwell et al.: the first integrates the areas under $[M+H]^+$ and $[DAG]^+$ fragments separately (with or without the $1 \times {}^{13}C$ isotopic peaks), and the second groups all $[M+H]^+$ and $[DAG]^+$ fragments together (with or without the 1 x ^{13}C isotopic peaks) and integrates all ions for a given TAG together. The first approach requires more peaks to be integrated, but allows the Critical Ratios [228, 229] to be calculated from the separate integrated areas of $[M+H]^+$ and $[DAG]^+$ fragments, for determination or quantification of regioisomers. The second approach does not immediately allow identification of regioisomers, but this can be done manually by obtaining the peak list for the average spectrum across the integrated range and using the ratios of the fragments to identify/quantify regioisomers. Thus, the approach can take more or less time, depending on the level of information desired.

Holčapek et al. [80] have used an approach to quantification that involved APCI-MS analysis of 23 monoacid TAGs (C8:0 to C22:1, including C18:2, α-C18:3, and γ-C18:3), construction of linear calibration equations ($y = a{\cdot}x + b$) in the range 50–500 mg/l, and calculation of RFs for each monoacid TAG as the slope of a_{OOO}/a_{TAG}, where OOO is triolein, tri-18:1. RFs for monoacid TAGs are shown in Figure 11. Then, RFs for mixed-acid TAGs (C14:0 and higher) were calculated from the arithmetic mean ($RF_{123} = [RF_1 + RF_2 + RF_3]/3$) of the RFs for the three monoacid TAGs containing the appropriate FA chains. In this way, RFs for 133 TAGs were calculated and applied to plant oils including walnut, hazelnut, cashew, almond, poppy seed, yellow melon, mango stone, fig seed, and date seed. The FA compositions calculated from the TAGs were compared to the FA compositions determined as FAMEs by GC-FID (as weight %). Readers should assess the agreement of FA compositions calculated from the different TAG quantification approaches to the GC-FID results, as well as the amount of synthesis and preparation and analysis of standards required, to decide which approach best suits their needs and capabilities.

The most recent applications of APCI-MS to fat and oil analysis include the following. In 2017, TAGs in four African sesame oils were analyzed using isocratic

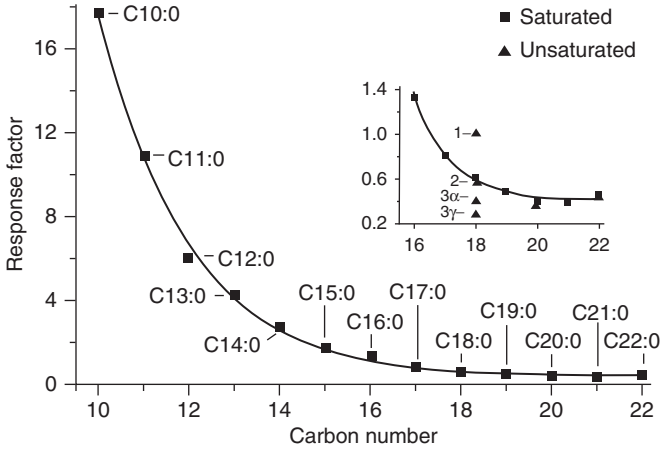

Figure 11. *Dependence of response factors of saturated single-acid TGs measured by APCI-MS on the carbon number in the acyl chain (fitted with the equation* $y = 29256\,exp(-0.5134x) + 0.3824$, $R^2 = 0.999$*). Unsaturated single-acid TGs are shown in detail in the inset. Source: Reprinted from Ref. [80] with permission. Copyright 2005 John Wiley & Sons, Inc.*

NARP–UHPLC–APCI-QqQMS [238]. The results were compared to a 2014 study on analysis of plant oils using HPLC–APCI–QqLITMS on a mixed-mode phenyl-hexyl column and an Ag-HiSep OTS column for separation of soybean, peanut, corn, and sesame oils [239]. Also in 2017 was the report by Byrdwell that employed NARP–HPLC–APCI-QqQMS in the first dimension along with 2 × ESI-MS and APPI-MS in a comprehensive 2D-LC analysis with four MS detectors in an LC1MS2 × LC1MS2 = LC2MS4 arrangement for analysis of normal TAGs, those containing *cis* and *trans* 18:3 isomers, and also oxo-TAGs in parinari seed oil [199]. In 2017, Rezanka et al. [231] published their valuable review of the analysis of regioisomers and enantiomers, which included a summary of APCI-MS approaches, which included their 2016 reports on separation of algal TAG regioisomers [235, 240]. In 2016, an article on analysis of TAGs in eggs [241] and a review of TAG analysis by APCI-MS [242] appeared in Chinese language journals. Also in 2016 was the report by Byrdwell [243] that generalized the previous work [215, 228, 229] that described the use of Critical Ratios from APCI-MS for determination of the structural characteristics of TAG described earlier, using soybean oil in a dietary supplement as an example. In addition to providing the desired structural information, the Critical Ratios constitute a reduced data set from which a library of APCI-MS mass spectra can be reproduced.

In 2015, Beccaria et al. [244] used comprehensive 2D-LC (LC × LC) employing Ag-ion HPLC in the first dimension and NARP–HPLC in the second dimension in stopped-flow and also offline configurations for analysis of the TAGs in fish (menhaden) oil, as pictured in Figure 12. In addition to that, they used comprehensive online GC × GC–MS and GC × GC-FID for the analysis of the FAs as FAMEs, with qualitative and quantitative analysis, respectively. Literature RFs from Holčapek and coworkers [80, 245] were applied for semiquantitative TAG analysis. The need for such 2D-LC and 2D-GC approaches highlights the complexity of some samples, such as fish oil. Geng et al. [246] used a lipidomics approach with RP-UHPLC coupled to HRAM Qorbitrap MS in both (±)ESI and (±)APCI modes, with up to MS^3, for qualitative analysis of wheat bran and germ. They identified a wide range

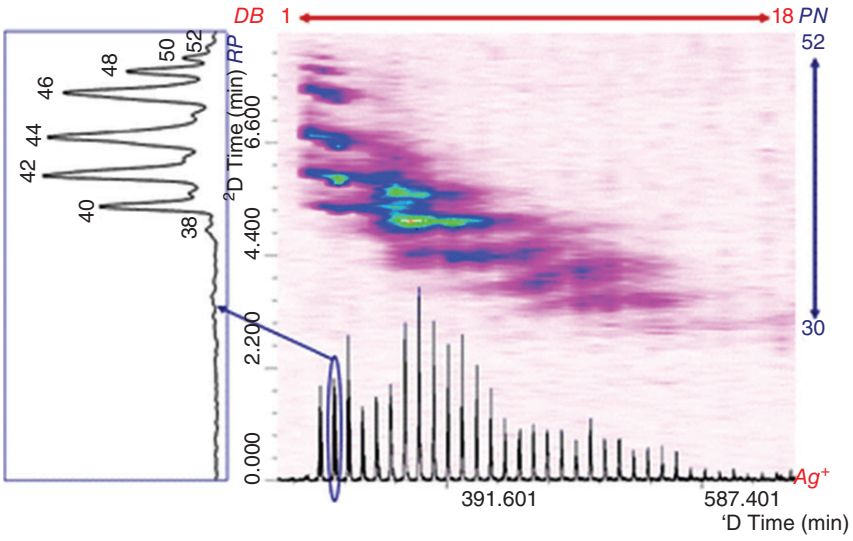

Figure 12. *Top: stop-flow Ag^+-LC × RP-LC-APCI-MS plot of the TAGs in menhaden oil. Bottom: modulated detector response. Left: 2D analysis of a selected fraction. Source: Figure reproduced from Ref. [244] with permission. Copyright 2015 Springer Nature.*

of lipid classes, as well as others, including PLs, galactolipids, oxylipins, ceramides, FAs, DAGs, and TAGs. Whole wheat was differentiated from refined wheat using PCA. One note of caution, however, is that the TAG fragmentation mechanism proposed by those authors is not in accord with the detailed work of Hsu and Turk [123] and Grossert [126].

Also in 2015, Hakova et al. [247] demonstrated the use of a small amount of $[M+55]^+$ adduct formed from acetonitrile (added by tee for infusion of standards, and in the mobile phase for the HPLC–APCI-MS of olive oil) as precursors for MS/MS, which produced characteristic ions for DB localization. Pulsed-Q CID (PQD) gave access to low-mass ions that are normally below the cutoff for such ion traps, but with some loss of sensitivity. While interpretation of mass spectra for simple monoacid TAGs was straightforward, spectra of polyunsaturated TAGs were much more complicated. And while the specific fragment ions could be picked out of the large number of other fragment ions from known polyunsaturated TAGs, the use of the technique for TAGs about which little was already known was more problematic. Dong et al. [248] reported the use of isocratic HPLC–APCI-QqLITMS (on a mixed-mode silver-modified HiSep OTS 2D) for analysis of 58 TAGs in 12 samples of refined peanut oils, including normal and high-oleic acid varieties, with oleic acid being as high as 80%. The column combined features of a C8 column and Ag-ion chromatography, but with polyunsaturated TAGs eluting before those containing monounsaturated and saturated FAs, indicating that the RP mechanism was predominant. Normal versus high-oleic acid varieties were easily differentiated by PCA. Fanali et al. [249] used NARP–HPLC–APCI-MS for analysis of TAGs in quinoa, combined with GC-FID of FAMEs plus 2D-GC–MS + FID and also GC-HR-TOF-MS of 66 unsaponifiable lipids (hydrocarbons (linear alkanes, squalene), alcohols, tocopherols, sterols, and free FAs). Quantification of TAGs was done using the literature RFs of Holčapek [80]. Wei et al. [250] used offline 2D HPLC–APCI–QqLITMS on a mixed-mode (phenyl-hexyl) column for analysis of TAGs, which was compared to offline 2D-LC using NARP–HPLC and Ag-ion

HPLC. The different characteristics of the mixed-mode column were successively brought forward in two separations using complementary solvent systems, with one mode utilizing hydrophobicity and the other utilizing π–π interactions with sites of unsaturation. Fractions from the ^{1}D were collected and subjected to analysis in the ^{2}D. Quantification of TAGs was done using RFs calculated from a series of 12 monoacid TAGs, according to the method of Holčapek et al. [80]. TAGs in six varieties of soybean oil, peanut oil, and "lord" oil were analyzed and separated into clusters using PCA. Several reports by Byrdwell [215, 229, 251] came out in 2015 that described the use of Critical Ratios for characterization of the structural properties of TAGs. Most of the results of these reports were described earlier in the section on trends in APCI-MS spectra of TAGs, since the purpose of the Critical Ratios was to elucidate trends, allow easy quantification of TAG regioisomers, and to be able to reproduce mass spectra from fewer values than the raw data. That work did finally characterize the pattern behind $[sn\text{-AB}]^+$ versus $[sn\text{-BC}]^+$ fragments in ABC TAGs, as described before.

In 2014, the last year covered here, Hu et al. [239] described their work, mentioned before, using HPLC with a mixed-mode phenyl-hexyl column and an Ag-HiSep OTS column coupled to APCI-QqLITMS for separation of six of each type of soybean, peanut, corn, and sesame oils. The authors developed a proprietary method for converting the MS data into barcodes for data comparison. PCA was also used to show the facile separation of clusters based on the very different TAG compositions, with loadings showing the TAG species that accounted for the greatest differences, as typical for PCA. Beccaria et al. [252] reported a thorough qualitative analysis of TAGs in goat, cow, and human milk and buffalo mozzarella cheese samples separated on one, two, or three fused core C18 columns in series, for NARP–UHPLC–APCI-IT-TOF-MS. Between the four samples, 243 TAGs were identified. Linderborg et al. [253] also reported an analysis of human milk TAGs using an update of the group's previously reported [117] NARP–UHPLC-negative-ion APCI-QqQMS approach with ammonia as the nebulizer gas [232] and a core–shell column, in which $[(M-H)-RCOOH-100]^-$ and $[RCOO]^-$ ions were used with their lab-written proprietary software MSPECTRA [254] to identify TAG regioisomers. They also used NARP–UHPLC–(+)ESI-QqQMS with ammonia as the nebulizer gas to form $[M+NH_4]^+$ adducts. More than 60 TAGs were analyzed by this group, including 37 regioisomers.

The above references between 2018 and 2014 provide an indication of what are the recent trends in APCI-MS analysis of TAGs. These fall into several broad categories, some of which have already been discussed. The newest column technologies for UHPLC are being implemented to reduce analysis times compared to HPLC or to provide better separations in the same time. New generations of instruments, including the increased use of hybrid instruments and especially HRAM (mostly TOF and Orbitrap) instruments, are being brought to bear on TAG analysis. Several approaches to TAG quantification have been reported, some of which use RFs calculated for a subset of TAG molecular species based on analysis of TAG standards, and others that calculate RFs from comparison of the FA composition from calibrated GC-FID to that determined from the TAG composition. The important point is that RFs are necessary due to differences in response according to degree of unsaturation and chain length. Quantification of regioisomers continues to factor heavily into analyses, and researchers who are new to the field need to recognize that fragment ratios from one ABA/AAB/BAA regioisomer is not sufficient for identification of regioisomers, unless the ratio is very low ($\sim$<0.3 for the $[AA]^+/[AB]^+$), which indicates the ABA isomer. Some researchers use the $[AA]^+/[AB]^+$ ratio, while others use $[AA]^+/([AA]^+ + [AB]^+)$, and these provide different values. Note that these are related by $[AA]^+/([AA]^+ + [AB]^+) = 1/(1 + 1/([AA]^+/[AB]^+))$. DB localization continues to be an area

in need of further development. While some approaches are available, they are not convenient enough yet to be widely adopted. The use of PCA continues to be more widespread, as efforts are made to derive more information with less manual processing of data and to elucidate trends that are not readily apparent. The use of lipidomics approaches, with library-based identification of an ever-growing number of lipids (with libraries constantly expanding), continues at a steady pace. But neophytes need to be especially careful when making assignments based solely on library match results.

2.4. Atmospheric Pressure Photoionization Mass Spectrometry (APPI-MS)

The development of modern APPI-MS seems to date back to 2000, with the report of Robb et al. [255], who employed a lab-made APPI source customized from an APCI source. Applied Biosystems soon marketed a commercially available APPI source (the Photospray™ source). At about that time, Syage and coworkers developed an APPI source commercialized by Syagen Technology (the Photomate™ source) for Agilent, Thermo Scientific, and Waters instruments, which then started appearing in the literature [256]. In 2001, the first of many reports appeared that compared APPI to ESI and APCI [257]. Kostiainen's group played a key role in the early literature of APPI, and reported several comparisons of API sources [257–259], as well as a review of the principles involved [260], including negative-ion APPI-MS [261]. An early review of the subject covered the history, principles, and early applications [262].

Early applications of APPI-MS to lipids were for analysis of phytosterols [263] and PC molecular species [264]. Cai and Syage reported APPI-MS for MAG, DAG, and TAG standards and for FFAs and FA ethyl esters [265, 266]. Gómez-Ariza et al. [267, 268] reported several early applications of APPI-QqTOF-MS, by infusion, to olive oil analysis and authentication, including analysis of hazelnut, sunflower, and corn oils. They reported that APPI-MS gave greater sensitivity than ESI-MS for MAGs and DAGs, whereas ESI-MS was preferred for TAGs. Cai and Syage have raised concerns over the linearity of calibration lines for TAG quantification by LC–ESI-MS [265].

While some early reports demonstrated comparisons without dopant, it has now become widely recognized that LC-APPI-MS sensitivity is increased by the addition of a dopant to the flow stream, and that the solvent has a substantial effect on sensitivity [266], with one report focusing specifically on the solvent effects on TAG analysis by NARP-HPLC-MS [269]. APPI-MS mass spectra of several TAGs from that report are shown in Figure 13 to demonstrate that the mass spectra appear similar to APCI-MS mass spectra. Figure 14 shows the ionization potentials for common RP-HPLC solvents plus acetone and toluene. It was demonstrated that the use of dopant can, to a great extent, equalize the disparate effects of various solvents in the mobile phase. On the basis of this and other work, acetone and toluene have become the preferred dopants for TAG analysis by LC-APPI-MS, although a recent report [270] cites chlorobenzene as having benefits, and indicates that additional work remains to be done in this nascent field.

Early [271] and recent [272] reviews have summarized the developments in the field of APPI-MS over the past decade or more, and summarized issues related to dopants and LC solvents. As discussed in our recent article [273], a surprising number of reports have appeared comparing all three API techniques. Examination of tables in Ref. 272 shows many examples in which multiple API techniques were applied and compared. Most of these are performed sequentially, although multimode sources are also available (though they do not yet allow switching between ionization modes during a single analysis).

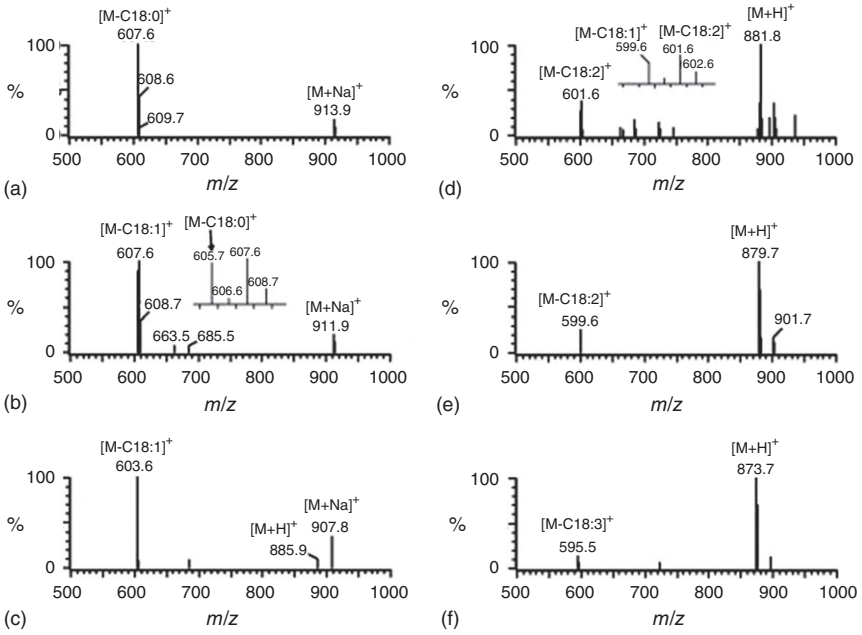

Figure 13. *APPI + full-scan mass spectra of TAGs. Injection amount: 10 ng each. (a) SSS, C18:0/C18:0/C18:0; (b) SSO, C18:0/C18:0/C18:1; (c) OOO, C18:1/C18:1/C18:1; (d) LLO, C18:2/C18:2/C18:1; (e) LLL, C18:2/C18:2/C18:2; and (f) LnLnLn, C18:3/C18:3/C18:3. As the degree of unsaturation increases, the intensity of protonated molecules increases. Source: Reprinted from Ref. [269] with permission. Copyright 2007 Elsevier, Inc.*

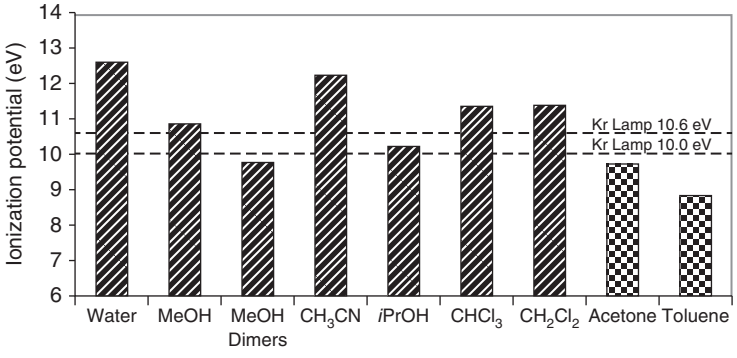

Figure 14. *Ionization potentials of solvents and dopants. The 10.0 and 10.6 eV Kr lines are shown as dashed lines. Source: Reprinted from Ref. [269] with permission. Copyright 2007 Elsevier, Inc.*

One approach pioneered by Byrdwell is the use of multiple API techniques simultaneously in parallel on multiple mass spectrometers [273, 274]. Using NARP-HPLC-MS, Byrdwell [215] showed that APPI-MS agreed better than ESI-MS with the identification of regioisomers by APCI-MS, which is generally accepted to provide good identification and quantification of regioisomers as discussed earlier. The pinnacle of the multiple parallel MS approach

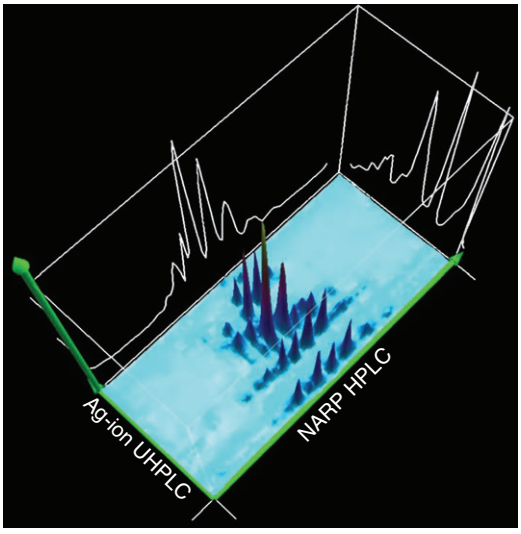

Figure 15. *3D plot of comprehensive 2D-LC separation of* P. curatellifolia*. The first-dimension separation uses NARP–HPLC, while the second dimension employs Ag-ion–UHPLC–QqQ–APPI-MS. Source: Reprinted from Ref. [199] with permission. Copyright 2017 American Chemical Society.*

was recently reported, in which APPI-MS was used in combination with APCI-MS and ESI-MS in a comprehensive 2D-LC experiment with detection by four mass spectrometers simultaneously, two in each dimension, including the first example of Ag-ion UHPLC as the second dimension (shown in Figure 15), for analysis of *trans*-DB-containing TAGs in *Parinari curatellifolia* (African mobola plum) kernel oil [199]. The issue of TAG regioisomer analysis and quantification by APPI-MS needs more attention and additional reports in the literature. Ideally, similar work to that of Holčapek et al. [108] on APCI-MS for regioisomer analysis would be performed using APPI-MS. A 2016 report described desorption atmospheric pressure photoionization (DAPPI)-LIT-orbitrap MS (on an LTQ Orbitrap XL) used for detection of HP-TLC separations of TAGs and wax esters from caraway, parsley, safflower, and jojoba oils [275]. APPI-MS was one of the detection methods used for the optimization of lipid class separation using NP-HPLC-LITorbitrapMS (on an LTQ Orbitrap Velos Pro) cited earlier [149].

While APPI-MS is a rapidly growing field, the number of applications to food, fat, and oil analysis is still rather limited, and applications since 2009 [271] easily fit into one table along with biological applications. All of those applications are more than a couple of years old, so they are not included here, and interested readers should consult the cited reviews [271, 272]. The preponderance of recent applications seems to be related to analysis of petroleum and its by-products, a field where APPI-MS has found particular utility. However, the usefulness for edible fat and oil analysis should not be underestimated, and analysts are missing out on a valuable tool if this approach is overlooked.

ACKNOWLEDGMENT

This work was supported by the USDA Agricultural Research Service. Mention or use of specific products or brands does not represent or imply endorsement by the USDA.

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