Journal of Chromatography A, 1217 (2010) 3908–3921

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Review

Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules

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ARTICLE INFO

Article history:
Available online 1 March 2010

Keywords:
Mass spectra
Mass spectrometry
Interpretation
Electrospray ionization
Atmospheric pressure chemical ionization
Atmospheric pressure photoionization

ABSTRACT

This review summarizes the basic rules for the interpretation of atmospheric pressure ionization (API) mass spectra of small molecules written with the style primarily intended for beginners and low-experienced researchers with the mass spectra interpretation. The first and basic step in any interpretation of mass spectra is always the determination of molecular weight, which is relatively easy in case of soft ionization techniques due to the limited extend of fragmentation and the prevailing presence of (de)protonated molecules in the full scan mass spectra. These $[M+H]^+$ and $[M-H]^-$ ions are often accompanied by low abundant molecular adducts, which can be used as the supplementary information for the unambiguous determination of molecular weights. In certain cases, adduct ions may dominate the spectra. The subsequent interpretation of full scan and tandem mass spectra is more complicated due to a high number of possible functional groups, structural subunits and their combinations resulting in numerous competitive fragmentation pathways. Typical neutral losses and the effect of individual functional groups on the fragmentation are discussed in detail and illustrated with selected examples. Modern mass analyzers have powerful features for the structural elucidation, for example high resolving power, high mass accuracy, multistage tandem mass spectrometry, dedicated softwares for the interpretation of mass spectra and prediction of their fragmentation. Background information on differences among individual ionization techniques suitable for the HPLC–MS coupling and basic types of mass analyzers with consequences for the data interpretation is briefly discussed as well. Selected examples illustrate that the right optimization of chromatographic separation and the use of other than mass spectrometric detectors can bring valuable complementary information.

Contents

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doi:10.1016/j.chroma.2010.02.049

1. Introduction

An enormous growth in the field of mass spectrometry (MS) and its coupling with separation techniques in last decades is still continuing, which generates a huge number of new HPLC–MS systems installed worldwide in academic, industrial, pharmaceutical and clinical laboratories. New HPLC–MS users often lack previous experiences with the interpretation of mass spectra or even with MS itself resulting in unfavorable situation of top-class expensive HPLC–MS instrumentation operated by users with little knowledge of MS. This discrepancy is partially caused by the lack of appropriate literature about the interpretation of soft ionization mass spectra. The goal of this review is to provide basic rules for the interpretation of mass spectra measured by common atmospheric pressure ionization (API) techniques used in HPLC–MS, but these rules are mostly applicable also for other soft ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI). This article is intended as a starting point in this area suitable for beginners, however all exceptions occurring during the interpretation cannot be covered in one review. This summary is based on almost 15 years of experiences of the first author with the interpretation of HPLC–MS data. During this time, an extensive amount of organic, organometallic and bioorganic compounds from different chemical classes has been analyzed and their spectra have been interpreted within the scope of research projects and mass spectrometric analytical service at our faculty. In addition to the citation of our papers and works published by other groups, many described observations are supported by our unpublished results and personal experiences. Procedures for the API spectra interpretation are often derived from the rules known for electron ionization (EI) mass spectra [1–3] either applied directly as it is or in a slightly modified form. The scope of this review is limited to small organic molecules but excluding biopolymers (peptides, proteins, nucleot(s)ides), where specific interpretation procedures are extensively described in the literature. Rather specific area is the field of organometallic compounds and metal complexes, which will be reviewed later on [4].

The coupling of separation techniques with MS has been of a great interest for long time due to the potential of MS in the identification of chromatographic peaks. The first gas chromatography–mass spectrometry (GC/MS) coupling was realized already in 1957 [5], but the development of really useful hyphenation of HPLC–MS has required longer time. Initial attempts with the implementation of EI in HPLC–MS concept [6] were not successful due to severe limitations concerning the mobile phase flow rate, application range and robustness of such devices. Later on, the thermospray as the first soft ionization technique specially designed for HPLC–MS coupling [7] still suffered from technical constraints. The real breakthrough has been the invention of electrospray ionization (ESI) [8,9]. The application of ESI for the analysis of large biomacromolecules by Fenn [10] has been awarded Nobel Prize for chemistry in 2002 together with Koichi Tanaka for the inspiration of MALDI [11]. The significant contribution of Michael Karas and Franz Hillenkamp in the MALDI development should not be overlooked [12]. In past, several other individuals were awarded Nobel Prizes for chemistry or physics for their principal achievements in mass spectrometry, such as Wolfgang Paul in 1989 for the theoretical description of ion trap and Francis W. Aston in 1922 for the accurate measurement of most elemental isotopes in the periodic table [11]. At present time, the most appreciated recent accomplishment is the construction and commercial introduction of Orbitrap as a new type of mass analyzer by Makarov [13], which is also the topic of one review in this special issue [14].

Two angles of view can be applied for the present state-of-art in HPLC–MS. The optimistic view can highlight routine applications of HPLC–MS over many branches of chemistry, biochemistry, pharmacy and medicine with only minor limitations concerning the choice of optimal chromatographic conditions, superior sensitivity, selectivity, extremely low sample consumption, three basic API techniques applied in both polarity modes and several vendors selling different types of mass spectrometers for HPLC coupling. On the other hand, the pessimist can argue that mass spectrometer is still rather sophisticated device generating huge data files, which is often not properly understood and interpreted by numerous chromatographists. Both views are true and the goal of this paper is to be a small contribution in a better understanding of mass spectra interpretation.

2. Atmospheric pressure ionization techniques

In our opinion, ESI is the softest ionization technique at all followed by MALDI with the similar application range. The application field of ESI covers the range of polarities from medium polar to ionic compounds (Fig. 1), but the ionization problems can occur for low polar and nonpolar compounds. There are some hints, how to ionize rather nonpolar compounds with ESI, such as the promotion of the formation of molecular adducts listed in Table 1 (mainly with alkali metal, ammonium and silver ions), which may significantly extent the polarity range of ESI shown in Fig. 1. The molecular weight (MW) range of ESI is significantly wider than for other API techniques due to the formation of multiply charged ions for proteins and other biopolymers (MWs >100,000), but even for singly charged ionic organic species the range of ESI is somewhat wider (MWs in the range of thousands) than for atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI).

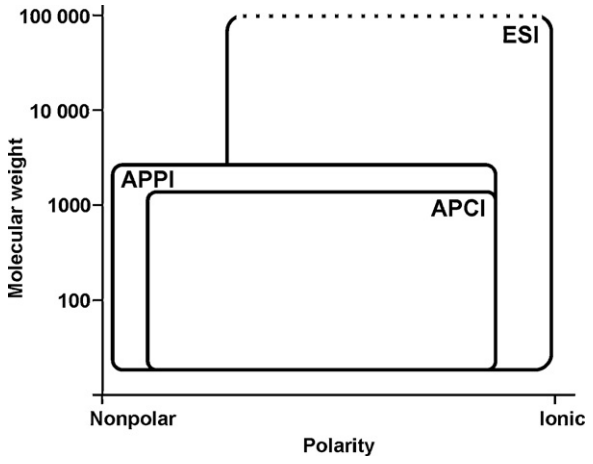

Fig. 1. General scheme of applicability of API techniques for small molecules based on polarity and molecular weights.

Table 1
Singly charged molecular adducts in positive-ion API mass spectra.

Molecular adduct	Nominal mass shift[a] [ΔDa]	Exact mass shift[a] [mDa]	References
$[M+Li]^+$	6	+8.2	[119]
$[M+NH_4]^+$	17	+26.5	Numerous citations in the literature
$[M+H+H_2O]^+$	18	+10.6	[45]
$[M+Na]^+$	22	−18.1	Numerous citations in the literature
$[M+H+CH_3OH]^+$	32	+26.2	[45,47,49,50]
$[M+K]^+$	38	−44.1	Numerous citations in the literature
$[M+H+CH_3CN]^+$	41	+26.5	[31,45–47]
$[M+H+H_2O+CH_3OH]^+$	50	+36.8	[45]
$[M+Na+CH_3CN]^+$	63	+8.5	[45]
$[M+Ag]^+$	106	−102.7	[107,120–122]
$[2M+H]^+$	–	–	[34,46]
$[2M+Na]^+$	–	–	[34,41,92,93]
$[2M+K]^+$	–	–	[41,92,93]
$[3M+Na]^+$	–	–	[41]
$[3M+K]^+$	–	–	[41]

[a] Mass shifts correspond to the difference between $[M+H]^+$ and particular molecular adduct, e.g., $[M+Na]^+ - [M+H]^+ = 22$.

Typical applications of ESI are various classes of bio(macro)molecules studied in proteomics, metabolomics and lipidomics, e.g., peptides, proteins, nucleot(s)ides, polysaccharides, phospholipids. In addition to these well-known applications, ESI is also an excellent tool for the analysis of wide range of polar to ionic "small" organic and organometallic compounds starting from very low masses up to several thousands. The attention must be paid to the ionization of rather nonpolar analytes, where APCI and APPI typically have a better performance within common mass range up to m/z 1500 (sometimes up to m/z 3000). It is often not realized that APCI and APPI techniques do not suffer so much from ion suppression/enhancement effects [15], because the ionization process takes place solely in a gas phase. Relative abundances of alkali metal and other ionic adducts are much lower than with ESI, which may cause a sensitivity increase for compounds suffering from the formation of various adducts. The limitation of APCI and APPI is the analysis of biopolymers, organometallics, ionic compounds and other labile analytes. Typical application areas of APCI and APPI are almost identical, such as drugs, nonpolar lipids, natural compounds, pesticides and various organic compounds [16]. To look for small differences between their application range, APPI shows quite often the formation of radical ions [17,18], has a slightly softer character and is more successful for highly nonpolar compounds as polyaromatic hydrocarbons [16,18,19]. The useful alternative for improved ionization efficiency can be a combined dual sources, where APCI/APPI is used for low polar compounds and ESI/APPI is applicable for the full range of compounds [18].

3. Mass analyzers

At present, six basic types of mass analyzers are available on the market – quadrupole, ion trap, time-of-flight (TOF), double focusing magnetic analyzer, Orbitrap, ion cyclotron resonance (ICR) – together with numerous hybrid combinations and variants of these analyzers, most of them applicable also for HPLC–MS coupling. It is not a goal of this review to provide the full survey of currently used mass analyzers, because it can be found in the specialized literature [20]. From the view of this review, the important issue is parameters affecting the appearance of mass spectra, such as resolving power, mass accuracy, mass range of analyzer, dynamic range used for the quantitation, types of tandem mass experiments. Typically, the first important question is the division on mass analyzers into two groups providing low vs. high mass resolution, which is related to the mass accuracy. The group of low resolution mass analyzers typically includes quadrupole analyzers and various types of spherical and linear ion traps with the resolving power in the range of few thousands using the full width at half maximum (FWHM) definition and without the possibility of accurate determination of m/z values. In common speech it is often said that they have unit resolution, which means that they are able to resolve ions differing by one mass unit (in current practice, it is about five times better), but this is not the correct definition of resolving power. High resolution (the range of tens of thousands, TOF based analyzers and double focusing magnetic sector analyzer) and ultrahigh resolution (>100,000 resolving power, Fourier transform based Orbitrap and ICR analyzers) analyzers give the highest confidence in the spectra interpretation and identification, because possible artefacts are reduced at the increasing resolving power. High mass accuracy (at minimum <5 ppm, TOF based analyzers) or even better ultrahigh mass accuracy (<1 ppm, Orbitrap and ICR analyzers) significantly reduce the number of possible elemental compositions for the ion within a given mass tolerance. The benefit of high resolving power and high mass accuracy can be illustrated on distinguishing phosphates vs. sulfates and phosphonates vs. sulfonic acids (see Table 3), where identical mass shifts and very similar fragmentation behavior occur, but it can be identified based on different exact masses. If accurate relative abundances can be recorded (preferably with analog-digital convertor), then the number of possible elemental compositions is again reduced [21,22], in favorable cases to only one correct hit. HPLC–MS systems are equipped with advanced software tools, which can speed up and semi-automate the interpretation, for example by integrating individual subgroups of information (e.g., positive and negative-ion modes, full scan and tandem mass spectra, information obtained for individual fragment ions). The field of dedicated softwares is growing very fast and appropriate software tools can provide valuable help even for experts.

4. Importance of chromatographic separation and other detection techniques

The basic rules described in this article are of course valid for any API mass spectra regardless the fact, whether they were measured by the direct infusion or HPLC–MS, but several important issues should be considered concerning the possibilities of coupling to liquid-phase separation techniques [23,24]. When the measured sample is pure or relatively pure compound, then there is no reason why to waste time and solvents on the chromatographic separation and this pure sample should be measured by the direct infusion (more time for MS^n experiments) or the flow injection analysis (the solvent background can be subtracted). In all other cases, the MS coupling to the separation brings some advantages depending on the sample complexity and the separation selectivity of developed chromatographic method. It is also important to keep in mind limitations of MS structural characterization, where well-optimized

chromatography can do an excellent job. For various types of positional isomers, MS mostly does not provide any distinction, but the chromatographic resolution of isomers is achievable. For enantiomers, there is no distinction by MS except for the special kinetic method based on the fragmentation of ternary complexes [25] and similar methods based on the use of chiral selectors [26], but the separation of enantiomers can be done on chiral stationary phases. If the separation is not properly optimized, then obtained mixed spectra complicate the interpretation and trace compounds may not be detected at all due to the signal overlap with coeluting peaks. For highly complex biological and natural mixtures, the use of 2D separation techniques coupled to MS can increase the number of identified compounds, especially in the off-line arrangement [27].

HPLC–MS technique provides different types of signal monitoring (e.g., total ion current (TIC) chromatogram, selected ion monitoring (SIM), selected reaction monitoring (SRM), etc.) depending on the data acquisition setting, but the highest importance for the mass spectra interpretation has MS^n scans and reconstructed (or extracted) ion current (RIC) chromatograms. The reconstruction of selected m/z values (or regions of m/z values) depending on the time processed after the analysis from TIC data can be used for various purposes: (A) the identification what ions are related to the particular chromatographic peak and what is the background contamination based on the comparison on RIC profiles for the region around the peak of interest, (B) the identification of coelution of two compounds based on the detailed comparison of their RIC records, the coelution can be detected even for very small differences of retention times (about 0.2 min or even less for UHPLC), (C) the detection of suitable regions for the signal averaging and background subtraction, (D) the discovery of ultratrace peaks in the noise using either targeted m/z values or narrow regions of m/z step-by-step to detect hidden trace peaks, e.g., m/z 100–150, 150–200, 200–250 and so on.

Another important issue is the compatibility of HPLC conditions optimal for the best separation with the MS detection. Nowadays the compatibility of both techniques is relatively good due to the technical developments, so we can operate at common chromatographic flow rates with all API techniques (up to 1 mL/min), common mobile phases in reversed-phase and HILIC systems. For normal-phase systems, ESI suffers from problems with the ionization unless the content of polar organic modifier is at least 5%, so APCI or APPI is a better choice for normal-phase systems. The only important limitation is the use mobile phase additives, where the use of nonvolatile agents discourages and even the concentration of volatile additives should be kept as low as possible [28]. Nonvolatile additives participate in the ion suppression and matrix effects (in some cases the sensitivity may drop to zero response) [15,29,30] and they may cause severe contamination of ion optics of mass spectrometer. The most dangerous contaminants are ion-pairing agents based on tetraalkylammonium salts [28], which should never enter the mass spectrometer due to severe memory effects apparent for several months even after the repetitive cleaning of the whole system from the sample injector until the mass analyzer (unpublished experiences of our group and others known from personal communications). In most cases, nonvolatile inorganic agents can be replaced by volatile organic alternatives with similar or slightly worse separation efficiency [31–33], but also generally accepted volatile additives such as ammonium acetate or formate, formic acid and acetic acid can cause certain suppression effects [28], so their concentration should be always kept at the lowest level necessary for the satisfactory chromatographic performance.

Other detection techniques can provide valuable supplementary information in some specific cases. The photodiode-array UV detector provides useful data in case of strong chromophoric systems. A good example is the identification of metabolites formed from the drug dimefluron (Fig. 2) with highly conjugated chromophoric system containing two aromatic rings conjugated through the additional carbonyl group. The parent drug and related metabolites without the change of this conjugated system have identical UV spectra, while all metabolites formed by the reduction of this carbonyl have completely different UV spectra, because the number of conjugated double bonds is reduced from nine to five [34]. This is complementary information, which is not evident from mass spectra, so the supporting information from UV detector significantly simplifies the structure elucidation of these metabolites. The similar approach can be applied for sensitive fluorescence detection [35]. In general, the mass spectrometer is considered as the most sensitive detector for liquid-phase separation techniques, which is true in most cases except for certain applications of electrochemical detection for compounds with redox properties or fluorescence detection for compounds with a high quantum yield.

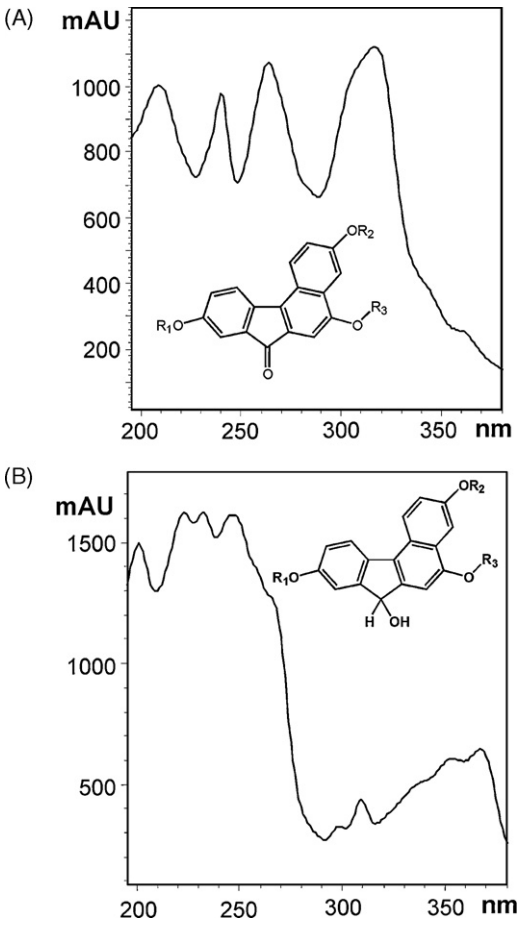

Fig. 2. UV spectra of two groups of metabolites of drug dimefluron: (A) conjugated system with carbonyl groups is retained and (B) reduction of carbonyl group to hydroxyl reduces the extent of conjugation. (Reproduced from Ref. [34] with permission.)

5. Interpretation of API mass spectra

Concerning the interpretation of mass spectra of unknowns, we can differentiate two types of unknown compounds. The first type is totally unknown where absolutely no information on the possible molecular structure and other background information is available, which is the most complicated case in the untargeted screening. In such situation, the combination of several spectral techniques is usually essential for the full structure elucidation, at minimum high resolution MS/MS and two-dimensional NMR. In practice, at least

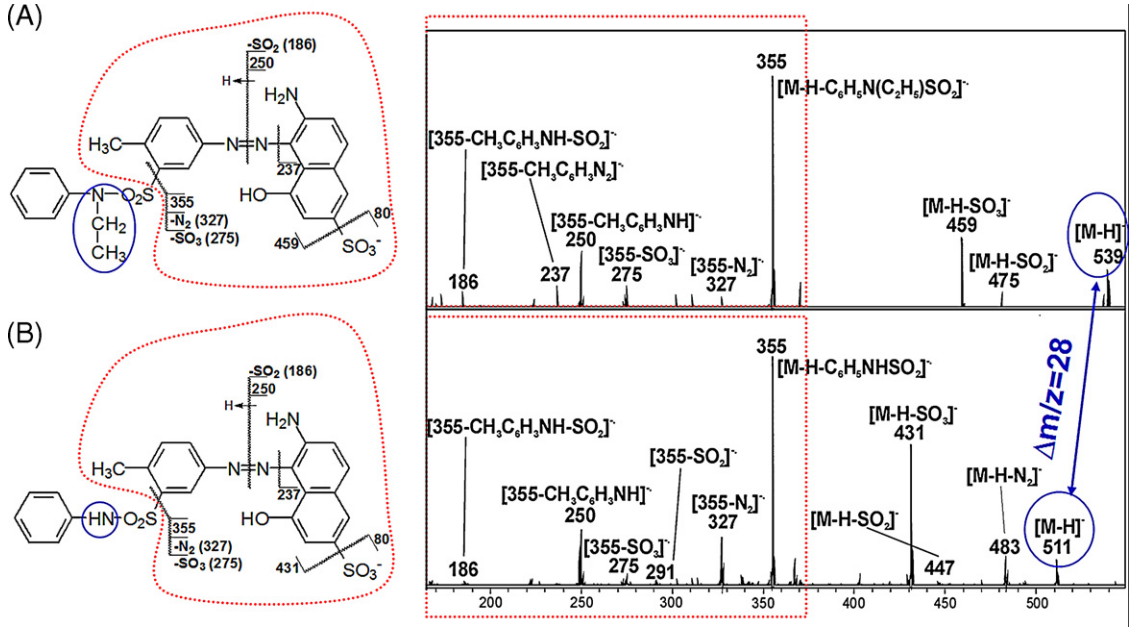

Fig. 3. Identification of unknown impurity based on the comparison of tandem mass spectra of parent compound (C.I. Acid Red 118) and impurity: (A) ESI-MS/MS of *m/z* 539 for parent compound and (B) ESI-MS/MS of *m/z* 511 for the impurity. Structures of parent compound and identified impurity are shown.

some supporting information can be deduced (e.g., sample history, sample preparation, typical metabolites for a given plant or animal, etc.) to simplify the interpretation. The second class of unknowns, in our experiences more common, is the situation, where the structure of "parent compound" (main product, initial drug, etc.) is known and the standard may be available for preliminary studies of ionization, fragmentation and retention behavior [34,35]. The task is the identification of minor, trace or ultratrace species somehow related to the parent compound, e.g., metabolites, degradation products, impurities, synthetic intermediates, by-products, etc. Obviously, such interpretation is easier than for completely unknowns, as illustrated in Fig. 3. The full scan negative-ion ESI spectra (not shown) exhibit only $[M-H]^-$ ions used for the MW determination of parent compound and impurity differing by 28 Da. Detailed examination of MS/MS spectra for both parent and unknown compound (Fig. 3) shows that the part of spectra from *m/z* 160 to 320 is almost identical and therefore the corresponding part in the structure must be identical as well. In the remaining part of molecule, the only logical difference corresponding to $\Delta m/z = 28$ is the absence of ethyl substituent in case of impurity. This example demonstrates the importance of detailed interpretation of parent compound mass spectra measured with all available techniques in the laboratory, because this learned knowledge can be later applied for the identification of related unknown analogs. Another alternative is when we know that studied compound belongs to the certain compound type (phospholipid, synthetic polymer, etc.) and we need to identify the length of alkyl chain and/or the number of repeating oligomeric units, which is probably the simplest case concerning the spectra interpretation [36,37].

5.1. Molecular weight determination

The MW determination is always the first and very important step in the interpretation of mass spectra regardless of the ionization technique used. API techniques belong to the group of so-called soft ionization techniques, so it is evident already from this name that the ionization process has a softer character in comparison to the conventional EI, which results in mass spectra less rich in fragment ions. There are some principal differences between EI and API mass spectra. The first difference is the type of primarily formed ions related to the MW, which is odd-electron (OE) molecular radical cation $M^{+\bullet}$ in case of EI, while even-electron (EE) protonated molecules $[M+H]^+$ or deprotonated molecules $[M-H]^-$ are characteristic for API and other soft ionization techniques. Please note

Table 2
Singly charged molecular adducts in negative-ion API mass spectra.

Molecular adduct	Nominal mass shift[a] [ΔDa]	Exact mass shift[a] [mDa]	References
$[M-H+H_2O]^-$	18	+10.6	[48,123]
$[M+F]^-$	20	+6.2	[124]
$[M-H+CH_3OH]^-$	32	+26.2	[50]
$[M+Cl]^-$	36	−23.3	[40,70,123–125]
$[M+HCOO]^-$	46	+5.5	[61]
$[M+NO_2]^-$	47	+0.7	[40,116]
$[M+CH_3COO]^-$	60	+21.1	[45]
$[M+NO_3]^-$	63	−4.4	[40,123]
$[M+Br]^-$	80	−73.8	[123,124]
$[M+HSO_4]^-$	98	−32.6	[123]
$[M+H_2PO_4]^-$	98	−23.1	[123]
$[M+CF_3COO]^-$	114	−7.1	[70]
$[M+I]^-$	128	−87.7	[123]
$[2M-H]^-$	–	–	[34,126]

[a] Mass shifts correspond to the difference between $[M-H]^-$ and particular molecular adduct.

Table 3
Typical fragmentation behavior observed in MS and MS/MS spectra for individual functional groups.

Functional group[a]	Nominal mass shift[b] [ΔDa]	Exact mass shift[b] [mDa]	Fragment/product ions	References
Phosphate (RPO_4H_2)	+96	−38.8	$[M-H-HPO_3]^-$ $[M-H-H_3PO_4]^-$ $[H_2PO_4]^-$ $[PO_3]^-$	[60] [60] [59–61,64] [59–61,64]
Phosphonate (RPO_3H_2)	+80	−33.7	$[H_2PO_3]^-$ $[PO_3]^-$ $[PO_2]^-$	[63] [62,63] [62,63]
Sulfate (RSO_4H)	+96	−48.3	$[M-H-SO_3]^-$ $[M-H-H_2SO_4]^-$ $[HSO_4]^-$	[33,43] [33,43] [33,43]
Sulfonic acid (RSO_3H)	+80	−43.2	$[M-H-SO_2]^-$ $[M-H-SO_3]^-$ $[SO_3]^{-\bullet}$ $[SO_2]^{-\bullet}$	[31,33,43,67,74,101] [31,33,43,67,74,101] [33,43] [43]
Sulfoxide ($R^1SO_2R^2$)	+64	−38.1[c]	$[M+H-OH]^{+\bullet}$ $[M+H-R^i]^{+\bullet}$	[68] [68]
Nitrate (RNO_3)	+61	−20.0	$[M-H-NO]^{-\bullet}$ $[M-NO_2]^-$ $[M-H-NO_2]^{-\bullet}$ $[M-H-ONO_2]^{-\bullet}$	[116] [40] [40,116] [116]
Nitro (RNO_2)	+45	−14.9	$[M+H-OH]+^{\bullet}$ $[M-OH]^-$ $[M+H-H_2O]+$ $[M+H-NO]+^{\bullet}$ $[M+H-NO_2]+^{\bullet}$ $[M-NO]^-$ $[M-H-NO]^{-\bullet}$ $[M-H-OH]^{-\bullet}$ $[M-H-HNO]^-$ $[M-NO_2]^-$ $[M-H-NO_2]^{-\bullet}$ $[M-H-HNO_2]^-$ $[NO_2]-$	[67,104,127] [116] [72] [67,127] [67,101,104,127] [71,116] [43,72,74] [67] [67] [71,116] [67,72,74] [67,73,128] [72]
Nitroso (RNO)	+29	−9.8	$[M+H-NO]^+$	[46,75,129]
N-oxide (RN^+O^-)	+29	−9.8	$[M+H-O]^+$ $[M+H-OH]^{+\bullet}$ $[M+H-H_2O]^+$	[77–83] [34,79,81,99,101] [79,80]
Azo ($R^1N=NR^2$)	+28	+6.1[c]	$[M+H-N_2]^+$ $[M+H-R^i]^+$ $[M+H-R^iNH]^+$ $[M+H-R^iN_2]^+$	[43,86,103]
Amide, alkylamide (R^1CONH_2, R^1CONHR^2, $R^1CONR^2R^3$)	+43	+5.8	$[M+H-NH_3]^+$ $[M+H-R^2NH_2]^+$ $[M+H-R^2R^3NH]^+$	[67,85]
Amine (R^1NH_2, R^1NHR^2, $R^1NR^2R^3$)	+15	+10.9	$[M+H-NH_3]^+$ $[M+H-R^2NH_2]^+$ $[M+H-R^2R^3NH]^+$ $[M+H-HCN]^+$	[67,86,88,99,101,130–133] [67,86]
Nitrile (RCN)	+25	−4.8	$[M+H-HCN]^+$	[67,86]
Hydroperoxide (ROOH)	+32	−10.2	$[M+H-H_2O]^+$ $[M+H-H_2O_2]^+$	[105,106] [105,106]
Epoxides (RO)	+16	−5.1	$[M+H-H_2O]^+$	[105]
Carboxylic acid (RCOOH)	+44	−10.2	$[M+H-H_2O]^+$ $[M+H-CO_2]^+$ $[M+H-H_2O-CO]^+$ $[M-H-CO_2]^-$	[67] [67,96] [41,67] [35,41,43,67,74,92,93,96,101]
Alcohol (ROH)	+16	−5.1	$[M+H-H_2O]^+$	[67,101]
Phenol (ROH)	+16	−5.1	$[M+H-H_2O]^+$ $[M+H-CO]^+$ $[M-H-H_2O]^-$ $[M-H-CO]^-$	[90,96,97] [103] [33,43,134] [43,103]
Methoxy ($ROCH_3$)	+30	+10.6	$[M+H-CH_3]^{+\bullet}$ $[M+H-CH_3O]^{+\bullet}$ $[M+H-CH_3OH]^+$ $[M+H-HCOH]^+$	[86,97–100] [100,101] [86,97,98] [97,102]

Table 3 (*Continued*)

Functional group[a]	Nominal mass shift[b] [ΔDa]	Exact mass shift[b] [mDa]	Fragment/product ions	References
Ester (R^1COOR^2)	+44	−10.2[c]	$[M+H-R^2OH]^+$	[67,99,101,131,135]
			$[M+H-R^2OH-CO]^+$	[67,135]
Ketone (R^1COR^2)	+28	−5.1[c]	$[M+H-H_2O]^+$	[104]
			$[M+H-CO]^+$	[67,97,104]
			$[R^iCO]^+$	[67]
Aldehyde (RCHO)	+28	−5.1	$[M+H-CO]^+$	[67]
			$[M-H-CO]^-$	[67]
Halides (RX)	+18 (F), +34 (Cl), +78 (Br), +126 (I)	−9.4	$[M+H-X]^{+\bullet}$	Cl—[67,101,104]
		−39.0		Br—[67,101]
		−89.5	$[M+H-HX]^+$	F—[67,99,101]
		−103.4		Cl—[67,84,102,104]
				Br—[43]
			$[M-H-X]^{-\bullet}$	Br—[43]
				I—[45]
			$[M-H-HX]^-$	Cl—[43,67,101,128,136]
				Br—[43,136]
			$[I]^-$	[45,102]
			$[Br]^-$	[43,45,102]
			$[Cl]^-$	[43,102]

[a] R or R^i means alkyl/aryl.

[b] Mass shifts correspond to the difference between the molecule with a particular functional group and the same molecule but without any substituent, e.g., the mass shift for sulfonic acid = $RSO_3H-RH = SO_3 = 80$ or the mass shift for nitro group = $RNO_2-RH = NO_2-H = 45$.

[c] For R^1XR^2 functional groups (sulfoxide $R^1SO_2R^2$, azo $R^1N=NR^2$, ester R^1COOR^2 and ketone R^1COR^2), mass shifts are compared to the parent molecule R^1R^2.

that terms "*protonated molecular ions*" and "*deprotonated molecular ions*" are incorrect and their use is discouraged [38]. In addition to (de)protonated molecules, there are some characteristic molecular adducts useful for the verification of MW determination.

In the positive-ion mode (Table 1), the most typical adduct is sodiated molecule $[M+Na]^+$ often accompanied by less abundant potassiated molecule $[M+K]^+$. The relative abundance of the adduct with ammonium ion $[M+NH_4]^+$ strongly depends on the presence (or absence) of ammonium additives in the mobile phase and the history of ammonium use on the particular instrument. Adducts with other inorganic cations (e.g., Li, Ag and less frequently other metal ions) can be generated, when these ions are added to the solution to support the formation of desired ions. Some types of adducts have special applications, such as the determination of double bond positions in long fatty acid chains from the characteristic fragmentation of lithium adducts [39]. Other special application of metal ion adducts is the fragmentation of ternary complexes with the copper ion used for the chiral recognition [25].

In the negative-ion mode (Table 2), the typical base peak $[M-H]^-$ may be in certain cases accompanied or replaced by adduct ions with small inorganic ions. The presence and relative abundances of these adducts usually strongly depend on the presence and concentration of these anionic species in the solution, so the selective adduct formation can be supported by adding the selected anion to the solution. The special application is the use of chloride (or other small inorganic anions) to determine MWs of aliphatic nitrates used as explosives [40], where no MW information can be obtained without this interesting hint. In case of amines and substituted amines, the formation of chloride adduct is common.

Other less common ions can occur in both polarity modes. Dimeric or in general polymeric ions are common especially in case where the analyte concentration is too high or the compound has an affinity to form dimeric or polymeric ions [41]. The most common dimeric ions are $[2M+H]^+$ or $[2M-H]^-$ depending on the measured polarity, but other adducts analogous to monomeric ions can be also expected, e.g., with sodium ion (Table 1). Other possible MW related ions are doubly and multiply charged ions, which can be easily determined by the fact that distances between isotopic peaks are equal to one half for doubly charged ions or in general $1/n$ for multiply charged ions with n charges. Moreover, peaks of multiply charged ions occur at m/z corresponding to $(MW + nH)/n$. Except for biomacromolecules not covered in this review, typical examples for small molecules are polyaromatic compounds providing doubly charged ions in the positive-ion mode and polysulfonated compounds providing multiply charged ions in the negative-ion ESI [32,42,43]. Non-covalent adducts with solvents coming from the mobile phase usually have low to negligible relative abundances with some exceptions. The most common adducts are with acetonitrile [31,44–46], methanol [45,47–50] or water [45,48]. The importance of these adducts should not be underestimated regardless of low relative abundances, because if they are positively detected and correctly interpreted, then they serve as a valuable confirmation of MW determination.

The last example of unusual MW related ions is the formation of OE molecular ions, which is known for example from the negative-ion APCI of compounds containing some functional groups, such as nitro group(s) on the polyaromatic system (preferably heterocycles containing nitrogen or sulfur). It is supposed that the electron capture mechanism is responsible for the formation of $M^{-\bullet}$ ions, but the situation can be rather complex and more structural subunits may contribute to this mechanism [51,52]. For positive-ion APCI and ESI modes, the formation of molecular radicals $M^{+\bullet}$ is known for highly conjugated systems found in polyaromatic compounds [17,43,52–55].

5.2. Typical fragmentation behavior for individual functional groups

Table 3 lists common functional groups sorted approximately in the order of their influence on the fragmentation and clustered in groups according to the type of heteroatom: (1) phosphorous containing functional groups (phosphate, phosphonate), (2) sulfur-containing functional groups (sulfate, sulfonic acid, sulfoxide), (3) nitrogen containing functional groups (nitrate, nitro, nitroso, N-oxide, azo, amide, alkylamide, amine, nitrile), (4) oxygen containing functional groups (hydroperoxide, epoxide, carboxylic acid, alcohol, phenol, methoxy, ester, ketone, aldehyde), (5) halogen substituents, and (6) other structural types (alkyl/aryl substitution on the aromatic ring, polycyclic aromatic hydrocarbons, alkene, alkyne). In general, the highest impact on the fragmentation has functional groups formally derived from strong inorganic acids, e.g., phosphate can be considered as a monoester of phosphoric acid,

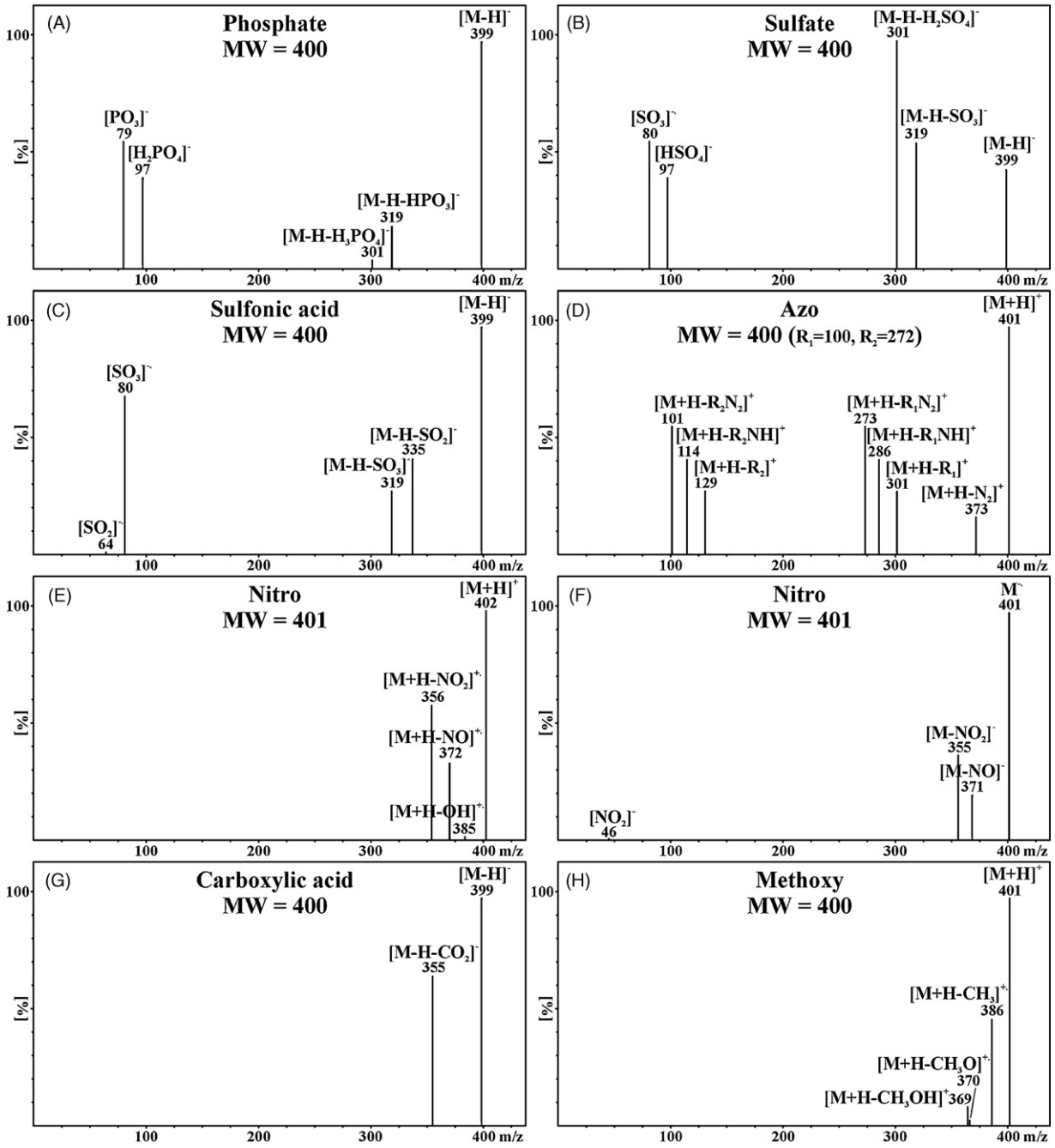

Fig. 4. Characteristic fragmentation behavior for a hypothetical molecule with one functional group for negative-ion (A, B, C, F, G) or positive-ion (D, E, H) modes: (A) phosphate, (B) sulfate, (C) sulfonic acid, (D) azo, (E) nitro, (F) nitro, (G) carboxylic acid, and (H) methoxy group.

sulfate as the monoester of sulfuric acid and nitrate as the ester of nitric acid to list three functional groups with the most distinct fragmentation. We have tried to exclude cases, where the fragmentation behavior of one functional group is substantially influenced by other functional group in the molecule to present a clear link between the functional group and its fragmentation. In practice, the presence of more functional groups and their mutual effects on the fragmentation are very common and highly complex, but such discussion is out of the scope of this introductory review on the interpretation of API mass spectra. In accordance with Ref. [56], the term "fragment ion" is used here to describe all ions originating from the fragmentation in the full scan mode and tandem mass spectra to simplify the discussion. Fig. 4 illustrates the characteristic mass spectra for hypothetical molecules containing single functional group for a better visualization of fragmentation behavior described in Table 3. In practice, the relative abundances of individual ions may differ significantly and some fragment ions may be absent depending on the structure of whole molecule, especially other functional groups. Fig. 5 shows proposals for mechanisms of common NLs and the formation of fragment ions described in the following chapters and Table 3. These mechanisms are proposed based on the knowledge of starting point (i.e., $[M+H]^+$ or $[M-H]^-$ ions depending on the polarity mode) and final product ions (Table 3) taking into account basic fragmentation mechanisms described for EI [1]. Some mechanisms are not listed, because analogous mechanisms can be found, e.g., the NL of ROH from ester has

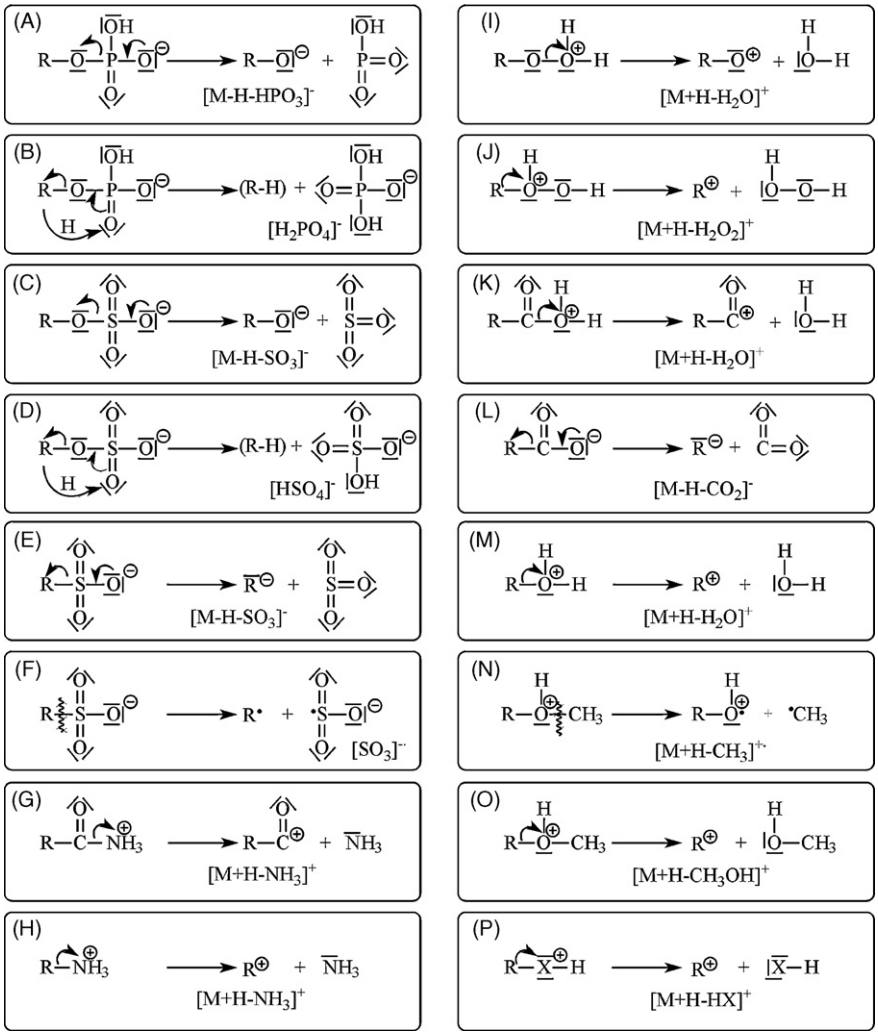

Fig. 5. Proposed mechanisms of characteristic NLs and the formation of fragment ions for selected functional groups in the negative-ion mode (A–F and L) from $[M-H]^-$ ion and in the positive-ion mode (G–K and M–P) from $[M+H]^+$ ion, R means (substituted) alkyl or aryl: (A) NL of HPO_3 from phosphate, (B) formation of $[H_2PO_4]^-$ from phosphate, (C) NL of SO_3 from sulfate, (D) formation of $[HSO_4]^-$ from sulfate, (E) NL of SO_3 from sulfonic acid, (F) formation of $[SO_3]^{-\bullet}$ from sulfonic acid, (G) NL of NH_3 from amide, (H) NL of NH_3 from amine, (I) NL of H_2O from hydroperoxide, (J) NL of H_2O_2 from hydroperoxide, (K) NL of H_2O from carboxylic acid, (L) NL of CO_2 from carboxylic acid, (M) NL of H_2O from phenol or alcohol, (N) NL of $CH_3^\bullet$ from methoxy group, (O) NL of CH_3OH from methoxy group, and (P) NL of HX from halides.

the identical mechanism as the NL of H_2O from alcohol (Fig. 5M), only hydrogen is replaced by R.

5.2.1. Phosphorous containing functional groups

Organophosphorous compounds are common especially in the chemistry of phosphorylated proteins (out of scope of this review), phospholipids [57] and pesticides [58]. In this review, we focus only on phosphate and phosphonate monoesters. Diesters are very common for phospholipids, but their fragmentation is governed by the type of other substituent of the phosphate group [56]. Due to the presence of two acidic protons in phosphate and phosphonate monoesters and quite labile character of these groups, the only recommended ionization mode is the negative-ion ESI [59–64]. The characteristic ions are small negatively charged phosphorous containing fragment ions: $[PO_3]^-$ for both phosphates [59–61,64] and phosphonates [62,63], $[H_2PO_4]^-$ for phosphates [59–61,64], $[H_2PO_3]^-$ and $[PO_2]^-$ for phosphonates [62,63]. The neutral loss (NL) of HPO_3 [60] and H_3PO_4 [60] from $[M-H]^-$ can occur as well.

5.2.2. Sulfur-containing functional groups

Sulfur-containing functional groups (especially sulfates and sulfonic acids) have a very high impact on the fragmentation behavior of compounds carrying these functional groups. It is typical even for compounds with multiple different functional groups that the fragmentation process starts from the NLs associated with these two groups, i.e., the NL of H_2SO_4 for sulfates [33,43], SO_3 for both sulfates and sulfonic acids [33,43,65] and SO_2 for sulfonic acids [43]. The complementary ions $[HSO_4]^-$ and $[SO_3]^{-\bullet}$ are often observed in the spectra as well, rarely also low abundant $[SO_2]^{-\bullet}$ for sulfonic acid. The NL of SO_2 has been also reported for arylsulfonyl esters [66]. It is interesting to note that the radical ion $[SO_3]^{-\bullet}$ is highly diagnostic, while its EE analog $[HSO_3]^-$ is not common in the spectra. The negative charge of both sulfate and sulfonic groups logically determines the use of negative-ion ESI mode, while negative-ion APCI (or APPI) can be applied with a reduced sensitivity only for compounds with up to two sulfonic acid groups in the molecule [42]. The positive-ion mode is not convenient and only weak signals for monosulfonic acids containing other proton-acceptor groups can be expected, e.g., dyes containing hydroxyl, amino group or anthraquinone skeleton [42,67]. The important group of water soluble organic dyes carries multiple anionic groups (sulfonic acid, carboxylic acid and sulfate). Their negative-ion ESI spectra show the series of multiply charged negative-ions $[M-xH]^{x-}$ and their sodiated adducts $[M-(x+y)H+yNa]^{x-}$ [31–33,42,43,65]. The total number of acid groups is equal to the highest observed charge or the highest number of acid protons replaceable by sodium ions in

$[M-(x+y)H+yNa]^{x-}$ series. The radical losses of OH• and R• have been published for sulfoxides [68]. For thiols, the only known NL is H_2S similarly as for EI spectra [1].

5.2.3. Nitrogen containing functional groups

The first thing related to the nitrogen containing functional groups must be the knowledge of so-called nitrogen rule. If the EE rule [69] is valid without any exception for API mass spectra, then all ions with even *m*/*z* values have an odd number of nitrogen atoms, while ions with odd *m*/*z* values have even number or zero nitrogen atoms. Unfortunately, exceptions from EE rule cannot be neglected, because OE ions are relatively common for some nitrogen containing groups, e.g., nitro, nitroso, N-oxide or nitrogen containing heterocyclic compounds.

The nitrate is rather labile group typical for explosives. Extremely strong tendency of this functional group for the spontaneous fragmentation even at the softest possible ESI conditions can result in the absence of $[M-H]^-$ ions in the full scan spectra showing only fragment ions $[NO_3]^-$ and $[NO_2]^-$ in the negative-ion mode without any information on the MW. There is one interesting hint applied in the negative-ion APCI that (poly)nitrated aliphatic and aromatic explosives can be detected in the form of molecular adducts with small inorganic anions, such as chloride, bromide, acetate, formate [40,70]. The source of halide ions can be either the addition of small amount of inorganic salt or chlorinated solvent (for example CH_2Cl_2 or $CHCl_3$). The trace concentration of these ions even in chromatographic solvents is often sufficient to form these stable adducts without the deliberate addition.

Nitro compounds have a quite complex fragmentation and ionization behavior starting from the initial MW related ions. It is quite often that negative-ion API spectra of nitro and especially polynitro compounds show the radical ion $M^{-\bullet}$ [70,71], but the formation of deprotonated molecule $[M-H]^-$ is also known depending on the whole structure of particular nitro compound [72–74]. The typical NLs are radicals NO• and NO_2• often occurring as the starting fragmentation process on condition that no phosphorous or sulfur-containing functional groups are present. These NLs can occur for both $M^{-\bullet}$ and $[M-H]^-$ initial ions, which brings a complex survey of possible fragment ions for nitro compounds (see Table 3). The complementary radical ion $[NO_2]^{-\bullet}$ can be observed as well [72]. The third NL known for the nitro group is the loss of oxygen itself (at very low abundance, if at all), which is something rather unusual for all other functional groups except for N-oxides. Nitroso group is quite rare except for certain applications and the only predictable NL is NO [46,75].

N-oxide is a common metabolic oxidation product for some nitrogen containing drugs [34,76–78]. The characteristic fragmentation of N-oxides is deoxygenation occurring only in the full scan mass spectra [77–83] and it is explained by the thermal degradation in the APCI source [77]. Some authors reported that the NL of oxygen in APCI is replaced by NLs of OH• and H_2O in ESI [77], while others [81] observed the loss of oxygen also in ESI mass spectra. In all cases, the $[M+H-O]^{+\bullet}$ is reported only for the full scan mass spectra but not for tandem mass spectra. It is likely that the prevailing fragmentation pathway strongly depends on the setting of tuning parameters and API technique used for the measurement. The second unusual fragmentation pathway known for tertiary N-oxides is the elimination of aldehyde or ketone after Meisenheimer N–R to N–O rearrangement [78,79]. Another fragmentation of N-oxides (i.e., N,N-dimethylamino-N-oxide) is the cleavage of the nitrogen–carbon bond resulting in the NL of $(CH_3)_2NOH$ [34]. Two isobaric oxidation metabolites, hydroxylated vs. N-oxide, can be distinguished based on the characteristic fragmentation of both groups, where the dehydration is typical for hydroxyl, while the deoxygenation is a clear marker of N-oxide [77,79].

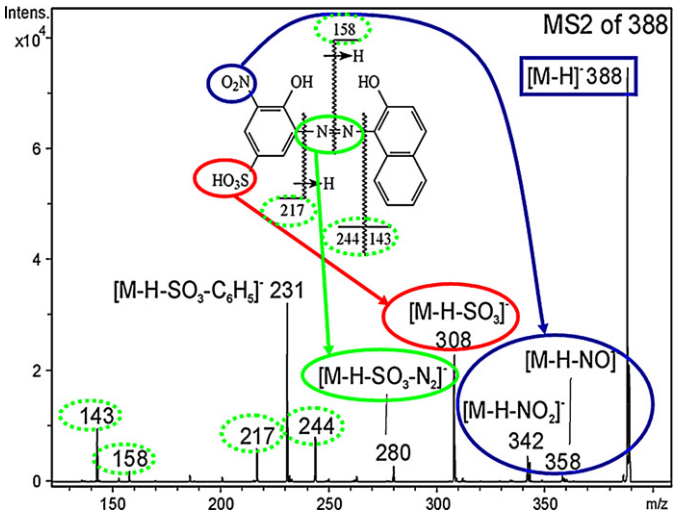

Fig. 6. Correlation between the fragmentation behavior and multiple functional groups (1 sulfo, 1 nitro, 2 hydroxyl and 1 azo groups) for dye C.I. Mordant Black 15 in the negative-ion MS/MS spectrum of $[M-H]^-$.

The azo group is the characteristic functional group for a wide range of organic dyes, often accompanied by other functional groups including sulfonic acid(s) because of a better solubility in water. There are several possibilities of cleavages of azo group together or without the transfer of hydrogen atom(s), i.e., cleavages between nitrogen and neighboring carbon atoms and between two nitrogen atoms in the azo group [43], as illustrated on the example in Fig. 6. The rearrangement loss of N_2 is known for both EI and API spectra [43]. Azo compounds without any other functional group provide signal only in the positive-ion mode, but in practice organic azo dyes carrying anionic functional groups are measured in the negative-ion ESI mode with the observation of the rearrangement loss of N_2 as well [31,33,43,84].

Amide compounds without other electronegative functional groups are suitable for the measurement in the positive-ion mode, where the characteristic NL of NH_3 (RNH_2 for alkylamides and R^1NHR^2 for dialkylamides) for amides results in the formation of stable carbonyl ions [85]. This is an analogy of peptide cleavage leading to the formation of *y* and *b* ions. The same NL of NH_3 can be expected for amines, but in fact this loss is typical only for aliphatic amines, but not so common for aromatic amines, where it occurs only in case of absence of other functional groups [67,86]. The NL of HCN is characteristic for both nitriles [67,87] and aromatic amines without other functional groups [67,86]. It is interesting to note that very similar behavior is known for aliphatic alcohols vs. phenols, where H_2O loss is common for alcohols but rare for phenols. Substituted amines provide losses of alkyl- and dialkylamines, such as methyl amine [34,88], $CH_3N{=}CH_2$ [34,89], dimethylamine [34,88–90]. Nitrile compounds containing other electronegative substituents can provide the signal in the negative-ion mode with common NL of HCN or even the nitrile ion $[CN]^-$ can be detected [91]. For compounds with multiple functional groups, the fragmentation pathways of amides, amines and nitriles can be suppressed by other competitive fragmentation reactions.

5.2.4. Oxygen containing functional groups

The fragmentation behavior of oxygen containing functional groups has many common features with EI. Regardless the fact that the initial ion in API mass spectra is EE $[M+H]^+$ or $[M-H]^-$ unlike to OE ion $M^{+\bullet}$ in EI spectra, the resulting fragment ions are often the same or at least part of them. The strongest effect on the fragmentation among common oxygen containing functional groups has carboxylic acids and alcohols, if we neglect relatively

unusual hydroperoxides and epoxides. Oxygen containing functional groups are often present together with other functional groups, so it is not easy to recommend the most suitable ionization mode for individual functional groups. The negative-ion mode is more sensitive for carboxylic acids, while the positive-ion mode is generally more convenient for other functional groups. Esters, ketones and ethers may not provide any signal in the negative-ion mode, unless other suitable functional group improves the ionization efficiency.

Carboxylic acids have quite specific behavior with the characteristic NL of CO_2 observed in all types of spectra, i.e., both positive and negative modes, full scan and tandem mass spectra [35,43,92,93]. We know only one common isobaric NL corresponding to $\Delta m/z = 44$, which is the radical $NH_2CO^{\bullet}$ occurring for some nitrogen containing heterocycles with the carbonyl group next to nitrogen [94,95], but this loss never occurs in both polarity modes unlike to the carboxylic acid. In principle, the carboxylic acid can also provide $[HCOO]^-$ ion at m/z 45 in the negative-ion mode, but it is rarely reported due to the fact that the mass range is mostly set from m/z 50 and for some manufacturers it is the instrument limit. In the positive-ion mode, the typical non-specific NL is water [67], often followed by the loss of carbon monoxide ($H_2O + CO$) [31,67]. It is interesting to note that Levsen et al. [67] excludes the possibility of CO_2 NL in the positive-ion mode, while we have some examples on this fact [92,93,96], but it is less frequent than for the negative-ion mode.

Aliphatic alcohols provides the abundant NL of water in any measurement mode as expected, but unfortunately this NL is not specific and can occur almost for any oxygen containing functional group, but the relative abundance of water loss is the highest for alcohols and always present already in the full scan mode. It is frequently the base peak in tandem mass spectra. The situation is rather different for phenols, where the most typical is the rearrangement loss of CO and the loss of water is less common.

Concerning the ether functional group, the fragmentation pathways should be similar as with EI, however most examples of API mass spectra in the literature deal only with the aromatic methoxy group, so we have focused only on this group. Ether compounds without other functional group usually do not provide a signal in the negative-ion mode. There are four possible NLs in the positive-ion mode, radicals $CH_3^{\bullet}$ [35,86,97–100] and less frequently $CH_3O^{\bullet}$ [100,101] and the neutral molecule of methanol [86,97,98,102] or methanal [97,102]. The presence of OE ions is typical for the methoxy group [58,67].

Ester functional group has a relatively low polarity and therefore not so high importance on the fragmentation if other more polar groups are present. The characteristic fragmentation is the NL of alcohol (methanol for methylesters, ethanol for ethylesters, etc.) often followed by the NL of CO. If ester is the only functional group, then the positive-ion APCI (or APPI) is the most convenient and the negative-ion mode does not provide any signal. Products of the fragmentation in API mass spectra of ketones are identical as for EI, i.e., $[R^1CO]^+$ and $[R^2CO]^+$ [67,103]. Low abundant NL of water may occur as well [104]. For aldehydes, the only reported NL is CO [67].

Epoxy and especially hydroperoxy groups are not often detected in organic molecules, because they are known as unstable oxidation intermediates. Epoxides are more stable, so their detection is more likely. Both groups induce an extensive fragmentation with the typical NL of water for both groups or H_2O_2 for hydroperoxides [105,106]. The extensive fragmentation and also the adduct formation may complicate the right determination of MWs [49]. Their MW can be also determined with the positive-ion APCI mode at the special soft setting of tuning parameters (low temperatures and low flow rates) based on [M+H + methanol]$^+$ adducts [49] and by silver-ion adduct formation in the positive-ion ESI [107].

5.2.5. Halogen substituents

Possible fragmentation pathways for halides are relatively easy with NLs of HX or radical $X^{\bullet}$. The probability of radical ion appearance increases in the order F < Cl < Br < I (Table 3). For polyhalogenated compounds, repetitive NLs are observed and the relative abundance of (de)protonated molecules can be significantly decreased. Halides can be, in principal, measured in both polarity modes with all API techniques depending on other functional groups present in the molecule. Chlorine (^{35}Cl:^{37}Cl = approximately 3:1) and bromine (^{79}Br:^{81}Br = approximately 1:1) atoms have highly characteristic isotopic doublets enabling their easy identification in individual ions in the spectra, while fluorine (^{19}F) and iodine (^{127}I) are monoisotopic. If more Cl/Br atoms are present in the molecule, then the ratio of isotopic peaks differing by two units is calculated according to the binomial equation. For example for two chlorine and one bromine atoms, the equation is $(3a + b)^2(a + b)$ and the calculated coefficients are equal to relative abundances of M: M + 2: M + 4, etc. In the negative-ion mode, anionic halides can be observed, especially with the quadrupole analyzer [43,45,102]. The formation of radical molecular anions $M^{-\bullet}$ has been reported for polybrominated diphenyl ethers followed by NLs of $Br^{\bullet}$ and Br_2 [108] and for brominated polyaromatic hydrocarbons [43]. If the fluorine atom is present next to other functional groups, then the NL of HF is often suppressed.

5.2.6. Other structural types

It is worthy to comment in this chapter also the fragmentation behavior of some structural subunits without heteroatoms, such as alkyl/aryl substitution, polycyclic aromatic hydrocarbons and the presence of double and triple bonds. Very common structural feature is the alkyl or aryl substitution on the aromatic and other cyclic and also acyclic structures. The typical NL for the alkyl substituent is alkene [95,102,109], for example the NL of butene for the butyl substitution. The NL of alkane is also known [94], if hydrogen is sterically available, but based on the literature and own experiences, the NL of alkene is generally preferred. The radical loss of alkyl is reported mainly in the negative-ion APCI [94,95]. For the phenyl (in general aryl) substitution, the logical NL is C_6H_6 [104] or C_6H_4 [95], less frequently radical $C_6H_5^{\bullet}$ [95]. For organometallic compounds containing a carbon–metal bond, the cleavage of this bond resulting in NLs of alkene and alkane for the alkyl substitution [48,84,92] and C_6H_6 for the phenyl substitution [48,89,93,102] is typical.

Aromatic compounds with highly conjugated structures (preferably nitrogen [17,52,54] or sulfur [55] containing heterocyclic systems) have an ability to stabilize an unpaired electron resulting in the molecular radical cation $M^{+\bullet}$ formation [17,35,43,52,54,55], which is typical for polycyclic aromatic hydrocarbons, porphyrins, etc. The ability to delocalize the unpaired electron can be retained also for some fragment ions. In practice, there is often a competition between the formation of radical cation $M^{+\bullet}$ and protonated molecule $[M+H]^+$ depending on various parameters, such as other functional groups, the alkyl chain length, used solvents, flow rates, the type of instrument, API ionization technique [43,54].

The presence of double or triple carbon–carbon bonds results in corresponding shifts of MWs, which may help to determine the number of unsaturations, but there is no as significant consequences for the fragmentation of alkenes and alkynes as for heteroatom containing functional groups. If the position of unsaturation has to be determined (e.g., for unsaturated fatty acids), then there are several derivation procedures (additions on the double bond) resulting in the subsequent fragmentation used for the localization of the double bond due to the characteristic fragmentation behavior. These approaches are known for both EI [110] and API techniques [111]. The difference between isobaric monounsaturated and cyclic saturated compounds is not so difficult to recognize, because monounsaturated compounds have very sim-

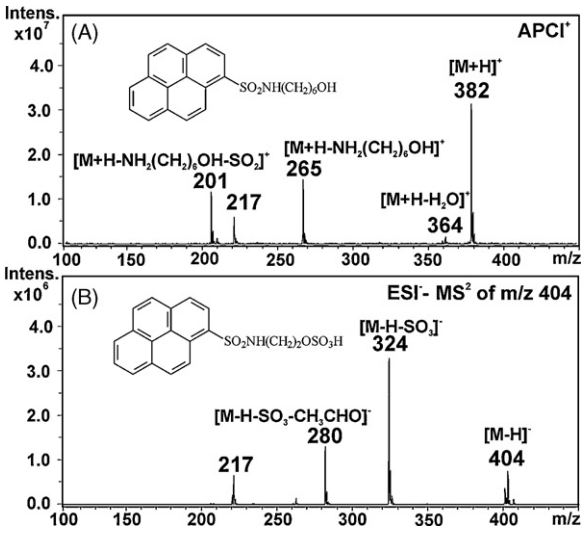

Fig. 7. Comparison of mass spectra of structural analogs without and with ionic sulfate functional group: (A) full scan positive-ion APCI spectrum of compound without sulfate and (B) negative-ion ESI-MS/MS spectrum of compound with sulfate functional group. Please note that the alkyl chain length of both compounds differs by 4 methylene units, but it has not substantial effect on the ionization and fragmentation behavior.

ilar fragmentation behavior as the saturated analogs, while cyclic compounds produce new cleavages related to the side loss from the cycle. For analogous groups of compounds (e.g., triacylglycerols), the increasing number of double bonds for the identical molecular skeleton brings two important changes: (1) better ionization efficiency results in better sensitivity [112] and (2) more double bonds means better stabilization and lower level of fragmentation in comparison to the saturated analog [36,113]. The position of double bond may also play rather significant role in this respect [114].

5.2.7. Molecules with multiple functional groups

In practice, analyzed molecules often have a higher complexity than only one functional group and they can bear multiple functional groups with different effects on the ionization and fragmentation behavior. As a rule of thumb, higher the polarity of the functional group is, stronger effect on the MS behavior is expected. One example is ionic sulfur-containing functional groups sulfo and sulfate. If one of these ionic functional groups is present, it completely changes both the ionization and fragmentation behavior in comparison to the analog without this functionality, as illustrated in Fig. 7. The molecule without sulfate (Fig. 7A) can be easily ionized in the positive-ion APCI mode yielding several structure relevant fragment ions already in the full scan mass spectrum, while after the introduction of sulfate group, the negative-ion ESI mode is the most convenient and products ions are observed only in MS/MS spectra dominated by the NL of SO_3 (Fig. 7B). The following order of common functional groups is listed according to their impact on the fragmentation behavior in API mass spectra based on our experiences: nitrate > phosphate ~ sulfate ≫ sulfonic acid > carboxylic acid > hydroxyl > nitro > halogens > other functional groups. It should be emphasized that this order is a rough guide, which can be altered depending on the application. The fragmentation behavior of compounds containing multiple functional groups is shown in Fig. 6. More discussion on the competition of individual fragmentation pathways has been published in our previous work on API mass spectra of organic dyes [42]. Some general comments are valid in most cases and their knowledge can simplify the interpretation:

(A) Chlorine and bromine have characteristic isotopic patterns easily recognized for both (de)protonated molecules and product ions.

(B) The knowledge of nitrogen rule, briefly the odd value of MW means the odd number of nitrogen atoms in the molecule.

(C) The presence of anionic functional groups (i.e., phosphate, sulfate, sulfonic and carboxylic acid) strongly shifts the choice of most suitable ionization technique towards the negative-ion ESI mode with very little regard to the rest of molecule (except for the presence of cationic functional group, such as the presence of quarternary amine). Similarly, the presence of cationic functional group makes the positive-ion ESI ideal for the analysis of such molecules.

(D) The presence of multiple functional groups typically does not represent new fragmentation pathways in addition to these listed here, but rather the competition among fragmentation pathways. It is almost impossible to give exact rules for the preference of individual fragmentation valid generally for complex molecules and their fragmentation has to be interpreted based on the personal expertise and the literature data for similar compounds.

5.3. Odd-electron ions in API mass spectra

The characteristic MW related ions in API techniques are EE (de)protonated molecules [56,67]. If there are no exceptions from the even-electron rule [69], then no OE ions should be found in API spectra. In practice, this rule is followed in most cases, as illustrated for example by recent excellent paper on the formation of EE vs. OE ions in positive-ion ESI mass spectra [56], where about 93% of all observed ions are assigned as EE with only 7% OE ions. The authors advertise in the last paragraph of their work that their preliminary work has shown that the amount of OE ions is slightly higher (about 14%) in the negative-ion ESI mode. To our best knowledge, no systematic investigation has been published so far for APCI, but our practical experiences with many different classes of small molecules show that the situation is similar or slightly favored towards radical ions compared to ESI. Quite different situation is with APPI [16,19,115], where OE ions are more common due to the different ionization mechanism favoring the formation of radical

Table 4
Typical radical neutral losses leading to the formation of odd-electron ions in API spectra (sorted approximately in the order of decreasing frequency of their occurrence).

Radical neutral loss	References			
	ESI^+	ESI^-	$APCI^+$	$APCI^-$
$NO_2^•$	[56,67,101,127]	[67,71,74]	[67,104]	[67,71,72]
$NO^•$	[67,127]	[71,74]	[67]	[71,72]
$CH_3^•$ (alkyl)	[56,67,68,97–99,101,118]	[34,35,136–139]	[67,68,100,104]	[134,136]
$OH^•$	[34,56,67,68,99,101,127]	[67]	[67,68,79]	[67,71]
$Br^•$	[67,99,101]	–	[43,67,140]	[67,71,72]
$Cl^•$	[56,67,99,101]	–	[67,104]	–
$CH_3O^•$	[101]	–	–	–
$CH_3S^•$	[56,118]	–	–	–
$CH_3SO_2^•$	[56]	–	–	–

The missing citation for particular ionization mode does not automatically mean that this radical NL cannot occur, but the suitable reference is not found.

ions. Concerning OE ions, APPI is somewhere in between soft ionization techniques and EI. In certain cases, molecular radical ions can be observed due to the charge exchange mechanism in both polarity modes and the electron capture mechanism in the negative-ion mode [16,19,52].

Table 4 summarized the most common radical NLs leading to the formation of OE ions in API spectra sorted approximately in the order of their importance based on our experiences and other references as well. In our opinion, the most typical functional group for the formation of OE ions is the nitro group starting from the formation of the radical molecular ion $M^{-\bullet}$ by the electron capture mechanism [52,70] and three common radical NLs, $NO_2^\bullet$, $NO^\bullet$ and $OH^\bullet$ occurring in both polarity modes (Table 4). Similar losses are known also for nitrates [70,116]. Typically, nitro group characteristic NLs are observed as the initial fragmentation step even for compounds containing multiple functional groups [43]. The same NL (NO) is known for the nitroso group, but this functional group is relatively rare [75]. The radical NL is common for the methyl substitution on the aromatic ring or other cyclic structures, N-methyl substitution [56,67], (poly)methoxylated flavonoids [97,100], drugs containing methoxy group [34,35], etc. For alkyl/aryl substitution, NLs of EE alkene/arene or alkane are characteristic, but the radical alkyl loss can be also observed, especially in the negative-ion APCI mode and for heterocyclic compounds [94,95,109,117]. The presence of methoxy group sometimes exhibits the second radical $CH_3O^\bullet$, but it is not as common as the methyl radical loss. The analogous radical loss is known for $CH_3S^\bullet$ [118]. The radical loss can also occur for poly- and perhalogenated compounds (Table 4), while the NL of HX is usually preferred, if sterically available hydrogen is present. The radical NL of $OH^\bullet$ has been reported for certain functional groups, e.g., nitro group, N-oxide, sulfoxide, while we have not found any report for this radical loss from alcohol, where the NL of water has a strong preference. If CH_3SO_2 group is present in the molecule (e.g., pesticides), then the corresponding radical NL leads to the formation of OE ion with relative abundances up to 100% [56].

6. Concluding remarks

Nowadays, HPLC–MS and other liquid-phase separation techniques coupled to MS are routinely used in analytical laboratories, but the potential of MS is often not fully explored due to the absence of basic knowledge of mass spectra interpretation partially caused by the lack of appropriate literature for soft ionization techniques. This review describes the basic rules for the interpretation of API mass spectra of small molecules, especially their ionization and fragmentation behavior related to the type of particular functional groups. On the other hand, the broad range of functional groups included in our review may cause that the information on particular functional groups may not be enough detailed. It should be emphasized that the expertise in the mass spectra interpretation cannot be obtained by studying the literature only, but the practical everyday interpretation of mass spectra is essential. Experiences with the similar or identical class of compounds either from previous own measurements or from the literature are invaluable in solving practical problems. The most sophisticated Fourier transformation instruments Orbitrap and ICR with the ultrahigh resolving power and excellent mass accuracy below 1 ppm provides the most reliable data for the structural elucidation of complex structures, but on the other hand even the simplest low resolution quadrupole analyzer can – in certain cases – be applied for the identification of simpler compounds derived from the parent structure, e.g., drug metabolites, degradation products and technological impurities. Limitations of MS in the structure elucidation should be also kept in mind (e.g., positional isomers, enantiomers) and – if possible – the complementary data from other spectral techniques and the knowledge of retention behavior should be used together with the basic chemical sense. The final step in the unambiguous structural elucidation should be the comparison with the identical standard either commercial available or synthesized.

Acknowledgments

This work was supported by the grant project no. MSM0021627502 sponsored by the Ministry of Education, Youth and Sports of the Czech Republic and project no. 203/08/1536 sponsored by the Czech Science Foundation. We are obliged to anonymous reviewers for their valuable comments how to improve this manuscript.

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