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Review

Sensitivity enhancement in liquid chromatography/atmospheric pressure ionization mass spectrometry using derivatization and mobile phase additives

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Abstract

High performance liquid chromatography with atmospheric pressure ionization (API) mass spectrometry has been essential to a large number of quantitative analytical applications for a variety of compounds. Poor detection sensitivity however is a problem observed for a number of analytes because detection sensitivity can be affected by many factors. The two most critical factors are the chemical and physical properties of the analyte and the composition of the mobile phase. In order to address these critical factors which may lead to poor sensitivity, either the structure of the analyte must be modified or the mobile phase composition optimized. The introduction of permanently charged moieties or readily ionized species may dramatically improve the ionization efficiency for electrospray ionization (ESI), and thus the sensitivity of detection. Detection sensitivity may also be enhanced via introduction of moieties with high proton affinity or electron affinity. Mobile phase component modification is an alternative way to enhance sensitivity by changing the form of the analytes in solution thereby improving ionization efficiency. pH adjustment and adduct formation have been commonly used to optimize detection conditions. The sensitivity of detection for analytes in bio-matrices could also be enhanced by decreasing ion-suppression from the matrix through derivatization or mobile phase addition. In this review, we will discuss detection-oriented derivatization as well as the application of mobile phase additives to enhance the sensitivity of detection in liquid chromatograph/atmospheric ionization/mass spectrometry (LC/API/MS), focusing in particular on the applications involving small molecules in bio-matrices.

Keywords: Reviews; Sensitivity improvement; Derivatization; Mobile phase additives

Contents

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1. Introduction

Since API was introduced in the 1980s, the combination of high-performance liquid chromatography and mass spectrometry (HPLC/MS) has become widespread, and has had a significant impact on all major areas of analytical science [1]. Along with many successful applications, a number of limitations of API using either electrospray ionization or atmospheric pressure chemical ionization have been noted [2–4]. Poor sensitivity using ESI has been observed for compounds, which lack an ionic, functional group or which cannot transfer ions from solution into the gas phase efficiently [2,3]. Sensitivity constraints using atmospheric pressure chemical ionization (APCI) have been observed for compounds which have low proton affinity [3,4]. In order to increase the detection sensitivity for ESI or APCI with various compounds, many research groups have used derivatization to introduce functional groups into analytes or have used mobile phase additives to improve ionization efficiency.

The process of electrospray involves transferring ions which are present in solution into the gas phase in the presence of a strong electrical field [5]. Therefore, pre-formation of ions is very important in the ESI detection mode. In the APCI detection mode, solvent molecules form reagent gas ions first, followed by gas-phase ion-molecule reactions of reagent ions with analytes. The analyte molecule can be protonated as long as it's proton affinity exceeds that of the protonated solvent molecule. The analyte molecule can be deprotonated however when the gas phase acidity of the analyte molecule is larger than that of the deprotonated solvent molecule. In addition to these proton transfer reactions, other possible reactions in APCI can be involved such as adduct formation and electron capture reactions [6]. For both API detection modes, the sensitivity of detection is critically related to the solution environment as well as the properties of the analyte.

Many interrelated parameters can affect the generation of ions in LC/API/MS detection. Nebulization and ionization of analytes in the ion source are determined by instrument parameters, such as the flow rate of nebulizer gas and dissolvent gas, desolvation temperature, electrospray capillary voltage among others. Secondly, solution phase factors play an important role in ionization, such as pH, mobile-phase composition, surface tension, and the concentration of electrolytes and analytes [7–9]. The chemical and physical properties of an analyte are perhaps the most critical parameters for superior sensitivity in the various modes of ionization. Ionization status, surface activity and other factors, which are directly related to the properties of the analytes, determine the ionization efficiency. Generally, ESI is considered to be more useful for compounds which can form ionic species in solution; while APCI is more useful for low to medium polarity compounds having high proton-affinity atoms, such as oxygen and nitrogen [1]. Derivatization changes the structure of the analyte and therefore changes its physical and chemical properties, resulting in high ionization efficiency. The chromatographic retention of the analyte will also be changed after derivatization, therefore decreases in suppression of ionization related to the co-elution of biomatrix components may be realized.

Although derivatization is a useful way to improve the sensitivity of detection, it can be a time-consuming endeavor. The most common advantage of LC/MS over gas chromatography/mass spectrometry (GC/MS) is the elimination of a derivatization step. However, this advantage is not the only advantage. When a derivatization step is necessary for both GC/MS and LC/MS, often the sample handling step for LC/MS is easier than for GC/MS. Also, the run time for the LC/MS is generally shorter than that of GC/MS and LC coupled with tandem MS may offer better selectivity than GC/MS.

Compared to derivatization, the adjustments of the mobile phase in many cases is easier. Solvent composition has proved to be a vital factor for achievement of high sensitivity in electrospray ionization because of its important role in the various steps of the nebulization and ionization process. Corcia et al. reported a 60-fold increase in the ion signal for 3,4-dichloroaniline by simply substituting methanol for acetonitrile in the mobile phase solution [10]. The pH of the mobile phase or the presence of mobile phase additives has

also been shown to have a significant effect on the formation of protonated or adduct ions [7]. The ionization efficiency of a particular analyte could also be dramatically changed as a result of formation of an adduct ion.

Derivatization has been successfully applied to a number of biomolecules such as peptides and proteins, and a review covering these applications has been published recently [11]. Reviews involving derivatization of neutral steroids have also been reported [12,13]. This work will present an overview of chemical derivatizations which have been applied to small compounds. The application of mobile phase additives to improve the sensitivity of detection will be discussed as well. This review focuses in particular on the bioanalytical areas.

2. Analyte derivatization

With increased application of liquid chromatography and mass spectrometry (LC/MS), some scientists have utilized LC/MS detection for certain compounds even although they may not be readily amenable to MS analysis or may lack sufficient detection sensitivity. Derivatization may be employed to improve the sensitivity of detection for these analytes. Most of the derivatization techniques used for ESI focus on introduction of a permanently charged moiety or readily ionizabled group into analytes; whereas the derivatization techniques for APCI tend to introduce moieties with high proton affinity or electron affinity into the analytes. Table 1 summaries applications of derivatization to API. The analyte functional groups that were derivatized are also shown in the table.

2.1. Alcohols

Most of the derivatization methods published in the bioanalytical area were applied to the analysis of steroids, which generally possess hydroxyl, phenolic or ketone groups. According to the summary of Table 1, the application of derivatization for alcohols has involved all detection modes in API, and employs the principle of the introduction of ionization-active moieties.

2.1.1. Positive ESI/MS

Unmodified simple alcohols are not readily detectable in ESI/MS because of the absence of an ionizable functional group. Although some steroids may be ionized using the ESI or APCI detection modes, the detection sensitivity would not generally meet the requirements for trace analysis. Two major types of chemical derivatizations have been utilized to improve the detectability of alcohols: chemical addition of an ionic species or introduction of ionizable moieties into the alcohol.

Typically used ionic species are phosphonium, quaternary ammonium and pyridinium salts. Tris(trimethoxyphenyl) phosphonium (TMPP) reagents have been used to react with the N-terminus of peptides for sequence elucidation in fast atom bombardment (FAB), matrix-assisted laser desorptionionization (MALDI) and ESI [11]. Ionic phosphonium moieties can also be introduced into alcohols under non-aqueous conditions. Barry et al. discussed the synthesis of two TMPP regents: *S*-pentafluorophenyl tris(2,4,6-trimethoxyphenyl)phosphonium acetate bromide (TMPP-AcPFP) and (4-hydrazino-4-oxobutyl) [tris(2,4,6-trimethoxyphenyl)phosphonium bromide (TMPP-PrG). They applied TMPP-AcPFP for derivatization of alcohols and TMPP-PrG for aldehydes and ketones [14]. Most of the model compounds selected, which were not detectable previously, demonstrated strong signals and good chromatographic behavior. The response of the alcohol derivatives did not show significant differences among the different alcohols studied, regardless of their aromaticity/aliphaticity or the presence of other substituents. Signals for steroids possessing alcohol groups, moiety, except β-estradiol, however did not demonstrate signals as high as the test alcohols. 2-Fluoro-*N*-methylpyridinium *p*-toluene sulfonate can impart a permanently charged pyridinium moiety into analytes with the base catalyst triethylamine under non-aqueous conditions. Detection limits for fatty alcohol and alcohol ethoxylate surfactants in wastewater samples were typically less than 10 ppt [15].

Van Berkel noted that the typical ESI ion source is a two-electrode system, which consists of a metal sample capillary with a high voltage applied and an atmospheric sampling aperture plate, therefore he proposed that an electrolytic process must be present in the ESI ion source for successful ionization according to the law of conservation of charge [16]. Consequently, a series of derivatization methods to enhance the sensitivity of electrospray mass spectrometry (ESI/MS) based on electron transfer reactions were developed by Berkel's group [3,17]. Enhanced sensitivity for analysis of alcohols, sterols and phenols was demonstrated using a ferrocene-based "electrochemically ionizable" derivatization process [3]. After derivatized using ferrocenoyl azide, cholesterol was converted to the ferrocene carbamate ester, which selectively oxidized in the high electronic field to form a radical cation. 25 fg (41 amol) of cholesterol could be detected with an excellent signal-to-noise ratio (S/N) by this technique.

Introduction of an ionizable moiety can also be achieved by reaction with derivatization reagents possessing nitrogen atoms, especially those possessing an amine group. Derivatization with mono(dimethylaminoethyl)succinyl (MDMAES) imidazolide was used to efficiently improve the sensitivity of detection for cholesterol and dehydrocholesterol [18]. The limit of quantification (LOQ) for dehydrocholesterol was shown to be 0.12 mM for clinical samples. Dansylation may lead to an impressive improvement for the detection for steroids with hydroxyl groups [19] or phenol groups [20–21]. The limit of detection (LOD) for brassinosteroids was 125 amol following derivatization with dansyl-3-aminophenylboroinc acid, based on a S/N ratio of 3 [19].

Table 1
Derivatization reagents and the functional groups they have been applied to

Functional group	Derivatization reagents			
	(+) ESI	(−) ESI	(+) APCI	(−) APCI
Alcohols	TMPP [14]	Sulfur trioxide–pyridine complex [LOQ:5 pmol, 23]	Benzyl chloride [0.27 μg/mL, 25]	ECNAPCI: boronic acid derivatives possessing nitro or trifluoromethyl groups, such as 2NFP-APB [1 fmol, 30]
	2-Fluoro-*N*-methylpyridinium *p*-toluenesulfonate (Pyr+) [0.1−22.7 ng/L, 15]	Sulfur trioxide *N*,*N*-dimethyl-formamide complex in dimethylformamide/pyridine [200 amol, 24]	Acetic anhydride [10 pg/mL, 25]	
	Ferrocenoyl azide [41 amol, 3] MDMAES [LOQ: 0.12 mM, 18] DAPB [125 amol, 19] Propionyl or benzoyl acid anhydride [fmol to low pmol, 22]			
Phenols	Dansyl-Cl [0.2 fpg/mL, 20,21]		Propionic anhydride [LOQ, 0.2 ng/mL, 33]	ECNAPCI:PFB [170 amol, 29]
Ketones, aldehydes	TMPP [14]		DNPH [1.0 nM, 36], MNBDH [100 nM, 37]	DNPH [0.3 nM, 36] ECNAPCI: NFPH [3.2 fmol, 32]
	GirP [10 ng/mL, 34] and GirT TSH [0.1 ng/mL, 35]			
Thiols	DMAE [38]	AMDSA [38]		
Carboxylic acids	TMPP [44]		Esterification: hydrochloric acid/methanol [LOQ: 20 ng/mL, 40], BF_3–methanol [LOQ: 0.5 ng/mL, 41], ethereal diazomethane [5 ng/mL, 42], HCl/*n*-butanol [0.07 pmol, 43]	
Amines	TMPP [44]	PFPA [0.2 fmol, 47], 4-nitrobenzyl-chloroformate [LOQ: 10 ng/mL, 48]		ECNAPCI: PFB-Br [29], NBDCl [20–100 ng/mL, 49], NBDPZ [20–100 ng/mL, 49]
	NIT [0.12–0.25 ng/μL,45] NBD-F [0.6 ng/mL, 46]			
Amide			BMC [LOQ: 1 ng/mL, 50]	
Vitamin D_3			Cookson-type reagents: MBOTAD [18 fmol, 26], DMEQTAD [10 fmol, 51]	NPTAD [10 fmol, 31], boronic acid derivatives possessing nitro or trifluoromethyl groups [1 fmol, 30]

Reported LODs and references are listed in brackets. AMDSA: 2-acrylamino-2-methyl-propanesulfonic acid; BMC: 4-bromomethyl-7-methoxycoumarin; DAPB: dansyl-3-aminophenylboronic acid; DMAE: 2-(*N*,*N*-dimethyl-amino)ethyl acrylate; DMEQTAD: 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione; DNPH: 2,4-dinitrophenylhydrazine; GirP: 1-(carboxymethyl)pyridium chloride hydrazide; GirT: 1-(carboxymethyl)trimethylammonium chloride hydrazide; MBOTAD: 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione; MDMAES: mono(dimethylaminoethyl)succinyl imidazolide; MNBDH: *N*-methyl-4-hydrazino-7-nitrobenzofurazan; NBDCl: 4-chloro-7-nitro-2,1,3-benzoxadiazole; NBD-F: 7-fluoro-4-nitrobenzoxadiazole; NBDPZ: 4-nitro-7-piperazion-2,1,3-nitrobenzoxadiazole; 2NFP-APB: [3-(2-nitro-4-trifluoromethylphenyl)aminophenyl]dihydroxyborane; NFPH: 2-nitro-4-trifluoromethylphenylhydrazine; NPTAD: 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione; NIT: naphthylisothiocyanate; PFB: pentafluorobenyl bromide; PFPA: pentafluoropropionic acid; TMPP: tris(trimethoxyphenyl) phosphonium reagents; TSH: *p*-toluenesulfonhydrazide.

In addition to the derivatization approaches introduced above, esterification has proved to be another useful tool for improving the detection for small polar compounds possessing hydroxyl groups. Better chromatographic behavior was achieved for adenine bases, ribosides/glycosides, nucleotides, AMP, ADP and ATP without adding ion-pairing reagents [22]. The response of the analytes was increased as well, due to the increases in surface activity after derivatization. Detection limits for cytokinins were improved 10–100-fold compared to that without derivatization.

2.1.2. Negative ESI/MS

The introduction of an acidic group, such as a carboxyl or sulfonic acid group, is a commonly used strategy to improve detection sensitivity in the negative ESI/MS mode. These acidic groups are readily deprotonated, therefore high ionization efficiency may be obtained. A sulfur trioxide-pyridine solution was used to convert cholesterol into its sulfate, and resulted in low picomole determination of cholesterol using nano-electrospray ionization [23]. A comparable sensitivity of detection for pregnenolone was demonstrated by Chatman et al. using a sulfur trioxide *N*,*N*-dimethyl-formamide complex in dimethylformide/pyridine as the derivatization reagent [24]. The LOD was reported to be 200 amol/μL and was achieved for biological samples using nano-electrospary.

2.1.3. Positive APCI/MS

Alcohols are not only problematic with ESI, but also show poor ionization efficiency using the APCI detection mode [25]. This may be due to poor proton transfer in the gas-phase reaction. Introduction of high proton-affinity atoms such as oxygen and nitrogen has been shown to improve the capability of proton transfer for several compounds [4,26]. Gao et al. used benzoyl chloride to derivatize propylene glycol and established a quantitative assay for detection of propylene glycol in rat plasma and lung tissue with an LOD of 0.27 μg/mL and 1.1 μg/g, respectively. They proposed that the gas-phase basicity and proton transfer ability of propylene glycol was improved via introduction the benzoyl moiety. Introduction of the acetate group into steroids may also improve ionization efficiency using the APCI detection mode. A mixture of 12.5% acetic anhydride and 12.5% triethylamine was employed as a derivatization reagent to produce acetyl derivatives [27]. The limits of detection were established as 25 pg/mL for budesonide and 10 pg/mL for fluticasone propionate [27].

2.1.4. Negative APCI/MS

Many successful applications of electron-capture negative APCI/MS (ECNAPCI/MS) have been employed to enhance sensitivity of detection and are drawing significant attention in recent years. After electron capture negative chemical ionization (ECNCI)/MS was introduced for gas chromatographic detection by Hunt et al. [28], it became a method of choice for trace analysis of many drugs and biomolecules. Singh first introduced the ECNCI technique for LC/MS [29]. He applied electron capture atmospheric pressure chemical ionization mass spectrometry with a liquid chromatography interface to establish attomole sensitivity for steroids, prostaglandins and DNA-adducts. It was postulated that the low-energy electrons generated in the APCI source could potentially ionize suitable analytes through dissociative electron capture. The sensitivity of detection was enhanced by tagging analytes with an electron-capturing group. In the original study, pentafluorobenzyl bromide (PFB-Br) was used as the derivatization reagent [29].

Higashi et al. investigated the application of ECAPCI/MS to a variety of compounds, including the analysis of Vitamin D_3 derivatives [30–32]. Because the nitro group represents a high electron affinity group, the authors predicted that the derivatization reagents with nitro groups or other high electron affinity groups could be employed in ECAPCI/MS. They synthesized various boronic acid derivatives possessing nitro and trifluoromethyl groups and applied these derivatization reagents to the analysis of 1,2-diol compounds [30]. Although [3-(2,3,4,5,6-pentafluorobenzyl)aminophenyl] dihydroxyborane (PFB-APB) has been used to increase the sensitivity of detection for 24,25$(OH)_2D_3$ 32-fold, other derivatization reagents with nitro group(s) were more effective in improving detection. In comparison with underivatized steroids, the response of steroids after derivatization with [3-(2-nitro-4-trifluoromethylphenyl)aminophenyl] dihydroxyborane (2NFP-APB) was increased by more than 200-fold. Limits of detection lower than 1 fmol could be readily obtained.

2.2. Phenols

ECNAPCI has been used to improve the sensitivity of detection for phenolic groups. Estrone and 2-methoxyestrone were model compounds in Singh's work [29]. The LODs of estrone and 2-methoxyestrone were 740 and 170 amol, respectively, after derivatization with PFB-Br.

Dansyl chloride, however, is more commonly used in improving the detection of steroids with phenolic groups in the ESI detection mode. A number of assays have been reported utilizing fluorometric detection of estrogens after dansyl derivatization. Due to possession of a basic secondary amine moiety, dansyl chloride has been shown to be valuable in improving the detection of some steroids in ESI as well. Anari introduced an analytical method using dansyl chloride to derivatize ethinyl estradiol. The LOD of this method was reported to be 0.2 fg/mL in monkey plasma when 50 μL plasma was used. The LOQ, however, was 5 pg/mL, 25,000 times higher than LOD because of a carryover problem [20]. Shou et al. reported the validation of an LC/MS/MS assay for ethinyl estradiol using dansyl chloride in human plasma. The LOQ was established as 2.5 pg/mL when using a 1 mL sample [21].

Esterification has also been successfully applied to phenolic groups for decreasing matrix effects and baseline noise. A LOQ of 0.2 ng/mL was achieved for the metabolite of ri-

vastigmine NAP 226-90 after derivatization with propionic anhydride [33].

2.3. Ketones and aldehydes

Ketone and aldehydes groups are good target functionalities for derivatization to improve ionization efficiency. The TMPP [14] and 1-(carboxymethyl)pyridium chloride hydrazide (GirP) reagents [34] can react with ketones and aldehydes to form hydrazones with a permanently charged moiety. TMPP derivatives of aldehydes showed stronger signals than the ketones possibly due to the lower reactivity of the ketones [14]. Direct determination of 17-hydroxyprogesterone in dried blood specimens has been developed with a detection limit of 10 ng/mL following derivatization with the GirP reagent [34].

p-Toluenesulfonhydrazide (TSH) is a reagent used for imparting an ionizable moiety into steroids using the ESI detection mode [35]. After derivatization with TSH, a hydrazone at the carbonyl group was formed. The lower limits of quantitation for two catechol estrogens 2- and 4-hydroxyestrone were 0.1 ng/mL in urine samples.

2,4-Dinitrophenylhydrazine (DNPH), a commonly used derivatization reagent in absorbance detection, was employed to derivatize aldehydes in exhaled breath condensate. The APCI detection mode showed a higher response than the ESI detection mode in this study. The hydrazones of DNPH can generate $[M–H]^-$ more efficiently due to the relatively high electron affinity of the nitro groups. The limits of detection were established in the 0.3–1.0 nM range for acrolein, several α,β-unsaturated hydroxylated aldehydes and saturated aldehydes in negative APCI, except for malondialdehyde which demonstrated a LOD at 1.0 nM in the positive APCI mode [36]. In comparison with DNPH, *N*-methyl-4-hydrazino-7-nitrobenzofurazan (MNBDH) produces less interference from other oxidants (such as ozone and nitrogen dioxide) when utilized for carbonyl compound derivatization. The LOD for the acetone MNBD-hydrazone was 100 nM in tobacco smoke using positive APCI [37]. Higashi et al. found that the 2-nitro-4-trifluoromethylphenyl (NFP) group possess better electron capturing ability than PFB-Br [30], therefore they developed the high electron-affinity reagent, 2-nitro-4-trifluoromethylphenylhydrazine for the ECNAPCI detection of steroids with ketone groups. The sensitivity of detection for 20-oxosteroids increased 20-fold when compared to detection using positive APCI for the intact steroids [32]. The LODs of pregnenolone and progesterone were 19 and 3.2 fmol, respectively based on a S/N ratio of 5.

2.4. Thiols

The major issue in the analysis of thiols is their instability, since thiols are readily oxidized during and after collection of biologic samples. The reported analytes which are involved in derivatization of thiols are generally cysteine-derived analogs. Tiopronin (TP) derivatives of thiols can be formed after being subjected to the Michael addition reaction with acrylic acids. This derivatization method stabilized the thiol groups as well as introduced an ionizable functional group. Seven TP derivatives of structurally diverse acrylic acid analogues were investigated by Matsuura [38]. The ion responses depended on the functional group of the acrylic acid of the derivatives. $[M + H]^+$ ions of TP-2-(*N*,*N*-dimethyl-amino)ethyl acrylate derivatives showed the highest response among the seven derivatives studied because of the introduction of a high proton affinity dimethylamino group. $[M–H]^-$ ions of TP-2-acrylamino-2-methylpropanesulfonic acid derivatives were strong in the negative mode due to their sulfonic acid groups.

2.5. Carboxylic acids

Although carboxylic acids can be detected in the negative ESI mode, the detection of some carboxylic acids may not meet detection requirements. This may be due to matrix effects and high background noise. APCI is less affected by matrix suppression than ESI [39]. Most of the applications using derivatization techniques reported for carboxylic acids involve esterification following APCI detection [40–43]. The carboxylic acids are generally aliphatic acids, such as modified tyrosine, and derivatives of glucuronic acid. Methylation is the most commonly used esterification reaction. Hydrochloric acid/methanol [40], BF_3–methanol [41] and ethereal diazomethan [42] have all been used as the methylation reaction reagent.

Leavens et al. synthesized TMPP reagents to derivatize carboxylic acids and amines [44]. 1-Chloro-4-methylpyridinium iodide and triethylamine were used to activate the carboxyl groups. Good peak shape and strong response of target compounds were observed with the LC/MS system after derivatization. A review summarizing the derivatization of peptides [11], utilizing C-terminal- and N-terminal-charged derivatives, is also a good reference for derivatization of carboxylic acids and amine.

2.6. Amines and amides

Difficulties in establishing an analytical method for volatile amines include their high volatility, polarity, basic character and high water solubility [45]. Problems associated with matrix effects and high background noise also exist for amines. Generally, the background is lower in the high mass range and increasing the molecular weight may be achieved through derivatization. Ultraviolet (UV) absorbance or fluorescence detection tags can be successfully used for the derivatization of primary and secondary amines in the API detection mode. 7-Fluoro-4-nitrobenzoxadiazole (NBD-F) [46] has been used in positive electrospray detection, while pentafluoropropionic anhydrides [47] have been used in negative ESI. The limit of detection was found to be 0.2–3 fmol for several diamines in the negative ESI detection mode. 4-Nitrobenzylchloroformate was used to form

carbamate ester derivative with primary amines; the LOQ was found to be 10 ng/mL using negative ESI [48]. The sensitivity of detection for amines has been improved using ECNAPCI after derivatization with PFB-Br [29]. Making use of the electron-attracting nitro group, nitrobenzoxadiazole (NBD) based derivatization reagents were employed to improve the response of amines in ECNAPCI [49] as well. The LOD ranged from about 20–100 nM for model compounds. 4-Bromomethyl-7-methoxycoumarin has been used to improve the proton affinity for 5-fluorouracil (5-Fu), which demonstrated no response in the positive APCI or ESI modes. The response of 5-Fu significantly increased after derivatization and an assay was validated in the range of 1.0–200 ng/mL [50].

2.7. Vitamin D_3 derivatives

The derivatization of Vitamin D_3 using ECNAPCI has been previously discussed with alcohol and ketone groups [30–32]. High detection sensitivity can also be achieved using APCI after reaction with a Cookson-type reagent, 4-[4-(6-methoxy-2-benzoxazolyl) phenyl]-1,2,4-triazoline-3,5-dione (MBOTAD) [25] or 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD) [51]. The sensitivity of Vitamin D_3 was increased dramatically after introduction of the high proton-affinity atoms, resulting in a limit of detection in the fmol range. DMEQTAD was found to be the most effective Cookson-type reagent for derivatization of Vitamin D_3. The limit of quantitation for 1α-hydroxyvitamin D_3 was established as 25 pg/mL for a 1.0 mL plasma aliquot after derivatization with DMEQTAD [51].

3. Mobile phase additives

Mobile phase additives can be used to improve the sensitivity in both the ESI and APCI detection modes.

Various factors can affect the ionization of analytes in ESI, such as: pH, mobile phase additives, flow rate, solvent composition, concentrations of electrolytes, viscosity, and surface tension of the solution. It is favorable if the ion can be formed in the solution phase for ESI detection. Adjustment in the composition of the mobile phase can result in the formation of ions in the solution phase and therefore have a significant affect on the sensitivity of detection. Mobile phase composition may also play an important role in APCI, especially in negative adduct formation which could improve detection significantly. Table 2 summarizes commonly used mobile phase additives in API detection modes.

Table 2
General strategy for choosing detection mode and mobile phase additives

Analyte	Basic samples, e.g. amines	Positive ion detection:
		pH at 2 units below pK_a of samples
		Acetic acid pH (3–4)
		Formic acid pH (2–3)
		Trifluoroacetic acid pH (1–2)
	Acetic samples, e.g. carboxylic acids	Negative ion detection:
		pH at 2 units above pK_a of samples
		Ammonium hydroxide
	Others, formation of adduct ions	Positive ion detection:
		Alkali and other metal adducts (Li^+, Na^+, K^+, Ag^+, Mg^{2+})
		Amine adduct (NH_4^+, RNH_3^+)
		Negative ion detection:
		Halide adduct (Cl^-, F^-, Br^-)
		Carboxylate adduct (X^-)

3.1. pH adjustment and buffers in mobile phase

The pH of the mobile phase can affect the sensitivity of analytes significantly. A novel indolocarbazole compound could be detected using the negative mode because negative ions were formed by loss of a proton from phenol groups. The intensity of deprotonated ions increased by a factor of 4 when the pH of the mobile phase was increased from 8 to 10 using 2 mM ammonium hydroxide [52]. The ionization efficiency of flavonoids also was significantly affected by the pH of the mobile phase [53]. The sensitivity of detection was increased up to 10-fold for isorhamnetin with the decrease in pH of the mobile phase ranging from 4.5 to 2.3 in positive mode.

Ammonium hydroxide, acetic acid, formic acid, ammonium acetate and ammonium formate are commonly used mobile phase additives [7,54]. Mallet et al. evaluated ion suppression/enhancement by using several mobile phase additives and ion-pairing agents at different concentrations for some test analytes [55]. Formic acid, ammonium hydroxide and acetic acid could increase the response for the majority of the test compounds at lower concentration (0.05% by volume); while trifluoroacetic acid (TFA) and ion-pairing agents showed ion suppression for both basic and acidic compounds. Marwah et al. investigated the influence of TFA and other volatile organic acids on the sensitivity of some steroids because they hypothesized that the oxygen atom present in the ester functionality might act as a substrate for the proton. They found the response of steroids increased several fold when adding these acids at very low concentrations, the order being: formic acid (50–200 ppm, v/v) > acetic acid (100–500 ppm) > trifluoroacetic acid (5–20 ppm). The signal response for several of the ergosteroids increased more than 10-fold at TFA concentrations about 10 ppm [56]. The addition of formic acid allowed the signal of cholesterol acetate to increase more than 20-fold and made it possible to analyze the compound at the subnanogram level.

The buffer added to the mobile phase also has a significant effect on the signal suppression commonly observed in complex matrices. Choi compared the response of ana-

lytes dissolved in pure solvent to that of analytes extracted from matrices when using different mobile phase additives [57]. The response of hydroxyl-fenozide in matrix was only 12.5% of that in pure solvent without any mobile phase additives. 1 mM ammonium formate was the optimal mobile phase additives in comparison to 0.1% formic acid (FA) and 0.01% ammonium hydroxide (NH_4OH), with the response of hydroxyl-fenozide in matrix shown to be 81.6% of that in pure solvent.

3.2. Adduct formation

Although non-nitrogen containing compounds have difficulty in forming a protonated molecule, formation of a metal adduct ion may offer another way to improve sensitivity of detection. Metal adduct ions are commonly observed in the positive detection mode. The formation of metal adduct ions has been used to improve sensitivity of detection through adding metal ions into the mobile phase. Ammonium and alkylamine adducts have been used to suppress the formation of multiple molecular ions in the positive ESI mode.

The negative detection mode has been far less amenable to sensitivity enhancement through adduct formation because electrical (corona) discharge may be generated before achieving enough voltage for ion evaporation [58]. Moreover, the anionic adducts once formed may be not as stable as the cationic adducts [59]. The detection sensitivity of the negative detection mode however sometimes may be expected to be higher because of a restively lower background. The anion-attachment mechanism is important in the electrospray process as well as the atmospheric pressure chemical ionization process [6]. Negative adduct ion formation could be used to improve the sensitivity of detection for some analytes which do not have sites with permanent charge or ionizable groups. Electron-deficient atoms, which bind to electronegative atoms such as the hydrogen present on hydroxyl groups, could offer binding sites for anions [59]. The most commonly used negative adducts are: carboxylate adducts and halide adducts.

3.2.1. Sodium and other metal adducts

Alkali metal adduct ions of organic compounds are observed in FAB, MALDI and ESI mass spectrometry of various compounds. The formation of metal adduct ions can be very useful in the identification and elucidation of the mass spectrum of analytes. Previous studies have investigated the binding site of sodium with various analytes [60,61] and proposed that the sodium ion is bound to the oxygen atoms of the analyte. Jemal et al. successfully applied the sodium adduct to quantitate a thiol compound at 2 ng/mL and proposed that good candidates for formation of the sodium ion adduct would be compounds with functional groups such as a methyl ester, a carboxylic acid, a thioether, a carboxylic acid amide, alcohols and lactones [62]. With multiple oxygen atoms in the structure, some steroids and polyether ionophores will readily form sodium adduct ions [63,64].

Ma and Kim divided steroids into three groups (I) containing 3-one and 4-ene functional groups; (II) containing at least one ketone group without conjugation; and (III) containing hydroxyl group(s) only. The capability of forming sodium adducts was (I) > (II) > (III). They reported that detection limits in the 5–15 pg range using ESI/LC/MS [63] could readily be achieved.

Sodium and potassium ions are ubiquitous in the experimental environment, $[M + Na]^+$ and $[M + K]^+$ are therefore commonly observed adducts using the ESI/MS detection mode. Higher ionization efficiency has been observed after the addition of metal salts. The response for 100 ng of lassalocid increased 75-fold after the addition of sodium chloride (5 mM) to the mobile phase [65]. In many cases however, the response of the sodium adduct did not increase with an increase in the concentration of sodium ions in the mobile phase. Although $[M + Na]^+$ adducts were formed for steroids and polyether ionophores [63], the addition of sodium salts of octanoic acid or dodecanoic acid at 10–50 μM into mobile phase did not improve sensitivity further. The ubiquitous content of sodium from glass and reagents was found to be about 2–10 μM, which is enough to form the $[M + Na]^+$ adduct with analytes. Methanol has demonstrated better sensitivity than acetonitrile as the organic component of the mobile phase, whereas the addition of formic acid or formate acetate will suppress the response of the $[M + Na]^+$ adduct [63]. This is due to differences in the ionization process. The sodium adduct is rarely observed in the APCI detection mode [66].

In addition to the formation of sodium adduct ions and potassium adduct ions, ionophores readily form complexes with other polar cations, such as Li^+, NH_4^+, Ca^{2+}, Mg^{2+}. Karlsson evaluated post-column addition of lithium, sodium, potassium, rubidium and cesium ions to form charged adduct ions [67]. Target compounds included modified cyclodextrins, oligosaccharides, bafilomycins and 18-crown-6. The optimum concentration which was independent of the type of alkali metal ions, was about 5×10^{-5} M. The relative complexation constants for cations and analytes were shown to increase with the size of the cation except for 18-crown-6 which forms a crown cavity. When the metal ion fits into the cavity more optimally, it will form stronger binding and therefore result in higher sensitivity [68]. In contrast to what was reported by Karlsson, Kohler reported a different order of cation size for sensitivity improvement when applying 1 nmol/μL of LiCl, NaCl, KCl, RbCl, CsCl to analysis four oligosaccharides [69]. Although the difference between the coordination affinity of the four investigated oligosaccharides for lithium and sodium is small, the affinity dropped sharply with an increase of the alkali metals ionic radii. Compared with $[M + H]^+$, the $[M + Li]^+$ demonstrated the highest ionization efficiency which was approximately 70-fold higher than that of the $[M + H]^+$ ion. Although $[M + Li]^+$ and $[M + K]^+$ adduct ions could be formed, it was difficult to displace ubiquitous Na^+ by adding the metal ions Li^+, or K^+. Sensitivity of detection therefore was not improved due

to the formation of mixture adducts of $[M + Na]^+$ and other $[M + Me]^+$. Marwah found that Ag^+ could suppress the formation of other metal adduct ions and formed an intense Ag adduct with the analyte androst-5-ene-3β,17β-diol [56]. Because higher voltage was needed to form fragment ions, the baseline noise was decreased and resulted in further improvement of detection. More than a 10-fold increase in response was obtained using post-column infusion of silver nitrate at 1–100 nM.

Other metal adduct ions have also been found to be useful to improve sensitivity of detection. Ag^+, Cu^+, Ni^{2+}, Pd^{2+}, Pt, all metals of the first and eighth transition groups have been investigated by Bayer et al. [70]. They proposed a technique they referred to as coordination-ionspray mass spectrometry which ionizes the analytes as charged complexes by introducing a suitable central atom to the analytes. Some unsaturated compounds which could not be detected or only with poor sensitivity, formed highly stable π or π-allyl complexes with the introduced central atom such as Ag, Pd and other metal atoms. In Bayer's work, the formation of Ag^+ complexes were demonstrated for 38 compounds, which included steroids, vitamins of the D and E families, carotinoids, polystyrenes, terpenes, and unsaturated fatty acids. This method can also be employed in the negative ion mode to form adducts such as $[M + BF_3–H]^-$.

3.2.2. Alkylamine adduct ions

Although formation of adduct ions were found useful for sensitive detection as discussed above, adduct formation can make sensitive and reproducible quantitation difficult as well. Molecular ions of analytes $[M + H]^+$ or $[M + Na]^+$ are commonly used as precursor ions when employing tandem mass spectrometry detection; however, the formation of multiple molecular ions such as $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ and $[M + NH_4]^+$ may disperse the detected signal and decrease the sensitivity and reproducibility of detection [71,72]. Ammonium acetate, ammonium formate and ammonium hydroxide can suppress the formation of $[M + Na]^+$, $[M + K]^+$ ions by promoting the formation of $[M + H]^+$ [72] or $[M + NH_4]^+$ [73]. Recently, the successful suppression of formation of multiple molecular ions was achieved by formation of $[M + alkylamine + H]^+$ adduct ions. Stephenson et al. first introduced the use of alkylamines to suppress the formation of multimers under ESI conditions through the formation of predominant molecular amine adduct ions [74]. Among a variety of amines which were investigated, primary amines showed the strongest multimer suppression effect and led to an improvement of detection sensitivity. Zhao et al. [75] employed 1 mM (pH 4.5) methylammonium acetate as mobile phase additives to suppress the formation of multiple molecular ions when developing sensitive quantification methods for simvastatin. The simplified mass spectrum resulted in a higher sensitivity of detection. 1-Hexylamine with 0.05 mM increased the response of the analytes five-fold in comparison to that without amine addition [76]. Mortier et al. investigated the effect of different mobile phase additives on adduct formation such as $[M + Na]^+$, $[M + H]^+$, $[M + K]^+$ and [M + primary amine]$^+$ for paclitaxel [77]. They completed a thorough comparison of different mobile phase additives including acetic acid, formic acid, ammonium formate and a range of primary amines. One major molecular ion could be obtained with either dodecylamine/acetic acid (0.1 mM/0.1 mM) or acetic acid/sodium acetate (1.7 mM/20 μM). Gao et al. studied competition between the formation of $[M + Na]^+$ and $[M + alkylamine + H]^+$ including their binding sites and established a quantitation method for paclitaxel with LOD at 0.5 ng/mL [78].

3.2.3. Carboxylate adducts

$[M + RCOO]^-$ ions have been observed for corticosteroids in both ESI and APCI detection [72,79,80]. Triamcinolone acetonide, budesonide, beclomethasone dipropionate, its metabolites beclomethasone-17-propionate and beclomethasone, formed abundant carboxylate adduct ions, whereas there was no apparent $[M–H]^-$ ion formed in the negative detection mode. Unfortunately, not all negative adduct ions produced intense product ions other than the $[M–H]^-$ ion [72]. The transition from $[M + RCOO]^-$ to $[M–H]^-$ is not favored for the selected reaction monitoring (SRM) detection mode, because the selectivity of detection will not be increased during the second MS stage. The high background accompanying the low selectivity of detection made it impractical for biological samples. This method may be limited to corticosteroid compounds with a 17α-hydroxyl group such as betamethasone, desoxycortisone, fludrocortisone, prednisone, triamcinolone and so on [81,82]. An intense fragment ion $[M–H–CH_2O]^-$ could be produced in these compounds by cleavage of the $C_{20–21}$ bond. Sensitive quantitation has been established for budesonide in plasma samples with a limit of quantitation reported as 15 pM after addition of 0.2% acetic acid into the mobile phase [80]. Savu et al. achieved limits of detection for 10 corticosteroids at 50 pg/ml to 1.0 ng/mL in urine using ammonium acetate 0.1 M/acetaonitrile (60/40, v/v) as the mobile phase additive [83]. In Kim's study of corticosteroids using theromospray ionization in the negative mode, the abundance of the $[M + RCOO]^-$ adduct ion for five organic acids (R-COOH with R=H–, CH_3CH_2–, $HOCH_2$–, or $HSCH_2$–, and $HFCH_2$–) was found to be increased when the pK_a value of the acid decreased [84]. Antignac et al. evaluated the effect of formic acid and acetic acid on the detection of dexamethasone in the ESI negative detection mode. $[M + acetate]^-$ was observed to be more favorable than $[M + formate]^-$ [82]. The sensitivity of detection increased with an increase in the concentration of acetic acid, and then was constant from 0.01% to 0.5%. When the concentration was more than 1%, the sensitivity of detection started to drop. No further explanation of this interaction, such as the mechanism of interaction or the possible binding sites was offered. Kim however, hypothesized that only the 17α-hydroxyl group could form hydrogen bond with the functional group (–OH or –SH) of the organic acid.

3.2.4. Halide adducts

Although most of the reported methods using halide adducts have been for sugars and nitrate esters in the APCI detection mode, halide adducts have also been used in the ESI mode for lipid analysis [85]. Target compounds are generally neutral molecules having some polar functional group, such as a hydroxyl or carboxyl group. Halide adduct ions are formed through a hydrogen bonding interaction between the analyte and the halide ion [59,86]. Bromoform, dichloromethane, chloroform, carbon tetrachloride and ammonium chloride are commonly used additives in forming halide adducts [87,88]. After post-column addition of 1% chloroform in methanol, the ionization efficiency of polyols was improved and $[M + Cl]^-$ ions were detected as base peaks. The limit of quantitation for polyols in serum was 0.2 μg/mL using the selected ion monitoring (SIM) mode [89]. Bromine adduct ions demonstrated more sensitivity than chlorine adducts for β-lactam antibiotics when 1% (v/v) of bromoform or chloroform was added to mobile phase [87]. Among 13 cephalosporins, nine compounds could be detected as bromine adduct ions whereas only four compounds could be detected as chlorine adducts. The compounds which could not form $[M + Br]^-$ and $[M + Cl]^-$ ions possessed negative groups within their chemical structures. In comparison with the $[M–H]^-$, $[M + Br]^-$ was demonstrated an increase of up to 23.74-fold for carbenicillin. The $[M + Cl]^-$ ion however, was increased only 2.07-fold. $[M + Br]^-$ ions were more stable and sensitive than the $[M + Cl]^-$ which may have been due to their higher electronegativity. Another advantage of the formation of halide adducts is that it is easier to recognize the adduct ions due to their isotopic ratios (1:1 for bromine and 3:1 for chlorine adducts).

The formation of chloride adducts also has been successfully applied to lipid analysis in a lipidomics study. Han and Cheng used 1:1 chloroform/methanol to analyze cerebrosides, which could form the $[M + Cl]^-$ adduct. The quantification range for a mixture of cerebrosides was established as 1.0–1000 mM [90].

3.3. Ion-pairing reagents and post-column addition

The majority of separations of small polar compounds utilized reverse-phase (RP) packing materials, although these analytes generally lack sufficient retention on RP columns. Interfering peaks or matrix suppression may make sensitive detection of analytes difficult [43]. In order to increase the retention of small polar compounds on octadecyl (C_{18}) packing materials, derivatization procedures or the addition of ion-pairing reagents may be required. Extensive use of LC/MS for a wide variety of pharmaceutical separations dictates the use of volatile ion-pairing reagents for the separations of small polar molecules. Most of the applications of volatile ion-pairing reagents with LC/MS have been limited to biomolecules such as oligonucleotides and amino acids. Triethylamine, tri-*n*-butylamine, and tetrabutylammonium acetate are the most commonly used ion-pairing reagents for native or chemically modified oligonucleotides and other anions in the negative detection mode [91–93]. The concentrations of amine additives are generally several mM in the mobile phase. Perfluorinated carboxylic acids provide good ion-pairing reagents for cations in LC/MS analysis. Petritis et al. investigated the effect on retention and selectivity of different perfluorinated carboxylic acids at 0.25–2 mM for the analysis of underivatized small peptides [94,95]. Although good retention and separation of polar compounds was obtained, ion suppression was observed in some cases [96]. Since a strong ion-pair formed between analytes and the perfluorinated carboxylic acids such as TFA, poor dissociation of the ion-pair may decrease the formation of protonated sample cations and may reduce their ionization efficiencies. Post-column infusion of a weaker acid could be used to solve this problem, which has been referred to as the “TFA Fix” [96]. Post column addition of a mixture of propionic acid–2-propanol, 75:25, v/v overcame the ion suppression of TFA and heptafluorobutyric acid, resulting in a 10–100-fold improvement in the sensitivity of detection. Another very useful practical solution related to the a “TFA Fix” is to add 0.5% acetic acid or 1% propionic acid directly into the mobile phases containing either 0.025% or 0.05% TFA. The sensitivity of eight basic compounds have been found to be increased 2–5-fold using this approach. Since this approach does not involve a post-column infusion, it is desirable for high throughput samples analysis [97].

The major advantage of post-column infusion is that ionization conditions for the analytes could be optimized without changing the chromatographic separation. The application of post-column infusion has involved (1) pH adjustment; (2) improvement of ion-suppression (e.g. the TFA Fix) and (3) improvement of ionization efficiency through the formation of an adduct ion, or improving nebulization and desolvation. In fact, the adduct ions discussed above ($[M + Me]^+$ and $[M + AC]^-$, $[M + Cl]^-$) could be formed through post-column addition.

Separations for acidic compounds may be achieved with an acidic mobile phase, but poor MS detection sensitivity may be observed. Post-column addition of a basic solution such as ammonium hydroxide, dimethylamine or trimethylamine could raise the solution phase pH and improve the sensitivity. This post-column addition has successfully been applied to analysis of acidic endocrine-disrupting compounds [98]. The signal to noise ratio increased more than 200-fold for 4-*tert*-butylphenol when using 1,8-diazabicyclo-(5,4,0)undec-7-en in methanol as a post-column addition solution.

Ibuprofen demonstrated poor ESI response when using 50 mM ammonium acetate/acetic acid buffer (pH 4.4) as the mobile phase. The poor ionization efficiency may have been due to both the high surface tension of the mobile phase and the ion-suppression effect of the acetate anion in the mobile phase. Post column addition of 2-(2-methoxyethoxy)ethanol (50 μL/min) increased the signal of analyte approximately 100-fold [99] and dramatically improved the ESI responses of all urinary metabolites as well. This method was also used

to enhance the signal of rat urinary and biliary metabolites for a novel antirheumatic drug esonarimod [100]. Isopropanol is also one of the best post-column infusion solvents to improve nebulization, desolvation and the ion evaporation process.

Postcolumn infusion can also be used to improve the detection of organoselenium compounds by reducing fragmentation. Generally, small fragile low-mass (<250 Da) organoselenium compounds are prone to fragmentation even at the mildest in-source collision-induced dissociation (CID) conditions. The response of the molecular ion can therefore be low and the sensitivity of detection poor. Post column addition of a polyether, 18-crown-6(at 30 μL/min), can overcame this extensive fragmentation. Shou applied this approach to analysis of 4-hydroxyphenyl 2-methyl-2-aminoethyl selenide in human urine with a limit of detection about 5 pg/μL [101]. This method can be potentially applied to other amine-containing compounds.

4. Conclusion

LC/API/MS has proved to be a powerful research tool due to its sensitivity, high selectivity, and high throughput efficiency. Derivatization techniques to improve the detectability for LC/API/MS have been successfully used in the analysis of biomacro-molecules such as peptides, and are becoming practical methods to overcome poor detectability with small molecules. The overall goal of derivatization is the introduction of permanently charged moieties or readily ionized moieties into the analyte for the ESI detection mode, and introduction of high proton affinity or high electron affinity moieties for the APCI detection mode. Derivatization has also been used for the introduction of hydrophobic moieties into small polar compounds to decrease background noise and increase the ionization efficiency. Derivatization methods applied to steroid analysis have drawn the most attention because of the challenges involved in method development. Electron-capture negative APCI/MS and dansylation both have produced impressive sensitivity of detection for steroids. Although detection-oriented derivatization in LC/API/MS is a somewhat minor endeavor at present, it is expected to be more important in the future as the demand for ultra sensitive analysis increases. Many of the derivatization methods that have been summarized here have been used in other analytical areas, such as PFB-Br in GC/MS and dansyl chloride in UV/fluorescence detection. Other well characterized derivatization reactions which have been developed for other analytical methods may therefore be potentially applied to API/MS as well as new reagents that may be synthesized.

The same issues involved with derivatization in other analytical areas still exist however. Time-consuming sample extraction procedures and clean-up steps after derivatization cannot be avoided in many cases. The technical steps involved with derivatizations are demanding and the purity of reagents is often critical. Non-aqueous conditions are often needed for derivatization reactions making it necessary to carefully dry solvent extracts of aqueous samples. Other pitfalls involved in derivatization for LC/MS analysis area may also be significant. Derivatives lack distinguishing product ions specific to the analyte of interest due to the ease of loss of the added derivatizing reagent moiety. Excess derivatizing reagents can cause ion suppression or interference, extra extraction and separation procedure therefore may be required. Isotopically-labeled internal standards may be required to track the analyte during the derivatization and the stability of analyte is of concern when using harsh derivatization conditions.

Compared to derivatization reactions, addition of various compounds to the mobile phase is a more convenient way to improve detection in LC/API/MS. pH adjustment of the mobile phase has been a routine technique to optimize detection conditions. Although $[M + H]^+$ and $[M + Na]^+$ are still the most useful ions, formation of adduct ions in the positive or negative detection mode has become an important approach to improvement of sensitivity. The majority of applications for involving adduct formation have been focused on qualitative studies and semi-quantitative applications have been limited to a few compounds possessing multiple oxygen atoms. More extensive application of mobile phase additives is expected since the processes involved are closely related to ion formation in the solution and gas phases and a better understanding of the ionization process in API will lead to further developments in this area.

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