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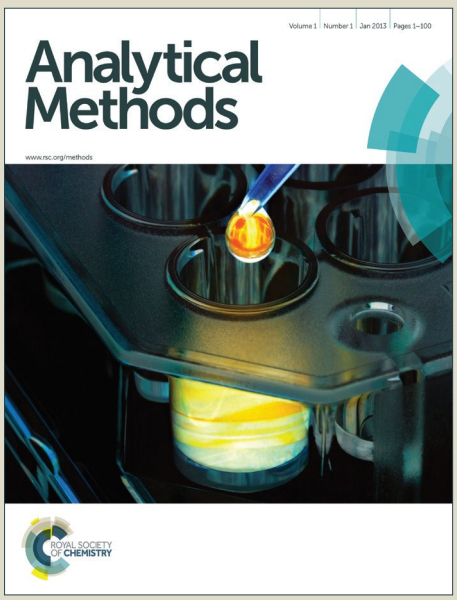

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Coupling normal phase liquid chromatography with electrospray ionization mass spectrometry: strategies and applications

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Abstract

Normal phase liquid chromatography (NPLC) is widely applied in the analysis of lipids, plant extracts, chiral molecules, and petroleum. NPLC alone is not powerful enough to handle the complexity of some samples. Electrospray ionization mass spectrometry (ESI-MS) coupled with NPLC enables characterization of complex samples. However, the mobile phases commonly used in NPLC are not compatible with ESI-MS due to their low polarity and dielectric constant. There are five pathways for coupling NPLC with ESI-MS. In limited cases, where the NPLC eluent contains significant quantities of polar solvents, the eluent can be directly introduced into the ESI-MS. More commonly, NPLC effluent fractions are collected and reconstituted off-line in an ESI-compatible solvent. Alternately, there are three emerging on-line approaches that enable direct coupling of NPLC and ESI-MS. A make-up solvent that is ESI compatible can be added through a T union or sheath liquid interface to the NPLC effluent to generate a stable ESI spray. Alternatively, analytes separated by normal phase TLC plates can be sampled into ESI-MS by ambient ionization techniques. This review discusses the five strategies for hyphenation of NPLC with ESI-MS and the application of NPLC-ESI-MS for lipids, enantiomers and fuel analysis. The comparison between different strategies for NPLC-ESI-MS is also briefly discussed.

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1. Introduction

Normal phase liquid chromatography (NPLC) and mass spectrometry (MS) are complementary to one another. MS offers greater sensitivity and specificity than conventional NPLC detectors such as ultraviolet absorbance or refractive index detection. On the other hand, NPLC can resolve isomers that cannot be differentiated by MS, as isomers have the same mass-to-charge ratio (*m/z*). Of the commonly used ionization techniques, electrospray ionization (ESI) is more amenable to characterization of polar compounds than atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI).[1, 2] ESI provides higher ionization efficiency for example for azaarenes[3] and pharmaceuticals[4] than does APCI or APPI. Furthermore, ESI generates an intact molecular ion, while APCI and APPI yield more fragment ions due to thermal decomposition.[5] For complex samples, the simpler spectrum offered by ESI is a benefit.

However, the coupling of NPLC to ESI-MS is challenging. Common NPLC mobile phases contain large amounts of low polarity solvents such as hexane or heptane, which cannot be easily sprayed to promote analyte ion formation.[6, 7] Thus, several strategies have been developed to combine NPLC with ESI-MS. **Fig. 1** summarizes the off-line and on-line methods that have been reported. In an off-line method, fractions of the NPLC effluent are collected, the ESI-MS incompatible solvents are removed by evaporation, and the analytes are re-dissolved in an ESI compatible solvent and analyzed by ESI-MS. The off-line mode has been widely used in petroleum analysis.[8-11] On-line coupling of NPLC separations with ESI-MS can be done by direct effluent introduction into the ESI interface; use of post column solvent addition or a sheath liquid interface; or

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using an ambient ionization source. Each of the strategies in **Fig. 1** and their applications are discussed in the following sections.

2. Solvents in ESI-MS

The ESI process involves multiple complex steps: formation of the Taylor cone; generation of charged droplets; shrinkage of the droplets by evaporation; and production of gas phase ions.[12] The ions are formed through four mechanisms: self-dissociation; electrochemical redox reactions caused by the high voltage; adduct formation; and gas phase proton transfer reactions.[13] Various instrumental parameters (voltage, capillary dimensions and flow rate) and solution characteristics (solvent surface tension, conductivity, dielectric constant, ionic strength and volatility) affect the stability of ESI operation.[13, 14] The formation of a stable spray and stable ESI response is dependent on the balance of these parameters. For example, when the solvent is changed from methanol (MeOH) to water, the onset voltage for Taylor cone formation and charge emission increases from 2.2 kV to 4.0 kV.[12, 15] For positive mode ESI, MeOH, acetonitrile (ACN), IPA, ethanol (EtOH) and their aqueous mixtures (organic composition > 50%) are commonly used.[13] For negative mode, halogenated solvents or MeOH/halogenated solvents provide optimal performance.[13]

With some solvent systems, formation of a stable spray is difficult. For example, to form a stable spray for pure water, which has high surface tension, corona discharge occurs due to the high voltage required.[16] Also, pure water is not suitable for ESI because its low volatility hinders shrinkage of the droplets.[17] Conversely, it is not possible to form

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a stable spray with non-polar solvents such as hexane, benzene, and toluene due to their low conductivity, surface tension, and dielectric constant.[18]

Additives in the solvent, such as formic acid, acetic acid, and ammonium hydroxide, also affect the ESI response by affecting one or more of the mechanisms of ion formation.[13, 14, 19] First, additives affect the conductivity of the solution. At a given voltage and flow rate, there is an optimum conductivity for the formation of a stable spray.[6, 15] Too low a conductivity will prevent the formation of the Taylor cone, while too high a conductivity will inhibit the generation of charged droplets.[6] Second, weakly acidic or basic additives change the solution pH. In ESI, the charged analyte ions are often formed in the solution by self-dissociation. In this case, acidic analytes are best ionized in basic solutions and basic analytes in acidic solutions.[13, 14] For instance, tetramethylammonium hydroxide has higher basicity than ammonium hydroxide, and so the addition of tetramethylammonium hydroxide to petroleum samples yields deprotonation of six times more acidic analytes.[20] Third, for polar analytes that possess no acidic or basic functionality, solution phase ions can be generated through adduct formation with additives, *e.g.*, addition of NH_4^+ can generate $[M+NH_4]^+$.[13] Last, for analytes that are neutral in solution, the additives may carry charge that is transferred to the analyte in the gas phase.[13, 14] For example, protonated valine ions have been observed in ESI-MS for valine in a basic solution adjusted using ammonium hydroxide. During the ESI process, both neutral valine analyte and NH_4^+ are present in the droplets. In the gas phase, protons transfer from NH_4^+ to valine because of the greater proton affinity of valine than NH_3.[21]

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3. Direct infusion of NPLC eluate into ESI-MS

There are several cases in the literature where NPLC eluent has been directly introduced into an ESI-MS. As discussed in **Section 2**, NPLC eluents containing predominantly nonpolar solvents such hexane or heptane are not compatible with ESI-MS because of their low polarity. However, the intensity of analyte ions by direct infusion ESI increases upon addition of polar solvents (**Section 2**). For instance, an ESI-MS signal for protonated acridine was observed only when ≥ 20% IPA was present in an IPA/hexane mixture.[22] In some NPLC separations there is sufficient polar solvent in the eluent to enable direct ESI ionization. For example, propranolol enantiomers eluted using 20/80 IPA/hexane were ionized by ESI-MS without the need to add any make-up solvent.[23] Similarly, direct coupling of NPLC with ESI-MS has been achieved without make-up solvent for drug enantiomer separations in 23/77/0.1 EtOH/hexane/trifluoroacetic acid[24] and clevudine in 68/28/10/0.02 MeOH/EtOH/hexane/trifluoroacetic acid.[25]

Alternatively a more ESI-compatible weak solvent can be used in the NPLC mobile phase. Ethoxynonafluorobutane (ENFB, a mixture of $CF_3CF_2CF_2OCH_2CH_3$ and $(CF_3)_2CFCF_2OCH_2CH_3$) offers similar NPLC separation selectivity as hexane.[26, 27] But ENFB possesses much lower flammability which would reduce any explosion hazard in ESI-MS.[26] However, higher detection limits were observed for ESI-MS than UV absorbance when NPLC separation of diphenylmethyl phenylsulfoxide and diaminocyclohexane acrylamide was performed using 10/90 EtOH/ENFB eluent.[26]

Many other papers were found in *ISI Web of Science* when searching "normal phase" in the title and "electrospray ionization " and "mass spectrometry" in the topic.

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However, the vast majority of these 84 papers used normal phase adsorbents with mobile phases that contained MeOH, ACN, chloroform and 5-25% water.[28-34] Strictly speaking, these mobile phases are better termed as hydrophilic interaction chromatography (HILIC),[35] and are beyond the scope of this review.

4. Off-line NPLC-ESI-MS

4.1. Approaches to off-line NPLC-ESI-MS

The solvent incompatibility of most NPLC mobile phases with ESI makes direct introduction of the effluent into an ESI challenging. Thus, for a long time, coupling of NPLC to ESI-MS was performed in the off-line mode. For off-line NPLC-ESI-MS, effluent fractions are typically collected based on retention time windows defined by the peaks observed with an absorbance detector.[9, 11, 36] The non-ESI compatible solvents in each fraction are evaporated down, and the analytes are redissolved in ESI compatible solvents, *e.g.*, petroleum samples were redissolved in <50% toluene in MeOH.[11, 37] MeOH promotes a stable ESI response, while toluene assists the dissolution of the petroleum fractions. The reconstituted analytes are then directly introduced into the ESI-MS. The sample reconstitution process concentrates the analytes to yield higher sensitivity, which enhances the signal intensities and makes many additional compositional features visible in crude oil samples.[38] Also, higher sensitivity has been achieved by combining fractions from 3-5 replicate runs.[9, 38] However, off-line NPLC is time consuming and may cause loss of compositional information or compositional biases. For instance, off-line NPLC identified only one third of the molecular

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compositions that were identified within the same retention time window of a petroleum separation as were identified by on-line LC-MS.[39]

Alternatively, the collected fractions can be introduced into an ESI-MS after addition of an ESI-compatible solvent, such as MeOH/H_2O or MeOH/toluene.[22] This strategy is more time effective compared to sample reconstitution, but dilutes the analytes and is more labor intensive than on-line LC-MS (**Section 5**). Our experience is that the most stable and greatest ESI response are obtained if the ESI-compatible solvent is miscible with the solvent in the fractions.[22]

Off-line reconstitution has been used with NPLC-ESI-MS for complex samples such as petroleum.[9-11, 37, 40] Crude oil contains tens of thousands of compounds, from non-polar saturates to polar nitrogen, sulfur or oxygen containing organics.[41, 42] The peak capacity of state-of-the-art 2D NPLC × reversed phase liquid chromatography (RPLC) is 300-1500.[43, 44] Even with such a technique, it is not possible, nor practical, to separate all of the individual compounds in crude oil. More commonly petroleum is isolated with fractionation or class separation, often by NPLC.[45] Nonetheless, the subfractions of petroleum are still too complex to be analyzed by low resolution MS. Thus, high resolution MS techniques such as Fourier transform ion cyclotron resonance MS (FT-ICR-MS) are used,[46, 47] most commonly with ESI. Commonly the target analytes are polar compounds such as nitrogen containing compounds and acids.[41, 42, 48]

It should be noted that there is some disagreement as to whether pre-separation is needed for petroleum analysis by FT-ICR-MS. Rodgers and co-workers claim that saturates, resins and asphaltenes do not affect the identification of polar compounds in the aromatics fraction by positive ESI.[8] Also, ESI FT-ICR-MS can identify basic polar

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nitrogen compounds in heavy gas oil without interference by other compound classes (*e.g.*, polycyclic aromatic hydrocarbons, sulfur containing compounds).[8] However, ion suppression has been observed amongst polar nitrogen containing compounds with the same functionality in ESI-MS analysis. For instance, the ESI signal intensity for pyridine decreased 10 fold in the presence of acridine.[22] Thus, a pre-separation is needed to simplify complex samples such as petroleum.[22] In addition, a much larger number of molecular classes can be identified by FT-ICR-MS after pre-separation than by direct infusion.[10, 49]

4.2. Applications of off-line NPLC-ESI-MS

Off-line NPLC-ESI-MS methods for petroleum analysis fall into two categories. First, conventional stationary phases may be used for preparative fractionation of crude oil. One such NPLC separation is SARA, which separates petroleum into Saturates (S), Aromatics (A), Resins (R) and Asphaltenes (A) fractions. SARA usually serves as the first step of petroleum purification before subsequent NPLC classification and ESI FT-ICR-MS analysis.[9, 50, 51] In SARA, Asphaltenes (A) are precipitated from crude oil using pentane, hexane or heptane. The alkane soluble supernatant is then separated on a column consisting of a clay layer atop a mixture of silica and clay. Sequential elution with hexane, toluene/acetone and finally toluene yields the Saturates (S), Aromatics (A) and Resins (R) fractions, respectively. After SARA fractionation, each fraction is evaporated down to dryness under nitrogen. Total recoveries of 92-95% have been reported.[8, 9] Redissolving the SARA fractions in 90/10 MeOH/toluene enabled analysis by ESI-MS.[2, 9, 37] Nitrogen containing compounds in the SARA fractions have been analyzed by ESI FT-ICR-MS under both positive[2, 9] and negative mode.[37] Preparative scale NPLC based

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on a silica gel open column has also been applied to resolve subfractions of various petroleum extracts.[40, 52, 53] Commonly, 6-7 subfractions were obtained from each extract by stepwise elution with anhydrous cyclohexane, dichloromethane (DCM), chloroform, toluene, MeOH and EtOH. The subfractions were reconstituted in 50/50 MeOH/toluene for ESI-MS analysis. Six known types and three new types ($C_nH_mN_4VO_2$, $C_nH_mN_4VO_3$ and $C_nH_mN_4VO_3$) of petroleum vanadyl porphyrins[40, 52, 53] were detected from the subfractions using ESI FT-ICR-MS. In addition, the fractions from preparative NPLC have been further separated by ion exchange chromatography (IEX)[54] or ligand exchange chromatography[55] for petroleum separation and characterization. For example, pre-fractionation of petroleum by NPLC-IEX increased the ESI-FT-ICR-MS signal-to-noise ratio for nickel porphyrins about 100 fold in Ref. 54. Analytical scale NPLC has also been used for class type separation of crude oil, followed by off-line ESI FT-ICR-MS of the fractions collected.[9, 11, 36] For instance, charge transfer phases such as 3-(2,4-dinitroanilino)propyl (DNAP) separate polycyclic aromatic hydrocarbons based on their aromatic ring number[56] and also can separate polycyclic aromatic hydrocarbons from polar compounds.[56, 57] **Fig. 2** shows four fractions that were collected for treated and untreated heavy gas oil using DNAP with a CH_2Cl_2/hexane eluent.[11] The fractions were re-concentrated in 75/25 MeOH/toluene for characterization of nitrogen containing compounds by ESI FT-ICR-MS.[11] Alternately, alumina with a MeOH/chloroform eluent has been used to generate a nitrogen enriched petroleum fraction.[58] This nitrogen fraction was further separated into neutral and basic nitrogen containing fractions on a silicic acid column using a toluene or toluene/diethyl ether mobile phase. MeOH was added to the final fractions, without solvent evaporation, prior to ESI.[58]

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Off-line NPLC-ESI-MS has also been applied to procyanidins analysis from apple juice extracts[59] and peanut skins.[60] Separations were performed on silica with a MeOH/ethyl acetate/hexane gradient mobile phase. Fractions were collected and rotary evaporated down and lyophilized prior to RPLC-ESI-MS analysis. High peak capacity, resolution and specificity were achieved.[59, 60] In another study, crude Ginsenoside-Ro fractions obtained by NPLC on silica were purified by high-performance counter-current chromatography with ethyl acetate/IPA which was directly analyzed by ESI-MS/MS.[61]

5. Post column solvent addition for on-line NPLC-ESI-MS

Post column addition after HPLC separations has been conventionally used to modify the eluent and enhance the detectability of various analytes.[62-64] The addition of reagent to the effluent may change the chemical properties of the analytes, *e.g.*, post column derivatization.[62, 64] Alternately, the addition of reagent may merely cause changes to the properties of the effluent. This latter role is the primary purpose of post column solvent addition for on-line NPLC-ESI-MS.[1, 65-67] **Fig. 3** shows that the post column solvent addition is usually achieved through a zero dead volume (ZDV) T union positioned after the NPLC column and prior to the ESI-MS. The NPLC eluent compromised of non-ESI compatible solvents is connected to one arm of the T union. An ESI-compatible solvent or solvent mixture, called the make-up solvent, is introduced typically at 90° to the eluent, and the mixed stream passes through a short narrow connecting tubing to be introduced into the ESI-MS inlet. This ESI process is usually assisted by a nitrogen drying gas. Addition of the make-up solvents enables formation of a more stable spray during the ionization process.

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5.1.Common NPLC eluents and make-up solvents

Common NPLC mobile phases contain large amounts (>50%) of n-alkanes such as n-hexane or heptane. Post column solvent addition of polar solvents has been used to overcome the incompatibility of the eluent with the ESI process. **Table 1** lists common make-up solvents. For a given NPLC eluent, the composition of the make-up solvent affects the degree of ESI signal enhancement.[23] For example, for propranolol in 20/80 IPA/hexane, the use of IPA as make-up solvent doubles the signal-to-noise relative to that achieved using 1/1 IPA/water and 4-times that when pure water is used.[23] The greater enhancement with IPA was attributed to better distribution of analyte in the IPA due to greater miscibility between the eluent and IPA.[23]

However, the composition of the make-up solvent has generally not been optimized nor compared. For example, MeOH,[1] IPA/MeOH,[67] and MeOH/chloroform[68] have all been used as make-up solvent for ionization of different analytes in IPA/hexane eluent (**Table 1**). Similarly, make-up solvents consisting of MeOH/chloroform have been used to promote generation of analyte ions for lipids in either methyl tert-butyl ether (MTBE)/hexane or IPA/hexane eluent.[68, 69]

Table 1 shows that the choice of make-up solvent depends on the analyte type and the NPLC eluent. However, some general conclusions can be made. First, a good make-up solvent commonly contains an alcohol (such as MeOH, EtOH or IPA). Often this alcohol is also a component of the mobile phase.[23] For example, a make-up solvent consisting of IPA enables more stable ionization than MeOH for propranolol in 20/80 IPA/hexane.[23] Second, a make-up solvent that is miscible with the NPLC mobile phase generates a higher and more stable signal-to-noise ratio.[22] Third, only in very rare cases

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does the make-up solvent consist of only solvents.[70] More commonly, a good make-up solvent contains minor additives, such as formic acid (0.1%-2% v/v),[71, 72] ammonia (0.15-2.5% v/v)[69] or ammonium acetate (NH_4Ac) (2-40 mM),[73-75] as was discussed in **Section 2**. The type of additive has not usually been optimized, but can have profound effects. For instance, for felodipine determination by NPLC-ESI-MS, ion intensity increased six fold upon addition of NH_4Ac *vs.* that with formic acid.[76] This is because the ionization process is through adduct-formation with NH_4^+ rather than direct protonation.[76] Hence, NH_4Ac appears more frequently in **Table 1** than formic acid.

Table 1 also lists the relative flow rate between the sample and the make-up solvent. Both higher[69, 74] and lower[71-73, 75] flow rates of make-up solvent *vs*. NPLC eluent has been applied to real sample analysis, but it is not apparent that these flows were optimized. In the few cases where the effect of the relative flow rate on ionization efficiency was studied, an optimum flow rate of make-up solvent relative to the effluent flow was observed.[22, 70, 77] For example, 2-10 times higher signal intensity was obtained for jasmonic acid in 5/95 IPA/hexane when the ratio of eluent flow rate to make-up solvent (IPA with 0.1% ammonia) was 1:1.[58] Similarly, optimal signal intensity for progesterone in pure hexane was achieved with addition of 49/49/2 MeOH/H_2O/HOAc at a relative flow rate of 1:1.[22] Finally, for Prostaglandin $F_{1\alpha}$ in 0.3 mL/min 3/97 IPA/hexane, a study of 0 to 0.4 mL/min of 3/2 IPA/H_2O make-up solvent observed optimal ionization at 0.2 mL/min make-up solvent.[70]

Overall, if the make-up solvent flow is too low, there will not be enough solvent to solubilize the non-polar component of the NPLC eluent and a low signal-to-noise results presumably due to less stable ionization.[22] Too high of a make-up solvent flow

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dilutes the effluent and at extreme make-up flows will cause instability in the ESI spray.[13, 77]

5.2. Applications of NPLC-ESI-MS using post column solvent addition

On-line NPLC-ESI-MS using post column solvent addition has been successfully applied to lipids analysis,[69, 78, 79] enantiomer differentiation for drugs,[80] petroleum characterization,[1] and detection of reaction intermediates.[67]

5.2.1 Lipids

Lipids are a diverse group of natural occurring small molecules including fatty acids (FA), glycerolipids, glycerophospholipids, sterol lipids, phenol lipids, saccharolipids and polyketides that exist in various concentration ranges in cells, tissues, and biofluids. Lipids are usually hydrophobic or amphiphilic molecules which constitute the membrane, store energy and are involved in cell signaling.[81] Typically LC-MS is used for lipids analysis.[82, 83] RPLC separates based on the hydrophobicity of the FA chain. In contrast, NPLC resolves lipids into classes based on the polarity of the function group.[29, 84] For instance, silica with a MTBE gradient in hexane separated neutral lipids into cholesterol esters (CE), monoalkylether diacylglycerols (MeDAG), diacylglycerols (DAGs), and triacylglycerols (TAGs) within 30 minutes.[81] Post column addition of 10 mM NH_4OH in 45/45/5/5 IPA/ACN/DCM/H_2O facilitated the formation of ammonium ion adducts with the neutral lipids $[M+NH_4]^+$ which enabled ESI-MS analysis. Within each class, some individual molecular species were resolved, identified and quantified, especially within the cholesterol esters. The ionization enhancement achieved by make-up solvent addition enabled identification of a previously unknown neutral lipid, ubiquinone-9, in cells.[81]

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NPLC-ESI-MS and MS/MS has also been applied for regioisomeric[69, 78, 79] and diastereomeric[68] analysis of triacylglycerols (TAGs). The regioisomerism of TAGs affects their physico-chemical and nutritional properties. Regioisomers of short-[69] medium- or long-chain TAGs in butterfat were separated on a series of two or three silica columns with a MTBE/hexane gradient.[69, 78, 79] Addition of 20/10/3 chloroform/MeOH/(25% ammonia in water) after the NPLC separation yielded strong ammonium adduct ions $[M+NH_4]^+$. Based on the relative intensity of adduct ions, the position of the FA chain in TAGs could be identified.[78] Diastereomers of TAGs can also be resolved on silica with 0.37/99.63 IPA/hexane and analyzed by ESI-MS through post column solvent addition of 24.5/75/0.5 MeOH/chloroform/(30% ammonia in water).[68]

NPLC-ESI-MS has also been used to separate and detect intermediates and products of the transformation of lipids.[67] For example, the ozonolysis of model lipids[67] was monitored using NPLC on silica with an IPA/hexane gradient elution. Addition of a make-up solvent consisting of 40 mM NH_4Ac in 1/3 IPA/MeOH charged the secondary ozonide by formation of adducts with NH_4^+ in ESI-MS. Another example was the separation and negative ESI detection of keto fatty acids, epoxyalcohols, and allylic epoxyalcohols derived from linoleic and α-linolenic acids.[70] Better separation of epoxyalcohols was observed with silica using NPLC than with RPLC. Post column solvent addition of 3/2 IPA/H_2O provided a stable spray for MS and MS/MS analysis.

5.2.2 Enantiomer separation and impurities mapping in pharmaceutical process

Differentiation and quantification of individual enantiomers for drug or drug metabolites is essential during drug discovery and development. Most enantiomeric separations are performed with NPLC-like eluents.[85, 86] Post column solvent addition is

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the most common method used to connect chiral separations with ESI-MS.[80] **Table 2** summarizes the mobile phases and make up solvents used in chiral-ESI-MS. The typical NPLC-like mobile phase for chiral separation consists of an alcohol (*e.g.*, EtOH or IPA) and an alkane (*e.g.*, hexane or heptane). The make-up solvent is generally NH_4Ac in the same alcohol as in the mobile phase. The final two rows in **Table 2** are examples of detection of impurities during pharmaceutical activities. Particularly, the impurities detected in ABT-578 are reactive in aqueous environments. A non-aqueous separation like in NPLC is required to identify these impurities.[75] With THF/hexane gradient, aqueous reactive impurities were separated from ABT-578 on silica column. With post column addition of 10 mM ammonium acetate in MeOH/ACN to the effluent, impurities were identified using negative ESI through the formation of the adduct ion $[M-H+CH_3COOH]^-$. Similarly, the de-fluorinated analogue of Casopitant mesylate (impurity) was separated from Casopitant mesylate on a Chiralpak AD-H column with 95/5 EtOH/hexane.[72] But the sensitivity of UV detector is insufficient to meet the regulated value, which demands the use of ESI-MS. Addition of 0.1% formic acid in MeOH overcomes the incompatibility between NPLC solvent and ESI-MS and enables detection of the impurity.

5.2.3 Petroleum characterization

As stated in **Section 4,** coupling of NPLC with ESI-MS for petroleum analysis is mostly performed in the off-line mode. Nonetheless, there are examples where petroleum or petroleum subclasses have been analyzed by NPLC-ESI-MS with on-line post column solvent addition.

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High resolution ESI-FT-ICR-MS enables greater understanding of petroleum.[46, 47] On-line NPLC-FT-ICR-MS provides faster, better structure separation, and more molecular composition identifications than off-line NPLC-FT-ICR-MS.[39] An extra challenge to on-line NPLC with FT-ICR-MS is the balance between the time available across an NPLC peak and the scanning time required for sensitive detection.[87] For NPLC-ESI-FT-ICR-MS analysis of petroleum, the crude oil was baseline resolved into two peaks on an aminocyano-bonded silica column with a gradient of IPA/hexane mobile phase. Addition of formic acid in IPA after elution promoted generation of protonated analytes ions $[M+H]^+$ in positive ESI, but the ESI-MS trace is very noisy. More nitrogen containing compounds in crude oil were detected with ESI than with other ionization techniques (APCI and APPI).[1] On-line NPLC-ESI-MS also allowed two-dimensional analysis of the nitrogen containing isomers. The two peaks with the empirical formula $C_{29}H_{43}N$ could be identified as alkylated carbazoles and polar quinoline or carbazole derivatives based on the retention time of standard compounds.[1]

Low resolution ESI-MS has also been used for analysis of simplified petroleum analogs. Nine azaarenes were baseline resolved on a 3-(2,4-dinitroanilino)propyl (DNAP) column and coupled to ESI-MS through post column solvent addition.[88] The protonation of the azaarenes in ESI was enhanced by addition of 98/2 IPA/HOAc to the 20/80 IPA/hexane eluent. Ten times lower detection limits than UV detection were achieved.

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6. Sheath liquid interface for on-line NPLC-ESI-MS

A sheath liquid interface is another strategy for on-line NPLC-ESI-MS. The sheath liquid interface was originally developed for post column derivatization of carbohydrates for ESI-MS,[89] and has subsequently been widely used for capillary electrophoresis-ESI-MS.[90-92] The sheath liquid interface uses a triaxial flow scheme (**Fig. 3**) to mix the separation eluent with a sheath liquid at the tip of the ESI capillary. The effluent from the separation is introduced into a capillary situated in the center of a metal tube (red tube in **Fig. 3**). An appropriate sheath liquid, also called the make-up liquid is added via a syringe pump through the metal tube. N_2 gas is delivered through a third concentric outer tube to assist ionization. The sheath liquid forms an external layer around the separation eluent during spray formation.[93] Analytes in an effluent consisting of a solvent that is incompatible with ESI-MS are ionized in the outer layer of the spray.[93, 94]

The sheath liquid usually consists of ESI-compatible solvents (mainly MeOH) with minor additives (*e.g.*, 3-60 mM NH_4Ac). In the absence of a sheath liquid or with a sheath liquid without additives, there is no signal. The signal intensity increases significantly with the addition of any amount of NH_4Ac to the sheath liquid.[93] The signal intensity is also increases with sheath flow rate to an optimum, and then decreases with further increases in the sheath flow,[93, 94] presumably due to dilution effects. Thus, the dependence of ionization efficiency on sheath flow is similar to that in post column solvent addition (**Section 5**).

However, some differences exist between post column (**Section 5**) and sheath liquid solvent addition for on-line NPLC-ESI-MS. First, in post column solvent addition,

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the use of a make-up solvent that is miscible (*i.e.,* IPA) with the IPA/hexane NPLC eluent enhanced ionization more than using an immiscible make-up solvent (*i.e.*, MeOH/H_2O).[22] The opposite dependence has been observed with the sheath liquid interface, where an immiscible solvent (*i.e.*, MeOH) was the optimal sheath liquid for the 3/97 IPA/isooctane eluent.[93] Second, in a direct comparison (**Fig. 4**) post column solvent addition generated higher ionization efficiency for inophyllum P in IPA/isooctane than a sheath liquid interface when using 60 mM NH_4Ac in MeOH as the make-up solvent.[93] However, the signal from post column solvent addition (**Fig. 4(b)**) is much noisier than that from the sheath liquid interface (**Fig. 4(a)**). This noise is attributed to an instable spray caused by a poor mixing between the make-up solvent and NPLC eluent.[93]

The lower ionization enhancement is probably why the sheath liquid interface has not been widely used for on-line NPLC-ESI-MS. The sheath liquid interface has been applied for neoflavonoids[93] and amitrole[94] NPLC analysis. Optimum ionization of neoflavonoids separated on silica with a IPA/isooctane gradient was observed with a sheath liquid of 60 mM ammonium acetate in MeOH.[93] Optimal analysis of amitrole was observed using an amine column with 60/40 EtOH/hexane and a sheath liquid of 3 mM NH_4Ac in MeOH.[94]

7. Ambient ionization (AI) for non-ESI-MS compatible eluents

As shown in **Fig. 1**, another means to couple on-line ESI-MS with non-ESI compatible NPLC eluents are the ambient ionization (AI) techniques.[95] AI are a group of ionization techniques that require little or minimum sample preparation. AI methods usually consist of: a desorption step; followed by a post-ionization step. The desorption

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can be by nebulization, laser desorption, thermal evaporation, or direct desorption. Common post-ionization methods are ESI and APCI.[95, 96] Various ESI-based AI techniques have been developed over the past decade.[95]

7.1. Background on ambient ionization methods

Desorption electrospray ionization (DESI) was the first and the most popular AI technique.[97] In DESI (**Fig. 5(a)**), charged solvents emitted from an electrospray nebulizer impinge on the sample surface or on samples that are deposited on an insulating surface.[97, 98] Direct desorption and ionization occurs simultaneously, and the generated ions are introduced into an MS analyzer. DESI is most suitable for direct analysis of solid or liquid samples, particularly for samples on flat surfaces[99, 100] or biological tissue imaging.[101] Two other techniques similar to DESI isolate the desorption step from the ionization step. Electrospray-assisted laser desorption ionization (ELDI)[102] uses UV- or IR-laser irradiation to desorb samples deposited on flat surfaces.[102] Laser-induced acoustic desorption (LIAD)[103] focuses a laser pulse at the back of the surface onto which the sample was deposited. The resultant acoustic and shock waves cause sample desorption.[103] The desorbed samples from both techniques then enters the electrospray solvent produced by a nebulizer positioned above the sample surface.[102, 103] The analytes are ionized in the electrospray solvent and analyzed by the mass spectrometer.

Solvent-assisted electrospray ionization (SAESI, **Fig. 5(b)**) is a recent ambient ionization technique that utilizes two electrospray nebulizers that are touching.[104] The intimacy of the electrosprays allows immediate contact of the charged solvent droplet and the sample.[104] Continuous flow-extractive desorption electrospray ionization (CF-EDESI, **Fig. 5(c)**) is a comparable technique which was recently reported by Schug and

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coworkers.[105] Instead of using a sprayer to nebulize the samples as in SAESI, CF-EDESI utilizes a needle to provide a continuous flow of the sample. In CF-EDESI there is some distance between the tips of the sprayer and the needle. Charged solvent (MeOH/water mixture) droplets produced by an electrosprayer assists desorption and extraction of analytes in the continuous flow of sample. **Fig. 5(c)** is the original scheme of CF-EDESI, based on an on-axis ESI-MS system. A modified CF-EDESI source based on an off-axis ESI-MS instrument has recently been reported.[22]

All of the above ESI-based ambient ionization techniques have been reported for ionization of samples in non-ESI compatible solvents, either in normal phase thin layer chromatography (TLC)[106, 107] or with NPLC-ESI-MS.[22, 23, 108]

7.2. Online normal phase TLC-ESI-MS with ambient ionization

AI methods have been predominantly used with thin layer chromatography (TLC). Thin layer chromatography is a simple, fast, inexpensive and low solvent consumption separation and purification technique for chemical and biological samples. TLC also allows analysis of multiple samples simultaneously.[106] Resolved bands on TLC plates are usually visualized using optical or spectroscopic methods. Ambient ionization techniques such as DESI, ELDI and LIAD allow direct sample analysis on planar surfaces, and so serve as ideal interfaces for TLC with MS. A comprehensive discussion on combining TLC with MS is beyond the scope of this review, and but can be found in Ref. 107. Here we focus on on-line normal phase TLC-ESI-MS through ambient ionization.

Silica gel pre-coated plates developed with normal phase solvents have been used for the separation of *Salvinorin* species in *Salvia divinorum* leaves[109] and for small

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molecules in *Excedrin tablets*.[110] Direct and rapid scanning of the separated bands using 3/1 MTBE/hexane or 99/1 ethyl acetate/HOAc by DESI-MS using 80/20 ACN/H_2O or pure MeOH resulted in comparable resolution and quantification to that by optical detection. The resolution and sensitivity are highly dependent on the spray to tip distance, solvent flow rate and scanning rate.[111] On-line TLC-ESI-MS has also been observed with ELDI[102] using 1/1 MeOH/H_2O for characterization of drug extracts on a silica gel TLC plate developed using 98/1/1 ethyl acetate/DCM/HOAc. Similarly, LIAD using 1/1 MeOH/H_2O was applied for separation of rosemary essential oils on a silica gel TLC plate using 9/1 ethyl acetate/toluene.[103] The various ambient ionizations expand ESI-MS's application for normal phase TLC.

7.3. On-line NPLC-ESI-MS with SAESI or CF-EDESI

SAESI (**Fig 5(b)**) has been used for on-line chiral-NPLC-ESI-MS analysis of benzoin[104] and propranolol.[23] Enantiomers in the IPA/hexane effluent were ionized with high efficiency using an immiscible ESI compatible electrospray solvent.[23, 104] **Fig. 6** indicates that SAESI-MS using electrospray solvent 0.1% formic acid in water provided 200-400 fold higher response than direct NPLC-ESI-MS.[23] This SAESI-MS technique also provided higher specificity than direct NPLC-ESI-MS and comparable sensitivity to post column solvent addition ESI for propranolol enantiomers in 20/80 IPA/hexane.[23]

CF-EDESI (**Fig. 5(c)**) also improves ionization for analytes in non-ESI compatible solvents.[22, 108] For direct infusion of progesterone in hexane, CF-EDESI with 49/49/2 MeOH/H_2O/HOAc as the electrospray solvent generated the highest ion intensity[108] comparing to 98/2 MeOH/HOAc or 98/2 H_2O/HOAc. The optimized electrospray solvent to eluent flow was 1/1.[22] On-line NPLC-ESI-MS through CF-EDESI

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has been used for chiral separations and identification of amine-containing compounds.[112] Limits of detection (0.02-0.1 ng/mL) were comparable to UV absorbance. Off-axis CF-EDESI with NPLC of azaarenes exhibited an irregular response which made analysis semi-quantitative for low molecular weight azaarenes such as quinoline and isoquinoline, and impossible for later eluting azaarenes such as benz(a)acridine and benz(c)acridine.[88]

8. Conclusions

Various strategies including direct hyphenation, off-line mode, post column solvent addition, sheath liquid and ambient ionization have been used for coupling NPLC with ESI-MS. Our analysis of the NPLC-ESI-MS literature provides some conclusions. First, post column solvent addition is a simpler, more time efficient and more sensitive method compared to other methods of coupling NPLC to ESI-MS. Second, there is an increasing use of ambient ionization techniques, predominantly for TLC separations. The success of these methods warrants further exploration of ambient ionization methods for NPLC separations. Standardization of the configuration of ESI-based ambient ionization sources and optimization in the performance are needed for further exploration.

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Figure Captions

Fig. 1 Strategies to couple NPLC to ESI-MS.

Fig. 2 Chromatogram of four petroleum fractions collected on the DNAP column. Reprinted with permission from Ref. 11. Copyright (2013) American Chemical Society.

Fig. 3 Scheme for sheath liquid interface for coupling of NPLC to ESI-MS.

Fig. 4 Chromatograms for 1 μg/mL inophyllum P standard (a) With sheath liquid interface (60 mM ammonium acetate in MeOH at 5 μL/min) (b) With post column solvent addition (60 mM ammonium acetate in MeOH at 20 μL/min). Reprinted with permission from Ref. 93. Copyright (2004) Wiley-VCH.

Fig. 5 Configuration for (a) DESI. Reprinted with permission from Ref. 98. Copyright (2013) Royal Society of Chemistry. (b) SAESI. Reprinted with permission from Ref. 105. Copyright (2014) American Chemical Society. and (c) CF-EDESI. Reprinted with permission from Ref. 108. Copyright (2013) Elsevier B.V.

Fig. 6 Chromatograms of 1 μg/mL R- and S- propranolol in 20/80 IPA/hexane (a) direct NPLC-ESI-MS (b) with SAESI (electrospray solvent: 0.5% formic acid in H2O) (c) with post column solvent addition (make-up solvent: 0.5% formic acid in IPA). Reproduced with permission from Ref. 23. Copyright (2004) Elsevier B.V.

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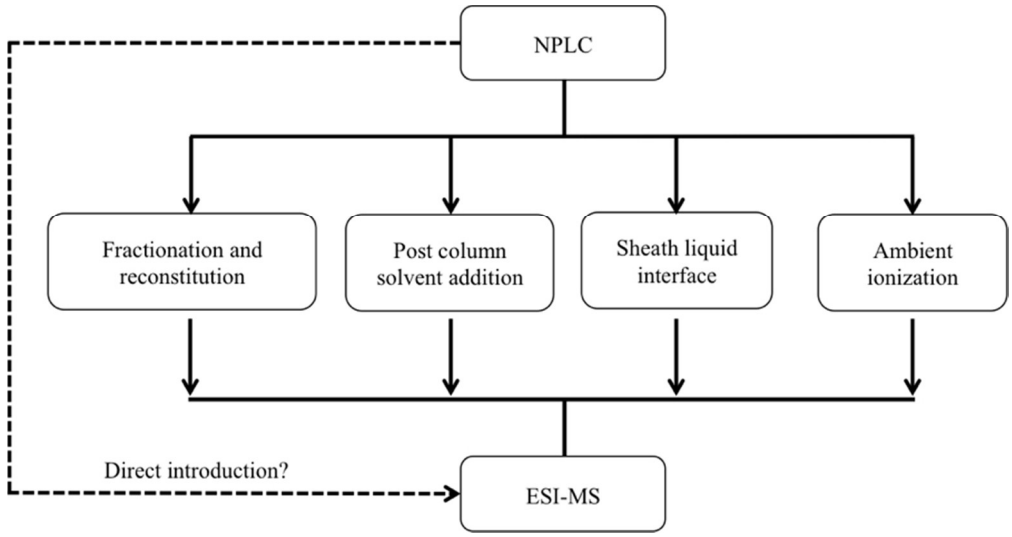

Fig. 1 Strategies to couple NPLC to ESI-MS.
152x80mm (150 x 150 DPI)

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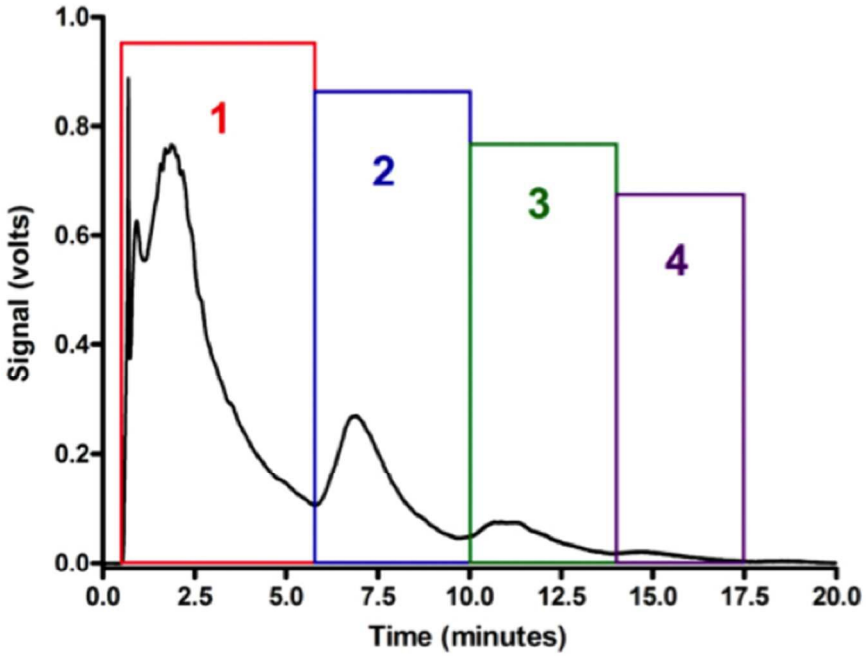

Fig. 2 Chromatogram of four petroleum fractions collected on the DNAP column. Reprinted with permission from Ref. 11. Copyright (2013) American Chemical Society.
133x90mm (150 x 150 DPI)

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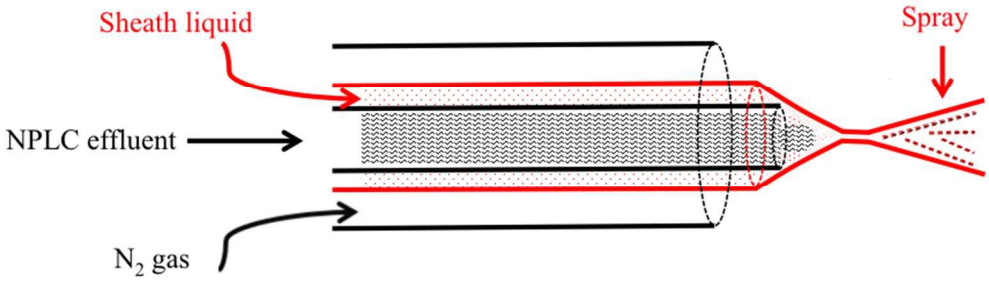

Fig. 3 Scheme for sheath liquid interface for coupling of NPLC to ESI-MS.
146x43mm (150 x 150 DPI)

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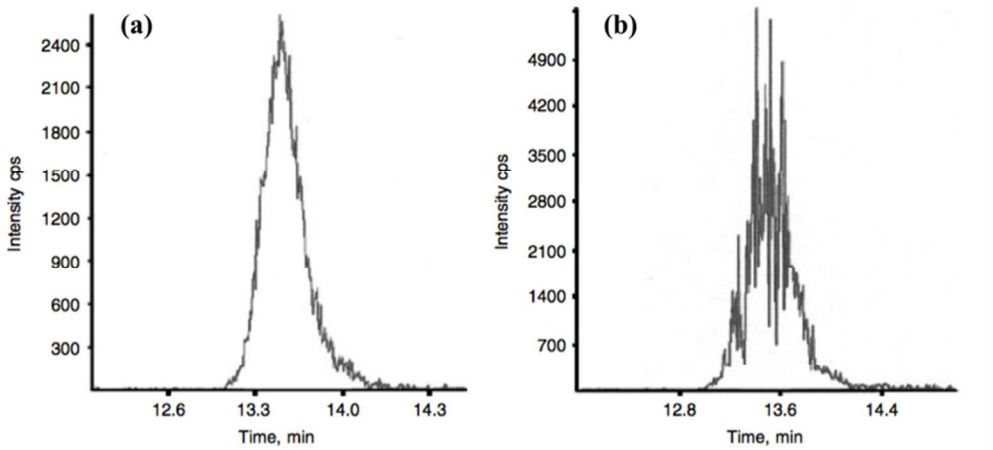

Fig. 4 Chromatograms for 1 µg/mL inophyllum P standard (a) With sheath liquid interface (60 mM ammonium acetate in MeOH at 5 µL/min) (b) With post column solvent addition (60 mM ammonium acetate in MeOH at 20 µL/min). Reprinted with permission from Ref. 93. Copyright (2004) Wiley-VCH.
152x73mm (150 x 150 DPI)

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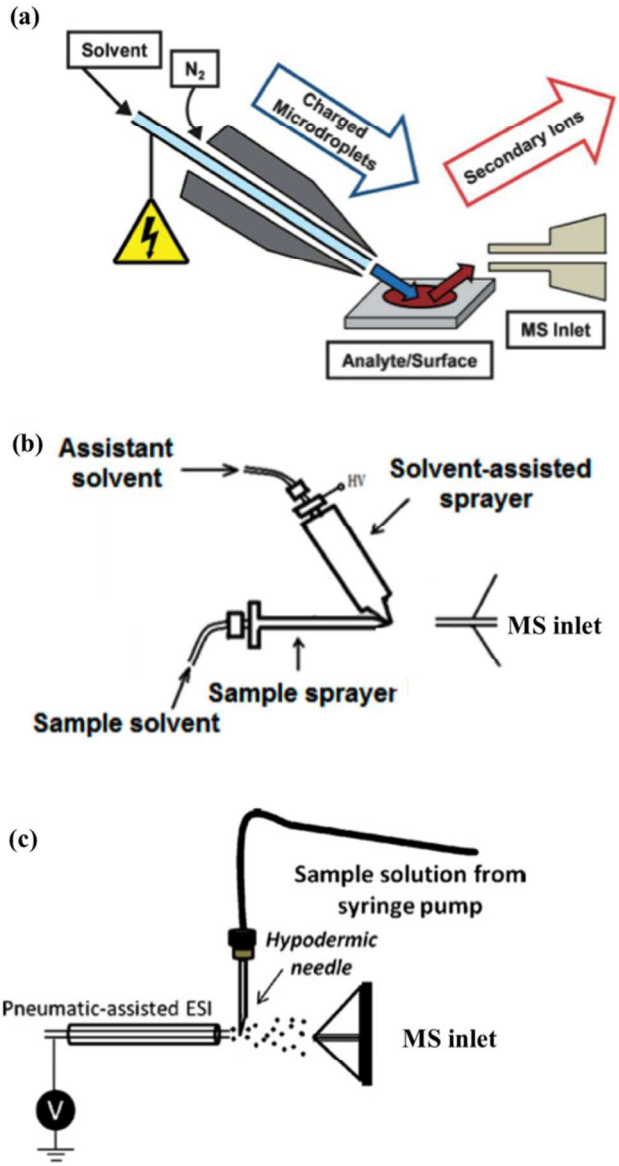

Fig. 5 Configuration for (a) DESI. Reprinted with permission from Ref. 98. Copyright (2013) Royal Society of Chemistry. (b) SAESI. Reprinted with permission from Ref. 105. Copyright (2014) American Chemical Society. and (c) CF-EDESI. Reprinted with permission from Ref. 108. Copyright (2013) Elsevier B.V.
137x206mm (150 x 150 DPI)

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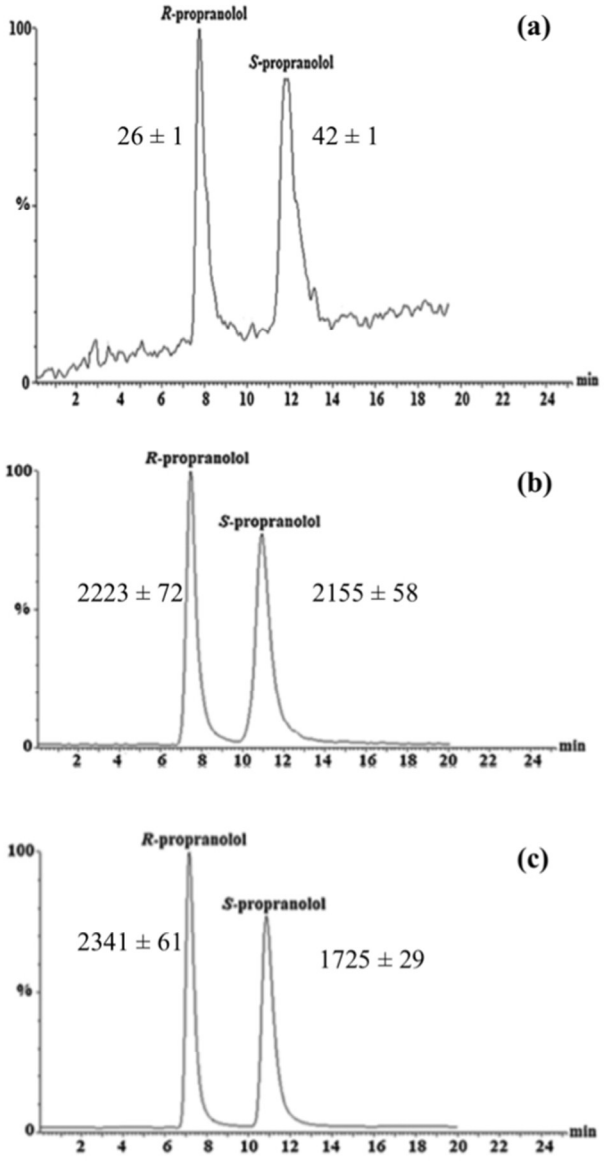

Fig. 6 Chromatograms of 1 µg/mL R- and S- propranolol in 20/80 IPA/hexane (a) direct NPLC-ESI-MS (b) with SAESI (electrospray solvent: 0.5% formic acid in H2O) (c) with post column solvent addition (make-up solvent: 0.5% formic acid in IPA). Reproduced with permission from Ref. 23. Copyright (2004) Elsevier B.V.
133x182mm (150 x 150 DPI)

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Table 1 Common make up solvents and the relative flow rate used in post column solvent addition

Analytes	Mobile phase	Flow rate (mL/min)	Make-up solvent	Additives	Flow rate (mL/min)	Ref.
Triacylglycerols	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	69
Triacylglycerols	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	78
Diacylglycerol	IPA/hexane	0.7	MeOH/chloroform	0.15% NH_4OH	0.6	68
Triacylglycerols	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	79
Neutral lipids	MTBE/hexane/HOAc	0.1 or 0.5	MeOH/chloroform	2.3% NH_4OH	0.6	66
Neutral lipids	MTBE/hexane/HOAc	0.1	IPA/ACN/DCM/H_2O	10 mM NH_4Ac	0.03	81
Methyl oleate	IPA/hexane	NA	MeOH/IPA	40 mM NH_4Ac	0.025	67
Petroleum	IPA/hexane	1.0	MeOH	2% HCOOH	NA	1
Drug	THF/heptane	0.05	MeOH/ACN	10 mM NH_4Ac	~0.01	75
Epoxyalcohols	Hexane-IPA	0.3	IPA/Water	_	0.2	70

HOAc: acetic acid; THF: tetrahydrofuran; NA indicates not available; "-" indicates no additives was added. Note: All the separation listed above used silica columns except in Ref. 1, where a aminocyano-bonded silica column was used. Positive mode in ESI has been used except in Ref. 69.

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Table 2 Representatives of chiral-ESI-MS with post column solvent addition for drug analysis

Pharmaceutical	Chiral column	Mobile phase	Make-up solvent	Ref.
Omeprazole	Chiralpak AD	EtOH /ACN/isohexane	EtOH/HCOOH	71
Felodipine	Chiralcel OJ-R	IPA/isohexane	EtOH /H_2O/NH_4Ac	76
Terazosin	Chiralpak AD	EtOH /IPA/DEA	IPA/ NH_4Ac	74
Lercandipine	Chiralpak AD	EtOH /hexane/DEA	EtOH / NH_4Ac	73
Verapamil	Chiralpak AD	IPA/hexane/DEA	IPA/ NH_4Ac	65
Sotalol	Chiralpak AD	EtOH /IPA/DEA	IPA/ NH_4Ac	65
Doxazosin	Chiralpak AD	IPA/hexane/DEA	IPA/ NH_4Ac	65
Oxybutynin	Chiralpak AD	IPA/hexane/DEA	IPA/ NH_4Ac	65
Jasmonic acid	Cellulose tris(4-methylbenzoate) coated silica	IPA/hexane	IPA/ NH_4OH	77
Propranolol	Chiral Art SB	IPA/hexane/NH_4OH	IPA	23
Mexiletine	Chiralpak AD	EtOH /IPA/hexane/TFA	Ethanol/ NH_4Ac	86
ABT-578	YMC-Pack SIL	THF/heptane	MeOH/ACN/ NH_4Ac	75
Impurities in ABT-578	YMC-Pack SIL	THF/heptane	MeOH/ACN/NH_4Ac	75
Casopitant Methylate	Chiralpack AD-H	EtOH/heptane	MeOH/HCOOH	72

DEA: diethylamine. Note: The identification of ABT-578 is performed in negative ESI, while the others are performed in positive ESI.

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