JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2005; **40**: 75–82
Published online 7 December 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.777

JMS

Sheath liquid interface for the coupling of normal-phase liquid chromatography with electrospray mass spectrometry and its application to the analysis of neoflavonoids

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Received 19 August 2004; Accepted 16 October 2004

A novel interface that allows normal-phase liquid chromatography to be coupled with electrospray ionization (ESI) is reported. A make-up solution of 60 mM ammonium acetate in methanol, infused at a 5 μl min^{-1} flow-rate at the tip of the electrospray probe, provides a sheath liquid which is poorly miscible with the chromatographic effluent, but promotes efficient ionization of the targeted analytes. Protonated molecules generated in the ESI source were subjected to tandem mass spectrometric experiments in a triple-quadrupole mass spectrometer. The main fragmentation reactions were characterized for each analyte and specific mass spectral transitions were used to acquire chromatographic data in the multiple reaction monitoring detection mode. Results obtained during optimization of the sheath liquid composition and flow-rate suggest that the electrospray process was mainly under the control of the make-up solution, and that it forms an external charged layer around a neutral chromatographic mobile phase core. This sheath liquid interface was implemented for the analysis of some neoflavonoid compounds and its performance was evaluated. Limits of detection were established for calophillolide, inophyllum B, inophyllum P and inophyllum C at 100, 25, 15 and 100 ng ml^{-1}, respectively. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: sheath liquid interface; normal-phase liquid chromatography; electrospray ionization mass spectrometry; neoflavonoid

INTRODUCTION

In crude extract or complex mixture analysis, it is often necessary to be able to separate isomeric compounds. This can be very challenging when using methodology based on conventional reversed-phase (RP) chromatography. As an alternative, normal-phase (NP) chromatography can provide dramatic improvements in the resolution of enantiomers.[1] However, in NP liquid chromatographic (LC) methods, detection is mainly performed by UV spectrophotometry and lacks specificity, a key parameter in complex mixture analysis. Mass spectrometry would allow a selective screening of targeted compounds, particularly when operated in the tandem mass spectrometric (MS/MS) mode.

Atmospheric pressure chemical ionization (APCI) is precluded for thermally labile compounds. The main drawback to using an electrospray interface to implement a coupling between NPLC and MS is the incompatibility of non-polar solvents, used as eluents in NPLC, and the mechanism of electrospray ionization (ESI).[2] As a consequence, compound separation is often first carried out by normal-phase chromatography operated on a semi-preparative scale and the collected fractions are further subjected to mass analysis after reversed-phase chromatography.[3,4]

We have developed a new interface to perform on-line coupling of NPLC with MS that allows the ionization conditions to be optimized independently from compound chromatographic separation. This methodology has been applied to the analysis of targeted compounds extracted from *Calophyllum inophyllum*, an evergreen tree mainly found in tropical parts of the indo-pacific area. Different parts of this plant have been widely used in folk medicines, suggesting a rich source of bioactive secondary metabolites. In particular, neoflavonoids isolated from *Calophyllum inophyllum* were recently shown to be representatives of a distinct class of non-nucleoside HIV-1 specific reverse-transcriptase inhibitors under clinical trials as an AIDS chemotherapeutic.[5–7]

To overcome difficulties arising when chromatographic conditions do not favour ESI, the introduction of a liquid gap between the column and the sprayer has been proposed to introduce a polar buffer in the chromatographic effluent.[8] This so-called liquid junction may allow adequate conditions in the ionization source[9,10] but dilution effects and loss of chromatographic resolution have been reported because of

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the introduction of a gap in the system.[11] Alternatively, the ionization conditions can be optimized without affecting the chromatographic separation by adding a make-up solution at the tip of the electrospray probe. The idea of using coaxial flow, originally developed for the post-column derivatization of analytes,[12] is now widely applied for the coupling of capillary electrophoresis (CE)[13–17] or electrochromatography[10,18] with MS, where the sheath liquid assists the electrospray process and completes the electrical circuit. Such a make-up solution has also been introduced to enhance the amount of organic solvent in the electrospray source, as reported in the mass analysis of nucleic acids separated by capillary ion-pair RPLC,[19,20] or as a supply of ammonium formate to promote ammonium adduct formation in RPLC/MS.[21,22]

Although a sheath liquid interface appears to be particularly relevant for coupling NPLC with MS, to our knowledge this configuration has never been reported. We describe here a methodology that uses a sheath liquid to allow ESI of some neoflavonoid compounds separated by NPLC.

EXPERIMENTAL

Chemicals and reagents

Solvents used for sample and standard preparation (i.e. *n*-hexane, ethyl acetate, methanol, dichloromethane and isooctane) were of Chromanorm grade from VWR International (West Chester, PA, USA). Sulfuric acid–vanillin solution used for thin-layer chromatographic (TLC) detection was prepared with Rectapur-grade vanillin and Normapur-grade ethanol and H_2SO_4 (95%), purchased from VWR International. HPLC-grade isooctane and 2-propanol used in analytical LC were supplied by SDS (Peypin, France). Sheath liquid solutions were prepared with ammonium acetate (NH_4OAc) from Sigma-Aldrich (St. Louis, MO, USA) and HPLC-grade methanol from SDS.

Sample preparation

Apparatus

Sample fractionation and purification were performed with a low-pressure LC system using a Gamma 4-W pump (Prominent, Strasbourg, France), Chromaflex glass columns (Kontes, Vineland, NJ, USA) filled with Kieselgel 60 (40–63 μm) silicagel (Merck, Darmstadt, Germany) and a circular fraction collector (Eldex, San Carlos, CA, USA).

Preparative chromatography was performed with an HP 1100 system and a HP 1047A refractometer coupled with a recorder unit as a detector (Hewlett-Packard, Palo Alto, CA, USA), using a Varian (Walnut Creek, CA, USA) Dynamax Si column (250 × 21.4 mm i.d., film thickness 5 μm).

TLC was performed on Empore Si TLC sheets (3M, Maplewood, MN, USA) with *n*-hexane–ethyl acetate (60 : 40, v/v) as migration solvent; spots were detected using sulfuric acid–vanillin (3 g of vanillin in 100 ml of absolute ethanol containing 1 ml of H_2SO_4).

Procedures

Neoflavonoid standard preparation was adapted from a methodology developed by Patil *et al*.[5] and is briefly described below.

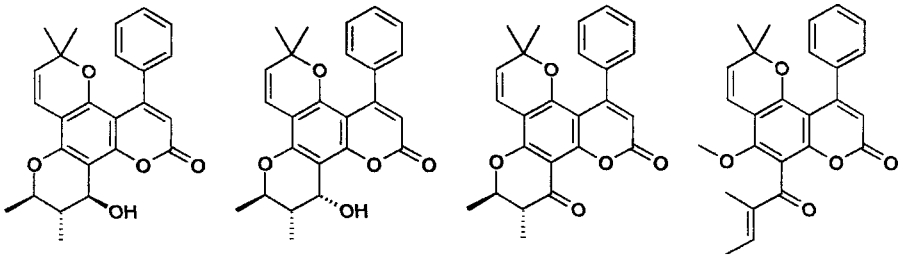
Three successive Soxhlet extractions were performed for 8 h on a 2 kg sample of Tamanu leaves (*Calophyllum inophyllum* Linn., from French Polynesia), using a 2 l volume of *n*-hexane, ethyl acetate and methanol, respectively. Solvent evaporation of the second extract yielded 140 g of crude extract, which was further triturated in 1 l of dichloromethane. The soluble part of the crude extract was then evaporated to yield a very viscous, dark-green oil (52 g), which was further purified. Three fractions were successively eluted from the purification column (0.5 m × 5 cm i.d.) using *n*-hexane, ethyl acetate and methanol. The ethyl acetate fraction (14 g) was further fractionated into 150 parts using a second silica gel column (1.2 m × 2 cm i.d.), performing a gradient elution from *n*-hexane–ethyl acetate (85 : 15, v/v) to ethyl acetate in 12 h at 5 ml min^{-1}.

A total of 11 fractions could be selected after TLC analysis and were submitted to preparative LC. Compounds were eluted isocratically at 10 ml min^{-1} using isooctane–ethyl acetate (80 : 20, 70 : 30 or 60 : 40), v/v, depending on fraction polarity. Isolated neoflavonoid standards were monitored by NMR analysis.

ESI-MS and coupling with NPLC

All experiments were performed using an API III Plus triple-quadrupole mass spectrometer (Perkin-Elmer SCIEX, Concord, ON, Canada), equipped with an atmospheric pressure ionization (API) source. A sheath flow connection was implemented on the source, via a stainless-steel tee mounted on a Teflon part for electrical insulation, to allow a make-up solution to be introduced at the tip of the electrospray probe. This interface utilizes a triaxial flow arrangement where the chromatographic effluent, split down to 50 μl min^{-1} using a zero dead volume tee connector, is introduced in the atmospheric region of the electrospray source via a silica capillary inserted in a narrow metal tube which delivers the sheath liquid to the capillary exit; a third concentric tube delivers a gas flow to assist spray formation. Ultra-high purity (UHP, 99.999%) nitrogen was used as the curtain gas in the API source at a flow-rate of 0.6 l min^{-1} and zero-grade air as the nebulizing gas, at a flow-rate of 0.8 l min^{-1}. The interface temperature was held at 54 °C. Positive mode ESI was performed at 5 kV and the orifice voltage was set at 70 V. The resolution for both quadrupoles was set at 0.7 u full width at half-height (FWHH). Mass calibration was performed on poly(propylene glycol) solution. MS/MS measurements were based on collision-induced dissociation (CID), using UHP argon as the target gas, at a collision gas target of 90×10^{15} molecules cm^{-2}. Data acquisition was done in the multiple reaction monitoring (MRM) mode. The API III Hyperspec workstation and API software version 2.6 were used on a Power Macintosh 8100/80 for instrument control, data acquisition and data processing.

A syringe pump (Harvard Apparatus, South Natick, MA, USA) was used for direct introduction experiments and for pumping sheath liquid. Flow injection analysis (FIA) and NPLC were performed using an Agilent Series 1100 instrument (Agilent Technologies, Palo Alto, CA, USA). LC analyses were carried out using a QS LiChrosorb Si



Scheme 1. Structure and molecular masses of the four neoflavonoids tested.

column (250 × 4.6 mm i.d., film thickness 5 μm) purchased from Interchim (Montluçon, France). Solvent A was pure isooctane and solvent B was isooctane–2-propanol (90 : 10, v/v). The following linear gradient was performed at a 1 ml min^{-1} flow-rate: 0 min, 90% A (held for 5 min); 20 min, 60% A; 25 min, 10% A (held for 5 min); 35 min, 90% A (held for 5 min).

RESULTS AND DISCUSSION

Mass spectrometry of neoflavonoids

Our study was focused on the performance of the NPLC/ESI interface as evaluated by examining four neoflavonoid compounds. These compounds were chosen on the basis of their therapeutic relevance. As can be seen from Scheme 1, which shows the structure of the analytes, inophyllum B and inophyllum P are two epimers that differ from each others only by the configuration of the carbon atom holding the hydroxy group. NPLC conditions were particularly required to separate these isomers.

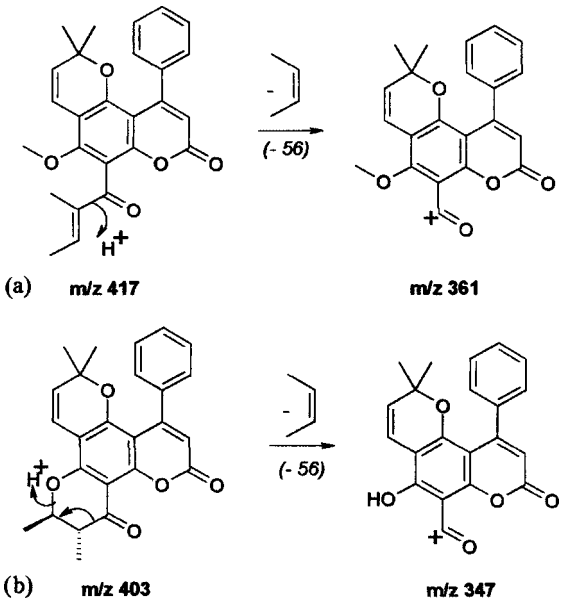
In positive mode ESI, protonated molecules $[M + H]^+$ could be generated from acidic standard solutions. However, these ions were observed with a greater abundance in the mass spectra when arising from in-source dissociation of ammonium adducts, as observed while infusing individual standard solutions in methanol–3 mM NH_4OAc and applying a high orifice voltage (70 V).

Protonated molecules generated in the electrospray source were submitted to CID in the collision cell of the triple-quadrupole mass spectrometer. MS/MS experiments showed that the major fragmentation route of protonated molecules formed from compounds containing a hydroxy function, i.e. inophyllum B and inophyllum P, consists of the loss of a water molecule. Accordingly, the mass spectral transition m/z 405 → 387 was selected to monitor these two isomers. A 56 u neutral loss was found to be the main fragmentation reaction of $[M + H]^+$ ions of both inophyllum C and calophillolide. This reaction was interpreted as the loss of a 2-butene molecule in both cases, although it would proceed via two different dissociation mechanisms, as shown in Scheme 2. The proposed fragmentation mechanisms are further confirmed by a subsequent CO loss giving rise to small peaks, observed at m/z 333 and 319 in the tandem mass spectrum of m/z 417 and 403 precursor ions, respectively. The mass spectral transition m/z 403 → 347 was therefore selected for the specific detection of inophyllum C whereas m/z 417 → 361 was used in selected reaction monitoring of calophillolide. The optimum signal was obtained from the selected spectral transitions with a 30 eV collision energy (laboratory frame).

Optimization of sheath liquid composition and flow-rate

FIA experiments were performed to investigate the effects of sheath liquid composition and flow rate on MS detectability without the complexity of LC separation. Once established in FIA, these experimental conditions were then checked in the LC/MS configuration. Optimization experiments were carried out with inophyllum P as a model compound, using a 3 μg ml^{-1} standard solution. Isooctane–2-propanol (97 : 3, v/v) was isocratically delivered at 50 μl min^{-1} in the electrospray source to fit the mobile phase composition at the expected retention time for this analyte.

In the absence of any sheath liquid or when infusing sheath liquid consisting of pure organic solvent, no signal could be obtained, showing respectively that the binary solvent system used in the chromatographic method was not compatible with electrospray and ionization of the targeted



Scheme 2. Proposed mechanisms for the 56 u neutral loss observed in the fragment ion mass spectrum of $[M + H]^+$ from (a) calophillolide and (b) inophyllum C.

analytes did require the addition of an electrolyte. As a chromatographic mobile phase component, 2-propanol was first tested as the organic solvent for supplying ammonium acetate at the tip of the electrospray probe but gave very poor results. This is in contrast with studies reporting the suitability of this solvent in a sheath liquid used for CE/MS coupling.[15,23,24] However, these findings were understood in terms of electrical connection and for the ionization of acidic compounds via deprotonation. Amongst the organic solvents tested, the most suitable was found to be methanol in spite of the fact that it is not miscible with the mobile phase main component, isooctane. A set of experiments were then conducted with different concentrations of ammonium acetate (3, 10, 30 and 60 mM) in methanol, introduced in the ionization source as a sheath liquid at six different flow-rates.

When describing sheath liquid effects, signal-to-noise (S/N) ratios were often found to be more appropriate than absolute signal intensity to report MS data, owing to the high background levels induced by some organic solvents.[15,25] However, MS/MS reaction monitoring used here is a very specific data acquisition mode and exhibits extremely low noise levels. Hence similar conclusions can be drawn by plotting either peak area or S/N ratios.

Figure 1 shows that the same trend is observed as the sheath liquid flow-rate is increased, regardless of its composition: the signal first increases until a maximum is reached, then decreases. However, the flow-rate from which the signal discrepancy is obtained varies as a function of ammonium acetate content in the sheath liquid: the lower the concentration, the higher is the flow-rate required for sensitivity to drop off. For example, the maximum signal is reached at 5 μl min^{-1} for a 60 mM NH_4OAc sheath liquid whereas the signal decrease is only observed from 25 μl min^{-1} when the make-up solution contains 3 mM ammonium acetate. On the other hand, up to 15 μl min^{-1}, higher sensitivity is obtained with more concentrated sheath liquid. For instance, a fourfold increase in the peak area is measured as the NH_4OAc concentration is increased from 3 to 60 mM in the sheath liquid infused at 5 μl min^{-1}. These experimental results can be rationalized in terms of charge supply: increasing the amount of ammonium cations in the electrospray source, by increasing either the electrolyte concentration in the sheath liquid or the solution flow-rate, first improves the ionization yield. The extent of analyte dilution with the make-up solution at the tip of the electrospray probe, and the amount of methanol reaching the ionization source, are directly proportional to the sheath liquid flow-rate. Therefore, these two parameters cannot account for the observed signal decrease as it is measured at different flow-rates depending on NH_4OAc concentration. The loss in sensitivity might rather reflect a less stable spray induced by overcharging of the liquid surface. Indeed, if too many charges are available, the surface charge can exceed the Rayleigh limit before small droplets can be formed, resulting in spray instability.[26] This effect would account for the early signal decrease (from above 5 μl min^{-1}) observed when using the most concentrated make-up solution (60 mM ammonium acetate in methanol). These results suggest that, on the time-scale of the electrospray process, the two liquid phases do not completely mix but rather form a system where charges in the sheath liquid forms an external layer around a neutral LC mobile phase core. This effect might be further favoured by the limited miscibility of the sheath liquid solvent (methanol) in the LC effluent (97% isooctane). Interaction of the analyte from the inner phase with a surface charge to form ammonium adducts would then occur as the droplet size decreases during the evaporation–explosion sequences.

Keeping the electrospray source operating conditions unchanged, the general sensitivity loss observed for the highest tested infusion flow-rates of the make-up solutions suggests that a stable spray cannot be obtained from such a quantity of liquid.

The dependence of optimal signal on both the composition and flow-rate of the sheath liquid suggests that sensitivity might be under the control of charge supply rate. Figure 2 shows that the best signal is obtained when the charge supply regime is around 400 nmol NH_4^+ min^{-1} under the operating conditions. However, this optimal regime seems to depend greatly on the sheath liquid composition. For less concentrated solutions, it can be predicted that overflowing effects would dominate long before the optimal regime is reached as a signal decrease is observed from 60 and 200 nmol NH_4^+ min^{-1} using 3 and 10 mM NH_4OAc sheath liquid, respectively. On the other hand, although the same

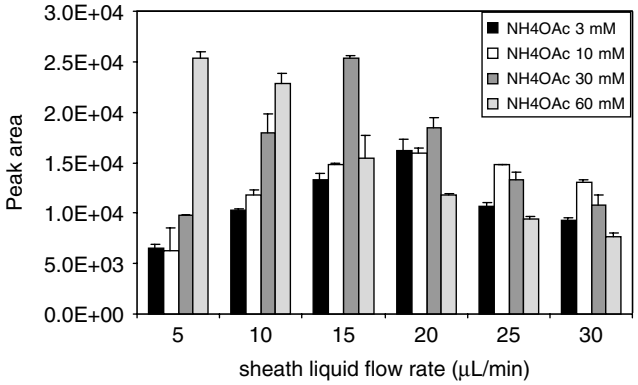


Figure 1. Effects of sheath liquid composition and flow-rate on inophyllum P signal, monitored as the selected MS/MS reaction *m/z* 405 → 387.

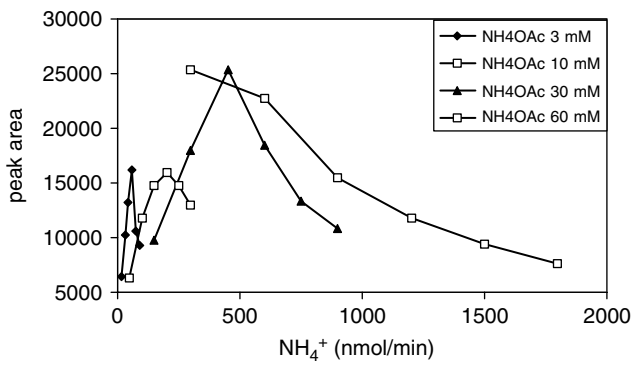


Figure 2. Influence of charge supply rate on inophyllum P signal, monitored as the selected MS/MS reaction *m/z* 405 → 387.

charge supply rate (300 nmol NH_4^+ min^{-1}) can be reached using either the 60 or 30 mM NH_4OAc solution, while infused at 5 or 10 µl min^{-1}, respectively, the signal is 1.5 times higher with the most concentrated sheath liquid. One could argue that a less stable spray is formed when a make-up solution is infused at 10 µl min^{-1} as compared with 5 µl min^{-1}, but this would not be consistent with the observed signal enhancement as the flow-rate of the 30 mM NH_4OAc solution is increased from 10 to 15 µl min^{-1} (Fig. 1). Enhanced sensitivity measured with the 60 mM NH_4OAc sheath liquid would rather be due to the higher conductivity of this solution, allowing thinner charged droplets to be formed and thus a faster ion evaporation rate.[26]

Sheath liquid vs liquid junction

The performance measured with the sheath liquid interface was compared with results obtained using a liquid junction configuration, where the make-up solution is mixed with the LC effluent prior to reaching the electrospray source. The best signal that could be obtained when introducing such a liquid gap in the system was obtained by infusing a 60 mM NH_4OAc methanolic solution at 20 µl min^{-1} in the chromatographic effluent. FIA experiments of the same standard solution performed in each configuration shows that a higher response was measured with the liquid junction (peak height ~4500 in Fig. 3(b)) than when using the optimized sheath liquid (peak height ~2500 in Fig. 3(a)). This is consistent with results that showed that the H/D exchange efficiency using a deuterated sheath liquid was not as high as when samples were dissolved in D_2O.[27] However, the peak resolution was hardly affected in the liquid junction experiments. The generation of a very unstable spray can account for the signal shown in Fig. 3(b), as the result of variations in the composition of the liquid phase at the tip of the electrospray probe arising from very poor mixing between the electrolyte solution and the FIA effluent. In contrast, good electrospray conditions were reached in the sheath liquid experiments, as illustrated by Fig. 3(a), although the system had to accommodate the same two liquid phases. This result further confirms that, as a sheath liquid, NH_4OAc solution does not mix with the LC effluent and charged droplet formation would be under the control of the make-up solution.

NPLC/MS coupling

As depicted in Fig. 1, 60 mM NH_4OAc in methanol infused at a 5 µl min^{-1} flow-rate was found to be the optimal sheath liquid system and was therefore implemented in NPLC/MS coupling. A neoflavonoid standard mixture was injected on to the column and, by monitoring the MS/MS transition defined for each compound, the total ion current (TIC) chromatogram presented in Fig. 4 was obtained. All compounds are baseline resolved and assignment of the chromatographic peaks was based on both the extracted chromatograms shown in Fig. 4 and the retention times (t_R) obtained from individual standard injections: inophyllum C (4 µg ml^{-1}) at t_R 27.7 min in the m/z 403 → 347 trace, inophyllum B (5 µg ml^{-1}) at t_R 14.6 min and inophyllum P (3 µg ml^{-1}) at t_R 15.7 min in the m/z 405 → 387 trace and calophyllolide (1 µg ml^{-1}) at t_R 13.5 min in the m/z 417 → 361 trace.

The high sensitivity of MS/MS allows additional peaks to be observed in the chromatograms as compared with data obtained in UV detection, suggesting that individual standard solutions were not very pure. Each chromatographic trace extracted from the TIC obtained in the MRM detection mode is highly specific to a mass spectral transition. Therefore, any additional peaks observed in an extracted chromatogram can be considered as arising from isomers of the studied analyte. These impurities could have been sampled together with the targeted compounds during preparative LC. For example, low amounts of callophyllum P were detected in callophyllum B individual standard solutions, and vice versa. Some impurities could also form during storage: the intensity of the peak detected at 6.3 min in the m/z 403 → 347 extracted chromatogram was found to

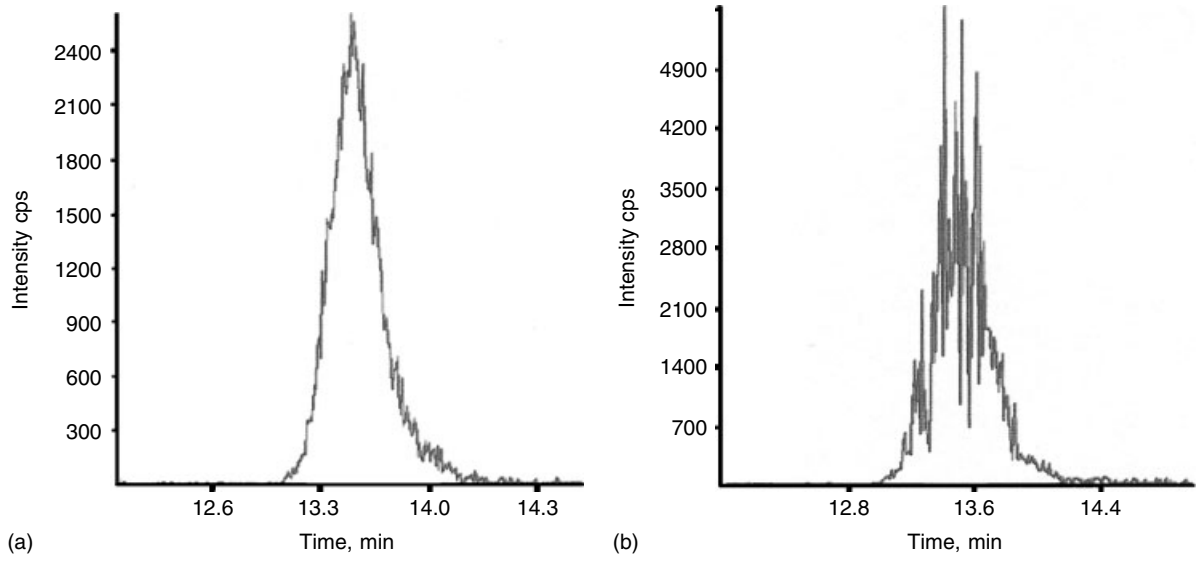


Figure 3. Flow injection analysis of a 1 µg ml^{-1} inophyllum P standard (a) in the sheath liquid configuration (60 mM NH_4OAc in methanol at 5 µl min^{-1}) and (b) using a liquid junction (60 mM NH_4OAc in methanol at 20 µl min^{-1}).

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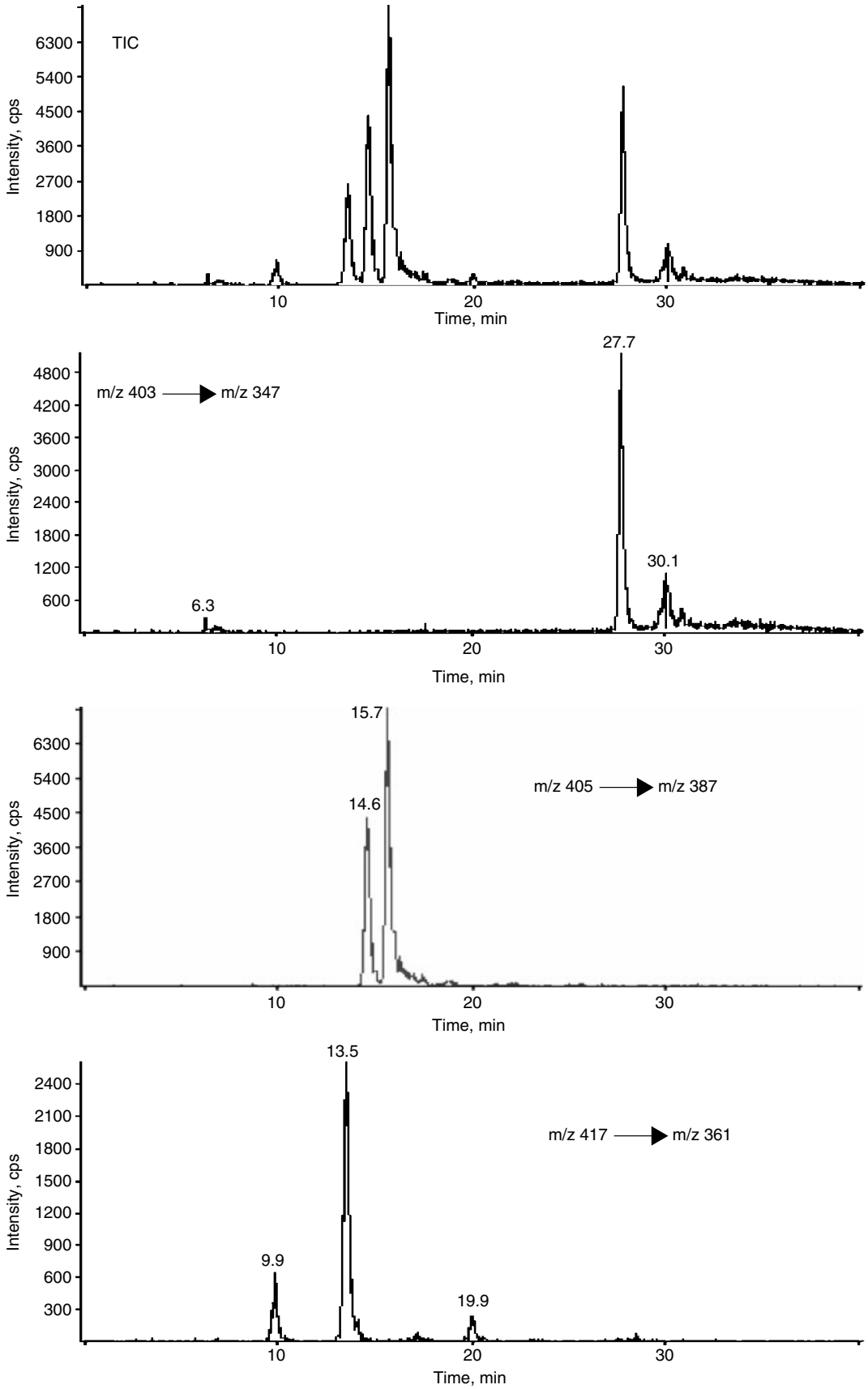

Figure 4. Top: TIC chromatogram of a neoflavonoid standard mixture recorded in the MRM detection mode and using a 60 mM NH_4OAc methanolic sheath liquid infused at 5 μl min^{-1} in the electrospray source. Analytes are identified in the extracted chromatogram associated with their specific MS/MS transition: inophyllum C at t_R 27.7 min in the *m/z* 403 → 347 trace; inophyllum B at t_R 14.6 min and inophyllum P at t_R 15.7 min in the *m/z* 405 → 387 trace and calophyllolide at t_R 13.5 min in the *m/z* 417 → 361 trace.

Table 1. Analytical performance of the NPLC/ESI-MS/MS coupling for the analysis of four neoflavonoid compounds using the MRM detection mode

Compound	Mass spectral transition (m/z)	LOD (ng ml^{-1})[a]	LOQ (ng ml^{-1})[a]
calophyllolide	417 → 361	100	250
inophyllum B	405 → 387	25	60
inophyllum P	405 → 387	15	50
inophyllum C	403 → 347	100	400

[a] Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3σ and 10σ of a blank signal, respectively.

increase as inophyllum C standard solutions were aged. These results indicate that quantitative analysis would require further purification of the standards and a more extensive study of their stability to define optimal storage conditions. Consequently, the analytical performances presented in Table 1 are underestimated as they were evaluated assuming that pure standards were used.

CONCLUSION

A unique method has been developed to couple NPLC with MS via an ESI interface modified to accommodate the introduction of a make-up solution at the tip of the electrospray probe. This sheath liquid was shown to promote successful analyte ionization although it was poorly miscible with the LC mobile phase, conferring great flexibility to this configuration.

This method will be further used to control standard purity and storage conditions, as some impurities were shown to form during solution aging, and to perform neoflavonoid screening in biodiversity studies. It is expected that it should be possible to extend this methodology to the analysis of any complex natural extracts requiring normal-phase separation.

REFERENCES

1. Lee SH, Williams MV, DuBois RN, Blair IA. Targeted lipidomics using electron capture atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 2003; **17**: 2168.
2. Wang G, Cole RB. In *Electrospray Ionization Mass Spectrometry—Fundamentals, Instrumentation and Applications*, Cole RB (ed). Wiley: New York, 1997; 137.
3. Almudaris A, Ashton DS, Ray A, Valko K. Trace Analysis of Impurities in 3′-azido-3′-deoxythymidine by reversed-phase high-performance liquid-chromatography and thermospray mass-spectrometry. *J. Chromatogr. A* 1995; **689**: 31.
4. Ashton DS, Ray AD, Valko K. Detection of the principal synthetic route indicative impurity in lamotrigine. *Int. J. Pharm.* 1999; **189**: 241.
5. Patil AD, Freyer AJ, Eggleston DS, Haltiwanger RC, Bean MF, Taylor PB, Caranfa MJ, Breen AL, Bartus HR, Johnson RK, Hertzberg RP, Westley JW. The inophyllums, novel inhibitors of HIV-1 reverse-transcriptase isolated from the Malaysian tree, *Calophyllum inophyllum* Linn. *J. Med. Chem.* 1993; **36**: 4131.
6. Ishikawa T. Anti HIV-1 active *Calophyllum* coumarins: distribution, chemistry, and activity. *Heterocycles* 2000; **53**: 453.
7. Creagh T, Ruckle JL, Tolbert DT, Giltner J, Eiznhamer DA, Dutta B, Flavin MT, Xu ZQ. Safety and pharmacokinetics of single doses of (+)-calanolide A, a novel, naturally occurring nonnucleoside reverse transcriptase inhibitor, in healthy, human immunodeficiency virus-negative human subjects. *Antimicrob. Agents Chemother.* 2001; **45**: 1379.
8. Lee ED, Muck W, Henion JD, Covey TR. Liquid junction coupling for capillary zone electrophoresis ion spray mass-spectrometry. *Biomed. Environ. Mass Spectrom.* 1989; **18**: 844.
9. Shang DY, Ikonomou MG, Macdonald RW. Quantitative determination of nonylphenol polyethoxylate surfactants in marine sediment using normal-phase liquid chromatography–electrospray mass spectrometry. *J. Chromatogr. A* 1999; **849**: 467.
10. Choudhary G, Apffel A, Yin HF, Hancock W. Use of on-line mass spectrometric detection in capillary electrochromatography. *J. Chromatogr. A* 2000; **887**: 85.
11. Pleasance S, Thibault P, Kelly J. Comparison of liquid-junction and coaxial interfaces for capillary electrophoresis mass-spectrometry with application to compounds of concern to the aquaculture industry. *J. Chromatogr. A* 1992; **591**: 325.
12. Kohler M, Leary JA. LC/MS/MS of carbohydrates with postcolumn addition of metal chlorides using a triaxial electrospray probe. *Anal. Chem.* 1995; **67**: 3501.
13. Tsai CY, Her GR. Capillary zone electrophoresis electrospray mass spectrometry of priority phenols. *J. Chromatogr. A* 1996; **743**: 315.
14. Barry JP, Muth J, Law SJ, Karger BL, Vouros P. Analysis of modified oligonucleotides by capillary electrophoresis in a polyvinylpyrrolidone matrix coupled with electrospray mass spectrometry. *J. Chromatogr. A* 1996; **732**: 159.
15. Hagberg J. Analysis of low-molecular-mass organic acids using capillary zone electrophoresis–electrospray ionization mass spectrometry. *J. Chromatogr. A* 2003; **988**: 127.
16. McCourt J, Bordin G, Rodriguez AR. Development of a capillary zone electrophoresis–electrospray ionisation tandem mass spectrometry method for the analysis of fluoroquinolone antibiotics. *J. Chromatogr. A* 2003; **990**: 259.
17. Von Brocke A, Freudemann T, Bayer E. Performance of capillary gel electrophoretic analysis of oligonucleotides coupled on-line with electrospray mass spectrometry. *J. Chromatogr. A* 2003; **991**: 129.
18. Zheng J, Shamsi SA. Combination of chiral capillary electrochromatography with electrospray ionization mass spectrometry: method development and assay of warfarin enantiomers in human plasma. *Anal. Chem.* 2003; **75**: 6295.
19. Premstaller A, Oberacher H, Huber CG. High-performance liquid chromatography–electrospray ionization mass spectrometry of single- and double-stranded nucleic acids using monolithic capillary columns. *Anal. Chem.* 2000; **72**: 4386.
20. Huber CG, Krajete A. Analysis of nucleic acids by capillary ion-pair reversed-phase HPLC coupled to negative-ion electrospray ionization mass spectrometry. *Anal. Chem.* 1999; **71**: 3730.
21. Byrdwell WC. Dual parallel liquid chromatography/dual mass spectrometry (LC2/MS2) of bovine brain total lipid extract. *J. Liq. Chromatogr. Relat. Technol.* 2003; **26**: 3147.
22. Byrdwell WC, Neff WE. Electrospray ionization MS of high MW TAG oligomers. *J. Am. Oil Chem. Soc.* 2004; **81**: 13.
23. Ahrer W, Buchberger W. Determination of haloacetic acids by the combination of non-aqueous capillary electrophoresis and mass spectrometry. *Fresenius' J. Anal. Chem.* 1999; **365**: 604.
24. Vuorensola K, Kokkonen J, Siren H, Ketola RA. Optimization of capillary electrophoretic–electrospray ionization–mass spectrometric analysis of catecholamines. *Electrophoresis* 2001; **22**: 4347.
25. Huber CG, Krajete A. Sheath liquid effects in capillary high-performance liquid chromatography–electrospray mass spectrometry of oligonucleotides. *J. Chromatogr. A* 2000; **870**: 413.

26. Hayati I, Bailey AI, Tadros TF. Investigations into the mechanisms of electrohydrodynamic spraying of liquids 1. Effect of electric-field and the environment on pendant drops and factors affecting the formation of stable jets and atomization. *J. Colloid Interface Sci.* 1987; **117**: 205.

27. Lam W, Ramanathan R. In electrospray ionization source hydrogen/deuterium exchange LC–MS and LC–MS/MS for characterization of metabolites. *J. Am. Soc. Mass Spectrom.* 2002; **13**: 345.