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High-performance liquid chromatography–mass spectrometry (pneumatically assisted electrospray) of hydroxy polycyclic aromatic hydrocarbons

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Abstract

Electrospray (ES) in both positive and negative ionization mode and coupling high-performance liquid chromatography (HPLC) to mass spectrometry (MS) for the characterization of hydroxy polycyclic aromatic hydrocarbons (hydroxy-PAHs) are studied in this work. A series of reference hydroxy-PAHs were separated on a reversed-phase column and their ES spectra were analyzed in both ionization modes. $[M + H]^+$ and $[M - H]^-$ were obtained for positive and negative ES. Reduction of quinones was also observed in negative ES. Suitable masses for single-ion monitoring were used in HPLC–MS analysis. Flow injection of solute standards in isocratic mode and LC analyses in gradient mode using methanol–formic acid/ammonium formate 10 m*M*, pH 3 as eluent were performed.

1. Introduction

Electrospray ionization mass spectrometry (ES-MS), since its introduction in the early 1980s [1,2] has emerged as a technique for the analysis of a variety of compounds ranging from high-molecular-mass biopolymers to metal ions [3–7]. The studies of Fenn and co-workers [1,2,4,8] demonstrated the potential of ES ionization as an interface for capillary liquid chromatography (LC)–MS. Henion and co-workers [9–11], using a modification of the ES method which they called ion spray (IS), where the nebulization of the liquid effluent is assisted by a turbulent gas (nitrogen) flow, have also described a number of applications of this technique for the detection of many analytes of biological and pharmaceutical interest. Smith and co-workers [12–14] using ES as the interface for capillary electrophoresis were able to demonstrate the suitability of ES for the detection of bioorganic ionic analytes.

The capabilities of the spray methods have created also a high interest in the mechanism by which the gas-phase analyte ions are produced. It has been found empirically that the best analytical results are obtained for compounds that are ionic in solution (i.e., preformed ions). Such species include metal salts, organic salts, and compounds (e.g., peptides and proteins) which can be ionized [3–7]. The latter species are typically observed as protonated, or otherwise positively ionized molecules such as sodium adducts in positive-ion mode and as the deprotonated molecules or as molecular adduct anions in negative-ion mode.

There are two important stages in the pro-

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duction of gas-phase ions: the production of the charged droplets and the production of gas-phase ions from the charged droplets. The mechanism by which the droplets are charged is assumed to be electrophoretic [15–17], although the electrospray process may also involve electrochemical reactions at the metal–liquid interface of the ES capillary tip [18–20].

The production of gas-phase ions from the charged droplets can be produced as the result of a large electrical field applied to the surface of the liquid [21–24]. When the electrical field exceeds a critical value the solute ions escape ("evaporate") from the liquid phase into the gas phase. Also ionization in the gas phase can take place via reactions identical to those occurring in chemical ionization sources. Since ions and neutral molecules are formed close together at the atmospheric pressure source, many ion/molecule collisions occur.

Few data are available on LC–MS of hydroxy polycyclic aromatic hydrocarbons (hydroxy-PAHs). Ionization of neutral, relatively non-polar compounds, such as PAHs was inefficient [19], but ES can be used in the analysis of hydroxy-PAHs because of their functional groups. Here we discuss the applicability of ES with both positive and negative ionization to the analysis of hydroxy-PAHs, developing the MS conditions suitable for the identification of these products. Calibration conditions for low-molecular-mass compounds were established. The hydroxy-PAH spectra were interpreted using the mechanisms described in the literature and coupling LC with MS was studied.

2. Experimental

2.1. Reagents

The compounds studied were 5-hydroxyindole (5-HI), 1-indanol (1-HI), 2-hydroxy-1,4-naphthoquinone (2-H-1,4-NQ), 9-hydroxyfluorene (9-HFL), 9-hydroxyphenantrene (9-HF) and 2-hydroxy-9-fluorenone (2-H-9-FLO) and were provided by Aldrich (Milwaukee, WI, USA). LC solvents, methanol (HPLC grade; Merck, Darmstadt, Germany) and water purified with a Culligan system were used. All solvents were degassed in an ultrasonic bath for 15 min.

A stock solution of the compounds was prepared containing 1 mg ml^{-1} of each in methanol. The 100 μg ml^{-1} solutions in mobile phase were obtained by dilution of appropriate volumes of this stock solution.

2.2. Instruments and procedures

MS was performed using a VG Quattro (FISON Instruments, VG Biotech, Altrincham, UK) triple quadrupole mass spectrometer equipped with an ES interface, which was assisted pneumatically, with nitrogen at a flow-rate of 10 l h^{-1}. Drying nitrogen was heated to 80°C and introduced into the capillary region at a flow-rate of 200 l h^{-1}. The electrospray needle was held at a potential of +3.8 kV and −3.2 kV relative to the potential at the counter electrode for positive and negative modes respectively. The focus potential was 60 V.

For data acquisition the mass spectrometer operated over the mass range m/z 10.0–600.0 in centroid mode at a cycle time of 2.00 s and at inter scan time of 0.10 s. Ion intensity was optimized using the mobile phase ion clusters, and calibrations were performed with these clusters in positive mode and NaI (2 μl ml^{-1} in mobile phase) in negative mode. To improve cluster formation a drying nitrogen flow of 50 l h^{-1} and a focus potential of 40 V were used.

Flow injection analysis (FIA) was used using methanol–formic acid/ammonium formiate 10 mM (pH 3) (55:45) at 35 μl min^{-1} was performed in an HPLC system with two Phoenix 20 (Carlo Erba, Milan, Italy) syringe pumps, a master (A) and a slave (B) pump. A ODS-Hypersil C_{18} (5 μm particle size, 100 × 1 mm I.D.) reversed-phase column (Shandon Scientific, Cheshire, UK) was used for hydroxy-PAH LC separation; in this case the mobile phase was run in the gradient mode. Mixtures of standards were prepared in mobile phase and 10-μl aliquots were injected in the FIA mode and 200 nl were injected in the chromatographic mode.

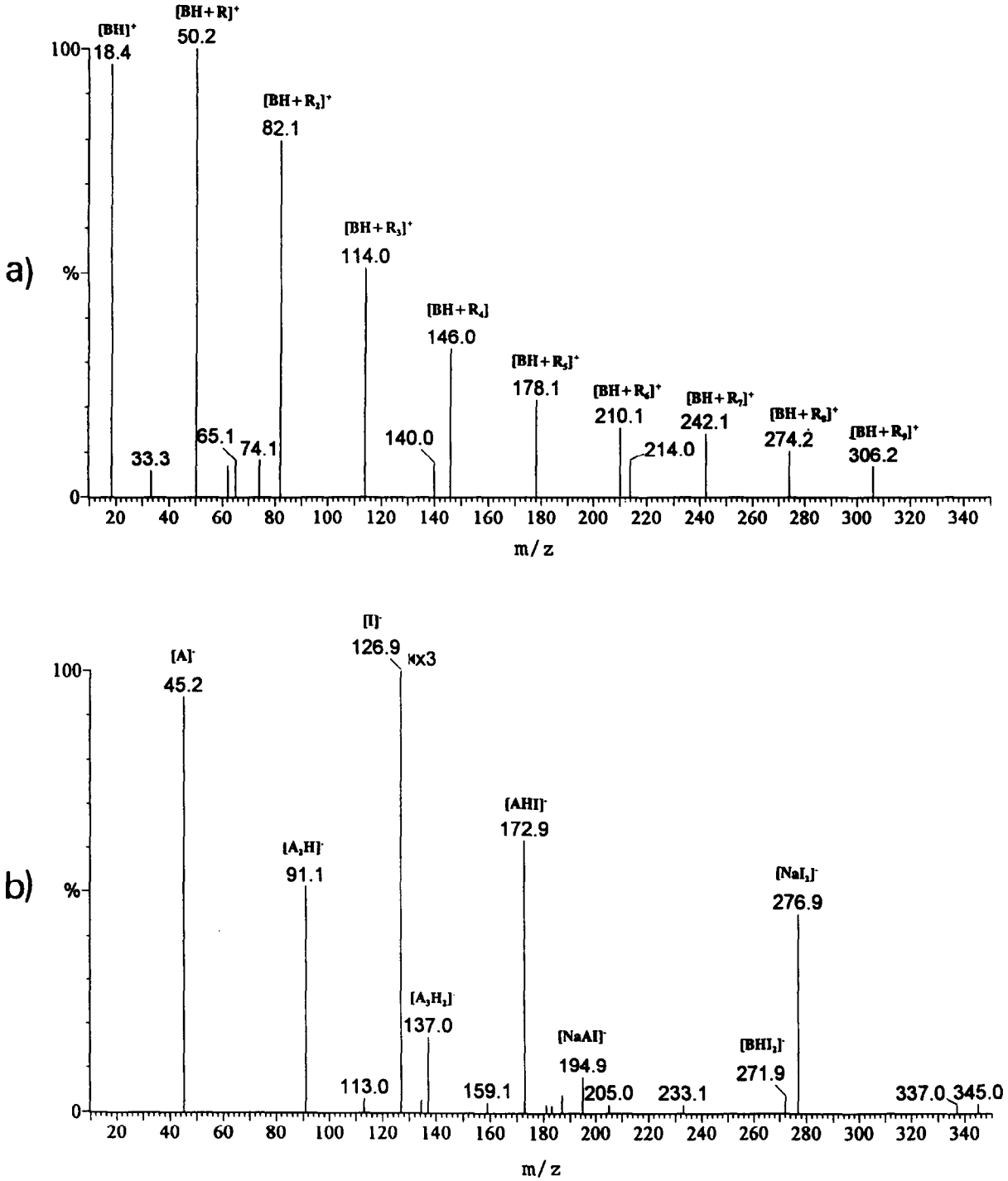


Fig. 1. ES spectra of (a) mobile phase in positive ionization and (b) NaI in negative ionization. A = $HCOO^-$; B = NH_3; R = CH_3OH.

3. Result and discussion

3.1. Calibration

One of the problems associated with "soft" ionization techniques like IS and thermospray (TSP), is that common MS reference compounds, such as perfluorokerosene (PFK), cannot be used to calibrate the mass spectrometer. Poly(ethylene glycol)s (PEGs) and poly(propylene glycol)s (PPGs) were therefore studied as reference standards [25–28]. Unfortunately, PEG and PPG mixtures are not the most suitable candidates for reference compounds, since they cause rapid contamination of the ion source due to the deposition of non-volatile material [29,30]. In addition, it is often difficult to identify individual PEG and PPG

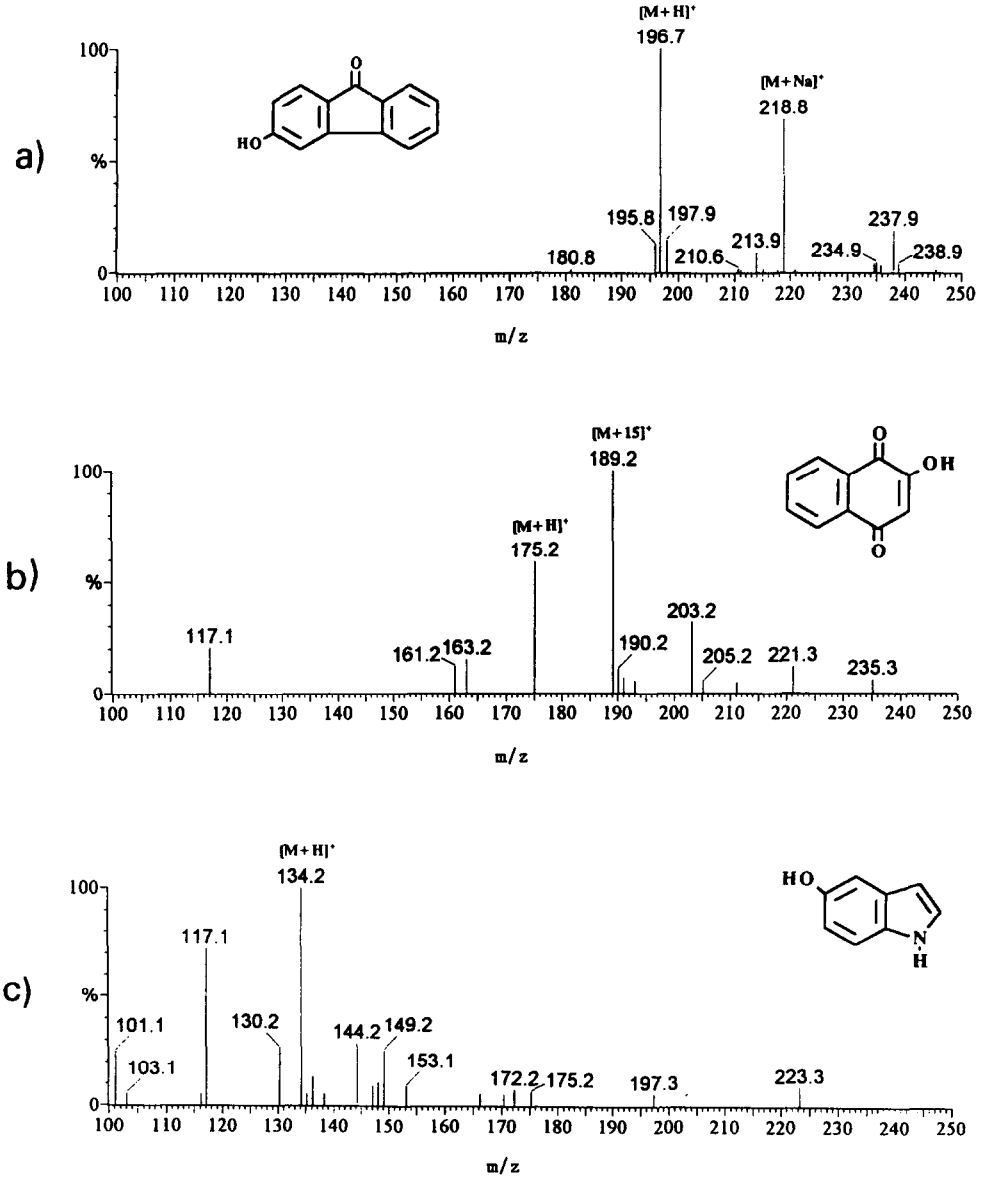


Fig. 2. Positive-ion ES ionization mass spectra of (a) 2-H-9-FLO (M_r = 196), (b) 2-H-1,4-NQ (M_r = 174) and (c) 5-HI (M_r = 133).

oligomer peaks in the complex spectra obtained with the mixtures used to cover the entire mass range.

Cesium iodide cluster ions $[Cs_nI_{n-1}]^+$, and other alkali metal halide salts, are routinely used as mass calibration standards in fast atom bombardment (FAB) and liquid secondary ion (LSI) MS. Anacleto et al. [31] reported, however, that cesium iodide was difficult to remove completely from the ES ionization source, and provided a source of contamination that hindered the ES process. There is a clear need for a more universal calibration method in ES.

An alternative approach to calibration for LC–MS is to use the mobile phase itself. It has been shown that acetic acid–ammonia cluster ions $[(CH_3COOH)_x + (NH_3)_4 + NH_4]^+$ can be generated under certain conditions in TSP ionization to provide a convenient mass calibration over the mass range m/z 100–1000 [29–31].

In positive ES, calibration was performed using the mobile phase ions. Fig. 1a shows the spectrum obtained for the calibration in which the ammonium–methanol cluster ions predominate.

The spectrum shown in Fig. 1b was obtained by FIA of NaI and the $[Na_nI_{n+1}]^-$ ion series is observed. In addition satellite peaks at lower masses are also observed for each of the $[Na_nI_{n+1}]^-$ clusters. These satellite peaks corresponded to a progressive replacement of iodide (127 u) by the formiate anion ($HCOO^-$; 45 u) of the mobile phase. Sodium iodide as calibrant has the advantage that both sodium and iodine are monoisotopic. Formic cluster ions were also used for calibration purposes.

3.2. Mechanisms of ionization

Organic compounds introduced into the source as sample components can be ionized in a variety of ways. Proton addition to form $[M + H]^+$ and proton loss to form $[M - H]^-$ are common routes of ionization for gas-phase bases and gas-phase acids, respectively [32,33].

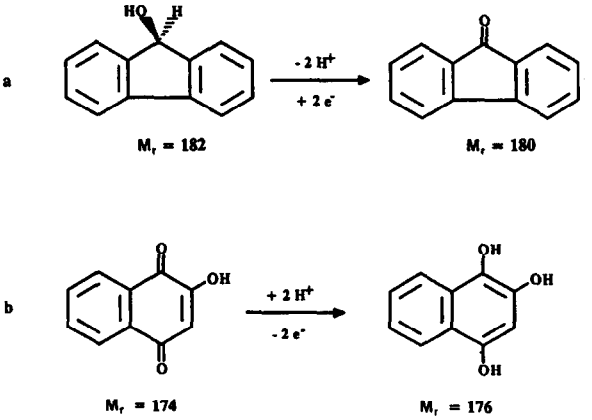
The general reaction to form $[M + H]^+$ occurs whenever M is a stronger gas-phase base than AH and can be written as:

$$M + AH \rightarrow [M + H]^+ + A^-$$

So α-β-unsaturated ketones (2-H-9-FLO and 2-H-1,4-NQ) and amines (5-HI) are stronger gas-phase bases than methanol and formic acid, and their mass spectra (Fig. 2) are dominated by abundant $[M + H]^+$ species and some $[M + NH_4]^+$ and $[M + Na]^+$ species. Because sodium was not introduced into the reference mixture, the $[M + Na]^+$ could come from contamination of the samples, the solvents, or the glassware.

Both 9-HFL and 1-HI are hydroxy non-aromatic compounds and their acidity and probably their proton affinities are low. Both of them gave the $[M - 17]^+$ ion in positive electrospray as base peak (Fig. 3). This fragmentation is similar to that obtained when positive chemical ionization with methane as reagent gas was used (Fig. 4). The $[M + H]^+$ ions obtained from cyclic alcohols are unstable and the loss of H_2O is the most probable path of fragmentation [34].

Electrochemical oxidation reaction may be occurring at the metal–liquid interphase of the capillary tip. The actual oxidation reaction(s) depend on the electrical potential at given locations of the metal–liquid interface and on the electrochemical oxidation potential for the given reaction(s). Oxidation may explain the ion m/z 181 in the spectrum of the 9-HFL (Fig. 3a), which corresponds to the protonation of the oxidation product (Scheme 1a).



Scheme 1. Electrochemical reactions of (a) 9-HFL and (b) 2-H-1,4-NQ.

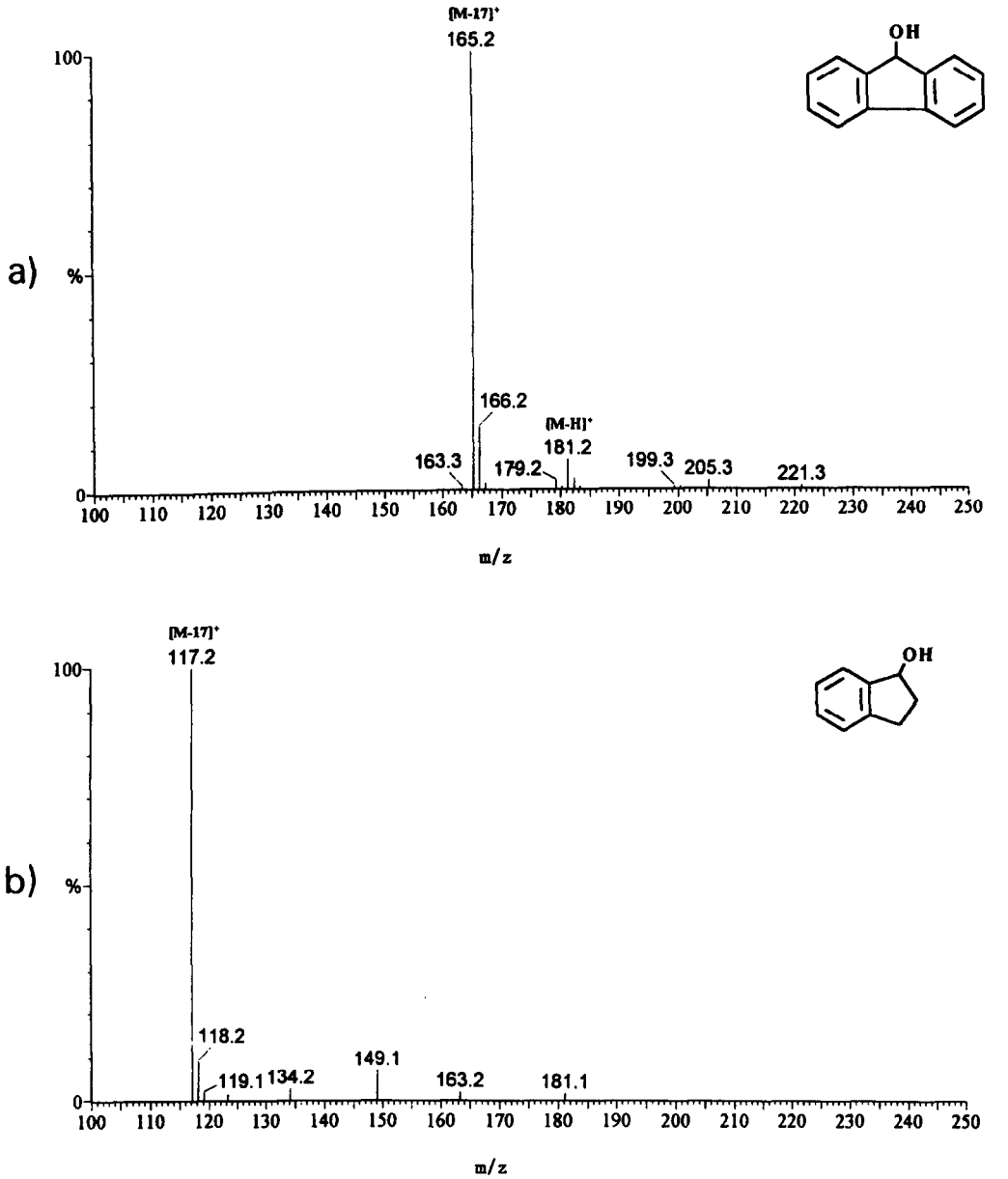


Fig. 3. Positive-ion ES ionization mass spectra of (a) 9-HFL (M_r = 182) and (b) 1-HI (M_r = 134).

For the 2-H-1,4-NQ the $[M + 15]^+$ ion (m/z 189) (Fig. 2a) presumably arises from oxidation of the phenol to a ketone, followed by the addition of CH_3.

In negative ES the reduction of quinone group for the 2-H-1,4-NQ can be observed (Fig. 5a). The $[M + H]^-$ (m/z 175) could be the reduction product (Scheme 1b).

As has been mentioned proton loss $[M - H]^-$ is a common route of ionization for gas-phase acids in negative ES if ions of sufficient basicity are present. The general reaction is:

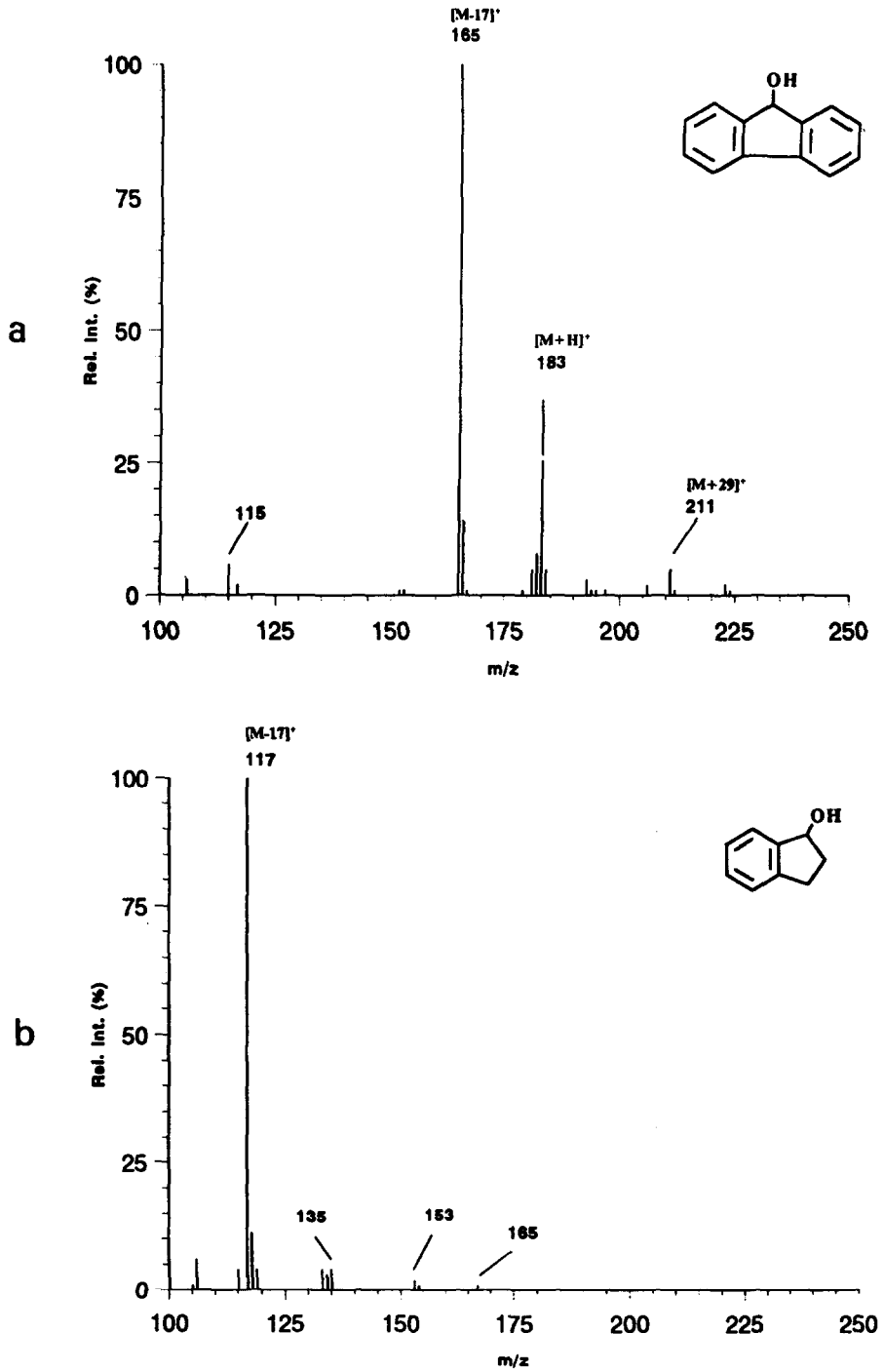


Fig. 4. Positive chemical ionization mass spectra, with methane as reagent gas, of (a) 9-HFL (M_r = 182) and (b) 1-HI (M_r = 134).

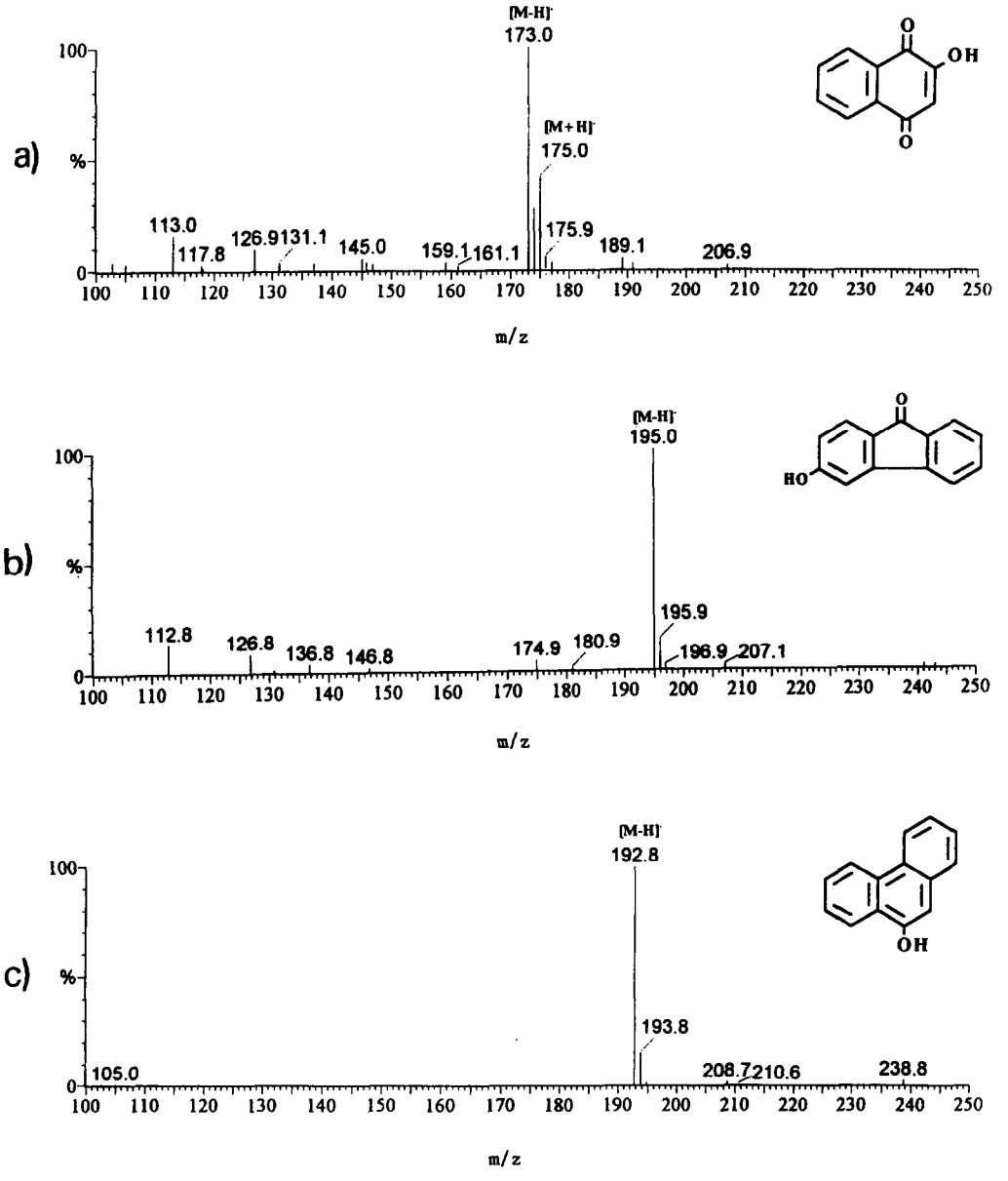


Fig. 5. Negative-ion ES ionization mass spectra of (a) 2-H-1,4-NQ ($M_r = 174$), (b) 2-H-9-FLO ($M_r = 196$) and (c) 9-HF ($M_r = 194$).

$$M + A \rightarrow [M - H]^- + [AH]^+$$

and will occur whenever A is a stronger acid than M. Dissociation constants for organic compounds in different solvents have hardly any relevance for gas-phase work. When a formic stream is vaporized through the ES source $[HCOO]^-$ ions are formed. If a gas-phase acid is introduced, ionization to $[M - H]^-$ occurs if M is a stronger gas-phase acid than HCOOH. Moreover, non-aromatic alcohols (9-HFL, 1-HI) and some phenols (5-HI) are weaker than formic acid, and do not ionize under these conditions. Fig. 5 shows the negative ES spectra for 2-H-1,4-

NQ, 2-H-9-FLO and 9-HF which are dominated by the $[M-H]^-$ ion.

3.3. HPLC–MS

Few studies are available on the separation of these compounds by HPLC, only some naphthols have been separated using C_8 column with acetonitrile–acetic buffer as mobile phase and UV detector [35]. A synthetic mixture of the six hydroxy-PAHs was prepared and used for HPLC on a C_{18} reversed-phase column. Satisfactory separation and well shaped peaks for the aromatic compounds were only obtained at low pH. The system was run in the gradient mode at 35 μl min^{-1}. Solvent A was methanol and solvent B was formic acid–ammonium formiate 10 m*M* (pH 3). The gradient was: 45% A from 0 to 10 min, a linear increase to 60% A from 10 to 25 min and isocratic elution at this composition for 5 min. The chromatogram obtained using UV detection is given in Fig. 6. Low resolution between 1-HI and 2-H-1,4-NQ can be observed and coelution of 9-HFL and 2-H-9-FLO occurred.

Poor resolution can be avoided using a specific detection method such as MS using the hydroxy-PAHs spectra to select the monitoring masses for each compound. In positive ES single-ion monitoring (SIM) of $[M+H]^+$ ion at *m/z* 197 and *m/z* 134 for the 2-H-9-FLO and 5-HI respectively, the $[M-17]^+$ fragment ion at *m/z* 165 and *m/z* 117 for the hydroxy non-aromatic com-

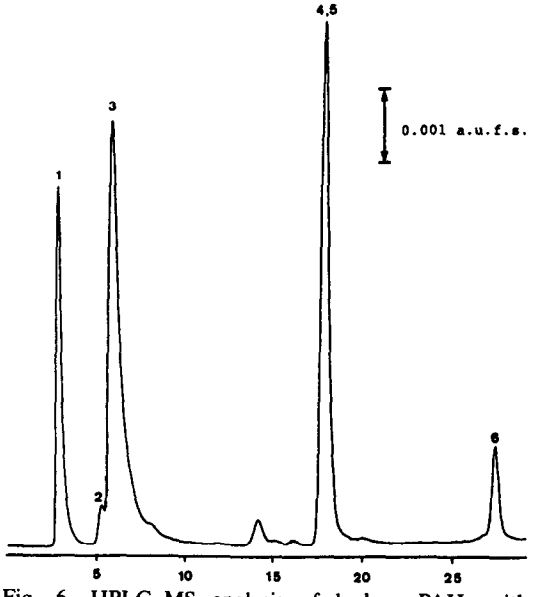


Fig. 6. HPLC–MS analysis of hydroxy-PAHs with UV detector (280 nm). Components in the mixture were (1) 5-HI, (2) 2-H-1,4-NQ, (3) 1-HI, (4) 9-HFL, (5) 2-H-9-FLO and (6) 9-HF.

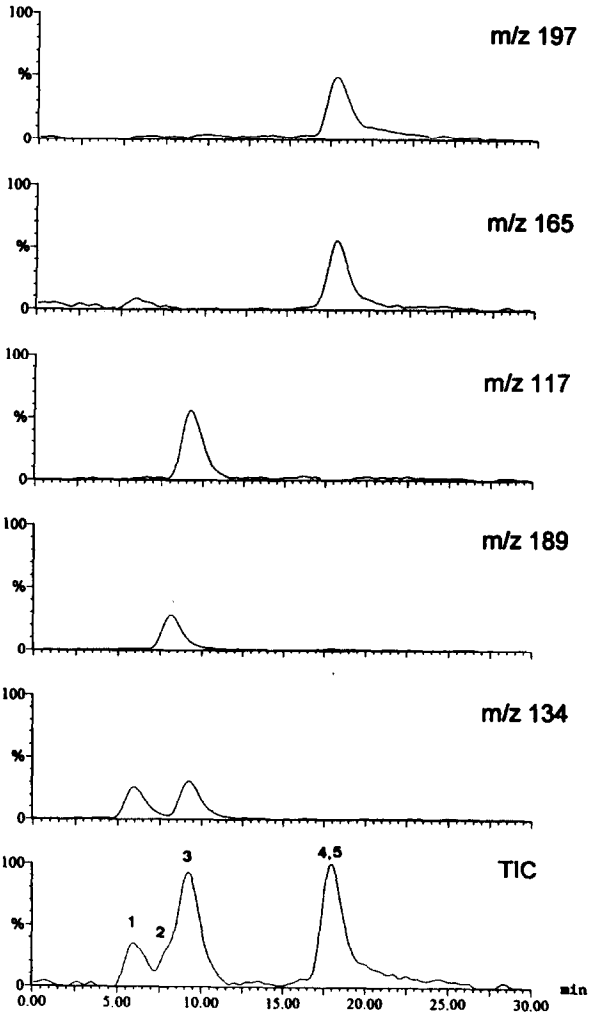


Fig. 7. HPLC–MS analysis in positive electrospray ionization of hydroxy-PAHs. The lower trace is the TIC obtained by summing all ions above. Components in the mixture were (1) 5-HI, (2) 2-H-1,4-NQ, (3) 1-HI, (4) 9-HFL and (5) 2-H-9-FLO.

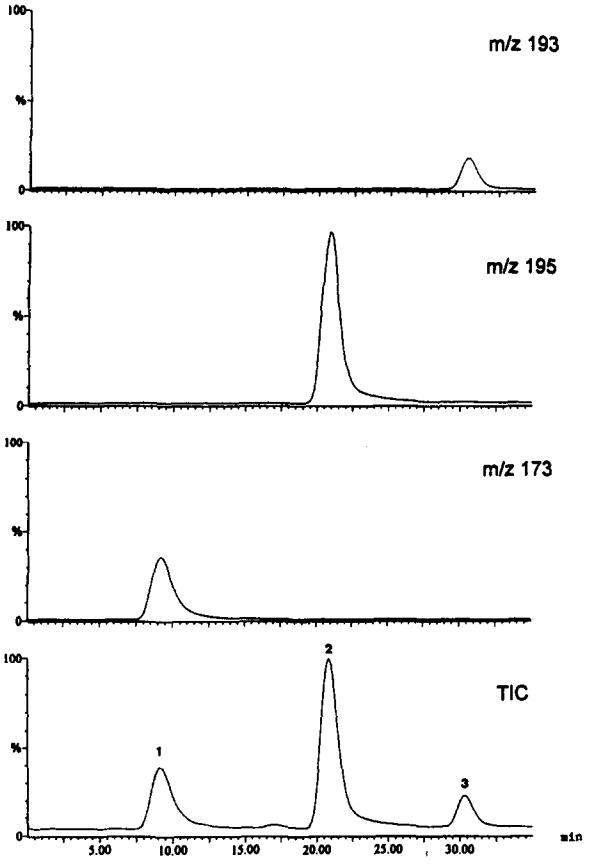


Fig. 8. HPLC–MS analysis in negative electrospray ionization of hydroxy-PAHs. The lower trace is the TIC obtained by summing all ions above. Components in the mixture were (1) 2-H-1,4-NQ, (2) 2-H-9-FLO and (3) 9-HF.

pounds (9-HFL, 1-HI) and $[M + 15]^+$ ion at m/z 189 for the 2-H-1,4-NQ were used. The reconstructed ion liquid chromatograms for each mass and the total ion chromatogram (TIC) are given in Fig. 7, showing that quantification at the 50-ng level is possible.

In negative ES the $[M - H]^-$ ion was monitored for each hydroxy aromatic compound 9-HF, 2-H-9-FLO and 2-H-1,4-NQ. The reconstructed-ion liquid chromatograms are shown in Fig. 8.

Negative ES showed lower background current and the response in negative ES was 15-fold that for the 2-H-9-FLO and 2-H-1,4-NQ than positive ES which would lead to improved sensitivity.

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