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Investigation of the separation of heterocyclic aromatic amines by reversed phase ion-pair liquid chromatography coupled with tandem mass spectrometry: The role of ion pair reagents on LC–MS/MS sensitivity

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Abstract

Reversed phase ion-pair chromatography (RP-IPC) of seven heterocyclic aromatic amines encompassing quinoline (IQ, MeIQ), quinoxaline (MeIQx), pyridine (PhIP) and carboline derivatives (AαC, Harman, Norharman) was carried out with formate as counter ion in an aqueous eluent with acetonitrile as organic modifier. TSKgel ODS-80TS was used as the stationary phase. With the aim of acquiring a better insight into the mutual influence of ion-pair reagent and the organic modifier upon solute retention, the study was performed by using an experimental design approach able to evidencing the effect of the simultaneous variation of the two factors. A model for the chromatographic behavior of the amines is proposed that includes classical ion-pair mechanism involving formate in the case of MeIQx, PhIP, Harman and Norharman. A competitive ion-exchange mechanism was hypothesized to govern retention of quinoline compounds, whereas electrostatic interactions and hydrogen bond formation with the silanols of the stationary phase were judged to be responsible for the retention of AαC. Further, the chromatographic behavior of the analytes using the formic acid-ammonium formate buffer in the mobile phase was compared with that observed using acetic acid-ammonium acetate buffer. The method based on the use of RP IPC with tandem mass spectrometry when the eluent contained formate buffer at pH 2.8 exhibited higher detectability with respect to that achieved using the acetate buffer.
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Keywords: Heterocyclic aromatic amines; Liquid chromatography–mass spectrometry; Ion-pair reagents

1. Introduction

Heterocyclic aromatic amines (HAAs) are biologically active substances identified in a wide variety of food [1]. These compounds are known to possess a high mutagenic activity in the Ames test [2] and are recognized as possible human carcinogens. The major pathway for the metabolic activation of HAAs starts with the hydroxylation of the hexocyclic amino group, catalyzed mainly by cytochrome P4501A2, followed by acetylation or sulphation to form direct-acting reactive mutagens that alter DNA and genome [3].

As illustrated in a review devoted to the determination of HAAs in foods [4], high-performance liquid chromatography (HPLC) and gas chromatography (GC) are the techniques predominantly used for analysis of these substances, GC being less convenient than HPLC because of the need of chemical derivatization. As regards to HPLC, separation of HAAs is commonly performed on a reversed-phase (RP) C_{18} column [5–15]. However, a drawback of ordinary RPLC is that organic solute ions exhibit poor peak shapes and inadequate retention. In this context, ion pair RPLC represents a more effective technique for retention

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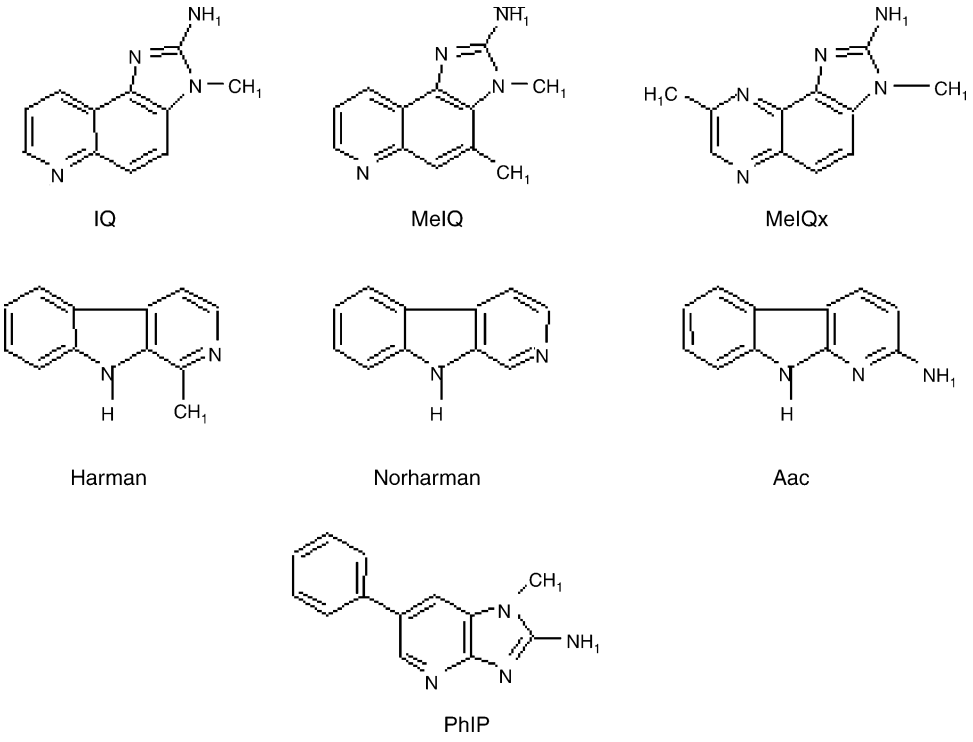

Fig. 1. Chemical structures of heterocyclic amines investigated.

of basic compounds because of the improvement in selectivity and peak shape of ionizable solutes. In spite of this, to our knowledge this mechanism has been scarcely investigated for separation of HAAs [16]. In that paper the development of a method for determination of some HAAs by ion-pair chromatography on a new phenyl-hexyl stationary phase with coulometric electrode array detection is described [16].

In the past few years many papers dealing with the analysis of these mutagenic compounds by LC coupled with mass spectrometry (MS) and tandem mass spectrometry (MS/MS) have been published [5,7,8,10,12–17]. In most of these studies, LC has been performed under RP conditions using buffered mobile phases, salts used as buffers likely acting as ion-pair reagents. However, taking into account that in LC–MS systems the performance of electrospray (ESI) and atmospheric pressure chemical ionization (APCI) is known to be affected by the solvent composition, scarce attention has been paid to the evaluation of the ion pair reagent capable of providing the highest sensitivity when MS and MS/MS detection is used.

In the present investigation we studied the influence of the concentration of a common volatile ion pairing reagent suitable for LC–MS separation and the percentage of the organic modifier on the retention of seven HAAs encompassing quinoline (IQ, MeIQ), quinoxaline (MeIQx), pyridine (PhIP) and carboline derivatives (AαC, Harman, Norharman) (Fig. 1). With the aim of acquiring a better insight into the mutual influence of these chromatographic factors upon solute retention, the study was performed by using an experimental design approach able to evidencing the effect of the simultaneous variation of the two factors. Further, the chromatographic behavior of the analytes using the formic acid-ammonium formate buffer was compared with that observed using acetic acid-ammonium acetate buffer in the mobile phase. LC–MS/MS method based on the use of formate-based eluent exhibited higher detectability with respect to that achieved using the acetate buffer.

2. Experimental

2.1. Chemicals

Harman (2-methyl-β-carboline) and norharman (9H-pyrido[3,4-*b*] indole) were from Sigma–Aldrich (Germany). IQ (2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline), MeIQ (2-amino-3,4-dimethyl-3H-imidazo[4,5-*f*]quinoline), MeIQx (2-amino-3,8-dimethyl-3H-imidazo[4,5-*f*]quinoxaline), PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine), AαC (2-amino-9H-pyrido[2,3-*b*]indole) were from Toronto Research Chemicals Inc. (Canada).

Stock standard solutions of HAAs at concentrations of 0.5 mg/ml were prepared in methanol and stored in the dark at 4 °C. Working standard solutions were prepared daily by diluting stock solutions with HPLC-grade water. Ammonium formate, formic acid, ammonium acetate, acetic acid, HPLC-grade acetonitrile and HPLC-grade water were from Carlo Erba (Milan, Italy).

2.2. Instrumentation and mass spectrometry parameters

An Alliance 2690 liquid chromatograph (Waters, Milford, MA, USA) equipped with a 120-vial capacity sample management system was used.

A Quattro LC triple quadrupole-mass spectrometer (Micromass, Manchester, UK) with a pneumatically-assisted electrospray interface was used. Data acquisition was performed in positive ion (PI) mode. Interface parameters were set as follows: capillary voltage 2.8 kV, cone voltage 50 V (IQ, MeIQ, MeIQx, Norharman, Harman, PhIP) and 40 V (AαC), extractor lens voltage 2 V, source temperature 110 °C, desolvation temperature 240 °C, rf lens 0.1 V. The nebulizer and desolvation gases (nitrogen, 99.999% high-purity) were delivered at 60 and 550 l/h, respectively.

Experiments for optimization of ESI interface parameters were performed by directly infusing solutions into the ESI–MS system at a flow-rate of 6 μl/min. Operating in MS/MS mode, selected reaction monitoring (SRM) analyses were performed as follows: m/z 199 → 184 (collision energy, CE 26 eV) for IQ, m/z 213 → 198 (CE 27 eV) for MeIQ, m/z 214 → 131 (CE 40 eV) for MeIQx, m/z 169 → 115 (CE 32 eV) for Norharman, m/z 183 → 115 (CE 32 eV) for Harman, m/z 225 → 210 (CE 30 eV) for PhIP, m/z 184 → 134 (CE 30 eV) for AαC. The dwell time and the interchannel delay were set at 0.25 and 0.01 s, respectively. For data acquisition and processing the Masslynx v4.0 software was used.

All statistical analyses and tests were performed by using the statistical package SPSS v. 9.0 for Windows (SPSS, Bologna, Italy).

2.3. Influence of pH on chromatographic retention of HAAs

The experiments were carried out on aqueous standard solutions of IQ, MeIQ, MeIQx, Norharman, Harman, PhIP and AαC each at the concentration of 100 μg/l. HAAs were classified in two groups depending on their lipophilicity, the most polar analytes IQ-compounds being grouped into the group I, whereas the most lipophilic amines Norharman, Harman, PhIP and AαC being classified in the group II. The pK_{a1} values of HAAs were estimate to be in the 5–9 range by using NIST pK_a database.

Separation was carried out on the TSKgel ODS-80TS (250 mm × 2.0 mm, 5 μm) (TOSOH BIOSEP, Germany) column. The effect of pH on the chromatographic behavior of HAAs was investigated by using a mixture of ammonium formate-formic acid aqueous solution at three different pH values (pH 2.8, 3.7 and 4.7) (eluent A) and acetonitrile (eluent B). The mobile phase was delivered under isocratic conditions at a flow-rate of 0.2 ml/min and two different compositions were used: aqueous solution of ammonium formate/formic acid at various pH/acetonitrile 90/10 (v/v) for the separation of IQ, MeIQ and MeIQx, and aqueous solution of ammonium formate/formic acid at various pH/acetonitrile 80/20 (v/v) in the case of Harman, Norharman, PhIP and AαC. The salt concentration was kept constant at 3.03 mM.

2.4. Study of the ion-pair chromatographic behavior of HAAs by experimental design

Separation of the two groups of HAAs each at the concentration of 100 μg/l was carried out on the TSKgel ODS-80TS column under isocratic elution using the aqueous solution of ammonium formate/formic acid buffer at pH 2.8 and acetonitrile as eluent delivered at a flow-rate of 0.2 ml/min. The nominal level of ammonium formate concentration was 3.03 mM, whereas the nominal level of acetonitrile percentage was 10% for the IQ-compounds and 20% for the analytes of group II.

A 2^2 two-levels full factorial design (FFD) [19] was performed to investigate the effects of the percentage of acetonitrile (ACN) as the organic modifier and the content of ammonium formate (FOR) in the aqueous phase on retention of HAAs. Low and high levels were: ACN = 7–13% (v/v), FOR = 1.01–5.05 mM for group I and ACN = 15–25% (v/v), FOR = 1.01–5.05 mM for group II. The measured response was the capacity factor k'.

The order of experiments was randomized in order to avoid possible memory effect of the analytical apparatus.

A F-test comparing the experimental and calculated responses at the centre of the experimental domain was performed to evaluate the existence of relevant quadratic effects [20]:

$$F_{\text{calc}} = \frac{(y_f - y_o)^2}{s_o^2(1/N_f + 1/N_o)}$$

where y_f is the mean of the estimated values obtained for the experiments of the FFD, i.e. the calculated response at the centre of the experimental domain; y_o the mean of the replicated measurements at the centre of the experimental domain; N_f the number of factorial experiments; N_o the number of experiments at the centre of the experimental domain and s_o the experimental error estimated through the replicated measurements at the centre point. A 95% confidence level was chosen.

A star design [20] was added to the factorial design experiments since some analytes showed relevant quadratic effects.

The final regression models were then calculated using the central composite design (CCD) experiments obtained both from the full factorial design and the star design.

For each compound the following polynomial model was fitted:

$$\hat{y} = b_0 + \sum_{i=1}^{2} b_i x_i + \sum_{i=1}^{2}\sum_{j=1}^{2} b_{ij} x_i x_j$$

where $\hat{y}$ is the predicted response and the x_i variables are the coded values of the factors. The b values are the estimated polynomial coefficients: b_0 the intercept term, b_i coefficients

represent the main effect for each variable, b_{ij} coefficients in the quadratic terms are responsible for the curvature effects and $b_{ij(i\neq j)}$ coefficients describe the interaction effects.

The best regression models were obtained by a forward search step-wise variable selection algorithm with a $F_{\text{to enter}} = 2$.

2.5. Influence of buffers on the ion-pair chromatography of HAAs and on sensitivity of the LC–ESI–MS/MS methods

To compare the chromatographic behavior of HAAs in dependence of the type of buffer, two different mobile phases were used, i.e. ammonium acetate and ammonium formate buffer-based eluents, and elution of HAAs was obtained under gradient conditions.

In the case of formate buffer, the mobile phase consisted of a mixture of ammonium formate-formic acid buffer (3.03 mM ammonium formate, pH 2.8) aqueous solution (eluent A) and acetonitrile (eluent B). In the case of acetate buffer, the mobile phase consisted of a mixture of ammonium acetate-acetic acid buffer (3.03 mM ammonium acetate, pH 4.0) aqueous solution (eluent A) and acetonitrile (eluent B). In both cases, the gradient was as follows: at 0 min 5% B, in 20 min 54.5% B hold for 3 min, back to 5% B in 3 and 10 min to re-equilibrate the column. The mobile phase was delivered at 0.2 ml/min.

For the two different chromatographic systems the instrumental detection limits (LOD) of the LC–ESI–MS/MS methods were calculated. Following a statistical approach [21], detection limit (y_D) were calculated as signals based on the mean blank ($\bar{y}_b$) and the standard deviation (s_b) of the blank signals as follows:

$$y_D = \bar{y}_b + 2ts_b, \quad y_Q = \bar{y}_b + 10\,s_b$$

where t is a constant of the t-Student distribution (one-sided) depending on the confidence level and the degrees of freedom ($\nu = n - 1$, n = number of measurements). Ten blank measurements were performed to calculate $\bar{y}_b$ and s_b. A solvent mixture of water/acetonitrile (85/15, v/v) was used as the blank solution. To estimate LODs, y_D were converted from the signal domain to the concentration domain using an appropriate calibration function.

3. Results and discussion

3.1. Chromatographic behavior of heterocyclic aromatic amines as a function of pH

Under ESI(+)–MS conditions, mass spectra of all compounds showed predominant $[M + H]^+$ ions without fragmentation; no adducts with alkali metals were observed.

The product-ion MS/MS spectra as obtained by low-energy collision-induced dissociation of protonated molecules showed fragmentation patterns for IQ, MeIQ, MeIQx, Harman and PhIP dominated by the loss of the methyl group leading to product ions at m/z 184, m/z 198, m/z 199, m/z 168 and m/z 210, respectively. Additional loss of 27-amu fragments accounted for the release of the HCN moiety from the $[M + H\text{-}CH_3]^+$ ions of IQ and MeIQ. In addition, peaks at m/z 142 and m/z 115 resulting from the elimination of one and two molecules of HCN, respectively, from the $[M + H]^+$ ion were detected in the MS/MS spectrum of Norharman. In the case of AαC, the $[M + H\text{-}NH_3]^+$ and $[M + H\text{-}2HCN\text{-}NH_3]^+$ fragment ions were visible.

The separation of the investigated ionizable analytes was performed by ion-pair reversed liquid chromatography. A TSKgel ODS-80TS with a full endcapping was chosen to perform separation of HAAs and the mobile phases containing ammonium formate at pH 2.8, 3.7 and 4.7 were considered in order to investigate the influence of pH on retention of amines. As reported in Section 2, HAAs were divided in two groups on the basis of their different lipophilicity. The effects of pH were investigated by maintaining constant acetonitrile percentage and salt concentration in the mobile phase. It was observed that under isocratic conditions pH values higher than 3.7 produced broad peaks of all analytes (Fig. 2a–d). At pH 4.7, this effect was marked for IQ-compounds, probably due to a strong interaction with the residual silanols of the stationary phase in spite of the complete endcapping of the column (Fig. 2a). At this pH a change in selectivity as for the elution of MeIQ and MeIQx was also observed. In fact, by varying pH in the 2.8–4.7 range the retention time of MeIQx was not influenced by this parameter, whereas increasing pH, MeIQ was significantly retarded. In addition, at pH 4.7 the carbolines Harman and Norharman coeluted (Fig. 2b) and their peaks were baseline resolved at pH 3.7. By decreasing pH from 4.7 to 2.8, a reduction of the retention time for almost all analytes together with the improving of peak shape was observed. At lower pH, the number of SiO^- groups of the stationary phase significantly decreases by reducing interaction with the analytes. In particular, pH reduction affected significantly retention time of PhIP and AαC, the first eluting at 26.0 and 13.5 min at pH 4.7 and pH 2.8, respectively, the latter eluting at 63.3 min (pH 4.7) and at 18.6 min (pH 2.8) (Fig. 2b and f). It could also be observed that PhIP and AαC were the most retained compounds among the HAAs investigated, showing particularly at higher pH retention not rationalizable on the basis of the lipophilicity of the analyte. When operating at pH 2.8, this behavior could be explained on the basis of the capability of both compounds having N–H-acceptor and N–H-donor groups on the same part of the molecule to form strong hydrogen bonds with the SiOH and the $Si–O^-$ groups of the stationary phase. Electrostatic interactions between deprotonated silanols and protonated molecules could be responsible for the late elution of the two compounds when operating at pH 4.7.

The improved peak shape shown by all the HAAs when operating at pH 2.8 could be useful for ensuring higher detectability for a LC–MS determination of the analytes if compared with the other mobile phase conditions investigated.

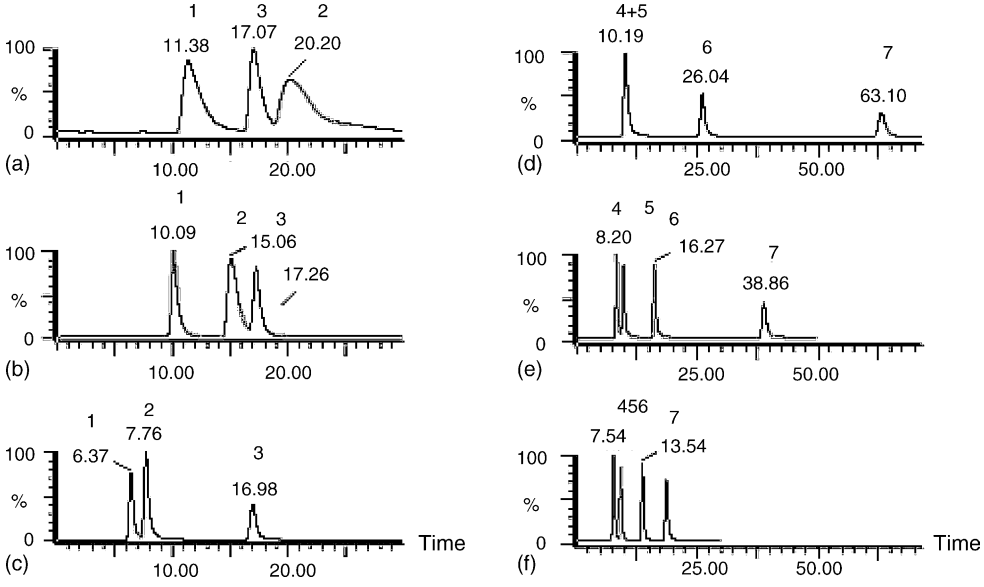

Fig. 2. LC–ESI(+)–MS/MS chromatograms of HAA standard solution (100 μg/l). Eluent: ammonium formate/formic acid at different pH values: (a and b) pH 4.7; (c and d) pH 3.7; (e and f) pH 2.8. Peaks: (1) IQ, (2) MeIQ, (3) MeIQx, (4) Norharman, (5) Harman, (6) PhIP, (7) AαC.

3.2. Chromatographic behaviour of heterocyclic aromatic amines as a function of ion pair reagent concentration and organic modifier percentage

An experimental design was then used to study the effect both of ACN and formate on the chromatographic behavior of HAAs. The nominal conditions, i.e. the centre of the experimental domain for all the analytes were chosen on the basis of the previously defined chromatographic separation. In particular, all the experiments were performed at pH 2.8 and by using isocratic elution. The experimental domain was defined taking into account instrumental and operative limits, namely ACN percentages lower than 7 and 15% for group I and II, respectively, were found to determine long analysis times, whereas values higher than 13 and 25% were found to cause the co-elution of some compounds. Finally, FOR concentrations lower than 1.01 mM were considered not adequate for the ion-pair formation, since concentrations of about 5 mM are commonly used.

In order to evaluate repeatability of the measurements, eight replicates at the centre of the experimental domain were added before and after performing the factorial design experiments for each group under investigation. The main and interaction effects were then calculated for each analyte. The presence of curvature was tested by the *F*-test described in Section 2. All the amines showed a significant curvature, F_{calc} values being higher than the $F_{tab(1,7,\alpha=0.05)} = 5.59$, thus indicating that quadratic models have to be used. Additional measurements corresponding to a star design were then added in order to investigate which variables were responsible for the quadratic effects.

The regression models calculated by forward regression analysis are described in Table 1. From this Table, it can be observed that:

- the coefficients of ACN were negative for all the analytes;
- the coefficients of formate were positive for all the amines with the exception of IQ and MeIQ, whereas this factor resulted to be not significant for AαC at 5% significance level.

As expected, the negative values of the coefficients of the organic modifier in the regression model indicate a lower re-

Table 1
Coefficients of the significant factors ($\alpha < 0.05$) for each of the components obtained after the two-factor central composite design study[a]

Analytes	IQ	MeIQ	MeIQx	Norharman	Harman	PhIP	AαC
Constant	0.910 ± 0.007	1.287 ± 0.009	3.888 ± 0.048	1.312 ± 0.013	1.808 ± 0.021	3.038 ± 0.030	4.569 ± 0.041
Coefficients							
CAN	−0.832 ± 0.007	−1.259 ± 0.009	−4.098 ± 0.043	−1.152 ± 0.013	−1.733 ± 0.021	−3.085 ± 0.03	−4.779 ± 0.041
FOR	−0.042 ± 0.007	−0.100 ± 0.009	0.468 ± 0.043	0.198 ± 0.013	0.277 ± 0.021	0.417 ± 0.003	–
ACN × FOR	0.059 ± 0.009	0.106 ± 0.011	−0.331 ± 0.053	−0.133 ± 0.016	−0.203 ± 0.025	−0.324 ± 0.037	–
ACN × CAN	0.390 ± 0.010	0.598 ± 0.012	2.099 ± 0.065	0.469 ± 0.019	0.761 ± 0.029	1.388 ± 0.042	1.874 ± 0.058
FOR × FOR	–	–	−0.262 ± 0.065	–	–	–	–
r^2	0.999	0.999	0.998	0.997	0.997	0.998	0.999

[a] Not significant ($\alpha = 0.05$).

tention of HAAs with increasing of the ACN percentage. In particular, the main effects attributable to the organic modifier content were characterized by the highest absolute values for AαC and MeIQx, thus evidencing that hydrophobic interactions are also important for retention of these amines.

The effect of formate was different for the various amines investigated: in the case of MeIQx, Norharman, Harman and PhIP, k' values were found to increase with increasing concentrations of the ion pair reagent. In general, for all the analytes with the exception of AαC, formate coefficients were significant and lower than those obtained in the case of ACN, thus suggesting that ACN played an important role in the amine separation. AαC exhibited an anomalous behaviour, since the main effect referred to formate was found to be not significant and the linear term including the coefficient relative to formate did not appear in the regression model. These findings confirm that the chromatographic behavior of AαC is not ruled by a classical ion-pair mechanism, as discussed previously. Instead, competitive interactions with the column packing surface residual silanols of the stationary phase and, in particular, hydrogen bonds and ionic interactions between the protonated AαC and the free silanols could be responsible for the retention of this amine, as already observed by varying pH. At pH 2.8, AαC being protonated, the ion-pair formation should not be favored because of the possible delocalization of the positive charge on the three nitrogen atoms of the molecule. Accordingly, with the exception of AαC significant interactions between ACN and FOR were found for all the analytes (Table 1). In the case of MeIQx, Norharman, Harman and PhIP the calculated coefficients were negative showing a concurrent effect of ACN and FOR, whereas positive values were obtained for IQ and MeIQ, thus evidencing an opposite effect between the two factors.

Since the analytes showed different behaviors regarding the effect of the pairing ion concentration, further experiments were carried out following the one-variable-at-a-time (OVAT) approach by focusing attention on this factor. In particular, the capacity factor of the ionized solutes was investigated with changing pairing ion concentration in the 0.2–20 mM and 1–10 mM range for group I and group II amines, respectively. As depicted in Fig. 3, retention of MeIQx, Norharman, Harman and PhIP increases with increasing the concentration of the ion pair reagent until a plateau is reached. The trend observed for AαC confirmed that retention of this amine is virtually independent of the pairing ion content under the given conditions, whereas in accordance with the regression model calculated by experimental design a decrease in k' of IQ and MeIQ was observed.

The chromatographic behavior exhibited by the HAAs is in accordance with the studies and the models described in literature [22–27]. According to the solvophobic theory proposed by Horvath [23], solute retention in IPC is determined not only by the charge but also by solute size and configuration. Similarly, Karger et al. expressed the ion-pair association constant K_{IP} mainly as a function of the non-coulombic contributions to K_{IP} and of the ion charge and ion size [24].

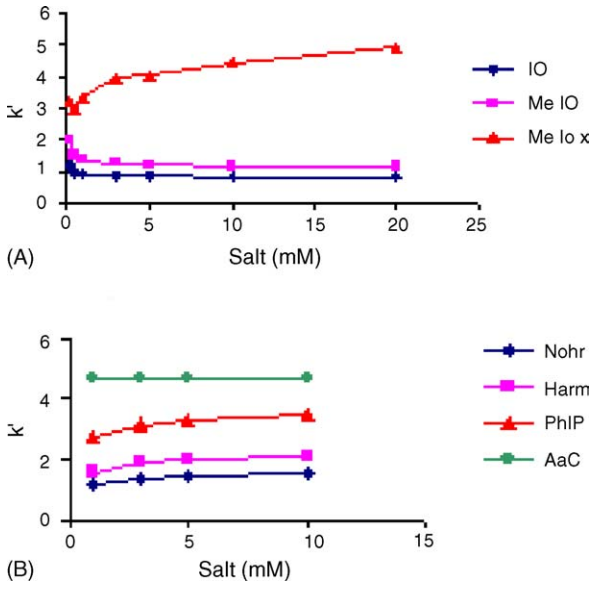

Fig. 3. Plot of k' against the concentration of the ion pairing reagent.

Therefore, various effects like hydrophobic interactions, the presence of substituent groups and salt addition are able to modify solute retention in IPC. In the present case, an ion-pair mechanism as well as an ion-exchange interaction and non-coulombic contributions could be proposed to describe the amine separation. The retention of MeIQx, Norharman, Harman and PhIP could be explained by an ion-pair mechanism involving the formate ion, the ion-pair formation occurring mainly in the mobile phase. In the case of these amines, the increase of k' until the achievement of a plateau could be ascribed to the decreased capacity of the stationary phase to retain the ion-pairs owing to a reduction of the available surface area. In fact, with increasing formate concentration ammonium concentration increases, competing thus with the analytes for interactions with the stationary phase.

The results obtained for AαC confirmed the behavior previously observed from the model calculated from the experimental design.

The opposite behavior exhibited by IQ and MeIQ could be explained taking into account that a competitive ion-exchange mechanism governs their retention. In fact, on the basis of their chemical structure it is possible to observe that IQ and MeIQ can carry a second positive charge on the N-quinoline group (pK_a 3–4.5) that can interact with the SiO^- of the stationary phase or the formate in the mobile phase in a mechanism involving ion-exchange. Increasing the ion pair reagent increases the concentration of ammonium ions, which then mediate in this ion-exchange interaction. Since the silanols available for the ion-exchange interaction decreases with increasing concentration of the pairing ion reagent, k' values will decrease at higher concentration of the latter. Under these conditions, the other IQ-compound MeIQx did not exhibit this behavior because of the lower pK_a value of the N of the pyrazine ring (typically lower than 2) with respect to quinoline group. Among the amines investi-

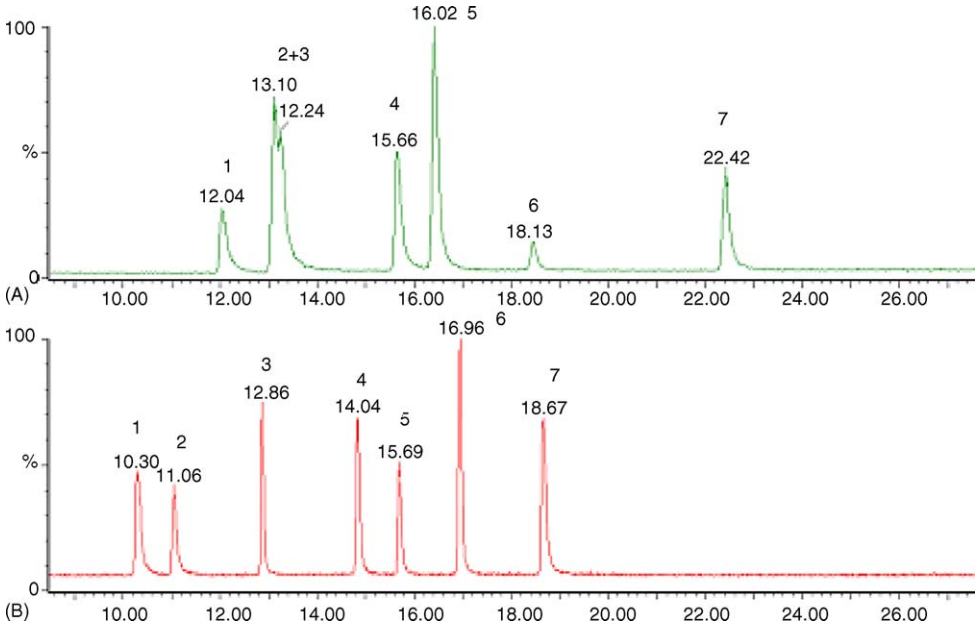

Fig. 4. LC–ESI(+)–MS/MS extracted ion chromatograms of HAA standard solution (50 μg/l) obtained under gradient elution by using (A) acetate buffer and (B) formate buffer in the mobile phase. Peaks: (1) IQ, (2) MeIQ, (3) MeIQx, (4) Norharman, (5) Harman, (6) PhIP, (7) AαC. For chromatographic conditions see Section 2.

gated, in addition to IQ and MeIQ only PhIP could carry a second positive charge, but its retention was governed by the classical ion-pair mechanism involving the formate ion. Such results could find an explanation taking into account that for IQ and MeIQ the two positive charges are on the opposite site of the molecules, whereas in the case of PhIP they are on the same part and hence more masked by the counter ion of the ion pair reagent.

3.3. Influence of buffers on the ion-pair chromatography of HAAs and on sensitivity of the LC–ESI–MS/MS methods

In the last part of the study, the ammonium acetate (pH 4.0), usually employed in HAA chromatographic separation [5,7,14,17,18], was evaluated for the study of the amine retention by IPC and the results in terms of peak shape, analysis time and detectability of the analytes were compared with those obtained with the ammonium formate/formic acid buffer. The same eluent gradient as reported in Section 2 was run for both buffer solutions. In general, when using formate as the pairing ion reagent the analytes exhibit better peak shape than that shown in the case of the use of acetate (Fig. 4). The α values for all peaks were greater than 1, implying that a good selectivity of mobile phase to sample components was achieved in the case of formate. By contrast, the complete overlapping of MeIQ and MeIQx peaks was observed when using acetate buffer.

Advantages of using formate buffer compared to acetate buffer include also faster analyses, i.e. ca. 18 min instead of ca. 22 min, and higher detectability of all the amines analyzed, as illustrated by the LOD values quoted in Table 2.

Table 2
LOD values calculated for HAAs using the LC–ESI(+)–MS/MS method

Analyte	LOD (μg/l)[a]	
	Acetate buffer	Formate buffer
IQ	1.3	0.4
MeIQ	0.8	0.2
MeIQx	2	0.5
Norharman	0.7	0.4
Harman	0.6	0.2
PhIP	3.6	1.5
AαC	1.2	0.9

[a] Calculated as concentration corresponding to signal: $y_D = \bar{y}_b + 2ts_b$.

Even though the analytes had different retention times and sensitivity could not be properly compared, LODs of IQ compounds were four-times lower than those calculated with a mobile phase based on the use of acetate buffer.

Under ESI conditions, the eluent entering the MS interface and the presence of organic modifiers is of paramount importance in the ionization efficiency of individual compounds. In this application, the use of formate buffer did not exhibit any suppression effect on the ESI signals of HAAs with respect to those obtained using the acetate buffer. For these reasons, ion-pair chromatography with the use of formate instead of acetate buffer is recommended when developing a LC–MS or LC–MS/MS method for the determination of HAAs at trace level.

4. Conclusions

Different mechanisms for the separation of seven heterocyclic aromatic amines by reversed phase ion-pair liquid

chromatography with formate as counter ion in an aqueous eluent with acetonitrile as organic modifier are proposed. Both pH and organic modifier percentage had an important role for all the analytes, whereas the effect of formate was different for the amines investigated. Retention of IQ and MeIQ was governed by competitive ion-exchange mechanism, whereas hydrogen bonds and electrostatic interactions with the column packing surface silanols could be responsible for the retention of AαC.

The use of formate buffer proved advantageous with respect to acetate buffer for the gradient separation and detection by ESI-tandem MS of HAAs. In fact, a better chromatographic behavior and sensitivity were observed when using a mobile phase made up of the formate buffer at pH 2.8. The detection limits reported using formate buffer attest applicability of the method for trace analysis of HAAs.

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