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Separation of *N*-nitrosoamino acids by C_{18} reversed-phase ion-pair high-performance liquid chromatography and compatible detection by electrospray ionization mass spectrometry

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Abstract

Four non-volatile *N*-nitrosoamino acids, namely *N*-nitrososarcosine, *N*-nitrosoproline, *N*-nitrosothiazolidine-4-carboxylic acid and *N*-nitroso-2-methyl-thiazolidine-4-carboxylic acid were separated by C_{18} reversed-phase ion-pair high-performance liquid chromatography (HPLC) using 1.4 m*M* C_{16}-cetyltrimethylammonium chloride in methanol–water–acetonitrile (60:35:5, v/v) as the mobile phase. The *N*-nitrosoamino acids were sensitively detected by negative electrospray ionization mass spectrometry (ESI-MS) in the form of the deprotonated carboxylate anion, $[M-H]^-$. Compatibility problems associated with HPLC separation and ESI-MS detection, such as formation of solvent cluster ions and the effects of co-eluting anions of the ion-pairing reagent, were systematically investigated. The optimized experimental conditions for separation and detection of *N*-nitrosoamino acids were described.

Keywords: Ion-pairing reagents; Nitrosamino acids

1. Introduction

N-nitroso compounds (NOCs) are potent carcinogens; 90% of over 300 NOCs tested have been found to be carcinogenic in laboratory animals. In recent years, non-volatile NOCs have been increasingly found to be prevalent in some foods, tobacco products and body fluid [1]. *N*-Nitrososarcosine (NSAR), *N*-nitrosoproline (NPRO), *N*-nitrosothiazolidine-4-carboxylic acid (NTCA) and *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA) are the four most common non-volatile NOCs found in foods. NSAR is a proven carcinogen in rats [2]. NTCA has been reported to induce diabetes in laboratory animals [3]. Urinary levels of NPRO, NSAR, NTCA and NMTCA have been used as indexes of endogenous nitrosylation in epidemiological studies linked to occurrence of cancer in humans [4].

The analytical methodology for non-volatile NOCs is not as well established as that of volatile NOCs [5,6]. For the four *N*-nitrosoamino acids (NAAs) mentioned above, a commonly employed procedure is to convert them to volatile trimethylsilyl or methyl derivatives, which were then separated by gas chromatography (GC) and detected by thermal energy analysis (TEA). High-performance liquid chromatography (HPLC) separation has the advantage of

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avoiding the derivatization step. However, attempts to separate NAAs by reversed-phase C_{18} or normal-phase cyano/amino columns failed to yield satisfactory and reproducible separation [7–9]. Even if both normal and reversed-phases were used together, only partial separation of NPRO and NTCA was achieved [10]. Consequently, the NAAs had to be methylated and separated by cyano or amino bonded phases at elevated column temperatures (90–100°C) to obtain satisfactory HPLC separation [11]. However, the need of derivatization prior to HPLC separation defeated the primary objective of directly analysing non-volatile NOCs in the first place.

The *syn* and *anti* conformers of NTCA, NSAR and NPRO were individually separated using an α-cyclodextrin bonded silica gel column and 0.01 *M* triethylammonium acetate in aqueous acetonitrile as the mobile phase, but not for a mixture of these three NAAs. Microgram amounts of NAAs were analyzed because the less sensitive UV absorption detector at 238 nm was employed [12].

Although TEA is the established specific detection method for NOCs, it is incompatible with aqueous mobile phases associated with reversed-phase HPLC separation [8]. The TEA detector response was found to be susceptible to slight changes in experimental conditions and high background noises. On the other hand, mass spectrometric detection by electrospray ionization (ESI) is compatible with aqueous HPLC mobile phases; methanol–water or acetonitrile–water (50:50, v/v) are in fact the common solvent systems used in ESI mass spectrometry (MS) [13]. ESI-MS is also sensitive to pre-formed ions in solution at the nanomole to picomole level, and in the present case, the carboxylate anions of NAAs. Furthermore, ESI-MS is ideally suited to detect thermally labile NOCs as it is normally operated at ambient or temperatures below 100°C. However, ESI-MS detection and sensitivity is subjected to interferences, sometimes seriously, due to formation of solvent cluster ions and the presence of co-eluting anions or cations. Compatibility between HPLC mobile phases and ESI-MS detection is a very important problem that must be solved if HPLC–ESI-MS is to become a widely applicable technique for analysis of thermally labile compounds such as NOCs.

In this study, the separation of NAAs by C_{18} reversed-phase ion-pairing high-performance liquid chromatography (RP-IP-HPLC) and their compatible ESI-MS were systematically investigated. The optimized experimental conditions for HPLC separation and ESI-MS detection of NAAs were found. The ability of different tetraalkylammonium ion-pairing reagents to promote efficient separation of NAAs, and the effects of mobile phase compositions, solvent cluster ion formation, co-eluting anion of the ion-pairing reagent and other compatibility issues associated with ESI-MS detection were also addressed.

2. Experimental

2.1. Chemicals

The *N*-nitrosoamino acids, NSAR and NPRO, were synthesized by the method of Lijinsky et al. [14]. NTCA was synthesized according to the method of Tahira et al. [15], while NMTCA was synthesized by the procedure of Riemschneider and Hoyer [16]. The synthesized NAAs were purified by re-crystallization, and their structures were verified by infrared (IR) spectroscopy, 90 MHz nuclear magnetic resonance (NMR) spectra and mass spectral analysis. The purity of the synthesized NAAs was checked by GC–MS analysis of their methyl ester derivatives prepared by the diazomethane method [17]. No significant impurity peaks were found. The estimated purity of all the synthesized NAAs was>98%.

Tetraethylammonium chloride (C_2-TEACl), tetrabutylammonium chloride (C_4-TBACl), dodecyltrimethylammonium bromide (C_{12}-DTMABr), dodecyltrimethylammonium chloride (C_{12}-DTMACl), and cetyltrimethylammonium chloride (C_{16}-CTMACl) were obtained from Acros (Geel, Belgium). Hexyltriethylammonium bromide (C_6-HTEABr), HPLC grade methanol and acetonitrile were purchased from Aldrich (Milwaukee, WI, USA). Other reagents used were all analytical grade and also obtained from Aldrich. Double deionized water generated from a Millipore Milli-Q system was used throughout this study also.

NSAR, NPRO, NTCA and NMTCA stock standard solutions in methanol (1 mg/ml) were prepared and stored in 10-ml brown vials at 4°C. Working

standard solutions containing 0.5–40 μg/ml of NAAs were prepared freshly from the stock standard solutions by serial dilution.

2.2. HPLC conditions

The HPLC system consisted of a Bischoff (Leonberg, Germany) Model 2250 HPLC pump (flow-rate range 0–0.999±0.001 ml/min) and a Brownlee (Applied Biosystems, Foster City, USA) C_{18} reversed-phase HPLC column (RP-18, 5 μm, 15 cm× 2.1 mm I.D.). The mobile phase was made up of 0.0–2.2 m*M* tetraalkylammonium ion-pairing reagent in methanol–acetonitrile–water mixture solvents. The NAAs were separated by isocratic elution with a mobile phase flow-rate of 200 μl/min. In HPLC–ESI-MS analysis, the eluent from the HPLC was split, and 1/10 of the eluent was allowed to flow into the ESI interface. The hold up time (t_0) was measured by injection of pure methanol into the HPLC column, and with the mass spectrometer operated in the scanning mode, a sudden drop in the total ionization current (TIC) was observed when the methanol plug eluted from the HPLC column. The measured hold up time was in the 1.2–1.3 min range.

Before carrying out the HPLC analysis, the column was allowed to equilibrate for more than 1 h with the mobile phase. The NAA standard solution (10 μg/ml unless otherwise stated) was injected via a 5-μl sample loop.

2.3. Electrospray ionization mass spectrometry conditions

A VG Micromass (Manchester, UK) Platform quadrupole mass spectrometer (mass range *m/z* 10–2000) equipped with a VG electrospray ionization interface was used in the present study. Instrument control, data acquisition and processing were conducted via the VG Masslynx 2.0 system software.

Typical negative ESI-MS conditions were: capillary voltage −3.0 kV, skimmer cone voltage −20 V, flow-rate of solvent/mobile phase 20 μl/min, nitrogen nebulising gas flow-rate at 15 l/h and drying gas flow-rate at 350 l/h. NAA standard (0.5–40 μg/ml) and sample solutions were introduced by injection via a 5-μl sample loop. ESI mass spectra were obtained in the *m/z* 30–200 range at a scan rate of 200 u s^{-1}.

Sensitivities in the negative electrospray ionization mode were maximized by tuning and optimizing the carboxylate anion intensity of NPRO at *m/z* 143. Quantitative measurement of ion abundances was carried out by selected ion monitoring (SIM) of the deprotonated carboxylate molecular anions $[M-H]^-$ with ion measuring (dwell) time of 0.1 s. The peak areas of SIM mass chromatograms were integrated by the use of the Masslynx software.

The relative ion-evaporation rate constants of Cl^-, Br^-, I^- and acetate ions were measured according to the procedure of Tang and Kebarle [18] by comparing the ion abundances derived from equimolar solutions ($5\cdot10^{-4}$ *M*) of chloride and another anion in methanol–water (50:50, v/v).

3. Results and discussion

3.1. Electrospray ionization mass spectra of N-nitrosoamino acids

Fig. 1a–d shows the negative ESI mass spectra of NSAR, NPRO, NTCA and NMTCA, respectively, obtained from solutions containing 10 μg/ml NAA standards in methanol–water (50:50, v/v). All the mass spectra were clean and simple; they showed the deprotonated carboxylate molecular anion, $[M-H]^-$, as the only major and base peak in the spectrum. The high abundance of the carboxylate anion is typical of the ESI-MS spectra of organic acids, and their generation shows clearly that ESI is a suitable ionization technique for detecting NAAs. In this study, the ion abundance of the $[M-H]^-$ ion was measured in all RP-IP-HPLC–ESI-MS experiments.

3.2. Separation of NAAs by C_{18} reversed-phase ion-pair HPLC

NAAs are small and ionic molecules. Due to its highly polar properties, it is very difficult to be separated by normal-phase HPLC. Also, due to their short alkyl chains, they are not easily retained by non-polar reversed-phase stationary phases. Since ion-pairs with longer alkyl chains are better retained by reversed-phase stationary phases, a reversed-

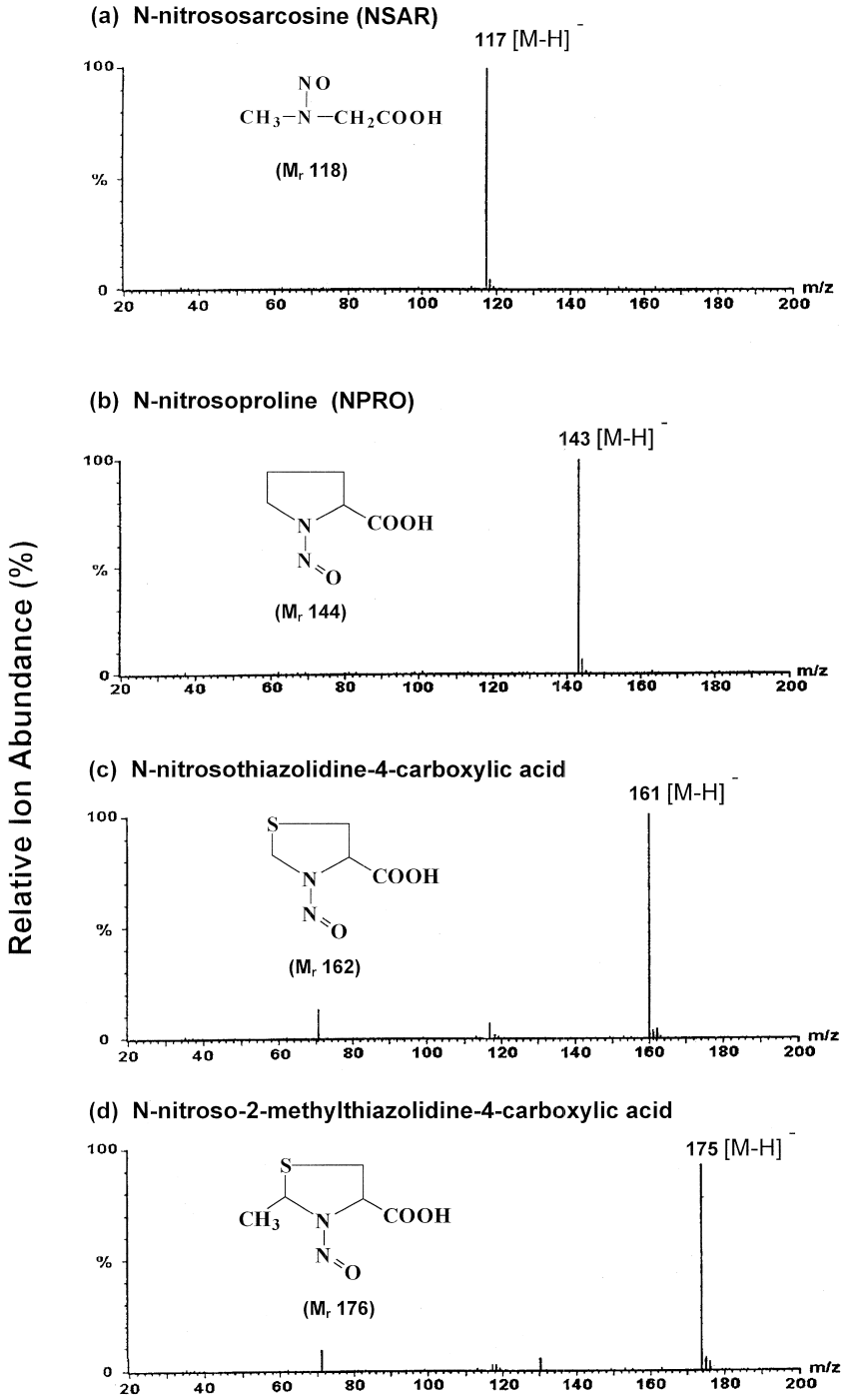

Fig. 1. Negative electrospray ionization mass spectra of (a) NSAR, (b) NPRO, (c) NTCA and (d) NMTCA. The mass spectra were obtained from 10 μg/ml standard solutions of NAA in methanol–water (50:50, v/v) at a skimmer cone voltage of 20 V.

phase ion-pair chromatographic method was attempted in the present study to separate the NAAs.

Most *N*-nitrosoamino acids are ionized in aqueous solvents, and their carboxylate anions are expected to be able to form ion-pair complexes with positive tetraalkylammonium counter ions. This was indeed experimentally observed. Fig. 2 shows the HPLC separation of NPRO, NSAR, NTCA and NMTCA using a reversed-phase C_{18} column and a mobile phase of methanol–water (60:40, v/v) containing 1.4 m*M* C_{16}-cetyltrimethylammonium chloride. The NPRO, NSAR, NTCA and NMTCA peaks were completely separated at retention times of 10.5, 11.7, 14.9 and 20.2 min, respectively. However, the retention times were too long, and the ESI-MS sensitivity was not optimized because the background ion intensity traces included contributions from interfering solvent cluster ions with overlapping *m/z* values (refer to Section 3.3.2 and Table 5 below).

In order to optimize the experimental conditions for analysis of the four NAAs, the major factors affecting the HPLC separation, i.e., the length of the alkyl chain of the tetraalkylammonium ion-pairing reagent, chemical composition of the mobile phase, and the concentration of the ion-pair reagent, were systematically investigated in the first stage of our study.

3.2.1. Effects of alkyl chain length of tetraalkylammonium ion-pairing reagent on separation efficiency

The separation of NAAs using tetraalkylammonium ion-pairing reagents and C_{18} HPLC stationary phase was first tested with methanol–water (50:50, v/v) mobile phase, the most commonly used solvent system used in electrospray ionization. The retention of NAAs by the C_{18} stationary phase, in terms of the capacity factor (k'), was found to be

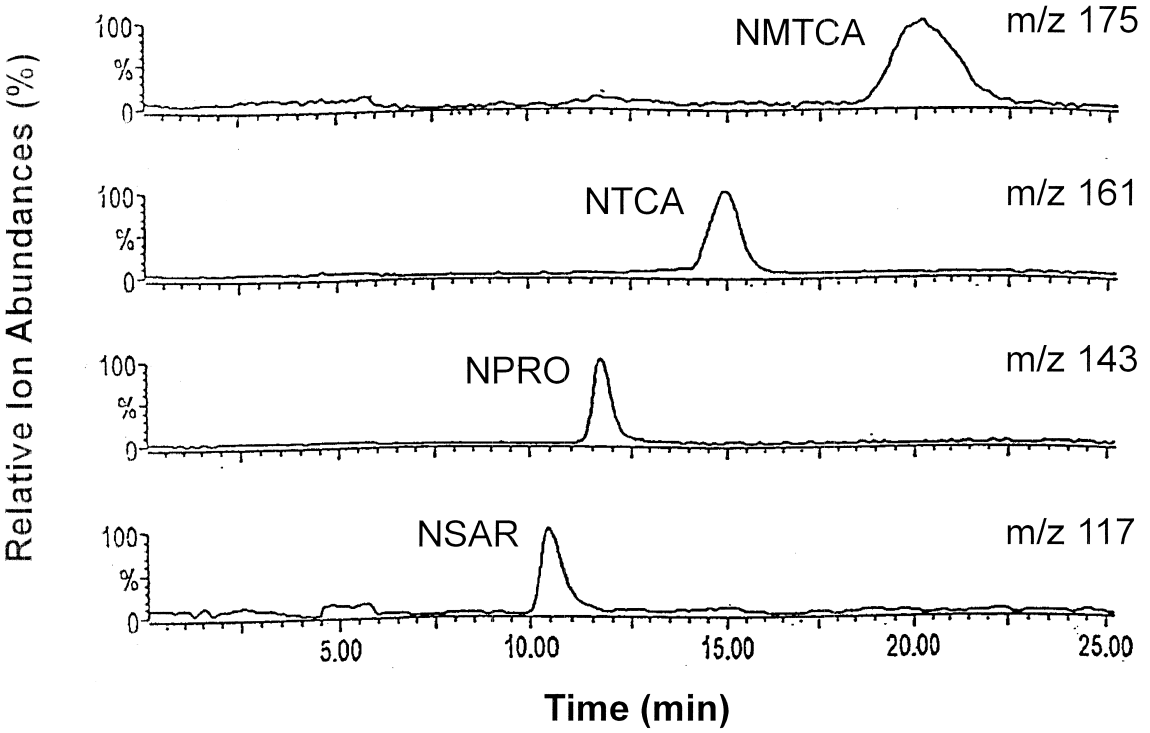

Fig. 2. HPLC–ESI-MS $[M-H]^-$ mass chromatograms of four *N*-nitrosoamino acids (10 μg/ml). HPLC conditions: 150 mm×2.1 mm C_{18} column (Brownlee), mobile phase 1.4 m*M* C_{16}-cetyltrimethylammonium chloride in methanol–water (60:40, v/v) at a flow-rate of 200 μl/min and a split ratio of 1:10 to the ESI interface. The broad widths of the NMTCA and NTCA peaks were due to partial separation of the *syn* and *anti* isomers (Section 3.2.3).

Table 1
Effect of alkyl chain length of ion-pairing reagent on retention of NAAs[a]

Ion-pair reagent	Capacity factor (k')			
	NSAR	NPRO	NTCA	NMTCA
C_{16}-CTMA	10.5	14.4	–[b]	–[b]
C_{12}-DTMA	3.8	4.4	4.8	8.9
C_6-HTEA	0.3	0.3	0.3	0.4
C_4-TBA	0.4	0.4	0.4	0.4

[a] The experimental conditions used were: Brownlee 15 cm×2.1 mm C_{18} (5 μm spheri, RP-18, 5 μ) reversed-phase column, mobile phase containing 1.8 m*M* ion-pairing reagent in methanol–water (50:50, v/v) and flowing at 200 μl/min. C_{16}-CTMA, cetyltrimethylammonium chloride; C_{12}-DTMA, docecyltrimethylammonium chloride; C_6-HTEA, hexyltriethylammonium bromide, C_4-TBA, tetrabutylammonium chloride.

[b] The analyte was not detected after 30 min due to enhanced retention of the ion-pair complex on C_{18} stationary phase.

highly dependent on the alkyl chain length of the tetraalkylammonium ion-pairing reagent (Table 1). In the presence of C_4 (tetrabutylammonium) and C_6 (hexyltriethylammonium) ion-pairing ions, the four NAAs eluted roughly at the same time as the solvent front (about 1.5 min), and hence were totally not retained by the C_{18} stationary phase. A longer alkyl chain would impart a higher lipophilic property onto the ion-pair complex, leading to greater retention on the non-polar C_{18} stationary phase. Our retention data suggested that at least a C_{12} or longer alkyl chain in the ion-pairing reagent was needed to induce significant ion-pair complex formation and enhancement in retention of NAAs. With 1.8 m*M* C_{12}-dodecyltrimethylammonium ion, NSAR, NPRO, NTCA and NMTCA were all well retained with acceptable capacity factors of 3.8, 4.4, 4.8 and 8.9, respectively (Table 1). With the longer alkyl chain C_{16}-cetyltrimethylammonium ions, the retention times of NSAR and NPRO increased considerably to 15 and 20 min, respectively, while the NTCA and NMTCA peaks were so retained that they were not observed even 30 min after injection.

3.2.2. Effects of methanol concentration in aqueous mobile phase on the retention of NAAs

In reversed-phase ion-pair chromatography, the concentration of the organic modifier (methanol) in the aqueous mobile phase usually has a significant effect on the separation efficiency and resolution. As shown in Table 2, the capacity factors (k') of the four NAAs were reduced mostly in the 1–10 acceptable range when the methanol content was increased to 60–75% (v/v) of the mobile phase, and a lower concentration of 1.4 m*M* C_{16}-cetyltrimethylammonium chloride ion-pairing reagent could be used to effect separation. In particular, reasonably good separation in the $[M-H]^-$ mass chromatograms of

Table 2
Effect of methanol concentration in aqueous mobile phase on the retention of NAAs

Mobile phase composition (%, v/v)			Capacity factor (k')			
MeOH	Water	MeCN	NSAR	NPRO	NTCA	NMTCA
[a]75	25	0	1.4	1.5	1.6	1.8
[a]70	30	0	2.3	2.5	2.8	3.0[c,e] 3.7[d,e]
[a]65	35	0	3.9[c,e] 4.9[d,e]	4.3	4.9	6.9
[a]60	40	0	7.1	8.0	8.8[c,e] 10.5[d,e]	14.5
[a]60	35	5	3.6	3.9	4.6	6.2
[b]60	35	5	0.80	0.9	1.0	1.1

[a] 1.4 m*M* C_{16}-cetyltrimethylammonium chloride in mobile phase.

[b] 1.4 m*M* C_{12}-dodecyltrimethylammonium chloride in mobile phase.

[c] *anti* Isomer.

[d] *syn* Isomer.

[e] This is our proposed assignment of *anti* and *syn* isomers based on projected chromatographic behavior of ion-pair formation of the *syn* and *anti* isomers. The identity of the isomers has not been verified experimentally because the equilibrium between *syn* and *anti* isomers could be affected by many experimental factors.

the four NAAs and economy of analysis time were achieved with methanol–water (65:35, v/v), or a methanol–water–acetonitrile (60:35:5, v/v) solvent system (Fig. 3). However, the capacity factors tended to level off at C_{16}-CTMA concentrations greater than 1.4 m*M*. Since sensitivity in electrospray ionization was reduced by higher concentration of ion-pairing reagents (refer to Section 3.3), 1.4 m*M* C_{16}-cetyltrimethylammonium chloride is a better choice than 1.8 m*M* C_{12}-dodecytrimethylammonium chloride (as shown in Table 1) as the ion-pairing reagent in the actual RP-IP-HPLC–ESI-MS analysis. Under similar mobile phase conditions, the NAAs were not retained at all when 1.4 m*M* C_{12}-dodecyltrimethylammonium chloride was used.

The retention order: NSAR<NPRO<NTCA<NMTCA was found to remain the same irrespective of changes in the ion-paring reagents or the mobile phase compositions, suggesting that the retention was mainly determined by the hydrophobicities of the analyte ion-pairs.

3.2.3. Separation of syn and anti isomers

The co-existence of the *E*-(*syn*) and *Z*-(*anti*) conformers of NAAs in different solvent systems was demonstrated by NMR studies [19]. As a result of our systematic study, we found that the *syn* and *anti* isomers of three NAAs could be separated by C_{18} RP-IP-HPLC, but the separation was highly dependent on the composition (%, v/v) of the organic modifier, i.e., methanol, and the concentration of the ion-pairing reagent in the mobile phase. Thus the *E*- and *Z*-conformers of NSAR and NMTCA were almost completely separated using 1.4 m*M* cetyltrimethylammonium (C_{16}-CTMA) chloride in methanol–water (65:35) and methanol–water

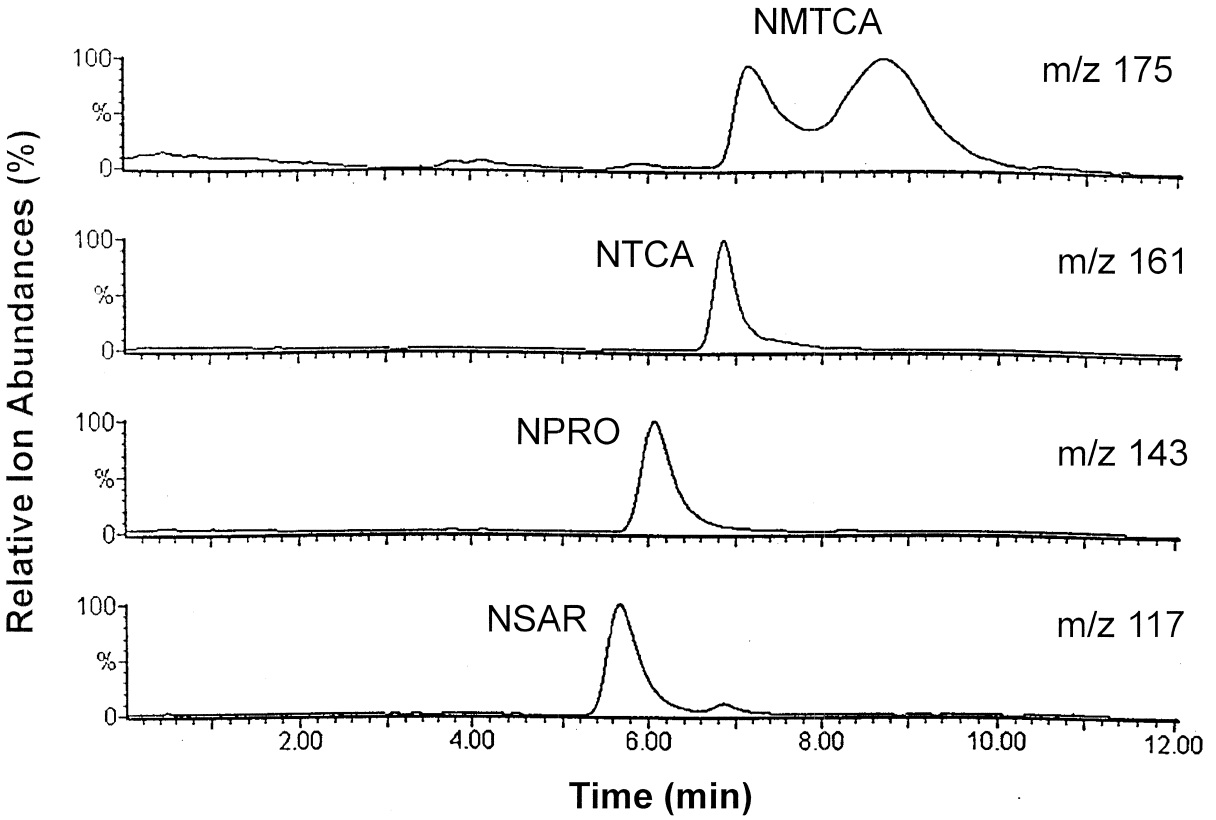

Fig. 3. HPLC–ESI-MS $[M-H]^-$ mass chromatograms of four *N*-nitrosoamino acids (10 μg/ml). Mobile phase: methanol–water–acetonitrile (60:35:5, v/v). Other experimental conditions as in Fig. 2.

(70:30, v/v) solvent systems, respectively (Fig. 4a and b). The NTCA conformers were only partially separated using a high concentration of C_{16}-CTMA (2.2 m*M*) and a methanol–water–acetonitrile (60:35:5, v/v) mobile phase (Fig. 4c). On the other hand, the conformers of NPRO could not be separated over a wide range of methanol and ion-pairing reagent concentrations.

The ability to separate the *syn* and *anti* conformers of NTCA, NMTCA and NSAR by varying the concentrations of methanol and C_{16}-CTMA ion-pairing reagent in the mobile phase is a useful means of confirming the presence of these NAAs in unknown samples.

3.3. Detection by electrospray ionization: compatibility and sensitivity studies

Under ESI-MS conditions, the analyte ion current is reduced by the presence of co-analytes or electrolytes present in the sample solution. Based on the work of Tang and Kebarle [18], the ESI generated ionization current is competitively divided between the analyte anion, X^-, and the co-eluting anion, Y^-, according to Eq. (1):

$$I_{X^-} = fp \cdot \frac{k_{X^-}[X^-]}{k_{X^-}[X^-] + k_{Y^-}[Y^-]} \cdot I \quad (1)$$

where I is the capillary current, I_{X^-} is the mass spectrometrically detected ion current of X^-, f is the efficiency of conversion of electrospray charged droplets to gas-phase ions, p is a constant expressing the transmission efficiency of the gas phase ions to the mass analyser, $[X^-]$ and $[Y^-]$ are the concentrations of the ions (with $[X^-]$ and $[Y^-] \geq 10^{-5}$ *M*), and k_{X^-} and k_{Y^-} are the ion-evaporation rate constants relating to the relative efficiencies of ejection of X^- and Y^- from the electrospray charged droplet. Hence, it is of practical importance to study the effect of the co-eluting anion, $[Y^-]$, of the tetraalkylammonium ion pairing reagent on ESI-MS sensitivity under actual RP-IP-HPLC analysis conditions. In the present study, the effect of co-eluting anions on sensitivity of ESI-MS detection, i.e., on I_{X^-}, was studied by comparing the ion intensity of the NAA carboxylate anion, $[M-H]^-$, with and without the co-eluting anion present.

The results of the sensitivity study are summarized in Table 3. Tetraalkylammonium ion-pairing reagents are commercially available in the Cl^-, Br^-, I^- and acetate form. The ESI-MS detection sensitivity for NAAs was found to decrease in the order $I^- > Br^- > CH_3COO^- > Cl^-$. The iodide ion showed the greatest reducing effect (80–90%), while the reduction by chloride ion was least at about 40–50%.

According to Eq. (1), the reduction in ESI-MS sensitivity is related to the ion-evaporation rate constant, k_{Y^-} of the co-eluting anion: a greater value of k_{Y^-} would lead to enhanced reduction of I_{X^-}. The relative ion-evaporation rate constants of these anions have not been reported in the literature. Hence, the relative ion-evaporation rate constants of Cl^-, Br^-, I^- and acetate ions were measured in this work according to the procedure of Tang and Kebarle [18], in which the anion intensities derived from equimolar solutions ($5 \cdot 10^{-4}$ *M*) of chloride and another anion in methanol–water (50:50, v/v) were measured and compared. The experimentally measured ion-evaporation rate constants are summarized in Table 4: they were found in the order of $I^- > Br^- > CH_3COO^- > Cl^-$, which is in good agreement with the order of decreasing relative response factors or analyte ion intensities already shown in Table 3. In fact, a plot of (relative response factor) versus the reciprocal of (relative ion-evaporation rate constants of anions) for the case of NPRO yielded an approximate straight line ($y = 0.21x + 0.18$, $r^2 = 0.92$) (Fig. 5). Similar linear plots were obtained for NSAR, NTCA and NMTCA, suggesting that the response factors are roughly inversely proportional to the relative ion-evaporation rate constants of the co-eluting anions.

Under RP-IP-HPLC conditions described in this report, the sample concentration, $[X^-]$, is usually in the range 10^{-6}–10^{-9} *M* (less than 10^{-5} *M*) while $[Y^-]$ is equal to $1.4 \cdot 10^{-3}$ *M*. Then Eq. (1) may be modified to:

$$I_{X^-} = fp \cdot \frac{k'_{X^-}[X^-]}{k'_{X^-}[X^-] + k_{Y^-}[Y^-]} \cdot I \quad (2)$$

$$\approx fp \cdot \frac{k'_{X^-}[X^-]}{k_{Y^-}[Y^-]} \cdot I \quad (3)$$

where k'_{X^-} ($k'_{X^-} < k_{X^-}$) represents the ''reduced'' ion-evaporation rate constant under dilute analyte con-

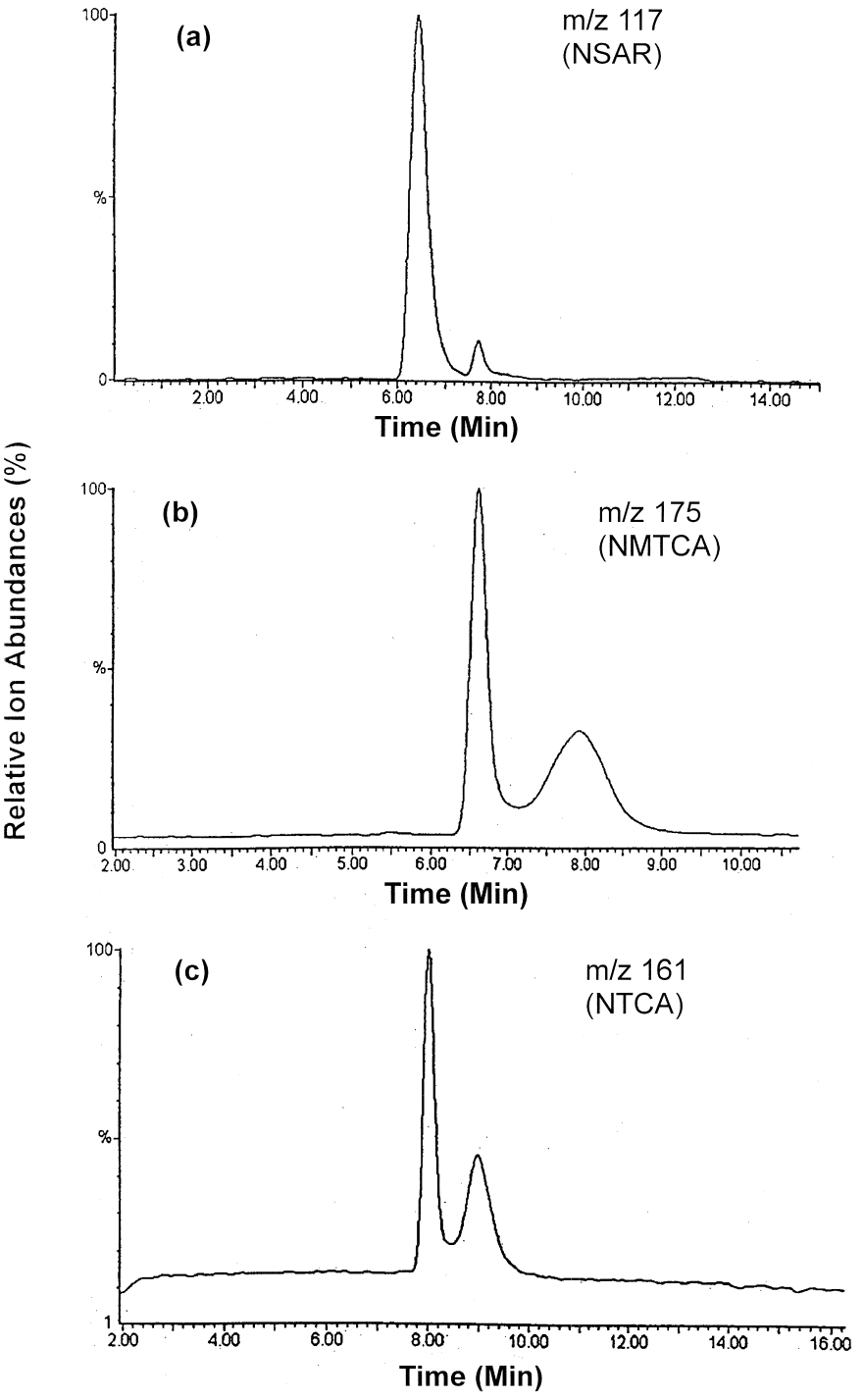

Fig. 4. Separation of *syn* and *anti* conformers of *N*-nitrosoamino acids: $[M-H]^-$ mass chromatograms of (a) NSAR; 1.4 m*M* C_{16}-CTMA in methanol–water (65:35, v/v); (b) NMTCA; 1.4 m*M* C_{16}-CTMA in methanol–water (70:30, v/v) and (c) NTCA; 2.2 m*M* C_{16}-CTMA in methanol–water–acetonitrile (60:35:5; v/v). Other experimental conditions as in Fig. 2.

Table 3
Effect of anions derived from ion-pairing reagent on electrospray ionization sensitivity of *N*-nitrosoamino acids [mobile phase composition: methanol–water–acetonitrile (60:35:5, v/v); average of $n=5$ measurements, the relative deviation is $<\pm 6.8\%$][a]

Anions	Relative response factor[b]			
	NSAR	NPRO	NTCA	NMTCA
Cl^-	0.48	0.41	0.41	0.43
Br^-	0.18	0.23	0.18	0.20
I^-	0.12	0.17	0.10	0.14
CH_3COO^-	0.31	0.32	0.29	0.31

[a] The intensity of the deprotonated carboxylate anion, $[M-H]^-$ derived from the analyte NAA (5 μg/ml) was measured.

[b] Relative response factor is the $[M-H]^-$ ion intensity of the NAA analyte measured in the presence of 1.4 m*M* concentration of the co-eluting anion derived from the sodium salt, and compared to that of the control solution containing no co-eluting anion, which is assigned a value of 1.00.

centration conditions, when the droplet surface concentration of $[X^-]$ in the charged droplet is not readily replenished in the successive ion ejection steps during the ESI process. Furthermore, $k_{Y^-}[Y^-] >> k'_{X^-}[X^-]$ as $[Y^-]$ is much greater than $[X^-]$, so Eq. (2) can be approximated to Eq. (3), leading to the simple correlation experimentally observed and depicted in Fig. 5. It should be stressed here that Eq. (3) is not intended to be applied in a quantitative manner, but only serves as a semi-quantitative guide to estimate or predict ESI-MS sensitivity under RP-IP-HPLC conditions.

The ion-evaporation rate constants of cations and anions are dependent on their physical properties such as solvation energy, ionic radius and surface activity [18]. In general, ions with smaller ionic radii have greater solvation energies, and are less efficiently formed in the ESI process as demonstrated by our measured k_{Y^-} values: I^- (2.16 Å), $24.8 > Br^-$ (1.95 Å), $8.1 > Cl^-$ (1.81 Å), 1.0. Based on the results of our sensitivity study, the chloride form of tetraalkylammonium ion-pairing reagent should be preferred in RP-IP-HPLC separation because it suppresses ESI-MS sensitivity the least.

Table 4
Experimentally determined relative ion-evaporation rate constants of anions (average of $n=3$ measurements, the relative deviation is $<\pm 8.2\%$)

Anions	Ion-evaporation rate constant[a]
Cl^-	1.0
Br^-	8.1
I^-	24.8
CH_3COO^-	1.2

[a] The ion-evaporation rate constant was measured relative to that of chloride, which is assigned a value of 1.00. Ion intensities were not corrected for mass discrimination effect of the quadrupole mass analyser.

3.3.1. Effects of concentration of co-eluting anion (Cl^-) on ESI-MS sensitivity

In RP-IP-HPLC, increasing the concentration of the ion-pairing reagent would generally lead to enhanced retention of the analytes onto C_{18} the non-polar stationary phase. However, this would also lead to a corresponding increase in the concentration of the co-eluting anion which, according to Eq. (3), would lead to a reduction in the ion intensity of the analyte. To study the concentration effect of co-eluting anions, the ESI-MS generated $[M-H]^-$ ion intensities derived from 5 μg/ml NAA standard solutions were measured as a function of chloride concentration (as NaCl in the 0.35 to 56 m*M* range) in methanol–water–acetonitrile (60:35:5, v/v).

The result, illustrated for the case of NMTCA, is shown in Fig. 6. The analyte ion intensity was found to decrease rapidly with added chloride, and was reduced to 20% and 5% of its initial intensity at 5 m*M* and 56 m*M* of $[Cl^-]$, respectively. In order to obtain good sensitivity in ESI-MS detection, the lowest concentration of ion-pair reagent which could, on the other hand, provide adequate chromatographic separation and resolution should be adopted. In this study, a concentration of 1.4 m*M* C_{16}-CTMA ion-pairing reagent was finally adopted, leading to about 50% reduction in the analyte NAA ion intensity compared to a control solution containing no ion-pairing reagent at all.

3.3.2. Interference due to formation of solvent cluster ions in ESI-MS detection

In electrospray ionization, association of solvent molecules with cations/anions frequently occurs, leading to formation of solvent cluster ions and complex ESI mass spectra. These cluster ions can be eliminated by the de-clustering effect of an additional flow of a sheath gas, or increasing the sampling skimmer cone voltage of the ESI interface. However,

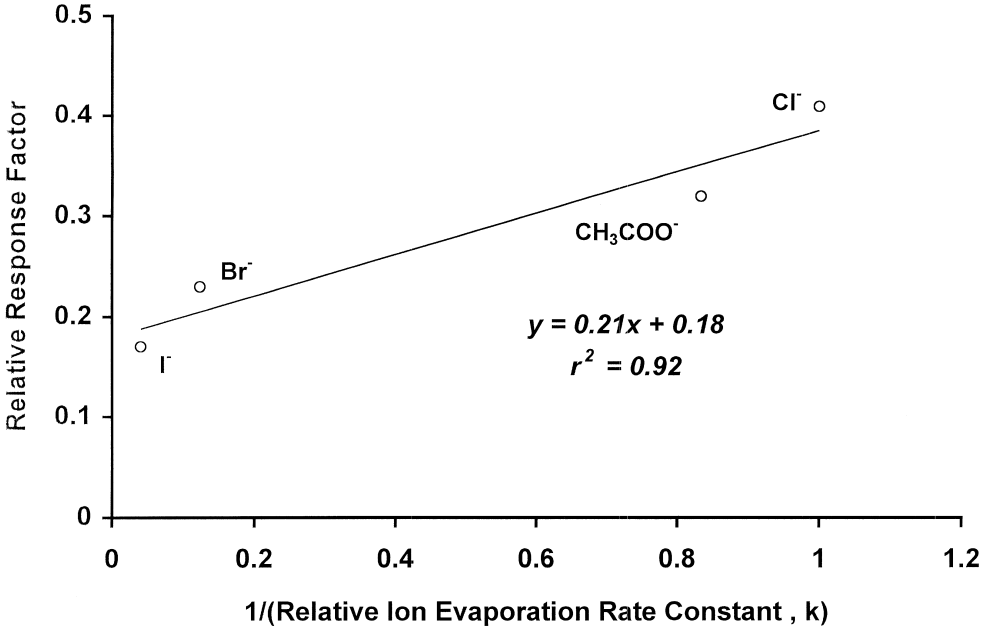

Fig. 5. The dependence of relative response factor of NPRO on the reciprocal of the relative ion-evaporation rate constants of co-eluting anions derived from ion-paring reagents.

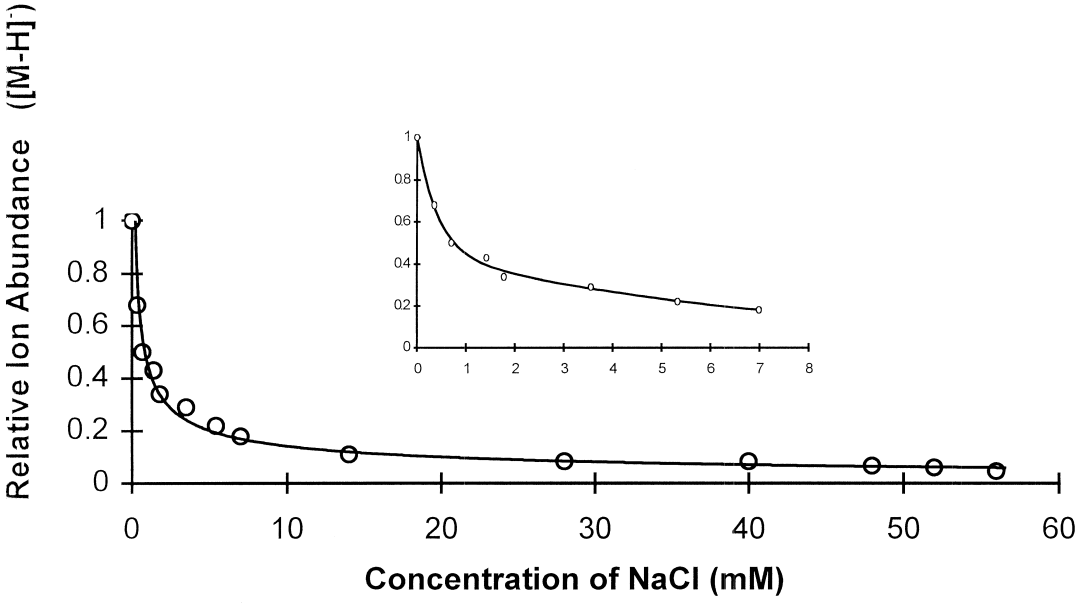

Fig. 6. The relative ion intensity of carboxylate anion, $[M-H]^-$, of NMTCA as a function of chloride concentration in methanol–water–acetonitrile (60:35:5, v/v).

under such de-clustering conditions, a decrease in the ESI-MS sensitivity of detection is often observed.

In the present study, Cl^- and Br^- anions were found to form cluster ions with methanol, water and acetonitrile. The ESI mass spectra displaying the $(H_2O)_nCl^-$ and $(H_2O)_nBr^-$ cluster ions are shown in Fig. 7 ($n=1$ to 9 and 6, respectively). Some of these cluster ions had the same m/z values as the deprotonated molecules, $[M-H]^-$, of NAAs (Table 5). In particular, the high intensity cluster ions at m/z 117 $[Cl^-(H_2O)(MeOH)_2]$, m/z 143 $[Cl^-(H_2O)_6]$ and m/z 161 $[Cl^-(H_2O)_7]$ interfered seriously with the detection of NSAR, NPRO and NTCA, respectively. Increasing the ESI interface temperature from 60°C to 90°C, and increasing the drying gas flow-rate from 250 l/h to 350 l/h could partially decrease the intensity of the cluster ions, but they were still present in the background spectrum. A better solution is to eliminate completely the formation of cluster ions by varying the composition of the mobile

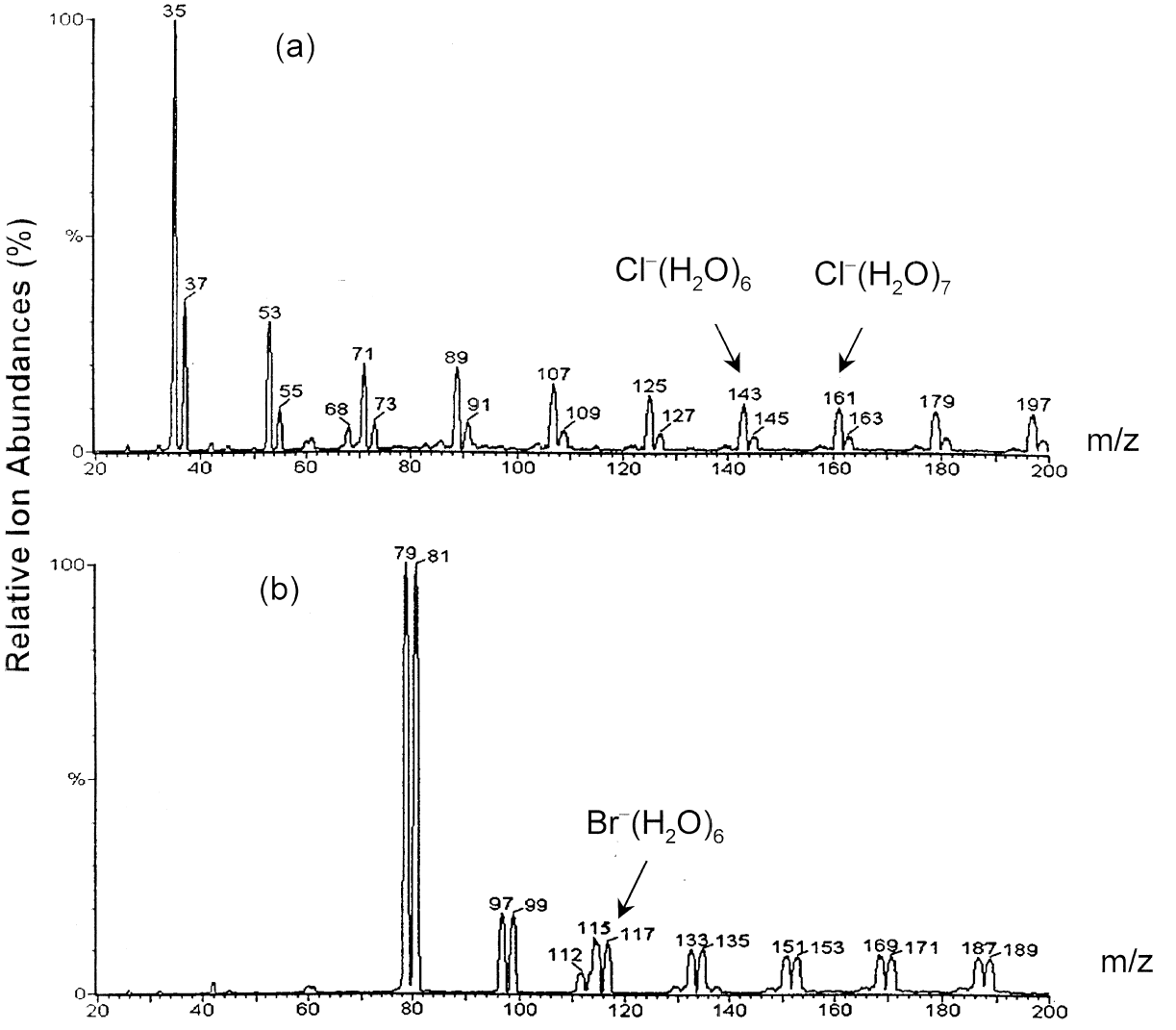

Fig. 7. Solvent cluster ion formation under different mobile phase conditions: (a) 1.0 m*M* C_{16}-cetyltrimethylammonium (C_{16}-CTMA) chloride in water–acetonitrile (70:30, v/v) (b) 1.8 m*M* C_{12}-dodecyltrimethylammonium (C_{12}-DTMA) bromide in water–acetonitrile (50:50, v/v).

Table 5
Effect of methanol, water and acetonitrile content of HPLC mobile phase on formation of solvent cluster ions with chloride and bromide

N-Nitrosoamino acid	$[M-H]^-$ analyte ion	Solvent cluster ion[a,b]
NSAR	*m/z* 117	$Cl^-(H_2O)(MeOH)_2$; (>65%, v/v, MeOH) $Cl^-(MeCN)_2$; (>50%, v/v, MeCN) $Br^-(H_2O)_2$; (>70%, v/v, water)
NPRO	*m/z* 143	$Cl^-(H_2O)_6$; (>50%, v/v, water) $Br^-(MeOH)_2$; (>50%, v/v, MeOH)
NTCA	*m/z* 161	$Cl^-(H_2O)_7$; (>50%, v/v, water) $Br^-(MeOH)_2(H_2O)$; (>50%, v/v, MeOH)

[a] Solvent cluster ions with the same *m/z* value as the carboxylate anion, $[M-H]^-$.
[b] The threshold values (%, v/v) at which solvent cluster ions appeared for individual solvents are shown in parentheses.

phase solvent. After systematic tests, we found that formation of solvent cluster ions with Cl^- and Br^- could be avoided by limiting the water content to <40% (v/v), methanol to <65% (v/v), and acetonitrile to <50% (v/v) in the mobile phase. The composition of the mobile phase finally adopted was methanol–water–acetonitrile (60:35:5, v/v), which could yield reasonable separation of the NAAs in the mass chromatograms of the $[M-H]^-$ ions, but avoiding formation of solvent cluster ions in the background ESI spectra.

3.4. Optimized HPLC–ESI-MS conditions: linearity and detection limits

As a compromise between separation efficiency, ESI-MS sensitivity and analysis time, the final optimized HPLC–ESI-MS conditions adopted in this study was 1.4 m*M* C_{16}-cetyltrimethylammonium chloride in methanol–water–acetonitrile (60:35:5, v/v) mobile phase, with the ESI interface operated in the negative mode. As shown in Fig. 3, the four NAAs were eluted out in about 10 min. Linear calibration plots were obtained ($r^2 \geq 0.97$) for 250 pg–20 ng NSAR, NPRO NTCA and NMTCA standards injected into the ESI interface (5 μl injection onto the HPLC system, and a split ratio of 1:10 to the ESI interface), corresponding to a working range of 0.5–40 μg/ml of standard or sample concentrations injected into the HPLC. The ESI-MS detection limit for NSAR, NPRO, NTCA and NMTCA achieved under the prescribed experimental conditions were found to be 250 pg injected at a $S/N \geq 3$. Although the sensitivity and detection limits could be improved further by adjusting HPLC conditions such as injection volume and split ratios, the quoted values are adequate for most analysis of NAAs in foods.

4. Conclusion

For the first time, we have successfully developed a C_{18} reversed-phase ion-pair HPLC method capable of separating four non-volatile *N*-nitrosoamino acids, i.e., NSAR, NPRO, NTCA and NMTCA. The HPLC separation is very sensitive to experimental conditions, i.e., the chain length of the tetraalkylammonium ion-pairing ion, and the concentration of the methanol in the aqueous mobile phase. These experimental parameters have been systematically studied, evaluated and optimized with regard to chromatographic resolution and analysis time.

The four *N*-nitrosoamino acids were found to be easily and sensitively detected by ESI-MS. However, ESI-MS sensitivity is reduced by the presence of co-eluting anions derived from the ion-pairing reagent. The chloride form of tetraalkylammonium ion-pairing reagent is recommended to be used in actual RP-IP-HPLC–ESI-MS analysis because of its relatively small ion-evaporation rate constant compared to other commonly available anions. The mobile phase composition should also be adjusted so that the formation of anion–solvent cluster ions is minimized and does not overlap and interfere with the *m/z* of the analyte anions.

In recent years, because of the inadequacy of HPLC–TEA method of analysis, a variety of dedi-

cated instrumental set-ups and procedures have been reported for HPLC determination of non-volatile NOCs based on post-column denitrosation and detection of the liberated nitric oxide radical or resulting nitrite ion [20–22]. Commercial supply of bench-top, relatively low cost LC–ESI-MS systems are readily available today. The RP-IP-HPLC–ESI-MS method described in this report could provide an alternative analytical solution to the challenging problem of analysis of non-volatile NOCs.

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