Abstract of thesis entitled

"Analytical Studies on N-nitroso and Related Compounds"

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for the degree of Master of Philosophy in Chemistry

at The Hong Kong Polytechnic University in September, 1998.

Abstract

N-nitroso compounds (NOCs) are putative carcinogens the majority of which have been shown to be carcinogenic in laboratory animals. While the analytical methodology for volatile NOCs, namely N-nitrosamines, was well established, there is a lack of progress in the development of sensitive analytical methods for determination of non-volatile NOCs in foods. Furthermore, existing methodology relies mainly on the application of gas chromatography-thermal energy analyser (GC-TEA), which is susceptible to interference from other nitrogen-containing compounds. Development of alternative methods for their analysis is needed to confirm and validate qualitative and quantitative results derived from GC/TEA analysis.

In this study, we have successfully developed an alternative analytical method based on combined use of reverse-phase ion-pair high performance liquid chromatography (RP-IP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). Four non-volatile N-nitrosoamino acids, namely nitrososarcosine (NSAR), nitrosoproline (NPRO), N-nitorsothiazolidine-4-carboxylic acid (NTCA), N-nitroso-2-methyl-thiazolidine-4-carboxylic acid (NMTCA) were completely

separated by isocratic elution using a 2.1 mm x 150 mm C_{18} (5μ) HPLC column and 1.4 mM C_{16}-cetyltrimethylammonium chloride in 60 : 35 : 5 (*v/v*) MeOH : H_2O : MeCN mobile phase. The HPLC conditions, such as alkyl chain length and concentration of tetraalkyl ammonium ion-pairing reagent and mobile phase composition were systematically studied, evaluated and optimized with regard to chromatographic resolution, economy of analysis time and sensitivity of detection by electrospray ionization mass spectrometry.

Non-volatile N-nitrosoamino acids were sensitivity detected by negative electrospray ionization in the form of the deprotonated carboxylate molecular anion, $[M-H]^-$. The effect of co-eluting anion derived from the ion-pairing reagent was evaluated and optimized. Interfering cluster ion formation was avoided by careful manipulation of the mobile phase composition. A linear working range of 250pg to 20ng/injection of N-nitrosoamino acids was established for quantitative analysis. The detection limit was 250 pg/injection for NSAR, NPRO, NTCA and NMTCA at a S/N ratio of ≥ 3. Our study is the first report on the development and application of RP-IP-HPLC/ESI-MS method to analysis of non-volatile NOCs in food.

Relatively high levels of NSAR and NPRO at 2,200 ± 260 and 154 ± 15 μg/kg, respectively, was found in Chinese salted fish. This is also the first time that non-volatile N-nitroso compounds were analysed in an example of preserved Chinese food.

As a supplementary study a RP-IP-HPLC method for simultaneous of nitrosating agents, i.e. nitrite and nitrate, and inhibitor of nitrosation, i.e. ascorbic acid in food was also developed. The method makes use of 0.010 M

octylammonium orthophosphate as the ion interacting reagent and 20% (v/v) aqueous methanol as the mobile phase. The content of nitrite, nitrate (expressed as nitrite ion and nitrate ion, respectively) and ascorbic acid in commercial brands of canned tomato, carrot and mixed vegetable juices were surveyed. The quantitative results indicated that nitrite was very probably added as a preservative in the canned vegetable juices. The total amount of nitrite and nitrate ion per can in two commercial brands of vegetable juices was found to exceed the acceptable daily intake recommended by the 1996 Joint FAO/WHO Expert Committee on Food Additives for an average adult of 60 kg body weight.

The Hong Kong Polytechnic University

Analytical Studies on N-nitroso and Related Compounds

Submitted in Partial Fulfillment of the Requirements of the Degree of

Master of Philosophy

in

Chemistry

by

Cheng Chi Fai, Raymond

5-10-98

Declaration

This is to declare that this work has been done by the author in the Department of Applied Biology and Chemical Technology of The Hong Kong Polytechnic University and this thesis has not been submitted to this or other institution for any academic qualification.

Cheng Chi Fai, Raymond

Acknowledgment

I wishes to express my gratitude to all academic and technical staffs of the Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University who have helped in various ways during the course of this study.

In particular, I would like to thank Dr. C.W. Tsang, my supervisor, for his supervision and his critical suggestion for this project as well as his comments on the draft of this thesis. He also gave me a lot of freedom in the development of this project.

I would like to thank Mr. P.M. Yeung for the synthesis and supply of NAAs standards.

I would like to thank my parents and sisters for their encouragement and unflagging moral support during the course of this study.

Finally, I would like to acknowledge the support of the Research Degrees Committee of The Hong Kong Polytechnic University (Grant No. 350/668) and the Research Grant Council (RGC) of Hong Kong (Grant No. HKP/130/92M) for the funding of this project.

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List of Abbreviations

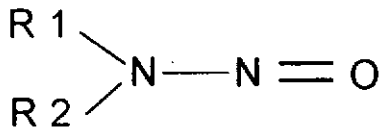
C_2-TEA	Tetraethylammonium
C_4-TBA	Tetrabutylammonium
C_6-HTEA	Hexyltriethylammonium
C_{12}-DTMA	Dodecytrimethylammonium
C_{16}-CTMA	Cetyltrimethylammonium
CI	Chemical Ionization
CID	Collision Induce Dissociation
EI	Electron Impact
ESI	Electrospray Ionization
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
IPC	Ion-pair Chromatography
IP	Ion-pair
MeCN	Acetonitrile
MeOH	Methanol
MS	Mass Spectrometry
NAA	N-nitrosoamino Acid
NDMA	N-nitrosodimethylamine
NMTCA	N-nitroso-2-methylthiazolidine-4-carboxylic acid
NOC	N-nitroso Compounds
NPIC	N-nitrosopipecolic acid
d_3-NPRO	deuterated N-nitrosoproline

NPRO	N-nitrosoproline
NSAR	N-nitrososarcosine
NTCA	N-nitrosothiazolidine-4-carboxylic acid
NVNOC	Nonvolatile N-nitroso Compounds
RP	Reverse-phase
SPE	Solid Phase Extraction
TEA	Thermal Energy Analyser
VNA	Volatile N-nitrosamine

Chapter 1 Introduction

1.1 N-Nitroso Compounds (NOCs)

N-nitroso Compounds (NOCs) are a group of organic compound of diverse chemical structures, but all contain the nitroso functional group N-NO. The general structure of NOCs is shown below:



Broadly speaking, NOCs are divided into three major groups : (a) N-nitrosamines in which R_1, R_2 are alkyl or aryl, or they can form the part of a heterocyclic ring ; (b) N-nitrosamides, in which one substituent group, R_1, is alkyl or aryl, but the other R_2 is an acyl or substituted acyl functional group ; and (c) N-nitrosoamino acids, in which R_1 is an alkyl or aryl group, and R_2 is an alkyl group with a carboxylic acid functional group, i.e. $-CH_2CO{\cdot}OH$

The first evidence that N-nitrosodimethylamine (NDMA), the simplest member of NOCs, could be hazardous to humans came from the observation that industrial workers exposed to NDMA developed cirrhosis of the liver (Freund, 1937). Later studies by Magee and Barnes (1954) established that NDMA was indeed a potent hepatotoxic agent, as well as a potent carcinogen in laboratory animals (Magee and Barnes, 1956). Since this notable finding, intensive research studies on the carcinogenic activity not only of the NDMA, but also on a wide variety of nitroso compounds have been and are being conducted. The majority of

over 300 NOCs tested to-date for carcinogenicity on laboratory animals have been positive (Preussmann and Stewart, 1984; Djordjevic *et al.*, 1994).

Both the carcinogenic potency and the target organ seem to vary with the chemical structure of the NOCs, the species of the animal being tested, their age, sex, as well as nutritional status. Besides being carcinogenic, many NOCs are mutagenic and some are teratogenic (Sen, 1992).

N-nitrosamines, on their own, are believed to be non-carcinogenic. They require metabolic activation before being converted to reactive alkylating agents, which are the ultimate carcinogens (Preussmann and Stewart, 1984). The N-nitrosamides, however, are direct-acting carcinogens requiring no metabolic activation.

Human are exposed to carcinogenic NOCs through ingestion or inhalation of performed nitrosamines in the environment or from precursors in the body. The result of extensive research showed that NOCs are commonly found in a various kinds of food, especially those preserved by nitrite, such as nitrite cured meat, bacon, ham, salted fish, skim milk power and beer (Sen and Kubacki, 1987). NOCs, notably nitrososarcosine (NSAR), 3-(methylnitrosamino)propionic acid (MNPA) and 4-(methylnitrosamino)butyric acid (MNBA), are also found in tobacco and tobacco smoke (Djordjevic *et al.*, 1994). Since the findings by Magee and Barnes in 1956, many researchers attempted to investigate the possible link between various human cancers and exposure to N-nitroso compounds. Exposure to endogenously formed NOCs has been associated with increased risk of oesophagus, stomach, bladder and kidney cancer, although convincing epidemiological evidence is still lacking (Ohshima and Bartsch, 1988). One of the

reasons is the lack of reliable and sensitive analytical methods to estimate the extent of *in vivo* formation of NOCs.

The potent carcinogencity of NOCs has prompted extensive research towards developing sensitive and specific analytical methods for detecting and quantifying NOCs in a variety of sample types, e.g. in foods and biological samples.

For analytical purposes, such methodologies have been divided into two broad categories: (a) methods for volatile N-nitrosamines (VNAs) and (b) those for nonvolatile N-nitroso compounds (NVNOCs). The term VNA is usually designated for all nitrosamines that can be isolated in good yields (>70%) from food matrices by aqueous distillation (steam, atmospheric or vacuum). Most of published methods for determination of VNAs are based on gas-chromatography and detection by the thermal energy analyser (GC-TEA). At present, the analytical methodologies for VNA have been developed and their reliability established by international collaborative studies (Scanlan and Reyes, 1985). On the contrary, the methodologies for non-volatile NOCs, like N-nitrosoamino acids, are much less developed. In particular, there are few reported methodologies employing either HPLC separation and mass spectrometric methods of detection (Sen, 1992).

The lack of analytical methods for quantifying NOCs could be due to a number of reasons. First and foremost is the fact that, due to the relative ease in which the N-nitroso bond is cleaved to yield NO, NOCs are thermally and photolytically unstable. Thus spectroscopic methods employing UV irradiation could not be employed to detect NOCs. Secondly, most of NOCs are small, highly polar molecules which are difficult to be separated efficiently by chromatographic

techniques, which include gas chromatography (GC) and high performance liquid chromatography (HPLC). The unique success of the thermal energy analyser (see Section 2.1.1, p.13) for specific detection of NOCs also dampen the drive for alternative methods of detection. However, GC-TEA analysis does have its limitation, particularly for non-volatile NOCs. These is also the need for confirmatory and validation analysis, which is the basis of good analytical chemistry and practice. The present project was initiated to develop alternative methods of separation and detection of non-volatile NOCs. In the process, methods for analysis of the precursors and inhibitors of *in vivo* NOC formation was also developed.

1.2 Non-Volatile N-Nitroso compounds - N-Nitrosoamino Acids (NAAs)

1.2.1 Occurrence, structure and properties of NAAs

The most common non-volatile compounds found in preserved food are four N-nitrosoamino acids (NAAs) : N-nitrososarcosine (NSAR), N-nitrosoproline (NPRO), N-nitrosothiazolidine-4-carboxylic acid (NTCA) and N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA) (Figure 1.1).

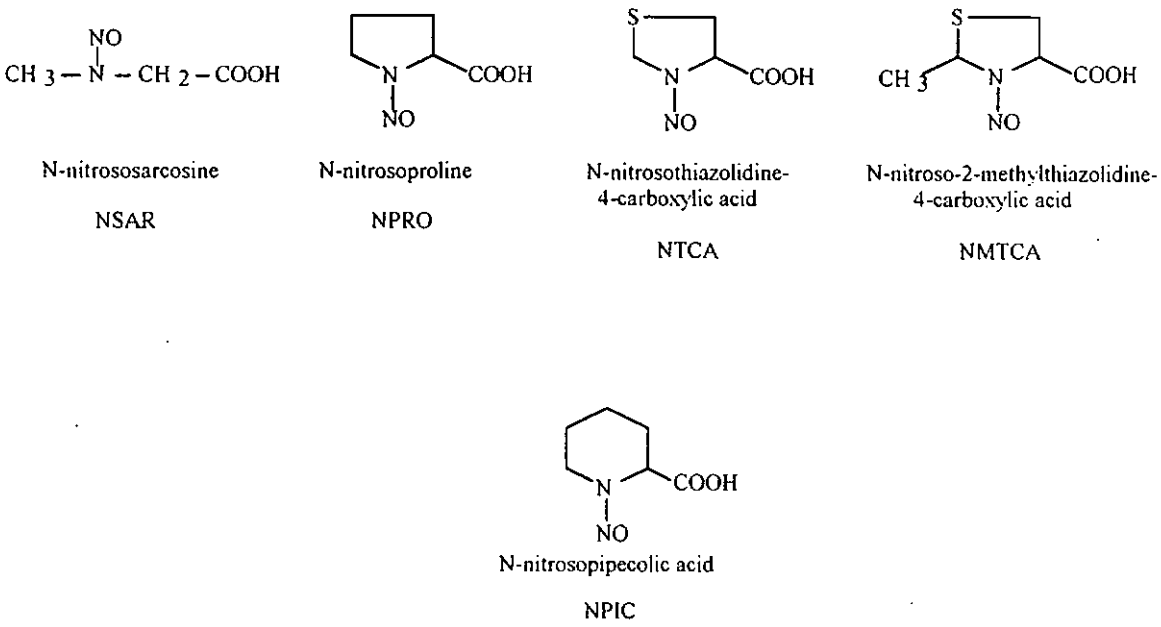
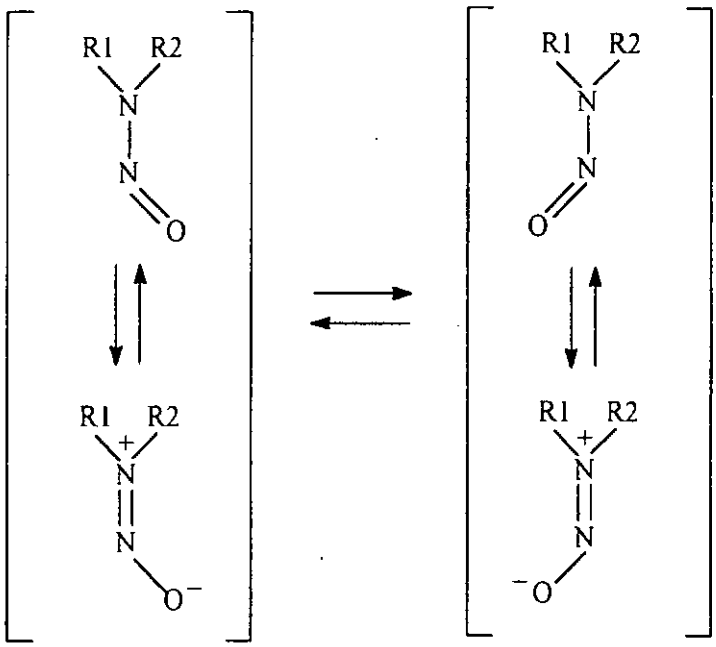


Figure 1.1 : Chemical structure of N-nitrosoamino acids

N-nitrosoamino acids are precursors of carcinogenic nitrosamines (Hamburg *et al.*, 1992). They are formed by nitrosation of free amino acids or peptides, followed by hydrolysis of the peptide bonds (Dunn and Stich, 1984). With the exception of NSAR, most NAAs have been reported to be non-carcinogenic. Quantitative determination of the amount of NAAs in food or biological sample would provide an estimation of nitrosation potential *in vivo* and *in vitro*. Of all the NAAs, N-nitrosoproline (NPRO), N-nitrososarcosine (NSAR) and N-nitrosothiazolidine-4-carboxylic acid (NTCA) have been mostly widely detected in cured meats, beer, malts, fish and tobacco products (Pensabene and Fiddler, 1990). The amount of NAAs found in food sample is a safe and reliable method for estimation of human exposure to exogenous NOCs. Another major contributory source of human exposure to non-volatile NOCs is tobacco smoking (Tricker *et al.*, 1994). Aside from exposure to exogenously NOCs, NPRO formation *in vivo* is widely used as a reasonable indicator for the exposure of endogenously formed NOC (Chakradeo *et al.*, 1994). Urinary excretion of NPRO has been used as an indicator of endogenous nitrosation (Ohshima and Bartsch, 1988).

All the common N-nitrosoamino acids are crystalline solids which melt sharply with decomposition. Those compounds are quite stable at room temperature. They are highly soluble in water and most organic solvents. The N-nitrosoamino acids are strong organic acids with $pk_a \approx 3$.

Due to overlap interactions of nitrogen non-bonding and p-π electrons in the N-nitroso group, a resonance character was found to occur at the N-N=O bond linkage. Consequently a partial double bond is formed between the N-N single bond (Scheme 1). Because of the partial double bond character of the N-N linkage, the nitrosoamino group of the NOCs, and NAAs in particular, assume an essentially planar conformation, in which the O atom is syn to one substituent and anti to the other. Syn and anti conformations are possible when $R_1 \neq R_2$.



Scheme 1

Lijinsky *et al.* showed that most of NAAs were preferentially crystallized in a conformation in which the C atom bearing the carboxyl group in syn to the nitroso O atom (Lijinsky *et al.*, 1970). NMR studies of NAAs in solution showed that some of NAAs isomerize to a mixture of the syn and anti isomers, whose composition appear to depend on steric factors (Chow *et al.*, 1981).

N-nitrosoamino acids undergo typical reactions of both the N-nitroso group and the carboxylic acid group. One remarkable reaction of NAA with possible toxicological consequence is their decarboxylation in dilute alkali solution to form N-nitrosamines, which are known to be both acutely toxic and carcinogenic (Lijinsky *et al.*, 1970). Also, the NAAs are characteristically photosensitive and the nitroso group is split by exposure to ultra-violet light. Ohshima *et al.* found that NTCA and NMTCA are particularly acid labile and were found to decomposed in considerable amounts (~90%) when they were dissolved in an acidic solution (0.1 and 1.0 mol/L hydrochloric acid) after 24-hr incubation at 50 ^{0}C (Ohshima *et al.*, 1984).

1.2.2 Carcinogenicity and Toxicity of NAAs

Unlike most NOCs, the majority of NAAs do not exhibit carcinogenic properties in laboratory animals. Only NSAR is proven to be a weak carcinogen in rats (Wogan *et al.*, 1975). NTCA has been shown to induce diabetes in laboratory animals. Nevertheless, Lin *et al.* indicated that NTCA are neither cytotoxic nor mutagenic (Lin *et al.*, 1990). Although NPRO, NTCA and NMTCA are not potent carcinogens, they are the precursor of carcinogenic nitrosoamine. Under high temperature conditions like those encountered during cooking processes, they can be converted to carcinogens by decarboxylation. For instance, NPRO forms N-nitrosopyrrolidine (Nixon *et al.*, 1976), NSAR forms N-nitrosodimethylamine (Janzowski *et al.*, 1978), and NTCA forms N-nitrosothiazolidine (Sen *et al.*, 1985).

NPRO is one of the non-carcinogenic and non-mutagenic NAAs. This may be related to the fact that it appears to be neither absorbed nor metabolized *in vivo* and is excreted directly into the urine. As it is metabolized to only a small extent inside our body and is excreted unchanged, monitoring the levels of urinary N-nitrosoamino acids appears to be a useful way for estimation of the extent of endogenous nitrosation (Ohshima *et al.*, 1982). The NPRO test has been applied to human subjects in clinical and epidemiological studies, and the kinetics and dietary modifiers of endogenous nitrosation have been investigated. Results obtained after application of the NPRO test to subjects at high risk of cancers of the stomach, oesophagus, oral cavity and urinary bladder showed that in most instances, higher exposures to endogenous NOC were found in high-risk subjects (Bartsch *et al.*, 1989). But individual exposure was greatly affected by dietary modifiers or disease states.

1.3 Interaction of Nitrosating Agents and Ascorbic acid on NOC formation

Nowadays, in many epidemiological and clinical studies, NOCs in food and biological samples were determined in conjunction with their nitrite, nitrate and ascorbic acid content. It is because nitrite and nitrate are precursors, or nitrosating agents, leading to formation of NOCs, while ascorbic acid is an inhibitor of nitrosation *in vivo* (Mirvish *et al.*, 1995). Their levels in food and biological samples are often used to deduce the likely sources of formation of NOCs.

The addition of nitrite and /or nitrate salts to meats, poultry and fish at low concentration (up to a few 100 ppm) has been a common method of preservation for centuries (Binkerd and Kolari, 1975). Nitrite addition to meat products results in the formation of characteristics pink-red coloration in cured meats (Clydesdale and Francis, 1971), prevents rancidity occurring under normal storage conditions (Gray *et al.*, 1981) and weight against the risk from outgrowth and toxin production by *Clostridium botulinum* growth (Woods and Wood, 1982). However, nitrite, and nitrate after being metabolized or reduced to nitrite, can react with amines, currently occurring in the meat product, to form N-nitroso compounds (Magee *et al.*, 1982). Cancer could be induced at specific sites in animals by nitrosamines and nitrosamides as well as by nitrite administered together with the corresponding amine or amide. Moreover, too much intake of nitrite and nitrate in the diet may cause toxic effects since methaemoglobinaemia is produced by oxidation of hemoglobin by nitrite (Chan, 1996). Therefore, the use of nitrite and nitrate as meat and poultry curing agent are strictly controlled by legal regulation in some countries, e.g. U.K.

On the basis of safety evaluation, the maximum Acceptable Daily Intake (ADI) for nitrite have been suggested by the joint 1996 FAO/WHO Expert Committee on food additives to be 0 - 0.06 mg/kg body weight/day (expressed as nitrite ion) (FAO/WHO, 1996). Based on similar criteria, the ADI for nitrate is recommended to be 0 - 3.7 mg/kg body weight/day (expressed as nitrate ion).

Ascorbic acid is an inhibitor for *in vivo* formation of N-nitroso compounds by competing with the precursors for nitrosating agents (Helser *et al.*, 1992). This may explain why the intake of ascorbic acid-rich fruits and vegetables is negatively

correlated with many types of cancer, although it may also react with free radicals in tissues, thereby blocking promotion of radical species. Mirvish reported that ascorbic acid inhibits the endogenous formation of carcinogenic N-nitroso compounds (NOCs) in the gastric lumen, thereby lowering the risk of developing a gastric neoplasm (Mirvish, 1983). Also, ascorbic acid was shown to inhibit NPRO formation in most of the quoted NPRO studies. Addition of ascorbic acid to nitrite-preserved food is required by USDA regulations (Joseph *et al.*, 1987). Since nitrite, nitrate and ascorbic acid are closely related in their ability to induce or prevent formation of carcinogenic N-nitroso compounds, it is worthwhile to develop an analytical method for simultaneous quantitation of nitrite, nitrate and ascorbic acid in food.

In this project, canned vegetable juices were chosen as the food sample for analysis of nitrite, nitrate and ascorbic acid because they are one of the likely major and common source of nitrite and nitrate, in modern day diet especially for city dwellers, and also because they are commercial products that may be required to be regulated.

Chapter 2 Analytical Methods for Measuring N-Nitrosoamino Acids

2.1 Gas chromatography

Gas chromatography (GC), in the last 30 years, is the most utilized chromatographic technique for the separation of N-nitroso compounds, especially the volatile N-nitrosamines. The popularity of GC separation is not only due to its common availability, but also due to its good resolution on the separation of NOCs compared to HPLC. For non-volatile NOCs, they are usually converted to volatile chemical derivatives followed by GC analysis. GC analysis is compatible with the commonly used NOC detector, the thermal energy analyser (TEA), a specific, qualitative and quantitative measuring device specifically designed for NOCs. Although N-nitrosoamino acids are non-volatile NOCs, most of their analysis to-day are carried out with their volatile methyl ester or silyltrimethyl derivatives rather than in the underivatized form. According to the authoritative review by Sen and Kubacki, this could be due to two main reasons : (1) the four NAAs, including NSAR, NPRO, NTCA and NMTCA commonly found in food and biological samples, have a high ~85% derivatization yield, and (2) the four NAAs can be completely separated with a simple GC temperature programming condition (Sen and Kubacki, 1987). There are, however, disadvantages associated with GC analysis of NAAs in their derivatized forms. First, an additional derivatization step is involved, which is time consuming and is an additional source

of experimental error. The execution of the derivatization step requires experimental skills and experience ; problems of side reactions and partial derivatization was reported in a number of literature reports. The derivatization process lengthens the analytical time and it is not convenient for routine analysis. Furthermore, with the exception of NMTCA, the syn and anti conformers of NAAs cannot be differentiated by GC separation.

At present, the thermal energy analyser (TEA) and mass spectrometric detection are usually used in conjunction with gas chromatography for qualitative and quantitative analysis of volatile and non-volatile NOCs.

2.1.1 GC-Thermal Energy Analyser (TEA) Analysis

As mentioned earlier, TEA is most widely used for determination of NOCs in all types of samples. Figure 2 shows the basic component of the TEA detector. Basically, it is a chemiluminesence detector. When an N-nitrosamine is introduced into the TEA, it enters first into a pyrolysis chamber, where the N-NO bond in the molecule is cleaved. Nitrosyl (NO) radicals produced from the bond cleavage are swept by a carrier gas into cold traps or Tenax cartridges. Various pyrolysis products, other than NO, are either adsorbed on a Tenax cartridge or in suitable cold traps (-140 ^{0}C to - 160 ^{0}C). Therefore, the TEA is highly selective for the determination of NOCs. After trapping interference substances in the effluent stream, the NO radicals enter the reaction chamber where they are allowed to react with ozone to form excited NO_2 molecules. The excited NO_2^* afterwards returned

to the ground state with emission of light in the near infrared region (600-3000 nm).

$$NO + O_3 \longrightarrow NO_2^* + O_2$$

$$NO_2^* \longrightarrow NO_2 + h\nu$$

The emitted light in the 600-800 nm range is detected by a photomultiplier tube and amplified electronically to give an output response. A vacuum pump is used to maintain the instrument under moderate vacuum.

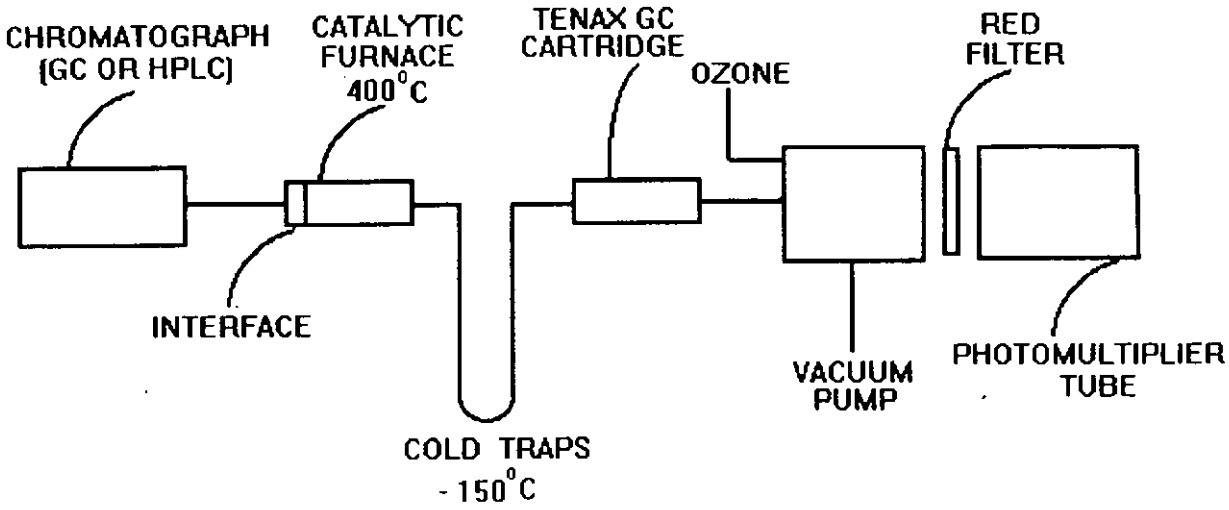


Figure 2.1 : Schematic diagram of the TEA Analyzer

TEA is an extremely useful detector for the determination of NOCs in food and biological samples. It is both highly selective and sensitive, and the response is linear over a wide range (2-3 orders of magnitude). However, some other compounds such as nitrite, C-nitroso compounds and some olefins give

positive response to TEA. A list of TEA responsive interference compounds is shown in Table 2.1. Thus, several clean-up procedures should be carried out to removed these potential interference compounds before the GC-TEA analysis either by first treating the samples with nitrite scavengers, or taking the sample extract through suitable clean-ups step. Another problem has been lack of information on the identity and structure of nitrosamines likely to be encountered, or unknown NOCs. For this reason, some laboratories or research groups prefer the determination of the "total" nitroso compounds, i.e. the sum of the volatile and nonvolatile NOC contents of samples. Therefore, alternative methods of NOCs analysis are needed to validate the results of GC-TEA analysis. This is especially important for identifying new NOCs, and also confirming their presence in un-tried sample matrices.

Table 2.1 : TEA responsive compounds that are not N-nitroso compounds

Compound	Molar Response Ratio[a]
Sodium Saccharin	1×10^{-4}
C-nitro Compounds	
Tetranitromethane	3.1
2,2',4,4',6,6'-Hexanitrodiphenylamine	1.4
2,3-Dimethyl-2,3-dinitrobutane	1.1
2,2-Dinitropropanol	9×10^{-1}
1,5-Difluoro-2,4-dintirobenzene	1×10^{-1}
2,4,6-Trinitro-3,5-dimethyl-t-butylbenzene	1×10^{-1}
2-Nitro-2-bromo-1,3-propanediol	8×10^{-2}
2-Nitro-1-propanol	2×10^{-2}
2,6-Dinitrochlorobenzene	2×10^{-2}
Pentachloronitrobenzene	4×10^{-3}
C-nitroso Compounds	
2-Nitroso-2-acetoxymethylpropane	(b)
2-Nitroso-2-methylpropane	(b)
2-Nitroso-2-chloromethylpropane	(b)
O-nitro Compounds	
Ethylene glycoldinitrate	2×10^{-1}
N-propylnitrate	7×10^{-1}
Isopropylnitrate	7×10^{-1}
N-butylnitrate	8×10^{-1}
Isopentylnitrate	9×10^{-1}
O-nitroso Compounds	
n-Pentylnitrite	1.0
Isopentylnitrite	1.0
Sodium nitrite	1.0
Nitric acid	1.0

a) Molar response ratio = $\frac{\textit{TEA response per mole of compound}}{\textit{TEA response per mole of NDMA}}$

b) Due to a faulty pyrolysis chamber, the response ratios were estimated incorrectly. The response ratios should be less than 1.0 because each compound contains only one nitroso group.

Taken from Fine, D.H., Rufeh, F., Lieb, D., and Rounbehler, D.P., 1975, Analytical Chemistry, Vol. 47, pp. 1188

2.1.2 GC-Electron-Impact Mass spectrometry

Gas Chromatography/electron-impact mass spectrometry (GC/EI-MS) is usually used to qualitatively validate the result obtained from GC-TEA. To the best of our knowledge, there are few reports on the validation of GC-TEA quantitatively results by GC/EI-MS. This is probably due to the fact that severe interference are usually present in the sample matrix so that different clean-up procedures are needed for GC-TEA and GC/EI-MS analysis. Wagner *et al* reported that additional clean-up steps were needed for urine samples for GC/MS analysis when compared to GC/TEA detection (Wagner *et al.*, 1985). The additional steps included passage through an anion exchange column, and further HPLC clean-up steps (amino-NH_2 column and C_{18} column) were needed to remove other organic acids in the urine sample prior to NAAs analysis. For this reason, GC-MS analysis of NAAs has not been routinely used.

For the methyl ester derivative of NAAs, only weak molecular ions can be found in their respective electron impact mass spectra. The most common fragment ion found is the $[M\text{-}COOCH_3]^{+\cdot}$ ion, which can be used to identify the NAAs (Ishibashi *et al.*, 1980).

At present, EI spectra are still commonly used for confirmation and structural studies of nitrosamines. While confronted with some unknown nitrosamines in the food or biological sample, EI-MS still plays an important role in the structural identification.

2.2 High Performance Liquid Chromatography (HPLC)

GC, GC-TEA and GC-MS are the established methods for analysing NOCs. The need for developing HPLC related methods of analysis for non-volatile NOCs was noted in the early 1990s (Sen, 1992), but successful development in this direction has been limited. In theory, there are a wide variety of chromatographic modes that can be employed for the HPLC determination of NAAs compound, but only a few have been tried for the determination of NAAs. These included partition/adsorption on silica gel, liquid-liquid partition on polar-bonded phase (e.g. cyano and amino bonded phases) or non-polar hydrophobic-bonded phase (e.g. C_{18} reverse phase), and anion exchange chromatography (Sen and Kubacki, 1987).

In practice, the choice of the proper stationary and mobile phases for HPLC separation is dependent on several factors such as the nature (polarity, stability in mobile phase) of the NAAs analyzed, and the availability / compatibility of the detector used. For example, if only a TEA is available as a detector, the use of an ion exchange or a reverse-phase system is ruled out because both required aqueous mobile phase for proper operation. Moisture in the mobile phase causes freezing-up of cold traps in the TEA, and also results in noisy response due to interference during chemiluminescence detection (for details, refer to Section 2.2.3 p.27). Similarly, if one is using the newly developed hydrogen-iodide (HI)-catalysed denitrosation-TEA or the photolytic cleavage-TEA as the detector, a reverse phase HPLC system using aqueous mobile phase would be the method of choice.

2.2.1 Separation by Normal and Reverse phase HPLC

Analysis of non-volatile NOCs by HPLC offers the advantage direct analysis in the underivatized state, consequently time and man-power can be saved. Unfortunately, there are only limited references in the literature on HPLC determination of non-volatile NOCs in foods. The problem is mainly because of the incompatibility between the aqueous mobile phase and the TEA detector due to solvent trap-freezing. Therefore, reported HPLC methods for analysis of NAAs were limited to the use of normal phase systems like silica (Si), cyano (CN) and amino (NH_2) as stationary phases and mixtures of organic solvents of low polarity as eluent. However, incomplete separation and peak tailing usually resulted.

In one of the studies, Tricker *et al.* used hexane-acetone-acetic acid (81 : 18 : 1 by *v/v*) with cyano-bonded column unable to differentiate between NTCA, NSAR and NPRO, especially when one of these is present in much higher concentrations than the other (Tricker *et al.*, 1984). In another study, Sen *et al.* used an organic buffer containing only ~2 % *v/v* water, and were able to separate the syn and anti isomers of NSAR and NPRO on a silica column (Sen *et al.*, 1980) The technique was, however, susceptible to slight changes in experimental conditions and often resulted in high background noise (Kubacki *et al.*, 1984). To avoid this problem, Kubacki *et al.* derivatized the NAAs to their methyl esters before analysis by HPLC-TEA using only organic solvents (e.g. n-hexane and dicholomethane) (Kubacki *et al.*, 1989) On the amino ($-NH_2$) column, NSAR and NPRO were unresolved, while on the cyano column they were just separated at the top of peaks. In order to enhance separation efficiency, the temperature of the

HPLC column was increased to 90^0C , then baseline separation was achieved. However, the need of derivatization prior to HPLC analysis defeated the aim of directly analysing these compounds in the underivatized state. Besides, the HPLC system has to be heated.

Based on the ionic nature of the NAAs compound, it is the best to apply the ion-exchange or reverse phase HPLC for their analysis. Thus, either reverse-phase or normal phase HPLC are satisfactory for the analysis of NAAs. In a recent study, both normal and reverse phase HPLC were used in series, but only partial separation of NPRO and NTCA was achieved (Massey *et al.*, 1991). A better separation of the N-nitrosoamino acid carboxylate anions can be achieved by ion-exchange or reverse phase HPLC, but that requires the use of an aqueous mobile phase which is incompatible with the operation of the TEA (Kubacki *et al.*, 1984). NSAR and NPRO could be successfully separated by the ion-suppression RP-HPLC (10 mM trifluoroacetic acid) with 254-nm UV detection (Conboy and Hotchkiss, 1989).

The syn and anti conformers of NPRO, NSAR and NTCA are reported to be separated with an α-cyclodextrin-bonded silica gel column using acetonitrile : triethylammonium acetate buffer as the mobile phase. Nevertheless, it could not separate a mixture of these three NAAs, and the column is not commonly available (Issaq *et al.*, 1988). Also, microgram amounts of NAAs were analysed because the less sensitive UV absorption detector at 238 nm was employed (Issaq *et al.*, 1988).

Thus, only limited success was achieved in the separation of NAAs by HPLC methods, and there is ample room for development both in terms of

separation efficiency and compatibility with chosen method of detection. In this project, we propose a new approach to the separation of N-nitrosoamino acids by the application of C_{18} reverse-phase ion-pair chromatography, the details of which are discussed in detail in Section 5.

2.2.2 Reverse-phase Ion-pair Chromatography

Since C_{18} reverse-phase ion-pair chromatography (RP-IPC) was extensively applied in the separation and analysis of NAAs and related compounds in this project, a brief introduction of this chromatographic technique is given in this sub-section. Reverse-phase ion-pair chromatography is quite a board term to describe all chromatographic separation of hydrophilic ionic solutes on lipophilic stationary phases. It includes the chromatographic process of ion-pair chromatography, ion-exchange chromatography, ion interaction chromatography, and a variety of similar chromatographic techniques. The mechanism by which separation takes place in ion-pair chromatography is not fully understood. The following retention models are proposed to explain the separation processes involved.

(a) The ion-pair model

In this model, an ion-pair is envisaged to form between the solute ion and the ion-interaction reagent. This occurs in the aqueous-organic eluent and the resultant neutral ion-pair can be absorbed onto the lipophilic stationary phase in the same manner as any neutral molecule with lipophilic character is retained in

reverse-phase chromatography. Retention therefore results solely as a consequence of reaction taking place in the eluent. The degree of retention of the ion-pair is dependent on its lipophilicity, which in turn depends on the lipophilicity of the pairing ion itself. An increase in the percentage of organic solvent in the eluent decreases the interaction of the ion-pairs with the stationary phase and therefore reduces their retention.

(b) The dynamic ion-exchange model

The dynamic ion-exchange model proposed that a dynamic equilibrium is established between ion-interaction reagent adsorbed onto the stationary phase, as follows :

$$\text{Ion-interaction reagent}^{\pm}_{\text{(In eluent)}} \rightleftharpoons \text{Ion-interaction reagent}^{\pm}_{\text{(In stantionary phase)}}$$

The adsorbed ion-interaction reagent imparts a charge to the stationary phase, causing it to behave as an ion-exchanger. The total concentration of pairing ion adsorbed onto the stationary phase is dependent on the percentage of the organic solvent in the eluent, with higher percentages of solvent giving lower concentrations of pairing ion on the stationary phase. Thus, for a given eluent composition, the concentration of adsorbed ion-interaction reagent remains constant. The constant interchange of ion-interaction occurs between the eluent and the stationary phase, so the stationary phase can be considered to be a dynamic ion-exchanger.

(c) The ion-interaction model

The ion-interaction model can be viewed as intermediate between the two previous models in that it incorporates both the electrostatic effects which are the basis of the ion-pair model and the adsorptive effects which form the basis of the dynamic ion-exchange model. The lipophilic ion-interaction reagent ions are considered to form a dynamic equilibrium between the eluent and stationary phase, as described in the above equation. This results in the formation of an electrical double-layer at the stationary phase surface.

The adsorbed ion-interaction reagent ions are expected to be spaced evenly over the stationary phase due to repulsion effects, which leaves much of the stationary phase surface unaltered by the ion-interaction reagent. The adsorbed ion-interaction reagent ions constitute a primary layer of charge, to which is attracted a diffuse, secondary layer of opposite charge ions. The secondary layer of charge consists chiefly of the counter-ions of the ion-interaction reagent. The amount of charge in both the primary and secondary charged layers is dependent on the amount of adsorbed pairing ion, which in turn depends on the lipophilicity of the pairing ion, the pairing ion concentration, and the percentage of organic solvent in the eluent. Transfer of solutes through the double-layer to the stationary phase surface is a function of electrostatic effects and of the solvophobic effects responsible for retention in reverse-phase chromatography.

The three models are illustrated schematically in the Figure 2.2 All proposed mechanisms fully explained the experimental observation that neutral solutes molecules are unaffected by the presence of the ion-interaction reagent.

The three mechanisms differ in the relative effects of the nature and size of the ion-pairing counter ion, and the percentage organic solvent in the aqueous mobile phase on the retention of the analyte, which is also related to the chemical properties of the analyte. These effects are described in more detail in Section 5.1.1 (p. 44).

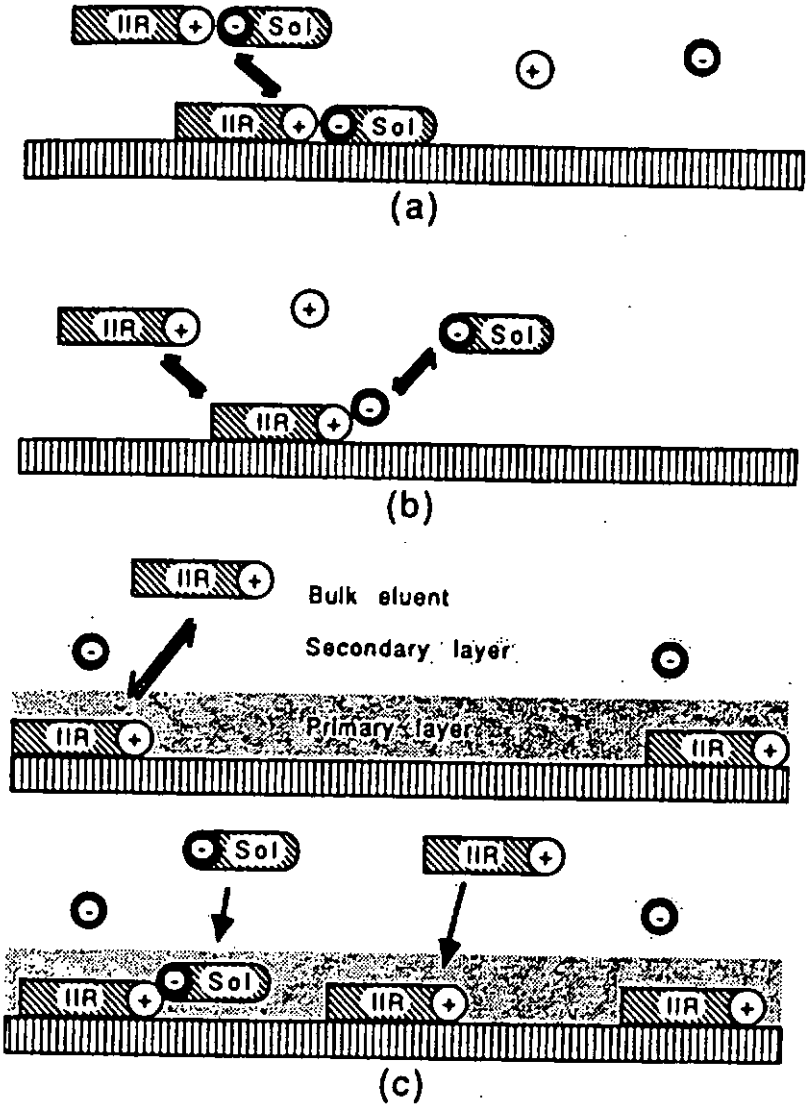


Fig 2.2 : Schematic illustration of (a) the ion-pair, (b) the dynamic ion-exchange and (c) the ion-interaction models for the retention of anionic solutes in the presence of a lipophilic cationic ion-interaction reagent. The solute and the ion-interaction reagent (IIR) are labeled on the diagram. The large, hatched box represents the lipophilic stationary phase, the black circle with the negative charge represents the counter-anion of the ion-interaction reagent, whilst the white circle with the positive charge represents the counter-cations of the solute.

In spite of many mechanistic studies, the theory of ion-pair chromatography has remained a subject debate over many years. In a fundamental study on the ion-pair and the dynamic ion-exchange model (so-called stoichiometric models), Knox and Hartwick pointed out that formally both models lead to identical retention equations (Knox and Hartwick, 1981). They have provided an easy-to-understand qualitative picture of solute retention for many analytes and promoted the practical use of ion-pair chromatography (IPC). For these reasons, the concepts and qualitative trends predicted by these two models will be used to rationalized our observed results in Section 5.3 on the separation of NAAs by C_{18} reverse-phase ion-pair HPLC.

The ion-interaction, or the non-stoichiometric model describes the retention of ionic analytes without the formation of chemical complexes. These models assume that the retention of solute ions is partly determined by their interaction with the electric field created by the adsorbed pairing ion. Therefore, the effect of the pairing ion is assumed to be indirect and acts through establishing a certain electrostatic surface potential. The development of the ion interaction model was largely stimulated by experimental evidence of the adsorption of the ion-pair reagent on the hydrophobic stationary phase. A qualitative description of electrostatic interactions in IPC systems was given by Bidlingmeyer in the ion-interaction theory (Bidlingmeyer, 1980).

A number of attempts have been made to formulate the model quantitatively (Stranahan and Deming, 1982). However, none of them provided a rigorous description of the system, and some even reduced to a stoichiometric model (Zou *et al.*, 1991). Based on this model, Stahlberg and Hagglund were able

to explain the effects of different mobile phase electrolytes (Stahlberg and Hagglund, 1988) ; Bartha and co-workers have extended the model to include the effect of organic solvent and eluent pH (Bartha *et al.*, 1991). However, the quantitative ion-interaction model has been considered to be too complex for practical work. For this reason, the ion interaction model is used to rationalized our experimental observations in Section 7.3 only, in which the adsorption of octylamine phosphate, the ion-pair reagent used, on the C_{18} stationary phase was used in previous studies to rationalize the retention of nitrite and nitrates in aqueous mobile phases.

2.2.3 Incompatibility of HPLC separation with Thermal Energy Analyser detection

The working principle of TEA is described in Section 2.1.1. However, reported studies up to now indicate that HPLC-TEA analysis of non-volatile NOCs has not been successful. In the HPLC-TEA analytical system, most solvent vapors derived from the HPLC mobile phase and pyrolysis products are trapped in a series of two cold traps (dry ice + ethanol). This has been found to be quite efficient in removing most organic solvent vapors, but is unworkable with aqueous mobile phase because of freeze-up and blockage of the cold traps. Also, traces of moisture entering the TEA detection chamber interfere with the chemiluminescence detection. In addition, mobile phases containing inorganic buffers are totally incompatible with the detector because of possible coating of the catalyst in the pyrolyzer tube, thus affecting the final sensitivity. All these factors

make TEA incompatible with HPLC separation, and become a major obstacle to analysis of non-volatile NOCs without chemical derivatization.

2.2.4 HPLC Analysis with Postcolumn Denitrosation Detectors

Because of the failure of HPLC-TEA method of analysis, a variety of alternative instrumental set-ups have been reported for HPLC determination of NOCs based on postcolumn denitrosation and detection of the liberated nitric oxide radical or resulting nitrite ion. These specialized methods use custom-built instruments and differ in both the determination procedures and the final determinative techniques.

Conboy *et al.* suggested a UV photolysis method that is similar to normal HPLC-TEA (Conboy *et al.*, 1989). First, the >N-N=O bond cleavage is done photolytically by UV light, thus making it amenable to the determination of thermolabile NOCs, especially nitrosamides since they give poor yields of NO under normal TEA pyrolytic conditions. Second, by omitting the TEA furnace, this detector allows one to use aqueous mobile phases necessary for reverse-phase or ion-exchange chromatography. However, the composition (ratio of the organic modifier to water) of the mobile phase seemed to have a marked effect on the response of various NOC tested. The response was linear between 1 and 100 ng NPRO injected, but fell off at > 100ng.

Based on the UV photolysis, Righezza *et al.* described another method that the NOCs, after HPLC separation, were photolyzed by a UV lamp, and the charged nitrite species was determined amperometrically (Righezza *et al.*, 1987).

The de-nitrosation reaction was found to be dependent on wavelength of the UV light, lamp intensity, exposure time, and pH of the solution.

Alternatively, postcolumn de-nitrosation using HBr or HI as the dentirosating agent was reported by several researchers. Havery offered a method that employed KI solution as de-nitrosating agent for NOCs (Havery, 1990). The liberated NO was finally detected by TEA. This system worked well with aqueous mobile phase but not with normal-phase solvents. The temperature of the reaction coil was critical for the determination of N-nitrosamines. While a temperature of 23 ^{0}C was adequate for the denitrosation of NPRO, a much higher (up to 70 ^{0}C) temperature was required for comparable response from other NOCs.

UV photolysis-TEA detection was subjected to positive interferences from alkyl nitrites and C-nitroso compounds. In this regard, it is less specific than the HX-denitrosation techniques, which showed negligible or no response for such compounds. In fact, the post-column de-nitrosation methods mentioned above for the determination of NAAs in foods found limited applications by other laboratories. In a recent review, Sen (1992) suggested that the usefulness of these methods would largely depend on the resulting chromatographic resolution of the system employed as well. For example, cured smoked meats may contain as many as 8-10 NAAs or other NOCs. Therefore, it would be imperative that the chromatographic system used, including the HPLC stationary and mobile phase accepted, give acceptable resolution of these NAAs and other NOCs (e.g. NDMA, NPIP, NHPYR) that might be present in the food sample.

From these reported studies, it can be seen that analysis of non-volatile NOCs is a difficult and challenging problem, both in terms of separation efficiency, as well as choice and compatibility of the detector with the HPLC mobile phase.

2.3 HPLC Analysis with detectors other than TEA

2.3.1 HPLC Analysis with UV Detectors

As most NAAs absorb strongly in the UV region (220 - 235 nm and 330 - 375nm), UV detectors could be very useful for the determination of NAAs in foods. However, the detector is quite non-specific, and there is a possibility of false-positive results from other UV-absorbing compounds present in food extracts. For this reason, the use of such detectors has been limited to the analysis of NOC standards only (Issaq et al., 1988). An UV detector should still be useful for analyzing very clean extracts or studying N-nitrosamine formation in model systems. Also, a UV absorption detector is less sensitive than the TEA, and could only detect NAAs in the μg/ml or ppm levels.

2.3.2 Analysis by Moving Belt Liquid Chromatography/Mass spectrometry

Mass spectrometry is generally considered to be the most reliable tool for the characterization and quantitation of trace levels of toxic organics in food. In this regard, GC-MS has been much more widely used than HPLC-MS techniques mainly because of its relative ease of operation, the advanced state of commercial instrumentation, and the high resolving power of capillary GC column that allows one to carry out specific detection at extremely low nanogram to picogram levels.

GC-MS techniques are, however, not applicable to all NOCs, especially some thermally labile non-volatile NOCs.

Although the potential benefits of the HPLC-MS are obvious, the main difficulties lie in interfacing an HPLC system with a MS. With the advancement of developed LC-MS techniques, and the availability of commercial LC-MS instruments, it is an opportune time to develop a HPLC-MS technique for analysis of NOCs. However, there is a paucity of data in the literature on the application of LC/MS to the determination NOCs in foods.

The only report on LC/MS analysis of non-volatile NOCs was that by Beattie et al. (1985). A moving polyamide belt interface and chemical ionization (ammonia as the ionizing medium) were used for this purpose. However, the NAAs were found to be thermally decomposed during the solvent heating and vaporization step. Although the technique provided useful structural information, it had limited sensitivity and was unsuitable for trace analysis of NAAs in complex food or biological extracts (for details, refer to Section 6.1.3, p.104).

2.3.3 Electrospray Ionization Mass Spectrometry

The use of electrospray (ESI) for the ionization of a very wide variety of biochemical and chemical compounds with low or high molecular weight is now well established in mass spectrometry. Electrospray is a method that allows ions to be transferred from solution to the gas phase and subjected to mass spectrometric analysis. In fact, ESI-MS is probably the most versatile soft ionization technique and allows direct interfacing with HPLC. Through the advances made in the last 10

years, ESI is becoming the most popular interfacing ionization technique for the coupling between the liquid chromatography, in particular, reverse-phase liquid chromatography, with mass spectrometric detection. It is because the most common eluent in reverse-phase liquid chromatography is aqueous methanol or acetonitrile, which are also the most used solvents in electrospray mass spectrometry. Furthermore, thermally labile and polar samples can be ionized without thermal degradation since input of heat to the electrospray capillary is not required in electrospray operations.

As far as we know, ESI/MS has not been applied to the analysis of NAAs. As ESI operates only at mild temperatures, the NAAs can be easily ionized without thermal decomposition. Qualitative and quantitative analysis of NAAs by HPLC/ESI-MS is carried out in this project. The developed method is also used to assay the NAA content in food sample as a demonstration of its practical applicability.

2.3.3.1 The Electrospray Ionization Process

The sample solution is injected via a HPLC sample loop, pumped through a electrospray capillary which is maintained at a potential of several kilovolts (2.5 - 3.5 kV) relative to the surrounding chamber walls, and sheared by a stream of N_2 nebulizing gas (Figure 2.3). A mist of highly charged droplets with the same polarity as the capillary voltage is generated. The charged droplets are continuously reduced in size due to evaporation of solvent, which is aided with another stream of nitrogen drying gas, leading to an increase of surface charge

density and a decrease of the drop radius. Finally, the electric field strength reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the liquid to be ejected into the gas phase (Figure 2.4). The emitted ions are then accelerated onto the mass analyser for analysis.

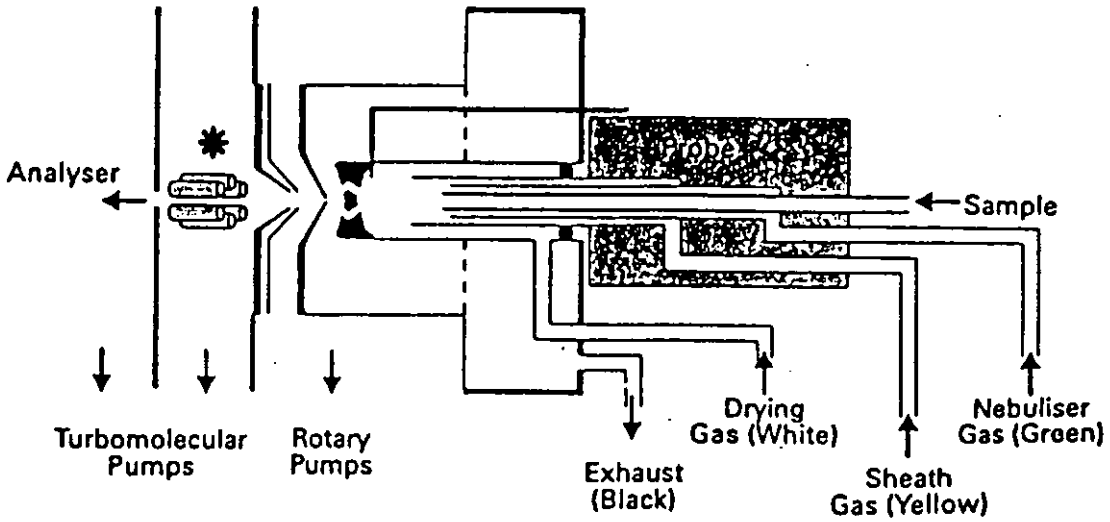


Fig. 2.3 : Diagram of ESI interface

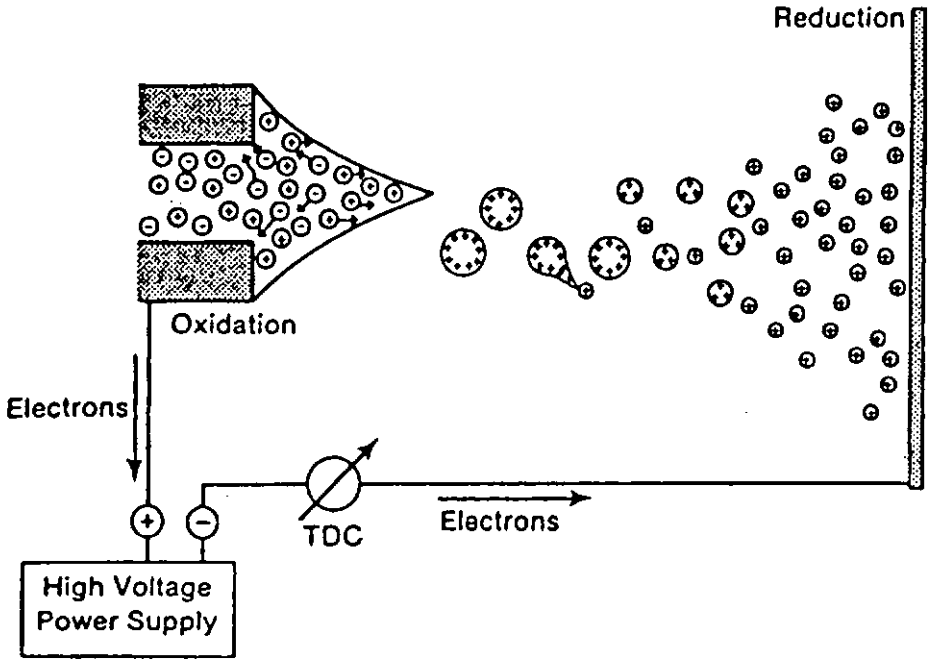


Fig. 2.4 : Schematic of major process occurring in electrospray. Taken from Kebarle and Tang, Analytical Chemistry, 1993, 65, 972A.

The electrospray process is a complex process. The mechanism of droplet formation and ion evaporation has been treated in detail by Kebarle and Tang (Kebarle and Tang, 1993). The ion intensity of the analyte is dependent on a variety of parameters, including its solvation energy, ionic radius and presence of co-eluting cations/anions. The parameters affecting ESI detection of NAAs are discussed in more details in Section 5.1.2.

2.3.3.2 Solvent Cluster Ion Formation in Electrospray Ionization

Electrospray ionization (ESI) is one of the ionization techniques available for an ion source operating at atmospheric pressure, whereas mass separation and detection is working under vacuum conditions. In ESI/MS, the ions were transported from an atmospheric pressure region into the vacuum system of the mass spectrometer so that the strong cooling of a mixture of gas and ions occur when expanding into vacuum. The resulting condensation of polar neutrals (notably water and solvent vapor) on analyte ions produces cluster ions.

$$X^- + nH_2O \longrightarrow X^-(H_2O)_n$$

In any design of an atmospheric pressure ionization source, in particular electrospray, the problem of formation of cluster ions must be addressed. Polar molecules that tend to cluster with ions are water and solvent vapor present in air, and are usually generated by the evaporation of the eluate when a liquid chromatography is coupled to an ESI source.

To prevent cluster ion formation, one way is to impose a countercurrent flow of drying gas which pushes water, solvent molecules and neutral contaminants

away from the source region. Thus, the ESI generated ions have passed through a so-called dry nitrogen gas curtain. As a result, cluster ions are not formed, in spite of the strong cooling due to the expansion into the vacuum system, since nitrogen lacks the ability to form hydrogen bridges with solvent clusters. It is a very elegant solution for solving the clustering problem. Another way of preventing cluster ion formation is to increase the ion source temperature so that the temperature of gas and ions remains high enough after expansion and clustering can be avoided.

Despite the instrumental design to reduce formation and observation of cluster ions in the ESI process, cluster ions are frequently observed in many ESI experiments due to the fact that the clustering reaction are energetically and kinetically favorable. In our study, cluster ion formation between the co-eluting anion of the ion-pairing reagent and the solvent molecules was indeed found to be very serious and directly interfered in the detection of NAAs. Since adjustment of instrumental parameters would lower the detection sensitivity, we have undertake a systematic study on the effect of solvent composition on reducing cluster ion formation. This part of the study is discussed in Section 5.3.3.3 (p. 94).

Chapter 3 Analysis of Nitrosating Agents and Ascorbic acid

Nitrite and nitrate are established nitrosating agents, which react alkylamines under suitable conditions to yield NOCs. Various methods for determination of nitrite and nitrate by ion selective electrode, chemiluminescence, colorimetry, gas chromatography, HPLC and ion chromatography have been reported (Utermahlen *et al.*, 1992). The availability of analytical methods for nitrite and nitrate quantitation are sample dependent as various degrees of interference may be encountered in different types of samples. Nitrite, and nitrate after reduction to nitrite, are routinely measured in food by a spectrophotometric absorption method involving diazotization/coupling reaction between sulphanilamide and N(1-naphthyl)ethylenediamine (Hunt, 1994). However, residual ascorbate was found to interfere positively with the diazotization and coupling reaction (Fox *et al.*, 1984). Significant interference in the nitrite measurement due to reduction by ascorbic acid under acidic conditions were reported (Davis *et al.*, 1985 ; Norwitz and Keliher, 1987 ; Riise and Berg-Nielsen, 1990).

Nitrite is usually analysed together with nitrate. At present, a more direct approach for simultaneous analysis of nitrite and nitrate is by employing anion exchange or reverse phase ion-pair HPLC. An ion chromatography method using Dionex Ionpac AS5 anion exchange column and eluting with 2.1 mM sodium bicarbonate for quantitation of nitrite and nitrate in gastric juice was reported by Uternanlen *et al.* (Uternanlen *et al.*, 1992). Also, Ewa *et al.* used a reverse-phase C_{18} column with 2.0 mM nonylammonium phosphate at pH 6.5 as mobile phase for the determination of nitrite and nitrate in natural waters (Ewa *et al.*, 1993). The

problem with analyzing nitrite and nitrate by these techniques in environmental or biological samples is the high concentration of other anions, especially chloride ions, which interfere with the determination of nitrite due to lack of chromatographic resolution and instability of retention times. Removal of interference anions by using pretreatment columns, and precipitation of chloride ion by silver affinity column was sometimes adopted.

Ascorbic acid is usually determined separately by chemical analysis or HPLC methods (AOAC, 1989 edition). The chemical method is based on the reducing power of ascorbic acid. 2,6-Dichlorophenol indophenol has been used as the oxidizing agent for the reaction with ascorbic acid. Ferrozine (3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)-1,2,4-triazine disodium salt), which form chelates with ascorbic acid, has been employed for the analysis of ascorbic acid (Levine and Morita, 1985). All these methods were followed by spectrophotometric determination of the complex formed with ascorbic acid. Unfortunately, the chemical methods may give false positive or negative results due to the presence of reducing or oxidizing substances in the samples. Hence these methods are not suitable for analysis of ascorbic acid in complex food samples.

High performance liquid chromatography is increasingly used for the analysis of ascorbic acid since it offers increased specificity and sensitivity in comparison with chemical methods. Ion-exchange chromatography has been used for the analysis of ascorbic acid in food by using an Aminex HPX-87 ion-exchange column with UV detection (Ashoor *et al.*, 1984). However, reversed phase ion-interaction chromatography is the most common technique used for the analysis of the ascorbic acid. A bondapak C_{18} column coupled with UV detection has been

employed for the analysis of ascorbic acid in urine (Wagner *et al.*, 1979), in fruit juices and vitamin C tablets(Gennaro and Bertolo, 1990). Ascorbic acid and benzoic acid have been analyzed in fruit juices by using a Bondapak-CN column with UV detection (Ling *et al.*, 1992). Furthermore, reverse phase ion pair chromatography has been applied to the analysis of ascorbic acid with other organic acid analytes. Quaternary ammonium salts have been employed, usually as ion-pairing reagents for the analysis of ascorbic acid. Tri-n-butylamine, cetyltrimethyl ammonium bromide, tridecyl ammonium formate, hexadecyltrimethyl ammonium bromide, tetra-butyl ammonium hydrogen sulphate, octadecylamine orthophosphate, octylamine salicylate, and myristylmethyl ammonium bromide have been used as ion-pairing reagents for the analysis of ascorbic acid. (Iqbal, 1995).

Despite the extensive work on HPLC analysis of nitrite and nitrate, and for ascorbic acid described above, a high performance liquid chromatography (HPLC) method for simultaneous determination of nitrite, nitrate and ascorbic acid in food has not been reported in the literature. As noted in Section 1.3, simultaneous determination of these three species is desirable because of their promotion and inhibitory roles in the *in vivo* formation of NOCs. Thus, a reversed-phase ion interaction HPLC method for simultaneous determination of nitrite, nitrate, and ascorbic acid was developed in the present project, and its application was demonstrated by the measurement of nitrite, nitrate, and ascorbic content in commercial canned vegetable juices.

Chapter 4 Objectives of Study

The main theme of this project is the development of specific and sensitive analytical methods for the determination of non-volatile NOCs, namely N-nitrosoamino acids in foods. High performance liquid chromatography - mass spectrometry (HPLC-MS) was chosen because it could provide the specificity and sensitivity required for unequivocal identification and direct measurement of NAAs in foods down to the nanogram levels. Also, a complementary and related method for quantitative measurement of nitrosating agents, i.e. nitrite and nitrate, and ascorbic acid in food samples was also developed.

The specific objectives of this project are :

4.1 Studies on separation of NAAs by C_{18} reverse-phase ion-pair high performance liquid chromatography (RP-IP-HPLC) and compatible detection by electrospray ionization mass spectrometry, including

1. to evaluate the ability of different tetraalkyl ammonium ion-pairing reagents in the separation of NAAs by C_{18} RP-IP-HPLC, and
2. to study the compatibility of HPLC mobile phase composition with electrospray ionization detection.

4.2 Analysis of NAAs in Food by High Performance Liquid Chromatography-Electrospray Ionization Mass Spectrometry (HPLC / ESI-MS), including

1. to study the mass spectral characteristics of NAAs in both the positive and negative electrospray ionization mode ;
2. to develop a clean-up procedures for efficient isolation of target NAAs from the food matrix ; and
3. to measure quantitatively the levels of NAAs in Chinese salted fish samples.

4.3 Simultaneous determination of nitrite, nitrate and ascorbic acid in canned vegetable juices by reverse-phase ion-interaction HPLC, including

1. To develop a reversed-phase ion-interaction HPLC method for complete separation of nitrite, nitrate and ascorbic acid ; and
2. To apply the developed HPLC method for analysis of nitrite, nitrate and ascorbic acid in commercial bands of canned tomato, carrot and mixed vegetable juices.

Chapter 5 Studies on Separation of NAAs by C_{18} Reverse-phase Ion-pair HPLC and Compatible Detection by Electrospray Ionization Mass Spectrometry

5.1 Background

The analytical methodology for non-volatile NOCs is not as well established as that of volatile NOCs (Sen and Kubacki, 1987). For N-nitrosoamino acids, a commonly employed procedure is to convert them to volatile trimethylsilyl or methyl chemical derivatives, which were then separated by gas chromatography and detected by the thermal energy analyser (TEA). HPLC separation has the advantage of omitting the derivatization step. However, NAAs are small, highly polar or ionic molecules which are difficult to be efficiently separated by normal or reverse phase HPLC.

Although the TEA is the established specific detectors for NOCs, it is incompatible with aqueous mobile phases associated with ion exchange or reverse phase chromatography. On the other hand, mass spectrometric detection by electrospray ionization (ESI) is compatible with aqueous HPLC mobile phase; 1 : 1 (*v*/*v*) methanol/water or acetonitrile/water solvent systems are widely used in ESI. ESI is also sensitive to pre-formed ions in solution at the nanomole to femtomole level, in this case, the carboxylate anions of NAAs. Since the ESI-MS interface is normally operated at ambient or temperatures below 100 ^{0}C, it is ideally suited to detect thermally labile NOCs. However, ESI detection and sensitivity is subjected

to interference, sometimes seriously from formation of solvent cluster ions, and co-eluting anions or cations. Compatibility between HPLC mobile phase and ESI detection is a wide-ranging 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 part of this thesis, the separation of NAAs by C_{18} reverse-phase ion-pair high performance liquid chromatography (RP-IP-HPLC) and their compatible mass spectrometric detection by electrospray ionization (ESI) were systematically investigated and optimized. The ability of different tetraalkyl ammonium ion-pairing reagents to promote efficient separation of NAAs, and the effects of mobile phase composition, solvent cluster ion formation, co-eluting ion-pairing anions and cations, and other compatibility issues associated with ESI detection are also addressed.

5.1.1 Enhancement of Retention by Ion-pair Formation in Reverse-phase High Performance Liquid Chromatography

Reverse-phase ion-pair high performance chromatography (RP-IP-HPLC) is widely used for the separation of mixtures of ionic and/or ionizable compounds on lipophilic stationary phases. The technique is based on the addition of amphiphilic (surface-active) ions to the mobile phase in order to enhance the retention of ionic sample components.

The high flexibility in adjusting the chromatographic conditions to a given separation problem is the main advantage of ion-pair chromatography. This flexibility results from the great variety of experimental parameters that affect retention. They include

(i) the lipophilicity of pairing ion ;

(ii) the concentration of the pairing ion in the eluent ; and

(iii) concentration of organic solvent in the mobile phase.

5.1.1.1 Effect of the Lipophilicity of the Pairing ion

The retention of the analyte in HPLC is generally expressed as :

$$t_R = t_o (1 + k') \qquad [5.1]$$

where t_R is retention time of solute, t_o is the retention time of the non-retained species, and $k' = M_S / M_M$ is the ratio of the amount of analyte in the stationary phase (S) and the mobile phase (M), respectively.

One of the important parameters to control retention in ion-pair chromatography is the lipophilicity of the pairing ion, which is mainly determined by the number of its carbon number, i.e. the length of its alkyl group. Generally, the retention of analyte ions increases with increasing lipophilicity, i.e. the alkyl chain length, of the pairing ions when they are used at identical mobile phase concentrations. This is illustrated in Figure 5.1 which shows k' value of adrenaline increase with the chain length of C_4 - C_{10} alkylsulphate pairing ion on separation with a C_{18} HPLC column (Horvath *et al.*, 1977). According to the ion-pair model, such a dependence may be accounted for the fact that the lipophilicity or the hydrophobic character of the formed ion-pair complexes, which increase with C_8 octyl and C_{10} decyl pairing ion. This results in both a stronger interaction between the ion pair and the stationary phase, and a reduced solubility in the eluent. Therefore, in reverse-phase ion-pair chromatography systems, the most efficiency way to adjust the selectivity of the chromatographic system is to change the alkyl chain length of the pairing ion.

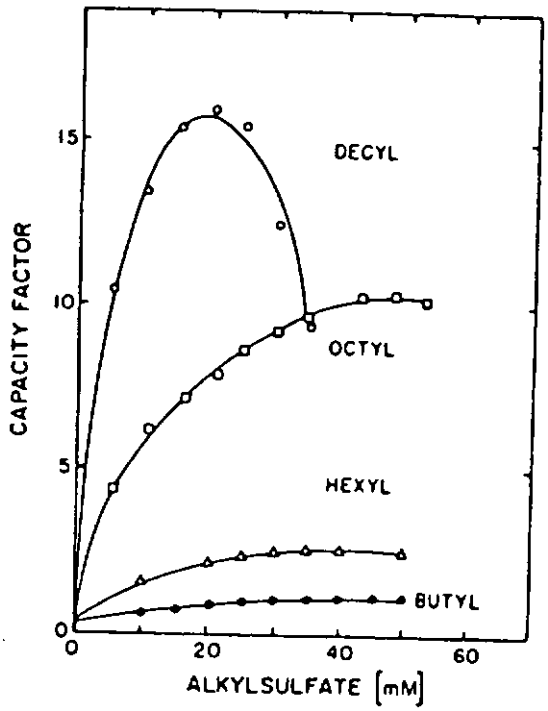


Figure 5.1 : The dependence of the capacity factor of adrenaline on the carbon number of n-alkyl sulfates as the ion-pairing reagent (Taken from Horvath et al., Analytical Chemistry, 1977, Vol. 49, pp. 2295).

5.1.1.2 Effect of Concentration of Pairing ion

Another means for controlling retention is by varying the concentration of the pairing ion. Figure 5.2 shows the dependence of the k' value of inorganic anions on the concentration of tetrabutylammonium iodide, using C_{18} as the stationary phase (Xianren and Baeyens, 1988). Two opposing effects on the retention of solute ion with increasing the concentration of pairing ion are observed. The retention of the solute ion generally increases with the concentration of pairing ion in the eluent, but there is a threshold concentration above which solute retention decreases with further increases in the concentration of pairing ion. The first is an increase in analyte retention due to either increase

ion-pair complex formation in the mobile phase or the adsorption of pairing ion on the lipophilic stationary phase. The second is a decrease in analyte retention due to the fact that the stationary phase surface becomes saturated with the tetrabutylammonium pairing ion, and any further addition of ion-pairing reagent to the eluent results in increased concentration of the iodide ion, which competes with the analyte ions for the binding with the adsorbed tetrabutylammonium cation.

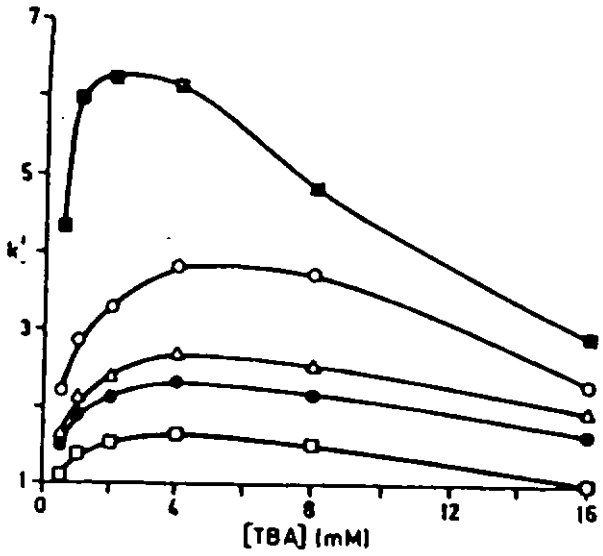


Figure 5.2 : The dependence of the capacity factor of inorganic anions on the concentration of tetrabutylammonium iodide in the eluent (Taken from Xianren and Baeyens, Journal of chromatography, 1988, Vol. 456, pp 267).

5.1.1.3 Effect of Organic Solvent Concentration in the Aqueous Mobile Phase

The percentage *w/v* of organic solvent in the aqueous eluent is another significant factor that affect the retention of solute ions in reverse-phase ion-pair chromatography. Because the organic solvent molecules can also be adsorbed at the surface of the stationary phase, they are in a competing equilibrium with lipophilic ions for active centers on the stationary phase available for adsorption. Therefore, an increase in the concentration of organic solvent in the aqueous mobile phase would lead to a decrease in both the adsorption of the pairing ions and the retention of the analyte. To illustrate this effect, the retention of two aromatic sulfonic acids - toluene-p-sulfonic acid and naphthalene-2-sulfonic acid - was investigated as a function of percentage of the organic solvent in the mobile phase (Weiss, 1995). In this experiment, an acetonitrile/water mixture was used to which tetrabutylammonium hydroxide (TBAOH) was added as the ion-pair reagent. Plotting k' versus the acetonitrile content in the mobile phase yields the parabolic dependence shown in Figure 5.3.

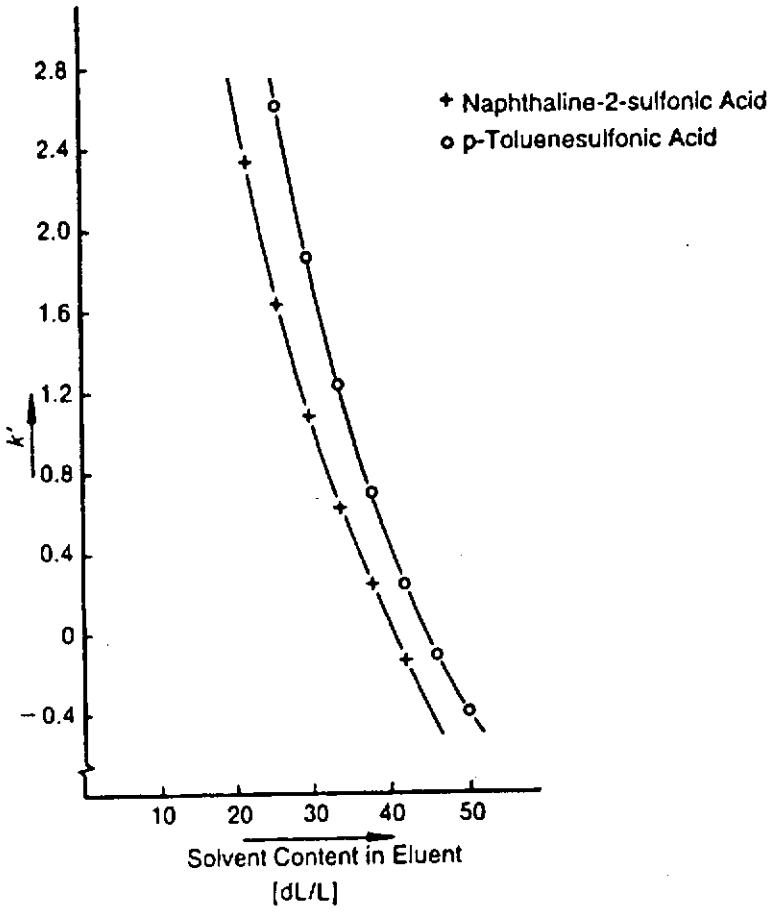


Figure 5.3 : The dependence of the capacity factor of aromatic sulfomic acids on the organic solvent content in the mobile phase (Taken from Weiss, Ion chromatography, 2 nd edition, 1995, Chapter 5, pp. 239).

All of the above three parameters, alkyl chain length, concentration of the pairing ion, and concentration of organic solvent are important variables on the separation and selectivity of solute ions in reverse-phase ion-pair chromatography. It is worthwhile to examine those effects while we develop a new chromatographic method for the separation of NAAs. For this reasons, the effect of (i) alkyl chain length and (ii) concentration of tetraalkyl ammonium pairing ions, and (iii) the concentration of methanol in aqueous mobile phase on separation of NAAs by C_{18} RP-IP-HPLC are systematically investigated in the present study.

5.1.2 Effect of Co-eluting Cations/Anions on ESI Sensitivity

In electrospray, a current flows through the capillary tube which is produced by reduction of an equivalent amount of ions and electrolytically discharged on the wall of the capillary, or electrolytically provided by formation of negative ions from the capillary wall. Thus, the negative electrospray process can be viewed as an electrolysis of a special kind where reduction occurs at the capillary cathode, or oxidation occurs at the capillary anode if operated in the positive mode. At this point, it should be pointed out that the bulk of the charged droplet is made up of solution electrolytes with equivalent amount of cations and anions.

The current, I, leaving the ES capillary is a measure of the rate at which excess electrolyte ions leave the capillary. This current is easily measured and is found to be a weak function of the conductivity, σ, of the solution (Kebarle and Tang, 1993).

$$I = H\sigma^n \qquad n \approx 0.2 - 0.3 \qquad [5.2]$$

H is a constant which can be determined experimentally and depends on experimental parameters such as electric field at the tip, radius of the capillary, surface tension of the solvent, etc. When strong electrolytes are used at concentration not exceeding 10^{-2} M, the capillary current, I, can be expressed as (Kebarle and Tang, 1993) :

$$I = H\sigma^n = H\lambda_m^{\circ n} C^n \qquad n \approx 0.2 - 0.3 \qquad [5.3]$$

where λ_m° is the equivalent molar conductivity, and C is the molar concentration.

Under RP-IP-HPLC/ESI-MS conditions, the analyte positive/negative ions will be co-eluting with the cations/anions of the ion pairing reagent. Hence it is a fundamental problem to investigate the effect of the co-eluting ion-pairing reagent on ESI sensitivity of detection of the analyte. In the following discussion, the negative electrospray process will be considered because formation and detection of the deprotonated carboxylate molecular anions, $[M-H]^-$, of NAAs were evaluated in the present study. When two electrolytes such as A^+X^- (the analyte) and B^+Y^- (the ion-pairing reagent) are present in solution, both X^- and Y^- will be present as excess negative ions at the surface of the charged droplets. However, because of the weak dependence of I on the total electrolyte concentration, addition of B^+Y^- and A^+X^- will not materially increase the current, i.e., the total excess charge. Instead, Y^- will compete with X^- for the excess surface charges on the droplets. This means that the amount of gas-phase analyte ions X^- produced from the charged droplets will decrease as the ion-pairing reagent B^+Y^- is added to the solution.

Based on the work of Kebarle and Tang (1993) the ESI intensity-concentration relationship for negative ions is given by :

For single anion system at 10^{-4} M or above :

$$I_{X^-\,ms} = p\, I_{X^-,\,g} \qquad [5.4]$$

For 2 anions system at 10^{-4} M or above:

$$I_{X^-\,ms} = pf \frac{k_X[X^-]}{k_X[X^-] + k_Y[Y^-]} I = \frac{pf\,[X^-]}{[X^-] + k_Y/k_X\,[Y^-]} I \qquad [5.5]$$

where $I_{X^-\,ms}$ is the mass spectrometrically detected ion current of X^-, p is a constant expressing the efficiency of the mass spectrometer of sampling the gas phase ion current, $I_{X^-,g}$, due to gas-phase ion X^- produced from the charged droplets (see equ. 5.5), and f is the efficiency of conversion of droplet charge to gas-phase ions, and $[X^-]$ and $[Y^-]$ are the concentration of the analyte and ion-pairing ions, X^- and Y^- respectively, and k_X and k_Y are ion evaporation rate constants relating to the relative efficiencies of ejection of X^- and Y^- from the charged droplets. For a solution containing both X^- and Y^- anions, $I_{X^-\,ms}$ depends on the ratio of ion-evaporation rate constants, k_Y/k_X, and not on the individual values of k_X and k_Y. $k_X[X^-]$ and $k_Y[Y^-]$ could be regarded as proportional to the surface concentration of X^- and Y^-. Upon ion ejection, the surface concentration is rapidly replenished because the bulk concentrations, $[X^-]$ and $[Y^-]$, are sufficiently high to allow rapid transport of ions to the droplet surface. The ratio expresses a "fractionation" factor in the electrospray conversion of ions in solution to ions in the gas phase. It should be noted that k_X and k_Y are also dependent on the chemical composition of the solvent.

For lower concentration solutions in the range of 10^{-5} - 10^{-9} M, the ESI current is limited by the rate of diffusion of the analyte and ion-pairing ions to the droplet surface, and hence is diffusion-controlled. A typical plot ESI current versus electrolyte concentration for different cations is shown in Figure 5.4 (Kebarle and Tang, 1993). The ratio of ESI currents at concentrations greater than 10^{-4} M is equal to k_{A^+}/k_{B^+}, the ratio of ion evaporation constants. At very low concentrations (e.g. < 10^{-6} M) the ratio of ESI current is equal to D_{A^+}/D_{B^+}, where D is the diffusion coefficient of the cations. As can be seen from Figure 5.4, the

difference in relative diffusion coefficients is smaller than that of ion evaporation constants for cations.

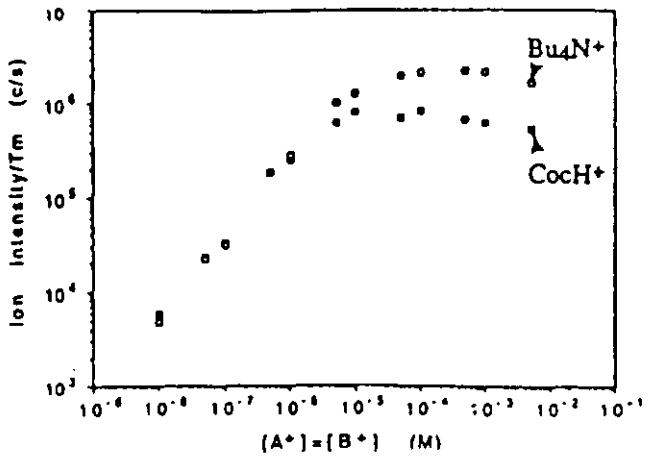


Figure 5.4 : Ion intensities observed in experiment where [Pen_4N^+] = [Bu_4N^+] is increased from 10^{-8} - 10^{-2}. [Pen_4N^+] = Tetrapentylammonium ion ; [Bu_4N^+] = Tetrabutylammonium ion (Taken from Tang and Kebarle, Analytical Chemistry, 1993, Vol. 65, pp. 3654)

Under RP-IP-HPLC/ESI-MS conditions, the analyte concentration is usually in the range 10^{-5} - 10^{-9}, while the ion-pairing reagent concentration is usually > 10^{-4} M. Thus equation (5.5) is not strictly valid for the experimental conditions generally encountered in analytical LC/MS conditions. Qualitatively, the analyte ESI current will be mainly determined by the rate of migration of the analyte ion to the droplet surface, while the ESI current due to the ion-pairing ion will be determined by its ion evaporation constant. Thus the analyte ESI current and sensitivity will be decreased by the presence of the ion-pairing ion, and the

sensitivity reducing effect of the ion-pairing ion is in term dependent on its concentration and ion evaporation constant.

While the ion evaporation constants of common cations have been systematic investigated (Kebarle and Tang, 1993), the relative rates of evaporation of anions has not been studied and reported. Hence, in this project, the sensitivity reducing effect of co-eluting ion-pairing anions on ESI detection sensitivity of NAAs was systematically investigated. Finally, we attempted to correlate the observed analyte ion intensity with the evaporation rate constant of co-eluted ion-pairing anions.

5.2 Materials and Methods

5.2.1 Equipment

The major equipment used for this part of the project were : -

1. VG platform quadrupole mass spectrometer (Manchester, U.K.) equipped with an electrospray ionization interface (mass range m/z 10 - 2000) ; instrument control, data acquisition and processing were conducted via the VG Masslynx 2.0 system software ;
2. Alcott HPLC pump (model 760A), flow rate range 0 - 0.999 ± 0.001 ml/minute;
3. Brownlee (Applied Biosystems, 850 Lincoln Centre Drive Foster City, CA99404 USA) C_{18} reverse phase HPLC column (RP-18, 5μm, 15 cm x 2.1 mm I.D);
4. Mettler UM3 microbalance (± 0.001 mg)
5. Vortex Mixer (Thermolyne Maxi Mix II)

5.2.2 Reagents and Materials

1. The following analytical grade chemicals (purity > 99%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) :

   methanol (MeOH)
   acetonitrile (MeCN)
   hexyltriethylammonium bromide (C_6-HTEABr)

2. The following analytical grade chemicals (purity > 99%) were bought from Acros Chemical Co. (Geel West Zone 2, Janssen Pharmaceuticalaan 3a, 2440 Geel, Belgium) :

   sodium chloride (NaCl)
   sodium bromide (NaBr)
   sodium iodide (NaI)
   sodium fluoride (NaF)
   sodium acetate ($NaCH_3CO{\cdot}O$)
   tetraethylammonium chloride (C_2-TEACl)
   tetrabutylammonium chloride (C_4-TBACl)
   dodecyltrimethylammonium bromide (C_{12}-DTMABr)
   dodecyltrimethylammonium chloride (C_{12}-DTMACl)
   cetyltrimethylammonium chloride (C_{16}-CTMACl)

3. Double deionized water was generated from a Milli-Q system (Millipore) system.

5.2.2.1 Synthesis of N-nitrosoamino Acid Standards

a) Synthesis of N-nitrosoproline (NPRO)

The N-nitrosoamino acids used in this work were synthesised by Mr. P.M. Yeung (Yeung, 1992). The nitrosoamino acids, NSAR, NPRO and NPIC were synthesized by the method of Lijinsky et al. (Lijinsky *et al.*, 1970). 4 g of proline were dissolved in 20 ml HCl (2M) cooled in ice and 3.5 g of $NaNO_2$ were added slowly. After reacting for 3 hours at ice-cooled temperature, the water was removed by rotatory evaporation and further dried in a vacuum oven. The NPRO was extracted with 2 x 10 ml pure acetone and filtered through filter paper. The acetone in the pale yellow filtrate was removed in the cold by blowing with a gentle stream of N_2 and pale yellow solids were obtained. Colorless crystals were formed with m.p. 97.5 - 98 ^{0}C after crystallization from chloroform.

Mass spectrum : parent peak at 144 amu, fragments at m/z 99 (loss of COOH) and 69 (loss of COOH, NO) were observed.

NSAR and NPIC were synthesized according to the above method by just replacing with sarcosine and pipecolic acid as the reacting reagent, respectively. The structure of the synthesized product was further verified by 90 MHz NMR. The purity of the synthesized product was checked by GC/MS analysis of their methyl ester derivatives synthesized by the diazomethane method (Ishibashi *et al.*, 1980). No significant impurity peaks were found.

b) Synthesis of N-nitrosothiazolidine-4-carboxylic acid (NTCA)

NTCA was synthesized based on the method of Tahira *et al.* (Tahira *et al.*, 1984). 1.32 g of L-thioproline were dissolved in 100 ml water and acidified to pH 2-2.5 with $HClO_4$ (2 M). 1.35 g of $NaNO_2$ were dissolved in 50 ml water and adjusted to pH 2-2.5 with $HClO_4$ and allowed to reacted with acidified L-thioproline solution for 1 hour. The reaction product was extracted three times with 100 ml ethyl acetate. After drying the extract over anhydrous sodium sulfate, the ethyl acetate extract was reduced to dryness by rotatory evaporation at room temperature. The solid crude product was purified by dissolving in dichloromethane, then adding n-pentane until the solution became cloudy, and crystallizing the NTCA from dichloromethane : n-pentane of 3 : 1 (v/v). White crystals with m.p. 105-106^{0}C were obtained.

Mass spectrum : parent peak at 162 amu, Fragment at m/z 132 (loss of NO) , m/z 87 (loss of NO, COOH) and m/z 60 $(C_2H_4S]^+$ were observed.

c) Synthesis of N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA)

2-Methylthiazolidine-4-carboxylic acid (MTCA) was prepared according to the method of Riemschneider and Hoyer (Riemschneider and Hoyer, 1962). 11.4 g of L-cystine were dissolved in 60 ml water and acidified with 6 ml of acetic acid. 75 ml of ethanol was added to precipitate out the L-cystine. 7.5 ml acetaldehyde freshly prepared by distilling triacetaldehyde was reacted with the L-cystine precipitate for 1 hours. The unreacted substances were filtered and the filtrate was

dried by rotatory evaporation. The solid residue (MTCA) was washed by cold ethanol, following by isopropanol. The solid crystals were dried again in a vacuum oven. The purity and identity of the MTCA product (m.p. 162 ^{0}C) were verified by MS and NMR. The synthesized 2-Methythiazolidine-4-carboxylic acid (MTCA) was nitrosated by $NaNO_2$ to produce NMTCA in a similar manner as in the case of NPRO.

Mass spectrum : parent peak at 176 amu, fragment at m/z 146 (loss of NO) , m/z 101 (loss of NO, COOH) were observed.

All the synthesized NAAs were confirmed by IR spectroscopy, NMR spectroscopy and MS spectroscopy. These analytical results agreed with published data in the literature. The purity was checked by GC/MS analysis of the methyl ester derivatives. The estimated purity for all the NAAs is > 98%. The mass spectra of the methyl ester derivatives are shown in Appendix 1.

5.2.3 Instrumentation

The following high performance liquid chromatographic / mass spectrometric conditions were used in the analysis of N-nitrosoamino acids.

5.2.3.1 HPLC Conditions

The NAAs were separated by reverse-phase ion-pair high performance liquid chromatography with isocratic elution.

All solvents were purified by re-distillation. The mobile phase was freshly prepared up each day and filtered through a 0.45 μm Nylon-66 filter before use. The HPLC mobile phase was ultrasonically de-gassed and flowing at 0.2 ml/ min. Before carrying out the HPLC analysis, the column was allowed to equilibrate for more than 1 hour with the eluent. The sample solution (10 mg/L) was injected via a 5 μl sample loop. The NAAs were separated by isocratic elution with different mobile phase compositions as shown in Table 5.1 :

Table 5.1 : Variation of mobile phase compositions for separation of NAAs

Elution profile	Ion-pair Reagent	Conc. of Ion-pair Reagent (mM)	Solvent composition (% v/v)		
			Methanol	Water	Acetonitrile
1	C_{16}-CTMA	1.40	60	35	5
2	C_{12}-DTMA	1.40	60	35	5
3	C_{16}-CTMA	1.80	50	50	0
4	C_{12}-DTMA	1.80	50	50	0
5	C_6- HTEA	1.80	50	50	0
6	C_4-TBA	1.80	50	50	0
7	C_{16}-CTMA	1.40	75	25	0
8	C_{16}-CTMA	1.40	70	30	0
9	C_{16}-CTMA	1.40	65	35	0
10	C_{16}-CTMA	1.40	60	40	0
11	C_{16}-CTMA	0.00	65	35	5
12	C_{16}-CTMA	0.35	65	35	5
13	C_{16}-CTMA	0.70	65	35	5
14	C_{16}-CTMA	1.00	65	35	5
15	C_{16}-CTMA	1.80	65	35	5
16	C_{16}-CTMA	2.20	65	35	5

5.2.3.2 ESI-MS Conditions

The NAA analytes were analysed by using the HPLC system linked to the VG Platform quadruple mass spectrometer equipped with an electrospray ionization interface. The eluent from the HPLC column was splitted, and 1/10 of the eluent was allowed to flow into the electrospray interface. The ESI-MS conditions were :

Source Temperature : 90 ^{0}C

Flow rate of nebulising gas (N_2) : 15 L/hr

Flow rate of drying gas (N_2) : 350 L/hr

Flow rate of mobile phase : 20 µl/min.

Capillary voltage : -3.0 kV

HV Lens : 0.0 kV

Skimmer Cone voltage : -20V

Skimmer Lens offset : 5.0V

LM resolution : 5.5

HM resolution : 5.5

Ion Energy : 1.0 V

Ion Energy Ramp : 1.8

Multiplier : 650

Scan mode : Scan range 30 - 200 Da ; 200 Da s $^{-1}$

SIM mode : Dwell time 0.1 s

Sensitivities in negative electrospray ionization (-ESI) modes was maximized by tuning and optimizing the ion intensities of NPRO. Quantification was done by selective ion monitoring (SIM) of the deprotonated carboxylate molecular anion $[M-H]^-$, and the characteristic fragment ions. All ESI mass spectral data were processed by the Masslynx 2.0 software and the peak areas of the SIM mass chromatogram were integrated by the use of the Masslynx software.

5.2.4 Preparation of Analytes and Co-analytes Standard Solutions

NSAR, NPRO, NTCA, NMTCA and d_3-NPRO stock standard solutions in methanol (1000 mg/L) were prepared and stored in 10 ml brown vials at 4 ^{0}C. Working standard solution containing 5, 10, 20, 50 and 100 mg/L were prepared from the stock standard solutions by serial dilution.

2.8 mM solutions of sodium chloride, sodium fluoride, sodium iodide, sodium bromide, sodium acetate, tetraethylammonium chloride, tetrabutylammonium chloride, dodecyltrimethylammonium chloride and cetyltrimethylammonium chloride in MeOH (60) : H_2O (35) : MeCN (5) *v/v* were prepared.

A series of 0.70, 1.4, 2.8, 3.6, 7.0, 10.8, 14, 28, 56, 80, 96, 104, 112 mM sodium chloride solutions were prepared by serial dilution

5.2.5 Preparation of Mixed Standard Solution for ESI Sensitivity Study

In the ESI sensitivity study, equal volumes of 10 mg/L NAA standard solutions were well mixed with various kinds of inorganic salt solution (e.g. NaCl, NaBr, NaI, NaF, etc) at suitable concentrations according to the purpose of study. The details of preparation of mixed standard solution were described in each part of the Result and Discussion of Section 5. 5 μl of pre-mixed standard solution was introduced by flow injection into the ESI interface via a Peek tubing at a flow rate 20 μl/min.

5.3 Results and Discussions

5.3.1 Electrospray Ionization (ESI) Mass Spectra of N-nitrosoamino Acids

Figure 5.5 (a) to (d) shows the negative ESI mass spectra of NSAR, NPRO, NTCA and NMTCA, respectively, obtained from solutions containing 20 mg/L NAA standards in 50 : 50 *v/v* MeOH : H_2O. All the 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 in the ESI spectra of organic acid, and their generation shows clearly that ESI is a suitable ionization technique for detecting NAAs.

In this study, the ion intensity of the $[M-H]^-$ ion was monitored in experiments to evaluate separation efficiency and sensitivity of detection of NAAs by RP-IP-HPLC/ESI-MS.

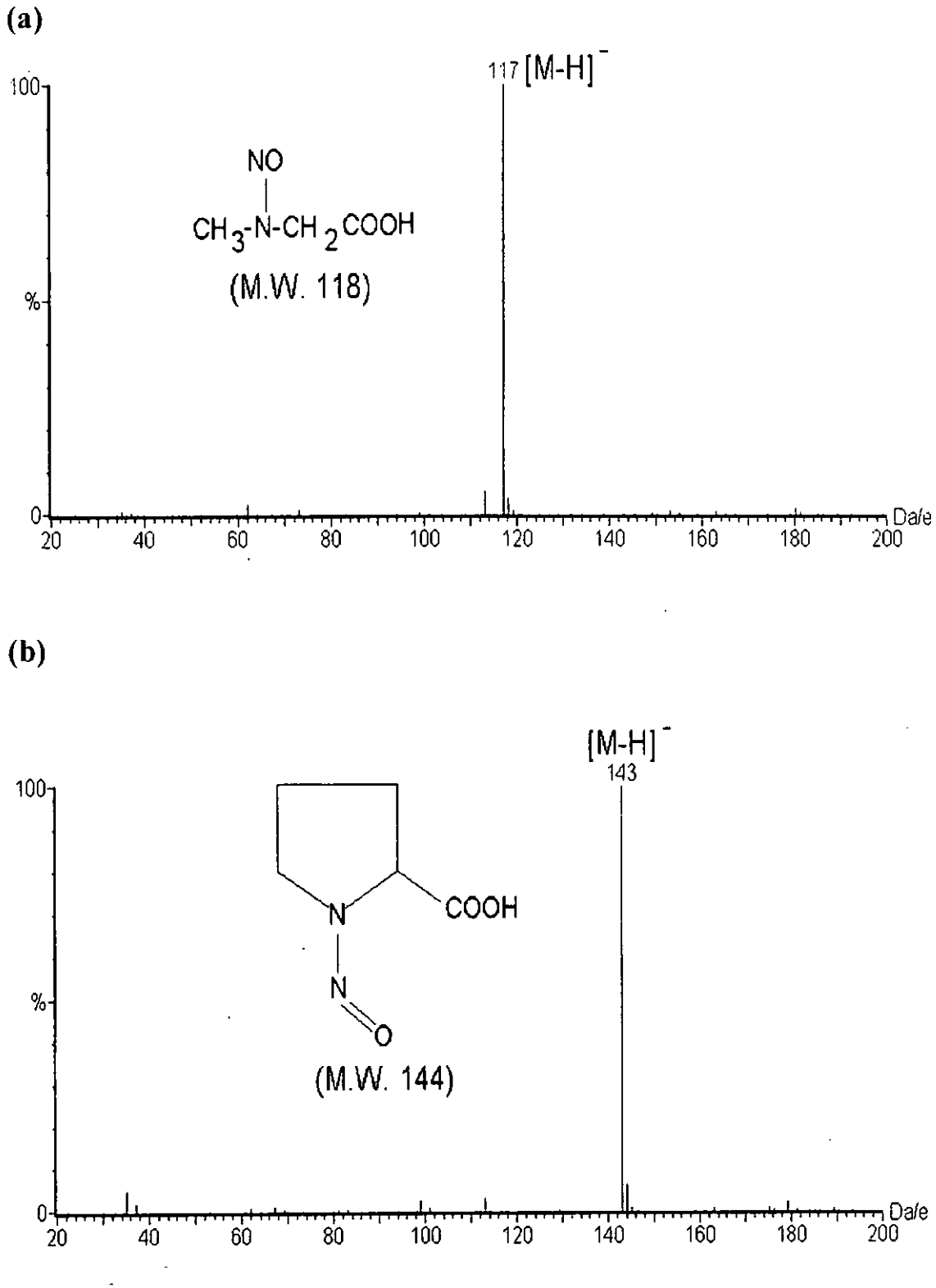


Figure 5.5 : Electrospray ionization mass spectra of (a) NSAR, (b) NPRO. The spectra were obtained for 20 mg/L standard solutions of NAA in H_2O : MeOH (50 : 50 % v/v) at skimmer cone voltage of 20 volt.

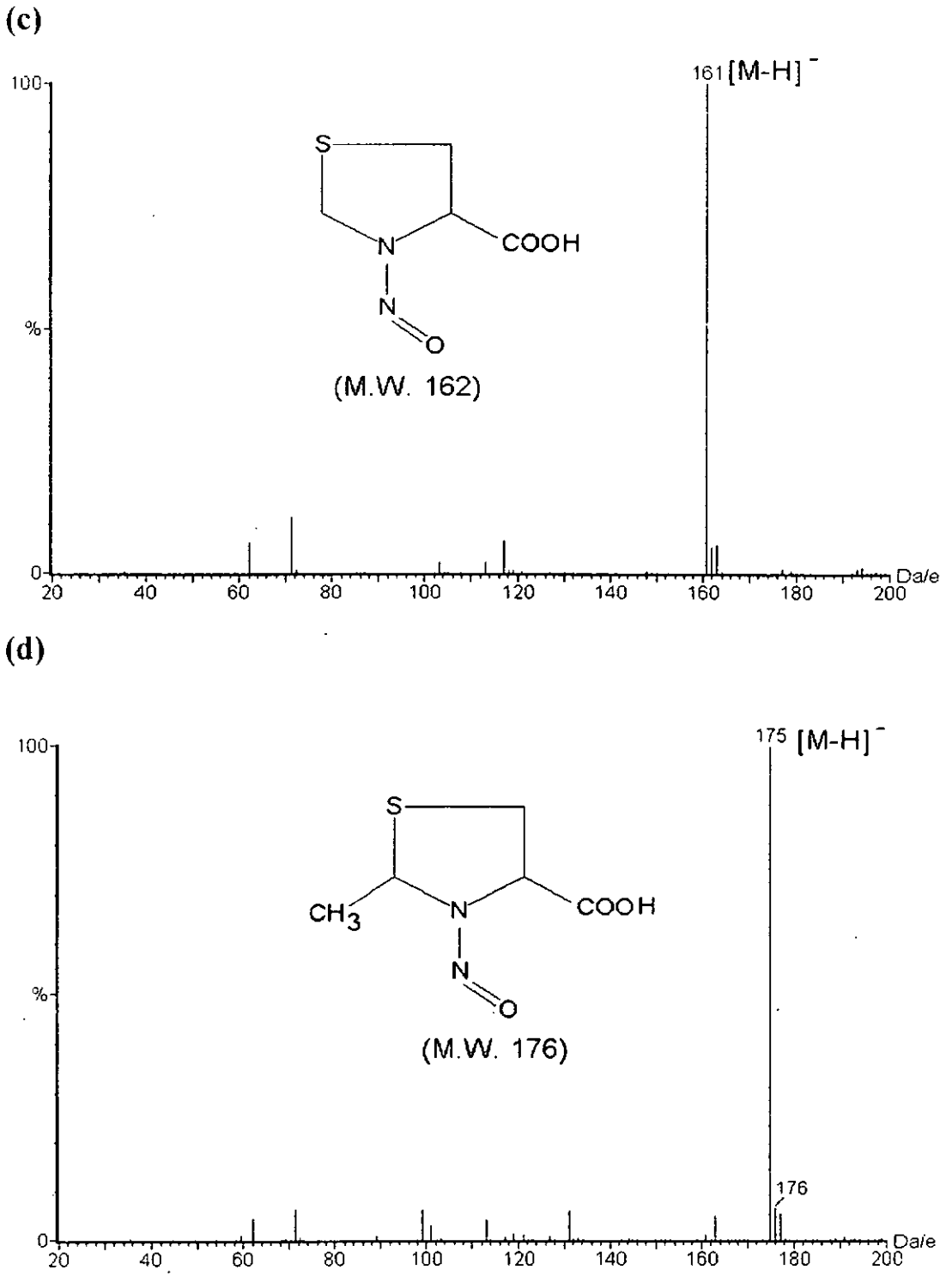


Figure 5.5 : Electrospray ionization mass spectra of (c) NTCA, (d) NMTCA. The spectra were obtained for 20 mg/L standard solutions of NAA in H_2O : MeOH (50 : 50 % v/v) at skimmer cone voltage of 20 volt.

5.3.2 Separation of NAAs by C_{18} Reverse-phase Ion-pair HPLC

NAA are small and ionic molecules. Due to its highly polar properties, it is very difficult to separated by normal phase HPLC. Also, due to their short alkyl chains, they are not easily retained by non-polar reverse phase stationary phases.

Since ion-pairs with longer alkyl chains are better retained by reverse phase stationary phase, a reverse-phase ion-pair chromatographic method was employed in this study to separate the NAAs. This work deals mainly with the separation of NSAR, NPRO, NTCA and NMTCA, which are the most abundant and commonly found non-volatile NOCs in food. They are also produced endogenously, mainly in the stomach of humans (Stillwell *et al.*, 1991).

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 tetraalkyl ammonium counter ions. This was indeed experimentally observed. Figure 5.6 shows the HPLC separation of NPRO, NSAR, NTCA and NMTCA using a reverse phase C_{18} Column and a mobile phase of methanol : water (60 : 40 *v/v*) containing 1.4 mM 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 minutes, respectively. However, the retention times were too long, and the experimental conditions have not been optimized.

To optimize the HPLC conditions for efficient separation of the four NAAs, the major factors affecting the HPLC separation, including the length of the alkyl chain of the ion-pair reagent, chemical composition of the mobile phase, and the concentration of the ion-pair reagent, were investigated.

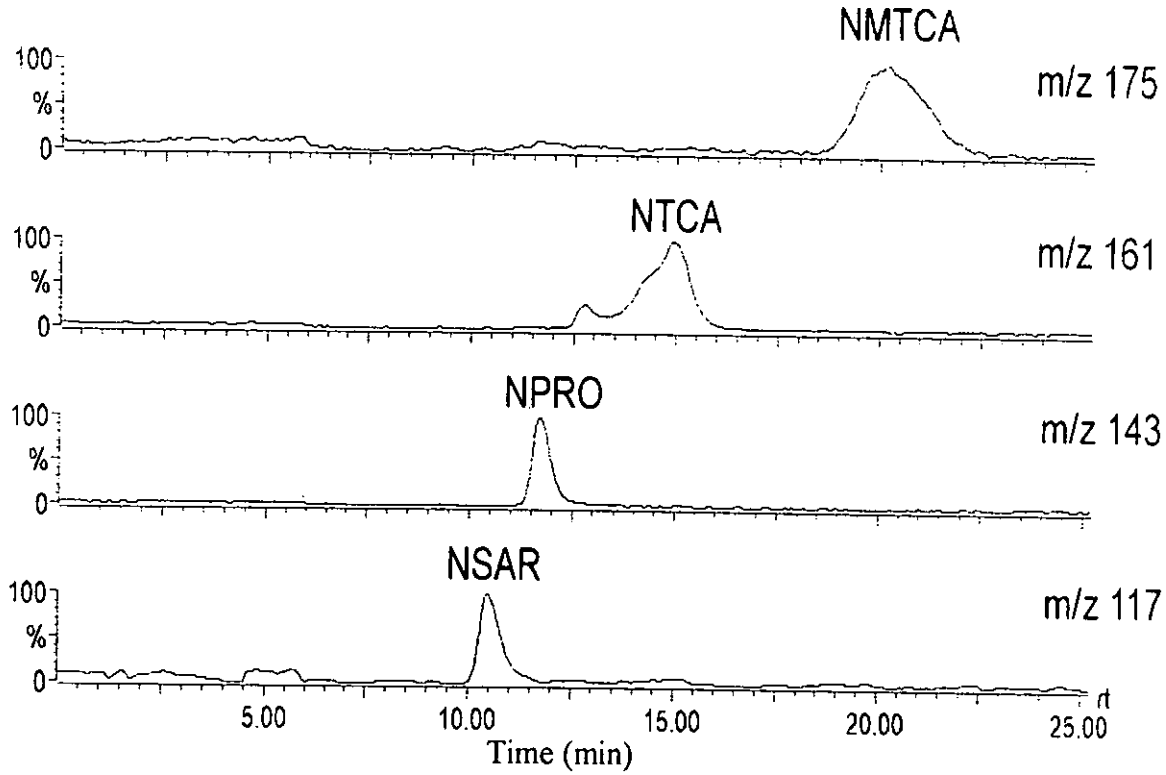


Figure 5.6 : HPLC/ESI-MS mass chromatogram by monitoring the pesudomolecular ion, [M-H]⁻, of four N-nitrosoamino acids standards (10 ppm) using 2.1 mm x 150 mm C_{18} column (Brownlee); mobile phase : 1.4 mM C_{16}-cetyltrimethylammonium chloride in 60 : 40 (% v/v) MeOH : H_2O ; at a flow rate of 200 µl/min and a split ratio of 1 : 10 to the ESI interface.

5.3.2.1 Effects of Alkyl Chain Length of Tetraalkyl Ammonium Ion-pairing Reagent on Separation Efficiency

The separation of NAAs using 1.8 mM C_4 (tetrabutylammonium) to C_{16} (cetyltrimethylammonium ion) ion-pairing reagents (Figure 5.7) and C_{18} HPLC stationary phase was first tested with MeOH : H_2O (50 : 50 *v/v*) mobile phase, the most commonly used solvent system used in electrospray ionization.

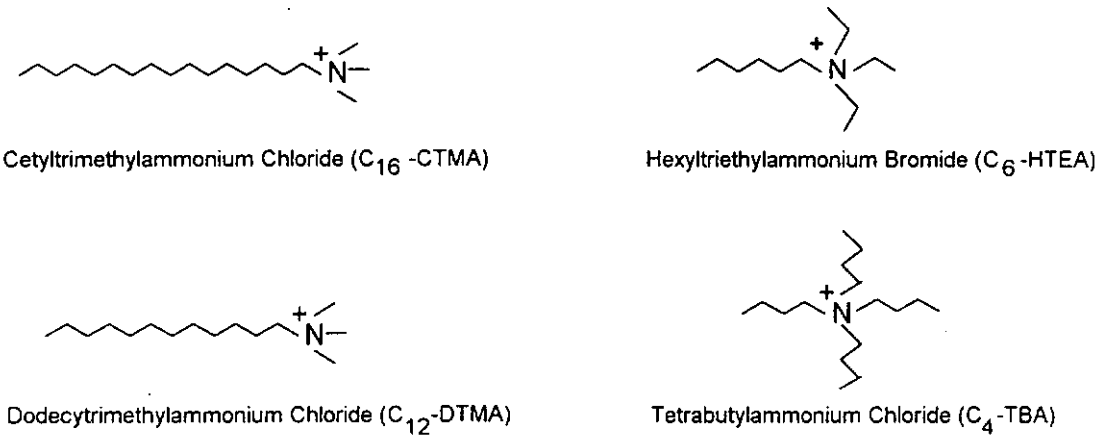


Figure 5.7 : Chemical structure of tetraalkyl ammonium ions

The retention of NAAs by the C_{18}, in terms of the capacity factor (k'), was found to be highly dependent on the alkyl chain length of the tetraalkyl ammonium ion-pairing reagent (Table 5.2). In the presence of C_4 (tetrabutylammonium) and C_6 (hexyltriethylammonium) pairing ions, the four NAAs eluted out roughly at the same time as the solvent front (about 1.5 minutes), and hence were totally not retained by the C_{18} stationary phase.

Table 5.2 : 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 : 15 cm x 2.1 mm (5 μ spherical) C_{18} reverse phase column ; mobile phase containing 1.8 mM ion-pairing reagent in MeOH : H_2O (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 minutes due to strong attraction of the ion-pair complex on C_{18} stationary phase

With 1.8 mM 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 (Figure 5.8). With the longer C_{16}-cetyltrimethylammonium ions, the retention times of NSAR and NPRO increased considerably longer to 15 and 20 minutes, respectively, while the NTCA and NMTCA peaks were so retained that they were not observed even 30 minutes after injection.

Qualitatively, our findings were in general agreement with literature reports, as exemplified in Figure 5.1 (p.46). The lipophilicity of the tetraalkyl ammonium pairing ion is mainly determined by the chain length or carbon number of its longest hydrocarbon alkyl group. 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.

Since the NAAs investigated were small in size, a C_{12} or longer alkyl chain in the pairing ion was found to be needed to induce ion-pair complex formation and significant enhancement in retention.

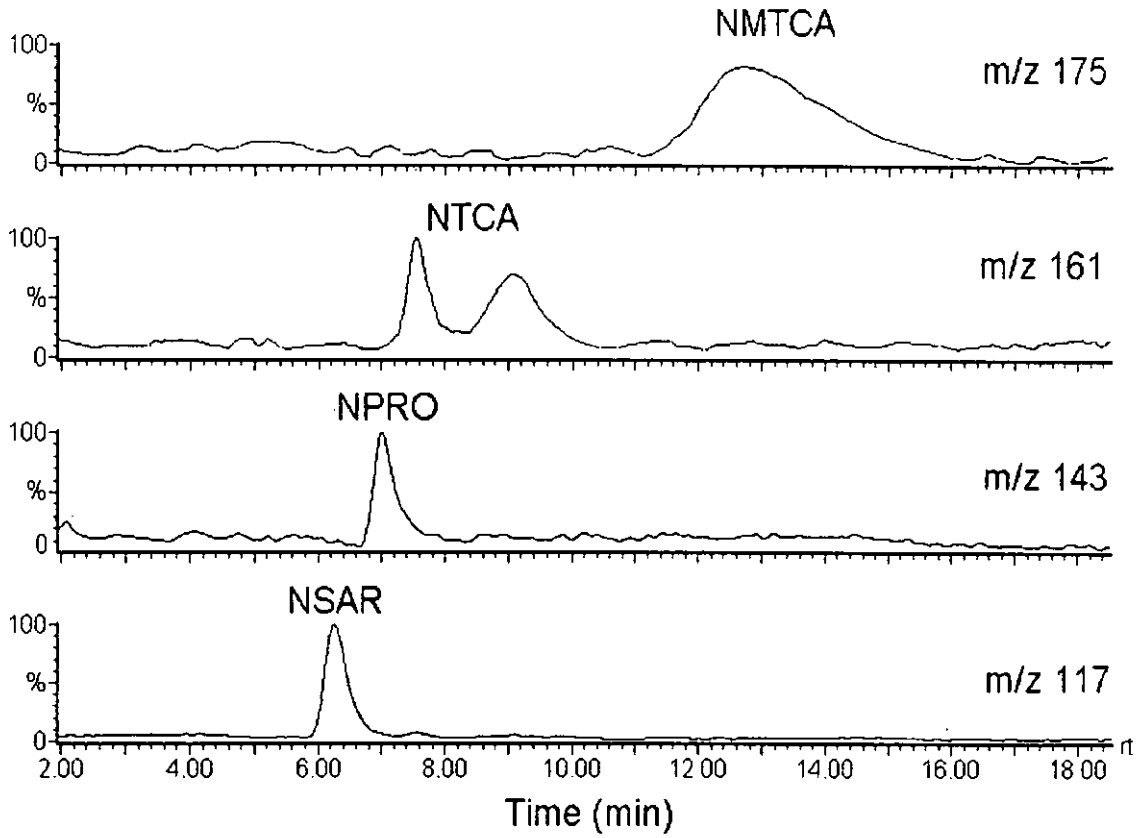


Figure 5.8 : HPLC/ESI-MS mass chromatogram of four N-nitrosamino acids standards (10 ppm). Mobile phase : 1.8 mM C_{12}-dodecytrimethylammonium chloride in 50 : 50 (% v/v) MeOH : H_2O. Other experimental conditions were the same as shown in Figure 5.6.

5.3.2.2 Effects of Methanol Concentration in Aqueous Mobile Phase on the Retention of NAAs

In reverse-phase ion-pair chromatography, the concentration of organic modifier (methanol) in the aqueous mobile phase usually has a significant effect on the separation efficiency and resolution. As shown in Table 5.3, the capacity factor (k') of the four NAAs were reduced mostly to 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 mM C_{16}-cetyltrimethylammonium chloride ion-pairing reagent was used. The decrease in capacity factor and retention time with increased methanol content is in agreement with reported trends as exemplified in Figure 5.3 (p.49). In particular, reasonably good separation in the $[M-H]^-$ mass chromatogram of the four NAAs and economy of analysis time were achieved with 65% *v/v* aqueous methanol (Figure 5.9), or a 60 : 35 : 5 methanol : water : acetonitrile solvent system (Figure 5.10). Under similar mobile phase conditions, the NAAs were not retained at all (k' equal to 1.0) when 1.4 mM 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.

Another notable finding was that separation of the syn and anti isomers of NSAR, NTCA, and NMTCA became observable at various but different methanol concentrations of the mobile phase (Table 5.4). The separation of syn and anti

isomers allows their molecular properties to be studied under HPLC separation conditions, and it is a matter of interest for future studies.

Table 5.3 : Effect of methanol concentration in aqueous mobile phase on the retention of NAAs

Mobile phase composition (% v/v)			Capacity factor (k')			
MeOH	H_2O	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 mM cetyltrimethylammonium chloride (C_{16}) in mobile phase

b) 1.4 mM dodecyltrimethylammonium chloride (C_{12}) in mobile phase

c) anti-isomer

d) syn-isomer

e) This is our proposed assignment of anti- and syn- isomers based on projected chromatographic behaviour of ion-pair formation of the syn- and anti-isomers. The identity of the isomers has not been verified experimentally because the equilibria between syn- and anti- isomers could be affected by many experimental factaors.

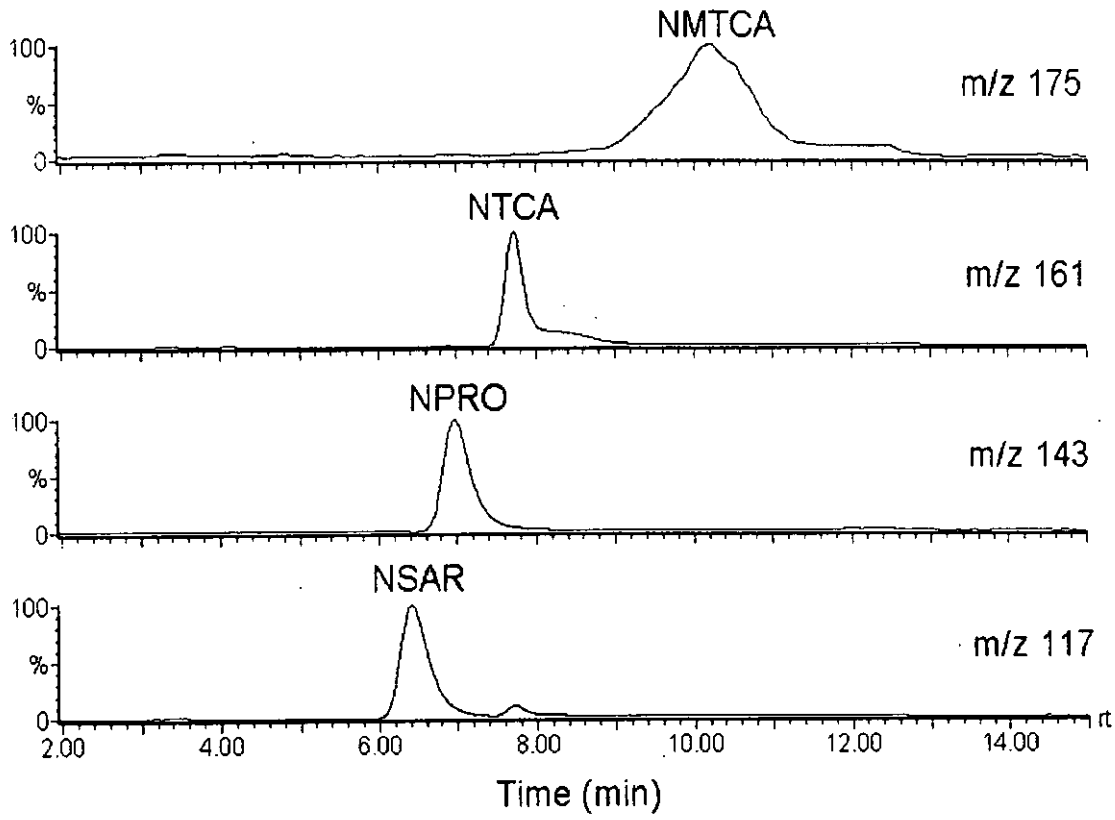


Figure 5.9 : HPLC/ESI-MS mass chromatogram of four N-nitrosoamino acids standard (10 ppm). Mobile phase : 1.4 mM C_{16}-cetyltrimethylammonium chloride in 65 : 35 (% v/v) MeOH : H_2O. Other experimental conditions were the same as shown in Figure 5.6.

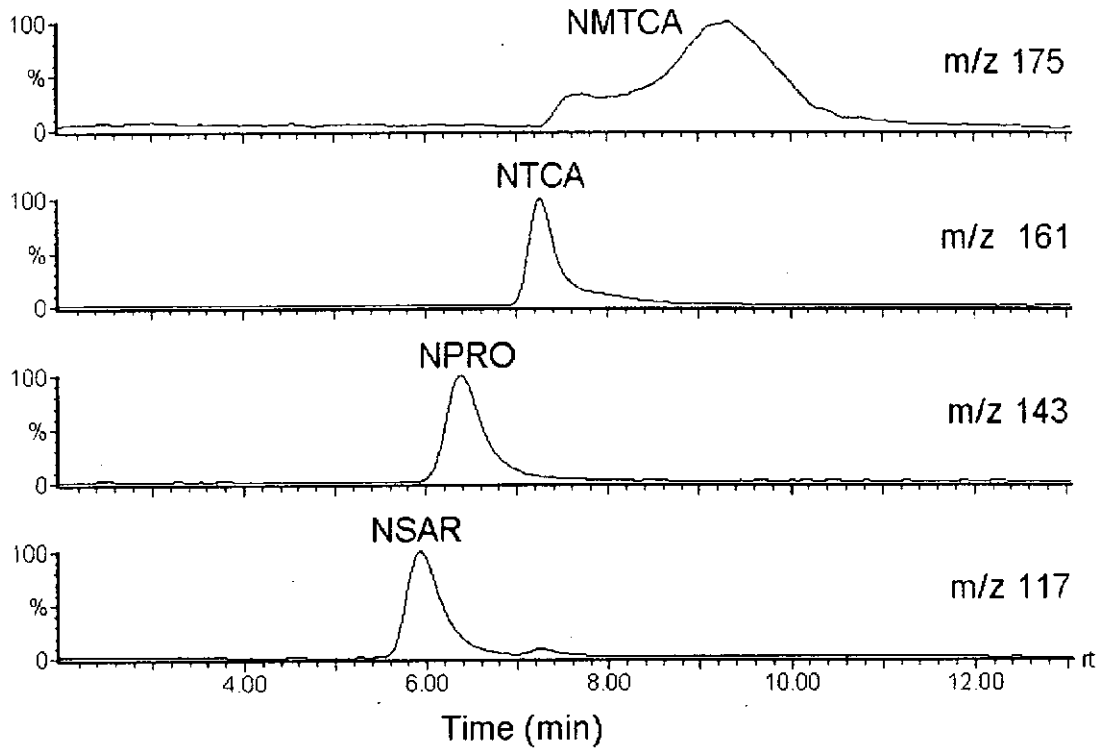


Figure 5.10 : HPLC/ESI-MS chromatogram of four N-nitorosoamino acids standard (10 ppm). Mobile phase : 1.4 mM C_{16}-cetyltrimethylammonium chloride in 60 : 35 : 5 (% v/v) MeOH : H_2O : MeCN . Other experimental conditions were the same as shown in Figure 5.6 .

5.3.2.3 Effects of Ion-pairing Reagent Concentration

The concentration of the ion-pairing reagent is another variable parameter for optimization separation efficiency in RP-IP-HPLC. The C_{16}-cetyltrimethylammonium (CTMA) concentration was varied from 0 to 2.20 mM in 60 : 35 : 5 MeOH : H_2O : MeCN in the present study. The variation of capacity factors of the four NAAs under investigation as a function of the concentration of C_{16}-CTMA are shown in Figure 5.11. The results illustrate typically ion-interaction chromatographic behavior. As the concentration of the pairing ion was increased from 0.35 to 1.40 mM, a considerable increase in capacity factors due to increased ion-pair complex formation or adsorption of the pairing ion onto the stationary phase was observed. The effect is greater for NMTCA, which is the most lipophilic and retained among the four NAAs. However, as the concentration of pairing ion was increased further the stationary phase become saturated with pairing ion, causing the retention factor to level off. Since sensitivity in electrospray ionization was affected by higher concentration of ion-pairing reagents, 1.4 mM C_{16}-cetyltrimethylammonium chloride is a better choice than 1.8 mM C_{12}-dodecytrimethylammonium chloride as the ion-pairing reagent in the RP-IP-HPLC/ESI-MS analysis. The observed trend is similar to a reported case shown in Figure 5.2 (p.47)

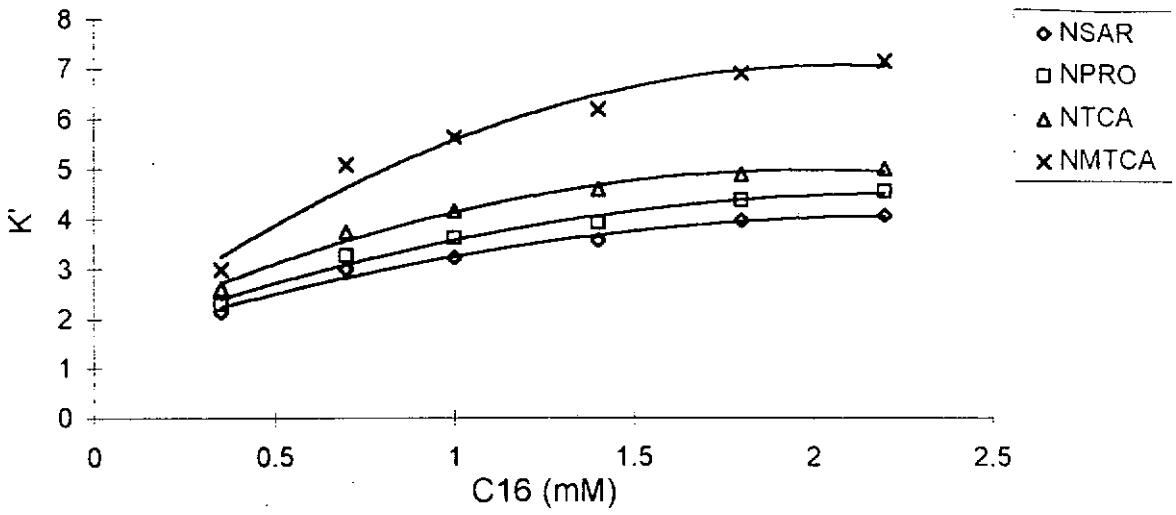


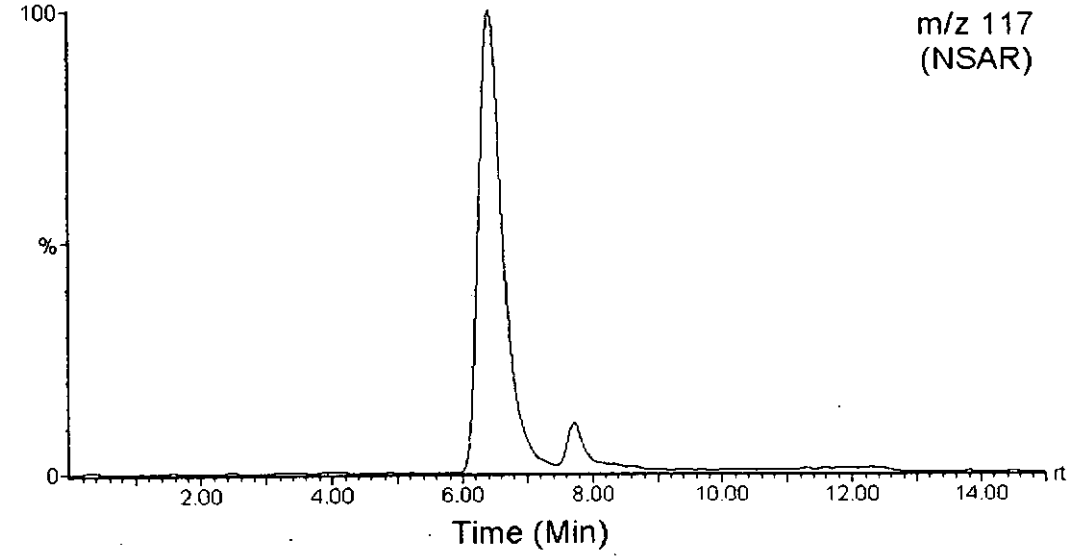
Figure 5.11 : Effect of C_{16}-cetyltrimethylammonium chloride concentration in the eluent on the capacity factor (k') of NSAR, NPRO, NTCA and NMTCA

5.3.2.4 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 (Chow and Polo, 1981). Therefore, it is of interest to investigate whether the E- and Z- isomers can be separated under HPLC conditions. The separation of E- and Z- isomers of NTCA, NSAR, NPRO and N-nitrosohydroxyproline (NHPRO) by β-cyclodextrin bonded silica HPLC column and 0.01 M tetraethylammonium acetate in acetonitrile mobile phase was reported (Issaq *et al.*, 1988). In this work, the ability of C_{18} RP-IP-HPLC to separate the syn and anti isomers of NAAs was investigated by systematically varying the methanol content of the mobile phase.

Our results showed that the separation of the syn and anti isomers of NAAs by C_{18} RP-IP-HPLC was highly dependent on the % composition (*v/v*) of the organic solvent, i.e. methanol, and the concentration of the ion-pairing reagent in the mobile phase. Thus E- and Z- conformers of NSAR and NMTCA were almost completely separated using 1.4 mM cetyltrimethylammonium (C_{16}-CTMA) chloride in 65 : 35 MeOH : H_2O and 70 : 30 MeOH : H_2O (*v/v*) solvent systems, respectively (Figure 5.12 (a) and (b)). The NTCA conformers were only partially separated using a high concentration of C_{16}-CTMA (2.2 mM) and a 60 : 35 : 5 MeOH : H_2O : MeCN (*v/v*) solvent system (Figure 5.12 (c)). On the other hand , the conformers of NPRO could not be separated over a wide range of methanol and ion-pairing reagent concentrations.

(a)



(b)

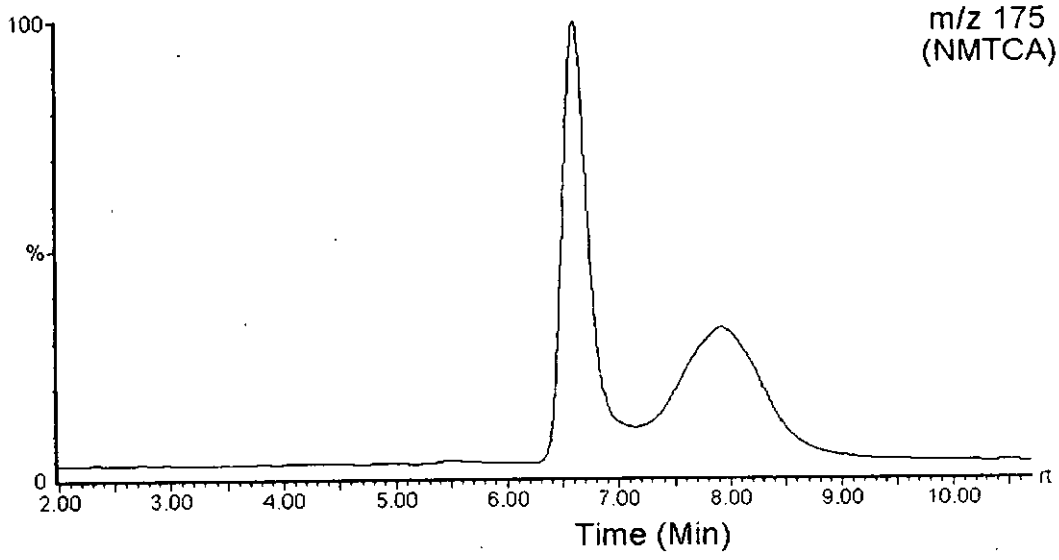


Figure 5.12 : Separation of syn and anti conformers of N-nitrosoamino acids : mass chromatogram of (a) NSAR ; 1.4 mM C_{16} -CTMA in 65 : 35 MeOH : H_2O ; (b) NMTCA ; 1.4 mM C_{16} -CTMA in 70 : 30 MeOH : H_2O. Other conditions were the same as shown in Figure 5.6.

(c)

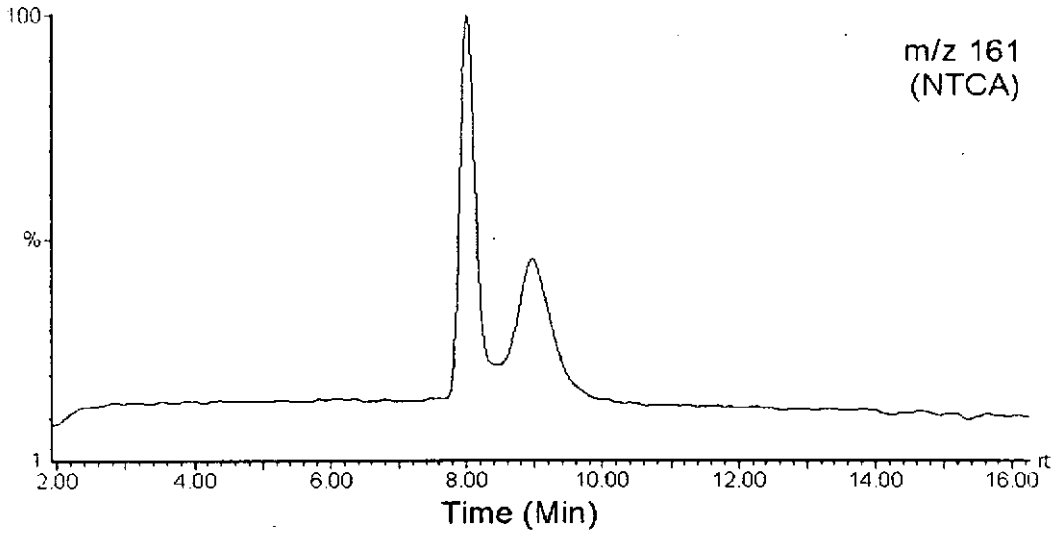


Figure 5.12 : Separation of syn and anti conformers of N-nitrosoamino acids : mass chromatogram of (c) NTCA ; 2.2 mM C_{16} -CTMA in 60 : 35 : 5 MeOH : H_2O : MeCN. Other conditions were the same as shown in Figure 5.6.

The separation of syn and anti isomers by RP-IP-HPLC has been rationalized by Bruzzoniti *et al.* using the stoichiometric model and the various chemical equilibria involved in the chromatographic process (Bruzzonti *et al*, 1996) :

$$A_S + [N^+]_m + [X^-_{syn}]_m \overset{K_{syn}}{\rightleftharpoons} (Qx_{syn})_S \qquad [5.6]$$

$$A_S + [N^+]_m + [X^-_{anti}]_m \overset{K_{anti}}{\rightleftharpoons} (Qx_{anti})_S \qquad [5.7]$$

where A_S is the number of free adsorption sites on the lipophilic stationary phase, N^+ is ion-pair reagent, $[X^-_{syn}]$ and $[X^-_{anti}]$ are the analytes of syn and anti isomers, respectively, and according to Xianren and Baeyens, the round ( ) and square [ ] brackets refer to the stationary and mobile phase, respectively (Xianren and Baeyens, 1988). The association equilibrium constants corresponding to equation [5.6] and [5.7] are denoted by K_{syn} and K_{anti} , respectively. The values of K_{syn} and K_{anti} are a measure of analyte retention by the non-polar stationary phase. In order to completely separate the conformers, the difference between K_{syn} and K_{anti} values should be sufficient large under the critical mobile phase conditions. The value of equilibrium constants can be influenced by electrostatic interaction between the hydrophobic pairing ion and analytes, which are mainly determined by the concentration of organic modifier in the eluent (Zou *et al.*, 1991). This was indeed found to be the case for separation of the NSAR and NMTCA isomers. Compared to NSAR and NMTCA, NTCA has lower valued K_{syn} and K_{anti}, and hence a higher concentration of C_{16}-CTMA was needed to drive the chemical equilibrium in favour of adsorption or partition onto the non-polar stationary phase.

On the other hand, unsuccessful resolution of the syn- and anti-conformers of NPRO means that K_{syn} and K_{anti} are nearly identical over a wide range of methanol and C_{16}-CTMA concentrations.

The fact that the syn- and anti- conformers of NTCA, NMTCA and NSAR could be separated from each other by adjusting the concentration of methanol and C_{16}-CTMA pairing ion of the eluent makes this chromatographic separation useful as a possible confirmation step for the presence of these NAAs in unknown samples.

5.3.3 ESI detection - Compatibility and Sensitivity studies

RP-IP-HPLC and electrospray ionization mass spectrometric detection are compatible because aqueous solvent systems are used by both. As shown in Section 5.1.2, the analyte ion current, and hence ESI detection sensitivity, is dependent on the chemical properties and concentration of co-analytes or other electrolytes present in the sample solution. The important work by Kebarle and Tang are mainly concerned with co-analyte effects on formation of charged droplet and gas phase ions in the ESI process (Kebarle and Tang, 1993). They chose cations as the object of their study. The relative ion evaporation rates of common cations were measured and explained on the basis of their ionic properties.

Under RP-IP-HPLC conditions, the cations and anions of the ion-pairing reagents co-elute with the analytes. Hence, from the analytical point of view, it is of practical importance to study the effect of co-eluting cations or anions on ESI sensitivity under experimental conditions. In the presence study, the deprotonated carboxylate anions of N-nitrosoamino acids, $[M-H]^-$, were generated and detected under negative ESI conditions. Hence, the reduction in ESI sensitivity is expected to be derived mainly from the chemical nature and the relatively high concentration of the anion of the tetraalkyl ammonium ion-pairing reagent.

5.3.3.1 Effects of Co-eluting Anion Derived from the Ion-pair Reagent on ESI Sensitivity

Tetraalkyl ammonium ion-pairing reagents, including many employed in the present study, are usually commercially available in the form of the chloride, bromide, iodide and acetate salts. Hence, choice of anions accompanying the tetraalkyl ammonium cation are available, and this is another factor in achieving optimal ESI detection of anionic analytes, including that derived from NAAs under RP-IP-HPLC conditions.

In the present study, the effect of co-eluting anions on sensitivity of detection was studied by comparing the ion intensity signal of the NAA carboxylate anion with and without the anion present. HPLC conditions for actual analysis of NAAs in food, i.e. solutions containing four NAA standards (5 ppm, 5 μg/ml) and 1.4 mM of the anion (F^-, Cl^-, Br^-, I^- and acetate derived from their respective sodium salts) in MeOH : H_2O : MeCN (60 : 35 : 5 %*v/v*) solvent, were tested in the sensitivity study.

The results of the sensitivity study are summarized in Table 5.4. In the presence of 1.4 mM of co-eluting anions, the ion intensities of the deprotonated molecular anion, $[M-H]^-$, of the analyte NAAs were found to decrease significantly by 50-90% compared to that of a control containing no co-eluting ion at all. Iodide showed the greatest reducing effect (80-90%), while the reduction fluoride was least at about 50%. The ESI detection sensitivity for NAAs was decreased in the order $I^- > Br^- > CH_3COO^- > Cl^- > F^-$.

Table 5.4 : Effect of counter anion derived from the ion-pair reagent on electrospray detection sensitivity of N-nitrosoamino Acids[a]

Anions	Relative Response [b]			
	NSAR	NPRO	NTCA	NMTCA
F^-	0.57	0.74	0.52	0.54
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 deprotonated carboxylate anion, $[M-H]^-$ derived from analyte NAA (5μg/ml) was measured.

b) Relative response is the ESI current of the NAA analyte measured in the presence of 1.4 mM concentration of the anion derived from the sodium salt, and compared to that of the control solution containing no co-eluting anion.

c) Mobile phase composition : MeOH : H_2O : MeCN (60 : 35 : 5 v/v).

d) Average of n=5 measurements, the relative deviation is $< \pm 6.8$ %.

As noted in Section 5.1.2, the ESI generated ion current is competitively divided between the analyte, X^-, and the co-eluting anion, Y^-, according to equation below (Kebarle and Tang, 1993):

$$I_{X^-_{ms}} = pf \frac{k_X[X^-]}{k_X[X^-] + k_Y[Y^-]} I \qquad [5.5]$$

where $I_{X^-_{ms}}$ is the mass spectrometrically detected ion current of X^-, p is a constant expressing the transmission efficiency of the mass analyser for sampling the gas phase ion current produced from the charged droplets, and *f* is the efficiency of conversion of droplet charge to gas-phase ions, and $[X^-]$ and $[Y^-]$ are the concentration of the ions, (with $[X^-]$ and $[Y^-] \geq 10^{-4}$ M), k_X and k_Y are ion evaporation rate constants relating to the relatives efficiencies of ejection of X^- and Y^- from the charged droplet. Therefore, the co-eluting anion, Y^-, will take up a bigger share of the ESI ion current if its ion evaporation rate constant, k_Y, is greater, leading to a corresponding lower ion intensity for the analyte, X^-. Under RP-IP-HPLC conditions, $[X^-]$ is in the range 10^{-5} - 10^{-9} M and is less than 10^{-4} M, equation [5.5] may be modified to :

$$I_{X^-_{ms}} = pf \frac{k_X[X^-]}{k_X[X^-] + k_Y[Y^-]} I = pf \frac{k'_X[X^-]}{k'_X[X^-] + k_Y[Y^-]} \qquad [5.8]$$

where k'_X ($k'_X < k_X$) represents the 'reduced' ion evaporation rate constant under dilute concentration conditions, when the surface concentration of $[X^-]$ in the charged droplet is not readily replenished during the successive ion ejection steps in the ESI process.

The ion evaporation rate constants of F^-, Cl^-, Br^-, I^- and acetate ion were measured according to the procedure of Kebarle and Tang (Kebarle and Tang, 1993), in which the anion intensities derived from equimolar solutions (5×10^{-4} M) of chloride and another anion in 50 : 50 (% *v/v*) MeOH : H_2O were measured and compared, and are summarized in Table 5.5. The ion evaporation rate constants were found in the order of $I^- > Br^- > CH_3COO^- > Cl^- > F^-$, which is in good agreement with the order of decreasing analyte ion intensity if these anions were used as the co-eluting ions in the ESI sensitivity study experiments (Table 5.4).

Table 5.5 : The relative ion-evaporation rate constants of anions

Anions	Ion-evaporation rate constant, $k^{(a)}$
F^-	0.1
Cl^-	1.0
Br^-	8.1
I^-	24.8
CH_3COO^-	1.2

a) The ion-evaporation rate constant is measured relative to that of chloride, which is assigned a value of 1.0

b) Average of n = 3 measurements, the relative deviation is $< \pm 8.2\%$

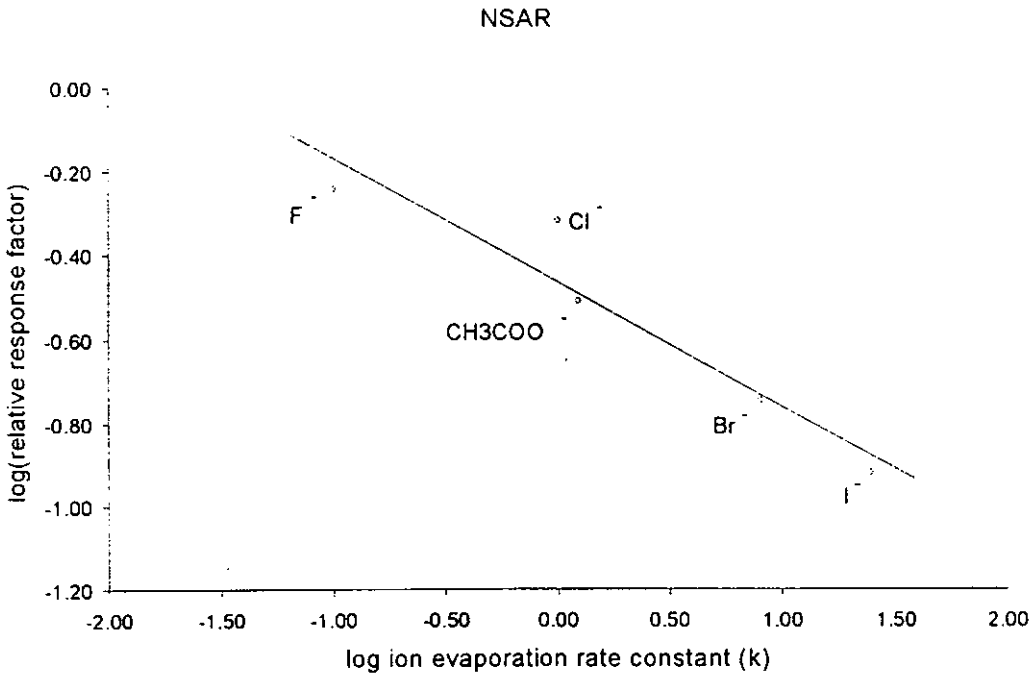
In fact, as shown in Figure 5.13, the relative response factor for the NAA analytes were found to be roughly proportional to the reciprocal of the measured ion evaporation rate constant, k_Y, of the co-eluting anion. Under RP-IP-HPLC conditions, if $k_Y [Y^-] >> k'_X[X^-]$, then equation [5.8] can be approximated to equation [5.9] :

$$I_{X^-\,ms} = pf \frac{k'_X[X^-]}{k'_X[X^-] + k_Y[Y^-]} I \approx pf \frac{k'_X[X^-]}{k_Y[Y^-]} I \qquad [5.9]$$

Hence, the analyte ion intensity, $I_{X^-\,ms}$ would be proportional to $1/k_Y$, and this was indeed experimentally verified. The ion evaporation rate constant of cations and anions is dependent on their physical properties such as solvation energy, ionic radius and surface activities (Kebarle and Tang, 1993). In general, ions with smaller ionic radius 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) > Br^- (1.95 Å) > Cl^- (1.81 Å) > F^- (1.36 Å).

Tetraalkyl ammonium ion-pairing reagents are usually available in the Cl^-, Br^-, I^- and acetate form. Based on the results of our sensitivity study, the chloride form should be preferred in RP-IP-HPLC separation because it has the least reducing effect on ESI sensitivity and it is commonly available.

(a)



(b)

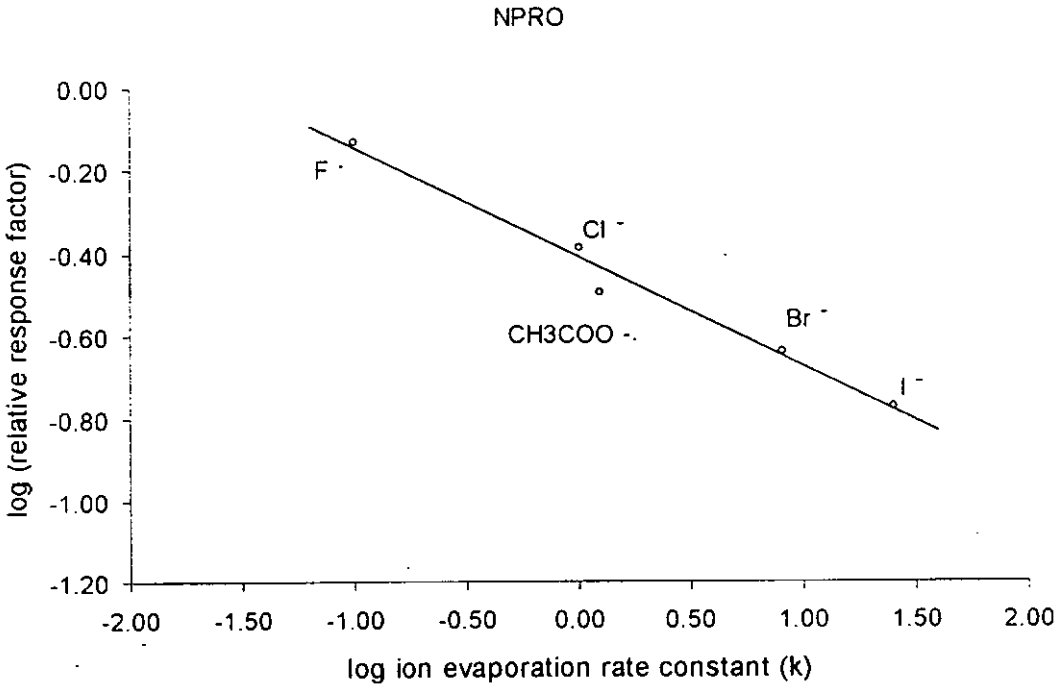
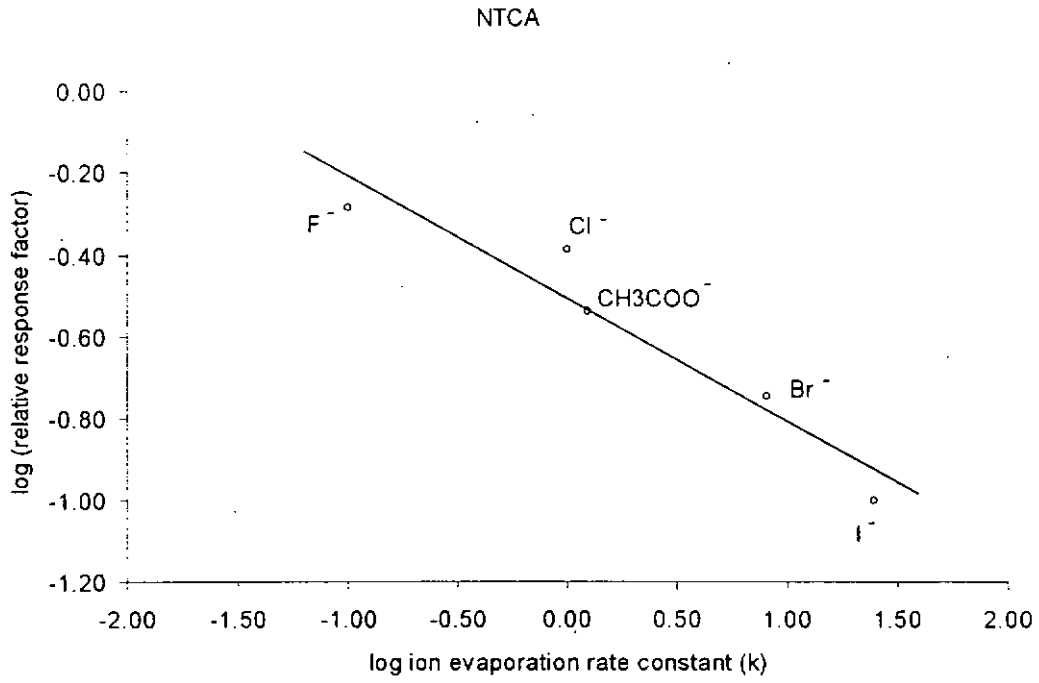


Figure 5.13 : The dependence of relative response factors of (a) NSAR, (b) NPRO on the ion evaporation rate constants of co-eluting anion derived from ion-pairing reagent.

(c)



(d)

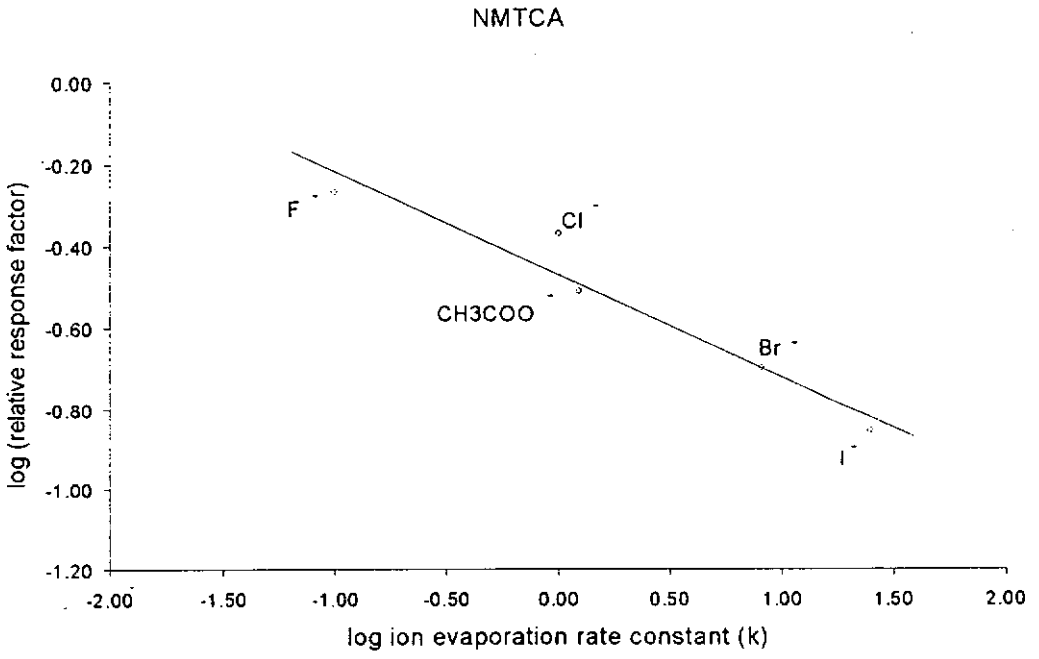


Figure 5.13 : The dependence of relative response factors of (c) NTCA, (d) NMTCA on the ion evaporation rate constants of co-eluting anion derived from ion-pairing reagent.

5.3.3.2 Effects of Concentration of Co-eluting Anion (Cl^-) on ESI 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, increasing the ion-pair reagent would also lead to corresponding increase in the concentration of the anion which co-elute with the analytes. As shown in equation [5.9], this would lead to a reduction in the ion intensity of the analyte. To study the concentration effect of co-eluting anions, the ESI generated $[M-H]^-$ ion intensities derived from a standard 5 ppm NAA standard solutions were measured as a function of chloride concentration (as NaCl) in the 0.35 to 56 mM range in MeOH : H_2O : MeCN (60 : 35 : 5 %*v/v*).

The results , illustrated for the case of NMTCA, is shown in Figure 5.14. The analyte ion intensity was found to decrease rapidly with added $[Cl^-]$, and reduced to 20% and 5% of its initial intensity at 5 mM and 56 mM of Cl^-, respectively. In order to obtain good sensitivity in ESI 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 mM 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.

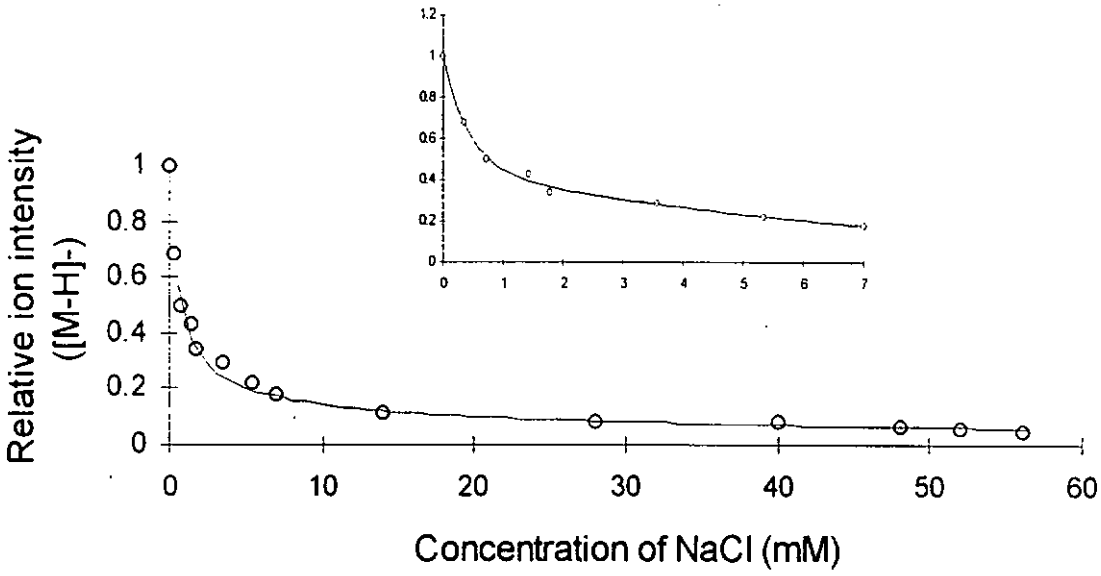


Figure 5.14 : The relative ion intensity of NMTCA ([M-H]⁻) as a function of chloride concentration in MeOH : H_2O : MeCN (60 : 35 : 5 %v/v)

5.3.3.3 Interference due to Formation of Solvent Cluster Ions in ESI Detection

In electrospray ionization, association of solvent molecules with cations/anions frequently occur, leading to formation of 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 cone voltage of the ESI interface. However, under such de-clustering conditions, a decrease in the ESI 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 (Figure 5.15). Some of these cluster ions had the same m/z values as the deprotonated molecular ions, $[M-H]^-$, of NAAs (Table 5.6). 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 ^{0}C to 90 ^{0}C, and increasing the drying gas flow rate from 250 L/hr 350 L/hr could decrease, to some extent, 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 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 60 : 35 : 5 *v/v* MeOH : H_2O : MeCN, which could yield reasonable separation of the NAAs in the mass chromatogram of the $[M-H]^-$ ion, but avoiding formation of solvent cluster ions in the background ESI spectra.

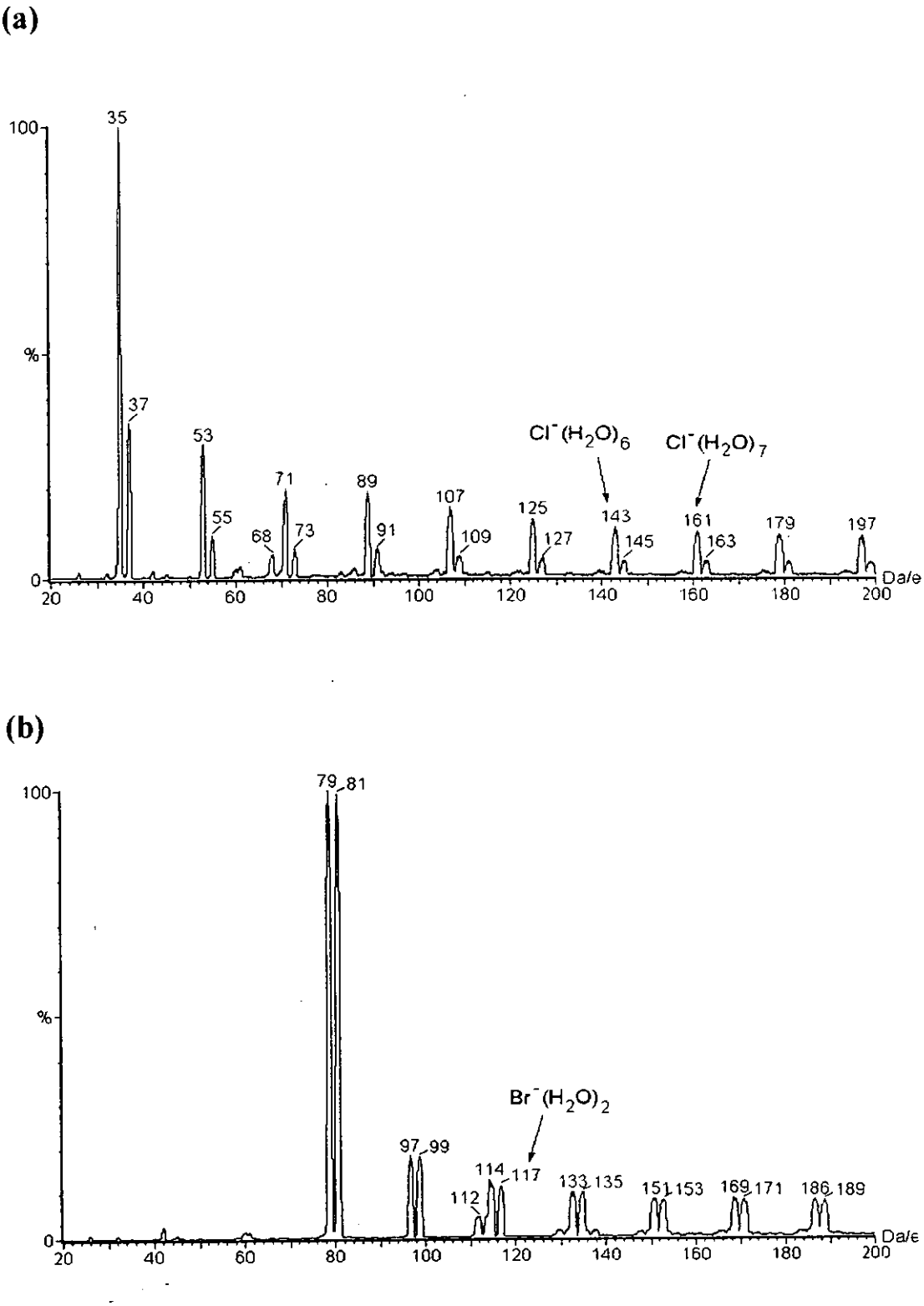


Figure 5.15 : Cluster ion formation under different mobile phase conditions : (a) 1.0 mM C_{16}-CTMA chloride in H_2O : MeCN (70 : 30 % v/v) (b) 1.8 mM C_{12}-DTMA bromide in H_2O : MeCN (50 : 50 % v/v).

Table 5.6 : Effect of methanol, water and acetonitrile content in the mobile phase on formation of 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* H_2O)
NPRO	m/z 143	$Cl^-(H_2O)_6$; (>50% *v/v* H_2O) $Br^-(MeOH)_2$; (>50% *v/v* MeOH)
NTCA	m/z 161	$Cl^-(H_2O)_7$; (>50% *v/v* H_2O) $Br^-(MeOH)_2(H_2O)$; (>50% *v/v* MeOH)

a) Solvent cluster ions with the same m/z value as the carboxylate pseudo-molecular anion, [M-H]⁻.

b) The threshold values (% v/v) for individual solvents at which solvent cluster ions were observed are shown in parenthesis.

5.3.3.4 Effects of Tetraalkyl Ammonium Cation on Anion ESI Sensitivity

Although the carboxylate anions of NAAs were detected in this study, the stability of the tetraalkyl ammonium-carboxylate ion pair could affect the efficiency of ESI anion formation and sensitivity of detection. Consequently, the $[M-H]^-$ ion intensity signal for 5 ppm (μg/ml) NAA standard solutions were measured and compared in the absence / presence of 1.4 mM tertaethylammonium chloride (C_2-TEA), tetrabutylammonium chloride (C_4-TBA),

dodecyltrimethylammonium chloride (C_{12}-DTMA) and cetyltrimethylammonium chloride (C_{16}-CTMA) in MeOH : H_2O : MeCN (60 : 35 : 5 % v/v).

The quantitative comparison of results are shown in Table 5.7. Differences in the relative $[M-H]^-$ ion intensities were indeed observed for different ion-pairing reagents and NAAs, but the variation was moderate, in the range of 0 - 45% reduction of ion intensity when compared to that of a control solution containing 1.4 mM NaCl, which is assumed not to be involved in the ion-pairing process. The effect of adding tetraethylammonium chloride was almost the same as sodium chloride, suggesting that the C_2^+(TEA) cation could not form stable ion-pair complexes with the carboxylate anions of NAAs. For C_{12}-DTMA and C_{16}-CTMA, the relative ion intensities were found in the order NSAR > NPRO > NTCA > NMTCA, which is also the order of elution of these four NAAs from the C_{18} column under RP-IP-HPLC conditions. The C_{12}-DTMA-NMTCA and C_{16}-CTMA-NMTCA ion-pair complexes were the most retained on the C_{18} stationary phase, and their associated relative ion intensities were found to be lowest at 0.56 and 0.62, respectively.

Table 5.7 : Effect of ion-pairing tetraalkyl ammonium cations on ESI detection of carboxylate anions [M-H]⁻ of NAAs

	Relative ion intensity[a]			
Ion-pair reagent	NSAR	NPRO	NTCA	NMTCA
C_2^+ (TEA)	1.0_6	1.0_6	1.0	1.0
C_4^+ (TBA)	0.81	0.76	0.85	0.72
C_{12}^+ (DTMA)	0.90	0.73	0.68	0.56
C_{16}^+ (CTMA)	1.0	0.78	0.78	0.62

a) Relative ion intensity of deprotonated carboxylate molecular anion, [M-H]⁻, derived from 5 ppm NAA standard solutions containing 1.4 mM tetraalkyl ammonium ion-pairing reagent in MeOH : H_2O : MeCN (60:35:5 % v/v). The ion intensity obtained from control solution containing 5 ppm NAA standard and 1.4 mM NaCl was arbitrarily assigned a value of 1.0.

b) Average of n=5 measurements , the relative deviation is < ± 6%

Thus, our results suggest that the more retained, and presumably the more stable ion-pair complexes will yield lower ESI sensitivity of detection. In the present study, the differences among C_{12}-DTMA and C_{16}-CTMA ion-pair complexes on ESI detection sensitivity were found to be small. Since lower concentration of C_{16}-CTMA could be used to effect reasonable separation of the

four NAAs, the final and optimal reverse phase ion-pair HPLC condition adopted was 1.4 mM C_{16}-cetyltrimethylammonium chloride in MeOH : H_2O : MeCN (60 : 35 : 5 % *v/v*) mobile phase.

To our knowledge, this is the first report on successful separations of NAAs by reverse-phase ion-pairing chromatography employing a commonly available C_{18} HPLC column. To demonstrate the practical application of the developed HPLC method, the NAA content in Chinese salted fish was analysed. The electrospray mass spectrometry of NAAs were also discussed in details.

Chapter 6 Analysis of NAAs in Food by High Performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry (HPLC/ESI-MS)

6.1 Background - Mass Spectrometry of N-nitroso Compounds

6.1.1 Electron-Impact Mass Spectrometry

The wide occurrence of trace quantities of N-nitrosamines in food has prompted extensive efforts directed towards their detection and identification. Generally, the method of choice for confirmation of the presence of N-nitroso compounds in food has been the use of electron impact (EI) mass spectrometry (Eisenbrand, 1974).

Extensive studies on the electron mass spectral behaviour of N-nitrosamines and their derivatives have been carried out by high-resolution measurement of fragment ions, deuterium labelling studies and metastable ion measurements (Billet *et al.*, 1970 ; and Rainey *et al.*, 1978).

On the basis of such extensive studies, some of the common features in the 70 eV EI mass spectra of N-nitroso compounds, mainly N-nitrosamines (R_1 R_2 NNO), can be summarized as follows :

(i) molecular ions, $M^{+\cdot}$, occur with varying intensity ;

(ii) alkyl cations and NO^+ (m/z 30) are present in the mass spectra ; and

(iii) $[M\text{-}NO]^+$ ($[M\text{-}30]^+$) and $[M\text{-}HNO]^{+\cdot}$ ($[M\text{-}31]^{+\cdot}$) fragment ions are commonly observed, presumably due to the labile nature of the N-NO bond.

The EI mass spectra of N-nitrosoamino acids had been reported (Rainey *et al.*, 1978). Fragmentation from the molecular ion was found to be extensive. Aside form the characteristic peaks mentioned above, other prominent peaks include the $[M\text{-}COOH]^+$, $[M\text{-}COOH\text{-}NO]^{+\cdot}$ and $[M\text{-}COOH\text{-}NOH]^+$. With these structurally specific fragment ions, NAAs are quite easily identified by their respective EI mass spectra.

However, a review of the literature shows that confirmation of various non-volatile N-nitroso compounds in food were mostly carried out by GC/MS analysis after suitable derivatization (Sen and Kubacki, 1987). In the case of N-nitrosoamino acids, conversion to the methyl ester derivative is the most commonly adopted method (Ishibashi *et al*, 1980). The EI mass spectra of the methyl ester derivative showed abundant and analyte-specific $[M\text{-}NO]^+$, $[M - COOCH_3]^+$ and $[M - NO - COOCH_3]^{+\cdot}$ fragment ions, which could be used in identification and selected ion monitoring (SIM) measurements of NAAs, but no molecular ion.

In summary, EI mass spectrometry has been mostly applied to qualitative analysis and confirmation of non-volatile N-nitroso compounds in food and environmental samples. Discrepancies between GC-TEA and GC-MS results of NAAs in food have been reported (Sen and Kubacki, 1987). This emphasizes the

urgent need to develop alternatives HPLC/MS methods for confirming and quantifying non-volatile NOCs, including NAAs, in food and environmental samples.

6.1.2 Chemical Ionization (CI) Mass Spectrometry

There is no systematic study on the chemical ionization mass spectra of N-nitrosoamino acids, or other non-volatile NOCs. The formation of the protonated molecular ion, MH^+, and the adduct ion, $[M+C_2H_5]^+$, in the partial methane CI mass spectra of NSAR and NPRO was noted, but not investigated in detail. Surprisingly, the negative ion chemical ionization mass spectrum of some N-nitroso compounds, again mainly on N-nitrosamines, with CO_2 as reagent gas was reported (Suzuki and Brandenberger, 1986). The major ions observed were : $[M-H]^-$, $[M-NO]^-$ and $[M-H-NOH]^-$. However, the origin and advantages/limitation of using CO_2 negative ion chemical ionization was not discussed. There is no report yet on the application of CI mass spectrometry to analysis of N-nitroso compounds.

6.1.3 Liquid Chromatography/Mass Spectrometry (LC/MS)

Very little work has been done on development of LC/MS methods to the analysis of non-volatile NOCs. The only report that has appeared so far in the literature was that by Beattie *et al.* (Beattie *et al.*, 1985). A standard polyimide moving belt interface was used on which the eluent from the LC column was deposited and the solvent allowed to evaporate before entering the ion source. The mass spectrometer was operated in the chemical ionization (CI) mode using ammonia as the ionizing medium. Most compounds studied produced $(M+H)^+$ and $(M+NH_4)^+$ peaks. The base peaks for the N-nitrosoamino acids usually corresponded to loss of both COOH and NO fragments i.e. $[MH-COOH-NO]^+$. Comparison of the HPLC mass spectra with those obtained by probe introduction revealed the presence of some spurious fragments in the former technique. For example, two fragments ion at 101 ($[MH-CO\cdot O]^+$) and 116 ($[MH-NO+H]^+$) for NPRO were present only in the spectra obtained by LC-MS. These fragment ions were thought to have been produced by thermal decarboxylation during solvent evaporation. Also, a few microgram of NAAs were required to provide a reliable and recognizable spectra. These researchers (Beattie *et al.*, 1985) felt that, although the technique produced informative spectra, it had limited sensitivity and was unsuitable for trace analysis of NVNCs in food samples, which contain NAAs at the ng/g level.

Thus, it can be said that the analytical capabilities of mass spectrometry have not been fully utilized in the qualitative and quantitative analysis of N-nitroso compounds, including N-nitrosoamino acid. As bench-top LC/MS systems are now

commonly available, and the price of LC/MS system is decreasing to a level that is comparable to a GC-TEA system, we felt it may be the right time to revive the application of LC/MS techniques to the analysis of NOCs. As mentioned in Section 2.3.3, electrospray ionization (ESI) mass is very suitably applied to thermally labile compounds. In this part of our report, the ESI mass spectral behaviour of NAAs was investigated in both the positive and negative ESI mode, and the application to analysis of NAA contents in Chinese salted fish is demonstrated.

6.2 Material and Methods

6.2.1 Equipment

The major equipments used for this part of the project are the same as those listed in Chapter 5 (refer to section 5.2.1 p.55).

6.2.2 Reagents and materials

1. The following analytical grade chemicals (purity > 99%) were purchased from Acros chemical Co. (Geel West Zone 2, Janssen Pharmaceuticalaan 3a, 2440 Geel, Belgium)

   tirfluoracetic acid
   sodium hydroxide
   sodium sulfate
   ammonium sulfamate
   hydrochloric acid

   Other chemicals employed in this part of study are the same as shown in Chapter 5 (refer to Section 5.2.2).

2. The solid phase extraction (SPE) C_{18} cartridges and Nucleopore nylon filter were supplied by Waters China Limited (Connaught Road West, Hong Kong)

3. Chinese salted fishes were bought from the local market in Hong Kong.

6.2.3 Instrumentation

6.2.3.1 HPLC conditions

Based on the studies on separation efficiency and ESI sensitivities in Chapter 5, RP-IP-HPLC/ES-MS analysis was carried out under isocratic conditions with 1.4 mM cetyltrimethylammonium chloride in methanol : water : acetonitrile (60 : 35 : 5 % *v/v*) and the mobile phase flowing at 200 µl/min with a split ratio of 1/10 into ESI interface.

6.2.3.2 ESI-MS conditions

At the initial stage of our study, the mass spectra of NAAs obtained under positive electrospray ionization (+ESI) conditions were studied. The NAA standard solutions (20 mg/L) were made up in methanol : trifluoroacetic acid (TFA) 99 : 1 *v/v* solution. The positive ESI-MS conditions were :

capillary voltage : +3.0 kV

skimmer cone voltage : +20V

In studying the fragmentation behaviour of protonated NAAs, collision induce dissociation (CID) mass spectra were obtained by varying the skimmer cone voltage in the range of 30 - 70 V. The other MS instrumental parameters are the same as that described in Section 5.2.3.2 (p.62).

To obtain the negative ESI mass spectra of NAAs, the voltage of the capillary sprayer was set at - 3.0 kV, and the skimmer cone voltage was - 20V. Methanol was used to transfer the sample solution from the sample loop to the electrospray interface. Negative ESI-CID (sample cone voltage 30 - 70V) mass spectra of all NAAs were also obtained.

The negative ESI mass spectra of NAAs were considered as a better choice for the qualitative and quantitative analysis of NAAs because more abundant $[M-H]^-$ molecular ions were generated with less background interferences (Section 5.3.1, p.66). Therefore, quantification of NAAs was carried out by selective ion monitoring (SIM) of the $[M-H]^-$ molecular anion (dwell time 0.1 s) and its characteristic fragment ions in the negative ESI mode.

6.2.4 Preparation of Standard Solutions of N-nitrosoamino Acids

Individual stock solution containing NSAR, NPRO, NTCA, NMTCA, NPIC and d_3-NPRO, each at a concentration of 1 mg/ml, were prepared by

weighting accurately about 10.0 mg of each NAA standard dissolved in methanol and made up to 10 ml solution. The stock solution was kept at 4^0C. A series of working standard solution containing 10 - 800 μg/ml were freshly prepared by serial dilution. Also, a mixture standard solution containing NSAR, NPRO, NTCA and NMTCA in the concentration range of 1 - 80 μg/ml were prepared. 1 ml of the mixture standard solution was vortex mixed with 1 ml of 10.0 μg/ml d_3-NPRO (deutrated internal standard) in 5 ml capped V-vials. The concentrations of mixture solutions were 0.50, 1.25, 2.5, 5.0, 10, 20, 40 μg/ml for each of the NAA anlaytes with 5 μg/ml d_3-NPRO (internal standard) for obtaining the standard calibration curve. These solutions could be stored at 4 ^{0}C for at least 3 months without any evidence of degradation. Corresponding solutions using another internal standard, NPIC, were prepared in a similar way.

6.2.5 Preparation of Food Samples

The following sample extraction and clean-up procedure was developed by us after trying out several other procedures, and adopted in the analysis of food samples.

About 5.0 ± 0.1 g of fish sample was accurately weighted and pulverized for 3 minutes in a blender. The pulverized sample was transferred to an extraction thimble and Soxhlet extracted with chloroform for 2 hours to remove fatty contents. After solvent extraction, the fish sample was placed in an evaporating dish to allow residual chloroform to evaporate. The de-fatted sample was then homogenized with 50 ml of methanol for 2 hours and 100 μl of 10 μg/ml d_3-

NPRO (deuterated NPRO internal standard) were added. The sample was then centrifuged at ~ 3,000 g_{av} for 10 minutes. The chloroform extracts was evaporated *in vacuo* at temperatures below 40 ^{0}C and re-dissolved in 8 ml of distilled water. The pH of the aqueous extract solution was adjusted to 9 - 9.5 with dropwise addition of 0.01M sodium hydroxide. The aqueous sample extract was passed through a 500 mg Sep-Pak C_{18} cartridge that had been pre-wetted with MeOH and H_2O. The cartridge was washed with 2 ml of water and the combined eluent acidified with 1.0 M HCl to pH 1. 1 g of ammonium sulfamate was added to the solution, followed by saturation with sodium chloride. The aqueous phase was then extracted 6 times with 10% methanol in dichlormethane. The organic phases were combined, dried by mixing with anhydrous sodium sulfate, and evaporated to dryness. The sample residue was re-dissolved in 2 ml of water. The aqueous sample extract was then passed through a Nucleopore nylon filter and 100 µl of 0.02 M cetyltrimethylammonium chloride (C_{16}-CTMA) was added to the eluent. The whole sample solution was transferred again to a 100 mg Sep-Pak C_{18} cartridge (pre-conditioned by MeOH, water and 0.001 M C_{16}-CTMA) and rinsed with 3 ml of methanol-water (30 : 70 *v/v*). The NAAs were eluted out by 3 ml of methanol onto a 5 ml v-vial and gently blown to dryness with nitrogen. Finally, 100 µl of methanol-water-acetonitrile (60 : 35 : 5 *v/v*) were added to the residue and 5 µl of this sample solution was injected via a sample loop for HPLC/ESI-MS analysis.

6.3 Result and Discussions

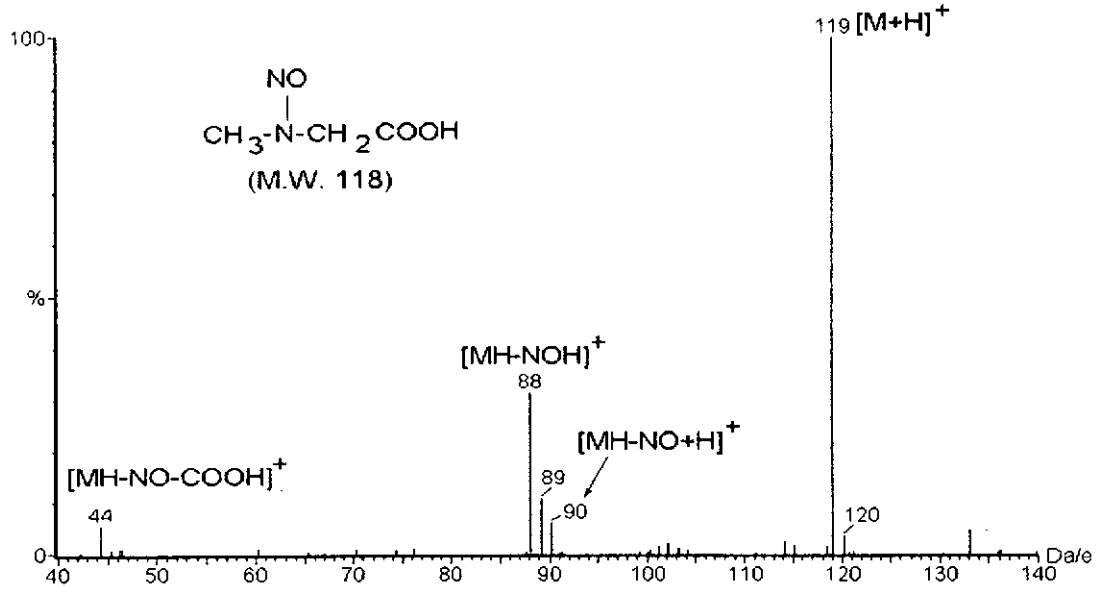
6.3.1 ESI Mass Spectrometry of NAAs

6.3.1.1 Positive Electrospray Ionization (+ESI) mass spectra of NAAs

The positive ESI mass spectra of NAAs were obtained by flow injection of standard solution (20 ng/μl in 99 : 1 *v/v* MeOH : trifluoroacetic acid (TFA)) at a flow rate of 20 μl/min. The +ESI mass spectra of NPRO, NSAR and NTCA are shown in Figure 6.1, while the ion abundances are quantitatively tabulated in Table 6.1.

(a)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 40 volt

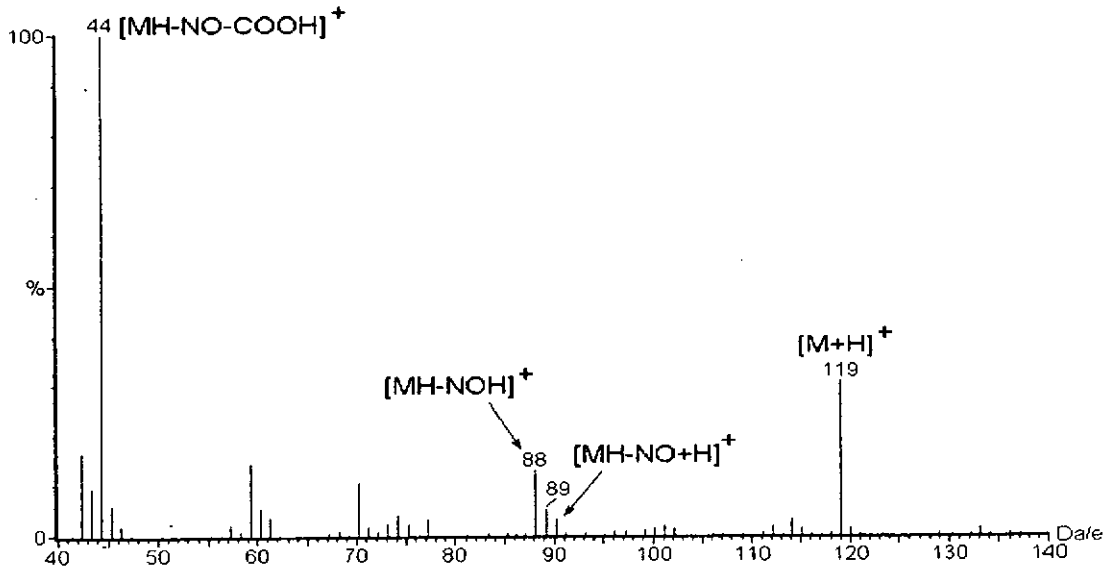
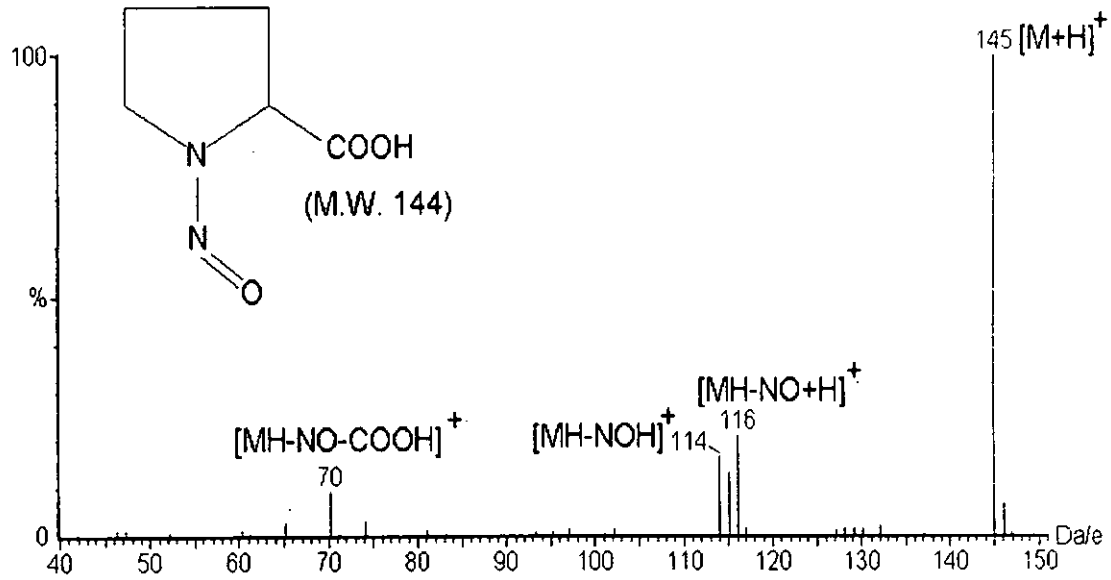


Figure 6.1 (a) : Positive ESI mass spectrum of NSAR. Experimental conditions : (i) 20 and (ii) 40 skimmer cone voltage

(b)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 40 volt

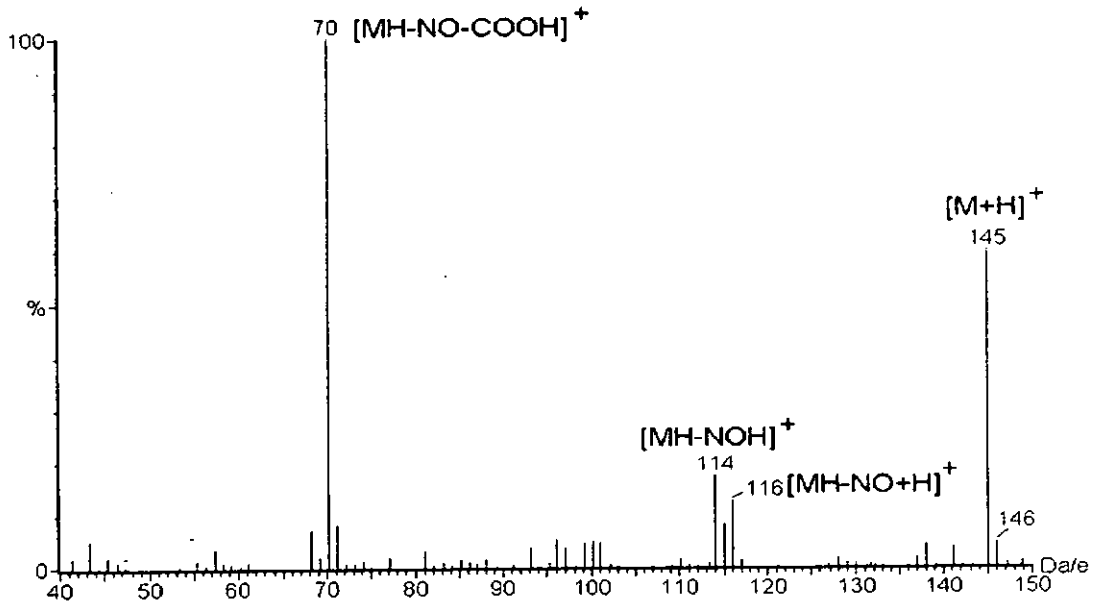
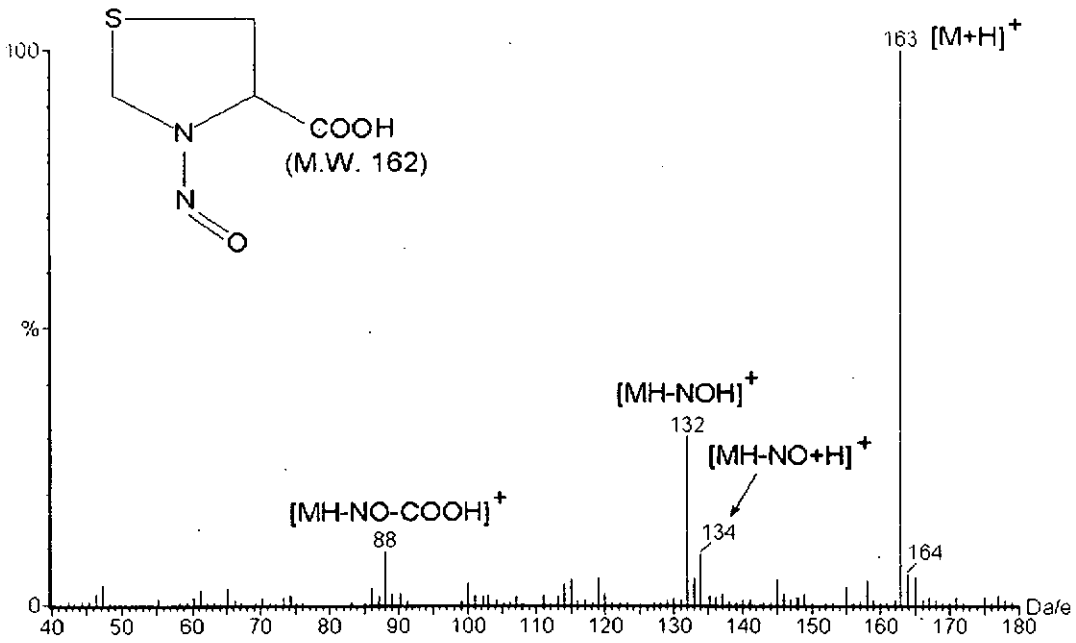


Figure 6.1 (b) : Positive ESI mass spectrum of NPRO. Experimental conditions : (i) 20 and (ii) 40 skimmer cone voltage

(c)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 40 volt

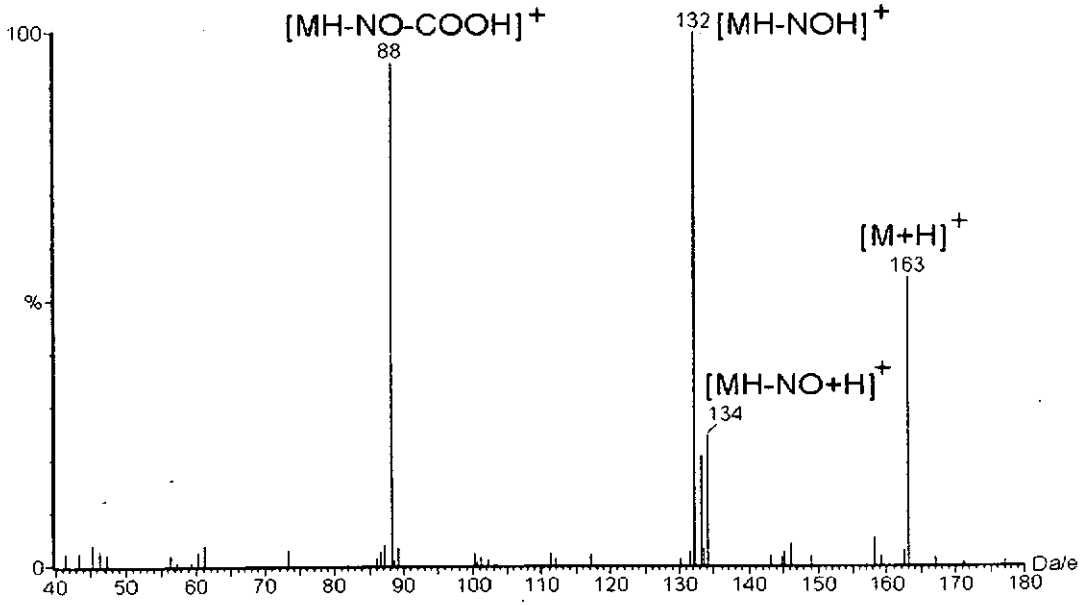


Figure 6.1 (c) : Positive ESI mass spectrum of NTCA. Experimental conditions : (i) 20 and (ii) 40 skimmer cone voltage

Table 6.1 : Positive electrospray ioniziation mass spectra of N-nitrosoamino acids[a]

Compound	Mol. Wt.	$[M+H]^+$	$[MH-NO]^{+\cdot}$	$[MH-NOH]^+$	$[MH-NO+H]^+$	$[MH-NO-CO{\cdot}OH]^+$	other ions : % relative intensity(m/z)
NSAR	118	100(*119*)	10(*89*)	31(*88*)	5(*90*)	5(*44*)	
NPRO	144	100(*145*)	14(*115*)	19(*114*)	24(*116*)	10(*70*)	
NTCA	162	100(*163*)	5(*133*)	47(*132*)	18(*134*)	4(*88*)	16(*102*)
NMTCA	176	100(*177*)	33(*147*)	26(*146*)	69(*148*)	14(*102*)	43(*159*) $[MH-H_2O]$

a) Skimmer cone voltage at 20 volt

As shown in Figure 6.1, at low sampling cone voltage (20 volts), the protonated molecular ion, MH^+, is the only major and base peak in the +ESI mass spectra. In the previous report by Beattie et al., the MH^+ peak of NPRO was lower in intensity than the $[MH\text{-}CO\cdot O]^+$ (m/z 101) and $[MH\text{-}NO+H]^+$ (m/z 116) peaks ascribed to derive from thermal decomposition product of NPRO. The abundant MH^+ protonated molecular ions observed indicated that under +ESI conditions, thermal decomposition of NAAs did not occur or occurred only to a minor extent. As shown in Table 6.1, $[MH\text{-}NO]^{+\cdot}$, $[MH\text{-}NOH]^+$ and $[MH\text{-}NO\text{-}CO\cdot OH]^+$ fragment ions were found in moderate intensities (mostly 10 - 30% of the base peak). These fragment ions were formed by simple bond fission at the N-NO bond and at the carboxylate functional group. The loss of NO, NOH and NO+CO·OH neutral fragments were also found in the EI mass spectra of NAAs, suggesting that the stability of the leaving neutrals is the driving force for the decomposition of both $M^{+\cdot}$ and MH^+. The fragmentation behaviour of the MH^+ protonated molecular ion could be revealed by studying its collision-induced dissociation (CID) obtained by increasing the skimmer (sampling) cone voltage, i.e. increasing the collisional frequency of ESI generated MH^+ with the nebulizing N_2 gas molecules. A plot of the % total ion abundance of the fragment ions versus the skimmer cone voltage for NPRO is shown in Figure 6.2.

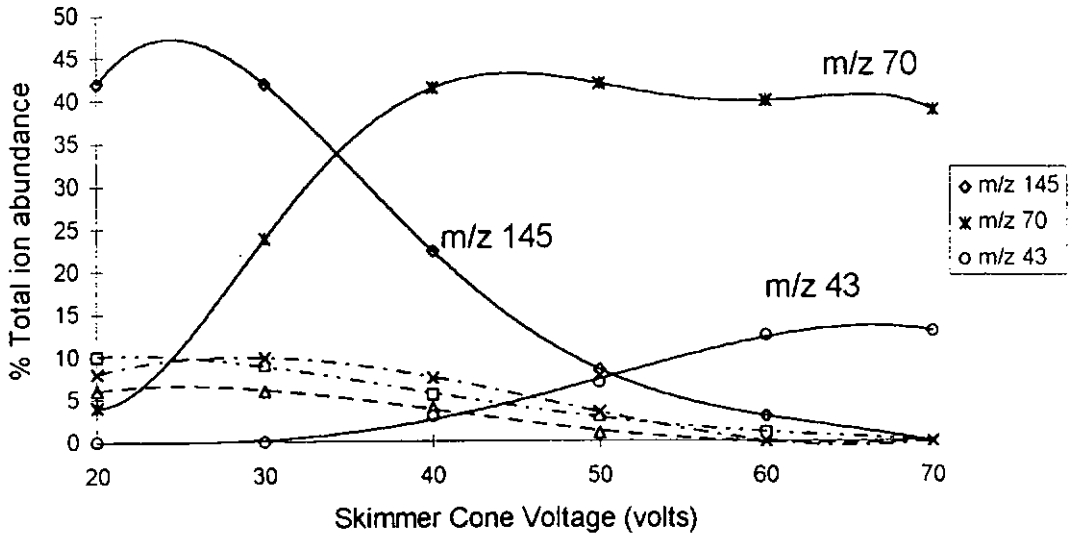


Figure 6.2 : Plot of % total ion abundance (% I/Σ_I) versus skimmer cone voltage in the +ESI mass spectra of N-nitrosoproline

The plot clearly shows that upon collisional addition, the protonated molecular ion (m/z 145), MH^+, decomposed mainly to yield the $[MH\text{-}NO\text{-}CO\cdot OH]^+$ (m/z 70) fragment ion, while the abundances of other fragment ions varied only to a small extent. This is further confirmation that the fragment ion intensities observed in the +ESI mass spectra of NAAs were derived from fragmentation of MH^+ ions, and no significant thermal decomposition of the analyte had occurred. The +ESI mass spectra of NAAs are simple and easy to interpret. They could be used in supplementary or complementary tests for identification of NAAs in food or unknown samples. However, when used in conjunction with RP-IP-HPLC separation, positive ESI is not the preferred mode of detection for NAAs. Firstly, the protonating agent, trifluoroacetic acid, must be added post-column to the HPLC eluent because an acidified mobile phase would affect the separation efficiency and causes damage to the stationary phase.

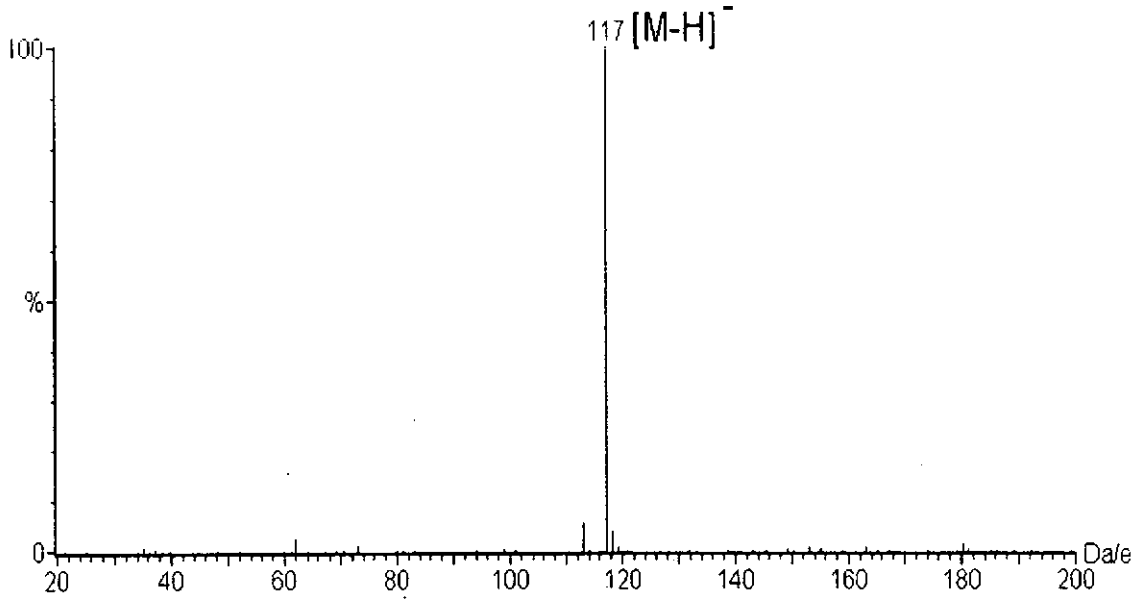
Secondly, NTCA and NMTCA are known to be unstable under acidic conditions, thereby introducing additional sources of error in the analysis. We have also found that much higher background noises were observed if NAAs in food samples were detected in the +ESI mode, presumably because many of the interfering substances present were also easily protonated. Since a relatively high concentration of ion-pairing reagent (1.4 mM C_{16}-cetyltrimethylammonium chloride) was present in the HPLC eluent, the fragmentation of the tetraalky ammonium cation containing a C_{16} alkyl chain also yields many low m/z fragment ions which masked the presence of the MH^+ ions derived from the NAA analytes. Hence, the negative ESI mode was investigated and finally adopted for detection of NAAs eluted out from a RP-IP-HPLC separation.

6.3.1.2 Negative Electrospray Ionization (-ESI) Mass Spectra of NAAs

The negative ESI mass spectra of N-nitrosoamino acids are shown in Figure 6.3. Under -ESI conditions, the only and base peak in the mass spectrum is the deprotonated carboxylate molecular anion, $[M-H]^-$. As mentioned in Section 5.3.1, the high abundance of the deprotonated molecular anion can be suitably employed for sensitive detection of NAAs eluted from a RP-IP-HPLC separation.

(a)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 50 volt

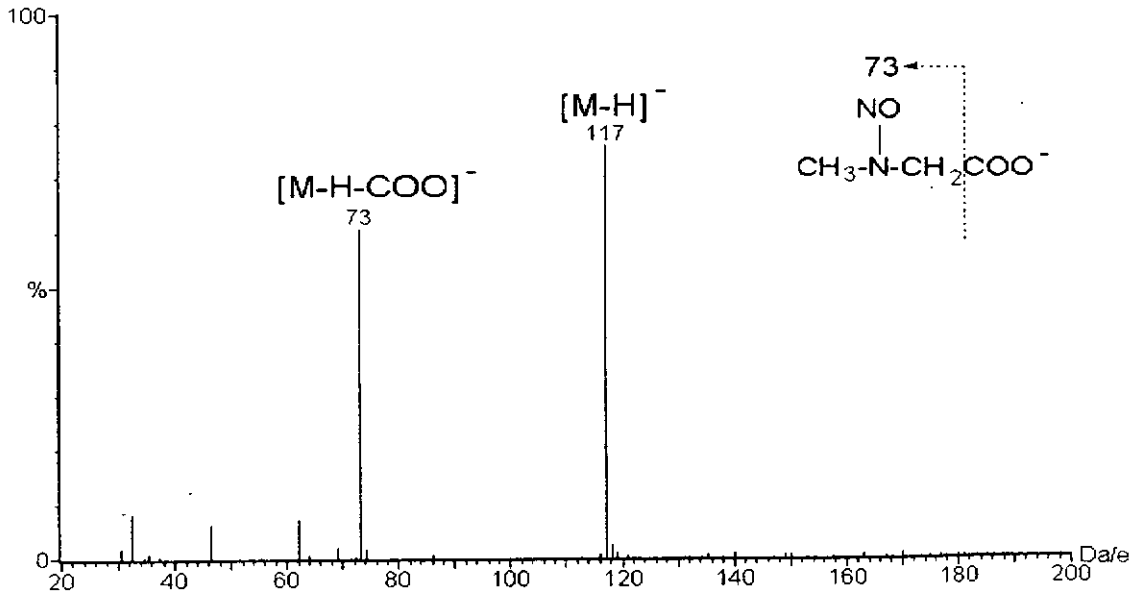
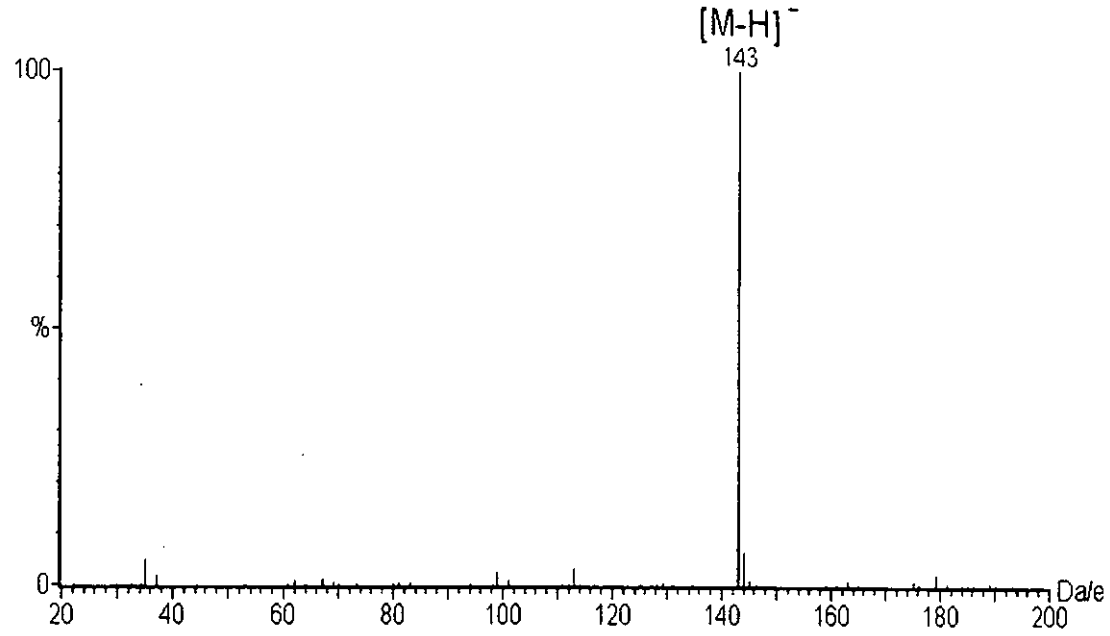


Figure 6.3 (a) : Negative ESI-MS (skimmer cone voltage 20 volt) and ESI-CID-MS (skimmer cone voltage 50 volt) of NSAR.

(b)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 50 volt

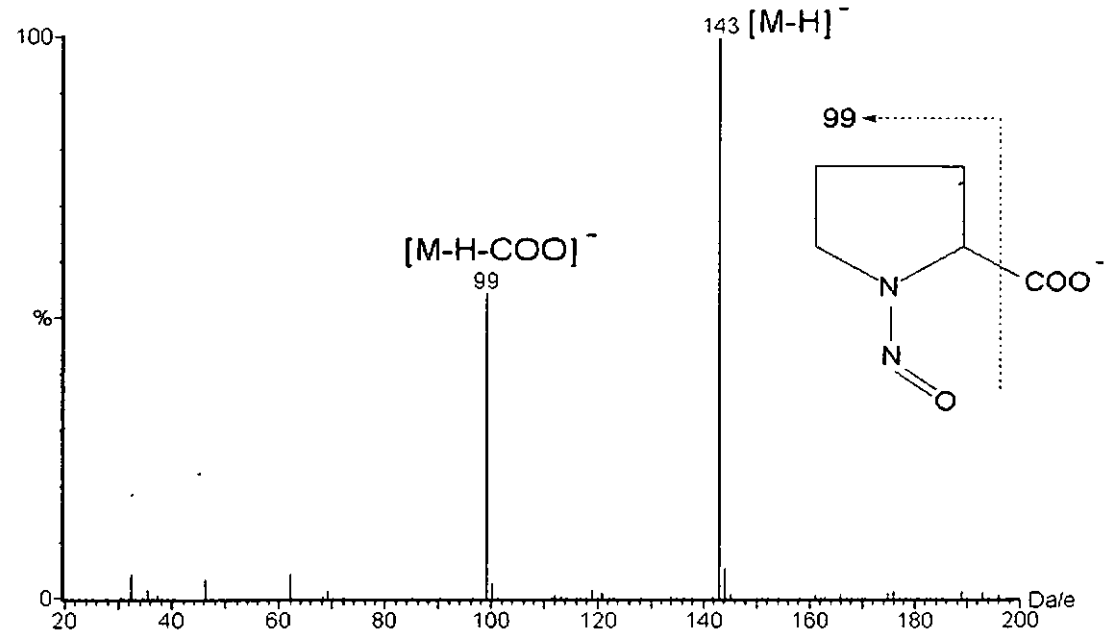
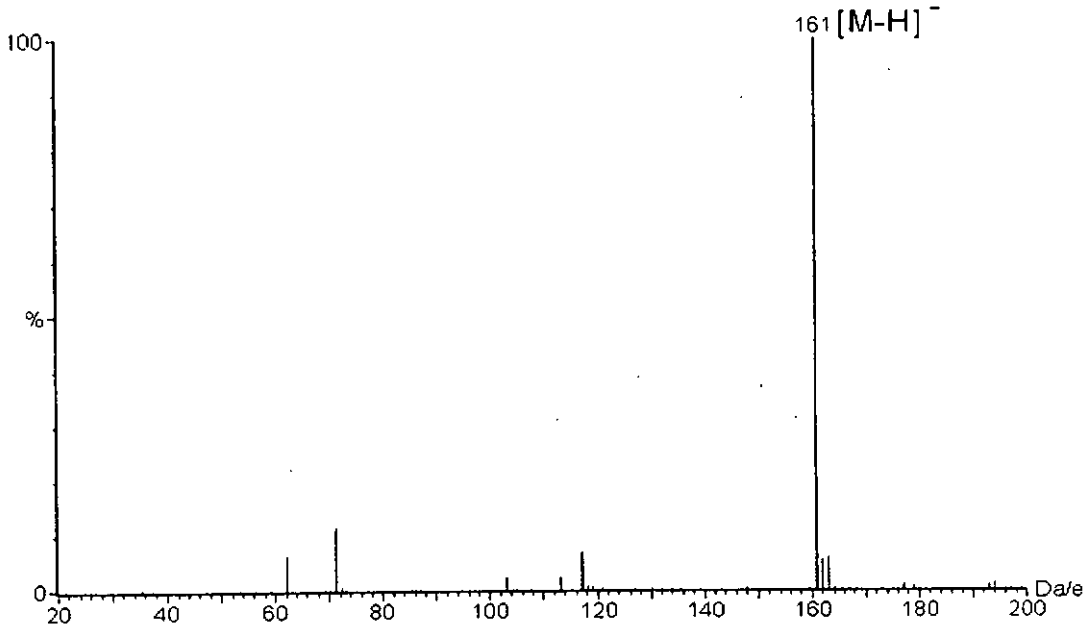


Figure 6.3 (b) : Negative ESI-MS (skimmer cone voltage 20 volt) and ESI-CID-MS (skimmer cone voltage 50 volt) of NPRO

(c)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 50 volt

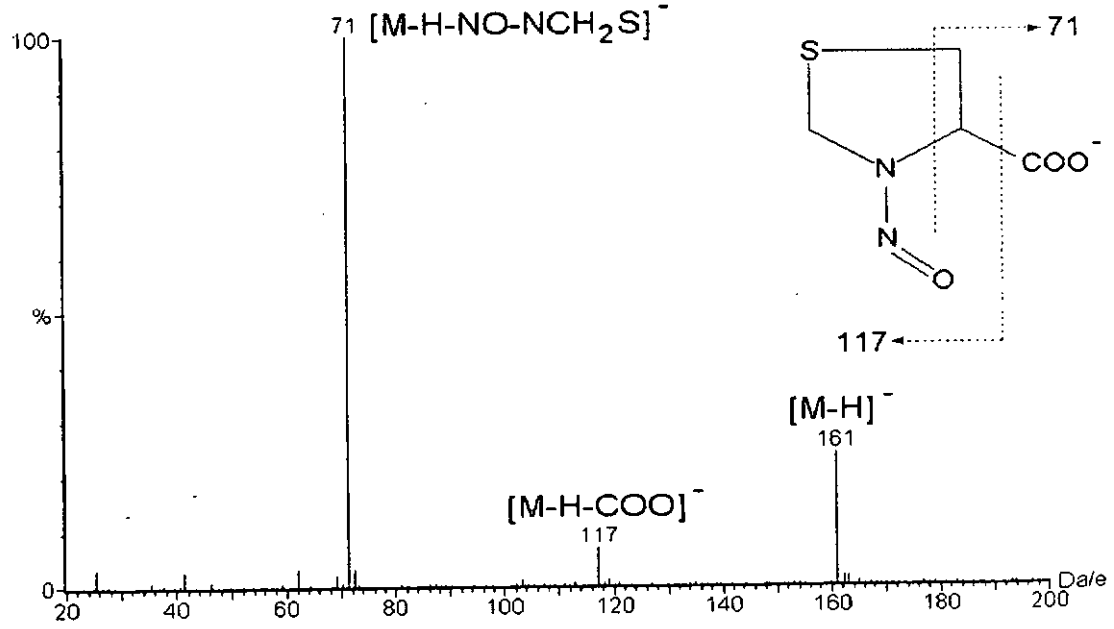
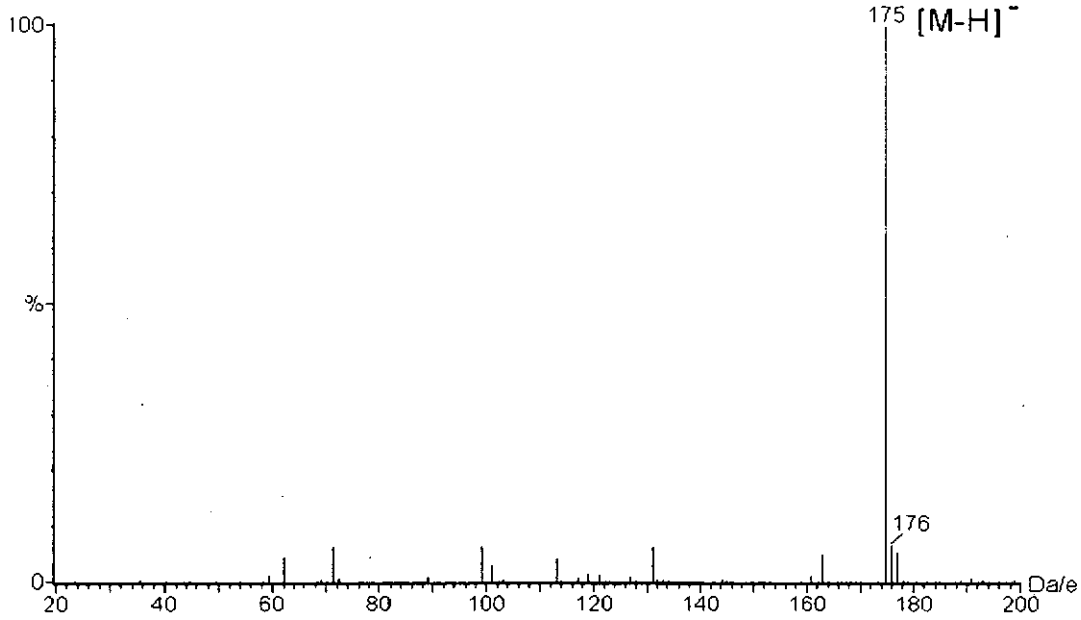


Figure 6.3 (c) : Negative ESI-MS (skimmer cone voltage 20 volt) and ESI-CID-MS (skimmer cone voltage 50 volt) of NTCA

(d)

(i) Skimmer cone voltage 20 volt



(ii) Skimmer cone voltage 50 volt

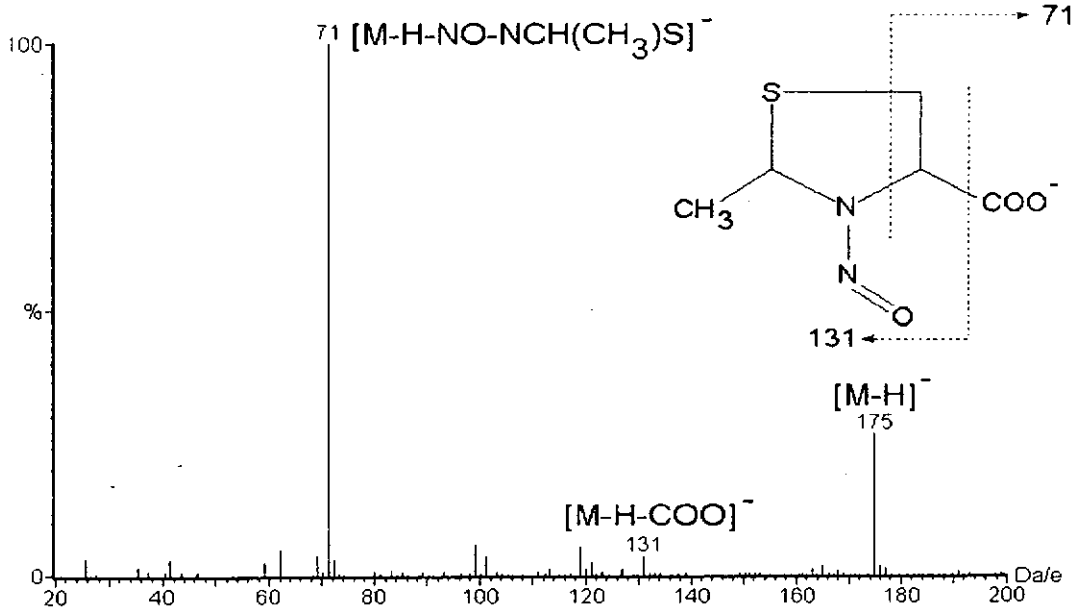
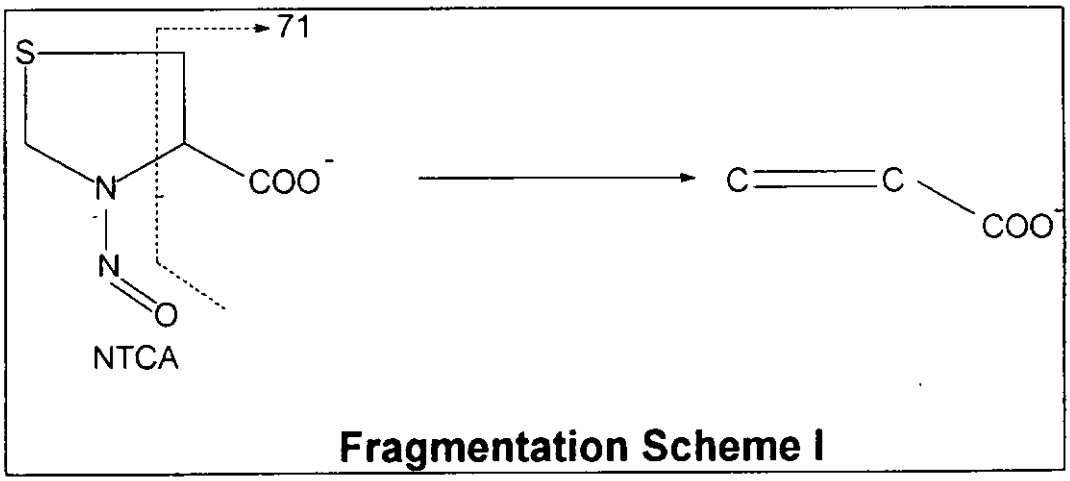


Figure 6.3 (d) : Negative ESI-ms (skimmer cone voltage 20 volt) and ESI-CID-MS (skimmer cone voltage 50 volt) of NMTCA

In fact, the formation of the deprotonated molecular ion, $[M-H]^-$, is a commonly observed feature in the -ESI mass spectra of organic acids. Its formation is independent of the % composition of the aqueous methanol solvent, which is an added advantage in the optimization of experimental conditions for sensitive detection of NAAs under RP-IP-HPLC conditions. Since other interfering substances are not easily deprotonated, background noises are much less found in -ESI compared to +ESI spectra.

The fragmentation of the deprotonated carboxylate anions can be studied by their collision-induced dissociation (CID), i.e., by increasing the skimmer cone voltage of the ESI interface. As shown in Figure 6.3 (a) and (b) for NSAR and NPRO, the only major fragment ions formed were by further loss of CO_2, i.e. the $[M-H-CO\cdot O]^-$ ion at m/z 73 and m/z 99, respectively. For both NTCA and NMTCA, a major and common fragment ion at m/z 71 was observed. The m/z 71 ion, characteristic of sulphur containing cyclic NAAs, is proposed to have the $[C=C-CO\cdot O]^-$ structure, and is formed by simultaneous cleavage of the cyclic C-S and N-C bonds as shown in Fragmentation Scheme I.



Fragmentation Scheme I

The $[C{=}C\text{-}CO\cdot O]^-$ (m/z 71) fragment ions were observed in abundance when the skimmer cone voltage was increased to 30 volt, which was lower than the 50 volt cone voltage required for observation of the $[M\text{-}H\text{-}CO\cdot O]^-$ fragment ions for NSAR and NPRO. This suggests that the presence of a sulphur atom weakens the cyclic C-S and N-C bonds, leading to the characteristic fragmentation pathways of $[M\text{-}H]^-$ ions of NTCA and NMTCA as shown in Fragmentation Scheme I.

The ESI-CID spectra of NAAs contain additional structure-specific ions which could be used to confirm the identity of the analyte NAAs. These diagnostic fragment ions, including m/z 73 [M-H-CO·O] for NSAR, m/z 99 [M-H-CO·O] for NPRO, m/z 71 $[M\text{-}H\text{-}NO\text{-}N\text{-}NCH_2S]^-$ for NTCA and m/z 71 $[M\text{-}H\text{-}NO\text{-}NCH(CH_3)S]^-$ for NMTCA, were used in subsequent HPLC/MS analysis of salted fish samples to confirm the presence of NAAs and to provide supporting evidence on the presence or absence of co-eluting interfering substance (refer to Section 6.3.4).

6.3.2 HPLC-RP-IP/ESI-MS Analysis

The optimal chromatographic conditions adopted for analysis was 1.4 mM of C_{16}-cetyltrimethylammonium chloride in MeOH : H_2O : MeCN (60 : 35 : 5 %*v*/*v*) with a flow rate of 200 µl/min. Under these condition , NSAR, NPRO, NTCA and NMTCA were not completely separated. However, if these NAAs were detected by the m/z values of their respective deprotonated carboxylate molecular anion, $[M\text{-}H]^-$, then the resulting mass chromatogram displayed good

resolution within a reasonable analysing time of 10 minutes. As mentioned in Section 5.3.2 (p.68), the four NAAs could be completely separated by using alternative ion-pairing reagent (1.8 mM C_{12}-dodecyltrimethylammonium chloride) and mobile phase compositions (H_2O : MeOH 50 : 50 % *v/v*), but the ESI detection sensitivity would be reduced and analysis time extended to 15 minutes. The adopted condition is an optimal compromise between sensitivity of detection, chromatographic resolution and analysis time.

A pre-requisite for successful application of the developed RP-IP-HPLC/ESI-MS method is a thorough sample extraction and clean-up procedure which is able to get rid of most of the interfering substances and yet retain satisfactory recovery of the target analytes.

We have spent considerable time and effort in establishing an extraction and clean-up procedure compatible for use in conjunction with the HPLC/ESI-MS analysis. The final procedure, outline in Section 6.2.5, involved (i) pre-clean-up using chloroform and C_{18} solid phase extraction (SPE) column, (ii) solvent extraction using 10% methanol in dichloromethane at pH 1, and (iii) post extraction clean-up using reverse-phase (C_{18}) ion-pairing (C_{16}-CTMA) SPE was developed. Reverse-phase ion-pairing chromatography was adopted in the final sample clean-up step, which is not reported previously. The extensive use of SPE clean-up procedures saves time and labour in the sample preparation steps.

6.3.2.1 Intra-assay Precision

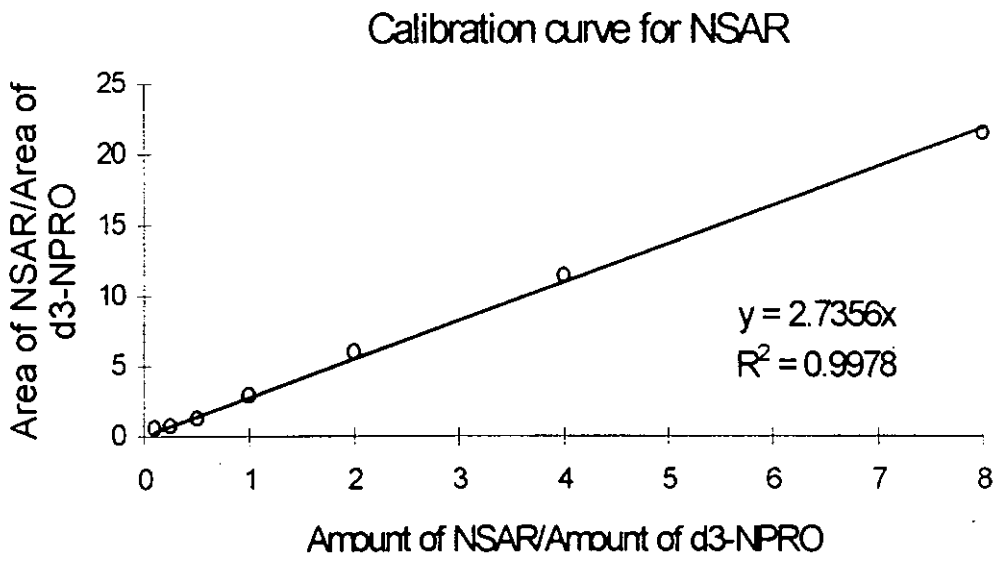
The intra-assay precision were evaluated with respect to the retention behaviour of four NAAs and their ESI detection output signal under RP-IP-HPLC chromatographic conditions using 10 μg/ml standard sample solutions. The HPLC retention times were very reproducible for NASR, NPRO, NTCA and NMTCA at 5.9 ± 0.3 , 6.0 ± 0.3, 6.9 ± 0.3 and 8.5 ± 0.2 minutes (mean ± S.D., n=8), respectively. The ESI detection sensitivity are 30,354 ± 3,250, 11,011 ± 1,210, 14,096 ± 1,365, 8,632 ± 1051 peak area counts (arbitrary units)/ng (mean ± S.D., n= 8). The coefficients of variation were 5% and 11% (n=8) for retention time and detection sensitivity, respectively, which could be regarded as very satisfactory.

6.3.2.2 HPLC/ESI-MS : Linearity and Limit of Quantification

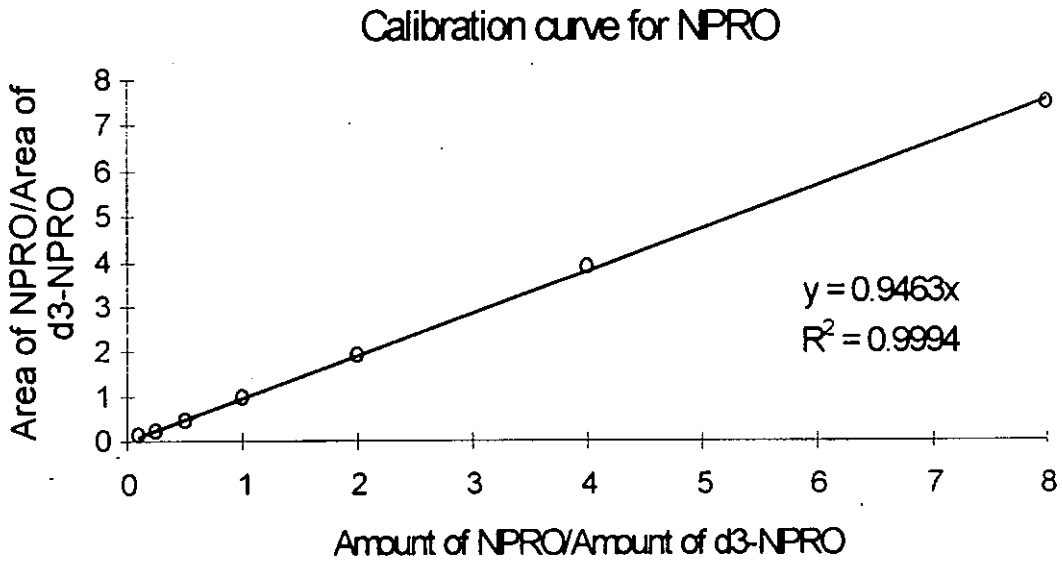
The range of the standard calibration curve was chosen according to the concentration expected in the food samples to be analysed. A concentration range 0.5 - 40 ng/μl (via 5 μl injection and a split ratio 1 : 10 to the ESI interface), corresponding to 250 pg to 20 ng injected proved to be sufficient for determination of NSAR, NPRO, NTCA and NMTCA in food samples. The carboxylate molecular anion $[M-H]^-$ was detected in the selected-ion-monitoring (SIM) mode for quantitative analysis of NAAs. An internal standard was used for better accuracy and against fluctuation of output ion intensities with time. Initially, we chose NPIC as internal standard and the ratio of the amount of target and internal

standard injected is plotted against the ratio of the chromatographic peak areas of the NAAs and NPIC. The standard calibration plots were all linear with $r^2 \geq 0.97$. However, when we attempted to use NPIC internal standard in the Chinese salt fish samples, severe interfences due to co-elution of substances at m/z 157, the $[M-H]^-$ of NPIC internal standard, were observed. Consequently, a deuterated internal standard (d_3-NPRO) with $[M-H]^-$ observed at m/z 146 was used. Again, excellent linearity was found in the same concentration range for all NAAs analysed (Fig 6.4). For NPRO, the standard calibration curve passed through the origin with a slope of 1.0, confirming the linearity of the assay and the absence of non-labeled compounds in the trideutrated internal standard.

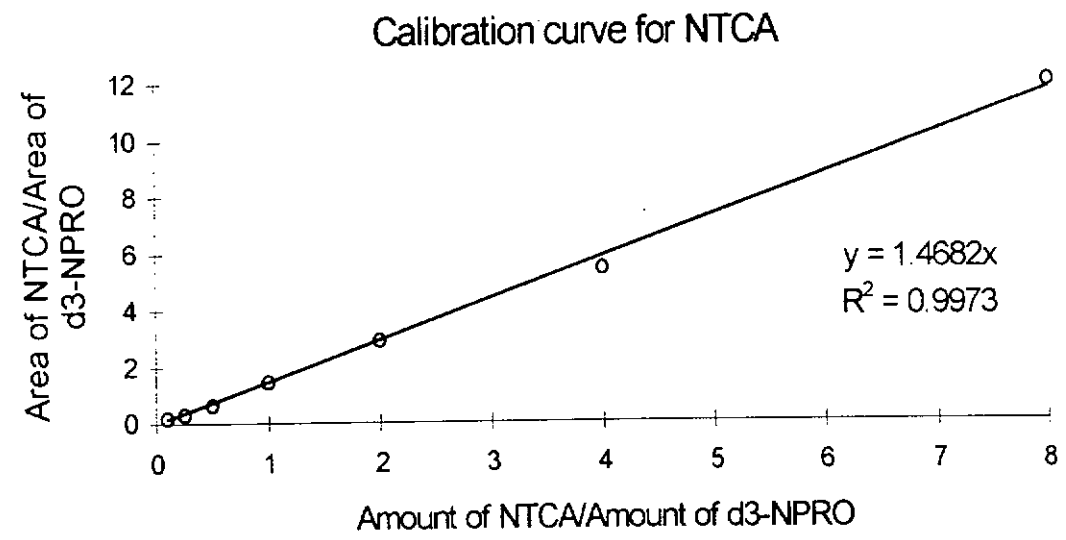
(a)


Calibration curve for NSAR
Area of NSAR/Area of d3-NPRO
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
y = 2.7356x
R2 = 0.9978
Amount of NSAR/Amount of d3-NPRO

(b)


Calibration curve for NPRO
Area of NPRO/Area of d3-NPRO
8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
y = 0.9463x
R2 = 0.9994
Amount of NPRO/Amount of d3-NPRO

(c)



(d)

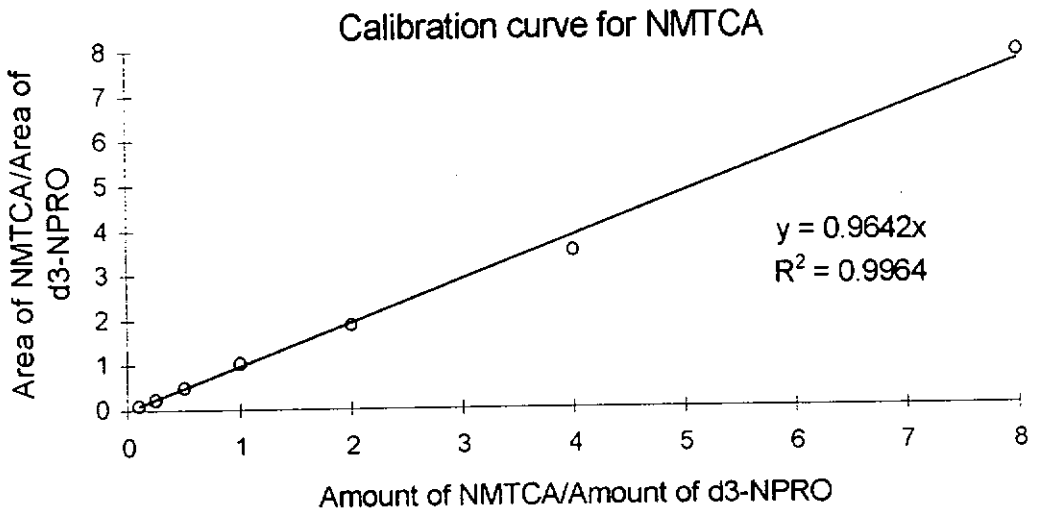


Figure 6.4 : Standard calibration curve for NAAs under -ESI/MS conditions (a) NSAR (b) NPRO (c) NTCA (d) NMTCA

The detection limit for NASR, NPRO, NTCA, and NMTCA achieved under the prescribed experimental conditions were found to be 250 pg at a S/N ratio of ≥ 3 (Figure 6.5). The detection limit could be improved further by lowering the concentration of the ion-pairing reagent used in the mobile phase at the expense of poorer chromatographic resolution. However, the sensitivity and detection limits achieved is adequate for most analysis of NAAs in foods.

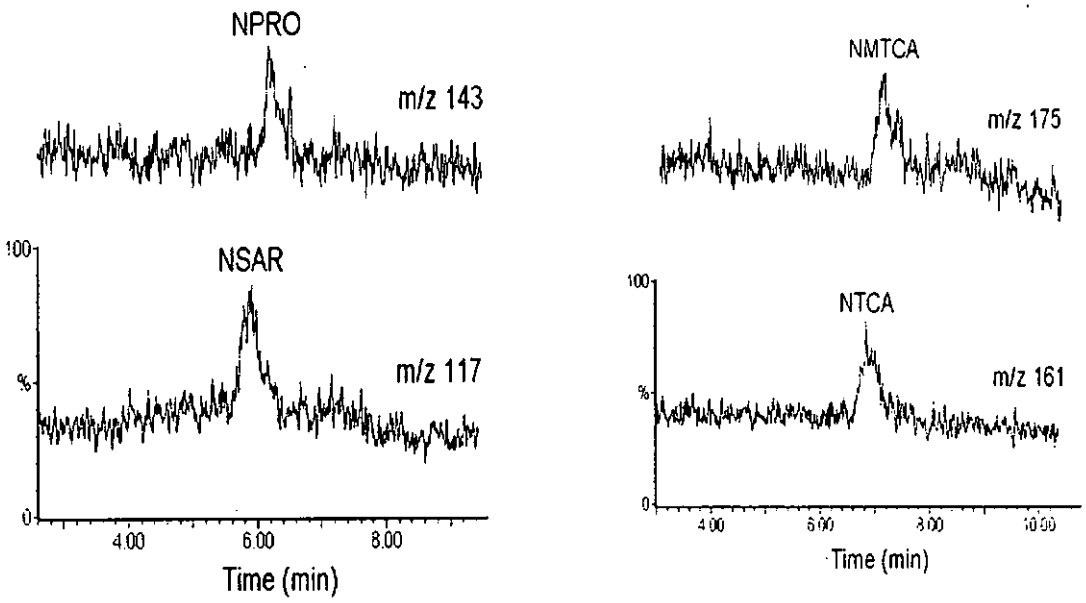


Figure 6.5 : HPLC/ESI-MS mass chromatogram and detection limit (S/N ≥ 3) for NSAR (250 pg), NPRO (250 pg), NTCA (250 pg) and NMTCA (250 pg) standard. HPLC conditions are same as shown in Figure 5.6 (p.69). The signal intensity scale is the same for all chromatographic traces.

6.3.2.3 Percentage Recovery in Sample Preparation

The % recovery (mean ± S.D, n=5) of nitrosoamino acids spiked to fish samples at a level of 200 μg/kg were found to be 56.2 ± 5.2% for NSAR, 83.1 ± 6.2% for NPRO, 83.4 ± 5.0% for NTCA and 91.4 ± 6.9% for NMTCA, respectively. NSAR has the shortest carbon chain and is the most hydrophilic among the four NAAs studied. It is the most difficult NAA to be extracted, which could explain why it displays the lowest percentage recovery in the sample extraction procedure. Fortunately, the signal response of NSAR in negative ESI-MS is two times stronger than other NAAs under identical experimental condition, which could compensate for the relatively low percentage recovery of NSAR so that comparable and satisfactory sensitivity of detection could be achieved in quantitative analysis. The recovery of the deuterated internal standard (d_3-NPRO) was not investigated ; it was assumed to be equivalent to its non-labeled analogues (NPRO).

Low levels of NAAs in food matrixes are known to be difficult to be extracted efficiently. Compared to previously reported sample preparation procedure, our procedure could be considered very satisfactory in terms of time spent and percentage recovery achieved.

6.3.3 Quantitative Analytical Results of Chinese Salted Fish

The developed RP-IP-HPLC/ESI-MS method was applied to the determination of NAAs in Chinese salted fish samples. Salted fish containing N-nitroso compounds was reported to be probably one of the major dietary factors for the occurrence of nasopharyngeal tumors among Chinese in Hong Kong (Weng *et al.*, 1992). The occurrence and levels of N-nitrosamines in salted fish samples were reported, but there is no report yet on N-nitrosoamino acids and non-volatile NOCs.

Figure 6.5(a) shows the ESI-SIM mass chromatogram of a salted fish sample acquired at low skimmer cone voltage (20v). The chromatogram clearly showed the presence of NPRO and NSAR in the sample. A sample spiked with 1000 ng of each of the NAA standards showed the same retention times and peak shapes as the unspiked sample, confirming the identity of the NAA peaks (Figure 6.5(b)).

Under the chromatographic condition employed in the actual analysis, the syn and anti conformers of NMTCA standard were only partially separated, but a near complete separation was observed for the NSAR standard which had a very low concentration for one of the conformers versus a high concentration of the other compound. However, while we analysed the spiked and non-spiked food samples, the resolution between the conformer was lost, probably due to near equal concentration of the syn and anti conformers of NSAR in the fish sample. One possibility is that the relative concentrations of the syn and anti conformers in the fish samples were different from that of the pure standard due to sample matrix

effects. It was also reported that syn and anti conformers were at equilibrium in the solution phase and their relative concentrations were influenced by the polarity of solvents and the time they spend in this solvent (Chow *et al.*, 1981). Since the clean-up procedure for food sample involved many solvent extraction steps and the solvent composition was different from the mobile phase in the RP-IP-HPLC separation, it is possible that equilibrium concentrations of the syn and anti isomers were change during this extraction procedure and the chromatographic process. This effect was noticeably observed for NSAR and NMTCA, but not for other NAAs analysed in this study.

The levels of NSAR and NPRO in the salted fish sample analysed were 2,200 ± 260 μg/kg and 154 ± 15 μg/kg (mean ± S.D, n=4), respectively. No NTCA and NMTCA were detected in the sample.

The relatively high content of NSAR in salt fish sample is probably due to long salting process, which partial hydrolysis of proteins occurs. Although, there is not any direct evidence supporting the NOCs as the responsible agents to nasopharyngeal carcinoma and the formation of non-nitroso mutagens remain a possibility, it is still a matter of concern for the high level of NSAR occurred in the Chinese traditional salted fish.

(a)

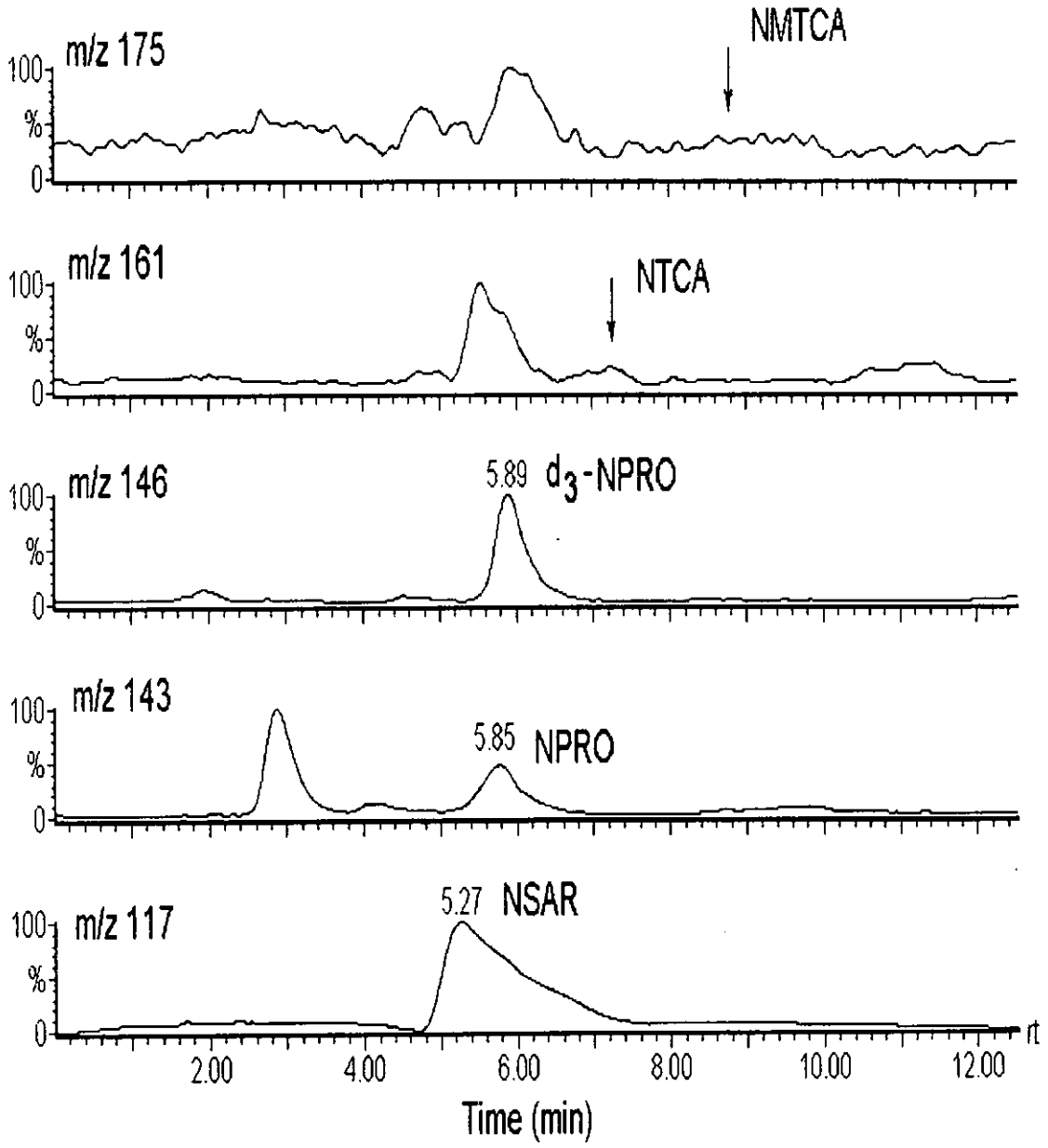


Figure 6.6 (a) : Mass chromatogram of Chinese salted fish sample

(b)

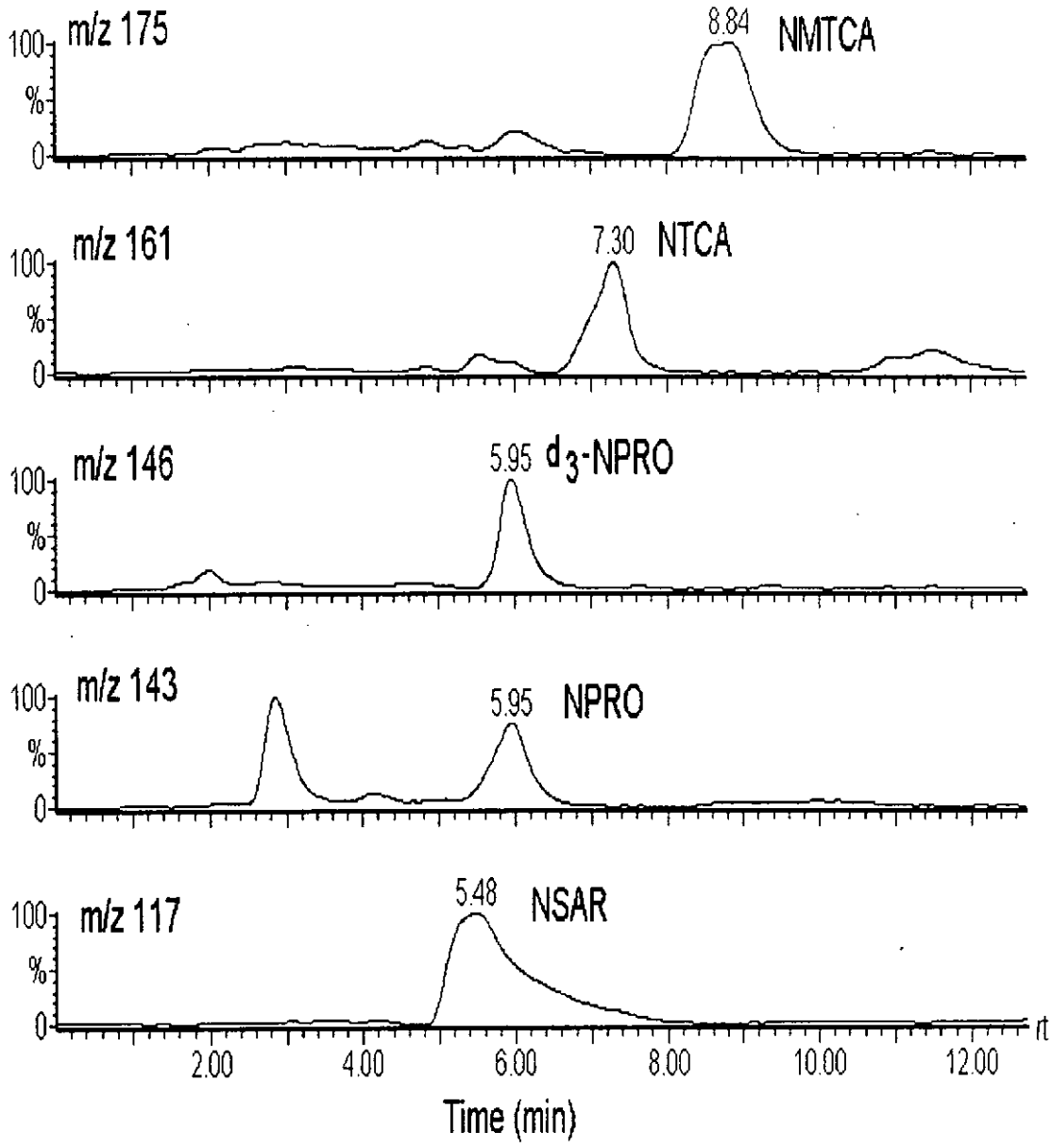
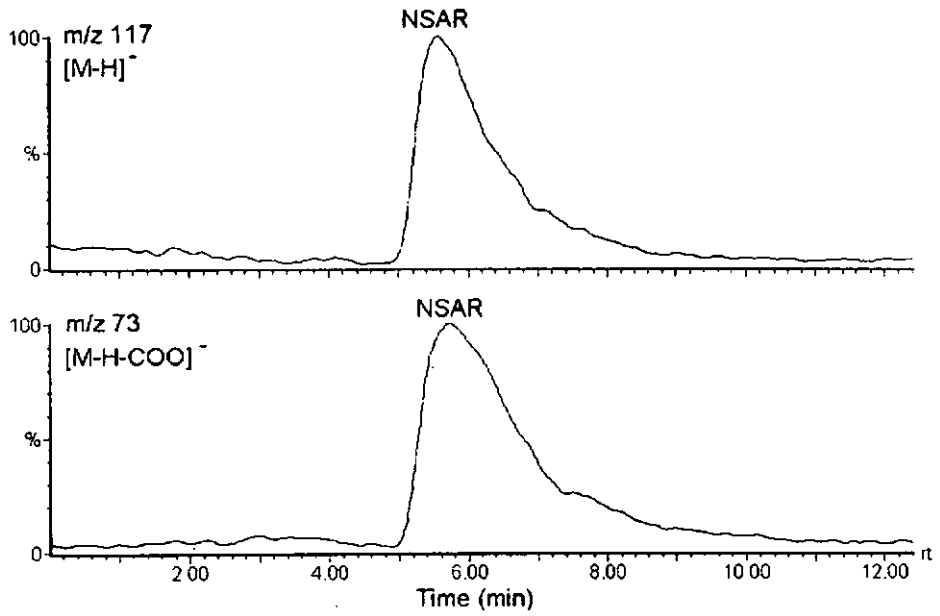


Figure 6.6 (b) : Mass chromatogram of Chinese salted fish sample spiked with 200 μg/kg NAA standards

6.3.4 Confirmation of Analytical Specificity

In order to further confirm if any co-eluted substances masked the NSAR and NPRO peaks, the $[M-H]^-/[M-H-CO_2]^-$ ion intensity (peak area) ratios in the ESI-CID mass spectra of the fish sample were compared to that of authentic NSAR and NPRO standards at 50 volt skimmer cone voltage (Figure 6.6). Good agreements in the ion intensity ratio of the fish sample and the authentic standard were obtained, with relative deviations of 7% and 9% observed for NSAR and NPRO, respectively, suggesting that interference due to co-eluents was only minor in the sample analysed. The success of the confirmation test indicated also the advantage of using the HPLC/ESI-MS method. It is more specific because the quantitative analytical results could be verified by measuring ion intensities at two different m/z values. In conclusion, the RP-IP-HPLC/ESI-MS method developed in the present study was successfully applied to the analysis of non-volatile N-nitrosoamino acids in Chinese salted fish. The developed method, including the sample clean-up steps, in relatively simple and less time consuming compared to previous reports employing HPLC-TEA method of analysis. Relatively high level of NSAR was found in one Chinese salted fish sample, suggesting that the presence of non-volatile NOCs in the Chinese diet, especially in preserved foods, should be a matter of concern and warrants further monitoring by the Chinese scientific community.

(a)



(b)

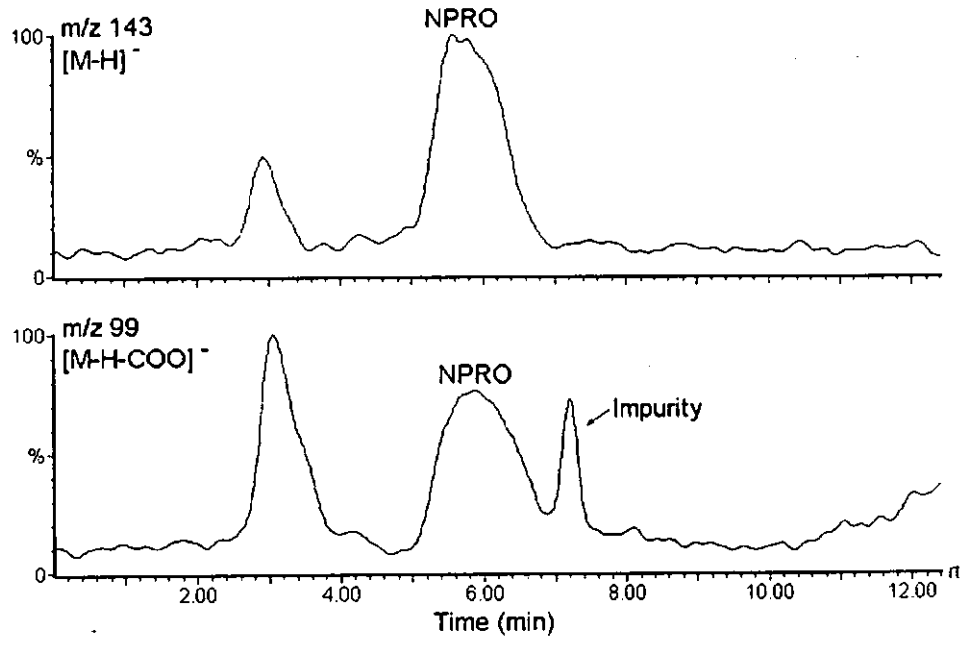


Figure 6.7 : Fragmentation mass chromatogram of fish sample obtain at 50 cone voltage (a) NSAR, (b) NPRO

Chapter 7 Simultaneous Determination of Nitrite, Nitrate and Ascorbic acid in Canned Vegetable Juice by Reverse-phase Ion-interaction HPLC

7.1 Background

According to published surveys, most of nitrate intake in our diet are derived from vegetables which contribute 75-80 % of the total daily intake (Maff, 1987 ; Walker, 1990). The nitrite and nitrate content of fresh vegetables and vegetable juices have been reported (Wootton *et al.*, 1985 ; Walker, 1990 ; Omieljaniuk *et al.*, 1995). Since high levels of nitrate and nitrite in vegetable and vegetable juices are harmful to our health, methods to minimize their content in fresh and processed vegetable products have been investigated (Keeney, 1970). Meanwhile, most vegetables and vegetable juices also contains high level of ascorbic acid (Gennaro and Bertolo, 1990). As nitrite, nitrate and ascorbic acid are related in their ability to induce or prevent formation of carcinogenic N-nitroso compounds, the content of nitrite, nitrate and ascorbic acid in food is directly related to the formation of NOCs *in vivo*. Hence, it is worthwhile to develop an analytical method for simultaneous quantitation of nitrite, nitrate and ascorbic acid in food sample.

Ion interaction HPLC using 0.005 M aqueous octylammonium orthophosphate in conjunction with reverse-phase C_{18} stationary phase has been applied to efficient separation of anions (Gennaro *et al.*, 1990) and organic acids

(Gennaro and Abrigo, 1990 ; Kathryn *et al.*, 1992). The hydrophobic carbon chain of octylamine is adsorbed onto the C_{18} stationary phase and the protonated amine group creates a positive charged primary ion layer, which holds the negatively charged orthophosphate by electrostatic attraction, forming a secondary ion layer. When nitrite and nitrate anion are introduced, they could compete with and replace the orthophosphate, leading to retention in the secondary ion layer. Organic anion such as ascorbate are also expected to behave similarly.

This part of the thesis describes the development and application of an ion interaction reverse phase HPLC method which permitted identification and simultaneous determination of nitrite, nitrate and ascorbic acid in canned vegetable juices. The method makes use of octylamine ortho-phosphate as the ion-interaction agent in the functionalization of C_{18} HPLC stationary phase and 20 % aqueous methanol (*v/v*) as the mobile phase. In addition, the nitrite, nitrate and ascorbic acid contents of commercial brands of canned vegetable juices were surveyed using this method. Unless otherwise stated, concentrations or amounts of **nitrite ion** and **nitrate ion** (**not** as sodium nitrite and sodium nitrate) are adopted in this report.

7.2 Materials and Methods

7.2.1 Equipment

The major equipment used for this part of the project were :-

1. Hewlett Packard 1050 series HPLC system, equipped with a photodiode array detector (190 - 600 nm) and a HPLC 3D Chromatographic Chemstation for data acquisition and processing ;
2. Alltech-Applied Science Spherisorb C_{18} reverse phase HPLC column (ODS-2, 5 μm, 250 x 4.6mm i.d.) ;
3. Spectrophotometer, Milton Roy, Spectronic 601
4. Corning PS30 pH meter and a combined glass-calomel electrode
5. Beckman J2M1 High Speed Centrifuge
6. Mettler UM3 microbalance (± 0.001 mg)

7.2.2 Reagent and Materials

1. The following analytical grade chemicals (purity > 99%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) :

   octylamine
   orthophosphoric acid
   sodium nitrite
   sodium nitrate
   L-ascorbic acid
   methanol

2. Double deionized water from a Milli-Q system (Millipore) system.
3. The Nucelopore nylon filters (25 mm, 0.45μm) were from Waters Associates (Connaught Road West, Hong Kong).
4. Canned vegetable juices were imported brands commonly available from local supermarkets.

7.2.3 Preparation of Ion Interaction Reagent and Standard Solutions

A 0.010 M octylammonium orthophosphate reagent solution was prepared by dissolving 0.64 g of octylamine in 500 ml of aqueous 20% (v/v) methanol. The pH of the solution was adjusted to 6.4 ± 0.4 by addition of orthophosphoric acid. This solution was freshly prepared every 3 days.

Sodium nitrite, sodium nitrate and ascorbic acid standard solutions (1,000 mg/l) were prepared and stored at 4 ^{0}C. Working standard solutions were prepared from the stock standard solutions daily by serial dilution. Standard solutions containing 1.2, 5.0, 10, 20, 40, 60, 100 and 200 mg/l of sodium nitrite, sodium nitrate and ascorbic acid were used to obtain the spectrophotometric calibration curve.

7.2.4 Preparation of Sample

The vegetable juice sample was shaken for 1 min, opened, centrifuged at 27 $200g_{av}$ for 15 min and filtered through a Nucleopore nylon filter (Waters Associates, 25 mm, 0.45 μm). The filtered samples were stored in 50 ml plastic centrifuge tubes at 4 ^{0}C in a refrigerator prior to analysis. All samples were analyzed within one hour after can opening.

7.2.5 HPLC Analysis

Prior to analysis, the ion-interaction reagent solution was allowed to pass through the HPLC column until a stable baseline signal was obtained. Generally, one hour of stabilization time before analysis was required, then reproducible retention times were observed throughout the working day (8-12) hours of analysis. At the end of the working day, the HPLC column was regenerated by passing 1 : 1 water - methanol overnight at a flow rate of 0.1 ml / min. In this

way, no degradation in column performance or decrease in column lifetime was observed.

The juice samples were diluted 4 times and introduced into the HPLC column via a 20 μl sample loop. Isocratic separation was achieved with the 0.010 M octylamine orthophosphate mobile phase flowing at 0.5 ml/min. Peak identification, retention time and peak area integration measurements were performed using the Hewlett Packard-HPLC chemstation chromatographic data processing software.

7.3 Result and Discussion

7.3.1 Choice of Wavelength for Spectrophotometric Detection

The molar absorptivities of the target analytes and chloride were determined in the wavelength range from 210 nm to 280 nm, and the data are tabulated in Table 7.1.

Table 7.1 : Molar absorptivities (ε, mol^{-1} cm^{-1}) in the wavelength range 220 to 270 nm

Wavelength	210 nm	220 nm	230 nm	240 nm	250 nm	260 nm	270 nm
Nitrite	4838	3812	1592	360	38	8	8
Nitrate	5422	3276	770	106	10	2	2
Ascorbic acid	1430	2495	4780	7320	8720	8440	6640
Chloride	2	1.2	0.4	0.4	0.4	0.4	0.4

Obviously, the best spectrophotometric detection should be made at 210 nm for nitrite and nitrate. However, even for the low ε value, chloride absorbance is not zero for practical samples due to its high concentration in food sample. Therefore, detection wavelength for quantitative analysis of nitrite and nitrate was chosen to be 230 nm. Ascorbic acid found its highest absorptivity at the

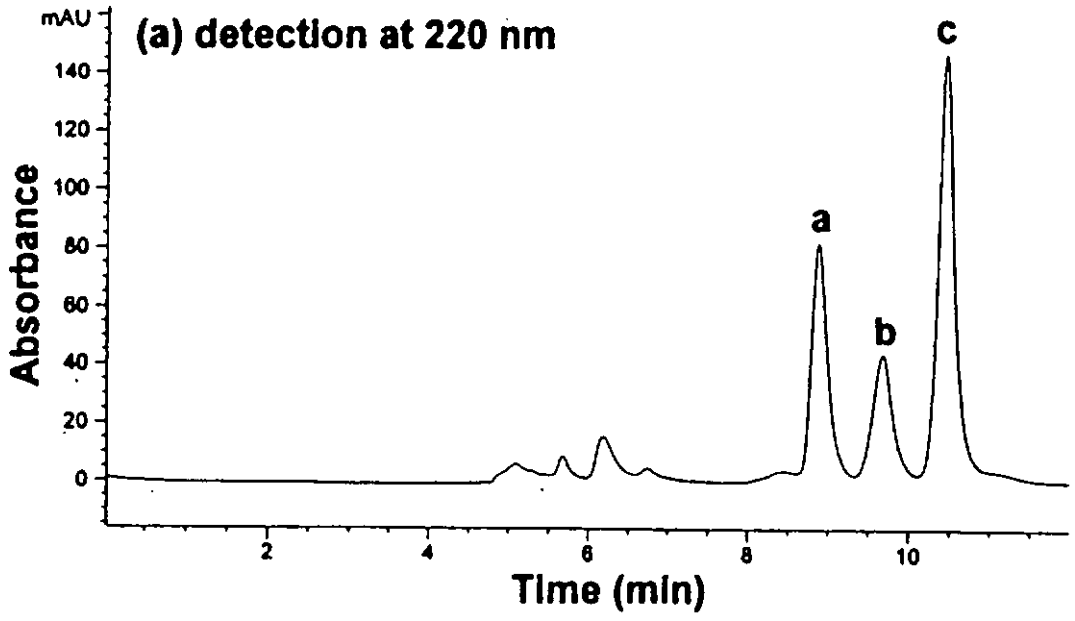
wavelength range 250 nm to 260 nm, therefore UV detection was made at 254 nm, the absorbance maxim.

7.3.2 Optimization of HPLC parameters

Using 0.005 M aqueous octylammonium orthosphate, the retention time of nitrite in reverse-phase ion interaction HPLC using C_{18} stationary phase was found to vary and dependent on the molar ratio of chloride and nitrite in the sample (Gennaro *et al.*, 1990). This was indeed observed in the early stage of this work when vegetable juice samples containing varying amounts of chloride and nitrite were analyzed. The accuracy and precision of analysis was affected because overlapping of chromatographic peaks could occur. Abundant chloride ion in the samples injected could compete effectively with the orthophosphate in the formation and stability of the secondary ion layer, which in turn altered significantly the retention of the nitrite ion (Gennaro *et al.*, 1990).

The retention time shift problem was solved in the present study by using a higher concentration of octylammonium orthophosphate (0.010 M) and addition of 20 % (*v/v*) of methanol to the aqueous mobile phase. The HPLC separation conditions were optimized with respect to concentration of the ion interaction reagent, chemical composition of the mobile phase, flow rate, chromatographic resolution and analysis time. Under the experimental conditions described, the retention time of the target analytes became very reproducible, with that of nitrite found at 9.7 ± 0.2 (mean ± S.D., n=10).

Figure 7.1 shows the chromatograms of a canned vegetable juice one hour after the can was opened and kept at 4 ^{0}C. The ascorbic acid (peak a), nitrite (peak b) and nitrate (peak c) peaks were well separated at retention times of 9.1, 9.7 and 10.2 minutes, respectively. In this study, the absorption of the analytes in the 190-600 nm wavelength range was recorded simultaneously by a photodiode array detector.



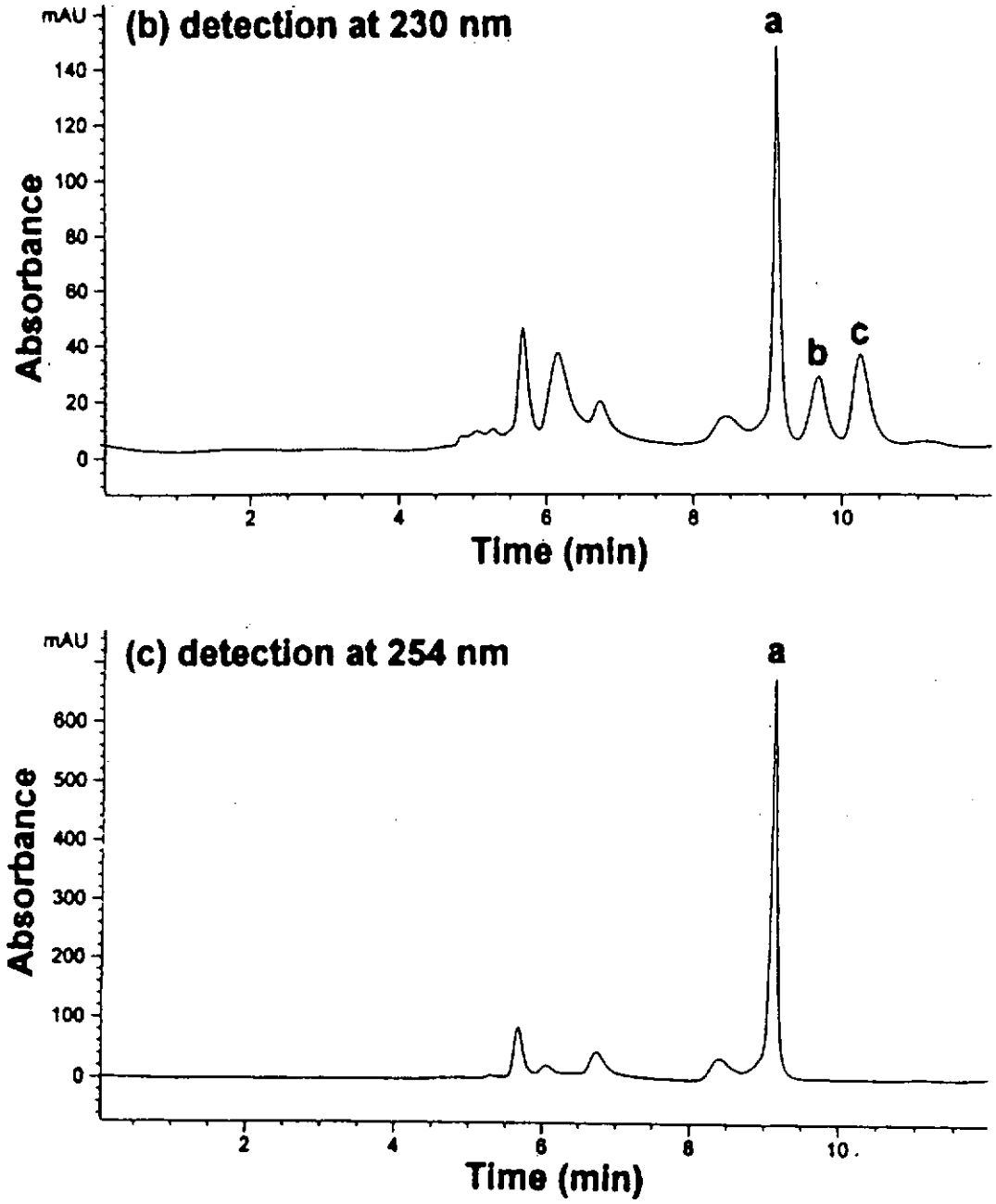


Figure 7.1 : Ion interaction HPLC chromatograms of a commercial canned mixed vegetable juice with UV detection at (a) 220 nm, (b) 230 nm and (c) 254 nm. Peaks : a ascorbic acid ; b nitrite ; c nitrate.

HPLC conditions : Alltech-Applied Science Spherisorb ODS-2 column (250.0 x 4.6 mm, 5 μm) ; ion interaction reagent, 0.010 M octylamine ortho-phosphate ; mobile phase, 20 % v/v aqueous methanol ; flow rate, 0.5 ml/min ; 20 μl of sample injected.

In the first stage of our study, the quantitative results obtained by absorption measurements at 220 nm and 230 nm were compared for nitrite and nitrate, while the absorption measurements at 230 nm and 254 nm were compared for ascorbic acid. The comparison results for nitrite, nitrate and ascorbic acid in four commercial brands of vegetable juices are shown in Table 7.2.

Table 7.2 : Simultaneous analysis of nitrite, nitrate and ascorbic acid in canned vegetable juices - average deviations obtained by comparing quantitative results measured at two different wavelengths (220 nm versus 230 nm for nitrite and nitrate ; 230 nm versus 254 nm for ascorbic acid)

	Average % Deviation[a]		
Canned Vegetable Juice	Nitrite	Nitrate	Ascorbic Acid
Mixed vegetable juice (Brand A)	3.8%	6.8%	5.6%
Tomato juice (Brand B)	4.2%	3.2%	4.8%
Tomato juice (Brand C)	5.0%	not detected	6.1%
Carrot juice (Brand D)	3.0%	4.9%	6.3%

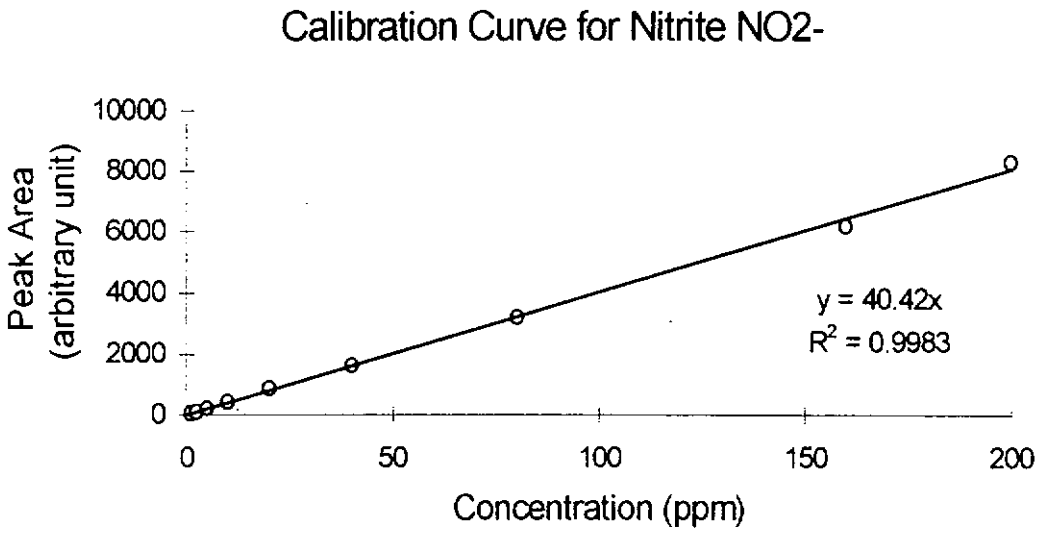
a) average % deviation is defined as $= \frac{100}{n}\sum\left|X_{\lambda 1} - X_{\lambda 2}\right| / X_{mean}$, *where* $X_{\lambda 1}$ = *result obtained by measurement at* λ_1 *(220 nm for nitrite and nitrate, 230 nm for ascorbic acid),* $X_{\lambda 2}$ = *result obtained by measurement at* λ_2 *(230 nm for nitrite and nitrate, 254 nm for ascorbic acid)* $X_{mean} = 0.5\ (X_{\lambda 1} + X_{\lambda 2})$, *and* $n = 6$ = *number of independent juice samples analysed.*

Within each pair of measurement wavelengths, the quantitative results were in close agreement ; the average percent deviations were within experimental error limits, in the range of 3.0 - 6.8% (n = 6). In practice, it is highly unlikely that interfering substances, if present, would show the same relative absorption at two different wavelengths as that for nitrite, nitrate and ascorbic acid. Thus our results showed that even if interfering substances co-eluted with the analytes, they were not detected at the chosen absorption wavelengths. Chloride ions showed negligible absorption at these wavelengths, therefore no chloride interference was expected in the analysis. It is interesting to note that the width of the ascorbic acid peak measured at 220 nm (Figure 7.1(a)) was visibly wider than that measured at 230 nm and 254 nm (Figure 7.1 (b) and 7.1 (c)), indicating that co-elution of interfering substance(s) with ascorbic acid indeed was observed for a sample of mixed vegetable juice. For this reason, absorption at 220 nm was not chosen for quantitative measurement of ascorbic acid. In all the sample runs, no peak broadening was observed for ascorbic acid at 230 nm and 254 nm. Co-elution or overlapping of interfering peaks were not found at the chosen wavelengths for the other three brands of vegetable juices analysed in this study.

For subsequent samples, the measurement wavelength for nitrite and nitrate was set at 230 nm, while that of ascorbic acid was set at 254 nm. These wavelengths were chosen because they provided sufficient sensitivity for quantitative analysis, and minimal absorption from interfering substances, including chloride. Quantitative analysis was carried out by comparing sample peak areas against a standard calibration curve of peak areas versus the concentration of a series of mixed standard solutions of authentic sodium nitrite, sodium nitrate and

ascorbic acid. The calibration plots measured at these absorption wavelengths were linear (average correlation coefficient equal to 0.997) in the 1.25 to 200 mg/l range for sodium nitrite, sodium nitrate and ascorbic acid (Figure 7.2).

(a)



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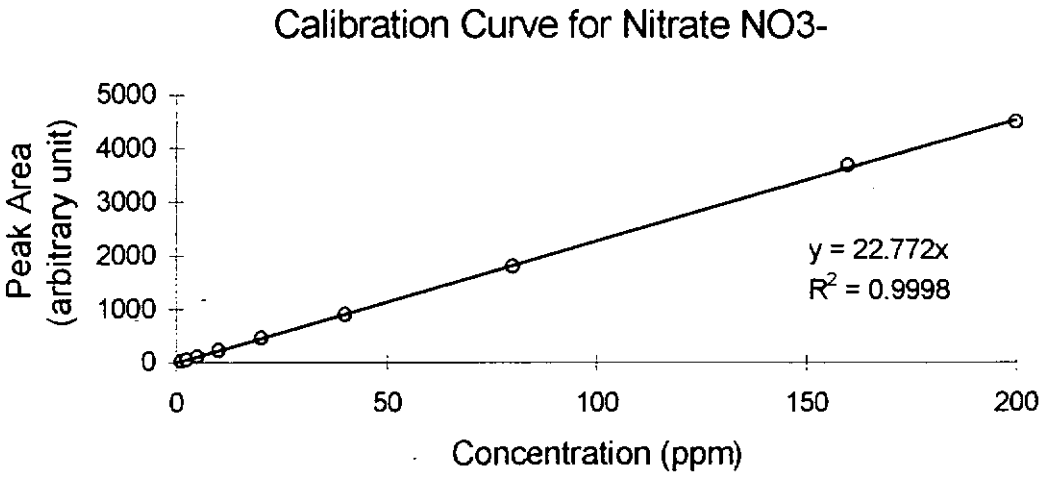

(c)

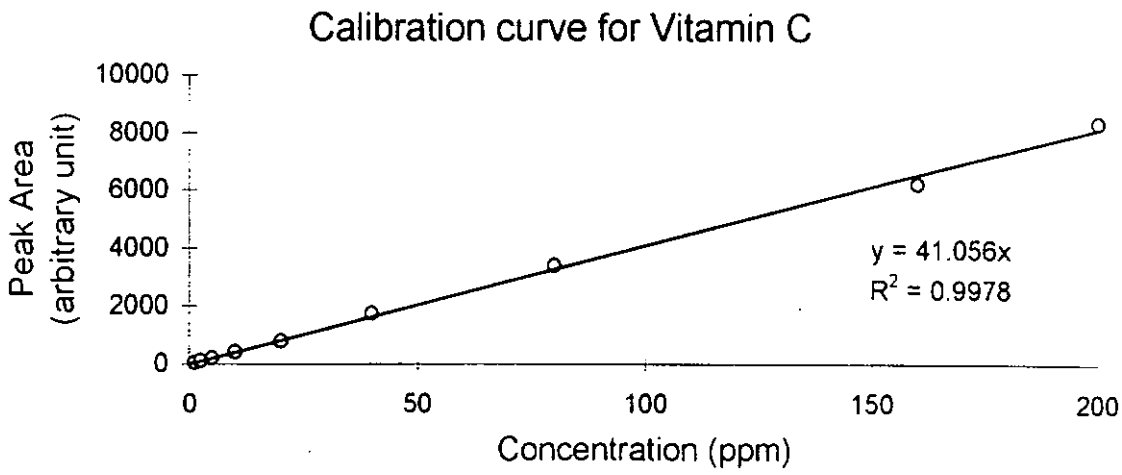

Figure 7.2 : HPLC/UV calibration plot of (a) NO_2^- at 230 nm (b) NO_3^- at 230 nm (c) Vitamin C at 254 nm

The analytical results of selected samples (about one third of samples analyzed) were further verified by standard addition analysis in which known amounts of analyte standards were added to the samples. The added standards, served to identify the analyte peaks, appeared at the same retention times as the sample peaks, and the plots of peak area against added standard concentrations showed good linearity (average correlation coefficient > 0.998) and with slopes equaled to that obtained by standard calibration. For mixed vegetable juice, the average deviation of the results obtained by the standard calibration and standard addition methods were 4.2%, 3.9% and 4.4% (n = 5) for nitrite, nitrate and ascorbic acid, respectively. Similar results were obtained for other types of vegetable juices (Table 7.3). Thus, no serious interference was observed in our analysis of vegetable juices at the chosen wavelengths and according to the experimental procedure adopted.

Table 7.3 : Simultaneous analysis of nitrite, nitrate and ascorbic acid in canned vegetable juices - average deviations obtained by standard calibration and standard addition methods

	Average % Deviation[(a)]		
Canned Vegetable Juice	Nitrite	Nitrate	Ascorbic Acid
Mixed vegetable juice (Brand A)	4.2%	3.9%	4.4%
Tomato juice (Brand B)	2.3%	4.0%	2.6%
Tomato juice (Brand C)	6.7%	not detected	6.5%
Carrot juice (Brand D)	5.2%	3.1%	6.8%

(a) average % deviation is defined as $= \frac{100}{n}\Sigma|X_{DC} - X_{SA}| / X_{mean}$, *where* X_{DC} = *result obtained by direct standard calibration,* X_{SA} = *result obtained by standard addition method,* $X_{mean} = 0.5\ (X_{DC} + X_{SA})$, *and* $n = 5$ = *number of independent juice samples analysed.*

Practically, the detection limits for sodium nitrite, sodium nitrate (at 230 nm) and ascorbic acid (at 254 nm) standards were found to be 0.3, 0.3 and 0.6 mg/l (S/N ≥ 2), respectively, which were more than adequate for the present study (Figure 7.3).

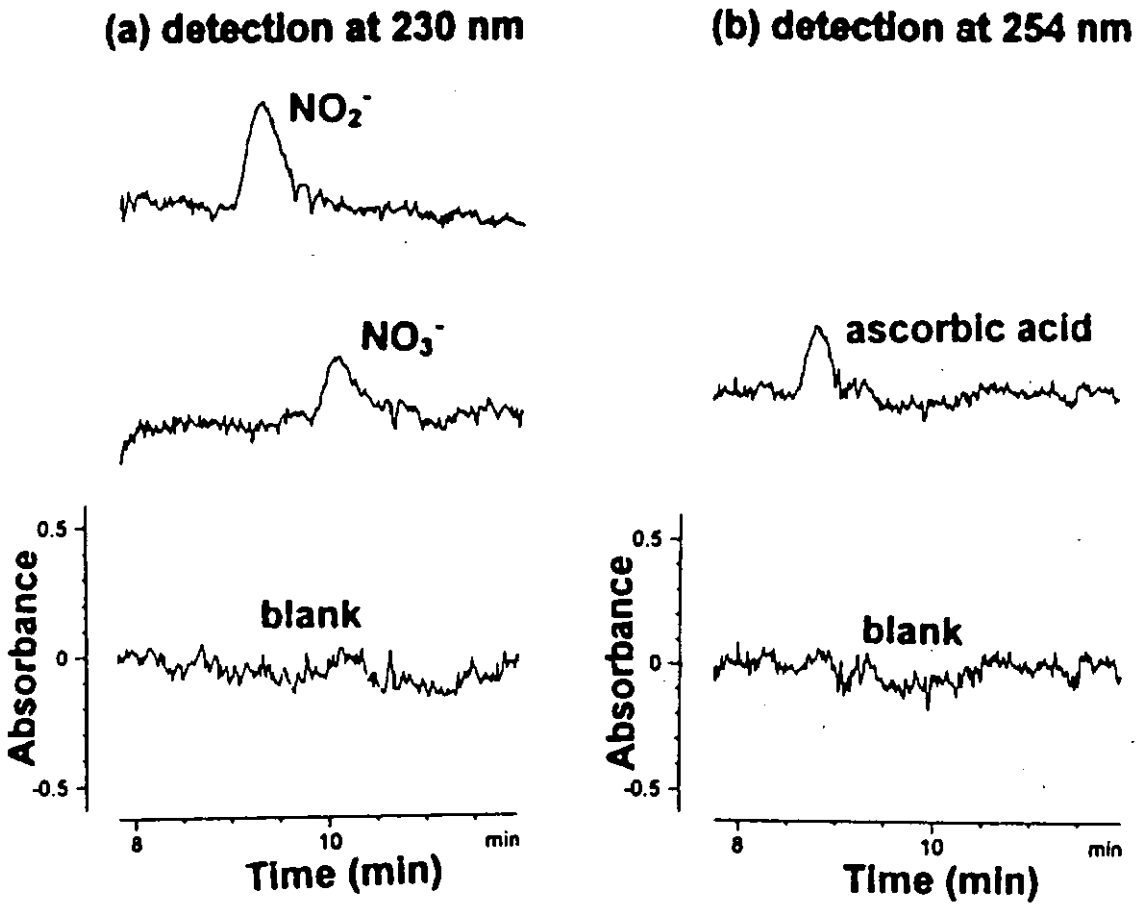

Figure 7.3 : HPLC chromatograms and detection limits for sodium nitrite (0.3 mg/l), sodium nitrate (0.3 mg/l) and ascorbic acid (0.6 mg/l) standard. HPLC conditions are the same as in Figure 7.1. The absorbance scale is the same for all chromatographic traces.

Ascorbic acid in fruit juices is known to decompose under prolonged exposure to air at room temperature (Gennaro and Bertolo, 1990). Hence in this study, all canned vegetable juices were analyzed within one hour of can opening. The sample juices were kept at 4 ^{0}C except during the sample preparation steps. The intra-assay reproducibility for nitrite, nitrate and ascorbic acid obtained from analysis of the same sample of mixed vegetable juice was found to be 5.1 %, 5.5 % and 4.1 % , respectively (C.V., n = 6). Our results showed that under the prescribed experimental conditions, the levels of nitrite, nitrate and ascorbic acid in

vegetable juices did not change significantly. In other words, minimal reduction of nitrite and degradation of ascorbic acid had occurred, which fell within the limits of experimental error. Our findings were in agreement with that of Norwitz et al., who reported that there was no reaction between nitrite and ascorbic acid at mild pHs (Norwitz *et al.*, 1987). The pH of the vegetable juices were found in the 5.0 - 6.5 range.

7.3.3 Determination of Nitrite, Nitrate and Ascorbic acid in Vegetable Juices

The total amounts of nitrite, nitrate (expressed as nitrite ion and nitrate ion, respectively) and ascorbic acid per can, and their respective concentrations found in four commercial brands of tomato, carrot and mixed vegetable juices are shown in Table 7.4.

Table 7.4 : Total amount (mg per can) and concentrations (mg/l, in parenthesis) of nitrite, nitrate[a] and ascorbic acid in four brands of commercial canned vegetable juices.

Sample	n[b]	Can Volume (ml)	Nitrite	Nitrate	Ascorbic Acid
Mixed[c] Vegetable Juice (Brand A)	15	355	6.8 ± 0.5 (19.0 ± 1.5)	27 ± 2 (75 ± 7)	126 ± 19 (355 ± 53)
Tomato Juice (Brand B)	9	163	3.2 ± 0.2 (20.2 ± 1.5)	1.2 ± 0.2 (7.3 ± 1.3)	18 ± 2 (110 ± 14)
Tomato Juice (Brand C)	15	355	5.4 ± 0.4 (15.3 ± 1.1)	not detected	90 ± 6 (254 ± 16)
Carrot Juice (Brand D)	9	180	2.8 ± 0.2 (15.6 ± 1.4)	52 ± 4 (290 ± 21)	94 ± 5 (522 ± 26)

a) nitrite and nitrate are expressed as nitrite ion and nitrate ion, respectively.

b) n = number of canned samples, quoted values are mean ± S.D.

c) The mixed vegetable juice was labeled with ingredients of tomato, carrots, celery, beets, parsley, lettuce, watercress, spinach, salt, ascorbic acid, spice extract and citric acid.

For an average adult of 60 kg body weight, the 1996 FAO/WHO recommended acceptable daily intake for nitrate and nitrite ion are 0 - 222 and 0 - 3.6 mg per day, respectively. The nitrate contents per can were found to spread over a relatively wide range (1.2 - 52 mg per can), depending on the type and brand of vegetable juices. On the other hand, similar concentrations of nitrite ion were found in the four different brands of vegetable juices, in the range of 15.3 to 19.0 mg / liter. Nitrite was reported not found in fresh tomatoes, while extreme low levels of nitrite ion (~ 0.9 mg/kg) were found in carrots (Hotchkiss *et al.*, 1992 ; Walker 1990). The nitrite : nitrate concentration ratio for fresh carrots was reported to be 0.005 : 1 ; yet in the commercial canned carrot juice, nitrite was found at a much higher (18 times) ratio of 0.05_5 : 1. Thus nitrite was very probably added as preservatives in the four commercial brands of vegetable juices examined in this study. The total amount of nitrite in two commercial brands (355 ml cans) of mixed vegetable (brand A) and tomato (brand C) juices were found to exceed the recommended limits for an average adult of 60 kg body weight. Except for brand B tomato juice, relatively high levels of ascorbic acid (about 100 mg) were found in other brands of vegetable juices surveyed, including brand A and C. The high levels of ascorbic acid present may reduce the potential harmful effects of nitrite due to its inhibitive effects on nitrosamine formation *in vivo*.

In conclusion, a simple and fast method for simultaneous measurement of nitrite, nitrate and ascorbic acid in canned vegetable juices was developed. The sample pretreatment steps involved only filtration and centrifugation. The pH of the samples (5.0 - 6.5) were not significantly altered, and no chemical derivatization was needed. Interference by other species and anions were

excluded. The analysis time is less than 12 minutes per sample. The method is useful for analysing food samples in which the ascorbic acid and nitrite are unstable. The method also displays good sensitivity, accuracy and precision for the canned vegetable juices studied.

In the UK, as elsewhere, the maximum amounts of nitrate and nitrite that may present in food are controlled by legislation. (The Preservatives in Food Regulations 1989). The tendency throughout the world is to impose the concentration limits permitted for the general use. Our results showed that the total amount of nitrite in some commercial brands of canned vegetable juice exceeded the FAO/WHO ADI criterion for an average adult of 60 kg body weight, and their consumption has to be taken judiciously. On the positive side, the potential harmful effect of nitrite may be alleviated by the relatively high levels of ascorbic acid present.

Chapter 8 Conclusions

8.1 Summary of Major Findings

Non-volatile N-nitrosoamino acids could not be separated efficiently by normal-phase nor reverse-phase high performance liquid chromatography due to its small size and highly polar and/or ionized nature. For the first time, we have successfully developed a C_{18} reverse-phase ion-pair high performance liquid chromatography method capable of separating four N-nitrosoamino acids, i.e. N-nitrososarcosine (NSAR), N-nitrosoproline (NPRO), N-nitrosothiazolidine-4-carboxylic acid (NTCA), and N-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA).

The HPLC separation is very sensitive to experiment 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.

Furthermore, the syn- and anti- isomers of NSAR, NTCA and NMTCA were completely separated or near completely separated under suitable HPLC conditions, which could be used to further confirm the identity of the N-nitrosoamino acids in unknown samples.

The four N-nitrosoamino acids were found to be easily and sensitively detected by electrospray ionization mass spectrometry. Both the positive and negative ESI mass spectra were found to be clean and simple, showing abundant

protonated, $[M+H]^+$, and deprotonated, $[M-H]^-$, molecular ions, respectively. No thermal decomposition of the NAAs was found to occur. The fragment ions were derived from loss of small simple neutrals, and are structurally specific of the parent N-nitrosoamino acid. Again, their relative ion intensities could be used to confirm the presence of N-nitrosoamino acids in unknown samples. Negative electrospray ionization was finally adopted in reverse-phase ion-pair HPLC/ESI-MS detection of N-nitrosoamino acids in food samples because of its relative ease of operation, ability to produce abundance deprotonated carboxylate molecular anions, and lower background interferences observed. Interfering cluster ion formation in negative electrospray ionization was avoided by careful manipulation of the mobile phase composition to 60 : 35 : 5 (% *v/v*) MeOH : H_2O : MeCN.

The RP-IP-HPLC/ESI-MS conditions adopted for practical analysis of NSAR, NPRO, NTCA, NMTCA in Chinese salted fish were : isocratic elution with 2.1 mm x 150 mm C_{18} (5 μ) HPLC column 1.4 mM C_{16}-cetyltrimethylammonium chloride in 60 : 35 : 5 (% *v/v*) MeOH : H_2O : MeCN mobile phase flowing as 200 μl/min.

A linear working range of 250 pg to 20 ng/injection of NAAs was obtained. The detection limits were 250 pg/injection for NSAR, NPRO, NTCA and NMTCA at a S/N ratio ≥ 3. Relatively high level of NSAR and NPRO at 2,200 ± 260 and 154 ± 15 μg/kg, respectively, were found in Chinese salted fish. This is also the first time that non-volatile N-nitroso compounds was analysed in an example of preserved Chinese food.

As a supplementary study, a RP-IP-HPLC method for simultaneous analysis of nitrosating agents, i.e. nitrite and nitrate, and inhibitor of nitrosation, i.e. ascorbic acid in food was also developed. The method was applied to analysis of nitrite, nitrate and ascorbic acid in commercial brands of canned vegetable juices. The total amount of nitrite and nitrate ion per can in two commercial brands of vegetable juices was found to exceed the acceptable daily intake recommended by the 1996 Joint FAO/WHO Expert Committee on Food Additives for an average adult of 60kg body weight.

Chapter 9 Suggestion for Further Studies

We have shown that electrospray ionization mass spectrometry provides a sensitive simple and easy way of detecting non-volatile N-nitrosoamino acids. It is a specific method of detection, and could be used as an alternative method to GC-TEA or HPLC-TEA for analysis of N-nitroso compounds in unknown samples.

By suitably manipulating ion-pairing and HPLC experimental conditions, reverse-phase ion-pair HPLC was demonstrated to be an efficient chromatographic technique for separation of non-volatile N-nitrosoamino acids. Therefore, it is proposed that the RP-IP-HPLC/ESI-MS method could be applied to analysis of other types of non-volatile N-nitroso compounds such as non-volatile N-nitrosamines, N-nitrosamides, N-nitrosoamino sugars, and other non-volatile NOCs recently found in tobacco and tobacco smoke. This is a largely un-explored area of method development for a difficult and challenging analytical problem.

Our preliminary quantitative results showed relatively high levels of non-volatile NOCs in Chinese salted fish. While there were surveys of levels of volatile NOCs, mainly, N-nitrosamines, in Chinese preserved foods, the corresponding non-volatile NOCs have not been monitored. For health and safety considerations, surveys of non-volatile NOCs in preserved Chinese food should be conducted, though it is beyond the resources of a university research group.

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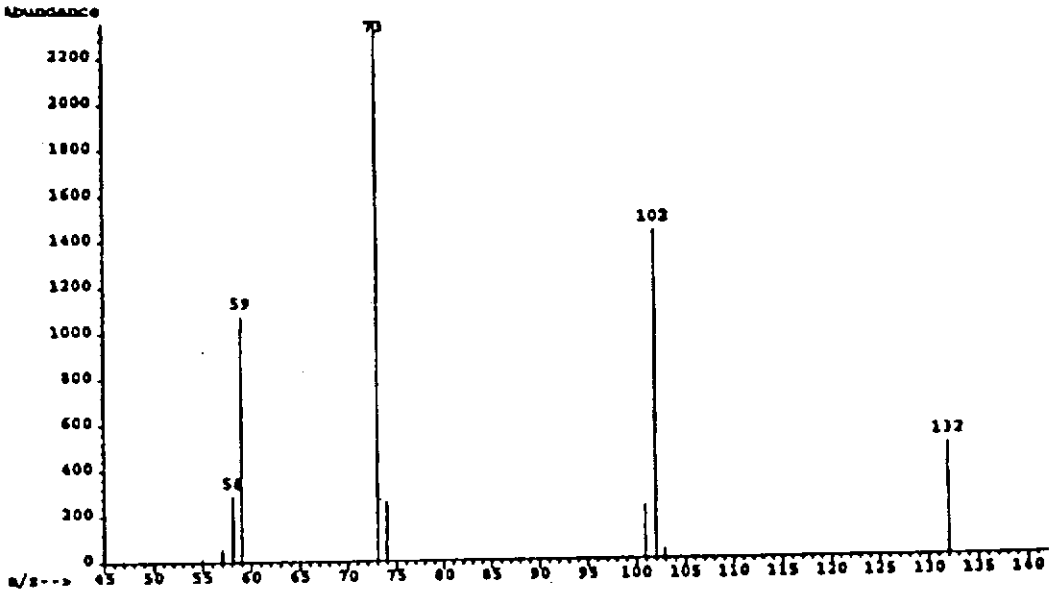
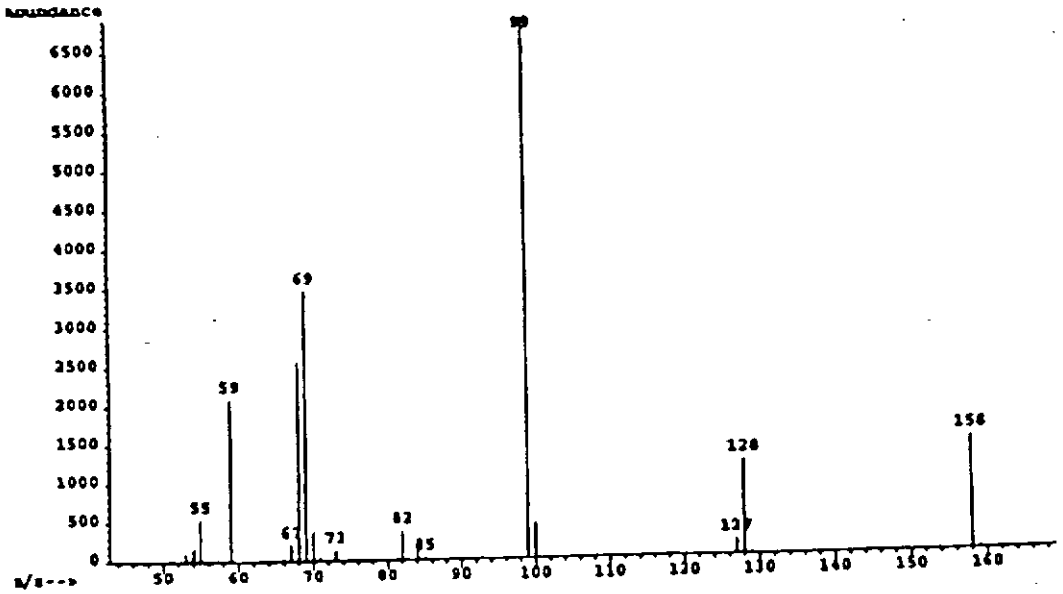
Appendix 1

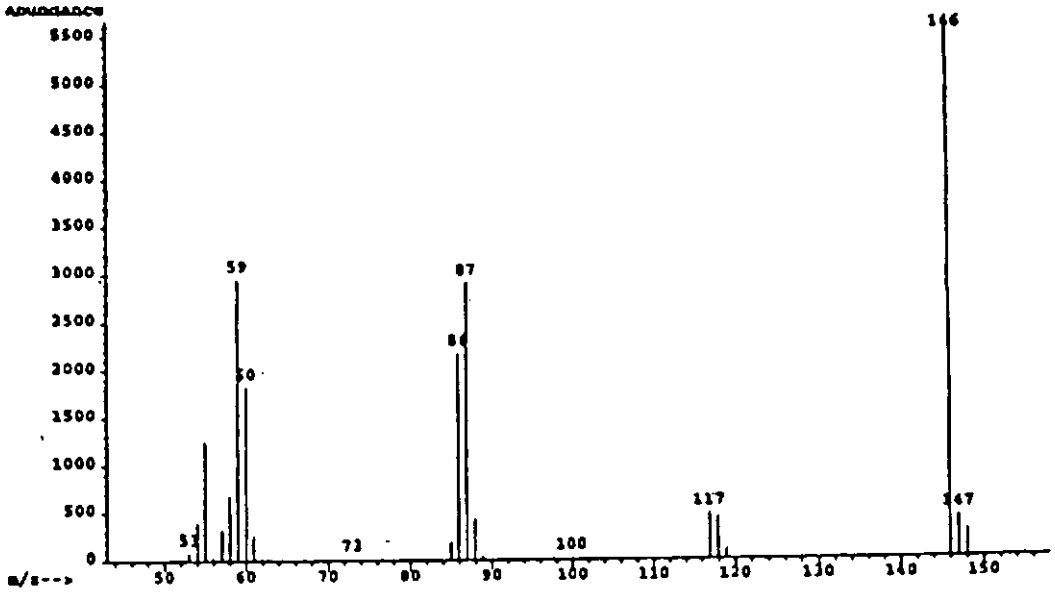
(a) GC/EI-MS mass spectrum of methyl-ester derivatives of N-nitrososarcosine (NSAR)



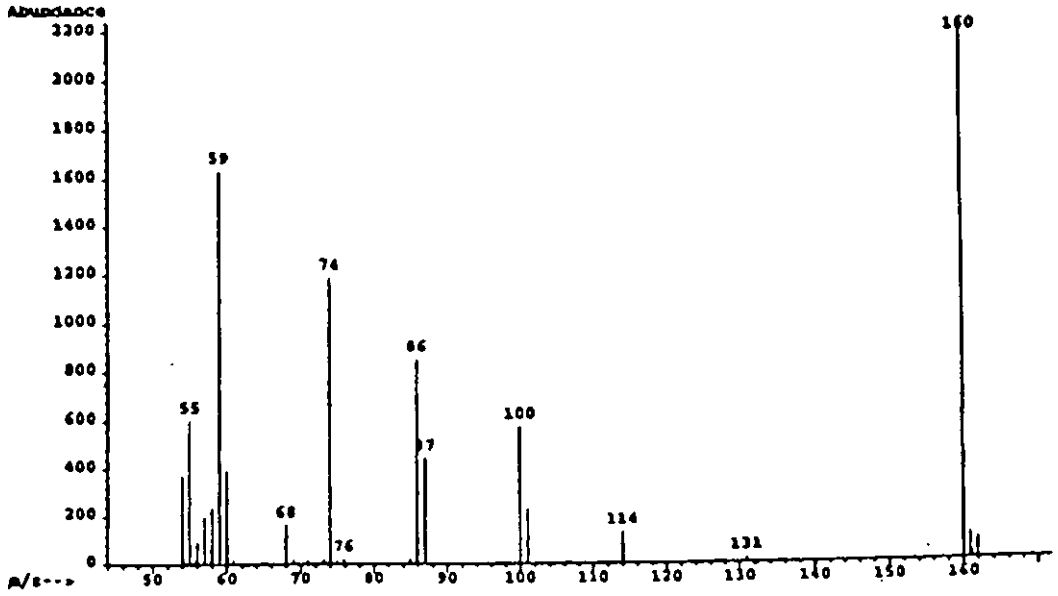
(b) GC/EI-MS mass spectrum of methyl-ester derivatives of N-nitrosoproline (NPRO)



(c) GC/EI-MS mass spectrum of methyl-ester derivatives of N-nitrosothiazolidine-4-carboxylic acid (NTCA)



(d) GC/EI-MS mass spectrum of methyl-ester derivatives of N-nitroso-2-methyl-thizaolidine-4-carboxylic acid (NMTCA)



(e) GC/EI-MS mass spectrum of methyl-ester derivatives of N-nitrosopipecolic acid (NPIC)