# ANALYSIS OF *N*-NITROSAMES IN WATER USING MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC) AND DEVELOPMENT OF ONLINE AND OFFLINE METHODS OF EXTRACTION AND ENRICHMENT OF NEUTRAL POLAR ANALYTES

by

© Ali Modir-Rousta

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## ABSTRACT

Evaluation of the quality of the environment is essential for human wellness as pollutants in trace amounts can cause serious health problem. Nitrosamines are a group of compounds that are considered potential carcinogens and can be found in drinking water (as disinfection byproducts), foods, beverages and cosmetics. To monitor the level of these compounds to minimize daily intakes, fast and reliable analytical techniques are required. As these compounds are relatively highly polar, extraction and enrichment from environmental samples (aqueous) are challenging. Also, the trend of analytical techniques toward the reduction of sample size and minimization of organic solvent use demands new methods of analysis. In light of fulfilling these requirements, a new method of online preconcentration tailored to an electrokinetic chromatography is introduced. In this method, electroosmotic flow (EOF) was suppressed to increase the interaction time between analyte and micellar phase, therefore the only force to mobilize the neutral analytes is the interaction of analyte with moving micelles. In absence of EOF, polarity of applied potential was switched (negative or positive) to force (anionic or cationic) micelles to move toward the detector. To avoid the excessive band broadening due to longer analysis time caused by slow moving micelles, auxiliary pressure was introduced to boost the micelle movement toward the detector using an in house designed and built apparatus. Applying the external auxiliary pressure significantly reduced the analysis times without compromising separation efficiency. Parameters, such as type of

surfactants, composition of background electrolyte (BGE), type of capillary, matrix effect, organic modifiers, etc., were evaluated in optimization of the method. The enrichment factors for targeted analytes were impressive, particularly; cationic surfactants were shown to be suitable for analysis of nitrosamines due to their ability to act as hydrogen bond donors. Ammonium perfluorooctanoate (APFO) also showed remarkable results in term of peak shapes and number of theoretical plates. It was shown that the separation results were best when a high conductivity sample was paired with a BGE of lower conductivity. Using higher surfactant concentrations (up to 200 mM SDS) than usual (50 mM SDS) for micellar electrokinetic chromatography (MEKC) improved the sweeping.

A new method for micro-extraction and enrichment of highly polar neutral analytes (*N*-Nitrosamines in particular) based on three-phase drop micro-extraction was introduced and its performance studied. In this method, a new device using some easy-to-find components was fabricated and its operation and application demonstrated. Compared to conventional extraction methods (liquid-liquid extraction), consumption of organic solvents and operation times were significantly lower.

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I also owe my deepest gratitude to faculty and staff in department of chemistry, C-CART and the School of Graduate Studies of Memorial University and the National Sciences Research Council of Canada (NSERC) for funding.

## Dedication

I am honored to dedicate this work to my lovely wife, daughter, and son in appreciation to their unconditional support.

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## List of Symbols, Nomenclature or Abbreviations

| | |
|---|---|
| ACN | acetonitrile |
| APFO | ammonium perfluorooctanoate |
| BGE | background electrolyte solution |
| CE | capillary electrophoresis |
| CFME | continuous-flow micro-extraction |
| CMC | critical micelle concentration |
| CZE | capillary zone electrophoresis |
| CTAB | cetyltrimethylammoniumbrmide |
| CTAC | cetyltrimethylammoniumchloride |
| DAD | diode array detector |
| DLLME | dispersive liquid-liquid micro-extraction |
| DMSO | dimethyl sulfoxide |
| DBP | disinfection by-product |
| DL | detection limit |

| | |
|---|---|
| EI | electron ionization |
| EOF | electroosmotic flow |
| ESI-MS | electrospray ionization- mass spectrometry |
| FASS | field amplified sample stacking |
| GC-MS | gas chromatography- mass spectrometry |
| HBA | hydrogen bonding acceptor |
| HBD | hydrogen bonding donor |
| HF-LPME | hollow-fibre liquid-phase micro-extraction |
| HPLC | high performance liquid chromatography |
| HS-LPME | Headspace liquid-phase micro-extraction |
| ID | internal diameter |
| IEF | isoelectric focusing |
| IPA | isopropanol |
| LED | high-emitting diode |
| LSER | linear solvation energy relationship |

| | |
|---|---|
| LVSS | large volume sample stacking |
| MEKC | micellar electrokinetic chromatography |
| MD | micro-dialysis |
| MS | mass spectrometry |
| MS-MS | tandem mass spectrometry |
| NDBA | *N*-nitrosodibutyl amine |
| NDEA | *N*-nitrosodietyl amine |
| NDMA | *N*-nitrosodimetyl amine |
| NDPA. | *N*-nitrosodipropyl amine |
| NMOR | *N*-nitrosomorpholine |
| NPIP | *N*-nitrosopiperidine |
| NPYR | *N*-nitrosopyrrolidine |
| OD | outer diameter |
| PEG | polyethylene glycol |
| PSP | pseudostationary phase |

| | |
|---|---|
| RM-EKC | reversed migration-electrokinetic chromatography |
| RSD | relative standard deviation |
| S/N | signal to noise ratio |
| SDS | sodium dodecylsulfate |
| SLMs | supported liquid membranes |
| SDME | single drop micro-extraction |
| SFE | supercritical fluid extraction |
| SPE | solid phase extraction |
| SPME | solid phase micro-extraction |
| VOC | volatile organic compounds |
| U.S. EPA | U.S. environmental protection agency |
| UV | ultraviolet |
| UV-vis | ultraviolet-visible |

## Co-authorship Statement

Chapter 2 and 3 have already been published in a peer reviewed Journal by Ali Modir-Rousta as first author and Dr. C. S. Bottaro as corresponding author. However, at that time of thesis submission Chapter 4 and 5 were being prepared for publication and are in publication format.

Dr. C. Bottaro proposed the idea of using capillary electrophoresis for analysis of the disinfection byproducts in drinking water and wastewater. The first author proposed the analysis of *N*-nitrosamines in environmental samples. The idea of new pressure assisted sweeping RM-EKC and single-drop micro-extraction concepts were introduced by the first author, with suggestions made during the research by Dr. C. S. Bottaro.

All experimental work was performed by the first author; interpretation and modification of experiments were applied under Dr. C. S. Bottaro's supervision.

All manuscripts were prepared by the first author and Dr. C. S. Bottaro edited the manuscripts and responded to reviewers.

# Chapter 1 Introduction and Overview

## 1.1. Introduction

A complete review of nitrosamine chemistry and methods of analysis for nitrosamines is presented in this chapter. After introducing information about nitrosamines including physical, chemical, and structural characteristics – which are key to understanding their importance and executing research described in this thesis – an overview on the current methods for chemical analysis of nitrosamines in the literature (including GC, HPLC, and CE) is presented. This overview highlights the importance of CE as a reliable and powerful tool in analytical chemistry. The theory of CE and related techniques are explained, particular attention is given to the issue of the poor sensitivity in CE and the ways of solving this problem, such as using better detection systems and sample enrichment. A technical review of existing detection systems and a detailed discussion of methods of sample enrichment (e.g. online and offline methods) is presented. Since offline sample enrichment techniques are most common and are the main competition for online preconcentration, theories and mechanism of these methods are reviewed. In particular, micro-extraction as a new frontier in the green analytical chemistry in the literature is investigated and latest advances in this field are reviewed.

## 1.2. Nitrosamines

*N*-Nitrosamines were reported for the first time in 1863 as synthetic intermediates in rubber industries [1]. Toxicity in humans was not established until nearly a century later. Barnes and Magee reported that when fed to rats *N*-Nitrosodimethylamine (NDMA)

induced cancer of the liver [2]. Later it was found that alkylation of cellular DNA by NDMA was the main reason for induction of a variety of cancers [3-4]. Nitrosamines are now widely recognized as potential carcinogens and as a serious threat to public health.[5] Generally, nitrosamines are polar compounds that are found in food and beverages [6], drinking water [7], and air [8]. They are often formed as disinfection by-products (DBPs) [9], thus they can occur in drinking water. Since they have been identified as probable carcinogens [10], they are an important public health concern. Nitrosamines are relatively stable compounds in environment [11]. They are formed by a reaction between secondary or tertiary amines [12] and nitrous agents such as nitrous acid or nitrogen oxides [13] and they don't decompose easily. Nitrosamines have been found widely in the daily diet (foods and beverages) [14-15], cosmetics [16-17], pharmaceuticals [18], biological systems [19], air (e.g. tobacco smoke) [20], contaminated ground water (e.g. from rocket fuel) [21], rubber [16], and in drinking water and wastewater [10, 22-23]. The presence of secondary amines in food and nitrite as a food preservative leads to the formation of nitrosamines in the digestion system due to the acidic nature of stomach fluid.

Recently, The U.S. EPA announced a new regulation for water contaminants (Section 304(a) of the Clean Water Act (33 U.S.C. 1314(a)) [24]. It "*requires EPA to publish and periodically update water quality criteria. These criteria are to reflect the latest scientific knowledge on the identifiable effects of pollutants on public health and welfare, aquatic life, and recreation*". It presents 'safe' concentrations for humans, and in the case of

suspected or proven carcinogens, gives various levels of incremental cancer risk. The *Section 304(a) water quality criterion* is a qualitative or quantitative estimate of the concentration of a water constituent or pollutant in ambient waters which, when not exceeded, will ensure a water quality sufficient to protect a specified water use. Under the Act a criterion is based solely on data and scientific judgment (Document EPA#: 440580064). For the starting point, a group of contaminants including 16 carcinogen volatile organic compounds (VOC) were introduced. It is important to know that nitrosamines were a part of the first group. This highlights the degree of importance of the presence of these compounds in the environment for human health risk. Currently, *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodipropylamine (NDPA) and *N*-nitrosodibutylamine (NDBA), are included in the Unregulated Contaminant Monitoring Regulation (UCMR 2), listed in the recently proposed Contaminant Candidate List 3 (CCL 3) by the U.S. EPA. This organization has also established ng $L^{-1}$ control levels in drinking water. [25-26]

### 1.2.1. Nitrosamines formation during chlorination of water

In 2003 Choi and Valentine proposed a pathway for nitrosation during chlorination of drinking water and waste water [27]. In this pathway, formation of $ClNO_2$ is described by transferring $Cl^+$ from chlorine or chloramines to nitrite. Nitrosating agent ($N_2O_4$) is generated by further reaction between $ClNO_2$ and nitrite. In an experiment, 0.1 mM free

chlorine, 0.1 mM dimethylamine and 0.2 mM nitrite were mixed at pH=7 and 4,000 ng/L NDMA was produced after 2 hours. When chlorine replaced by monochloramine, the NDMA formation via this pathway was insignificant. This pathway is slow and reaction is halted by formation of nitrate. In 2006, Chen and Valentine proposed the enhanced nitrosation pathway that explains the formation and accumulation of nitrosamines via choloramine compare to chlorine [28-29] (See Figure 1-1).

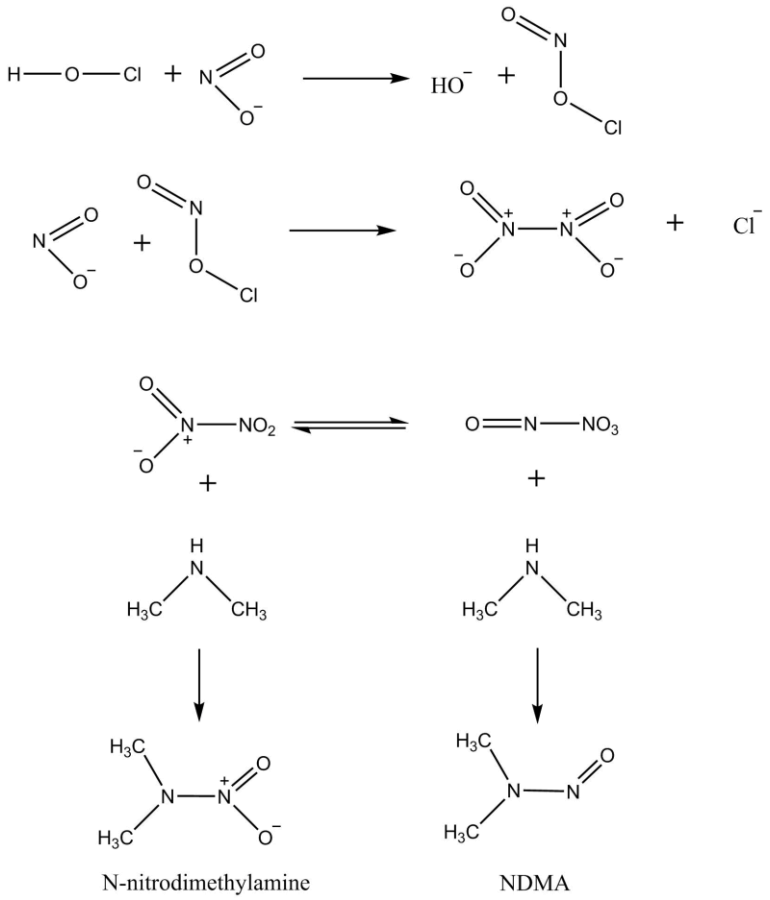


Figure 1-1 Formation of NDMA and N-nitrodimethylamine adopted from Schreiber, I.M., Mitch, W.A., *Environ Sci. Technol.* 2007, 41, 7039-46. Without permission)

According to the recent pathway, NDMA is formed from reaction between monochloramine and natural organic matter (NOM) at lower pH[29].

### 1.2.2. Physical and Chemical Characteristics

The properties of the simplest members in the broader class of *N*-nitrosodialkylamines (e.g., *N*-nitrosodimethyl- and *N*-nitrosodiethyl-amines) (figure 1-4) have been most fully investigated [30-31]. The smaller *N*-nitrosodialkylamines are yellow or yellow-green, non-hygroscopic liquids, which are fairly soluble in water and readily soluble in organic solvents. *N*-Nitrosodialkylamines have relatively low melting points and their boiling points lie in the range of 150-220 °C (Table1-1). The densities of nitrosamines are typically in the range of 0.9-1.2 g $cm^{-3}$, increasing with molecular weight [32]. The dipole moments of *N*-nitrosodialkylamines are evidence of considerable polarity of the molecules. For example, the dipole moment of *N*-nitrosodimethylamine is 3.98 D [33-34] ( $H_2O$ is 1.8 D).

The dissociation energy of the N-N bond in *N*-nitrosodimethylamine was reported 134 kJ $mol^{-1}$ that is consistent with the hypothesis of presence of delocalized electrons or conjugation system in the nitrosamine group [35]. This also confirmed by XRD results, which showed that the molecules of aliphatic nitrosamines have planar structures in the crystalline states (both nitrogen atoms are $sp^2$ hybridized) (see Figure 1.2).

A summary of XRD data follows: the bond angles of NDMA in $CuCl_2$ complexes are $C_1$-N-N: 120.3°, $C_2$-N-N: 116.4° and N-N-O: 113.6°; and bond lengths are 1.235 Å for N-O, 1.344 Å for N-N, 1.461 Å for C-N, and 1.129 Å for C-H. It is interesting that the N-N bond in *N*-nitrosodimethylamine is shorter by 0.04 A than in *N*-nitrodimethylamine, which has been attributed to the greater electron-withdrawing activity of the nitroso-group compared to nitro-group [36]. Considering above data (bonds angle and length) for amino nitrogen, it can be concluded that this nitrogen has the characteristics of $sp^2$ hybridization.

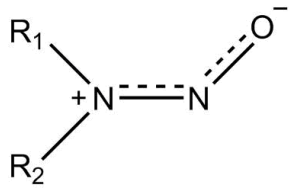


Figure 1-2- Configuration of *N*-nitroso functional group

In a number of studies the internal rotation about the N-N bond with partial double bond character was investigated. The barrier to rotation about this bond was estimated to be approximately 100 kJ $mol^{-1}$ [37]. This rotation leads to the appearance of *cis-trans* isomerism in nitrosamines (Figure 1.3). The presence of the isomers of a large number of *N*-nitroso-derivatives has been demonstrated by NMR spectroscopy [38].

Optical study of nitrosamines functional group shows two absorption peaks in the UV region; a broad peak in range of 350-400 nm, and a stronger and narrower peak in range of 220-240). The band at 350-400 nm [39] is attributed to $n$-$\pi^*$ transition ($n$ is for non-

bonding or lone pair electrons) and one at 220-240 nm to $\pi$-$\pi$* transitions [40] that is considered nitroso-chromophores in aqueous solutions. The absorption wavelength increases with the addition of an electron donating substitute and decreases with the addition of an electron withdrawing substitute. In aqueous solution of *N*-nitrosodimethylamine the $\pi$-$\pi$* transition has a $\lambda_{max}$ 228 nm and the *n*-$\pi$* transition at 332 nm [41].

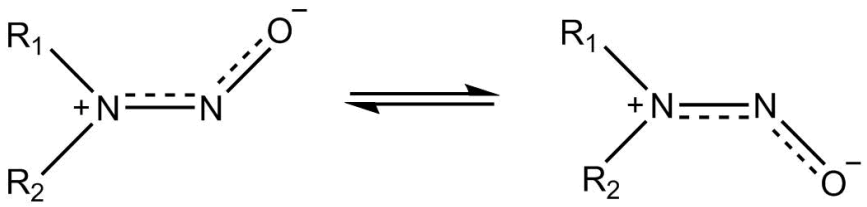


Figure 1-3- Nitrosamine geometric isomers, $R_1$, $R_2$ are different alkyl groups

*N*-Nitrosamines act as potential Lewis bases. The conjugated system between nitrogen atoms and oxygen with the withdrawal of the electron cloud towards the oxygen atom is responsible for many interesting reactions of nitrosamines such as the formation of metal complexes, reaction with inorganic acids, reduction to *N*,*N*-substituted hydrazines, oxidation, nitration, cyclization, and photochemical reactions. These reactions could be considered as potential methods of derivatization of nitrosamine, however in most derivatization cases the first step is elimination of nitroso group to form amines which limits selective derivatization.

Since identification of NDMA as a water disinfection by-product in 1989 [42], drinking water in Ontario, Canada and California, USA has been monitored for NDMA, with maximum allowable concentrations of 9 and 10 $ngL^{-1}$, respectively.

Table 1.1. Physical properties of nitrosamines [43]

| **Compound** | **Abbreviation** | **Molecular Formula** | **Molar mass ($gmol^{-1}$)** | **BP (°C)** | **LogP (octanol/ water)** |
|---|---|---|---|---|---|
| *N*-nitrosodimethylamine | NDMA | $C_2H_6N_2O$ | 74.048 | 152 | -0.57 |
| *N*-nitrosomorpholine | NMOR | $C_4H_8N_2O_2$ | 116.059 | 224 | -0.44 |
| *N*-nitrosopyrrolidine | NPYR | $C_4H_8N_2O$ | 100.064 | 214 | -0.19 |
| *N*-nitrosopiperidine | NPIP | $C_5H_{10}N_2O$ | 114.145 | 219 | 0.36 |
| *N*-nitrosodiethylamine | NDEA | $C_4H_{10}N_2O$ | 102.079 | 176.9 | 0.48 |
| *N*-nitrosodi-n-propylamine | NDPA | $C_6H_{14}N_2O$ | 130.187 | 206 | 1.36 |
| *N*-nitrosodi-n-butylamine | NDBA | $C_8H_{18}N_2O$ | 158.241 | 116 | 2.63 |

In a study in the Netherlands, the average daily intake of NDMA was estimated to be 0.38 μg per day. The main intake of NDMA came from beer, which is 71% of daily intake of an average consumer [44]. In a similar study in Germany the total intake of NDMA was estimated to be 1.1 μg /day in which 64% of daily intake came from beer [45]. However, the latest study shows this portion has significantly decreased due to improvement in beer processing [15].

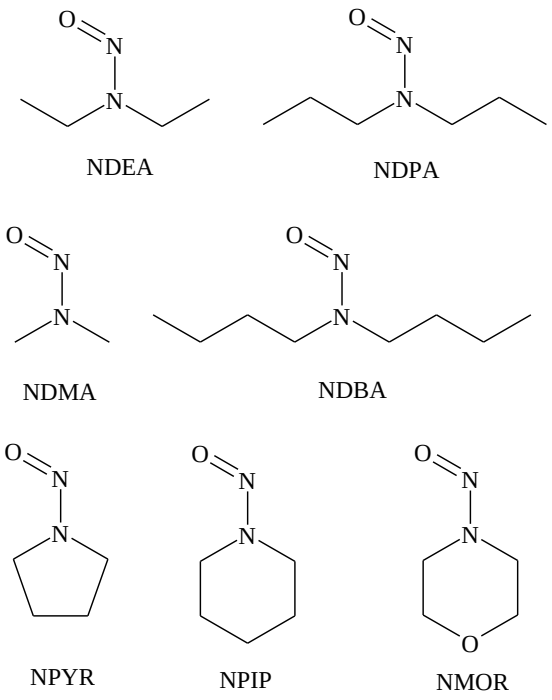


Figure 1-4- Structures of nitrosamines that have been identified in drinking water.

The daily intake of nitrosamines in Japan was estimated as 0.5 μg per day and 88% of this intake came from fish products [46]. In a similar study, it has been shown that seafood has a major contribution in daily intake of nitrosamines in China [47]. A summary occurrence of volatile nitrosamines in food products and beverages in some countries is presented in Table 1.2.

Table 1.2 Sample levels of NDMA in food and drinks[a]

| **Source** | **ng.g$^{-1}$** |
|---|---|
| Squid, dried broiled (Japan) | 313 |
| Dried Fish (Greenland) | 38 |
| Smoked meat (Canada) | 2 |
| Salted meat (USSR) | 54 |
| Cheese (Canada) | 0.7 |
| Cheese (Germany) | 5 |
| Beer (Canada) | 3 |
| Beer (US) | 14 |
| Beer (Germany) | 68 |

a- Adapted from Leoppkey and Michejda, 1994, Nitrosamines and related N-Nitroso compounds chemistry and biochemistry.

### 1.2.3. Current Methods of Analysis of Nitrosamines

A number of different separation and detection techniques have been used for the analysis of nitrosamines. GC and HPLC with different methods of detection are mainly considered as traditional approaches. GC-MS with electron ionization (EI) has been accepted as the most common technique for the determination of nitrosamines [48-49]. However, since environmental samples are mostly aqueous, tedious work is required for sample preparation (solid phase extraction or liquid-liquid extraction) prior to CG analysis. Nitrosamines are highly polar compounds which also makes them unsuitable for GC

techniques. Conventional HPLC methods using UV-Vis detector are not sensitive enough to detect trace amounts of nitrosamines in environmental samples. Fluorescence detectors have high sensitivity but require fluorescent substances. For nitrosamines that are not fluorescent, an additional step (fluorescent label derivatization) is needed [50-51]. Similar to HPLC, not many reports on application of capillary electrophoresis (CE) for analysis of nitrosamines have been published due to the low detection limit in conventional CE methods. Despite these limitations, application of online preconcentration methods can make CE a powerful technique for analysis of environmental samples.

## 1.3. Capillary Electrophoresis

### 1.3.1. History of Capillary Electrophoresis (CE)

Tiselius was the first (1930s) to show utility of electrophoresis for separation of proteins in solutions [52-54]. Hjerten introduced the zone electrophoresis in a horizontal, rotating polyethylene tube (0.5 cm ID) in a homogeneous medium for separation of pigmented proteins [55]. Later, Jorgenson and Lukacs performed a zone electrophoresis in open-tubular capillaries (75 μm ID and a length of 100 cm) in combination with high applied potential voltage (30 kV) and a special fluorescence detector to detect the fluorescent derivatives of amino acids, peptides, and urinary amines [56].

Capillary electrophoresis separates ions based on their electrophoretic mobility under the influence of an applied electric field. The electrophoretic mobility is dependent upon the

charge of the molecule, the hydrodynamic volume, the viscosity of solution, and presence of additional solvent(s). The rate of the ion movement is directly proportional to the magnitude of the applied electric field. If two ions are the same size, the one with greater charge will move the fastest. For ions of the same charge, the smaller volume experiences less friction and has overall faster migration. The greatest advantages of CE are speed of analysis, separation resolution, wide range of available detection methods, low reagent consumption; and running cost [57]. In light of these factors, along with a performance for separations in aqueous phase, CE is considered as an environmentally-friendly technique. Employing a capillary for electrophoresis has solved some common problems in traditional electrophoresis. For example, the narrow dimensions of the capillaries greatly increased the surface to volume ratio, which minimizes heating associated with high applied voltages (Joule heating). The increased efficiency and remarkable separating capabilities of CE has led to a growth of interest in the scientific community to further develop the technique.

### 1.3.2. Theory of CE

Since a major theme of this thesis involves CE, it is useful to consider some fundamentals of CE theory. As stated previously, electrophoresis is the process in which sample ions move under the influence of an applied potential (Figure 1-6). The induced electrical force is proportional to the net charge and the electric field strength (E). It is also affected

by the friction due to viscosity of the solution and the velocity of the ion. This leads to the expression for electrophoretic mobility, $\mu_{EP}$:

$$\mu_{EP} = \frac{q}{f} = \frac{q}{6\pi\eta r} \qquad 1\text{-}1$$

where $f$ for a spherical particle is given by Stokes' law; η is the viscosity of the solvent; and r is the radius of the ion. The rate at which these ions migrate is dictated by the charge to hydrodynamic volume. The actual velocity, *v*, of the ions is directly proportional to E, the magnitude of the electrical field and can be determined by the following equation:

$$v = \mu_{EP} E \qquad 1\text{-}2$$

This relationship shows that a greater voltage will quicken the migration of the ionic species.

The movement of the bulk solution or EOF results when high-voltage is applied to an electrolyte solution inside a glass capillary with a charged inner wall. For fused silica capillary EOF occurs when the BGE has pH>4 so that the SiOH functional groups are at least partially ionized to become negatively charged as $SiO^-$ (Figure 1.5). For a negatively charged capillary wall, a double layer develops comprised of a stationary inner cationic layer (Stern layer) and a diffuse layer with an excess of cations which is free to move along the capillary.

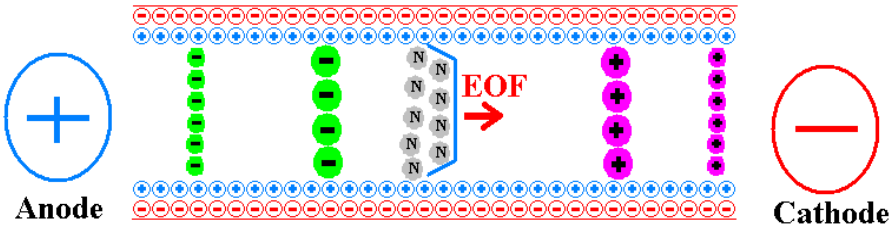


Figure 1-5. Schematic view of EOF and electrophoretic mobility of charged particles

The applied electric field causes the solvated cations in the diffuse layer to move toward the cathode creating a powerful bulk flow. The rate of the electroosmotic flow is defined by the following equation:

$$\mu_{EOF} = \frac{\varepsilon\zeta}{4\pi\eta} \qquad 1\text{-}3$$

where ε is the dielectric constant of the solution; η is the viscosity of the solution; E is the field strength; and ζ is the zeta potential. EOF is strongest with a large zeta potential between the cationic layers. A large diffuse layer of cations, a consequence of lower ionic strength, drags more solute molecules towards the cathode. In low resistance from the surrounding solution, and buffer with pH of 9 so that all the SiOH groups are ionized to $SiO^-$, all work together to give a strong EOF (Figure 1.5).

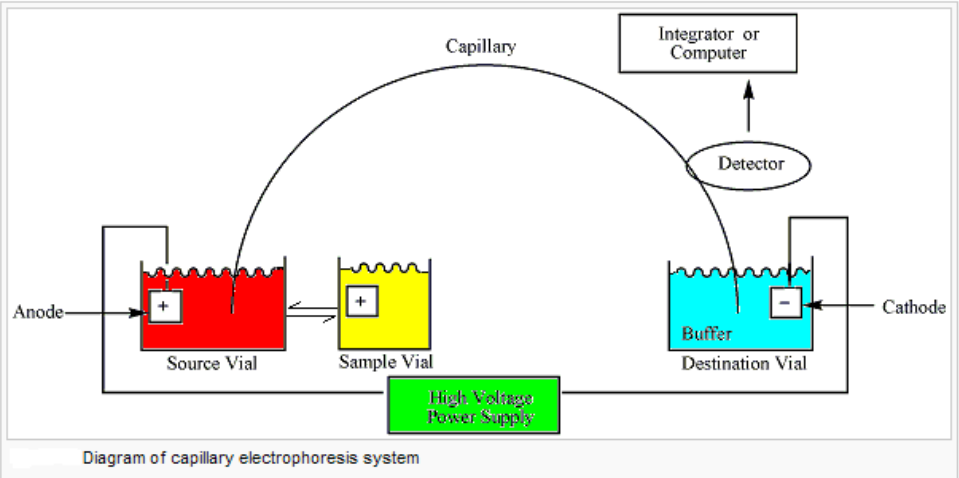


Figure 1-6. Diagram of a typical CE presented with positive applied potential.

### 1.3.3. Micellar Electrokinetic Chromatography (MEKC)

Conventional CE or capillary zone electrophoresis (CZE) works well for separation of charged compound but is incapable of separation of uncharged analytes. To overcome this weakness, a technique based on combination of electrophoresis and chromatography was developed by Terabe. This technique is called electrokinetic chromatography (EKC). When surfactants at concentrations higher than critical micelle concentration (CMC) are used, micelles form and act as a pseudostationary phase (CMC of each surfactant may vary based on conditions such as temperature, ionic strength of solution, etc). This technique is called micellar electrokinetic chromatography (MEKC). In MEKC, analytes are distributed between an aqueous electrophoretic phase and a micellar phase (Figure 1.7). MEKC is different than chromatography because the micellar phase is dynamic and mobile, thus it is described as the pseudostationary phase (PSP). The separation principles

of MEKC have been described by Terabe *et al.* [58]. Since its invention, MEKC has seen significant advancement in sensitivity and separation efficiency. Enhancements in sensitivities have been achieved using advanced detection systems (e.g. MS, LIF, electrochemical detectors, etc.) and online enrichment techniques (stacking, sweeping, etc.). Separation efficiencies have been improved using different surfactants, buffers, and other additives to the BGE.

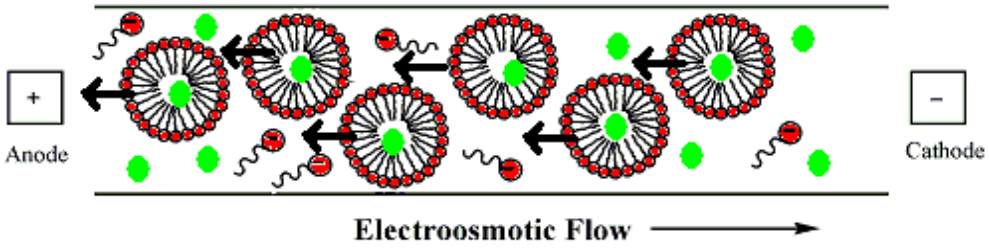


Figure 1-7. Mobility of micelles and neutral analytes in MEKC when anionic surfactants are used.

The anionic surfactant, sodium dodecyl sulfate (SDS) is the most commonly used anionic surfactant for MEKC; thus it is convenient to use it in an explanation of the principles of MEKC. When SDS is dissolved in the BGE at concentrations higher than CMC, micelles are formed and experience anodic electrophoretic mobility upon application of potential across the capillary anionic micelles in the solution. EOF causes the bulk solution to flow toward the cathode (against the mobility of anionic micelles). The apparent mobility of the micelles is sum of EOF and electrophoretic mobility of the micelle. In general, the magnitude of the EOF is much higher than the electrophoretic mobility of micelles and opposite in direction thus the net movement of micelles is toward the detector (cathode).

The micelles are dynamic, which means that they are continuously breaking down and reforming [59]. Analyte molecules will partition between the aqueous and micellar phase and they will migrate with different mobilities depending upon their partition coefficient, where the maximum velocity would be the same as the EOF and minimum would be that of the micelles [60]. The apparent velocities fall between these two extremes. Molecules that do not interact with micelles migrate with EOF, $t_0$, while highly hydrophobic compounds spend most of their time in the micellar phase and migrate close to the migration time of micelles, $t_{mc}$. For the other analytes, the partitioning process dictates the migration time, $t_R$. The capacity factor ($k$) can be calculated based on above mentioned migration times as [61] :

$$k = \frac{t_R - t_0}{t_0(1 - \frac{t_R}{t_{mc}})} \qquad 1\text{-}4$$

Although the original goal of MEKC was to perform CE separation for uncharged or weakly anionic compounds, the scope of the method was expanded to the analysis of ionic species, including a large variety of low-molecular-weight ions [62]. Equation 1.4 is generally used for neutral analytes but a similar expression can be deriven for charged species. The capacity factor has been used to quantify the analyte-stationary phase interactions in chromatography and electrokinetic chromatography [63-64]. To study the solute-micelle interaction, the value of $k$ can be calculated and any change in $k$ can be attributed to the change in solute-micelle behavior.

### 1.3.4. Solute Micelle Interaction

Since the first introduction of MEKC [60], SDS has been the most widely used surfactant. However, several reports have demonstrated the important role of surfactant type in MEKC [65-66]. The variation of selectivity and migration behavior with surfactant type is evidence of the selective nature of solute interactions with micelles. The hydrophobic interaction is the main driving force of solute retention by the micelles in MEKC, yet the influence of different forces such as hydrogen-bonding and dipole-dipole interactions have significant effects on migration behavior and selectivity. The mechanisms of the effects of these selective forces on migration behavior are not fully known, though theory has been developed to provide insight. Linear salvation energy relationship (LSER) modeling has been used to describe and quantify the relationships between solutes structure, and retention behavior in RP-HPLC [63], and similarly for migration behavior in MEKC [64]. LSER models are built to describe the influence of solute-solvent interactions in terms of nonspecific and specific interactive forces. In this model, the solubility-related property (*SP*) is described by three main terms:

$$SP = SP_o + \ cavity\ term + dipolar\ term + hydrogen\ bonding\ term \qquad 1\text{-}5$$

In the case of MEKC migration, *SP* would be logarithm of the capacity factor, *log k***,** and the three terms show the net effects of solute interactions with the two interactive phases (bulk aqueous solution and micellar phase). A multi-parameter equation can then be

written with four system coefficients ***(m***, **s**, ***b***, ***a)*** and four solute descriptors ***(***$V_I$, $\pi^*$ , $\beta$, $\alpha$***)*** as:

$$SP = SP_o + \boldsymbol{m}V_I/100 + \boldsymbol{s}\pi^* + \boldsymbol{b}\beta + \boldsymbol{a}\alpha \qquad 1\text{-}6$$

where, $\boldsymbol{m}V_I/100$ is the cavity term and thermodynamically unfavorable; $\boldsymbol{s}\pi^*$ is the dipolar term, a measure of dipole-dipole and dipole-induced dipole interactions and thermodynamically favorable; both the $\boldsymbol{b}\beta$ and $\boldsymbol{a}\alpha$ are hydrogen bonding terms and thermodynamically favorable. The cavity term represents the energy that is required to overcome solvent-solvent interactions in order to provide a suitably sized cavity for the solute. This term is a measure of nonspecific interactions. Intrinsic molar volume (Van der Waals volume), $V_I$, describes the solute effect, and coefficient ***m*** shows the solvent contributions. The other two terms (dipolar and hydrogen bonding) are used to account for specific interactions, where $\pi^*$ is the polarizability value of solutes, while ***s*** is the equivalent term to describe the solvent involvement. The hydrogen-bonding terms represent the interactions involving sharing of a proton, term for solutes accepting and solvent(s) donating as well as for solutes donating and solvent(s) accepting. Therefore, ***b*** is to account for the strength of solvent hydrogen bond donating acidity, $\beta$ is solute hydrogen bond accepting basicity, ***a*** is solvent hydrogen bond accepting basicity, and $\alpha$ is solute hydrogen bond donating acidity. The $SP_0$ term includes information about the chromatographic systems such as phase ratio [67].

The chemical composition of the hydrophobic moieties and ionic head groups in surfactants significantly influences their interactions with solutes as well as the migration velocity of micelles. In other words, type of surfactant has a major impact on at least three of the four factors that influence resolution in MEKC, i.e., capacity factor ***(k),*** selectivity （$\alpha$）, and size of the elution window ($t_{mc}/t_m$). In many cases, efficiency is also affected. Consequently, the characterization of different surfactants is of great interest for a better understanding of the separation process in MEKC.

Nitrosamines are generally known as $(R_1R_2)$-*N*-*N*=*O*, where $R_1$ and $R_2$ are usually small alkyl groups. Therefore, specific terms in LSER equation such as the hydrogen bond accepting (HBA) term is as important as the nonspecific term. In case of selecting surfactants for MEKC separation of nitrosamines, a hydrogen bond donating (HBD) head group seems to be enhancing nitrosamine-micelle interaction.

### 1.3.5. Limitations of CE

Unlike HPLC, CE requires very small sample volume. This advantage is accompanied by poor sensitivity due to the small light path length (25 to 100 μm ID) and low sample volume (nL). Consequently, detection sensitivity is significantly compromised compared to other separation techniques such as HPLC [68]. This hinders the applicability of CE for the analysis of dilute analyte mixtures. Thus, development of more sensitive methods is indispensable to improve the detection limit (i.e., increasing concentration sensitivity and

solving the problems associated with low concentration sensitivity has been the emphasis in various reports). These investigations include the installation of capillaries equipped with extended detection path length [69] (e.g. Z-shaped, multi-reflection, and bubble cell), the use of highly sensitive detection methods (i.e., laser-induced fluorescence [70], electrochemical detection [71]), and sample enrichment methods (i.e., liquid-liquid [72] and/or solid phase extraction [73]). However, all these methods require rather expensive and somewhat complex hardware or time consuming procedures.

### 1.3.6. Detection Methods in CE and MEKC

MEKC, as other types of CE, suffers from low detection sensitivity in comparison with HPLC [74]. Capillaries with longer optical path length (e.g. Z-shape and bubble cell), detectors with high sensitivity [75], and offline enrichment methods such as liquid–liquid extraction or SPE prior to injection are also still applied [76]. However, the most applied approach for improving sensitivity in CE is online sample preconcentration technique, for which CE offers some unique opportunities.

UV-Vis detection is the conventional and cost-effective detection technique in CE, is available in any commercial CE systems and can be used to detect large number of UV or visible light absorbing molecules. The main drawback is the relatively poor concentration sensitivity, which has been mentioned already.

MS offers several advantages over UV-Vis detection [77], for example, for analytes with weak UV absorption, MS offers higher sensitivity [78], and provides information about the molecular weight and structure [79]. However, the potential interferences from salts in the BGE and non-volatile surfactants in MEKC affect the MS signal intensity. Non-volatile surfactants reaching the interface deteriorate ionization efficiency, particularly for electrospray ionization (ESI) where is most widely applied in MEKC-MS [80]. Different approaches have been employed to overcome MEKC-MS incompatibility, including partial filling-MEKC-ESI-MS [81], BGEs containing volatile surfactants and buffers [82-83], novel interfaces that are tolerant to non-volatile salts and surfactants [84], atmospheric pressure chemical ionization source [85], atmospheric pressure photoionization source [86-87].

Laser-induced fluorescence (LIF) is an important detection technique for MEKC, as it provides higher sensitivity compared to other detection techniques in MEKC. It has even been effectively applied for non-fluorescent molecules via fluorescence labeling, though derivatization adds extra preparation time. Interference from excess labeling reagent, as well as impurities and structural similarities of some related solutes after derivatization can also complicate analysis [88]. The application of light-emitting diodes (LEDs) in MEKC detection attracted attention [89-90]. LEDs can operate with a battery and give a better output stability than LIF even over a wide spectrum range (420–950 nm). However, the sensitivity of LED detector is lower than that of LIF detection due to photo flux from the LED light and the nature of fluorescence detection itself.

## 1.4. Online Analyte Enrichment Techniques in CE

Online preconcentration refers to a process where an analyte electromigrates in such a way that the length of its zone is decreased compared with that of the original sample zone. As a result, concentration increases and also detection sensitivity improves [91]. There is little consensus in the literature regarding the naming of some approaches, particularly those involving stacking. Furthermore, improvements or slight variation in one approach often results in generating a new name which can lead to confusion. Some attempt has been made here to consolidate these approaches based on the principles underlying the preconcentration mechanism.

To have a better understanding of various online enrichment techniques, their similarities and differences, a complete review of existing techniques in the literature is presented. A brief description of each of the online preconcentration techniques, their names, abbreviations and typical sensitivity enhancements are listed in Table 1.3. To improve the detection sensitivity in CE, online sample concentration techniques, such as sample stacking and sweeping have been examined individually or in combination [92] as well as the most recently introduced micelle collapse preconcentration technique [93-94]. Among the significant number of online preconcentration techniques, only a limited number of them can be used for neutral compounds in MEKC (those use surfactants, e.g. sweeping). Stacking and sweeping are the two well known preconcentration techniques for CE and many reviews for these online concentration techniques and different applications can be

found in the literature [95-97]. Despite many successful reports on preconcentration of neutral analytes in MEKC, only a small number of research works have been focused on highly polar compounds neutral compounds.

In stacking when conductivities of sample zone is less than BGE, the electrophoretic velocities of the ions in sample zone are higher than BGE. As a result velocities of ions reduce when they stack when rich the boundary between two zone. Different stacking modes have been defined for MEKC such as normal stacking mode (NSM) [98], reversed electrode polarity stacking mode (REPSM) [99], stacking with reversed migration micelles (SRMM) [100], stacking using reversed migration micelles and a water plug [101], field-enhanced sample injection [102], and field-enhanced sample injection with reverse migration micelles [103]. Stacking continues to be an important preconcentration technique for MEKC in many applications and was reviewed by Kim and Terabe [68].

Sweeping was first introduced by Terabe [99] and has been effectively used for hydrophobic analytes that interact strongly with the PSP [100]. In sweeping BGE consist of high concentration of surfactant while sample zone is micelle free. Under applied electric field micelles pass though sample zone and sweep the analytes. Remarkable improvements in detection sensitivity up to several thousand fold have been achieved by sweeping [95, 102].

Table 1.3. Summary of various online and offline preconcentration techniques in capillary electrophoresis [50]

| | **Name** | **Description** | **Sensitivity improvement** |
|---|---|---|---|
| FASS | Field-amplified sample stacking | Hydrodynamic injection<5% capillary volume Conductivity of sample < 1/10 BGE | 10- 20 |
| FASI | Field-amplified sample injection | Electrokinetic injection <5% capillary volume<br>Conductivity of sample < 1/10 BGE | 100-1000 |
| LVSS<br>LVSEP | Large volume sample stacking<br>Large volume stacking with matrix removal with EOF | Hydrodynamic injection up to 100%<br>Conductivity of sample < 1/10 BGE<br>Matrix removal by polarity switching | 100-500 |
| FAEP<br><br>FESI | Field-amplified Sample injection with matrix removal<br>Field-enhanced injection | Hydrodynamic injection of low-conductivity plug, electrokinetic injection<br>Conductivity of sample < 1/10 BGE | 1000-10000 |
| SEI | Selective exhaustive injection, pH mediated FASS,<br>Base mediated stacking | Sample matrix removed by EOF pump<br>Electrokinetic injection of sample<br>High-conductivity sample | 10-100 |
| t-ITP<br>EKS | Transient ITP<br>Electrokinetic supercharging | Hydrodynamic injection<50%<br>Leading and terminating electrolytes | 100-500000 |
| - | Counter-current electro-capture (Electro-capture, or Electrophoretic focusing) | Hydrodynamic injection with counterbalancing electrophoretic velocity<br>Hi and low-conductivity samples | 100-20000 |
| MRB | Moving reaction boundary (Dynamic pH junction) | High- and low-conductivity samples<br>Sample has different pH than BGE | 10-500 |
| Sweeping | Sweeping | Hydrodynamic injection <50%<br>High- and low-conductivity samples<br>Sample has no pseudophase | 100-5000 |

| | Name | Description | Sensitivity improvement |
|---|---|---|---|
| FAEP-Sweeping<br><br>ASEI-sweeping<br>CSEI-Sweeping | Field-amplified sample injection with matrix removal by EOF pump<br>Anions-selective exhaustive<br><br>Cations-selective exhaustive | Hydrodynamic injection of low conductivity plug, electrokinetic injection of sample<br>Conductivity of sample < 1/10 BGE<br>Low conductivity plug removed by EOF pump. electrolyte has pseudophase for sweeping | 100000-1000000 |
| - | Dynamic pH junction-sweeping | Hydrodynamic injection<br>High- and low-conductivity samples<br>Samples has different pH than BGE<br>Electrolyte has pseudophase for sweeping | 100-2000 |
| - | Membrane filtration | Electrokinetic injection<br>Semi-permeable membrane for physical exclusion of analytes<br>High- and low-conductivity samples | 100-1000000 |
| SPE | Solid phase extraction | Hydrodynamic or electrokinetic injection<br>heterogeneous phase for adsorption<br>High- and Low-conductivity samples | 100-10000 |
| LLE | Liquid-liquid extraction | Hydrodynamic or electrokinetic injection<br>extraction through immiscible liquid phase, usually a 3-phase system<br>High- and Low-conductivity samples | 100-1000 |

Analyte focusing by micelle collapse (AFMC) is another technique that has been recently introduced shows promising results for sample concentration in MEKC, but is still not fully proven [93]. In this technique saple zone consist of surfactants slightly above CMC in proximity of a non-micellar BEG. Then moving micelles enter the BGE zone and

concentration of surfactant drops below CMC Therfore analytes relesen and since they have no electrophoretic mobility they accumulate in this boundary. Dawood et al. compared and reported the efficiencies of sweeping, AFMC, and simultaneous field amplified sample stacking (FASS) and sweeping. When compared with a hydrodynamic injection (5 s at 50 mbar, 0.51% of capillary volume to detection window) of drug mixture prepared in the separation BGE, improvements of detection sensitivity of 60-, 83-, and 80-fold were obtained with sweeping, AFMC and simultaneous FASS and sweeping, respectively [104].

Online preconcentration of neutral analytes in MEKC techniques have been reported widely but only for analytes with high affinity for PSP using anionic [68] and cationic surfactants [105].

### 1.4.1. Stacking in CE

The purpose of stacking is to reduce the distribution of analytes within the sample zone and its vicinity before separation is initiated. If the sample matrix does not affect distribution of the analytes in the sample zone prior to separation, the analyte distribution will be equal to the injected plug length, and this length will become the minimum peak width at detection. There are many techniques available that share the principle of

stacking. A brief discussion about mechanism of each method is presented in following paragraphs.

#### 1.4.1.1. FASS

Chien and Burgi first introduced the field-amplified sample stacking (FASS) of charged analytes for capillary zone electrophoresis (CZE) [106]. The FASS method is considered to be the simplest technique for online sample pre-concentration. In this mode, charged analytes are injected into the capillary in a low-conductivity matrix. After application of separation voltage, the charged analytes experience enhanced velocity in the lower conductivity (amplified field) sample zone and are stacked at the sample zone/BGE interface. The electrophoretic velocity, $v_{ep}$, of analyte in free solution is described as:

$$v_{ep} = \mu_{ep} E = \mu_{ep} \frac{V}{L} \qquad 1\text{-}7$$

where $E$ is electric strength; $L$ is capillary length; $V$ is applied voltage; and $\mu_{ep}$ is electrophoretic mobility. It has been assumed the field strength through the capillary is uniform. As mentioned, the sample and BGE zones in FASS have different conductivities, the ratio of the electrophoretic velocities of the ions, $\gamma$, between the two zones is given:

$$\gamma = \frac{v_{ep1}}{v_{ep2}} = \frac{E_1}{E_2} = \frac{\rho_1}{\rho_2} \qquad 1\text{-}8$$

Where $\rho$ is the resistivity of zone 1 and zone 2. Stacking is roughly proportional to the ratio of ionic strength between the sample matrix and separation buffer [106].

If sample solution is prepared in high resistivity solution (or low ionic concentration) and BGE is a low-resistivity solution (or high ionic concentration), the analyte in sample solution will be concentrated at the boundary of two zones

$$C_{stacked} = \gamma C_{injected} \qquad 1\text{-}9$$

In practice, sample is dissolved in diluted of BGE, resulting in a low-conductivity solution. In the initial step, the capillary is conditioned and filled with a high-conductivity BGE and an appropriate length of the sample solution is then injected into the capillary.

Then a high positive voltage is applied, a proportionally greater electric field develops across the sample zone causing the ions to migrate faster (Figure 1-8). Once the ionic analytes reach the boundaries between the sample zone and the BGE, the electric field strength suddenly decreases and migration becomes slower, causing the sample analytes to be focused near the boundaries. Since the mobility of EOF is greater than those of the charged analytes, all analytes will finally move toward the detection window (the cations migrate faster than the anions). The analytes are separated by the CZE mode. In this method, the sample injection volume must be optimized because separation does not begin until after focusing and there must be enough length of capillary left for separation, otherwise analytes reach the detector unresolved.

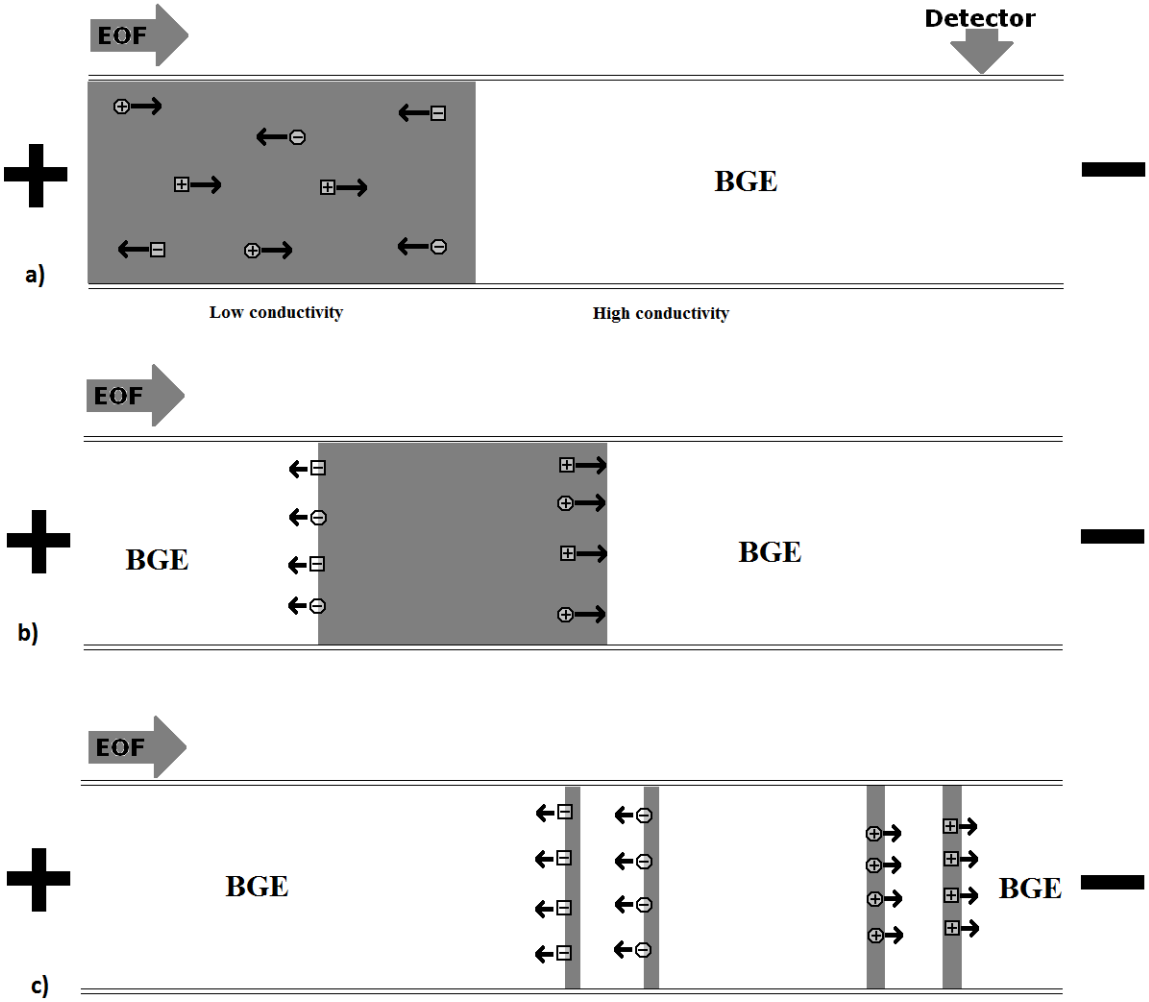


Figure 1-8. Schematic diagrams of the FASS technique. (a) The capillary is conditioned with a BGE (a high conductivity buffer), the sample, prepared in a low-conductivity matrix, is then injected to a certain length, and a high positive voltage is applied; (b) focusing of the analytes occurs near the boundaries between the sample zone and the BGE because of its mobility changes; (c) stacked ionic analytes only migrate and are separated by the CZE mode.

The optimal conditions for sample stacking is to prepare the sample in a buffer that concentration is at least 10 times less than BGE and a sample plug length up to 10 times the diffusion-limited peak width [107]. This technique has not been used for the neutral compounds because it relies on mobility of charged compounds. Since neutral analytes gain their electrophoretic mobility by forming complex with micelles, their velocities are always less than the micelles. Therefore, even at the optimum condition it is expected that the enrichment factor will be only a fraction of that is usually seen for charged compounds (less than one order of magnitude).

#### 1.4.1.2. Large Volume Sample Stacking (LVSS)

As it was explained before, injection of large volume of sample may result poor separation due to lack of required capillary length for separation. Chien and Burgi introduced a method in which larger volumes of sample could be injected into the capillary without adverse effect on separation efficiency. In their proposed method, the analytes would remain stacked while the sample matrix was removed.

This is essentially large-volume FASS with matrix removal, which is known within the literature as large-volume sample stacking (LVSS) [108]. LVSS and FASS are different in arrangement of voltage application and polarity. For example, in LVSS of anionic analytes, the electrode polarity is negative at the beginning to acquire a reversed EOF (Figure 1.9). The sample can be dissolved either in a low-conductivity buffer or water. When the capillary is filled with a high-conductivity BGE, a large amount of the sample

solution is then injected into the capillary and a negative polarity is applied. The anionic analytes move toward the detection end (outlet) and stack at one side of the boundary between the sample zone and the BGE while the direction of the EOF is toward the inlet. Only anions concentrated and the rest are lost, thus it is critical that the polarity of the system is reversed before the stacked anionic analyte zone exits the inlet of the capillary. The level of electrophoretic current is an indication of end of focusing process. In fact, the current should be monitored carefully until it reaches approximately 95–99% of its original value (current when capillary is filled just with BGE).

At this point the polarity is switched to positive to redirect the EOF toward the detector. There is always risk of losing analytes and poor reproducibility if the current is not well-monitored. Separation occurs by CZE. Compared to FASS, this method can provide a much larger sample injection without any significant loss in separation efficiency.

Later, Burgi [109] introduced a method in which polarity switching was not necessary. In this approach the direction of the EOF changes during the stacking and matrix removal process using an EOF reversal agent. The BGE containing an EOF reversal agent enters the capillary from the detection end of the capillary up to the point where the EOF in BGE is greater than the sample matrix. This is simpler than the polarity switching approach, because monitoring of the current is not necessary and no analytes are lost from the inlet. By using the same concept of manipulating EOF in the sample matrix and BGE,

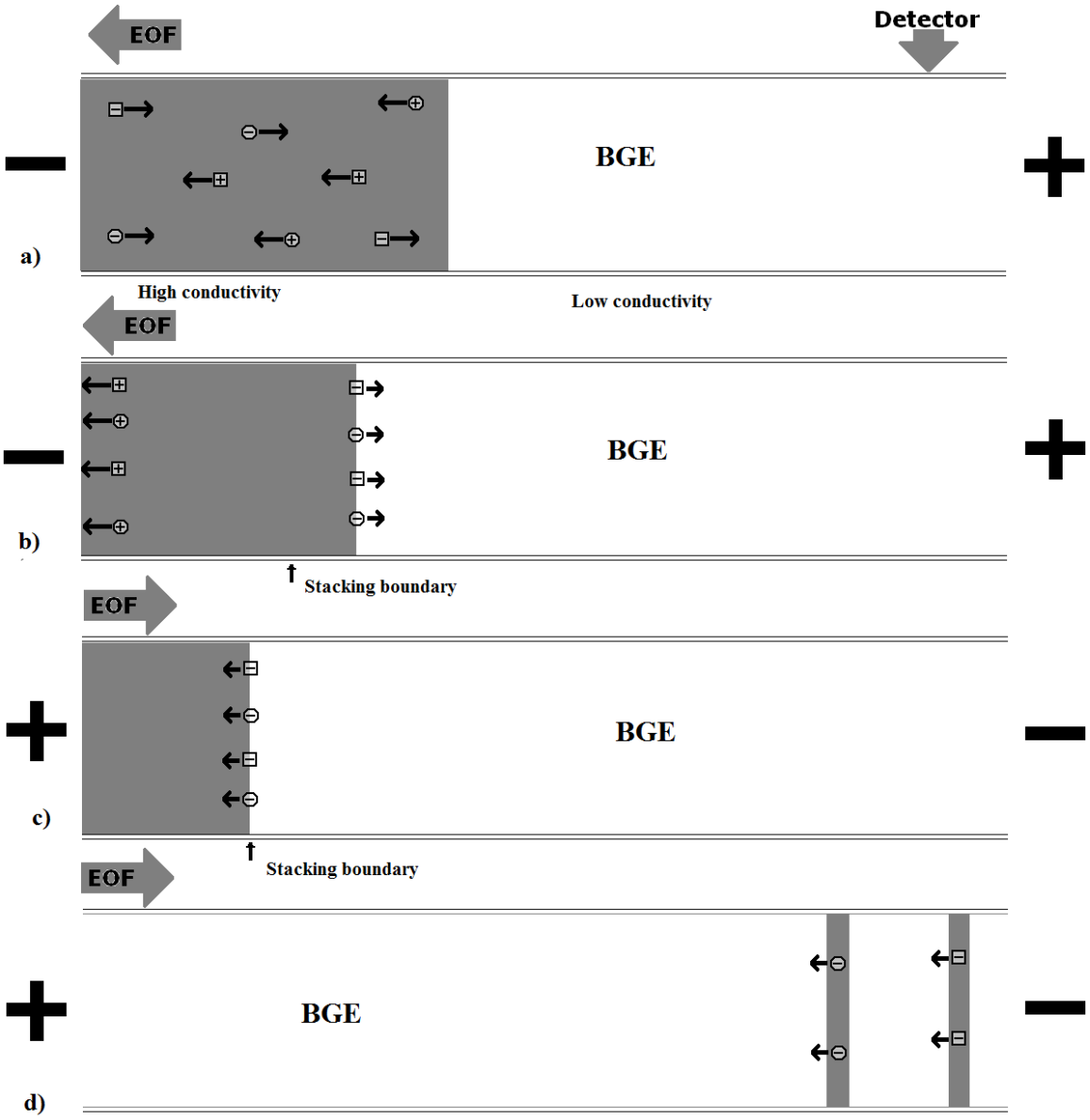


Figure 1-9. Schematic diagrams of the LVSS technique for anionic analytes.(a) The capillary is conditioned with a BGE (a high-conductivity buffer), the sample, prepared in a low-conductivity matrix, is then injected to a certain length, and then a high negative voltage is applied (EOF is toward the inlet); (b) the anionic analytes move toward the detection end (outlet) and stack at one side of the boundary, whereas the cations and

neutral species move and exit the capillary at the injection end (inlet); (c) the electrophoretic current is carefully monitored until it reaches approximately 95–99% of its original value, and the polarity is then quickly returned to positive (EOF is reversed); (d) the following separation occurs by CZE mode.

He and Lee [110] used a low pH separation electrolyte in combination with a high-pH sample zone to control matrix removal, Baryla and Lucy [111] used a zwitterionic surfactant, Macia *et al.* [112] used a high concentration of methanol (70%) to suppress the EOF, and Han et al. [113] used the phenomenon of pH hysteresis to control the EOF.

#### 1.4.1.3. pH-mediated Stacking

As described above, in FASS and LVSS methods the sample is prepared in low conductivity matrix, however; this is not always possible because some samples, such as sea water, urine or blood analytes, which contain salts, are usually highly conductive which limiting the application of above techniques. A pH-mediated stacking method has been developed for the samples in a high-ionic strength medium. In this method sample is injected into the capillary using electrokinetic injection (Figure 1.10) followed by a plug of strong acid, then a positive separation potential is applied. The strong acid neutralizes the sample solution to create a high-resistance zone (less ionic). As a result, a greater field will develop across the neutral zone, causing the ions to migrate faster. The analytes (positive charge ions) then are stacked at the boundary between the titrated zone and the BGE, and separation is by CZE[114].

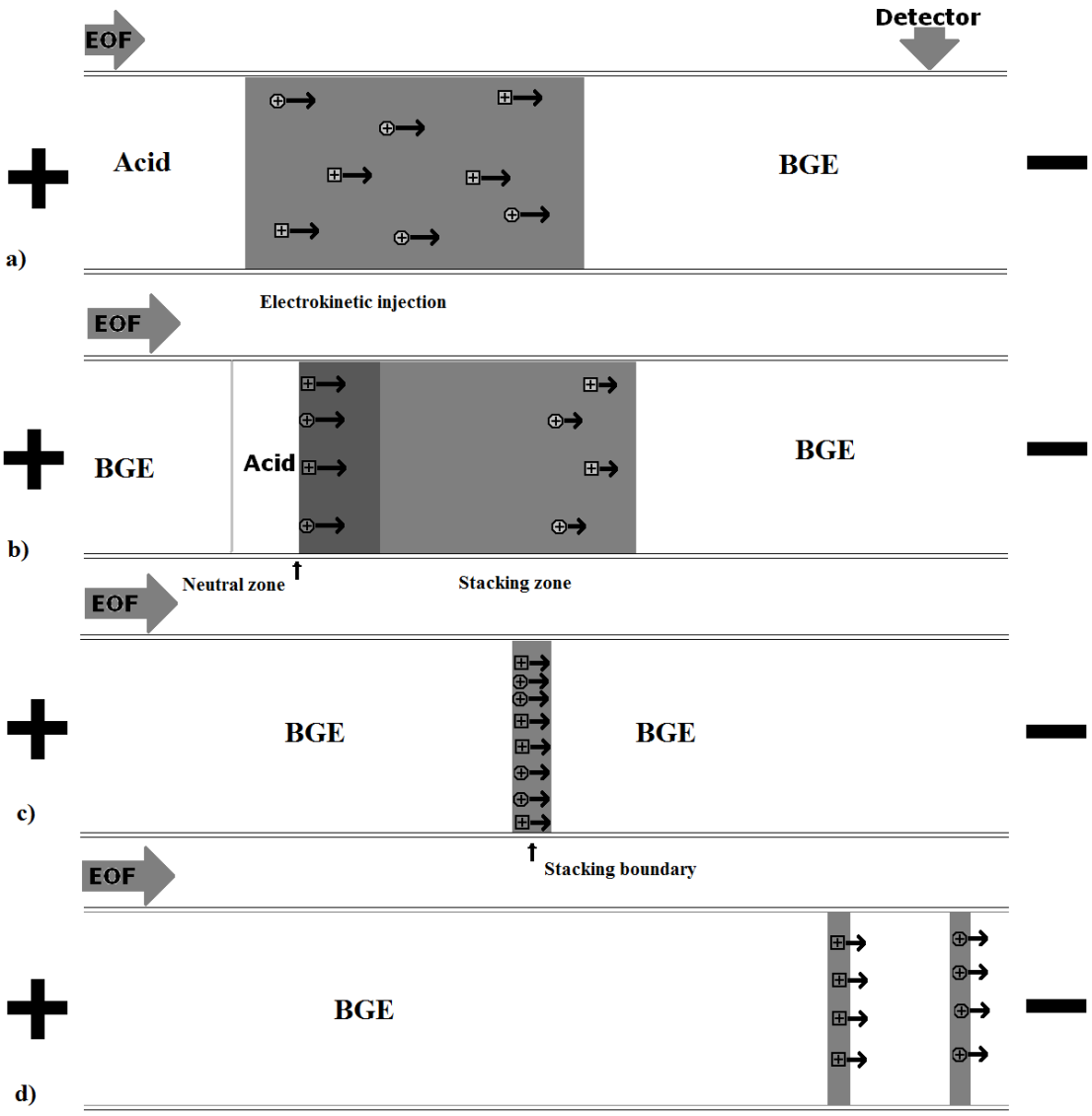


Figure 1-10. Schematic diagrams of a pH-mediated stacking technique. (a) The capillary is conditioned with a high conductivity BGE, the cationic analytes dissolved in a low-conductivity buffer and a plug of strong acid are injected into the capillary. (b) a positive separation potential is applied; (c) the strong acid titrates the sample solution to create a neutral zone causing the ions to migrate faster and become stacked; (d) the subsequent separation occurs by the CZE mode.

### 1.4.2. Stacking in MEKC

Stacking techniques have also applied to MEKC and are categorized into two modes, normal stacking method (NSM) and reversed electrode polarity stacking mode (REPSM). In first method, injection and separation are carried out in same polarity, while the second method requires switching of the polarity [115].

#### 1.4.2.1. Normal-stacking in MEKC

The stacking techniques described above are performed based on the CZE mode. Stacking in the MEKC mode is also possible when micelles are employed. Figure 1.11 shows schematic diagrams of the normal stacking-MEKC separation when SDS is used as surfactant. The sample is dissolved in a low-conductivity buffer or water; the BGE contains SDS (at concentration above the CMC) to form the micelles. After the BGE and sample solutions are injected in sequence, a positive voltage is applied. The SDS micelles enter the sample zone from the detector-side boundary, interact with analytes and the micelle-analyte complex undergoes stacking in the sample zone. Since the mobility of EOF is greater than that of the SDS micelles, the sample zone moves toward the detector. Following this, the SDS-analytes are separated by MEKC. Based on this principle, a cationic surfactant can also be used but the electrode polarity must be switched.

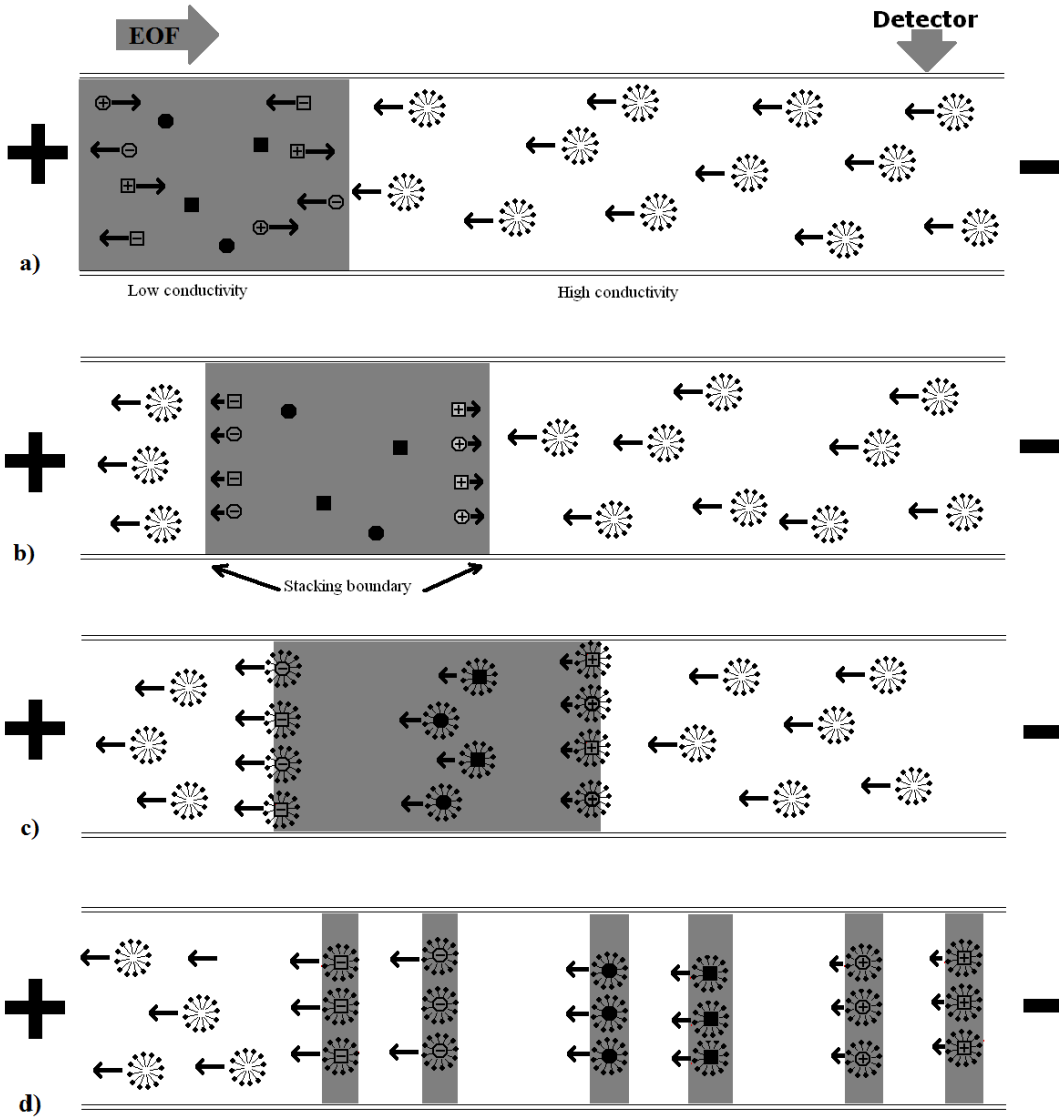


Figure 1-11. Schematic diagrams of a normal-stacking MEKC technique. (a) The sample is dissolved in a low-conductivity buffer. BGE consisting of SDS to form the micelles, after the background and sample solution are injected, respectively, a positive voltage is applied; (b) the SDS micelles from the detector-side enter the sample zone and then permit the analytes to migrate and become stacked; (c) then the SDS-analytes are separated by the MEKC mode. (d) separation is based on interaction of charged and uncharged analytes with micelles.

These methods can be used to separate either charged or neutral analyte molecules, and several different modes have been proposed MEKC mode when SDS micelles are used. Terabe *et al.* [116] published a comprehensive review of the conditions for various stacking modes.

#### 1.4.2.2. Reversed-stacking MEKC

The schematic diagrams for reversed-stacking MEKC, also called reversed electrode polarity stacking mode (REPSM), are shown in Figure 1.12. The sample is dissolved in a low-conductivity buffer, whereas a high-conductivity micellar buffer (such as SDS) is used as BGE. When a negative polarity is applied, the EOF moves toward the inlet; the anionic analytes move toward the outlet and stack at one side of the boundary, whereas the cations and neutral species move and exit the capillary at the injection end (inlet). When electrophoretic current reaches approximately 95–99% of its normal value, the polarity is quickly returned to positive, leading to the reversal of EOF. The micelles from the BGE will carry and stack the analytes at the stacking boundary and the analytes are separated by MEKC. Instead switching the electrode polarity, an acidic BGE can be used to reduce the EOF [103]. Thus, this method, the so-called stacking with reverse migrating micelles (SRMM), needs only negative polarity needed. As a result, a better reproducibility can be achieved, since a polarity-switching step is no longer necessary.

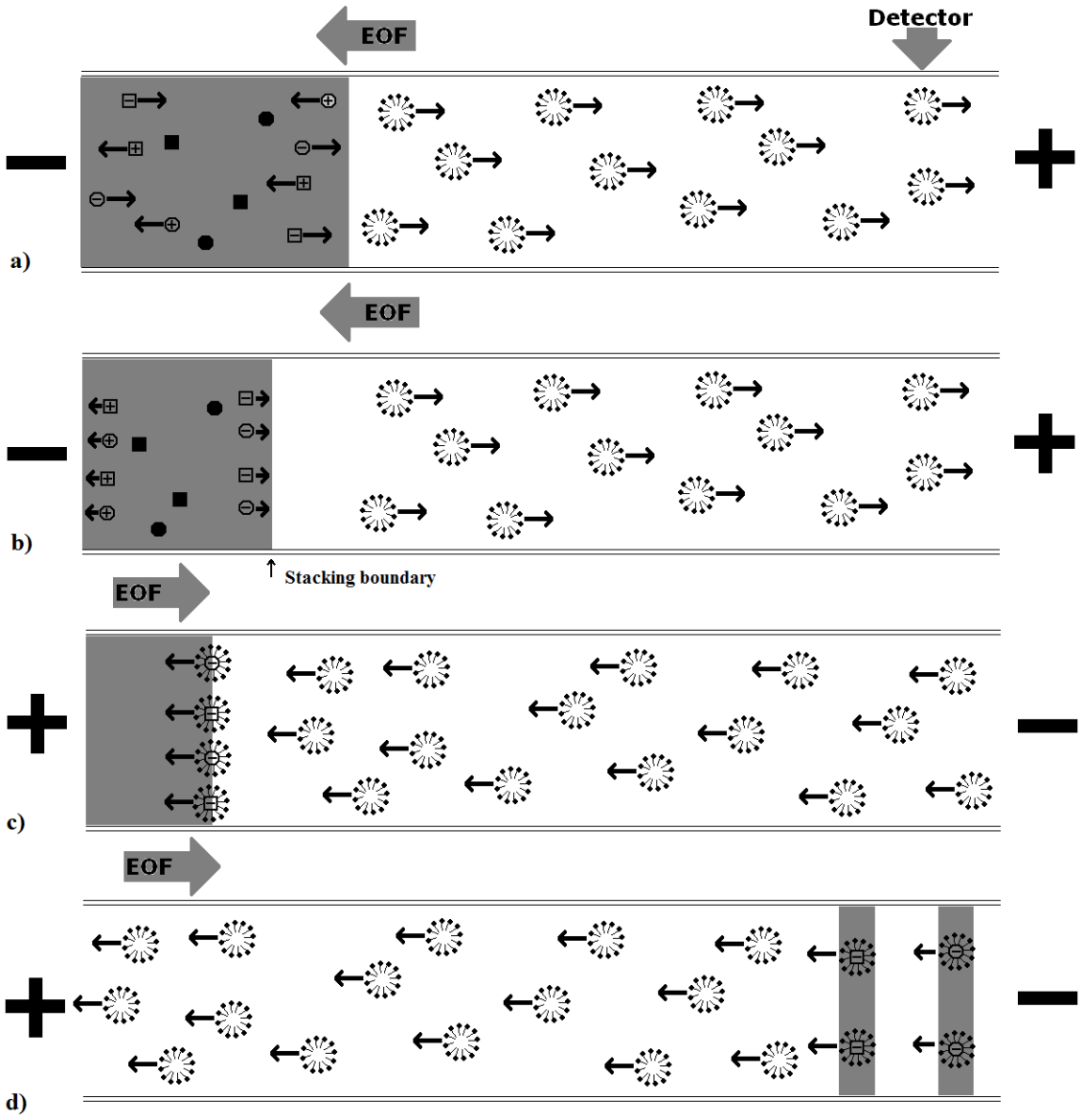


Figure 1-12 Schematic diagrams of a reversed-stacking MEKC technique. (a) The sample and BGE are prepared as described in Figure 1.11(A) but a negative polarity is applied; (b) the EOF moves toward the inlet, the anionic analytes move toward the outlet and stack at one side of the boundary; (c) the electrophoretic current reaches approximately 95–99% of its original value, the polarity is quickly returned to positive, reversing the EOF; (d) then the SDS-analytes are separated by the MEKC mode.

A series of reversed-stacking modes have been reported by Terabe *et al.*, including stacking using reversed-migrating micelles and a water plug (SRW) [101], field-enhanced sample injection (FESI) [102], and field-enhanced sample injection with reverse migrating micelles (FESI-RMM) [103]. Each of these methods has unique advantages and disadvantages with respect to sensitivity, precision, and simplicity of use.

### 1.4.3. Sweeping in MEKC

Sweeping is a simple and convenient on-line sample concentration method for either charged or neutral analytes. The efficiency of the sample enrichment relies on how the pseudostationary phase (PSP) interacts with the analytes when PSP enters the sample solution zone. In this method, sample matrix is free of surfactant while the BGE consists of relatively large amount of surfactant. A fused silica capillaries is normally filled with a low-pH BGE (usually <2) to suppress the EOF. A sample plug is injected and then a negative polarity is applied. As a result, anionic SDS micelles enter the capillary at the inlet and migrate toward the detector and sweep the analytes. Once the analytes are completely swept by micelles, the separation starts by MEKC (see Figure 1.13). In sweeping, the length of the resulting zone after sweeping ($l_{sweep}$) is given by [117]:

$$l_{sweep} = l_{inj} \frac{1}{1+k} \qquad 1\text{-}10$$

where $l_{inj}$ is the length of the injected sample zone and $k$ is the retention factor of the analyte for the given PSP. The $k$ value is assumed to be the same in the sample and the separation zone.

Concentration enhancement factors of 2 to 3 orders of magnitude have been reported for sweeping. In this method enhancement is highly depend on $k$ value, therefore it works better only if the analytes have a large $k$ value. This thesis will present possible ways to improve the $k$ value.

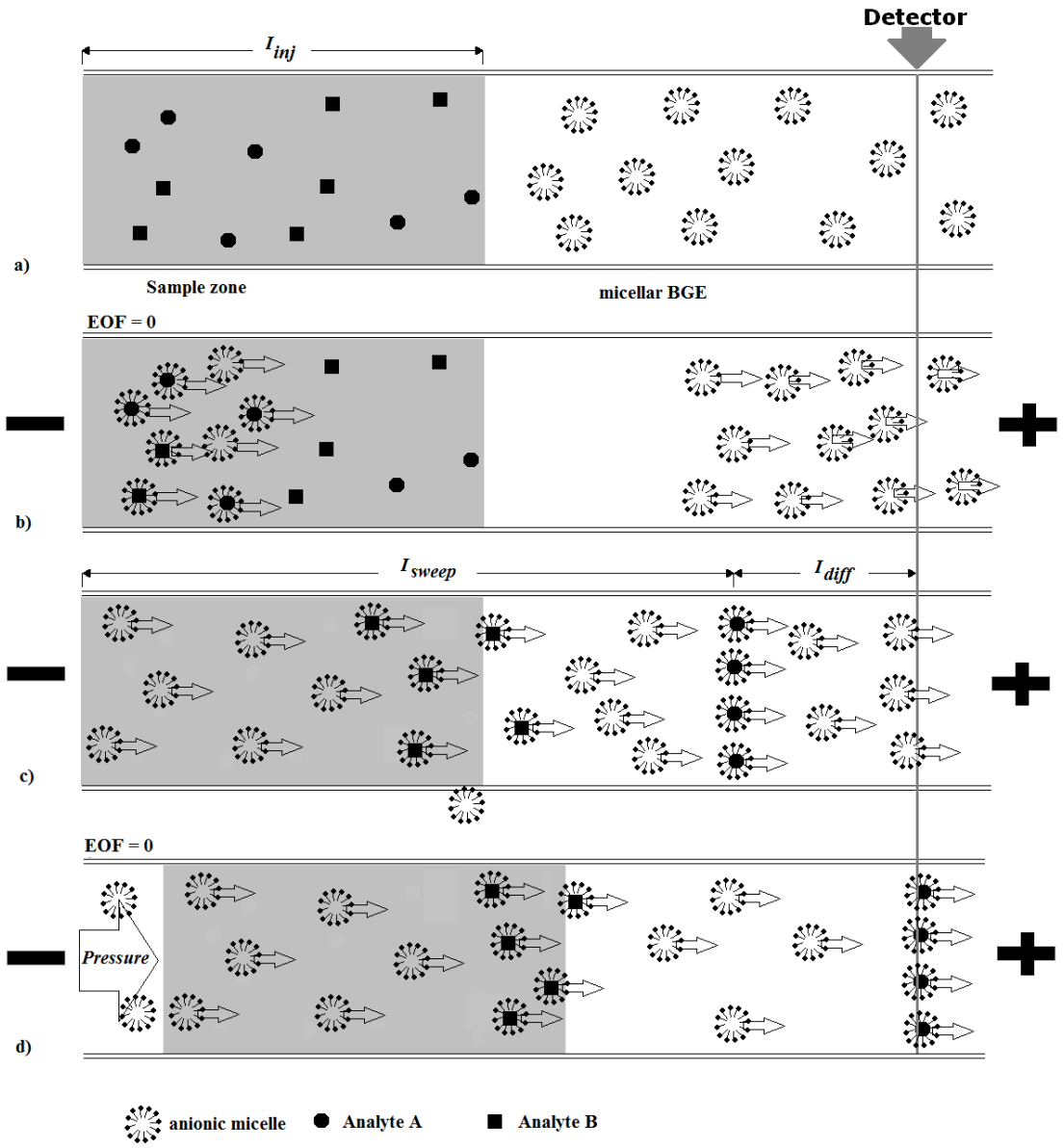


Figure 1-13. Schematic of a reversed-sweeping MEKC technique. The BGE consists of an anionic surfactant (for example, SDS) and a low-pH buffer solution, but the samples are dissolved in a micellar free buffer; (a) Capillary is conditioned and filled with the BGE and then sample solution is injected, (b) a negative polarity is applied to power the CE separation; (c) anionic SDS micelles are sweeping the analytes; the subsequent separation occurs by MEKC

#### 1.4.3.1. Cation-selective exhaustive injection seeping (CSEI-sweeping)

The CSEI-sweeping-MEKC technique was first reported by Terabe *et al.* [118]. This method provides for a more sensitive detection than sweeping for positively chargeable analytes (an increase in the detection limit of more than $10^5$-fold or more have been reported). First, the capillary is conditioned with a non-micellar BGE, followed by the injection of a high-conductivity buffer zone free of organic solvent, and finally the injection of a short plug of water (Figure 1-14). The cationic analytes are prepared in a low-conductivity matrix or water. Electrokinetic injection (a positive polarity) is used to introduce sample into the capillary. The cationic analytes enter the capillary through the water plug at high velocities, and are then focused (or stacked) at the interface between the water zone and the high-conductivity buffer. The continuous electrokinetic injection provides a high efficient sample concentration. Once an optimized injection time is reached, the injection is then stopped and the micellar BGEs are replaced at both ends of the capillary and the separation voltage is applied with a negative polarity. Therefore, micelles enter from the cathodic vial into the capillary to sweep the stacked analytes. Finally, the separation can be performed using MEKC in the reversed-migration mode.

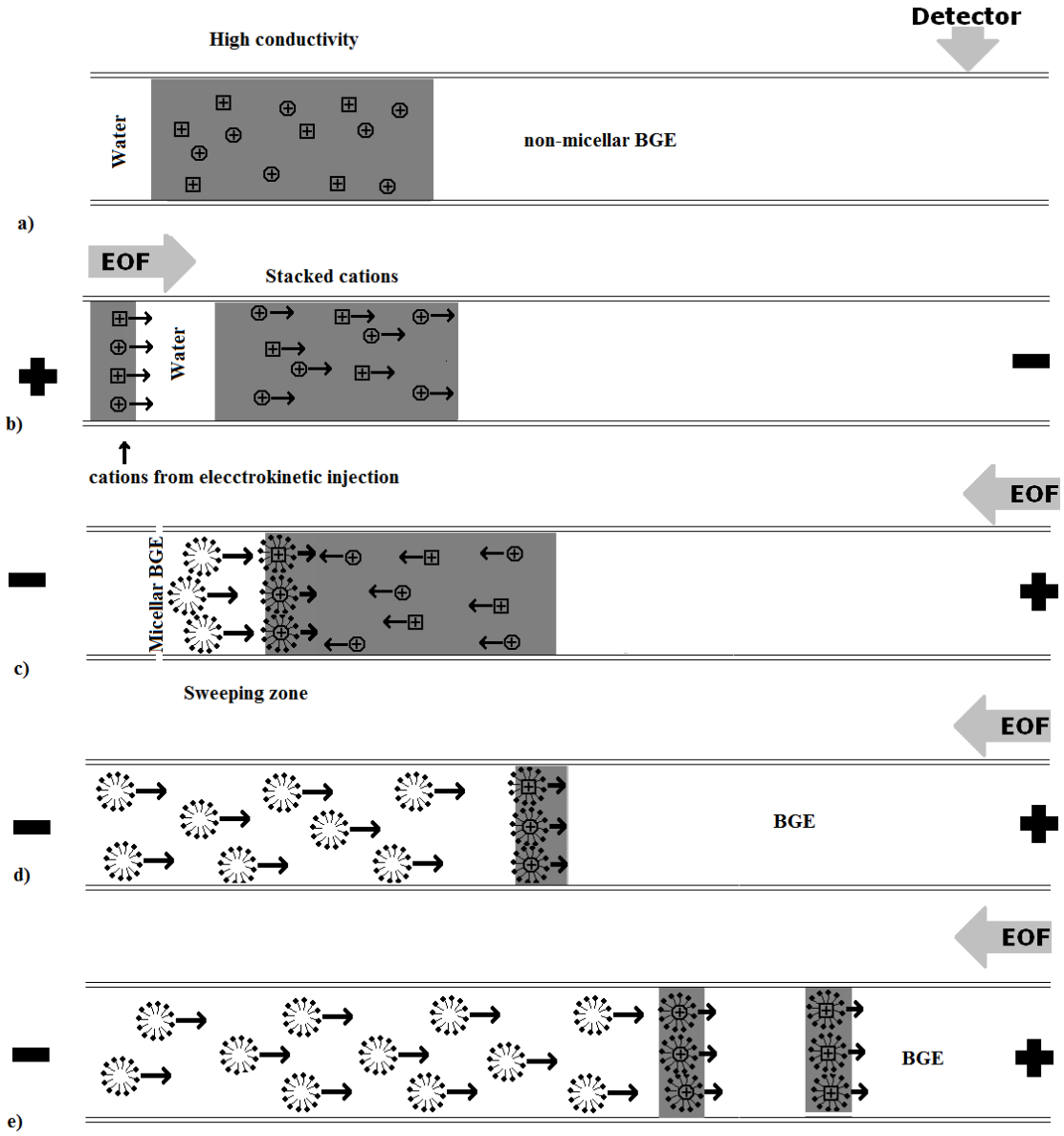


Figure 1-14- Schematic diagrams of CSEI-sweeping-MEKC technique. (a) The capillary is conditioned and filled with a non-micellar BGE, and then a high-conductivity buffer and a short plug of water are injected; (b) the a low-conductivity cationic analytes is electrokinetically injected. (c) Micellar BGEs are placed at both ends and the negative polarity voltage is applied; (d) the micelles enter the capillary to sweep the stacked analytes into narrow bands; (e) the following separation is achieved by the reversed MEKC mode.

### 1.4.3.2. Anion-selective exhaustive injection sweeping (ASEI-sweeping)

The principle of ASEI-sweeping-MEKC differs from CSEI-sweeping-MEKC in terms of the type of surfactant; a cationic surfactant is used instead of anionic one. Also, a polyacrylamide-coated (PAA) capillary is used to suppress the EOF. About 1000- to 6000-fold increases in detection sensitivity were obtained in terms of peak heights by ASEI-sweep–MEKC [119]. Figure 1-15 shows the steps of this technique. Application of cationic surfactant in PAA capillary is adapted from this method to be able to use cationic surfactants under suppressed EOF. In this method, a PAA-coated (low/zero EOF) capillary is conditioned with a non-micellar BGE, and then a plug of high conductivity buffer solution and a short water plug are injected. Sample is dissolved in water of a low-conductivity buffer solution and introduced by electrokinetic injection method under the negative polarity. Anionic analytes enter the capillary and pass the water plug and focused at the interface between the water and HCB zones. After completion of injection, BGEs containing cationic surfactant are placed at both ends of the capillary and then the voltage at the positive polarity is applied. Cationic micelles move toward the detector and sweep the analytes then MEKC separation is performed

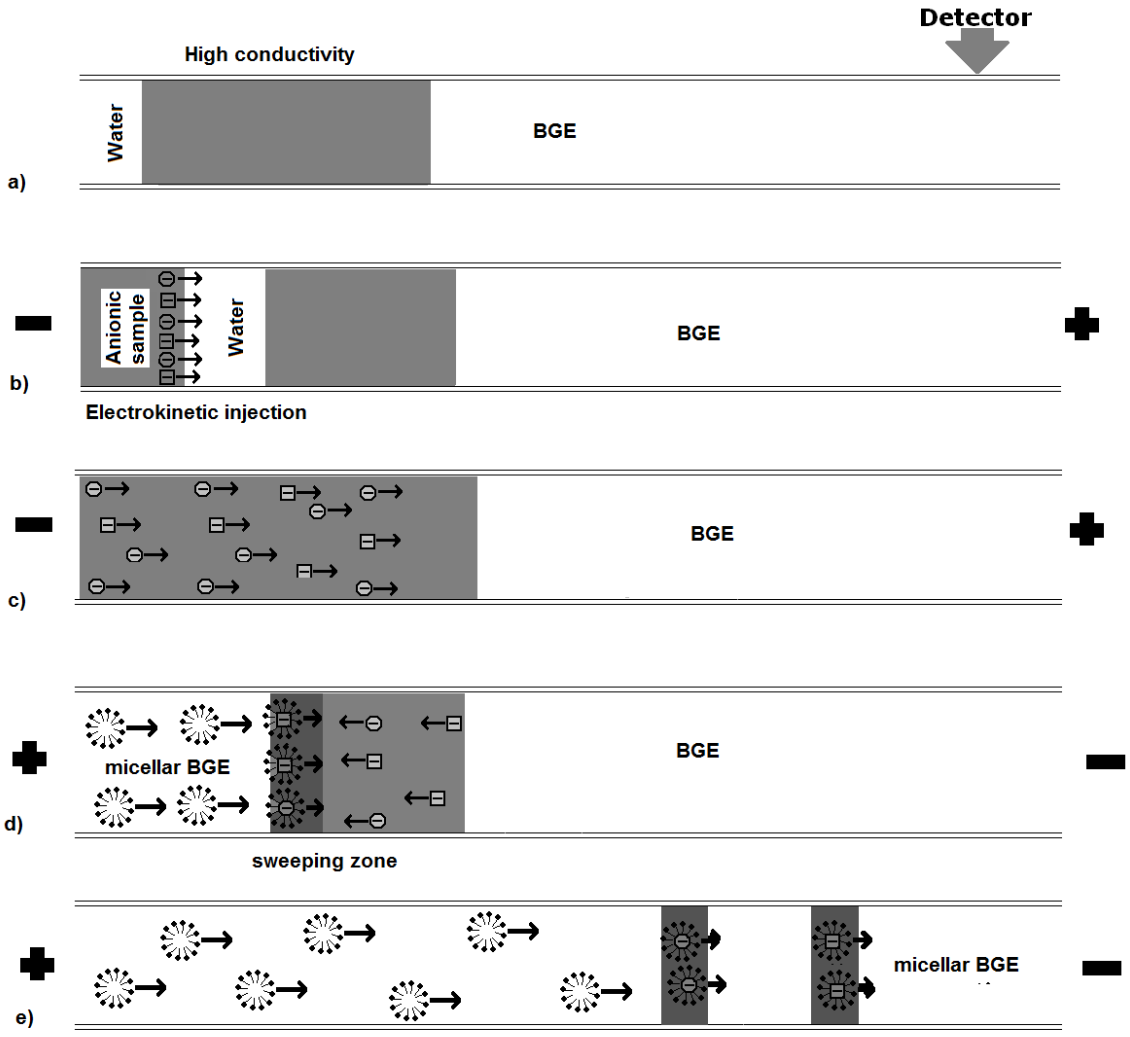


Figure 1-15. Schematic diagram of the ASEI-sweep-MEKC method: (a) PAA-coated (low/zero EOF) capillary is conditioned with a non-micellar BGE, then a high conductivity (HCB) buffer free of micelles is injected, followed by the injection of a short water plug. (b) Electrokinetic injection with negative polarity (c) Anionic analytes focus at the interface between the water and HCB zones. (d) Micellar BGEs are placed at both ends of the capillary followed by the application of voltage with positive polarity. (e) Separation by MEKC.

### 1.4.3.3. Dynamic pH junction

The main target analytes of this method are weak acidic or zwitterionic analytes by altering the mobility of the analytes at the interface of two solutions with different pH [120]. Figure 1.16 shows schematic diagrams of the dynamic pH junction method. The BGE consists of a high pH buffer solution, while the sample is dissolved in low pH (pH< $pK_a$ of analyte). First, the capillary is filled with the high pH BGE (borate buffer) followed by injection of the sample solution (lower pH). At the interface of the two solutions a pH junction forms. The analytes (weak acids) are neutral in acidic solution, while they are ionized (negatively charged) in alkaline solution. When a high positive voltage is applied, the non-uniform electrolyte zones form and $OH^-$ and $B(OH)_4^-$ ions from BGE, which move toward the sample zone and generate a reversed EOF. The analytes are focused at the pH junction. Subsequently, the analytes are separated by the CZE after focusing. The focusing effect is dependent on the pH, the concentrations (both of the BGE and the sample matrix), and p*K*a values of zwitterionic analytes.

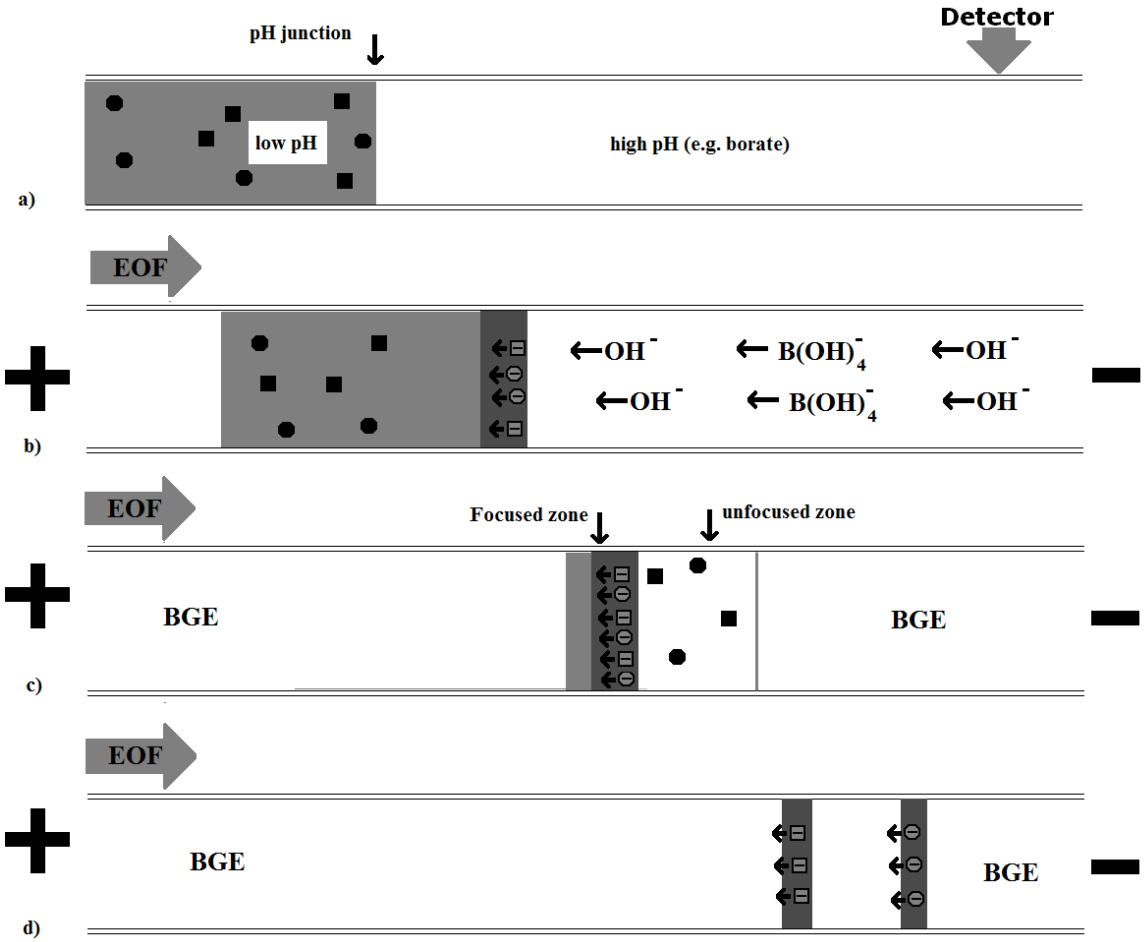


Figure 1-16. Schematic diagrams of a dynamic pH junction technique. (a) The capillary is filled with a high pH-BGE and a section of sample solution (prepared in a lower-pH buffer); (b) a high positive voltage is applied, resulting in a discontinuous electrolyte zone; (c) the anionic analytes are focused on the boundary of the pH junction; (d) separation of the analytes occurs by the CZE.

### 1.4.3.4. Dynamic pH junction and Dynamic pH junction sweeping

The dynamic pH junction- sweeping technique is very similar to the dynamic pH junction technique (The main target analytes of this method are weak acidic or zwitterionic analytes) except a surfactant is incorporated into the BGE. The sample is prepared in a non-micellar buffer solution and injected into the capillary filled with micellar BGE. A positive polarity for anionic surfactants or negative polarity for cationic surfactants are used. The $OH^-$ ions and anionic SDS micelles enter the capillary and sweep the sample zone. The neutral analytes (weak acids) are converted to anions and are swept by the SDS micelles followed by MEKC separation (Figure 1.17). Since the mobility of the EOF is greater than that of the SDS micelles, MEKC separation proceeds after focusing. These methods are potentially effective for conventional sweeping (using anionic micelles) and the dynamic pH junction for hydrophilic and neutral analytes [121].

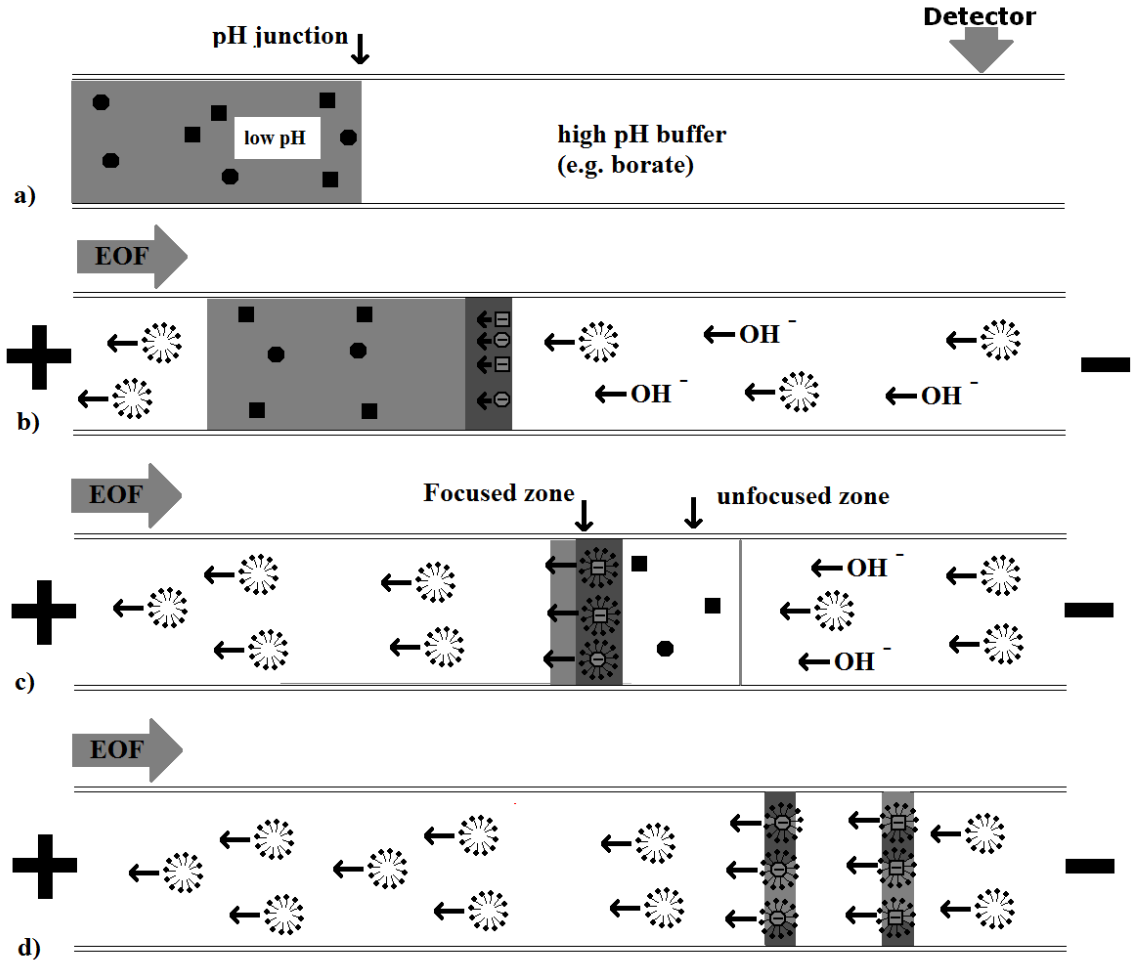


Figure 1-17. Schematic diagrams of the dynamic pH junction- sweeping technique. (a) The micellar (such as SDS) BGE and the sample solution (a non-micellar buffer) are injected into the capillary, respectively; (b) when the injection is complete, a positive polarity is applied (if a negatively charged SDS surfactant is used) to power the CE separation; (c) the neutral analytes are converted to anions and are swept by the SDS micelles; (d) separation occurs by MEKC.

### 1.4.4. Analyte Focusing by Micelle Collapse

In addition to above mentioned on-line sample concentration strategies, other on-capillary approaches has also been reported [122]. Micelle collapse focusing is based on the transport by micelles followed by release and accumulation of analytes. The sample molecules are carried first by micelles under an applied electric field in a micellar electrolyte solution containing an anionic surfactant along with an additional anion with a high electrophoretic mobility (small anion such as $Cl^-$). In this technique the capillary is filled by non-micellar BGE, and the sample is diluted in a micellar BGE. Micelles carry the analyte(s) into the capillary. The surfactant micelles are continuously diluted and at the boundary between micellar and non-micellar BEGs. When concentration of surfactant is below the CMC, micelles start to collapse. Therefore, as it shown in Figure 1.18 analytes are released and accumulate at the boundary and then a micellar BGE with high concentration of surfactant in placed at the inlet to introduce high concentration of surfactant and increase the concentration of surfactants above the CMC and separation begins. An enrichment of over two orders of magnitude has been reported in detection sensitivity for steroidal compounds using SDS [121].

The influence of the parameters affecting the performance of this approach has been evaluated by Quirino [123]. To maximize the volume of injected samples without compromising the enhancement factors, the concentration of surfactant micelle in the sample must be kept low and only slightly above the CMC, and the conductivity ratio

(BGE/sample) must be kept low. This method was employed in PF-MEKC (partially filling) with UV and ESI-MS detection. The results suggest that this strategy could provide better enhancement factors than sweeping for some less hydrophobic analytes [93, 124].

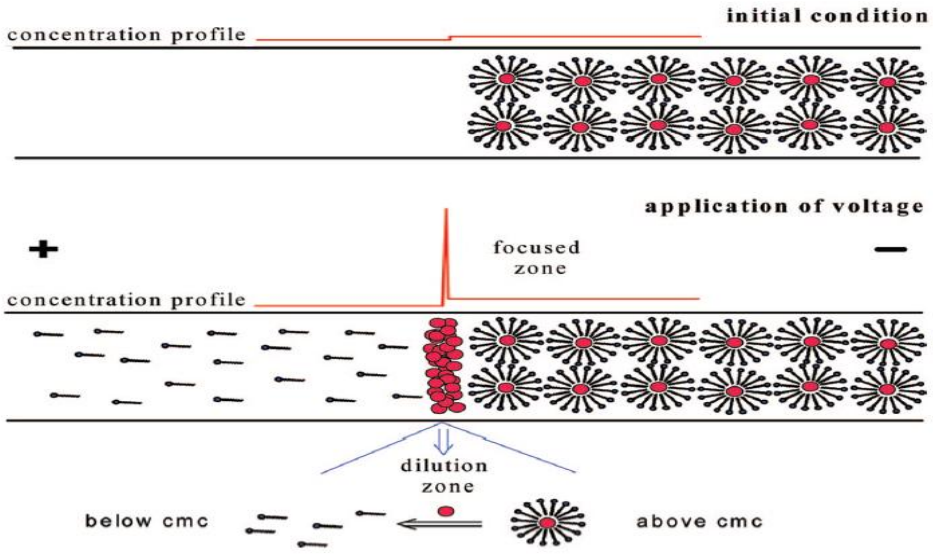


Figure 1-18. Analyte focusing by micelle collapse method.[122]

While current electrophoretic separation methods can provide high resolution separation for some analytes, the whole analytical process may be jeopardized if an inappropriate method is chosen for sample preparation prior to CE analysis. It is widely accepted that in case of complex matrices, sample preparation may be needed prior to CE or chromatography. The aim of the sample treatment is to enhance the analytical signal in order to improve the typically poor limits of detection (LODs) of CE-UV/Vis methods or to minimize potential interferences from the sample matrix.

## 1.5. Offline Sample Enrichment

The health risks of environmental contaminants have raised public awareness and led to intensive environmental research to monitor toxic contaminants in air, water, and soil. Highly selective and sensitive instruments for quantification still require the sample preparation step when the simple approach of "dilute and shoot" is not applicable for trace quantitation in environmental samples. Although improvements of detection limits may be accomplished by both detector improvement and in-capillary analyte focusing, analyte enrichment during a sample preparation step normally is the most practical concept to overcome the sensitivity problems of CE. Traditional sample-preparation techniques are frequently used, even today. Conventional solvent extraction methods often consume large amounts of solvents and creating more environmental and occupational hazards. The volume reduction step used in most extraction procedures can result in release of the solvents into the atmosphere. Chemists have responded by increasing research on sorbent traps, solid-phase extraction (SPE), supercritical $CO_2$ fluid extraction (SFE), pressurized fluid extraction (PFE), and microwave-assisted extraction. Micro-extraction techniques have earned their place in modern analytical laboratories, and solid-phase micro-extraction (SPME) was the first of these [125]. Since then, several other modern versions of solvent micro-extraction, including single solvent drop approaches and other related techniques [124]. The two most popular are single-drop micro-extraction (SDME) [126] and hollow-fibre liquid-phase micro-extraction (HF-LPME) [127]. The invention and the development of dispersive liquid-liquid micro-

extraction (DLLME) in 2006 is one of the latest examples [128-129]. Combination of offline sample enrichment techniques with CE, including solid phase extraction (SPE), SPME, micro-dialysis, liquid- liquid extraction [130] and recently single drop micro-extraction [131-132]have been reported.

Other areas of analytical science have been influenced by these developments in extraction technologies. For example, development of polydimethylsiloxane (PDMS)-coated SPME fibres led to the development of PDMS-based sensors [133]. Miniaturization of the extraction process (i.e. micro-SPE) is also attractive for use in on-site analytical measurements [134].

Understanding the fundamental principles of extraction is very important in the development of new and novel approaches in sample preparation. However, analytical researchers must also pay close attention in new technologies developed by engineers to find new and unique opportunities and applications [135-136]. For example, recent advancements in micro-machining and micro-fabrication are expected to have a profound impact on future analytical devices. The incorporation of sample preparation into miniaturized devices can result in simple elegant and efficient sample preparation and analysis systems. In next section, the theory common to the different extraction techniques will be presented, and future research opportunities in integration and miniaturization trends will be discussed.

### 1.5.1. Classification of Extraction Techniques

There is a fundamental similarity among the extraction techniques used in the sample preparation process. In all techniques, the extraction phase is in contact with the sample matrix and analytes are transported between the phases. There are three major extraction regimes namely, flow through, batch, and steady state (Figure 1.19). Each category has exhaustive and non-exhaustive methods. In principle, exhaustive extraction approaches do not require calibration, because analytes are transferred quantitatively (i.e. high recovery efficiencies) to the extraction phase by employing large volumes of receiving phase or phase with a phase with a high affinity for the analyte. In practice, however, to confirm the recovery, surrogate standards are usually used. In general, some exhaustive batch extraction methods such as liquid-liquid extraction (LLE) consume more solvent than any other methods, therefore to save solvent and time, they are frequently replaced by flow-through techniques such as sorbent trap techniques, e.g. SPE. Large volumes of sample can be passed through a small cartridge to increase the efficiency of mass transfer. Only a small volume of solvent is required to elute analytes from sorbent. As a result enrichment of analytes is achieved by using less solvent [137]. Similarly, solid samples can be packed in the bed and the extraction phase (liquid or gas) can be used to convey the analytes to the collection phase. In dynamic solvent extractions such as Soxhlet apparatus, the fresh solvent continuously elutes the analytes from the solid matrix at the boiling point of the solvent. Recently, smaller solvent volumes at higher temperatures and high pressures have been used. In these conditions, the solvent capacity and elution

strength is increased therefore greater enrichment at the same time of extraction can be achieved [138]. In SFE, compressed inert gas is used to extract the analytes from the sample matrix function in purge-and-trap modes.

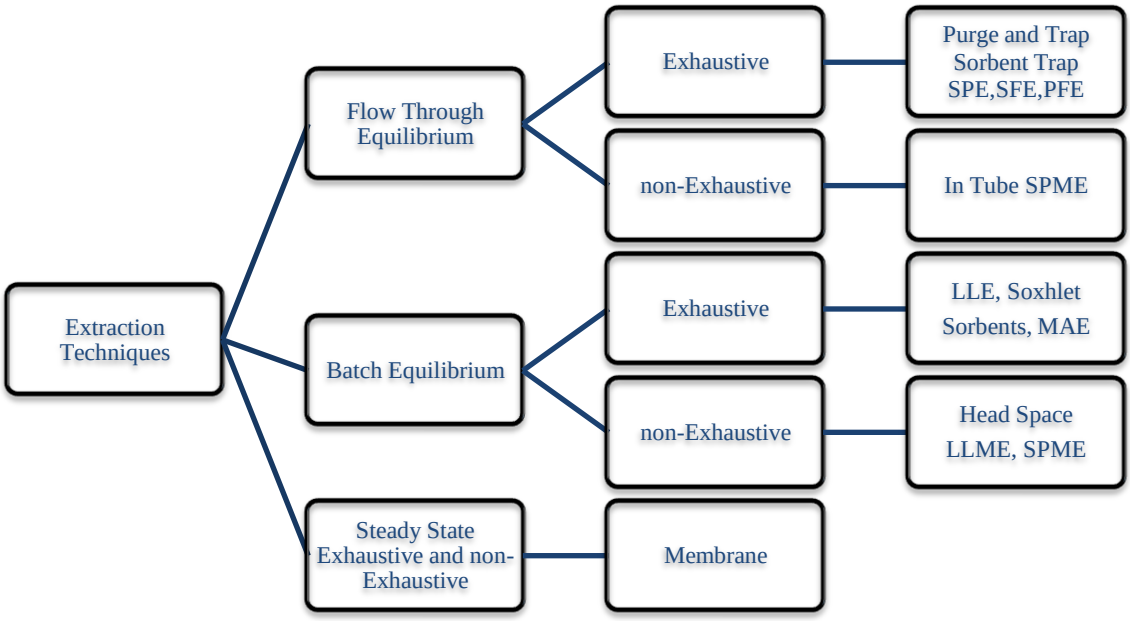


Figure 1-19. Extraction method classification [139]

Non-exhaustive methods mainly work based on the principles of equilibrium, pre-equilibrium, and permeation [140]. The equilibrium non-exhaustive techniques are similar to equilibrium-exhaustive techniques in principle, but due to the use of a small volume of the extracting phase relative to the sample volume, the capacity of the extraction phase is smaller. For example, in solvent micro-extraction and SPME techniques [141-142] the capacity of the extraction phase is usually insufficient to remove most of the analytes from the sample matrix.

### 1.5.2. Micro-extraction

Classical sample pre-treatment techniques (e.g., liquid-liquid extraction (LLE) and solid-phase extraction (SPE)) are time consuming, labor intensive, and use substantial amounts of hazardous organic solvents. Nevertheless liquid–liquid extraction (LLE), based on transfer of analyte between two immiscible solvents, is widely employed for sample preparation. The main disadvantage of LLE in ultra-trace analysis is the necessity of using large volume of very clean solvents and the inevitable subsequent solvent evaporation step needed to obtain significant preconcentration. Thus, this technique is both expensive and environmentally unfriendly. Miniaturization of this extraction technique can be achieved by a drastic reduction of the extracting phase volume. Based on this premise, several micro-extraction techniques have been developed, the most popular are single-drop micro-extraction (SDME) [126-127, 143], hollow-fibre liquid-phase micro-extraction (HF-LPME) [127], dispersive liquid-liquid micro-extraction (DLLME) [128-129], solid-phase micro-extraction (SPME) and liquid-phase micro-extraction (LPME). New LPME techniques based on SDME have been introduced in combination with GC, HPLC, and CE analysis. The high enrichment factor, low running cost, simple operation set-up, and trace-solvent consumption are part of LPME advantages. All this has been done with a single micro-syringe of several μL volume, which serves as both solvent holder and sample injector to perform the extraction procedure and extract injection [143-144]. Headspace liquid-phase micro-extraction (HS-LPME) [145], micro-dialysis (MD), supported liquid membranes (SLMs) were developed

to extend the scope of the applications in GC, HPLC, and CE analysis [146]. Nevertheless, all these extraction techniques have advantages and disadvantages. One of the objectives of the work presented in this thesis is to introduce a new micro-extraction technique for highly polar neutral compounds for application in modern analytical instruments such as GC, HPLC, and CE.

#### 1.5.2.1. Liquid Phase Micro-extraction (LPME)

Analytes extracted by LLE are dissolved in a water-immiscible organic phase which is of poor compatibility with analysis using reversed phase chromatography, CE, ESI-MS, etc. In CE, the use of aqueous electrophoretic buffers requires evaporating the most common solvent from LLE and subsequently re-dissolving the analyte in an appropriate aqueous or a miscible non-aqueous medium. One important goal in developing new sample preparation techniques is to automate the entire analytical process. For analytical extractions in miniaturization of conventional liquid–liquid extraction several systems such as liquid droplets at the tip of a capillary [147-150], supported liquid films/droplets [151] and continuous forming and falling drop systems [152-153], have been reported.

#### 1.5.2.2. Single Drop Micro-extraction (SDME)

SDME is a simple, low-cost, fast and virtually solvent-free sample preparation technique based on a great reduction of the extracting phase volume. SDME is not exhaustive, and only a small fraction of analyte(s) is extracted and pre-concentrated for analysis. From the

first publication in 1997 [143], SDME has been used as an extraction technique for numerous analytes, mostly organic compounds.

In SDME, a micro-drop of water-immiscible extracting phase suspended from the tip of a micro-syringe needle to a stirred aqueous sample. The drop is retracted back into the micro-syringe needle when extraction is done and finally injected into an analytical instrument or a detector to obtain the corresponding analytical signal. Since two liquid phases are in direct contact when one of the phases is mechanically stirred, the other will also experience convective mixing. In fact, the momentum transfers from first phase to the second phase as a result of frictional drag at the LL interface. Mass transfer of the analytes between two phases continues until thermodynamic equilibrium is attained or extraction is stopped. Jeannot and Cantwell introduced a theoretical model based on film theory of convective-diffusive mass transfer for SDME [143]. In the film theory, convective mixing exists in the bulk solution to some distance away from the liquid–liquid interface. There are some limitations for SDME; for example, extracting phase must be water-immiscible and analytes more soluble in the extracting phase than in the sample donor solution [154]. The instability of the drop at high stirring rates or temperatures, especially when samples are not perfectly clean is the main drawbacks of this technique. The presence of large amounts of non-polar species can saturate the organic phase.

Other modes of SDME have been reported, namely, headspace single drop micro-extraction (HS-SDME), liquid–liquid–liquid micro-extraction (LLLME) and continuous-flow micro-extraction (CFME) which will be discussed in more details.

#### 1.5.2.3. Headspace Single Drop Micro-extraction (HS-SDME)

Theis *et al.* first introduced the HS-SDME [145]. In this technique a micro-drop exposed to the headspace above the sample. Micro-extraction, preconcentration and derivatization in a single drop can be performed by exposing a hanging drop containing the derivatizing agent to the gaseous phase [155]. Mass transfer in the headspace is a fast process owing to the large diffusion coefficients in the gas phase (~$10^4$ greater than corresponding diffusion coefficients in condensed phases). Therefore, thermodynamic equilibrium between the aqueous and vapor phases can be achieved rapidly. The overall rate of mass transfer is limited by both the aqueous-phase stirring rate and the diffusion of analytes within the extraction phase [145]. Since non-volatile compounds and high molecular weight species are not extracted in the drop placed in the headspace, a high degree of extract clean-up can be achieved. When this micro-extraction mode is employed in conjunction with atomic detectors without prior GC separation, solvents do not need to have high vapor pressure.

#### 1.5.2.4. Liquid−Liquid−Liquid Micro-extraction (LLLME)

LLLME was developed by Ma and Cantwell in 1999 [156] under the name “solvent micro-extraction with simultaneous back-extraction”. LLLME is based on the extraction of analytes from the aqueous stirred sample into an organic layer or membrane with lower density than water and simultaneous back-extraction into an aqueous micro-drop. It is suitable for ionizable analytes. Direct convection (stirring) in one phase (aqueous sample) results in indirectly induced convection in the other two phases (organic layer and aqueous micro-drop) as a result of momentum transfer across both LL interfaces. First the pH of the aqueous solution is adjusted to obtain the neutral form of analytes (e.g. acidic), therefore analytes like carboxylic acids transfer to the organic phase. The pH of the aqueous interior micro-drop can be adjusted to obtain the ionized form of the analyte, extractable by the micro-drop aqueous phase. The concept can also be applied using complexing agents, where one is added to the sample solution and the other dissolved in the aqueous drop. In this way, the formation of a neutral complex allows its extraction into the organic layer. If the aqueous micro-drop contains a complexing agent which forms a stronger complex with the analyte, it can be back-extraction into the drop. Although LLLME is more specific and so difficult to implement compared to other SDME modes, it is very selective and can be combined with separation techniques such as RP-HPLC or CE.

### 1.5.2.5. Continuous-Flow Micro-extraction (CFME)

Continuous-flow micro-extraction first was reported by Liu and Lee in 2000 [157]. The extraction is carried out in a glass extraction chamber and sample is pumped continuously at a constant flow rate; when the extraction chamber is full of sample, a drop is formed at the tip of a micro-syringe needle. The rate of extraction increases with increasing flow rate of the aqueous solution, consistent with a decrease in thickness of the Nernst diffusion layer. Sample flow rate should be optimized to obtain an effective micro-extraction of analytes without drop dislodgement or bubble formation. To insure the stability of the drop at the tip of the needle samples should be perfectly clean. Cyclic-flow micro-extraction is a modification of this technique [158]. In this technique sample is pumped into the extraction chamber and after passing the chamber returns into the sample reservoir. There is no waste container and sample flows in a closed loop.

### 1.5.2.6. Hollow Fibre Liquid-phase Micro-extraction (HF-LPME)

In HF-LPME a hydrophobic hollow fibre is used to protect and expose a certain volume of extracting phase to the sample. The extraction process occurs in the pores of the hollow fibre, where the solvent is immobilized [127]. Hollow fibre liquid-phase micro-extraction is a simple and inexpensive technique which allows extraction and preconcentration of analytes from complex samples. In the two-phase LPME mode (HF-LPME), a water-immiscible extracting phase immobilized in the pores of a hollow fibre and in contact

with the analyte in an aqueous phase [159]. Hollow fibre typically made of polypropylene and supported by a micro-syringe. In this technique, the liquid acceptor phase is compatible with GC and HPLC to allow for coupling to chromatographic separation techniques. In the three-phase sampling mode, the analytes are extracted into an organic layer that fills the pores of the hollow fibre, and then are back-extracted into an aqueous phase placed inside the fibre. The three-phase mode (HF-LLLME) is limited to analytes with ionizable functionalities. As the acceptor phase is aqueous in this micro-extraction mode, the technique should be compatible with hyphenated techniques involving HPLC or CE. The pores of a porous hydrophobic polymer membrane are filled with an organic liquid [124]. The extracting phase should have a polarity matching that of the hollow fibre to be immobilized within its pores. In general, the extraction efficiency achieved with HF-LPME is higher than with SDME, because hollow fibres allow the use of vigorous stirring rates to accelerate the extraction kinetics. Moreover, the contact area between the aqueous sample and the extracting phase is higher than the drop in SDME. The use of the hollow fibre provides protection for the extracting phase, therefore; extraction of analytes from complex and unclean samples( e.g. blood, urin) is feasible. In fact, the small pore size of hollow fibre acts as micro-filters to clean the extracts. It should be noted that the manipulation of the hollow fibre at the time of placement at the tip of the needle could be a source of contamination.

### 1.5.2.7. Dispersive Liquid‑Liquid Micro-extraction (DLLME)

DLLME was introduced by Rezaee et al. in 2006 [129]. In DLLME, solvent is quickly injected into the aqueous sample to form a cloudy solution. The water-immiscible extracting phase solvent usually has a density higher density than water. Organic solvents with high density such as tetrachloromethane, chloroform, carbon disulfide, nitrobenzene, bromobenzene, chlorobenzene or 1,2-dichlorobenzene can be used. The fine droplets of extraction solvent are dispersed throughout the aqueous sample, creating high surface area for the phase boundary which allows equilibrium to be achieved quickly. A disperser solvent or emulsifier with high miscibility in both extracting phase and aqueous phase (such as methanol, ethanol, acetonitrile or acetone) is mixed with extracting phase. When rapidly injected into the sample, high turbulence is produced causing the formation of small droplets dispersed throughout the aqueous sample. The disperser solvent influences droplet size distribution, the mean droplet size, and emulsion viscosity. The cloudy solution is centrifuged to separate the extract. This technique is limited to a small number of extracting phases that efficiently extract the analytes of interest and have a higher density rather than water. This micro-extraction technique has been reported as difficult to automate.

In a new DLLME approach called “cold-induced aggregation micro-extraction” (CIAME) [160] an ionic liquid (IL), a non-ionic surfactant and a derivatizing reagent, if necessary, are added to an aqueous sample placed into a conical-bottom centrifuge tube. The

dissolution of the IL in the sample is achieved by heating the centrifuge tube in a thermostated bath. The centrifuge tube is then placed in an ice bath, and like DLLME, a cloudy solution forms. The subsequent procedure is similar to that for DLLME. Compared to DLLME, CIAME avoids the use of a disperser solvent, but it is time consuming because it introduces several steps before centrifugation [161].

#### 1.5.2.8. Characteristic of Extracting Phase

In micro-extraction techniques, selecting a suitable extracting phase is very important. For different solvents various properties should be considered. For example, a low phase ratio between extracting phase and sample and high distribution ratio, K, produce highest enrichment factors and extraction efficiencies. The K parameter depends on the type and nature of the extracting phase and analytes. Physical properties of the extracting phase such as boiling point, vapor pressure, water solubility, density, viscosity, surface tension, dipole moment and dielectric constant must be compared to select the best option for each method of extraction. Physical properties of some solvents are presented in Appendix A [132].

The selection of the extracting phase should be based on a comparisons of selectivity, extraction efficiency, incidence of drop loss, rate of drop dissolution (especially for faster stirring rates and extended extraction times; and level of toxicity [154]. A high boiling point reduces evaporative loses and bubble formation, which can take place inside the drop when a solvent with a low boiling point such as benzene is used. A high surface

tension increases the cohesive forces at the interface, hence reducing solvent solubilization.

In HS-SDME, in theory any extracting phase could be chosen to use as a micro-drop, but in practice the extracting phase should have a high boiling point and low vapor pressure, in addition to the ability to extract the analytes. In the case of HF-LPME, the extracting phase should have a low solubility in water to avoid its dissolution; low volatility to reduce evaporation of the solvent during extraction; a polarity matching that of the fibre to be (typically polypropylene) strongly immobilized within the pores of the hollow fibre to prevent leakage; and high extraction efficiency for the target analytes. In DLLME, the extracting phase should have low solubility in water; higher density than water; high affinity for the targeted compounds; and form a stable dispersion.

After learning from existing techniques and by focusing on advantages of individual techniques, two new approaches namely multi-phase single drop micro-extraction (MP-SDME) and continuous flow micro-extraction (CFME) are presented in this thesis. Since single drop micro-extraction is based on liquid-liquid extraction, in addition to general theory and some practical points of high importance are presented (Chapter 5). This review has been limited to enrichment of neutral polar compounds from aqueous samples prior to CE analysis.

Liquid-liquid extraction (LLE) is based on partitioning of solutes between two immiscible liquid phases. Our main focus is based on aqueous environmental samples as CE is one of

the best suited to aqueous samples. First, analytes are extracted to an organic phase then a second extraction that transfers analytes from organic phase to a fresh aqueous phase is carried out. This sample treatment technique enables the concentration and purification of analytes by aqueous to organic and back to aqueous phase with minimum solvent consumption.

## 1.6. Summary of Thesis Objectives

The main theme of this work is based on application of CE as an analytical technique for the analysis of highly polar contaminants, particularly *N*-nitrosamine, in aqueous environmental samples. Application of CE to this group of compounds has had some limitations.

First, due to the lack of intrinsic charge, an MEKC method must be developed and optimized for separation of *N*-nitrosamine; poor interaction between the analytes and micellar phase (due to high polarity of selected nitrosamines) demands an efficient surfactant system; different types of surfactants can be selected and their effects on separation efficiency compared.

Second, in general, CE suffers from poor sensitivity. This deficiency is highly-problematic when concentrations of target analytes (*N*-nitrosamine) are low, typical of environmental samples (ng $L^{-1}$). To overcome this limitation, online and offline preconcentration of analytes were studied. CE is not only a good technique for analysis of

contaminants - like *N*-nitrosamines - in aqueous environmental matrices, but also offers several online preconcentration methods. However, not all preconcentration techniques are compatible with MEKC and the polar, uncharged *N*-nitrosamines. To choose an existing online preconcentration technique or develop a new technique, the mechanism of existing online techniques as along with their similarities and differences were reviewed. Only those techniques that were compatible with the nature of our target analytes - neutral polar nitrosamines - were selected in this study. Since the interaction between analytes and micelles play the major rule in MEKC, understanding the mechanism of solute-micelle interactions is important. Based on the structure of nitrosamine it can be assumed that hydrogen bond interaction between analyte and PSP plays the major role. To develop a new approach of online preconcentration in MEKC for highly polar neutral compounds, a sweeping technique was selected as our foundation. After identifying the weakness of this technique for enrichment of highly polar compounds, the effect of different factors on the performance of this technique was studied. In sweeping of neutral compounds, the enrichment was more efficient when EOF was suppressed. Since the micelles are the only source of movement inside the capillary, stronger interactions between analytes and micelles would be required to avoid a large analysis time and consequently, excessive peak broadening for highly polar compounds. Since that is not easily attainable using simple RM-EKC, the application of auxiliary pressure in sweeping RM-EKC is presented in this research to overcome this problem and achieve a better enrichment.

Finally, since most of the environmental samples are not ready-to-inject samples, an extra clean up is sometimes required. The sample clean up is usually performed by an extraction method that is also considered as an offline preconcentration technique. Offline preconcentration techniques can be considered as an alternative or complementary technique for enrichment of *N*-nitrosamines. Many micro-extraction techniques have been introduced for sample treatment and enrichment. After comparing advantages and disadvantages of different micro-extraction techniques, single drop micro-extraction was selected for further study. A new single-drop multiphase micro-extraction compatible with MEKC is introduced and sustained by presenting a new technique called continuous flow single drop micro-extraction.

After developing a combination of offline- and/or online-MEKC techniques, analysis of seven nitrosamines in environmental samples (waste water) using the proposed method was implemented and compared with a conventional sample preparation technique (SPE) followed by MEKC.

## 1.7.References:

[1] A.L.Fridman, F. M. M., and S.S.Novikov, *Russ. Chem. Rev.* 1971, *40*, 34.

[2] Mageep, N. M., P. N., *ACTA UNIO INTERNATIONALIS CONTRA CANCRUM* 1959, *15*.

[3] Hadidian, Z., Fredrickson, T. N., Weisburger, E. K., Weisburger, J. H., Glass, R. M., Mantel, N., *J Natl Cancer Inst* 1968, *41*, 985-1036.

[4] Drabløs, F., Feyzi, E., Aas, P. A., Vaagbø, C. B., Kavli, B., Bratlie, M. S., Peña-Diaz, J., Otterlei, M., Slupphaug, G., Krokan, H. E., *DNA Repair* 2004, *3*, 1389-1407.

[5] Wagner, E. D., Osiol, J., Mitch, W. A., Plewa, M. J., *Environmental Science & Technology* 2014, *48*, 8203-8211.

[6] Bhangare, R. C., Sahu, S. K., Pandit, G. G., *Journal of Food Science and Technology* 2015, *52*, 507-513.

[7] Qian, Y., Wu, M., Wang, W., Chen, B., Zheng, H., Krasner, S. W., Hrudey, S. E., Li, X.-F., *Analytical Chemistry* 2015, *87*, 1330-1336.

[8] Simcox, N. J., Bracker, A., Ginsberg, G., Toal, B., Golembiewski, B., Kurland, T., Hedman, C., *Journal of Toxicology and Environmental Health, Part A* 2011, *74*, 1133-1149.

[9] Gan, X., Karanfil, T., Kaplan Bekaroglu, S. S., Shan, J., *Water Research* 2013, *47*, 1344-1352.

[10] Taguchi, V. Y., *Canadian Journal of Applied Spectrometry* 1994, *39*.

[11] Zhu, X. Q., He, J. Q., Li, Q., Xian, M., Lu, J., Cheng, J. P., *J Org Chem* 2000, *65*, 6729-6735.

[12] Kemper, J. M., Walse, S. S., Mitch, W. A., *Environ Sci Technol* 2010, *44*, 1224-1231.

[13] Zolfigol, M. A., Zebarjadian, M. H., Chehardoli, G., Keypour, H., Salehzadeh, S., Shamsipur, M., *J Org Chem* 2001, *66*, 3619-3620.

[14] Lijinsky, W., *Mutat Res* 1999, *443*, 129-138.

[15] Tricker, A. R., Preussmann, R., *Mutation Research/Genetic Toxicology* 1991, *259*, 277-289.

[16] Ikeda, K., *Journal of the Society of Cosmetic Chemists* 1990, *41*.

[17] Burdock, G. A., *International Journal of Toxicology (Taylor & Francis)* 2007, *26*, 51-55.

[18] Schuller, H. M., *Life Sciences* 2007, *80*, 2274-2280.

[19] Bryan, N. S., *Free Radical Bio Med* 2006, *41*, 691-701.

[20] Cui, M., Burton, H. R., Bush, L. P., Sutton, T. G., Crafts-Brandner, S. J., *J Agr Food Chem* 1994, *42*, 2912-2916.

[21] Lars Carlsen, B. N. K., Svetlana Ye. Batyrbekova, *Environ Health Insights* 2008, *1*, 11-20.

[22] Dai, N., Shah, A. D., Hu, L., Plewa, M. J., McKague, B., Mitch, W. A., *Environ Sci Technol* 2012, *46*, 9793-9801.

[23] Shah, A. D., Mitch, W. A., *Environ Sci Technol* 2012, *46*, 119-131.

[24] EPA, U., United State Environmental Protection Agency 2012.

[25] Kristiana, I., Tan, J., Joll, C. A., Heitz, A., von Gunten, U., Charrois, J. W. A., *Water Research* 2013, *47*, 535-546.

[26] Qian, Y., Wu, M., Wang, W., Chen, B.-B., Zheng, H., Krasner, S. W., Hrudey, S. E., Li, X.-F., *Analytical Chemistry* 2014.

[27] Choi, J., Valentine, R. L., *Environmental Science & Technology* 2003, *37*, 4871-4876.

[28] Chen, Z., Valentine, R. L., *Environmental Science & Technology* 2006, *40*, 7290-7297.

[29] Chen, Z., Valentine, R. L., *Environmental Science & Technology* 2007, *41*, 6059-6065.

[30] Dai, N., Mitch, W. A., *Environmental Science & Technology* 2013, *47*, 3648-3656.

[31] Mestankova, H., Schirmer, K., Canonica, S., von Gunten, U., *Water Research* 2014, *66*, 399-410.

[32] Fridman, A. L., Mukhametshin, F. M., Novikov, S. S., *Usp. Khim.* 1971, *40*, 64-94.

[33] Cowley, E. G., Partington, J. R., *J. Chem. Soc.* 1933, 1255-1257.

[34] George, M. V., Wright, G. F., *J. Am. Chem. Soc.* 1958, *80*, 1200-1204.

[35] Korsunskii, B. L., Pepekin, V. I., Lebedev, Y. A., Apin, A. Y., *Izv. Akad. Nauk SSSR, Ser. Khim.* 1967, 525-528.

[36] Rademacher, P., Stoelevik, R., Luettke, W., *Angew. Chem., Int. Ed. Engl.* 1968, *7*, 806.

[37] Looney, C. E., Phillips, W. D., Reilly, E. L., *J. Am. Chem. Soc.* 1957, *79*, 6136-6142.

[38] Karabatsos, G. J., Taller, R. A., *J. Am. Chem. Soc.* 1964, *86*, 4373-4378.

[39] Djerassi, C., Lund, E., Bunnenberg, E., Sjoberg, B., *J. Am. Chem. Soc.* 1961, *83*, 2307-2312.

[40] Layne, W. S., Jaffe, H. H., Zimmer, H., *J. Am. Chem. Soc.* 1963, *85*, 435-438.

[41] Layne, W. S., Jaffe, H. H., Zimmer, H., *J. Am. Chem. Soc.* 1963, *85*, 1816-1820.

[42] Taguchi, V. Y., *Canadian Journal of Applied Spectrometry* 1994, *39*.

[43] Weast, R. C., *CRC Handbook of Chemistry and Physics - 66th Edition*, CRC Press, Boca Raton, Florida 1985.

[44] Stephany, R. W., Schuller, P.L., *Oncology.* 1980, *37*, 203-210.

[45] Spiegelhalder, B., Eisenbrand, G., Preussmann, R., *Oncology* 1980, *37*, 211-216.

[46] Yamamoto, M., Iwata, R., Ishiwata, H., Yamada, T., Tanimura, A., *Food and Chemical Toxicology* 1984, *22*, 61-64.

[47] Song, P. J., Hu, J. F., *Food and Chemical Toxicology* 1988, *26*, 205-208.

[48] Ng, C. L., Ong, C. P., Lee, H. K., Li, S. F. Y., *Journal of Chromatographic Science* 1994, *32*, 121-125.

[49] Bell, L. M., Murray, G. M., *J Chromatogr B* 2005, *826*, 160-168.

[50] Breadmore, M. C., *Electrophoresis* 2007, *28*, 254-281.

[51] Dabek-Zlotorzynska, E., Celo, V., Yassine, M. M., *Electrophoresis* 2008, *29*, 310-323.

[52] Tiselius, A., Kabat, E. A., *Science* 1938, *87*, 416-417.

[53] Tiselius, A., *Biochem J* 1937, *31*, 313-317.

[54] Tiselius, A., *Transactions of the Faraday Society* 1937, *33*, 524-531.

[55] Hjerten, S., *Arkiv foer Kemi* 1958, *13*, 2.

[56] Jorgenson, J. W., Lukacs, K. D., *Clin Chem* 1981, *27*, 1551-1553.

[57] Kim, J. B., Terabe, S., *J Pharm Biomed Anal* 2003, *30*, 1625-1643.

[58] Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T., *Analytical Chemistry* 1984, *56*, 111-113.

[59] Cui, X., Mao, S., Liu, M., Yuan, H., Du, Y., *Langmuir* 2008, *24*, 10771-10775.

[60] Terabe, S., Otsuka, K., Ando, T., *Analytical Chemistry* 1985, *57*, 834-841.

[61] Jandik, P. B., Gunther; Drossman, Howard, *Capillary Electrophoresis of Small Molecules and Ions*, VCH, New York, N. Y. 1993.

[62] Nishi, H., Tsumagari, N., Terabe, S., *Analytical Chemistry* 1989, *61*, 2434-2439.

[63] Sadek, P. C., Carr, P. W., Doherty, R. M., Kamlet, M. J., Taft, R. W., Abraham, M. H., *Anal Chem* 1985, *57*, 2971-2978.

[64] Yang, S. Y., Khaledi, M. G., *Journal of Chromatography A* 1995, *692*, 301-310.

[65] Bumgarner, J. G., Khaledi, M. G., *Electrophoresis* 1994, *15*, 1260-1266.

[66] Nishi, H., Fukuyama, T., Matsuo, M., Terabe, S., *J Pharm Sci* 1990, *79*, 519-523.

[67] Carr, P. W., Doherty, R. M., Kamlet, M. J., Taft, R. W., Melander, W., Horvath, C., *Anal Chem* 1986, *58*, 2674-2680.

[68] Kim, J. B., Okamoto, Y., Terabe, S., *J Chromatogr A* 2003, *1018*, 251-256.

[69] Threeprom, J., Som-aum, W., Lin, J.-M., *Chinese Journal of Chemistry* 2006, *24*, 1747-1753.

[70] Zhang, X., Stuart, J., Sweedler, J., *Analytical and Bioanalytical Chemistry* 2002, *373*, 332-343.

[71] Hilmi, A., Luong, J. H. T., *Analytical Chemistry* 2001, *73*, 2536-2540.

[72] Sun, J., Xu, X., Wang, C., You, T., *Electrophoresis* 2008, *29*, 3999-4007.

[73] Ramautar, R., de Jong, G. J., Somsen, G. W., *Electrophoresis* 2012, *33*, 243-250.

[74] Heiger, D. N., Kaltenbach, P., Sievert, H. J., *Electrophoresis* 1994, *15*, 1234-1247.

[75] Jing, P., Kaneta, T., Imasaka, T., *Electrophoresis* 2002, *23*, 550-555.

[76] Jing, P., Kaneta, T., Imasaka, T., *J Chromatogr A* 2002, *959*, 281-287.

[77] Stutz, H., *Electrophoresis* 2005, *26*, 1254-1290.

[78] Akbay, C., Rizvi, S. A., Shamsi, S. A., *Anal Chem* 2005, *77*, 1672-1683.

[79] Silva, M., *Electrophoresis* 2007, *28*, 174-192.

[80] Molina, M., Wiedmer, S. K., Jussila, M., Silva, M., Riekkola, M. L., *J Chromatogr A* 2001, *927*, 191-202.

[81] Stubberud, K., Forsberg, A., Callmer, K., Westerlund, D., *Electrophoresis* 2002, *23*, 572-577.

[82] Petersson, P., Jornten-Karlsson, M., Stalebro, M., *Electrophoresis* 2003, *24*, 999-1007.

[83] Van Biesen, G., Bottaro, C. S., *Electrophoresis* 2006, *27*, 4456-4468.

[84] Tseng, M. C., Chen, Y. R., Her, G. R., *Anal Chem* 2004, *76*, 6306-6312.

[85] Isoo, K., Otsuka, K., Terabe, S., *Electrophoresis* 2001, *22*, 3426-3432.

[86] Mol, R., de Jong, G. J., Somsen, G. W., *Anal Chem* 2005, *77*, 5277-5282.

[87] Mol, R., de Jong, G. J., Somsen, G. W., *Electrophoresis* 2005, *26*, 146-154.

[88] Serrano, J. M., Silva, M., *Electrophoresis* 2006, *27*, 4703-4710.

[89] Tseng, W. L., Hsu, C. Y., Wu, T. H., Huang, S. W., Hsieh, M. M., *Electrophoresis* 2009, *30*, 2558-2564.

[90] Tsai, C. C., Liu, J. T., Shu, Y. R., Chan, P. H., Lin, C. H., *J Chromatogr A* 2006, *1101*, 319-323.

[91] Gebauer, P., Bocek, P., *Electrophoresis* 2009, *30 Suppl 1*, S27-33.

[92] Kartsova, L. A., Bessonova, E. A., *Journal of Analytical Chemistry* 2009, *64*, 326-337.

[93] Quirino, J. P., Haddad, P. R., *Electrophoresis* 2009, *30*, 1670-1674.

[94] Quirino, J. P., *Electrophoresis* 2009, *30*, 875-882.

[95] Kazarian, A. A., Hilder, E. F., Breadmore, M. C., *Journal of Separation Science* 2011, *34*, 2800-2821.

[96] Aranas, A. T., Guidote Jr, A. M., Quirino, J. P., *Analytical & Bioanalytical Chemistry* 2009, *394*, 175-185.

[97] Sentellas, S., Puignou, L., Galceran, M. T., *Journal of Separation Science* 2002, *25*, 975-987.

[98] Quirino, J. P., Terabe, S., *J Capillary Electrophor* 1997, *4*, 233-245.

[99] Quirino, J. P., Terabe, S., *Journal of Chromatography A* 1997, *791*, 255-267.

[100] Quirino, J. P., Terabe, S., *Analytical Chemistry* 1998, *70*, 149-157.

[101] Quirino, J. P., Otsuka, K., Terabe, S., *J Chromatogr B Biomed Sci Appl* 1998, *714*, 29-38.

[102] Quirino, J. P., Terabe, S., *Journal of Chromatography A* 1998, *798*, 251-257.

[103] Quirino, J. P., Terabe, S., *Anal Chem* 1998, *70*, 1893-1901.

[104] Dawod, M., Breadmore, M. C., Guijt, R. M., Haddad, P. R., *Journal of Chromatography A* 2010, *1217*, 386-393.

[105] Kim, J.-B., Quirino, J. P., Otsuka, K., Terabe, S., *Journal of Chromatography A* 2001, *916*, 123-130.

[106] Chien, R. L., Burgi, D. S., *J. Chromatogr.* 1991, *559*, 153-161.

[107] Burgi, D. S., Chien, R. L., *Anal. Chem.* 1991, *63*, 2042-2047.

[108] Chien, R. L., Burgi, D. S., *Anal. Chem.* 1992, *64*, 1046-1050.

[109] Burgi, D. S., *Anal. Chem.* 1993, *65*, 3726-3729.

[110] He, Y., Lee, H. K., *Anal Chem* 1999, *71*, 995-1001.

[111] Baryla, N. E., Lucy, C. A., *Electrophoresis* 2001, *22*, 52-58.

[112] Macia, A., Borrull, F., Aguilar, C., Calull, M., *Electrophoresis* 2004, *25*, 428-436.

[113] Han, J. H., Chun, M. S., Riaz, A., Chung, D. S., *Electrophoresis* 2005, *26*, 480-486.

[114] Osbourn, D. M., Weiss, D. J., Lunte, C. E., *Electrophoresis* 2000, *21*, 2768-2779.

[115] Quirino, J. P., Terabe, S., *Anal Chem* 1998, *70*, 149-157.

[116] Simpson, S. L., Jr., Quirino, J. P., Terabe, S., *J Chromatogr A* 2008, *1184*, 504-541.

[117] Quirino, J. P., Terabe, S., *Analytical Chemistry* 1999, *71*, 1638-1644.

[118] Quirino, J. P., Terabe, S., *Analytical Chemistry* 2000, *72*, 1023-1030.

[119] Kim, J.-B., Otsuka, K., Terabe, S., *Journal of Chromatography A* 2001, *932*, 129-137.

[120] Kim, J. B., Britz-McKibbin, P., Hirokawa, T., Terabe, S., *Anal Chem* 2003, *75*, 3986-3993.

[121] Britz-McKibbin, P., Markuszewski, M. J., Iyanagi, T., Matsuda, K., Nishioka, T., Terabe, S., *Analytical Biochemistry* 2003, *313*, 89-96.

[122] Quirino, J. P., Haddad, P. R., *Anal Chem* 2008, *80*, 6824-6829.

[123] Quirino, J. P., *J Chromatogr A* 2008, *1214*, 171-177.

[124] Pawliszyn, J., Editor, *Comprehensive Analytical Chemistry, Volume 37. (Sampling and Sample Preparation for Field and Laboratory: Fundamentals and New Directions in Sample Preparation.)*, Elsevier Science B.V. 2002.

[125] Risticevic, S., Lord, H., Gorecki, T., Arthur, C. L., Pawliszyn, J., *Nat Protoc* 2010, *5*, 122-139.

[126] Jeannot, M. A., Cantwell, F. F., *Anal Chem* 1996, *68*, 2236-2240.

[127] Pedersen-Bjergaard, S., Rasmussen, K. E., *Anal Chem* 1999, *71*, 2650-2656.

[128] Berijani, S., Assadi, Y., Anbia, M., Milani Hosseini, M. R., Aghaee, E., *J Chromatogr A* 2006, *1123*, 1-9.

[129] Rezaee, M., Assadi, Y., Milani Hosseini, M. R., Aghaee, E., Ahmadi, F., Berijani, S., *J Chromatogr A* 2006, *1116*, 1-9.

[130] Boone, C. M., Waterval, J. C., Lingeman, H., Ensing, K., Underberg, W. J., *J Pharm Biomed Anal* 1999, *20*, 831-863.

[131] Pena-Pereira, F., Lavilla, I., Bendicho, C., Vidal, L., Canals, A., *Talanta* 2009, *78*, 537-541.

[132] Pena-Pereira, F., Lavilla, I., Bendicho, C., *Anal Chim Acta* 2009, *631*, 223-228.

[133] Stahl, D. C., Tilotta, D., *Infrared Spectroscopic Detection for SPME*, Royal Society of Chemistry, U.K. 1999.

[134] Smith, W. D., *Anal Chem* 2002, *74*, 462A-466A.

[135] Auroux, P. A., Iossifidis, D., Reyes, D. R., Manz, A., *Anal Chem* 2002, *74*, 2637-2652.

[136] Reyes, D. R., Iossifidis, D., Auroux, P. A., Manz, A., *Anal Chem* 2002, *74*, 2623-2636.

[137] Thurman, E. M., Mills, M. S., *Solid-Phase Extraction: Principles and Practice.*, Wiley 1998.

[138] Dean, J., *Extraction Methods for Environmental Analysis*, John Wiley, New York 1998.

[139] Pawliszyn, J., *Analytical Chemistry* 2003, *75*, 2543-2558.

[140] Handley, A., *Extraction Methods in Organic Analysis*, Sheffield Press, Sheffield, UK 1999.

[141] Pawliszyn, J., Editor, *Applications of Solid Phase Microextraction*, Royal Soc. Chem. 1999.

[142] Pawliszyn, J. b., *Solid Phase Microextraction: Theory and Practice*, VCH 1997.

[143] Jeannot, M. A., Cantwell, F. F., *Anal Chem* 1997, *69*, 2935-2940.

[144] He, Y., Lee, H. K., *Anal. Chem.* 1997, *69*, 4634-4640.

[145] Theis, A. L., Waldack, A. J., Hansen, S. M., Jeannot, M. A., *Anal Chem* 2001, *73*, 5651-5654.

[146] Xie, H.-Y., He, Y.-Z., *TrAC, Trends Anal. Chem.* 2010, *29*, 629-635.

[147] Cardoso, A. A., Liu, H., Dasgupta, P. K., *Talanta* 1997, *44*, 1099-1106.

[148] Milani, M. R., Stradiotto, N. R., Cardoso, A. A., *Electroanal* 2003, *15*, 827-830.

[149] Pretto, A., Milani, M. R., Cardoso, A. A., *J Environ Monit* 2000, *2*, 566-570.

[150] Ugucione, C., Cardoso, A. A., *Anal Bioanal Chem* 2007, *389*, 1647-1650.

[151] Cardoso, A. A., Dasgupta, P. K., *Anal. Chem.* 1995, *67*, 2562-2566.

[152] Liu, H., Dasgupta, P. K., *Anal. Chem.* 1995, *67*, 4221-4228.

[153] da Silva Borges, S., Reis, B. F., *Anal Chim Acta* 2007, *600*, 66-71.

[154] Psillakis, E., Kalogerakis, N., *TrAC, Trends Anal. Chem.* 2002, *21*, 53-63.

[155] Fiamegos, Y. C., Stalikas, C. D., *Anal Chim Acta* 2007, *599*, 76-83.

[156] Ma, M., Cantwell, F. F., *Anal. Chem.* 1999, *71*, 388-393.

[157] Liu, W., Lee, H. K., *Anal Chem* 2000, *72*, 4462-4467.

[158] Xia, L., Hu, B., Jiang, Z., Wu, Y., Liang, Y., *Anal Chem* 2004, *76*, 2910-2915.

[159] Psillakis, E., Kalogerakis, N., *TrAC, Trends Anal. Chem.* 2003, *22*, 565-574.

[160] Baghdadi, M., Shemirani, F., *Anal Chim Acta* 2008, *613*, 56-63.

[161] Baghdadi, M., Shemirani, F., *Anal Chim Acta* 2009, *634*, 186-191.

# Chapter 2 New Pressure Assisted Sweeping Online Preconcentration for Highly Polar Environmentally-Relevant Nitrosamines: Part 1. Sweeping for Polar Compounds and Application of Auxiliary Pressure[1]

## 2.1.Introduction

Capillary electrophoresis employs electrophoretic, electroosmotic, and chromatographic phenomena, and results in an unparalleled degree of flexibility in achieving separation, which has lead to a wide range of applications for problems as simple targeted analysis of pharmaceutical compounds to complex analytical problems such as the mapping of the human genome [2-6]. Although CE can easily separate very complex mixtures, it suffers from low sensitivity arising from small sample volumes (e.g., 2–10 nL) and short optical path lengths (e.g., 25–100 μm). On-line focusing methods for enrichment in CE have been developed based on chromatographic partitioning, sorption or electrophoretic effects for neutral and charged analytes [7]. Sweeping can be employed with EKC for analysis of neutral compounds, where enrichment mainly based on affinity for the micellar phase [7-10]. In EKC neutral analytes are separated only due to partitioning, while charged analytes benefit from both partitioning and electrophoretic effects [8-9]. Depending on the type of surfactants, micelles can be charged or neutral lending flexibility to the approach, however applications using neutral surfactants exclusively can only be used for charged analytes [10-12].

*N*-nitrosamines are potentially-carcinogenic, neutral polar compounds [13], which makes them important from a human health standpoint and challenging from the analytical perspective. The seven nitrosamines used in this study are listed in Table 2.1 along with data on their water solubility and log P values. The log P is defined by partitioning of analytes between 1-octanol and water and gives an indication of polarity of analytes; it is

commonly used as a measure of lipophilicity and as a predictive tool for analytical separations of all types including chromatography. The data indicates that the nitrosamines should be very polar and highly soluble in water with a low affinity for micellar phases. Since other online preconcentration methods can successfully enrich the nonpolar compounds, the main focus of this study are the analytes with log P<1, for which traditional sweeping is not a viable choice.

Table 2.1. Physical properties and structures of nitrosamines in water[a]

| **Name of compounds** | **Abbreviation** | **Solubility in water (mgL-1)** | **log P** |
|---|---|---|---|
| N-nitrosodimethylamine | NDMA | $1x10^{6}$ | -0.57 |
| N-nitrosomorpholine | NMOR | $1x10^{6}$ | -0.44 |
| N-nitrosopyridine | NPYR | $1x10^{6}$ | -0.19 |
| N-nitrosodiethylamine | NDEA | $1.06x10^{5}$ | 0.48 |
| N-nitrosopipyridine | NPIP | $7.65x10^{4}$ | 0.36 |
| N-nitrosodipropylamine | NDPA | $1.3x10^{4}$ | 1.36 |
| N-nitrosodibuthylamine | NDBA | 1270 | 2.63 |

a- Data obtained from CRC Handbook of Chemistry and Physics 66th edition

In general, sweeping is very similar to normal MEKC in terms of compositions of the BGE and the sample matrix. The sample should be micelle-free, while the BGE should contain a relatively large amount of surfactant, and conditions should be such that EOF is low or absent. Movement of analytes is mainly due to their association with migrating micelles [14]. For an injection zone containing a neutral analyte between two zones containing

charged pseudostationary phase, the length of the enriched analyte zone (the sweep-zone) is narrowed to a fraction of the injection zone, which can be estimated from:

$$\boldsymbol{l_{sweep} = l_{inj}\frac{1}{1+k}} \qquad 2\text{-}1$$

where $l_{inj}$ is the length of the injected sample zone and $k$ is the chromatographic retention factor [15-16]. According to equation 2.1, sweeping is most effective for analytes with large $k$, resulting in the narrowest sweep zones and therefore the highest concentration factors. Although the injection zone can be increased in a particular capillary to improve the detection limit, some fraction of the capillary must be left with the BGE where separation occurs. This suggests that the capillary could be made infinitely long to maximize sample loading and decrease limits of detection, however, the drive toward long capillaries for sensitivity improvements must be balanced with other factors such as analysis times and excessive band broadening for, polar compounds with low $k$ values.. Rather than using a shorter capillary to speed up analysis times, the use of auxiliary pressure with longer capillaries was described for the first time in this study.

## 2.2.Materials and Methods

### 2.2.1. Apparatus

All electropherograms were obtained using an Agilent 3D Capillary Electrophoresis 1600 with Agilent ChemStation software (Waldbronn, Germany) using fused silica polyimide coated capillaries 50 µm ID and 375 µm OD and coated capillaries (Zero EOF), MicroSolv

(USA). Capillaries were loaded in a temperature controlled compartment held at 25°C. To increase the capacity of cartridge (normal length is up to 64.5 cm), an adapter was designed and fabricated that can be added to Agilent capillary cartridge to accommodate capillaries up to 130 cm. Application of capillaries longer than this is limited by the maximum applicable electric field. UV detection was performed at wavelengths 230 nm for nitrosamines and 210 nm for DMSO, the EOF marker and for dodecanophenone, the micelle marker. Absorbance maxima for all analytes fell between 225 and 235 nm. The auxiliary pressure device made in our lab to control is described later in the chapter and precise control of pressure between 0 and 200 mbar ± 0.5% was possible; all parts were obtained from OMEGA Engineering (New Jersey, USA).

### 2.2.2. Materials

Individual nitrosamine (Table 2.1 and 1.1) standards at 2000 $mgL^{-1}$ in methanol were purchased from Supelco (Ontario, Canada), stock solutions of 1000 $mgL^{-1}$ in methanol were prepared and stored at 4°C and working solutions were made fresh daily. All solvents were HPLC grade or higher, sodium dodecyl sulfate 99.0% (SDS) was purchased from Fluka, DMSO (99.0%), dodecanophenone (98.0%) and ammonium phosphate (99.99%) from Sigma-Aldrich (Oakville, Canada). Ultrapure water was obtained from Barnstead NanoPure Diamond (18 MΩ), (Ontario, Canada). The micelle marker (dodecanophenone) and the EOF marker (DMSO) were spiked as required. Stock solutions of 500 mM of SDS and 1000 mM of phosphate buffer were made weekly and used in making the BGE

solutions as required. The pH and conductivity were adjusted after final dilution. Solutions were filtered with 0.22 µm nylon filters and degassed prior to use.

### 2.2.3. Conductivity Measurements

Typically, the conductivities of BGE and sample solutions were measured with a VWR-Symphony potentiometer/ pH meter model SB70C. For small volume samples, conductivities were also determined by filling the capillary with the solution of interest and applying a potential of 5 kV for a limited time and current at a fixed temperature (25 °C) was recorded. The conductivity ($\delta$) was calculated from:

$$\boldsymbol{\delta = \frac{IL}{VA}} \qquad 2\text{-}2$$

where $I$ is the measured current (A), $L$ is the total length of the capillary (m), $V$ is the applied voltage, and $A$ is the cross-sectional area ($m^2$) [17].

## 2.3.Results and Discussion

Eq. 2-1 suggests that EOF has no effect on analyte focusing in sweeping, while in practice elimination of the EOF is preferred as a strong EOF often leads to a reduced concentration efficiency [16]. However, in the absence of EOF the analyses are typically lengthened by the limited mobility of micelle, which is the only vector for migration of neutral analytes. Thus, some of the advantage gained in sweeping-based focusing is lost when the analyte has too much time for diffusion related band broadening. The analysis time can be reduced

by using shorter capillaries; this was attempted using 33.5 cm capillary, which is the minimum length of capillary allowed by the Agilent CE. The total analysis time for the analytes of interest (*N*-nitrosamines) was relatively long at nearly 30 min, and the improvement in sensitivity was limited by a concomitant decrease in the injection volume. The analysis time may also be improved by increasing the applied voltage, though there is a practical limit, for example most commercial instruments operate at 30-40 kV.

If a method carried out at an instrument's maximum voltage is transferred to a longer capillary, the analytes must both migrate further and more slowly because of the diminished micelle velocity under the reduced electric field strength. This effect was studied for various capillary lengths with a fixed internal diameter (Figure 2.1). It was also found that the relationship between pressure and total analysis time was not linear, and is likely related to viscosity and the frictional force at the capillary walls (Figure 2.2).

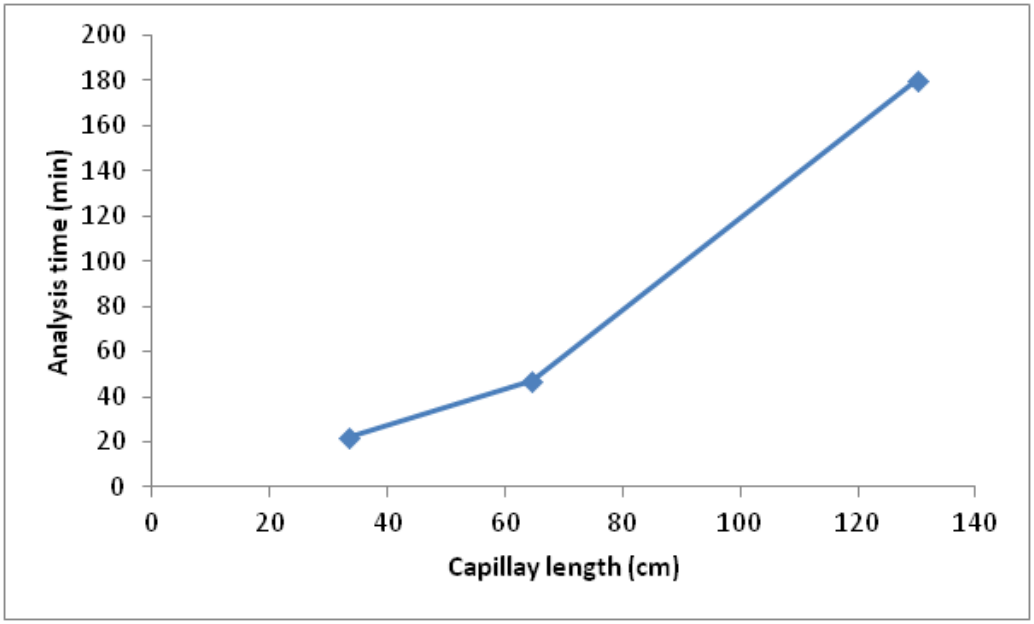

Figure 2-1. Effect of capillary length on analysis time in absence of auxiliary pressure

Capillaries with larger internal diameter showed better migration time reproducibility and. longer capillaries gave substantial increases in the total analysis time with broader and flatter peaks. Despite the reduction in S/N and hard earned gains in sensitivity, especially for analytes with small *k*, longer capillaries are necessary to accommodate the larger sample volumes needed to improve detection limits. The application of auxiliary pressure to push the analytes toward the detector was a workable solution to minimize the time analytes spend in the capillary, which as will be shown, improves the key peak characteristics.

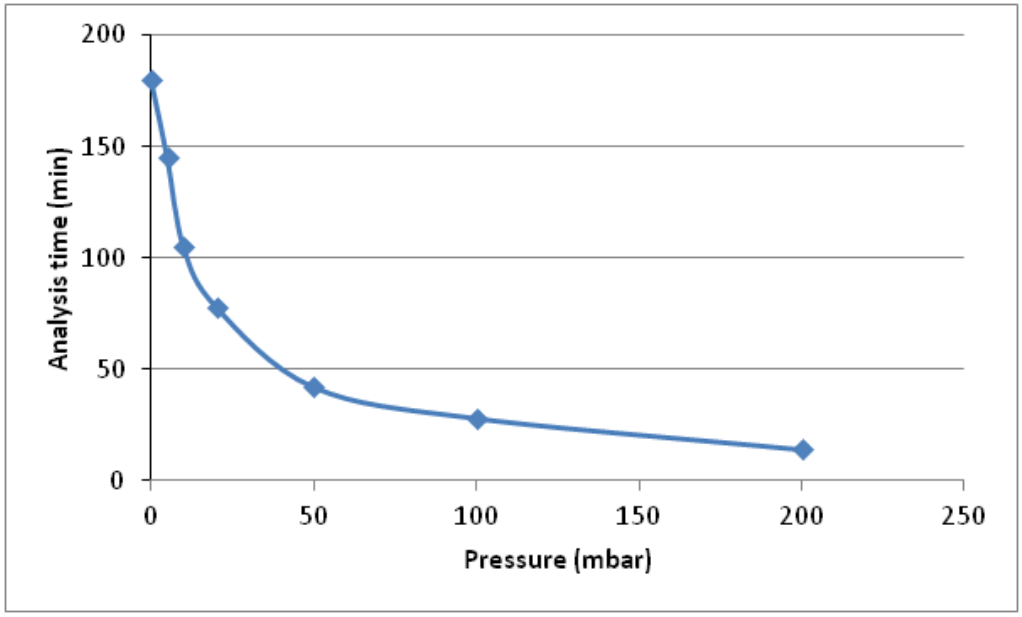


Figure 2-2. Effect of pressure on analysis time. Capillary: length 130 cm, ID 50 µm

### 2.3.1. Application of Pressure

The effect of pressure was assessed using the variable system pressure on our Agilent CE, noting that the upper limit for this pressure was 50 mbar. Figure 2.3A shows normal

sweeping with RM-EKC under acidic conditions when no pressure was applied. The total analysis time was about 60 minutes and significant band broadening for last two peaks was observed. These peaks correspond to NDMA and NMOR; both have negative log P values and so have low association with the micellar phase. The electropherograms B and C in Figure 2.3 show that the application of external pressure shortens the analysis time and peak shapes improve dramatically. An unexpected result of these experiments was the noticeable decrease in peak height (see Figure 2.3) for first two compounds (NDBA and NDPA) as the applied pressure was increased. With sweeping, compounds with higher log P values should demonstrate better concentration efficiencies with taller and narrower peaks; this was evident in the case of these early migrating peaks; however it does not explain the loss in signal intensity with the applied pressure. A number of reasons for this effect were examined. One factor that was considered was the relationship between the application of auxiliary pressure and the time interval for interaction between the analyte and the micellar phase. If the analytes were categorized into two sets, high interaction and low interaction with micellar phase, then it can be predicted that for the analytes with high interaction with the micelles, migration is fast, less time is spent in the bulk aqueous phase and narrower peak can be achieved.

For small polar analytes with low interaction with the micellar phase, the time spent in the capillary was long and diffusion was problematic; therefore, it was useful to apply pressure to ensure these compounds reach the detector before excessive band broadening can occur. From Figure 2.3A (no pressure applied), it can be seen that it takes about 15 minutes for the

most hydrophobic nitrosamines to reach the detector solely through migration when associated with the micelle; whereas it took more than 50 min before the last analyte peak appears and with poor peak characteristics.

If the pressure was applied early (Figures 2.3B and 2.3C), the problem with signal loss was apparent for the faster migrating peaks. However, when the auxiliary pressure was applied after 15 minutes (Figure 2.3D), there was no signal loss for first two analytes and good peak shapes are achieved for the rest of the compounds, give optimal concentration efficiencies. While pressure improved the separation from an analytical standpoint, the reason for the loss in signal when the pressure was applied early is still not clear. It is possible that in sweeping analytes with high interaction with micelles, very narrow bands smaller than the height of the slit in the detection cell may result. When the focusing effect of sweeping is very efficient, the analyte zone may be very concentrated and narrow, filling only part of the detection window. In this case, absorbance of light by the analyte occurs in only one part and in the other part the absorbance is essentially zero. If the analyte concentration in that zone exceeds the upper limit of the linear range, the net signal measured will be lower than expected for a similar mass loading but in a broader, shorter peak. Some of these issues have been explained in more detail by Patrick Kaltenbach (Hewlett-Packard Journal, June 1995, pp 20-24). A detection window with shorter height from Agilent (Part G1600-60132) was employed and the same experiments repeated, but no significant improvement was observed (results are not shown).

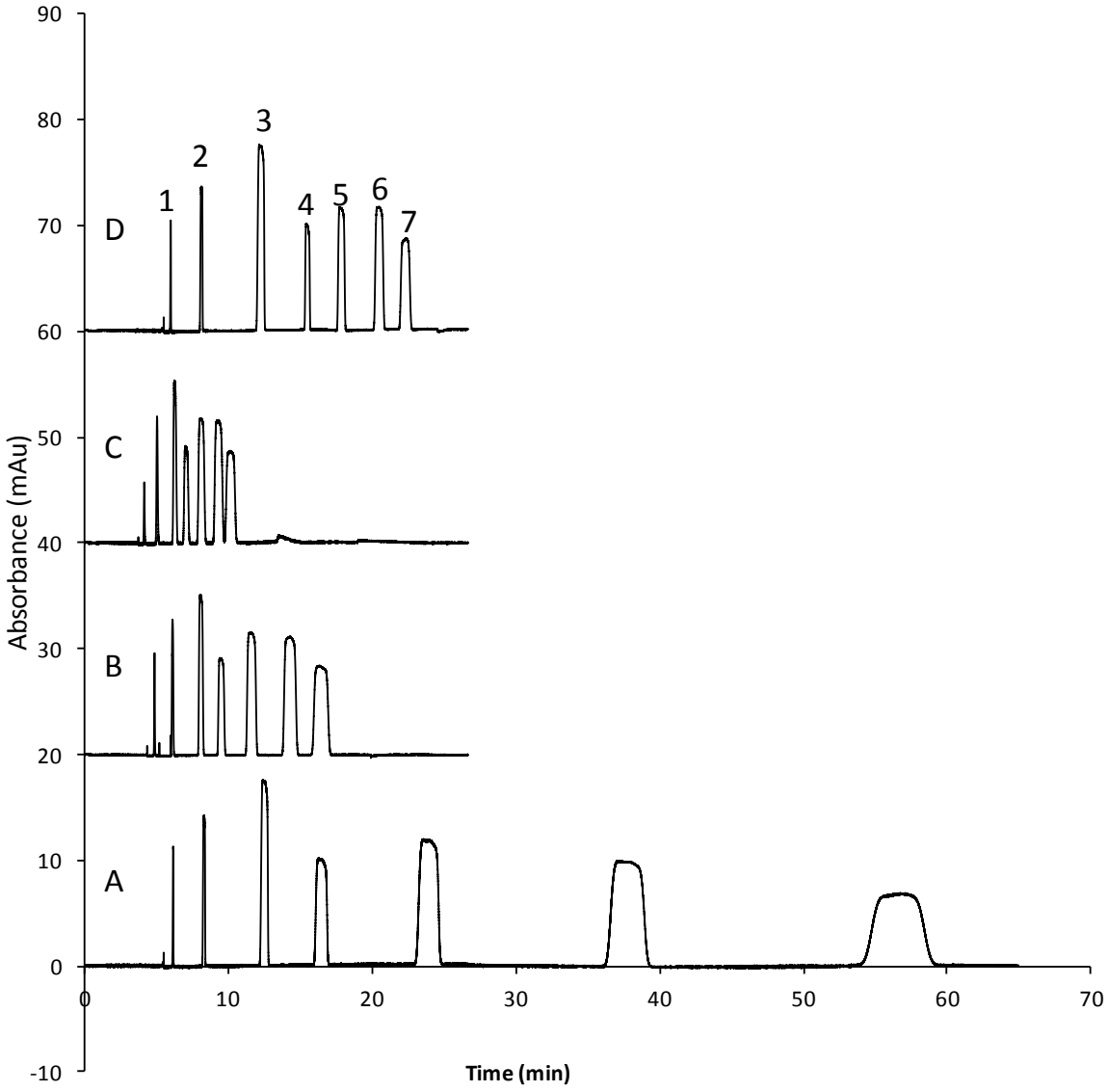


Figure 2-3. Effect of using auxiliary pressure on migration time and peak shapes in sweeping RM-EKC. Analytes: 1) NDBA, 2) NDPA, 3) NPIP, 4) NEDA, 50 NPYR, 6) NMOR, 7)NDMA. BGE 50 mM phosphate buffer pH 1.9 and 100mM SDS (conductivity 7.2 mS). Capillary length 64.5 cm and 50 µm id. Voltage -230V/cm; hydrodynamic injection 50 s at 50 mbar pressure at inlet during analysis: A 0 mbar, B 25 mbar, C 50 mbar, D 50 mbar applied after 5 min delay.

### 2.3.2. Device for Application of Auxiliary Pressure

Experimentally, it was found that longer capillaries (>64.5 cm) require higher applied pressure (>50 mbar) for the most effective separations. However, pressure controls are limited in commercially available instruments like the Agilent CE-1600, which has available pressure from -50 to +50 mbar. Even this pressure cannot be maintained at a stable level for more than a few minutes, at which point there is a cyclic fluctuation in the applied pressure. In some CE instruments application of external pressure is designed for high pressures of 2 bar to 12 bar, and it cannot be precisely controlled. With the appropriate equipment, it should be possible to accurately control the magnitude and direction of the applied pressure for good reproducibility of linear flow. Out of necessity, a device was developed that can easily be added to instruments to supply the required pressure. A schematic of the device is shown in Figure 2.4. This device consists of a small nitrogen cylinder (C), pressure regulators (G1 and G2), solenoid valves (S1, S2) and a pressure transducer (T). The first regulator (G1) sets the upper limit of applied pressure and regulator (G2) is responsible for fine tuning the pressure. Pressure was monitored and recorded during the analysis using pressure transducer (T) which has a USB connection to the computer. The device was placed between the instrument pressure source and vial in the existing instrument. The pressure switch (S1) engages the device. The presence of a three-way valve (S2) in this design facilitates the activation of pressure without interruption in

instrument function. When the device was inactive, the connection between the internal pressure control system (P) and the inlet vial (v) was open and the instrument was able to carry out its normal functions such as flushing and hydrodynamic injection. When the device was activated the internal pressure control was shut down and the connection between the external pressure and the vial was opened, allowing for pressure to be delivered to the inlet vial (v). This device was triggered by the external relay which was preinstalled in the CE instrument and it can be automatically controlled with the software.

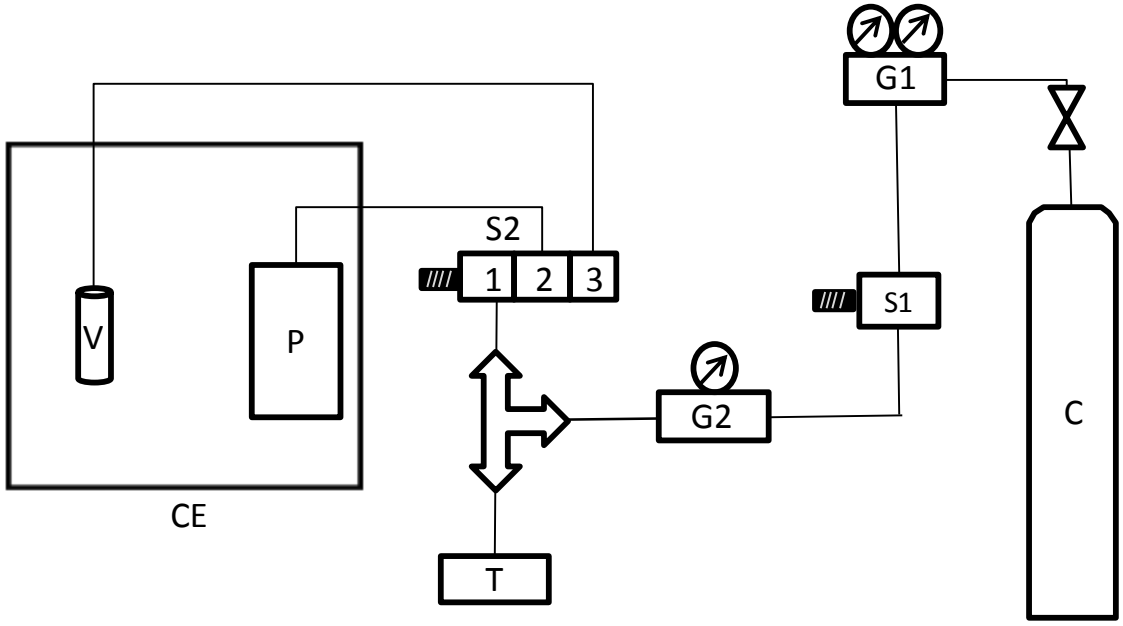


Figure 2-4. Diagram of portable device) for application of external pressure Components are: C, nitrogen gas container; G1 and G2, regulator, S1 and S2, solenoid valves; T, pressure sensor; P, pressure components in CE and V is vial.

### 2.3.3. Explanation of Analyte Behavior with Pressure Assisted Sweeping

It is clear that the main advantage of sweeping is the high focusing efficiency for analytes with large *k* values [18]. However, polar analytes with smaller *k* values show broader peaks, poorer preconcentration efficiency, and have long migration times due to their low affinity for the migrating micelles. This has meant that the use of sweeping is generally avoided for polar compounds, and there are few effective alternatives for online preconcentration in CE that are not limited by a low affinity for the micelles. Broad peaks are attributable to inefficient focusing as the micelles move through the large injection zone, which is made worse in the time after sweeping where low affinity for the micelles leads to extra time for diffusion. [19-20]. Since sweeping is already hindered by small injection volumes compared to other online focusing techniques (e.g. such as stacking), the capillary needed to be as long as is practical to accommodate higher loading volumes, but long columns mean more time for diffusion related peak broadening.

By using an auxiliary pressure, the compounds with the lower affinity for the micelles can be pushed toward the detector, maintaining some of the advantage of the focusing while minimizing the time for diffusion. Figure 2.5 illustrates the behavior of analytes in sweeping-RM-EKC with the auxiliary pressure applied after the sweeping process has been initiated. The capillary has been filled with BGE (contains micellar phase), then hydrodynamically-injected sample (gray zone, $l_{inj}$, Figure 2.5A), and the ends of the capillary are placed in sample vials containing BGE. Initially, sample solutions and BGE

conductivities were made nearly equal, to maintain uniform electric field across the capillary; this is considered ideal for optimal peak shapes.

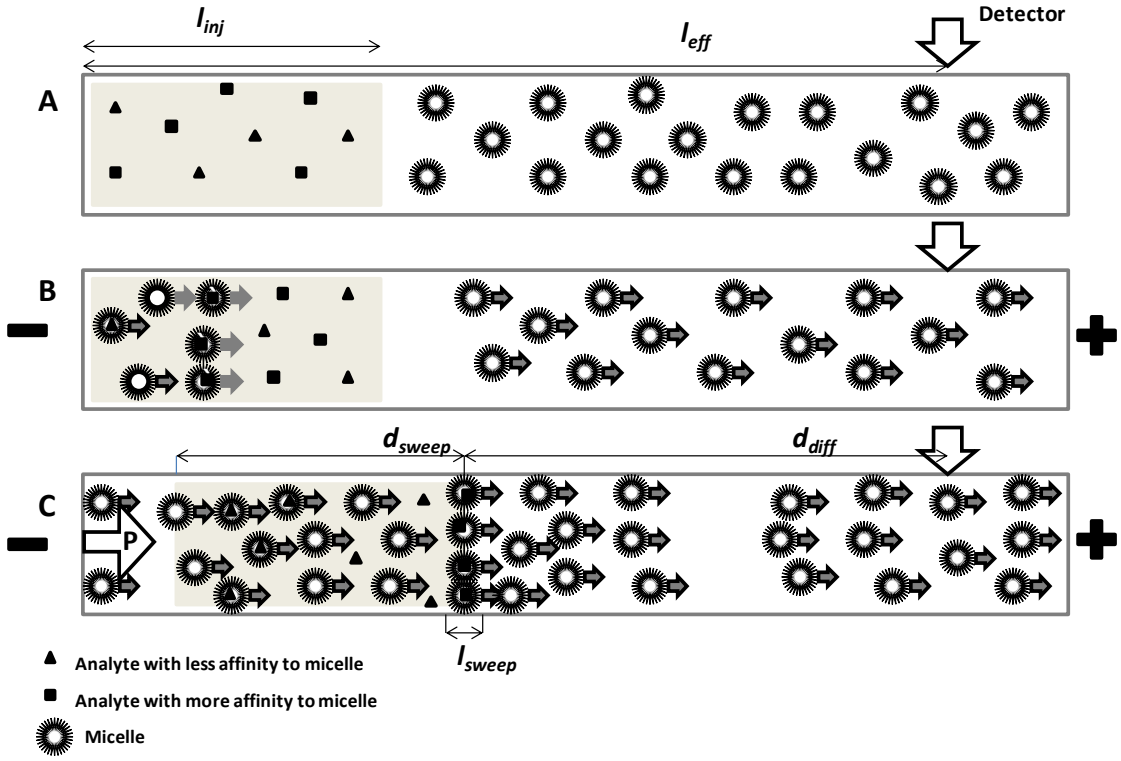


Figure 2-5 . Conceptual representation of analyte behavior with pressure assisted sweeping. A) Capillary is filled with BGE consist of SDS (anionic surfactant) and sample in injected. B) negative voltage applied, in absence of EOF, micelles are moving toward the detector passing through the sample zone and sweep the analyte. C) auxiliary pressure applied to move the bulk solution toward the detector.

In this study, the anionic surfactant SDS was used, therefore negative polarity was applied (the anode is at the detector end). With both ends of the capillary in vials containing BGE, the separation voltage was applied and external pressure applied, immediately or with a delay. Negatively charged micelles migrate in the electric field from inlet vial and pass through the sample zone (Figure 2.5B). Analytes are carried toward the detector based on

their interaction with micelles. The first peak belongs to the micelle marker that defines the leading edge of the plug of the swept micelles. Peaks are narrowest immediately after sweeping, and though there must be space for the analytes to be resolved through the RM-EKC mechanism, the distance ($d_{diff}$) between the end of the sweeping zone ($d_{sweep}$) and detector window should minimized to avoid band broadening. With normal sweeping, the injection zone ($l_{inj}$) is essentially stationary with micelles migrating toward the detector. Whereas with the application of pressure the flow of the bulk solution, including sample zone, is in the same direction as the micelles, toward the detector (Figure 2.5C). Under ideal conditions maximum focusing can be observed when $d_{sweep} = l_{eff}$, ($d_{sweep}$, sweeping zone and $l_{eff}$, effective length of capillary). When $d_{sweep} < l_{eff}$, there is more time for diffusion after completion of sweeping. With no auxiliary pressure was applied, we call the distance between $d_{sweep}$ and effective length of capillary, $l_{eff}$ the diffusion zone $d_{diff}$. When pressure was applied $d_{sweep}$ and effective length of capillary, $l_{eff}$ are unchanged, but $d_{diff}$ decreases. Since analysis time was proportional to the distance that the micelles must travel, the analysis time was the sum the sweeping time ($t_{sweep}$) and the diffusion time ($t_{diff}$). Typically, the $t_{sweep}$ and $t_{diff}$ depend on the $k$ value of each analyte and the length of their respective zones. For analytes with high $k$ values, $t_{diff}$ will be relatively short, but more time in $d_{diff}$ be necessary for resolution of analytes with similar $k$ values, meaning the longest $d_{diff}$ was achieved when no pressure was applied. However, for compounds with relatively low $k$ values, $t_{diff}$ was longer than necessary to resolve peaks and led to unnecessary band broadening. If simultaneous analysis of compounds with a wide range of $k$ values is desirable, then the capillary cannot be physically shortened without sacrificing

separation of the more lipophilic compounds. Only through the application of pressure, can the conditions be varied for separation of such different analytes, where the $d_{diff}$ was effectively shortened when pressure was applied, reducing the $t_{diff}$ and time for analytes to diffuse. Ideally the applied pressure should be adjustable with respect to magnitude and time of application, allowing the user to balance distance necessary for separation of certain analytes with minimization of the $d_{diff}$ as needed.

It is possible that EOF can continue to play a role in these separations, thus the magnitude of the EOF was measured according to Williams method [21]. The relevant linear velocities under different conditions were measured by injecting sample under constant pressure and recording the time when the sample peak was detected. BGE composition, auxiliary pressure, and capillary size (length and internal diameter) were varied. Our results showed that for fused silica capillary and acidic conditions (pH 1.9) EOF was present, though it very low and variable. When external pressure was applied, the presence of the small EOF was insignificant compared to the net flow.

### 2.3.4. Effect of Conductivity of Sample Matrix

A difference in conductivities for sample and BGE can have a substantial effect on peak quality. This effect was investigated for pressure assisted sweeping-RM-ECK. A series of buffered samples containing two nitrosamines with very different log P values (NPIP, NPYR) were prepared with conductivities of 0.5 to 16.9 mS. The BGE was similar to those used in the other experiments (100 mM SDS, 25 mM phosphate buffer adjusted to pH 1.9,

measured conductivity 7.2 mS). To minimize the time of the experiments, the shortest capillary possible (33.5 cm) was used, and analysis was carried out with an applied potential was 230 $Vcm^{-1}$, and auxiliary pressure of 25 mbar. Examples of the pertinent electropherograms are presented in Figure 2.6. The results show that when the sample conductivity was lower than that of the BGE, increases in the sample volume result in significant degradation of peak shape.

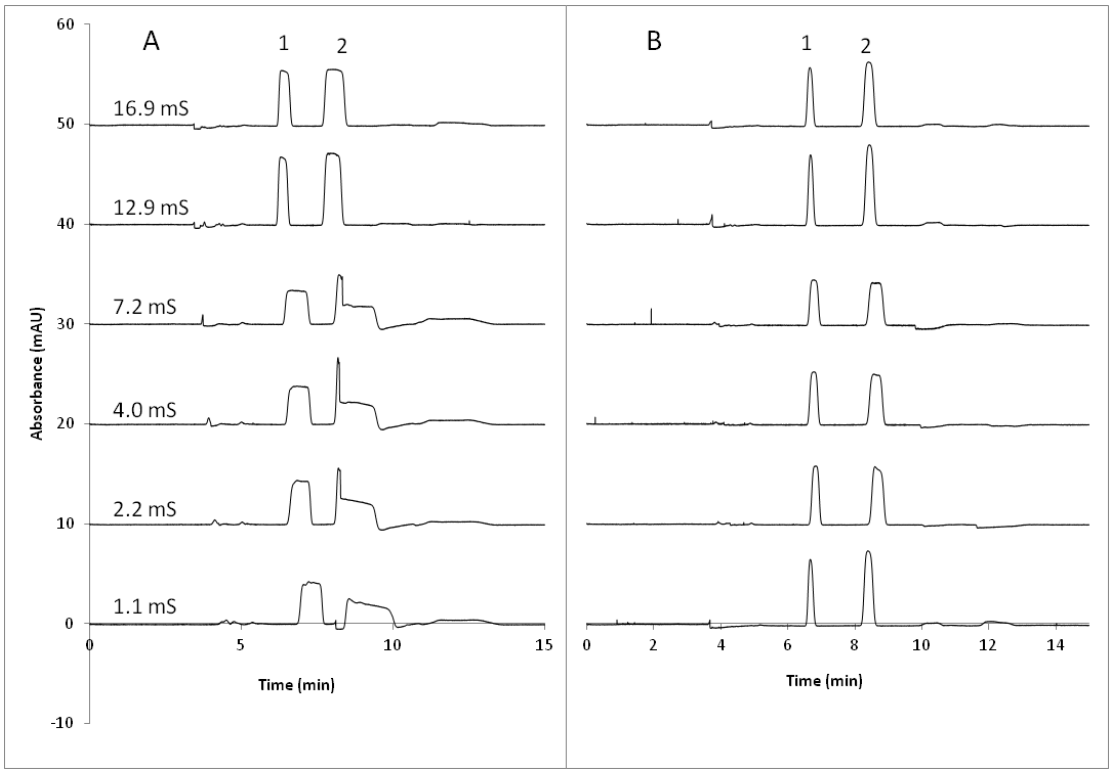


Figure 2-6. The effect of conductivity of sample matrix and injection volume on peak shapes. Analytes: 1) NPIP and 2) NPYR; BGE: 50 mM phosphate buffer pH 1.9 and 100 mM SDS, conductivity 7.2 mS; Capillary is 33.5 cm long (25 cm effective length) and 50 µm ID; voltage -7.7 kV; auxiliary pressure 25 mbar; injection at 50 mbar A) 100 sec, B) 50 sec;

This was contrary to what would be expected, where sharpening usually occurs for analytes in matrices with lower conductivities than the BGE; usually due to the higher electric field across the sample zone. Conversely, peak shapes improved when sample matrix conductivity was the same or higher than BGE. The explanation for this has not been investigated thoroughly, however higher conductivity is associated with higher ionic strength, which can affect micelle dynamics (e.g. CMC) and the partition coefficients for the analytes. When the injection volume was large (Figure 2.6A), the difference between low conductivity and high conductivity matrix was profound, where much better focusing was observed when the conductivity of sample matrix was higher than the BGE. This was an interesting phenomenon and contrary to what is normally expected through the stacking mechanism [22]. It can be very useful in instances when the sample matrix is highly saline, e.g. sea water. This also implies that by increasing sample ionic strength, the injection volume could be increased without losing good peak characteristics.

### 2.3.5. Effect of Surfactant Concentration in BGE

Experiments were performed to assess the effect of the BGE SDS concentration on peak width and peak height using the same conditions as described in section 3.4 and a range of concentrations of SDS (50 mM-300 mM).. Increasing the concentration of SDS up to 200 mM increased the peak height and decreased the peak width, while further increases in surfactant concentrations had a negative impact on peak height and area for the analytes with large *k*, such as NDBA.

### 2.3.6. Evaluation of the Performance

Calibration curves were generated for selected nitrosamines using pressure assisted sweeping RM-EKC, and the performance of the method was evaluated for the traditional analytical figures of merit and compared to results using MEKC. Points in the calibration curves cover two orders of magnitude and are the average of peak areas from three sequential injections. Samples were injected hydrodynamically, for MEKC 50 mbar for 2 s, and sweeping-RM-EKC, 50 mbar for 100 s. The LODs reported are based on three times the standard deviation of the intercept-to-slope ratio, and LOQs are calculated as ten times standard deviation of the same ratio. Key data from these analyses are shown in Table 2.2. In all cases sweeping leads to lower detection limits, where improvements in concentration efficiency result in more concentrated sample zones with larger signals. It was only when the data from this large number of experiments was being processed. It was noticed that analytes with higher log P, such as NDBA, showed lower signals when pressure was applied at the same time as the potential (as discussed earlier in this chapter).

The decision was made not to repeat these experiments with the delayed pressure protocol, as compounds like NDBA, and to a limited extent NDPA, do not usually suffer from the problems in sweeping that have been tried to address in this work. No doubt, the LODs would be improved by using the delayed pressure; in future work these will be reported accordingly. As can be seen in Table 2.2, improvements of up to 34 times in LOD were achieved. The $R^2$ values of sweeping-MEKC show better linearity over concentration range 0.05- 6.25 mg $L^{-1}$. This means that sub-mg$L^{-1}$ detection was possible using online

preconcentration, without excessive sample handling or derivatization, as is seen with the typical GC-MS methods (e.g. US-EPA Method 521).

Table 2.2. Statistical data for analysis of nitrosamines

| | MEKC | | | | Pressure assisted sweeping-RM-EKC | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | slope | Intercept | LOD ($mgL^{-1}$) | $R^2$ | slope | intercept | LOD ($mgL^{-1}$) | $R^2$ | Factor |
| NDBA | 0.4210 | 0.4576 | 2.26 | 0.9992 | 4.817 | 0.4804 | 0.53 | 0.9962 | 4.3 |
| NDPA | 0.9094 | 1.688 | 2.61 | 0.9989 | 9.799 | -0.0733 | 0.14 | 0.9996 | 18.6 |
| NPIP | 2.229 | 4.082 | 2.84 | 0.9987 | 21.99 | -0.0504 | 0.12 | 0.9998 | 23.7 |
| NDEA | 1.853 | 3.559 | 2.64 | 0.9989 | 19.03 | -0.0599 | 0.11 | 0.9998 | 24 |
| NMOR | 3.123 | 6.006 | 2.91 | 0.9986 | 31.43 | -0.0525 | 0.09 | 0.9998 | 32.3 |
| NPYR | 3.669 | 7.519 | 2.75 | 0.9988 | 38.01 | -0.0455 | 0.08 | 0.9999 | 34.4 |
| NDMA | 3.124 | 6.665 | 2.93 | 0.9986 | 33.60 | -0.0366 | 0.11 | 0.9998 | 26.6 |

## 2.4. Conclusions

According to Equation 1, injection length, $l_{inj}$, and retention factor are the two major parameters that control the sweeping technique. Since sweeping was performed under zero EOF conditions and analyte migration was limited by the affinity for the micelles and by the linear velocity of the micelles, migration times can be long, especially for polar analytes. Short capillaries can make analysis times reasonable and reduce peak broadness, but they limit the maximum injection volume and thus the concentration factors. Borne from necessity, a system was built for application of controllable, uniform pressure that can

be easily integrated into a commercial CE system. The upper limit for fine control regulator used here was 100 mbar; different regulator configurations can allow for higher pressure control. It has been demonstrated that by using an external auxiliary pressure device, analysis times can be reduced without compromising separation efficiency allowing for use of longer capillaries to accommodate larger sample volumes. It was also shown that there was a significant effect on peak shapes and separation efficiency when the conductivity of sample solution was different than BGE. Separation results were best when a high conductivity sample was paired with a BGE of lower conductivity; e.g., if sample salinity was high, large volumes of sample could be injected without compromising the separation. Using higher surfactant concentrations (up to 200 mM SDS) than usual for MEKC (50 mM SDS) improved the sweeping, but increasing beyond optimal values led to signal loss for many analytes. It was shown that by optimizing all parameters that influence sweeping-RM-EKC (e.g., surfactant loading and type, application of pressure, changes to composition of sample, etc.), good online preconcentration methods can be developed for polar analytes that are typically are poorly suited for normal sweeping.

## 2.5.References:


[1] Modir-Rousta, A., Bottaro, C. S., *Electrophoresis* 2013, *34*, 2553-2560.

[2] Ban, E., Park, S. H., Kang, M. J., Lee, H. J., Song, E. J., Yoo, Y. S., *Electrophoresis* 2012, *33*, 2-13.

[3] Britz-McKibbin, P., *Methods Mol Biol* 2011, *708*, 229-246.

[4] Cortes, D. F., Kabulski, J. L., Lazar, A. C., Lazar, I. M., *Electrophoresis* 2011, *32*, 14-29.

[5] Rose, C. M., Hayes, M. J., Stettler, G. R., Hickey, S. F., Axelrod, T. M., Giustini, N. P., Suljak, S. W., *Analyst* 2010, *135*, 2945-2951.

[6] Desiderio, C., Rossetti, D. V., Iavarone, F., Messana, I., Castagnola, M., *J Pharm Biomed Anal* 2010, *53*, 1161-1169.

[7] Knudsen, C. B., Beattie, J. H., *J Chromatogr A* 1997, *792*, 463-473.

[8] Nishi, H., Tsumagari, N., Kakimoto, T., Terabe, S., *J Chromatogr* 1989, *477*, 259-270.

[9] Nishi, H., Tsumagari, N., Kakimoto, T., Terabe, S., *J Chromatogr* 1989, *465*, 331-343.

[10] Cao, J., Li, B., Chang, Y. X., Li, P., *Electrophoresis* 2009, *30*, 1372-1379.

[11] Cao, J., Yi, L., Li, P., Chang, Y. X., *J Chromatogr A* 2009, *1216*, 5608-5613.

[12] Monton, M. R., Quirino, J. P., Otsuka, K., Terabe, S., *J Chromatogr A* 2001, *939*, 99-108.

[13] Haidian, Z., *Journal of the National Cancer Institute* 1968, *41*.

[14] Quirino, J. P., Terabe, S., *J Chromatogr A* 1999, *850*, 339-344.

[15] Quirino, J. P., Otsuka, K., Terabe, S., *J Chromatogr B Biomed Sci Appl* 1998, *714*, 29-38.

[16] Quirino, J. P., Terabe, S., *Analytical Chemistry* 1999, *71*, 1638-1644.

[17] Giordano, B. C., Horsman, K. M., Burgi, D. S., Ferrance, J. P., Landers, J. P., *Electrophoresis* 2006, *27*, 1355-1362.

[18] Sera, Y., Matsubara, N., Otsuka, K., Terabe, S., *Electrophoresis* 2001, *22*, 3509-3513.

[19] Musheev, M. U., Javaherian, S., Okhonin, V., Krylov, S. N., *Analytical Chemistry* 2008, *80*, 6752-6757.

[20] Evenhuis, C. J., Musheev, M. U., Krylov, S. N., *Analytical Chemistry* 2011, *83*, 1808-1814.

[21] Williams, B. A., Vigh, G., *Anal Chem* 1996, *68*, 1174-1180.

[22] Guillaume L. Erny, A. C., *Analytical Chemistry* 2006, *78*.

# Chapter 3 New Pressure Assisted Sweeping Online Preconcentration for Highly Polar Environmentally-Relevant Nitrosamines: Part 2. Cationic and Anionic Surfactants with Zero-Flow Capillaries [1].

## 3.1.Introduction

Capillary electrophoresis is a powerful separation method, offering applications for an impressive array of compounds from small organic and inorganic ions [2] to more complex systems like proteins [3] and DNA [4]. However, small sample volumes (e.g., 2–10 nL) and short optical path lengths (e.g., 25–100 μm) limit trace level detection, particularly by UV-Vis detection. Several on-line preconcentration methods have been developed to overcome this drawback; for neutral compounds with no inherent electrokinetic mobility, this can be accomplished using sweeping preconcentration with separation by electrokinetic chromatography (EKC) [5],[6]. Such methods (e.g., sweeping-RM-EKC) rely on the interaction between analyte and micellar phase for preconcentration and separation.

In sweeping preconcentration, a large volume of sample can be swept by migrating micelles and analytes with a high affinity for the micellar phase will be concentrated in a narrow segment at the leading edge of a zone rich in surfactant. Quirino and Terabe [7] have introduced a mechanism for sweeping that has been defined by the following equation:

$$\boldsymbol{l_{sweep} = l_{inj}\frac{1}{1+k}} \qquad 3\text{-}1$$

The minimum length of the capillary occupied by enriched analyte and micelles following sweeping, $l_{sweep}$, is related to the length of the injected sample zone ($l_{\mathrm{inj}}$) and the retention factor ($k$). Under fixed conditions, the peak width for any analyte is indirectly proportional to $k$ and sweeping is most effective for analytes with large $k$. Polar analytes with low $k$ show less focusing and consequently less improvement in signal-to-noise. Similarly, these

polar compounds also show poorer recoveries using offline methods like solid phase extraction. Although sweeping is inherently less efficient for focusing of very polar analytes like nitrosamines, it is superior to off-line preconcentration since sample handling is minimized and outright loss of analytes is eliminated. Furthermore, the relatively weak affinity of small polar neutral compounds (e.g. nitrosamines) for the micellar phase can be in part mitigated by careful selection of the surfactant phase and by using high concentrations of the surfactant to optimize sweeping preconcentration and separation. This is illustrated in the following expression of the retention factor given in terms of the partition coefficient ($K$) and the phase ratio ($\varphi$):

$$\boldsymbol{k = K\varphi = \frac{n_{mc}}{n_{aq}} = \frac{C_{mc}}{C_{aq}}\varphi} \qquad 3\text{-}2$$

Where moles of the analyte in the aqueous phase is $n_{aq}$ and in the micellar phase is $n_{mc}$; $c_{mc}$ is the concentration of the solute in the micellar phase and $c_{aq}$ is the concentration of solute in the aqueous phase [8-9].

Selection of the micellar phase is complicated by the lack of data ($k$ or $K$) for nitrosamines in any surfactant system. And although there are a number of ways to measure $K$ or $k$ (*e.g.* two phase solvent-solvent extraction, chromatography) [10] including CE-based techniques, it is not practical to test all surfactant systems amenable to MEKC. To ensure that surfactants with a broad range of characteristics that influence $k$ are chosen, one can look to linear solvation energy relationships (LSER) studies, which express $k$ as a sum of interactions between the solute and micellar phase. Properties such as ability to act as a

hydrogen bond donor/acceptor (acidity/basicity), effort required form a cavity in the buffer or micellar phase for the solute [11] and various factors that influence the descriptors, as such, the effects of surfactant chain length [12], head-group [13], and counter ion [14] on the intermolecular interactions controlling retention have been studied. Fuguet *et al.* [15] used principle component analysis on published data for a large selection of micellar systems (single and mixed surfactants) to determine which system parameters were the most important in assessing the selectivity of the micellar phase. Fuguet's results confirm that hydrophobicity and ability to participate in hydrogen bond formation account for most of the differences in selectivity of surfactants. This information has guided the choice of surfactants for this work. The anionic surfactant SDS is an obvious candidate as it is the most common surfactant used in MEKC [16-18] and provides the benchmark by which the others are judged. The ammonium salt of perfluorooctanoic acid (APFO) was chosen because fluorinated anionic surfactants have been shown to exhibit unique characteristics when evaluated by LSER [15] and are compatible with ESI-MS [19]. The anionic surfactant bile salts (cholic acid-deoxycholic acid sodium salt) was selected for the ability to act as a good hydrogen bond donor [16, 18]. Cetyltrimethylammonium bromide (CTAB) and cetyltrimethylammonium chloride (CTAC) are two salts of the same cationic surfactant with a positively charged quaternary amine head and a relatively long hydrophobic chain (C16). The influence of the counter ion makes CTAC more soluble than CTAB, but in both cases the surfactant acts as a good hydrogen bond donor [18].

Regardless of type of surfactant, the phase ratio ($\varphi$) has important contribution in calculation of *k* and has been defined as follows:

$$\boldsymbol{\varphi} = \frac{\boldsymbol{V_{mc}}}{\boldsymbol{V_{aq}}} = \frac{\boldsymbol{v(C_{surf} - CMC)}}{\boldsymbol{1 - v(C_{surf} - CMC)}} \qquad 3\text{-}3$$

where $V_{mc}$ and $V_{aq}$ are the volume of micellar and aqueous phases, respectively, which are in turn related to partial specific molar volume ($v$), *CMC*, and concentration of surfactant ($C_{surf}$) [9]. Eqs. 3.2 and 3.3 show that increased surfactant concentration will increase the phase ratio ($\varphi$), and should increase *k*, and sweeping efficiency. Since the partial specific molar volume ($v$) is constant (0.25 L/mol for SDS [20]), only concentration of the surfactant and *CMC* can be controlled. Though *CMC* is considered a constant under fixed conditions, ionic strength, the presence of organic modifiers, and temperature influence its magnitude.

In assessing the performance of the sweeping-RM-EKC it is useful to calculate the retention factors for each micellar system. In MEKC, *k* is normally calculated in presence of EOF as illustrated in Eq. 3.4 [8, 21-22]:

$$\boldsymbol{k} = \frac{\boldsymbol{t_s - t_{EOF}}}{\boldsymbol{t_{EOF}(1 - t_s/t_{mc})}} = \frac{(\frac{\boldsymbol{t_s - t_{EOF}}}{\boldsymbol{t_{EOF}}})}{(\frac{\boldsymbol{t_{mc} - t_s}}{\boldsymbol{t_{mc}}})} = \frac{\boldsymbol{t_s - t_{EOF}}}{\boldsymbol{t_{mc} - t_s}} * \frac{\boldsymbol{t_{mc}}}{\boldsymbol{t_{EOF}}} \qquad 3\text{-}4$$

Where $t_s$ , $t_{EOF}$ , and $t_{mc}$ are the migration times for the solute, EOF marker, and micelle markers, respectively. Though Eq. 3.1 suggests that the sweep zone ($l_{sweep}$) is independent of EOF and only affected by *k*, the presence of a strong EOF usually reduces the

enrichment efficiency [6]. Thus, sweeping of neutral compounds is usually carried out under reduced or no EOF, and analyte migration toward the detector relies on association with an electrophoretically mobile micellar phase to lend mobility to the analytes. In this case, the system and calculations are simplified, and the system described is analogous to normal phase chromatography with a polar pseudostationary phase and a more hydrophobic micellar mobile phase (Eq. 3.5, where $t_R$ is the migration time of analyte and $t_{mc}$ is the migration time of a marker with highest interaction with the micellar phase). A similar observation was made in 1996 by Janini *et al.* [23-24].

$$k' = \frac{t_R - t_{mc}}{t_{mc}} \qquad 3\text{-}5$$

Since, slow moving micelles and low affinity of the analyte for the micellar phase leads to long analysis times and excessive band broadening of very polar compounds [8], a sweeping preconcentration RM-EKC was modified to use auxiliary pressure to achieve faster analyses without compromising the sweeping efficiency and resolution [25].This chapter follows the previous chapter in a series of studies on the method, and is primarily focused on the role of surfactants and their interactions with the target analytes. Seven *N*-nitrosamines have been selected for this study (see Figure 1.1): *N*-nitrosodimethylamine (NDMA), *N*-nitrosomorpholine (NMOR), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodiethylamine (NDEA), *N*-nitrosopiperidine (NPIP), *N*-nitrosodipropylamine (NDPA), *N*-nitrosodibutylamine (NDBA). These are of interest analytically as they are

relatively difficult to analyze at environmentally relevant concentrations, particularly by CE, and are important contaminants due to their potential as human carcinogens [26].

## 3.2.Material and Methods

### 3.2.1. Apparatus

All electrochromatograms were obtained using an Agilent 3D 1600 CE system with Agilent ChemStation software, (Waldbronn, Germany). Capillaries used in this work included polyimide coated fused silica capillaries of 50 µm and 75 µm, and Zero-EOF and Controlled-EOF capillaries of 50 and 75 100 µm ID obtained from MicroSolv (Eatontown, USA). Zero-EOF capillaries were coated (bonded) with linear polyacrylamide (LPA), which eliminates EOF. Polyethylene glycol (PEG) coated and polyacrylamide (PAAm) coated capillaries of 75 µm were obtained from Sepax Technologies (Delaware USA). Capillaries were conditioned as recommended by manufacturers before first use and conditioned prior to use daily. All CE experiments were carried out at a constant 25°C. Absorbance was measured at 230 nm to 240 nm for *N*-nitrosamines and at 210 nm for the EOF marker (DMSO) and micelle marker, *N*-nitrosodiphenylamine (NDPhA).

### 3.2.2. Materials

Standard solutions of 2000 mg $L^{-1}$ of NDMA, NPYR, NMOR, NDEA, NPIP, NDPA, NDBA, and NDPhA were purchased from Supelco (Ontario, Canada). Stock solutions at concentration of 1000 mg $L^{-1}$ in methanol were prepared from standard solutions and stored

at 4°C. Working solutions were made fresh daily. All solvents were HPLC grade or higher from Sigma-Aldrich (Oakville, Canada), sodium dodecyl sulfate (SDS)( 99.0%) and bile salts (sodium cholate (50%), sodium deoxycholate (50%)) were purchased from Fluka (Sigma Aldrich), pentadecafluorooctanoic acid ammonium salt (APFO) (98%), cetyltrimethylammonium chloride (CTAC) (25 wt %( solution in water, hexadecyltrimethylammonium bromide (CTAB) (99.0%), DMSO (99.0%), dodecanophenone (98.0%) and ammonium phosphate (99.99%) from Sigma-Aldrich (Oakville, Canada). Ultrapure water was obtained from Barnstead NanoPure Diamond (18 MΩ), (Ontario, Canada). The micelle marker (NDPhA) and the EOF marker (DMSO) were spiked to sample solutions as required. The conductivity of the sample matrix was in some instances adjusted to obtain the same conductivity as background electrolyte (BGE). Stock solutions of 500 mM of SDS and 1000 mM of phosphate buffer were made weekly. BGE solutions were made through dilution of stock solutions with ultrapure water. pH and conductivity of the BGE solutions were adjusted after dilution. Each solution was filtered with 0.22 µm nylon filters and degassed prior to use.

## 3.3.Results and Discussion

### 3.3.1. Suppression of EOF

As was discussed in the previous chapter [25], when analyzing polar analytes this preconcentration method works best with suppressed EOF. Minimization of EOF is usually achieved by decreasing the charge on the surface of the capillary either using low pH BGE

or coated controlled flow capillaries. To assess the efficacy of EOF suppression, a series of MEKC experiments were conducted using various combinations of pH (acidic and neutral, 1.9 and 7.4) and polarity (+/-) for cationic (CTAC) and anionic (SDS) surfactants in fused silica capillaries (Table 3.1). Given that analytes only reach the detector when the mobility toward the detector exceeds opposing forces, there were only four combinations that allowed for analyte detection and of these only the anionic surfactants in acidic buffer with negative polarity met the requirements for efficient sweeping.

Table 3.1. Assessment of suitability of various combinations of micellar phase, pH and polarity for sweeping in fused silica capillary

| **Surfactant type[a]** | **pH of BGE** | **Polarity[b]** | **EOF** | **Micelle migration** | **Net migration[c]** | **Peak Observed** | **Suitable for sweeping** |
|---|---|---|---|---|---|---|---|
| Anionic | 1.9 | + | ⇨ | ⇦ | ⇦ | no | no |
| Anionic | 1.9 | - | ⇦ | ⇨ | ⇨ | yes | yes |
| Anionic | 7.4 | + | ⟹ | ⇦ | ⇨ | yes | no |
| Anionic | 7.4 | - | ⟸ | ⇨ | ⇦ | no | no |
| Cationic | 1.9 | + | ⟸ | ⇨ | ⇦ | no | no |
| Cationic | 1.9 | - | ⟹ | ⇦ | ⇨ | yes | no |
| Cationic | 7.4 | + | ⟸ | ⇨ | ⇦ | no | no |
| Cationic | 7.4 | - | ⟹ | ⇦ | ⇨ | yes | no |

a- Anionic surfactant SDS and cationic surfactant CTAC

b- + polarity when detector side is at the cathode end

c- Right arrow is toward the detector, and the left arrow is toward the inlet (injector) and length of each arrow indicates relative magnitude of mobility

Cationic surfactants were ruled out for use in bare fused silica because strong EOF reversal was observed at all pH values in untreated capillaries. These results were in agreement with Kim *et al.* [27] and showed that for bare fused silica the selection of surfactants is limited to anionic surfactants. In addition, the use of the acidic pH necessary for EOF suppression imposed further limits on the choice of surfactants, since solubility of anionic surfactants like APFO and bile salts were decreased substantially.

Coated capillaries can give zero or low flow under a range of conditions. These coatings chemically modify the internal surface of the capillary, altering generation of the electric double layer and EOF. Using BGEs containing each of the surfactants, the EOFs in four commercially-available coated capillaries (PSA, LPA, PAAm and PEG coated) were compared to the EOF in bare fused silica capillaries. The BGEs used included ammonium phosphate buffer (50 mM, pH 7.8), and similarly buffered SDS and CTAC solutions at 50 mM. Samples were diluted and conductivity adjusted with buffer to maintain a uniform electric field across the capillary. All capillaries had same dimensions (75 cm length and 75 μm ID). The Williams' method was employed for EOF measurement [28]. One plug of sample was injected hydrodynamically (50 mbar) for 30 s, 10 kV was applied for 5 min and another sample plug was injected. Pressure was then applied until signals for each sample plug were recorded. Based on the separation between the two peaks, EOF was calculated (Table 3.2). According to the certificate of performance of capillaries provided by suppliers, mobility of EOF below $0.3 \times 10^{-4}$ $cm^2 V^{-1} s^{-1}$ is considered suppressed EOF. Negative numbers in this table indicate reversed EOF.

In fused silica capillaries, the use of the cationic surfactant led to reversed EOF that was 4 times higher than seen with the buffer only. With SDS, the direction of the EOF was unchanged, but the magnitude was increased substantially (>200%). Generally, coated capillaries designed to reduce EOF were effective in the absence of surfactants, though only PSA fully eliminated EOF. Both PSA and LPA were effective for use with the surfactants, and worked especially well with SDS (no EOF detected). The LPA capillary was more effective with SDS than with simple buffer solution. The PAAm and PEG coated capillaries were unable to suppress the EOF when surfactants were used; furthermore EOF was substantially higher than that measured in fused silica with buffer only. This was evidence of significant interactions between the coatings and surfactants, which were strongest for cationic surfactants. One other important conclusion can be drawn from these results, and that was in general, modification of the capillary surface by surfactants significantly improved the reproducibility of migration times in MEKC (see Table 3.2, RSD in brackets).

Table 3.2. Effect of surfactants on EOF in capillaries with bonded phases.

| | **Mobility of EOF $\mu_{EOF}$ x$10^{-4}$ $cm^2 V^{-1} s^{-1}$ (RSD)** | | |
|---|---|---|---|
| **Capillary** | **Buffer** | **Buffer+SDS** | **Buffer+CTAC** |
| Fused silica | 1.0 (21.6) | 3.3 (1.4) | -4.0 (0.1) |
| PSA | ND | ND | -0.1 (5.7) |
| LPA | -0.6 (14.8) | ND | -0.4 (66.1) |
| PAAm | -0.2 (33.5) | 2.8 (0.6) | -1.5 (2.2) |
| PEG | -0.1 (115) | 2.6 (2.4) | -1.3 (1.0) |
| ND – Not detected | | | |

### 3.3.2. Optimization of Conditions with Regards to Surfactants

#### 3.3.2.1. Critical micelle concentration (CMC)

CMC contributes to the phase ratio component of the retention factor (Eq. 3.3) and there are several factors influence the CMC of surfactants including ionic strength, organic solvents, and temperature. To study the effect of ionic strength, CMCs of each surfactant in buffer at a range of concentrations (phosphate buffer 0-50 mM at pH 7.4) were measured at 25 °C using the conductivity method [29] (Table 3.3). Predictably, the CMC decreased as ionic strength of solution increased for the anionic surfactants. For the cationic surfactants CTAC and CTAB, CMCs were low even with no additional electrolyte. Increased electrolyte concentration led to only minor decreases for CTAB, and a small increase in CTAC.

Table 3.3. Effect of ionic strength of solution on CMC[a]

| | CMC (mM) ± %RSD | | | |
|---|---|---|---|---|
| **Surfactant** | **0 mM** | **12.5 mM** | **25 mM** | **50 mM** |
| SDS | 4.1 ± 0.2 | 3.4 ±0.2 | 2.6 ±0.2 | 1.8 ±0.1 |
| APFO | 25.1 ± 0.6 | 24.3 ±0.5 | 23.8 ±0.5 | 22.4 ±0.6 |
| Bile salt | 14.2 ± 0.5 | 13.3 ±0.4 | 12.9 ±0.2 | 12.3 ±0.2 |
| CTAB | 0.9 ± 0.1 | 0.9 ±0.1 | 0.8 ±0.1 | 0.6 ±0.1 |
| CTAC | 1.5 ± 0.1 | 2.2 ±0.1 | 2.0 ±0.1 | 1.9 ±0.1 |

a- experiments conditions: Phosphate buffer pH=7.4 at 25 °C. each experiment repeated three times

Since CTA compounds have longer hydrophobic tails than the other surfactants studied and tend to have relatively strong associations with their counter anions (bromide and chloride) in the solvation sphere, barriers to micelle formation are diminished, which can explain the lower CMC. Since the fractional charge on these micelles is already low, the impact of increased electrolyte concentration is not as significant.

#### 3.3.2.2. Concentration of surfactants

To examine the effect of surfactant concentration on pressure assisted-sweeping, SDS as a representative surfactant was used (50 mM to 300 mM in 25 mM phosphate buffer at pH 1.9 in fused silica capillary) and electrochromatograms are presented in Figure 3.1. Peak width decreased with increasing SDS concentration, but for analytes with large logP the change in peak width at concentrations above 200 mM was minimal (See Figure 3.2). For these analytes (e.g. NDPA and NDBA), the peaks were already very narrow and retention and sweeping was fairly efficient. The narrowing was most profound for compounds with small log P, which are also the compounds for which sweeping tends to be least efficient. In a previous chapter it has been shown that increase in concentration of surfactant results in better separation and enrichment [25], however; the ionic strength of the BGE increased with concentration of ionic surfactants, which led to undesirable Joule heating and put a practical limitation on the concentration of surfactant that was lower than the limit of solubility. In this study, concentrations of surfactants were increased while the conductivity and current level were monitored to ensure the power never exceeded 1 W. By using longer

conditioning times for capillaries and employing lower temperature (20 °C) the effect of Joule heating was minimized. As a result, the reproducibility and peak characteristics were acceptable. Results obtained using coated capillaries were better than those with bare fused silica capillaries.

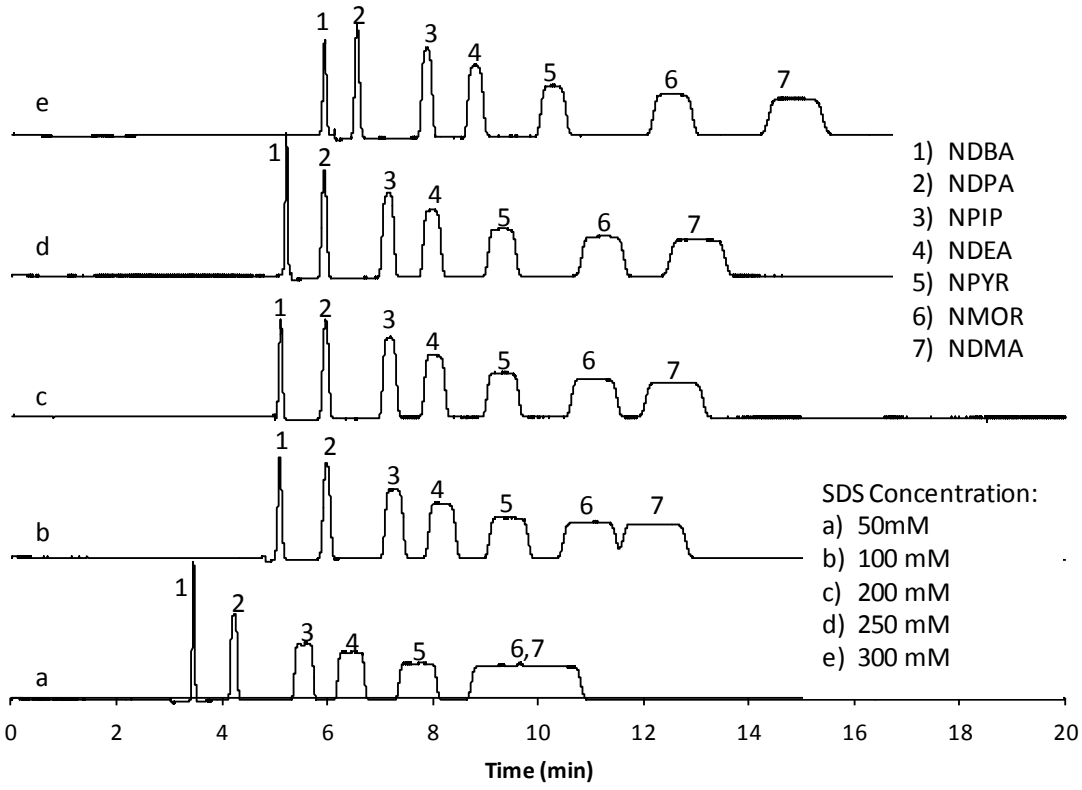


Figure 3-1. Effect of concentration of SDS on peak shape in sweeping-RM-EKC. BGE: 50-300 mM of SDS in 25 mM phosphate buffer (pH 7.8), capillary: PSA coated 75μm ID and 33.5 cm, potential: +180Vcm$^{-1}$, injection: 100 mbar.s.

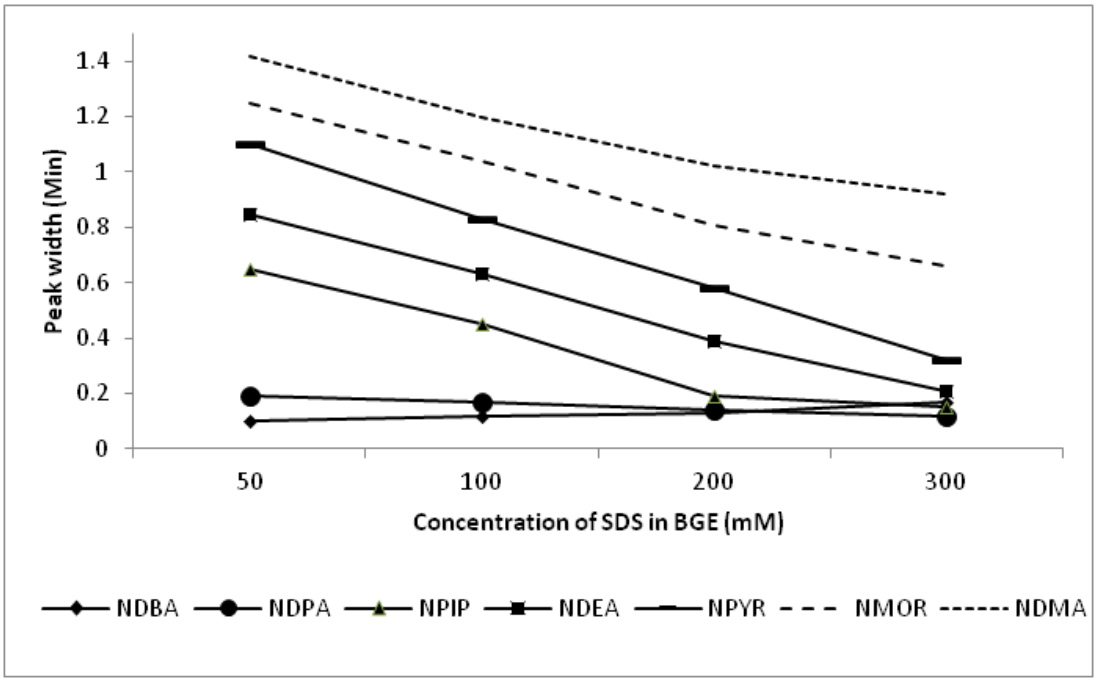


Figure 3-2. Effect of concentration of SDS on peak width in MEKC. CE condition: fused silica capillary with 98.5 cm length and 50 μm diameter, ID, electric field 230 $Vcm^{-1}$, hydrodynamic injection (50 s at 50 mbar), auxiliary pressure 70 mbar, BGE: 25 mM of phosphate buffer at pH 1.9 and 50-300 mM of SDS. Sample is a mixture of seven nitrosamines in buffer solution with same conductivity as BGE.

To ascertain the ideal surfactant concentrations for this work, a series of BGEs in a low buffer concentration (25 mM phosphate buffer, pH 7.4) and a range of surfactant concentrations (25 mM to 300 mM) were used in conventional MEKC experiments to determine *k* according to Eq. 3.4. The results are presented in Table 3.4. Due to the lower solubility of CTAB (less than 50 mM) no data were reported. For surfactants with high CMCs, such as bile salts and APFO, analytes co-migrated at low concentrations of surfactant and no data were reported. High retention factors demonstrate that the analyte

have a strong association with the micellar phase and should afford the best enrichment factors. The *k* values from MEKC were also suitable for pre-determination of suitability to resolve closely migrating pairs.

Although increasing the concentration (which increases phase ratio) of the surfactant should increase retention and enhance differences in retention behavior, this effect is not always predictable. The relationship between *k* and phase ratio was tends to be non-linear, resulting from a change in the micelle (i.e. size, shape, fractional charge, etc.) and consequently a change in the partitioning of the analyte between the bulk and stationary phases (i.e., *K*). Using the migration of the NDEA and NPIP as an example, the *k* values (Table 3.4) that these two compounds are baseline resolved or nearly baseline resolved in all cases except when using APFO, regardless of concentration. With both SDS and CTAC, increases in surfactant concentration result in significant improvements in retention and resolution. Increases in the concentration of bile salts from 100 to 200 mM yielded only incremental improvements for NPIP and no improvement for NDEA. As the concentration of the bile salts was increased to 300 mM, all *k* values increase, but the effect for the cyclic NPIP was more pronounced. The bile salts showed different trends than the other surfactants for the five most polar nitrosamines, with little practical use at concentrations below 100 mM and no improvement in retention behavior at higher concentrations for NDMA, NMOR and NPYR.

Table 3.4. MEKC-based estimation of *k* for nitrosamines with different PSPs[a]

| ***SDS*** | | | | | | ***Bile* salts** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **25 mM** | **50 mM** | **100 mM** | **200 mM** | **300 mM** | **25 mM** | **50 mM** | **100 mM** | **200** | **300 mM** |
| *NDMA* | 0.05 | 0.23 | 0.26 | 0.41 | 0.62 | | | 0.14 | 0.05 | 0.07 |
| *NMOR* | 0.08 | 0.30 | 0.37 | 0.58 | 0.92 | | | 0.18 | 0.10 | 0.14 |
| *NPYR* | 0.11 | 0.43 | 0.60 | 0.92 | 1.54 | | | 0.21 | 0.15 | 0.21 |
| *NDEA* | 0.15 | 0.67 | 0.97 | 1.49 | 2.65 | | | 0.27 | 0.25 | 0.38 |
| *NPIP* | 0.24 | 1.03 | 1.61 | 2.38 | 4.63 | | 0.12 | 0.32 | 0.35 | 0.53 |
| *NDPA* | 0.74 | 3.15 | 6.47 | 7.63 | 27.14 | | 0.28 | 0.63 | 0.91 | 1.41 |
| *NDBA* | 6.15 | 36.83 | 46.41 | 82.99 | 122.76 | | 1.42 | 3.39 | 6.15 | 9.19 |
| | ***APFO*** | | | | | ***CTAC*** | | | | |
| *NDMA* | | 0.08 | 0.19 | 0.33 | | | | 0.09 | 0.09 | 0.15 |
| *NMOR* | | 0.12 | 0.29 | 0.55 | | | 0.09 | 0.15 | 0.20 | 0.31 |
| *NPYR* | | 0.28 | 0.47 | 1.28 | | 0.08 | 0.12 | 0.19 | 0.27 | 0.42 |
| *NDEA* | | 0.55 | 1.04 | 2.46 | | 0.21 | 0.30 | 0.44 | 0.75 | 1.10 |
| *NPIP* | | 0.55 | 1.06 | 2.48 | | 0.24 | 0.40 | 0.68 | 1.18 | 1.73 |
| *NDPA* | | 2.32 | 4.58 | 8.90 | | 0.78 | 1.46 | 2.65 | 4.75 | 6.87 |
| *NDBA* | | 30.81 | 43.07 | 57.25 | | 5.25 | 10.89 | 20.54 | 32.41 | 46.00 |

a-MEKC conditions: BGE: phosphate buffer 25 mM, pH 7.8 and surfactants 25 mM-300 mM, potential: 230 $Vcm^{-1}$ (in positive mode, except for CTAC), injection: 100 mbar.sec, sample: mixture of seven nitrosamines (~20 µg $mL^{-1}$), EOF and micelle markers, capillary: fused silica 50 µm ID, 56 cm effective length.

### 3.3.3. Calculation of $k'$ and Separation Characteristics from Sweeping-RM-EKC Experiments

Given that sweeping-RM-EKC differs from MEKC in terms of the mode for analyte migration and the relative magnitudes of the $t_{EOF}$ and $t_{mc}$, $k$ determined by MEKC may not be the best expression of the analyte interaction with the system, particularly with respect to the separation. Moreover, though the highest $k$ values should give the best results for sweeping, $k$ does not account all the variables involved. In absence of EOF, the micellar phase acts as a mobile phase and bulk solution is stationary, thus we suggest that the traditional chromatographic retention factor, $k'$ (Eq. 3.5) is a better representation of the system. In the revised version of $k'$, $t_{mc}$ for a compound strongly retained by the micellar phase compound (i.e. NDPhA) replaces $t_0$, and $t_R$ the migration time of analyte. In Table 3.5, calculated $k'$ values are listed along with peak width and number of theoretical plates as an indication of performance consistent with chromatographic theory. Here, a small $k'$ shows higher interaction with the micellar phase and larger $k'$ indicates higher retention by the stationary phase, the bulk electrolyte solution. The lowest values were for the least polar components, which were weakly retained in the pseudostationary phase (i.e., bulk BGE). Taken with electrochromatograms for each system (Figure 3.3), good performance characteristics were seen for all systems but the bile salts, though some interesting migrations behaviors were seen with the bile salts that could be further exploited.

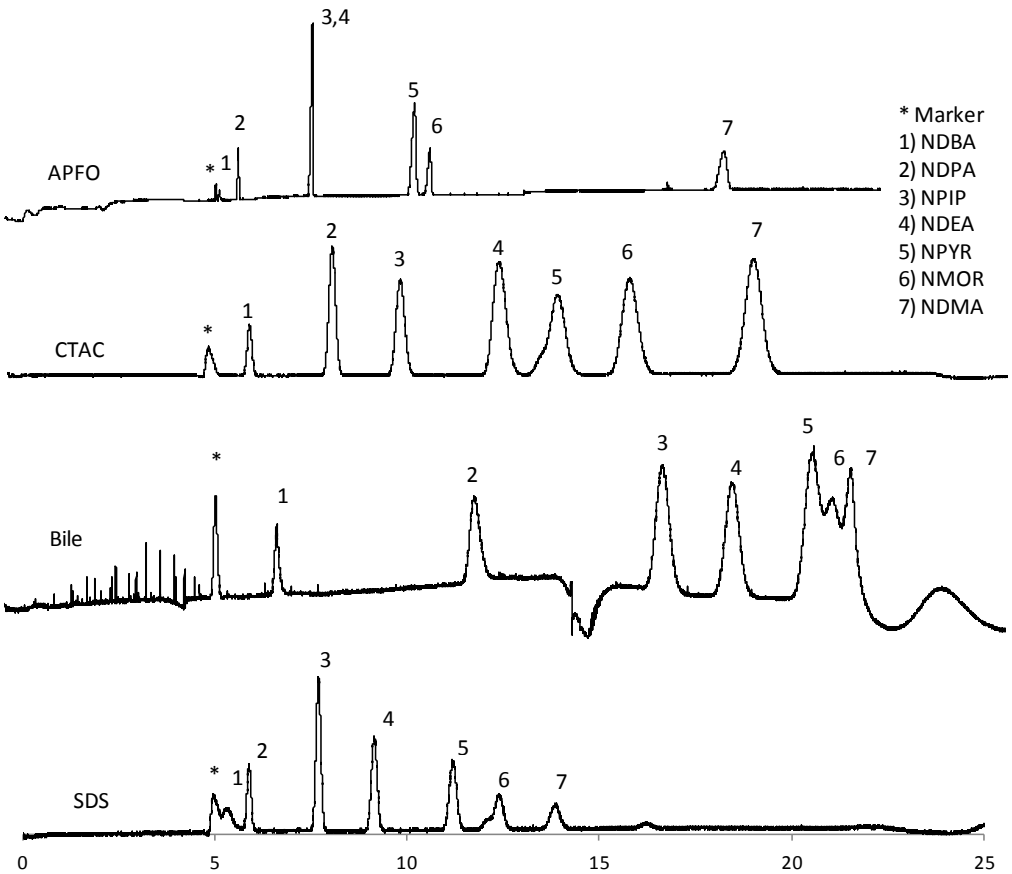


Figure 3-3. Comparison of the selectivity and peak shapes in sweeping-RM-EKCSDS, CTAC, bile salts and APFO, BGE: 200 mM surfactant in 25 mM phosphate buffer (pH 7.0) capillary: PSA coated 50 μm ID and 34 cm length, potential: 180 V/cm, injection: 250 mbar.s and 5 mbar external pressure. Sample: mixture of seven nitrosamines at concentration of 10 mg $L^{-1}$

Peak widths and theoretical plate numbers for the four surfactant systems are comparable, the exception being results for APFO, which were outstanding even though NPIP and NDEA were not resolved - as was the case in the MEKC studies. APFO is unique among the surfactant systems given that the perfluorinated tail is neither hydrophilic nor lipophilic; here it can be concluded that the associations with

the nitrosamines were primarily through hydrogen-bond type interactions with the head-group.

If nonpolar interactions between the nitrosamines and the hydrophobic surfactant tail (micelle core) were significant, nitrosamines would interact more weakly with the aqueous BGE in the presence of CTAC (C16) than SDS (C12). This was contrary to the data which shows lower $k'$ values for SDS. This confirms the idea that interactions with the head group dominate with small polar compounds like nitrosamines under these conditions, even for nitrosamines with significant hydrophobic character such as NDBA.

Table 3.5- Sweeping-RM-EKC estimation [a] of *k′*

| | **Calculated *k′*** | | | | **Peak Width (min)** | | | | **Number of Theoretical Plates (x10$^4$)** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **SDS** | **Bile** | **CTAC** | **APFO** | **SDS** | **Bile** | **CTAC** | **APFO** | **SDS** | **Bile** | **CTAC** | **APFO** |
| *NDBA* | 0.07 | 0.29 | 0.21 | 0.02 | 0.206 | 0.169 | 0.139 | 0.035 | 1.4 | 8.9 | 10.5 | 133.0 |
| *NDPA* | 0.18 | 1.23 | 0.62 | 0.11 | 0.130 | 0.451 | 0.192 | 0.034 | 11.4 | 3.8 | 9.9 | 161.3 |
| *NPIP* | 0.55 | 2.12 | 0.96 | 0.46* | 0.145 | 0.489 | 0.258 | 0.057* | 15.6 | 6.2 | 8.0 | 101.7* |
| *NDEA* | 0.84 | 2.45 | 1.45 | 0.46* | 0.177 | 0.518 | 0.379 | 0.057* | 14.9 | 6.8 | 5.8 | 101.7* |
| *NPYR* | 1.25 | 2.84 | 1.74 | 0.94 | 0.226 | 0.503 | 0.468 | 0.106 | 13.6 | 8.9 | 4.8 | 51.7 |
| *NMOR* | 1.49 | 2.92 | 2.10 | 1.01 | 0.263 | | 0.480 | 0.093 | 12.4 | | 5.8 | 72.8 |
| *NDMA* | 1.79 | 3.01 | 2.72 | 2.40 | 0.287 | | 0.500 | 0.216 | 13.0 | | 7.8 | 38.2 |

*Unresolved components

a-Peak width and theoretical plates for nitrosamines with various surfactants at 200 mM. BGE: phosphate buffer 25 mM, pH 7.8, potential: 180 V/cm (in negative mode, except for CTAC), injection: 250 mbar.sec, sample: mixture of seven nitrosamines (~20 µg mL$^{-1}$), EOF and micelle markers, capillary: PSA 50 µm ID, 25.5 cm effective length and 5 mbar external pressure.

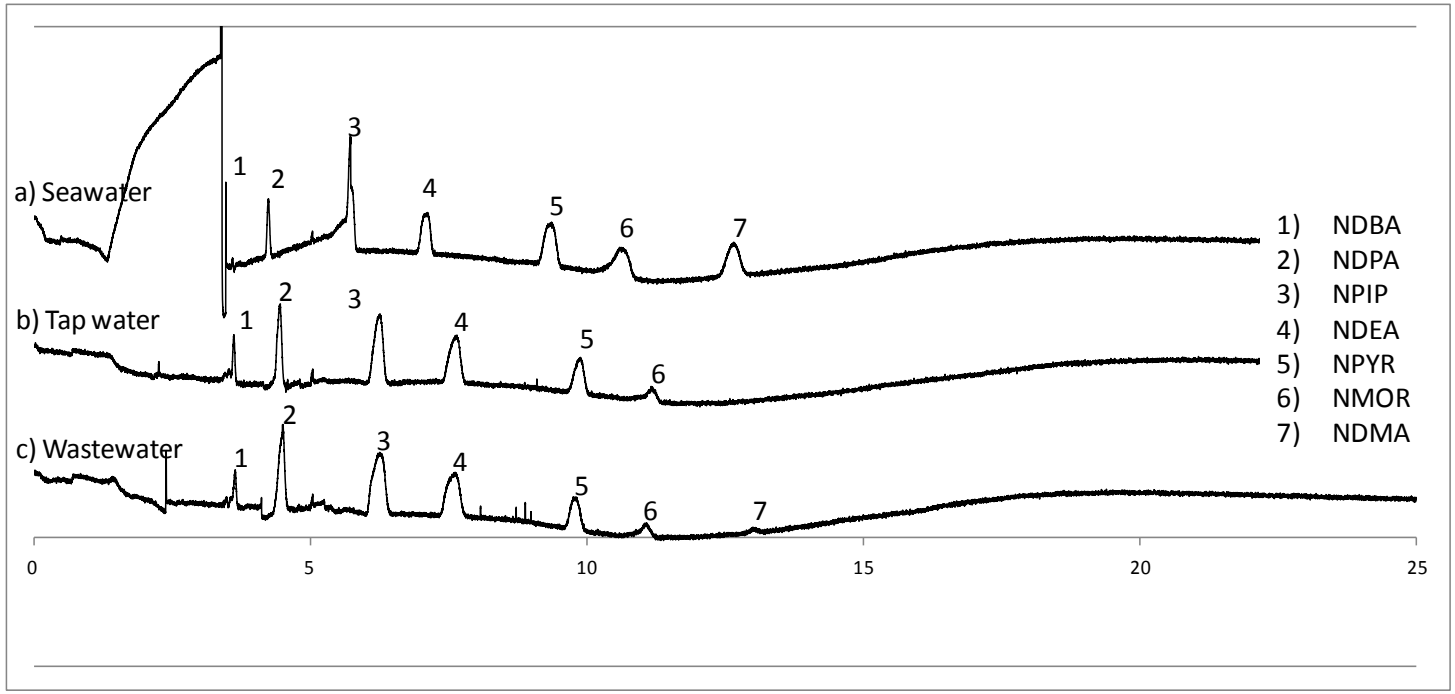


Figure 3-4. Sweeping-RM-EKC of fortified samples Sweeping-RM-EKC using SDS under optimized conditions with fortified samples a) seawater, b) tap water, and c) wastewater. BGE: 125 mM SDS in 25 mM phosphate buffer (pH 7.8), capillary: PSA coated 50 μm ID and 34 cm length, potential 180V/cm, injection 1000 mbar.s and 20 mbar external pressure after 5 min. Concentration of each nitrosamines in each sample is 0.5 mg $L^{-1}$

To demonstrate the application of this method in different sample matrices, fortified samples of nitrosamines in tap water, seawater and wastewater were prepared and results are shown in Figure 3.4. The ionic strength of the matrices was not adjusted. The Electrochromatogram a in Figure 3.4 clearly shows that peak shapes were considerably better due to high concentration of salt in the seawater sample matrix. Similarly, low ionic strength in tap water sample resulted in a decrease in the efficiency of focusing and the peak for NDMA was not detected. For wastewater, electrochromatogram c (Figure 3.4), which the ionic strength was higher than tap water, and though significantly lower than seawater, it was possible to identify all peaks. Since the proposed method is compatible with highly saline matrices, addition of salt to the low conductivity samples is recommended.

The use of programmed auxiliary pressure to augment the slow migration of analytes weakly retained by the mobile micellar phase was optimized; pressures ranging from 2 to 30 mbar were applied at various time intervals (1 to 15 min). Electrochromatograms presented in Figure 3.4 were obtained using the optimal 20 mbar external pressure applied at 5 min after sweeping was commenced. The time delay was used to allow analytes with large *k* (e.g. NDPA and NDBA) have enough time to be resolved.

## 3.4. Concluding Remarks

A number of factors have been studied to improve the enrichment of polar neutral nitrosamines by pressure-assisted sweeping-RM-ECK, including ways to suppress the EOF (by pH and coated capillaries) and maximize the retention factor by changing parameters that influence $K$ and $\varphi$. In fused silica capillaries, the EOF can be suppressed at pH<2 but this limits our selection of surfactants. Coated capillaries were better alternative for suppression of the EOF. Among different coated capillaries studied in this work, the sulfonic acid (PSA) coated capillaries were capable of suppressing the EOF over the pertinent pH range (2-8) and with any type of surfactant. In general, SDS showed reasonable performance however; application of cationic surfactants was shown to be suitable for analysis of nitrosamines due to their ability to act as a hydrogen bond donor. APFO, showed remarkable results in term of peak shapes and number of theoretical plates, however, NDEA and NPIP remained unresolved despite of increasing concentration of surfactant. Nevertheless, the compatibility of APFO with mass spectrometry makes it attractive, and use of mixed micelle systems can be exploited to improve resolution of these co-migrating species.

## 3.5.References:


[1] Modir-Rousta, A., Bottaro, C. S., *Electrophoresis* 2015, *36*, 1016-1023.

[2] Chen, R., Cheng, H., Wu, W., Ai, X., Huang, W., Wang, Z., Cheng, J., *Electrophoresis* 2007, *28*, 3347-3361.

[3] Glavač, N., Injac, R., Kreft, S., *Chromatographia* 2009, *70*, 1473-1478.

[4] Falck, E., Groenhagen, A., Mühlisch, J., Hempel, G., Wünsch, B., *Analytical Biochemistry* 2012, *421*, 439-445.

[5] Quirino, J. P., Terabe, S., *Anal Chem* 1998, *70*, 149-157.

[6] Quirino, J. P., Terabe, S., *Analytical Chemistry* 1999, *71*, 1638-1644.

[7] Quirino, J. P., Terabe, S., *Science* 1998, *282*, 465-468.

[8] Terabe, S., *Chem Rec* 2008, *8*, 291-301.

[9] Kelly, K. A., Burns, S. T., Khaledi, M. G., *Anal Chem* 2001, *73*, 6057-6062.

[10] Lambert, W. J., *Journal of Chromatography A* 1993, *656*, 469-484.

[11] Poole, S. K., Poole, C. F., *J Chromatogr A* 2008, *1182*, 1-24.

[12] Weckwerth, J. D., Carr, P. W., Vitha, M. F., Nasehzadeh, A., *Anal Chem* 1998, *70*, 3712-3716.

[13] Trone, M. D., Khaledi, M. G., *Electrophoresis* 2000, *21*, 2390-2396.

[14] Trone, M. D., Mack, J. P., Goodell, H. P., Khaledi, M. G., *J Chromatogr A* 2000, *888*, 229-240.

[15] Fuguet, E., Rafols, C., Bosch, E., Abraham, M. H., Roses, M., *Electrophoresis* 2006, *27*, 1900-1914.

[16] Yang, S. Y., Khaledi, M. G., *Analytical Chemistry* 1995, *67*, 499-510.

[17] Yang, S. Y., Khaledi, M. G., *Journal of Chromatography A* 1995, *692*, 301-310.

[18] Quina, F. H., Alonso, E. O., Farah, J. P. S., *J Phys Chem-Us* 1995, *99*, 11708-11714.

[19] Van Biesen, G., Bottaro, C. S., *Electrophoresis* 2006, *27*, 4456-4468.

[20] Burns, S. T., Khaledi, M. G., *Anal Chem* 2004, *76*, 5451-5458.

[21] Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T., *Analytical Chemistry* 1984, *56*, 111-113.

[22] Terabe, S., Otsuka, K., Ando, T., *Analytical Chemistry* 1985, *57*, 834-841.

[23] Janini, G. M., Muschik, G. M., Issaq, H. J., *Electrophoresis* 1996, *17*, 1575-1583.

[24] Janini, G. M., Muschik, G. M., Issaq, H. J., *J Chromatogr B Biomed Appl* 1996, *683*, 29-35.

[25] Modir-Rousta, A., Bottaro, C. S., *Electrophoresis* 2013, *34*, 2553-2560.

[26] Haidian, Z., *Journal of the National Cancer Institute* 1968, *41*.

[27] Kim, J.-B., Otsuka, K., Terabe, S., *Journal of Chromatography A* 2001, *912*, 343-352.

[28] Williams, B. A., Vigh, G., *Anal Chem* 1996, *68*, 1174-1180.

[29] Bakshi, M. S., *J Chem Soc Faraday T* 1993, *89*, 4323-4326.

# Chapter 4 Analysis of *N*-Nitrosamines in Wastewater Samples Using SPE-MEKC

## 4.1.Introduction

*N*-Nitrosamines are relatively stable compounds found in the environment [1-2] formed by reactions between amines, or their derivatives, and nitrous agents such as nitrous acid or nitrogen oxides[3]. Nitrosamines, reported for the first time in 1863, have been detected in a variety of matrices as byproducts of food and beverage production and preservation, water and wastewater treatment and as intermediates in industrial processes [2]. Toxicity in humans was not established until nearly a century after their first discovery, Magee reported that when fed to rats *N*-nitrosodimethylamine (NDMA) induced cancer of the liver [4]. Later it was found that alkylation of cellular DNA by NDMA was the main reason for induction of a variety of cancers [5]. Nitrosamines are now widely recognized as potential carcinogens and a serious threat to public health.

*N*-Nitrosamines have been found widely in foodstuffs, beverages [6], cosmetics[7], pharmaceuticals, biological material, air (e.g. tobacco smoke), contaminated ground water (from rocket fuel), rubber [7], and recently in drinking water and wastewater[8]. Since identification of NDMA as a water disinfection byproduct in 1989, drinking water in Ontario, Canada and California, USA has been monitored for NDMA, with maximum allowable concentrations of 9 and 10 ng $L^{-1}$, respectively [8]. Discovery of other nitrosamines in drinking water has been reported, and with the addition of *N*-nitrosopyrrolidine and *N*-nitrosomorpholine by Charrois *et al*. [9].List of *N*-

nitrosamines detected in drinking water is listed in Table 1.1. Based on the boiling points of the nitrosamines (Table 1.1), these compounds can be categorized as volatile. GC/MS is known as the most reliable technique for analysis of volatile nitrosamine and has been introduced as a routine technique by U.S. Environment Protection Agency (EPA 521). In this method, SPE extraction is required, which is tedious and uses a significant amount of solvents. At the end of extraction, analytes are isolated in an organic solvent ($CH_2Cl_2$) and method requires that the range of recovery must be 50% - 150%. The method tolerance for such an unimpressive range of recoveries speaks to the limitations in efficiency of the extraction methods. Furthermore, the absence of non-volatile nitrosamines on this method- list is notable, and is most likely explained by limitations of the GC techniques used in accommodating highly polar or high-mass compounds [8-9]. HPLC has also been used as an alternative technique for relatively high molecular weight compounds and wide range of contaminants in drinking water including nitrosamines [10-11].

While analysis of small molecules such as pharmaceuticals and agrochemicals using HPLC is routine, CE has gradually become more widely-accepted as a complementary technique for HPLC in the analysis of hydrophilic analytes. Advances in capillary electrophoresis (CE) techniques, such as capillary electrochromatography (CEC) and micellar electrokinetic chromatography (MEKC), have greatly extended the capability and efficiency of CE [12]. The utility of MEKC for separation of small neutral polar compounds, such as phenols, PCBs, PAHs, chairals, and pesticides has been

established [13-14]; however, only a few references are available on applications of CE in analysis of nitrosamines [13-17].

The versatility of CE and related techniques, particularly EKC, has led to a growth in interest in CE for environmental analysis, and several major reviews on the subject have been published in past number of years [16, 18-19]. CE and EKC efforts in *N*-nitrosamine analysis have been focused on improvement of detection methods and sample enrichment techniques [18]. The most common detector in CE is on-column UV-Vis absorbance with typical detection limits in the μmol $L^{-1}$ range for a typical analyte [19]. Fluorescence detection has been used to lower the detection limits for nitrosamines , however, derivatization is required [16]. Electrochemical detection has some advantages such as low cost, high sensitivity (sub-μg $L^{-1}$) and suitability to miniaturization. Amperometric detection has been used for electrochemically active compounds [20] and conductivity for charged analytes [21], though none of the electrochemical detection methods have been used in the analysis of nitrosamines. The application of MS for detection in CE has represented an area of ongoing interest and led to improvements in selectivity and detection limits. However most interfaces rely on ionization sources designed for LC-MS flow rates (>μL $min^{-1}$) that are much higher than those of CE (nL $min^{-1}$) necessitating addition of sheath liquid that dilutes the sample and reduces sensitivity [22-25]. Further complicating this marriage, particularly with electrospray ionization, is the use of non-volatile compounds and

salts in CE BGE solutions that interfere with the ionization method. In this work, seven nitrosamines, NDMA, NDEA, NDPA, NDBA, NMOR, NPYR, and NPIP, were chosen on the basis of their frequent identification in environmental studies of drinking water, wastewater and food. Based on the structure of *N*-nitrosamines, amino nitrogen (as with tertiary amines) could be protonated at acidic pH and be candidate for analysis by capillary zone electrophoresis (CZE).To examine an attempt has been made to determine the $pK_a$ value of *N*-nitrosamines. A method of MEKC also developed for analysis of *N*-nitrosamines. Finally, a solid phase extraction (SPE) method was optimized using different types of sorbent. SPE-MEKC experiments were performed on fortified samples.

## 4.2.Materials and Methods

### 4.2.1. Instruments and Operating Conditions

Electrochromatograms were obtained using an Agilent 3D 1600 capillary electrophoresis system with Agilent ChemStation software (Waldbronn, Germany). Electrophoresis experiments were performed in polyimide coated fused silica capillaries of 50 µm i.d. from MicroSolv Technologies, Eatontown, NJ, USA. All CE experiments were carried out at 25 °C unless otherwise stated. Absorbance data of *N*-nitrosamines were collected using CE system diode array detector with signal processing Chemstation software. Conductivity of BGE and sample matrix was

measured with a VWR conductivity meter when the volume of solution prepared was sufficient, otherwise the conductivity measurements were carried as described in previously [26].

### 4.2.2. Materials

All solvents (HPLC grade or higher) except water, sodium dodecyl sulfate (SDS) (99.0%), dodecanophenone (98.0%) and ammonium acetate (99.9%), ammonium formate (99.5%), ammonium hydrogen phosphate (99.9%), ammonium dihydrogen phosphate (99.9%), sodium tetraborate (99 %) and ammonium hydroxide solution (30%) were purchased from Sigma-Aldrich (Oakville, Canada). Formic acid (88%) ACS grade was from Baker Analyzed and phosphoric acid (85%) ACS grade was from EM Science. Ultrapure water was obtained from a Barnstead NanoPure Diamond water purification system (18 MΩ), (Barnstead, Ontario, Canada). SPE cartridges: 200 mg/6 mL of Strata-X 33µm, Strata-XC, Strata-XCW, Strata-XAW were obtained from phenomenex, California, USA; 150 mg/6 mL of Oasis MAX, Oasis AWX, Oasis CWX from Waters, Milford, MA, USA and Resprep from Restek, Bellefonte, PA, USA.

### 4.2.3. Standards and Samples

Standard solutions of 2000 mg $L^{-1}$ of NDMA, NPYR, NMOR, NDEA, NPIP, NDPA, and NDBA were purchased from Supelco (Ontario, Canada). Stock solution were prepared from standard solutions at a concentration of 200 mg $L^{-1}$ in methanol and stored at 4°C. Working solutions were made fresh daily. The micelle marker (dodecanophenone) and the EOF marker (DMSO) were spiked to standards and sample solutions as required. Each sample was filtered with 0.22 µm nylon filters and degassed prior to use.

### 4.2.4. Background Electrolyte solutions (BGE)

Stock buffer solutions were prepared at various pHs ranging from pH 3 to 12; details of the specific salts and pH used are shown in Table 4.1. The pH and conductivity of the electrolyte solutions were measured after final pH adjustment and dilution. Electrolyte and solutions were prepared each day by dilution of the corresponding stock and SDS surfactant solutions with ultrapure water. BGE were prepared using buffer solution at concentration from 50 mM to 100 mM and SDS at concentration from 50 mM to 300 mM.

Table 4.1-Buffer solutions (50 mM) for pH ranging from 2 to 12

| pH | Buffer solution | pKa |
|---|---|---|
| 2.0 | Phosphate | 2.12 |
| 3.0 | Formate | 3.72 |
| 4.0 | Acetate | 4.75 |
| 5.0 | Acetate | 4.75 |
| 6.5 | Phosphate | 7.20 |
| 7.0 | Phosphate | 7.20 |
| 8.5 | Borate | 9.14 |
| 9.5 | Borate | 9.14 |
| 10.0 | Ammonium | 9.15 |
| 11.5 | Phosphate | 12.35 |
| 12.0 | Phosphate | 12.35 |

## 4.3.Procedures

### 4.3.1. Capillary Conditioning

Prior to first use, each capillary was conditioned by flushing (1 bar) the following sequence of solutions: methanol (15 min), ultrapure water (15 min), sodium hydroxide 1.0 M (15 min), ultrapure water (15 min), and finally BGE, (15 min). Conditioning was performed at the beginning of each day by flushing with NaOH 0.1 M, ultrapure water, and BGE for 5 min each. All sample injections were performed

hydrodynamically at the inlet end of the capillary. The capillary was conditioned between each run by flushing with NaOH 0.1 N (1 min) and BGE (3 min). At the end of each day, post-run conditioning of the capillary was carried out by rinsing (flushing) with ultrapure water, methanol and air for 5 min each. For quantitative analysis each experiment was repeated at least three times. In case of pressure assisted sweeping RM-EKC, Zero-Flow coated capillaries were conditioned using ultrapure water and BGE for two and five minutes respectively. Other operating conditions are specified in the text or figure captions.

## 4.4.Results and Discussion

The structure of *N*-nitrosamines (Figure 1.3), suggests that it might be possible to protonate the amino nitrogen (as with tertiary amines) at acidic pH and thereby make them amenable to analysis by capillary zone electrophoresis (CZE). Since CE has been used widely for determination of $pK_a$ [27-29] and no data has been reported for the $pK_a$ of nitrosamines in the literature, a set of CZE experiment in a wide range of pH was performed. A series of buffers with pH ranging from 2.0 to 12.0 (pH beyond these ranges are rarely used in CE given their effects on the silica capillary) were prepared (see Table 4.2). Conditions for CE were set as follow: separation voltage at 20 kV, hydrodynamic injection of approximately 2 nL (20 mbar for 5 seconds), and wavelength 236 nm. A mixture of seven nitrosamines including: NDMA, NMOR, NPYR, NDEA, NPIP, NDPA, and NDBA in water were injected. All of nitrosamines

co-migrated along with the EOF marker at all pH values, indicating that these compounds remained uncharged in the range of pH studied.

Since CZE was not applicable for separation or determination of $pK_a$ of these nitrosamines, micellar electrokinetic capillary electrophoresis was assessed as an alternative separation technique in CE. LogP is a common measure of polarity and is defined on the basis of the partitioning of a given compound from water to octanol. The logP values for nitrosamines (see Table 1.1), are typically small or negative and reflect the tendency of those compounds to stay in the aqueous phase. The small differences of logP between NDMA, NMOR, NPYR and NMEA demand highly selective separation conditions. MEKC can afford the needed selectivity.

Initial MEKC investigation involved addition of 50 mM SDS as a pseudostationary phase to the BGE solutions used in the $pK_a$ study with the same conditions as used for CZE were repeated.

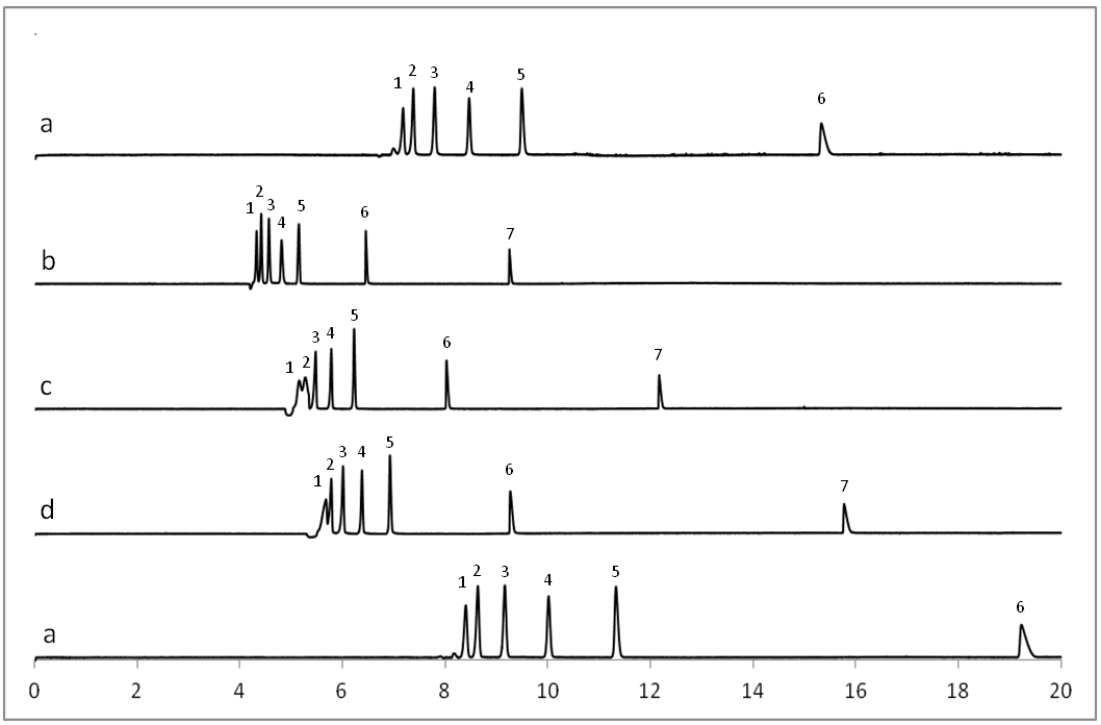


Figure 4-1. Electrochromatograms MEKC of nitrosamines at different pH, 50 mM of SDS in buffer solutions: a) pH 11, b) pH 10, c) pH 9, d) pH 8, and e) pH 6 capillary length 64.5cm, potential +25 kV, and injection volume 2.0 nL. Sample: 1) NDMA, 2) NMOR, 3) NPYR, 4) NDEA, 5) NPIP, 6) NDPA, 7) NDBA at concentration of 50 mg $L^{-1}$.

Results shown in Figure 4.1 indicate that regardless of pH, the presence of SDS facilitated separation. At pH 10, the peak characteristics such as peak height, area, width and symmetry, resolution, capacity are best and the fastest analysis is achieved. All MEKC parameters such as concentrations of buffer solution, SDS, and organic modifiers; temperature, applied potential, injection volume, and capillary length were then systematically optimized and the ranges studied and optimal conditions are summarized in Table 4.2.

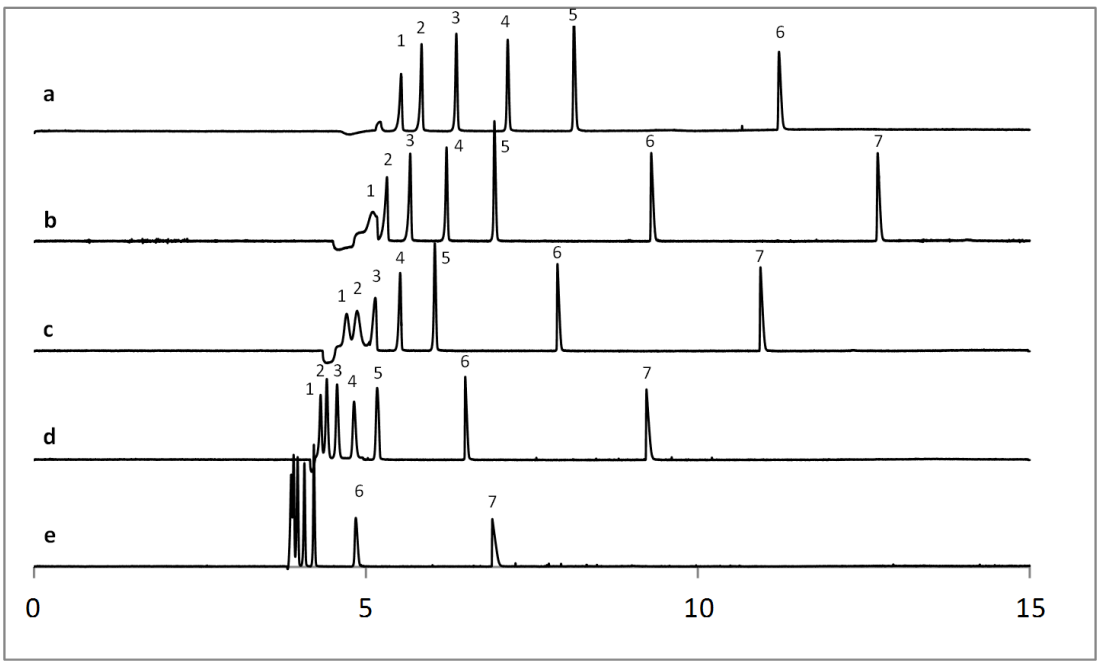


Figure 4-2. Effect of concentration of SDS on separation 50 mM of buffer solutions pH 10 and SDS concentration of: a) 150mM, b) 125 mM, c) 100 mM, d) 50 mM, and e) 25 mM capillary length 64.5cm, potential +25 kV, and injection volume 2.0 nL. Sample: 1) NDMA, 2) NMOR, 3) NPYR, 4) NDEA, 5) NPIP, 6) NDPA, 7) NDBA at concentration of 50 $mgL^{-1}$.

To avoid the Joule heating, it was preferable to keep the concentration and ionic strength of the buffer solution in BGE as low as possible. Increasing of concentration of SDS enhanced the resolution, but elevated the current. When concentrations of SDS were below 50 mM, three nitrosamines, NDMA, NMOR, and NPYR, were not resolved completely. Furthermore, co-migration of the analytes with methanol (solvent) and the EOF marker (DMSO) had significant peak broadening effect which also lowered the peak height (see Figure 4.2b-d). At higher concentrations of SDS (125 mM), all peaks were completely resolved; including NDMA, EOF marker and

methanol (Figure 4.2a-b) and peak 7 (NDBA) migrates within the desired time window. When concentration of SDS increased to 150 mM, peak shape of NDMA improved but NDBA peak after 25 min appeared.

Table 4.2. The optimized MEKC conditions

| **Conditions** | **Range** | **Optimized** |
|---|---|---|
| Voltage | 0-30 kV | 25 kV |
| Capillary length | 33-100 cm | 64.5 |
| Temperature | 10-40 °C | ≤ 25 °C |
| Injection volume | 1-80 nL | 4 nL |
| BGE | | |
| Buffer pH | 2-12 | 8-10 |
| Buffer concentration | 0-100 mM | 25-50 mM |
| SDS concentration | 0-300 mM | 125 mM |
| Organic modifiers | | |
| Methanol | 0-30% | 0% |
| Ethanol | 0-30% | 0% |
| n-propanol | 0-30% | 0% |
| n-butanol[a] | 0-30% | 0% |
| Acetonitril | 0-30% | 0% |

Increasing concentration of buffer component of the BGE had no significant effect on separation; however, ionic strength of BGE is elevated and peak broadening observed due to the Joule heating problem. On the other hand reducing the concentration of buffer below 25 mM has negative impact on reproducibility of migration times. Therefore, the buffer was kept at 25-50 mM.

The effect of organic modifiers on the separation was also studied. Organic modifiers used in this study were methanol, ethanol, n-propanol, n-butanol, and acetonitrile (ACN). The organic modifiers had a number of effects on separation and EOF. First, they change the partitioning behavior by changing the CMC of SDS. Second, they change the viscosity of BGE and modify the magnitude of the EOF. Although, subtle effects were noted in the electropherograms for separations using the various organic modifiers, there was no evidence of any improvement in separation after using an organic modifier. On the other hand, the negative effects were not substantial and organic modifiers can be used as necessary.

### 4.4.1. Effect of Sample Matrix

The sample matrix had significant effect on separation especially on very polar nitrosamines (such as NDMA, and NMOR). When methanol was used as a solvent for elution of samples in SPE, it interfered with separation of EOF marker, NDMA, and NMOR led to a poor base line resolution whereas, the use of water or BGE as dilution solvents gave the best separations. Two methods were applied to overcome the interferences those associated with methanol in particular. First, the effect of the methanol in a sample can be eliminated by increasing the concentration of SDS above 100 mM in BGE. Second, addition of methanol up to 30% to BGE can also suppress the interference of methanol in the sample matrix. It should be noted that addition of

methanol into BGE only improved the separation when methanol was present in sample matrix. Combination of both remedies had a synergistic effect.

### 4.4.2. Effect of Temperature

Capillary temperature can have a significant effect on separation therefore the effect of the capillary temperature was studied from 10 °C to 40 °C. At temperatures below 25 °C resolution improved, which was mostly attributable to the increased viscosity, and decreased mobility and thermal diffusion. When shorter capillary (33.5 cm) was used at 40 °C, the total analysis time was less than 4 min but NDMA and NPYR co-migrated with the EOF marker. Under the same conditions at 20 °C, the EOF marker, NDMA, and NPYR were completely resolved and total analysis time increased only to 6 min and peak characteristic were improved (see Appendix C)

### 4.4.3. Solid Phase Extraction (SPE)

A number of different types of commercially available sorbents (solid phase) were employed (cationic, weak cataionic, anaionic and weak anionic). Strata-X 33 µm, Strata-XC, Strata-XCW, and Strata-XAW from Phenomenex; Oasis MAX, Oasis AWX, and Oasis CWX from Waters; and Resprep from Restek (suggested by EPA method 521) were selected. For SPE and preconcentration of nitrosamines from water, conditions were systematically optimized. The percent absolute recoveries were

determined by comparison with directly injected standards to quantify the efficiency of extraction during method optimization. The recovery for each system using the best conditions for each sorbent were compared. Among sorbents studied, the Resprep (coconut charcoal) from Restek showed superior performance (see table 4.3 and figure 4.3).

Table 4.3 Performance of selected SPE sorbents for extraction of nitrosamines

| | % Recovery | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Resprp | Strata (Phenomenex) | | | | | Oasis (waters) | | |
| | | X | XC | XCW | XA | XAW | MAX | AWX | CWX |
| NDMA | 85.2 | 39.9 | 59.9 | 52.2 | 26.8 | 49.9 | 33.2 | 30.8 | 44.2 |
| NPYR | 84.4 | 44.2 | 56.2 | 48.9 | 32.1 | 50.1 | 46.2 | 39.2 | 41.9 |
| NMOR | 88.7 | 59.1 | 61.5 | 61.1 | 39.9 | 55.4 | 56.6 | 34.7 | 55.1 |
| NPIP | 89.1 | 65.1 | 68.3 | 66.5 | 51.2 | 61.6 | 61.2 | 48.8 | 51.6 |
| NDEA | 86.6 | 68.2 | 66.1 | 72.2 | 58.8 | 68.5 | 60.1 | 52.9 | 61 |
| NDPA | 81 | 71.5 | 72.9 | 77.4 | 63.2 | 71.1 | 67.3 | 58.3 | 74.8 |
| NDBA | 81.3 | 79.2 | 78.8 | 79.9 | 68.8 | 74 | 74.9 | 66.6 | 77.7 |

The optimized conditions are listed in Table 4.4. Nitrosamines with small and negative logP values show low recoveries. For some phases the percent recoveries for NDMA and NMOR were below 30% that can be attributed to the lower affinity of these analytes toward the sorbents. The recoveries of nitrosamines with large log P, such as NDPA and NDBA for all type of sorbents were satisfactory. The following procedure was optimized for the Resprep SPE phase. Each cartridge was conditioned with 2 x 2-mL portions methylene chloride followed by the same amounts of methanol and water.

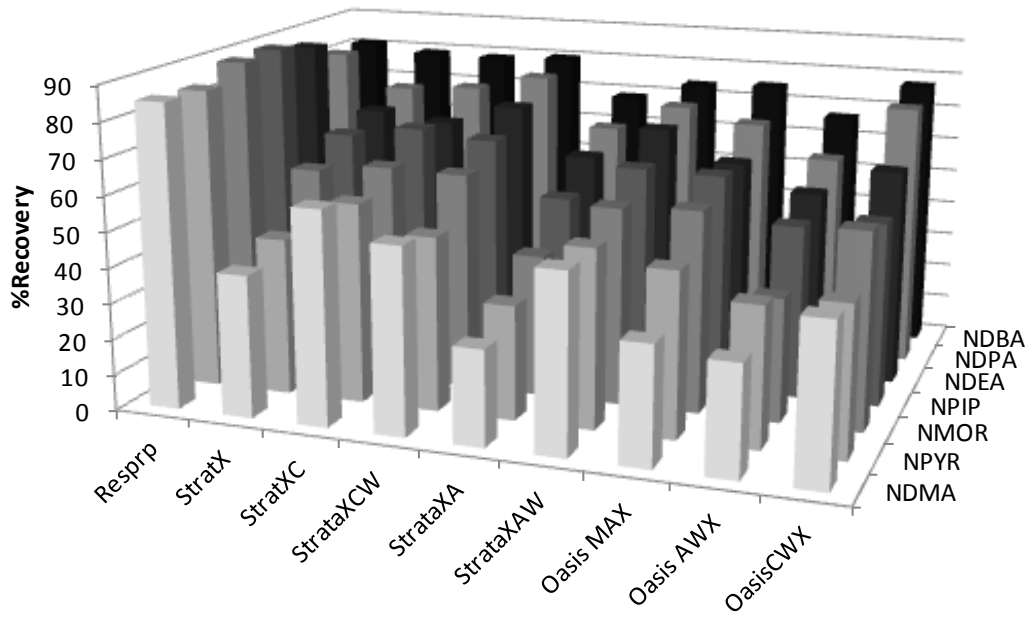


Figure 4-3 Performance (% Recovery) of selected SPE sorbent for extraction of seven nitrosamines.

The analytes were then extracted onto the SPE phase by passing 500-mL of aqueous sample through a SPE cartridge containing 2 g of 80-120 mesh coconut charcoal (Resprep). The cartridge was dried under vacuum for 10 min and compounds are eluted with a small volume of methylene chloride. Though this sample could be run directly with GC-MS, methylene chloride is not compatible with CE, and the solvent was exchanged to water by evaporation either vacuum drying or under nitrogen flow and re-dissolution in water. At this stage extreme care was required to avoid poor recovery for volatile nitrosamines like NDBA. In the solvent exchange step, when the volume of eluent was large, more analyte loss occurred. To reduce this effect, the

minimum amount of solvent was used (2 mL) and the eluent was dried in the ice bath under gentle flow of nitrogen (≤5 psi). In general, SPE cartridges such as Strata-X (from Phenomenex) and Oasis (from Waters) demonstrate clean extract (flat base line in MEKC) with low matrix effects, but poorer recoveries.

Table 4.4. Optimized conditions for SPE method (Resprep from RESTEK)

| Process | Experimental range | Optimized conditions |
|---|---|---|
| *Conditioning* | | |
| $CH_2Cl_2$ | 2-12 mL | 4 mL |
| $CH_3OH$ | 2-12 mL | 4 mL |
| $H_2O$ | 2-12 mL | 4 mL |
| *Equilibrium* | | |
| $CH_3OH$ | 0-10 mL | 2 mL |
| $H_2O$ | 0-30 mL | 6 mL |
| *Sample* | | |
| Extraction Volume | 100-1000 mL | 500 mL |
| Flow rate | 1-20 mL/min | 10 mL/min |
| *Drying* | | |
| Method | Vacuum, nitrogen flow | Vacuum |
| Drying time | 5-30 min | 10 min |
| *Elution* | | |
| Solvent | $CH_3OH$, $CH_2Cl_2$ | $CH_2Cl_2$ |
| Flow rate | 1-10 mL/min | 1 mL/min |
| Volume | 1-15 mL | 2 mL |

For both Waters and Phenomenex brands, the weak cation exchange and weak anion exchange sorbents gave the best performance within each company. However, even these suffered from poor recovery for NDMA, NMOR, and NPYR. On the other hand, the coconut charcoal and activated carbon performed better in terms of recovery for all nitrosamines, but they were non-selective and matrix effects were high resulting in very noisy baselines in the electropherograms.

### 4.4.4. Quantitative Analysis

Calibration curves for the seven nitrosamines were constructed. Five calibration data points were used over two orders of magnitude; each point was calculated as the average peak area of three sequential injections. The limit of detection (LOD) was considered as three times the standard deviation of the intercept-to-slope ratio, and LOQ was calculated as a ten times the standard deviation of the same ratio. The LOD and LOQ values for both MEKC without preconcentration and SPE-MEKC are shown in Table 4.5. In MEKC case, the efficiency dictates the detection limits, as increased separation efficiency will decrease peak width and increase peak height and improve signal to noise. Based on calibration curves excellent $R^2$ values for range of 2.5-50 mg $L^{-1}$ for MEKC method were achieved from 0.9986 to 0.9992. Similarly, offline preconcentration, SPE, was performed using the Restek SPE sorbent followed by MEKC. Calibration curves showed excellent $R^2$ values for range of 0.25-10 mg $L^{-1}$ obtained (from 0.9962 to 0.9999).

Percent recoveries (accuracy) and RSDs (precision) for each analyte at any given point were calculated and listed in Table 4.6. According to the results in Table 4.6, there was some loss in recoveries for all nitrosamines, this is partly related to solvent exchange step. This effect was more profound for those nitrosamines with lower boiling point (i.e. NDBA).

A number of fortified samples of nitrosamines in reagent water, drinking water, wastewater, and seawater were prepared and extracted via optimized SPE method. The extracts were analyzed with optimized MEKC method and results are listed in Table 4.7. Concentration of analytes in each sample is approximately close to the LOQ (0.5 mg $L^{-1}$). Recoveries for Reagent water are lower and for seawater are higher than other samples. This can be explained due to the presence of salt in the samples (drinking water, wastewater, and seawater) that enhances the affinity of analytes to sorbent. Results showed that this effect was more profound when the concentration of salt in the sample increased (i.e. seawater has highest concentration of salt).

Table 4.5. Analytical characteristic of MEKC and SPE-MEKC

| | MEKC | | | SPE-MEKC | | | Enrichment |
|---|---|---|---|---|---|---|---|
| | LOD (mg $L^{-1}$) | LOQ (mg $L^{-1}$) | $R^2$ | LOD (mg $L^{-1}$) | LOQ (mg $L^{-1}$) | $R^2$ | Factor |
| NDBA | 2.3 | 7.7 | 0.9992 | 0.15 | 0.50 | 0.9962 | 15 |
| NDPA | 2.6 | 8.7 | 0.9989 | 0.12 | 0.40 | 0.9996 | 22 |
| NPIP | 2.8 | 9.3 | 0.9987 | 0.97 | 0.32 | 0.9998 | 31 |
| NDEA | 2.6 | 8.7 | 0.9989 | 0.11 | 0.33 | 0.9998 | 24 |
| NMOR | 2.9 | 9.7 | 0.9986 | 0.13 | 0.43 | 0.9998 | 22 |
| NPYR | 2.8 | 9.3 | 0.9988 | 0.14 | 0.47 | 0.9999 | 20 |
| NDMA | 2.9 | 9.7 | 0.9986 | 0.13 | 0.43 | 0.9998 | 22 |

Table 4.6. Analytical data obtained from spiked reagent water (SPE-MEKC)

| Analytes | Fortified reagent water samples | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.25 mg $L^{-1}$ (N=5) | | 0.50 mg $L^{-1}$ (N=5) | | 1.0 mg $L^{-1}$ (N=5) | | 5 mg $L^{-1}$ (N=5) | | 10 mg $L^{-1}$ (N=5) | |
| | %Recovery | %RSD[a] | %Recovery | %RSD | %Recovery | %RSD | %Recovery | %RSD | %Recovery | %RSD |
| NDMA | 36.2 | 15 | 66.0 | 10 | 79.1 | 8.1 | 81.6 | 7.8 | 85.2 | 8.0 |
| NMOR | 45.8 | 8.2 | 69.2 | 9.3 | 82.5 | 7.5 | 84.1 | 10 | 84.4 | 8.2 |
| NPYR | 58.3 | 12 | 72.5 | 8.1 | 85.6 | 8.2 | 87.7 | 8.3 | 88.7 | 7.3 |
| NPIP | 72.0 | 9.1 | 75.1 | 9.0 | 79.0 | 9.3 | 86.3 | 8.1 | 89.1 | 9.1 |
| NDEA | 68.8 | 8.6 | 68.3 | 11 | 78.1 | 11 | 86.0 | 9.6 | 86.6 | 8.6 |
| NDPA | 42.6 | 13 | 65.7 | 13 | 75.6 | 8.0 | 83.5 | 9.4 | 81.0 | 8.2 |
| NDBA | 38.1 | 15 | 62.4 | 11 | 72.3 | 12 | 80.1 | 11 | 81.3 | 12 |

Table 4.7. Analytical data obtained from fortified water samples (SPE-MEKC)

| Analyte | Fortified water samples 0.5 mg $L^{-1}$ (N=5) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Reagent water | | Drinking water | | Wastewater | | Seawater | |
| | %Recovery | %RSD | %Recovery | %RSD | %Recovery | %RSD | %Recovery | %RSD |
| NDMA | 66.0 | 10 | 71.7 | 10 | 82.3 | 8.0 | 86.3 | 7.6 |
| NMOR | 69.2 | 9.3 | 79.0 | 9.2 | 81.2 | 12 | 88.7 | 8.2 |
| NPYR | 72.5 | 8.1 | 81.4 | 8.6 | 85.9 | 9.3 | 84.2 | 6.4 |
| NPIP | 75.1 | 9.0 | 82.2 | 11 | 86.1 | 9.1 | 85.3 | 8.2 |
| NDEA | 68.3 | 11 | 85.0 | 11 | 88.0 | 11 | 87.7 | 10 |
| NDPA | 65.7 | 13 | 73.3 | 9.0 | 79.1 | 9.6 | 80.1 | 9.3 |
| NDBA | 62.4 | 11 | 68.6 | 10 | 77.8 | 11 | 79.0 | 12 |

## 4.5. Conclusions

Nitrosamines are neutral and polar compounds that are not capable of being separated via CZE without modification. Thus, a MEKC method has been developed. For this method, the effects of the key parameters in the BGE were studied and optimized. In this method 125 mM of SDS in 25-50 mM of ammonia buffer at pH=10 were shown to be optimal. The performance of this developed method was excellent over the range of 0.25-10 mg $L^{-1}$ ($r^2$>0.99) studied. The LODs were significantly improved using a pre-concentration method (SPE) with the MEKC method. After comparing a wide range of commercially available sorbents, the best results were obtained by using coconut charcoal as the sorbent (Resprep from Restek), though the poor retention by the SPE sorbents for some of the nitrosamines compromised the method. The SPE recoveries for the method were at 66%-75% for 0.5 mg $L^{-1}$ of individual nitrosamines in reagent water, and 68.2%-85.0% for wastewater, 79%-89% for seawater.

## 4.6.References

[1] Zhu, X. Q., He, J. Q., Li, Q., Xian, M., Lu, J., Cheng, J. P., *J Org Chem* 2000, *65*, 6729-6735.

[2] A.L.Fridman, F. M. M., and S.S.Novikov, *Russ. Chem. Rev.* 1971, *40*, 34.

[3] Zolfigol, M. A., Zebarjadian, M. H., Chehardoli, G., Keypour, H., Salehzadeh, S., Shamsipur, M., *J Org Chem* 2001, *66*, 3619-3620.

[4] Magee, P. N., *Food and Cosmetics Toxicology* 1971, *9*, 207-218.

[5] Hadidian, Z., Fredrickson, T. N., Weisburger, E. K., Weisburger, J. H., Glass, R. M., Mantel, N., *J Natl Cancer Inst* 1968, *41*, 985-1036.

[6] Lijinsky, W., *Mutat Res* 1999, *443*, 129-138.

[7] Ikeda, K., *Journal of the Society of Cosmetic Chemists* 1990, *41*.

[8] Taguchi, V. Y., *Canadian Journal of Applied Spectrometry* 1994, *39*.

[9] Charrois, J. W., Arend, M. W., Froese, K. L., Hrudey, S. E., *Environ Sci Technol* 2004, *38*, 4835-4841.

[10] Haeberer, A. F., Scott, T. A., *Abstr Pap Am Chem S* 1980, *180*, 85-Envr.

[11] Macmillan, W. D., *Anal Lett Pt A* 1983, *16*, 957-968.

[12] Terabe, S., Otsuka, K., Ando, T., *Analytical Chemistry* 1985, *57*, 834-841.

[13] Liu, X., Zhao, Y. Y., Chan, K., Hrudey, S. E., Li, X. F., Li, J., *Electrophoresis* 2007, *28*, 1327-1334.

[14] Dabek-Zlotorzynska, E., *Electrophoresis* 1997, *18*, 2453-2464.

[15] Ng, C. L., Ong, C. P., Lee, H. K., Li, S. F. Y., *Journal of Chromatographic Science* 1994, *32*, 121-125.

[16] Bell, L. M., Murray, G. M., *J Chromatogr B* 2005, *826*, 160-168.

[17] Zuccher, S., Luchini, P., Bottaro, A., *J Fluid Mech* 2004, *513*, 135-160.

[18] Breadmore, M. C., *Electrophoresis* 2007, *28*, 254-281.

[19] Dabek-Zlotorzynska, E., Celo, V., Yassine, M. M., *Electrophoresis* 2008, *29*, 310-323.

[20] Muna, G. W., Quaiserova-Mocko, V., Swain, G. M., *Analytical Chemistry* 2005, *77*, 6542-6548.

[21] Masar, M., Wojcik, L., Kaniansky, D., Trojanowicz, M., *Journal of Separation Science* 2005, *28*, 1271-1277.

[22] Klampfl, C. W., *Electrophoresis* 2006, *27*, 3-34.

[23] Richardson, S. D., *Analytical Chemistry* 2006, *78*, 4021-4045.

[24] Lazar, I. M., *Electrophoresis* 2009, *30*, 262-275.

[25] Van Biesen, G., Bottaro, C. S., *Electrophoresis* 2006, *27*, 4456-4468.

[26] Modir-Rousta, A., Bottaro, C. S., *Electrophoresis* 2013, *34*, 2553-2560.

[27] Caliaro, G. A., Herbots, C. A., *J Pharm Biomed Anal* 2001, *26*, 427-434.

[28] Koval, D., Kasicka, V., Jiracek, J., Collinsova, M., *Electrophoresis* 2006, *27*, 4648-4657.

[29] Buckenmaier, S. M., McCalley, D. V., Euerby, M. R., *J Chromatogr A* 2003, *1004*, 71-79.

# Chapter 5 Three-phase Single Drop Micro-Extraction (SDME) and Continuous Flow Micro-Extraction for N-Nitrosamines (Polar Neutral Organic Compounds)

## 5.1. Introduction

Sample preparation is perhaps the most challenging and time consuming part of any analytical technique when the sample matrix is complex and analytes are in trace amounts. Elimination of interferences and enrichment of target analyte(s) are the two main goals in sample preparation. Elimination of interferences can be achieved by extracting analytes from the matrix. The liquid-liquid extraction (LLE) is one of the oldest and widely used techniques for extraction of many different types of compounds from liquid matrices; it can also act as an enrichment technique. The process is based on transfer of analytes between two immiscible phases. The major drawback for conventional LLE is high consumption of organic solvents that are not environment friendly. Demands on sustainable techniques and miniaturization of analytical techniques dictate the reduction of sample and solvent volumes. Development of micro-extraction techniques is one of the new trends toward the greening and miniaturization of analytical techniques. Within the past decade, a significant number of research works devoted to development of micro-extraction techniques and several reviews on two of the major micro-extraction techniques, solid-phase micro-extraction (SPME) [1-4] and liquid micro-extraction (LPME), much like LLE have been published [5-10].

LPME is based on the solubility of a given analyte in two immiscible liquid phases, which reaches equilibrium through partitioning phenomena. The target analyte(s) should have superior solubility in extracting phase if extraction is to be efficient. The polarity,

molecular size of the analyte, and the physicochemical interactions between the analyte and extracting liquid phase are major factors in solubility. In addition, depending on chemistry of analytes, the solubility can be altered by liquid phase modification, e.g. changing pH, or adding chelating agents.

Single drop micro-extraction using μL syringe (SDME) is one of the most commonly used LPME techniques. This technique is used to extract analytes from a sample solution into a drop of acceptor phase. After extraction, the drop is withdrawn from donor phase and injected into an analytical instrument such as GC [11-12], LC [11], CE [7, 13-14], atomic absorption Spectrometer (AAS) [15], etc. Depending on the number of phases involved in extraction, the modes of SDME can be classified as two-phase and three-phase techniques. Two-phase SDME is considered to be a miniaturized form of traditional liquid-liquid extraction technique. This technique is inexpensive and easy to operate. There is no need for any specific or complex equipment, and practically, any solvent drop is enough for extraction. This method was first introduced by Liu and Dasgupta [16-17], then the mass transfer mechanism was studied by Jeannot [18] and He groups [19]. Notable development and applications of SDME over the past decade have been reviewed [9-10, 20-22].

The organic solvents typically used as extracting phase in SDME-GC methods are water-immiscible and compatible with GC [20]. This is considered a major limitation in application of other analytical techniques such as RP-HPLC and CE. To overcome this limitation, solvent exchange or back extraction is required. In addition, the small volume

of the sample (one drop) is not enough for conventional HPLC or increase in sample volume is necessary resulting in dilution.

Liu and Lee introduced continuous-flow micro-extraction (CFME) for the first time in 2000 [23]. In this technique, the sample solution is continuously pumped into a chamber and a drop of extracting phase is injected and held inside the chamber. Thus, the drop is continuously in contact with a fresh sample solution. Therefore, this method should give a higher concentration factor than static LPME method. The performance of CFME, is directly related to parameters such as type of solvent, size of droplet, flowrate, volume of sample solution, ionic strength [24] as well as characteristics of the solute (analyte). However, extracting and enrichment of neutral polar organic compounds, such as nitrosamines, from aqueous phase becomes more challenging as they tend to stay in the aqueous phase. Since nitrosamines have polar characteristics and are relatively non-volatile, even variation on the SDME theme that would help overcome solubility issues such as headspace SDME, seem to be inefficient for these compounds.

Immersed SDME method shows better overall mass-transfer coefficient compared to headspace SDME because the extracting phase is in direct contact with the solvated analyte. Immersed SDME has been successfully employed for extraction of drugs [25] vaccines [12], metals [15, 26], hormones [11, 27] proteins [28] in biological fluids [26, 29], pesticides [30-31]and environmental contaminants [9, 32] herbicides [33-35] in water samples and in foods [36]. When an analyte is extracted from an aqueous donor phase of

volume $V_{aq}$ to an organic acceptor phase of volume $V_o$ by SDME, the enrichment factor (*EF*) is given by [37-38]

$$EF = \frac{C_{o,eq}}{C_{aq,ini}} = \frac{1}{1/D + V_o/V_{aq}} \qquad 5\text{-}1$$

where $C_{aq,init}$ and $C_{o,eq}$ are the initial concentration of the analyte in the aqueous (donor phase) and the concentration of analyte in organic phase at equilibrium, respectively, and *D* is the distribution coefficient defined by:

$$D = \frac{C_{o,eq}}{C_{aq,eq}} \qquad 5\text{-}2$$

where $C_{aq,eq}$ is the concentration of analyte in the aqueous phase after equilibration. The mass transfer of analyte molecules from the aqueous sample solution to the organic phase (micro-drop) is generally affected by temperature, viscosity and diffusion rates of the analyte molecules in the aqueous and/or organic phases. Agitation of the solution by stirring or mechanical vibration increases the amount of convective mixing. Agitation also reduces the time required to reach equilibrium, which could be anywhere from seconds to hours. It is important to note that due to massive difference between volume of aqueous phase and organic phase, even if equilibrium is reached, most of the analyte will remain in the aqueous (donor) phase [8]. Most of micro-extraction techniques such as SPME and SDME are not exhaustive extraction techniques and their recoveries are low (from 0.1% to 10%) [39]. SDME is often performed under kinetically controlled

conditions to avoid excessive analysis times. Calibration is based on aqueous phase standards and extractions of standards and unknown are performed under same conditions. Therefore, this technique is functional analytically even if only a trace amount of analyte is recovered from the sample solution.

## 5.2. Material and Methods

### 5.2.1. Materials

Individual nitrosamines standards at 2000 mg $L^{-1}$ in methanol were purchased from Supelco (Ontario, Canada), stock solutions of 1000 mg $L^{-1}$ in methanol were prepared and stored at 4 °C and working solutions were made fresh daily. All solvents were HPLC grade or higher from Sigma-Aldrich (Oakville, Canada), dichloromethane was purchased from Sigma-Aldrich (Oakville, Canada). Ultrapure water was obtained from a Barnstead Nano Pure Diamond (18 MΩ) system, (Ontario, Canada).

### 5.2.2. Instruments

A digital temperature and flow controlled gas heater was built using parts purchased from Omega Canada (Ontario, Canada). Syringes and needles were purchased from Hamilton Company (NV, USA). An Agilent GC 6850 system with FID and TCD detectors and an HPLC 1100 system with diode array detector were used for analysis of samples.

## 5.3. SDME of Polar Neutral Analytes

In liquid-liquid extraction methods, normally two immiscible solvents are used. In such a case, small neutral polar compounds in environmental samples have tendency toward the aqueous phase rather than organic phase. In conventional liquid extraction large volume of organic solvent is used to overcome this problem. Then, extra steps are necessary to remove excessive solvent and enrich the analytes. As it was explained earlier, extraction efficiency is directly related to the distribution coefficient (solute-solvent interaction) and phase ratio (Equation 5-1). In case of highly polar compounds such as *N*-nitrosamines, none of these terms are in favor of extraction from aqueous phase into organic phase. In our proposed three-phase extraction method phases are aqueous-organic-aqueous respectively (Figure 5-3). The phase ratio of first stage ( phase1/phase2 extraction, from bulk aqueous sample solution into the organic phase outer droplet) is about $10^{-3}$ while for second extraction (from organic phase outer droplet to aqueous inner droplet) phase ratio is about $10^{3}$ and distribution coefficient is in favor of extraction (highly polar *N*-nitrosamine from organic phase into the aqueous phase). Moreover, evaporation of organic solvent can be considered as an extra boost for final extraction. Since the trend in new analytical methods is toward the use of less organic solvents, the method described here can be used to reduce the volume of organic solvent from many mL to a few μL.

### 5.3.1. Apparatus

The steps involved in the execution of this method are presented schematically in figure 5.1. First, a syringe is filled with a plug of air; 1-2 μL of water; and about 5 μL dichloromethane (a low boiling point organic solvent immiscible with water) respectively (figure 5.1A). Then concentric droplets were formed using positive pressure (figure 5.1B, C). This process was visually monitored using magnifying glass or digital camera (microscope). The maximum size of each droplet was estimated by the volume of solvent in the syringe. This size was directly related to the wall thickness, shape of the tip, and material of the needle. In practice, the size of each droplet was kept smaller than the maximum to avoid drop loss due to physical movement. To increase the stability, the drops should have the maximum interface with the tip of needle. Therefore, needles with thicker walls were used when larger drops were needed. Gravity is one the most important cause of detachment of the large drops from the tip of needles. Formation and stability of the droplet(s) inside the solution is easier due to the presence of hydrostatic forces which oppose the gravity. Droplets must be retracted into the syringe prior to entering or removing from a solution to avoid drop detachment from the syringe.

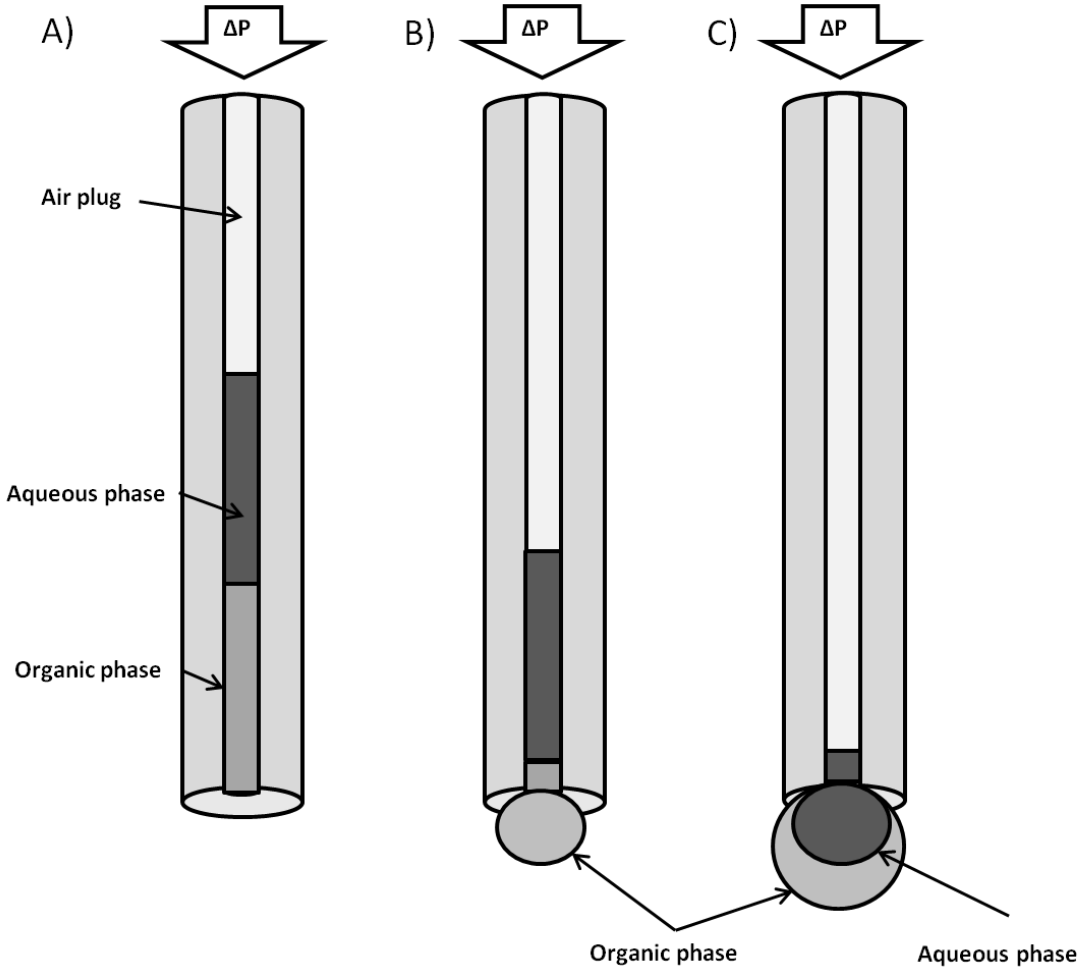


Figure 5-1. The schematic of formation of two-phase concentric droplets.

## 5.4.Methods and discussion

In this work different size of syringes and different type of needles were employed and compared. Among different style of needles the blunt tip needles showed better drop adhesion and needles with thicker walls were able to hold larger droplets. A 22s gauge (0.5 mm O.D) needle with blunt point was chosen because it afforded a thicker wall (large interface between drop and flat tip of needle) and less dead volume (less than 0.05

μL inch$^{-1}$). To generate larger drops, the wall thickness of the needle could be increased by using a P-TFE sleeve. PTFE material gave better results in term of lower residual contamination when retracting the droplets. Sleeves with different external diameters and fixed internal diameter were made to fit the external diameter of the needle (0.5 mm). To increase the stability of droplet, it was assumed that droplets with hemispherical shape can form stronger binding forces between droplet and tip of needle (needle wall + sleeve) due to a larger interface (diameter of droplet ≤ O.D. of needle wall thickness + sleeve). Therefore, for any given syringe size, dimensions of a half-droplet (half of sphere) was calculated and droplet volume were used accordingly. Then sleeves with corresponding diameters to the closest 0.5 mm were made. In Table 5.1, the size of syringe with suggested dimensions of sleeves and nominal droplet sizes are listed. This information was used to select the right sizes of sleeves for corresponding syringe sizes (volume). Based on the total volume of the syringe, the maximum size of droplet and dimension of sleeve were determined. For example a 5 μL syringe needs a sleeve with 2.5 mm O.D to hold a 5 μL droplet (see Table 5.1).

The volumes within the syringe dictate the volume of outer droplet (organic droplet) plus inner aqueous drop and air plug. The size of air plug (see Figure 5.1) is slightly more than the dead volume (volume inside the needle) as this is needed to push the water plug out completely. Later this air plug is used to make sure all the aqueous droplet is injected to a given analytical instrument.

Table 5.1. sleeve size based on calculated values for half drop for Hamilton needle blunt point gauge 22s (0.474 mm O.D.and 0.178 mm wall thickness).

| Size of syringe (µL) | External drop diameter (mm)[a] | Sleeve O.D. (mm)[b] | ½ External drop volume(µL)[c] | Wall thickness (mm)[d] |
|---|---|---|---|---|
| 5 | 2.7 | 2.5 | 7.1 | 2.3 |
| 10 | 3.4 | 3.5 | 11.2 | 3.2 |
| 25 | 4.6 | 4.5 | 23.6 | 4.3 |
| 50 | 5.8 | 6 | 56.5 | 5.8 |
| 100 | 7.3 | 7.5 | 110.4 | 7.3 |
| 250 | 9.8 | 10 | 261.8 | 9.8 |

a-calculated diameter of half of a sphere (half drop) Corresponding to the volume of the syringe

b-PTFE sleeves external diameter.

c-The calculated volume of half a drop corresponding to the O.D. of sleeve.

d-Wall thickness= O.D. of sleeves- I.D. of needle.

The size of water plug is very important because it is considered as the extracting phase volume. A larger air plug could form a third drop inside the inner drop, which can cause an increase in the surface area of both drops and interface between the two co-centric droplets, thereby increasing the mass transfer rate. But in practice, presence of large air bubble inside the co-centric droplets caused distortion and instability of droplets. Formation of an inner droplet during extraction was not necessary, but it was formed to expedite the equilibrium. Any technique usually used with conventional LLE to improve the extraction can be applied to this method. For example, since droplets were fairly stable, vigorous stirring of solution was possible.

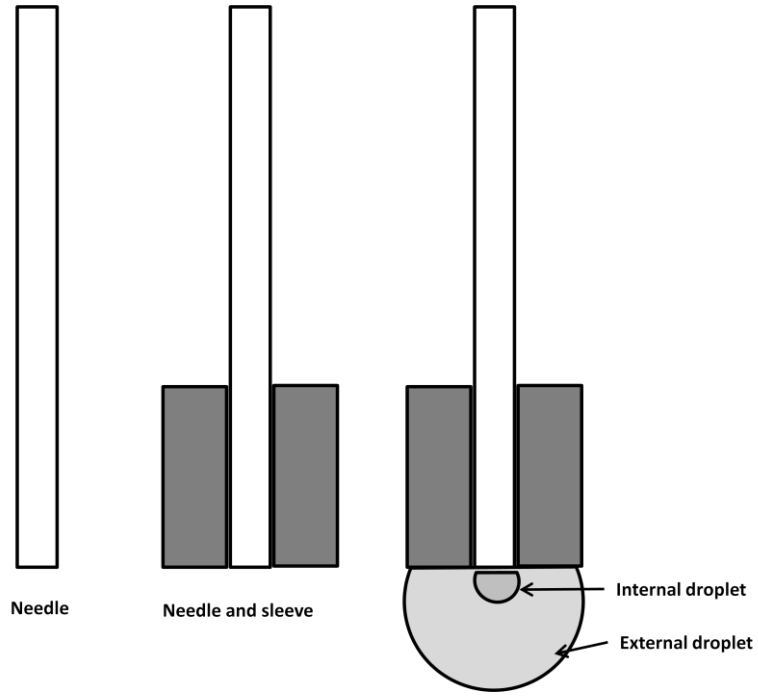


Figure 5-2. Schematic of using PTFE sleeve to increase the size of droplets

### 5.4.1. Results

100 mL of a fortified sample containing mixture of seven nitrosamines (1 mg $L^{-1}$ ) was place in a beaker and a 25 µL syringe was filled with 0.5 µL air, 1 µL water and 20 µL of dichloromethane respectively and the syringe needle was inserted into a PTFE sleeve (4.5 mm O.D.). The assembly was immersed into the sample solution. Droplets were formed by gently pushing the plunger; the drop and assembly stayed in the solution while it was agitating with magnetic stirring at a rate of 100 rpm for 30 min (see Figure 5.3). The droplets were then retracted into the syringe by pulling the plunger back gently. The PTFE sleeve was replaced by a clean smaller one (2 mm O.D.). The syringe was inserted into a nitrogen drying chamber (designed and fabricated by the author) and the external

droplet was reformed gradually. Due to smaller size of the sleeve, the external droplet was formed by pushing only 2 µL of the extract phase out each time and visually inspected the drop as it evaporated to prevent complete dryness.

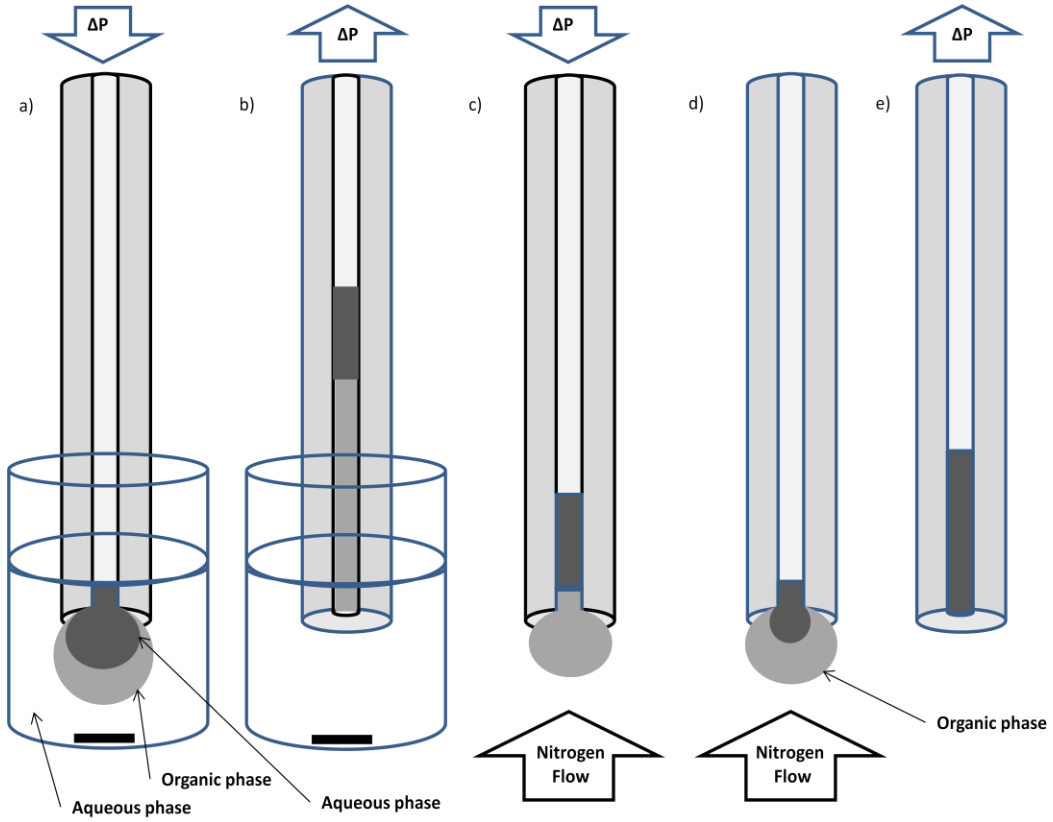


Figure 5-3. Illustration of formation of concentric droplets inside and outside a solution. a) formation of co-centric two phase drops inside the sample solution b) retracting drops into the syringe, c) formation of two-phase droplets outside the solution d) evaporation of outer layer using nitrogen flow, e) collecting enriched sample into the needle.

In practice, using larger sleeve caused analyte(s) loss during evaporation step. The evaporation rate was experimentally measured and set by changing flow rate and temperature of the nitrogen gas using our device made in-house. For example, in this

experiment a flow rate 0.2 L $min^{-1}$ at 35 °C gave evaporation rates of about 3 µL $min^{-1}$. It must be mentioned that drying gas temperature was kept below the boiling temperature of organic solvent otherwise the solvent boiled and bubbles formed causing distortion and loss of droplets (see Figure 5.3). When the last part of the outsise drop was formed (last 2 µL of organic phase + water), 30 sec was added to the drying time and aqueous droplet was retracted into the syringe and injected into a GC. The enrichment for highly polar compounds (NDMA, NPYR, NMOR, NPIP and NDEA) was significant but low (see Table 5.2). This result was attributed to low portioning of these compounds into the organic phase (outer drop) or some loss of analytes when drying the organic solvent. The size could be increased; however the volume of organic phase is limited by the size of syringe, needle, and sleeve.

Table 5.2 Analytical data obtained from three-phase single drop micro-extraction

| **Analytes** | **BP (°C)** | **Log($P_o/P_w$)** | **Concentration (µg $mL^{-1}$)** | | **Enrichment** | **%RSD[a]** |
|---|---|---|---|---|---|---|
| | | | **Initial** | **Final** | | |
| NDMA | 152 | -0.57 | 0.1 | 0.25 | 2.5 | 9.2 |
| NMOR | 224 | -0.44 | 0.1 | 0.28 | 2.8 | 7.5 |
| NPYR | 214 | -0.19 | 0.1 | 0.33 | 3.3 | 6.9 |
| NPIP | 219 | 0.36 | 0.1 | 0.56 | 5.6 | 4.5 |
| NDEA | 176.9 | 0.48 | 0.1 | 0.45 | 4.5 | 6.8 |
| NDPA | 206 | 1.36 | 0.1 | 2.1 | 21 | 3.1 |
| NDBA | 116 | 2.63 | 0.1 | 1.8 | 18 | 5.3 |

a-(n=6)

When a larger syringe is used the size of aqueous phase must increase as well. For example using a 5 mL syringe requires at least 0.1 mL of aqueous phase (water) to form a separate layer inside the syringe otherwise formation of aqueous drop may not be possible.

## 5.5. Continuous Flow Single Drop Micro-extraction (CFSDME) of Neutral Polar Analytes

To increase the volume of organic phase, a device was designed to provide continuous flow of organic phase in single drop micro-extraction (see Figure 5.4). The main components of this device consist of a tee, PEEK tubing, needle, PTFE sleeve and syringe. The needle was inserted from one port of tee and passed through PEEK tubing in the other end of the tee (see Appendix B). The external diameter of needle was smaller than I.D. of PEEK tubing thus a concentric system of tubing was formed to allow two different solutions to run simultaneously and form two concentric droplets. From the 3$^{rd}$ port of the tee which was perpendicular to other two ports, another piece of PEEK tubing connected the device to a syringe pump. The inner drop (aqueous) was formed by syringe needle. The organic phase was pumped through the PEEK tubing to form the external drop. (see Figure 5.4).

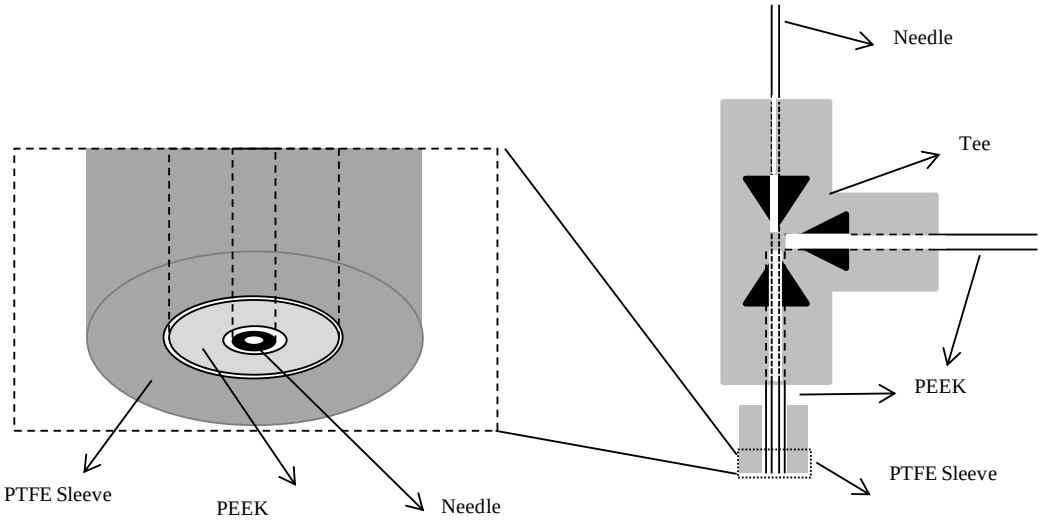


Figure 5-4. Schematic of a device for formation of concentric droplets with continuous flow.

In this method, in a 25 mL syringe, 5 mL of dichloromethane was added to the equal amount of sample (aqueous) and vigorously agitated for 15 min. Then solution was left for 5 min to allow two phases form. Then extract (organic phase) was pumped into the device using a syringe pump. The device was put under the flow of heated nitrogen gas and the flow rate of the pump was adjusted to match the evaporation rate of external droplet (organic phase). The internal droplet was formed by injection of 5 μL of water using a 10 μL syringe. After complete evaporation of 5 mL of organic phase, aqueous droplet was retracted to the syringe and kept in fridge prior to injection into a given analytical instrument. The total process took less than 30 minutes.

## 5.6. Quantitative Analysis

To evaluate the method quantitatively, calibration curves were constructed then two blanks and two fortified samples were extracted simultaneously and analyzed using GC. These experiments were repeated three times and results are listed in Table 5.3. The GC conditions were as follow: temperature of injector was set to 250 °C and 1:5 split, injection volume was 1 µL and FID detector was employed at 300 °C; oven temperature program set to 50-250 °C at a rate of 10 °C $min^{-1}$, The GC and BD-225 column 30 m x 0.53 mm were from Agilent.

Table 5.3. Analytical data obtained using continuous flow single drop micro-extraction

| **Analytes** | **Concentration (µg $mL^{-1}$)** | | **Enrichment** | **%RSD[a]** |
|---|---|---|---|---|
| | **Initial** | **Final** | | |
| NDMA | 0.1 | 0.72 | 7.2 | 3.2 |
| NMOR | 0.1 | 1.3 | 13 | 4.5 |
| NPYR | 0.1 | 1.9 | 19 | 6.8 |
| NPIP | 0.1 | 3.4 | 34 | 3.9 |
| NDEA | 0.1 | 3.0 | 30 | 4.4 |
| NDPA | 0.1 | 4.4 | 44 | 5.1 |
| NDBA | 0.1 | 2.6 | 26 | 9.1 |

a-(n=6)

To evaluate the performance of the method in a real sample matrix, samples were prepared by spiking a mixture of nitrosamine into tap water and seawater. Since extraction and enrichment were combined, the enrichment factor may be a better description of the performance. The enrichment factor is calculated based on the ratio

between final concentration (by GC) and initial concentration. Based on the results that are listed in Table 5.2 and Table 5.3, SDME and CFSDME methods show enrichment factor range 2.5-21 and 7.2-44 for CFSDME. The enrichment factors for NDMA, NMOR, and NPYR are lower than others analytes due to their negative log P. Enrichment factor trend is in agreement with log P values (see Table 5.2) except NDBA. Low boiling point (or high vapor pressure) of NDBA is the main cause of loss during the drying step. In general, the enrichment factor values obtained from SDME are smaller than those from CFSDME, however, reproducibility of SDME method is better.

## 5.7. Conclusion

Efficient extraction and enrichment of neutral polar compounds from environmental samples are challenging steps in any analytical method. Regardless of the type of analytical technique, there has been a trend toward lower consumption of organic solvents and smaller sample size. In this work, the main focus has been on developing a device for enrichment of neutral polar compound in environmental samples. The methods proposed are applicable to most of the analytical techniques for which small sample sizes are preferred or easily accommodated including GC, UHPLC, CE, etc. three-phase single drop microextraction was performed using syringe and dryer gas but due to small volume of organic acceptor phase limited enrichments were achieved (upto 6 for highlypolar nitrosamines and upto 20 for the rest). To increase the volume of organic phase) simple and inexpensive components, such as syringes, PEEK tubing and fitting and syringe

pump were combined into devices that are easy to fabricate. PTFE sleeves were used to increase the volume of extractant (drop size). To avoid the possibility of loss of drops, the drop volume was kept low to maximize the ratio of contact area. This method was applied to extract and enrich seven *N*-nitrosamines as a representative group of highly polar neutral compounds and the results were promising. Enrichment factors up to 34 fold for highly polar nitrosamines and 44 times for the rest were achieved in less than 30 min.

## 5.8.References:

[1] Spietelun, A., Kloskowski, A., Chrzanowski, W., Namieśnik, J., *Chemical Reviews* 2012, *113*, 1667-1685.

[2] Souza Silva, E. A., Risticevic, S., Pawliszyn, J., *TrAC Trends in Analytical Chemistry* 2013, *43*, 24-36.

[3] Bojko, B., Cudjoe, E., Gómez-Ríos, G. A., Gorynski, K., Jiang, R., Reyes-Garcés, N., Risticevic, S., Silva, É. A. S., Togunde, O., Vuckovic, D., Pawliszyn, J., *Analytica Chimica Acta* 2012, *750*, 132-151.

[4] Wardencki, W., Curyło, J., Namieśnik, J., *J Biochem Bioph Meth* 2007, *70*, 275-288.

[5] Dehghani Mohammad Abadi, M., Ashraf, N., Chamsaz, M., Shemirani, F., *Talanta* 2012, *99*, 1-12.

[6] Demeestere, K., Dewulf, J., De Witte, B., Van Langenhove, H., *Journal of Chromatography A* 2007, *1153*, 130-144.

[7] Jain, A., Verma, K. K., *Analytica Chimica Acta* 2011, *706*, 37-65.

[8] Jeannot, M. A., Przyjazny, A., Kokosa, J. M., *J Chromatogr A* 2010, *1217*, 2326-2336.

[9] Lambropoulou, D. A., Konstantinou, I. K., Albanis, T. A., *Journal of Chromatography A* 2007, *1152*, 70-96.

[10] Psillakis, E., Kalogerakis, N., *TrAC, Trends Anal. Chem.* 2003, *22*, 565-574.

[11] Jeannot, M. A., Cantwell, F. F., *Anal Chem* 1997, *69*, 2935-2940.

[12] Bagheri, H., Ghambarian, M., Salemi, A., Es-Haghi, A., *J Pharm Biomed Anal* 2009, *50*, 287-292.

[13] Choi, K., Kim, S. J., Jin, Y. G., Jang, Y. O., Kim, J. S., Chung, D. S., *Anal Chem* 2009, *81*, 225-230.

[14] Choi, K., Kim, Y., Chung, D. S., *Anal Chem* 2004, *76*, 855-858.

[15] Bagheri, H., Naderi, M., *J Hazard Mater* 2009, *165*, 353-358.

[16] Liu, H., Dasgupta, P. K., *Anal. Chem.* 1995, *67*, 4221-4228.

[17] Liu, H., Dasgupta, P. K., *Anal Chem* 1996, *68*, 1817-1821.

[18] Jeannot, M. A., Cantwell, F. F., *Anal Chem* 1996, *68*, 2236-2240.

[19] He, Y., Lee, H. K., *Anal. Chem.* 1997, *69*, 4634-4640.

[20] Nerín, C., Salafranca, J., Aznar, M., Batlle, R., *Analytical & Bioanalytical Chemistry* 2009, *393*, 809-833.

[21] Psillakis, E., Kalogerakis, N., *TrAC Trends in Analytical Chemistry* 2002, *21*, 54-64.

[22] Xu, L., Basheer, C., Lee, H. K., *Journal of Chromatography A* 2007, *1152*, 184-192.

[23] Liu, W., Lee, H. K., *Anal Chem* 2000, *72*, 4462-4467.

[24] Li, Y., Zhang, T., Liang, P., *Analytica Chimica Acta* 2005, *536*, 245-249.

[25] Pedersen-Bjergaard, S., Rasmussen, K. E., *J Chromatogr B Analyt Technol Biomed Life Sci* 2005, *817*, 3-12.

[26] Xia, L., Hu, B., Jiang, Z., Wu, Y., Liang, Y., *Anal Chem* 2004, *76*, 2910-2915.

[27] Fiamegos, Y. C., Stalikas, C. D., *Anal Chim Acta* 2007, *597*, 32-40.

[28] Ho, T. S., Pedersen-Bjergaard, S., Rasmussen, K. E., *Analyst* 2002, *127*, 608-613.

[29] Pedersen-Bjergaard, S., Rasmussen, K. E., *Anal Chem* 1999, *71*, 2650-2656.

[30] Berijani, S., Assadi, Y., Anbia, M., Milani Hosseini, M. R., Aghaee, E., *J Chromatogr A* 2006, *1123*, 1-9.

[31] Pakade, Y. B., Tewary, D. K., *Journal of Separation Science* 2010, *33*, 3683-3691.

[32] Ridgway, K., Lalljie, S. P. D., Smith, R. M., *Journal of Chromatography A* 2007, *1153*, 36-53.

[33] Psillakis, E., Ntelekos, A., Mantzavinos, D., Nikolopoulos, E., Kalogerakis, N., *J Environ Monit* 2003, *5*, 135-140.

[34] Rezaee, M., Assadi, Y., Milani Hosseini, M. R., Aghaee, E., Ahmadi, F., Berijani, S., *J Chromatogr A* 2006, *1116*, 1-9.

[35] Shen, G., Lee, H. K., *Anal Chem* 2002, *74*, 648-654.

[36] Asensio-Ramos, M., Ravelo-Pérez, L. M., González-Curbelo, M. Á., Hernández-Borges, J., *Journal of Chromatography A* 2011, *1218*, 7415-7437.

[37] Ho, T. S., Pedersen-Bjergaard, S., Rasmussen, K. E., *J Chromatogr A* 2002, *963*, 3-17.

[38] Choi, K., Jin, Y. G., Chung, D. S., *J Chromatogr A* 2009, *1216*, 6466-6470.

[39] López-Blanco, M. C., Blanco-Cid, S., Cancho-Grande, B., Simal-Gándara, J., *Journal of Chromatography A* 2003, *984*, 245-252.

# Chapter 6 Summary and Future Work

MEKC is a powerful CE mode for analysis of charged and uncharged analytes. However, MEKC suffers from the same limitation as CE with respect to detection limits, particularly for detection with UV-vis (most common in commercial CE instruments). Many solutions have been offered to overcome this problem either using more advanced detectors or analyte enrichment. Application of different detection system can be costly and, in some cases, are not applicable for all analytes. Online and offline sample enrichment techniques are more popular ways to improve the sensitivity. There is no general technique for analyte enrichment and techniques are often limited to use with a group of compounds that share similar physical or chemical characteristics. Enrichment of highly polar neutral compounds found in environmental samples such as drinking water, wastewater, and seawater are more challenging. In light of finding a method of enrichment for neutral polar analytes, *N*-nitrosamines were selected as a model due to shortage of methods of analysis in the pertaining literature. CE rather than traditional GC was selected as an analytical method because of the possibility of miniaturization (lab-on-chip) and availability of several online preconcentration techniques.

First, an attempt was made to protonate the amino nitrogen using an acidic pH, but all nitrosamines were not protonated based on CE results in the wide pH range (Chapter 4). The lack of intrinsic electric charge eliminated many of the usual CE-based online preconcentration techniques, including stacking. To compensate for the lack of mobility of nitrosamines (uncharged) in an electric field, surfactants were used and methods based on MEKC principles were developed. Application of surfactants in sweeping (a

preconcentration technique in CE) was a guide to designing a new sweeping technique for preconcentration of highly polar neutral compounds, such as *N*-nitrosamines. Sweeping, is one of the few techniques applicable to neutral compounds, here the surfactant imparts mobility and enrichment for neutral compounds, but a strong interaction between analytes and micelles is normally required. For highly polar compounds weak interactions with the micelles means that these analyte spend more time in the capillary before reaching the detector, thus diffusion causes excessive band broadening. In Chapter 2, a new method was introduced to mediate this problem. In this method, which we call pressure assisted sweeping RM-EKC, an auxiliary pressure was applied to boost the velocity of slow moving analytes and minimize the band broadening.

To optimize the method, all the parameters that may improve the efficiency were examined. The effect of different factors, such as concentration of surfactants, type of surfactants and ionic strength of background electrolyte, on separation were studied and results presented in Chapter 2 and 3. Different types of surfactants including SDS, CTAC, CTAB, bile salts, and APFO (at various concentrations) were selected and their associated *k* values were studied. Interactions between analytes and surfactants were compared to find the best pseudostationary phase for nitrosamines (Chapter 3). This technique was implemented for real samples and results were compared to an offline enrichment technique (SPE) in Chapter 4. Considering the amount of time spent for sample preparation, the SPE-MEKC method compared to the proposed online preconcentration technique's enrichment was not impressive.

Since sweeping was performed under zero, EOF conditions and analyte migration was dictated only by the affinity for the micelles and the linear velocity of the micelles, resulting in migration times can be long, especially for polar analytes. Short capillaries can make analysis times more reasonable and reduce peak broadness, but they limit the maximum injection volume and thus concentration factors. Born from necessity, a system for application of controllable, uniform pressure that could be easily integrated into a commercial CE system was designed and fabricated. The introduction of different regulator configurations can allow for better pressure control; the upper limit for fine control regulator used here was 100 mbar. It was demonstrated that by using an external auxiliary pressure device, analysis times can be reduced without compromising separation efficiency and allowing for use of longer capillaries to accommodate larger sample volumes. Peak shape and separation efficiency was impacted when the conductivity of sample solution was different than BGE. Separation results were best when a high conductivity sample was paired to a BGE of lower conductivity; i.e., if sample salinity was high, large volumes of sample could be injected without compromising the separation. Using higher surfactant concentrations (up to 200 mM SDS) than usual for MEKC (i.e. 50 mM SDS) improved the sweeping. By optimizing all parameters that influence sweeping-RM-EKC (i.e., surfactant loading and type, application of pressure, changes to composition of sample, etc.), good online preconcentration methods can be developed for polar analytes that typically are poorly suited for normal sweeping.

A number of factors have been studied to improve the enrichment of polar neutral nitrosamines by pressure-assisted sweeping-RM-ECK, including ways to suppress the EOF (by pH and coated capillaries). In fused silica capillaries, the EOF can be suppressed at pH<2 but this limits our selection of surfactants. Coated capillaries were better alternatives for suppression of the EOF. Among different coated capillaries studied in this work, the sulfonic acid (PSA) coated capillaries were capable of suppressing the EOF - with any type of surfactant - over the pertinent pH range (2-8).

To maximize the retention factor, parameters such as type and concentration of surfactants that influence $K$ and $\varphi$ were studied. In general, SDS showed reasonable performance however; application of cationic surfactants were shown to be a useful alternative surfactant for analysis of nitrosamines due to their ability to act as a hydrogen bond donors. APFO, showed remarkable results in terms of peak shapes and number of theoretical plates, however, NDEA and NPIP remained unresolved despite of the increasing concentration of surfactant. Nevertheless, the compatibility of APFO with mass spectrometry makes it attractive, and use of mixed micelle systems can be exploited to improve resolution of these co-migrating species.

An MEKC method for *N*-nitrosamines has been developed and results are presented in Chapter 4. For this method, the effects of concentration of surfactant, pH, concentration of buffer solution, organic modifiers, capillary size, injection volume, and temperature were systematically studied and optimized. In this method, 125 mM of SDS in 25-50 mM of ammonia buffer at pH=10 were used. To improve the limit of detection and

quantitation, a pre-concentration method based on SPE was optimized and combined with the MEKC method. After comparing a wide range of commercially-available sorbents, the best results were obtained using coconut charcoal for sorbent. Inability of the SPE sorbents to retain some of the nitrosamines such as NDMA, NMOR, and NPYR compromised the method.

As mentioned before, during our literature survey, we noticed that there was a lack of linear solvation energy relationship, LSER, study on *N*-nitrosamines, but to carry out extensive LSER studies was out of the scope of research presented in this thesis. This can be an interesting research topic for future work. This study can be used for prediction of partitioning behavior of this group of compounds in different micellar systems. Also, it can be useful for selecting novel and more efficient surfactant systems [1]. Our present work was limited to using a small group consisting of different surfactants based on information found from literature. However, using a limited number of surfactant systems, has identified that the structure of surfactant can play a major role in the interaction between analytes and micelles. To obtain a better understanding regarding the interaction between solute and micellar system, a larger selection of surfactants would be required. One approach is to extend the previous LSER studies by adding nitrosamines to the list of model compounds. Application of ionic liquid for sweeping [2] can also be considered.

In Chapter 5, the main focus was on developing a device for enrichment of neutral polar compounds in environmental samples. A method was proposed that was applicable to

most of the analytical techniques for which sample sizes as low as few micro-litres, can be accommodated, such as GC, UHPLC, and CE. Using a device that was easy and inexpensive to construct, a continuous flow single-drop micro-extraction method was developed. Dichloromethane was used as primary extractant due to its immiscibility with aqueous solution and low boiling point. Later, the primary extractant was introduced into the proposed device as the outer portion of a two-phase concentric droplet with a drop of water as the inner droplet. To increase the volume of extractant, PTFE sleeves were used to increase the area to which the drop can adhere. To avoid the possibility of loss of droplets, the drop volume was kept at half the maximum volume of a sphere defined by the outer diameter of PTFE sleeves. As the outer droplet evaporated, the analyte was transferred to inner water droplet as acceptor phase, which was used for analysis. This method was applied to extract and enrich *N*-nitrosamines as a group of highly polar neutral compounds and gave enrichment factors range of 7.2-44 times.

In this present work, due to the small volume of extract, we were only able to evaluate the performance using GC. Sample collection and transfer were the most important limitations to our choice of analytical instrument. For example, using HPLC or CE at least a few-hundred microlitres of sample volume must be collected into a vial. To overcome this problem, an automated device tailored to a commercially available analytical instruments is required. This device can provide and transfer the sample directly to the injection port of a given instrument. For example, in Figure 6.1 a design for automation of the above method is demonstrated. In this figure, the continuous single

drop micro-extraction is combined to a solid phase extraction cartridge and the final extractant is pumped into an analytical instrument. In this device, a combination of two multiport valves, a nitrogen tank, and pumps are used. Sample, water and solvent are introduced into a SPE cartridge for sample clean up and enrichment and then the effluent is directed toward our proposed device for further enrichment and phase transfer.

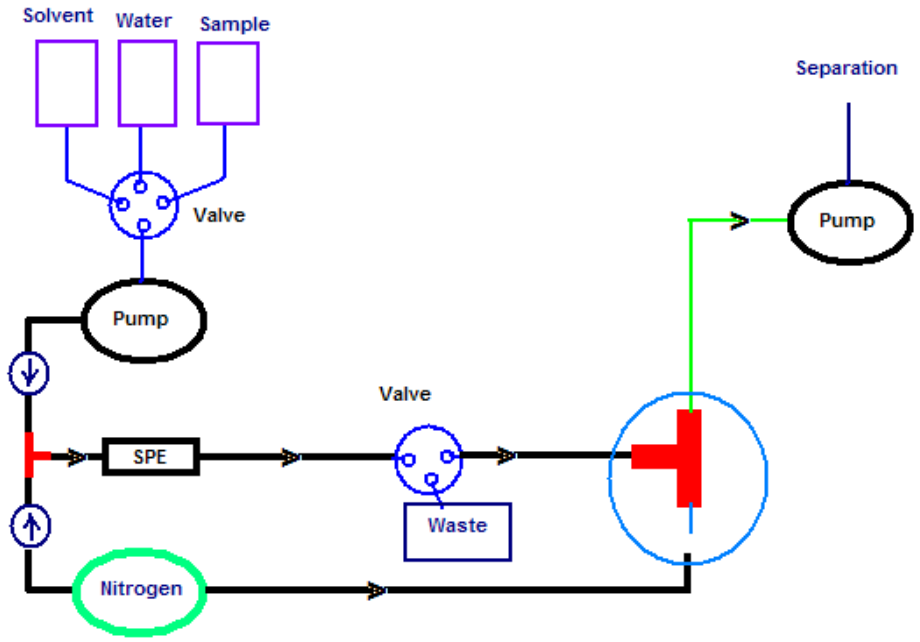


Figure 6-1. Schematic an automated continuous flow single drop micro-extraction. Water, sample, and solvent (eluent) can be pumped into a SPE cartridge accordingly and flow of nitrogen gas is used for drying the sorbent in SPE and extractant in single drop micro-extraction. Finally extractant is pumped to an analytical instrument.

To enhance the selectivity, application of molecular imprinted solid phase extraction (MISPE) [3]instead of SPE is also recommended. Finally following phase transfer and final preconcentration the enriched extract is transported to a given analytical instrument.

This device can be used as an external module or added to an analytical instrument. Most of the CE-enrichment techniques are applicable to the lab-on-chip technologies [4] including the continuous flow single drop micro-extraction method. All tubing, tees, syringes and needles can be replaced by chan8nels. In this case, the amount of solvent will be reduced to a nanolitre scale. Using a three-phase system and stabilizing larger volume drops using hollow fibre [5] in combination with MEKC [6] is another suggestion for future research. In this instance, the organic phase can be formed inside the fibre while aqueous drop can be formed and supported by hollow fibre.

In this work, the overarching theme is analysis of highly polar compounds, but only *N*-nitrosamine was used as model compound. Based on physical and chemical properties of selected *N*-nitrosamines (Table 1.1), the method performance by CE was impressive; the use of these methods for enrichment and analysis of less polar neutral compounds may show even better results. These methods are more suitable for analysis of pharmaceuticals, pollutants, pesticides, etc.

## 6.1.References:

[1] Lu, J., Ni, X., Cao, Y., Ma, X., Cao, G., *Electrophoresis* 2015, *36*, 312-318.

[2] Abd El-Hady, D., Albishri, H. M., Rengarajan, R., Wätzig, H., *Electrophoresis* 2014, *35*, 1956-1964.

[3] Sun, H., Lai, J.-P., Fung, Y. S., *Journal of Chromatography A* 2014, *1358*, 303-308.

[4] Proczek, G., Augustin, V., Descroix, S., Hennion, M.-C., *Electrophoresis* 2009, *30*, 515-524.

[5] Tajabadi, F., Ghambarian, M., Yamini, Y., *Analytical Methods* 2015, *7*, 2959-2967.

[6] Zhou, X., He, M., Chen, B., Hu, B., *Analyst* 2015, *140*, 1662-1671.

# Appendix A

Table A.1. Physical properties of most common used solvent as extracting phase for micro-extraction [a]

| Organic solvents | Boiling point (°C) | Vapour pressure (Torr) | Water solubility ($mgL^{-1}$) | Density ($gcm^{-3}$) | Viscosity (cP) | Surface tension (dyn $cm^{-1}$) | Dipole moment (D) | Dielectric constant |
|---|---|---|---|---|---|---|---|---|
| **Cyclohexane** | 80.7 | 97.8 | 55 | 0.78 | 0.90 | 24.65 | 0.00 | 2.02(20°C) |
| **n-Hexane** | 68.7 | 151.3 | 1.2 | 0.65 | 0.29 | 17.94 | 0.08 | 1.88 |
| **Octane** | 125.7 | 14.0 | $6.6x10^{-3}$ | 0.70 | 0.51 | 21.18 | 0.00 | 1.95(20°C) |
| **Iso-octane** | 99.2 | 49 | 2.4 | 0.69 | 0.50(20°C) | 18.8 | 0.00 | 1.94(20°C) |
| **Decane** | 174.2 | 1.3 | 0.05 | 0.73 | 0.86 | 23.37 | 0.00 | 1.99(20°C) |
| **Benzene** | 80.1 | 95.2 | 1791 | 0.87 | 0.60 | 28.2 | 0.00 | 2.27 |
| **Toluene** | 110.6 | 28.8 | 515 | 0.86 | 0.55 | 27.92 | 0.31 | 2.38 |
| **o-Xylene** | 144.4 | 6.6 | 175 | 0.88 | 0.76 | 29.49 | 0.45 | 2.57(20°C) |
| **m-Xylene** | 139.1 | 8.3 | 146 | 0.86 | 0.58 | 28.10 | 0.30 | 2.37(20°C) |
| **p-Xylene** | 138.4 | 8.7 | 156 | 0.86 | 0.60 | 27.76 | 0.02 | 2.27(20°C) |
| **Ethylbenzene** | 136.2 | 9.6 | 152 | 0.86 | 0.64 | 28.48 | 0.37 | 2.40(20°C) |
| **Methanol** | 64.5 | 127.0 | ∞ | 0.79 | 0.55 | 22.30 | 2.87 | 32.66 |
| **Ethanol** | 78.3 | 59.0 | ∞ | 0.79 | 1.08 | 22.32(20°C) | 1.66 | 24.55 |
| **1-Octanol** | 195.2 | 0.08 | 538 | 0.82 | 7.36 | 26.92 | 1.76 | 10.34(20°C) |
| **Benzyl alcohol** | 205.4 | 0.11 | 800 | 1.04 | 4.65(30°C) | 39.44 | 1.66 | 13.1(20°C) |
| **Ethylene glycol** | 197.5 | 0.09 | ∞ | 1.11 | 13.76 | 48.49(20°C) | 2.31 | 37.7 |
| **Acetone** | 56.1 | 231.1 | ∞ | 0.78 | 0.30 | 22.68 | 2.69 | 20.56 |

Continued:

| Organic solvents | Boiling point (°C) | Vapour pressure (Torr) | Water solubility ($mgL^{-1}$) | Density ($gcm^{-3}$) | Viscosity (cP) | Surface tension (dyn cm−1) | Dipole moment (D) | Dielectric constant |
|---|---|---|---|---|---|---|---|---|
| 4-mthyl-2-pentanone | 117.4 | 18.8 | 17000 | 0.80 | 0.55 | 23.64(20°C) | - | 13.11(20°C) |
| α,α,α-Trifuorotoluene | 102.0 | 38.5 | - | 1.18 | 0.57(19.80°C) | 23.41(20°C) | 2.56 | 9.03 |
| Chlorobenzene | 131.7 | 11.7 | 327 | 1.10(20°C) | 0.72(20°C) | 31.98(20°C) | 1.62 | 5.62 |
| Dichlorometane | 39.6 | 435.8 | 1980 | 1.32 | 0.39(30°C) | 26.54(20°C) | 1.14 | 8.93 |
| Chloroform | 61.2 | 194.8 | 8500 | 1.48 | 0.54 | 26.53 | 1.15 | 4.81(20°C) |
| Tetrachlorometane | 76.6 | 115.2 | 770 | 1.58 | 0.90 | 26.13 | 0.00 | 2.23 |
| 1,2 dichloroethane | 83.5 | 83.2(20°C) | 8100(20°C) | 1.25 | 0.73(30°C) | 30.84(30°C) | 1.83 | 10.37 |
| 1,2 dichlorobenzene | 180.5 | 1.3 | 156 | 1.30 | 1.32 | 26.48(20°C) | 2.14 | 9.93 |
| Tetracholoroethylene | 121.1 | 18.5 | 150 | 1.61 | 0.80(30°C) | 31.30 | 0.00 | 2.28 |
| Bromobenzen | 155.9 | 4.2 | 424 | 1.48 | 1.01(30°C) | 35.09(30°C) | 1.55 | 5.40 |
| Nitrobenzene | 210.8 | 0.28 | 1900(20°C) | 1.20 | 1.62(30°C) | 42.17(30°C) | 4.00 | 34.78 |
| Acetonitrile | 81.6 | 88.81 | ∞ | 0.78 | 0.34 | 28.25 | 3.53 | 35.94 |
| Pyridine | 115.2 | 20 | ∞ | 0.98 | 0.88 | 36.33 | 2.37 | 12.91 |
| N´N”-dimethyl-formamide | 153.0 | 3.7 | ∞ | 0.94 | 0.80 | 36.42 | 3.24 | 36.71 |
| Carbon disulfide | 46.2 | 361.6 | 2100(20°C) | 1.29 | 0.36(20°C) | 32.25(20°C) | 0.06 | 2.64 |

a- Data obtained from CRC Handbook of Chemistry and Physics 66th edition

# Appendix B

## Single-drop microextraction apparatus

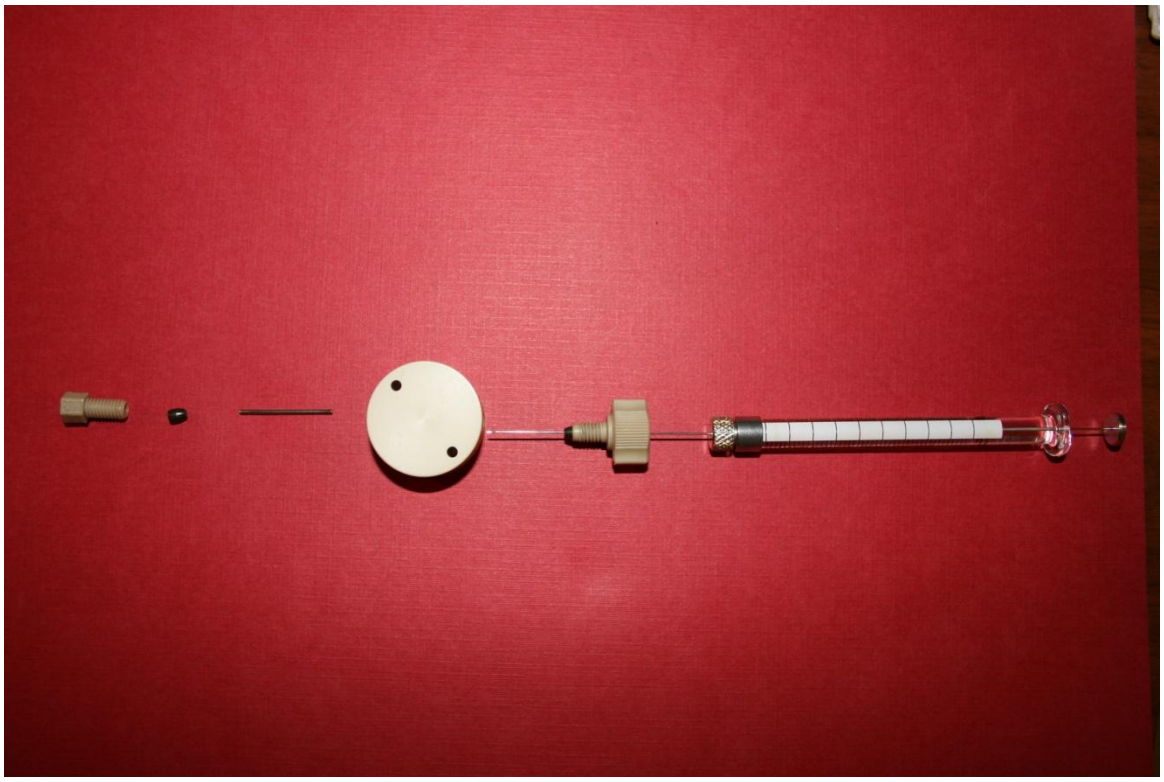

Figure B-1 Single-drop extraction assembly for making co-centric droplets using 10 µL syringe and PEEK tubings and fittings.

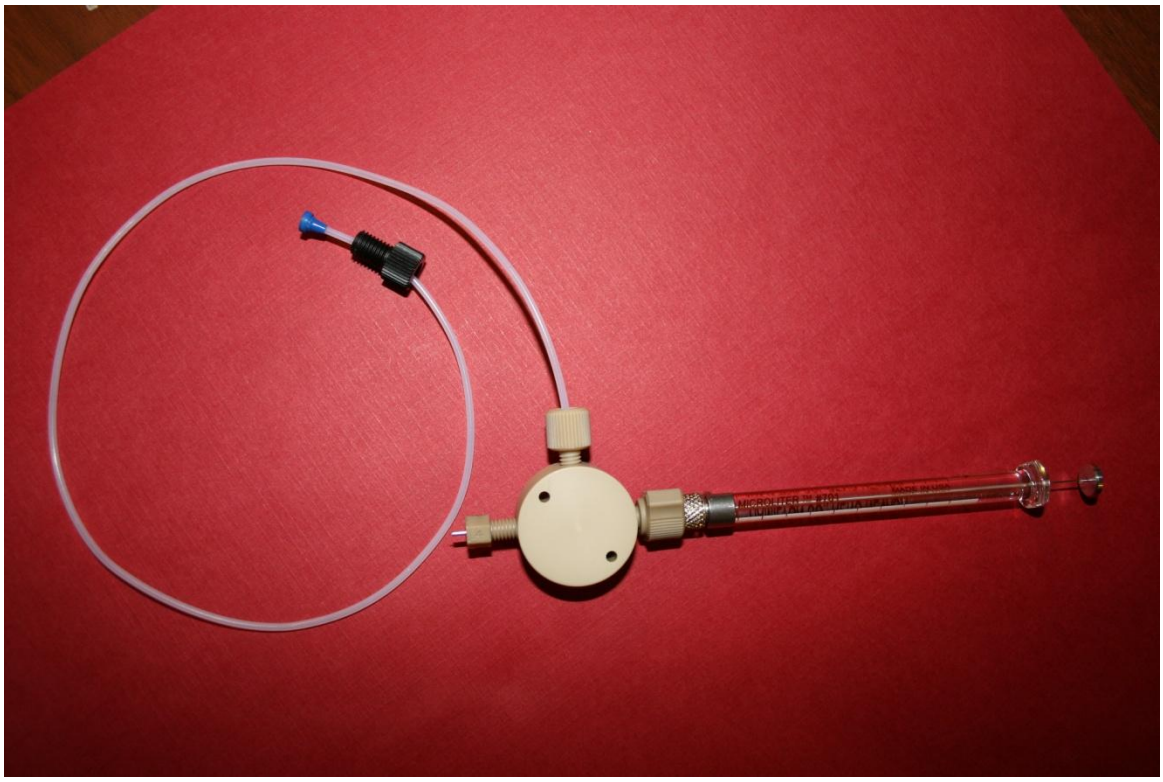

Figure B-2 Configuration for continuous flow single-drop extraction  using 10 µL syringe and PEEK tubings ad fittings

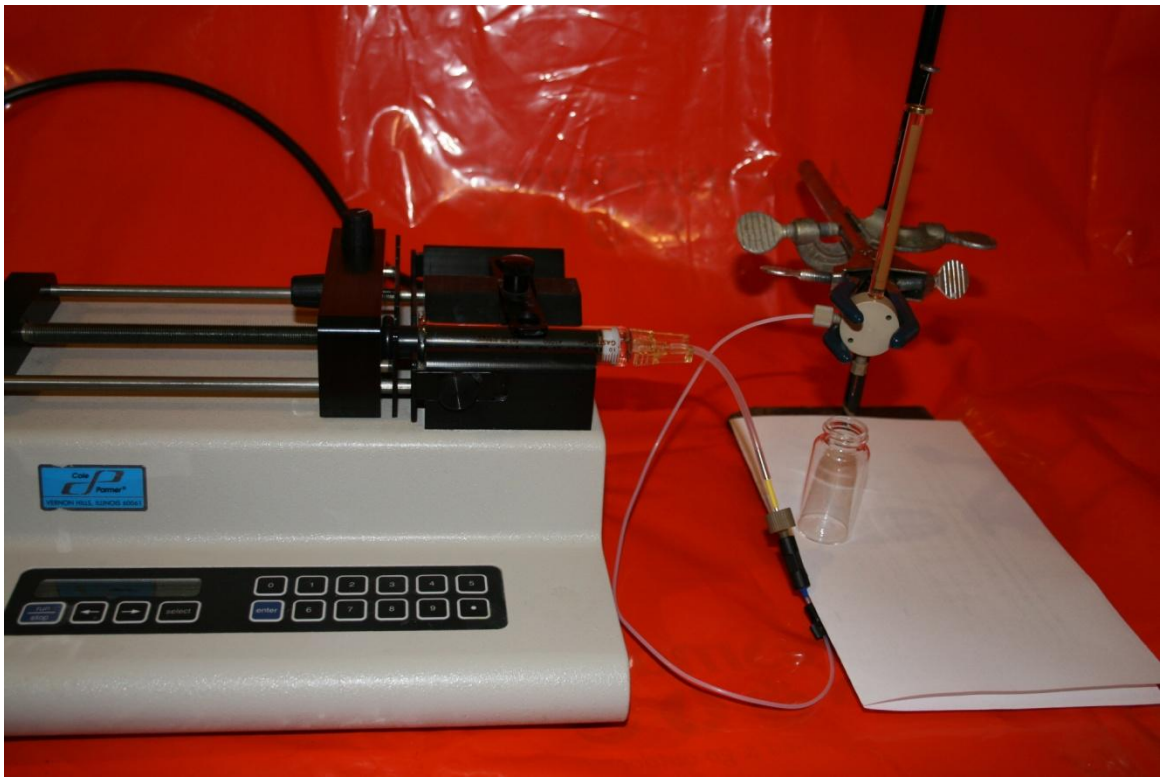

Figure B-3 Configuration for continuous flow single-drop extraction  using syringe pump.

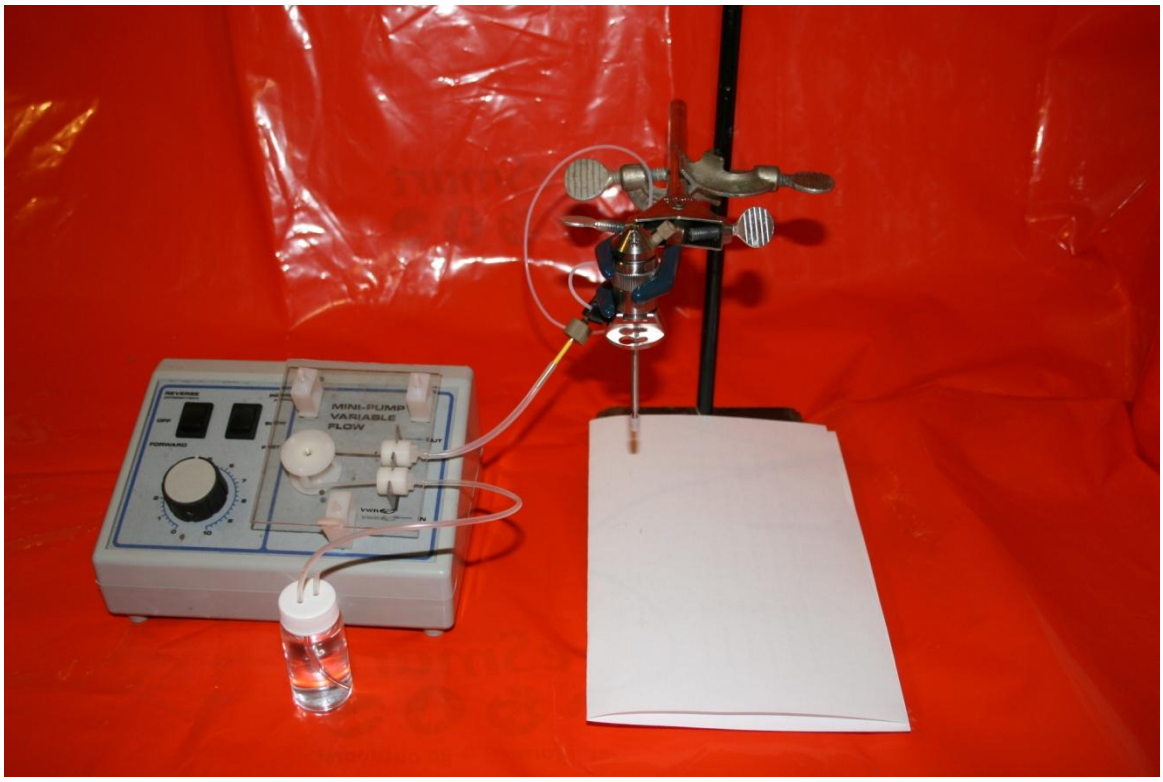


Figure B-4 Configuration for contious flow single-drop extraction  using pristaltic  pump

Figure B-3 Configuration for continuous flow single-drop extraction using three sets simultaniously.