ON-LINE PRE-CONCENTRATION TECHNIQUES IN CAPILLARY ELECTROPHORESIS FOR ENVIRONMENTAL ANALYSIS

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7. Chuanhong Tu, Lingyan Zhu, Hian Kee Lee, The effect of counter-ions of the background electrolyte on large volume sample stacking of phenoxy
acids in capillary electrophoresis with phosphate background at pH 6.0. Manuscript under preparation.


SUMMARY

This work focused on the development of on-line sample pre-concentration techniques to improve detection sensitivity in capillary electrophoresis (CE). Several on-line enrichment methods were established for acidic compounds with various pKₐ values, including strong acid (nitrate), weak acids (haloacetic acids, phenoxy acids, pKₐ 0.6-4.8) and very weak acids (phenols, pKa 7.5-10.6), in different sample matrices.

For nitrate in seawater sample, a zwitterionic surfactant was added into the background electrolyte (BGE) to increase the mobility difference between chloride and nitrate, so that a leading-type sample self-stacking could be employed to pre-concentrate low concentration nitrate in seawater using native chloride in the sample as the leading ion, and the co-ion in the BGE as terminating ion. Thus, a highly conductive sample could be injected in a large volume with about 4-fold sensitivity enhancement compared to large volume sample stacking in which nitrate was dissolved in pure water. A detection limit of nitrate of 35µg/L was achievable for seawater with relatively low concentration BGE. At an analyte concentration near the limit of detection (LOD), the mole ratio between the matrix and the analyte was around $10^6$:1.

Organic solvent is often used for sample extraction during off-line sample pretreatment. Unfortunately, samples in common organic solvents, such as hexane, cannot be analyzed directly by CE. Aqueous alkaline solutions are usually employed to back-extract organic weakly acidic compounds from organic solvent in sample pretreatment. We developed three on-line pre-concentration methods for acidic compounds dissolved in NaOH solution,
which can be coupled with off-line sample pretreatment steps to increase the sensitivity further.

For haloacetic acids, hydroxide-induced dual transient isotachophoresis was used to compress the injected large volume sample to a very small volume. After sample stacking, NaOH was neutralized by the $H^+$ in the low-pH BGE, the analytes were separated in capillary zone electrophoresis (CZE) mode. More than 100-fold sensitivity enhancement was obtained. Combined with off-line solvent extraction, sub-ppb level haloacetic acids were determined in drinking water samples.

For phenoxy acids, when diethylenetriamine (DETA) was used as electroosmotic flow (EOF) suppressor and counter-ion of the BGE at pH 6.0, sample dissolved in NaOH solution could be injected in a large volume into capillary, the analytes were focused at the initial state of electrophoresis and then separated in CZE mode. 75-fold sensitivity enhancement was achieved. Combined with liquid phase microextraction, the limit of detection reached 0.1 ppb level in water samples.

For phenols with high $pK_a$ values, the above methods cannot work due to the lack of suitable EOF modifier in high-pH BGE. A field amplification sample injection was used to introduce a large amount of analytes into capillary followed by micellar electrokinetic chromatography for separation. Since a low pH BGE was used, the injected analytes accumulated at the interface between the BGE and the pre-injected water plug by the dynamic pH junction. More than 2,000-fold sensitivity enhancement was obtained.

All the developed methods were as simple and convenient to implement as conventional CE operation with a hydrodynamic or electrokinetic injection
(except for longer injection times) to improve sensitivity. In addition, these methods can be coupled with off-line sample pretreatment steps and applied to real samples, as demonstrated in this work.
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1.1 Brief History of Capillary Electrophoresis

Electrophoresis is a liquid phase separation technique based on ionic mobility differences of species in an electric field. Capillary electrophoresis (CE) is electrophoresis performed in a capillary tubing with typical internal diameter (i.d.) of 20-150 μm. The principle underlying most kinds of electrophoresis is the same, which can be described in general as the migration of charged substances in solution under the influence of an applied electrical field. The research on the principle of electrophoresis dates back to more than one hundred years ago when Kohlrausch derived his basic equations for ionic migration in an electrolyte solution in 1897 [1]. In 1930, Tiselius achieved some pioneering separations of blood plasma proteins in free solution and demonstrated that the electrophoretic mobility of proteins was related to their electric charge and molecular weights [2].

One common problem in the early practice of electrophoresis was band broadening due to thermal effects (e.g. convection) caused by Joule heating. The most important solution to this problem was the introduction of supporting media such as paper, cellulose acetate, starch and polyacrylamide gels [3,4]. Nevertheless, running gel electrophoresis involves gel preparation, sample application and staining. All these steps were time-consuming and labor-intensive. Additionally, interactions between the analytes and the gel matrix affected the separation. Although this was often desirable, for example, in the
molecular sieving effect of polyacrylamide gels in zone electrophoresis, the electrophoretic behavior of the separated compounds was overlaid by chromatography. Hence, many attempts were made to perform electrophoresis in free solution without any stabilizing media to overcome the effect of convection.

Zone electrophoresis in free solution was described by Hjertén in 1967 [5]. He performed zone electrophoresis in tubes of quartz glass, of 1-3 mm i.d. and coatings of methylcellulose to prevent electroosmosis. Convection was reduced by rotating the separation chamber around its longitudinal axis. Zone detection was accomplished with a UV detector, which scanned the length of the tube.

Another approach to reduce convection was the use of narrow-bore capillary tubes of sub-millimeter internal diameters. Due to the high ratio of the cross-section of the separation compartment to its surface area, heat dissipation was enhanced in these systems. Based on this so-called anticonvective wall effect, Everaerts and coworkers developed capillary isotachophoresis (CITP) in narrow-bore Teflon tubes in the mid 1970s [6, 7]. The use of Teflon instead of glass tubes had the advantage of minimizing electroosmosis, which would distort the isotachophoretic separation. Although commercial equipment for this technique has been available since then, the interest in CITP among the scientific world is rather low in comparison to other techniques.

In 1974, Virtanen reported zone electrophoresis in glass tubes with 200-500µm i.d. [8]. The separated compounds were detected by potentiometry.
Several years later, Mikkers, Everaerts and Verheggen performed zone electrophoresis in narrow-bore Teflon tubes with 200 µm i.d.[9]. The separation of 16 small anions employing conductometric detection within 10 minutes was demonstrated. Plate heights of less than 10 µm were achieved. Nevertheless, this detection mode was relatively insensitive and required large volume sample loading.

Two major problems were not completely solved at that time, namely the low sensitivity of the detection systems for narrow-bore tubes, and electroosmosis. It was Jorgenson and Lukacs who addressed these issues in the 1980s [10-12]. They employed 75 µm i.d. glass capillaries, which could efficiently dissipate Joule heating and permit the use of high voltage. Instead of suppressing electroosmosis by using electrically inert capillaries, they took advantage of the unique plug flow profile of the electroosmotic flow, which was generated in glass capillaries of very narrow internal diameters, to move the analytes through a capillary with much less dispersion than observed in high performance liquid chromatography (HPLC). In their work, on-column fluorescence detection was used to increase sensitivity [10-12].

It is worth mentioning the two breakthroughs in the development of CE techniques after Jorgenson’s work. One was miceller electrokinetic chromatography (MEKC), which was first reported by Terabe in 1984. It expanded the application of CE to neutral compounds [13]. Another was CE-on-a-chip. In 1992, Manz et al integrated all the CE parts into a microchip system that could reduce the analysis time further [14,15].
1.2 Basic Principles of CE

1.2.1 Electrical migration of charged species

The migration velocity $v$ of a charged species such as ion or particle is proportional to the electrical field strength $E$.

$$v=\mu E \quad (1-1)$$

The electric field strength is expressed as the electrical potential gradient in volts per unit length. The constant of proportionality $\mu$ is called the electrophoretic mobility. If the ion is a sphere with radius of $r$, according to Stokes law, the electrophoretic mobility is

$$\mu=q/6\pi\eta r \quad (1-2)$$

Where $q$ is the charge that the ion carries, $\eta$ is the viscosity of the solution, and $r$ is the radius of the hydrated ion. Considering the influence of chemical equilibria on mobility, Tiselius defined the concept of effective mobility [2]. The substance, present in the solution in more than one form, whose molar fractions are $x_0$, $x_1$, ...$x_n$, with mobilities $\mu_0$, $\mu_1$, ...$\mu_n$ and the individual forms are in a rapid dynamic equilibrium with one another, migrates through the electric field as one substance with a certain effective mobility, $\mu_{\text{eff}}$, defined as

$$\mu_{\text{eff}}=x_0\mu_0+ x_1\mu_1 + ...+x_n\mu_n \quad (1-3)$$

From the macroscopic point of view, the mixture of different forms of the given substance thus appears during migration as a uniform substance with a defined mobility and a defined charge. The definition indicates that the effective mobility of substance can be changed through altering molar fractions and/or mobilities of its individual forms. In practice, acid-base and complex
equilibria are often used to modify the effective mobility to improve CE separation.

1.2.2 The electroosmotic flow (EOF)

In general, there is a charge segregation at the interface between the solid phase and the aqueous solution. The solid surface can become electrically charged by a variety of mechanisms, including ion dissociation, ion adsorption, etc. [16]. The first theory for the charge distribution at the solid-liquid interface was the electrical double layer theory suggested by Helmholtz in 1879 [17]. He assumed that a layer of counter ions would be immobilized on the surface by electrostatic attraction such that the surface charge was exactly neutralized. Later, Gouy [18] and Chapman [19] pointed out that ions were subject to random thermal motion and thus would not be immobilized on the surface. They suggested that the ions which neutralize the surface charge were spread out into solution, forming what was called a diffuse layer. Stern [20] suggested a combination of the two models to account for the properties of the double layer. Thus some ions were indeed immobilized on the surface, but usually not enough to exactly neutralize the surface charge; the remainder of the charge was neutralized by a diffuse double layer extending to the solution. In CE, the interface between the surface of capillary tubing and an aqueous buffer of electrolytes is a good example of electric double layer. The surface of the fused silica capillary becomes negatively charged owing to dissociation of acidic surface silanol groups when in contact with a solution of pH above about 3 [21]. This surface charge influences the distribution of ions in the solution in
the vicinity of the capillary surface; counter-ions are attracted towards the surface and ions of the same charge sign (co-ions) are repelled away from the surface. The balance of mixing tendency from thermal motion and static electrical interaction leads to the formation of the electrical double layer made up of the charged surface and a neutralizing excess of counter-ions over co-ions distributed in the solution close to the surface.

Electroosmotic flow (EOF), one of the known electrokinetic phenomena, refers to the bulk movement of liquid inside capillary system under the influence of an electric field along the capillary. In a fused silica capillary filled with an aqueous solution of pH above 3, the native EOF is cathodic, i.e. towards the cathode. It can be simply explained using the electrical double layer theory. When an electric field is applied parallel to the capillary surface, the mobile positively charged counter-ions in the diffuse layer migrate toward the cathode together with the solvent molecules held in their primary solvation shell [22]. This movement spreads out immediately over the whole liquid through frictional forces among the solvent molecules. Electroosmotic mobility $\mu_{eo}$ can be described by the Smoluchowski equation [8].

$$\mu_{eo} = \frac{(\varepsilon \zeta)}{(4\pi \eta)}$$  \hspace{1cm} (1-4)

where $\varepsilon$ is dielectric constant of the liquid, $\zeta$ is the zeta potential of the interface, $\eta$ is the viscosity of the liquid.

For the small diameter tube, the EOF profile is plug-like, which is quite different from the parabolic flow profile of hydrodynamic flow in a pressure-driven liquid phase separation system, such as HPLC. For capillaries with internal diameter of 5 $\mu$m to 100 $\mu$m the flow profile can be regarded as
essentially flat, which results in substantially less band broadening than the hydrodynamic flow of a parabolic flow profile.

The apparent mobility of an ion in CE is the combination of EOF and electrophoretic mobility. Therefore, EOF will affect the separation time and resolution. A simple way to regulate EOF in CZE is to control buffer pH since EOF is strongly dependent on pH in the range of 3-8 [23]. However, pH changes alter the effective mobility of a weak acidic or basic compound at the same time. Another dynamic approach to regulation EOF is to add oligoamine [24,25], zwitterionic [26] or cationic surfactant into the CE buffer [27]. To eliminate EOF, the capillary can be coated with non-ionic polymers, such as polyacrylamide [28] and polyethylene glycol [29].

1.3 INSTRUMENTAL SETUP OF CE

The basic instrumental setup of CE system is shown in Figure 1-1. The close circuit is composed of a high voltage power supply, two electrodes, two buffer vials and the separation capillary. Sample is introduced into the capillary from one end by pressure or by voltage. A detector is used to monitor the separated analytes. Commonly, a computer system is devoted to data acquisition and instrument control.
Figure 1.1  Schematic diagram of basic CE instrumental setup.

1.3.1 Capillary

Fused silica is the most popular material for use as CE capillary tubing, although there are some reports on the uses of other materials such as borosilicate glass [10] or synthetic polymers [30]. The typical internal diameter of a capillary tubing used in CE is in the range of 25-150 μm. Fused silica capillary provides good performance in terms of thermal conductivity, flexibility
and ruggedness with its external polyimide coating, ultraviolet radiation transparency for detection when the external coating is removed. A shortcoming with fused silica capillary is that it can interact irreversibly with some analytes such as proteins. In those cases an appropriate inert coating, either permanent or dynamic, may be helpful in preventing this type of interaction. Since CE can be performed on a microchip [14,15], the micro-channel etched on the planar chip takes the role of capillary tubing as in conventional CE. CE-on-a-chip has the potential to be multiplexed for high-throughput applications.

1.3.2 Sample Introduction

Sample can be introduced into the capillary in two common ways in CE. One is the hydrodynamic injection, and the other is electrokinetic injection. In hydrodynamic injection, a pressure difference between the inlet and outlet is applied to move the sample into the capillary. The injection volume, V, can be calculated by following equation:

\[ V = \frac{\Delta p \pi r^4 t}{8 \eta L} \]  

(1-5)

where \( \Delta p \) is the pressure difference; \( r \) is the inner diameter of the capillary; \( t \) is the injection time; \( \eta \) is the viscosity of buffer; \( L \) is the total length of the capillary. In general, the injected sample plug is usually 1% of the total capillary length.

In electrokinetic injection, a voltage is applied across the capillary. Sample solute enters the capillary due to electrophoretic migration and/or electroosmotic flow under the influence of an electric field. The injection quantity, Q, of a component can be represented by
Q=(\mu_{ep}+\mu_{eo})\pi r^2 v t c / L \quad (1-6)

where \mu_{ep} and \mu_{eo} are the electrophoretic and electroosmotic mobility respectively, c is the concentration of the component in the sample solution, r is the inner diameter of the capillary, v is the applied voltage, t is the injection time, L is the capillary length.

1.3.3 Detection

Almost all detection techniques used in liquid chromatography can be modified to be used in CE. The commonly used detectors include ultraviolet-visible (UV) detection, fluorescence detection, electrochemical detection and mass spectrometric detection.

UV detection is the most popular detection techniques in CE since most analytes absorb some UV radiation. It is performed on-capillary for minimized detection cell volume and convenience in operation, but the optical path length is defined by the inner diameter of the capillary tubing. This limits the sensitivity of absorbance detection techniques since the signal strength is proportional to the optical pathlength according to Beer-Lambert’s Law.

Fluorescence detection is also performed on-capillary like UV detection. In fluorescence, there two types of excitation sources, one is lamp-based, the other is laser-based [31]. For analytes with a fluorophores, lamp-based fluorescence detection provides one to two orders of magnitudes higher sensitivity than UV detection. With laser induced fluorescence (LIF), very high sensitivity can be obtained, but the excitation wavelength is limited by the availability of commercial laser sources.
Electrochemical detection (ECD) can be carried out in either on-capillary or end-capillary format in CE. It can be classified into amperometry, conductivity and potentiometry according to operation principles. Amperometry is the most sensitive ECD, but it is only responsive to electro-active analytes.

Mass spectrometry (MS) is a universal detection technique in CE. MS can be coupled to CE in either on-line mode or off-line mode. The interface for on-line CE-MS can be electrospray ionization (ESI) \[32\] or continuous-flow fast atom bombardment (CF-FAB) \[33\]. For off-line CE-MS, matrix-assisted laser desorption/ionization (MALDI) is commonly used \[34\].

### 1.4 OPERATING MODES OF CE

Different modes of capillary electrophoresis can be performed using a standard CE instrumental set-up with different electrophoretic media. In the continuous system, electrophoretic buffer forms a continuum along the migration path. In contrast, in discontinuous system, the composition of the electrophoretic buffer changes along the migration path. A variety of electrophoretic media render versatility to CE. The versatility in operation makes it quite flexible to select a proper CE mode for a specific sample separation. The distinct CE modes include capillary zone electrophoresis (CZE), capillary isotachophoresis (CITP), capillary isoelectric focusing (CIEF). They are three fundamental modes of CE \[35\]. When the electrophoresis media contains gel, micelles, or stationary bed, they can be sub-categorized
into capillary gel electrophoresis (CGE), micellar electrokinetic chromatography, capillary electrochromatography [36].

In environmental analysis, the widely used modes of CE are CZE and MEKC. CGE and CIEF are commonly used for separating biological macromolecules such as DNA and proteins.

CZE separates ions or electrically charged particles on the basis of the differences in their effective mobilities in a uniform electrophoresis medium. This is the most common and the simplest mode in CE. CZE can be performed in either free solution or anticonvective media, such as gels. A uniform, or homogeneous, carrier electrolyte system is used to fill the capillary, both anodic and cathodic buffer reservoirs. The sample is introduced as a narrow zone (band) into the inlet of the capillary surrounded by the carrier electrolyte solution. As the electric field is applied, each substance begins to migrate according to its own effective mobility independently of the others. Ideally, each substance will eventually separate from the others and form a pure zone. Analytes suitable for CZE separation range from small inorganic and organic ions to cells [36].

MEKC was designed for the separation of neutral compounds by making use of partition equilibria of solutes between the surrounding aqueous buffer and a charged pseudo-stationary phase (commonly micelles formed by ionic surfactants). The partition equilibria are quickly established and hence neutral analytes are separated by the differences in partitioning themselves between the two phases. Other reported pseudo-stationary phases include microemulsions, charged cyclodextrin, polymer ions, dendrimer, suspended
chromatographic particles, etc. Any factor affecting partition of solutes between the pseudo-stationary phase and the aqueous buffer will change the selectivity of the separation in electrokinetic chromatography. Those factors include the structures and properties of pseudo-stationary phase, temperature, organic modifier, etc.

1.5 ONLINE PRECONCENTRATION TECHNIQUES IN CE

Although the separation efficiency of CE is higher than that of HPLC, the limits of detection (LOD) for capillary electrophoresis are constrained by the dimensions of the capillary. For example, the small volume of the capillary limits the total volume of sample that can be injected into the capillary. In addition, the reduced path length decreases the sensitivity of common optical detection method such as UV detection.

To increase the sensitivity of CE, many methods involving modifications of capillary internal diameter at the detection window have been reported, which included the use of bubble cell [37,38], Z-shaped cell [39-41] with about 10 times sensitivity enhancements and a slight sacrifice of separation efficiency.

Other detection improvement can be obtained by using high sensitivity detection techniques such as laser induced fluorescence (LIF) [42-45] and electrochemical detection [46,47]. However, these detection techniques respond only to fluorescence- or electrochemically-active compounds. For example, LIF detection provides extremely high mass sensitivity with single molecule detection being reported [48,49]. Currently, direct LIF is only applicable to analytes as laser sources are only commercially available with
wavelength of 325nm or 488nm. An alternative to direct detection is derivatization of the analytes with fluorescent tags. Although theoretically, the improvement on detection techniques is a direct way for sensitivity enhancement, the performance of these improvements is not good enough for routine analysis.

A more practical way to increase the sensitivity of CE is the on-line concentration techniques, which is effected by manipulating the composition of the sample and background electrolyte together with simple injection procedures without modification of commercially available instrumentation. This topic has been reviewed by a few authors with different emphasis [50-58]. Most on-line preconcentration procedures utilize some forms of transport phenomena to achieve enrichment. According to the types of transport phenomena, they can be categorized into three groups [59]. The first one is using physical barriers such as gels to selectively retain macromolecules. The second one uses a chromatographic trap (e.g. solid stationary phase or micellar pseudo-stationary phase) to enrich the analytes from a large-volume diluted sample to a small volume. The third involves manipulating the electrophoretic velocity of the analytes in different zones to accomplish the concentration.

1.5.1 On-line preconcentration using physical barrier

The principle of on-line pre-concentration using a physical barrier is the same as in the classical technique of ultrafiltration. The leading species are stopped by the physical barrier, allowing the molecules following behind to
eventually reach the same physical space, thus increasing concentration. The commonly available physical barriers can be a gel, hollow fiber or other semi-permeable membrane.

Hjerten [60,61] inserted a short plug of gel at the tip of the capillary after filling the capillary with the sample diluted in the leading buffer. An electric field was applied with a suitable polarity so that migration of the proteins proceeds in the direction of the gel. Since the pores of the gel were very small, the proteins accumulated on its surface. Once the concentration process was completed, the polarity of voltage was reversed. A mobilization step using a terminating buffer for a short period of time was employed to avoid peak broadening. After mobilization, the separation proceeded under CZE mode by replacement of the trailing buffer for a vessel containing leading electrolytes. Thus, ~400-1000 fold sensitivity enhancement was achieved.

Hollow fiber was also used by both Zhang [62] and Wu et al [63] to concentrate proteins in diluted samples. A short hollow fiber with a suitable molecular weight cutoff value was connected to the inlet end of a capillary. A voltage was applied across the hollow fiber. The proteins migrated to the hollow fiber and accumulated on it. A 1000-fold increase in signal with UV-detection was observed. Recently, Yeung and Wei [64] used hydrofluoric acid to etch the part of the fused-silica capillary to form a semi-permeable porous membrane to selectively concentrate peptides and proteins.

Although an on-line pre-concentration method using physical barrier provides effective sensitivity enhancement, its application is limited to the
macromolecules with significantly different molecular weight from the other ions in the sample matrix.

1.5.2 On-line chromatographic preconcentration techniques

1.5.2.1 On-line solid phase extraction

Solid phase extraction (SPE) is commonly used in off-line sample pretreatment. Solid adsorbents are used to retain the analytes in a large volume of a low-concentration sample; the analytes are then eluted in a small volume to achieve sample enrichment. The concentrated sample can be injected directly into a CE system. Since this technique and its off-line combination with CE obviously consume more analysis time, many researchers are trying to incorporate SPE into a CE system for on-line concentration. One method was to pack a short segment, about 2 mm, of the injection end of the capillary with LC stationary phase [65-76]. The sample was loaded onto the stationary phase by hydrodynamic injection, and then eluted by injection of a second solvent [77]. Theoretically, solid phase normally used for off-line extraction can be applied in on-line mode, but there may be some problems with the packing. One main problem was the increased back pressure disturbing the EOF due to the glass frits and the packing material [78]. A small frit and shorter packing material had to be used to reduce the back pressure [79]. To overcome the problems arising from the packing, alternatively, Cai and El Rassi [80,81] have developed an open-tubular preconcentrator for CE. In this approach the wall of a 20-cm capillary was modified with a C18 phase for herbicide analysis, or a metal chelate phase for
protein analysis. The diluted analytes could be concentrated by 10-35 times. Breadmore and Haddad [82-84] developed an open-tubular ion-exchanger preconcentrator for inorganic anions by coating part of the capillary with ion-exchange resin. A gradient elution was realized by a transient isotachophoresis. 100-fold sensitivity enhancement was obtained. Tomlinson et al [78] reported a different technique for on-column partitioning-based preconcentration, termed membrane preconcentration. They used C18-impregnated styrene-divinylbenzene membranes installed in a Teflon cartridge system. This technique could reduce the volume of organic solvent necessary to elute the analytes from the enrichment device and reduce the back pressure in comparison to glass frit-based packing, resulting in a more reproducible EOF and better resolution. In these techniques, the preconcentration capillary was connected in series with the separation capillary. A number of problems may arise, including tailing, loss of separation efficiency, and interference between the organic elution solvent and the CE electric field [55].

To solve the above problems, many attempts have been made to use multiple-capillary system to separate the enrichment capillary from the separation capillary. These include the use of a double-capillary system [69], and an on-line switching valve [85]. In the double-capillary system, two capillaries were connected with a T connector. The pre-concentration by SPE was carried out in one capillary; the separation was performed in the other capillary. In the on-line switching valve design, the analytes were retained on a stationary phase within the valve. The retained analytes were transferred to the separation capillary by valve switching. The on-line coupling of LC with CE
provides a possible technique for on-line pre-concentration in addition to a two-dimensional separation \[86-89\]. In the multiple-capillary scheme, detection enhancements of 400- to 500-fold \[90,91\] and as high as 7000-fold \[92\] have been reported. These techniques were limited by their complexity, which could lower the reproducibility of the methods.

Although on-line SPE methods can provide high sensitivity enhancement factors, the SPE devices are not part of any commercially available CE instrument and their fabrication is tedious. Generally, these SPE methods are not rugged enough for routine analysis.

1.5.2.2 Pseudo-stationary phase partition-based techniques

In on-line SPE techniques, the analytes are mobilized to go through the stationary phase and retained there. In pseudo-stationary phase partition-based preconcentration techniques, neutral analytes are stationary, the charged pseudo-stationary phase (e.g. ionic surfactant) migrates through the sample zone. The analytes partition into the pseudo-stationary phase and are focused. Although the pseudo-stationary phase itself may be concentrated when it migrates through sample zone, the focusing of analytes mainly depends on the partitioning process during which the diluted analytes are transferred from a relatively large volume of sample to a smaller volume of pseudo-stationary phase.

The idea for concentrating neutral compounds with a pseudo-stationary phase was first proposed by Liu et al \[93\]. They injected a sample in a low-conductivity micellar solution into a capillary containing a high conductivity
micellar background electrolyte (BGE). After applying voltage, the micelles migrated to the boundary between the sample solution and the BGE, and accumulated there. Since the analytes partitioned into the micelles, they were stacked at the boundary. This technique could be operated at either normal or reverse polarity mode with similar stacking efficiencies with a small injection volume. For a large injection volume, the reverse polarity provided better focusing effect. An 85-fold increase in sensitivity was reported using these methods.

Quirino and Terabe have reported a series of methods for concentrating neutral analytes with micelles, similar to Liu et al’s except that the analytes were dissolved in the low-conductivity matrix without micelles [94,95]. The micelles used for stacking were from the BGE. Due to the high-field strength across the sample zone when the separation voltage was applied, micelles migrated rapidly across the sample zone, incorporating the neutral analytes. Once the micelles reached the boundary between the sample zone and the BGE, they were stacked into a narrow band. These methods could be realized in normal- and reverse-polarity mode. When reverse-polarity mode was used with high-pH BGE, careful monitoring the current was required for switching the polarity [95]. The analytes incorporated in the micelles could migrate to the detection window with suppressed EOF by low pH BGE using reverse polarity. No polarity switching was needed [96]. Quirino and Terabe have also explored the possibility to stack neutral analytes with micelles in field-amplified electrokinetic injection [97], field-amplified electrokinetic injection with reverse migrating micelles [98], and reverse migrating micelles with the injection of a
water plug [99]. The sensitivity enhancement in terms of peak heights can be improved by 20-, 75-, and 100-folds, respectively.

Later, Quirino and Terabe found that in stacking of neutral analytes with micelles, the low-conductivity sample matrix was not necessary. Samples in a buffer with a similar conductivity to that of the BGE, but in absence of a pseudo-stationary phase, could be injected in a large volume into the capillary. The charged micelles would go through the sample zone after the application of the voltage. The analytes partitioned into the micelles and were concentrated. This method is called sweeping. The effectiveness of sweeping was dependent on the analytes’ affinity for the pseudo-stationary phase. 80- to 5000-fold enhancements were reported [100,101]. The sweeping method was also used in micro-emulsion electrokinetic chromatography using micro-emulsion as pseudo-stationary phase [102].

Palmer and Landers reported that a high-conductivity matrix in the sample zone could help the micelles in the BGE to concentrate the neutral analytes in the sample. The mechanism was that the micelles in BGE were concentrated at the boundary between the BGE and sample zone due to the field amplification across the BGE zone since the conductivity of BGE was less than that of sample zone [103]. Utilizing high-conductivity sample matrices to invoke sample stacking was promising, but required the limited use of sample solubilizing agents such as alcohols in the sample matrix. Munro et al [104] reported that simple replacement of the sample solvent (methanol) with a solution of sulfated β-cyclodextrin allowed a significant increase in the sensitivity of detection of model hydrophobic analytes. This increase in
sensitivity was accompanied by significant peak sharpening. Sulfated CDs in the sample matrix allowed for effective solubilization of hydrophobic analytes without the use of organic solvents such as methanol.

Recently, Palmer and Landers reported a scheme for stacking neutral analytes in high-salt sample matrix with electrokinetic injection. The analytes were injected into the capillary by EOF and was stacked at the inlet due to their partitioning into negatively charged micelles. This scheme could be performed on conventional CE or CE on a microchip [105,106].

In summary, the on-line pre-concentration methods based on partition into pseudo-stationary also have limitations. They are not effective for concentrating the analytes with weak affinity for the pseudo-stationary. For analytes with higher affinity, although they can be effectively concentrated, the separation by MEKC that follows may encounter a problem due to overly strong partitioning in the micellar phases. Therefore, a compromise has to be reached to address the sensitivity and selectivity.

1.5.3 Online electrophoretic pre-concentration techniques

To concentrate the analytes in a large sample plug, the velocities of the analytes in the direction of their movement should be reduced. The analytes in the leading part slow down, those in the tailing part will catch up. Thus, the analytes are accumulated into a small volume. This principle is applicable to all on-line sample pre-concentration techniques.
In electrophoresis, the velocity of an ionic analyte is dependent on its mobility $\mu$ and the electric field strength $E$ that it experiences. Therefore, there are two ways to manipulate the velocities of analytes to achieve on-line pre-concentration. One is to control the electric field, which includes field-amplified sample stacking, large-volume sample stacking, pH-mediated stacking, isotachophoresis etc. The other is to change the ionic mobility with acid-base or complex equilibrium to manipulate the velocity of the analyte.

1.5.3.1 On-line pre-concentration based on electric field enhancement

1.5.3.1.1 Field-amplified sample stacking

Field-amplified sample stacking (FASS) is the simplest method for on-line pre-concentration. It can be induced by injecting a large volume of sample dissolved in a low conductivity sample matrix. The effects of injecting samples in a low-conductivity matrix were first reported by Mikkers in 1979 [9]. In general, this method is based upon the idea that ions migrating through a low conductivity solution into a high conductivity solution slow down dramatically at the boundary of the two solutions and stack into a narrow zone due to the high electrical field strength in the sample zone.

In 1990s, Burgi and Chien [57,107,108] investigated FASS thoroughly. They found that, theoretically, the peak width in sample stacking was proportional to the ratio, $\gamma$, of buffer concentration in the original sample solution to that in the BGE. This difference in the concentrations inside the capillary tubing generated an electroosmotic pressure originating at the concentration boundary. The laminar flow resulting from the electroosmotic
pressure caused peak broadening. Sample stacking and broadening due to laminar flow worked against each other, resulting in an optimal point relating to the sample buffer concentration, the BGE concentration, and the sample plug length. Experiments confirmed that the optimal condition for sample stacking was to prepare the sample in a buffer concentration that was about 10 times less than that of the BGE and a sample plug length up to 10 times the diffusion-limited peak width. With this condition, over 10 times sensitivity enhancement could be achieved [109]. Beckers and Ackermans investigated the effect of field amplification sample stacking on the resolution, calibration graphs and pH in CZE [110].

Furthermore, Chien and Burgi [111] extended the field-amplification technique into electrokinetic injection mode with a sample dissolved in low-conductivity matrix. With polarity switching, both cations and anions can be injected with field amplification [112]. They described enhanced stacking and sample loading by injection of a water plug into the capillary immediately prior to electrokinetic injection. Further study by Thormann et al [113-116] has shown that injection of a high viscosity plug, such as ethylene glycol, before the plug of water, acted as a trap to slow the electrophoretic velocity of the analytes. Stacking efficiencies were doubled using this procedure. Quirino et al [53] found that the presence of a water plug did not improve the peak shape or the corrected peak areas when the directions of the EOF and electrophoretic migration were the same. Zhu and Lee [117] reported a field-amplified sample injection method with a long water plug. The anionic analytes migrated against the suppressed EOF which pumped the water plug out of the
capillary during sample stacking. With this method, 3,000-fold sensitivity enhancement was obtained. Kuban et al developed an on-line flow sample stacking method in a flow injection-CE system, obtaining 2000-fold enhancement of detection sensitivity for priority phenol pollutants [118]. They also found that a water pre-plug before electrokinetic injection did not increase the pre-concentration efficiency significantly. Therefore, the effect of a water plug needs further investigation. In general, sample stacking with electrokinetic injection provides higher concentration factors compared with hydrodynamic injection, since in hydrodynamic injection, the maximum injection volume is the volume of the entire separation capillary, there is no such limitation in electrokinetic injection, but the reproducibility is not as good as in the former [119].

In FASS, the uneven voltage distribution can cause the temperature of the sample zone to increase dramatically. This was investigated by Vinther and Burgi [120-122]. Vinther [123] observed a thermal-degradation of protein in capillary electrophoresis under sample stacking conditions. This is one of the limitations of FASS technique.

The direct applications of FASS to real samples are limited because these samples are seldom in low-conductivity matrices. For example, biological samples consist of around one percent of salts. To pre-concentrate the analytes in biological samples, Lunte et al developed a technique termed pH-mediated sample stacking. This method required the counter-ion of the BGE to be a weak electrolyte. First, a sample in a high ionic strength biological matrix was electrokinetically injected. As the sample was injected, the counter-ion in
the sample matrix, e.g. Cl⁻ was replaced by the counter-ion of the BGE, such as acetate. Next, a strong acid was injected electrokinetically. The H⁺ from the acid injection migrated quickly through the sample zone, neutralizing the acetate ions and creating a region of high resistivity. This allowed the cationic analytes to migrate quickly through the titrated zone to the boundary with the BGE, where they stacked into a narrow band [124,125]. This method could also be used for the determination of anions by incorporating an EOF modifier such as CTAB into a basic BGE and running in reverse polarity [126]. In pH-mediated sample stacking, FASS was triggered by titrating the injected sample zone to neutrality, thus creating a low conductivity region. Applications of pH-mediated stacking have been reported for the analysis of pharmaceuticals as well as for DNA sequencing [127]. To increase the sample loading capacity, a double-capillary system was introduced with a “T” connector. One capillary was used for stacking, the other was used for separation. A 300-fold enhancement in detection limits has been reported using pH-mediated stacking [126]. In this method, the ratio of injection times for sample and acid or base should be optimized experimentally; the precision was not good due to the double electrokinetic injections.

Addition of organic solvent to the sample matrix is another alternative to reducing the conductivity of the sample matrix, resulting in a field-amplification effect for sample stacking [128,129].

Sample stacking for non-aqueous CE [130-134] has also been performed. However, a limitation of FASS is that the ionic strength of the sample must be
significantly lower than that of the BGE. This requirement may cause problems for analysis of some physiological solutions such as dialysates.

1.5.3.1.2 Large volume sample stacking

In FASS, after the analytes are concentrated, the low conductivity sample matrix is still in the separation capillary. Since the sample matrix is less conductive, the electric field is more distributed across the sample zone. The electric field strength used for the separation is reduced, resulting in longer separation times and lower separation efficiency.

To overcome this problem, Chien and Burgi [135] designed a method to remove the sample matrix from the separation capillary after completing the stacking. The sample, dissolved in low-conductivity matrix, was injected hydrodynamically into capillary, filling up the whole capillary volume. After injection, both ends of the capillary were put into the BGE vials. Then, a negative volatage was applied. As a result, the EOF pushed the sample plug out of the capillary from the inlet while anionic analytes moved towards the detection end and stacked at the interface with the BGE. The electrophoretic current was monitored. Since the low-conductivity sample matrix was pumped out, the current increased slowly. When it reached 95-99% of the value when the entire capillary was filled with the BGE, the polarity was reversed, and the separation proceeded in the conventional fashion. Since compared to FASS, a significantly large volume could be injected into the capillary, this method was termed large volume sample stacking (LVSS). Using this method, the analytes have to migrate against the EOF. To stack cationic analytes, a surfactant,
tetradecyltrimethylammonium bromide (TTAB) was added to the buffer to reverse the EOF. McGrath and Smyth used cationic surfactant cetyltrimethylammonium bromide (CTAB) to reverse the direction of EOF. The cationic analytes were separated under a negative voltage (the anode at the detection end) [136,137]. Other applications of this technique include arsenic compounds [138] and phenols [139].

In LVSS for concentrating either anionic or cationic compounds, careful monitoring the current to select the appropriate time for switching the polarity was needed to avoid some analyte loss. Later, Burgi [25] developed a new LVSS method that did not require monitoring the current and switching of the polarity, using diethylenetriamine (DETA) as the EOF suppressor. After applying negative voltage, the anionic analytes migrated against the suppressed EOF towards detection window and stacked into a narrow zone, while the EOF pushed the sample matrix out of the capillary from the inlet. The whole procedure was the same as conventional CE separation except for a longer injection time. Other methods for suppressing EOF to achieve LVSS without polarity switching include using a cationic surfactant [140], zwitterionic surfactant [26] and a low-pH BGE [141,142]. Baryla and Lucy [143] used a zwitterionic surfactant, coco(amidopropyl) ammoniumdimethyl(dimethylsulfo)betaine to suppress the EOF, with various concentrations of salts containing different anions to control the direction of EOF, LVSS was achieved for either cationic or anionic analytes. Quirino and Terabe [144] used CTAB to reverse the EOF that was suppressed by the low-pH BGE to stack cationic compounds, resulting 100-fold detection sensitivity enhancement. Chun [145] used LVSS to
enrich low-abundance proteins with a polymer-coated capillary to suppress the EOF. LVSS can also be performed with non-aqueous BGE.

Like FASS, LVSS performance is also dependent on the conductivity ratio between the BGE and the sample. Although the sample can be injected in a larger volume, the application of LVSS is still limited to low conductivity samples.

1.5.3.1.3 Isotachophoresis

In isotachophoresis (ITP), the sample is sandwiched by a leading ion and a terminating ion. When high voltage is applied, a potential gradient develops and each of the analytes zone migrates with the same velocity. Where ions of slower mobility are present, the electric field is stronger, making the velocity of the zone match the rest of the sample. If a solute moves too slowly and enters the band behind it, a region of higher field strength, the analyte will accelerate until it re-enters its own zone. Eventually, a steady state is reached where each analyte moves as a discrete band according to its mobility. The concentration in individual analyte zone is independent on the original sample concentration prior to applying voltage, but is adjusted according to the Kohlrausch regulating function (KRF) [54]. For diluted analytes, enrichment can be obtained. Although pre-concentration based ITP is quite different from FASS or LVSS, from the above discussion, we know that the principle underlying the concentrating is also uneven electric field distribution (field amplification).
In classic ITP experiments, the separated zones are in contact with one another, resulting in plateaus in detection signals, not like the peaks in chromatography or zone electrophoresis. Although many people practise capillary ITP for separation, more often, ITP is used in combination with CZE as the first stage for concentrating the analytes into a narrow starting zone for CZE.

The combination of ITP and CZE can be accomplished in a single- or dual-capillary system. The single-capillary system can be applied in most commercial instruments. Hjerten et al modified the Ornstein and Davis discontinuous buffer system to adapt it to CZE in order to achieve automatic sharpening of the starting zone, obtaining very high resolution of serum proteins [146]. Later, Schwer and Lottspeich designed a series of stacking methods termed “three-buffer”, “two-buffer” and “one-buffer” stacking systems. In these methods, sample was sandwiched by leading and terminating zones, the analytes were isotachophoretically concentrated [147].

Another ITP-based pre-concentration method uses the sample matrix ion as leading or terminating ion, and the co-ion of BGE as another part of ITP. This is often called sample self-stacking [148-151]. Sample self-stacking is especially suitable for concentrating trace amounts of analytes in biological samples in which sodium and chloride occur in high concentrations. Sodium and chloride are of high mobility, and are good leading ions in ITP for cationic and anionic analytes respectively. Gebauer et al described the ITP process in sample self-stacking, and established a general theoretical model for sample self-stacking and proposed several possible stacking modes. Sample self-
stacking has been applied for concentrating trace proteins, adenosine, and inorganic ions [36,152-155]. This method was also used to concentrate oligonucleotides followed by CZE separation in a poly(ethylene glycol) sieving medium [156].

The dual-capillary ITP system used two capillaries connected with a “T” junction, one for ITP, the other for CZE separation. Since the ITP and the following CZE were run independently, it was easier to select buffer composition than in single capillary system. The analytes were enriched in the first capillary after the ITP process, and then were transferred on-line to the second capillary for separation. Besides the pre-concentration of analytes, the ITP step had several other specific features that were advantageous for CZE, such as the high sample loadings. A theoretical description of the electrolyte systems in ITP-CZE was given by Krivankova et al [157]. A classification of these systems was presented based on the type of electrolyte used for the CZE separation step and a few electrolyte systems were recommended [158,159]. Using this technique, bulk, and trace sample components can be determined simultaneously in a concentration ratio up to $10^4:1$ [152,160]. Krivankova compared the dual-capillary ITP-CZE system with one-capillary sample self-stacking system for the determination of hippurate in serum. the former provided better performance for real samples [159]. This technique can be applied to protein analysis and environmental analyzes [152,161].
1.5.3.2 On-line pre-concentration based on varying mobility

Since the velocity of analyte depends on the electric field strength and ionic mobility, many efforts were made to manipulate the velocity of analyte through changing its mobility to achieve on-line pre-concentration. The mobility of ion can be changed by acid-base equilibrium or ion-association.

A good example for pre-concentration by changing mobility was the isoelectric focusing (IEF) of amphoteric compounds such as proteins and peptides [59]. The analytes were focused according to their isoelectric points in a pH-gradient. Cao et al designed a method for concentrating amino acids based non-steady IEF followed by CZE separation [162]. Since IEF can complete the separation and enrichment in one step, it is seldom used in combination with CZE.

Britz-McKibbin and Chen used a dynamic pH junction to selectively focus catecholamine, weakly acidic compounds and nucleotides. The focusing was caused by the dramatic changes in the analyte mobility within the sample and BGE zones, resulting from the differences in pH and borate concentration within these two zones [163,164].

Recently, Wei and Yeung [165] reported a one-step concentration method based on the mobility change caused by a dynamic pH gradient. A short platinum wire was inserted into the 75-µm-i.d. separation capillary. When a high voltage was applied for CE separation, a sharp pH gradient along the capillary was created dynamically by the electrolysis of water in the BGE. The pH gradient changed the charge that the analyte carried, thus influencing its mobility in different zones in the capillary. The concentration of a large volume
of injected analytes was accomplished for either anions or cations. Several hundredfold concentration factors were achieved.

Since ionic mobility is dependent on the temperature due to the variation of the solvent viscosity and degree of solvation, temperature gradient was used by Ross and Locascio for focusing ionic compounds [166]. The technique was demonstrated for a variety of analytes, including fluorescent dyes, amino acids, DNA and proteins. It was shown to be capable of greater than 10,000-fold concentration of dilute analytes.

1.6 THE OBJECTIVES OF THIS PROJECT

Research in the field of CE is continuously growing and CE is becoming a popular analytical tool in many application areas. Thus, CE is faced with samples of increasing complexity, and a need for improving its sensitivity. Of special interest is the employment of the concentrating properties of electrophoretic systems. Because electrophoretic pre-concentration is an inherent feature of electromigrational mass transport, it offers a way to both simple operation and consistent understanding of the whole analytical procedure.

Various online pre-concentration methods have been reported to improve CE sensitivity, but their applications in real sample analysis have been limited for reasons such as the requirement of low conductivity sample matrix, complicated optimization procedures etc. This project focuses on developing simple sample stacking methods that can be easily combined with common
sample pretreatment procedures for environmental analysis. The stacking mechanisms are also investigated.
Chapter Two
Experimental

This chapter describes the instrumentation, chemicals and procedures used throughout this work. Unless specifically reported otherwise in particular chapters and sections, the same parameters were used for all experiments.

2.1 INSTRUMENTATION

Two capillary electrophoresis (CE) systems were employed in the course of this project. The first was a Hewlett Packard (HP, Waldbronn, Germany) 3D CE system equipped with a diode array detector (DAD). An HP Chemstation (revision A. 06. 03) software was used for instrument control, data acquisition and data processing.

The second instrument was a Prince (Prince Technologies, Emmen, The Netherlands) CE system with a Bischoff (Leonberg, Germany) Lambda 1010 UV detector. Data were collected and processed with DaX software (Prince Technologies).

Either hydrodynamic or electrokinetic injections were used in this project. The injection amount is described in individual chapters.

Capillary tubing used for CE was supplied by Polymicro Technologies (Phoenix, AZ, USA). The total capillary length and the effective length of the capillaries, as well as the applied voltage are indicated in individual chapters.
pH measurements were performed using a Metrohm (Harisau, Switzerland) 692 pH/ion meter and a Metrohm probe, calibrated with pH 4.00, 7.00 or 9.00 buffer solutions (Fluka Chemicals, Buchs, Switzerland).

2.2 GENERAL CE PROCEDURES

The separation capillary was pre-conditioned prior to use with 1 M NaOH solution for 20 min; followed by water for 20 min, and finally the BGE for 5 mins. The capillary was flushed using 0.1 M NaOH solution for 2 mins and the BGE for 5 mins between runs. All solutions were filtered through 0.45 μm filters prior to CE experiments.

2.3 MOBILITY MEASUREMENT

Dimethylsulfoxide (DMSO) was used as the neutral electroosmotic flow (EOF) marker in this project. Because the EOF was suppressed by zwitterionic surfactant, low pH BGE or oligoamine, it took considerable time for the neutral marker to reach the detection window from the inlet. Since the distance between outlet and detection window was 8.5 cm, much shorter than the effective length of capillary, the DMSO (dissolved in the BGE) was injected at the outlet after sample was injected at the inlet. Once the high voltage was applied, the DMSO was carried to the detection window by the EOF. The anionic analytes migrated to the detection window from the inlet under the influence of the high electrical field. Thus, in one electrophoresis run, the migration times of both the EOF marker and analytes were obtained. The EOF mobility and apparent mobilities of analytes were calculated with the following equations:
\[ \mu_{\text{EOF}} = -\frac{L_T (L_T - L_{\text{EFF}})}{tV} \]  
\[ \mu_{\text{APP}} = \frac{L_T L_{\text{EFF}}}{tV} \]

where \( L_T \) and \( L_{\text{EFF}} \) are the total and effective lengths of the capillary, respectively; \( t \) is the migration time of the EOF marker or analytes, and \( V \) is the applied voltage. The anionic analytes migrate in the reverse direction to the EOF, so the effective mobilities of analytes were calculated using the equation:

\[ \mu_{\text{EFF}} = \mu_{\text{APP}} - \mu_{\text{EOF}} \]

If a large volume sample was injected into the capillary, especially in the case that the sample matrix was reactive with the composition of the BGE, the influence of the sample plug on the global EOF was significant, the EOF determined using above method was an average value during first 8.5 cm, it might be considerably different from the value when the capillary was filled with the BGE.

2.4 REAGENTS AND MATERIALS

All common reagents used in this study are listed in Table 2-1. The analytes used are detailed in the respective chapters. The water used is ultrapure water prepared on a Nanopure (Barnstead Thermolyne Corp., Dubuque, IA, USA) system.

Polypropylene hollow fiber used in liquid phase microextraction was purchased from Membrana GmbH (Wuppertal, Germany). The inner diameter
of the fiber was 600 μm, the thickness of the wall was 200 μm, and the pore size was 0.2 μm.

### TABLE 2-1  LIST OF COMMON CHEMICALS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Grade</th>
<th>Company</th>
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<tr>
<td>Phosphoric acid</td>
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<td>AnalytiCals, Caro Erba, Milan, Italy</td>
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<td>Sodium dihydrogenphosphate</td>
<td>AR</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>AR</td>
<td>BDH Chemicals, Poole, England</td>
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<td>Sodium chloride</td>
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<td>BDH</td>
</tr>
<tr>
<td>Sodium dodecylsulfate (SDS)</td>
<td>AR</td>
<td>Merck</td>
</tr>
<tr>
<td>Anhydrous sodium sulfate</td>
<td>GR</td>
<td>BDH</td>
</tr>
<tr>
<td>3-(N,N-dimethyldodecyl-ammonio) propane sulfonate (DDAPS)</td>
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<td>Raschig, Ludwigshafen, Germany</td>
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<tr>
<td>Diethylenetriamine (DETA)</td>
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<tr>
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<td>Fisher</td>
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3.1 INTRODUCTION

Many capillary electrophoresis (CE) approaches have been proposed for the determination of inorganic anions in various sample matrices for its high separation efficiency in comparison with ion chromatography (IC) [167-170]. However, for high conductivity samples such as seawater, the determination of minor ionic components is challenging because the mobile salts in the sample will induce electrodispersion, resulting in distortion and broadening of analyte peaks [154]. To overcome these problems, sample dilution has been used [171]. However, sample dilution causes direct sensitivity loss along with the decrease of matrix concentration. Some researchers have used high concentration background electrolytes (BGEs) [172-175]. Although they can alleviate electrodispersion, highly concentrated BGEs lead to high electrical current and excessive Joule heating which are not favorable in CE.

Another problem often encountered in inorganic anionic analysis by CE is the low concentration sensitivity due to the short pathlength with online UV detection and the low extinction coefficients of analyte anions [176]. Breadmore and Haddad reviewed sensitivity enhancement techniques for the determination of inorganic and small organic anions by CE [51]. For a sample in a low conductivity matrix, field amplified sample stacking or field amplified electrokinetic injection can be used to improve the sensitivity.
Okemgbo et al. determined nitrate and nitrite in a low concentration sample matrix with rapid reverse polarity capillary zone electrophoresis (CZE). The detection limits were 271 nM nitrite and 143 nM nitrate using on-column sample stacking [178]. Field amplified sample stacking requires considerable conductivity difference between the BGE and the sample. To preconcentrate trace anions in seawater, the BGE concentration will be unacceptably high.

One possible method for preconcentrating analyte ions in a high concentration ionic matrix is the use of sample self-stacking [152,179]. This can be realized by creating a transient isotachophoresis step in the initial state of a CZE separation [155,180]. The native matrix ion or intentionally added ion can function as a leading or terminating ion and the co-ion of BGE as the other part of isotachophoresis. Gebauer et al. have described the criteria for both leading- and terminating-type sample self-stacking [149-151].

Theoretically, analytes with mobilities ranging between those of the major sample matrix ion and the BGE co-ion can be preconcentrated by this method. However, when the mobility difference between the matrix ion and analyte ions is small, the transient isotachophoresis time is so long that the analytes are very close to the matrix ions at the detection window and cannot be detected as individual peaks [151,154,180].

In seawater of 35‰ salinity, the chloride concentration is about 0.56 M with high mobility [181]. It is easy to find a BGE with a slow co-ion to satisfy the transient isotachophoresis conditions for nitrate using chloride as the leading ion. However, to our knowledge, no one has reported leading-type sample self-
stacking for the determination of nitrate in seawater due to the above mentioned reason. Recently, Fukushi et al [182] determined nitrite and nitrate in seawater with artificial seawater as the BGE, with high-concentration chlorate added into the sample to induce terminating-type sample self-stacking. The sensitivity was improved by 3-fold. However, highly conductive artificial seawater used as BGE resulted in high current and excessive Joule heating.

The aim of this study is to establish a CE method to determine trace nitrate in seawater with a relatively low concentration BGE. Nitrate is pre-concentrated on-line using chloride-induced leading-type sample self-stacking. To overcome the problem of excessive transient isotachophoresis time due to the small mobility difference between chloride and nitrate, a zwitterionic surfactant was added into the BGE for its selective interaction with anions [171]. Thus, the high concentration of chloride in seawater does not interfere with the determination of nitrate, but functions as a leading ion in the transient isotachophoresis for the pre-concentration of the latter ion.

3.2 EXPERIMENT

3.2.1 Chemicals

Sodium nitrite, nitrate, chloride and bromide were purchased from Merck (Darmstadt, Germany). All standards and buffers were prepared in 18 MΩ ultrapure water.
3.2.2 Apparatus and procedures

All experiments were performed on the HP 3D CE system. The detection wavelength for nitrate was set at 210 nm according to the spectrum obtained with the DAD. For monitoring chloride, a wavelength of 195 nm was used.

The capillary used was 64.5 cm × 50 μm i.d. fused silica capillary (56 cm effective length). Samples were hydrodynamically injected into the capillary with a pressure of 50 mbar; a 1-second injection corresponded to 0.067 cm in length of the sample plug. The capillary temperature was maintained at 20°C. The BGE consisted of 0.1 M sodium phosphate and 150 mM DDAPS (pH 6.2). All measurements were performed at a constant voltage of −25 kV, with the current of -88 μA.

3.2.3 Sample collection and pretreatment

The seawater was collected from the western coast of Singapore, 2 meters offshore. Samples were stored under 4°C prior to analysis. Before analysis, the sample was filtered through a 0.45 μm membrane filter. The standard addition method was used for quantitation, to overcome the influence of the variation of chloride concentration in the sample.

3.3 RESULTS AND DISCUSSION

3.3.1 Chloride-induced leading-type sample self-stacking

Sample self-stacking may occur if the analyte transiently migrates in the stack within the sharp boundary between the major sample matrix ion and the co-ion of the BGE, as shown in Figure 3-1A. In leading type sample self-
stacking, the major sample matrix ion is the ion with the highest mobility, and the co-ion of the BGE is the least mobile one. The minor analyte ion will be stacked at the sharp rear boundary of the sample matrix. The analyte ion migrate transiently at same velocity with the sharp rear boundary of the matrix ion. After applying the electric field for a period of time, the analyte ion get separated from the rear boundary of matrix ion, migrating in background electrolyte in CZE mode, as shown in Figure 3-1. In terminating-type sample self-stacking, the converse situation applies. The front boundary is sharp, and analyte ions will be stacked at the front boundary.

![Figure 3.1 Chloride-induced leading-type sample self-stacking. Peak 1: nitrate. The broad peak with sharp rear boundary is chloride. Sample: 0.4 mg/L nitrate in 200 mM NaCl. BGE: A: 0.1 M sodium phosphate, pH 6.2. B: 0.1 M sodium phosphate pH 6.2 with 0.15 M DDAPS. Detection wavelength: 195 nm. Injection: 124 nL. Separation voltage: -25 kV.](image)

In seawater of 35‰ salinity, chloride concentration is about 0.56 M [181]. Its mobility in 0.1 M sodium dihydrogen phosphate (pH 6.2) is $79.4 \times 10^{-5}$ cm$^2$V$^{-1}$s$^{-1}$. 

1. Nitrate occurs in very low concentration of less than 1 mg/L with mobility of $67.2 \times 10^{-5}$ cm$^2$V$^{-1}$s$^{-1}$, so leading-type sample self-stacking will be expected for the pre-concentration of nitrate with dihydrogen phosphate as the co-ion of the BGE. Leading-type sample self-stacking has at least two advantages over the terminating-type for minor analytes in seawater. Firstly, there is no deliberate need to add additional ions to the sample to induce transient isotachophoresis. Thus, the chance of introducing contaminant will be much reduced or eliminated. Secondly, the co-ion of the BGE functions as terminating ion whose mobility is the lowest; thus, the current for the CZE will be lower, obviating excessive Joule heating.

Due to the small mobility difference between chloride and nitrate, the transient isotachophoresis time for nitrate in the sharp boundary between chloride and the co-ion of BGE is rather long. According to Boden and Bachmann [154],

$$t_{ITP}=L_0 \kappa_s (\mu_{M} - \mu_{E}) / [i (\mu_{M} + \mu_{A})^2]$$

(3-1)

where $t_{ITP}$ is the time of transient isotachophoresis, $\kappa_s$ is the conductivity of sample zone, $i$ is the current, $\mu_A$, $\mu_E$ and $\mu_M$ is the mobility of the analyte ion, the co-ion of BGE and the matrix ion respectively. $L_0$ is the length of sample zone. Figure 3.1A shows the electropherograms of 0.4 ppm nitrate in 0.2 M NaCl solution with 0.1 M sodium phosphate (pH 6.2) as BGE. When nitrate migrates to the detection window, it is within the sharp boundary between the chloride and the BGE co-ion, and no separated peak can be detected. EOF for the BGE without DDAPS is three-fold higher than in the presence of DDAPS. The migration direction of anionic analyte is against the direction of EOF, the
separation time is much longer in the former situation due to the larger EOF, but the nitrate cannot be separated from the sharp rear boundary of chloride. On the other hand, in the presence of 0.15 M DDAPS in the BGE, nitrate can be separated from chloride boundary due to the larger mobility difference, as Figure 3.1B shows.

3.3.2 Optimization of sample self-stacking

3.3.2.1 Effect of DDAPS concentration on mobility and sample self-stacking

DDAPS is a zwitterionic surfactant with oppositely charged functional groups in close juxtaposition with its hydrophilic part. It can selectively interact with anions as in electrostatic ion chromatography (EIC). A few interaction mechanism have been proposed [24-28]. As a consequence, this interaction can selectively control the mobilities of anionic analytes [24]. In addition, DDAPS can suppress EOF, which can also be used to improve separation efficiency in CZE [29]. Figure 3.2 shows the influence of DDAPS on the mobilities of nitrate, nitrite, chloride and EOF in 0.1 M sodium phosphate (pH 6.2). The mobilities of the ions decreased with the increasing of concentration of DDAPS at different paces. When DDAPS concentration reached 150 mM in the BGE, the mobility difference between chloride and nitrate reached $18.7 \times 10^{-5}$ cm$^2$V$^{-1}$s$^{-1}$ in comparison with $13.2 \times 10^{-5}$ cm$^2$V$^{-1}$s$^{-1}$ in the absence of DDAPS. Transient isotachophoresis time was shortened according to equation 3-1. Thus, nitrate could migrate away from the sharp boundary between the chloride and the co-ion (dihydrogen phosphate) of the BGE when it reached the detection window. This is shown in Figure 3.1B. At 250 mM DDAPS, the
mobility of nitrate decreased further to approach the effective mobility of the co-ion (dihydrogen phosphate) in the BGE. The conditions for sample self-stacking were therefore violated, resulting in the broadening of the nitrate peak.

Figure 3.2 Effect of DDAPS on mobility of anions and EOF. Experimental conditions: BGE: 0.1 M sodium phosphate (pH 6.2) with various concentrations of DDAPS.

3.3.2.2 Effect of BGE concentration.

In sample self-stacking, not only must the mobility of the BGE co-ion satisfy the condition of the terminating or leading ion, but its concentration also plays an important role in the preconcentration process. At the initial state of transient electrophoresis, the sample concentration is readjusted to the conditions of BGE according to Kohlrausch regulating function [20]. In principle,
a higher BGE concentration leads to a higher preconcentration factor. On the other hand, from equation 3-1, the isotachophoresis time is inversely proportional to the electrical current which is determined by the concentration of the BGE. A higher BGE concentration produces a larger electrical current and shorter isotachophoresis time, so the analytes will migrate in CZE mode for a longer time before it reaches the detection window, resulting in a more diffuse sample zone. When the BGE concentration was increased from 0.05 M to 0.1 M, the peak height of nitrate increased by 3-fold. The peak height reached its maximum at 0.1 M BGE. Further increasing the BGE concentration caused larger electrical current, shorter isotachophoresis time, greater Joule heating, and loss of sensitivity.

3.3.2.3 Influence of chloride concentration

The chloride in the sample functioned as a leading ion in the transient isotachophoresis system. The concentration of chloride determined the specific conductivity of the sample zone. According to equation 3-1, a higher chloride concentration in the sample zone resulted in a longer isotachophoresis time. During transient isotachophoresis, the analyte zone was almost free of diffusion due to electromigrational sharpening effect [20]. When the isotachophoresis condition did not exist any more, the analyte migrated in the BGE under CZE mode, and the analyte zone would broaden due to longitudinal diffusion. Longer isotachophoresis time resulted in a shorter CZE migration time, less diffusion and higher sensitivity. Figure 3.3 shows the influence of chloride concentration on the peak height of nitrate. With the same
injection volume, the peak height increased with the chloride concentration in the sample due to a longer isotachophoresis time. The sensitivity could be enhanced about 4-fold in the presence of 200 mM NaCl compared to that when nitrate was dissolved in pure water, in which field amplification sample stacking took place.

![Graph showing the effect of NaCl concentration on nitrate peak height.](image)

**Figure 3.3.** The effect of NaCl concentration in sample on nitrate peak height. Experimental conditions: BGE: 0.1 M sodium phosphate, pH 6.2, 0.15 M DDAPS. Sample: 0.4 μg/L nitrate in various concentrations of NaCl. Injection: 50 mbar×200 s (248 nL). Separation voltage: -25 kV. Detection wavelength: 210 nm.

It should be noted that the migration time interval between the analytes and the sharp rear boundary of the chloride decreased with chloride concentration. This was especially obvious for nitrite because of its small mobility difference with chloride. In the presence of 10 mM NaCl in the sample, the peak of nitrite appeared close to the rear boundary of chloride (see Figure 3.4B). The peak
height was about 3-fold higher than that in pure water. When chloride concentration was increased to 25mM, the nitrite could not be separated from the chloride sharp boundary. Therefore, this method could not be used to determine nitrite in the presence of a high concentration of chloride.

Another influence of chloride was in the variation of migration time. As shown in Figure 3.4, the presence of chloride in sample resulted in shorter nitrate migration time. In their computer simulation, Gebauer et al showed that the migration times of analyte ions increased with the sample matrix concentration for leading-type sample self stacking [20]. This was different from our results. This difference came from the injection length and the EOF. In Gebauer's model, the injection volume was 3% of the total capillary volume, and the EOF was assumed to be zero. In our experiment, the injection volume (248 nL) was 20 percent of the capillary volume, with suppression of the EOF. The effect of sample length was not negligible. The electrical field distribution between the sample zone and the BGE should be taken into account for the prediction of the migration time. Before the sample matrix zone was pushed out of the capillary by the EOF, the specific conductivity of the sample zone was lower than that of the BGE in case of low concentration of chloride in the sample. The electrical field strength distributed across BGE zone was weaker, resulting in lower migration velocity and longer migration time.

It is worth noting that the concentration of chloride in the sample was correlated to the maximum injectable volume. According to equation 3-1, both injection volume and chloride concentration influenced the transient isotachophoresis time. A longer injection time resulted in more analytes being
introduced, a longer isotachophoresis time, and so a greater peak height, but nitrate could not be separated from the boundary between chloride and the co-ion of the BGE at the detection window. For a sample with high concentration of chloride, a satisfactory electropherogram could be obtained with a smaller injection volume. Figure 3.5 shows an electropherogram of filtered seawater analyzed without any dilution.

Figure 3.4. The effect of chloride concentration on migration time of analytes. Peak 1: nitrite; 2: nitrate. The broad peak with sharp rear boundary is chloride. Experimental conditions: sample: 0.4 mg/L nitrite and nitrate in: (A) pure water, (B), 10 mM , (C) 50 mM , (D) 100 mM , (E) 200 mM NaCl. Detection wavelength:195 nm.
Figure 3.5. The electropherogram of undiluted seawater. Peak 1: bromide, 2: nitrate. Sample: filtered seawater, injection 50 mbar×60 s (74 nL), BGE: 0.1 M sodium phosphate, pH6.2, 0.15 M DDAPS. Other conditions as in Figure 3.3.

3.3.3 Current change during the sample self-stacking

Since the applied voltage was constant during the process of electrophoresis (-25 kV), the current was a function of the total conductivity of the electrolytes in the capillary. Figure 3.6B presents the current change during the electrophoresis.

From 0 to 0.8 min the current (absolute value) decreased from 79 to 77 μA. This was caused by the displacement of the original sample by the adjusted BGE according to the Kohlrausch regulating function (KRF). Since the mobility (denominator in KRF) of chloride was higher than that of dihydrogen phosphate, the adjusted BGE concentration was lower than that of sample.
The adjusted BGE zone was less conductive than the original sample. The total conductivity of electrolytes in the separation capillary decreased a little.

From 0.8 min to 3.7 min, the current increased from 77 to 87 μA. The EOF accounted for this increase. If there was no EOF, the current became constant after the adjusted BGE displaced the sample in the separation capillary according to the KRF in the electrophoresis. In this case, the suppressed EOF moved from the outlet to inlet, and was superimposed on the concentration adjustment process. The EOF pushed the newly formed BGE zone (less conductive) out of the capillary from the inlet slowly and sucked the BGE into capillary from the outlet vial. Therefore, the total conductivity of the electrolytes increased.

From 3.7 min to 7.35 min, the current decreased again from 87 μA to 78 μA and after 7.3 min the current remained constant. This was caused by the migration of chloride out of the capillary from the outlet. From the electropherogram at 195 nm, we know that at 3.45 min, the front edge of chloride passed the detection windows, at 3.70 min it migrated out of the capillary from the outlet. Then, the current started to decrease. It took 0.25 min for the front edge of chloride to migrate from the detection window to outlet (8.5 cm). The velocity of the chloride front edge during detection window pass-by to the outlet was 34 cm/min (8.5 cm/0.25 min). At 6.49 min, the sharp rear edge of the chloride passed by the detection window. At 7.35 min, all the chloride had migrated out of the capillary. After that, the capillary was filled entirely with the BGE and the current remained constant. It took the rear edge of the chloride 0.86 min to migrate from the detection window to the outlet at
an average velocity of 9.9 cm/min. The diffuse front edge migrates around 3.5-fold faster than the sharp rear boundary. The difference in the front and rear edge velocities was a characteristic of isotachophoresis due to the uneven electric field strength distribution.

![Graph showing electropherogram and current trace during sample self-stacking.](image)

Figure 3.6 The electropherogram (A) and the current trace (B) during sample self-stacking.

3.3.4 Determination of nitrate in seawater sample

The major anions in seawater of 35‰ salinity are chloride (ca. 0.56 M), sulfate (0.0114 M), bicarbonate (0.00143 M), and bromide (67 mg/L). The
minor anions are borate, fluoride and nitrite and nitrate at mg/L or μg/L levels according to the literature [23]. The chloride concentration in our sample was roughly 0.46 M measured by titration with silver nitrate. To overcome the peak height changes due to the possible chloride concentration variation in the sample, standard addition was used for nitrate quantitation. The sample was diluted 3.5-fold with water. 100, 200, 400 and 800 μg/L nitrate were spiked separately into the samples. Both peak areas (As) and heights (Hs) were linear with the concentrations (c). The calibration equations for areas and heights were 

\[ A = 0.0241c + 5.82 \quad (r^2=0.9983) \]

and 

\[ H = 0.0139c + 3.30 \quad (r^2=0.9997) \]

respectively. The standard deviations for the slopes of the calibration curves with areas and heights were \(1.13 \times 10^{-7}\) and \(0.22 \times 10^{-7}\), respectively, while for intercepts, they were \(19.2 \times 10^{-3}\) and \(3.7 \times 10^{-3}\), respectively. The determined concentration of nitrate in seawater sample was 0.60 mg/L. The relative standard deviations (RSDs, n=5) in term of migration time, peak area and height were 0.1%, 3.0%, 1.5% respectively. The limit of detection at S/N=3 was 35 μg/L, close to the naturally occurring concentration of nitrate in seawater [23], which was comparable with the results of terminating type sample self-stacking [10], but with a relatively low concentration BGE.

The mobility of bromide was also less than that of chloride in the presence of DDAPS in the BGE (data not shown). Thus, bromide in seawater could also be determined simultaneously with this method (as shown in Figure 3.5). The enrichment using sample self-stacking was not necessary due to the relatively high concentration of this anion in seawater, although sample self-stacking did occur in the present case.
3.4 CONCLUSION

Zwitterionic surfactant DDAPS was used to enlarge the mobility difference between chloride and nitrate, so that leading-type sample self-stacking could be employed to preconcentrate low concentration nitrate in seawater using native chloride in the sample as the leading ion, and the co-ion in the BGE as terminating ion. Thus, a highly conductive sample could be injected in a large volume with about 4-fold sensitivity enhancement compared to that of field amplification sample stacking in which nitrate was dissolved in pure water. The relative standard deviations (RSDs, n=5) of migration time, peak area, peak height were 0.1%, 3.0%, 1.5% respectively. A detection limit of nitrate of 35µg/L was achievable for seawater with relatively low concentration BGE. At the concentration of LOD, the mole ratio between the matrix and the analyte was around $10^6$:1. The overall procedure consisting of online preconcentration and separation was as simple as routine CZE except for a slightly longer sample injection time (3-4 minutes).
4.1 INTRODUCTION

4.1.1 Occurrence, toxicity and analysis of haloacetic acids

Haloacetic acids (HAAs) are disinfection byproducts (DBPs) formed in the process of chlorination of drinking water. Studies carried out in the 1970s revealed that chlorine can react with humic and fulvic acids to form a variety of potentially toxic organochlorines [183,184]. Organobromine compounds may be formed in bromide-containing raw water. HAAs constitute the second most prevalent group of known DBPs after trihalomethanes (THMs). Nine HAAs may be formed during chlorination, namely, monochloroacetic acid (MCAA), monobromoacetic acid (MBAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), bromochloroacetic acid (BCAA) and dibromoacetic acid (DBAA), tribromoacetic acid (TBAA), bromodichloroacetic acid (BDCAA) and dibromochloroacetic acid (DBCAA).

Epidemiological studies have suggested a weak association between drinking chlorinated water and the occurrence of bladder, rectal and colon cancer [185]. DCAA and TCAA, have been shown to be rodent herptocarcinogens [186-188]. DCAA is believed to be more potent than THMs [189]. Under the Stage I D/DBP Rule proposed by the US Environmental Protection Agency (EPA) in July 1994, the maximum contaminant level (MCL) for the sum of the concentration of five selected HAAs (MCAA, MBAA, DCAA,
DBAA and TCAA) was established to be 60 μg/L. It was suggested that this value would be reduced to 30 μg/L under the Stage II D/DBP Rule [190]. Quantitative target levels for haloacetic acids have also been set by the World Health Organization (WHO): 50 μg/L for DCAA and 100 μg/L for TCAA [191]. Thus, an accurate and sensitive analytical method is needed for their monitoring.

The USEPA publishes three analytical methods for HAAs in water samples, including EPA methods 552.1, 552.2 and 6251. In these methods, liquid-liquid extraction (LLE) or solid-phase extraction (SPE) was used for sample pretreatment. HAAs were determined with gas chromatography-electron capture detection (GC-ECD) after derivatization of the analytes with diazomethane or acid-alcohol to esters. Other analytical methods include gas chromatography-mass spectrometry (GC-MS) [192-195] and ion chromatography (IC) [196-198].

4.1.2 CE for HAAs analysis

CE is a good alternative technique for the determination of HAAs. Since HAAs are moderately strong acids, they exist in the ionic state across a wide range of pH and can be separated in CZE mode, providing high separation efficiency. On the other hand, its concentration sensitivity is generally lower than in HPLC or IC because only a small volume of sample (~nanoliter) may be loaded into the capillary. CE-MS has been applied to determine all nine HAAs, and detection limits between 0.3 and 7.6 μg/L for real water samples were achieved [199]. However, caution in the optimization of CE and MS
parameters was required in order to achieve sufficient resolution and high mass spectrometric sensitivity. Considering the cost of instrumentation, CE with indirect or direct UV detection may offer more practicality. When common optical detection was utilized, CE suffers from a reduced pathlength as compared to HPLC. Low detection sensitivity could be overcome by pre-concentration techniques (on-capillary and off-line enrichment).

The feasibility of CE analysis of HAAs with indirect UV detection has been demonstrated by Martinez et al. [193,200,201]. Electrokinetic injection was applied to pre-concentrate the HAAs on-capillary. Detection limits at low mg/L level were reported for spiked samples and analysis was completed within 8 minutes. However, this method was not successful when applied to real water samples due to matrix interferences. Offline sample pretreatment was still required to clean up the sample. The use of SPE for extraction was investigated but this usually required careful selection of sorbents. CE with direct UV detection has been applied, and determination of HAAs at 5 \( \mu \text{g/L} \) in tap water was achieved within 7 minutes, except for TCAA [202]. The HAAs were treated by liquid-liquid extraction and concentrated up to 2000 times. Pre-concentration by evaporation of solvent followed by reconstitution is, however, cumbersome and may contribute to sample losses. Nevertheless, both cases have demonstrated that the analysis time can be significantly reduced using CE and the tedious derivatization step can be avoided. As direct UV detection usually yields a more stable baseline than that of indirect UV detection [202], it is the most widely applicable detection mode for CE.
Although there are some attempts to determine HAAs using CE after sample pretreatment with LLE or SPE, the sensitivity has to be increased further to analyze real samples. The on-line pre-concentration and its combination with sample pretreatment provide a possibility for improving the sensitivity. In combining off-line and on-line pre-concentration methods, convenience is the main concern. For example, the analytes have to be dissolved in a low-concentration BGE using field amplification sample stacking, but the solubility of the analytes in the low BGE is limited.

The main objective of this study is to develop an on-line pre-concentration method that can be combined with the off-line pretreatment, for HAAs analysis. In our approach, the analytes are back-extracted into NaOH solution after liquid-liquid extraction. The extract is injected in a large volume into the CE system with a low-pH BGE. Sample stacking occurs at the interface between the sample zone and the BGE. The sample stacking mechanism is investigated.

4.2 EXPERIMENTAL

4.2.1 Instrument and procedures

Experiments were performed on the HP 3D CE system. Detection wavelength was set at 195 nm. The column was a bare fused-silica capillary of 64.5 cm of length, and 50 µm internal diameter with a detection window made by burning off a small part of the polyimide coating 8.5 cm from the outlet of the column. The sample was hydrodynamically injected into the capillary. A 1-second injection at 50 mbar was 0.067 cm in length. For conventional injection,
the sample was injected hydrodynamically for 4 seconds at 50 mbar, corresponding to 0.27 cm in length. In sample stacking, the sample dissolved in NaOH solution was injected for a prescribed time as noted below at a pressure of 50 mbar. The temperature of the capillary was maintained at 20ºC. Negative voltage (-25 kV, cathode at the inlet) was used for the anionic analytes since the EOF was suppressed with the low-pH BGE or with a zwitterionic surfactant.

4.2.2 Chemicals

The HAAs standards used were monochloroacetic acid (MCAA), monobromoacetic acid (MBAA), dichloroacetic acid (DCAA), dibromoacetic (DBAA), bromochloroacetic acid (BCAA), trichloroacetic acid (TCAA) and tribromoacetic acid (TBAA), purchased from Aldrich (Milwaukee, WI, USA) with purity higher than 95%. The structures and pKₐ values are listed in Table 4.1. Individual standard solutions of 10 g/L of each compound were prepared with ultrapure water. The BGE was composed of 0.1 M phosphoric acid, of which pH was adjusted with NaOH solution.

4.2.3 Off-line sample pretreatment

A solvent microextraction method was used to enrich the HAAs in tap water sample [203]. Briefly, a 30-mL sample was adjusted to pH <0.5 with conc. H₂SO₄. 3 mL MTBE, 3 gm CuSO₄·5H₂O and 12 gm anhydrous sodium sulfate were added to the sample. After 2 min extraction, exactly 1 mL of the MTBE extract was transferred to a 1.5-mL centrifuge tube, and 50 μL NaOH solution was used to back-extract the HAAs from the organic phase to the aqueous
phase. ~25 μL NaOH solution was transferred to a CE sample vial with a 100-μL microsyringe for analysis.

Table 4-1 Structures of Haloacetic Acids and Their pKₐ Values

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<th>Substitution Number</th>
<th>Compounds and pKₐ</th>
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<tr>
<td>0</td>
<td>CH₃COOH (AA) pKₐ=4.76</td>
</tr>
<tr>
<td></td>
<td>CH₂BrCOOH (MBAA) pKₐ=2.87</td>
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<td>CBr₃COOH (TBAA)</td>
</tr>
<tr>
<td>3</td>
<td>CCl₃COOH (TCAA) pKₐ=0.70</td>
</tr>
</tbody>
</table>

4.3 RESULTS AND DISCUSSION

4.3.1 Separation of HAAs

In previous attempts to determine HAAs in drinking water with CE, complete resolution could not be obtained due to the similarity of the structures and molecular weights of these compounds, or indirect UV detection had to be used for their lack of chromophoric groups. The HAAs absorb UV radiation at low wavelength with low extinction coefficients due to the substitution of...
chlorine and bromine. With a UV-transparent phosphate BGE, however, we could determine them with direct UV detection in this work.

In CE, the apparent electrophoretic mobility ($\mu_{\text{app}}$) of an ion was the vector sum of its electrophoretic ($\mu_{\text{ele}}$) and EOF mobilities ($\mu_{\text{eof}}$).

$$\mu_{\text{app}} = \mu_{\text{ele}} + \mu_{\text{eof}} \quad (4-1)$$

The EOF was from the anode to the cathode for a bare fused silica capillary. The $\mu_{\text{ele}}$ of anionic species was the opposite direction to that of the $\mu_{\text{eof}}$.

$$\mu_{\text{app}} = \mu_{\text{ele}} - \mu_{\text{eof}} \quad (4-2)$$

where both $\mu_{\text{ele}}$ and $\mu_{\text{eof}}$ were dependent on the pH of the BGE. The results are shown in Figure 4-1. For bare fused silica capillary, the $\mu_{\text{eof}}$ maintains a constant low value when the pH of the BGE was lower than pH 4.0. The $\mu_{\text{eof}}$ increased dramatically due to higher dissociation degree of silanol groups on the surface of the capillary when the BGE pH was increased from 4 to 5.8.

HAAs are weak acids with $pK_a$ values from 0.6 to 2.9. Their effective electrophoretic mobilities are dependent on the pH of the BGE according to

$$\mu_{\text{HA,eff}} = \frac{K_{\text{HA}}}{[H^+] + K_{\text{HA}}} \mu_A \quad (4-3)$$

where $\mu_{\text{HA,eff}}$, $\mu_A$ are the effective electrophoretic mobility of weak acid HA and electrophoretic mobility of its anionic form $A^-$, and $K_{\text{HA}}$ and $[H^+]$ are the ionization constant of HA and the proton concentration in the BGE, respectively. The $pK_a$ values of multi-substituted HAAs have lower values. They dissociated almost completely in the pH range of 2.4-5.8 with small
variances in their effective mobilities, as shown in Figure 4.1, but the small change of their mobilities in the low pH range was critical for the

![Graph showing the effect of BGE pH on mobility and EOF](image)

**Figure 4.1** The effect of the BGE pH on the mobility of analytes and EOF. BGE: 0.1 M H₃PO₄, the pH was adjusted with NaOH.

electrophoretic resolution. When the pH was higher than 3.4, DBAA and TCAA could not be resolved. Therefore, a lower pH BGE was preferred for the separation. Figure 4.2 shows the electropherogram of 100 mg/L standards with 0.1 M phosphate (pH 2.9) as the BGE. Due to the higher pKₐ values (about 2.9) for the mono-substituted HAAs (MCAA and MBAA), their effective mobilities were lower than the multi-substituted ones due to less dissociation, resulting in long migration times.
Figure 4.2 Electropherogram of HAA standards. BGE: 0.1 M sodium phosphate, pH 2.9. Sample: 100 ppm of each standard in pure water. Injection: 50mbar×4s. Capillary length: 64.5 cm (56 cm effective length). Voltage: -25 kV. Peak identification: 1. DCAA, 2. BCAA, 3. TCAA, 4. DBAA, 5. TBAA, 6. MCAA, 7. MBAA.

Organic solvents were often used for improving the selectivity of CZE due to changes in the solvation volume or dissociation constants (pKₐ), which in turn altered the effective charge-to-radius ratio of the anionic solutes [204]. In this experiment, it was found 2-propanol in the BGE did not improve the resolution of the analytes. In the presence of 20% 2-propanol in the BGE, DBAA co-migrated with the TCAA when they passed the detection window.

Zwitterionic surfactant was another type of additive used in the BGE for suppressing the EOF and improving the separation selectively due to its selective interaction with anionic analytes [205,206]. We examined the effect of 3-(N,N-dimethyldecyl-ammonio) propane sulfonate (DDAPS) on the separation of HAAs with pH 5.8 phosphate BGE. The results are shown in
Figure 4.3. The EOF decreased dramatically when the concentration of DDAPS varied from 0 mM to 4 mM, and remains constant in the presence of 4 to 10 mM DDAPS. This was in agreement with the results of Yeung and Lucy [207]. Since the critical micelle concentration (CMC) of DDAPS was around 3 mM, its EOF suppression effect was proposed to be related to the formation of hemi-micelles on the surface of the capillary tubing.

![Graph](image-url)

Figure 4.3 Effect of zwitterionic surfactant on mobility of EOF and HAAs. BGE: 0.1 M sodium phosphate, pH 5.8 with various concentrations of DDAPS.

The DDAPS also affected the mobility of HAAs, as shown in Figure 4.3. When DDAPS concentration was lower than its CMC value, it hardly influenced the migration of HAAs. From 0 to 4 mM of DDAPS, the mobility of HAAs increased slightly possibly due to the viscosity change induced by the
presence of DDAPS. When the surfactant concentration was increased from 4 to 10 mM, the mobility of HAAs was reduced, and was linear with the concentration of DDAPS in the range of 4 to 10 mM.

According to the literature [206], an association between an anion (X⁻) and the zwitterionic surfactant micelle (M) can be described by

$$X^- + M = XM^- \quad (4-4)$$

Where $XM^-$ is the anion-micelle associate. The association constant $K_{ass}$ is defined as

$$K_{ass} = \frac{[XM^-]}{[X^-][M]} \quad (4-5)$$

where $[X^-]$, $[M]$, and $[XM^-]$ are the concentrations of the anion, the micelle, and the anion-micelle associate, respectively. The micellar concentration ($[M]$) was calculated using

$$[M] = \frac{\text{total concentration of surfactant}}{\text{aggregation number}} - \text{CMC} \quad (4-6)$$

for DDAPS, the CMC and the aggregation number is 3 mM and 59 respectively. The association constant can be determined using Benesi-Hildebrand (B-H) plot.

$$\frac{1}{(1/\mu_a) - (1/\mu_{XM})} = \frac{K_{ass}[M]}{(1/\mu_X) - (1/\mu_{XM})} + \frac{1}{(1/\mu_X) - (1/\mu_{XM})} \quad (4-7)$$

where $\mu_a$, $\mu_{XM}$, $\mu_X$ are the measured effective electrophoretic mobility, and the electrophoretic mobility of the anion-micelle associate and the analyte anion, respectively. For the first approximation and considering the effect of the ionic strength, $\mu_{XM}$ was assumed to be $2.8\times10^{-5}$ cm²/Vs for all HAAs since the radius of anion-micelle associate was predominantly determined by the micelle.
The $K_{\text{ass}}$ values are listed in Table 4-2. The association constants increased as more hydrogen atoms were substituted by the halogen atoms. According to the results for inorganic anions in electrostatic ion chromatography (EIC) and CE, the molecular polarizability was one of the most important parameters that dictated the interaction between the anions and micelles. The halogen-substitutions made acetate more polarizable due to the larger molecular volume and the inductive effect of halogen atoms. Therefore, the association constant was larger for multi-substituted HAAs.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MCAA</th>
<th>MBAA</th>
<th>DCAA</th>
<th>BCAA</th>
<th>DBAA</th>
<th>TCAA</th>
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<tr>
<td>$K_{\text{ass}}$</td>
<td>8.3</td>
<td>11.1</td>
<td>37.8</td>
<td>49.6</td>
<td>68.6</td>
<td>349.2</td>
</tr>
</tbody>
</table>

Figure 4.4 shows a typical electropherogram of HAAs in pH 5.8 phosphate BGE with 4 mM DDAPS. When the DDAPS concentration was increased to 6 mM, tailing peak was obtained for TCAA due to electrodispersion resulting from a larger mobility difference between TCAA and the co-ion ($\text{H}_2\text{PO}_4^-$) of the BGE.

Although good resolution could be obtained for HAAs with either the low pH BGE or with the BGE comprising zwitterionic surfactant DDAPS, the sensitivity was not good enough for the real drinking water sample in which HAAs occurred at ppb (part per billion) levels as disinfection by-products. Further enrichment was an obvious requirement for dealing with real samples.
Figure 4.4 Electropherogram of HAAs in pH 5.8 0.1 M sodium phosphate BGE in presence of 4 mM DDAPS. Peak identification: 1. MCAA; 2. MBAA; 3. DCAA; 4. BCAA; 5. DBAA; 6. TCAA.

4.3.2 Large volume sample stacking

LVSS was the first choice to improve the sensitivity of CE due to its easy implementation compared to off-line enrichment. Since the EOF was suppressed with a low pH BGE or zwitterionic surfactant DDAPS in this experiment, LVSS with EOF pumping was adopted for the on-line concentration according to He and Lee [141]. Samples dissolved in pure water or diluted BGE were injected into the CE system in large volumes. The analytes were stacked at the boundary between the BGE and the sample due to field amplification. The stacking effect for sample dissolved in different concentrations of BGE is shown in Figure 4.5. For a sample dissolved in pure
water or 1 percent of BGE, the sensitivity was improved ~20-fold compared to the conventional injection of 50mbar×4s. When the sample was dissolved in 5% of BGE, although there was an observable sensitivity enhancement, the resolution was worsened by peak broadening.

4.3.3 NaOH Effects in Large Volume Sample Stacking

4.3.3.1 BGE of pH 2.9

From the above results, we know that even with on-line pre-concentration, sensitivity still needs to be improved further to deal with real samples. Obviously, offline sample pretreatment can help lower the limits of detection. Acidic compounds usually have considerable solubility in alkaline solutions, which can be used to separate them from neutral compounds in sample pretreatment. In EPA method 552.1, HAAs were extracted and pre-concentrated into MTBE with LLE. We studied the possibility for stacking the sample dissolved in alkaline solutions, since HAAs can be easily stripped from the organic solvent such as MTBE into an aqueous NaOH solution.

Since the EOF was suppressed, anionic analytes could migrate to the detection windows under negative voltage (cathode at the inlet). No polarity switching was involved in the stacking process. Sample was injected as in conventional CE operation but with a longer injection time (e.g. 20% of capillary volume). The presence of NaOH in the sample could improve the sample stacking performance. Figure 4.6 presents the electropherograms with
Figure 4.5 Large volume sample stacking of HAAs with EOF pumping. BGE: 0.1 M phosphate, pH 2.9; sample: 1 ppm HAAs in (A). pure water; (B). 1% BGE; (C). 5% BGE. injection: 50 mbar*200s. voltage: -25 kV. peak identification: 1. DCAA; 2. BCAA; 3. TCAA; 4. DBAA; 5. MCAA; 6. MBAA.

different NaOH concentrations in the sample. The peak heights increased with the concentrations of NaOH from 1 to 100 mM in the samples. Further increasing NaOH concentration to 0.15 M produced overloading, and no peaks could be observed, this observation is explained below in the stacking mechanism section. This sample stacking scheme resembled LVSS without polarity switching, and the performance was better than LVSS due to the presence of NaOH in the sample. The sensitivity in terms of peak height could be improved by about 4-fold in the presence of 0.1 M NaOH compared to the case when the sample was dissolved in pure water. Comparing with conventional injection (4 seconds at 50 mbar), the sensitivity could be
enhanced about 60-fold when the sample was dissolved in 0.1 M NaOH with injection time of 200 seconds at 50 mbar (~20% of the capillary volume). The effect of NaOH concentration on the peak heights of HAAs is summarized in Figure 4.7. Since with 100 mM NaOH in the sample, BCAA, DBAA and TCAA could not be completely resolved, their values are not included in Figure 4.7. Although NaOH in the sample could increase the sensitivity in terms of peak heights, the peak areas did not vary significantly. The relative standard deviations of peak areas with different NaOH concentrations (0, 1, 5, 10, 25, 50 mM) in the samples were less than 10%. The calibration and quantitation based on peak areas were more reliable than these based on peak heights.

4.3.3.2 BGE of pH 5.8 in the presence of DDAPS

The effects of NaOH on LVSS with higher pH BGE were also investigated. With a phosphate BGE at pH 5.8, and 4 mM DDAPS used to suppress EOF, the anionic analytes could migrate to the detection window while the EOF pushes the sample matrix out of the capillary from the inlet. Figure 4.8 shows the HAAs electropherograms with different concentrations of NaOH. NaOH reacted with the co-ion of the BGE (H$_2$PO$_4^-$) to produce a broad fronting peak (HPO$_4^{2-}$) with a sharp rear boundary in front of the HAAs peaks (Figure 4.8A). When 5 mM NaOH was present in the sample, all HAA peaks could be separated from the leading HPO$_4^{2-}$. Compared with 50mbar×4s injection, ~60-fold sensitivity enhancements were obtained for all HAAs. When the concentration of NaOH was increased to 10 mM, the first two peaks could not
Figure 4.6 Electropherograms of HAAs in different concentration NaOH with low pH BGE. BGE: 0.1 M sodium phosphate, pH 2.9. Sample: 1 mg/L HAAs in: A. 0 mM; B. 1 mM; C. 5 mM; D. 10 mM; E. 25 mM; F. 50 mM; G. 100 mM; H. 150 mM NaOH. Injection: 50 mbar*300s. Voltage: -25 kV. Peak identification as in Figure 4.1.
Figure 4.7 NaOH concentration effects on peak heights. The conditions are as in Figure 4.6.

be separated from the sharp rear boundary of $\text{HPO}_4^{2-}$. For sample with 20 mM NaOH, only the last two peaks were observed close to the sharp rear boundary of $\text{HPO}_4^{2-}$. With the BGE of pH 5.8 in the presence of DDAPS, better resolution was observed for HAAs, but the concentration of NaOH that could be tolerated without any deterioration of separation was much lower than that of the BGE at pH 2.9.
Figure 4.8 Electropherograms of HAAs in different concentration of NaOH with pH 5.8 BGE. BGE: 0.1 M sodium phosphate, 4 mM DDAPS, pH 5.8. Sample: 1 mg/L HAAs in A. 5 mM; B. 10 mM; C. 20 mM NaOH. Injection: 50 mbar*200s. Voltage: -25 kV. Peak identification: 1. MCAA; 2. MBAA; 3. DCAA; 4. BCAA; 5. DBAA; 6. TCAA.

4.3.3.3 Optimization of the BGE for sample stacking.

From the above discussions, we know that in the BGE of pH 2.9, high concentration NaOH in the sample could provide better sample stacking performance, but the resolution was sacrificed, while in the BGE of pH 5.8 with DDAPS, better resolution was obtained, but the applicable NaOH concentration was limited to 5 mM. With the BGE of pH 2.9, the mobility of TCAA was faster than that of DBAA (Figure 4.1), if 4 mM DDAPS was added into the BGE, TCAA and DBAA co-migrated. If the pH value of the BGE was increased to 3.0, 4 mM DDAPS could help separate TCAA and DBAA. Under this condition, the mobility of TCAA was slower than that of DBAA, and 100
mM NaOH in the sample would produce overloading. Therefore, the maximum NaOH concentration in the sample should be 75 mM. The following experiments were performed with 0.1 M sodium phosphate at pH 3.0 in the present of 4 mM DDAPS.

4.3.4 Effect of injection volume

In standard CE operation, if the sample is introduced as a rectangular pulse of length L, then the variance of the sample injection, $\sigma_i^2$, can be expressed by the relationship

$$\sigma_i^2 = \frac{L^2}{12} \quad (4-8)$$

For suppressing the dispersion due to the injection, the initial length of the sample zone should be minimized [33], which is one of the reasons for the low concentration sensitivity of CE. We investigated the effects of the injection volume on the peak heights, areas, and widths in this scheme. Figure 4.9 shows the relative values normalized according to those of the smallest injection volume. The peak areas were linear with the injection volume in the range of 50-250 seconds injection time at 50 mbar (5-25% capillary volume). With further increase of the injection volume, complete separation of HAAs could not be obtained due to shorter separation length since a significant part of the capillary was occupied by the sample plug. Peak heights were not linear with the injection volumes, but increased faster. The peak widths became narrower with larger injection volumes which were in contrast with the results
of LVSS. This difference implies that the sample focusing mechanism may be different between these two sample stacking techniques.

![Graph showing the effect of injection volume on peak areas, heights, and widths of DCAA. The values are relative to the values with 50 s injection time. Sample: 1 mg/L DCAA in 0.1 M NaOH, other conditions as in Figure 4.6.]

4.3.5 Sample stacking mechanism

4.3.5.1 Current changes during sample stacking

In LVSS, the current increased steadily from a low value to constant value as the sample of low conductivity was pumped out of capillary by the EOF, as shown in Figure 4.10A. When NaOH was present in sample matrix, the current decreased sharply at first followed by a slow increase to a maximum value, and then decreased slowly again to a constant value, as shown in Figure 4.10B.
Figure 4.10 The electric current changes in the absence (A) and the presence (B) of NaOH during sample stacking. Sample: 1 mg/L HAAs in: A. 0 mM; B. 75 mM NaOH. The other conditions as Figure 4.2.

Since a constant voltage was applied in the experiment, the current was an indicator of overall conductivity of the solutions in the capillary according to Ohm’s Law. The composition changes in the capillary during sample stacking was illustrated in Figure 4.11 and verified in Figure 4.12. At the very beginning, there were two segments of solutions in the capillary (Figure 4.11B), one was the low pH BGE and the other is the NaOH solution (the contribution of analytes to the current was negligible). Both segments were highly conductive, so the initial current was large (60 μA), as shown in Figure 4.10B.
The current decreasing during the first 0.8 min came from the low conductivity BGE zone formed in the original position of the sample according to the Kohlrausch regulating function (KRF) [54, 154]:

\[
c_{\text{H}_2\text{PO}_4^{-}, A} = c_{\text{OH}^{-}} \frac{\mu_{\text{H}_2\text{PO}_4^{-}} \ast (\mu_{\text{OH}^{-}} + \mu_{\text{Na}^+})}{\mu_{\text{OH}^{-}} \ast (\mu_{\text{H}_2\text{PO}_4^{-}} + \mu_{\text{Na}^+})}
\]

(4-9)

where \(c_{\text{H}_2\text{PO}_4^{-}, A}\) and \(c_{\text{OH}^{-}}\) are the adjusted concentration of \(\text{H}_2\text{PO}_4^{-}\) and the concentration of \(\text{OH}^{-}\) in the sample, respectively; \(\mu_{\text{H}_2\text{PO}_4^{-}}, \mu_{\text{OH}^{-}}\) and \(\mu_{\text{Na}^+}\) are respective mobilities of \(\text{H}_2\text{PO}_4^{-}\), \(\text{OH}^{-}\) and the counter-ion, \(\text{Na}^+\). Although equation 4-9 cannot be used for accurate calculation, it can predict the appearance of a low conductivity zone in the original sample zone since the mobility of \(\text{OH}^{-}\) is much faster than that of \(\text{H}_2\text{PO}_4^{-}\). This low conductivity of adjusted BGE zone dominated the total conductivity of the solutions in the capillary, although there was a newly formed high conductivity zone due to the reaction of \(\text{OH}^{-}\) and the co-ion (\(\text{H}_2\text{PO}_4^{-}\)) of the BGE. Therefore the current decreased after the application of the voltage.

In the above discussion, the EOF was not considered for simplification. In fact, the EOF was initiated after applying voltage, moving in the direction from the outlet to the inlet (Figure 4.11C-E). It pushed the newly formed low-conductivity BGE zone out of the capillary from the inlet, resulting in an increase in total conductivity of the solutions in the capillary. Thus, the current increased slowly after 0.8 min. When the low conductivity BGE zone was
Figure 4.11. Schematic illustration of composition changes in the capillary during sample stacking. **A.** Capillary filled with the BGE before injection; **B.** Sample is hydrodynamically injected into capillary in a large volume. After injection, both ends of capillary were immersed into the BGE; **C.** High voltage was applied, OH\(^-\) migrated to anode and reacted with H\(_2\)PO\(_4\)\(^-\) of BGE to form a high conductivity zone. In the original sample zone, adjusted BGE zone was formed according to KRF. The EOF started to push the newly-formed adjusted BGE zone out of capillary from the inlet. **D.** The adjusted BGE zone is partially (pushed out of capillary from the inlet. **E.** The adjusted BGE zone was completely pushed out of capillary from the inlet. The high conductivity zone was almost consumed by the H\(^+\) in the BGE.
removed completely, the current reached the maximum value at 5.4 min, as shown in Figure 4.10B and illustrated in Figure 4.11D. From onset of the application of voltage, \( \text{OH}^- \) reacted with \( \text{H}^+ \) and \( \text{H}_2\text{PO}_4^- \) at the front boundary as it migrated forward. A higher pH phosphate zone was formed in front of the original sample zone (Figure 4.11C), which was verified by the strong absorbance as shown in Figure 4.12B. This high pH phosphate zone was more conductive and reactive with \( \text{H}^+ \) in low pH BGE. Its length would be reduced as the reaction proceeded, as shown in Figure 4.12B-F, resulting in a decrease in the current. When the higher pH phosphate zone disappeared, the current reached a stable value which was when the entire capillary was filled with the original BGE.

4.3.5.2 Sample stacking mechanism

It was obvious that this sample stacking mechanism was different from the reported methods such as LVSS and dynamic pH junction method, although it was very similar to the former (LVSS) except that NaOH was present in the sample matrix. In LVSS, the sample stacking came from the uneven electric field distribution (field amplification). In this method, the sample zone was initially more conductive than the BGE zone due to the presence of a high concentration of NaOH; no straightforward field amplification occurred. The \( pK_a \) values of multi-substituted HAAs were at least one unit lower than the pH of the BGE, and were almost completely dissociated in both the BGE and sample. Therefore, the contribution of effective mobility changes to the sample stacking was negligible, although there was a pH difference between the
sample and BGE (pH ~13.0 and 3.0 respectively). This was different from sample stacking with the dynamic pH junction to focus weakly acidic compounds [163,164,208].

To investigate the mechanism of sample stacking, we monitored the concentration profile of one analyte (DBAA) during sample stacking using the following method: at some time-points of the electrophoresis, the high voltage was aborted, and the sample zone was pushed with the BGE from the inlet to the detection window at a pressure of 50 mbar. The absorbance was recorded. Since both OH\(^-\) and phosphates at higher pH (HPO\(_4^{2-}\) or PO\(_4^{3-}\)) absorbed UV light at low wavelength, we could also monitor the OH\(^-\) concentration profile and the evolution of the formed high pH phosphate zone during sample stacking.

Since the mobility of DBAA was between those of OH\(^-\) and the co-ion of the BGE (H\(_2\)PO\(_4^{-}\)), DBAA could be isotachophoretically stacked at the rear boundary of the OH\(^-\). This was substantiated by its concentration profile as shown in Figure 4.12B. At the same time, the OH\(^-\) reacted rapidly with H\(_2\)PO\(_4^{-}\) in the BGE to form HPO\(_4^{2-}\) or PO\(_4^{3-}\). After undergoing electrophoresis for 3.3 min, the strong absorbance due to OH\(^-\) or PO\(_4^{3-}\) disappeared, as shown in Figure 4.12B-D. Due to the short life time of OH\(^-\) in the low pH BGE and to the large mobility difference between OH\(^-\) and DBAA, the transient isotachophoresis time for stacking DBAA was very short, according to [154]:

\[
 t_{ITP} = \frac{10 \cdot k_s \cdot (\mu_{OH} - \mu_{H_2PO_4})}{\overline{i} \cdot (\mu_{OH} - \mu_{DBAA})^2} \]  

(4-10)
Figure 4.12. Monitoring the DBAA concentration profile in the process of electrophoresis. The graphs were obtained by pushing the sample zone to the detection window after applying voltage for (A). 0 min, (B). 1.3 min, (C). 2.3 min, (D). 3.3 min, (E). 5.4 min, (F). 7.4 min. BGE: 0.1 M sodium phosphate, pH 3.0, 4 mM DDAPS. Sample: 50 mg/L DBAA in 75 mM NaOH. Peak identification: (1). DBAA. (2) formed HPO$_4^{2-}$ zone

where $l_0$, $k_s$, $i$ are sample length, electric conductivity of sample zone and current density, respectively; $\mu_{OH}$, $\mu_{H_2PO_4}$, and $\mu_{DBAA}$ are the mobility of OH$^-$, H$_2$PO$_4^-$ and DBAA, respectively. Therefore, this first tITP lasted for a very short period of time.

The second ITP occurred at the same time when DBAA left the sharp boundary between OH$^-$ and co-ion of the BGE, since the mobility of HPO$_4^{2-}$ or PO$_4^{3-}$ (which are possible reaction products of OH$^-$ and H$_2$PO$_4^-$) was faster
than that of DBAA. The tITP conditions were valid with HPO$_4^{2-}$ or PO$_4^{3-}$ as leading ion and H$_2$PO$_4^-$ as terminating ion. This was confirmed by the Figure 4.2D-F, the DBAA was concentrated at the sharp rear boundary of a high absorbance zone. This high absorbance zone should be HPO$_4^{2-}$ or PO$_4^{3-}$ since it was a reaction product of NaOH and H$_2$PO$_4^-$. As the electrophoresis proceeds, the HPO$_4^{2-}$ or PO$_4^{3-}$ reacts with H$^+$ or H$_2$PO$_4^-$ at the front boundary, which was confirmed by the narrowing of this high absorbance zone, as shown in Figure 4.12D-F. When this high absorbance zone was consumed completely, the analytes were de-stacked and separated in CZE mode. It is noted that although the mobility of HPO$_4^{2-}$ or PO$_4^{3-}$ was faster than the co-ion of the BGE, both its front and rear boundaries were sharp, as shown in Figure 4.12D-F, and no diffuse front boundary was observed. This is different from the diffuse front boundary in tITP with a non-reactive leading ion such as Cl$^-$ (see chapter 3). The difference might come from the reaction occurring at front boundary as depicted by Cao et al [162]. When higher pH phosphate BGE (pH 5.8) was used, a diffuse front boundary was observed (Figure 4.8), similar to the results reported by Boden and Bachmann [154].

In the second tITP, the amount of leading ions (HPO$_4^{2-}$ or PO$_4^{3-}$) was dependent on the amount of NaOH in the sample matrix, which was defined by the concentration of NaOH and the sample injection volume. If the amount of NaOH was too large, the second tITP persisted when the analyte passed by the detection window; the analytes did not leave the sharp boundary of leading ion to produce individual peaks. This accounted for the overloading effect when the NaOH concentration was increased to 150 mM, where no peaks was
observed, as shown in Figure 4.5E, and the insufficient resolution with a large injection volume. In addition, the persistence time of the second tITP was also dependent on the pH of the BGE, since a higher concentration $H^+$ titrated the formed high pH phosphate zone at a faster rate. Therefore, a higher NaOH concentration in sample and larger injection volume could be used with a lower pH BGE.

From the above discussion, we could conclude that the sample stacking came from dual tITP. $OH^-$ was the leading ion in the first tITP, and its reaction product with $H_2PO_4^-$ in the BGE, $HPO_4^{2-}$ or $PO_4^{3-}$ was the leading ion in the second tITP. $H_2PO_4^-$ in the BGE was the terminating ion in both cases. This was confirmed by further experiments with maleic acid (MA) as an analyte due to its faster mobility than DBAA. The results are shown in Figure 4.13 and Figure 4.14. When MA was dissolved in 75 mM NaOH solution with an injection length of 13.3 cm, the sensitivity could be enhanced 19-fold, while for DBAA, 90-fold enhancement was obtained, as shown in Figure 4.13A and 4.13B, compared with a conventional injection (0.27 cm). We carried out the MA concentration profile monitoring experiments as depicted above for DBAA, as shown in Figure 4.14. In the first tITP, MA behaved similarly with DBAA since the mobilities of both of them were between $OH^-$ and $H_2PO_4^-$. If the mobility of MA was faster than $HPO_4^{2-}$, the tITP condition was not valid for MA after the first tITP, and no second tITP pre-concentration took place. MA migrated in the CZE mode in the formed $HPO_4^{2-}$ zone first, as shown in Figure 4.14D. Since the mobility of $MA^{2-}$ was faster than that of $HPO_4^{2-}$, it continued its CZE migration in the low pH BGE as $MA^-$ (due to pH change of the media)
after leaving the formed HPO$_4^{2-}$ zone, as shown in Figure 4.14F. Comparing the limiting ion mobilities of MA$^{2-}$ (64.14×10$^{-5}$ cm$^2$/Vs), HPO$_4^{2-}$ (59.07×10$^{-5}$ cm$^2$/Vs), PO$_4^{3-}$ (96.16×10$^{-5}$ cm$^2$/Vs), we could conclude that the main anion is HPO$_4^{2-}$ in the formed high absorbance zone.

4.3.6 Method Validation

We validated the method under stacking conditions with a constant injection volume (50mbar×200 s). As shown in Table 4-3, peak areas were linear with concentrations in the range of 0.05 mg/L to 5 mg/L. The peak heights were also linear with concentrations, but within a narrower range from 0.05 mg/L to 2 mg/L (results not shown). The relative standard deviations (RSDs, n=6) of the migration times, peak area and heights are less than 1.8%, 6.0% and 8.0%, respectively. No irreproducibility was observed due to the discontinuous BGE with sharp difference of pH value. The limits of detection (LODs) at S/N=3 without off-line sample pre-concentration, for the multi-substituted HAAs, were ~0.06 mg/L. For the mono-substituted HAAs, they were ~0.2 mg/L. The results are summarized in Table 4-3.

4.3.7 Real Sample Analysis

The tap water sample was collected in our laboratory. After extraction with MTBE, HAAs were back-extracted into 75 mM NaOH with a total theoretical concentration factor of 200. The extract was injected into the CE system for 200 seconds at 50 mbar to determine the HAAs. A typical electropherogram is shown in Figure 4.15. The analytes were identified by comparing the migration
Figure 4.13. Comparison of conventional injection (A) and sample stacking effect with NaOH in the sample (B). BGE: 0.1 M phosphate, 4 mM DDAPS, pH 3.0. Sample: (A). 100 mg/L MA and DBAA in water, injection: 50mbar×4s; (B). 10 mg/L MA and DBAA in 75 mM NaOH, 50mbar×200s. Peak identification: 1. MA; 2. DBAA.

time with spiked individual standards. Only DCAA and TCAA were detected in the tap water sample at concentrations of 3.2 and 6.6 μg/L, respectively. The other HAAs were not detected. With the combination of off-line preconcentration and on-line enrichment, the LODs were ~0.4 μg/L for multi-substituted HAAs, and ~1 μg/L for MCAA and MBAA. These values are far below the maximum contaminant levels stipulated by the EPA (60 ug/L for five HAAs) for drinking water. To the best of our knowledge, these are the lowest LODs for HAAs using CE with direct UV detection. Although the method is not as sensitive as GC with electron capture detection (ECD), its sensitivity is sufficient for rapid and convenient screening of HAAs in drinking water.
Figure 4.14. Monitoring MA concentration profile in the process of electrophoresis. (1). MA, (2). The formed HPO$_4^{2-}$ zone. The other conditions as Figure 4.12.

Figure 4.15 Electropherogram of HAAs extracted from tap water and analyzed using the described CE procedure. Peak identification: 1. DCAA, 2. TCAA, u. unknowns from reagent blank. Injection: 50mbar*200s.
Table 4-3 The linearity of calibration curve and precisions

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<td>12.16</td>
<td>0.0936</td>
<td>0.9998</td>
<td>0.9</td>
<td>0.4</td>
<td>5.6</td>
</tr>
<tr>
<td>TCAA</td>
<td>0.05-5.0</td>
<td>11.73</td>
<td>0.1312</td>
<td>0.9998</td>
<td>1</td>
<td>0.4</td>
<td>5.6</td>
</tr>
<tr>
<td>MCAA</td>
<td>0.1-5.0</td>
<td>4.80</td>
<td>0.0626</td>
<td>0.9993</td>
<td>1.6</td>
<td>4.3</td>
<td>4.2</td>
</tr>
<tr>
<td>MBAA</td>
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<td>7.94</td>
<td>0.6034</td>
<td>0.9991</td>
<td>1.8</td>
<td>5.8</td>
<td>7.9</td>
</tr>
</tbody>
</table>
4.4 CONCLUSION

A new on-line sample preconcentration method for haloacetic acids (HAAs) dissolved in sodium hydroxide solution was developed in capillary zone electrophoresis (CZE) with low-pH BGE (background electrolyte, pH 2.9 phosphate). The sample in NaOH was hydrodynamically injected into the capillary in a large volume. The analytes could be compressed into a narrow zone, thereby increasing detection sensitivity. The sample enrichment performance was better than large volume sample stacking in which the sample was dissolved in pure water. Compared with conventional injection (~1% of capillary), 60-fold sensitivity enhancement was obtained without deterioration of separation with an injection amount equal to 20% of the capillary volume. The stacking mechanism is proposed to be a dual transient isotachophoresis. OH⁻ was the leading ion in the first tITP, and its reaction product with H₂PO₄⁻ in the BGE, HPO₄²⁻ or PO₄³⁻ was the leading ion in the second tITP. H₂PO₄⁻ in the BGE was the terminating ion in both cases. Since the pH of the BGE was low (pH 3.0), the leading ions (the OH⁻ and HPO₄²⁻) would be transformed into H₂PO₄⁻. The analytes migrated in the BGE and was separated in CZE mode. This method was applied to determine HAAs in drinking water by combination with liquid-liquid extraction followed by back-extraction into NaOH solution. Sub-ppb level limits of detection were obtained for real samples with direct UV detection, providing a reliable approach for the rapid and convenient screening of HAAs in drinking water.
5.1 INTRODUCTION

In Chapter 4, the on-line pre-concentration of HAAs with OH\textsuperscript{-}-induced dual transient isotachophoresis with a low pH BGE was discussed. After sample stacking, OH\textsuperscript{-} was neutralized by H\textsuperscript{+} in the BGE. The sample stacking performance was better than field amplification sample stacking. This method was limited to moderately weak acids since a low pH BGE was used. The CZE separation could not be achieved for weak acids with pK\textsubscript{a} values one unit higher than the pH of the BGE because the effective mobility of the weakly acidic compounds was very low due to the low dissociation constant.

To separate weakly acidic compounds, a higher pH BGE has to be used. Acidic compounds usually have considerable solubility in NaOH solution, which can be used to back-extract the analytes from an organic phase in off-line sample pretreatment [209]. In this chapter, the effort to enrich on-line weakly acidic compounds dissolved in NaOH solution with a medium pH BGE is discussed.
5.2 EXPERIMENTAL

5.2.1 Instrument and procedures

Experiments were performed on the Hewlett-Packard 3D CE system. The column was 48.5 cm×50 μm-i.d. bare fused silica capillary with a detection window 8.5 cm from the outlet of the column. Sample was introduced by a pressure of 50 mbar, 1-second injection corresponding to ~0.09 cm of the length of the sample plug. The capillary was thermostated at 20°C. Detection wavelength for the analytes was set at 210 nm.

The CE system was operated in the negative polarity mode (the cathode was at the inlet) with a suppressed electroosmotic flow (EOF). The BGE consisted of 50 mM sodium phosphate buffer and 9 mM diethylenetriamine (DETA) at a pH of 6.0. DETA was used to suppress the EOF [210]. The separation voltage was -25 kV. Under these conditions, all anionic analytes migrated to the detector end. The EOF was towards the capillary inlet, thus pumping out the sample matrix at this end.

For regular injection, the sample was injected hydrodynamically for 4 seconds at 50 mbar. For sample stacking, sample dissolved in NaOH solution was injected for a prescribed time as noted below at a pressure of 50 mbar.

5.2.2 Chemicals

3,5-Dichlorobenzoic acid (3,5-DCBA), 4-chlorophenoxyacetic acid (4-CPA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-amino-3,5,6-trichloropicolinic acid (Picloram), 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB), 2-(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP) were bought from Aldrich (Milwaukee, WI, USA). 2, 4-dichlorophenoxyacetic acid (2,4-D) and
pentachlorophenol (PCP) were from Fluka AG (Buchs, Switzerland). 2-(2,4-
Dichlorophenoxy)propionic acid (2,4-DP) was from Tokyo Kasei (Tokyo,
Japan). The structures of the herbicides are shown in Figure 5-1. The
herbicides were dissolved in methanol to generate individual stock solutions of
1 g/L. Working solutions were made by diluting the above solution with water
or sodium hydroxide solutions. Hexamethonium chloride, another type of EOF
suppressor [178] was purchased from Aldrich. The BGEs were prepared from
0.5 M sodium dihydrogen phosphate and 0.3 M DETA (its pH was adjusted to
5.7 with phosphoric acid), the pH of BGEs were adjusted to pH 6.0 with NaOH.

![Figure 5.1 Structures of the herbicides](image-url)
5.3 RESULTS AND DISCUSSION

5.3.1 Large volume sample stacking vs. base-aided large volume sample stacking

In base-aided large volume sample stacking method, the analytes dissolved in NaOH solution were injected in a large volume into the capillary which was pre-filled with the BGE (50 mM sodium phosphate, 9 mM DETA, at pH 6.0). After the voltage was applied, the analytes were focused. The stacking scheme resembled large volume sample stacking (LVSS) without polarity switching except that NaOH solution was the sample matrix instead of diluted BGE. The presence of NaOH in the sample improved the sample stacking performance. Therefore, we termed this stacking method base-aided large volume sample stacking (BA-LVSS). With the same injection volume, the peak heights increased with the concentration of NaOH from 1 mM to 20 mM. The concentration sensitivity in 20 mM NaOH was ~twofold higher than in pure water for all analytes. Further increase in NaOH concentration higher than 20 mM did not improve the sensitivity (Figure 5.2).

![Graph showing the effect of NaOH concentration on peak height. BGE: 50mM sodium phosphate, 9mM DETA, pH 6.0. Sample: 0.5 mg/L. Injection: 50mbar×100s. Voltage: -25kV.](image)

Figure 5.2 The effect of NaOH concentration on peak height. BGE: 50mM sodium phosphate, 9mM DETA, pH 6.0. Sample: 0.5 mg/L. Injection: 50mbar×100s. Voltage: -25kV.
We compared the stacking performance of large volume sample stacking and BA-LVSS with the same composition of BGE. The result is shown in Figure 5.3. In LVSS, we followed the scheme reported by Burgi and Albert [140,210]. The analytes were dissolved in 1% BGE. The injection amount was 40% of capillary volume. Enrichment factors of ~20 were obtained for all analytes. In BA-LVSS, the sample was dissolved in 20 mM NaOH, and the injection volume was the same as in LVSS. Around 50-fold enrichment factors were obtained for all analytes.

Figure 5.3 Base-aided large volume sample stacking (A) vs. large volume sample stacking (B). Sample: 0.2 mg/L analytes in (A). 20 mM NaOH; (B). 1% of BGE. BGE: 50 mM sodium phosphate, 9 mM DETA, pH 6.0. Injection: 50 mbar×200 s. Voltage: -25 kV. Peak identification: (1). 3,5-DCBA; (2). 4-CPA; (3). 2,4-D; (4). PCP; (5). Picloram; (6). 2,4,5-T; (7). 2,4-DP; (8). 2,4,5-TP; (9). 2,4-DB.
5.3.2 The effect of BGE composition on BA-LVSS

5.3.2.1 Effect of pH of the BGE

The pH value of the BGE influenced the protonation of DETA and the dissociation of silanol group on the surface of the capillary, affecting EOF suppression ability of DETA. When the pH was increased from 6.0 to 7.0, the EOF mobility increased from $14.04 \times 10^{-5} \text{ cm}^2/\text{Vs}$ to $20.82 \times 10^{-5} \text{ cm}^2/\text{Vs}$ at a constant concentration of DETA (9 mM) and phosphate (50 mM). Since the anionic analytes migrated in the direction against the EOF, their migration times increased significantly. At pH 6.0, all analytes migrated to the detection window within 20 min; while at pH 7.0, only five peaks could be detected within 60 min.

Figure 5.4 Effect of BGE pH on sample stacking performance. BGE: 50 mM sodium phosphate, 9 mM DETA at different pH values. Sample: 0.2 mg/L analytes in 20 mM NaOH. The other conditions were as in Figure 5.3.
The sample stacking performance with the BGEs of different pH values is shown in Figure 5.4. The enrichment factors for peak 1 decreased from 52 to 39, and further to 22, when the pH was increased from 6.0 to 6.7, and than to 7.0. At higher pH, the peaks were not as symmetrical as those at pH 6.0, and exhibited diffuse frontal edges.

5.3.2.2 Effect of DETA concentration

DETA is often used a modifier to suppress EOF in CE. Its suppression ability depends on its concentration and the pH of the BGE [140,210]. At pH 6.0, the EOF mobility decreased from $21.01 \times 10^{-5}$ cm$^2$/Vs to $5.56 \times 10^{-5}$ cm$^2$/Vs when DETA concentration was increased from 3 mM to 30 mM, as shown in Figure 5.5.

In addition to serving as an EOF modifier, the protonated DETA as counter-ion in BGE played an important role in the sample stacking procedure. The $pK_a1$, $pK_a2$, $pK_a3$ of the conjugate acid of DETA are 4.25, 8.98 and 9.78, respectively [211]. At pH 6.0, DETA was doubly protonated, and would react with OH$^-$ at the boundary between the BGE and sample during the sample stacking process since they migrated in opposite directions. The effect of DETA on stacking is shown in Figure 5.5. With 6 mM DETA in the BGE, tailing peaks were obtained for the analytes of low mobilities; at higher DETA concentration, fronting peaks were obtained for the all analytes. With 9 mM DETA in the BGE, all analytes were concentrated with good peak shape. Therefore, 9mM DETA concentration was selected for further experiments.
5.3.3 The effect of injection volume

The EOF is a concern when the injection volume is significantly large, influencing the global EOF. In LVSS without polarity switching, to make sure that all the anionic analytes could migrate to the detection window, the global EOF velocity should be less than the velocity of analytes in the sample zone. The global EOF is the combination of local EOFs in the sample and BGE zones. In a simple case, when the capillary is filled with two segments of the same composition at different concentrations, the global EOF velocity is a weighted average of the electroosmotic velocities of the individual zones [109]. In the present stacking scheme, a discontinuous buffer system was used. The sample matrix was 20mM NaOH, while the BGE was composed of dihydrogen phosphate and protonated DETA. When the voltage was applied, the chemical reactions (e.g. $H_2DETA^{2+} + OH^- \rightarrow DETA + H_2O$) were involved at the interface of the BGE and the sample zone. It was too complicated to calculate the global
EOF velocity from the EOFs of the individual zones. When the sample injection volume was 10% of the capillary volume (50 mbar×50 seconds), the global EOF velocity was dominated by the value of the BGE segment. The average EOF velocity was 0.073 cm/s during the time in which the EOF marker (injected at the outlet) moved from outlet to the detection window (8.5 cm long). This value was close to the average EOF velocity (0.072 cm/s) when the entire capillary was filled with the BGE. All analytes could migrate to the outlet without being carried out of the inlet by the EOF. For larger sample injection volumes, the peak areas were linear with the injection volumes up to 75% of the capillary volume, as shown in Figure 3.5A. It is justifiable to conclude that when a volume equivalent to 75% of the capillary was injected with the sample,
all the analytes could migrate to the detection window without being carried out of the capillary inlet by the EOF pumping. Peak areas rather than the corrected peak areas based on migration times were used for the calculation, although the migration times were not the same at different injection volumes. The reason was that the entire capillary was filled with the BGE when the analytes migrated to the detection window, and the migration velocity of the analytes passing by the detection window did not suffer from the influence of the sample matrix since the sample zone was completely pumped out by the EOF at this moment. This was indirectly verified by the value of current: when the analytes passed the detection window, the current was approximately equal to the value when the capillary was filled with the BGE (Figure 5.11). Peak heights of the analytes were also linear with the injection volumes when the latter ranged from 10% to 75% of the volume of the capillary except 2,4,5-DP and 2,4-DB (the last two peaks), as shown in Figure 5.5B. The sensitivity could be enhanced about 75-fold for analytes except 2,4,5-TP and 2,4-DB (about 50-fold) compared with an injection volume that was equivalent to 1% of the capillary.

When the injection volume was equal to the entire capillary, all analytes could migrate to the detection window without leakage from the inlet. This was demonstrated by the following experiment: Filling the capillary with 20 mM NaOH (sample matrix), then injecting the sample at 50 mbar for 4 seconds at the inlet; after injection, both ends of the capillary were placed in the BGE vials and voltage was applied, the analyte peaks could be observed. This confirmed that when the injection volume was equal to the whole capillary, no or negligible leakage from the inlet occurred.
5.3.4 Tolerance of salt

Another difference between BA-LVSS and LVSS is that high concentration of salt in the sample can be tolerated in BA-LVSS. In LVSS, high concentration of salt in the sample results in high conductivity, low electrical field strength distribution and electrophoretic dispersion. Therefore, high salt concentration in the sample zone should be avoided for LVSS. The influence of salt concentration on stacking performance in the presence and absence of NaOH is shown in Figure 5.8. When 10 mM NaCl was the sample matrix, the specific conductivity of sample zone might be lower than that of the BGE zone, and field amplification was applicable to stack the analytes (Figure 5.8A). When 20 mM NaOH co-existed in the sample with 10 mM NaCl, the sensitivity could be enhanced ~two-four-fold compared with the situation without NaOH (Figure 5.8B). In addition, with 20 mM NaOH in sample, the analytes could be focused even in presence of 40 mM NaCl in sample matrix (Figure 5.8D), although there were tailing peaks for the analytes with lower mobility. Therefore, this method is promising for the online preconcentration of acidic compounds of biological origin without desalting.

5.3.5 Linearity, precision and detection limits

We investigated the linearity of all the compounds with the injection volume at 75% of the entire capillary volume. In the range of 10-500 μg/L, the peak areas were linear with the concentrations of all analytes. The peak heights were also linear with concentration, but the concentration range exhibiting linear response for 2,4-DP, 2,4,5-TP, 2,4-DB (the last three peaks) was 10-
200 μg/L. The results are shown in Table 5-1 and Table 5-2. The precisions of migration times, peak areas, and peak heights were investigated with five

Figure 5.7 Effect of sample injection volumes on (A): peak areas and (B): peak heights.
Figure 5.8 Tolerance of salt in sample. Sample: 0.2 ppm analytes in (A) 10 mM NaCl, 0 mM NaOH; (B) 10 mM NaCl, 20 mM NaOH; (C) 20 mM NaCl, 20 mM NaOH; (D) 40 mM NaCl, 20 mM NaOH. BGE: 75 mM sodium phosphate, 9 mM DETA, pH 6.0. Injection: 50 mbar x 200 s, voltage: -25 kV. Detection wavelength: 195 nm. The first broad peak in (A)-(D) with a fronting edge is chloride. The second broad peak in (B)-(D) are due to HPO$_4^{2-}$.

Consecutive injections of 0.2 mg/L analytes in 20 mM NaOH. The relative standard deviations (RSDs) for migration times, peak areas and peak heights were 0.5-1.0%, 1.3%-2.3%, and 1.5-2.9%, respectively. The limits of detection (LODs) with injection time of 400 s at 50 mbar were less than 5 μg/L, as summarized in Table 5-1.
Table 5-1. Calibration Data (Peak area vs. Concentration) and LOD

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dynamic range (μg/L)</th>
<th>Slope</th>
<th>Intercept</th>
<th>R²</th>
<th>LOD (μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-DCBA</td>
<td>10—500</td>
<td>0.3402</td>
<td>0.1259</td>
<td>0.9998</td>
<td>3.7</td>
</tr>
<tr>
<td>4-CPA</td>
<td>10—500</td>
<td>0.0818</td>
<td>0.1063</td>
<td>0.9996</td>
<td>4.0</td>
</tr>
<tr>
<td>2,4-D</td>
<td>10—500</td>
<td>0.2199</td>
<td>-0.0366</td>
<td>0.9996</td>
<td>2.9</td>
</tr>
<tr>
<td>PCP</td>
<td>10—500</td>
<td>0.2989</td>
<td>1.2083</td>
<td>0.9995</td>
<td>3.0</td>
</tr>
<tr>
<td>Picloram</td>
<td>10—500</td>
<td>0.2697</td>
<td>-0.2449</td>
<td>0.9994</td>
<td>2.8</td>
</tr>
<tr>
<td>2,4,5-T</td>
<td>10—500</td>
<td>0.3711</td>
<td>-0.0448</td>
<td>0.9996</td>
<td>3.6</td>
</tr>
<tr>
<td>2,4-DP</td>
<td>10—500</td>
<td>0.2656</td>
<td>-0.0577</td>
<td>0.9991</td>
<td>3.4</td>
</tr>
<tr>
<td>2,4,5-TP</td>
<td>10—500</td>
<td>0.4147</td>
<td>-0.2274</td>
<td>0.9992</td>
<td>4.1</td>
</tr>
<tr>
<td>2,4-DB</td>
<td>10—500</td>
<td>0.3191</td>
<td>-0.6769</td>
<td>0.9979</td>
<td>4.4</td>
</tr>
</tbody>
</table>

a. BGE: 50mM phosphate, 9mM DETA, pH 6.0. Injection: 50mbar×400s, voltage: -25kV

Table 5-2 Calibration Data (Peak height vs. Concentration)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dynamic range (μg/L)</th>
<th>Slope(a)</th>
<th>Intercept(b)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-DCBA</td>
<td>10—500</td>
<td>0.1041</td>
<td>-0.1054</td>
<td>0.9989</td>
</tr>
<tr>
<td>4-CPA</td>
<td>10—500</td>
<td>0.0226</td>
<td>-0.0381</td>
<td>0.9986</td>
</tr>
<tr>
<td>2,4-D</td>
<td>10—500</td>
<td>0.0493</td>
<td>0.0973</td>
<td>1</td>
</tr>
<tr>
<td>PCP</td>
<td>10—500</td>
<td>0.0573</td>
<td>0.1991</td>
<td>1</td>
</tr>
<tr>
<td>Picloram</td>
<td>10—500</td>
<td>0.0502</td>
<td>0.1436</td>
<td>0.9995</td>
</tr>
<tr>
<td>2,4,5-T</td>
<td>10—500</td>
<td>0.0635</td>
<td>0.5511</td>
<td>0.9964</td>
</tr>
<tr>
<td>2,4-DP</td>
<td>10—200</td>
<td>0.0476</td>
<td>-0.0061</td>
<td>1</td>
</tr>
<tr>
<td>2,4,5-TP</td>
<td>10—200</td>
<td>0.0624</td>
<td>-0.2051</td>
<td>0.9993</td>
</tr>
<tr>
<td>2,4-DB</td>
<td>10—200</td>
<td>0.0269</td>
<td>-0.1169</td>
<td>0.9981</td>
</tr>
</tbody>
</table>

a. BGE: 50mM phosphate, 9mM DETA, pH 6.0. Injection: 50mbar×400s, voltage: -25kV
5.3.6 Investigation of stacking mechanism

In this method, the pH values of the BGE and sample were 6.0 and 12, respectively. The pK\textsubscript{a} of all the analytes were below 4.0 except 2,4-DB (pK\textsubscript{a}=4.8) and pentachlorophenol (pK\textsubscript{a}=4.71). They were almost completely dissociated in both the BGE and NaOH solution. The electrophoretic mobility was approximately constant in the BGE and NaOH solution without considering the effect of ionic strength. Therefore, it was apparent that BA-LVSS was not due to the mobility difference of the analyte in the BGE and the NaOH solution.

This method was different from pH-mediated field amplification stacking or base-stacking in which sample was electrokinetically injected into the capillary first, and then NaOH was injected to neutralize the weakly alkaline counter-ions in the sample zone to induce low conductivity and high local electrical field distribution.

At pH 6.0, almost all the phosphate in the BGE existed in form of H\textsubscript{2}PO\textsubscript{4}-

The mobility of the BGE co-ion was calculated by correcting the effect of ionic strength according to reference [212] to be 25.92×10\textsuperscript{-5} cm\textsuperscript{2}/Vs. The mobilities of the analytes in the BGE were in the range of 21.5 to 27.2×10\textsuperscript{-5} cm\textsuperscript{2}/Vs. The rigorous conditions of isotachophoresis were not valid for all analytes if the OH\textsuperscript{-} functioned as the leading ion and the co-ion of the BGE as the terminating one.

To investigate the stacking mechanism, we monitored the analyte concentration profile changes in the capillary after switching on the voltage with the following approach: the applied voltage was aborted at some time-points, and then the contents in the capillary were mobilized to the detection window by applying a low pressure at the inlet end. To view the analyte-
focusing process clearly, a high concentration (20 mg/L) of 2,4,5-T was used as the analyte. The detection wavelength was set at 230 nm to avoid the interference from the OH⁻ absorbance. The results are shown in Figure 5-9. The variation of analyte concentration profile indicated that the analyte was focused at both ends of the sample zone. In the first 0.4 min of electrophoresis, the length of the analyte zone was reduced from 19.4 cm to 13.6 cm (corresponding to 200 s and 140 s injection times at 50 mbar pressure, respectively). The analyte concentration increased at both ends of the sample zone, but remained approximately constant in the middle, resulting in two peaks, one at the front boundary and the other at the rear boundary. As the electrophoresis proceeded, the distance between these two peaks became smaller. At 0.8 min, the two peaks met at the middle point of the sample zone to form one peak (Figure 5.9). After then, the analyte zone was sharpened continuously. The sample plug compression process was significantly different from that in LVSS. The analyte-focusing process in LVSS is shown in Figure 5.10. In LVSS, the analyte was stacked at the front boundary between the sample and the BGE, with only one sharp edge. In a very short period of time (0.6 min in Figure 5.10), the stacking process was completed.
Figure 5.9 Concentration profiles of 2,4,5-T in BA-LVSS. BGE: 50 mM sodium phosphate, 9 mM DETA, pH 6.0. Sample: 20 ppm 2,4,5-T in 20 mM NaOH. Injection: 50mbar×200s. The traces were obtained by mobilizing the contents in the capillary with a low pressure from the inlet after they had undergone electrophoresis for the times as labeled.

Figure 5.10 Concentration profiles of 2,4,5-T in LVSS. Sample: 20 mg/L 2,3,4-T in 1% BGE. The other conditions are as in Figure 5.9.
Although the analyte concentration profiles obtained at 230 nm could clearly represent the compression of the sample plug during BA-LVSS, the effect of NaOH could not be observed directly. Since NaOH played an important role in the present sample stacking, the concentration profiles of NaOH or its reaction product with the BGE were monitored at the wavelength of 195 nm, as shown in Figure 5.11. After applying voltage for 0.4 min, the zone of NaOH (indicated by the strong absorbance at 0 min) disappeared very rapidly, and a short plug with a strong absorbance appeared at the interface between the sample and the BGE. The front edge of the analyte lay just behind the strong absorbance zone. The self-sharpening rear edge of the analyte (Figure 5.9) lay just in front of a low absorbance zone (the baseline dips in Figure 5.11). This low absorbance zone was supposed to be a low conductivity zone according to the Kohlrausch regulating function (KRF). This was verified by monitoring the current during sample stacking, as shown in Figure 5.12.

The current profile of BA-LVSS was different from that of LVSS, as shown in Figure 5.12 and Figure 5.13. For LVSS, the discontinuity came from the different concentration of BGE in the BGE and the sample zone. After the low conductivity sample zone was removed from the capillary by EOF pumping, the current increased to a constant value as the entire capillary was filled with the BGE (Figure 5.13). For BA-LVSS, the current change was more complicated. From the onset of voltage application to ~0.5 min (point A-B in Figure 5.12), the current decreased very rapidly due to a low conductivity zone formed in the original sample zone as described above. After 0.5 min (B-C in Figure 5.11), the current increased because the newly formed low conductivity zone was pumped out by the EOF from the inlet. This was confirmed by the
reduced length of the low absorbance zone from 0.6 to 2 min as shown in Figure 5.11. After 2.4 min, the low conductivity zone was removed completely,

Figure 5.11 Concentration profile of NaOH and 2,4,5-T monitored at 195 nm. Other conditions are as in Figure 5.9.

Figure 5.12 Current changes during BA-LVSS. Other conditions are as in Figure 5.3A
and the capillary consisted of a high conductivity zone (reaction product of OH\(^-\) and the BGE, as indicated by the high absorbance zone in Figure 5.3 and 5.11) and the original BGE. The current reached a relatively constant value and was larger than the value when the capillary was filled solely with the BGE. After 6.5 min (point D-F in Figure 5.12), the high conductivity zone was migrating out of the capillary from the outlet, and the total conductivity of the electrolytes in the capillary was reduced, the current began to decrease. After the high conductivity zone migrated out of the capillary completely, the entire capillary consisted purely of the BGE. The current was constant from then on. This was confirmed by the electropherogram in Figure 5.3A. The diffuse front edge of the broad peak prior to the peaks of the analytes passed by the detection window at \(~5\) min, it took another \(~1.5\) min to migrate from the detection window to the outlet (8.5 cm in length), corresponding to the onset of the
decrease in the current (point D in Figure 5.11). The rear sharp edge of the broad peak passed by the detection window at ~8.5 min. It took another ~1.7 min to migrate to the outlet of the capillary. After the rear boundary migrated out of the capillary, the current became constant.

An alternative EOF suppressor was used to investigate the role of DETA as a counter-ion in the BGE. 5 mM hexamethonium chloride (HMC) was added to 50 mM sodium phosphate at pH 6.0 to produce the EOF mobility of $11.60 \times 10^{-5}$ cm$^2$/Vs, similar to the suppression capability of 9 mM DETA. When the BGE was composed of 5 mM HMC and 50 mM sodium phosphate at pH 6.0, the analytes, dissolved in 20 mM NaOH solution, could not be well stacked, as shown in Figure 5.14A. With this BGE, if 10 mM Na$^+$ was replaced by NH$_4^+$, the analytes could be stacked as well as in case of H$_2$DETA$^{2+}$ as counter-ion, as shown in Figure 5.14B. When zwitterionic surfactant DDAPS was used as EOF suppressor, similar results were obtained, as shown in Figure 5.15. Due to the weaker EOF suppression capability of DDAPS and its interaction with anionic compounds, only three analytes were observed in 30 min. From the experiments with HMC and DDAPS as EOF suppressor, we it is clear that the weak electrolyte counter-ions in the BGE played an important role in BA-LVSS.

In BA-LVSS, the mobility of BGE co-ion was higher than that of the analytes. No ion could play the role of a terminating ion if the transient isotachophoresis was assumed to be the sample stacking mechanism, especially with the BGE using 5 mM HMC as EOF suppressor, since the Cl$^-$ was much more mobile than the analytes. According to the concentration profiles obtained at 195 nm, we could conclude that the analytes were focused
Figure 5.14 Effect of counter-ion of the BGE in BA-LVSS with Hexamethonium chloride (HMC) as EOF suppressor. BGE: (A) 50 mM sodium phosphate, 5 mM HMC at pH 6.0. (B) 40 mM sodium phosphate, 5 mM HMC, 10 mM NH₄H₂PO₄, at pH 6.0. Sample: 0.2 ppm analytes in 20 mM NaOH. Injection: 50 mbar×200s. Voltage: -25 kV.

by field amplification at the rear boundary since a low conductivity zone was formed after the OH⁻ migrated away. At the front boundary, the high conductivity zone formed by the reaction of OH⁻ and H₂PO₄⁻ also helped to focus analytes due to the field amplification mechanism.

Figure 5.15. Effect of counter-ion of the BGE in BA-LVSS with DDAPS as EOF suppressor. BGE: (A) 4 mM DDAPS, 50 mM sodium phosphate, pH 5.5. (B). 4 mM DDAPS, 40 mM sodium phosphate, 10 mM NH₄H₂PO₄, pH 5.5. Other conditions as in Figure 5.13. peak identification: 1. 4-CPA, 2. Picloram, 3. 3,5-DCBA.
5.3.7 Sample analysis

To demonstrate the applicability of this method, a tap water sample spiked with 1 ppb of each analyte was analyzed. Extraction method was based on Pedersen-Bjergaard’s work with some modification [213]. 1-Octanol was impregnated in the wall of a 50-cm porous hollow fiber after filling its channel with 120 μL of 20 mM NaOH (acceptor solution). The fiber was placed into 60 mL acidified water sample (pH ≤ 2.0) to extract the analytes for 1 hour. After extraction, the extract (acceptor solution within the hollow fiber channel) was flushed out into a CE sample vial with nitrogen gas, and then injected in a large volume into the capillary for analysis. Figure 5.16 is a typical electrophoreogram. It should be noted that the extraction procedure was not investigated in detail and therefore not optimized. The recovery of PCP and Picloram was not satisfactory, and further optimization might improve the recovery significantly. In fact, the analytes could be extracted with classic liquid-liquid extraction into the organic phase, back-extracted into alkaline solution, and then injected into the capillary in a large volume for analysis. The results indicated that the sample stacking procedure could be efficiently coupled with sample pretreatment step for acidic compounds.

5.4 CONCLUSION

A new sample stacking method was developed for acidic compounds dissolved in NaOH solution using phenoxy acidic herbicides as model analytes. With weak base diethylenetriamine (DETA) present in the background
Figure 5.16 Electropherogram of a tap water sample spiked with 1 µg/L analytes after liquid phase microextraction into 20 mM NaOH and on-line sample stacking. Other conditions and peak identification as in Figure 5.3A.

electrolyte (sodium phosphate, pH 6.0) as an electroosmotic flow (EOF) suppressor and counter-ion, the sample dissolved in NaOH solution was injected in a large volume without loss of separation efficiency. During and after sample stacking, the sample matrix was pumped out by the EOF. After sample stacking, the analytes was separated in zone electrophoresis mode. The stacking performance was better than large volume sample stacking in which the sample was dissolved in 1% of BGE or pure water. The sensitivity could be increased approximately 75-fold at maximum injection volume compared with injection volume of 1% of the capillary length. With coupling liquid phase microextraction with NaOH as the final acceptor solution, the method provided lower than 0.1 ppb detection limits for the herbicides in a fortified water sample. The stacking mechanism was investigated by monitoring the variation of the distribution of analyte concentration during the sample stacking.
6.1 INTRODUCTION

In previous chapters, on-line enrichment for weakly acidic analytes with $pK_a$ values of less than 5 with a low or medium pH BGE was proposed. The sample matrix can be easily removed from the separation capillary after completing sample stacking by modifying the EOF. Therefore, the separation that follows is independent of the influence of sample matrix. To pre-concentrate the weakly acidic analytes with high $pK_a$ values under CZE mode, a high pH BGE has to be used to make sure the analytes have significant mobility in the BGE to be separated. The procedures used in a low- or medium-pH BGE for removing the sample matrix after sample stacking is not applicable in a high-pH BGE due to the lack of suitable EOF modifier in a high pH BGE. For example, a pH-independent large volume sample stacking scheme has been reported by Baryla [143] with a zwitterionic surfactant as EOF suppressor. It is effective for most analytes with $pK_a$ values less than 10. For the analytes with high $pK_a$ values such as dimethylphenol ($pK_a=10.5$), this method is not applicable. Martinez et al [214] used LVSS with polarity switching to enrich phenols with a phosphate BGE at pH 10, but since the mobility of dimethylphenol was very low, it moved at a mobility close to the EOF.

Micellar electrokinetic chromatography (MEKC) is another mode of CE that can be used for both ionic and neutral compounds. With MEKC, the high pH
BGE is not necessary for compounds with high pKₐ values since the charged micelles can carry the neutral compounds to the detection window. The separation of neutral compounds is based on the partition of analytes between micelle and the surrounding media. MEKC also suffers from low sensitivity as the other operational modes of CE. In this chapter, a new online sample enrichment technique is described for phenols as examples of weakly acidic compounds, and the phenols were separated in MEKC mode after on-line enrichment.

6.2 EXPERIMENT

6.2.1 Instrument

The experiments were performed on a Prince CE system. A 70-cm×50 μm i.d. bare fused-silica capillary with a detector window at 55 cm from the inlet end was used for separation. The detector was a Lambda 1010 spectrophotometer. The detection wavelength was 210 nm.

6.2.2 Reagents and solutions

Stock solutions of sodium dodecyl sulfate (SDS) and urea were prepared at 200 mM and 10 M, respectively. The BGE was prepared fresh daily by dilution of phosphoric acid, SDS and urea stock solutions in ultrapure water. The buffer solution used in this study was 50mM H₃PO₄/80 mM SDS/2 M urea (pH 2.5). 2,4-Dimethylphenol (2,4-DMP, pKₐ 10.5); 2,3,5-trimethylphenol (2,3,5-TMP, pKₐ 10.57); 2,4-dichlorophenol (2,4-DCP, pKₐ 7.85); 3-chlorophenol (3-CP, pKₐ 9.10); 2-chlorophenol (2-CP, pKₐ 8.55); 2,4-dinitrophenol (2,4-DNP, pKₐ 4.08) were bought from Fluka (Buchs, Switzerland).
Phenol standards were prepared in methanol as 1000 μg/ml stock solutions, then diluted to give working solutions at different concentrations with ultrapure water or NaOH solution.

6.3 RESULTS AND DISCUSSION

6.3.1 Field amplified sample injection in MEKC

The sample stacking scheme is illustrated in Figure 6.1. Initially, the capillary was filled with the low-pH BGE containing SDS micelles. The EOF was suppressed by the low-pH BGE. A long plug of water was injected hydrodynamically into the capillary before sample injection (Figure 6.1a). Phenolic analytes prepared in water or NaOH solution were then electrokinetically injected into the capillary for a long period of time, e.g. 600 s at –10 kV (Figure 6.1b), with the application of negative voltage (the anode at the outlet end). The phenolic anions migrating rapidly toward the boundary of water and the BGE, and were neutralized when they entered the low-pH BGE zone, at which point they stopped migrating (Figure 6.1c). A short zone of focused analytes was formed at the boundary. The water plug was moving out of the capillary from the inlet at the same time because the EOF (toward the cathode, i.e. inlet) was dominated by the influence of the water plug (Figure 6.1d). The inlet end of the capillary was then placed into a vial containing the BGE, and a negative voltage (-20 kV, anode at the outlet) was applied across the capillary. The anionic micelles migrated into the sample zone, and separation was then achieved in the MEKC mode (Figure 6.1e).

The low pH of the BGE had two functions: (a), it suppressed the EOF, making the apparent movement of the anionic analytes in the direction towards the
outlet when a negative voltage was applied. At the same time, the water plug was pumped out by the EOF from the inlet; and (b), it served as a trap to accumulate the injected anions in their neutral form.

6.3.2 Effect of sample matrix

6.3.2.1 Water as sample matrix

When the phenolic compounds were prepared in water, they were partially dissociated due to their weak acidity. When the electrokinetic injection was initiated, the negatively charged ions migrated rapidly into the capillary under the influence of the electric field, passing through the water plug, and stopped when they encountered the low-pH BGE. The dissociation equilibrium of the analytes in the sample solution was then disrupted. The neutral phenols dissociated continuously to replenish the phenolate in the sample solution. Therefore, a great amount of anions could be injected and accumulated at the interface between the BGE and the water plug. After sample injection, anionic micelles were used to carry the neutral analytes to the detection window. Therefore, a very effective pre-concentration could be obtained for the phenolic compounds in water samples, as shown in Figure 6.2a, b. In Figure 6.2a, the electropherogram was obtained with conventional hydrodynamic injection. In Figure 6.2b, the analytes were diluted 10 times from the solution used to generate Figure 6.2a, yet the peak heights shown in the former were
Figure 6.1, Schematic illustration of FASI in MEKC with low-pH BGE.

Θ represents phenolic anion; ⊗ represents neutral phenol; a) after filling the capillary with low-pH BGE containing SDS micelles, a water plug was injected into the column to provide the high electric field at the injection point; b) electrokinetic sample injection into the capillary. Due to the high electric field, the anions migrated rapidly toward the outlet. At the same time, the water plug was moving out of the inlet of the capillary; c) when the phenolic anions reached the boundary of water and low-pH BGE, the phenols were neutralized and stopped migrating; d) the inlet was changed to low-pH BGE, and negative potential –20kV was applied. The water plug continued to move out of the inlet.; e) the SDS micelles migrated into the capillary and separation began in MEKC mode.

much higher than those in Figure 6.2a. Mobility discrimination occurred in electrokinetic injection of the analytes, which could be observed by comparing
ratios of peak heights in Figure 6.2a and 6.2b. For dinitrophenol (peak 6 in Figure 6.2), its effective mobility was higher than other phenols in water, and its peak height increased significantly more than the other analytes. It should be noted that the migration times in the electropherogram were not indicators of effective mobility of the analytes since the separation was carried out in MEKC mode.

![Electropherograms of phenolic compounds](image)

Figure 6.2, The electropherograms of phenolic compounds at different concentration in water. a) 25 μg/ml, hydrodynamic injection 100mbar x 0.1 min; b) 2.5 μg/ml; c) 0.025 μg/ml; for b, c, water plug length: 33cm; sample electrokinetic injection: –10 kV x 15 min. Peak identification: 1) 2,4-DMP; 2) 2,3,5-TMP; 3) 2,4-DCP; 4) 3-CP; 5) 2-CP; 6) 2,4-DNP

The enrichment factors with different concentrations of analytes are listed in Table 6-1. 2,4-DNP has the highest enrichment factor among all of the
phenolic compounds. This is probably because 2,4-DNP has the lowest pKa, and its dissociation degree is higher than other phenols. The concentration of free 2,4-DNP anion was much higher than the other compounds.

**Table 6-1 Enrichment factors for phenolic compounds at different concentrations in water**

<table>
<thead>
<tr>
<th></th>
<th>2,4-DMP</th>
<th>2,3,5-TMP</th>
<th>2,4-DCP</th>
<th>3-CP</th>
<th>2-CP</th>
<th>2,4-DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 μg/ml</td>
<td>399</td>
<td>394</td>
<td>482</td>
<td>408</td>
<td>625</td>
<td>1029</td>
</tr>
<tr>
<td>0.25 μg/ml</td>
<td>273</td>
<td>346</td>
<td>303</td>
<td>362</td>
<td>590</td>
<td>3056</td>
</tr>
<tr>
<td>0.025 μg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5987</td>
</tr>
</tbody>
</table>

Injection conditions: water plug length: 33cm; electrokinetic injection –10kV×15 min

Figure 6.3 shows the relationship of peak heights versus the sample concentration. The peak heights of the phenols were not linear with the sample concentrations. This is probably because the dissociation degrees of phenols were not the same at different concentrations of the analytes considering the equilibrium of water dissociation when the concentrations of the weak electrolytes were lower than $10^{-7}$ M. When the sample concentration was 25 ng/ml ($\sim10^{-7}$ M), the concentrations of anionic phenolate with higher pKa were too low to be detected, as shown in Fig 6.2c.
Figure 6.3 The relationship between peak heights and sample concentrations. Water plug length: 33cm, sample electrokinetic injection $-10\text{kV} \times 15\text{min}$.

2,4-DNP exhibited a slightly different curve from those of the other phenols. The enrichment factors for 2,4-DNP increased with the decrease in sample concentrations. This is probably because the much lower concentrations of free ions in the sample solution resulted in the decrease of sample conductivity. At the same time, the concentrations of other phenolic anions were too low, and the injection for these ions was suppressed. Thus, more 2,4-DNP ions could be injected. When the concentration was at 25 ng/ml, only peak of 2,4-DNP could be observed (Fig 6.2c).
6.3.2.2 NaOH solution as sample matrix

Since the ionization of phenols was possibly suppressed by the water dissociation when the concentrations of the analytes were lower than $10^{-7}$ M, a small amount of NaOH was added into the sample to facilitate the ionization of these compounds. At higher sample concentration, e.g. 2.5 μg/ml, when 6 mM NaOH was added into the sample solution, the preconcentration factor decreased compared to a sample dissolved in water. Figure 6.4a shows the electropherogram of phenols at concentration of 2.5 μg/ml each in 6 mM NaOH. Compared to Figure 6.2b, which is the electropherogram of analytes at the same concentrations dissolved in water, the preconcentration factor decreased around 50%.

For low concentration of analytes, the NaOH solution as sample matrix could improve the enrichment performance significantly, as shown in Figure 6.4b. When phenols at 25 ng/ml were dissolved in 6 mM NaOH solution, all analyte peaks could be observed. Due to the increased dissociation of phenolic compounds under alkaline condition, the concentration of anions in the sample solution increased as well.

In electrokinetic injection, the number of moles of ions introduced into the capillary in t seconds can be described by equation 6-1, developed by Rose and Jorgenson.

\[
\text{Moles injected} = \mu_{\text{app}} E (\kappa_b/\kappa_s) \pi r^2 C t
\]

(6-1)

where $\mu_{\text{app}}$ is the apparent mobility of the analyte ($\mu_{\text{app}} = \mu_{\text{ep}} + \mu_{\text{EOF}}$), $\mu_{\text{ep}}$, $\mu_{\text{EOF}}$ are the effective mobility of the analyte and the mobility of EOF, respectively. $E$ is the applied electric field, $r$ is the capillary radius, $C$ is the sample concentration, $\kappa_b/\kappa_s$ is the ratio of conductivities of the buffer in the capillary and the sample.
Figure 6.4 Electropherograms of phenols dissolved in NaOH. a). 2.5 μg/ml phenols in 6 mM NaOH, water plug length: 33 cm. Injection: -10kV×15min. b). 25 ng/mL phenols in 6 mM NaOH. Water plug length: 50 cm. Injection: -10×25 min. Peak identification as in Figure 6.2.

According to equation 6-1, NaOH exhibited two contrasting effects on the preconcentration of the analytes. On the one hand, it improved the preconcentration efficiency since it facilitated the dissociation of the phenols, which resulted in higher effective mobility of phenols in the sample solution. On the other hand, it decreased the field amplification effect because it decreased the factor $\kappa_b/\kappa_s$ by increasing the conductivity of the sample solution ($\kappa_s$); at the same time, it caused electrokinetic injection discrimination due to its much higher mobility. Thus, for maximum stacking efficiency, it is necessary to optimize the concentration of NaOH in the sample solution. Figure 6.5 shows
the effect of concentration of NaOH on the enrichment factor of each phenol. Sample solutions at 25 ng/ml concentration were studied for this purpose. As the concentration of NaOH increased from 0–8 mM, the enrichment of the first five compounds increased gradually. This was induced by the increased dissociation of these compounds at higher pH. As the NaOH concentration increased further, due to the higher ionic strength, the enrichment factor decreased slightly. 8mM NaOH was subsequently selected as the optimum concentration.

Figure 6.5 Effect of NaOH concentration on the enrichment factor. Sample: 25 ng/ml; Injection conditions: water plug length: 50cm; electrokinetic injection: –10kV×25min.
6.3.3 Method Validation

The repeatability, dynamic range and limits of detection were studied for the present pre-concentration method. Due to the presence of NaOH in the sample solution and the presence of the water plug, the repeatability of the peak heights was improved. The relative standard deviation (RSD) for 5 replicate runs was less than 6.4% for all of the analytes. The performance of this method is summarised in Table 6-2. However, the limits of detection (LODs) were not low enough to deal with real sample analysis. An off-line extraction procedure was needed before CE determination of these analytes. To validate the application of this pre-concentration method, an extraction step was incorporated for a spiked tap water sample as an example.

Table 6-2 Performance of FASI in MEKC

<table>
<thead>
<tr>
<th></th>
<th>Enrichment factor</th>
<th>Linear range (ng/mL)</th>
<th>RSD (%)</th>
<th>LOD (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-DMP</td>
<td>904</td>
<td>10-250</td>
<td>5.6</td>
<td>5.0</td>
</tr>
<tr>
<td>2,3,5-TMP</td>
<td>1137</td>
<td>10-250</td>
<td>4.2</td>
<td>5.0</td>
</tr>
<tr>
<td>2,4-DCP</td>
<td>1185</td>
<td>15-250</td>
<td>6.1</td>
<td>8.0</td>
</tr>
<tr>
<td>3-CP</td>
<td>1216</td>
<td>15-250</td>
<td>6.4</td>
<td>8.0</td>
</tr>
<tr>
<td>2-CP</td>
<td>1261</td>
<td>15-250</td>
<td>6.4</td>
<td>8.0</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>2692</td>
<td>5.0-500</td>
<td>3.6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The samples were prepared in 8 mM NaOH. Injection conditions: water plug length: 50 cm; sample electrokinetic injection: –10kV×25min
6.3.4 Sample analysis

Analytes were extracted from the spiked water sample by a liquid-liquid-liquid microextraction (LLLME) procedure using a hollow fiber membrane as a solvent support. This extraction served as an off-line sample cleanup and pre-concentration step.

The extractions were performed according to the following procedure: The phenols stock solution was spiked into 50 ml tap water such that the final concentration of each phenol was at 1.0 ng/ml. The pH of water sample was adjusted to pH 2 with 2 M HCl. A 535-mm length of hollow fiber was immersed into 1-octanol for 10 sec. This allowed the pores of the hollow fiber to be filled with the organic solvent. After impregnation, 150 μl of 8 mM NaOH was injected into the hollow fiber with a 250 μl syringe. The fiber was then placed in the sample solution. Both ends of the fiber were held out of the sample solution. During extraction, the solution was stirred at 1000 rpm with a magnetic stirrer. After extraction for 20 min, the acceptor solution inside the fiber was flushed into a 200-μl CE sample vial with a slow nitrogen gas flow. The extract in the sample vial was then analysed directly with the described stacking method. A typical electropherogram of an extract of water sample is shown in Fig 6.6. This pre-concentration method could be coupled very conveniently with the LLLME. The LODs achievable by combining LLLME with the CE preconcentration procedure were lower than 0.2 ng/ml.
6.4 CONCLUSION

We have demonstrated a simple and efficient sample stacking method for weakly acidic compounds such as phenols in MEKC analysis. A low-pH BGE was used to suppress the EOF and focus the sample molecules by neutralizing them during field amplification sample injection. After sample stacking, the analytes were separated under MEKC mode. The sensitivity was improved up to 2600–fold compared with normal hydrodynamic injection. This method can be applied to other weakly acidic compounds. The presence of a water plug during the FASI step and NaOH in sample solution helps improve the repeatability. Since the analytes in NaOH solution could be on-line pre-
concentrated, this method could be coupled with liquid-liquid-liquid microextraction. The extract in the NaOH acceptor matrix was analysed directly using the described stacking method.
Chapter Seven
Concluding Remarks

The results in this work illustrated that the low concentration sensitivity of capillary electrophoresis can be overcome by increasing the sample injection amount, with an appropriate arrangement of the BGE and the sample matrix constituents and manipulating the electroosmotic flow. Since no universal method is available for concentrating the analytes in all kinds of matrices, sample matrix effect is critically important when we develop on-line concentration methods.

To determine trace amounts of nitrate in seawater, a transient isotachophoresis process prior to CZE separation was used for pre-concentration. The large amount of natural chloride in the sample functioned as a leading ion in transient isotachophoresis with the co-ion of the BGE as a terminating ion, but the small mobility difference between nitrate and chloride resulted in too long an isotachophoretic time. When the nitrate migrated to the detection window, it was still in the rear boundary of the chloride, no individual peak could be detected. A zwitterionic surfactant was added into the BGE to enlarge the mobility difference between nitrate and chloride, so that nitrate could be separated from the sharp rear boundary of the chloride and be detected as a peak after the isotachophoretic concentrating effect.

It should be mentioned that despite the excellent on-line pre-concentration factor achievable by the CE techniques developed, at times these approaches
are still inadequate for practical world analysis. Thus, off-line sample pretreatment to preconcentrate analytes may still be necessary.

In sample pretreatment, organic solvent is generally used to extract organic compounds. The final extract is therefore not suitable for direct analysis by CE. For some applications, it is possible to back-extract analytes into an aqueous solution, making it possible to use CE for analysis. Following this approach, here, we developed methods for on-line concentration of acidic compounds involving NaOH solution as the matrix for back extracting the analytes into sample matrix.

For haloacetic acids (pKₐ 0.6-2.9) dissolved in NaOH solution, a phosphate background electrolyte (BGE) at pH 3.0 was used for separation. It was found that the peak heights of analytes were increased by the presence of NaOH in the sample with large injection volume. The mechanism underlying this sample stacking was proposed to be hydroxide-induced dual transient isotachophoresis. In the first stage of isotachophoresis, hydroxide played the role of a leading ion. Since the hydroxide was reactive with the low-pH BGE, the reaction product, HPO₄²⁻ was the leading ion in the second stage of isotachophoresis. In both stages, the co-ion of the BGE (H₂PO₄⁻) was the terminating ion. Finally, the analytes were destacked and separated in CZE mode when the leading ion, HPO₄²⁻, was consumed due to continuous reaction with H⁺ in the low pH BGE.

For the CZE separation of phenoxy acids (pKₐ 1.9-4.8), a higher pH BGE had to be used due to higher pKₐ values of these analytes. When diethylenetriamine (DETA) was used as EOF suppressor with phosphate BGE at pH 6.0, the sample dissolved in the NaOH solution could be injected into the
capillary in a large volume, and the analytes were concentrated on-line. Further study indicated that the presence of a protonated amine in the BGE as counter-ion was critical for this sample stacking. The EOF was suppressed by DETA, and the analytes migrated against the EOF. After sample stacking, the sample matrix was removed from the separation capillary by the EOF pumping from the inlet; thus had no influence on the CZE separation that followed.

The above methods cannot be applied for the online pre-concentration of phenols due to their high pK$_a$ values and lack of a suitable EOF modifier in a high-pH BGE. Micellar electrokinetic chromatography (MEKC) was therefore employed for the separation of these analytes with a low pH BGE to suppress the EOF. The pre-concentration was carried out by field amplified sample injection (FASI) of the sample dissolved in NaOH solution. FASI was implemented by pre-injecting a long water plug to produce a field amplification effect. As a result, the phenols accumulated at the interface between the water plug and the BGE since they were neutralized due to the dynamic pH junction. Since the EOF was from the anode to the cathode, the water plug would be pumped out of the capillary by the EOF from the inlet. MEKC separation was initiated by transferring the inlet from the sample vial to one containing the BGE after a period of injection.

In summary, in developing on-line pre-concentration techniques, the sample matrix, together with the EOF are important factors to be considered. The EOF can be used for the removal of the sample matrix after sample stacking if the migration direction of EOF is against the analytes.
Finally, further research may be suggested. The on-line preconcentration techniques described in this work are mainly based on the manipulation of the velocity through the electric field strength that the ions experience. In fact, the velocity of electrophoretic migration is a product of mobility and electric field strength. Therefore mobility manipulation is another possibility to consider in sample stacking. Theoretically, the mobility is influenced by the charge, molecular volume and the viscosity of the medium. Possibly, a high viscosity BGE can be used to retain the analytes along their migration path. Since the effect of viscosity on the mobility is not selective to the analytes, a universal method may be developed. In another future research possibility, the methods developed in this work may be transferred to microchip-based CE since electrophoresis in the fabricated micro-channels also suffers from the same low sensitivity problems as conventional CE.
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