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On-line concentration of neutral analytes for micellar electrokinetic chromatography
IV. Field-enhanced sample injection

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Abstract

Fundamental conditions for the on-line concentration of neutral analytes by field-enhanced sample injection (FESI) for micellar electrokinetic chromatography (MEKC) is presented. Long water plugs are injected into the capillary to effect an enhanced field during electrokinetic injection of samples at negative polarity. Optimization of the injection procedure is performed by modifying the composition of the micellar sample matrix containing one anionic surfactant. Stacking is found to be dependent on analyte retention factors and the nature of the pseudostationary phase. More than 20- and 100-fold improvements in peak height and peak area were confirmed experimentally. The applicability of the technique was tested using spiked urine samples following a simple liquid–liquid extraction step. © 1998 Elsevier Science B.V.

Keywords: Injection methods; Micellar electrokinetic chromatography; Sample stacking; Sample handling; Field-enhanced sample injection; Electrokinetic injection; Resorcinol; Naphthols; Dihydroxynaphthalene; Estrogens; Steroids

1. Introduction

In the last few years, capillary electrophoresis (CE) has been growing exponentially, with applications not only in the pure sciences but also in many diverse areas of expertise. The most common modes of CE are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). Pioneered by Terabe et al. [1], MEKC allows the analysis of both neutral and ionic constituents, the former being impossible to separate by CZE. Separation in CZE is based on the differences in electrophoretic mobilities of ionic compounds, whereas MEKC is based primarily on the partitioning of the analytes between the micellar phase and the aqueous phase. Efficiencies are astounding and method development is relatively simple. One of the major advantages of CE is the minute volume of samples, in the order of 1 nl, which is also its major disadvantage. Concentration sensitivity is therefore poor, being one to two orders of magnitude lower compared to high-performance liquid chromatography. Its application to more real samples necessitates the development of sensitive detection methods, such as hyphenation with a mass spectrometer or the development of on-line concentration techniques. On-line concentration by sample stacking in CZE has been exploited [2–6] and reviewed [7] by Chien and Burgi. Liu et al. [8], Nielsen and Foley [9] and, more recently, we [10–12] reported the utility of sample stacking in MEKC. Sample stacking arises from the abrupt changes in the electrophoretic velocity of samples across a concentration boundary. The major rationale is to increase the amount of sample loaded into the capillary without impeding the high efficiencies that can be obtained in CE.

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In this paper, we present another simple technique for the on-line concentration of neutral analytes in MEKC by sample stacking using field-enhanced sample injection (FESI). One anionic surfactant is utilized both in the sample and separation solutions. The effects of analyte retention factors and the nature of the pseudostationary phase are studied. Significant improvements in the detector response are realized. Finally, one of the ultimate goals of this research was to apply the technique to a real sample.

2. Theory

2.1. General remarks

The effective electrophoretic velocity of a solute can be greater than and opposite in direction to the bulk electroosmotic velocity; this can be achieved by injecting a water plug prior to electrokinetic injection at negative polarity. Chien and Burgi [4,5] have described, in detail, FESI with a water plug for CZE. The water plug introduced provides an enhanced electric field at the injection end of the capillary and acts like a fast freeway for the micelles that carry the neutral analytes.

2.2. Field-enhanced sample injection micellar electrokinetic chromatography (FESI–MEKC) model

The steps and mechanisms involved in FESI–MEKC are depicted in Fig. 1. The capillary is first filled with the micellar separation solution (BGS, heavily shaded area). After a long water plug has been injected into the capillary, the sample prepared in a micellar matrix is placed in the inlet position (Fig. 1A) and polarity is applied in the negative mode (Fig. 1B). The micellar separation solution (BGS) is found at the end closer to the detector. Due to the enhanced field in the water plug, which generates electrophoretic velocities that are greater than the bulk electroosmotic flow, micelles, and neutral analytes solubilized in them, enter the capil-

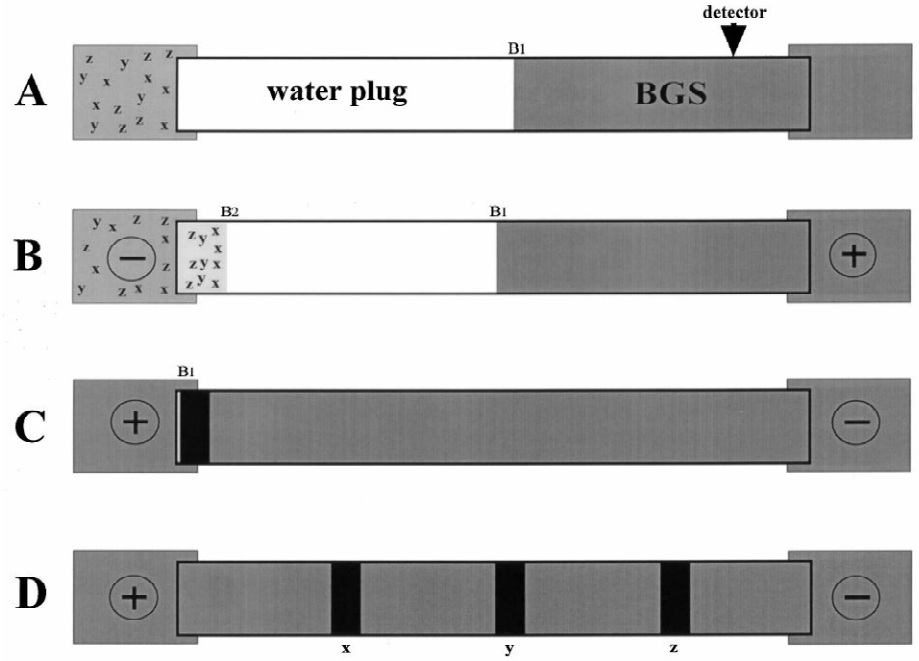

Fig. 1. Behavior of micelles and neutral analytes during FESI–MEKC. (A) initial situation (water plug, unshaded; BGS, shaded); (B) micelles enter the capillary and carry with them neutral analytes emanating from the cathodic vial, $k(x)>k(y)>k(z)$; (C) micelles and neutral analytes stacked at the concentration boundary, voltage is cut and the sample vial is replaced by another BGS vial when the measured current is approximately 97–99% of the predetermined current, voltage is then applied at positive polarity; (D) separation of zones occurs.

lary (Fig. 1B, lightly shaded part). B_2 is the boundary between the porions of the water plug occupied and unoccupied by micelles emanating from the sample reservoir. The migration order of neutral analytes is dependent on retention factors (k). While the neutral analytes are being brought to the water–BGS zone boundary (stacking boundary, B_1), the water plug is being pumped out from the capillary by the bulk electroosmotic flow. Neutral analytes concentrate upon reaching B_1. Once the current has reached 97 to 99% of the predetermined current at negative polarity, that is, when the water plug has been considerably removed from the capillary, the voltage is shut off. The sample vial is then replaced by another BGS vial and voltage is applied at positive polarity (Fig. 1C), to facilitate separation and detection (Fig. 1D).

2.3. On the amount of neutral analytes injected

In FESI for CZE, the total amount of ionic species (i) injected into the capillary after time (t), $N_i(t)$, was given by Chien [13] as

$$N_i(t) = \int_0^t AC_i[\nu_{ep}(i)(t) + \nu_{eof}(ave)(t)]\,dt, \quad (1)$$

where A is the cross-sectional area of the capillary, C_i is the concentration of i in the sample reservoir, $\nu_{ep}(i)$ is the electrophoretic velocity of i in the water plug, and $\nu_{eof}(ave)$ is the average bulk electroosmotic velocity. The total amount of neutral analyte (a) injected [$N_a(t)$] follows similarly and can be approximated by Eq. (2).

$$N_a(t) = \int_0^t AC_a(\nu^*_{ep}(a)(t) + \nu_{eof}(ave)(t))\,dt \quad (2)$$

where C_a is the concentration of a in the sample reservoir. The effective electrophoretic velocity of a neutral analyte $\nu^*_{ep}(a)$ is given by Eq. (3).

$$\nu^*_{ep}(a) = \mu^*_{ep}(a)E_s \quad (3)$$

where E_s is the field strength in the water plug and $\mu^*_{ep}(a)$ is the effective electrophoretic mobility of a. The latter is given as Eq. (4) [14].

$$\mu^*_{ep}(a) = \mu_{ep}(mc)\frac{k}{1+k} \quad (4)$$

The amount of injected neutral sample will therefore increase with increasing values of k, a scenario similar to the bias caused by electrokinetic injection of ions in CZE. Additionally, since very long water plugs are employed, neutral analytes incorporated in the micelle will only enter the capillary when x^*_{max} [11] is reached. x^*_{max} is the fraction of the capillary filled with low conductivity solution (in this case, plain water) that will allow the migration of neutral analytes into the capillary, and is also dependent on k. In summary, the amount of sample injected is very dependent on k.

3. Experimental

3.1. Apparatus

Capillary electrophoresis and on-line concentration were performed on a Hewlett-Packard 3D capillary electrophoresis system (Waldbronn, Germany) provided with fused-silica capillaries, 63.5 cm (55 cm to the detector)×50 μm I.D., obtained from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were thermostated at 20°C. Wavelengths of detection were selected using spectral absorbance curves that were recorded using a diode array detector. Conductivities were measured using a Horiba ES-12 conductivity meter (Kyoto, Japan). Capillaries were cleaned every day and in between runs using our previous method [10].

3.2. Samples, reagents and solutions

All reagents purchased were of the highest purity available from Nacalai Tesque (Kyoto, Japan). Butyl acrylate–butyl methacrylate–methacrylic acid copolymers, sodium salt (BBMA) supplied by Dai-ichi Kogyo Seiyaku (Kyoto, Japan) was purified by dialysis and solidified by freeze-drying. Buffers were prepared from stock solutions of sodium dihydrogenphosphate, disodium hydrogenphosphate, phosphoric acid and sodium hydroxide. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Stock solutions of resorcinol, 1,6-dihydroxynaphthalene and naphthols were prepared using purified water with concentrations depending on the water solubility of each. Stock solutions of the

estrogens (estrone, E_1 and estradiol, E_2) were prepared with spectrophotometric grade methanol and were stored in a refrigerator until used. All sample stock solutions were diluted initially with the BGS to procure sample solutions with analytes possessing comparable peak heights. Sample solutions for on-line concentration, with the concentration of each analyte that was decided upon, were then prepared with various micellar systems for optimization of the injection procedure. All solutions were filtered through 0.45-μm filters (Toyo Roshi, Japan) prior to electrophoretic analysis.

3.3. Procedure for FESI–MEKC

After conditioning the capillary with the micellar separation solution and injection of a long water plug, electrokinetic injection was performed at negative polarity. The capillary was flushed at high pressure with water for 1 min or until filled. The injection end of the capillary was placed on a micellar sample solution before electrokinetic injection. When the current reached 97–99% of the predetermined current at this configuration of a capillary filled only with BGS, the voltage was turned off and the inlet vial containing the sample prepared in a micellar solution was replaced by the BGS. Voltage was then applied at positive polarity.

4. Results and discussions

4.1. Effect of the retention factor on peak heights and corrected peak areas

Electropherograms obtained with FESI–MEKC using different concentrations of BBMA in the sample matrix are shown in Fig. 2. The same buffer was used to prepare the BGSs. With initial experiments involving the conditions given in Fig. 2B, detector responses for each analyte increased to a maximum as the water plug length was increased, the capillary was simply filled with water prior to electrokinetic injection for faster analysis, as done with the other electropherograms. Generally, for each concentration of BBMA, improvements in peak heights and corrected peak areas compared to the 2 s injection (Fig. 2A) were greater for compounds with higher *k* values. Peak areas were corrected for the migration time. Note that the value of *k* increases from peak 1 to peak 3. This is in accordance with Eq. (2); the effect of analyte retention factors on the amount of sample that can be injected and concentrated in the capillary.

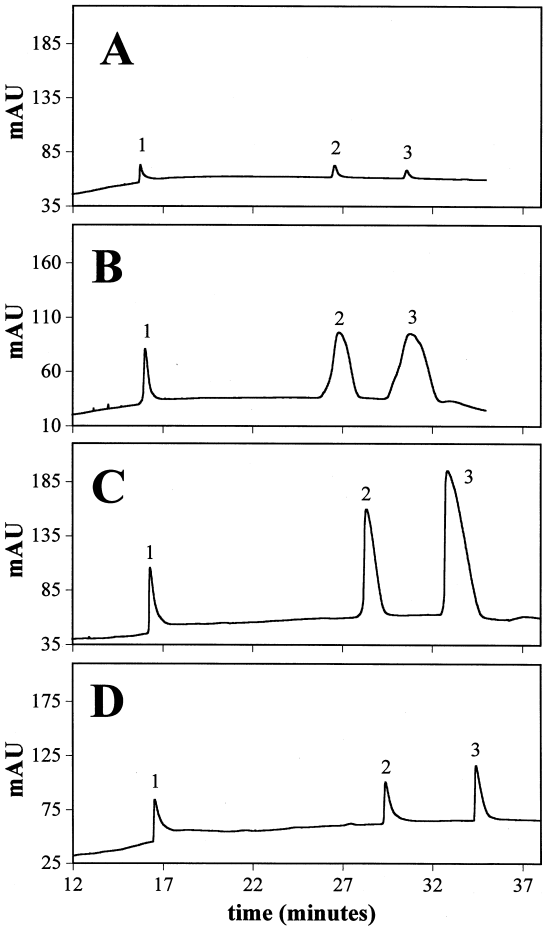

Fig. 2. Dependence of peak height and shape on the concentration of BBMA in the sample matrix. Sample matrix, BGS (A, B), 0.3% BBMA in separation buffer (C), 0.1% BBMA in separation buffer (D); BGS, 0.75% BBMA in separation buffer. Separation buffer, 50 m*M* sodium dihydrogenphosphate–100 m*M* sodium tetraborate (pH 8); injection, 2 s at 50 mbar (A), FESI at −20 kV, with the capillary filled with water (B, C, D); separation voltage, 20 kV. Peaks: 1,6-dihydroxynaphthalene (1), 2-naphthol (2) and 1-naphthol (3). Detection was at 210 nm.

4.2. Effect of sample matrix on peak shape

Let us keep in mind that the amount of analyte injected is directly proportional to k (Eq. (2)) and k is directly related to the concentration of surfactant. For each compound in the electropherograms shown in Fig. 2, corrected peak area, which is a good measure of the amount of sample injected, increased with increasing concentrations of BBMA in the sample matrix. This is reasonable, as stated in the opening statements of this paragraph. On the other hand, peak heights increased when the concentration of BBMA was lowered from 0.75 to 0.3%, especially for 2-naphthol and 1-naphthol (see Fig. 2C). Lowering the concentration of BBMA further, to 0.1% (Fig. 2D), is not useful, because peak heights are shorter compared to the higher concentrations of BBMA. The quite bizarre observation of greater peak heights with 0.3% BBMA compared to 0.75% BBMA in the sample matrix could not be explained simply. Several factors could have contributed to the resulting peak shape of the stacked analytes, such as probable changes in the conductivity of the zones during sample backout. At present, we are in the process of investigating this. In any case, improvement in detector responses compared to the injection using 0.3% BBMA in the sample matrix are 4.1, 9.2 and 18.1, in terms of peak height, and 5.4, 19.8 and 65.1, in terms of peak area, for 1,6-dihydroxynaphthalene, 2-naphthol, and 1-naphthol, respectively.

We studied further the effect of the concentration of surfactant in the sample matrix, using very hydrophobic samples (estrone and estradiol) and using a very low concentration of BBMA (0.04%) in the BGS. The concentrations of surfactant in the sample matrix were higher than the concentration of the surfactant in the BGS (0.1, 0.2 and 0.4%). No improvement in peak heights was observed at any of the concentrations tested, and peak shapes were heavily distorted when 0.4% BBMA was used in the sample matrix. Working with moderate concentrations of surfactant in the separation solution is therefore important for improving the loadability of the system and for obtaining reasonable peak shapes with FESI–MEKC. Using 0.75% BBMA in the BGS and twofold dilution of the BGS to prepare the samples (Fig. 3B), peak heights were improved by 14- and 23-fold, while corrected peak areas were improved by 60- and 106-fold, for estrone and estradiol, respectively. The skewed peaks observed (Fig. 3B) with these estrogens may be due to an overloading effect. A dilution of the BGS as used here was found to be more convenient than using lower concentrations of BBMA in the same buffer used for the BGS.

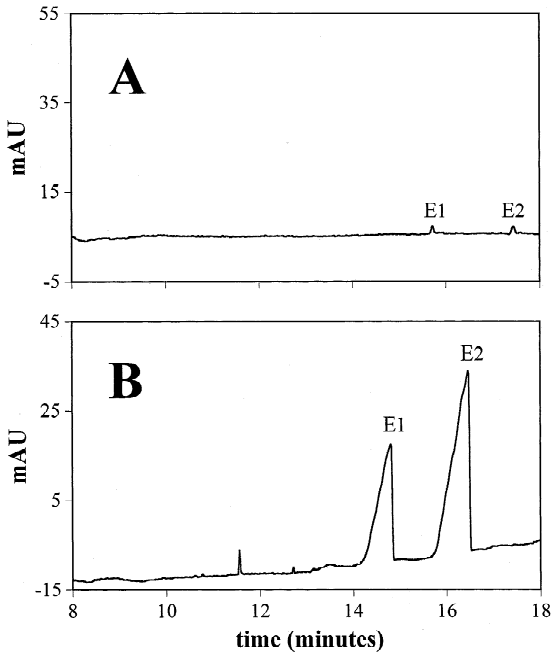

Fig. 3. On-line concentration of estrogens by FESI. Sample matrix, BGS (A), twofold dilution of BGS (B); BGS, 0.75% BBMA and 20% methanol in 50 m*M* sodium dihydogenphosphate–100 m*M* sodium tetraborate (pH 8.5); injection, 2 s at 50 mbar (A), FESI at −20 kV (the capillary had previously been injected for 1 min with a water plug; B, C); voltage, 20 kV. Peaks: Estrone (E_1) and estradiol (E_2). The concentration was 20 ppm and detection was at 200 nm.

4.3. Effect of the nature of the pseudostationary phase

Attempts using sodium dodecyl sulfate (SDS) as a pseudostationary phase for FESI–MEKC were of no avail. Micelles that had entered the water plug could have dispersed to their corresponding monomers due to enormous dilution. This is one disadvantage of low-molecular-mass surfactants versus high-molecu-

lar-mass surfactants with zero critical micelle concentration (CMC), such as BBMA. High concentrations of SDS (e.g. 100 m*M*) were studied with different concentrations of buffer solution (10 to 50 m*M* borate or phosphate at the same pH), and these resulted in very long migration times with high *k* test analytes and/or very poor peak shapes with all of the test analytes, which prevented us from optimizing the injection procedure.

4.4. Application to the analysis of estrogens

Estrogens were chosen because of their clinical relevance, specifically in the diagnosis of ailments related to their presence in biological fluids, such as urine. Urine was spiked with the selected estrogens. Direct dilution of the spiked urine with the chosen sample matrix (twofold dilution of the BGS) prior to electroinjection was not successful. Several other compounds were concentrated and migrated with the estrogen peaks, most likely urinary proteins. To eliminate these interfering substances, a clean-up scheme similar to that used in the high-performance liquid chromatography of estrogens was implemented [15]. Briefly, the estrogens were extracted with an equal volume of hexane (2 ml), the hexane extract was evaporated, the residue was dissolved in 0.3 ml of BGS and 0.3 ml of water, filtered and electrokinetically injected into the column. Since this was only a preliminary study, no further purification steps were employed. Fig. 4 shows the 2 s (A), FESI (B) and blank run (C) electropherograms. Hexane could be a selective liquid–liquid extraction solvent for the estrogens studied because no peaks that could have eluted with the test analytes were found in the blank run electropherogram. Improvements in detection were six- and sevenfold in terms of peak height and 23- and 29-fold in terms of peak areas for estrone and estradiol, respectively, compared to the results obtained following a 2-s injection. Improvements were obviously lower compared to those in standard samples, due to unknown interference. Further studies are in progress to optimize this procedure in the analysis of urinary steroids at levels of clinical relevance, including conjugated ones after conversion to the free forms.

In conclusion, FESI is a useful technique for the concentration of neutral analytes using only high-molecular-mass surfactants. The complex mechanisms involved will be the subject of further studies. Applicability is very simple and transparent, although, in this study, a clean-up step was required for a complex matrix (urine), as shown above.

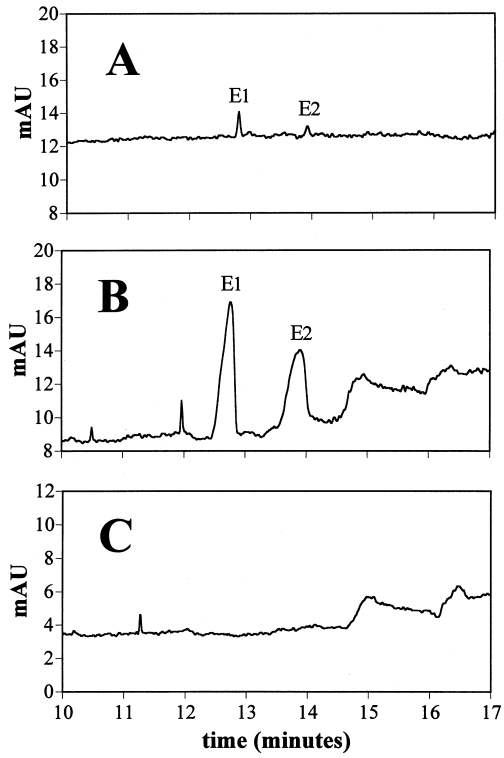

Fig. 4. On-line concentration of estrogens from spiked urine by FESI after liquid–liquid extraction. Sample: Urine containing 4 ppm estrogen (A, B), blank urine (C); Sample matrix, BGS diluted twofold; injection, 2 s at 50 mbar (A), FESI at −20 kV (the capillary had previously been injected for 1 min with a water plug; B, C); Detection was at 200 nm. Other conditions and the identity of peaks are the same as in Fig. 3.

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