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SEPARATIONS OF COMPOUNDS OF BIOLOGICAL AND ENVIRONMENTAL INTEREST BY MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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ABSTRACT

Important criteria for the effective separation of compounds of biological or environmental interest by micellar electrokinetic capillary chromatography are discussed. Efficiencies of approximately 100,000 plates/meter are achieved in the separations of samples of derivatized amines, aflatoxins, and hydroxy aromatic compounds. Laser fluorometric detection is shown to be capable of detecting subpicogram injected quantities. Organic solvents such as 2-propanol and acetonitrile are added to the aqueous mobile phases normally used to improve the separation of hydrophobic compounds, impart different selectivities, and provide a means for gradient programming. Column diameter is found to influence efficiency, analysis time, and detection.

INTRODUCTION

Because many samples of biological origin contain charged compounds (e.g., polypeptides, proteins, and polynucleotides), electrophoretic separation methods have been prominent in bioanalysis(1). A relatively new electrophoretic separation technique, capillary zone electrophoresis (CZE), has been demonstrated as an extremely efficient technique for the separation of charged biological compounds, particularly low

molecular weight compounds(2). The diminutive size of CZE columns (typically about 50 μm i.d. x 75 cm) facilitates the analysis of very small samples. Rapid separations are performed using large applied voltages (tens of kilovolts). The high efficiency of CZE can be directly attributed to the efficient dissipation of joule heat by the capillary. Temperature gradients that often disperse solute bands in electrophoresis are virtually nonexistent. The two main limitations with CZE are (1) detection is greatly complicated by the very small volumes of solute bands (typically in the nanoliter range) and (2) the technique is ineffective at separating uncharged solutes. Since many compounds of biological interest, and to a greater extent environmental interest, are normally uncharged, applications of CZE in those fields are restricted.

Two approaches have been directed at extending CZE to the separation of a wider range of compounds, including uncharged compounds. The first involves adding a large tetraalkylammonium ion to the mobile phase that imparts a charge to neutral solutes due to solvophobic interactions, thereby permitting electrophoretic separation(3). The second approach involves the addition of surfactant ions to the CZE mobile phase at concentrations above the critical micelle concentration (CMC)(4-16). We have termed this separation technique micellar electrokinetic capillary chromatography (MECC). With MECC, uncharged solutes are separated based on their differential partitioning between the electro-osmotically-pumped(17) mobile phase and the hydrophobic interior of the surfactant-formed micelles, which are moving at a velocity different from that of the mobile phase due to electrophoretic effects. Thus MECC exhibits characteristics common to both CZE and traditional partition chromatography. In particular the technique is capable of efficiencies comparable to CZE (hundreds of thousands of theoretical plates per meter) and is compatible with samples containing uncharged and/or charged solutes.

Fundamental studies that characterize the effects of experimental parameters on retention(5,6,8,15), efficiency(13), and selectivity(16) have been reported. MECC has been employed to separate a variety of compounds of biological or environmental interest. These separations include substituted benzenes(4,7, 16), derivatized amino acids(6,12), purine-type bases(11,4), metabolites of vitamin B_6(9), nucleosides and oligonucleotides(14), and derivatized amines(15).

To date, detection in MECC has been accomplished optically using either photometry or fluorimetry. Commercial HPLC absorbance detectors can be modified to be used in MECC by replacing the normal flow cell with a slit or pinhole assembly that is designed to limit radiation to a transverse path through the capillary along its diameter. Most of the studies and separations described above were performed using this type of detector. Short optical pathlengths, poor light throughput, and excessive stray radiation are problems with this mode of detection. Because of

the short pathlength, injected concentrations must be at least in the ppm range. This limits the utility of MECC for trace analysis. We have been able to achieve much better sensitivity (low to sub ppb minimum detectable injected concentrations) for fluorescent compounds using the laser excited fluorescence detection described herein.

While the examples of separations discussed above establish MECC as a technique that extends the applications of electrokinetic separation techniques beyond those possible in CZE, the full potential of MECC in biological and environmental analysis has not been realized because of the following; (1) the technique has a limited elution range (see results and discussion section) which limits resolution and peak capacity, (2) hydrophobic compounds all partition completely into the micelles (i.e., capacity factors are very large) and are not resolved, and finally, (3) current detection schemes for MECC are inadequate for most applications. In this paper we will discuss our use of laser excited fluorescence detection in MECC. The influence of column diameter on the technique will also be discussed. Further, we will discuss and illustrate how organic solvents can be added to the purely aqueous mobile phases normally used in MECC to improve mobile phase solubility of moderately hydrophobic compounds, thereby improving their resolution, provide some unique separation selectivities, and extend elution ranges.

EXPERIMENTAL

Apparatus and Procedures

The experimental configuration used in this work has been shown previously(10). Separations were performed using fused silica capillaries with dimensions of 50-75 μm i.d. x 75-150 cm obtained from Scientific Glass Engineering (Austin, TX). The capillaries were filled with surfactant/salt solutions (typically 0.05 M sodium dodecyl sulfate (SDS), 0.01 M Na_2HPO_4, and in some cases 0.005 M $Na_2B_4O_7$) and the ends of the capillaries suspended at equal heights in electrode reservoirs containing the surfactant/salt solution. Separations were performed by applying 8-30 kV potentials across the capillaries with a Model R40B Hipotronics regulated high voltage power supply.

Fluorometric and photometric detection was performed on-column by removing a short section of the protective polyimide coating from the capillary near the detection end to provide an optical window. When reporting column length, the dimension refers to the distance from the inlet to the detection zone. Photometric detection was accomplished using a commercial UV (254 nm) absorbance detector with a modified flow cell assembly that will be described elsewhere. This mode of detection was used for the separation of hydroxy aromatic compounds. Sensitive fluorometric detection was accomplished by using a Liconix Model

4230NB helium-cadmium laser (325 nm, 7 mW) or a Cyonics Model 2001-20BL argon ion laser (488 nm, 20 mW) for excitation. Fluorescence emission signals were isolated by an Instruments SA Model H-10 monochromator using one mm slits (6 nm bandpass) and detected with an RCA Model 1P28 photomultiplier tube. The emission wavelengths were 450 nm for the aflatoxins and 525 nm for the derivatized amines. Photocurrents were processed with a Pacific Precision Instruments Model 126 quantum photometer.

Sample plugs of 1-2 mm were injected at the inlet of the column using a previously described electromigration technique(11). Alternately, hydrostatic injections were accomplished by placing the inlet end of the capillary into the sample solution, then elevating the sample solution 20 cm for 10-15 seconds.

Step-wise mobile phase programs were accomplished by adding an appropriate aliquot of "strong" mobile phase (one containing organic solvent) to the surfactant/salt reservoir at the inlet of the column. This operation was performed with the solution stirred and the voltage momentarily shut-off.

Capacity factors (k') were calculated using equation 1(5)

$$k' = \frac{t_r - t_o}{t_o (1 - t_r/t_m)} \qquad (1)$$

where t_r is the retention time of the solute of interest, t_o is the retention time of a solute that is not solubilized by the micelles (generally marked by a solvent peak), and t_m is the retention time of a solute that is completely solubilized by the micelles. Elution orders were determined by injecting individual compounds or by spiking sample mixtures with the appropriate compounds.

Reagents

The surfactants used in this work were obtained from Sigma Chemical Co. Water used in preparing mobile phases was distilled and deionized. The Na_2HPO_4 and $Na_2B_4O_7$ were obtained from Fisher Scientific and the organic solvents that were sometimes added to the mobile phase were HPLC-grade.

A variety of classes of compounds were separated in this work. Aliphatic amines were obtained from Fisher Scientific and derivatized with the fluorescent label, 4-chloro-7-nitrobenzofuran (NBD-Cl) obtained from Sigma Chemical Co. The fluorescent laser dye Coumarin 153, used to mark t_m, was obtained from Exciton Corp. Other fluorescent compounds separated in this work include aflatoxins B_1, G_1 and G_2 (structures shown in Figure 4) and were purchased from Aldrich Chemicals. Separations involving

photometric detection were performed on mixtures of phenolic compounds, generally technical grade, that were obtained from various research laboratories within the chemistry department.

RESULTS AND DISCUSSION

Laser Fluorometric Detection

Two detection criteria that must be satisfied for the successful application of MECC to biological or environmental analysis are that (1) detector volume (for optical on-column detection this is the illuminated column volume) must be small enough not to disperse the MECC solute bands and (2) detection sensitivity must be adequate for trace analysis. Equation 2 can be used to calculate unretained solute band volumes, V, in terms of column radius, r, length, L, and plate count, N(18).

$$V = 4\pi r^2 L / N^{1/2} \qquad (2)$$

For a 50 μm i.d. x 100 cm column operating with a plate count of 500,000 (our best case) the calculated band volume is 11 nL. Detector volumes must be substantially less or solute band dispersion will occur.

We have found laser fluorometric detection meets the aforementioned criteria and can be used in those situations where the analytes of interest fluoresce or can be made to fluoresce through derivatization. The effective flow cell volume when the laser beam is focused to a 20 μm spot and passed through a 50 μm i.d column is a mere 20 pL. Although the pathlength is only approximately the diameter of the column, the sensitivity of fluorimetry, particularly with laser excitation, is sufficient for trace analysis. Figure 1 is a chromatogram of several aliphatic amines that were derivatized with NBD-Cl. The separation was accomplished by using a 50 μm i.d. x 150 cm column, a mobile phase that was 0.075 M SDS, 0.01 M Na_2HPO_4, and 0.006 M $Na_2B_4O_7$ and an applied voltage of 30 kV. The argon laser was used for fluorescence excitation. The first, fourth, and fifth peaks in the chromatogram are NBD-methylamine, NBD-butylamine, and NBD-cyclohexylamine, respectively. Calibration plots shown in Figure 2 for these compounds illustrate high sensitivity (absolute limits of detection based on a 5 nL injection volume(11) are less than 10 femtograms) and a linear dynamic range that extends over 5 orders of magnitude.

Two special considerations for fluorometric detection in MECC are fluorescence signal enhancement by the micelles(19) and heavy atom quenching by mobile phase components(20). The ordered environment of the micelle can reduce nonradiative deactivation of excited states and in some cases exclude quenching substances from

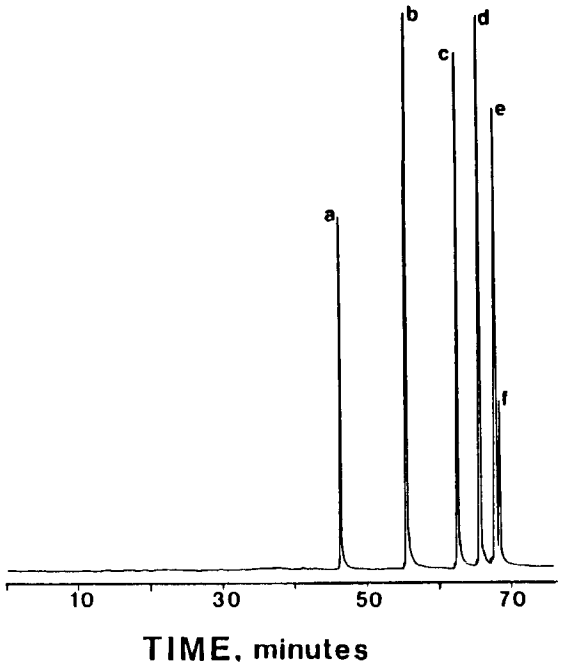

Fig. 1. Separation of NBD-Cl derivatives of the Aliphatic amines methylamine (a), n-propylamine (b), n-butylamine (c), cyclohexylamine (d), and n-hexylamine (e) and Coumarin 153 (f).

the vicinity of the fluorophor. This effect is illustrated in Figure 3. The peak height for NBD-methylamine is essentially the same with and without SDS micelles. The NBD-methylamine elutes very near t_o, indicating it is not solubilized very well by the micelles. In contrast, NBD-cyclohexylamine is solubilized quite well by the micelles and exhibits more than a ten-fold larger peak height when the micelles are present. Although this micelle enhancement may not be a general effect, it can be advantageous in certain situations when fluorescent compounds are separated. Adversely, care must be taken in MECC not to employ buffers or surfactants that contain heavy ions. For example, the peak height for NBD-methylamine was observed to be reduced by more than an order of magnitude when the cationic surfactant cetyltrimethylammonium chloride was replaced by the same surfactant bearing the heavier bromide counter ion.

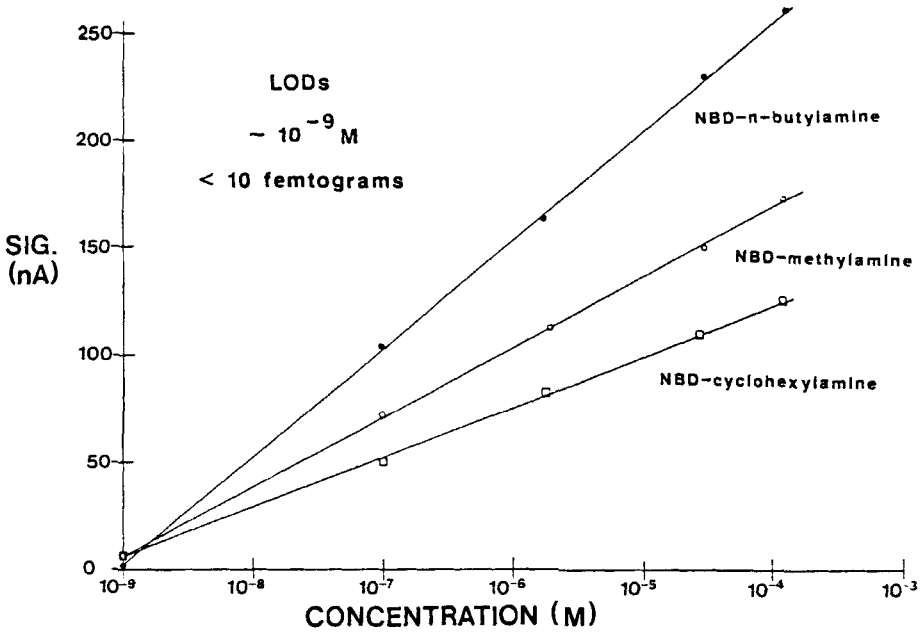

Fig. 2. Calibration plots for NBD-amines.

Influence of Column Diameter

An important experimental parameter in MECC is column diameter. It influences both the characteristics of the separation and the effectiveness of optical detection. Because less solute band dispersion is observed for electrokinetically-pumped capillaries(13), column diameters in MECC can be substantially larger than in hydrostatically-pumped capillary liquid chromatography (CLC). Typical column diameters in CLC are 5-20 μm, whereas MECC is performed with 25-100 μm i.d. capillary columns.

Although the influence of column diameter on efficiency is not as great in MECC as it is in CLC, better efficiency is observed with smaller i.d. columns. For example, we were not able to baseline resolve a mixture of 16 dinitrophenylhydrazene (DNP) amino acids in a one hour chromatogram using a 75 μm i.d. x 75 cm column, an applied voltage of 8 kV, and UV absorbance detection. Repeating the separation with a 50 μm i.d. x 75 cm column resulted in a lower current, thereby permitting the application of a higher voltage (20 kV) without the formation of dispersive temperature

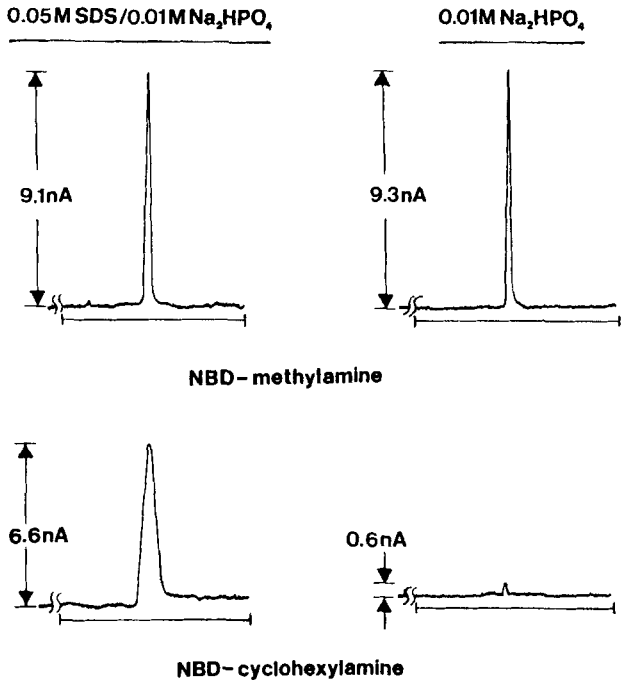

Fig. 3. Peak profiles illustrating micelle enhanced fluorescence detection.

gradients within the column(13). Furthermore, the efficiency roughly improved by an order of magnitude. The result was a near baseline resolved separation of the 16 DNP-amino acids in 25 minutes. Unfortunately, the smaller diameter column exhibited a large baseline noise level, presumably exacerbating the aforementioned problems of short optical pathlength and stray radiation.

Mixed (Organic-Aqueous) Mobile Phases

Adding organic solvents to the aqueous mobile phases normally employed in MECC can have beneficial effects. The first effect is to extend the elution range by reducing the electroosmotic flow velocity, V_{eo}, which is given by equation 3(17)

$$V_{eo} = \frac{-\epsilon \zeta E}{\eta} \qquad (3)$$

where ϵ and η are the permittivity and viscosity of the mobile phase, respectively, ζ is the so-called zeta potential at the capillary wall, and E is the applied field. Organic solvents affect ϵ and η but, more importantly, they can influence the ionic condition at the capillary wall (i.e., ζ). Alcohols such as 2-propanol interact strongly with silica surfaces and cause large reductions in V_{eo} (under certain conditions we have observed V_{eo} to be reduced by as much as 50% when the mobile phase is made 10% in 2-propanol), while solvents such as acetonitrile interact less with silica surfaces and have a much smaller effect on V_{eo}(21). Depending on the composition of the mobile phase we have observed slight increases or decreases in V_{eo} when acetonitrile is added to the mobile phase. In all our work the electroosmotic flow opposes the slower electrophoretic flow of the micelles. Therefore a reduction in V_{eo} can cause a dramatic reduction in the velocity of the micelles as the absolute magnitudes of the electroosmotic and electrophoretic flows approach the same value. For example, when the separation shown in Figure 1 was repeated using a mobile phase that contains 20% 2-propanol the last component eluted in 26 hours.

Another effect of adding organic solvent is to increase the mobile phase solubility of hydrophobic compounds and thereby reduce their capacity factors. In terms of resolution, the optimum capacity factor has been shown to be approximately 2(5). In certain cases specific interactions between organic solvents and solutes can also lead to beneficial changes in selectivity(21).

The theoretical importance of these organic solvent effects is manifested in the resolution equation derived by Terabe, et. al.,(5) for MECC (equation 4).

$$R_S = \frac{N^{1/2}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{k'_2 + 1}\right)\left(\frac{1 - t_o/t_m}{1 + (t_o/t_m)k'_1}\right) \qquad (4)$$

In this equation N is the average efficiency of the two components of interest, α is the selectivity coefficient, given by k'_2/k'_1, and t_o/t_m is an elution range parameter. Because of the last term in this equation compounds with large k's (compounds that elute near t_m, see equation 1) tend to be poorly resolved, particularly if the elution range is small (i.e., t_o/t_m is relatively large).

An example of an improvement in resolution with the addition of organic solvent is shown in Figure 4 for the separation of aflatoxins. Fluorescence detection was accomplished using the helium-cadmium laser for excitation. As seen from the figure the aflatoxins have very similar structures and are fairly hydrophobic. This separation was performed using a 50 μm i.d. x 65 cm column and an applied voltage of 25 kV. The second and

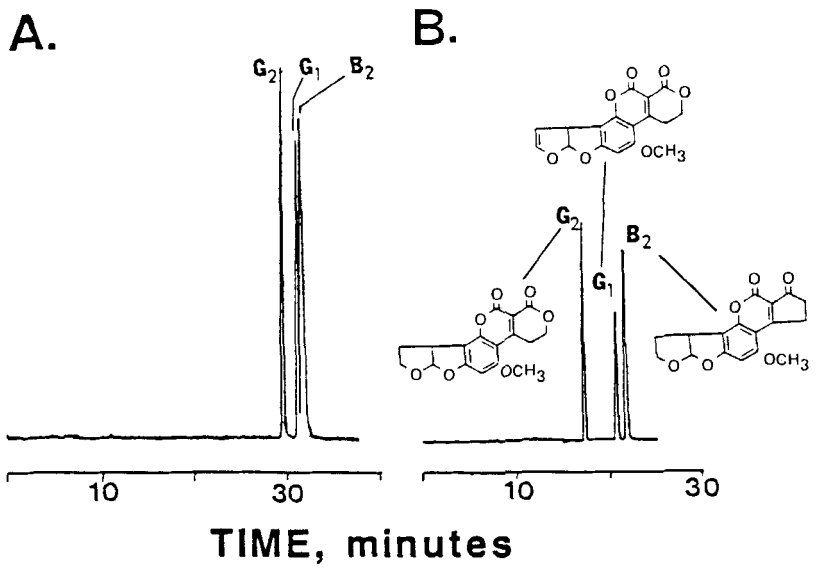

Fig. 4. Separation of aflatoxins. The injected amounts were approximately 10 pg. Separation and detection conditions given in text.

third aflatoxins to elute, G_1 and B_2, respectively, are not base line resolved (see Figure 4A) when the mobile phase is purely aqueous (0.10 M SDS, 0.02 M Na_2HPO_4). Markers for t_o and t_m were not included in this aflatoxin sample so capacity factors and the elution range parameter (t_o/t_m) can not be calculated. However, previous work with this system indicates that k's for the aflatoxins are very large (i.e., they are all eluting near t_m). When the mobile phase is made 10% in acetonitrile the k's are reduced to more optimum values. Under these conditions the aflatoxins are baseline resolved with a reduction in separation time (see Figure 4B).

The influence of the type of organic solvent on the MECC separation of hydroxy aromatic compounds was studied. When the separation was performed without organic solvent using a mobile phase that was 0.05 M SDS, 0.01 M Na_2HPO_4, and 0.005 M $Na_2B_4O_7$ several of the compounds were not resolved. This is shown in Figure 5A. The nitrophenol compounds (c, d, and e) and the dimethyl phenol compounds (f, g, and h) are not resolved under these conditions.

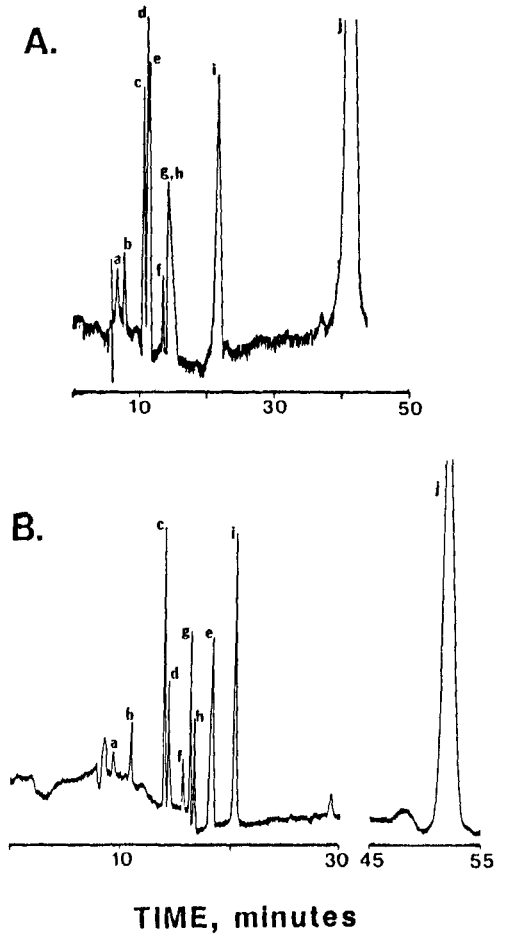

Fig. 5. Separation of resorcinol (a), phenol (b), o-nitrophenol (c), p-nitrophenol (d), 2,6-dibromo-p-nitrophenol (e), 2,6-dimethylphenol (f), 3,5-dimethylphenol (g), 3,4-dimethylphenol (h), β-naphthol (i), and 1-nitroanthracene (j) using a purely aqueous mobile phase (a) and a mixed aqueous-organic mobile phase with a step-wise solvent change (b). Separation and detection conditions given in text.

The elution characteristics of MECC resemble that observed in reversed phase liquid chromatography(16). Acetonitrile and 2-propanol have similar general eluting strengths in reversed phase liquid chromatography(22), and reduced k' values in MECC when added to the mobile phase. For example, the k' for β-naphthol was reduced from 6.4 to 3.1 when the hydroxy aromatic sample was separated using a mobile phase containing 10% acetonitrile. The retention time of the hydrophobic compound 1-nitroanthracene was used to mark t_m. In conventional reversed phase liquid chromatography, k' is reduced by organic solvents because of a reduction in the thermodynamic partition coefficient, K. In MECC organic solvents in the mobile phase reduce k' by reducing both K and the micellar phase volume(23). In this case the addition of 10% acetonitrile to the mobile phase also reduced t_o/t_m slightly from 0.13 to 0.10. Extending the elution range tends to spread-out the separated compounds. However, with acetonitrile the extension in elution range was not large enough to compensate for large reductions in k' values and the separated compounds were observed to "bunch-up" with short retention times. With 2-propanol the elution range is greatly extended. When this sample was separated with a mobile phase that contained 10% 2-propanol the 1-nitroanthracene did not elute after three hours.

We utilized the combined effects of 2-propanol and acetonitrile to effect a rapid baseline separation of the nine hydroxy aromatic compounds in this mixture (see Figure 5B). This was accomplished with a single step-wise solvent change during the separation. The initial mobile phase was 0.05 M SDS, 0.01 M Na_2HPO_4, and 0.005 M $Na_2B_4O_7$ in 95:5 H_2O: 2-propanol. The small percentage of 2-propanol served mainly to extend the elution range and provide conditions needed to resolve the early eluting compounds. Two minutes into the run an amount of acetonitrile was added to the inlet reservoir making its concentration 15% and slightly diluting the other components in the mobile phase. The acetonitrile served to reduce the k' values for the later eluting compounds (except for the anomaolous retention behavior of the 2,6-dibromo-p-nitrophenol discussed below). Baseline resolution of all the hydroxy aromatic compounds was accomplished in approximately 20 minutes.

Two other observations are apparent from Figure 5. First the mixed aqueous-organic mobile phase improved efficiency. Because of the poor sensitivity of the absorbance detector the concentrations of the compounds in this sample were near their solubility limits. The poor efficiency in Figure 5A is presumably due to phase overload. The mobile phase solubility of these compounds is improved with the addition of organic solvents. These solvents also influence selectivity as observed for 2,6-dibromo-p-nitrophenol (component e in Figure 5). Unlike the other compounds the k' of 2,6-dibromo-p-nitrophenol actually increased with the addition of these organic solvents. We offer no explanation for the anomalous retention behavior of this compound;

however, this illustrates that specific solvent-solute interactions can be exploited in MECC to affect selectivity.

CREDIT

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