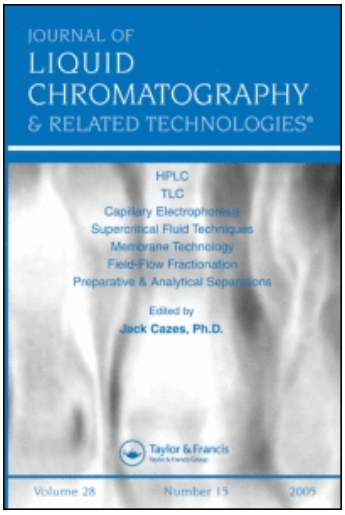

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Analysis of Antibiotics in Milk Using Open Tubular Capillary Electrochromatography

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Abstract: The analysis of five antibiotics (ampicillin, tetracycline, oxytetracycline, chlorotetracyline, and erythromycin) in milk by open tubular capillary electrochromatography is evaluated. The separation medium is an etched capillary chemically modified with the liquid crystal 4-cyano-4′-n-pentoxy-1-1′-biphenyl. A simple sample preparation method is used to produce a milk serum with a relatively small number of components and then the antibiotics are analyzed electrophoretically in this matrix. The effects of pH and addition of organic modifier to the mobile phase are also evaluated.

Keywords: Etched chemically modified capillaries, Stationary phases, Silanization/hydrosilation

INTRODUCTION

A capillary column configuration has been developed to improve the performance of open tubular capillary electrochromatography (OTCEC). The fabrication of this separation medium involves etching the inner wall of a fused silica capillary by heating it at a temperature of 300 or 400°C in the presence of ammonium hydrogen difluoride (NH_4HF_2) for three to four hours. Under these conditions, the surface area of the inner wall is increased by a factor of 1000 or more and radial extensions of up to 5 μm in length are created.[1,2] This process can partially alleviate the low capacity of the bare capillary, and shorten the distance solutes must travel to interact with a stationary phase

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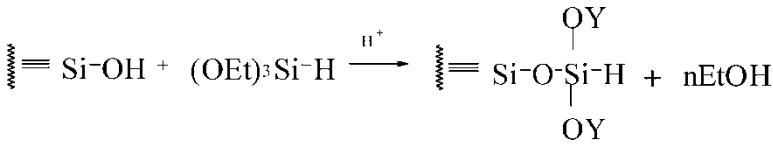
attached to the etched surface. Another feature is that elements from the etching reagents, nitrogen and fluoride, are also incorporated into the new surface matrix.[3] Their presence decreases some of the strong adsorptive properties of the silanols thus making the new surface more biocompatible. Further enhancements in capillary performance can be obtained from the bonding procedure used to modify the etched surface. In order to substantially eliminate the effects of residual silanols, a silanization reaction is used to create a new surface composed primarily of hydride moieties.[4,5]

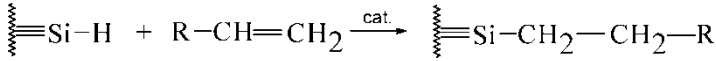
Silanization



Y = Si or H depending on the extent of crosslinking

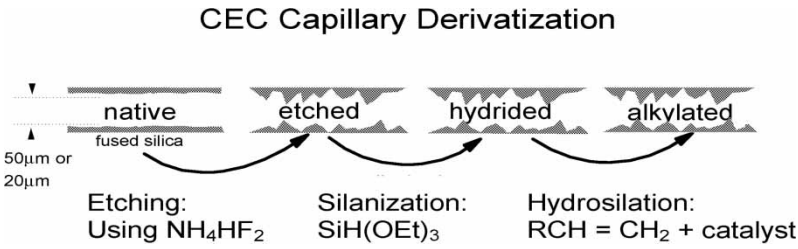
Under appropriate reaction conditions, the extent of crosslinking can be quite high (>95%) so that few residual silanols will remain on the surface. In order to attach an organic group to the surface that will determine some of the selectivity of the capillary, a hydrosilation reaction is used.[5,6]

Hydrosilation



cat = catalyst, metal complex such as hexachloroplatinic acid or free radical initiator such as *t*-butyl peroxide.

This process results in a stable silicon-carbon bond between the capillary wall and the organic moiety. The overall process is illustrated below.



As shown in the hydrosilation reaction and the diagram above, it is the "R" group that controls the chemical properties of the capillary surface.

Bonded materials with hydrophobic (octadecyl, butylphenyl), hydrophilic (diol), liquid crystal (cholesterol and cyanopentoxybipheynyl), and chiral (cycoldextrin and naphthethylamine) properties have been attached onto the etched inner wall.[7] These groups constitute the stationary phase that functions in the same manner as chemically modified silica particles in HPLC and CEC, and compounds physically coated on or chemically bonded to fused silica capillary walls in other formats of open tubular CEC.

The determination of antibiotics in a wide variety of biological samples has been an active area of research and method development for many years, due to the health implications of their use and subsequent residues in agricultural products for human consumption. The FDA has established guidelines for the allowable limits of a number of antibiotics such as tetracycline in food products like milk.[8] The focus of current analytical procedures and method development for monitoring antibiotics has been separation technology. HPLC using both diode array[9] and fluorescence[10] detection methods have been developed for the analysis of antibiotics in milk. More recently, the use of HPLC in conjunction with mass spectroscopy has provided more certain identification of species and better detection limits.[11,12] Capillary electrophoresis has also proven to be a viable method for the analyses of similar types of samples.[13,14] Some preliminary investigations using open tubular capillary electrochromatography (OTCEC), the technique described in this report, indicated that various tetracyclines could be effectively analyzed using etched chemically modified capillaries as the separation medium.[15] The advantages of OTCEC identified previously are studied further to determine how the method can be adapted to a milk matrix.

EXPERIMENTAL

Materials

The fused silica capillaries used had a 380 μm o.d. with a 50 μm i.d. inner channel and were obtained from Polymicro Technologies, Phoenix, Az, USA. Capillary dimensions for the etched modified capillaries were total length (L) = 50.0 cm and distance to the detection window (l) = 25.0 cm. The processes for etching a capillary with ammonium bifluoride[2] and bonding the stationary phase (4-cyano-4′-n-pentoxy-1-1′-biphenyl) have been described previously.[16]

Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ammonium bifluoride, the etching agent, was purchased from Sigma-Aldrich (St. Louis, MO/Milwaukee, WI, USA), triethoxysilane (Gelest, Morrisville, PA, USA), 1-octadecene, and hexachloroplatinic acid (Sigma-Aldrich) were used for the modification of the inner walls of the capillary by silanization/hydrosilation. The buffer materials were as follows: TRIS [tris [hydroxymethyl] amino methane], GABA (gamma-amino

butyric acid) (all from Sigma-Aldrich), and phosphoric acid (Fisher Scientific, Pittsburgh, PA, USA); and glacial acetic acid (Mallinckrodt, St. Louis, MO). The following were used as test solutes and were purchased from Sigma-Aldrich: ampicillin, chlorotetracycline, erythromycin, oxytetracycline, and tetracycline.

Instrumentation

The instrument used for high performance capillary electrochromatography (HPCEC) in this study was an Agilent (Waldbronn, Germany) 3D capillary electrophoresis system having a UV detector. The oven used for etching of capillaries was part of a Hewlett-Packard Model 5890 gas chromatograph. The GC oven was used for the control of the etching temperature and was modified so that multiple capillaries could be accommodated.

OTCEC Experiments

The following buffer compositions (diluted 1 : 10) and pH values were used in this study: pH 2.14, 0.3 mol/L H_3PO_4 and 0.19 mol/L TRIS; and pH 4.41, 0.3 mol/L acetic acid and 0.375 mol/L γ-amino butyric acid.

The milk samples were prepared as follows: To 100 mL of raw organic milk, 6 mL of 85% phosphoric acid was added. The sample was stirred for one to two minutes during protein precipitation. The sample was centrifuged for 10 min at 5000 G. The milk serum was vacuum filtered through a #2050 membrane filter. After 30 min, the sample was filtered again and the final filtrate was centrifuged at 10000 G. Under these conditions, the supernatant contains lactose and lactoglobulins as well as a number of other species. It is in this portion of the sample that antibiotics would be found, and where the test solutes were spiked for evaluation.

RESULTS AND DISCUSSION

The test samples used in this study are basic molecules that should be amenable to analysis by electrophoretic methods. The fact that these compounds can possess a positive charge below their pK_a leads to different migration rates in capillary electrophoresis measurements and the possibility of their separation and analysis. However, positively charged analytes can also adsorb on the capillary wall due to the negative charge on the silanols, even at relatively low pH. The previous CE analysis of tetracyclines[14] utilized high pH (8.5), so the compounds were neutral and a surfactant (SDS) to mitigate the surface effects. In a subsequent study utilizing an etched chemically modified capillary in the OTCEC format at low pH values so that the analytes were

positively charged, separation was possible without any additives in the electrolyte.[15] Therefore, a similar approach was used in this investigation, except that the chemical modification on the capillary surface was the liquid crystal 4-cyano-4′-n-pentoxy-1-1′-biphenyl (CPB) instead of octadecyl (C_{18}).

Figure 1 shows the electrochromatogram of a spiked milk plasma sample obtained on the CPB etched chemically modified capillary. Under these experimental conditions, each of the five test antibiotics is separated from the other milk components that are extracted in the serum sample. The first two peaks and the small peaks between components 3 and 4 are milk plasma components, identified by running the extract before the test

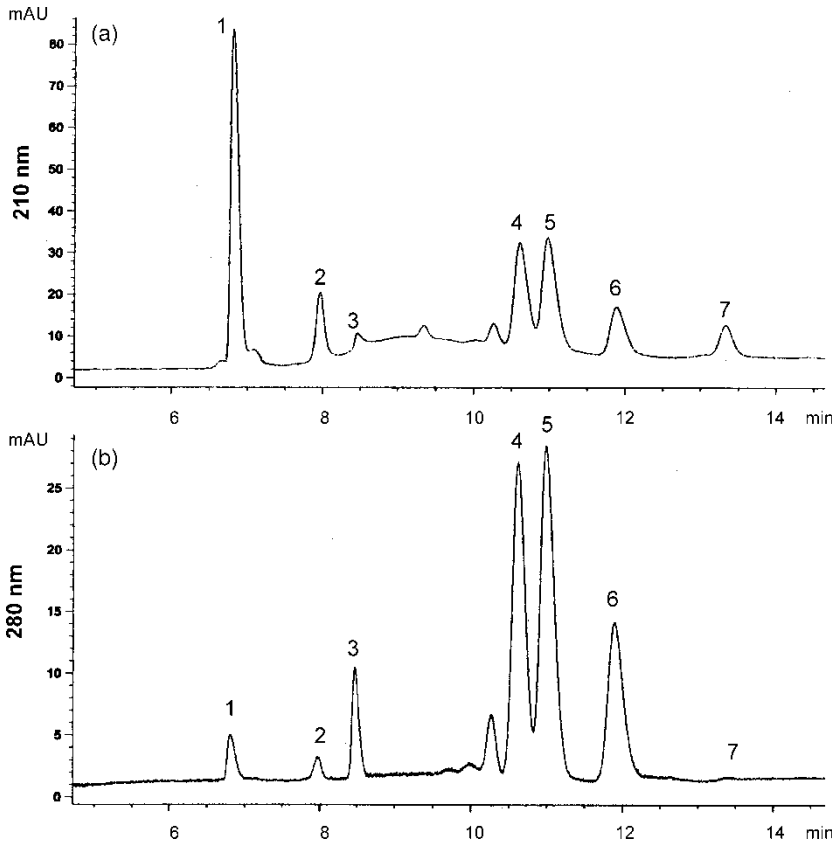


Figure 1. Capillary electrochromatogram of spiked milk serum sample at pH 2.14: A = detection at 210 nm and B = detection at 280 nm. V = 20 kV, injection for 3 s at 50 mm Hg. Solutes: 1 = plasma peak; 2 = plasma peak; 3 = ampicillin; 4 = tetracycline; 5 = chlorotetracycline; 6 = oxytetracycline; 7 = erythromycin; (all solutes at 90 ppm).

antibiotics are added. The five antibiotics and their migration times are verified by two means: 1) each compound is added individually into the serum extract; and 2) the UV-visible spectrum of each peak in the electrochromatogram is obtained and compared to the spectrum for the individual compounds run externally. The combined 3-D electrochromatogram/UV-visible spectral data for the spiked milk serum extract is shown in Figure 2.

In order to determine more about the OTCEC behavior of the antibiotics in milk serum samples as well as some characteristics of the CPB etched chemically modified capillary, some additional experimental conditions were tested. The most common variables in open tubular electrochromatographic experiments that can have a significant effect on migration, efficiency and peak symmetry are pH and addition of various organic modifiers to the mobile phase.[17–22] Figure 3 shows the result of changing the pH from 2.14 to 4.41. In this case, the migration times of all the antibiotics and plasma components decrease but resolution among the various analytes is maintained. The net result is that the same information provided in Figure 1 can be obtained with a decrease of about 10% in analysis time. The primary factor for the decrease in analysis time is most likely the change in electroosmotic flow. At low pH the EOF is anodic while at pH 4.41 it is near zero. Whatever the change in the overall charge on the analytes is for this pH change, it is not the controlling factor. With an increase in pH the charge should decrease and thus lower the electrophoretic mobility, leading to longer migration times. In addition, a significantly reduced charge on the analytes would likely lead to greater interaction with the stationary phase and, hence, longer migration times. Therefore, it seems that the changes observed in going from pH 2.14 to 4.41 are dominated by EOF variations.

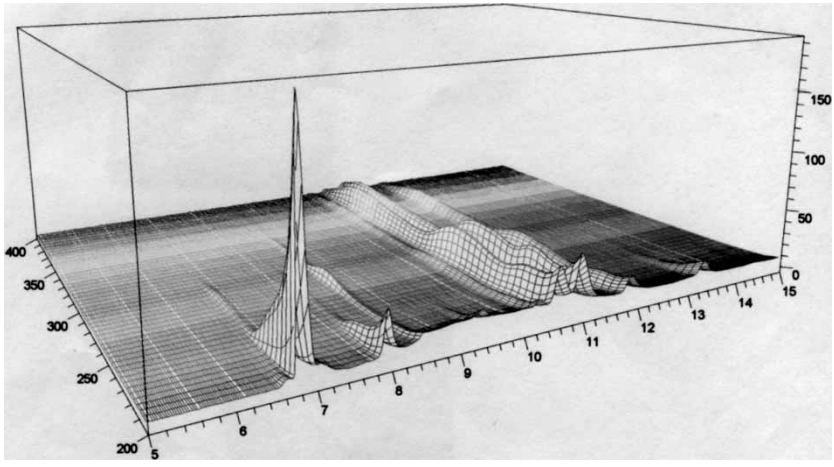


Figure 2. 3-D electrochromatogram/UV spectrum for the spiked milk serum sample. Conditions same as Figure 1.

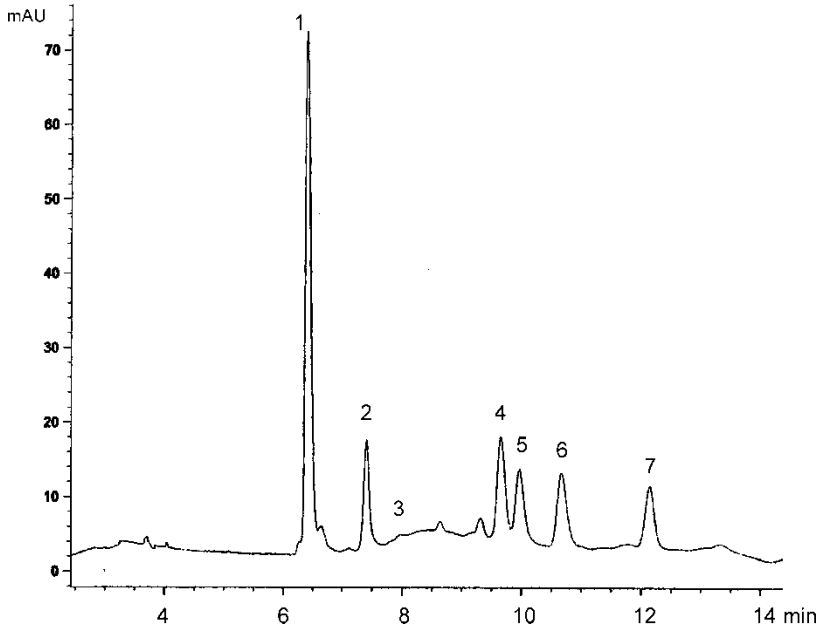


Figure 3. Capillary electrochormatogram of spiked milk serum sample at pH 4.41. Detection at 210 nm. Other conditions same as Figure 1.

Further increases in pH result in a loss of resolution among the analytes and an additional decrease in migration times.

The use of an organic modifier in the mobile phase was also assessed as a means of controlling retention and separation of antibiotics in OTCEC with the etched CPB modified capillary. Figure 4 shows the separation of the milk serum sample spiked with the test antibiotic mixture at pH 2.14 and with 10% MeOH added to the mobile phase. Several effects are evident here. First, the overall analysis time for the mixture is reduced from about 13 min in Figure 1 to about 8 min, with the MeOH in the mobile phase. This result can be explained by the decrease in retention (reversed phase chromatographic effect) as an organic modifier is added to the mobile phase. The second, a change in migration order, is seen with respect to the five antibiotic compounds in the test mixture. This could be due to either chromatographic or electrophoretic effects. Chromatographic effects can occur since each compound would have a different slope in a plot of $\log k'$ vs. % organic modifier in the solvent (Synder-Soczewinski equation) and thus, changes in elution order could result. Electrophoretic effects are possible since the relative degrees of ionization of the compounds can change with addition of organic modifier. The final effect observed, is that the number of peaks in the blank (plasma proteins) increases from two to three. As with the antibiotic test compounds, either chromatographic or electrophoretic effects could

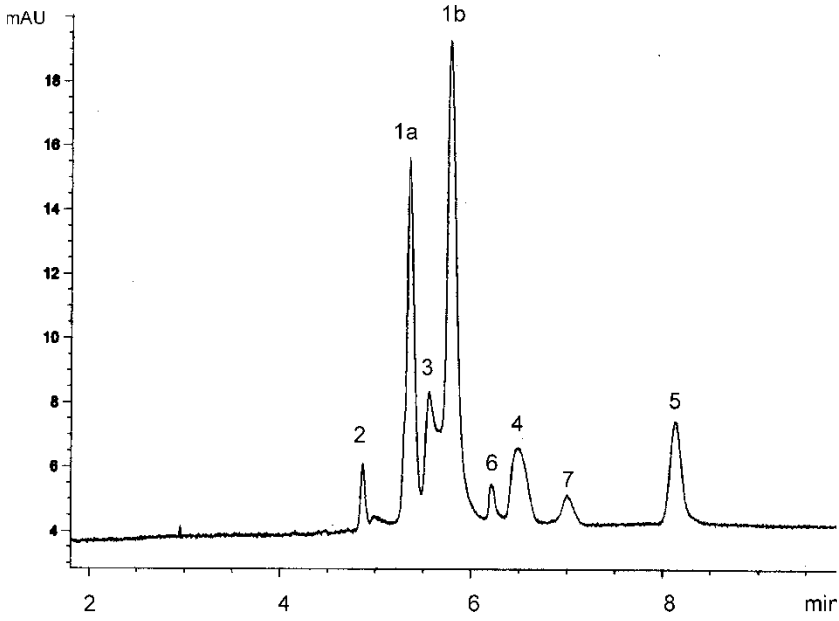


Figure 4. Capillary electrochromatogram of spiked mild serum sample at pH 2.14 with 10% MeOH in the mobile phase. Other conditions same as Figure 3 with peaks 1a, 1b and 2 being due to the serum blank.

explain this change in migration behavior. Thus, while it is clear that changes in EOF dominate the migration behavior when changing the pH from 2.14 to 4.41, it is not obvious if one mechanism, electrophoretic or chromatographic, or both in combination, control changes in migration as organic modifier is added to the mobile phase.

The net effect for the addition of MeOH to the mobile phase when analyzing antibiotics in milk serum samples is to reduce the overall analysis time. However, some loss in resolution has also occurred. The only problem with this set of experimental parameters is that ampicillin overlaps one of the plasma protein peaks. This might prevent detection at low concentrations and accurate quantitation, even at moderate concentrations (90 ppm) used in this example. However, the other four components are well separated (base line resolved) and could be accurately determined with the LOD being controlled by the method of detection.

CONCLUSIONS

It has been demonstrated that open tubular capillary electrochromatography, with an etched chemically modified capillary as the separation medium, is

feasible for the analysis of antibiotics in milk samples. With suitable sample preparation, relatively few peaks are obtained in the background, and these can easily be identified and separated from the five common antibiotics tested. Optimizing the separation can easily be accomplished through the use of two common experimental variables: pH and amount of organic modifier in the mobile phase. It was demonstrated that migration of the antibiotics in the method described in these experiments has both electrophoretic and chromatographic contributions.

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