Separation of Food Grade Antioxidants (Synthetic and Natural) Using Mixed Micellar Electrokinetic Capillary Chromatography

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A capillary electrophoretic method, for the determination of antioxidants present in food, has been developed using mixed micellar electrokinetic capillary chromatography. The buffer consists of sodium cholate (40 mM), sodium dodecyl sulfate (15 mM), 10% methanol, and 10 mM borate at pH 9.3. A separation was obtained for nine antioxidants (synthetic and natural) commonly found in food. High-performance liquid chromatography and capillary electrophoresis were applied to the analysis of sesame oil and wine. Ascorbic acid was identified in wine.

Keywords: *Mixed micellar electrokinetic chromatography; antioxidants; food analysis*

INTRODUCTION

Antioxidants in food are of considerable current interest. Not only do they play a major role in determining the quality of food products, but they may also participate in important biological processes that enhance health (Langseth, 1995). They are found either naturally or as additives in a large number of foods, where they prevent degenerative changes resulting from lipid oxidation.

A number of natural and synthetic antioxidants are permitted for use as food additives, the particular list varying from country to country. Those that may be used in Australia include propyl gallate (PG), octyl gallate (OG), dodecyl gallate (DG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (BHQ), ascorbic acid (AA) and its sodium salt, isoascorbic (erythorbic) acid (IAA) and its sodium salt, and the tocopherols (Australia New Zealand Food Authority, 1996). The permitted amount varies with the antioxidant and the food product. Australian regulations do not impose a limit on the amounts of tocopherols added to food, but maximum limits are imposed for the addition of other antioxidants (Australia New Zealand Food Authority, 1996).

Several methods of analysis have been developed to measure antioxidants in food, including high-performance liquid chromatography (HPLC) and gas chromatography (GC) (Grosset et al., 1990; Chen and Fu, 1995; Yentur et al., 1996; Gonzalez et al., 1998). Capillary electrophoresis (CE), a relatively new microseparation tool, has been applied very successfully to the analysis of food (Thompson et al., 1995; Kuo and Hsieh, 1997; Walker et al., 1997); however, its potential for the analysis of antioxidants in food has not been widely investigated. CE has developed rapidly over the past decade. It has fast analysis times when compared to HPLC, its separating capabilities exceed those of HPLC and are comparable with those of GC, and it consumes only small quantities of predominantly aqueous solvents (Terabe et al., 1984).

There are several modes of CE, including capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). CZE is an extremely useful tool for the separation of charged components and has been successfully employed to separate fully charged cations and anions (Jones and Jandik, 1992; Tangen et al., 1997; Boyce, 1998) as well as partially charged organics, such as phenolic acids at high pH (Cartoni et al., 1995). MECC, in contrast, is useful for the separation of neutral and partially charged species. In MECC a surfactant, commonly sodium dodecyl sulfate (SDS), is added to the buffer solution above its critical micelle concentration (cmc), so that the surfactant molecules aggregate to form micelles. The resulting micellar phase acts as a pseudostationary phase for neutral and partially charged organic solutes, and separation is dependent on the partitioning of the analytes between the aqueous and micellar phases. Selectivity can be altered to enhance separation by modifying the mobile phase and/or the micellar phase. The mobile phase is often modified through the addition of organic solvents such as methanol or acetonitrile. Organic solvents interact with the capillary wall, slowing the electroosmotic flow (eof) and extending the elution window. The migration factor or partition coefficient (k') is also altered as the polarity of the aqueous phase is reduced (Seifar et al., 1997; Kuhn and Hoffstetter-Khun, 1993). Another effective method of controlling selectivity is to modify the micellar phase directly. Although SDS is certainly the most popular anionic surfactant, other surfactants such as bile salts have proved to be very successful in separating nonpolar analytes that could not be resolved using SDS (Cole et al., 1991; Bumgarner and Khaledi, 1996). Combining surfactants with different structural and polar properties to generate mixed micelles has the potential to provide an infinitely variable micellar phase (Clothier and Tomellini, 1996). By tailoring the micellar environment in this way, solute−micelle interactions can be manipulated to generate the desired selectivity (Bumgarner and Khaledi, 1994).

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CZE and MECC have both been applied to the analysis of a wide range of foods and additives including vitamins, preservatives, and colorings (Choi and Jo, 1997; Kuo and Hsieh, 1997; Thompson et al., 1997; Walker et al., 1997). However, we could find only one paper dealing with the separation of antioxidants using CE. Hall et al. (1994) attempted to separate four synthetic antioxidants, BHA, BHT, BHQ, and PG, by both MECC and CZE. MECC was the preferred method as all four antioxidants are fully resolved, whereas components coeluted using CZE. However, using this MECC method, we found that DG and *dl*(α)-tocopherol, two other common antioxidants present in food, coeluted with BHT. The method described in this work separates natural and synthetic antioxidants, including DG, BHT, and *dl*(α)-tocopherol, in one analysis and has been applied successfully to food substances. The complementary nature of HPLC and CE is also highlighted.

MATERIALS AND METHODS

Chemicals. PG, OG, DG, BHA, BHT, BHQ, *dl*(α)-tocopherol, AA, IAA, sodium cholate (SC), and Sudan III were purchased from Sigma, Australia, and used as received. HPLC grade methanol, acetonitrile, and 2-propanol, analytical reagent grade disodium tetraborate, potassium hydroxide, and SDS were purchased from BDH Chemicals, Poole, England. The food samples were purchased locally.

Standards and Samples. The antioxidants were dissolved in a mixture consisting of 80:20 acetonitrile/water at concentrations of 600 μg/mL for *dl*(α)-tocopherol, BHA, BHT, and BHQ and 200 μg/mL for the remainder.

The antioxidants were extracted from sesame oil using the method described in the AOAC *Official Methods of Analysis* (AOAC, 1993). Oil (5 g) was dissolved in 20 mL of hexane (saturated with acetonitrile) and extracted three times with 50 mL aliquots of acetonitrile (saturated with hexane). The combined aliquots were evaporated to 3−4 mL, and the residue was transferred to a 10 mL volumetric flask using small portions of acetonitrile and then 2-propanol. The sample was filtered and applied to the HPLC and CE column.

Samples of white cask wine were filtered and applied directly to the CE column.

HPLC Conditions. HPLC analysis was carried out using a Varian 9010 gradient pump fitted with a Varian 9050 variable wavelength UV−vis detector and a Varian autosampler fitted with a 20 μL Rheodyne loop. The components were separated on an Altima C18, 5 μm column (250 mm × 4.6 mm, Alltech, Australia). A gradient elution method involving aqueous formic acid, methanol, and acetonitrile similar to that adopted by the AOAC (1993) was used. The flow rate was 2 mL/min, and the wavelength of detection used was 280 nm.

CE Conditions. MECC separations were carried out using a Waters Quanta 4000 system equipped with a 60 cm (52 cm effective length) × 75 μm i.d. fused silica capillary (Polymicro Technology, Phoenix, AZ). The capillary was conditioned daily by first washing with 0.5 M KOH (15 min), then with water (15 min), and finally with the running buffer (15 min). The running buffer consisted of 10 mM borate with differing concentrations of the surfactants SDS and/or SC added. Running buffers containing 5, 10, or 15% of additives including acetonitrile, methanol, and 2-propanol were also prepared and trialed. The samples were injected by hydrodynamic injection for 2 s unless otherwise stated and were run at ambient temperatures (23−25 °C) with an applied voltage of 18 kV. The detection wavelengths were 254 nm for the AAs and 214 nm for the other antioxidants. Acetonitrile and Sudan III were used to determine the retention time of a neutral unretained solute (t_o) and the migration time of the micelles (t_{mc}), respectively.

Determination of Chromatographic Data. The migration factor, k', is a measure of the ratio of the total moles of solute in the micellar phase versus those in the aqueous phase.

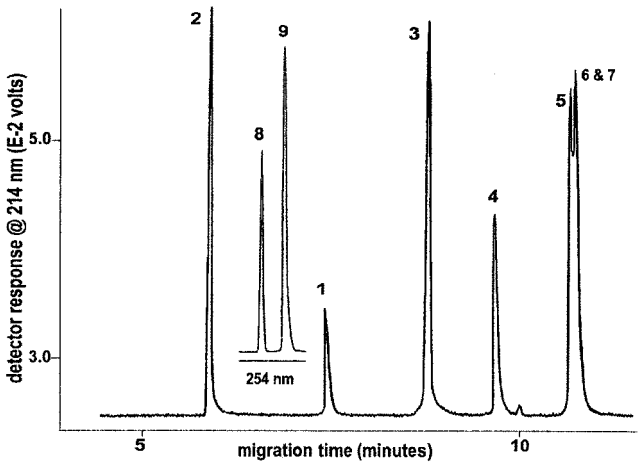

Figure 1. Separation of nine antioxidants: capillary, 52 cm effective length, 75 μm i.d.; applied voltage, 18 kV; buffer, 10 mM borate (pH 9.5) containing 50 mM SDS; temperature, ambient; detection, 214 and 254 nm. Peak identification: 1 = BHQ; 2 = PG; 3 = BHA; 4 = OG; 5 = BHT; 6 = DG; 7 = *dl*(α)-tocopherol; 8 = AA; 9 = IAA.

It was determined using

$$k' = \frac{t_r - t_o}{t_o[1 - (t_r/t_{mc})]} \quad (1)$$

where t_r is the migration time of the solute, t_o is the migration time of the electro-osmotic flow or a totally unretained component, and t_{mc} is the migration time of the micelles (Bumgarner and Khaledi, 1996). A component that is strongly retained by the micellar phase will have a large k value as its migration time approaches t_{mc}.

Theoretical plate efficiencies were calculated using the equation

$$N = 5.54(t_r/W_{0.5})^2 \quad (2)$$

where t_r is the retention time (in seconds) and $W_{0.5}$ is the width (in seconds) at half the maximum peak height (Weston and Brown, 1997).

RESULTS AND DISCUSSION

Factors that affect resolution (Rs) (eq 3) include efficiency (N), the migration factor (k'), selectivity (α), and the size of the elution window (t_{mc}/t_o) (Bumgarner and Khaledi, 1994). The migration factor and selectivity are influenced by the concentration and type of the surfactant and the presence of additives in the aqueous buffer. The surfactant type and concentration also influences the elution window as the mobility of the micelles is altered.

$$Rs = \frac{N^{1/2}}{4}\frac{\alpha - 1}{\alpha}\frac{k'_2}{1 + k'_2}\frac{1 - (t_o/t_{mc})}{1 + (t_o/t_{mc})k'_1} \quad (3)$$

Therefore, we explored the effect of varying the concentration and type of surfactant, and the effect of adding organic solvents to the buffer, on the separation of antioxidants.

Separation of Antioxidants Using SDS as the Micellar Phase. Initially, a 50 mM SDS borate buffer at pH 9.5, similar to that employed by Hall et al. (1994), was used to separate the antioxidant mixture. However, this buffer failed to resolve the nonpolar antioxidants BHT, DG, and *dl*(α)-tocopherol. The electropherogram is presented in Figure 1. The antioxidants BHT, DG,

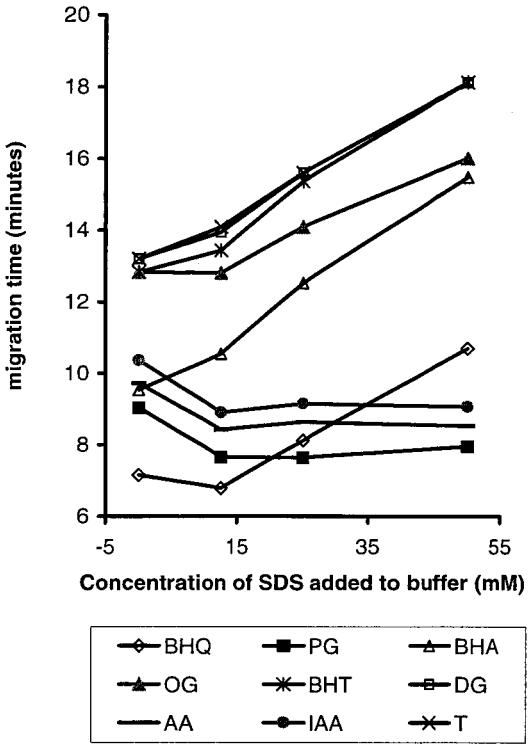

Figure 2. Effect of SDS concentration on the migration time of antioxidants. All other separation conditions were as in Figure 1.

and *dl*(α)-tocopherol eluted close to or with the micelles (t_{mc}). High capacity factors, typically 70−90, were recorded for these, indicating how strongly these antioxidants are retained by the micellar phase. The remaining antioxidants were fully resolved under these conditions, and optimal capacity factors in the range 1−5 were recorded for the more polar PG, BHQ, AA, and IAA.

The concentration of SDS added to the buffer was varied, to determine if surfactant concentration affected the resolution of the late eluting antioxidants. The migration time for BHT, BHA, BHQ, DG, OG, and *dl*(α)-tocopherol solutes increased with increasing SDS concentration (Figure 2). These solutes partition strongly with the micellar phase, and the increase in the migration times of these solutes can be attributed to the increased mobility of the SDS micelles as evidenced by the similar increases in t_{mc} (Figure 2). In contrast, the migration times for the more polar antioxidants PG, IAA, and AA did not change significantly with increase in the SDS concentration. These solutes favor the more polar aqueous phase, and as the mobility of the buffer is not influenced by the SDS concentration, as evidenced by the unchanging t_o in Figure 2, the mobility of these solutes is largely unaffected. As the concentration of SDS decreased from 75 to 25 mM, BHT was partially resolved from DG and *dl*(α)-tocopherol. However, *dl*(α)-tocopherol and DG coeluted at all concentrations of SDS due to their high affinity for the micellar phase. The reproducibility of the method for each concentration of SDS was good as the percent relative standard deviation (%RSD) for the migration times of the components was <0.6% over three runs.

The addition of organic solvents to the buffer can increase its polarity and reduce the capacity factor for less polar solutes. Organic solvents, and methanol in particular, also increase the elution window. The effect

Table 1. Migration Times for the Electro-osmotic Flow (t_o), the Micellar Phase (t_{mc}), and Elution Window (t_{mc}/t_o) Data for a 25 mM SDS Buffer Containing Various Amounts of Methanol

% methanol added to buffer	t_o	t_{mc}	t_{mc}/t_o
0	4.5	12.7	2.8
5	5.2	16.0	3.1
10	5.9	19.5	3.3
15	6.3	21.1	3.7

of adding organic solvents to the buffer on resolution was explored for BHT, DG, and *dl*(α)-tocopherol. Methanol was added in various amounts to a borate buffer containing 25 mM SDS buffer as this buffer slightly resolved BHT from DG and *dl*(α)-tocopherol. This addition increased the elution window (t_{mc}/t_o) (Table 1). BHT was increasingly resolved from DG and tocopherol as the percentage of methanol in the buffer increased. Fifteen percent methanol was sufficient to achieve baseline resolution of BHT from DG and *dl*(α)-tocopherol; however, *dl*(α)-tocopherol still eluted with DG and BHQ and PG also coeluted. The efficiency of the method was poor as the peaks were visibly broadened (separation not shown). This can be attributed to polydispersity. Polydispersity occurs when micelles have a range of velocities due to the presence of micelles with different aggregation numbers. The solutes associated with the micelles also have a range of velocities, which causes dispersion of the solutes and band broadening. Polydispersity is most pronounced when the concentration of surfactant is approaching its cmc (Cole et al., 1991). The cmc for SDS at ambient temperatures is ~8 mM; however, the addition of organic solvents raises the cmc for SDS (Cole et al., 1991). The low concentrations of SDS used in this experiment and the high percentage methanol (15%) added to the buffer are conducive to polydispersity.

When methanol was replaced with 2-propanol or acetonitrile, BHT and DG were partially resolved and band broadening due to polydispersity was again evident. The addition of organic solvents to buffers containing higher concentrations of SDS (50 and 75 mM) did not resolve BHT and DG. At these higher concentrations of SDS the nonpolar components remained largely solubilized in the micelle.

Separation of Antioxidants Using SC and Mixed Micellar Phases of SC and SDS. It is evident from the SDS results that the nonpolar antioxidants have a strong affinity for the SDS micellar phase. Although the addition of methanol reduced the affinity of the nonpolar antioxidants for the micellar phase, it was not sufficient to fully resolve the antioxidants. Bile salts have been used successfully in the separation of hydrophobic components (Nishi et al., 1990; Cole et al., 1991). Bile salts containing hydroxyl groups are more polar than SDS, and their use leads to a general reduction in capacity factors in MECC as the residence time of the nonpolar solutes in the micelle is reduced (Nishi et al., 1990).

The ability of a SC (50 mM) borate buffer to resolve the antioxidants was therefore investigated. DG coeluted with *dl*(α)-tocopherol, but BHT, although resolved from DG and *dl*(α)-tocopherol, coeluted with OG. The remaining antioxidants were fully resolved (separation not shown). The incorporation of a second or even a third surfactant into the micelle is a very effective method for tailoring the polarity of the micelle; therefore, the incorporation of SDS into the SC micelle was investigated.

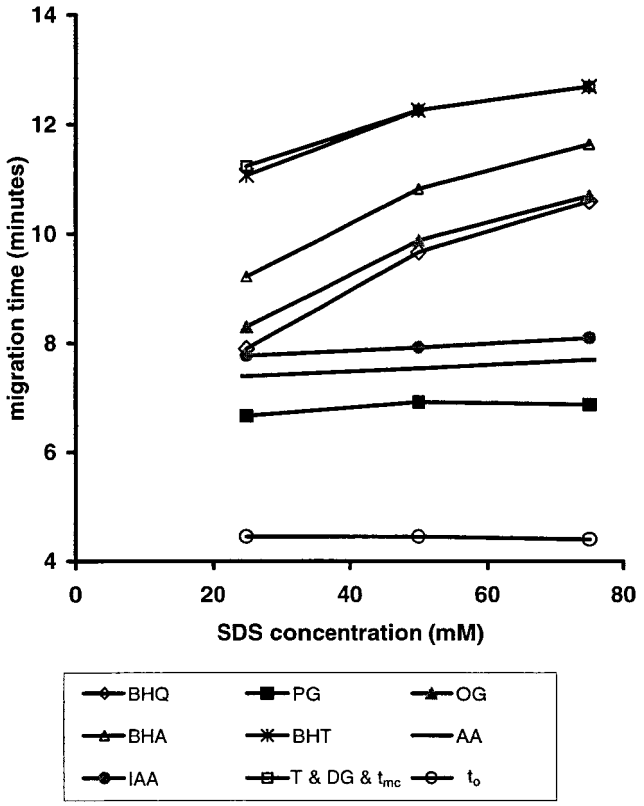

Figure 3. Effect of the presence of SDS in a buffer solution consisting of 50 mM SC and 10 mM borate on the migration time. All other separation conditions were as in Figure 1.

SDS (12.5, 25, or 50 mM) was added to 50 mM SC to determine if the resulting mixed micelle improved resolution. As the concentration of SDS increased in the micellar phase, the migration times for BHT, BHA, BHQ, DG, and *dl*(α)-tocopherol increased substantially (Figure 3). For the early eluting and more polar components (IAA, AA, and PG), incorporating up to ~15 mM SDS into the SC micelle decreased their affinity for the resulting micelle. Further additions of SDS had little effect on their migration times.

The antioxidants PG, AA, and IAA contain polar hydroxyl and/or carbonyl groups that interact weakly with the micellar phase, preferring the more polar aqueous phase. Migration times in the presence of the pure SC micelle were higher in response to their increased affinity for the more polar nature of the micellar phase. When the polarity of the micellar phase was decreased, with the addition of SDS, the solutes were retained less by the micelle and eluted earlier. In contrast, the migration times for BHT, BHA, and BHQ increased substantially as more SDS was added to the SC buffer. As the polarity of the micelles decreases, these antioxidants, possessing bulky nonpolar substituents, were readily incorporated into the nonpolar micelle and were retained longer.

At low concentrations of SDS, optimal resolution is achieved for the antioxidant mix. A borate buffer containing 50 mM SC and 12.5 mM SDS baseline resolves all but *dl*(α)-tocopherol and DG (Figure 4). The reproducibility of the method was good, and the %RSD for the migration times of the components was 0.4 or less (over three runs) (Table 2). The efficiency of the method was very good, with theoretical plate numbers in the range 10^4-10^5 (Table 2), which are typical values

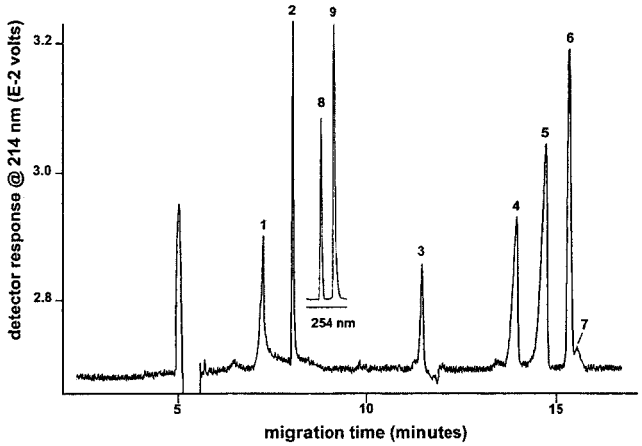

Figure 4. Separation of nine antioxidants: capillary, 52 cm effective length, 75 μm i.d.; applied voltage, 18 kV; buffer, 10 mM borate (pH 9.5) containing 12.5 mM SDS, 50 mM SC; temperature, ambient; detection, 214 and 254 nm. Peak identifications are as in Figure 1.

Table 2. Migration Time Data and Peak Efficiency Values for Selected Antioxidants Separated Using a Mixed Micellar System (50 mM SC and 12.5 mM SDS)

antioxidant	migration time, min (%RSD)	peak efficiencies, theoretical plates (SD)
BHQ	6.8 (0.4)	34280 (10160)
PG	7.65 (0.3)	168720 (21070)
BHA	10.56 (0.2)	61770 (310)
OG	12.83 (0.3)	17842 (1650)
BHT	13.47 (0.4)	64634 (16400)
DG	13.98 (0.3)	76353 (13090)
AA	8.488 (0.3)	127420 (1790)
IAA	8.934 (0.2)	122670 (3200)
tocopherol	14.21 (0.3)	

for MECC separations (Hall et al., 1994; Boyce and Bennett, 1996).

This method was successfully applied to wine samples. IAA and AA are often added to wines to prevent oxidative deterioration in color, flavor, and clarity (Madhavi et al., 1996). Spiking and comparison of retention data were employed to identify AA and IAA in several Australian white wines. Spiking is often advisable for MECC separations as the retention time of components in a sample mixture can differ from the retention time of the antioxidants in a standard mixture. However, for the wine samples the variation in retention time was minimal and the %RSD for the migration times of the antioxidants when real samples and standard solutions were compared was <0.4%. Figure 5 presents the electropherogram of a sample of white wine containing IAA. The efficiency of the separation for the real sample was consistent with that observed for the standard mixture. Theoretical plate counts in excess of 150000 were recorded for the IAA peak in the wine sample.

The method described above is not suitable for the simultaneous analysis of DG and tocopherol as they are not baseline resolved. The addition of 10% methanol to the 50 mM SC/12.5 mM SDS borate buffer fully resolved tocopherol and DG. The method was optimal with 40 mM SC, 15 mM SDS, and 10% methanol at pH 9.3 and 18 kV (Figure 6). The addition of methanol to this buffer did not reduce the efficiency of the method. A theoretical plate count in excess of 50000 was recorded for all components except BHQ, which recorded a theoretical plate count of ~15000 (Table 3). The reproducibility of

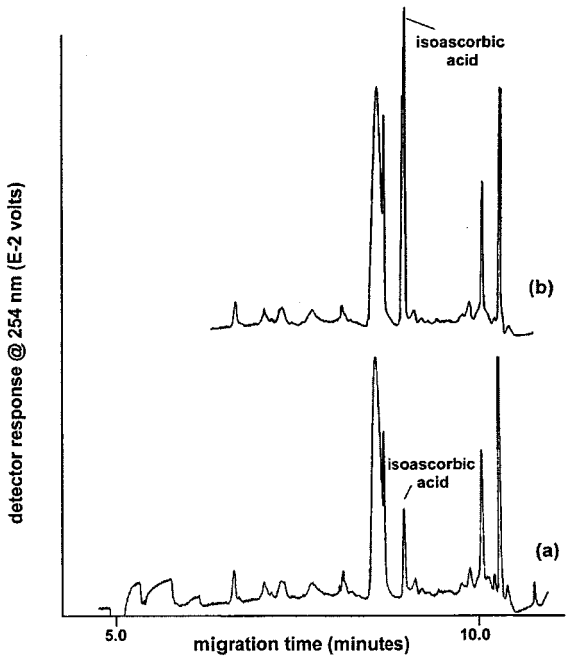

Figure 5. Electropherogram of a sample of wine: (a) wine sample only; (b) wine sample spiked with IAA. Operating conditions were as in Figure 4.

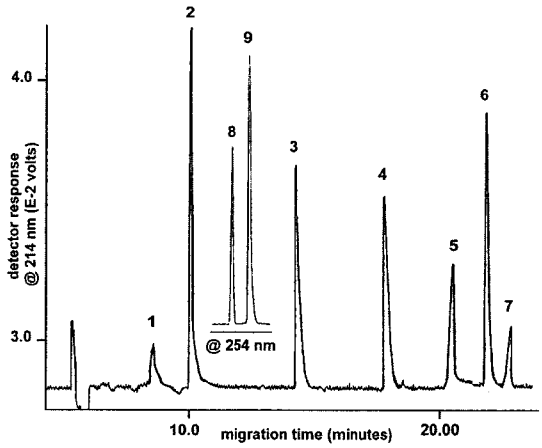

Figure 6. Separation of nine antioxidants: capillary, 52 cm effective length, 75 μm i.d.; applied voltage, 18 kV; buffer, 10 mM borate (pH 9.3) containing 15 mM SDS, 40 mM SC, 10% methanol; temperature, ambient; detection, 214 and 254 nm. Peak identifications are as in Figure 1.

Table 3. Migration Time Data and Peak Efficiency Values for the Antioxidants Separated Using a Mixed Micellar System (40 mM SC and 15 mM SDS) Containing 10% Methanol

antioxidant	migration time, min (%RSD)	peak efficiencies, theoretical plates (SD)
BHQ	9.18 (0.4)	13700 (490)
PG	10.54 (0.5)	172430 (2070)
BHA	14.28 (0.3)	55950 (16720)
OG	18.23 (0.3)	127020 (35540)
BHT	20.6 (0.1)	67030 (7810)
DG	21.05 (0.3)	180468 (9072)
tocopherol	21.85 (0.3)	70140 (15570)
AA	11.98 (0.1)	150340 (8500)
IAA	12.52 (0.2)	175430 (3495)

the method was good with %RSD for the migration times being <0.5% over three runs (Table 3).

The detection limit for these antioxidants, using this method and instrument, is in the range 1−10 μg/mL. However, the detection limits might be improved by using newer equipment and bubble capillaries. The light path length in HPLC is usually 1 cm, but in CE the path length is the width of the column, typically 50−75 μm. The use of a bubble capillary, where the width of the capillary is widened at the detection point, improves detection limits. Instruments that utilize a photodiode array detector, rather than filters, allow each component to be analyzed at the wavelength of maximum absorption, increasing the sensitivity of the method.

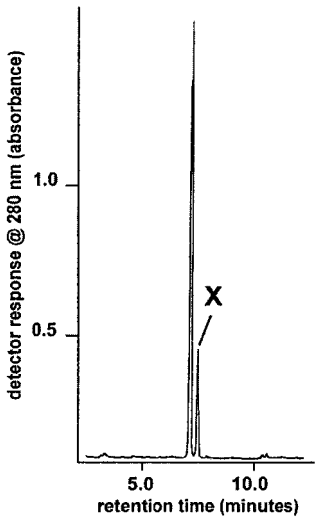

Figure 7. HPLC chromatogram of a sesame oil extract separated using a C18 analytical column (250 mm × 4.6 mm) and a mobile phase consisting of acetonitrile/methanol/water/formic acid run in gradient mode (see text for full details). Flow rate was 2 mL/min. Detection was at 280 nm. X is a compound with the retention properties of OG.

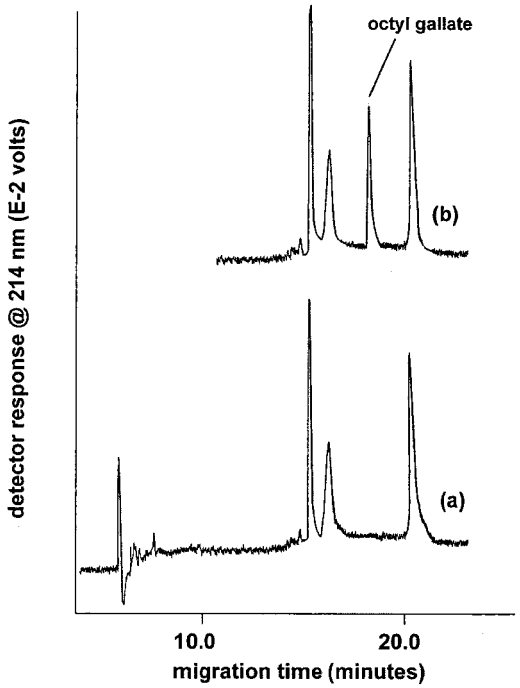

Figure 8. Electropherogram of a sesame oil extract: (a) sesame oil extract only; (b) sesame oil extract spiked with OG. Operating conditions were as in Figure 6.

Complementary Role of CE and HPLC. CE is a technique that can be used to advantage in conjunction with HPLC. For example, sesame oil was analyzed using the recommended AOAC HPLC method, and the resulting chromatogram had a compound (X) with migration

properties of OG (Figure 7). When the sample was analyzed by MECC, it was evident that OG was not present in the sample (Figure 8). CE provides a convenient second method of analysis for which no further sample preparation is required. In contrast, using GC in conjunction with HPLC requires derivitization of the sample prior to GC analysis.

Conclusions. Mixed micellar phases can be used effectively to separate a mixture of antioxidants, with similar and differing polarities. The composition of the micellar phase can also be modified to alter the solute−micelle interactions and obtain the desired separation. The addition of methanol to the buffer containing these mixed micellar phases improved resolution and did not reduce the efficiency of the method.

In the past 10 years there has been considerable effort to develop a range of microseparation techniques including micro-HPLC, CE, and capillary electrochromatography, which use lower quantities of organic solvents. We have shown that CE has the potential to be utilized in the determination of antioxidants in food as an alternative to HPLC or as a complementary technique.

ACKNOWLEDGMENT

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