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Journal of Chromatography A, 1079 (2005) 372–381
JOURNAL OF CHROMATOGRAPHY A
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Buffer system for the separation of neutral and charged small molecules using micellar electrokinetic chromatography with mass spectrometric detection

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Available online 19 March 2005

Abstract

An organic buffer system will be discussed that is suitable for the separation of neutral as well as charged molecules be means of micellar electrokinetic chromatography (MEKC). The buffers are based on the combination of a long chain alkyl acid, such as lauric acid with ammonium hydroxide or an organic base such as *tris*-hydroxymethylaminomethane (Tris). The resulting buffer system is able to separate neutral compounds based on its micellar properties. These buffers exhibit much reduced conductivity compared to traditional MEKC buffers, such as sodium dodecylsulfate (SDS), which contain inorganic salts. They also have inherent buffer capacity at high pH resulting from the basic buffer component, which in our studies had p*K* values from about 8–11. The separations that were observed showed high efficiency with plate counts in many cases above 500,000 plates per meter. The reduced conductivity allowed for the application of much higher electric fields, resulting in very fast analysis times. Alternatively, an increase in detection sensitivity could be achieved, as the reduced conductivity allowed for the use of capillaries with lager internal diameters. Combinations of different alkyl acids and organic bases provided for significant flexibility in selectivity tuning. Finally, the fact that the organic micellar buffer systems discussed here do not contain inorganic ions, allows for coupling with mass spectrometric (MS) detection. The possibility of MS detection combined with the high speed in analysis that can be obtained using these organic buffer systems, could make this approach an interesting option for high throughput analysis of combinatorial libraries.

Keywords: Micellar electrokinetic chromatography (MEKC); Lauric acid; Cholic acid; Organic buffer systems; High throughput analysis; Mass spectrometry (MS)

1. Introduction

Capillary based liquid separation methods have received wide attention over the past decade for their promise of improved separation efficiency and reduced sample needs. Capillary electrophoresis (CE) for proteins, capillary gel electrophoresis for DNA sequencing and chiral separations based on CE with chiral buffer additives have been some of the success stories in this field [1–6]. Recently, capillary electrochromatography (CEC), a hybrid technique between CE and high-performance liquid chromatography (HPLC) has attracted scientists as an alternative for the efficient separation of small molecules based on electrophoretic as well as hydrophobic selectivity [7]. This technique allows for the separation of neutral as well as ionic species because a stationary phase is utilized and high efficiency is achieved due to the replacement of pressure driven flow with electroosmotically generated flow. Despite its potential of high efficiency and tunable selectivity, CEC has not yet gained recognition as a rugged routine technique. This is mostly due to issues related to capillary technology that have not yet been adequately resolved relative to the mature technique of RP-HPLC.

Micellar electrokinetic chromatography (MEKC), displays similar features to CEC, as micelles establish a "moving stationary phase" and charged micelles allow the separation

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doi:10.1016/j.chroma.2005.03.015

of uncharged as well as ionic species. However, the micelle forming agents commonly used for this application are non-volatile and therefore not compatible with mass spectrometric (MS) detection.

Efficiency and speed are key success factors for the characterization of compounds made by automated parallel synthesis, a widely used approach to generate large numbers of pure compounds to be used in drug discovery efforts. The analytical method of choice for this application needs to be able to separate neutral as well as charged molecules in a generic fashion, since starting materials, potential side or degradation products and the final desired reaction product may have different characteristics. In addition, a mass spectroscopic detector is necessary to establish compound identity, because no calibration or reference standards are available a priori [8,9].

So far, the method of choice for this application has been HPLC–MS with additional UV detection for purity, despite the promise of capillary separation techniques. The dominance of HPLC in this field is due to the fact that CEC–MS is currently not rugged enough for a high throughput application that deals with large numbers of samples. MEKC on the other hand is rugged and can be easily automated, but currently prohibits the use of an MS detector, which is crucial in this application.

We have analyzed these current drawbacks in CEC and MEKC and believe that a buffer system, which allows MEKC separations with MS detection, could provide an alternative to the current LC–MS approach. The system that we will present is based on an aliphatic organic acid that is mixed with an organic amine or ammonia to establish a relatively volatile buffer system that forms micelles and therefore allows for the separation of neutral species. This approach combines the ruggedness and ease of use of MEKC with the MS compatibility of organic buffer additives commonly used with LC–MS methods. The potential of these buffer systems and their application to the fast separation of small molecules will be discussed in this paper.

2. Experimental

2.1. Chemicals and materials

All samples, such as phenyl alcohols, phthalic acid esters, and pharmaceutical compounds were of analytical grade and obtained from Sigma–Aldrich (Milwaukee, WI, USA). Lauric acid, cholic acid, sodium laurate, sodium cholate, *tris*-hydroxymethylaminomethane (Tris), triethylamine (TEA), diisopropylamine and ammonium hydroxide were purchased from Fluka (Milwaukee, WI, USA). The 100 mM sodium dodecylsulfate (SDS) buffer was purchased from Hewlett-Packard (Santa Clarita, CA, USA), and then diluted to 50 mM. The lauric acid/Tris buffer was prepared by adding 0.025 mole of lauric acid and 0.05 mole of Tris to 0.5 L of water, and cholic acid/Tris buffer was prepared using the same procedure. Sodium laureate buffer was prepared by adding 0.005 mole of sodium laurate to 100 ml 25 mM sodium tetraborate stock solutions in order to compare the current profile of the above buffer solutions, and sodium cholate buffer was prepared using the same procedure. All buffers were prepared fresh before use and pH was measured by an Accumet Research AR15 pH-meter (Fisher Scientific, Pittsburgh, PA, USA).

2.2. Instrumentation for CE

All separations were conducted on a Beckman P/ACE system MDQ (Beckman Instruments, Fullerton, CA, USA) controlled by P/ACE workstation version 2.0. The UV detector was operated at 214 nm. The cartridge coolant temperature was controlled at 25 °C. Fused silica capillaries of 50 and 75 μm I.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.3. MS infusion experiment

The infusion MS experiment was carried out on a Quattro I mass spectrometer (Micromass, UK) with electrospray ionization (ESI) interface in positive-ion mode. The ESI voltage was maintained at 3.5 kV. The cone voltage was set at 20 V. Samples were infused into the mass spectrometer through an infusion pump (Harvard Apparatus, Natick, MA, USA). A premixed sample of tetracaine in 50 mM cholic acid with 100 mM ammonium hydroxide buffer and another sample of tetracaine in 50 mM SDS with 25 mM sodium tetraborate buffer was prepared. Sheath liquid was a mixture of methanol and water (4:1) with 1% acetic acid. The sample and sheath liquid were introduced at 3 μl/min with a ratio of 1:2. The mass spectrometer scanned at a rate of 0.5 s/scan. Nebulizer gas flow (nitrogen) was maintained at 0.5 L/min.

2.4. Capillary electrophoresis–UV–MS experiment

The CE–UV–MS experiment was carried out on a Quattro I mass spectrometer (Micromass, UK) with ESI interface in positive-ion mode. The capillary voltage was maintained at 3.9 kV, and the cone voltage was set at 20 V. The sheath liquid, a mixture of methanol and water (1:1) with 0.5% acetic acid was infused through a syringe pump to the ESI interface at a rate of 20 μl/min. Ions at *m*/*z* 180.2, 195.2 and 223.1 were monitored using single-ion monitoring mode with a scan rate of 0.2 s/scan. Nebulizer gas flow (nitrogen) was maintained at 0.5 L/min. The voltage for the capillary electrophoretic separation was supplied by a Spellman power supply, CZE 1000R (Hauppauge, NY, USA) at 18 kV. Total length of the fused silica capillary was 90 cm (50 μm I.D.) with a length of 43cm between inlet and UV detector. An Accutec 500 UV–Vis detector was used in this experiment at 214 nm. Separation buffer was 50 mM cholic acid with 100 mM NH_4OH (pH 9.43). A mixture of caffeine (0.1 mg/ml), acetophenitidine (0.1 mg/ml) and flavone (0.1 mg/ml) was prepared in

water. The sample was introduced by manual pressure injection into the fused silica capillary.

3. Results and discussion

3.1. Proof of principle

MEKC separations based on SDS buffers have been reported to generate 100,000–300,000 plates per meter [10,11]. And the total actual efficiency generated in MEKC systems can be much higher compared to HPLC separations, as pressure is not a limiting factor here [12]. The commonly used MEKC buffer system, SDS to form micelles with borate as a buffer, is not compatible with mass spectrometric detection, due to suppression effects resulting from the high ionic strength and rapid contamination of the source resulting from the inorganic ions [13–15].

If SDS was replaced with an organic aliphatic acid and an organic amine were added to it, one should expect the resulting buffer system to have superior properties. The organic acid should form micelles at high pH and the organic base would provide the buffer capacity – all without introducing any inorganic ions. If the base were added in twice the concentration of the acid, the resulting pH can be estimated to be close to the p*K* of the base, as the acid will be completely deprotonated and the protonated and deprotonated form of the base will be present in equal parts. At that pH, one should also expect maximum buffer capacity of the organic base.

To prove this principle, lauric acid, the carboxylic acid homologue of dodecyl sulfate, was used in conjunction with Tris. The pK_a of lauric acid can be expected to be comparable to acetic acid (4.75). While lauric acid is insoluble at low pH, due to its hydrophobic alkyl chain, it dissolves upon addition of base, as the deprotonation of the carboxylic acid group renders it polar, and the solutions are stable as long as the pH is maintained about two units above the p*K* of the acid. The pH of the system was measured at 8.3, which is close to the p*K* of Tris (8.10) as expected. We also prepared cholic acid with Tris and the pH was found to be 8.5. For these preparations, the resulting pH will always be close to the p*K* of the base, independent of the carboxylic acid, as expected. To test the suitability of this buffer system for separating neutral compounds, we used a mixture of phenyl alcohols, which cannot be separated based on their electrophoretic mobility, unless interacting with a hydrophobic selector, such as a stationary phase or a micelle. The results of this experiment are shown in Fig. 1 together with the separation obtained in a conventional SDS buffer for comparison. The SDS separation efficiency for the second peak is about 430,000 plates per meter, much higher than usually reported to be possible in MEKC. The lauric/Tris system allows separation of the phenyl alcohols as well, which indicates that our hypotheses holds true, that micellar separations should be possible in such systems. The efficiency for the second peak (phenethyl alcohol) was about 575,000 plates per meter, which was even higher compared

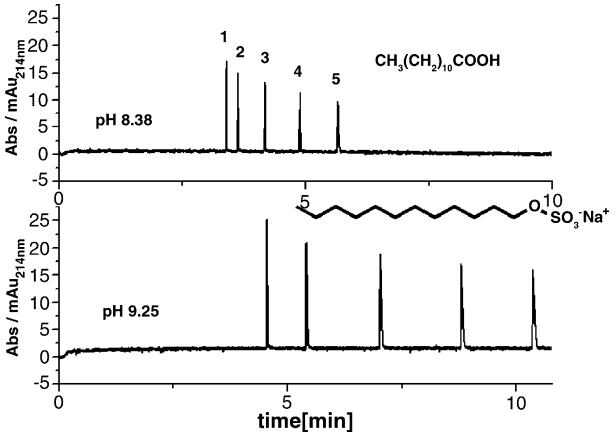

Fig. 1. Separation of a mixture of phenyl alcohols. Capillary: fused silica 60/50 cm with 50 μm I.D., 25 °C, separation voltage: 30 kV, sample: phenyl alcohols (1, benzyl alcohol; 2, phenethylalcohol; 3, 3-phenyl-1-propanol; 4, 4-phenyl-1-butanol; and 5, 5-phenyl-1-pentanol), pressure injection at 1.5 psi s, data acquisition rate: 32 Hz, top: 50 mM lauric acid/100 mM Tris, pH 8.38, bottom: 50 mM SDS/25 mM sodium tetraborate buffer, pH 9.25.

to the SDS system. The selectivity for the set of pheny alcohols is slightly different between the SDS system and the organic micellar system. The elution of this set of samples occurs faster in the organic micellar system. If we assume a similar mobility for the laureate micelles and the dodecylsulfate micelles, as well as a comparable electrosomotic flow in both systems (see Table 1), this would indicate a weaker interaction between the phenyl alcohols and the lauric acid micelles, compared to the SDS micelles. This example clearly demonstrates the utility of the organic micellar systems for the efficient separation of neutral compounds.

3.2. Electroosmotic flow (EOF) in MEKC buffers

EOF is the main factor that determines elution in systems using anionic micelles [11]. Analytes will be transported as a result of the bulk flow towards the cathode. Therefore, EOF is one of the key factors determining the separation speed. The organic micellar system as described above has shown very high theoretical plate heights. For applications in high throughput analysis the number of plates generated per time unit is an important measure, combining aspects of efficiency and speed. We decided to use DMSO as the marker for EOF, assuming that due to its polar nature it would not interact with the micelles. Any interaction of the DMSO with the micelles would delay its elution, and therefore, provide EOF values that are too low. The interaction, however, can be expected to be very similar for all the micellar systems investigated here, so that the values should definitely allow for a relative comparison between micellar systems. In Table 1, we compared EOF data for the SDS system, as well as a variety of lauric acid and cholic acid (another alkyl carboxylic acid investigated as a potential organic micellar system) based micellar systems. The EOF velocities can be as fast as 0.295 cm/s in 50 mM lauric acid with 100 mM TEA and 0.249 cm/s in

Table 1
EOF and linear velocity for various micellar buffer systems; capillary: 50/60 cm with 50 μm I.D., voltage 30 kV

Buffer	Buffer pH	Linear velocity (cm/s)	EOF ($(cm^2/V\,s) \times 10^{-4}$)
50 mM SDS/25 mM sodium tetraborate	9.2	0.251	5.03
50 mM lauric acid/100 mM Tris	8.68	0.285	5.72
50 mM cholic acid/100 mM Tris	8.25	0.227	4.56
50 mM lauric acid/100 mM TEA	10.85	0.295	5.92
50 mM cholic acid/100 mM TEA	10.81	0.249	5.00
50 mM lauric acid/100 mM ammoniumhydroxide	9.63	0.289	5.80
50 mM cholic acid/100 mM ammoniumhydroxide	9.58	0.248	4.98
50 mM lauric acid/100 mM diisopropylamine	11.26	0.260	5.22
50 mM cholic acid/100 mM diisopropylamine	11.14	0.213	4.27

Table 2
pH, current and conductivity for various micellar buffer systems; capillary: 50/60 cm with 50 μm I.D.

Buffer	pH	Voltage (kV)	Current (μA)	Conductivity (mS/cm)
50 mM SDS/25 mM sodium tetraborate	9.25	30	54.58	5.58
50 mM lauric acid/25 mM sodium tetraborate	9.23	30	57.10	5.84
50 mM cholic acid/25 mM sodium tetraborate	9.67	30	61.46	6.28
50 mM lauric acid/100 mM Tris	8.38	30	10.53	1.08
50 mM cholic acid/100 mM Tris	8.36	30	17.71	1.81

50 mM cholic acid with 100 mM TEA. There is some impact of the organic base as well, with ammonium hydroxide, TEA and Tris giving the highest values, while the use of diisopropylamine provided slightly lower EOF values. The EOF values found for the organic micellar systems are similar or slightly above the EOF value for the conventional SDS micellar system The slightly lower EOF observed for the cholic acid system may be the result of the higher molecular weight of this acid, and therefore, increased viscosity of this micellar agent, compared to lauric acid.

3.3. Conductivity

The maximum speed of liquid transport in the capillary is dependent on the EOF but also on the conductivity of the buffer systems. The lower the conductivity the higher the electric field that can be applied without sacrificing separation efficiency due to Joule heating, which is a key consideration when aiming for fast separations. Table 2 lists the measured conductivity values of some of the buffer systems under investigation.

The traditional SDS system with sodium tetra borate has a very high conductivity value with 5.58 mS cm^{-1}. Similar conductivity values are found, when the sodium salts of lauric acid and cholic acid were used. The lauric acid/Tris buffer system has a very low conductivity value with 1.08 mS cm^{-1}. A significant reduction of current is therefore observed when the inorganic cation is eliminated and replaced with an organic base, as in the lauric acid and cholic acid systems with Tris. This indicates that the sodium ion bears the major responsibility for the high conductivity observed in the traditional SDS system, not the micelle itself. Lauric and cholic acid mixed with organic bases have significantly lower currents at identical field strength. As can be seen in Fig. 2, a current above 50 μA was observed at 500 V/cm field strength by using a buffer of 50 mM of the sodium salt of lauric acid with 25 mM sodium tetraborate. However, a current less than 10 μA was registered under the same field strength when using 50 mM lauric acid with 100 mM Tris. At the same time, the system with lower conductivity can be expected to have even better buffer capacity. The buffer concentration is significantly higher (100 mM organic base, versus 25 mM tetra borate) and the system is operating right at the pH that corresponds to the p*K* of the organic base. It is well known that separation efficiency increases with increasing field strength within the liner range of Ohm's law [16]. By using low conductivity buffers, the analysis can be performed in a much higher electric field range,

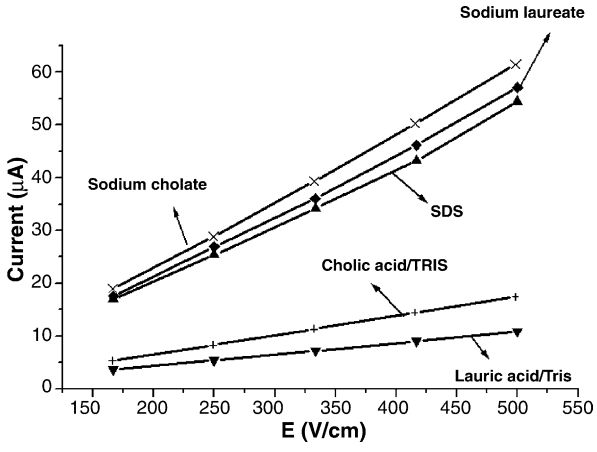

Fig. 2. Current profile of buffer systems. Capillary: 50/60 cm with 50 μm I.D., 25 °C, buffers as following: SDS: 50 mM SDS/25 mM sodium tetraborate, pH 9.25; sodium cholate: 50 mM sodium salt of cholic acid/25 mM sodium tetraborate, pH 9.67; sodium laurate: 50 mM sodium salt of lauric acid/25 mM sodium tetraborate, pH 9.23; cholic acid/Tris: 50 mM cholic acid/100 mM Tris, pH 8.36; lauric acid/Tris: 50 mM lauric acid/100 mM Tris, pH 8.38.

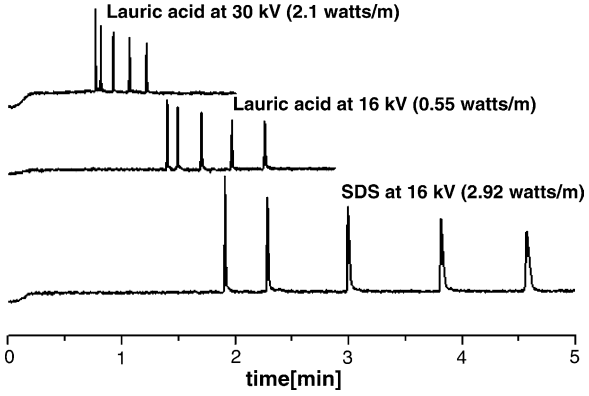

Fig. 3. Comparison of SDS and lauric acid system at the power limits of the instrument (3 W/m). Capillary: 30/20 cm with 50 μm I.D., 25 °C, sample: phenyl alcohols, pressure injection at 0.6 psi s, data acquisition rate 32 Hz, buffer systems: SDS with 25 mM sodium tetraborate, pH 9.25 and 50 mM lauric acid with 100 mM Tris, pH 8.38.

which therefore improves efficiency and speed of separation.

3.4. Fast separations

Fast separations can be achieved with buffer systems that provide high EOF and allow the application of high fields. Our previous investigations indicate that the organic micellar systems described here fulfill both requirements. The reduced power output generated by using less conductive buffer systems allows for the application of higher separation voltages, without exceeding the instrument performance range. Therefore, higher separation efficiency and shorter separation times should be achievable.

In Fig. 3, the separation of five phenyl alcohols using a 50 mM lauric acid/Tris system is compared to the separation using the traditional 50 mM SDS system with 25 mM sodium tetra borate. The two traces at the bottom compare the two systems under the same high voltage, using a 20/30 cm capillary with 50 μm I.D. At an applied field of 16 kV, the SDS system was running at 2.92 W/m, close to the recommended power limit of the instrument (manufacturer lists the power limit for the P/ACE_MDQ instrument at 3 W/m). The SDS system is therefore operating at its maximum speed and increasing the field would cause the separation to deteriorate due to excessive heating in the capillary. At a field of 16 kV, the lauric acid/Tris system only produced 0.55 W/m of power, significantly less, due to the reduced conductivity. The separation with the lauric/Tris system is faster under identical electric field strength due to the slightly higher EOF and decreased interaction of the probes with the micelle as discussed earlier. This is probably due to the higher polarity of lauric acid and it is known that a smaller separation window indicates a less lipophilic micelle. Because of its lower conductivity, the lauric acid/Tris system can be operated at the maximum voltage of the instrument (30 kV) with only 2.1 W/m of power generated. Increasing the field strength in this case enables the lauric acid/Tris system to provide an even faster separation. As can be seen in the top trace in Fig. 3, the last peak in the mixture was observed at 1.22 min under these conditions as compared to 4.56 min using the SDS system at the power limit of the instrument. This is an improvement in speed by almost a factor of 4. Furthermore, due to the decreased Joule heating, higher efficiency was achieved by using the lauric acid system over the SDS system, as can be seen in Table 3. For instance, the plate counts of benzyl alcohol were 621,000 for the SDS system at 16 kV, but almost 30% more for the lauric acid/Tris system, which generated 810,000 N/meter at the same field strength. If the plates generated were based on elution time, we can assess the efficiency that is generated per time unit, which is an important parameter that allows comparison of different separation systems with respect to their productivity. The SDS buffer system generated 1090 plates per second at 16 kV, while the lauric acid/Tris system generated 3080 plates per second at 30 kV, with both systems operating at the corresponding instrument limit as shown in Table 3. This translates into a gain of separation speed for the organic micellar system of almost a factor of 3, while providing the same separation efficiency.

Table 3
EOF, efficiency and separation productivity at the instrument limits of voltage (30 kV) and power (3 W/m); buffer: 50 mM SDS with 25 mM sodium tetraborate and 50 mM lauric acid with 100 mM Tris, capillary: 20/30 cm with 50 μm I.D.

Buffer system	SDS	Lauric acid/Tris	
Voltage (kV)	16	16	30
Power (W/m)	2.92	0.55	2.10
EOF (10^{-4} cm^2/V s)	5.0	5.7	5.7
Plates per meter (first peak)	621,000	810,000	708,000
Plates per second (first peak)	1090	1930	3080

Similar to fast HPLC applications, it is crucial to match the data acquisition rate and the detector response to the speed of fast eluting sample peaks to realize maximum peak resolution [9]. As demonstrated in Fig. 4, a mixture of five different phenyl alcohols can be separated within a very short time frame of less than 1 min. Such short analysis times can be achieved using organic micellar system based on lauric acid/Tris and injecting the sample on the short end side of the capillary [17–19], with the detection window closer to the capillary end (about 10 cm). With data acquisition rate set at 32 Hz, well-resolved, sharp peaks were obtained. Decreasing the acquisition rate causes peaks to broaden significantly in the detector trace. At a data acquisition rate of 2 Hz the resolution for this sample is almost completely lost. This effect is solely due to the detector settings, as the physical zone width has not changed at all, as all separations were performed under identical conditions. While the instrument parameter is referred to as acquisition rate in the instrument manual and software, it is obviously rather an electronic filter, most widely referred to as time-constant. Most detectors use electronic signal filtering to reduce background noise to improve signal-to-noise ratio. In conventional analysis this

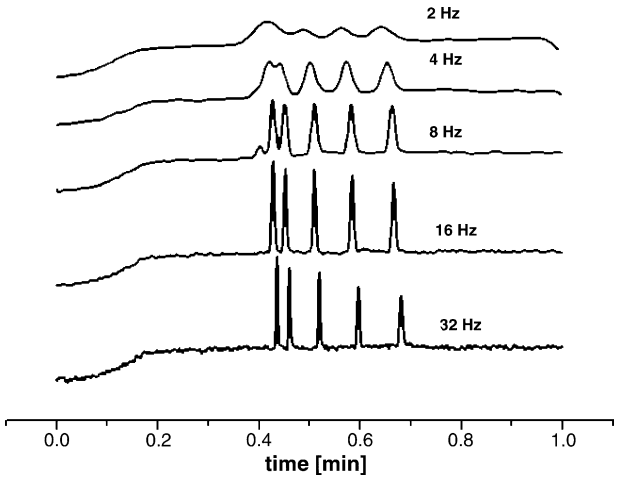

Fig. 4. Impact of data acquisition rate. Capillary: 30/20 cm with 50 μm I.D., 25 °C, sample: phenyl alcohols, pressure injection at 0.1 psi s (short end, 10 cm to detector), separation voltage 30 kV, buffer: 50 mM lauric acid with 100 mM Tris, pH 8.38.

feature results in improved sensitivity. However, it is detrimental to peak resolution, when dealing with fast separations, where peaks can elute within timeframes of a few seconds or less [9,20]. Using the highest setting for the time constant provided good resolution in our case, however we do not know if the peaks in reality might even be sharper. Utilizing short migration distances, as achieved with our commercial instrument by injecting from the detector side, very fast separations can be achieved that in many cases provide adequate resolution. Additional examples are shown in Fig. 5 where the high performance of the lauric acid/Tris system allowed complete separation of all sample constituents within less than 1 min. A sample of phthalates is shown as another example for the separation of neutral sample molecules. And a mixture of compounds of pharmaceutical interest illustrates that the system is well suited to deal with charged samples

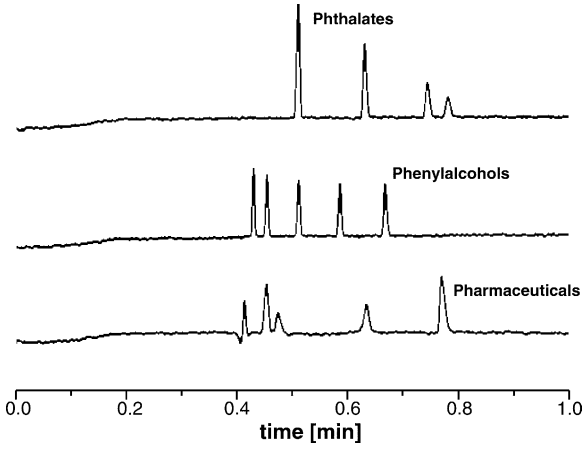

Fig. 5. Fast separations using lauric acid/Tris buffer. Separation conditions same as in Fig. 5 data acquisition rate 32 Hz (short end injection, 10 cm to detector) sample: phenyl alcohols, phthalates and pharmaceuticals (see Fig. 7).

as well, as can be seen in the bottom trace of Fig. 5. This demonstrates clearly the potential of this buffer system for fast separations and high throughput applications.

3.5. Improvement of detection sensitivity

In capillary separation technology, the detection sensitivity is known as one of the major limitations. While the detection of very small absolute amounts is possible in capillaries, the concentration sensitivity is rather poor. The reason for this is that with on-column detection, the UV–Vis detection path length is limited for the most part to the capillary internal diameter [21].

The organic micellar buffer systems described here have significantly lower conductivities compared to conventional SDS buffer systems, and therefore generate much lower currents. This feature allows the use of larger inner diameter capillaries. The increase in sensitivity that can be achieved based on this approach is proportional to the increase in the capillary inner diameter, according to the Lambert–Beer law [22,26]. This improvement in detection sensitivity is clearly demonstrated in Fig. 6, showing the separation of five phenyl alcohols in capillaries with 50 and 75 μm I.D., respectively. Electrokinetic injection was applied to deliver the same relative amount of sample into both capillaries. The signal for the analytes observed with the 75 μm capillary was significantly higher compared to the 50 μm capillary. Both separations were performed using the lauric acid/Tris buffer system at a voltage of 15 kV. The conventional SDS system would be operating at the power limit of the equipment under these conditions using a 50 μm capillary. This would not allow for the use of larger I.D. capillaries in this case and this option to improve detection sensitivity will therefore not be available with conventional SDS buffer systems.

3.6. Selectivity tuning

The organic micellar systems consisting of an alkyl acid and an organic base provide for a number of options to manipulate the selectivity of the separation system. The acid and base part of these systems can be varied independently of each other, such as using different bases to pair with lauric acid or cholic acid or investigate even further suitable alkyl acids with interesting properties.

An example of the impact of the organic base is seen in Fig. 7. The sample in this case is a mixture of caffeine, acetophenetidine, guaiacol glycerylether, procainamide and tetracaine. The buffer system consisted of 50 mM lauric acid with 100 mM of different bases, diisopropyl amine, triethyl amine and Tris in this case. The separation selectivity was different for each of the three combinations. This is in part due to the different pH values of the buffers, but also to the different degree to which these bases interact with the samples and the micelle itself. There is the potential for ion pairing of the charged species of the organic base with either the micelle

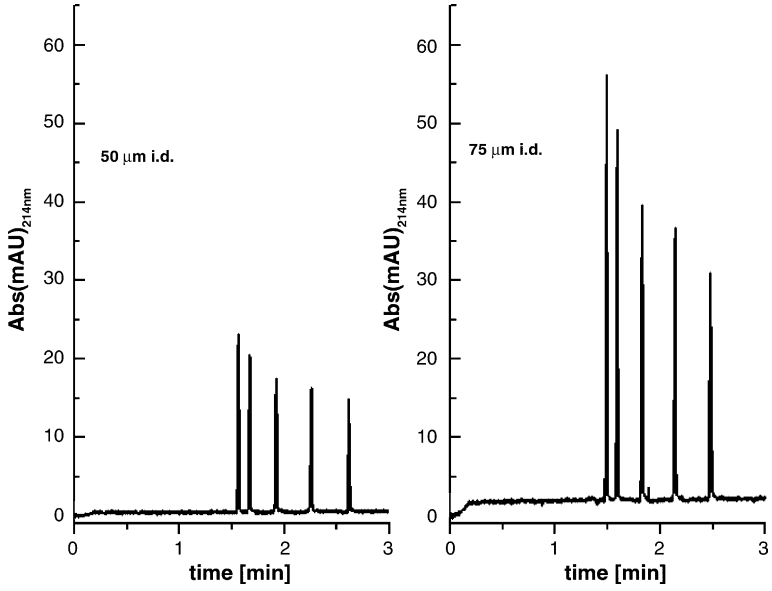

Fig. 6. Impact of capillary I.D. on sensitivity. Capillaries: 20/30 cm fused silica, left: 50 μm I.D. and right: 75 μm I.D., 25 °C, separation voltage 15 kV, data acquisition rate 32 Hz, sample: phenyl alcohols, electrokinetic injection at 5 kV for 3 s, buffer: 50 mM Lauric acid with 100 mM Tris, pH at 8.38.

or the sample molecules. The major difference in this case is seen for the separation of guaiacolglycerylether and acetophenetidine and overall shifts in elution are possible due to modifications of the capillary wall based on adsorption of these charged molecules. The fact that the peak shape for tetracaine, a strong basic molecule, is distorted when diisopropyl amine or triethyl amine are used as bases, maybe due to a mobility mismatch in free solution.

An example for the impact of the organic alkyl acid is shown in Fig. 8. In this case we used Tris as the base with two different types of micelle forming acids, lauric acid and cholic acid. In this example, the pH of the buffer system is almost identical, as the base that is chosen, as discussed in 3.1, largely determines the pH. Yet the different alkyl acids exhibit significantly different selectivity for the sample set under investigation. We observe a significant change in elution for procainamide, which seems to react very strongly to the nature of organic micelles employed. But also neutral samples, such as caffeine and guajacol glyceryl ether, show clearly different selectivities, when the organic micelle is changed. Cholic acid, which has a steroid backbone, is more polar than lauric acid and can therefore interact very differently with specific solutes.

These organic micellar systems allow for significant room to manipulate selectivity, using different combinations of

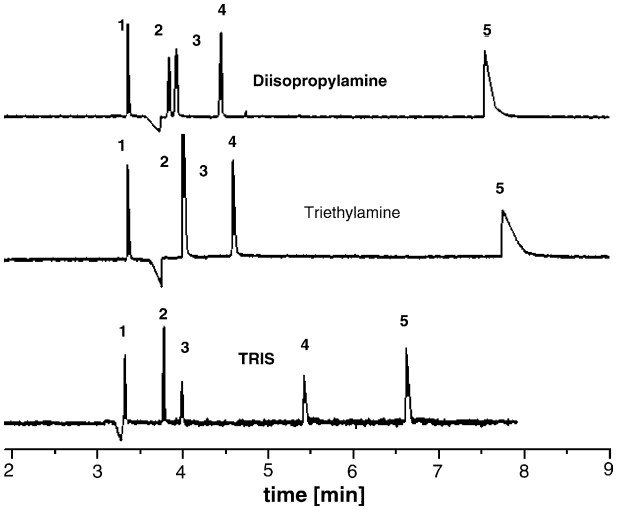

Fig. 7. Influence of organic base on selectivity for pharmaceuticals. Separation conditions as in Fig. 1 sample: pharmaceuticals (1, caffeine; 2, guaiacol glycerylether; 3, acetophenetidine; 4, procaineamide; 5, tetracaine), buffer: 50 mM lauric acid with 100 mM diisopropylamine, triethylamine (TEA) or Tris.

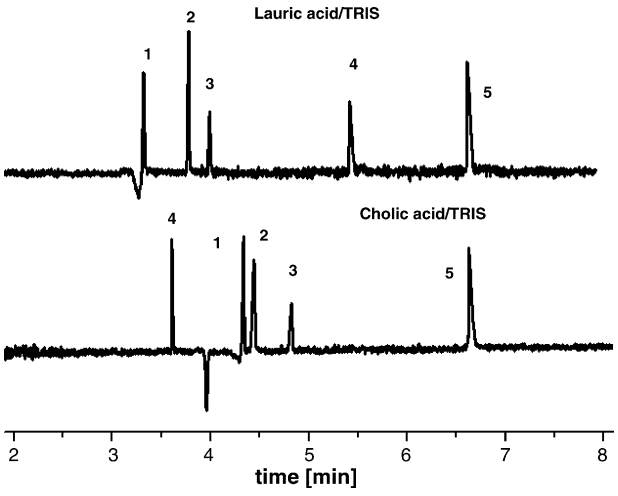

Fig. 8. Influence of organic acid on selectivity for pharmaceuticals. Separation conditions as in Fig. 1 sample: pharmaceuticals (see Fig. 8 buffer: 50 mM lauric acid and 50 mM cholic acid, each with 100 mM Tris (pH about 8.4).

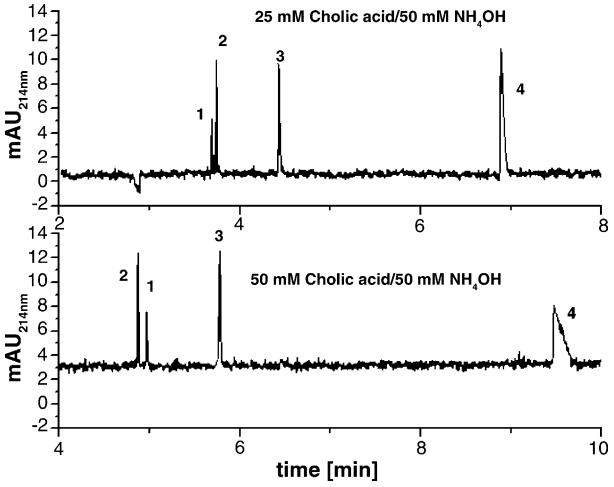

Fig. 9. Impact of micellar concentration. Separation conditions as in Fig. 1. Sample: acids (1, ibuprofen; 2, naproxen; 3, nicotinic acid; 4, phthalic acid).

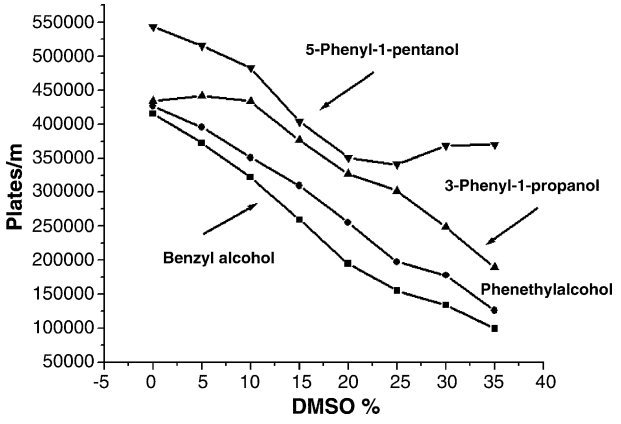

Fig. 10. Effect of sample matrix on efficiency. Capillary 50/60 cm with 50 μm I.D., 25 °C, separation voltage 20 kV, sample: phenyl alcohols in solution containing various amounts of DMSO, buffer: 50 mM lauric acid with 100 mM Tris, pH 8.70.

acids and bases in the buffer systems. Even the overall concentration of the micellar buffer can impact the selectivity and therefore the resolution. The example in Fig. 9 indicates a change in elution order by simply changing the buffer concentration. The samples in this case were four acids: ibuprofen, naproxen, phthalic acid and nicotinic acid and the buffer system consisted of cholic acid and ammonium hydroxide. The trace at the bottom is obtained based on the common formula used so far, of mixing 50 mM of the alkyl acid with 100 mM of the base. The trace on top is obtained by simply diluting this buffer system to half the concentration. The elution order of the first two peaks, ibuprofen and naproxen was reversed, when the micellar buffer was diluted to half of the original concentration.

Therefore it can be concluded that identity as well as concentration of organic micellar forming acids and the base used to adjust and control pH are parameters that can be exploited to tune the selectivity of these systems. This flexibility for method development is not available in SDS based systems. Further work needs to be done to fully understand and appreciate the potential of these buffer systems and to utilize them effectively.

3.7. Effect of sample matrix on the separation efficiency

The results above clearly indicate that the organic micellar systems discussed here could be of interest for high throughput applications, such as the quality control of parallel synthesis products. For these types of applications, separation speed and efficiency are of great importance and the systems discussed here provide just that. To be implemented for this type of assays, an analysis method needs to be able to deal with the sample matrix typically found in high throughput applications and the method also needs to be compatible with mass spectrometric detection.

The compounds produced in high throughput parallel synthesis cover a broad range of chemical space. Solubility issues are usually addressed by using dimethyl sulfoxide (DMSO) as a universal sample solvent, as it has the broadest capability to solubilize a wide variety of compounds. This could be a potential issue, as introduction of organic solvents into micellar systems can potentially interfere and disrupt the micelles. This effect has been reported previously for SDS systems [23]. To assess the applicability of the organic micellar systems to high throughput analysis of compounds in DMSO solutions, we need to understand the impact of the DMSO matrix on the separation. The effects of increasing amounts of DMSO in the sample solvent, when using a lauric acid/Tris buffer system, are shown in Fig. 10. The sample in this case is a mixture of five phenyl alcohols and the separation efficiencies were measured and plotted against the sample matrix composition. The peak efficiency is decreasing with increasing percentage of DMSO in the sample, and the effect is more pronounced for the early eluting sample constituents. At 20% DMSO, the first peak in the electropherogram measures about 200,000 plates per meter, which is about a 50% decrease compared to the efficiency found in plain micellar buffer without DMSO (due to the solubility of phenyl alcohols, they can be dissolved in plain separation buffer to allow for a direct comparison). While this is a significant reduction in separation efficiency, 200,000 plates per meter is still a much higher efficiency then available for example by HPLC. By increasing the percentage of DMSO in the sample beyond 20%, additional problems start to appear, such as peak distortion in addition to a further decrease inefficiency. An interesting observation is that the efficiency for the latest eluting peak does not decrease further, when percentage of DMSO in the sample is increased above 20%. It is too early to make any conclusion, but similar effects are known from HPLC where in gradient or isocratic separations, the late eluting components are less susceptible to interferences resulting from the injection solvent. More data needs to be acquired to understand this phenomenon better and this will be topic of further research.

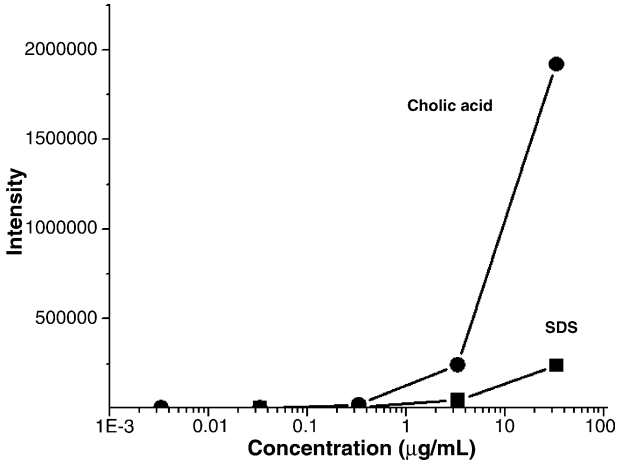

Fig. 11. Infusion MS analysis of tetracaine. Sample: tetracaine in 50 mM SDS with 25 mM sodium tetraborate, pH 9.25 and tetracaine in 50 mM cholic acid with 100 mM ammonium hydroxide, pH 9.5 (for more details see Section 2).

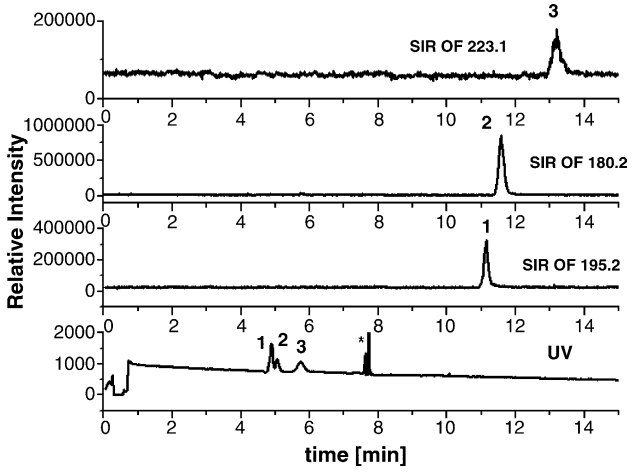

Fig. 12. MEKC separation with UV and MS detection. Sample: 1, caffeine; 2, acetophenetidine; 3, flavone (*, excipient) (for more details see Section 2).

3.8. Preliminary results from MEKC–MS coupling

Mass spectrometers have gained more and more popularity in recent years. They provide a wealth of important information about the sample and can be used to achieve superior detection sensitivity due to their selectivity, which is widely used in bioanalytical LC–MS applications. As mentioned above, MS detection is also very important for the analysis of new synthetic reaction products. Especially, when coupled with high efficiency separation techniques, this could be a key technology to deal with the ever-increasing sample load from high throughput chemistry operations. Being able to couple the high performance organic micellar separation systems described here with MS detection technology would therefore be very attractive. Many researchers have made efforts to combine conventional SDS systems for MEKC with MS detection [24]. However, the presence of nonvolatile salts, such as sodium and borate and the high concentrations of surfactants generate high background noise in the MS-signal and quickly lead to contamination of the ionization source of a mass spectrometer when being coupled directly [13,14,25].

Lauric acid or cholic acid combined with organic monoamines do not contain inorganic salts such as sodium and borates and should therefore cause less compatibility issues with MS detection and allow improved detection sensitivity relative to the conventional SDS system. A first indication of this is given in Fig. 11, showing a direct infusion experiment, where a tetracaine sample is infused into a MS detector from a cholic acid matrix and from a SDS matrix. This investigation shows that the MS signal intensity of the sample from the cholic acid matrix (lauric acid gave a comparable result, not shown) shows an about 10-fold higher signal intensity, while the concentration of both micellar agents is identical (50 mM in this case). This indicates that the ion suppression effects are much less severe in the organic micellar system compared to the traditional SDS buffer system.

We also made some preliminary attempts to use the organic micellar system to actually perform a MEKC separation coupled with MS detection. We used a UV detector in line, positioned before the MS detector, to provide a reference. The results of this experiment are shown in Fig. 12. A mixture of three compounds was injected and separated (including caffeine, acetophenetidine and flavone). The bottom trace results from the UV detector and the top traces are the single ion traces for the three sample constituents. The analysis was done on a manual instrument. The significant delay between the traces originates in the long distance between the UV detector and the sheath flow interface of the mass spectrometer. All three samples could be positively identified based on the MS information.

The results obtained here are preliminary and more research needs to be done to get a better understanding of how these organic buffer systems impact the MS ionization and long-term stability. In this case the whole system is cooled by air, so, the applied voltage to the separation system was limited and some extra band broadening was also observed. There was improvement to the sensitivity with this buffer system, however, there are still residual ion suppression effects observed. If this is due to the organic micelle or partially based on the base counterpart needs to be answered. Lower molecular weight carboxylic acids could improve this due to their increased vapor pressure. Further work will be done into quantitative comparisons of these buffer components and how they affect ionization efficiency.

4. Conclusion

In this paper, we describe a new way of preparing a micellar buffer system for the separation of neutral compounds based on MEKC. The substitution of the traditionally used alkyl sulfates, such as SDS, with carboxylates together with

the substitution of inorganic cations with organic bases, provides a different separation system with interesting properties.

We have shown that these buffer systems, based on lauric acid or cholic acid, buffered with organic monoamines can be used as micellar separation systems, allowing for the separation of neutral as well as charged molecules. They provide high efficiencies, due to their low ionic strength and therefore lower conductivity. The reduced conductivity compared to SDS buffers, provides more flexibility in separation conditions. We have shown that it can be either used to increase separation speed or alternatively to improve the detection limit by using larger I.D. capillaries. The considerable high EOF of such systems (almost 3 mm/s) can be used to support very fast separations. In short capillaries of 10 cm, the entire separation can be completed within less than one minute. In addition, these separation systems allow selectivity control based on the choice of organic acid as well as organic base. This provides much more flexibility in method development than is available with conventional SDS based micellar systems. It has been shown, that the elimination of inorganic buffer components allows coupling to MS detectors with improved MS sensitivity. Further work needs to be done to fully explore the potential of these organic micellar systems for MS detection.

Considering the need for ever faster separations, which is driven by the needs of high throughput parallel synthesis capabilities, the separation systems described in this paper could play an important role as an alternative to HPLC-based separations for purity assessments of synthetic organic compounds based on MEKC. We have also shown that with respect to selectivity, efficiency and overall flexibility they should be considered as a powerful alternative to the conventional SDS system, whenever MEKC seems indicated as the appropriate choice for separation.

Acknowledgments

The authors wish to acknowledge the contributions of colleagues Joe Twomey and Bailin Zhang.

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