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Counter-current chromatography using hexane/surfactant-containing water solvent systems

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Abstract

We developed a *n*-hexane/surfactant-containing water solvent system in counter-current chromatography (CCC) in order to separate hydrophobic compounds. By using the upper phase as the mobile phase, we have separated steroid samples. Retention times of steroids progesterone and Δ^4-androstene-3,17-dione increased slightly by increasing the concentration below the critical micellar concentration (CMC) of surfactant sodium 1-heptanesulfonate. However, the retention times increased drastically while the SHS concentrations were above the CMC. The partition of these two steroids in the two phases was significantly dependent on the interaction with micelles. Aromatic hydrocarbons were not retained by the lower phase no matter what the surfactant concentrations were. Their hydrophobic interaction with *n*-hexane greatly exceeded that with the micellar solution. The retention times of esters, however, were only slightly affected by the surfactant addition even above the CMC. The weaker interaction between esters and the micellar solution was probably due to their higher polarity. The micellar solvent systems provide an alternative way for hydrophobic sample separations in CCC, but the performance is limited.

Keywords: Counter-current chromatography; Solvent systems; Surfactant; Micelle

1. Introduction

Since the invention of the contemporary counter-current chromatography (CCC), the development of this technique mainly involves three aspects, i.e. the instrumentation, elution method, and solvent system. Various instruments [1,2] have been developed to meet the requirements for different applications. In addition to conventional methods, pH-peak-focusing and pH-zone-refining [3–5] elution techniques have been developed to greatly enhance separation resolution and sample capacity. Recently a new type of CCC elution, named centrifugal precipitation chromatography [6–8], was invented. Separation was achieved by repetitive precipitation and dissolving caused by a solvent gradient under a centrifugal force field. This technique can be considered as a combination of a new CCC instrumentation design and a new elution mode.

Selection of solvent systems plays an important role in CCC separations. Generally, the most commonly used solvent systems are multi-solvent two-phase systems in which water and organic solvents are their main components. By choosing organic solvents of different polarities or changing the ratio of the aqueous to the organic portions, the solvent systems can be used for separating samples of various polarities; for example, sugars [9], water-soluble azo dyes [10], alkaloids [11], hydroxyanthraquinones [12]. By adding chelate agents in the organic stationary phase, separation of metal ions was achieved [13,14]. In addition to the organic/water (aqueous) systems, aqueous two-phase and organic two-phase (non-aqueous) systems have also been explored. The former has been developed for separation and purification of hydrophilic compounds, such as proteins [15,16] that are unable to be separated in the organic/water solvent systems. Separation of cytochrome *c*, myoglobin and human serum albumin was attained by aqueous two-phase solvent systems using a XL cross-axis coil planet centrifuge [17]. Purification of horseradish peroxidase was achieved by using a polyethylene glycol (PEG)/phosphate system [18]. In addition, D,L-kynurenine was reported resolved using a PEG/phosphate solvent containing bovine serum albumin as the chiral selector [19]. Separation of cephalosporin from desacetyl cephalosporin C was accomplished in the recovery of cephalosporin from

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fermentation broth using PEG/ammonium sulfate systems [20]. On the other hand, hydrophobic compounds are the target samples using the organic two-phase systems. For example, lycopene was isolated from crude extract of tomato paste using a *n*-hexane/dichloromethane/acetonitrile nonaqueous solvent system [21]. Fat soluble vitamins, such as calciferol, Vitamin A acetate, and (+/−)-α-tocopherol acetate, were separated using an isooctane/methanol two-phase system [22]. Lutein was obtained from a crude extract of Marigold Flower Petals by preparative CCC using nonaqueous system [23]. In addition, application of nonaqueous solvent system enabled purification of fish oil ethyl esters in CCC [24].

Addition of chelate agent can greatly increase partition of metal ions in the organic layer, thus enable metal ion separation in the organic/water two-phase systems. Similarly, if partition of hydrophobic components into the aqueous layer can be increased through an appropriate modification, the system may be used for hydrophobic compounds separation. We, in this study, developed a solvent system using surfactant-containing aqueous solutions, attempting for hydrophobic component separations. Surfactants are amphiphilic molecules that have two distinct groups, i.e. hydrophobic and hydrophilic. When reaching critical micellar concentration (CMC), surfactant molecules aggregate and form micelles in aqueous solutions, and the center core of the micellar structure is in a hydrophobic environment [25]. Blending an organic solvent with surfactant-containing water may thus provide two hydrophobic phases for separating hydrophobic samples. Micellar liquid chromatography [26–31], has long been used as an alternative technique to the classical reverse-phase liquid chromatography. But micellar phase has not been applied in CCC separations. We developed a *n*-hexane/surfactant-containing water solvent system in this study to examine the separation feasibility.

2. Experimental

2.1. Reagents and solvent system preparations

Steroids ((+)-4-cholesten-3-one (CS) (>95%), progesterone (PS) (>98%), and Δ^4-androstene-3,17-dione (AS) (>99%) were purchased from TCI (Tokyo, Japan). Esters, including butyl acetate (99%), propyl acetate (98%), ethyl acetate (99.5%), and methyl acetate (99%) were all HPLC grade and purchased from Acros Organics (Fairlawn, NJ, USA). Anionic surfactants sodium 1-heptanesulfonate (SHS) (98%), sodium 1-hexanesulfonate (98%), and sodium 1-butanesulfonate were purchased from TCI. Sodium *n*-dodecyl sulfate (SDS) (99%) was obtained from Sigma (St. Louis, MO, USA). HPLC grade *n*-hexane was obtained from Mallinckrodt Baker (Philipsburg, NJ, USA), and deionized water from Milli-Q plus (Millipore, Bedford, MA, USA). Toluene (>99.9%) and ethylbenzene (99%) were purchased from Aldrich (Milwaukee,WI, USA); while naphthalene (>99%) and phenanthrene (>96%) from Sigma.

Solutions of surfactants in water of different concentrations were prepared and then mixed with *n*-hexane according to the desired proportion in a separatory funnel. The liquids were thoroughly shaken, and the two phases were clearly separated before use. Sample solutions were prepared by dissolving analytes in the upper phase of the solvent system.

2.2. Apparatus and procedures

The CCC used was a Model CCC-1000 (Pharma-Tech Research Company, Baltimore, MD, USA) high-speed counter-current chromatography (HSCCC), mounted in a temperature-controlled oven. A separation column was prepared by winding a 54 m long (3.2 mm O.D. and 1.6 mm in. I.D.) Tefzel tube onto three column holders forming three coiled layers with a total capacity of ∼108 ml. The CCC revolution radius was 85 mm with a β (the ratio between the radius of rotation and revolution) value of 0.59. During the chromatographic run, the multilayer coiled column was filled entirely with the lower phase after the oven reached to the desired temperature. The upper phase was then pumped into from the tail end of the column at a flow rate of 2.0 ml/min, while the counter-current chromatograph was rotating at 800 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the head outlet, an aliquot of 100 μl sample solution was injected through the sample port. The effluent from the head end of the column was continuously monitored with a Bio-Rad (Hercules, CA, USA) model 1801 UV–vis detector. After the experiment was completed, the column content was collected into a graduated cylinder by N_2 flushing. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

3. Results and discussion

3.1. Separation of steroids

We started with *n*-hexane–SDS in water (1:1) systems, since SDS is a very commonly used surfactant. Unfortunately, when the concentration of SDS in water reached 8 mM (the CMC of SDS [32,33]) the upper phase was highly emulsified and very viscous, and inadequate to be employed in CCC. Only when the concentration was as low as 0.83 mM, a stable and transparent two phases were achieved. A non-ionic surfactant triton X-100 was then examined. Emulsification also occurred even when the concentration was below the CMC. Either SDS or triton X-100 molecules possess a long alkyl chain; that may enhance its hydrophobic property and solubility in the organic phase, thus caused the formation of the emulsion. We therefore attempted other short-chain surfactants to prevent the solvent systems from emulsification. We tried surfactants sodium 1-heptanesulfonate (SHS, CMC = 0.3 M), and sodium 1-hexanesulfonate (CMC = 0.54 M) [34]. Although the solvent systems with these two surfactants above CMC did give stable and clear two phases, we chose surfactant SHS for all succeeding experiments due to its relatively lower CMC.

We first tried an arbitrary concentration 20 mM. The chromatogram obtained is shown in Fig. 1A. Steroids CS and PS were eluted out with the solvent front and separated from AS. As the surfactant concentration increased, the retention time of

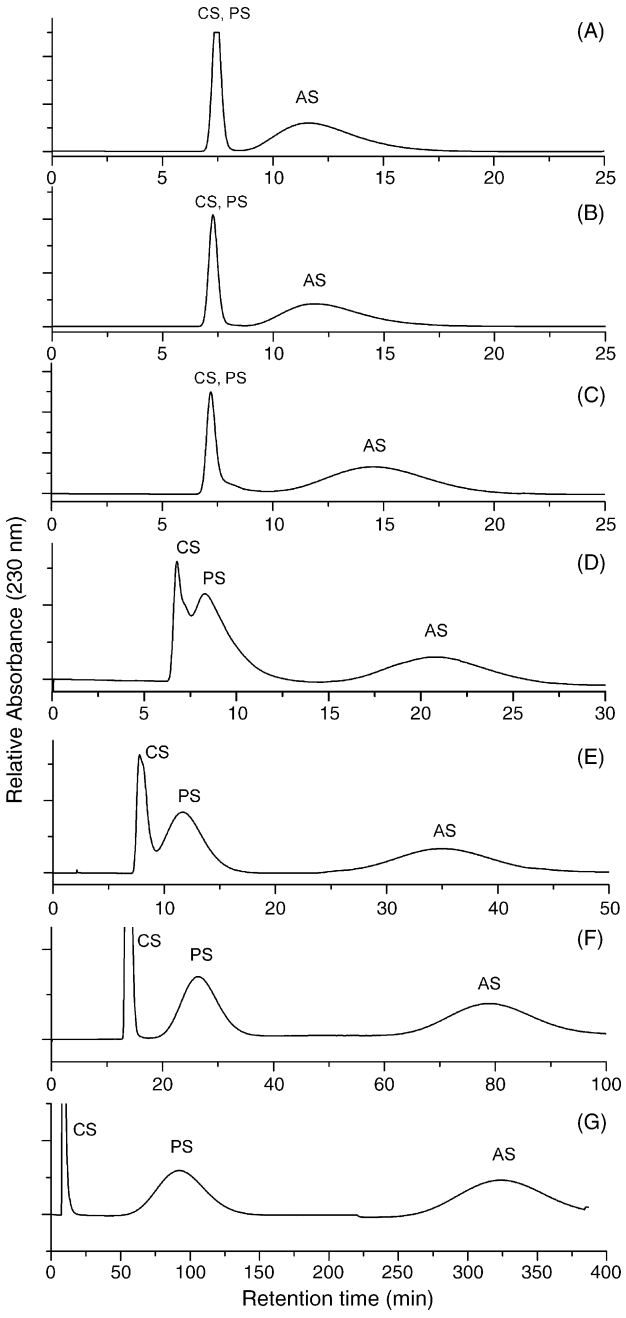

Fig. 1. Chromatograms for the mixture of CS (200 mM), PS (300 mM), and AS (400 mM) using solvent systems hexane–1-heptanesulfonate in water (1:2). Concentrations of 1-heptanesulfonate: (A) 20 mM; (B) 50 mM; (C) 200 nM; (D) 300 mM; (E) 350 mM; (F) 400 mM; and (G) 600 mM. Experimental conditions: total column volume = 108 ml; retention volume ratio ≈87% for all seven runs; flow rate = 2.0 ml/min; chromatograph rotation speed = 800 rpm; temperature = 20 °C; detection wavelength = 230 nm; and sample volume = 100 μl.

compound AS increased a little more notably and a small shoulder of PS appeared, shown in Fig. 1C. Solvent systems of even higher SHS concentrations were then examined. Steroid CS was still eluted out with the solvent front while PS and AS were retented in the stationary phase longer. At the CMC, steroids CS and PS were partially separated, shown in Fig. 1D. Three compounds were completely resolved at 400 mM, while the retention times of PS and AS increased drastically at 600 mM, shown in

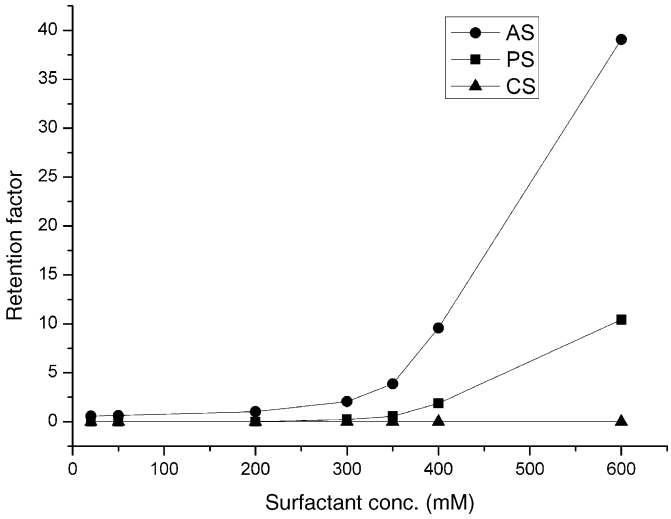

Fig. 2. Plot of the retention factors of steroids CS, PS, and AS as a function of the SHS concentration in the seven separation runs.

Fig. 1E–G. Apparently, higher surfactant concentration helped the separation for this sample.

When the concentration reached 200 mM, a small PS shoulder appeared while the retention of CS remained essentially the same. In addition, the retention time of AS increased by ∼3 min. Although the surfactant concentration was still less than the CMC, the weak interaction between solutes and the smaller surfactant aggregates [35–37] in the premicellar solution emerged. However, while the concentration of the surfactant was above the CMC, the retention times of PS and AS increased considerably. Apparently, formation of micelle would greatly influence the partitioning of these two analytes. It is interesting to explore that the plot (see Fig. 2) of the surfactant concentration as a function of the retention factor for the analyte shows two distinct regions for compounds PS and AS. The retention factors increased very slowly and almost linearly while the surfactant concentration was elevated from 20 to 300 mM, then the slope became very steep from 300 to 600 mM. This outcome reflected the very weak effect on the retention factor with the SHS concentration below the CMC, and the strong effect above the CMC. Apparently, formation of micelle significantly improved the partitioning of these two compounds in the lower phase. The interaction between these two compounds and micelles became the dominant factor for the separations. While the concentration was further increased, the size of micelle became greater and was able to solubilize more analyte molecules [34–36], therefore the retention factor was significantly increased. As for compound CS, the partition still significantly favored the mobile phase no matter how large the SHS concentration was. It should be noted that this solvent system was quite stable, no phase depletion was observed. The phase retention ratio remained ∼87% for all chromatographic runs.

3.2. Separation of aromatic hydrocarbons

In addition to steroids, we tried compounds of even lower polarity to examine the separation capability using this micellar

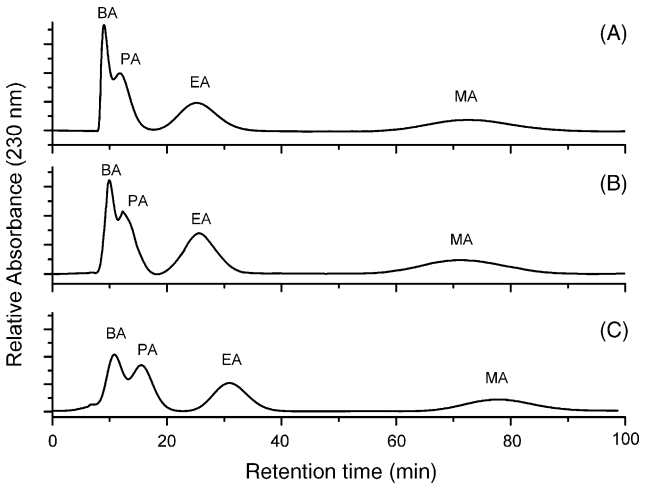

Fig. 3. Chromatograms of the mixture of four esters (MA: methyl acetate, EA: ethyl acetate, PA: propyl acetate and BA: 1-butyl acetate; 30 μg/ml for each component) using solvent systems hexane–SHS in water (3:1). Concentrations of SHS: (A) 0 mM; (B) 200 mM; and (C) 600 nM. Experimental conditions: total column volume = 108 ml; retention volume ratio ≈87% for all three runs; flow rate = 2.0 ml/min; chromatograph rotation speed = 800 rpm; temperature = 20 °C; detection wavelength = 230 nm; and sample volume = 100 μl.

system. Since extraction of polycyclic aromatic hydrocarbons (PAHs) using micelles of anionic and nonionic surfactants have been reported in the literature [38–41], these compounds or other aromatic hydrocarbons could be good target samples in this study. However, compounds including naphthalene, phenanthrene, methylbenzene, and ethylbenzene showed essentially no observed retention at all in this system. Although this outcome does not agree with what we expected, it does conform to a research [27] in micellar chromatography. In the study for steroids screening, the most hydrophobic steroids were significantly retented on the C_{18} column when eluted with SDS micellar solution. In our work, the aromatic hydrocarbons were all eluted out with the solvent front. Apparently these compounds were favorably partitioned in the nonpolar mobile phase. Without increasing the polarity of the mobile phase (for example, addition of ethanol in the solvent system), separation for compounds of very low polarity would not be possible.

3.3. Separation of esters

We also examined compounds of medium polarity. A mixture of methyl acetate, ethyl acetate, propyl acetate and butyl acetate was separated using the same solvent system. It should be noted that solvent systems of *n*-hexane–600 mM SHS in water (3:1) were employed in these experiments. Since the mutual solubility for the upper and low phases was very low, it would be advantageous to increase the ratio of the organic content in order to obtain more mobile phase in the solvent preparation.

As can be seen in Fig. 3B, the chromatogram obtained using 200 mM pre-micellar solvent system looked extremely similar to that using just hexane/water solvent system, as shown in Fig. 3A. Again, the premicellar solution did not affect the analytes' distribution between the upper and lower phases in any significant extent. The elution order agreed with that should be expected in common aqueous solvent systems. While the surfactant concentration (600 mM) exceeded the CMC, only small retention time increases for all four components were observed, shown in Fig. 3C. The interaction between these analytes and micelles was not as large as that between steroids and micelles. Instead, the interaction with the bulk water probably dominated the partitioning [41].

4. Conclusions

We attempted to separate hydrophobic components using a solvent system with a micellar phase instead of the organic two-phase solvent system in this study. Since micelle phases made of surfactants with long alkyl chains did not form stable two-phase solvent system required for CCC operations, we prepared the solvent systems using surfactant sodium 1-heptanesulfonate with a shorter alkyl chain. When the SHS concentrations were below the CMC, the premicellar lower phase did not significantly affect analytes' distribution. While the concentration was above the CMC, the interaction of the analytes with micelles turned out to be dominant, and significantly changed analytes' partition between the upper and lower phases. The retention times of the steroids could be fine-tuned using the micellar concentration. For very hydrophobic compounds, such as steroid CS and aromatic hydrocarbons, the partition appreciably favored the non-polar mobile phase, thus resulted in no retention. Compounds with medium polarity, such as short-chain esters, did not interact with micelles considerably; therefore the separation was not much affected by the surfactant addition even when the concentration was well above the CMC. The usefulness of this technique in its current form is limited.

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