ELSEVIER

Journal of Chromatography A, 876 (2000) 169–182

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

New pseudo-stationary phases for electrokinetic capillary chromatography
Complexes between bovine serum albumin and sodium dodecyl sulfate

L. Gaillon*, S. Cozette, J. Lelievre, R. Gaboriaud

Laboratoire Energétique et Réactivité aux Interfaces, Université Pierre et Marie Curie, 4 Place Jussieu, Bat. F Boite 39, 75252 Paris Cedex 05, France

Received 11 October 1999; received in revised form 7 February 2000; accepted 16 February 2000

Abstract

The complexes formed between a protein (bovine serum albumin, BSA) and a surfactant (sodium dodecyl sulfate, SDS) were studied as separation carriers in electrokinetic chromatography. Selectivities different from those with either SDS or BSA alone in the background electrolyte (BGE) were obtained. Separation performances were demonstrated to be closely related to the type of complex formed, as predicted by the isotherm curve of SDS on BSA. For each composition of background electrolyte, capacity factors and resolutions were calculated. We compared the results with these complexes to electropherograms using BGE containing either BSA or SDS alone. The separation of a mixture of phenols indicate that some compositions of the BSA–SDS complexes are efficient selectors.

Keywords: Pseudostationary phases; Electrokinetic chromatography; Background electrolyte composition; Albumin; Sodium dodecyl sulfate; Phenols; Chlorophenols; Nitrophenols

1. Introduction

The separation of neutral solutes, in electrokinetic chromatography (EKC), is achieved by interactions between each of the solutes and a charged pseudo-stationary phase in the running buffer. Thus the most widely used pseudo-stationary phases are ionic micelles, as originally described by Terabe et al. [1], alternative separation buffer carriers have been studied [2–10], but do not affect selectivity.

To change the type of interaction leading to the separation of neutral solutes, new types of pseudo-stationary phase with different affinities for the solutes can be used [11–29]; the technique is then called affinity electrokinetic chromatography (AEKC). Among them, proteins [23–29], because of their natural binding properties, are perhaps the most widely used. These properties have been frequently and successfully employed in HPLC with protein-immobilized stationary phases [30–34]. However, unlike many other selectors, proteins have relatively strong binding interactions with analytes. As it is generally desirable to have a moderate capacity

*Corresponding author. Fax: +33-144-273-035.
E-mail address: gaillon@ccr.jussieu.fr (L. Gaillon)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00234-X

factor (k') to obtain optimum resolution, apart from changing the selector concentration, the best way to tune k' is to weaken the strength of the binding interaction. A common way to achieve this is to add an organic modifier, generally an alcohol [35], or acetonitrile [36], to reduce the binding interaction.

Bovine serum albumin (BSA) is a protein which can be considered as a molecular sponge, binding and transporting a huge variety of ligands, with low to moderate affinity. This protein is currently successfully used as a chiral selector [26–28,37–45], because of small differences in affinity between structurally related ligands such as stereo isomers. The effect of BSA concentration on the separation has been shown [45], and this study suggests that concentration must be adjusted to achieve a compromise between detection sensitivity and efficiency or resolution.

The width of the retention window, another key parameter in AEKC, is directly correlated with the mobility of the selector, and can be changed in several ways. It is possible to slow down the protein by adding replaceable polymer networks to the background electrolyte (BGE). In this case, addition of dextran [27], for example, has made possible the resolution of chiral compounds, which was not successful by plain EKC with albumin. The use of such viscosifier probably not only modifies the viscosity of the running buffer [29], with all that entails, but also modifies the binding properties of the protein.

It is also possible to modify the protein mobility by altering the pH to make use of the protein's overall charge. It should also be possible to modulate the charge of the protein by adding a charged complexing agent. Such an approach has been already used with micelles of adjustable surface charge density [46], the overall charge of octylglucoside (OG) micelles being tailored by complex formation with borate ion. This last study showed that the retention window is easily manipulated, with this type of micelles, through t_{mc} (migration time of the selector) without major changes t_0 (migration time of the electrophoretic flow marker). It would be interesting to know whether it is possible with other types of selector.

Our method of investigation is a compromise between these two approaches. The addition of surfactant to the BGE which contains BSA, to form a protein–surfactant complex, make it possible to change the mobility of the protein if strong surfactant–protein interactions occur, or the overall charge if an ionic surfactant is used. This should lead either to a modulation of both the resolution and the retention window. Moreover, the linkage of surfactant monomers to the protein involves a change in the partitioning coefficients of all the solutes tested. The hydrophobicity of the selector is changed and a modulation of affinity can be reached by this way.

Such an approach was already used indirectly when Wu et al. [47] added sodium dodecyl sulfate (SDS) to an electrolytic buffer containing BSA, to minimize the protein–capillary wall interactions. It has been demonstrated [48,49] that SDS micellar solution facilitates proteins separation in capillary zone electrophoresis (CZE). In the same study [47], SDS was also compared with bile salt, a biological surfactant. The addition of BSA with either SDS or bile salt to the running buffer resulted in successful separation and even isomers were resolved efficiently and with short analysis times. So, the feasibility of using SDS (or bile salt) surfactant in conjunction with BSA as a novel modifier in capillary electrophoresis has been demonstrated at physiological pH using untreated fused-silica capillary. A running buffer with BSA alone did not separate the six solutes tested in this study. This was due not only to lower levels of protein adsorption to the capillary wall but also to a change in selectivity.

For electropherograms, Wu et al. [47] used buffer concentrations of 50 m*M* SDS and 0.015 m*M* BSA. Therefore, the SDS was in micelle form, because this concentration is much higher than the critical micellar concentration (CMC). However, BSA, even in micellar solutions of SDS, affects selectivity, because the resulting electropherogram is better resolved than with SDS alone.

However, the determinant role of BSA–SDS complexes in the separation (selectivity and resolution) was not demonstrated. As in other studies [50,51], SDS was used simply to prevent adsorption of the solute to the capillary wall, because SDS binds to any electrostatic or hydrophobic site. Thus, it is unclear whether various compositions of buffered electrolyte solution with different SDS and BSA concentrations were tested. The authors suggested

that BSA–SDS complexes may be very useful, based on their separation of six porphyrins, but they did not investigate further.

Our aim was to show that the behavior of BSA–SDS complexes as selectors differ, depending on the region of the isotherm in which the BGE composition is located. In the whole tested BGE with BSA, the concentration of SDS was always under the CMC in order to ensure that separation was due only to the complex BSA–SDS and not to micelles of SDS. BSA–SDS interactions are well known [52–66]. Detailed studies of the isotherm curve [58–66] generally defined two types of complex, depending on BSA concentration and on free SDS surfactant concentration. We found that the use of such complexes in BGE may lead to tremendous changes in the efficiency of the selector (or selectors, because the changes in composition give rise to a variety of species). We compared the results with these complexes to electropherograms using BGE containing either BSA or SDS above or below the critical micellar concentration, to demonstrate the particular behaviour of these complexes.

2. Experimental

A Europhore Prime Vision I capillary electrophoresis system (ATI Unicam, Cambridge, UK) was used. Detection was performed by on-column measurements of UV absorption at fixed wavelength: 280 nm for acetone, 354 nm for Sudan III, 310 nm for Coomassie blue dye and 280 nm for the other analytes. The total length of the fused-silica capillary tube was 50 cm, with 36 cm between injection and detection. The inner diameter was 75 μm. All injections were hydrostatic and lasted 1 s. The voltage applied was 15 kV unless otherwise specified. The electropherograms were recorded on a PC486 computer using Boreal software (Flotec, La Queue, France). All experiments were carried out at constant temperature (300 K).

The test solution consisted of an equimolar (10^{-4} mol l^{-1}) mixture of five different phenols: phenol (P1), 2-chlorophenol (P2), 2,6-dichlorophenol (P3), 4-chloro-2,6-dinitrophenol (P4), 2-chloro-4-nitrophenol (P5). These phenols are often found back during the synthesis of hair dyes and must be detected with accuracy. All these test analytes were purchased from Aldrich. Sudan III and acetone pro analysis grade, used as markers, were obtained from Aldrich and Merck, respectively. The protein marker Coomassie Brilliant blue G was from Aldrich.

Sodium dodecyl sulfate was purchased from Fluka. The buffer solutions were prepared with Tris [tris(hydroxymethyl)aminomethane] and hydrochloric acid (pH=pK_a=8.2) to give a Tris buffer concentration of 5 mmol l^{-1} in nanopure water. All buffered electrolyte solutions were filtered through membrane filters with 0.45-μm pores (Millipore, St-Quentin, France) before use. Sample solutions were made up in running buffer, with solute concentrations at or below 10^{-4} mol l^{-1}.

BSA used in this work was a single lot purchased from Sigma (ref A-7030; M_r 69 000) and stored at 277 K. Effects of alkaline conditions on BSA are generally considered as minor until pH 9. Thus a conformational change intervenes between pH 8 and 9, generally called ‘neutral transition’, it is one interesting advantage in our case since it enlarged the exposed surface area, from 39 000 $Å^2$ for *N* (*normal*) *form* to 47 000 $Å^2$ for *B* (*basic*) *form* [67]. Experimentally, no changes in the behavior of the BSA solutions were observed. All the results given here were reproducible overnight, for 1 day and more, with reproducibility in good agreement with that usually accorded to this experimental method.

The capillary was activated by rinsing with 1 mol l^{-1} potassium hydroxide for 15 min followed by rinses of 0.1 mol l^{-1} KOH, 0.1 mol l^{-1} HCl and Nanopure water. A much longer rinse was finally performed with the appropriate background electrolyte before the capillary was used. This process was also performed before each change of buffered electrolyte solution. Purges with the running buffer were also carried out for 3 min after each run.

3. Results and discussion

3.1. Characterization of BSA–SDS complexes

The binding of surfactants to proteins has been studied extensively over many years. BSA–SDS interactions have been particularly well studied [52–

66], and the binding isotherm is well established [58–66], with a variety of techniques (dialysis, dodecyl sulfate ion selective electrode, NMR and spectroscopic probe techniques). This isotherm curve [moles of detergent bound per mole of protein ($\bar{\nu}$) plotted as a function of free surfactant concentration (C_{free})] typically exhibit four regions (Fig. 1). In the first region (1), at the lowest concentrations of SDS, the surfactant binds to some specific sites of high energy on the protein. Then the curve reached a plateau or a region in which it increases slowly (2). Each molecule of BSA has about eight specific sites that interact with SDS molecules. The third region corresponds to the denaturation of the protein, illustrating the well-known denaturing characteristics of SDS [68]. There is a massive increase in binding at this point due to cooperative interactions, caused by the unfolding of the protein. This leads to saturation of all sites of the molecules of BSA. Beyond the saturation point, the binding isotherm shows a plateau (4), suggesting that there is no further binding of the surfactant to the protein and that normal micelle formation occurs if excess surfactant is added.

However, the key finding of all these studies is that the interactions between BSA and SDS, and consequently binding isotherms, are highly dependent on pH, ionic strength, temperature, and addition of neutral inorganic salts [58].

Concerning pH dependency, it has been shown that, for a given ionic strength and a fixed temperature, all the curves have the same shape [64]. Sharp rise of the binding curve [region (3)] was displaced to higher free SDS concentrations when pH increased. Compared to the curve at pH 7.5, data obtained at pH 8.3 are still further to the right. But the plateau ($\bar{\nu}$ comprised between 8 and 10) is a common part of all the curves for the different pH values.

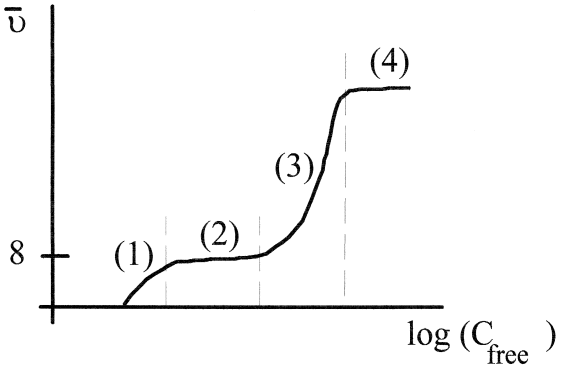

Fig. 1. Schematic plot of the number of bound SDS surfactant monomers per protein molecule ($\bar{\nu}$) versus log free SDS concentration (C_{free}).

For ionic strength, a similar modification of the curve has been demonstrated [65]. The isotherm curves obtained by Sen et al. [58], showing the effect of ionic strength, pH value, or temperature, are more difficult to compare. However, it can be also deduced that the plateau is not dramatically affected, neither for the value of $\bar{\nu}$ nor for its concentration zone.

It would have been interesting to establish the binding isotherm of SDS to BSA in our specific experimental conditions, by any methods. But it is possible to assume that this isotherm curve would be similar to those previously described with buffers of composition close to ours. Therefore, by comparison with binding curves at temperature, pH value and ionic strength close to ours, we assumed that the number of moles of surfactant per mole of protein is $\bar{\nu}=8$, and a free SDS concentration slightly above $C=10^{-4}$ mol l^{-1} corresponds to a composition located on the plateau. So that the composition of a solution at a peculiar point of the binding isotherm can be easily deduced.

3.2. Performance as pseudo-stationary phase in EKC

We varied the composition of the BGE in BSA and SDS, and tested the effect of such running buffer additives on the efficiency of separation by EKC of five phenols which composed the mixture we have to analyze. Taking into account the constant value of the pH (pH = pK_{Tris} = 8.20), one was neutral (phenol (P1), pK_a = 9.97), another was partially ionized (2-chlorophenol (P2), pK_a = 8.49) and the others were anionic (2,6-dichlorophenol (P3), pK_a = 6.79; 4-chloro 2,6-dinitrophenol (P4) pK_a = 3.00; 2-chloro 4-nitrophenol (P5) pK_a = 5.40). Therefore, they have electrophoretic mobility (μ_{ep}) towards the anode in the presence of an electric field (E). At a pH value of 8.2, BSA (isoelectric point 4.7 [69]), SDS, SDS micelles and their complexes are also anionic and have a μ_{ep} towards the anode. In all cases, the

electrophoretic mobility of the selector or the pseudo-stationary phase is higher than that of the ionic solute. Therefore, injection was performed at the anode. However, a solute, even totally ionic, will have at most the net velocity of the ionic selector or a solute totally retained by it, because, as it is always stated in such a case, the charge of the solute does not affect the mobility of the separation carrier. This was always true in our experiments, because no solute had a longer migration time than the selector. Furthermore, Tris buffer was probably not extracted by any of the selectors studied, as assessed by measuring pH.

It is often said to be preferable that the analytes and protein move in opposite directions, but we found that this was not absolutely necessary. Analyte and protein may move in the same direction provided they have significantly different mobilities, but this results in long analysis times.

3.2.1. SDS alone

The first BGE tested was a 10^{-4} mol l^{-1} SDS solution at pH 8.2. As this concentration is below the CMC, the separation observed with three peaks for the five products injected, is due to the charge of the substituted phenols (Fig. 2). Taking pH value into account, the only neutral solute was phenol and its migration time was the same as that of the electroosmotic flow marker (acetone). Increases in SDS concentration, up to the CMC, did not significantly improve separation.

We tested micellar SDS solutions with concentrations above the CMC (8×10^{-3} mol l^{-1}) varying up to 6×10^{-2} mol l^{-1} (Fig. 2). The retention time of each solute was longer than at concentrations of SDS below the CMC, and even phenol (P1) had a migration time different from that of acetone (Table 1). So that phenol, thus highly soluble in water, had a certain affinity for SDS micelles, as all other substituted phenols. However, the presence of micelles was not conducive to the separation of all solutes, despite the mechanism of separation based on micelle affinity and the difference in electrophoretic velocity of each charged solute. Four peaks

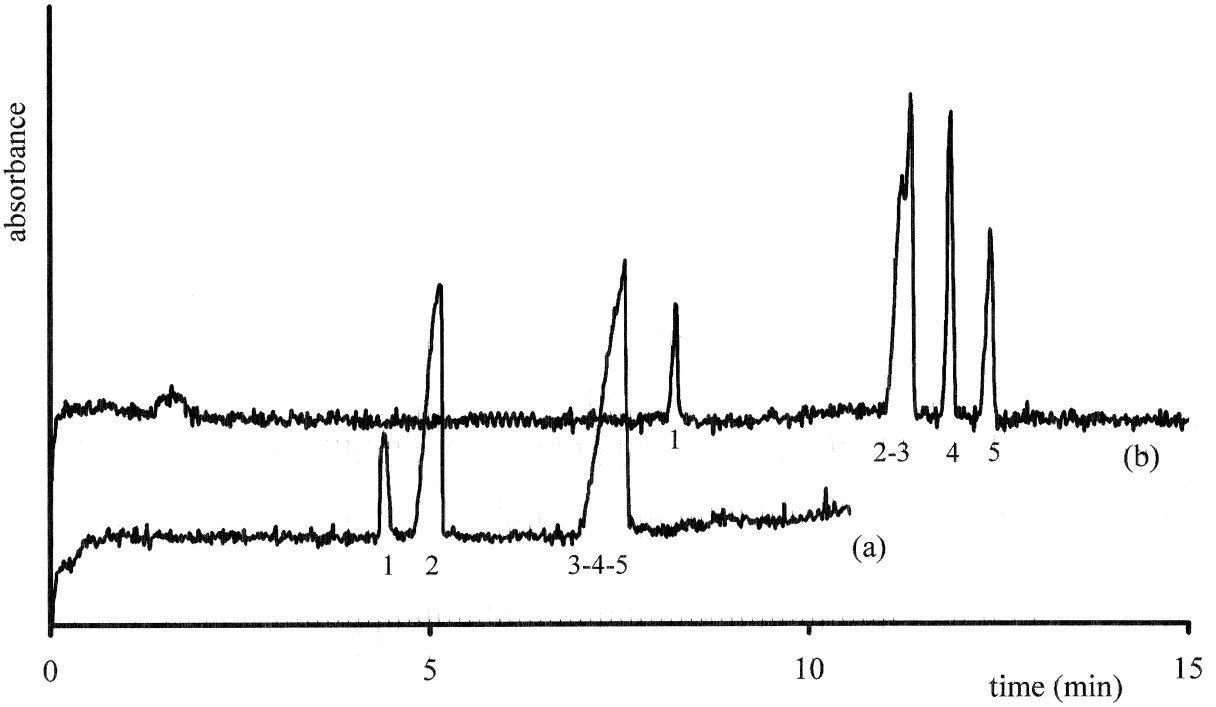

Fig. 2. Electropherograms with buffered SDS solution at (a) $C_{SDS}=10^{-4}$ mol l^{-1} submicellar or (b) $C_{SDS}=6\times10^{-2}$ mol l^{-1} super-micellar concentration. Migration time window, 5.75–25.65 min. Solutes P1–P5, see text.

Table 1
Net velocity (v_{net}) and capacity factor (k') for substituted phenols with SDS concentrations above and below the CMC in Tris-buffered electrolyte solution (pH 8.2)

Solute	v_{net} (cm s^{-1})		k'
	[SDS] = 10^{-4} mol l^{-1}	[SDS] = 6×10^{-2} mol l^{-1}	
v_0 (acetone)	0.132	0.101	
v_{mic} (Coomassie blue dye)	–	0.022	
(P1) Phenol	0.132	0.071	0.63
(P2) 2-Chlorophenol	0.112	0.052	1.4
(P3) 2,6-Dichlorophenol	0.075	0.051	0.83
(P4) 4-Chloro-2,6-dinitrophenol	0.075	0.049	0.98
(P5) 2-Chloro-4-nitrophenol	0.076	0.047	1.1

were still observed for five solutes injected. The migration time of micelles has been determined for the highest SDS concentration tested, using red Sudan III as a marker, to enable us to calculate the capacity factor k' (Table 1). For these calculations and followings, the electrophoretic mobility of the ionic solutes and the partial ionization of 2-chlorophenol (P2) at the working pH were taken into account in classical equations [11,26]. Although not high, the k' values were different enough between solutes for the separation of four of the five solutes. The order of increasing k' was not the same as that of migration times, further demonstrating that micelle affinity was not the only separation phenomenon involved.

Moreover, what can be underlined here is that, in the absence of micelles, both 4-chloro-2,6-dinitrophenol (P4) and 2-chloro-4-nitrophenol (P5) had the same migration time, and therefore the same electrophoretic mobility. However, with micelles in the BGE, as their capacity factors were slightly different, separation was achieved. In contrast, 2-chlorophenol (P2) which was well separated from the peak of 2,6-dichlorophenol (P3) in the absence of micelles, formed a single peak with P3 in the presence of micelles, because the difference in capacity factor (1.44 and 0.83) of the two molecules was counter balanced by the difference in their electrophoretic mobilities. In terms of capacity factors (Table 1), the anionic solutes were more distributed to the micelles than neutral solutes.

Decreasing the voltage applied from 15 to 5 kV had little effect on the electropherograms except that shoulder for the 2-chlorophenol (P2) peak was larger with no better resolution, but with a considerable lengthening of migration times.

3.2.2. BSA alone

We also tested running buffer consisting of an aqueous solution of Tris at pH 8.2 containing the protein BSA alone. We used a protein concentration of 3×10^{-6} mol l^{-1}. Protein selectors in solution have been demonstrated to give separation efficiencies of up to 200 000 plates [26,27] and it is the best figure.

We observed four peaks for five solutes on electropherograms (Fig. 3), as before. But in these conditions of buffered electrolyte solution, 2,6-dichloro- and 4-chloro-2,6-dinitrophenol (P3 and P4) had the same migration time. It was possible to determine the electroosmotic velocity from acetone and the net protein velocity from the migration time of Coomassie blue dye. We also took into account that some of the solutes were ionic, and calculated the capacity factor k' for each solute (Table 2). It is useful to know the net velocity of the charged phenols, and therefore we produced the electropherograms for the mixture of those solutes in the same conditions except that the BGE did not contain BSA. In this case, the electroosmotic flow was weaker, leading to slightly longer migration times (Table 2). This is probably due to BSA adsorption on the capillary wall rather than increase in ionic strength, because such an increase would compress the diffused double layer, and tend to suppress the electroosmotic flow; but the presence of BSA does not contribute so much to the ionic strength.

The electrophoretic mobility of BSA was 2.67×

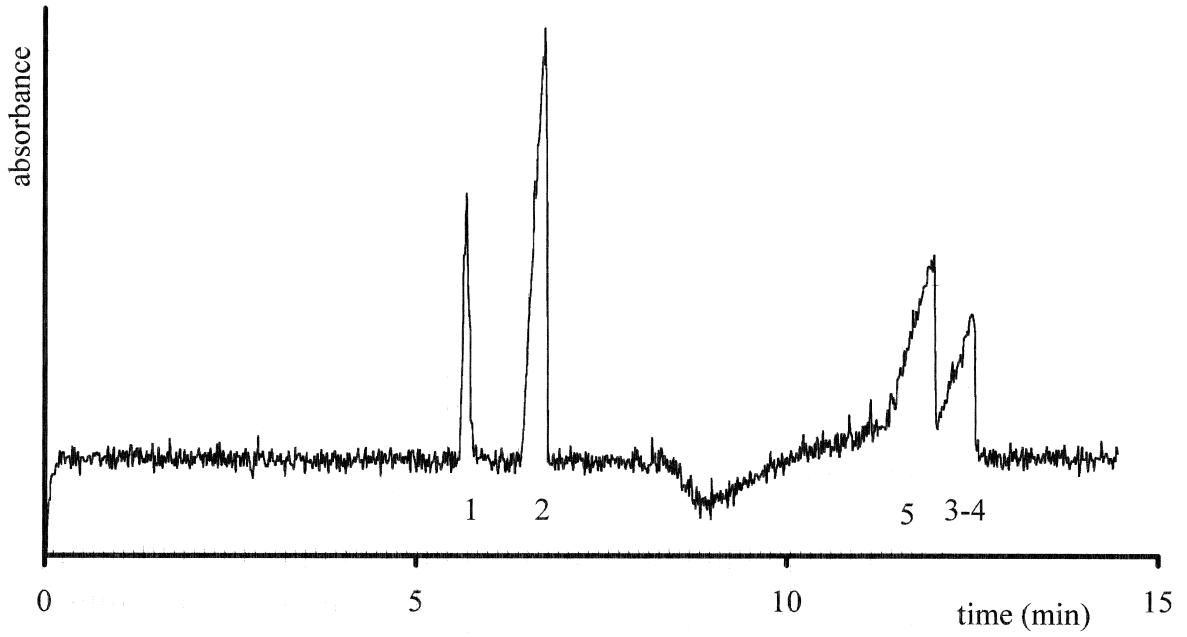

Fig. 3. Electropherogram with buffered BSA solution, $C_{BSA}=2\times10^{-6}$ mol l^{-1}. Migration time window, 5.6–12.45 min. Solutes P1–P5, see text.

10^{-4} cm^2 V^{-1} s^{-1}. The global charge of the protein is known to be about 25 when pH is close to 8, and the electrophoretic mobility of BSA has been found equal to 2.5×10^{-4} cm^2 V^{-1} s^{-1} [70] in experimental conditions similar to ours.

As a matter of comparison, SDS micelles are said to be associations of around 50 monomers and their counterion dissociation rate is close to 0.7. Therefore, they carry a lower ionic charge than BSA which has a charge close to (−25). However, the mobility of SDS micelles and BSA differs considerably because BSA is larger. Thus, in AEKC with BSA, in these experimental conditions, to a much narrower window (5.6–12.45 min) than micellar SDS buffer (5.75–25.7 min). The difference in capacity factors reported in Table 2 shows that the phenols could be separated if BSA was added to the Tris-buffered solution (all other conditions being identical), but 2,6-dichloro- (P3) and 4-chloro-2,6-dinitrophenol (P4) had the same net velocity, due to compensation between the affinity for BSA and electrophoretic velocity. It is not surprising that these

Table 2
Net velocity (v_{net}) and capacity factor (k') for substituted phenols with BSA electrolyte solution buffered with Tris, pH 8.2

Solute	v_{net} (cm s^{-1})		k'
	Tris alone	[BSA]=3×10^{-6} mol l^{-1} in Tris	
v_0 (acetone)	0.136	0.104	
v_{mic} (Coomassie blue dye)	–	0.047	
(P1) Phenol	0.136	0.102	0.03
(P2) 2-Chlorophenol	0.111	0.090	0.14
(P3) 2,6-Dichlorophenol	0.081	0.047	13
(P4) 4-Chloro-2,6 dinitrophenol	0.080	0.047	6.1
(P5) 2-Chloro-4-nitrophenol	0.085	0.049	1.7

three ionic solutes have large capacity factors, because it is known that this type of protein (albumins) preferentially retains solutes with negatively charged groups [71]. BSA has a low affinity for phenol (P1) and 2-chlorophenol (P2).

It was striking to observe that the addition of BSA to the BGE led to the well-resolved separation of 2-chloro- (P2) and 2,6-dichlorophenol (P3), solutes that were not separated in the presence of micellar SDS. But it also showed that it was impossible to separate 2,6-dichlorophenol (P3) from 4-chloro-2,6-dinitrophenol (P4), when using BSA as well as SDS micellar buffer.

We did not try to increase albumin concentration, although this has been shown to improve peak shape and resolution [24], because it also decreases selectivity. Moreover, increasing albumin concentration leads to a strong decrease in electroosmosis, due to albumin adsorption onto the wall of the non-coated capillary. It also causes some undesirable peak broadening.

None of these various buffered electrolyte solutions gave a well-resolved separation, so we tested the separation of these solutes with BSA–SDS complex as a selector.

3.2.3. BSA–SDS complex

The nature of the complex resulting from the association of the protein (BSA) and the surfactant (SDS) depends on the region of the isotherm corresponding to BGE composition. Thus, we varied the composition of the buffer in BSA and SDS, and tested the effect of such buffer additives on the efficiency of separation of the same five substituted phenols as above.

Each BGE selected had a known composition, in regard to the isotherm curve (Fig. 1). Each is characterized by its SDS concentration, protein concentration and amount of SDS bound. Corresponding to region (2) of the curve, we tested a buffer (BGE_1) with $C_{BSA}=2\times10^{-6}$ mol l^{-1} and $C_{SDS\ free}=8.4\times10^{-5}$ mol l^{-1}, $\bar{\nu}=8$ ($C_{SDS\ Total}=1\times10^{-4}$ mol l^{-1}) and a second buffer (BGE_2) with $C_{BSA}=3\times10^{-6}$ mol l^{-1} and $C_{SDS\ free}=7.6\times10^{-5}$ mol l^{-1}, $\bar{\nu}=8$ ($C_{SDS\ Total}=1\times10^{-4}$ mol l^{-1}). This second buffer had a higher BSA concentration and a weaker free SDS concentration since they have the same total SDS concentration and contained the same type of complex ($\bar{\nu}=8$). For BGE_3, we reduced the total SDS concentration, keeping the same concentration of complex ($C_{BSA}=2\times10^{-6}$ mol l^{-1}) in the buffer and the same number of moles of detergent bound per mole of protein ($\bar{\nu}=8$), in order to have the same free SDS concentration than in BGE_2. We also tested a buffer BGE_4 with $C_{BSA}=3\times10^{-6}$ mol l^{-1} and $C_{SDS\ total}=6\times10^{-7}$ mol l^{-1}, corresponding to the region of the isotherm curve where the binding is negligible. All these BGE compositions are collected in Table 3.

All experimental conditions were kept constant ($\Delta V=15$ kV, $T=300$ K, pH 8.2), such that any effect was due only to a change in the buffer composition. The solutes tested were injected separately and then as a mixture. The electroosmotic velocity was determined from the migration time of acetone and the migration time of the selector was determined from its high affinity for Coomassie blue dye.

In such experiments, it is important to show that changes in separation are due to changes in the capacity factor k', because this parameter is linked to

Table 3
Composition of the background electrolytes (BGE) which contained BSA–SDS complex

Solute	v_{net} (cm s^{-1})		k'
	[SDS] = 1×10^{-4} mol l^{-1}	BGE_1	
v_0 (acetone)	0.132	0.127	
v_{mic} (Coomassie blue dye)	–	0.067	
(P1) Phenol	0.132	0.124	0.05
(P2) 2-Chlorophenol	0.112	0.108	0.25
(P3) 2,6-Dichlorophenol	0.075	0.071	0.28
(P4) 4-Chloro-2,6 dinitrophenol	0.075	0.069	0.69
(P5) 2-Chloro-4-nitrophenol	0.076	0.068	22

Table 4
Net velocity (v_{net}) and capacity factor (k') for substituted phenols in BGE containing BSA–SDS complex (BGE_1), or SDS alone at the same concentration $C_{SDS}=1\times10^{-4}$ mol l^{-1} buffered with Tris, pH 8.2

Solute	v_{net} (cm s^{-1})		k'
	[SDS] = 1×10^{-4} mol l^{-1}	BGE_2	
v_0 (acetone)	0.132	0.102	
v_{mic} (Coomassie blue dye)	–	0.042	
(P1) Phenol	0.132	0.098	0.07
(P2) 2-Chlorophenol	0.112	0.088	0.12
(P3) 2,6-Dichlorophenol	0.075	0.047	25
(P4) 4-Chloro-2,6 dinitrophenol	0.075	0.044	1.2
(P5) 2-Chloro-4-nitrophenol	0.076	0.042	67

a specific separation carrier, for a given solute. We determined values of k' with these buffered solutions containing the BSA–SDS complex, by producing electropherograms without BSA. The net velocities and resulting capacity factors are given in Tables 4 and 5.

The first BGE tested (BGE_1) consisted of a Tris buffer containing protein ($C_{BSA}=2\times10^{-6}$ mol l^{-1}) and surfactant ($C_{SDS}=1\times10^{-4}$ mol l^{-1}), with $C_{SDS\ free}=8.4\times10^{-5}$ mol l^{-1} and $\bar{\nu}=8$. The presence of BSA in the buffer made several differences (Fig. 4a). P3 and P4, which were not separated if the buffer did not contain BSA were separated with a low resolution. However, calculation of these resolution values should be avoided because of difficulties to estimate the width of the peaks with such a peak shape.

BSA and SDS together created a new selectivity: P5 which was not well separated from P4, now had a separate peak, and the selectivity is greatly modified for this solute. It was the third solute, and the last one when the selector was BSA–SDS. This was also illustrated by the change in k' value. It is striking to observe that this solute had a high capacity factor with the BSA–SDS complex, whereas all the others have low values. This also suggests that the separation of the other phenols with similar k' was driven mainly by the difference in the electrophoretic velocities of these solutes.

As shown in Fig. 4, the total analysis time was still reasonably short and the separation of the five solutes was good, the worst resolution being that of P3 and P4 ($R=1.14$).

With this specific BGE_1 composition, the electrophoretic mobility of the BSA–SDS complex was $\mu_{BSA-SDS}=2.77\times10^{-4}$ cm^2 V^{-1} s^{-1}. The electrophoretic mobility of this complex is higher than the mobility of the protein alone in a buffer of the same pH. The surfactant molecules are fixed onto the protein by their hydrocarbon chain, interacting with specific high energy sites, hydrophobic patches [68]. The isotherm curve indicates that eight SDS monomers are attached to each protein in the first plateau of the isotherm curve, and many more are attached when the protein is denatured. As the protein bears a negative overall charge at this pH, the negative charges of the associated SDS monomers must be added. So that the complex produced by interaction between BSA and SDS, over the entire isotherm, bears a more negative charge than BSA alone. This

Table 5
Net velocity (v_{net}) and capacity factor (k') for substituted phenols in BGE containing BSA–SDS complex (BGE_2), or SDS alone at the same concentration $C_{SDS}=1\times10^{-4}$ mol l^{-1} buffered with Tris, pH 8.2

	C_{BSA} (mol l^{-1})	C_{SDS} (mol l^{-1})	$C_{SDS\ free}$ (mol l^{-1})	$\bar{\nu}$ (mol SDS/mol BSA)
BGE_1	2×10^{-6}	1×10^{-4}	8.4×10^{-5}	8
BGE_2	3×10^{-6}	1×10^{-4}	7.6×10^{-5}	8
BGE_3	2×10^{-6}	9.2×10^{-5}	7.6×10^{-5}	8
BGE_4	3×10^{-6}	6×10^{-7}	6×10^{-7}	ε

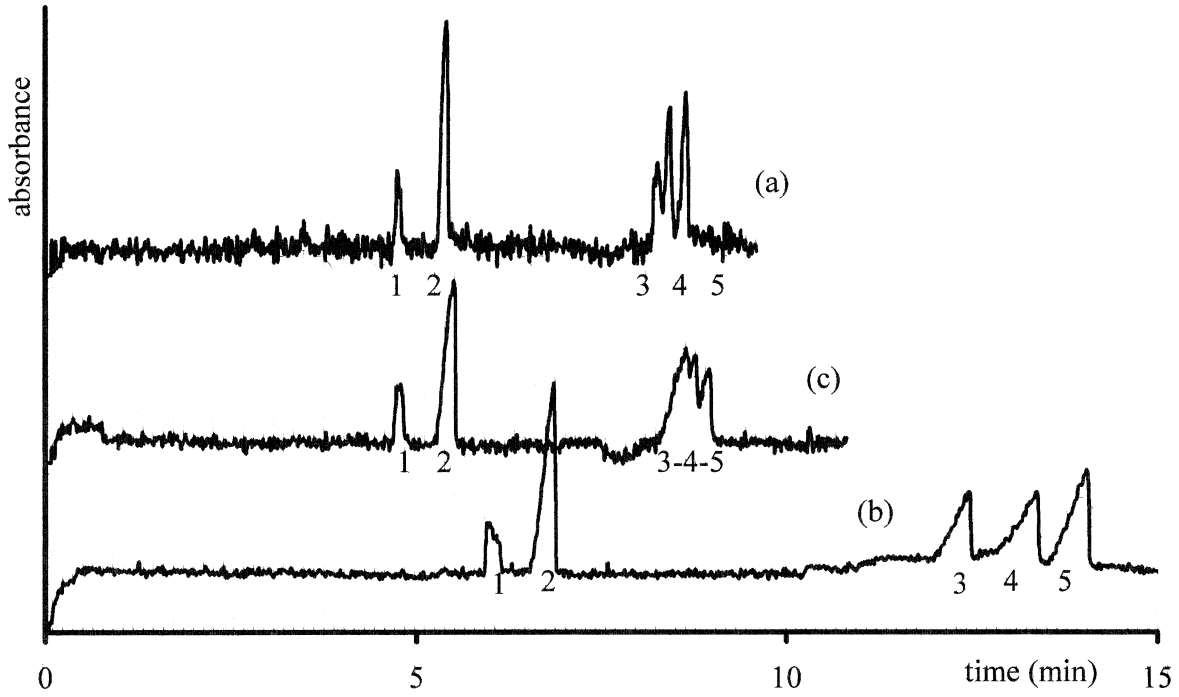

Fig. 4. Electropherograms with buffered BSA–SDS electrolyte solutions: (a) BGE_1; (b) BGE_2; (c) BGE_3, for solutes P1–P5, as defined in the text.

is consistent with the observed increase in electrophoretic velocity, considering that the increase in size of the BSA due to the bound SDS monomer is negligible.

Then we tried to improve the resolution. When the protein concentration was increased to $C^{BSA}=3\times 10^{-6}$ mol l^{-1}, keeping the total SDS concentration constant ($C_{SDS}=1\times 10^{-4}$ mol l^{-1}), the complex formed was the same as for BGE_1, as indicated on the isotherm (Fig. 1). With such a BGE, the separation was good (Table 5), because all the peaks were well resolved (Fig. 4b). Electroosmotic velocity was lowest in this case, and since the higher concentration of selector retaining the solutes brings also longer analysis time, the overall analysis time was longer. However the total time was still reasonable (14 min), and can be shortened by altering the voltage applied.

The k' values were different in this BGE_2, giving well separated peaks. The main difference with this complex was that there was high affinity for 2,6-dichlorophenol (P3). The electrophoretic mobility of the SDS complex calculated in BGE_2 was $\mu_{BSA-SDS}=2.83\times 10^{-4}$ cm^2 V^{-1} s^{-1}. This value of the mobility is similar to the value previously calculated ($\mu_{BSA-SDS}=2.77\times 10^{-4}$ cm^2 V^{-1} s^{-1}). This is consistent with the isotherm curve. The slight difference cannot be due to an increase in charge, because all sites were saturated, but is rather consistent with experimental uncertainty.

When the concentration of the complex in the buffer was increased, keeping the same concentration of free SDS, as is the case for BGE_3 and BGE_2 ($C_{BSA}=2\times 10^{-6}$ mol l^{-1} or $C_{BSA}=3\times 10^{-6}$ mol l^{-1}, with $C_{SDS\ free}=7.6\times 10^{-5}$ mol l^{-1} as a constant) the analysis was improved. These two buffered electrolyte solutions correspond to the same position on the isotherm curve. Comparison of the electropherograms with BGE_2 and BG_3 (Fig. 4b,c) showed that the time required for total analysis was increased from 9 min for the lower concentration to 14 min for the higher concentration. This was partially due to the decrease in the velocity of the electroosmotic flow. The resolution for the first two

peaks was the same in both cases. However, the resolution of the last three peaks was much better. With the lowest concentration, these peaks were not even baseline separated. With the second buffer, as the concentration of the separator increased, separations were achieved and resolutions were good.

Increasing the concentration of the BSA–SDS complex (all other conditions being identical) increased separation efficiency, indicating that it was effective. All the solutes interacted with this complex to some extent and the separation was based on differential interactions.

On the contrary, when the concentration of protein was fixed at $C_{BSA}=2\times10^{-6}$ mol l^{-1} and the free SDS concentration was increased (BGE_1 and BGE_3), separation efficiency was not modified (Fig. 4a,c). However, it was not surprising since these compositions are not close to threshold concentrations at which large changes might occur, and surfactant concentrations were well maintained. It has been shown previously that increase in the surfactant concentration below the CMC bring few modifications.

When the concentrations were fixed at $C_{BSA}=3\times10^{-6}$ mol l^{-1} for protein, and at $C_{SDS}=6\times10^{-7}$ mol l^{-1} for total surfactant concentration, the proteins do not fix any surfactant monomer, relative to the isotherm curve (Fig. 1). We observed that the separation efficiency was similar to that of the protein alone. In both cases, total separation of the solutes was not achieved, and only four peaks were observed for five solutes injected. The only difference between these two electropherograms was due to the faster electroosmotic flow velocity for the buffer with surfactant. In fact, such a small SDS concentration in regard to the BSA concentration had little effect.

One can think that other buffer compositions would have been interesting to study. For example, one can expect that composition in region (3) of the isotherm, where the charge of the complex could be greatly modified with little modification of the free SDS concentration, could lead to a better resolved separation. But, in practice, it is rather difficult to obtain such a BGE composition in the capillary with certainty since the slope of the isotherm, in this region of the curve, is rather straight. Moreover, free SDS concentration can be modified by any added adsorption phenomena.

For higher free SDS concentrations, protein is supposed to be denatured. In this case, the protein loses its globular shape, and this complex has already been studied as selector.

The capacity factor differed greatly between some of the solutes tested and some compositions of the BGE. Fig. 5 illustrates more clearly the large changes in selectivity shown by the calculated values of k' reported in Tables 1–4. It is clear that the k' values for P1 and P2 are not affected by the composition of the buffer. Moreover, even with BSA or SDS alone, the k' values for these solutes are very low. These two solutes have higher k' values in SDS buffers. We found back that P4, which has some affinity for BSA alone, gave, in BSA in the presence of SDS, a k' value slightly different from those of P1 and P2. P5 had rather high k' values in BGE with BSA–SDS complex but low k' values with BSA or SDS alone. P3, as P4, had k' values higher in BGE with BSA–SDS complex than with BSA alone. These experimental values, however, were highly sensitive to the precision of time measurements, so we have commented on relative results rather than on intrinsic values of k'. This suggests that the solutes that have some affinity for BSA alone also have an affinity for BSA in BSA–SDS complexes, although it may be weaker or stronger. These values of k' also show that P1 and P2 behave similarly, probably because they are non-ionic or partially ionized, and that the other three solutes, which are ionic at this pH may have strong affinity for anionic selectors.

Thus, this technique gives good separation, although the use of proteins in the BGE may have some disadvantages.

The major disadvantage when filling the buffer reservoirs and the whole capillary with the protein is that it is always present in the detection region. The analyte is thus always detected in the presence of a large background signal from the protein, leading to high limits of sensibility, even if the protein is used at low concentration. Wavelengths can be adjusted to achieve the best compromise in signal-to-noise ratio (250 nm is best). One possible interesting way to solve this problem is the partial-filling technique [25,42].

Many protein molecules do stick to the surface.

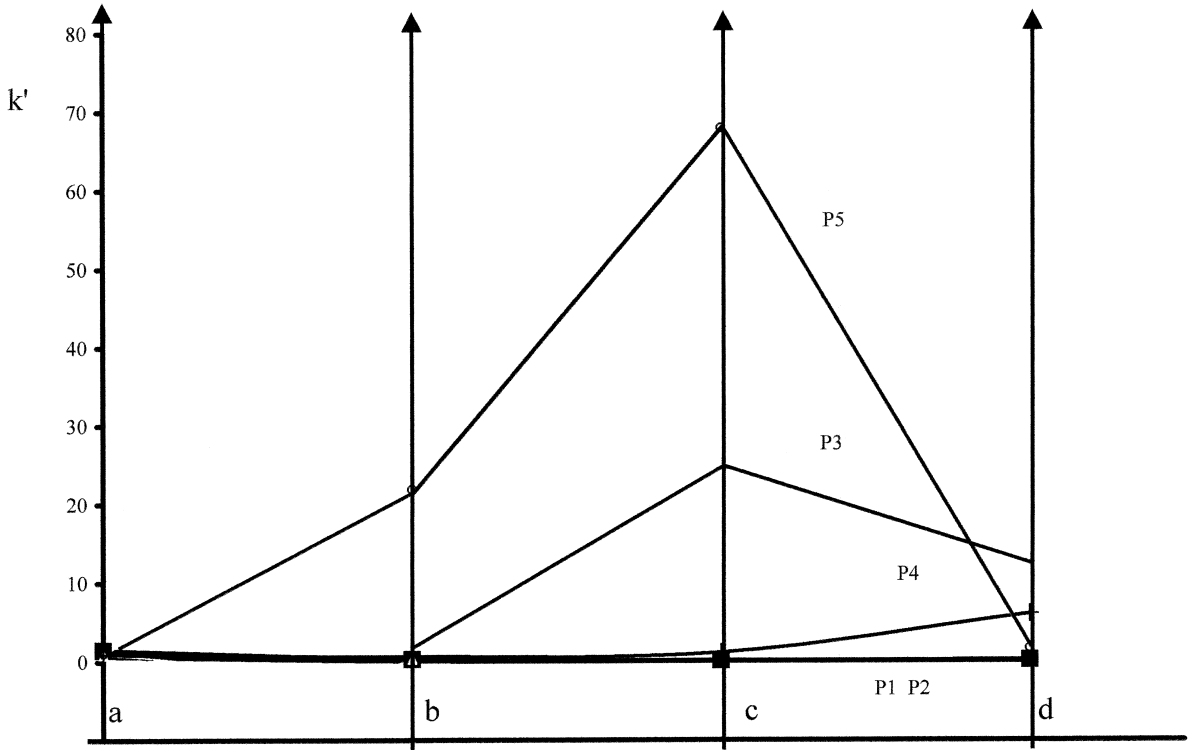

Fig. 5. Changes in k' values for solutes P1–P5 (see text) in various BGE compositions. (a) SDS alone ($C_{SDS}=6\times10^{-2}$ mol l^{-1}); (b) BSA–SDS complex ($C_{BSA}=2\times10^{-6}$ mol l^{-1}); (c) BSA–SDS complex ($C_{BSA}=3\times10^{-6}$ mol l^{-1}); (d) BSA alone ($C_{BSA}=3\times10^{-6}$ mol l^{-1}).

The capillary quickly becomes coated with a monolayer of protein. This is of major importance when protein concentration is determined by EKC analysis. This adsorption is generally prevented by the use of surfactant. Otherwise, this coating provides a retentive surface affecting resolution [72], but having a relatively small effect. It may cause some changes in electroosmotic flow. It is important to know whether this improves separation or not. If not, protein–wall interactions can be minimized using a poly-(ethyleneglycol)-coated capillary [26], which improves peak reproducibility and extends column lifetime.

The addition of surfactant to the protein-containing buffer also leads to changes in current, electroosmotic flow mobility, viscosity and effective mobility of the protein. This is why it is sometimes difficult to compare k' values.

The peak tailing may be due to an interaction of the solute with adsorbed BSA. Usually, this tailing is prevented by adding propanol [25]. It has been suggested that the extremely broad and asymmetrical peak observed [25] is due to strong hydrophobic interaction with the non-polar patches on the surface of the protein. Peak tailing is difficult to explain in our case, because the use of surfactant prevented BSA adsorption and saturated the hydrophobic patches on the protein. There is probably a screening effect due to the shape of the protein and the opposite directions of the flows.

4. Conclusion

As shown in Fig. 4b, substituted phenols can be separated on a bare silica capillary, using a Tris-buffered electrolyte solution that containing BSA and SDS.

Limitations of EKC, if protein is used, include the need to minimize protein–wall adsorption. This was

accomplished here by adding SDS surfactant, because the BSA–SDS complex bears a net negative charge, which considerably reduces its tendency to adhere to the wall of the fused-silica capillary. This property has also been successfully used to clean the capillary between injections of biological fluids, by rinsing the capillary with a washing solution containing SDS [73]. It is therefore not necessary to coat the capillary wall with polymers. Nevertheless, this should give more reproducible results, which are required for thermodynamic studies of substrate binding interactions.

In contrast, one study has shown that the capillary wall adsorption of the protein may improve separation that was not effective without this adsorption [72]. However, desorption is difficult to prevent and this often leads to a lack of reproducibility.

However, our results show that, using a biological molecule with high selectivity (BSA in this case) and a surfactant used as a BGE modifier, separations not achieved with surfactant alone were feasible. This EKC method, thus presenting many disadvantages, offers an advantage over some others in its simplicity to use. The biological molecule is simply added to the running buffer, with no other operations required. But the choice of the composition of the BGE, and the precise concentration of protein relative to the concentration of surfactant is of major importance. We have demonstrated that the separation behaviour of this selector is well correlated with the isotherm curve. Dramatic changes in the separation were observed for a minor change in composition. It provides useful alternative selectivities to SDS micellar electrokinetic chromatography. Our results show that the solutes studied which have low affinity for SDS micelles were well separated because of changes in affinity with BSA–SDS complexes. Even with a large decrease in the applied voltage, the separation with SDS micelles was not as good as that achieved with the complex, and the total analysis time was much longer. It was not even possible to determine the conditions of SDS concentration and applied voltage that will give the same separation efficiency as that with the BSA–SDS complex.

It would be interesting to know whether the pseudo-organic phase organized around the protein chains give this selector a peculiar selectivity. But if more surfactant molecules associated to the protein are required, another protein with a larger number of binding sites before protein denaturation should be used. With BSA, we obtained a larger number of associated surfactant monomers only when the protein lost its sponge-like properties.

Potentially, any protein associated with any surfactant may be used as a selective pseudo-phase, making this technique very general.

References

[1] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Anal. Chem. 56 (1984) 111.
[2] J. Gorse, A.T. Balchunas, D.F. Swaile, M.J. Sepaniak, J. High Resolut. Chromatogr. Chromatogr. Commun. 11 (1988) 554.
[3] C.P. Palmer, M.Y. Khaled, H.M. McNair, J. High Resolut. Chromatogr. 15 (1992) 756.
[4] C.P. Palmer, S. Terabe, J. Microcol. Sep. 8 (1996) 115.
[5] S. Yang, J.G. Bumgarner, M.G. Khaledi, J. High Resolut. Chromatogr. 18 (1995) 443.
[6] H. Harino, M. Tanaka, T. Araki, Y. Yasaka, A. Masayuma, Y. Nakatsuji, I. Ikeda, K. Funazo, S. Terabe, J. Chromatogr. A 715 (1995) 135.
[7] Y. Shi, J.S. Fritz, Anal. Chem. 67 (1995) 3023.
[8] P. Blatny, C.-H. Fischer, E. Kenndler, J. Fresenius, Anal. Chem. 352 (1995) 712.
[9] E.S. Ahuja, E.L. Little, K.R. Nielsen, J.P. Foley, Anal. Chem. 67 (1995) 26.
[10] N. Tanaka, T. Fukutome, T. Tanigawa, K. Hosoya, K. Kumata, T. Araki, K.K. Unger, J. Chromatogr. A 699 (1995) 331.
[11] M.-L. Riekkola, S.K. Wiedmer, I.E. Valko, H. Siren, J. Chromatogr. A 792 (1997) 13.
[12] R. Kuhn, S. Hoffstetter-Kuhn, Chromatographia 34 (1992) 505.
[13] R. Kuhn, F. Stocklin, F. Erni, Chromatographia 33 (1992) 32.
[14] S. Terabe, K. Otsuka, T. Ando, Anal. Chem. 57 (1985) 834.
[15] S.A. Wren, R.C. Rowe, J.Chromatogr. 603 (1992) 235.
[16] A. Shibukawa, D.K. Lloyd, I.W. Wainer, Chromatographia 38 (1993) 419.
[17] S.G. Penn, E.T. Bergström, D.M. Goodall, J.S. Loran, Anal. Chem. 66 (1994) 2866.
[18] Y.Y. Rawjee, G. Vigh, Anal. Chem. 66 (1994) 619.
[19] K.L. Rundlett, D.W. Armstrong, J. Chromatogr. A 721 (1996) 173.
[20] F. Lelievre, P. Gareil, A. Jardy, Anal. Chem. 69 (1997) 385.
[21] E. Gassmann, J.E. Kuo, R.N. Zare, Science 230 (1985) 813.
[22] P. Gozel, E. Gassmann, H. Michelsen, R.N. Zare, Anal. Chem. 59 (1987) 44.
[23] Y. Ishihama, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, J. Chromatogr. A 666 (1994) 193.

[24] R. Vespalec, V. Sustacek, P. Bocek, J. Chromatogr. 638 (1993) 255.
[25] L. Valtcheva, J. Mohammad, G. Pettersson, S. Hjerten, J. Chromatogr. 638 (1993) 263.
[26] G.E. Barker, P. Russo, R.A. Hartwick, Anal. Chem. 64 (1992) 3024.
[27] P. Sun, N. Wu, G. Baker, R.A. Hartwick, J. Chromatogr. 648 (1993) 475.
[28] S. Busch, J.C. Kraak, H. Poppe, J. Chromatogr. 635 (1993) 119.
[29] D.K. Lloyd, S. Li, P. Ryan, Chirality 6 (1994) 230.
[30] S.G. Allenmark, S. Andersson, J. Chromatogr. A 666 (1994) 167.
[31] N. Mano, Y. oda, N. Asakawa, Y. Yoshida, T. Sato, T. Miwa, J. Chromatogr. A 687 (1994) 223.
[32] G. Massolini, E. de Lorenzi, M.C. Ponci, C. Gandini, G. Caccialanza, H.L. Monaco, J. Chromatogr. A 704 (1995) 55.
[33] Z. Simek, R. Vespalec, J. Chromatogr. 629 (1993) 153.
[34] Z. Simek, R. Vespalec, J. Subert, J. Chromatogr. 543 (1991) 475.
[35] A. Ahmed, D.K. Lloyde, J. Chromatogr. A 766 (1997) 237.
[36] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801.
[37] S. Birnbaum, S. Nilsson, Anal. Chem. 64 (1992) 2872.
[38] G.E. Barker, W.J. Horvath, C.W. Huie, R.A. Hartwick, J. Liq. Chromatogr. 16 (1993) 2089.
[39] P. Sun, G. Barker, R.A. Hartwick, N. Grinberg, R. Kaliszan, J. Chromatogr. A 652 (1993) 247.
[40] T. Arai, N. Nimura, T. Kinoshita, Biomed. Chromatogr. 9 (1995) 68.
[41] T. Arai, M. Ichinose, H. Kuroda, N. Nimura, T. Kinoshita, Anal. Biochem. 217 (1994) 7.
[42] Y. Tanaka, S. Terabe, J. Chromatogr. A 694 (1995) 277.
[43] D. Wistuba, H. Biebold, V. Schurig, J. Microcol. Sep. 7 (1995) 17.
[44] X. Zhang, E.W. Davidian, T.H. Nguyen, R.W. Evans, S.J. Im, G.E. Barker, J. Chromatogr. A 745 (1996) 1.
[45] D. Eberle, R.P. Hummel, R. Kuhn, J. Chromatogr. A 759 (1997) 185.
[46] J. Cai, Z. El Rassi, J. Chromatogr. 608 (1992) 31.
[47] N. Wu, G.E. Barker, C.W. Huie, J. Chromatogr. A 659 (1994) 435.
[48] M.A. Strege, A.L. Lagu, J. Chromatogr. 630 (1993) 337.
[49] M.A. Strege, A.L. Lagu, J. Chromatogr. A 780 (1997) 285.
[50] R. Weinberger, E. Sapp, S. Moring, J. Chromatogr. 516 (1990) 271.
[51] Y.J. Yao, H.K. Lee, S.F.Y. Li, J. Chromatogr. 637 (1993) 195.
[52] J.A. Reynolds, C. Tanford, J. Biol. Chem. 245 (1970) 5161.
[53] O. Laurie, J. Oakes, J. Chem. Soc., Faraday Trans. 72 (1976) 1324.
[54] K. Takeda, M. Shigeta, K. Aoki, J. Colloid Interface Sci. 117 (1987) 120.
[55] W. Parker, P.-S. Song, Biophys. J. 61 (1992) 1435.
[56] J. Steinhardt, J. Krijn, J.G. Leidy, Biochemistry 10 (1971) 4005.
[57] A. Ray, J. Reynolds, H. Polet, J. Steinhardt, Biochemistry 5 (1966) 2606.
[58] M. Sen, S.P. Mitra, D.K. Chattoraj, Indian J. Biochem. Biophys. 17 (1980) 370.
[59] M.J. Pallansch, D.R. Briggs, J. Am. Chem. Soc. 76 (1954) 1396.
[60] J. Oakes, J. Chem. Soc., Faraday Trans. 1 (70) (1974) 2200.
[61] N.P. Turro, X.-G. Lei, K.P. Ananthapadmanabhan, M. Aronson, Langmuir 11 (1995) 2525.
[62] A. Chen, D. Wu, C.S. Johnson, J. Phys. Chem. 99 (1995) 828.
[63] R. Das, D. Guha, S. Mitra, S. Kar, S. Lahiri, S. Mukherjee, J. Phys. Chem. A 101 (1997) 4042.
[64] J.A. Reynolds, J.P. Gallagher, J. Steinhardt, Biochemistry 9 (1970) 1232.
[65] H.M. Rendall, J. Chem. Soc., Faraday Trans. 72 (1976) 481.
[66] J.A. Reynolds, S. Herbert, H. Polet, J. Steinhardt, Biochemistry 6 (1967) 937.
[67] T. Peters, in: All About Albumin Biochemistry, Genetics, Medical Applications, Academic Press, San Diego, CA, 1996, p. 59.
[68] C. Tanford, in: The Hydrophobic Effect: Formation of Micelles and Biological Membranes, Wiley, New York, 1980, pp. 156–159, Ch.14.
[69] T. Peters, in: C.B. Anfinsen, J.T. Edsall, F.M. Richards (Eds.), Advances in Protein Chemistry, Vol. 37, Academic Press, New York, 1985, p. 161.
[70] M.K. Menon, A.L. Zydney, Anal. Chem. 710 (1998) 1581.
[71] R.H. McMenamy, in: V. Rosenoer, M. Oratz, M. Rotshild (Eds.), Albumin Structure, Function and Uses, Pergamon, Elmsford, NY, 1977, p. 143.
[72] J. Yang, D.S. Hage, Anal. Chem. 66 (1994) 2719.
[73] D.K. Lloyd, H. Watzig, J. Chromatogr. B 663 (1995) 400.