ELSEVIER

Journal of Chromatography A, 757 (1997) 255–262

JOURNAL OF CHROMATOGRAPHY A

Capillary electrophoretic separation of acidic and basic proteins in the presence of cationic and anionic fluorosurfactants

E.L. Hult, Å. Emmer, J. Roeraade*

Royal Institute of Technology, Dept of Analytical Chemistry, S-10044 Stockholm, Sweden

Received 11 April 1996; revised 3 July 1996; accepted 29 July 1996

Abstract

We report the use of mixtures of cationic and anionic fluorosurfactants as additives for free-flow capillary electrophoresis of proteins. Effective deactivation of the capillary wall is obtained, which allows the use of raw fused-silica capillaries. The magnitude and direction of the electroosmotic flow is strongly affected by the composition of the surfactant mixture, and a suggested model for this behaviour in terms of micellation and formation of admicellar surfactant layers is described. By utilizing mixtures of the oppositely charged surfactants, it is possible to separate both positively and negatively charged proteins in the same run. Due to charge interactions of the fluorosurfactants with the proteins, it is possible to tune the separation selectivity, without having to change the buffer strength or pH. This was demonstrated in particular for the model substances myoglobin and ribonuclease, where the order of elution could be reversed, compared to their elution order in a normal buffer system. Another advantage of the fluorosurfactant additives is their effectiveness in low concentrations (<100 μg/ml) even when buffers of low ion strength are employed. Thus, rapid separations at a high field strength can be accomplished, without suffering from excessive Joule heating. This is demonstrated with an example, where a mixture of positively and negatively charged proteins is separated in less then 2 min in a 10 m*M* phosphate buffer at pH 7.

Keywords: Wall adsorption; Proteins; Fluorosurfactants

1. Introduction

Capillary electrophoresis (CE) is a powerful tool for analytical separation of biomolecules such as proteins. The features of the technique are high separation efficiency, automation possibilities, short analysis times and a very small consumption of sample and chemicals. However, a serious problem, recognised by many workers, is the tendency for proteins to adsorb onto the inner wall of the capillary column. Several methods have been proposed to reduce this problem, including the use of buffers with extreme pH and high ionic strength, by using buffer additives or permanent chemical modification of the capillary wall.

In earlier reports, we have proposed a concept where fluorinated surfactants were employed as buffer additives for improved CE separation of proteins [1–4]. The addition of a cationic surfactant causes a charge reversal of the silica surface, and this was shown to be particularly efficient in reducing adsorption of basic proteins at natural pH [1,2]. Further addition of nonionic and zwitterionic fluoro-

* Corresponding author.

0021-9673/97/$17.00
PII S0021-9673(96)00671-1

surfactants in different proportions also made it possible to tune the separation selectivity.

A fundamental requirement in this context is that the pH of the buffer is adjusted to a value, where the net charges of the proteins obtain the same sign as the fused-silica wall, otherwise the analytes will be tenaciously adsorbed onto the surface. Unfortunately this implies that it is not possible to obtain satisfactory conditions where both positively and negatively charged proteins can be separated in a single run.

Although a number of ways have been proposed to deal with this problem, these suggestions generally also include operational constraints. For example, it is possible to suppress adsorption by using buffers with a high salt concentration [5], but this can lead to excessive Joule heating and/or denaturation of proteins.

A permanent modification of the wall with a non-charged ligand such as acrylamide is an attractive and frequently employed way to reduce adsorption. However, such columns cannot be employed for a simultaneous analysis of positively and negatively charged proteins, since there is no electroosmotic flow to force all analytes through the capillary [6–8]. Some permanent wall coatings do not completely cover the negatively charged silanol groups at the capillary wall and, therefore, a substantial electroosmotic flow can be maintained. In other types of coatings, small ions are trapped in the bonded layer, which also results in an electroosmotic flow. If such coatings are shielded by a non-charged layer, which reduces the risk of the analytes adsorbing on the wall, it becomes possible to carry out a simultaneous analysis of both positively and negatively charged proteins [8–11]. However, often it is then necessary to utilize high-ionic-strength buffers, and operation is also restricted to certain pH intervals. Moreover, the preparation of permanent coatings can be time consuming and laborious, while the stability of such coatings is often limited.

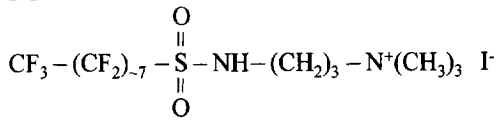
In this work, we have studied the effect of adding mixtures of an anionic and a cationic fluorosurfactant to the running buffer. It was shown that it is possible to alter the sign and the density of the charge at the capillary wall and in the vicinity of the proteins, which made it possible to carry out a CE analysis of both negatively charged and positively charged proteins in the same run, without any further pretreatments.

2. Experimental

A custom-made capillary electrophoresis apparatus was utilized, which consisted of a high-voltage power supply, constructed from a Spellman CZE100 unit (Plainview, NY, USA), delivering up to ±30 kV, a plexiglass box with safety interlocks and an injection device. Injections were performed by timer-controlled electromigration and rinsing of the capillaries was accomplished by the action of pressurized air. The detector used was a Linear Instruments UVIS 200 (Reno, NV, USA), equipped with an on-column capillary cell (Model 9550-9155). The wavelengths used were 254 nm for mesityloxide and 210 nm for proteins. Fused-silica capillaries with an I.D. of 50 μm and an O.D. of 360 μm were purchased from Polymicro Technologies (Phoenix, AZ, USA). The lengths of the capillaries were: 438 mm between the inlet and the detector, 600 mm total length (Figs. 4 and 6), 400 mm between the inlet and the detector, 550 mm total length (Fig. 5) and 390 mm between the inlet and the detector, 500 mm total length (Fig. 7). For data acquisition and processing either an integrator (D-2000 Chromatointegrator, Hitachi, Tokyo, Japan) or chromatography evaluation software (EZChrom, Scientific Software, San Ramon, CA, USA) was used.

The cationic fluorosurfactant FC134 and the anionic fluorosurfactant FC128 (for the structures of the surfactants, see Fig. 1) obtained from 3M Company (St. Paul, MN, USA) were dissolved in Milli-Q-deionized water (Millipore, Bedford, MA, USA) and made up to stock solutions with a concentration of 200 μg/ml (FC134) and 100 μg/ml (FC128).

FC 134:



FC 128:

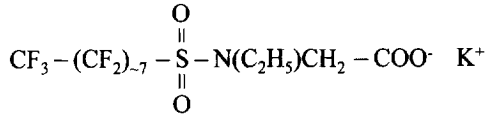


Fig. 1. General structures of the fluorosurfactants FC-134 and FC-128.

Table 1
Molecular mass and isoelectric points for the proteins

Protein	Molecular masses	Isoelectric point (pI)
Ribonuclease	13 700	9.6
Myoglobin	17 000	7.4
Carbonic anhydrase	29 000	6.3
Transferrin	88 000	5.4–5.9
β-Lactoglobulin	35 000	5.1/5.3
α-Lactalbumin	14 200	4.7
Albumin	66 000	4.6

Stock solutions of the proteins (Sigma, St. Louis, MO, USA), listed in Table 1 were prepared in Milli-Q-deionized water with a concentration of 2 mg/ml, and kept at 20°C until use. The samples injected had a concentration of 0.2 mg/ml. Throughout the work, a 0.01 *M* phosphate buffer of pH 7 was used as the background electrolyte.

New capillaries were rinsed with 1 *M* NaOH (15 min) and then with deionized water to neutrality before use. Finally, the capillaries were rinsed with the running buffer containing various concentrations and proportions of the two fluorosurfactants. When changing to another buffer composition the new solution was first passed through the capillary for about 30 min prior to CE. In certain cases, in order to obtain stable conditions, the capillary was equilibrated with the new buffer solution overnight.

Sometimes the fused-silica material showed variations in terms of osmotic flow between batches or even between different parts of the same batch (also observed in [12]). This can lead to reduced reproducibility between capillaries. This may be attended to by more extensive pretreatment of the capillaries before use.

3. Results and discussion

A proposed mechanism for the cationic fluorosurfactant as a dynamic coating agent in the CE separation of proteins has been described earlier [1,2]. In short, the positively charged fluorosurfactant adsorbs to the negatively charged fused-silica capillary wall. Due to a strong hydrophobic interaction between the fluorocarbon chains a second layer of surfactant molecules is adsorbed to the first layer, where the positively charged end of the second layer is oriented towards the bulk solution. Thus, the electroosmotic flow is reversed while positively charged proteins are electrostatically repelled from the surface.

In the present work, we started with a study of the influence of different mixtures of cationic and anionic surfactants on the electroosmotic flow, when these are added to a running buffer. First, the migration time for a neutral compound (mesityloxide) was measured, using a phosphate buffer system (pH 7), which only contained the cationic fluorosurfactant additive FC 134. Then, a series of subsequent measurements were made with an increasing fraction of the anionic fluorosurfactant additive FC 128. Another set of experiments, starting with FC 128 and ending with FC 134 was also carried out. Fig. 2 shows a graph of the recorded electroosmotic mobilities. These results are the average values of two experimental series. As can be seen, the electroosmotic flow reaches the highest value at a presence of 80% or more of either of the surfactants. However, the flow is in the opposite direction in the two cases. Thus, the net charge of the wall changes from positive to negative as the proportion of FC 128 is increased. A neutral surface is obtained when equal proportions of the fluorosurfactants are present. For a more detailed explanation of these results, it is necessary to consider the behaviour of mixed surfactant systems in terms of micellation and adsorption.

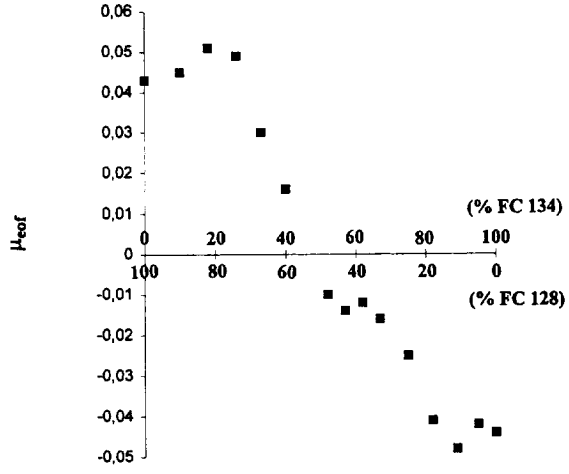


Fig. 2. Electroendosmotic mobility, μ_{eof} (cm^2/V min) as a function of the proportionality between the cationic and anionic fluorosurfactant additives. The total concentration of the additives was proportional between 50 μg/ml (only FC-128 added) and 100 μg/ml (only FC-134 added).

This issue has briefly been treated in one of our earlier reports, where zwitterionic and cationic surfactants were utilized as buffer additives [3].

It has been observed in several studies [13–15] that mixtures of surfactants with positively and negatively charged headgroups behave in a non-ideal manner, in contrast to mixtures of surfactants with the same sign of charge. Mixtures of the first-mentioned type show a deviation in critical micellar concentration (CMC) and the tendency of the surfactant molecules to adsorb onto surfaces is stronger than in the ideal case. Furthermore, it has been suggested that the surfactant molecules from a mixed cationic and anionic system can adsorb onto surfaces as ion pairs [13,16]. The cationic fluorosurfactant alone will readily adsorb onto fused-silica capillaries as described above, while the anionic fluorosurfactant alone will adsorb very weakly, due to electrostatic repellation as well as the extreme hydrophobicity of the fluorinated carbon chain.

In Fig. 3, a suggested model for the formation of the bilayer at the capillary wall in the presence of the different fluorosurfactant mixtures is shown. Starting with FC 128, only very few fluorosurfactant molecules adsorb onto the wall. The charge of the wall is, therefore, mainly due to the dissociated OH groups from the fused-silica and some scattered FC128 molecules. As the FC 128 is gradually exchanged by the cationic FC 134 additive, ion pairs and single cations will adhere to the surface. In this instance, a second fluorosurfactant layer is beginning to form on the wall due to the hydrophobic interaction. Eventually when the cationic surfactant fraction of the additive is sufficiently high, the wall obtains such a high concentration of positive charges that the EOF changes direction.

Subsequent experiments were then designed to study the behaviour of both acidic and basic proteins in a CE system, in the presence of mixtures of the fluorosurfactants. All separation studies were purposely carried out under very gentle conditions (pH 7 and a low buffer strength, 0.01 *M* phosphate buffer). Such separation conditions can be of interest when dealing with sensitive proteins and/or physiological fluids. However, this is challenging, since charge interactions between the capillary wall and the analytes is promoted under such conditions, and with bare fused-silica tubing it is normally not possible to obtain any useful separation efficiency. In fact, many proteins are irreversibly adsorbed onto the surface.

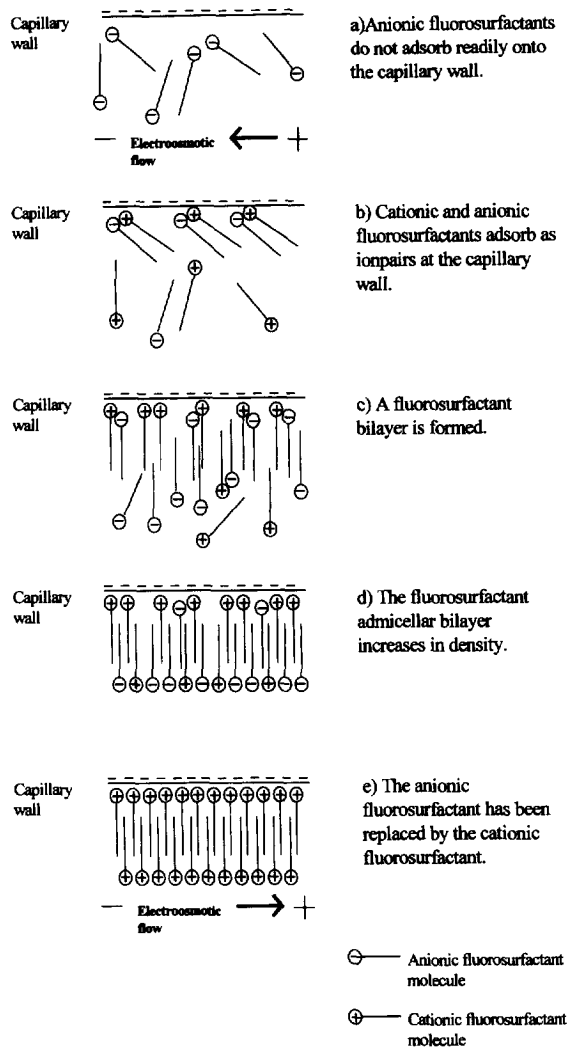


Fig. 3. Suggested model for the formation of an admicellar bilayer using mixtures of different fluorosurfactant additives, when progressively replacing the anionic (FC-128) for the cationic (FC-134) fraction.

The addition of the fluorosurfactants drastically affects the charge pattern of the proteins as well as the column wall. As we have shown earlier [1,2] excellent separation efficiency and peak symmetry is obtained for basic proteins at pH 7 when the cationic surfactant is added to the buffer due to the charge reversal of the wall and a complementary interaction

of the cationic surfactant. However, under these conditions the acidic proteins are tenaciously adsorbed on the wall and cannot be detected in the electropherograms. Addition of the anionic surfactant will create a reverse situation. The basic proteins are now adsorbed, while the acidic proteins are eluted from the column, but the peak shapes are very broad (low separation efficiencies). Apparently, the competing interaction of the positively charged sites of the analytes with the dissociated silanol groups of the silica is not sufficiently offset by the presence of the admicellar cloud of negatively charged surfactant molecules. However, when mixtures of the cationic and anionic fluorosurfactants are employed, it proved to be possible to perform a simultaneous separation of both acidic and basic proteins at natural pH. An example of this is shown in Fig. 4. The dominating additive of the buffer system was anionic, and the electroosmotic flow is, therefore, the normal direction (towards the cathode). It is important to note

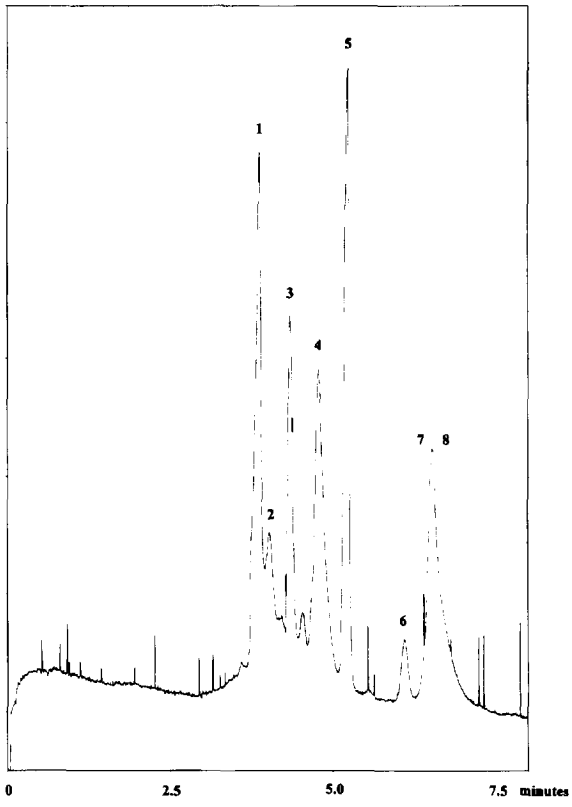


Fig. 4. Electropherogram of negatively and positively charged proteins: 1=myoglobin; 2=ribonuclease; 3=carbonic anhydrase; 4=transferrin; 5=α-lactalbumin; 6=β-lactoglobulin I; 7=β-lactoglobulin II; 8=albumin. Running buffer: 0.01 *M* phosphate, pH 7 with 59 μg/ml of a mixture of fluorosurfactants in the proportion 30% (w/w) cationic fluorosurfactant FC134 and 70% (w/w) anionic fluorosurfactant FC128. Running voltage: +20 kV. Injection: 15 s at +3 kV. The sharp spikes seen in the electropherogram are artefacts and are due to air bubbles.

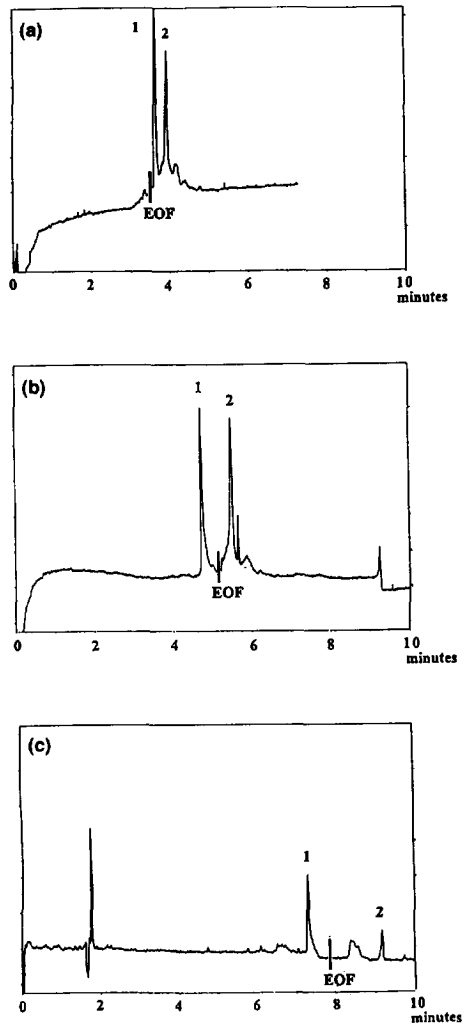


Fig. 5. Electropherograms of myoglobin (1) and ribonuclease (2), separated at pH 7, 0.01 *M* phosphate buffer, with different fluorosurfactant mixtures. (a) Ratio of FC-134 versus FC-128: 33% to 67% (w/w), total surf. conc.: 60 μg/ml; (b) ratio of FC-134 versus FC-128: 40% to 60% (w/w), total surf. conc.: 63 μg/ml; (c) ratio of FC-134 versus FC-128: 57% to 43% (w/w), total surf. conc.: 70 μg/ml. Running voltage: (a) +20 kV, (b) +20 kV and (c) −20 kV. Injection: 10 s at + resp. −4 kV.

that the migration order of the proteins is different from what is normally observed. For example myoglobin migrated at a higher velocity than ribonuclease, in spite of the fact that ribonuclease has a larger pI value. Several of the other proteins also show this elution anomaly. This suggests that the net charge of the proteins, and possibly also their 'electrophoretic size' has been altered by interactions with the fluorosurfactant molecules. These interactions are a dynamic equilibrium, where association/dissociation can occur with single surfactant molecules as well as various micellar structures [17,18].

A parameter of greatest importance for peak resolution is selectivity. The interactions between the protein molecules and the fluorosurfactant offers the possibility of selectivity tuning, when different proportions of the two fluorosurfactants are added to the buffer. This is shown in the electropherograms in Figs. 5a–c, where the basic proteins myoglobin and ribonuclease are separated, in the presence of a decreasing portion of the anionic surfactant. Significant changes in selectivity are obtained. The dominating part of the additive mixture in the experiments represented by Figs. 5a and b is anionic and the EOF is therefore towards the cathode. A slight increase in proportion of the cationic fluorosurfactant (Fig. 5b) accomplishes an increased electrophoretic mobility difference between the two proteins. This can be seen from the data in Table 2, where the mobilities of the proteins, the corresponding EOF and the ratios of the mobility differences and the EOF mobilities are shown. The migration time reproducibility (manual injections) was below 3% R.S.D. (relative standard deviation) for the neutral marker mesityloxide and below 5% R.S.D. for the proteins.

It is interesting to note that in all experiments (Figs. 5a–c), myoglobin has the shortest migration time, in spite of the change in direction and the electrode polarity (Fig. 5c).

Also the migration of acidic proteins are affected by varying the proportions between the fluorosurfactants. This is demonstrated in Figs. 6a–c, where carbonic anhydrase is subjected to CE with a small proportional increase of the cationic fluorosurfactant. While a 25/75% ratio of FC 128/FC 134 leads to a broad fused peak of the protein and its impurities (Fig. 6a), a change to 30/70 ratio leads to a resolution of the impurities and a much sharper protein peak (Fig. 6b). A further increase of the cationic surfactant (35/65 ratio) provides a still better separation of the impurities, but an increased peak dispersion, which cannot only be due to the decreased mobility of the analytes, can be noted. We rather believe this to be related to an increased interaction of the acidic protein with positively charged fluorosurfactant molecules present in the bilayer on the wall.

In conclusion, we have shown that it is possible to analyze both positively charged and negatively charged proteins at pH 7 in a phosphate buffer of only 0.01 *M* in concentration, by adding micromolar amounts of a combination of cationic and anionic fluorosurfactants in carefully selected proportions. Additionally, the separation selectivity can be tuned by changing the proportions of the surfactants, without having to adjust the pH or the buffer-ion strength. The low overall ionic strength of the separation medium offers the possibility of using a high field strength and of obtaining fast separations. Fig. 7 shows such an example, where a mixture of acidic and basic proteins were separated in less than

Table 2
Mobilities of the electroosmotic flow and the two proteins myoglobin and ribonuclease calculated from the results of the experiments, shown in Fig. 5

Electropherogram corresponding to Fig. 5	Electroosmotic mobility, μ_{eof} (cm^2/V min)	Mobility of myoglobin, μ_{myo} (cm^2/V min)	Mobility of ribonuclease, μ_{ribo} (cm^2/V min)	$(\mu_{myo}-\mu_{ribo})/\mu_{eof}$
(a)	0.030	0.030	0.028	0.079
(b)	0.021	0.023	0.020	0.16
(c)	0.014	0.015	0.012	0.22

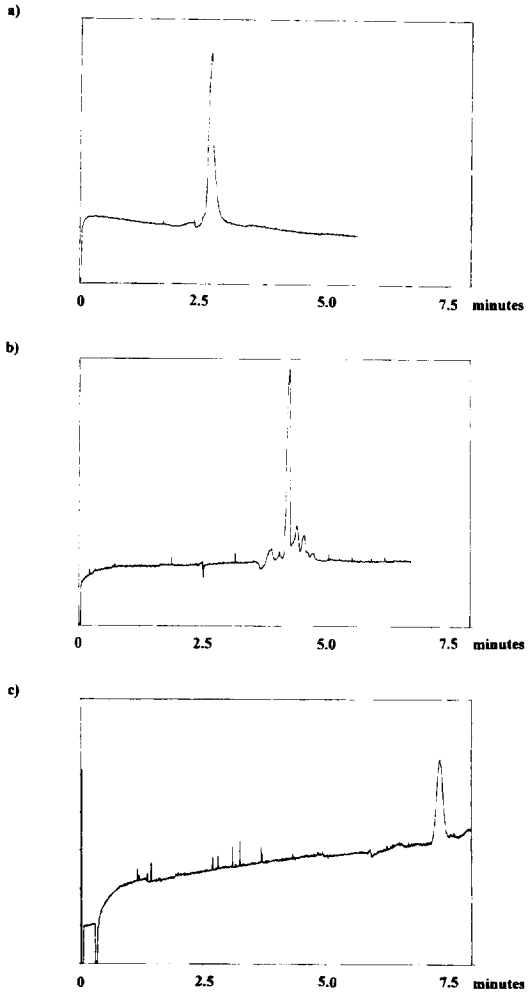


Fig. 6. Electropherograms of carbonic anhydrase with running buffers containing different mixtures of fluorosurfactants. Conditions as in Fig. 5. (a) Ratio of FC-134 versus FC-128: 25% to 75% (w/w); total surf. conc.: 56 μg/ml; (b) ratio of FC-134 versus FC-128: 30% to 70% (w/w); total surf. conc.: 59 μg/ml; (c) ratio of FC-134 versus FC-128: 35% to 65% (w/w); total surf. conc.: 61 μg/ml. Running voltage: +20 kV. Injection: 15 s at +3 kV.

2 min, using a field strength of about 600 V/cm, while the electrical current remained below 15 μA.

Acknowledgments

This project was financially supported by the Swedish Board for Technical Development and the Swedish Natural Science Council.

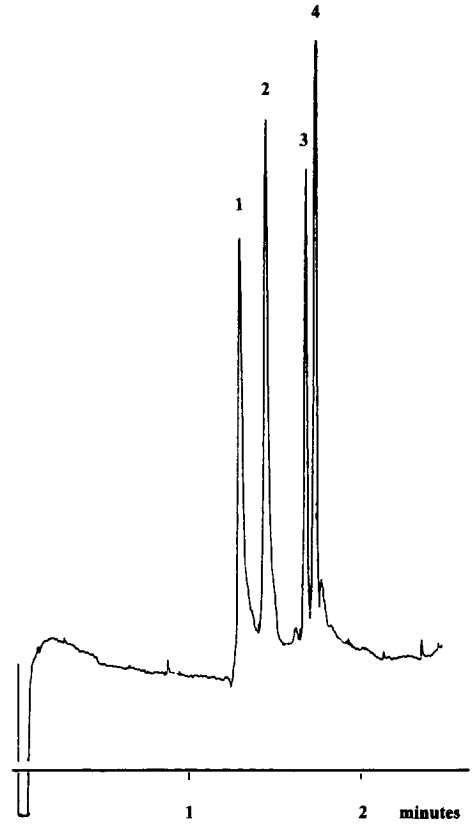


Fig. 7. Rapid electrophoretic separation of acidic and basic proteins: 1=myoglobin; 2=transferrin; 3=β-lactoglobulin I; 4=β-lactoglobulin II. Running buffer 0.01 *M* phosphate, pH 7, containing 20% (w/w) cationic (FC 134) and 80% anionic (FC 128) fluorosurfactant. The total concentration of the additives was 54 μg/ml. Running voltage: +30 kV. Injection: 10 s at +4 kV.

References

[1] Å. Emmer, M. Jansson and J. Roeraade, J. Chromatogr., 547 (1991) 544.
[2] Å. Emmer, M. Jansson and J. Roeraade, J. High Resolut. Chromatogr., 14 (1991) 738.
[3] Å. Emmer and J. Roeraade, J. Liq. Chromatogr., 17 (1994) 3831.
[4] Å. Emmer, M. Jansson and J. Roeraade, J. Chromatogr. A, 672 (1994) 231.
[5] J.S. Green and J.W. Jorgenson, J. Chromatogr., 478 (1989) 63.
[6] S. Hjertén, J. Chromatogr., 347 (1985) 191.
[7] K.A. Cobb, V. Dolnik and M. Novotny, Anal. Chem., 62 (1990) 2478.

[8] J.T. Smith and Z. El Rassi, Electrophoresis, 14 (1993) 396.
[9] S.A. Swedberg, Anal. Biochem., 185 (1990) 51.
[10] W. Nashabeh and Z. El Rassi, J. Chromatogr., 559 (1991) 367.
[11] M.H. Busch, J.C. Kraak and H. Poppe, J. Chromatogr. A, 695 (1995) 287.
[12] M. Gilges, M.H. Kleemiss and G. Schomburg, Anal. Chem., 66 (1994) 2038.
[13] J.H. Harwell and J.F. Scamehorn, in K. Ogino and M. Abe (Editors), Mixed Surfactant Systems, Marcel Dekker, New York, NY, 1993.
[14] M.R. Porter, in Handbook of Surfactants, Blackie Academic and Professional, London, 1994.
[15] J.F. Scamehorn, in J.F. Scamehorn (Editor), Phenomena in Mixed Surfactant Systems, ACS Symp. Ser. Vol. 311, American Chemical Society, Washington, DC, Washington, 1986.
[16] M.J. Schwuger, Kolloid Z.Z. Polym., 243 (1971) 129.
[17] J. Chen and E. Dickinson, Colloids Surfaces A, 100 (1995) 267.
[18] E.D. Goddard, Colloids Surfaces, 19 (1986) 301.