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Detection of bovine whey proteins by on-column derivatization capillary electrophoresis with laser-induced fluorescence monitoring

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Abstract

1-Anilinonaphthalene-8-sulfonic acid (1,8-ANS), 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) and 2-(*p*-toluidino)naphthalene-6-sulfonic acid (2,6-TNS) were evaluated as additives in different buffers for the detection of bovine whey proteins using laser-induced fluorescence (LIF) monitoring in capillary electrophoresis (CE). These *N*-arylaminonaphthalene sulfonates furnish a large fluorescence emission when associated to some proteins whereas their emission in aqueous buffers, such as those used in CE separations, is very small. To select the best detection conditions, the fluorescence of these probes was first compared using experiments carried out in a fluorescence spectrophotometer. Using bovine serum albumin (BSA) as a model protein, it was demonstrated that 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) buffer (pH 8 and pH 10.2) and the fluorescent probe 2,6-TNS gave rise to the highest increase in fluorescence for BSA. When the composition of these separation buffers was optimized for the electrophoretic separations, CHES buffer, pH 10.2 was chosen as the most suitable buffer to detect bovine whey proteins. The limit of detection obtained for some whey proteins in CE separations was about $6 \cdot 10^{-8}$ *M* for BSA, $3 \cdot 10^{-7}$ *M* for β-lactoglobulin A (β-LGA), $3 \cdot 10^{-7}$ *M* for β-lactoglobulin B (β-LGB), and $3 \cdot 10^{-6}$ *M* for α-lactalbumin (α-LA). These detection limits were compared to those achieved using UV detection under the same separation conditions. The results showed that the detection limits of BSA, β-LGA and β-LGB were twice as good using LIF than with UV detection. However, the limit of detection for α-LA was better when UV was used. The applicability of LIF detection to CE separation of whey proteins in bovine milk samples was also demonstrated. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Whey; Derivatization, electrophoresis; Proteins

1. Introduction

Capillary electrophoresis (CE) is well suited for protein analysis [1]. Although efficiencies close to 10^6 plates/m [2] were possible in the separation of these biopolymers in CE, the concentration sensitivity of this technique is usually limited to the μ*M* range for proteins if UV detection is used. To overcome this drawback, on-column sample pre-concentration [3–6] and fluorescence detection [7] are employed. The high sensitivity associated to fluorescence detection has increased the interest for CE in trace protein analysis. That is the case for instance of the detection of proteins coming from the host cell (HCPs) in the final formulation of bio-pharmaceutical products obtained by recombinant DNA technology [8]. Although, the use of lasers as

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light sources in fluorescence detection has led to limits of detection (LODs) of about 10^{-10} *M* [9] for proteins, this high concentration sensitivity is obtained only if rather expensive lasers with emission in the range of 270–280 nm are used to excite the intrinsic fluorescence of tryptophan, tyrosine or phenylalanine. Other less expensive lasers, such as He–Cd or non-frequency-doubled Ar^{+}, can also be used, but proteins have to be derivatized with appropriate dyes.

So far, pre-, on- and post-column derivatization of proteins have been used as derivatization methods in CE. In pre-column derivatization, covalent bonds are formed between the dye and the protein before injection. Although optimized reaction conditions are usually employed, multi-labeled products are formed causing band broadening or even the appearance of several peaks for pure proteins [10,11]. Post-column derivatization [11] requires complicated set-ups and the use of derivatizing reagents not fluorescent at the laser wavelength and capable of originating rapid reaction with proteins. The use of on-column derivatization for protein detection has been illustrated by Swaile and Sepaniak [12] and Colyer et al. [13] who have used fluorescence probes as a tool for non-covalent labeling of proteins for capillary electrophoresis using laser-induced fluorescence detection (CE–LIF).

Several *N*-arylaminonaphthlane sulfonate derivatives have been used for decades [14,15] as non-covalent binding fluorescent probes to obtain conformational information on proteins. Fluorescence excitation, emission and quantum yield of these probes are affected by their physical and chemical environment. Specifically, the quantum yield of these fluorescent dyes is enhanced when they are associated to some proteins [16].

The aim of this work is to explore the possibilities of on-column derivatization methods using fluorescent probes for CE–LIF of proteins. As a model system, we have selected the major bovine whey proteins: bovine serum albumin (BSA), β-lactoglobulin A (β-LGA), β-lactoglobulin B (β-LGB) and α-lactalbumin (α-LA). Bovine immunoglobulin was not considered due to its thermal instability [17]. At present, this model is particularly interesting because transgenic animals capable of producing therapeutic proteins in milk are under study as a cost-effective procedure for mass production of these proteins [18,19]. The immunogenic character associated to bovine β-lactoglobulins [20] and α-LA [21] increases the interest for the detection of these proteins at trace level.

2. Experimental

2.1. Instrumentation

A laboratory-made CE apparatus [7,22] was built using a Glassman (Whitehouse Station, NJ, USA) Model PS/EM 50R2 power supply and either a Linear (Reno, NE, USA) Model UVIS 200 detector or a LIF detector. The LIF detector consisted of an Omnichrom (Chino, CA, USA) Model 3074-20M He–Cd laser (325 nm, 20 mW); its output was focused by an Oriel (Stratford, CT, USA) $f=10$ mm quartz lens onto the detection window of the separation capillary. The fluorescence was collected normal to the excitation beam using an Oriel 10× microscope, filtered successively through a cut-off filter of 350 nm and a bandpass filter centered at 450 or 520 nm (all from Andover, Salem, NH, USA) and detected by a Hamamatsu (Hamamatsu City, Japan) Model R928 photomultiplier tube (PMT). Photocurrent was processed by an Oriel Model 7070 detection system photometer and a System Gold (Beckman, CA, USA) A/D converter. The photomultiplier detection system also included the photomultiplier power supply. Data were collected on a 486DX computer and handled with System Gold V8.10 software. The separation capillary was cooled to room temperature using a fan.

Separations were carried out using polyimide coated fused-silica capillaries of 75 μm I.D.×365 μm O.D. from Polymicro Technologies (Phoenix, AZ, USA).

Fluorescence spectrophotometric measurements were made using a Perkin-Elmer (Buckinghamshire, UK) LS 50B luminescence spectrophotometer. Slit widths of 2.5 nm were used. Quartz cells of 1×1 cm were employed. Experiments were carried out at room temperature.

2.2. Chemicals

All reagents employed were of analytical grade. 2-(*N*-Cyclohexylamino)ethanesulfonic acid (CHES)

and the potassium salt of 2-(*p*-toluidino)naphthalene-6-sulfonic acid (2,6-TNS) were purchased from Sigma (St. Louis, MO, USA). Disodium tetraborate decahydrate, boric acid, sodium hydroxide, trisodium citrate dihydrate and citric acid were from Merck (Darmstadt, Germany). Urea was from Fluka (Buchs, Switzerland). Potassium chloride was obtained from Panreac (Barcelona, Spain). Dipotassium salt of 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) and 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) were from Molecular Probes (Leiden, The Netherlands). Methylhydroxyethylcellulose 30000 (MHEC) was purchased from Serva (Heidelberg, Germany). Quinine sulfate monohydrate was from Aldrich (Milwaukee, WI, USA).

Standards of BSA, bovine β-LGA, bovine β-LGB and calcium depleted bovine α-LA were obtained from Sigma. Standard proteins were dissolved in Milli-Q (Millipore, Bedford, MA, USA) water at concentrations around $2\cdot10^{-5}$ *M* for α-LA, $9\cdot10^{-6}$ *M* for both β-lactoglobulins, and $2\cdot10^{-6}$ *M* for BSA.

2.3. Procedures

Buffers used in spectrofluorometric measurements and in CE separations were prepared in Milli-Q water as follows. For CHES buffers, pH 8 and 10.2: 20 m*M* CHES and 10 m*M* KCl were adjusted to either pH 8 or 10.2 with 1 *M* NaOH; for citrate buffer, pH 2.5: 10 m*M* citrate, 6 *M* urea and 0.05% MHEC were adjusted to pH 2.5 with 2.5 *M* citric acid; for citrate buffer, pH 3.2: 20 m*M* citrate, 6 *M* urea, and 0.05% MHEC were adjusted to pH 3.2 with 2.5 *M* citric acid; for borate buffers, pH 8 and 10.2: in spectrofluorometric measurements a 100 m*M* boric acid, 100 m*M* disodium tetraborate, 30 m*M* sodium sulfate (pH 8) buffer and a 100 m*M* disodium tetraborate, 30 m*M* sodium sulfate buffer, adjusted to pH 10.2 with 1 *M* NaOH, was used; in CE separations either 20 m*M* boric acid, 20 m*M* disodium tetraborate (pH 8) or 20 m*M* disodium tetraborate adjusted to pH 10.2 with 1 *M* NaOH was employed. The borate buffer used in spectrofluorometric measurements gave rise to currents as high as 100 μA in CE experiments; therefore, more diluted borate buffers were employed for electrophoretic separations.

Buffers were filtered through Millipore 0.5-μm Millex LCR_{13} filters and degassed with helium. For electrophoretic analyses, 10 kV were applied across uncoated fused-silica capillaries from Polymicro Technologies. Injections were made by siphoning.

In spectrofluorometric measurements, the increase in fluorescence signal (IFS) was calculated by dividing the area below the maximum (limited by a section of ±5 nm around the maximum) of the emission spectra curves (λ_{ex}=325 nm) obtained for a solution of each probe (concentration $2\cdot10^{-4}$ *M*) dissolved in a given buffer containing BSA at the concentration of 0.2 g/l, by the area corresponding to the same probe solution without BSA (background signal or BKS), in the same $\Delta\lambda$ range. IFS values were measured in this 10 nm range to simulate the bandwidth of the bandpass filter used in the CE–LIF experiments.

Optimization of PMT voltage polarization was carried out using the best signal-to-noise ratio (*S*/*N*) obtained in a CE separation of whey proteins using a 20 m*M* CHES, 10 m*M* KCl buffer (pH 10.2) containing $2\cdot10^{-4}$ *M* 2,6-TNS and applying increasing cathodic voltages to the PMT tube. A polarization potential of 300 V was selected. The capillary focusing was checked every day with 10^{-3} *M* quinine sulfate in 0.5 *M* sulfuric acid.

To increase migration time reproducibility and reduce the baseline noise of the electrolyte in CE measurements, the separation capillary and the inlet buffer reservoir were refilled with fresh, degassed buffer after every analysis and the buffer in the outlet reservoir was replaced every two separations. The capillary was rinsed successively with water, 0.1 *M* NaOH, water and fresh buffer between runs.

The bovine whey used was obtained from fresh raw milk by acidic precipitation (pH 4.6) of caseins using 2 *M* HCl [23].

3. Results and discussion

The selective binding of some *N*-arylaminonaphthalene sulfonate derivatives to specific sites of some proteins leads to an increase in the quantum yield and a blue shift in wavelength of the fluorescent probe emission. This effect has been attributed [16,24–26] to the fact that the energy of the excited state of these molecules is partly dissipated to solvent (through translation, rotation, chemical quenching, or by intersystem crossing to the

triplet state) by non-radiative deactivation processes when molecules of the solvent reorient around the more dipolar structure of the excited state of the probe. This non-radiative dissipation brings the probe to the equilibrium level of the excited state, from which the excited state dissipates the excess of energy by light emission to return to the ground state. In polar solvents, like aqueous CE buffers, the non-radiative dissipation process is more probable than in the non polar environment of the hydrophobic site of the protein. Consequently, a more energetic emission (blue shift) of higher intensity (higher quantum yield) can be produced by the probe when it is for instance associated to proteins.

For a given protein and separation conditions, each fluorescent probe gives rise to a different modification of its spectroscopic characteristics, such as quantum yield and wavelength shift [27]. Concomitantly, the spectral modifications of a given fluorescent probe are specific to each protein [28,29], and they also depend on the experimental conditions such as buffer [30,31], pH [16,28,31], and temperature [28,32,33]. Therefore, a preliminary spectrofluorometric study was carried out to compare the different probes in terms of quantum efficiency in CE buffers of different nature and pH.

3.1. Spectrofluorometric study

Several buffers have been reported to be useful for CE separation of whey proteins. In this work, two citrate buffers (pH 2.5 and pH 3.2) [34], a borate buffer, pH 8 [35], and a CHES buffer, pH 10.2 [12] were compared in terms of the increase in fluorescent signal (IFS) of the fluorescent probes 2,6-TNS, 1,8-ANS and bis-ANS for BSA. Moreover, borate buffer, pH 10.2 and CHES buffer, pH 8 were also employed to study the influence of pH and buffer nature.

From the emission spectra of the different probes in the buffers containing 0.2 g/l of BSA and in the same buffers without BSA, IFS and BKS values were obtained for each probe in the different buffers studied. These values are summarized in Table 1. The IFS value is related to the increase in the quantum yield of the fluorescent probe originated by its association to the protein in a particular separation buffer.

As a general trend, buffers with basic pH values furnish higher IFS values than those obtained with acidic buffers for the three probes studied. 2,6-TNS provides the highest values of IFS in basic buffers (CHES buffers, pH 8 and 10.2). 1,8-ANS and bis-ANS also show a fluorescence enhancement in basic buffers, but smaller than that obtained with 2,6-TNS. As for the BKS values, Table 1 shows a decrease of such values when the pH is increased from 2.5 to 10.2. 2,6-TNS resulted in smaller BKS values on CHES buffers (pH 8 and pH 10.2). Therefore, the higher IFS value observed for 2,6-TNS could be

Table 1
Increase in fluorescence signal (IFS) for 2,6-TNS, 1,8-ANS and bis-ANS for solutions of 0.2 g/l of BSA in several buffers[a]

	Citrate buffer pH 2.5	Citrate buffer pH 3.2	Borate buffer pH 8	CHES buffer pH 8	Borate buffer pH 10.2	CHES buffer pH 10.2
IFS[b] (2,6-TNS)	2	5	43	230	[d]	200
($\Delta\lambda_{max}$, nm)[c]	(423±5 nm)	(433±5 nm)	(438±5 nm)	(437±5 nm)	–	(433± 5 nm)
BKS[b]	1176	281	77	42	–	47
IFS (1,8-ANS)	2	2	22	40	72	57
($\Delta\lambda_{max}$, nm)	(491± 5 nm)	(492±5 nm)	(486±5 nm)	(477±5 nm)	(470±5 nm)	(468±5 nm)
BKS	727	532	250	184	117	130
IFS (bis-ANS)	[d]	[d]	21	22	16	22
($\Delta\lambda_{max}$, nm)	–	–	(498±5 nm)	(492±5 nm)	(497±5 nm)	(488±5 nm)
BKS	–	–	303	234	469	222

[a] Background signal (BKS) for the same fluorescent probes in the same buffers without BSA is reported for comparison. Probe concentration $2\cdot10^{-4}$ *M*.

[b] IFS and BKS values were measured as described in Section 2.3.

[c] As an indication, $\Delta\lambda_{max}$ gives the region where IFS has been measured for BSA in each buffer.

[d] 2,6-TNS precipitated in borate buffer, pH 10.2 and bis-ANS precipitated in citrate buffers, pH 2.5 and pH 3.2.

partly due to the lower BKS values observed for this dye in the CHES buffers. For the three probes investigated, it was shown that the influence of buffer nature was particularly relevant in the behavior of 2,6-TNS which resulted in higher IFS values and smaller BKS values for CHES buffer, irrespective of the buffer pH. In relation to the influence of buffer pH, the three fluorescent probes present very similar IFS and BKS values at pH 8 and 10.2, for the same buffer type.

The important enhancement in fluorescence emission observed for these dyes, particularly for 2,6-TNS, is in agreement with the fact that one molecule of BSA is capable of binding several molecules of the probe [30]. A smaller fluorescence enhancement is anticipated for other proteins such as β-lactoglobulins, which probably bind less probe molecules. Moreover, the increase in IFS values observed for the three dyes in basic buffers could be due to a modification in the BSA conformation at these pH values which could cause an increase in the affinity of the protein for the probe [30,36]. However, the effect of the nature and concentration of the buffers used on fluorescence emission cannot be ruled out [30,31].

In conclusion, these results seem to indicate that the probe 2,6-TNS in CHES buffers could give better detection limits in CE analysis of BSA using LIF monitoring than those obtained using the acidic buffers or the other probes studied.

3.2. CE separation with LIF detection of bovine whey proteins

In order to study the separation buffer's nature and pH effect on the resolution and LOD values obtained using CE with LIF detection for whey proteins, CHES and borate buffers at pH 8 and 10.2 were compared. In these experiments, the 2,6-TNS concentration was $2\cdot10^{-4}$ *M*.

Fig. 1 shows the separation of α-LA, β-LGB, β-LGA and BSA in CHES buffer and borate buffer, pH values 8 and 10.2. It should be noted a very noisy baseline for CHES buffer, pH 8. In general terms, the four whey proteins are well resolved ($R_S>0.9$) in all the buffers. However, BSA presents broad (buffers at pH 10.2) and split (buffers at pH 8) peaks in the conditions studied. It is known [37] that commercial preparations of BSA contain several microheterogeneities and aggregates which can differ slightly in their charge-to-mass ratio and, therefore, in their electrophoretic mobility. β-Lactoglobulins show sharp and symmetrical peaks in the buffers studied, although in CHES buffer, pH 8 the peak of β-LGB was distorted probably by one of the system peaks of the electropherogram. Finally, α-LA was also sharp and symmetrical in all the buffers studied except in CHES buffer, pH 8, where it appears as a broad peak due to the amount of sample necessary to detect this protein in this buffer (see Table 3). The peak efficiencies obtained for whey proteins in the different buffers studied (Table 2) show that borate buffer, pH 10.2 leads consistently to higher efficiencies than borate buffer, pH 8. Some bovine whey proteins, like BSA and β-lactoglobulins, show conformational changes around pH 8 [36,38] that could cause broad peaks in CE separations, whereas at pH 10.2, a unique, denatured conformation is the most probable species for these proteins, which gives rise to narrower peaks. By comparing the efficiencies obtained in CHES buffer, pH 10.2 and borate buffer, pH 10.2, it can be observed that α-LA and β-lactoglobulins present better efficiencies when borate buffer is used. This behavior could indicate that the nature of the buffer also affects the conformational states of some proteins as it has been reported for chromatographic separations [39].

Table 3 summarizes the LOD for a S/N ratio of 3 obtained for the whey proteins studied in the four buffers. As expected, LOD values depend on the protein and buffer used. For the buffers assayed, BSA has the best detection limit and α-LA the worst, the difference among them being 1–2-orders of magnitude. As indicated above, this result is consistent with the different quantum yields of the probe caused by their different interaction degree (binding constant and stoichiometry) with each protein [26,30]. CHES buffer, pH 8 resulted in significantly higher (worst) LOD values for all proteins. This result is mainly due to the higher baseline noise obtained with CHES buffer, pH 8 (noise level $2\cdot10^{-9}$ A) than that observed with CHES buffer, pH 10.2 (noise level $8\cdot10^{-11}$ A). This behavior does not agree with results shown in Table 1, where CHES buffers, pH 8 and 10.2 present similar IFS and BKS values. A noise level of $2\cdot10^{-9}$ A was observed for the solution containing 2,6-TNS in CHES buffer, pH 8, whereas the probe bis-ANS presents a noise level

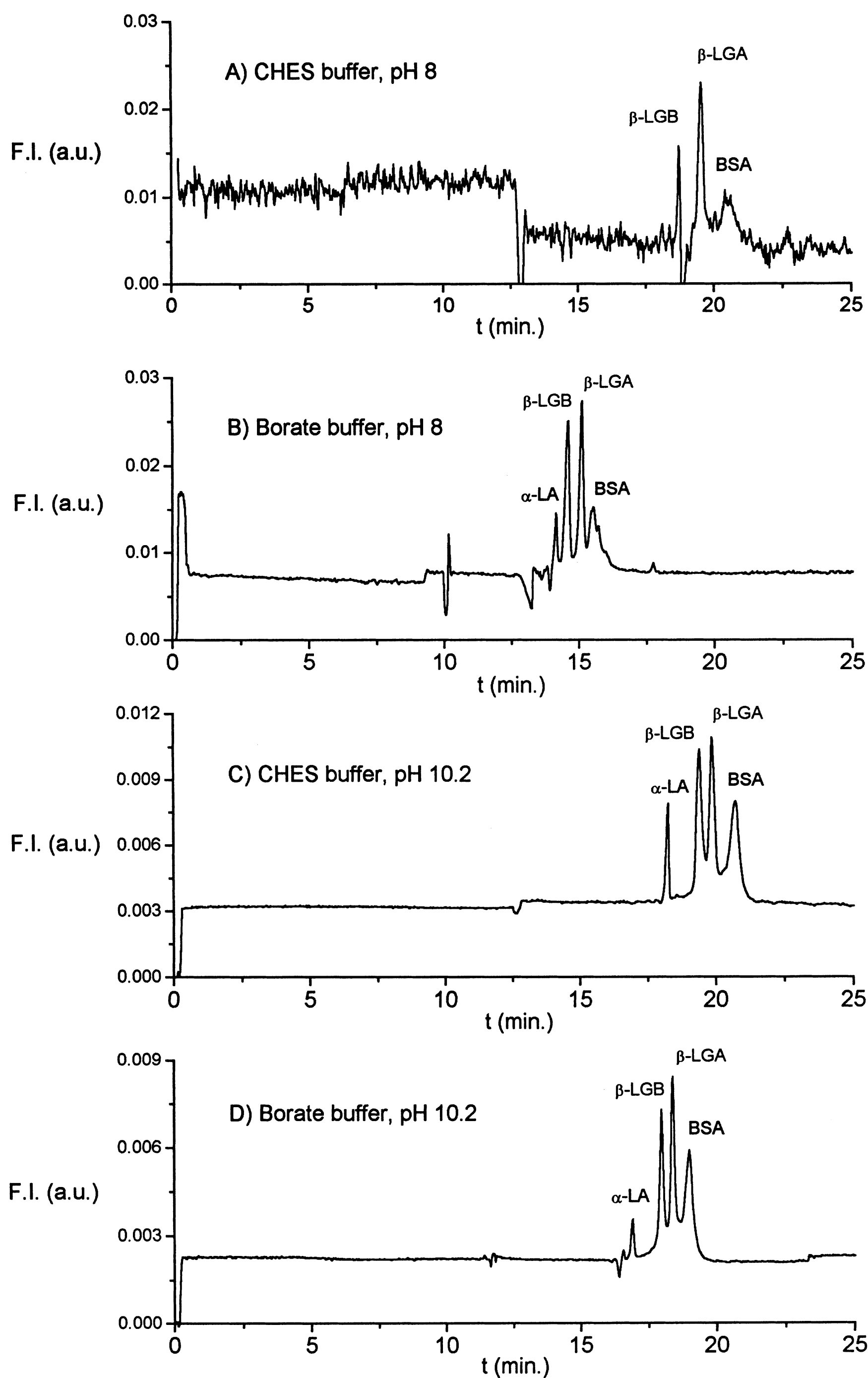

Fig. 1. Electrophoretic analyses of four bovine whey proteins under different conditions. (A) CHES buffer, pH 8; (B) borate buffer, pH 8; (C) CHES buffer, pH 10.2; (D) borate buffer, pH 10.2. Sample concentration: $9\cdot10^{-6}$ *M* β-LGA and β-LGB, $2\cdot10^{-6}$ *M* BSA and $2\cdot10^{-5}$ *M* α-LA; $2\cdot10^{-4}$ *M* 2,6-TNS in each buffer. Fused-silica capillary: 87 cm (effective length 57 cm)×75 μm I.D. Injection by siphoning: 23 cm, 5 s. Voltage: 10 kV. Room temperature.

Table 2
Efficiency[a] for the whey proteins studied obtained in borate and CHES buffers at pH 8 and pH 10.2[b]

Buffer	α-LA	β-LGB	β-LGA	BSA
CHES, pH 8	30 000	40 000	50 000	8000
Borate, pH 8	58 000	32 000	37 000	8000
CHES, pH 10.2	103 000	45 000	50 000	16 000
Borate, pH 10.2	141 000	69 000	72 000	16 000

[a] Number of theoretical plates calculated through peak width at half height using System Gold V8.10 software.
[b] $2\cdot10^{-4}$ *M* 2,6-TNS in each buffer. Fused-silica capillary: 87 cm (effective length 57 cm)×75 μm I.D. Injection by siphoning: 23 cm, 5 s. Voltage: 10 kV. Room temperature.

significantly lower, $1\cdot10^{-10}$ A, in the same CHES buffer. These results are not easy to understand because no electrochemical decomposition could be observed for 2,6-TNS in CHES buffer, pH 8. Furthermore, the instrumental differences of the spectrofluorometric and the CE–LIF set-ups used to obtain these data make the comparison rather difficult. Whey proteins in borate buffers at pH 8 and 10.2 show very similar LOD values although in the buffer at pH 8 the BSA peak appears as a split peak like in CHES buffer, pH 8.

It can be deduced from Tables 2 and 3 that both buffers of pH 10.2 give rise to good efficiencies and sensitivity for whey proteins using the CE–LIF technique. CHES buffer was selected for the next experiments because it resulted in a slightly better LOD value for α-LA. However, it should be highlighted that α-LA presented about a two-fold variation in LOD value depending on the care taken in the preparation and manipulation of the buffer and sample. Since the protein used in these experiments was Ca^{+2} depleted, small variations in the content of Ca^{+2} or some other metals in the buffers like Na^{+} could cause variations in the detection limit obtained for α-LA [29,40].

Since the fluorescence emission of 2,6-TNS increases with the probe/protein ratio [12,16,30], and the background noise also increases with the concentration of 2,6-TNS [12], a study of the variation in the LOD values of the proteins with the increasing concentration of 2,6-TNS was carried out to optimize the concentration of the probe in the buffer.

Table 4 summarizes LOD values ($S/N=3$) and their respective estimated standard error [41] (calculated for $n=6$) obtained for bovine whey proteins at different concentrations of 2,6-TNS in CHES buffer, pH 10.2. It can be observed that the best (smallest) LODs for these proteins are obtained at 2,6-TNS concentrations in the range $2\cdot10^{-5}$ *M* to $6\cdot10^{-5}$ *M*. Smaller concentrations of the dye give rise to little fluorescence enhancements for these proteins, whereas higher concentrations of fluorescent probe increase significantly the background noise of the buffer. In those experimental conditions the limit of detection obtained for bovine whey proteins was $6\cdot10^{-8}$ *M* for BSA, $3\cdot10^{-7}$ *M* for β-LGA and β-LGB, and $3\cdot10^{-6}$ *M* for α-LA.

To compare the detection limits achieved with CE–LIF with those obtained using UV detection, LOD measurements for standard whey proteins were carried out using UV detection (results not shown). The same separation capillary and separation buffer without the fluorescent probe were used. It was observed that for β-lactoglobulins and BSA, the sensitivity provided by LIF detection was twice as good than that provided by UV detection, whereas

Table 3
Limits of detection for the bovine whey proteins studied in borate and CHES buffers at pH 8 and 10.2 using CE–LIF[a]

Buffer	[α-LA] (*M*)	[β-LGB] (*M*)	[β-LGA] (*M*)	[BSA] (*M*)
CHES, pH 8	$>5\cdot10^{-5}$	$6\cdot10^{-6}$	$5\cdot10^{-6}$	$6\cdot10^{-6}$
Borate, pH 8	$8\cdot10^{-6}$	$6\cdot10^{-7}$	$6\cdot10^{-7}$	$2\cdot10^{-7}$
CHES, pH 10.2	$4\cdot10^{-6}$	$7\cdot10^{-7}$	$6\cdot10^{-7}$	$2\cdot10^{-7}$
Borate, pH 10.2	$7\cdot10^{-6}$	$6\cdot10^{-7}$	$5\cdot10^{-7}$	$2\cdot10^{-7}$

[a] Other conditions as in Table 2.

Table 4
Limits of detection (mean value±estimated standard error of mean[a]) for the bovine whey proteins studied[b]

[2,6-TNS] (M)	[α-LA]$\cdot 10^6$ (M)	[β-LGB]$\cdot 10^7$ (M)	[β-LGA]$\cdot 10^7$ (M)	[BSA]$\cdot 10^8$ (M)
$0.5\cdot 10^{-5}$	15±0.8	5.5±0.3	7.1±0.4	16±0.8
$1\cdot 10^{-5}$	4.7±0.2	4.2±0.2	4.5±0.2	10±0.4
$2\cdot 10^{-5}$	3.3±0.2	2.7±0.1	2.5±0.1	6.0±0.2
$4\cdot 10^{-5}$	2.8±0.1	2.5±0.1	2.3±0.1	5.8±0.2
$6\cdot 10^{-5}$	2.9±0.3	2.8±0.2	2.8±0.2	6.7±0.4
$8\cdot 10^{-5}$	2.9±0.1	3.5±0.2	3.5±0.2	8.5±0.4
$10\cdot 10^{-5}$	3.1±0.3	4.1±0.3	4.1±0.3	10±0.8
$14\cdot 10^{-5}$	5.4±0.6	5.7±0.7	5.9±0.7	16±2.4
$23\cdot 10^{-5}$	8.0±1.3	10±1.6	11±2	31±5.5

[a] Estimated standard error of mean values calculated using the formula $S/\sqrt{n}$, where S is the standard deviation and n is the number of measurements (in this case $n=6$).
[b] CHES buffer, pH 10.2, containing different concentrations of 2,6-TNS. Other conditions as in Table 2.

for α-LA the LOD value obtained with UV was ten-fold better than that obtained with LIF detection. It was also observed that the efficiency obtained with UV detection was smaller than that obtained for these proteins using LIF detection. This result seems to indicate that the adsorption/desorption kinetic of the fluorescent probe on the protein is fast enough not to increase the band broadening of the proteins. Conversely, the association of 2,6-TNS negatively charged molecules to proteins may have a beneficial effect on the separation efficiency, probably due to an increase in the electrostatic repulsion between the protein and the capillary wall.

It is interesting to compare the detection limits obtained in this work with those required for host cell proteins detection (10–100 mg/l) in recombinant pharmaceuticals [8]. For α-LA (M_r=14 175), β-LGA (M_r=18 368), β-LGB (M_r=18 277) and BSA (M_r=66 267) the low value in this range (10 mg/l) is equivalent to $7\cdot 10^{-7}$ M (α-LA), $5.5\cdot 10^{-7}$ M (β-lactoglobulins) and $1.5\cdot 10^{-7}$ M (BSA). Therefore, the results of this paper show that CE–UV is able to detect α-LA, and CE–LIF can detect β-lactoglobulins and BSA at the concentrations required for the quality control of biopharmaceutical products.

The LOD values obtained for whey proteins in the present work are of the same order of magnitude that those reported by Swaile and Sepaniak [12] for BSA and β-LGA using a similar detection system. Employing the minimum injectable amount and the data about hydrodynamic injection given in Ref. [12], the LOD values of $3\cdot 10^{-7}$ M for BSA and $6\cdot 10^{-7}$ M for β-LGA were estimated using Poiseuille's equation. The slight differences between these values and those obtained in our work can be attributed to the different stability of the laser and electronic used, different I.D. capillaries utilized, and different 2,6-TNS concentrations employed.

The on-column labeling method studied in this work is easier to develop than the post-column derivatization methods reported, although post-column derivatization methods are about an order of magnitude more sensitive for proteins [11].

3.3. Application of the method to bovine whey samples

Although the composition of proteins in cow's milk depends largely on factors such as animal breed, season, feeding, etc., a typical, approximate concentration of whey proteins in raw milk is: 1.2 g/l for α-LA, 3.2 g/l for β-lactoglobulins and 0.4 g/l for BSA [42]. This protein content is large enough to be detected with the UV detectors used in CE [34,35]. Nevertheless, as milk is a very complex matrix, we were concerned with other components that could interfere in the determination of whey proteins using the CE–LIF separation system. To check this point, the separation of whey proteins from several types of milk samples was undertaken using the method developed and LIF detection.

The electropherogram in Fig. 2 shows the separation of a bovine whey sample obtained from raw milk by CE–LIF. As it can be observed, this electropherogram showed only three major peaks that were identified, using a spiking technique, as

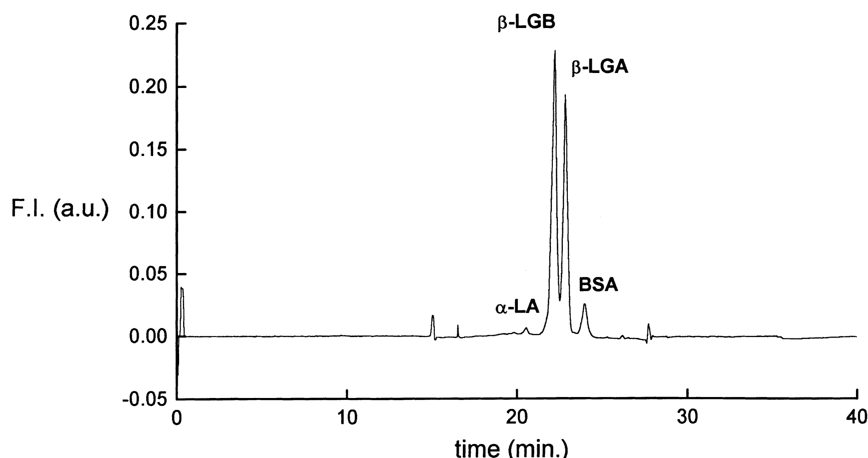

Fig. 2. Electropherogram of a bovine whey (without dilution) from raw milk using CE–LIF. Separation buffer: 20 m*M* CHES (pH 10.2), 10 m*M* KCl, $[TNS]=4\cdot10^{-5}$ *M*. Injection by siphoning: 15 cm, 3 s. Other conditions as in Fig. 1.

β-LGB, β-LGA and BSA. The small peak at migration time 21 min was identified as α-LA. The height of this peak indicate that detectability of α-LA in real samples ($4.6\cdot10^{-5}$ *M*) is smaller than that obtained in standard samples. This result could be related to the fact that α-LA in milk samples is complexed with Ca^{+2} which provides smaller fluorescence enhancement than Ca^{+2} depleted protein as discussed before [29,40]. The presence of immunoglobulin G (IgG) in this electropherogram was ruled out because the injection of a standard sample of bovine IgG in water ($3\cdot10^{-5}$ *M*) gave rise to only a minor, distorted peak at around 16 min. This small peak was probably due to water because it was also observed when a blank sample containing only water was injected.

4. Conclusions

CE–LIF, with on-column derivatization, is useful to detect some bovine whey proteins in raw milk samples. Although the *N*-arylaminonaphthalene sulfonate derivatives used as fluorescent probes in aqueous buffers have been described as non-fluorescent, they produce light emission to some extent, which increases the background noise and limits the sensitivity achieved. For β-lactoglobulins and BSA, LIF detection using $2\cdot10^{-4}$ *M* 2,6-TNS as the fluorescent probe and CHES buffer, pH 10.2 or borate buffers at pH 8 or 10.2 has furnished the best LOD values. When 2,6-TNS concentration was optimized in CHES buffer, pH 10.2 the detection limits for whey proteins were about $6\cdot10^{-8}$ *M* for BSA and $3\cdot10^{-7}$ *M* for both β-lactoglobulins. However, for α-LA, UV detection without 2,6-TNS in CHES buffer at pH 10.2 provided better sensitivity than LIF detection with a LOD of $3\cdot10^{-7}$ *M*. The results presented in this work demonstrate that on-column derivatization for LIF detection of proteins using *N*-arylaminonaphthalene probes should be developed in a case-by-case basis and results cannot be generalized. For a given protein, both the probe used and the separation buffer employed control the sensitivity obtained. For the same probe and the same CE conditions, different proteins give rise to different detection limits depending on the interaction degree of each protein with the probe. For those proteins giving high quantum yield, better sensitivity is generally achieved using this method than using UV detection. Compared to other LIF detection methods used for CE of proteins, on-column derivatization is a rather inexpensive, easy to implement method, although its sensitivity is somewhat limited.

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