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Journal of Chromatography A, 1025 (2004) 217–226
JOURNAL OF CHROMATOGRAPHY A
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Isoelectric point separation of proteins by capillary pH-gradient ion-exchange chromatography

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Received 10 July 2003; received in revised form 16 October 2003; accepted 22 October 2003

Abstract

In the present work, isoelectric point (p*I*) separation of proteins by pH-gradient ion-exchange chromatography (IEC) on packed capillary columns is demonstrated. The development of a miniaturized flow-through pH probe for reliable pH monitoring of the column effluent, which was an important technical challenge for adapting this technique to capillary dimensions, was solved by designing a low microliter per minute flow rate housing to a commercially available micro pH probe. Highly linear outlet pH-gradients within the pH range 8.5–4.0 were obtained when applying simple inexpensive buffers consisting solely of piperazine, *N*-methylpiperazine and imidazole on 10 cm × 0.32 mm i.d. fused silica capillaries packed with anion-exchange poly(styrene divinylbenzene)-based macroporous materials, i.e. 10 μm Mono P from Amersham Biosciences and 10 μm PL-SAX from PolymerLabs. Furthermore, when using a pH-gradient from 6.8 to 4.3, both columns were able to baseline separate the A and B genetic variants of β-lactoglobulin, which differ with two amino acid residues only, but the PL-SAX column provided almost a two-fold decrease in peak widths compared to the Mono P column. The influence of varying the buffer concentration, injection volume and column temperature on the peak widths and resolution of the β-lactoglobulins was investigated, e.g. a 100 μl sample of dilute β-lactoglobulins was injected directly on the column with practically no increase in peak width as compared to what obtained with conventional injection volumes. Finally, a pH-gradient from 6.8 to 4.3 was used to separate proteins in skimmed bovine milk on the PL-SAX column. The milk was simply diluted 1:10 (v/v) with water and filtrated before injection.

Keywords: Isoelectric points; pH-gradients; Milk; Food analysis; Proteins; Lactoglobulins

1. Introduction

Ion-exchange chromatography (IEC) is a versatile separation principle for proteins due to its applicability for several classes of proteins, high resolution, simplicity, good reproducibility, and not to forget, the possibility of performing the separation under nearly non-denaturing conditions. Nowadays, salt-gradient IEC is commonly applied for separation of proteins, but when compared to pH-gradient IEC, the latter technique has two prominent features: (1) the proteins are eluted roughly in order of their isoelectric points (p*I* values), and (2) the proteins are focused in narrow bands during the separation, which give better peak shapes than usually observed in salt-gradient IEC. By using a gradient pump to generate a pH-gradient over the ion-exchange column, the individual proteins will focus in narrow bands where pH is more or less equal to their p*I* values, i.e. retained when pH > p*I* and non-retained when pH ≤ p*I*, and thus finally elute in order of the p*I* values. In other words, pH-gradient IEC combines the characteristics of isoelectric focusing (IEF) and the simplicity of chromatographic techniques to yield a separation technique capable of high resolution of proteins. However, despite this features, the use of pH-gradient IEC has been relatively limited, primarily because it is a more delicate technique than salt-gradient IEC, but also because it generally has been difficult to obtain reproducible, linear outlet pH-gradients.

In the late 1970s, Sluyterman and co-workers [1–5] described a form of pH-gradient IEC that utilized the buffering capacity of the column to generate linear outlet pH-gradients without external mixing of the buffers, which they termed chromatofocusing (CF). CF is most commonly performed by titration of a weak anion-exchange column, e.g. DEAE-functionalized cellulose, which has initially been equilibrated with start buffer (high pH) and loaded

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doi:10.1016/j.chroma.2003.10.093

with the protein sample, with an elution buffer (low pH). To obtain as linear pH-gradients as possible, and hence larger peak capacity, the buffers usually contain mixtures of polyampholytes with different pK_a values to get an even buffering capacity over the chosen pH range. However, CF has unfortunately several important limitations. Primarily, polyampholyte buffers may cause poor chromatographic reproducibility because of batch-to-batch variability in the chemical composition of polyampholyte mixtures. They are also often difficult to remove from isolated proteins due to formation of association complexes [6], and they give high background absorption with UV detection. Secondly, CF also suffers from the inflexibility in choice of buffer concentrations. The technique is restricted to low concentrations of polyampholyte buffers in the mobile phase, i.e. typically 2.5–5 mM, since higher concentrations usually give steep pH-gradients and consequently poor resolution of the proteins. Thus, linear pH-gradients are not straightforward to generate [7], and moreover, possible chromatographic gains using higher buffer concentrations cannot be realized. Therefore, several researchers have investigated alternative buffer compositions for generation of linear pH-gradients without external mixing of the buffers. For example, Hearn and Lyttle [8], and Hutchens et al. [9,10] used a large number of carefully chosen low-M_r buffering species in the elution buffer to create multi-step pH-gradients that, in practice, performed as linear gradients. Bates and Frey [11], on the other hand, used amine buffering species with minimal adsorption on a hydrophilic weak-base anion-exchange column, and thus utilized solely the buffering capacity of the stationary phase to create retained linear pH-gradients.

While CF generally exploits the buffering capacity of the ion exchanger to obtain a retained intra-column pH-gradient, the opposite should be stressed in pH-gradient IEC [12]. Recently, Liu and Anderson [13,14], and Shan and Anderson [15,16] used pH-gradient IEC, which they termed gradient CF, to overcome many of the shortcomings of CF. They used ion-exchange materials with small buffering capacities in the applied pH range in combination with buffer components that are not adsorbed on the ion-exchange column, e.g. amine buffering species on anion-exchange materials. Under such conditions, the contribution from the column itself to the delay of the pH-gradient is minimal. Consequently, the outlet pH-gradient will roughly reflect the pump settings, which gives enhanced flexibility in controlling the slope of the pH-gradient [13–16]. In contrast to CF, pH-gradient IEC also allows the use of high buffer concentrations without affecting the slope of the pH-gradient. Accordingly, improved chromatographic performance can be obtained with only a few common buffer components, while still attaining the characteristic focusing effect of the protein bands.

During the last few years, packed capillary LC has obtained increasing attention since chromatographers are continuously confronted with applications where only minute sample amounts are available for analysis, e.g. in studies of differential expression of proteins from whole cell lysates. Although Hirose and Ishii [17–19] and Li et al. [20] have demonstrated capillary CF of proteins on particulate and monolithic anion-exchange materials, respectively, no studies, to the authors' knowledge, have been published on capillary pH-gradient IEC. Miniaturized pH-gradient IEC has the potential of becoming an important tool within proteomics, not only for off-line p*I* fractionation of small proteins amounts, but also as a highly efficient reversed-phase LC compatible dimension in capillary two-dimensional LC–LC–electrospray ionization MS [21–23]. Thus, the main objective of the present study was to explore the capabilities of this technique for high-performance, sensitive separation of proteins when using a combination of anion-exchange packed capillary columns and simple amine buffers. Bovine milk proteins were chosen as model proteins due to well-defined isoelectric characteristics and easy accessibility. Besides, a second objective was to develop a miniaturized flow-through pH probe for on-line pH monitoring of the column effluent.

2. Experimental

2.1. Chemicals and materials

Bovine β-lactoglobulins A and B were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Imidazole and 99% piperazine were purchased from Sigma–Aldrich Chemie, while 99+% *N*-methylpiperazine was purchased from Acros Organics (Geel, Belgium). Water was purified in the laboratory with a Milli-Q Ultrapure water system (Millipore, Bedford, MA, USA). 10 micron PL-SAX particles with an average pore diameter of 1000 Å were purchased from Polymer Labs (Church Stretton, UK), while 10 μm Mono P particles were obtained by emptying a Mono P 5/50 GL column purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Polyimide-coated fused silica capillaries (0.32 mm i.d. × 0.45 mm o.d.) were obtained from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Miniaturized flow-through pH probe

In order to achieve on-line pH monitoring of the effluent from the capillary IEC system, a miniaturized flow-through pH probe was developed by constructing a modified version of the plexiglass housing for the Micro Flow-through pH probe from Lazar Research Labs (Los Angeles, CA, USA) (Fig. 1). Unfortunately, the internal dead volume of the Plexiglass housing could not be made as small as desirable, since the diameter of the micro pH probe glass membrane was relatively large (approximately 8 mm). The internal dead volume, including the dead volume of the transfer line from the UV detector, which was calculated to approximately 15 μl, consequently caused a delay of the pH-gradient with respect to the UV chromatogram (e.g. a 2.5 min delay at 6 μl min^{-1}). With the pH-gradient slopes obtained in this study, a 2.5 min

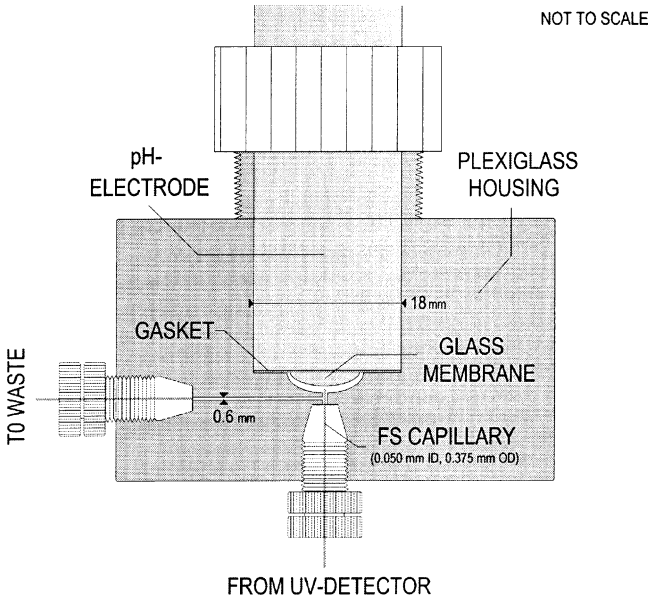

Fig. 1. The miniaturized flow-through pH probe.

delay at 6 μl min^{-1} generally corresponded to a 0.1 pH mismatch between the two curves, which has not been subtracted in the chromatograms presented. Nevertheless, the forced exchange of the effluent outside the glass membrane surface due to the positioning of the inlet and outlet ensured reliable and reproducible pH monitoring even at low flow rates. Approximately, once a week, the pH electrode was detached from the housing and calibrated, while the housing was flushed with Milli-Q water. The signal from the pH probe was translated with a Cl-6507 pH amplifier and monitored with Science Workshop Data Studio software (both from PASCO, Rosewille, CA, USA). This software allowed simultaneous monitoring of the signal from the pH probe and the UV detector.

2.3. Chromatographic instrumentation

A non-spliting Eldex Micro-Pro binary gradient pump (Eldex Labs, Napa, CA, USA) was used to deliver a constant flow rate throughout the study. Manual injections were performed with either a Valco ChemInert model C2 or C4 injection valve (Valco Instruments, Houston, TX, USA), which was mounted inside the column oven. The column oven was a Mistral 880 oven (Spark Holland, Emmen, The Netherlands), while detection was accomplished using a Spectra 200 UV–Vis Detector (Spectra-Physics, San Jose, CA, USA) equipped with a laboratory-built on-column optical cell. The detector was operated at 280 nm, while the response time was set to 1.0 s. For conversion of the recorded chromatograms from voltage (mV) to absorbance units (AU), the following conversion factor should be used: 1 mV = 0.001 AU. The column inlet was connected to the injector with a 10 cm × 100 μm i.d. fused silica capillary, while the column outlet was connected directly to the UV-detector with a 15 cm × 100 μm i.d. fused silica capillary. The miniaturized flow-through pH probe was coupled on-line to the UV detector with a 25 cm × 50 μm i.d. fused silica capillary. The capillary columns were prepared by packing the ion-exchange materials into 10 cm × 0.32 mm i.d. fused silica capillaries using a downward high-pressure liquid slurry technique that has been developed in the laboratory. Finally, the columns were equilibrated with start buffer for approximately 30 min before use.

2.4. Preparation of buffers and milk samples

Five hundred mM stock solutions of piperazine, *N*-methylpiperazine and imidazole were prepared by dissolving appropriate amount of each buffer in 100 ml Milli-Q water. The stock solutions were filtered through 0.45 μm Minisart-plus filters (Sartorius, Göttingen, Germany) and stored at 4–5 °C for no more than 2 weeks. Working buffer solutions were prepared by appropriate dilution of the stock solutions with Milli-Q water to desired buffer concentrations, and then titrated with 1.0 M HCl to obtain the desired pH. The working buffers solutions were degassed with 99.998% helium (AGA, Oslo, Norway) before use. Additionally, the buffer reservoirs were filled with argon (AGA) and sealed to reduce uptake of carbon dioxide during use or storage.

The milk samples were prepared by diluting 1 ml of pasteurized skimmed bovine milk (Tine, Oslo, Norway) in 9 ml of Milli-Q water. The pH of the diluted milk was measured to approximately 6.8 and could therefore be injected (and focused) directly on the column. The milk samples were filtered through 0.45 μm Minisart-plus filters and stored at 4–5 °C until use.

2.5. Calculations

The resolution R_s was calculated as described below:

$$R_s = \frac{2.354(t_2 - t_1)}{2(t_{w,1} + t_{w,2})}$$

where t_1 and t_2 are the retention times of the two peaks, while $t_{w,1}$ and $t_{w,2}$ are the corresponding peak widths at 50% peak height.

3. Results and discussion

3.1. Generation of linear pH-gradients on Mono P and PL-SAX

Hydrophilically modified polystyrene-divinylbenzene (PS-DVB) particles are inherently better suited for pH-gradient IEC of proteins than silica-based particles, e.g. PS-DVB particles are stable over the entire pH range and silica-based particles usually have several μmol m^{-2}

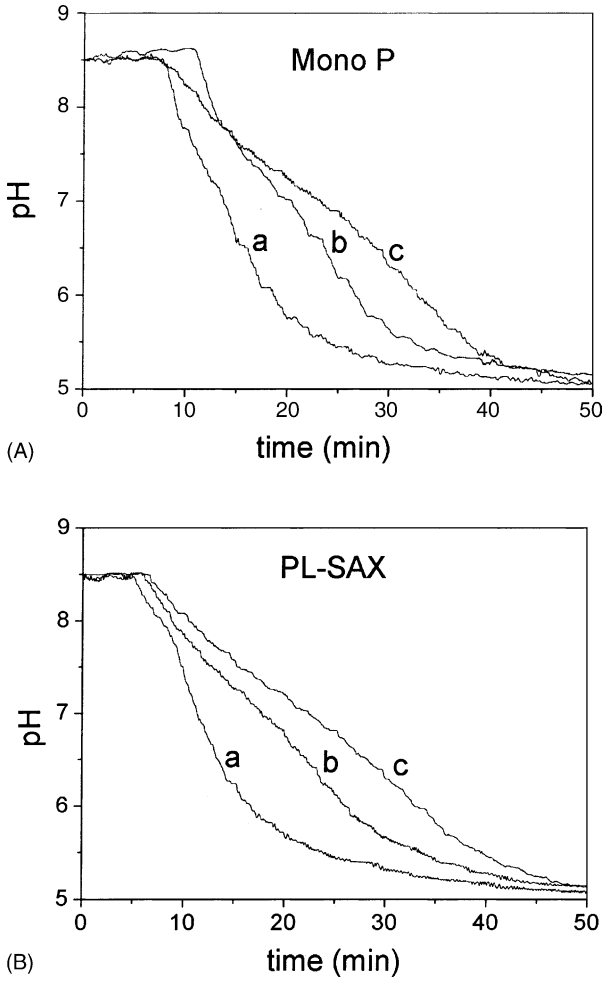

Fig. 2. Generation of linear pH-gradients on a 10 cm × 0.32 mm i.d. Mono P column and a 10 cm × 0.32 mm i.d. PL-SAX column. Start buffer (A): 10 mM piperazine; 10 mM *N*-methylpiperazine and 10 mM imidazole, pH 8.5, eluting buffer (B): same as start buffer, but pH 4.0. Gradient program: 0–100% B in (a) 10 min; (b) 20 min; and (c) 30 min. Flow rate: 8 μl min^{-1}. Column temperature: 30 °C. UV detection at 280 nm.

of non-reacted silanols that may provide buffering capacity or simply retain the amine buffering species. Consequently, the possibility of creating linear pH-gradients on two commercially available macroporous PS-DVB-based anion-exchange materials, i.e. Mono P (weak) and PL-SAX (strong), was initially investigated. The PL-SAX material is coated solely with polyethyleneimine, while the well-known Mono P material has both quaternary and tertiary amino groups on the surface. The buffering capacity of Mono P is well documented in the literature, e.g. Shan and Anderson [16] found that the principal buffering range of this material was 10.5–9, with only a small capacity at pH < 9, when titrating the material with 0.001 M HCl. Accordingly, both materials most likely have a small buffering capacity below pH 9, and should therefore, in combination with solely amine buffering species, affect the pump-generated pH-gradients little below pH 9. As shown in Fig. 2, linear pH-gradients with slopes that were slightly less steep the pump settings were obtained in the pH range between 8.5 and 4 with buffers consisting of only piperazine (pK_a = 5.76 and 9.73), *N*-methylpiperazine (pK_a = 4.94 and 9.09) and imidazole (pK_a = 6.95). For example, when the pump was set to deliver a pH-gradient to the PL-SAX column that decreased from pH 8.5 to 4 in 30 min (i.e. −0.15 pH units min^{-1}), this generated an outlet pH-gradient that decreased with approximately −0.1 pH units min^{-1} in the linear part of the curve. Since the obtained pH-gradients generally were slightly retained compared to the pump settings, this indicates that there was some buffering capacity present in the system.

3.2. Capillary pH-gradient IEC of genetic variants of β-lactoglobulin on Mono P and PL-SAX

When the already developed pH-gradients from 8.5 to 4 were applied for separation of β-lactoglobulins A and B, a rapid increase in column backpressure, which finally led to column clogging, was observed. This was believed to occur from precipitation of the proteins, since whey proteins, and particularly β-lactoglobulins, are becoming unstable above pH 8 [24]. However, when the pH of the start buffer was reduced to pH 6.8 (i.e. the pH of milk), a baseline separation of the two proteins was obtained on both the Mono P and the PL-SAX column, as shown in Fig. 3. These two genetic variants of β-lactoglobulin differ by two amino acid residues only, and are generally difficult to separate with reversed-phase liquid chromatography. In the applied pH range, the β-lactoglobulins exist as dimers (M_r 2 × 184,00) and each monomer contains 162 amino acids [25]. β-Lactoglobulins A and B eluted approximately 0.2 pH units closer to their actual p*I* values (p*I* = 5.13 and 5.23, respectively [26,27]) on the PL-SAX column than on the Mono P column. In addition, the PL-SAX material was found to be superior over Mono P material in terms of peak widths, with almost a two-fold decrease in peak width for both β-lactoglobulins A and B (both in terms of time and pH units) at roughly identical elution times (Table 1). There are at least two possible explanations for the difference in peak widths: (1) the PL-SAX material has a higher surface concentration of positively charged groups that interact in the retention mechanism than the Mono P material, or (2) the PL-SAX column is simply better packed than the Mono P column. Nevertheless, based on the results shown in Fig. 3, the PL-SAX column was preferred for further investigations. It is also worthy noting that the application of a pH range from 6.8 to 4.3 allowed the removal of imidazole from the buffers without affecting the shape of the pH-gradient. Thus, linear pH-gradients were obtained with simple buffers containing only two amine buffer species.

3.3. Influence of buffer concentration on peak width and resolution

The flexibility of pH-gradient IEC in allowing the use of a wide range of buffer concentrations (without markedly

Table 1
Figures of merit of β-lactoglobulins A and B in Fig. 3

	Elution time (min)		Peak width (min)		Peak width[a] (pH units)	
	Lact. A	Lact. B	Lact. A	Lact. B	Lact. A	Lact. B
Mono P	27.72 (0.8)	24.53 (0.5)	1.02 (1.9)	0.76 (1.6)	0.06	0.05
PL-SAX	23.49 (0.2)	20.68 (0.3)	0.40 (3.3)	0.37 (2.4)	0.04	0.03

The relative standard deviations (%) of the experimental measurements are given in parentheses ($n = 3$).
[a] Calculated from the column to the left. The slopes of the pH-gradient at the portion of the gradient where the peaks elute were calculated to −0.06 and −0.09 pH units min^{-1} for Mono P and PL-SAX, respectively.

affecting the slope of the pH-gradient) for optimizing the separation is a significant advantage over CF. The influence of the buffer concentration on peak width and resolution of β-lactoglobulins A and B was investigated by performing the separations at four different concentrations of piperazine and *N*-methylpiperazine, i.e. 5, 10, 20 and 40 mM. As shown in Fig. 4, the slopes of the pH-gradients were virtually not affected by using different buffer concentrations, which indicates that the buffering capacity of the column was small. Although shorter retention and somewhat larger spacing between the peaks were observed at higher buffer concentrations, the resolution was little affected due to increased peak widths, as shown in Table 2. However, slightly better resolution was obtained with 10 mM buffers than with the other concentrations.

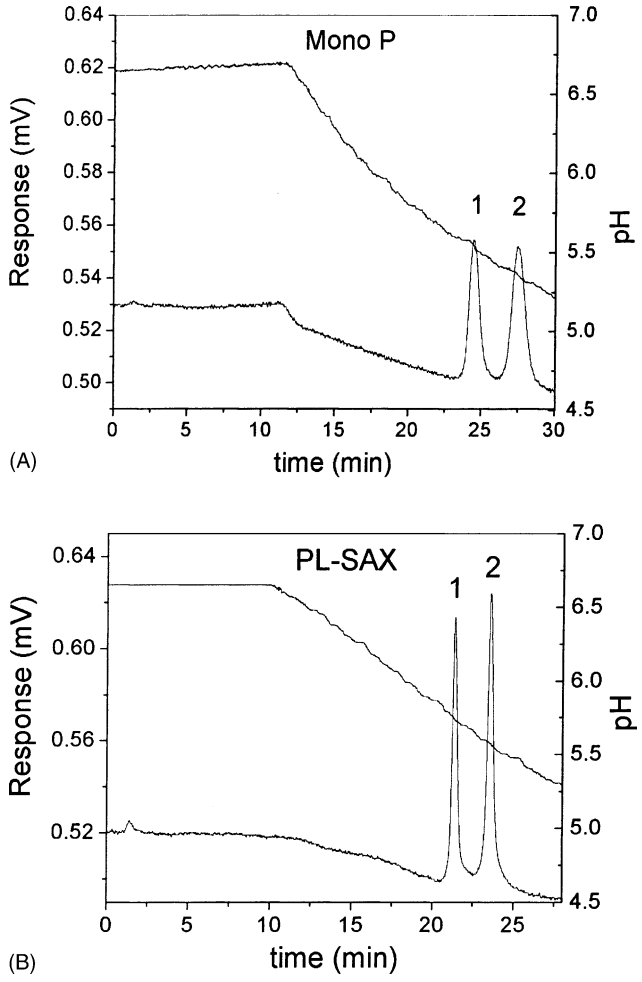

Fig. 3. Capillary pH-gradient IEC of β-lactoglobulins B (1) and A (2). Column: 10 cm × 0.32 mm i.d. Mono P column or 10 cm × 0.32 mm i.d. PL-SAX column. Start buffer (A): 10 mM piperazine and 10 mM *N*-methylpiperazine, pH 6.8, eluting buffer (B): same as start buffer, but pH 4.3. Gradient program: 0–100% B in 25 min, then 100% B for 5 min. Flow rate: 8 μl min^{-1}. Injection volume: 0.1 μl. Sample concentration: 5 mg ml^{-1} of each protein (diluted in start buffer). Column temperature: 30 °C. UV detection at 280 nm.

3.4. Influence of injection volume on peak width and resolution

The focusing effect in CF has been nicely demonstrated by Sluyterman and Widjenes [3], who obtained no increase in peak width, only double peak heights, when performing two subsequent injections instead of one during the chromatographic run. Hirose and Ishii [17,19] also exploited this effect to perform large-volume injections of sperm whale myoglobin on long 0.2–0.25 mm i.d. packed capillaries with good column efficiency. Furthermore, Liu and Anderson [13] observed the same focusing effect when 2 × 25 μl (after 0 and 1.5 min) of 0.2 mg ml^{-1} streptokinase was injected on a 5 cm × 5 mm i.d. Mono P column and eluted with a pump-controlled decreasing pH-gradient [13], which demonstrates that pH-gradient IEC has more or less the same focusing capabilities as CF. However, the situation becomes somewhat different when larger injection volumes are applied, since the injection of a large volume (e.g. larger than one void volume) of a sample dissolved in elution buffer will overwhelm the pump-generated gradient and most likely generate a too steep pH-gradient due to the minimized intra-column buffering capacity. When the samples are dissolved in start buffer, on the other hand, the sample solvent will retard the start of the pH-gradient and the proteins are trapped at the column inlet. Thus, large volumes can easily be pre-loaded, either on-column or with trace enrichment column-switching techniques, before the elution starts. Fig. 5 shows the chromatograms obtained when equal amounts of β-lactoglobulins A and B dissolved in 0.1–100 μl start buffer were pre-loaded on-column and then eluted with a linear pH-gradient. As shown in Table 3, only a minor increase in peak widths, and thus a small decrease

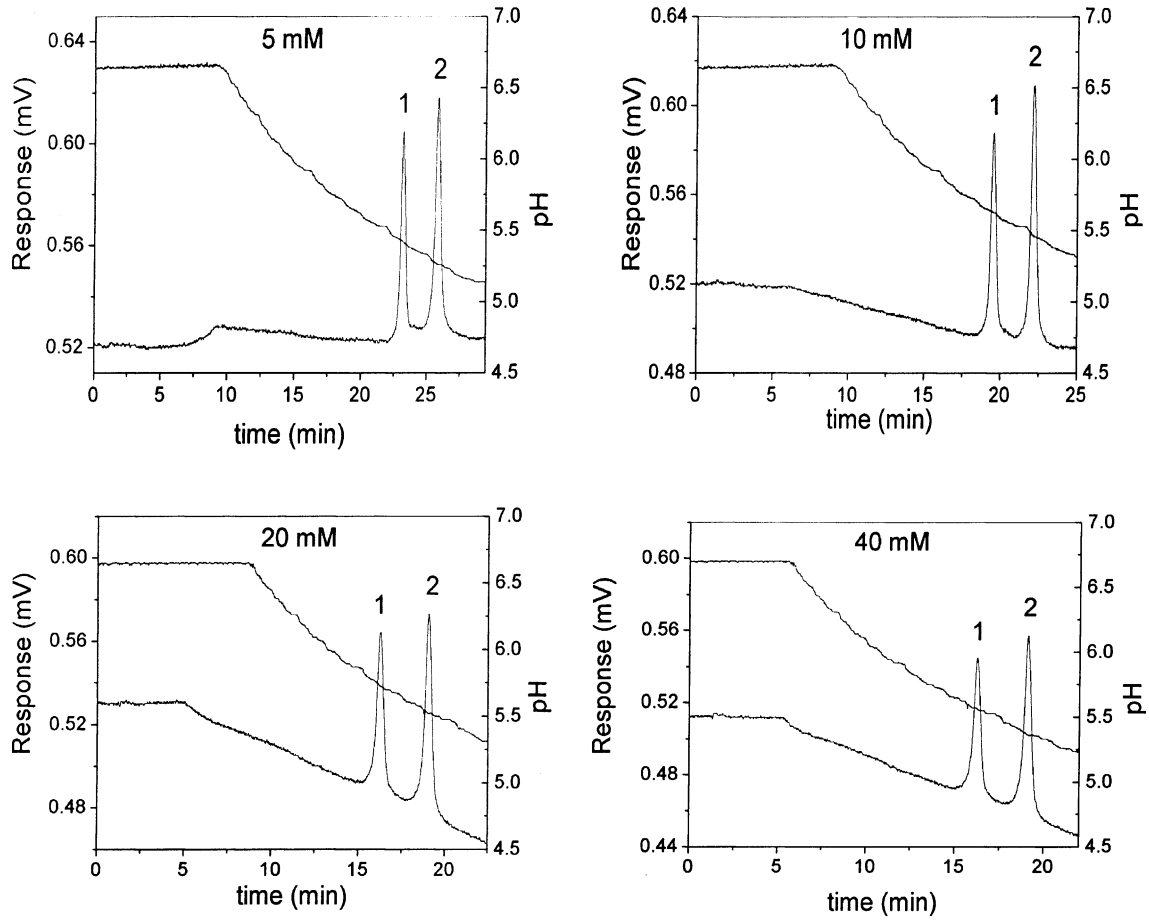

Fig. 4. Capillary pH-gradient IEC of β-lactoglobulins B (1) and A (2) using different buffer concentrations. Column: 10 cm × 0.32 mm i.d. PL-SAX column. Start buffer (A): piperazine and *N*-methylpiperazine, pH 6.8, eluting buffer (B): same as start buffer, but pH 4.3. Gradient program: 0–100% B in 25 min, then 100% B for 5 min. Flow rate: 8 μl min^{-1}. Injection volume: 0.1 μl. Sample concentration: 5 mg ml^{-1} of each protein (diluted in start buffer). Column temperature: 30 °C. UV detection at 280 nm.

in resolution, was observed when the injection volume was increased by a factor of 1000. An interesting feature of capillary pH-gradient IEC is that the focusing effect compensates for any initial band-broadening caused by sample overload. Thus, the sample capacity is more or less proportional to the column length, which is particularly useful when analyzing complex samples. Also, the focusing effect provides a significant advantage of capillary pH-gradient IEC over other techniques when using column-switching for trace enrichment, because the proteins bands are focused on the analytical column even when the same material is used in both the enrichment column and the analytical column.

3.5. Influence of temperature on peak width and resolution

Although column temperature has been recognized as an important parameter for separation optimization in salt-gradient IEC for more than 40 years [28], it has hardly been exploited in pH-gradient IEC. This is most likely due to the fact that the p*I* of proteins and the pK_a values of the buffers species are temperature-dependent and thus the separation becomes more difficult to control. For example, the dpK_a/dT (25 °C) = −0.026 for piperazine [29], which indicates that a 40 °C increment in column temperature will decrease the pK_a value about one pH unit, and

Table 2
Figures of merit of β-lactoglobulins A and B in Fig. 4, which illustrate the influence of buffer concentration on peak width (50% peak height) and resolution

	Buffer concentration (mM)			
	5	10	20	40
Peak width (min), β-lact. A	0.45 (1.9)	0.40 (1.5)	0.44 (1.4)	0.43 (2.5)
Peak width (min), β-lact. B	0.39 (2.0)	0.38 (1.8)	0.39 (1.6)	0.43 (3.7)
Resolution (R_s)	3.72 (1.7)	3.97 (1.9)	3.75 (5.5)	3.88 (3.0)

The relative standard deviations (%) of the experimental measurements are given in parentheses ($n = 3$).

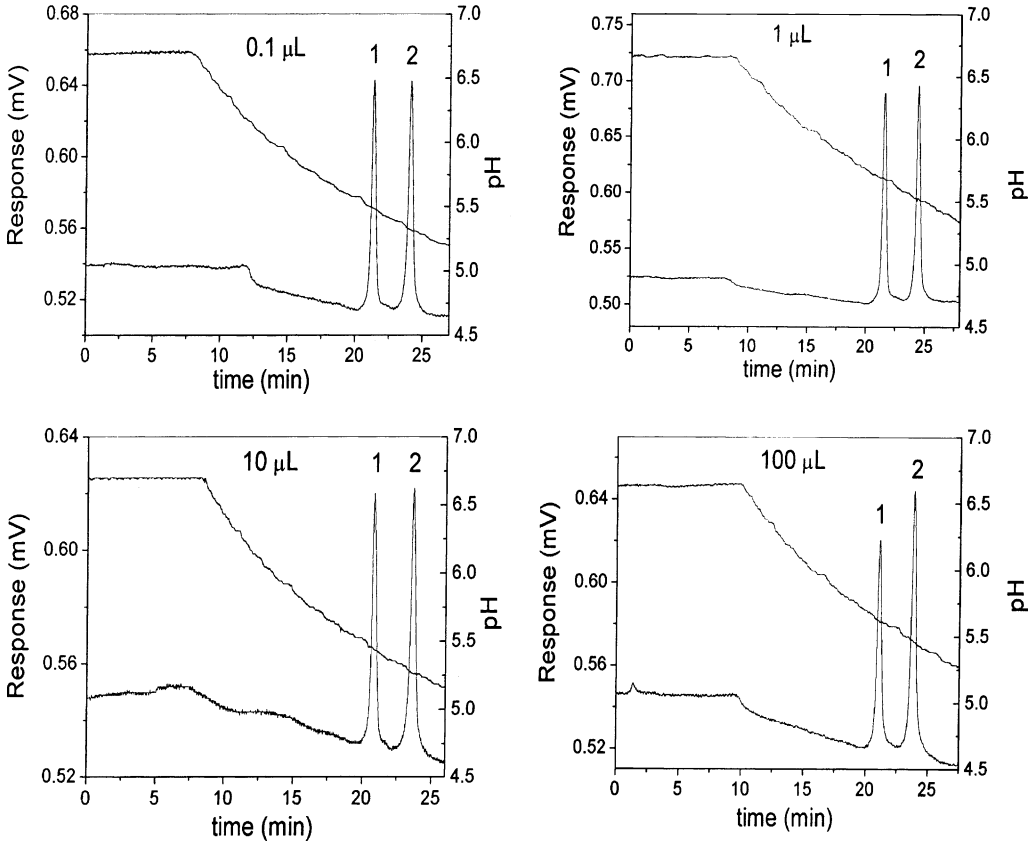

Fig. 5. Capillary pH-gradient IEC of β-lactoglobulins B (1) and A (2) using different injection volumes. The pH-gradient and the recording of the chromatograms were started after the sample was loaded. Column: 10 cm × 0.32 mm i.d. PL-SAX column. Start buffer (A): 10 mM piperazine and 10 mM *N*-methylpiperazine, pH 6.8, eluting buffer (B): same as start buffer, but pH 4.3. Gradient program: 0–100% B in 25 min, then 100% B for 5 min. Flow rate: 8 μl min^{-1}. Sample concentration: 5, 0.5, 0.05 and 0.005 mg ml^{-1} of each protein (diluted in start buffer). Column temperature: 30 °C. UV detection at 280 nm.

consequently, it may affect the pH of the mobile phase. Nonetheless, to investigate the influence of elevated column temperature on the chromatographic performance when separating β-lactoglobulins A and B, the separation was performed at four different column temperatures, as shown in Fig. 6. Generally, increased column temperatures gave reduced retention, e.g. increasing the temperature from 30 to 70 °C decreased the retention of β-lactoglobulins A and B with 21 and 24%, respectively, but also gave a significant increase in peak widths. Hence, as indicated in Table 4, the narrowest peaks and best resolution were obtained between 30 and 50 °C. This can probably be explained by the fact that an intermediate rise in temperature is the best compromise between increased transfer kinetics and denaturation of the proteins at the highest temperatures.

3.6. Capillary pH-gradient IEC of skimmed bovine milk

Besides water, fat, carbohydrates (lactose) and minerals, bovine milk contains 2–6 mass% of proteins and peptides [24]. Among the proteins, there are approximately 80% caseins, while the residual (i.e. whey proteins) mainly contains 10.2% β-lactoglobulins A and B, 5.0% α-lactalbumin, 1.3% bovine serum albumin (BSA), and 2.5% immunoglobulins [24]. However, since milk fat is not compatible with aqueous mobile phases, skimmed bovine milk that contained

Table 3
Figures of merit of β-lactoglobulins A and B in Fig. 5, which illustrate the influence of injection volume on peak width (50% peak height) and resolution

	Injection volume (μl)			
	0.1	1	10	100
Peak width (min), β-lact. A	0.37 (2.2)	0.36 (2.3)	0.38 (1.7)	0.39 (2.3)
Peak width (min), β-lact. B	0.35 (1.9)	0.35 (2.0)	0.36 (2.1)	0.38 (1.8)
Resolution (R_s)	4.60 (1.6)	4.81 (2.1)	4.27 (1.7)	4.18 (1.8)

The relative standard deviations (%) of the experimental measurements are given in parentheses ($n = 3$).

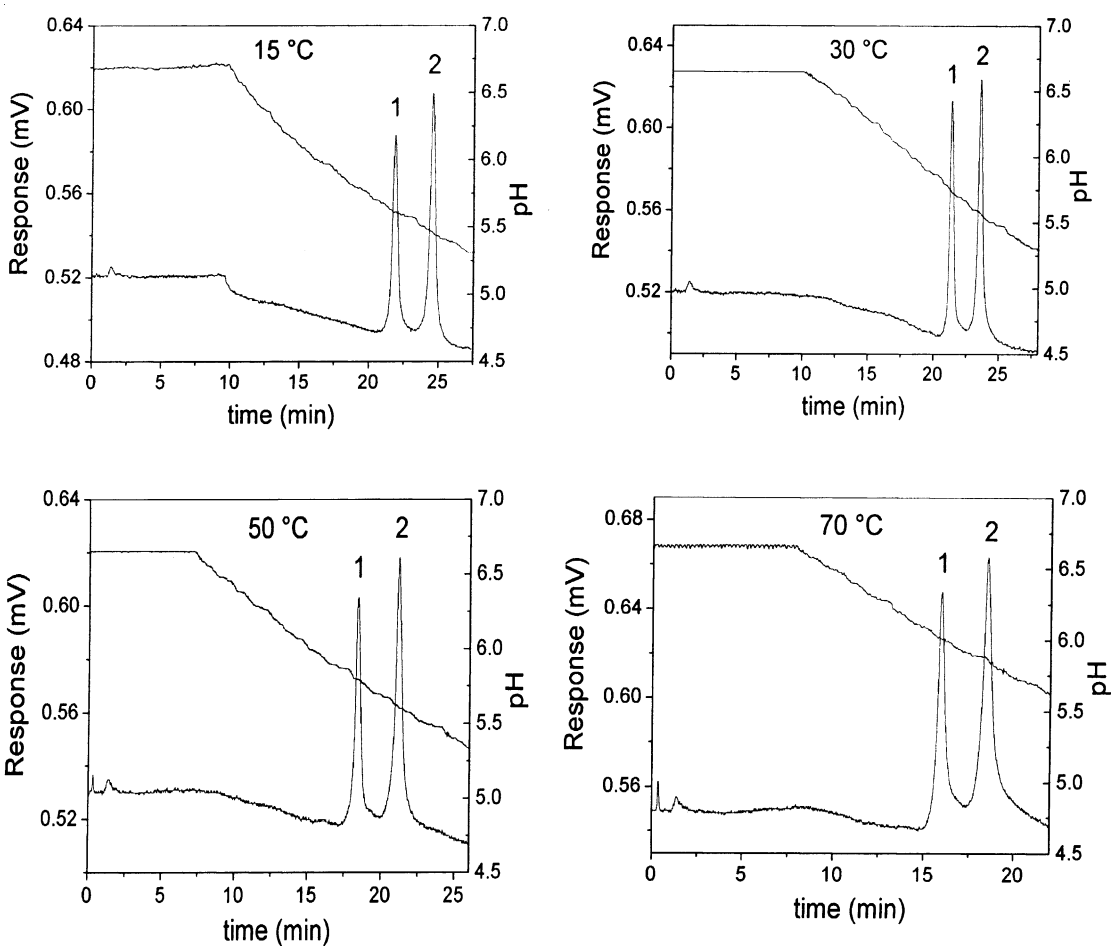

Fig. 6. Capillary pH-gradient IEC of β-lactoglobulins B (1) and A (2) at different column temperatures. Column: 10 cm × 0.32 mm i.d. PL-SAX column. Start buffer (A): 10 mM piperazine and 10 mM *N*-methylpiperazine, pH 6.8, eluting buffer (B): same as start buffer, but pH 4.3. Gradient program: 0–100% B in 25 min, then 100% B for 5 min. Flow rate: 8 μl min^{-1}. Injection volume: 0.1 μl. Sample concentration: 5 mg ml^{-1} of each protein (diluted in start buffer). UV detection at 280 nm.

less than 0.1% fat was chosen to avoid fat-removing sample preparation. Thus, the bovine milk samples were simply diluted with water and filtrated before injection. Fig. 7 shows the separation obtained when loading 5 μl of diluted milk (1:10 (v/v)) directly on the packed capillary PL-SAX column, followed by gradient elution from pH 6.8 to 4. When pure whey protein standards were injected, the retention time of α-lactalbumin (pI = 5.40) was identical to peak 2, while the retention time of β-lactoglobulins B and A were identical to peaks 3 and 4, respectively. Due to the delay between the UV detection and the pH-gradient, as discussed in Section 3.2, peaks 2, 3 and 4 appeared to elute at pI = 5.75, 5.50 and 5.40, respectively, which is in good agreement with the database pI values of the three suggested proteins [26,27]. The peaks eluting in front (i.e. peak 1) are most likely representing caseins, since most whey proteins have isoelectric points well below 6.8. However, it should be mentioned that some immunoglobulins have a pI value as high as 7.8 and will also elute in front, if present. The intra-day repeatability of the retention times, peak widths and peak areas of peaks 1–4 was investigated by performing six subsequent injections of the diluted milk,

Table 4
Figures of merit of β-lactoglobulins A and B in Fig. 6, which illustrate the influence of temperature on peak width (50% peak height) and resolution

	Temperature (°C)			
	15	30	50	70
Peak width (min), β-lact. A	0.43 (3.0)	0.40 (3.3)	0.40 (3.7)	0.52 (5.3)
Peak width (min), β-lact. B	0.43 (3.2)	0.37 (2.4)	0.38 (3.0)	0.42 (4.2)
Resolution (R_s)	3.79 (1.7)	4.28 (1.2)	4.30 (2.2)	3.03 (8.6)

The relative standard deviations (%) of the experimental measurements are given in parentheses (n = 3).

Table 5
Figures of merit of the peaks obtained in Fig. 7

	Peak no.			
	1	2	3	4
Retention time (min)	4.21 (1.7)	20.81 (1.0)	26.10 (0.8)	29.32 (0.3)
Peak width at 50% height (min)	0.25 (7.3)	0.52 (4.5)	0.43 (4.1)	0.44 (3.1)
Peak area (mV min)	0.065 (4.2)	0.023 (3.8)	0.020 (4.0)	0.013 (12.1)

The relative standard deviations (%) of the experimental measurements are given in parentheses ($n = 6$).

as shown in Table 5. The R.S.D.s of the retention times were highly acceptable and ranged from 0.3 to 1.7%, while the R.S.D.s of the peak widths and peak areas were somewhat higher. One possible explanation is that manual inspection of the automatically drawn peak baselines was not possible with the chromatographic software applied, leading to random errors in the calculated peak widths and peak areas.

The ruggedness of pH-gradient IEC has always been an issue that needs to be considered, particularly when injecting relative large volumes of complex samples directly on the column. Such samples often contain components that can affect the pH-gradient. However, if using column-switching techniques for fast loading of large sample volumes on a short enrichment precolumn, which is common in capillary LC, this step will also provide further sample clean-up and reduce the impact on the pH-gradient. Consequently, the ruggedness of capillary pH-gradient IEC when analyzing such samples, with emphasis on column-switching techniques, but also with direct injection, is currently under investigation in our laboratory.

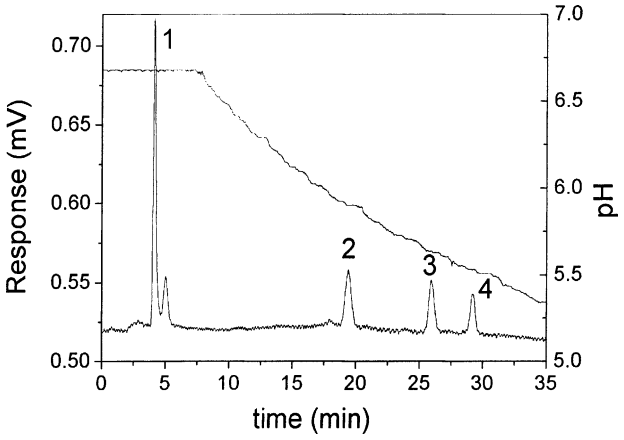

Fig. 7. Capillary pH-gradient IEC of diluted skimmed bovine milk (1:10, v/v) on a 10 cm × 0.32 mm i.d. PL-SAX column. Start buffer (A): 5 mM piperazine and 5 mM *N*-methylpiperazine, pH 6.8, eluting buffer (B): same as start buffer, but pH 4.3. Gradient program: 0–100% B in 25 min, then 100% B for 5 min. Flow rate: 6 μl min^{-1}. Injection volume: 5 μl. Column temperature: 30 °C. UV detection at 280 nm. The pH-gradient and the recording of the chromatograms were started after the sample was loaded.

4. Conclusions

Although CF is inherently more compatible with packed capillary columns than pH-gradient IEC due to the application of single step gradients, capillary pH-gradient IEC overcomes many of the limitations of CF. Besides, several reliable micro-flow pumping systems capable of delivering solvent gradients in the low microliter per minute range have become commercially available in recent years, such as the one applied in this study, which significantly simplifies the application of pH-gradient IEC on packed capillary columns. The developed miniaturized flow-through pH probe provided reliable pH monitoring of the effluent, but due to the relatively large diameter of the glass membrane the internal dead volume of the flow-through pH probe could not be made smaller than approximately 15 μl. Consequently, the authors hope to develop a truly dedicated flow-through pH probe for packed capillary effluent flow rates in the near future, since pH electrodes designed for smaller volumes become available all the time. Nevertheless, the pH monitoring of the effluents revealed that reproducible and highly linear outlet gradients from pH 8.5 to 4.0 were obtained with start and elution buffers containing the same amine buffering species. Furthermore, as demonstrated in this study with the on-column injection of 5 μl diluted milk, capillary pH-gradient IEC allows the focusing of proteins when the pH of the sample solution > pI, which, combined with the focusing of the protein bands, is highly beneficial in trace enrichment column-switching techniques. Hence, capillary pH-gradient IEC has the potential of becoming an important and sensitive separation technique in prospective proteomic studies, not only for off-line pI fractionation of low abundant proteins, but also as a highly efficient reversed-phase compatible dimension in two-dimensional capillary LC systems. Theoretically, capillary two-dimensional pH-gradient IEC × reversed-phase LC coupled to ESI-MS and NMR is capable of generating highly automated pI versus M_r plots of minute amounts of proteins, and is thus an attractive alternative to traditional two-dimensional gel electrophoresis.

Acknowledgements

T.A. acknowledges the financial support from the EUREKA project E!2410-HOT-SEP and the Norwegian Research Council (NFR). The authors also thank Inge

Mikalsen and Bjørn Dalbakk of the Instrumental Workshop, Department of Chemistry, University of Oslo for technical assistance.

References

[1] L.A.AE. Sluyterman, J. Wijdenes, in: B.J. Radola, D. Graesslin (Eds.), Proceedings of the International Symposium on Electrofocusing Isotachophoresis, Walter de Gruyter, Berlin, 1977, p. 463.
[2] L.A.A.E. Sluyterman, O. Elgersma, J. Chromatogr. 150 (1978) 17.
[3] L.A.A.E. Sluyterman, J. Wijdenes, J. Chromatogr. 150 (1978) 31.
[4] L.A.A.E. Sluyterman, J. Wijdenes, J. Chromatogr. 206 (1981) 429.
[5] L.A.A.E. Sluyterman, J. Wijdenes, J. Chromatogr. 206 (1981) 441.
[6] J.H. Scott, K.L. Kelner, H.B. Pollard, Anal. Biochem. 149 (1985) 163.
[7] G. Wagner, F.E. Regnier, Anal. Biochem. 126 (1982) 37.
[8] M.T.W. Hearn, D.J. Lyttle, J. Chromatogr. 218 (1981) 483.
[9] T.W. Hutchens, C.M. Li, P.K. Besch, J. Chromatogr. 359 (1986) 157.
[10] T.W. Hutchens, C.M. Li, P.K. Besch, J. Chromatogr. 359 (1986) 169.
[11] R.C. Bates, D.D. Frey, J. Chromatogr. A 814 (1998) 43.
[12] O. Kaltenbrunner, C. Tauer, J. Brunner, A. Jungbauer, J. Chromatogr. 639 (1993) 41.
[13] Y. Liu, D.J. Anderson, J. Chromatogr. A 762 (1997) 207.
[14] Y. Liu, D.J. Anderson, J. Chromatogr. A 762 (1997) 47.
[15] L. Shan, D.J. Anderson, J. Chromatogr. A 909 (2001) 191.
[16] L. Shan, D.J. Anderson, Anal. Chem. 74 (2002) 5641.
[17] A. Hirose, D. Ishii, J. Chromatogr. 387 (1987) 416.
[18] A. Hirose, D. Ishii, J. High Resolat. Chromatogr. 10 (1987) 360.
[19] A. Hirose, D. Ishii, J. Chromatogr. 438 (1988) 15.
[20] Y.-M. Li, J.-L. Liao, R. Zhang, H. Henriksson, S. Hjerten, Anal. Biochem. 267 (1999) 121.
[21] D.B. Wall, M.T. Kachman, S. Gong, R. Hinderer, S. Parus, D.E. Misek, S.M. Hanash, D.M. Lubman, Anal. Chem. 72 (2000) 1099.
[22] B.E. Chong, F. Yan, D.M. Lubman, F.R. Miller, Rapid Commun. Mass Spectrom. 15 (2001) 291.
[23] D.B. Wall, M.T. Kachman, S.S. Gong, S.J. Parus, M.W. Long, D.M. Lubman, Rapid Commun. Mass Spectrom. 15 (2001) 1649.
[24] H. Splitt, I. Mackenstedt, R. Freitag, J. Chromatogr. A 729 (1996) 87.
[25] K.M.G. Oliveira, V.L. Valente-Mesquita, M.M. Botelho, L. Sawyer, S.T. Ferreira, I. Polikarpov, Eur. J. Biochem. 268 (2001) 477.
[26] P.G. Righetti, T. Caravaggio, J. Chromatogr. 127 (1976) 1.
[27] D. Malamud, J.W. Drysdale, Anal. Biochem. 86 (1978) 620.
[28] P.B. Hamilton, D.C. Bogue, R.A. Anderson, Anal. Chem. 32 (1960) 1782.
[29] http://www.amershambiosciences.com, Aug. 2003.