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Perfusion liquid chromatography of whey proteins

Mercedes Torre[1], Maria Eugenia Cohen[2], Nieves Corzo[3], Miguel Angel Rodríguez[4], Jose Carlos Diez-Masa*

Instituto de Química Orgánica General (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Abstract

A perfusion reversed-phase (RP) HPLC method was developed for the rapid separation of the main bovine whey proteins: α-lactalbumin (α-LA), serum albumin (BSA) and the genetic variants of β-lactoglobulin (A and B) (β-LG A and β-LG B). For the method development, the influence of factors favouring structural changes of proteins (temperature and organic acid concentration in the mobile phase), gradient and other chromatographic conditions and the mass of protein injected was examined. The optimized method allowed the separation of proteins in about 1.5 min (cycle time 3.5 min) with resolution around 1.0 for the β-lactoglobulins. The method was applied to the determination of proteins in a whey from raw bovine milk. The precision of the determinations was ≤3.75 mg per 100 ml (S.D.). With respect to the accuracy, errors ≤7.0% in the determination on α-LA, β-LG A and β-LG B were obtained, compared with an RP-HPLC reference method. However, higher errors in the quantification of BSA were found owing to the lack of purity of the peak assigned. In addition, the proposed method has proved to be very useful in the detection of homologous whey proteins from different species (cow, sheep and goat) in milk mixtures.

Keywords: Perfusion chromatography; Proteins; Whey proteins; α-Lactalbumin; Albumin; β-Lactoglobulin

1. Introduction

The separation and determination of biopolymers (peptides, proteins, etc.) are frequently achieved by reversed-phase high performance liquid chromatography (RP-HPLC), although other HPLC methods such as ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), size-exclusion chromatography (SEC) and different types of affinity chromatography (AC) have also been used [1,2].

With the development of recombinant DNA techniques, HPLC has become an important tool in both quality control and process control in the production of recombinant proteins of pharmacological interest. This has contributed significantly to the development of RP-HPLC methods that could be used to carry out very rapid analyses for proteins with minimum complexity in both instrumentation and operating conditions [3,4].

Given the low diffusivity of biopolymers, the

* Corresponding author.

[1] Present address: Dept. Química Analítica e Ingeniería Química, Universidad de Alcalá de Henares, 28871 Alcalá de Henares, Madrid, Spain.

[2] Present address: Centro de Biomateriales, Universidad de la Habana, Havana, Cuba.

[3] Present address: Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain.

[4] Present address: Dept. Química Analítica, Bromatología y Toxicología, Universidad de la Laguna, 38204 S/C de Tenerife, Spain.

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need to achieve fast separations of proteins by RP-HPLC has led to the development of different types of packing materials such as non-porous microparticles ($d_p < 5$ μm) [5] or wide-pore materials [6,7], that solve the mass transfer problems in the stationary phase, allowing good separations within a reasonable time (<10 min).

Perfusion chromatography, a high-speed chromatographic method, was introduced a few years ago for the rapid separation and purification of proteins [8]. The particles used in perfusion chromatography have a bidisperse porous structure composed of 6000–8000 Å pores transecting the particles (through pores), and 500–1500 Å diffusive pores that line the through pores. The peculiar porous network of these poly(styrene–divinylbenzene)-based particles provides fast mass transfer for proteins while maintaining a large surface area [8–12]. Consequently, short analysis times (a few seconds) with moderate pressure drops can be achieved in the separation of biopolymers [12,13]. Perfusive particles also present good thermal, mechanical and chemical (wide pH range) stability.

Rapid, easy and accurate analyses for whey proteins are currently a matter of major interest for the dairy industry in the quality control of milks and related products. In recent years, some RP-HPLC methods for the determination of whey proteins have been used in dairy science [14–16]. However, these methods entail long analysis times [14,15] and strong interactions between proteins and the stationary phases currently used [14]. An additional limitation, which may be critical for the development of fast HPLC methods, is the incomplete separation of the two main genetic variants (A and B) of bovine β-lactoglobulin [8,14,16], whose primary structures differ only at two positions (A/B Asp64Gly and Val118Ala) of their 162 amino acid sequence.

The main goals in this work were to develop a perfusion RP-HPLC method for the rapid separation (analysis time about 1.5 min) of whey proteins, to explore the capability of the method for the determination of the major whey proteins and to evaluate this method for the separation of ovine and caprine whey proteins and the detection of milk mixtures from different animal species.

2. Experimental

2.1. Chemicals and samples

Acetonitrile (ACN) (HPLC grade; Scharlau, Barcelona, Spain), trifluoroacetic acid (TFA) (HPLC/Spectro grade; Pierce Europe, Oud Beijerland, Netherlands), formic acid (FA) (analysis grade; Merck, Darmstadt, Germany) and water obtained from a Milli-Q system (Millipore, Bedford, MA, USA) were used in the preparation of the mobile phases.

Bovine serum albumin (BSA), α-lactalbumin (α-LA), β-lactoglobulin A (β-LG A) and β-lactoglobulin B (β-LG B) were obtained from Sigma (St. Louis, MO, USA) and used without further purification. Standard protein solutions were freshly prepared in water. Aliquots of stock solutions (ca. 4 mg/ml) of each protein were weighed and dissolved in water in order to prepare dilute solutions of the desired concentration.

A raw bovine milk, used as reference material of the International Dairy Federation [17], was employed as a sample. The whey from bovine milk was prepared by acidic precipitation (pH 4.6) of caseins using 2 mol/l HCl [15,17]. Owing to the high protein concentration in bovine whey, samples were diluted 1:10 (w/v) in water before injection.

The whey from ovine and caprine milks was prepared by acidification of the milk to pH 4.6 (1.0 mol/l HCl) and subsequent centrifugation of caseins. Purified ovine β-LG A and B (each achieved from a homozygous animal) and caprine β-LG (all kindly donated by Professor M. Ramos, Instituto de Fermentaciones Industriales, CSIC) were obtained from the acidic wheys using a trichloroacetic acid (TCA) precipitation procedure (3.1 g of TCA in 100 ml of acidic whey) as described by Ebeler et al. [18]. The purity and identity of these samples were checked by isoelectric focusing [16].

Both the standard and whey solutions were filtered through 0.20-μm disposable sterile polysulfone filters (Acrodisc; Gelman Sciences, Ann Arbor, MI, USA) before injection.

All sample solutions were stored at −20°C until use. During analyses, samples and standards were kept in ice.

2.2. High-performance liquid chromatography

The chromatograph consisted of two Model 2150 pumps (LKB, Bromma, Sweden), an LKB Model 2152 gradient controller, an LKB Model 2152-400 static mixer, a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 20-μl sample loop and a Model 440 UV spectrophotometric detector (Waters, Milford, MA, USA) operated at 280 nm. The column, injector and a heat exchanger consisting of a stainless-steel capillary (200 cm × 0.5 mm I.D.) connecting the mixer and the injection valve were thermostated at the temperature indicated by immersion in a Haake F3-C thermostatic bath (Fisons Instruments, Karlsruhe, Germany). Data acquisition and chromatographic analysis were performed with a Model 406 analog interface (Beckman Instruments, Fullerton, CA, USA) and Beckman System Gold software (version 7.11).

For studies of the purity of the BSA peak, two Beckman Model 116 programmable pumps, a dynamic mixer (1.4-ml dead volume) and a Beckman Model 168 diode-array detector, all controlled by the Beckman System Gold software, were used. With this instrument a Rheodyne Model 7125 injection valve with a 20-μl sample loop was also employed.

A stainless-steel column (50 × 2.1 mm I.D.) was slurry packed in the laboratory using POROS 1 10R (PerSeptive BioSystems, Cambridge, MA, USA) as packing material and an 80:20 mixture of glycerol (Carlo Erba, Milan, Italy) and an aqueous solution of 2.5% (w/v) NaCl (Merck) as slurry liquid. Packing was performed at 180 atm (1 atm = 101 325 Pa) using an 80% solution of ACN (HPLC grade; Scharlau) in water as the packing solvent. Stainless-steel frits of 2 μm (0.62 in.) I.D. PEEK ring from Upchurch Science (Oak Harbor, WA, USA) were used.

After optimizing the experimental conditions (as described under Results and discussion), separations were carried out at a flow-rate of 3 ml/min and 50°C, using a linear binary gradient from 25% to 35% B in 1.5 min. To re-equilibrate the column, a linear reversed gradient from 35% to 25% B in 1.0 min followed by a 1.0-min isocratic step at 25% B was used. The mobile phases were prepared by mass as follows: phase A, ACN–water–FA (5:75:20, v/v/v); and phase B, ACN–water (93:7, v/v). The mobile phases were filtered through 0.45-μm filters (MSI, Westboro, MA, USA) and degassed by sparging with helium.

2.3. Calibration

Calibration of the chromatographic system for protein determination was carried out by the external standard method. For this purpose, each protein was calibrated individually by injection solutions of the standards (20 μl) with a concentration (corrected by the standard purity) ranging from 0.04 to 0.4 mg/ml. Before injecting a new solution a blank gradient was run to check that there was no carryover from a previous injection.

2.4. Data analysis

Resolution of overlapped peaks of β-LG A and B was determined according to the methods proposed by Snyder et al. [19]. Sample chromatograms were corrected by subtracting the chromatogram corresponding to a blank gradient in order to eliminate baseline drift before integration. Retention time and peak area were calculated in the corrected chromatograms for each protein peak using the System Gold software. For integration of overlapped peaks (β-LG A and B), a method using a vertical drop from the baseline to the deepest point of the valley was used.

3. Results and discussion

3.1. Separation of bovine whey proteins: method development

A previous study by our group [16] has shown that separation in ca. 7 min (cycle time 20 min) and quantification of the main whey proteins (α-LA, β-LG B, β-LG A and BSA) from bovine milks by RP-HPLC are possible at room temperature using a silica-based, wide-pore, C_4 column and mobile phases containing ACN–water–TFA–morpholine. However, it was observed that maybe owing to monomer–dimer equilibrium [20] and/or to the small structural differences between β-LG B and β-LG A, resolution at the baseline of these proteins was difficult to achieve in a shorter analysis time. This problem has also been reported by other workers [14,15,21]. Therefore, during the development of the method we aimed to achieve sufficient resolution for β-lactoglobulin A and B for the accurate determination of both genetic variants while maintaining a short analysis time (ca. 1.5 min).

Several gradients (various initial and final compositions and gradient times) of water–ACN containing TFA (0.1%) were first tried for the separation of the main whey proteins. Using the column described under Experimental, poor resolution of the two β-lactoglobulins ($R_s < 0.7$) was obtained when separation times below 3 min were attempted.

It is known that protein separations in RP-HPLC occasionally cause protein structural changes (i.e., changes in secondary, tertiary and/or quaternary structure) induced by the mobile phase, stationary phase and/or temperature. These changes can have a major impact on retention time, peak shape and separation selectivity of proteins [22,23]. It has recently been demonstrated [24,25] that for similar mobile phases, protein structural changes during separation are less favoured in non-alkylated polymeric packing materials than in alkyl-bonded silica-based stationary phases. We thought, therefore, that in this case the small capability of POROS material to induce structural changes on β-lactoglobulins could be the reason for the lack of selectivity observed for these proteins in the previous experiments. Therefore, other chromatographic conditions able to cause modifications in the structure of β-lactoglobulins (i.e., dimer $\rightleftharpoons$ monomer transition and maybe unfolding), such as column temperature [20] and type and concentration of acid in the mobile phase [26,27], that might increase selectivity for the separation of β-LG A and β-LG B, were explored. Once these variables had been optimized, the effect of the gradient (initial and final composition, flow-rate and gradient time) and the sample mass on the resolution of the β-lactoglobulins were investigated. The results are reported below.

Chromatographic conditions inducing structural changes

Temperature. The effect of temperature on the resolution of β-LG A and B was studied using mobile phases containing 0.1% TFA. It as observed (results not shown) that at room temperature a broad peak corresponding to the two unresolved proteins was obtained. However, increasing the column temperature in the range 40–60°C resulted in a steady increase in the resolution up to a value of $R_s \approx 0.8$. It was also observed that the detector noise remained almost constant at room temperature until 50°C and became 2–3 times higher at 60°C. This effect could be caused by temperature fluctuations in the detection cell. For this reason, a temperature of 50°C was selected for this separation.

This increase in resolution is consistent with the fact that some structural changes of proteins are thermally induced processes closely related to the lability of the protein involved. The heat stability of β-lactoglobulin A and B at the acidic pH used in this work (0.1% TFA, pH ≈ 2.0) has not been well established. However, some denaturation of β-lactoglobulins could occur at pH 2.5 when they are heated at 70–75°C for a short period of time [28]. On the other hand, the degree of dissociation of the dimeric forms of each β-lactoglobulin increases with increase in temperature [20] at this pH. These factors could

explain the increase in resolution observed for β-LG A and β-LG B with increase in temperature. Since the increase in temperature in the range studied led only to a limited resolution ($R_s \approx 0.8$) for these proteins, the effect of organic acid content in the mobile phase at high temperature on the separation of β-lactoglobulins was studied.

Organic acid. Although TFA (concentrations ranging from 0.1 to 0.3%) is the acid most often used in the reversed-phase chromatographic analysis of proteins, a high proportion of formic acid (FA) (up to 50–60%) has been very effective in the separation of large hydrophobic polypeptides and proteins [27–31], mainly owing to its strong dissolving and denaturing capacity [29,30]. Specifically, it has been observed [26] that the dissociation capacity for β-lactoglobulin aggregates increases on decreasing the pH.

In the experiments conducted in this work to study the ability of organic acids to improve the separation of a standard mixture of β-LG A and β-LG B, it was observed that FA provided a better resolution than TFA for these proteins at low acid concentrations (<0.3%) (results not shown). An increase in resolution of β-LG B and A was observed on increasing the FA concentration in the mobile phase up to 20% (Fig. 1). A further increase in the FA concentration did not cause an increase in resolution. However, it should be taken into account that the use of formic acid at this concentration prevents the detection of these proteins at 205–220 nm, owing to the strong absorption of this acid at these wavelengths.

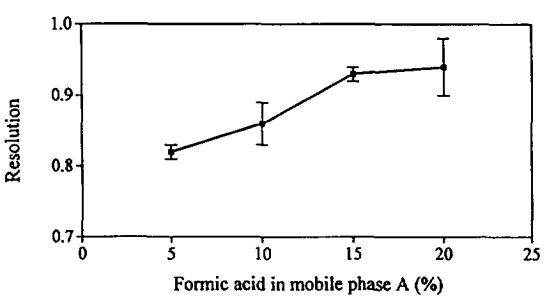

Fig. 1. Resolution of β-LG B and β-LG A as a function of the percentage of formic acid in mobile phase A. Experimental conditions: column, POROS 1 10 R (10 μm) (50 × 2.1 mm I.D.); linear gradient, 27–37% B, t_G = 3.0 min, flow-rate F = 1.0 ml/min; temperature, 50°C; detection at 280 nm.

Gradient and other chromatographic conditions

To optimize the gradient and other chromatographic conditions, the linear solvent strength (LSS) [32] model for this separation was assumed. Small I.D. columns (2.1 mm) were used to obtain a perfusive mobile phase velocity (superficial velocity >1000 cm/h) with moderate flow-rates (>0.6 ml/min). The gradient steepness was studied to improve the resolution of β-lactoglobulins. It was observed that shallow gradients produced better resolution than steeper gradients. Different gradient conditions (initial and final mobile phase compositions, gradient time and flow-rate) were investigated while maintaining the gradient steepness parameter constant to carry out the separation within 1.5 min. From these results (not shown), a linear gradient from 25% to 35% B in 1.5 min at a flow-rate of 3.0 ml/min was chosen.

Mass of protein injected

During the previous studies, it was observed that the resolution of β-LG B and β-LG A decreased with increasing mass of protein injected. The effect of sample amount on the resolution, at different flow-rates (while maintaining $Ft_G = 4.5$) to carry out the separation in 1.5 min for mixtures of β-LG B and β-LG A standards is illustrated in Fig. 2, which shows that at low loadings, good resolution ($R_s > 1.1$) is obtained. However, the resolution decreases with increasing sample mass up to ca. 10 μg, for which a decrease in R_s drop of 24% ($R_s = 0.88$) is observed. For amounts larger than 10 μg, no significant change in the resolution of the two proteins studied is observed. The loss in resolution is accompanied by a slight reduction in retention time (about 10%).

The overload of the POROS column for proteins follows the general trend observed with silica-based RP-HPLC columns [33]. Nevertheless, the load capacity of the perfusive support seems to be one order of magnitude smaller than that of silica-based stationary phases [33], proba-

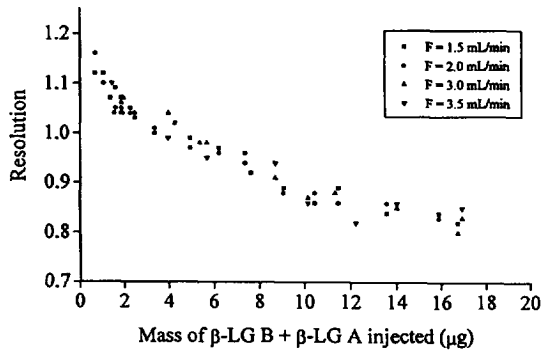

Fig. 2. Dependence of the resolution of β-LG B and β-LG A on the mass of protein injected. [β-LG B]/[β-LG A] ratio = 1.07. Experimental conditions: column, POROS 1 10 R (10 μm) (50 × 2.1 mm I.D.); mobile phases, A = ACN–water–FA (5:75:20, v/v/v) and B = ACN–water (93:7, v/v); optimized linear gradient, 25–35% B, Ft_G = 4.5 ml; injection volume, 20 μl; temperature, 50°C; detection at 280 nm.

bly owing to the smaller surface area of the POROS packing material.

Fig. 2 also shows that the resolution is almost independent of flow-rate in the ranges of flow-rates and sample amounts studied (from 1.5 to 3.5 ml/min and from 8.1 to 97 μg protein/ml column, respectively). This behaviour may be explained on the basis of the dominance of convective transport in the perfusion packing material [8,9,12]. In fact, as stated above, intraparticle perfusion would allow very high mobile phase velocities with little loss in resolution.

The importance of the detection limit in perfusion chromatography should be emphasized, since by using more sensitive detectors, the resolution may be improved by injecting smaller amounts of the analyte.

3.2. High-speed analysis of wheys

The optimized perfusion chromatographic method was applied to achieve rapid analyses for the main proteins of a whey from bovine milk (Fig. 3). Peak identification was carried out using standard proteins. It can be deduced that the analysis time for the four proteins is about 3,

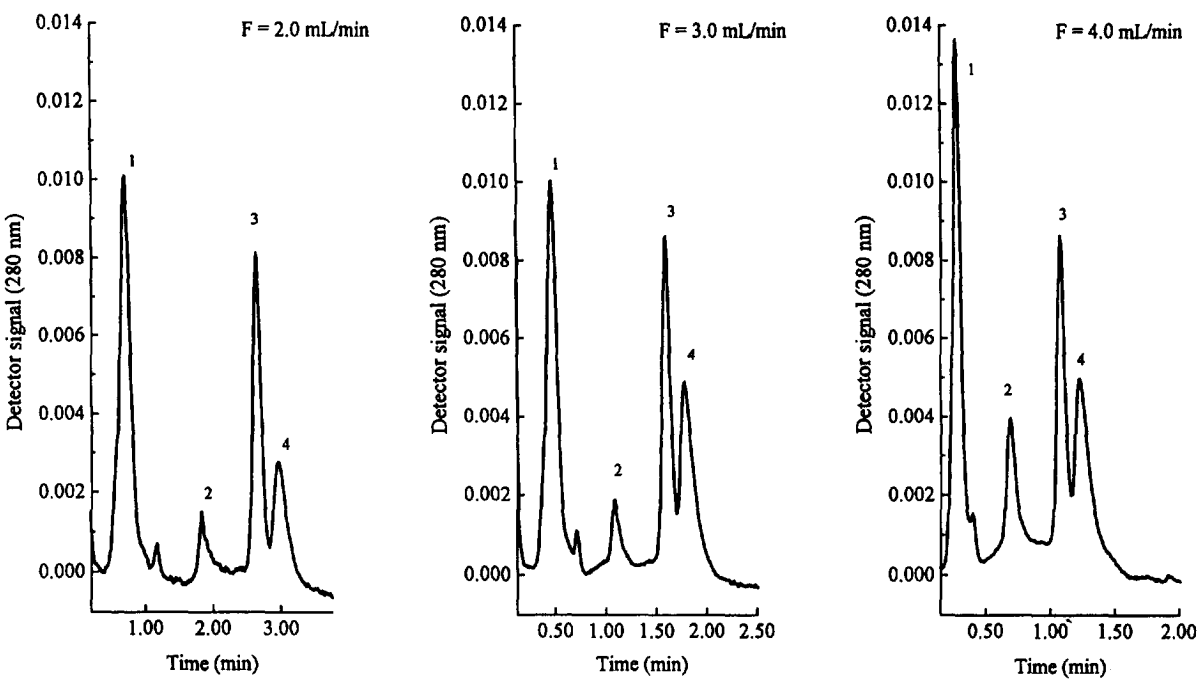

Fig. 3. Separation of a whey from raw bovine milk under perfusion reversed-phase conditions at different flow-rates, holding the gradient volume constant (Ft_G = 4.5 ml). Whey diluted in water, concentration 82.63 mg/ml. Other experimental conditions as in Fig. 2. Peaks: 1 = α-LA; 2 = BSA; 3 = β-LG B; 4 = β-LG A.

2, and 1.5 min at flow-rates of 2.0, 3.0, and 4.0 ml/min, respectively. This means that at a flow-rate of 4.0 ml/min, with a gradient cycle (run and column re-equilibration) of 3.1 min, this method would allow the injection of 460 samples in a 24-h period. Moreover, minimal decreases in peak efficiency and resolution are observed when the mobile phase flow velocity is increased from 2.0 to 4.0 ml/min. Specifically, the resolution values for β-LG A–β-LG B were 1.10, 1.05, and 1.00 at flow-rates of 2.0, 3.0 and 4.0 ml/min, respectively. An increase in the BSA peak area (corrected for flow-rate differences) was observed at higher speeds, for which we do not have any satisfactory explanation, although we are currently working on it.

3.3. Column stability

At the high temperature, very low pH and high flow-rate employed in these analyses, the perfusion column has been shown to be chemically stable. It was observed that after 700 whey injections, the retention time of β-LG B changed by less than 0.9% and its efficiency remained constant within 15%. Nevertheless, exposure of perfusion columns to very basic solutions such as 0.1 mol/l NaOH or KOH damaged the column irreversibly.

Under the experimental conditions used in the chromatographic separation of whey proteins, a peak of constant area for each protein was observed from the first injection into an unused column, which demonstrates that irreversible adsorption of proteins on the column is negligible.

3.4. Determination of whey proteins

Calibration and precision of the method

The chromatographic system was calibrated by the external standard method with solutions that contained each of the standard proteins (α-LA, β-LG A, β-LG B and BSA) in the range of protein concentrations from 0.04 to 0.4 mg/ml.

Each individual protein elutated as a single peak with the exception of BSA, which eluted as a peak with a shoulder (at low BSA concentrations) or as two peaks, of which the most retained represented 40% of the area of the main BSA peak. This chromatographic behaviour of BSA has been widely discussed in the literature and may be attributed to BSA aggregates [30,34]. Nevertheless, the peak with the shoulder was integrated as a whole.

The values of the slope, intercept, correlation coefficient and the theoretical detection limit calculated following the criteria of Miller and Miller [35] corresponding to the best straight line of regression to the calibration points are given in Table 1. From these results, it is evident that the calibration plots are linear in the concentration range studied and that the lines corresponding to β-LG A and β-LG B do no pass through the origin.

The precision of the perfusion method was

Table 1
Calibration by the standard external method of whey proteins by perfusion RP-HPLC

Protein	Concentration range (mg/ml)	n[a]	Slope	Intercept	r[b]	Detection limit (mg/ml)[c]
α-LA	0.049–0.176	5	1141.39 (39.34)	−5.42 (4.20)	0.9982	0.0143
β-LG B	0.065–0.281	7	515.57 (15.38)	−14.94 (2.65)	0.9977	0.0165
β-LG A	0.060–0.231	5	487.37 (14.94)	−14.06 (2.17)	0.9986	0.0125
BSA	0.064–0.417	6	379.31 (9.41)	−1.92 (2.36)	0.9988	0.0257

Flow-rate, 3.0 ml/min. Other experimental conditions as in Fig. 2.

[a] n = Number of points considered for the regression. Each point represents the average of eight injections of each standard solution. Standard deviation in the slope and intercept of the regression line are given in parentheses.

[b] r = Correlation coefficient.

[c] Detection limits were calculated according to the criterion of Miller and Miller [35].

measured using standards and a whey from raw bovine milk by analysing eight injections of each sample. Values of the relative standard deviation (R.S.D.) for measurements of retention time and peak area are given in Table 2. With the exception of the variation in retention time of α-La, eluting at shorter retention times, the within-day precision of the method seems to be good when standard concentrations higher than 0.10 mg/ml were assayed. On the other hand, the repeatability of retention times and peak areas between days is acceptable (R.S.D. <5–6%). The repeatability value observed for the peak area of BSA in wheys (9.2) could be due to the small area corresponding to this peak.

Accuracy of the method: analysis of proteins in a whey sample from raw bovine milk

The protein concentration in a whey sample from a raw bovine milk used as a reference material by the International Dairy Federation [17] was calculated using our perfusion method and an RP-HPLC reference method recommended by the Federation [17]. In the latter, the separation of the main whey proteins is accomplished by gradient elution using PLRP-S columns (40°C) and ACN–water–0.1% TFA mobile phases in a 21.5-min analysis time (cycle time 38 min). The concentration of proteins obtained by both methods and the accuracy of the results are given in Table 3.

The precision of the results, given as standard deviation, is good since it does not exceed 3.75 mg per 100 ml. As for the accuracy of the perfusion method, the analytical results in the determination of α-LA, β-LG B, β-LG A and β-LG (A + B) by this method closely agree with the values given by the reference method. However, the concentration of BSA found by perfusion chromatography in the whey sample was anomalously high. This result will be discussed below.

The variability between the results for α-LA content found by the two chromatographic methods may be due to the small peak eluted behind and close to the α-LA in the whey sample (Fig. 3), which was not considered in the integration of this protein because this peak was well resolved from the peak of α-LA using the reference method.

On the other hand, the relative errors in the determination of the content of either β-LG B or β-LG A are acceptable. The main source of error in this case may be attributed to problems in the peak-area integration of the incompletely resolved peaks of the genetic variants of β-lacto-

Table 2
Repeatability, expressed as relative standard deviation (R.S.D.), of the perfusion RP-HPLC determination of whey proteins in standard samples and in a whey from raw bovine milk

Sample	Assay	n[a]	α-LA		β-LG B		β-LG A		BSA	
			Time (%)	Area (%)	Time (%)	Area (%)	Time (%)	Area (%)	Time (%)	Area (%)
Standard										
0.060 mg/ml	Same day	8	6.1[b]	4.1	1.5	9.9	1.4	8.0	0.0	8.3
0.234 mg/ml	Same day	7	6.7[c]	1.5	1.5	2.4	2.0	3.5	1.8	2.8
	Between days[d]		5.2	2.1	1.4	1.9	1.7	3.1	0.4[e]	6.0[e]
Whey	Same day	8	4.9	3.1	2.6	4.3	3.0	3.9	1.9	6.3
	Between days[d]		5.8	3.2	1.6	2.8	0.8	0.5	1.4	9.2

Flow-rate, 3.0 ml/min. Other experimental conditions as in Fig. 2.
[a] n = Number of consecutive runs on the same day.
[b] Standard concentration: 0.050 mg/ml.
[c] Standard concentration: 0.176 mg/ml.
[d] Nine analyses performed on the same sample on two different days.
[e] Standard concentration: 0.180 mg/ml.

Table 3
Concentration of the main whey proteins in the raw milk of reference measured by the perfusion RP-HPLC method and by an RP-HPLC reference method [17]

Protein	Perfusion method[a]	Reference method[a]	Error (%)
α-LA	110.00 ± 0.87	118.24 ± 0.70	7.0
β-LG B	182.78 ± 3.19	194.98 ± 1.72	6.3
β-LG A	234.15 ± 3.74	219.79 ± 1.48	−6.5
β-LG (total)[b]	416.93	414.77	−0.5
BSA	62.30 ± 3.51	21.87 ± 0.53	−185.0

Flow-rate, 3.0 ml/min. Other experimental conditions for perfusion RP-HPLC as in Fig. 2.
[a] Results (mg protein per 100 ml milk) expressed as the mean value of three determinations ± standard deviation, except for α-LA and BSA measured by the reference method, where five determinations were carried out.
[b] β-LG content given as β-LG B + β-LG A concentrations.

globulin obtained by this procedure. The area of these peaks was integrated as a cluster by using the perpendicular-drop method. This integration method may overestimate and/or underestimate the area of each peak as a function of their symmetric or tailed shape with respect to the integration of symmetric single peaks, as has been widely discussed [36]. Nevertheless, the cancellation of errors in the individual determination of each variant of β-lactoglobulin gives a minimal relative error in the determination of the total content of β-lactoglobulin (0.5%).

In conclusion, the perfusion RP-HPLC method seems to be applicable to the determination of protein content in bovine wheys when the mode of calibration and integration of peak areas is correctly optimized.

Assessment of the BSA peak purity

The anomalous results obtained in the determination of BSA in the whey samples from raw milk led us to investigate on the purity of the assigned BSA peak.

By comparing the chromatograms corresponding to the whey from the raw milk of the reference (diluted 1:10) and that of a standard (concentration 7.9 mg per 100 ml) (Fig. 4), it is found that the area of the BSA peak in the whey is approximately the same as that of a pure standard BSA with a concentration approximately four times higher (the BSA content in this whey is 21.87 mg per 100 ml, according to the reference method used). Also the shape of the whey BSA peak (broad and tailed) was different from that of the peak of the standard. These results suggest that some impurities (other minor whey proteins or peptides) may co-elute with BSA under the chromatographic conditions of our method. In fact, in the chromatograms of the reference method, which provided higher resolution than the perfusion method, there was one unidentified peak between the α-LA and the BSA that has not been detected by our method, probably for the reason discussed above.

To confirm these results, diode-array UV detection was employed for determining the peak purity of BSA in the chromatograms of

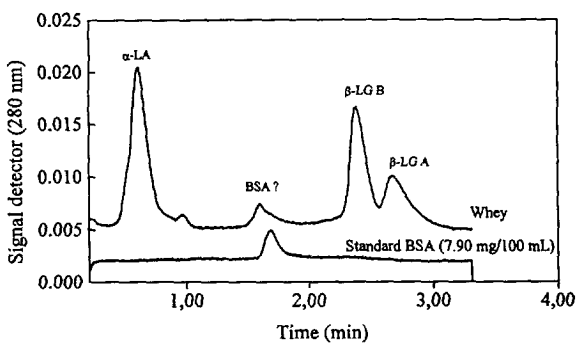

Fig. 4. Chromatograms corresponding to a standard BSA (7.90 mg per 100 ml) and the diluted (1:10) whey from the reference milk by perfusion RP-HPLC with diode-array detection. Experimental conditions: $t_G = 2.2$ min at a flow-rate of 2.0 ml/min. Other experimental conditions as in Fig. 2.

whey samples. We chose the algorithm of normalization of spectra from different BSA peak sections [37]. Data evaluation of normalization factors showed homogeneity of the peak of standard BSA (normalization factor >0.999 in three sections of the peak investigated: up-slope, apex and down-slope) and the lack of homogeneity (purity) of the BSA peak in the whey sample (normalization factor <0.999 in the above-mentioned peak positions).

These results may indicate the presence in the broad peak of the whey sample of multiple solutes. Nevertheless, the possibility of using rapid and precise methods of peak deconvolution may solve this problem when necessary, although the determination of BSA in wheys in the quality control of dairy products is not of interest at present.

3.5. Separation of whey proteins from milk of different animal species

The method developed in this work has also been used for separating homologous whey proteins from ewe and goat milk (Fig. 5). Three major peaks, which are attributed to α-LA, β-LG B, and β-LG A, were identified in whey from ovine milk by comparing their retention times with those of the purified whey protein fractions analysed separately. Similarly, two

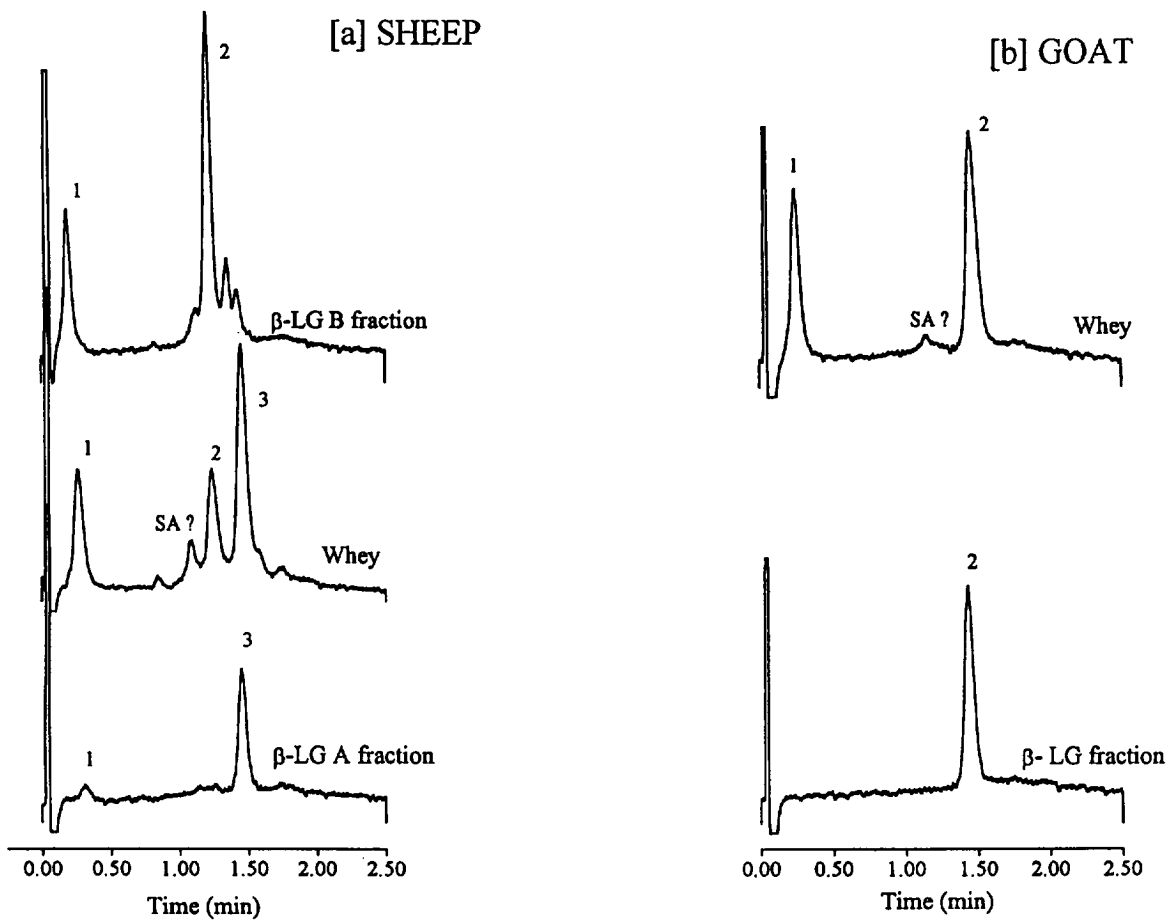

Fig. 5. Perfusion RP-HPLC of ovine and caprine wheys and purified fractions of ovine β-LG B and β-LG A and caprine β-LG. Flow-rate, 3.0 ml/min. Other experimental conditions as in Fig. 2. Peaks: (a) ovine: 1 = α-LA; 2 = β-LG B; 3 = β-LG A; (b) caprine: 1 = α-LA; 2 = β-LG.

major peaks, assigned to α-LA and β-LG, were found in the goat whey. The identification of the chromatographic peak of serum albumin in the wheys from ovine and caprine milks is not clear, since the identity of the minor peaks eluting between α-LA and β-LG has not been established.

It is noteworthy that the separation of the main whey proteins under investigation is accomplished in less than 2.0 min. Moreover, the ability of the perfusion method for separating ($R_s \approx 1.2$) the principal genetic variants (A, B) of ovine β-lactoglobulin, whose primary structures differ only in one amino acid, is illustrated [38].

This method is also suitable for the identification of homologous proteins in mixtures of milks from different species (Fig. 6). As is shown for the binary mixtures, it is possible to detect the presence of ovine milk in bovine milk or vice

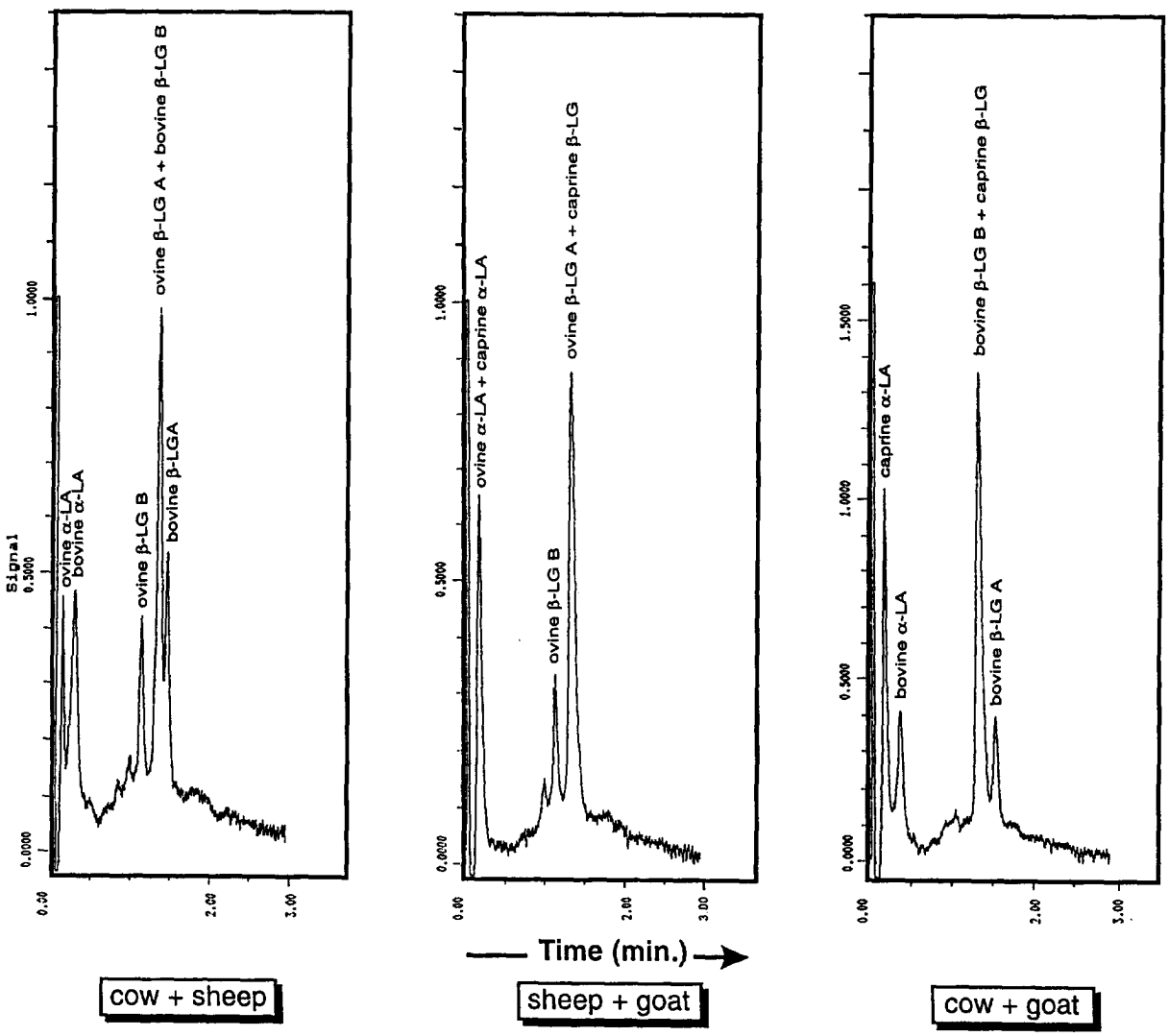

Fig. 6. Perfusion RP-HPLC for the separation of the following whey mixtures of different animal species (the ratio of species, by mass, is indicated in parentheses): cow and sheep (1:1), sheep and goat (2:1) and cow and goat (75:1). Flow-rate, 3.0 ml/min. Other experimental conditions as in Fig. 2.

versa by means of one of the two peaks of α-LA and the peaks of ovine β-LG B and bovine β-LG A, respectively. Addition of ewe milk to goat milk could be detected by the peaks of ovine β-LG B. Nevertheless, the addition of caprine milk to ovine milk cannot be detected. The addition of bovine milk to caprine milk can be detected by the peaks of bovine α-LA and β-LG A. By using the data in Fig. 6, the minimum amount detected in mixtures of different species can be calculated as 0.1 of cow in 1 of sheep, 0.1 of sheep in 1 of goat and 9 of cow in 1 of goat (values expressed in mass of the wheys).

The proposed method may be of great interest for routine analysis in dairy factories both in quality control of dairy products and to check fraudulent additions of milks from different species in the manufacture of these products [39,40].

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