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Identification and quantification of major bovine milk proteins by liquid chromatography

G. Bordin*, F. Cordeiro Raposo, B. de la Calle, A.R. Rodriguez

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg, B-2440 Geel, Belgium

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Abstract

In the field of food quality, bovine milk products are of particular interest due to the social and economic importance of the dairy products market. However, the risk of fraudulent manipulation is high in this area, for instance, replacing milk powder by whey is very interesting from an economic point of view. Therefore, there is a need to have suitable analytical methods available for the determination of all milk components, which is currently not the case, especially for the main proteins. The detection of potential manipulations requires then a clear analytical characterisation of each type of bovine milk, what constitutes the goal of this work. The separation of the major milk proteinic components has been carried out by ion-pair reversed-phase HPLC with photodiode array detection, using a C_4 column. The overall optimisation has been achieved using a statistical experimental design procedure. The identification of each protein was ascertained using retention times, peak area ratios and second derivative UV spectra. Quantification was based on calibration curves drawn using purified proteins. Major sources of uncertainty were identified and the full uncertainty budget was established. The procedure was initially developed using the skimmed milk powder certified reference material CRM 063R and then applied to various types of commercial milks as well as to raw milk. The method is able to separate and quantify the seven major proteins (κ-casein, α_{s2}-casein, α_{s1}-casein, β-casein, α-lactalbumin, β-lactoglobulin B and β-lactoglobulin A) in one run and also to provide precise determinations of the total protein concentration. These are important results towards the further development of a reference method for major proteins in milk. In addition, the use of a certified material reference is suggested in order to make comparisons of method performances possible.

Keywords: Milk; Food analysis; Statistical analysis; Proteins

1. Introduction

Quality of food products has recently become a major concern of legislators and producers in the European Union (EU) in view of protecting consumers. In this frame, the control of the origin and quality of agro-food products request the development of always more reference analytical methods to enforce the relevant regulations. This holds true for all categories of foodstuffs and particularly for milk products. Indeed dairy products constitute some of the major sources of proteins to human beings and therefore represent a considerable market for the EU, both for internal consumption and for export. However, dairy products receiving an export refund may contain legal (i.e. declared) additions such as lactose,

*Corresponding author. Tel.: +32-14-571-201; fax: +32-14-584-273.

E-mail address: bordin@irmm.jrc.be (G. Bordin).

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whey powder or casein/caseinate under certain schemes. In other cases, these components may have been added illegally. Effectively, the risk of fraud is rather high in this area, where addition of non-milk proteins to milk or mixing milks of different species, for example, are very valuable from an economic point of view. In both cases, it is necessary to determine these compounds analytically to avoid product evaluation being totally dependent on the declaration made by the producer. For all these reasons, reference analytical methods are needed as stated by several EU regulations 2721/95, 1854/96, 1997/97 or 881/1999.

However, from an analytical point of view, a major problem arises from the natural variation of milk composition, an important aspect which has to be taken into consideration when developing analytical procedures. Identification and quantification of all contributive proteins in milk is a challenging but difficult task due to the complex matrix that milk represents.

Bovine milk contains 3–3.5% (w/v) of proteins of which about 80% on average consist of caseins whereas the whey or serum proteins make up the remaining 20%. The casein (CN) group can be sub-divided into α_{s1}-CN, α_{s2}-CN, β (+γ)-CN and κ-CN occurring in the approximate proportions of 4:1:4:1. These proteins are insoluble at their isoelectric point (pH 4.6 at 20°C) and they form micellar complexes in milk. The whey proteins are soluble globular proteins and are mostly made up of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) in a ratio of 3:1, plus bovine serum albumin (BSA) and immunoglobulins as minor constituents. Furthermore genetic polymorphism within the caseins and the whey proteins has been demonstrated [1]. This polymorphism also seems to be correlated to milk composition and to influence some processing properties of milk [2–4].

The determination of milk proteins has been studied by several chromatographic and electrophoretic techniques [5–11], but none of them gave optimum separation of all the major bovine milk proteins, in particular for the whey proteins. In the present paper, we present the development of a method allowing the determination of the seven major milk proteins, optimised according to a statistical experimental design procedure. The identification of each protein was ascertained by using several parameters including second derivative UV spectrophotometry. Major sources of uncertainty were identified and the total uncertainty budget was established. The procedure was applied to various types of bovine milk. These results could constitute the first steps towards the establishment of a reference method for major milk protein analysis.

2. Experimental

2.1. Reagents, standards and samples

Guanidine hydrochloride (GdnHCL) [lot G-4505, purity>99%)], sodium citrate (trisodium salt, lot S-4641) and DL-dithiothreitol (lot D-5545) were purchased from Sigma (Sigma–Aldrich, Bornem, Belgium). Purified major proteins from bovine milk were also purchased from Sigma, purity checked by electrophoresis or by polyacrylamide gel electrophoresis (PAGE): κ-CN (lot C-0406,>80%), α-CN (lot C-6780,~85%), β-CN (lot C-6905,>90%), α-La (lot L-5385 type I,~85%), β-LgB (lot L-8005) and β-LgA (lot L-7880). Acetonitrile (HPLC grade> 99.8%) and trifluoroacetic acid (TFA) (lot T-6508) in 1 ml ampoules were purchased from Fluka (Bornem, Belgium). Analytical-reagent grade hydrochloric acid 25% was purchased from Merck (Darmstadt, Germany). Ultra pure water (Milli-Q Plus system >18.3 MΩ cm) was obtained in the laboratory. The certified reference material skimmed bovine milk in powder (CRM 063R) was obtained from the European Commission Community Bureau of References (BCR, Geel, Belgium). Commercial bovine milk samples from different types [Ultra High Temperature (UHT), pasteurised and powder milks] were purchased from local shops: one powder milk; five half skimmed UHT milks (labelled UHT 1, UHT 2, UHT 3, UHT 4, UHT 5) [of which two are from organic farming, UHT 4 and UHT 5]; one three quarts skimmed UHT milk (UHT 6); one whole UHT milk (UHT 7); one half skimmed pasteurised milk. Raw milk was obtained from a local farm situated in the neighbourhood of the institute.

2.2. HPLC instrumentation and procedure

The complete chromatographic system from Kontron (Zurich, Switzerland) was the following: a

HPLC pump model 525, an auto injector model 560, equipped with a polyether ether ketone (PEEK) injection loop and a titanium six-way valve from Valco (Schenkon, Switzerland) and a photodiode array detection (DAD) system model 440. The temperature of the column was adjusted and maintained by a HPLC column thermostat model 582 (Kontron). All data was treated by the chromatographic systems software KromaSystem 2000 Version 1.83 (Kontron). All connections between the different modules of the HPLC system were kept as small as possible. The sample vial was kept at constant low temperature (7°C) via a liquid refrigerator PolyScience. An injection loop of 20 μl was used. The separations were performed on a reversed-phase analytical column C_4 (150×2.1 mm), 300 Å pore diameter and 5 μm particle size (Vydac, Dionex, Mechelen, Belgium), with the following programme (optimised conditions): linear gradient from 26.5 to 28.6% B in 7 min (0.30% B min^{-1}), then from 28.6 to 30.6% B in 10 min (0.20% B min^{-1}) and from 30.6 to 36.1% B in 11 min (0.50% B min^{-1}), followed by an isocratic elution at 36.1% B during 10 min and a final increase to 43.3% B in 18 min (0.40% B min^{-1}), at a flow-rate of 0.25 ml min^{-1}, where eluent A is composed of 10% (v/v) acetonitrile and 0.1% TFA in ultrapure water and eluent B of 10% water and 0.1% TFA in acetonitrile. The column temperature was kept at 40°C.

2.3. Sample preparation

2.3.1. Purified protein

A certain amount of purified protein is weighed and a volume of a sample buffer consisting of 6 *M* guanidine–HCl, 20 m*M* dithiothreitol and 5 m*M* tri-sodium citrate (pH 7) is added. One hour of incubation is allowed at room temperature. A vortex mixer (Top-Mix 11118) from BioBlock Scientific (Tournai, Belgium) was used for sample dissolution without any noticeable foaming production. A 1:3 dilution into the chromatographic eluent A was then carried out. The actual amount of injected protein (between 0.3 and 8 μg) was subjected to an exhaustive uncertainty budget for combined uncertainties with the objective of finding the most relevant sources of error (e.g. weighing, pipetting, dilution, injection).

2.3.2. Powder milk

The milk powder is treated similarly to the purified proteins. A certain mass of powder milk is weighed and a volume of sample buffer is added. Prior calculations are carried out in order to not exceed the recommended injected mass for the analytical column (~50 μg of sample).

2.3.3. Liquid milk

Approximately 10 ml of liquid milk is poured directly from the commercial package to an ultracentrifugation tube in polycarbonate. The sample is then centrifuged at 14 800 rpm (16 000 *g*) at 4°C for 10 min using a LE-80 ultracentrifuge from Beckman Instruments (Palo Alto, CA, USA). The fat layer is removed and an aliquot of remaining liquid is weighed into a small glass vial. Pipettes used were subjected to a rigorous calibration procedure. The same treatment as for the powder milk is then carried out. The raw milk was also treated similarly.

2.4. Calculations of resolution and uncertainties

Resolution between two peaks 1 and 2 was calculated according to the equation:

$$R_{1,2} = 2(t_{R2} - t_{R1})/(W_2 + W_1)$$

where t_R refers to the retention time for the apex of the chromatographic peak and *W* refers to the base width of each of the peaks. The method of dropping a perpendicular line from the valley between each peak was used for calculations of the base width.

Uncertainty calculations for linear least square calibration curves were performed following the EURACHEM/CITAC guide [12]. Pipette validation was carried out by gravimetry.

2.5. Protein identification using peak area ratios and second derivative spectra

Due to the presence of the aromatic amino acids phenylalanine (Phe, F), tyrosine (Tyr, Y) and tryptophan (Trp, W) in their structures, casein and whey proteins absorb above 250 nm. Unfortunately the spectra of each of the major milk proteins, taken at the apex of the chromatographic peak, show similar patterns, lacking fine structure and therefore limiting their direct use for protein identification.

However, the calculation of the peak area ratio (area at 214 nm/area at 280 nm: A_{214}/A_{280}) for each protein gives a value indicative of the relative proportion of the total aromatic amino acid content and therefore allows a rather good distinction between most proteins.

Furthermore, the derivatisation of the UV–visible spectra has the advantage of revealing the fine structure of the spectra [13]. Second derivative in particular has the advantage of turning peaks and shoulders into minima, valleys into maxima and inflexion points into zero or interception points. For comparison purposes these second derivative spectra have to be overlaid and normalized at a certain wavelength (240 nm was used here). Fig. 1 shows the overlaid and normalized second-derivative spectra of some purified milk proteins. Both minima at 249.2 nm and at 259.4 nm are only due to the phenylalanine (F), the minimum at 272.4 nm is a side minimum for both Y and W. The maximum at 280 nm is a main maximum for W and a side maximum for Y, while the primary minimum at 290 nm is an indication of the presence of W. Therefore, the calculation of the second derivative ratios $\gamma = a/b$, where a and b are the amplitudes presented in Fig. 1, gives an indication of the ratio (Y+W)/W and should allow an efficient discrimination between the different proteins.

The use in parallel of these two parameters leads finally to a good characterisation of each protein (see Table 1).

3. Results and discussion

3.1. Optimisation of the separation

The optimisation of the major chromatographic parameters was carried out using a statistical design of experiments (DOE) approach based on the

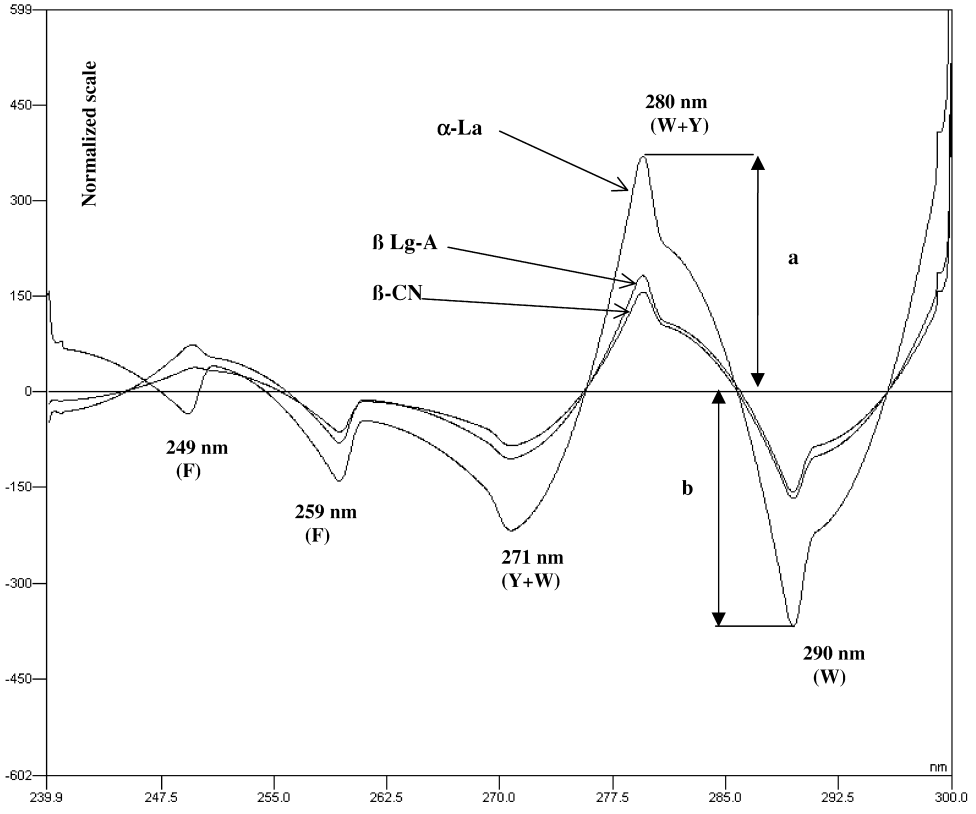

Fig. 1. Normalised second-derivative spectrum for some purified milk proteins (β-CN, α-La, β-LgA).

Table 1
Retention time (t_R), area (A_{214}/A_{280}) ratios and normalised second derivative γ ratios (normalisation at 240 nm) of individual major bovine milk proteins (definition in text) in standard solutions and some milks: mean values and standard uncertainties ($n=6$)

Sample	κ-CN[a]			α_{S2}-CN			α_{S1}-CN			β-CN[b]			α-La			β LgB			β LgA		
	t_R (min)	Area ratio	γ ratio	t_R (min)	Area ratio	γ ratio	t_R (min)	Area ratio	γ ratio	t_R (min)	Area ratio	γ ratio	t_R (min)	Area ratio	γ ratio	t_R (min)	Area ratio	γ ratio	t_R (min)	Area ratio	γ ratio
Purified proteins	11.16 ±0.21	19.25 ±0.55	1.54 ±0.05	17.06 ±0.30	15.74 ±1.03	1.19 ±0.10	30.17 ±0.13	16.07 ±0.37	1.41 ±0.05	35.87 ±0.30	35.55 ±2.38	1.07 ±0.10	46.13 ±0.36	12.51 ±0.44	1.08 ±0.05	47.50 ±0.36	16.33 ±0.28	1.18 ±0.05	50.50 ±0.40	17.77 ±0.62	1.18 ±0.05
CRM 063R	12.07 ±0.13	19.41 ±1.37	1.55 ±0.05	18.16 ±0.04	14.25 ±0.73	1.29 ±0.06	31.51 ±0.08	14.86 ±0.52	1.49 ±0.04	38.53 ±0.20	31.89 ±4.29	1.06 ±0.10	49.18 ±0.14	11.16 ±0.32	0.99 ±0.03	50.17 ±0.10	16.24 ±0.69	1.05 ±0.07	52.66 ±0.10	17.05 ±0.76	1.06 ±0.07
Powder milk	11.64 ±0.23	18.67 ±0.27	1.47 ±0.03	17.71 ±0.36	12.88 ±0.20	1.21 ±0.04	31.12 ±0.20	15.28 ±0.15	1.44 ±0.01	37.51 ±0.47	29.17 ±1.21	1.08 ±0.05	48.30 ±0.40	11.35 ±0.45	0.86 ±0.03	49.58 ±0.30	16.88 ±0.15	1.18 ±0.06	52.16 ±0.25	18.47 ±0.18	1.17 ±0.06
UHT 1	11.88 ±0.10	17.67 ±1.85	1.41 ±0.03	18.19 ±0.16	13.68 ±1.00	1.11 ±0.06	31.40 ±0.09	13.92 ±0.26	1.37 ±0.02	38.19 ±0.20	30.60 ±1.43	0.95 ±0.03	48.73 ±0.16	10.53 ±0.24	0.90 ±0.05	49.93 ±0.11	15.37 ±0.46	0.92 ±0.02	52.42 ±0.06	15.92 ±0.66	0.95 ±0.02
UHT 2	12.29 ±0.36	18.90 ±1.85	1.44 ±0.16	19.15 ±0.32	14.40 ±1.09	1.22 ±0.24	31.85 ±0.13	14.90 ±0.68	1.43 ±0.07	39.49 ±0.41	28.30 ±1.08	1.00 ±0.26	49.71 ±0.25	11.40 ±0.69	0.96 ±0.26	50.69 ±0.20	15.60 ±0.26	1.12 ±0.38	53.09 ±0.18	17.90 0.34	1.10 0.31
UHT 5[c]	13.67 ±0.41	19.12 ±1.59	1.56 ±0.29	21.37 ±0.74	11.78 ±1.76	1.28 ±0.16	32.52 ±0.21	13.58 ±0.80	1.46 ±0.06	42.29 ±1.02	27.27 ±1.21	1.04 ±0.23	51.01 ±0.36	10.81 ±0.99	1.00 ±0.09	51.83 ±0.30	15.24 ±0.40	1.04 ±0.14	54.08 ±0.29	16.92 ±0.78	1.06 ±0.15
UHT 7	11.14 ±0.14	19.01 ±0.37	1.44 ±0.11	17.03 ±0.40	12.90 ±0.22	1.17 ±0.11	30.85 0.21	14.00 0.37	1.42 0.02	36.87 ±0.26	28.20 ±0.79	1.05 ±0.12	47.50 ±0.14	11.66 ±0.37	0.90 ±0.08	48.92 ±0.12	16.20 ±0.56	0.94 ±0.08	51.61 ±0.13	18.10 ±0.52	0.99 ±0.07
Pasteurised	10.65 ±0.10	20.77 ±1.06	1.52 ±0.12	15.79 ±0.14	15.30 ±0.49	1.26 ±0.10	30.43 ±0.06	14.87 ±0.55	1.44 ±0.02	37.33 ±0.06	28.00 ±1.32	1.06 ±0.06	46.76 ±0.07	10.45 ±0.36	0.98 ±0.06	48.22 ±0.06	15.95 ±0.60	1.05 ±0.13	51.14 ±0.07	17.03 ±0.98	1.10 ±0.10
Raw milk	11.39 ±0.13	20.10 ±1.38	1.32 ±0.07	17.48 ±0.27	14.80 ±1.04	1.23 ±0.02	31.01 ±0.17	14.80 ±0.51	1.44 ±0.01	36.97 ±0.50	27.40 ±1.01	1.08 ±0.02	47.40 ±0.36	9.30 ±0.14	0.90 ±0.02	48.79 ±0.31	15.50 ±0.62	0.94 ±0.04	51.56 ±0.23	15.00 ±0.37	1.04 ±0.02

[a] Retention time (t_R), area and second derivative γ ratios are given for the second peak of κ-CN.
[b] Retention time (t_R), area and second derivative γ ratios are given for the second peak of β-CN.
[c] UHT 5 is a milk coming from organic farming.

MODDE 5 programme (Umetri, Umeå, Sweden). In this procedure, a screening is first done to determine which parameters have the strongest influence on the responses, followed by a finer optimisation where, by the use of a response surface modeling, real optimums can be achieved [14].

In the DOE approach, a carefully prepared set of experiments is constructed, in which all the relevant factors are varied simultaneously. By doing so, all interactions between the factors can be quantified. Full information on the variation of the responses due to changing various factors can be obtained by carrying out a small number of experiments. A full factorial design was selected, due to the following advantages: it requires relatively few runs per investigated factor, it can be upgraded to form composite designs which are needed for optimisation and it is easily interpreted [15].

The initial experimental design using a single protein (κ-casein) led to the conclusion that temperature had no significant impact on both retention time and resolution between the different peaks of κ-casein and was kept constant at 40°C. Flow-rate and gradient were optimised at 0.25 ml min^{-1} and 0.3% B min^{-1}, respectively. This approach has an enormous advantage over conventional optimisation procedures based on changing one separate factor at a time: it is much less time consuming and the present tool based on statistical experimental design permits the acquisition of reliable information in fewer experiments. The more challenging separation between κ-CN and α_{s2}-CN was then optimised separ-

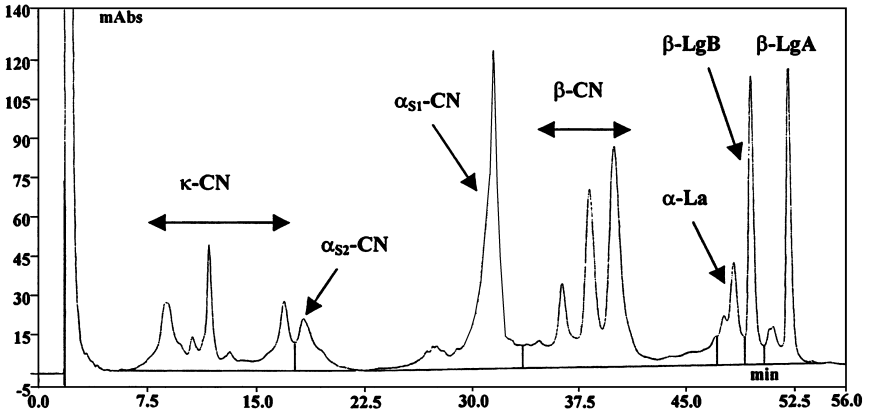

Fig. 2. RP chromatographic profile of a mixture of the major standard milk proteins using the optimised elution conditions: Vydac C_4 150×2.1 mm column; linear gradient from 26.5 to 28.6% B in 7 min, then from 28.6 to 30.6% B in 10 min and from 30.6 to 36.1% B in 11 min, followed by an isocratic step at 36.1% B during 10 min and a final increase to 43.3% B in 18 min, where eluent A is composed of 10% (v/v) acetonitrile and 0.1% TFA in ultra pure water and eluent B of 10% water and 0.1% TFA in acetonitrile; flow-rate 0.25 ml min^{-1} at 40°C; UV detection at 214 nm. Injected masses: κ-CN (1.95±0.01 μg), α-CN (4.25±0.02 μg), β-CN (4.25±0.02 μg), α-La (0.360±0.002 μg), β-LgB (1.50±0.01 μg), β-LgA (1.51±0.01 μg).

ately, resulting in the use of a gentle gradient of 0.2% B min^{-1}. A steeper gradient was used for the elution of α_{s1}-CN. Finally, achieving the separation of the three whey proteins led to further develop the gradient. A percentage of eluent B higher than 36.1% results in the co-elution of α-La, and β-LgB. The solution was therefore to keep isocratic conditions at this level for 10 min (which also improves the resolution of the three β-CN variants), followed by a final increase to 0.4% B min^{-1}.

3.2. Identification of the proteins in a CRM skimmed milk powder

A chromatogram showing the separation of a mixture of standard proteins (α-CN, β-CN, κ-CN, α-La, β-LgA, β-LgB) is shown in Fig. 2 while that of the proteins of a skimmed bovine milk in powder (CRM 063R) [42.5±0.2 μg injected] is depicted in Fig. 3. Retention times, peak area ratios (A_{214}/A_{280}) and second derivative UV spectrophotometry (γ

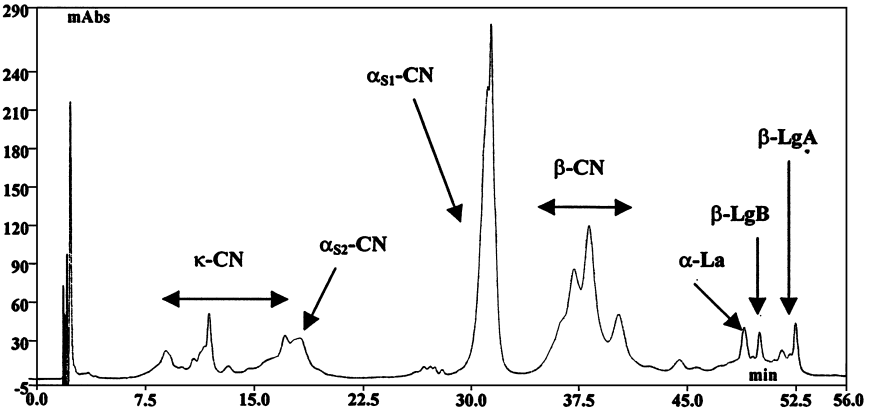

Fig. 3. RP chromatographic profile of a CRM 063R skimmed milk powder sample (42.51±0.19 μg injected) (conditions as in Fig. 2).

ratios defined in the Experimental section) allow the identification of the major proteins in this milk (Table 1). Injections of individual standard proteins were of course also performed but results are not shown here displaying no added value when compared to the mixture.

The standard κ-CN chromatogram exhibits three main peaks and several minor ones, which probably correspond to the main κ-CN variants A and B with different states of glycosylation [16,17]. In the skimmed milk, κ-CN is also the first protein to be eluted and exhibit three main peaks, again corresponding to glycosylated and unglycosylated forms of κ-CN A and κ-CN B. The values of the retention times and those of the area and γ ratios (all values are expressed as mean±expanded uncertainty U) confirm the identity of the peaks (Table 1): for the A_{214}/A_{280} ratio, 19.25±1.10 for each peak of the κ-CN standard and 19.41±2.74 for those attributed to κ-CN in the milk, while the corresponding γ ratios are 1.54±0.10 and 1.55±0.10 ($n=6$) respectively.

The peak eluted right after the last κ-CN peak in Figs. 2 and 3 has been identified as α_{s2}-CN by comparison with the α-CN standard. It should be noted that there is no standard for α_{s1}-CN and α_{s2}-CN separately but only for the mixture α-CN. Both A_{214}/A_{280} and γ ratios of α_{s2}-CN are significantly lower than those of the last peak of κ-CN, making the discrimination unambiguous (Table 1). Although the peak attributed to α_{s2}-CN is only partially separated from the last κ-CN peak, especially in the powder milk, and actually consists of a complex peak probably due to the presence of various phosphorylated forms as noticed by others [9,18], it is very reproducible. Furthermore, we did not notice any carry-over effects of traces of α_{s2}-CN in other protein separation as was previously observed [16,17]. This is probably due to the fact that the present chromatography is carried out on a C_4 column while most other authors used C_8 or C_{18} columns enhancing protein adsorption.

The species coming out next was clearly attributed to α_{s1}-CN eluted as a large peak. For α_{s1}-CN protein, the A_{214}/A_{280} and second derivative UV γ ratios are 16.07±0.74 and 1.41±0.10 respectively for the standard, while in the milk values of 14.86±1.46 and 1.49±0.08 ($n=6$) are found, thus confirming the identity of the peak. As also reported in previous works [9,17–19], variants B and C of α_{s1}-CN could not be separated although a shoulder can be noticed on the peak in both standard and powder milk chromatograms. On the whole, successive additions of α-CN to a milk solution confirmed the identification of both α_{s1}-CN and α_{s2}-CN.

The next group of peaks in Figs. 2 and 3 is attributed to the separation of the β-CN variants (B, A1, A2, A3), with A_{214}/A_{280} and γ ratios averaging respectively 35.55±4.76 and 1.07±0.20 for the standard protein and 31.89±8.58 and 1.06±0.20 ($n=6$) in the milk (Table 1). The separation is of very good quality for the standard (Fig. 2) while the peaks are badly resolved in the milk, although still identifiable (Fig. 3). γ-CN forms are assumed to be coeluted with β-CN, as it is currently reported [9,17,20], since the former are proteolytic products of the later.

The last group of peaks in Figs. 2 and 3 is due to the whey proteins α-La and β-Lg, which were known to be not easily separable [9,16,17]. However, in standard solutions, we have obtained excellent separation of successively α-La, β-LgB and β-LgA (Fig. 2), α-La and β-LgB displaying each one a single important peak, while β-LgA exhibits first a minor peak followed by a dominant one. All three proteins have γ ratios of the same order of magnitude, but α-La has a much lower A_{214}/A_{280} ratio than that of both β-Lg (due to the difference in their total aromatic amino acid content), making the discrimination possible (Table 1). In the milk (Fig. 3), the separation of the three whey proteins is also very good, with the same order of elution: α-La, β-LgB and β-LgA. The A_{214}/A_{280} and γ ratios of the proteins in the milk are similar to those of the standard proteins. The three whey proteins forms are therefore well separated and identified in the powder milk sample.

The difficulty of α-La separation has been often addressed. Groen et al. [17] wrote that α-La and β-LgA have a similar retention time in their method using a RP C_{18} column for analysing raw milk, while Bobe et al. [9] display a separation of proteins in skimmed milk where α-La and β-LgB are more or less co-eluted in a small but broad peak. Similarly, Léonil et al. [8] could not detect α-La using LC–electrospray ionization MS with a C_8 column. On the other hand, Parris et al. [20] working on non-fat dry

milk powder prepared from heat-treated skimmed milk found α_{s1}-CN, BSA and α-La eluting together, α-La being however only partially resolved. Also, according to Visser et al. [16], α-La should appear between the peaks of α_{s1}-CN and β-CN. On the whole, it appears that until now α-La could be separated from β-Lg by liquid chromatography only when working on whey proteins and not directly on milk samples [21–24]. Achieving the separation of α-La from the other whey proteins in one chromatographic run without the prior casein precipitation can therefore be considered as a major step in milk protein separation.

On the whole, there is the following order of elution for the major milk proteins with the present elution programme: κ-CN/α_{s2}-CN/α_{s1}-CN/β-CN/α-La/β-LgB/β-LgA. The retention time, A_{214}/A_{280} and γ ratios of the proteins allowed the precise identification of all seven proteins.

In Table 2 some of the performance characteristics of the separation of the proteins in the CRM milk are presented along with the precision on retention times and total peak areas. The repeatability of the separation is very high in terms of retention time (0.2%< R.S.D.<1.1%) as well as in response intensity (0.8%<R.S.D.<5.1%); the same holds true for the reproducibility over a 2-week period, 2.1%< R.S.D.<5.4% for the retention time and 4.1%< R.S.D.<9.8% for the total corrected peak area (the corrected peak area corresponds to the peak area divided by the injected mass defined in the next paragraph below).

3.3. Quantification of the proteins in the CRM skimmed milk powder

Calibration curves of the various proteins were obtained using mixtures of κ-CN, α-CN, β-CN, α-La, β-LgB and β-LgA. All figures of merit are given in Table 3, for a number of injections >20. The same calibration curve was used for α_{s2}-CN and α_{s1}-CN. In all cases, excellent linearity has been achieved ($R^2>0.99$) with low detection limits.

For the determination of the concentration C_o (μg mg^{-1}) of the various proteins in milk, the following equation has been used:

$$C_o = M_o \cdot 1000/M_i$$

where M_o (in μg) is the interpolated mass of protein found from the respective calibration curve and M_i (in μg) is the mass of sample injected on the column.

Table 2
Precision (repeatability and reproducibility) of retention times and peak areas of the major bovine milk proteins in the CRM 063R skimmed milk powder

Protein	Repeatability[a]		Reproducibility[b]	
	Retention time R.S.D.[c] (%)	Peak area R.S.D.[c] (%)	Retention time R.S.D.[c] (%)	Corrected peak area[d] R.S.D.[c] (%)
κ-CN	1.1[e]	0.8	5.4[e]	4.1
α_{s2}-CN	0.2	1.4	4.8	7.0
α_{s1}-CN	0.2	0.8	2.3	4.2
β-CN	0.5[f]	1.3	3.3[f]	5.1
α-La	0.3	5.1	2.4	7.1
β-LgB	0.2	4.7	2.3	9.8
β-LgA	0.2	1.9	2.1	4.7

[a] Six aliquots of the same CRM 063R milk preparation ($n=6$).
[b] Six aliquots of each of six CRM 063R milk sample preparations prepared over a 2-week period ($n=36$).
[c] Relative standard deviation (R.S.D., %).
[d] The corrected peak area corresponds to the peak area divided by the injected mass (definition given in the text).
[e] Second peak of κ-CN (similar value for other peaks of κ-CN).
[f] Second (main) peak of β-CN (similar value for other peaks of β-CN).

Table 3
Calibration lines for a mixture of standard bovine milk proteins (Figures of merit)

Protein	Intercept $a \pm u$ (a)	Slope $b \pm u$ (b)	R^{2} [a]	DL [b] (μg)	QL [c] (μg)	Range [d] (μg)	n
κ-CN [e]	−0.91±0.65	59.29±0.32	0.996	0.10	0.32	0.60–3.63	24
α_{s1}-CN	1.43±1.32	37.82±0.30	0.998	0.31	1.02	1.30–7.92	24
β-CN [e]	1.39±1.20	47.34±0.36	0.998	0.22	0.74	0.92–5.62	24
α-La	0.56±1.67	69.51±1.66	0.988	0.21	0.70	0.30–1.82	24
β-LgB	−0.03±0.33	35.29±0.22	0.998	0.08	0.28	0.46–2.78	24
β-LgA	2.64±0.67	40.41±0.43	0.998	0.14	0.48	0.46–2.81	24

[a] Regression coefficient (R^2).
[b] Detection limit (DL).
[c] Quantification limit (QL).
[d] Range of protein content used for calibration.
[e] For κ-CN and β-CN, the total area of all peaks was taken into account for building the calibration curves.
DL$=(Y_b - a)/b$ where $Y_b = 3S^2 + a$ and QL$=(Y_b - a)/b$ where $Y_b = 10S^2 + a$; a is the intercept, b the slope and S^2 the standard deviation for the residuals of the calibration line.

M_i is related to the weighted mass of sample M_w (in μg) by the following equation:

$$M_i = M_w D_f V_i / V_b$$

where V_b is the volume of buffer used to prepare the sample (in μl), D_f is the final dilution factor before injection (~1/3) and V_i is the volume of the injection loop (20 μl in this work).

As part of a more realistic determination of concentration in order to get the most reliable results as possible, complete uncertainty budgets were established for the injected mass of sample as well as for the final concentration of each protein in the milk sample. All potential sources of uncertainty were taken into account, especially the purity of the commercial purified proteins used for the calibrations, a parameter previously never considered as reported in available literature. All these statistical calculations are presented in detail elsewhere [25]. Concentrations will therefore be given with their expanded uncertainty ($C_o \pm U$, $k=2$).

In Table 4 the results for the skimmed milk powder CRM 063R are given ($n=6$), corresponding to the separation shown in Fig. 3. They show a very good repeatability of measurements, with relative uncertainty for each protein concentration lower than 3% (only α_{s2}-CN and α-La have higher relative uncertainty of 5 and 8% respectively). The reproducibility was also ascertained by preparing six milk samples, which were all injected six times consecutively, over a 2-week period. Results show excellent reproducibility with relative uncertainty for the proportion of protein lower than 2% for each protein.

In this skimmed milk powder sample CRM 063R, the caseins (α-, β-, and κ-CN) represent about 91% and the whey proteins 9% of the total proteins respectively. Within the caseins, α-CN represents less than the half and β-CN roughly one third of the total proteins, while κ-CN accounts for about 13%, results which could be expected from the average bovine milk values published in the literature, confirming therefore, as first approach, the validity of

Table 4
Concentration of the proteins in the CRM 063R skimmed milk powder (injected mass of milk powder: 41.11±0.18 μg) ($n=6$). Results are given with their expanded uncertainty U ($k=2$)

Protein	Co$\pm U$ [a] (mg g^{-1})	%$P \pm U$ [b]
κ-CN	53.76±6.92	12.60±1.82
α_{s2}-CN	23.35±5.20	5.47±1.26
α_{s1}-CN	180.25±24.20	42.25±6.30
β-CN	132.82±9.98	31.13±3.10
α-La	9.24±2.52	2.17±0.60
β-LgB	8.76±1.04	2.05±0.28
β-LgA	18.49±2.06	4.33±0.56
TMPC [c]	426.7±27.8	100.00

[a] Concentration (mg g^{-1}) of individual protein in milk.
[b] Proportion of individual protein in milk.
[c] Total major protein concentration (TMPC).

the separation procedure and of the quantification method. The lower value obtained for the proportion of whey proteins is obviously due to the heavy heat treatment to which the milk was submitted to get the powder [20]. However, it has to be stressed that, to our knowledge, this type of work has never been done, previous to our study, using a CRM powder milk, the use of which being however the only way in which to compare results. Other authors who have worked on milk protein separation normally use raw milks or commercially available milks from their local markets, e.g., [5,8,9,16,26], sometimes with even no mention at all of the milk provenance, e.g., [6,27], therefore limiting the comparisons on the respective methods characteristics and the absolute results. It is known that, not even taking industrial processing into account, the concentrations of individual proteins in natural milk are influenced by several genetic and environmental factors, seasons, nutritional regime of the cows, age of the cows, stage of lactation, etc. [26]. Therefore, although the skimmed milk CRM 063R is only certified for major and trace elements including nitrogen [28], it has the advantage of being available world-wide. On that condition, it is then possible to further investigate local milks.

3.4. Study of various types of raw and commercial milks

The whole procedure developed and previously applied to the CRM 063R skimmed milk has been applied to raw milk directly obtained from a farm located in Geel (Belgium) and to various commercial powder and liquid milks currently available on the Belgian market. A selection of the respective typical chromatograms is presented in Fig. 4. All of them display at first sight similar profiles, in agreement with that obtained for the CRM milk (Fig. 3), but the proportions of the various proteins obviously vary to some extent. The proteins were identified on the basis of the retention time and of the A_{214}/A_{280} and

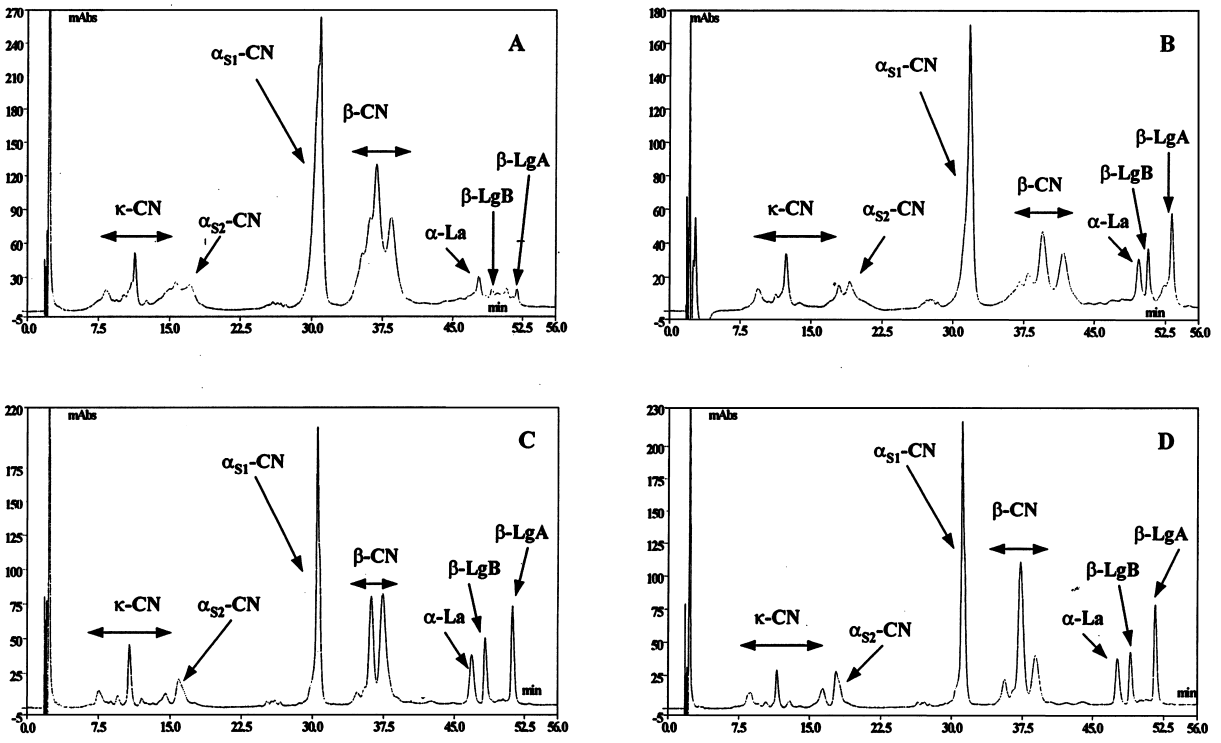

Fig. 4. RP chromatographic profiles of several types of commercial and raw bovine milks (conditions as in Fig. 2): (A) powder milk; (B) half skimmed UHT milk; (C) half skimmed pasteurised milk; (D) raw milk.

γ ratios of the proteins which are all given in Table 1. In each commercial and raw milk, the proteins are well characterised with good agreement between the values of the three parameters in standard solutions and in milk samples.

Obviously, the milk in powder form studied here shows a chromatogram (Fig. 4A) of poorer quality than any of the liquid milks and comparable to that of the CRM 063R powder milk (Fig. 3): the peak shapes are on the whole less well defined, especially those of β-CN and whey proteins. This is more than likely due to the high temperature treatment supported by the milk (spray drying dehydratation at 200 or 250°C), inducing some denaturation mostly of the thermosensitive whey proteins, confirming previous qualitative results [5]. We will see later that the quantitative analysis performed confirm this qualitative statement. Several possible mechanisms have been proposed to interpret these protein denaturations to which β-Lg largely contributes [29–32].

In the commercially available liquid milks (the cases of the half skimmed UHT 2 and half skimmed pasteurised milks are shown in Fig. 4B and C, respectively), κ-CN, α_{s2}-CN and α_{s1}-CN display similar profiles than in the milk powder, while the β-CN peaks are much better resolved that in the powder milks, always showing two main and almost equal peaks, preceded by two small peaks or shoulders, the whole sequence being probably the B, A1, A2 and A3 variants [16]. At the end, the three whey proteins are then very well separated, leading to three main peaks. Especially for the pasteurised milk, the quality of the overall separation is to be noted.

Finally, the raw milk, freshly collected from the cow, with no treatment, shows the nicest chromatogram, where all seven proteins are extremely well resolved (Fig. 4D). In particular, β-CN shows one very dominant variant out of the three peaks and α_{s2}-CN is now completely separated from κ-CN what was not fully achieved with UHT and powder milks: the resolution between the last peak of κ-CN and that of α_{s2}-CN is $R_{\kappa,\alpha S2} = 1.19 \pm 0.08$ ($n = 6$) for the raw milk and lower than 0.70 ± 0.02 ($n = 6$) for powder milks. The values in Table 5 show a trend indicating that a more intense heat treatment reduces the resolution between κ-CN and α_{s2}-CN.

The major profile differences of powder milks most likely result from the various kinds of heat treatment to which they have been submitted, resulting in the partial denaturation and complexation of whey proteins with various caseins (κ-CN and α_{s2}-CN) [20,33]. In their paper on proteins in non-fat dry milk powder prepared from heat-treated skim milk, Parris et al. [20] presented major results: they preheated milk to 63, 74 or 85°C for 30 min before spray-drying, stored the powders and rehydrated them before chromatography. They could show that after 10 days of storage, the powder pretreated at 85°C did not show the distinctive peaks of β-LgB and β-LgA anymore, and were instead replaced by one single broad peak containing β-Lg, α-La, κ-CN, α_{s2}-CN and BSA. This was accompanied by a decrease of the κ- and α_{s2}-CN peaks and the quasi disappearance of α-La co-eluting with α_{s1}-CN. All this was not observed with preheating at 63°C, and only slightly at 74°C. However, after a storage time of the powders for 120 and 240 days, they could clearly show the progressive increase of separate peaks for β-LgB and β-LgA, indicating some renaturation of the whey β-Lg proteins, resulting from the dissociation of the whey–casein complex. We may suggest that this is probably the case for the two powder milks studied here.

For each of the various milk samples the protein concentrations have been determined and a representative selection is given in Table 6, displaying values of all seven proteins. In all cases, the proportion of whey proteins is lower than 20% and even lower than 10% for powder milks. Although it is obviously too early to draw definite conclusions,

Table 5
Resolution between the last peak of κ-CN and α_{s2}-CN ($R_{\kappa,\alpha S2}$) for the different milks studied ($\pm U$, $n = 6$)

Milk type	$R_{\kappa,\alpha S2}$
CRM 063R skimmed milk powder	0.70 ± 0.01
Powder milk	0.65 ± 0.01
1/2 skimmed UHT 1	0.90 ± 0.02
1/2 skimmed UHT 2	0.95 ± 0.02
1/2 skimmed UHT 3	0.90 ± 0.01
1/2 skimmed UHT 4 (organic)	0.97 ± 0.02
1/2 skimmed UHT 5 (organic)	1.08 ± 0.05
3/4 skimmed UHT 6	1.04 ± 0.03
Whole UHT 7	1.05 ± 0.02
Pasteurised	1.15 ± 0.04
Raw milk	1.19 ± 0.04

Table 6
Concentration of the proteins in four different types of milk: powder milk, half skimmed UHT milk, half skimmed pasteurised milk and raw milk (injected mass of milk powder: 42.5±0.2 μg and of liquid milk: 330.1±1.2 μg) ($n=6$). Results are given with their expanded uncertainty U ($k=2$)

Protein	Powder milk		1/2 skimmed UHT milk 2		1/2 skimmed pasteurised milk		Raw milk	
	Co±U[a] (mg g^{-1})	%P±U[b]	Co±U[a] (mg g^{-1})	%P±U[b]	Co±U[a] (mg g^{-1})	% P±U[b]	Co±U[a] (mg g^{-1})	% P±U[b]
κ-CN	50.35±6.48	10.80±1.54	3.51±0.46	10.98±1.62	2.88±0.44	8.79±1.42	2.61±0.40	8.07±1.34
α_{S2}-CN	23.53±5.14	5.05±1.14	1.94±0.52	6.07±1.70	2.00±0.54	6.11±1.68	2.12±0.56	6.55±1.74
α_{S1}-CN	174.60±23.46	37.45±5.54	11.69±1.68	36.58±5.74	9.79±1.46	29.89±4.78	10.30±1.52	31.83±5.06
β-CN	183.30±13.90	39.33±3.84	9.97±0.84	31.20±3.28	11.36±0.98	34.69±3.64	11.42±0.88	35.29±3.44
α-La	6.82±1.72	1.46±0.38	0.82±0.22	2.57±0.68	1.21±0.32	3.69±1.00	1.00±0.30	3.09±0.92
β-LgB	13.88±1.34	2.98±0.34	1.39±0.14	4.35±0.54	2.21±0.20	6.75±0.72	1.79±0.18	5.53±0.62
β-LgA	13.65±1.74	2.93±0.42	2.64±0.28	8.26±1.00	3.30±0.32	10.08±1.12	3.12±0.30	9.64±1.10
TMPC[c]	466.1±28.6	100.00	32.0±2.0	100.00	32.8±1.90	100.00	32.4±1.9	100.00

[a] Concentration (mg g^{-1}) of individual protein in milk.
[b] Proportion of individual protein in milk.
[c] Total major protein concentration (TMPC).

some trends can be observed on the average proportions of whey proteins by types of milk (Table 7, first column): the two powder milks contain on average 8.0±0.9% whey proteins, while the seven UHT heat-treated milks contain 16.0±2.4%, the pasteurised milk contains 20.5±2.2% to be compared to the 18.3±1.6% in raw milk. The effect of intense heat-treatment for producing milk powder is demonstrated in these figures. Resmini et al. [24] working on isolated whey proteins and not directly on whole milk, found 18.5% of whey proteins in raw milks and 15.5% in pasteurised milks. And in their determination of whey protein in UHT milk from different Spanish areas using fourth derivative spectroscopy, Miralles et al. [34] found an average values of 18.1% whey proteins. It remains, however, obviously difficult to compare these results since the origin of the milks, before any industrial treatments, may influence considerably the protein content.

As also reported in Tables 4 and 6, the concentrations found for the various individual proteins were summed up to get the total major protein

Table 7
Proportion of whey proteins and comparison between the total major protein concentrations (TMPC) in the various milks determined in this work and the total protein concentration (TPC) given by the manufacturers. Results are given with their expanded uncertainty U ($k=2$)

Milk type	Whey proteins (%)	TMPC[a] in this work (in g/100 g)	TPC indicated by manufacturer[b]
CRM 063R skim milk powder	8.6±1.0	42.67±2.78	39.75±1.02[c]
Powder milk	7.4±0.8	46.61±2.86	34.5
1/2 skimmed UHT 1	17.1±1.6	3.16±0.19	3.20
1/2 skimmed UHT 2	15.2±1.6	3.20±0.20	3.30
1/2 skimmed UHT 3	16.0±1.6	3.27±0.20	3.20
1/2 skimmed UHT 4 (organic)	15.5±1.6	2.86±0.17	3.00
1/2 skimmed UHT 5 (organic)	12.8±1.6	2.91±0.24	3.30
3/4 skimmed UHT 6	20.6±1.8	3.23±0.19	3.73
Whole UHT 7	14.9±1.6	2.88±0.18	3.20
Pasteurised	20.5±2.0	3.28±0.19	3.30
Raw milk	18.3±1.6	3.24±0.19	–

[a] Total major protein concentration (TMPC).
[b] Total protein concentration (TPC); powder milks in g/100 g, liquid milks in g/100 ml.
[c] Certified value for N: 62.3±0.8 mg g^{-1}, hence TPC=62.3·6.38=397.5±10.2 mg g^{-1} or 39.75±1.02 g/100 g.

concentration (TMPC) in each milk. These values can then be compared to those provided by the manufacturers, with the following cautions: the milk powder CRM 063R is not certified in protein concentration, but only in Nitrogen (62.3 ± 0.8 mg g^{-1}). To derive the protein concentration from the nitrogen value, the average conversion factor of 6.38 is used [35], which gives an estimated protein concentration in this milk of 397.5 ± 10.2 mg g^{-1} (or 39.75 ± 1.02 g/100 g), allowing comparison to be made. For commercial milk, the protein concentrations mentioned on the labels of the packs are only indicative values corresponding to annual mean concentrations and not to those which would be actually measured in the bottles obtained from a shop. However, even taking into considerations these limitations, useful conclusions can be made when looking at Table 7 showing TMPC determined in this work and those given on labels. In all cases, there is good agreement, excluding the commercial powder milk, giving an average recovery of $99.9\pm13.9\%$ ($n=10$). We can then assume the value found for the local raw milk (3.24 ± 0.05 g/100 g) to be highly trustworthy.

4. Conclusion

In the present work, we have developed the basis for a method able to separate and quantify all the major milk proteins in one run, without the classically used prior sample treatment aiming at isolating whey proteins from caseins. To our knowledge, this is achieved for the first time. The whole process has been successfully optimised using a statistical design of experiments approach. Although not certified in total protein concentration, the use of the skimmed milk powder reference material CRM 063R has been shown to be extremely valuable in the development procedure and should be recommended in order to facilitate inter-laboratory comparisons and collaboration. The method has then been applied to various types of raw and commercially available milks allowing the separation of all proteins and their individual quantification taking into account the establishment of a full uncertainty budget. These are important results towards the further development of a reference method for the EU.

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