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Cloud point extraction applied to casein proteins of cow milk and their identification by mass spectrometry

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Abstract

This work describes the optimization of a cloud point extraction (CPE) method for casein proteins from cow milk samples. To promote phase separation, polyoxyethylene(8) isooctylphenyl ether (Triton® X-114) and sodium chloride (NaCl) were used as nonionic surfactant and electrolyte, respectively. Using multivariate studies, four major CPE variables were evaluated: Triton® X-114 concentration, sample volume, NaCl concentration, and pH. The results show that surfactant concentration and sample volume were the main variable affecting the CPE process, with the following optimized parameters: 1% (w/v) Triton® X-114 concentration, 50 μL of sample volume, 6% (w/v) NaCl concentration and extractions carried out at pH 7.0. At these conditions, 923 ± 66 and 67 ± 2 μg mL^{-1} of total protein were found in the surfactant-rich and surfactant-poor phases, respectively. Finally, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was then used to evaluate those target proteins (α_{s1}-casein, α_{s2}-casein and β-casein) separation as well as to check the efficiency of the extraction procedure, making a fingerprint of those target proteins possible.

Keywords: Milk; Protein; Cloud point; Extraction; Matrix-assisted laser desorption ionization-time of flight mass spectrometry

1. Introduction

Cow milk is important not only as a major nutritional source, but also for commercial and industrial reasons. Casein and whey proteins are major milk proteins that have received great attention owing to their various applications and high economical value [1]. Among them, caseins form the largest protein component in milk of industrial significance [2], which are fundamental to the production and characteristics of products, including yogurt, cheese, infant formulas, juices and others [3]. The caseins are capable of maintaining minerals (such as calcium) in soluble form in the intestine, preventing their precipitation and, consequently, making their absorption easy [3].

Casein and whey proteins differ mainly in hydrophobicity and abundance. Casein is the most abundant milk protein (*ca*. 80%) and shows predominant lipophilic properties. Whey proteins show amphiphilic character and constitute *ca*. 20% of total milk proteins [4,5]. Caseins are phosphoproteins that contain variable number of phosphate groups bound to the serine units of the polypeptide chains (P-ser). They are therefore more susceptible than whey proteins to proteolysis and diffuse faster in the milk micelle interfaces. Caseins are also classified in four subgroups, namely α, β, κ, and γ. Whey proteins display different structural characteristics and amino acid compositions, with high nutritional level. β-Lactoglobulin, α-lactalbumin, immunoglobulin, lactoferrin and bovine serum albumin are examples of whey proteins.

The development of efficient and fast methodologies for proteins extraction and purification has received great attention [6], and cloud point extraction (CPE) is an attractive alternative for such purpose [7–9]. CPE is based on the fact that, at a certain

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temperature and surfactant concentration (above to critical micellar concentration – c.m.c.), a homogeneous micellar solution separates into two macroscopic phases called surfactant-rich and surfactant-poor phases [10]. Nonionic surfactants are most frequently used owing to their lower cloud point temperature when compared to cationic or anionic surfactants. Certain additives can also be used to decrease the cloud point temperature, mainly for the separation of biomolecules. Electrolyte is the most used additive, and its presence decreases the surfactant solubility. The competition of salt ions for water effectively reduces the amount of free water available for surfactant solubilization.

Since its first application by Watanabe and Tanaka [11], CPE has been widely applied, such as for the extraction of metal chelates [12], viruses [13], herbicides [14] and vitamins [15]. Additionally, Bordier has suggested the application of CPE for the separation of membrane proteins [16].

Two-phase aqueous micellar systems have been increasingly used to separate or concentrate important biomolecules including proteins [6]. Micellar systems are suitable for this purpose when liquid–liquid extraction is used, with the attractive feature of ready scale-up [17]. Additionally, only few seconds are required to bring most two-phase systems into equilibrium, which makes their interactions with hydrophilic (aqueous phase) or lipophilic (surfactant phase) proteins compatible. Nonionic surfactants are preferable than ionic surfactants for CPE because they are less effective in denaturing proteins. Triton® X-114 is freqüently chosen because it promotes phases separation at low temperature range (4–30 °C) [10].

The present work describes the application of CPE to separate the two major classes of milk proteins, casein and whey proteins offering a new alternative for separation/extraction of these proteins. The separation was mainly based on their contrasting hydrophobicities, and our main goal was to achieve the highest concentration of casein in the surfactant-rich phase obtaining high efficiency of CPE method. Since separation of these proteins using CPE depends on several variables, such as type and concentration of surfactant, pH, net charge, size and sample volume [18,19], a multivariate methodology based on fraction fractional design was applied to optimize such variables. For this purpose, the experiments were carried out without heating, which could minimize denaturation of proteins. Finally, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) was successfully applied to highlight the separation of proteins after the optimization of the CPE method.

2. Experimental

2.1. Reagents and sample

All reagents (sodium chloride – NaCl, potassium dihydrogen phosphate – KH_2PO_4, sodium hydroxide – NaOH, coomassie brilliant blue – CBB G-250, Tris(hidroxymethyl)-aminomethane, hydrochloric acid – HCl, acetone, ammonium acetate, methanol, sinapinic acid, acetonitrile, and trifluoroacetic acid) were of analytical grade from Merck (Darmstadt, Germany), and J.T. Baker (Phillipsburg, NJ, USA). The nonionic surfactant polyoxyethylene(8) isooctylphenyl ether (Triton® X-114) was obtained from Sigma–Aldrich (Steinheim, Germany). The solutions were prepared with high purity water (18.2 MΩ cm) obtained through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Skim cow milk (ultra high temperature – UHT) was obtained at local market.

2.2. Extraction process

Amounts of solid NaCl (ranging from 6 to 12%, w/v) were dissolved into glass tubes containing 8.0 mL of surfactant solution (prepared with 0.1 mol L^{-1} KH_2PO_4/0.1 mol L^{-1} NaOH buffer) by utilizing a vortex mixer. The milk sample was added, and the solution was homogenized again. Finally, the glass tubes were centrifuged at 1780 × *g* during 15 min for accelerating phase separation. The temperature was *ca*. 25 °C in all experiments.

2.3. Optimization strategy

Multivariate studies were used as a tool for process optimization. The first step of multivariate optimization is accomplished for screening the factors studied (Triton® X-114 concentration, sample volume, NaCl concentration, and pH) in order to obtain their significant level on the partition coefficient. For this task, a fractional factorial design 2^{4-1} without replicates was performed in a random order. The assays are summarized in the Table 1. The temperature was not included in the optimization because for such values of the variables, Triton® X-114

Table 1
Experimental domain used in fractional factorial design 2^{4-1} for multivariate analyses of the milk proteins CPE

Assay	Triton® X-114 concentration (%, w/v)	Sample volume (μL)	NaCl concentration (%, w/v)	pH
1	2 (−1)[a]	100 (−1)	6 (−1)	6.4 (−1)
2	10 (+1)[b]	100 (−1)	6 (−1)	7.4 (+1)
3	2 (−1)	1000 (+1)	6 (−1)	7.4 (+1)
4	10 (+1)	1000 (+1)	6 (−1)	6.4 (−1)
5	2 (−1)	100 (−1)	12 (+1)	7.4 (+1)
6	10 (+1)	100 (−1)	12 (+1)	6.4 (−1)
7	2 (−1)	1000 (+1)	12 (+1)	6.4 (−1)
8	10 (+1)	1000 (+1)	12 (+1)	7.4 (+1)

[a] (−1): Minimum level factor.
[b] (+1): Maximum level factor.

allows to separate phases in a biocompatible condition (room temperature), which is one of the main goal of this work.

For evaluating the significant effects of each factor (in such case called as estimate of the contrast), the program ChemoMatrix (Quantum Chemistry and Chemometrics Laboratory, Unicamp, Campinas, São Paulo, Brazil) [20] was used.

After establishing the significant variables, design plus central point 2^2 was performed to achieve best results in the central point. In these assays, others factors were fixed at appropriated level. Additional design based on evolution operation (EVOP) was necessary by shifting the direction of variables to higher analytical response. Thus, test for significance of the regression model and test for lack of fit were performed by analysis of variance (ANOVA). Linear model was then evaluated. Finally, the domain experimental was shown as response surface (surface mapping system Surfer version 5.0, Golden Software Inc., Golden, CO, USA).

After establishing the optimum point (the best extraction condition), the extraction process was carried out in triplicate.

2.4. Partition coefficient (K)

As casein is the most abundant class of cow milk proteins, total protein concentration was assumed to approximately express casein concentration.

The partition coefficient was calculated by the ratio between the total protein concentration in the surfactant-rich phase and the total protein concentration in the surfactant-poor phase. Then, it was considered that for higher partition coefficient more casein was extracted in the surfactant-rich phase.

2.5. Total protein estimation

The concentration of total protein in both surfactant-rich and surfactant-poor phases was estimated according to the CBB G-250 method described by Bradford [21] using bovine serum albumin (BSA) as standard.

According to our experience, acetone at 1:4 (v/v) proportion was used to precipitate the proteins of the surfactant-rich phase as well as to remove the surfactant because the presence of high Triton® X-114 concentration interferes in the Bradford's method. The mixture (surfactant-rich phase aliquot and acetone) was incubated for 1 h at −20 °C. The supernatant was then removed, and the pellet was dried at room temperature. For total protein determination, the pellet was dissolved into 0.1 mol L^{-1} KH_2PO_4/0.1 mol L^{-1} NaOH buffer at pH 7.0. According to the literature [10], surfactant concentration in the poor phase is near to the c.m.c. (from 0.20 to 0.35 mmol L^{-1} Triton® X-114), which is considered negligible, being its removal unnecessary.

2.6. Identification of proteins by MALDI-TOF MS

Utilizing optimized extraction condition, the casein and the other proteins were identified by MALDI-TOF MS to determine the masses of the intact proteins. To remove potential interferences, such as Triton® X-114 residues, those proteins contained in 450 μL of the surfactant-rich and surfactant-poor phases were then precipitated (1:4, v/v proportion) using 0.1 mol L^{-1} ammonium acetate prepared in methanol. The mixture was incubated overnight for quantitative precipitation. The supernatant was then removed and the pellet was dried at room temperature. In order to maintain the protein structure conformation, the pellet was dissolved into 50 mmol L^{-1} Tris–HCl at pH 8.8 in the same collected phase volume prior to acquire MALDI-TOF MS.

After this procedure, the samples were purified and desalted for mass analysis by adding 100 μL of 25% trifluoroacetic acid (TFA) and passing the protein over miniature C_{18} columns (Millipore Corporation, Bedford, MA, USA), and spotted directly into the MALDI plate and dried. After spotting (1 μL) the sample or peptide (for calibration), a freshly prepared matrix solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, 1 μL) were added to the spots. Then, MALDI-TOF MS analysis using a Micromass MALDI-TOF instrument (Manchester, UK) in the linear ion mode was performed. The main settings were as follows: pulse voltage, 2100 V; delay extraction, 750 ns; accelerating voltage, 15 kV; Microchannel Plate (MCP), 1800 V. Mass spectra were generated by summing up 10 single spectra acquired over the *m/z* 10,000–50,000 range by shooting the laser at random positions on the target spot. The calibration was performed using peptide mass kit mixture II (Waters-Micromass, Manchester, UK) that consisted of bovine insulin (5734.59 Da), *E. coli* thioredoxin (11674.48 Da) and horse apomyoglobin (16952.56 Da). The values expressed are average mass and correspond to the $[M+H]^+$ ion. The matrix employed was a saturated solution of sinapinic acid (60:40, w/v, water containing 0.1%, v/v trifluoroacetic acid:acetonitrile).

3. Results and discussion

3.1. Effects of parameters in the separation process

For establishing an adequate system for each particular sample, several experiments are required, mainly when the univariate method is employed. Therefore, the multivariate studies performed to optimize the variables (Triton® X-114 concentration, sample volume, salt concentration, and pH, see Table 1) were aimed to evaluate the extraction efficiency of casein using CPE. The values of the variables (except for sample volume, which any report was found) selected were based on the results reported in the literature for CPE employing proteins [8,17,22]. It is interesting to comment that concentrations lower than 6% (w/v) NaCl were not tested once that a great volume of the rich phase could be obtained, producing smallest partition coefficient. The temperature was not included once that Triton® X-114 allows separating phases in a biocompatible condition (room temperature). To maintain the proteins in the native form, pH was varied within the physiological range (6.4–7.4) in order to assure that proteins and the surfactant micelle-protein complex would keep their conformational structures.

The partition coefficient was used as the parameter for methodology optimization. Table 2 presents screening results of the fractional factorial design 2^{4-1} and the partition coefficients obtained. In the brief evaluation, higher partition coefficients

Table 2
Results obtained after CPE according to fractional factorial design 2^{4-1}

Assay	Protein concentration (μg mL^{-1})[a]		K[b]
	Surfactant-rich phase	Surfactant-poor phase	
1	1273 ± 84	114 ± 11	11.2
2	475 ± 66	79 ± 10	6.0
3	8702 ± 198	1290 ± 30	6.7
4	4335 ± 292	969 ± 48	4.5
5	1387 ± 24	114 ± 2	12.2
6	563 ± 49	104 ± 3	5.4
7	7948 ± 146	1343 ± 45	5.9
8	3610 ± 228	789 ± 24	4.6

More details see Table 1.
[a] Detection limit (3σ/slope) = 5.0 μg mL^{-1}.
[b] Partition coefficients.

were obtained for assays 1 and 5 (see Table 2), which indicate that Triton® X-114 concentration and sample volume were the most significant variables. By increasing pH values and NaCl concentration, less significant alterations in the partition coefficients was observed. A graph related to probability cumulative (region of the variables codified for the program) versus estimate of the contrast was plotted using the results obtained in the fractional factorial design 2^{4-1}. Such behavior can be visualized in Fig. 1, which shows the probability cumulative related to estimate of the contrast. It is interesting to comment that the data for obtaining Fig. 1 were introduced in the ChemoMatrix program with no previous treatment, and the information given to such program was only related to the different assays (from 1 to 8) against the results obtained (K values for each one assay).

Effect of the variables related to partition coefficient and between the variables was observed. NaCl concentration (C), pH (D), and some interaction of variables (BC and AC) were not significant because the estimate of the contrast was around zero. On the other hand, sample volume (B), Triton® X-114 concentration (A) and their interactions were significant in the system (estimate of the contrast is far from the zero – positive or negative values).

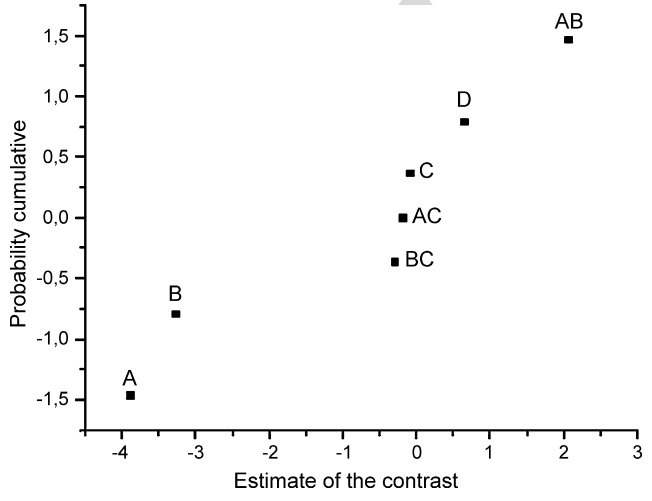

Fig. 1. Estimate of the contrast as a function of the probability cumulative. A = surfactant concentration; B = sample volume; C = salt concentration; D = pH value; AB, BC and AC = interactions between these variables.

For many analytes, the presence of electrolytes decreases the cloud point (salting-out effect) due to a reduction in the repulsive forces between the micelles, resulting in a more efficient extraction [9,12,23]. The salting-out effect is related to interaction of ions to the hydrophilic parts of the micelles [24,25], increasing inter-attraction between micelles and consequently leading to the precipitation of surfactant molecules. The lower cloud point temperature observed to Triton® series is attributed to electrolytes that promotes dehydration of the poly(oxyethylene) chains [26]. Although the electrolyte effects on the cloud point from nonionic surfactants play an important role, the lowest NaCl concentration used (6%, w/v) was enough to obtain good results, probably because it neutralizes all the surfactant micelles in the surfactant concentrations studied. Then, Fig. 1 shows that the NaCl concentration effect was not significant. Note that one of the goals of this work was to achieve phase separation without heating, minimizing denaturation of casein and others proteins. It is therefore important to observe that 6% (w/v) NaCl provides two-phase separation at room temperature. Fig. 1 also shows that any significant pH effect (D – estimate of the contrast was around zero) was observed for CPE; hence, pH 7.0 was selected as an optimal value.

Negative contrast was observed for surfactant concentration and sample volume (Fig. 1), i.e. higher partition coefficients were obtained for lower Triton® X-114 concentrations and lower sample volumes (partition coefficient varied around 12–11 factor for assays 1 and 5). Yu et al. [9] also observed that extraction factor increased by decreasing surfactant concentration when extracting nodularin-R employing the Aliquat-336/Na_2SO_4 system.

An increase in total protein concentration was also observed in the surfactant-poor phase for high protein amount (i.e. high sample volume), with a consequent decrease in the partition coefficient. This behavior indicates saturation of proteins in the surfactant-rich phase, being necessary the use of lower sample volumes to promote higher casein extraction. Such behavior can be observed by showing those results of Table 2 (assays 1 and 5) where higher K values were obtained.

Therefore, after establishing the most important parameters for CPE (Triton® X-114 concentration and sample volume), a factorial design based on 2^2 plus central point was evaluated by fixing 6% (w/v) NaCl concentration and pH 7.0 (Table 3). These results point out best partition coefficients for lower Triton® X-114 concentration and sample volume. Then, as the central point failed to show the best partition coefficient, a new experiment using EVOP based on 2^2 plus central point design was then evaluated to obtain the optimum region (Table 4). Once again, such region was not observed in the central point. However, new experiments were not possible (using different variable values range, i.e. sample volume and concentration) because some difficulties were achieved for precipitating proteins. Due to such difficulties, the experimental design was limited and the lowest condition (for both sample volume and surfactant concentration) was chosen.

In this way, tests for both significance of the regression linear model and lack of fit were then performed by analysis of variance (ANOVA). The ratio between average quadratic of

Table 3
2^2 plus central point design

Assay	Triton® X-114 concentration (%, w/v)	Sample volume (μL)	Protein concentration (μg mL^{-1})[a]		K[b]
			Surfactant-rich phase	Surfactant-poor phase	
1	2 (−1)[c]	100 (−1)	1700 ± 20	146 ± 8	11.6
2	10 (+1)[d]	100 (−1)	699 ± 62	97 ± 3	7.0
3	2 (−1)	1000 (+1)	9841 ± 272	1534 ± 11	6.4
4	10 (+1)	1000 (+1)	5653 ± 130	838 ± 34	6.7
5	6 (0)[e]	550 (0)	3832 ± 120	651 ± 13	5.9
6	6 (0)	550 (0)	3582 ± 58	659 ± 24	5.4
7	6 (0)	550 (0)	4172 ± 52	671 ± 29	6.2

NaCl concentration and pH were fixed at 6% (w/v) and 7.0, respectively.
[a] Detection limit (3σ/slope) = 5.0 μg mL^{-1}.
[b] Partition coefficients.
[c] (−1): Minimum level factor.
[d] (+1): Maximum level factor.
[e] (0) Central point.

Table 4
2^2 plus central point design using EVOP experiment

Assay	Triton® X-114 concentration (%, w/v)	Sample volume (μL)	Protein concentration (μg mL^{-1})[a]		K[b]
			Surfactant-rich phase	Surfactant-poor phase	
1	1 (−1)[c]	50 (−1)	923 ± 66	67 ± 2	13.0
2	3 (+1)[d]	50 (−1)	607 ± 26	52 ± 2	11.7
3	1 (−1)	150 (+1)	1986 ± 69	235 ± 9	8.4
4	3 (+1)	150 (+1)	1804 ± 75	222 ± 10	8.1
5	2 (0)[e]	100 (0)	1334 ± 11	144 ± 6	9.3
6	2 (0)	100 (0)	1134 ± 13	142 ± 7	8.0
7	2 (0)	100 (0)	1043 ± 29	135 ± 9	7.7

[a] Detection limit (3σ/slope) = 5.0 μg mL^{-1}.
[b] Partition coefficients.
[c] (−1): Minimum level factor.
[d] (+1): Maximum level factor.
[e] (0) Central point.

lack of fit (lof) and pure experimental error (pe) was compared to tabled values of F-distribution. The $F_{\text{lof/pe}}$ (3.21) was lower than the critical $F_{2,2,95\%}$ (19.0), which emphasizes the model significance. Then, the linear model was considered satisfactory.

Fig. 2 displays the response surface obtained from the equation of linear model:

$$K = 9.4531 - 0.4330\,(\text{Triton}^{®}\ \text{X-114 concentration}) - 2.1300\,(\text{sample volume})$$

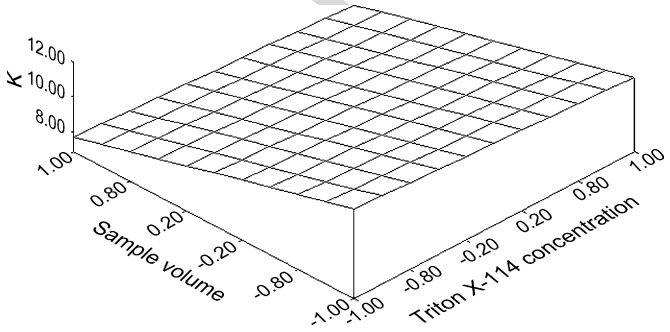

Fig. 2. Response surface obtained after optimized conditions.

This response surface shows a tendency and pointed out a maximum response for lower surfactant concentration and sample volume. But Triton® X-114 concentrations lower than 1% (w/v) could not be used because smaller surfactant-rich phase volumes would be obtained, and its removal being then laborious. At such condition, and as already previously commented, quantitative protein precipitation for surfactant removal could become difficult, because very low amount of protein was observed. In the same way, for sample volumes lower than 50 μL some difficulties were experienced in precipitating proteins. Then, surfactant concentration and sample volume were fixed at 1% (w/v) and 50 μL, respectively.

Finally, the optimized parameters were therefore: 1% (w/v) Triton® X-114 concentration, 50 μL of sample volume, 6% (w/v) NaCl concentration and pH 7.0. At such conditions, 923 ± 66 and 67 ± 2 μg mL^{-1} of total protein in the surfactant-rich and surfactant-poor phases were found, respectively. Such results indicate that the extracted proteins (probably from the casein class) strongly interact with the surfactant used, and most of these proteins migrate to the surfactant-rich phase (as expected from their high hydrophobicity). Note that higher partition coefficients have been already observed for a mixture of synthetic proteins [16,27] but only few applications to biological samples [9,28]. It is evident that the CPE efficiency of

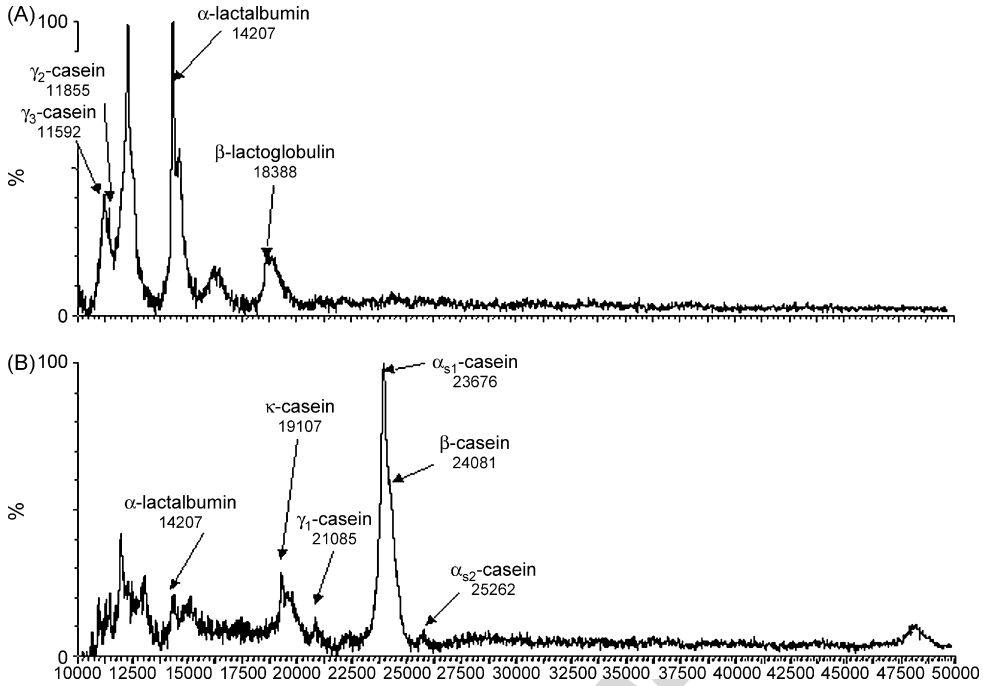

Fig. 3. MALDI-TOF mass spectra of (a) the surfactant-poor and (b) surfactant-rich phase after phase separations.

biomolecules is quite different when the target protein is in a "clean" medium or in a highly complex medium, such as milk, in which hundreds of proteins and other components are often present. In fact, it has been observed that lower partition coefficients are generally obtained when biological samples are employed [29,30]. Despite these problems, however, good partition coefficient (*ca.* 13) was obtained in this work.

3.2. MALDI-TOF MS analysis

To highlight the extraction of casein proteins from milk and their separation from whey proteins by CPE, MALDI-TOF MS to intact samples of the two protein fractions was applied. A simple sample treatment that only involved dilution of precipitated proteins [31,32] was performed.

Fig. 3 displays the mass spectra obtained, and Table 5 summarizes different proteins identified in the surfactant-poor and surfactant-rich phases. The slight differences between observed and theoretical molar masses are in agreement with those previously described for whole cow milk protein analyses by MALDI-TOF MS [31–34].

The mass spectra of Fig. 3A show ions of *ca. m/z* 11,592 and 11,855, corresponding to the γ_3-casein and γ_2-casein, respectively. Those ions of higher *m/z* values correspond to α-lactalbumin (*m/z* 14,207), and β-lactoglobulin (*m/z* 18,388). Finally, the ion of *ca. m/z* 14,531 (Fig. 3A) probably corresponds to species originating from lactose addition to α-lactalbumin. The main proteins identified in the surfactant-poor phase (Fig. 3A) were α-lactalbumin and β-lactoglobulin, which are fractions of the whey proteins [2]. Besides such proteins, γ_2- and γ_3-casein were also detected. CPE results in small amounts of the surfactant in the surfactant-poor phase, explaining why the γ_3- and γ_2-caseins as well as α-lactalbumin and β-lactoglobulin remain in such phase. In fact, both last proteins also present amphiphilic character [4,5], corroborating their presence in the surfactant-poor phase.

Table 5
Milk proteins identified in the surfactant-poor and surfactant-rich phases after CPE

Phase	Protein	Molar mass (Da)		Deviation (%)
		Reported values	MALDI-TOF MS	
Poor	γ_3-Casein	11500	11592	0.80
	γ_2-Casein	11800	11855	0.50
	α-Lactalbumin	14200	14207	0.05
	β-Lactoglobulin	18360	18388	0.20
Rich	α-Lactalbumin	14200	14207	0.05
	κ-Casein	19000	19107	0.60
	γ_1-Casein	21000	21085	0.40
	α_{s1}-Casein	23600	23676	0.30
	β-Casein	24000	24081	0.30
	α_{s2}-Casein	25250	25262	0.05

In the surfactant-rich phase (Fig. 3B), the main casein fractions identified were α_{s1}-casein (*m/z* 23,676) and α_{s2}-casein (*m/z* 25,262), β-casein (*m/z* 24,081), and κ-casein (*m/z* 19,107). The peaks associated with the detection of protonated α_{s1}-, β- and α_{s2}-caseins are quite broad owing perhaps to the detection of a mixture of related proteins with post-translational modifications. The presence of the α-lactalbumin (*m/z* 14,207) in such phase can be explained due to its amphiphilic character as already commented [4,5] as well as the slight amount of water present in such phase [35]. The abundant ion of *ca. m/z* 12,300 (Fig. 3B) may also indicate that, in such case, some Maillard reactions took place, denaturizing the high-mass milk proteins due to the experimental or the manufacturing process to which the milk was submitted [36].

4. Conclusions

CPE was efficiently employed to cow milk samples being able to extract and separate casein from whey proteins using only 50 μL sample volume without any sample pre-treatment. Biocompatible parameters in terms of pH and temperature were used for protein separations using 1% (w/v) Triton® X-114 and 6% (w/v) NaCl (salting-out effect) at pH 7.0 and room temperature. At such conditions, good partition coefficient (13) was obtained, allowing the separation of casein proteins (present in the surfactant-rich phase) from α-lactalbumin and β-lactoglobulin (present in the surfactant-poor phase) in only 15 min and with minimum costs, indicating that the proposed factorial design was successfully applied in the experimental domain employed and the main objective of this work attained. Although the partition coefficient was obtained, it is important to comment that some hydrophobic proteins were achieved in the surfactant-poor phase and vice-versa, as demonstrated through MALDI-TOF MS analysis. This behavior is inherent to the CPE, once that small amount of surfactant is found in the surfactant-poor phase as well as the surfactant-rich phase is somewhat hydrated.

After cloud point extraction and mass spectrometry identification, no significant differences (deviations less than 0.80%) were noted between the observed and theoretical molar mass for cow milk protein.

Finally, the adopted strategy could also be used for separating those low abundant proteins in milk after properly optimizing the CPE method for such task.

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