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Donkey's milk protein fractions characterization

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Abstract

Donkey's milk may be considered a valid alternative, for infant nutrition, to powdered milks, soybean milk or other formulas, since its composition in lipids and proteins is very close to human milk. The aim of the present study is to characterize "donkey's milk" product, especially with regard to the protein fractions. Three different chromatographic approaches for donkey's milk proteins separation are described, based on their different isoelectric points, hydrophobicity and molecular mass. The proteins obtained were further separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and identified by N-terminal sequencing. α_{S1}- and β-caseins, lysozyme, α-lactalbumin, β-lactoglobulin were identified. Furthermore, the content of α-lactalbumin, β-lactoglobulin, and lysozyme in donkey's milk was determined by a reversed-phase HPLC method. The β-lactoglobulin and α-lactalbumin content was 3.75 and 1.8 mg/ml, respectively (mean values). The present study showed the nutritional properties of donkey's milk characterized by low casein and high lysozyme content (1.0 mg/ml) compared with other kinds of milks.

Keywords: Donkey's milk; Protein fractions; N-terminal sequence

1. Introduction

In recent years, interest in donkey's milk was considerably increased due to its composition, which is very close to that of human milk, so much so that it may be considered a good substitute for dairy cow's milk derivatives in feeding children with severe Ig-E mediated cow's milk protein allergy (CMPA), as stated by Businco et al. (2000). Some clinical trials have demonstrated that infant formulae, which are mostly based on dairy cow's milk, are less adapted than donkey's milk for human nutrition in patients affected by CMPA (Carroccio, Cavataio, Montalto, D'Amico, & Alabrese, 2000; Iacono et al., 1992).

Donkey's milk is today used mainly in those countries where traditionally donkeys are still being bred, especially in Asia, Africa and eastern Europe. Recently it has been used as a possible substitute for human milk in some countries of western Europe.

Some authors suggested using donkey's milk also for probiotic purposes (Coppola et al., 2002) since it proved to be a good growth medium for probiotic lactobacilli strains, because of the high content of lysozyme and lactose. The high content of lactose is responsible for the good palatability and for optimising the intestinal absorption of calcium, that is essential for bone mineralization in infants (Schaafsma, 2003).

Furthermore, donkey's milk lipid fraction is comparable to that of human milk since it is characterized by high levels of linoleic and linolenic acid (Salimei et al., 2004). Several studies reported that the addition of linolenic acid in the diet is useful for the treatment of some atopic dermatitis; therefore, also donkey's milk could be used as a nutritional source of linolenic acid for children suffering from this pathology (Horrobin, 2000). The values of mineral composition in donkey's milk are very close to those of

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human and mare's milk, except for the highest level of calcium and phosphorus, but the Ca/P ratio is very close to that for human milk, as indicated by Salimei et al. (2004).

Donkey's milk total protein content is low (13–28 mg/ml), very close to the values for human (Salimei et al., 2004) and mare's milk (Malacarne, Martuzzi, Summer, & Mariani, 2002) and therefore it does not produce an excessive renal load of solute. The donkey's milk protein fraction is particularly rich in whey proteins; they represent 35–50% of the nitrogen fraction, while in cow's milk only 20% (Herrouin et al., 2000). The most important whey proteins in equid milks are α-lactalbumin (α-LA), β-lactoglobulin (β-LG) and lysozyme (LYS), as demonstrated by Fantuz et al. (2001). In Table 1, a comparison of the concentration of total caseins, whey proteins and non-casein proteins in human, mare's and donkey's milk is shown. The composition of mare's milk has been included in this table because of its resemblance with donkey's milk and the more abundant literature (Egito et al., 2002; Malacarne et al., 2002; Miranda, Mahé, Leroux, & Martin, 2004).

Donkey's milk α-LA presents two isoforms, A and B, with different isoelectric points (Giuffrida, Cantisani, Napoletano, Conti, & Godovac-Zimmerman, 1992). The amount of α-LA in mare's milk is 3.3 mg/ml, very close to bovine milk (Miranda et al., 2004). β-LG is the major whey protein found in bovine milk (Hambling, McAlpine, & Sawyer, 1992), and it is considered the principal milk allergen in neonates and children (Carroccio, Cavataio, & Iacono, 1999). β-LG is absent in the human milk (Chatterton, Rasmussen, Heegaard, Sørensen, & Petersen, 2004; de Wit, 1998; Miranda et al., 2004). Three genetic variants for donkey's milk β-LG were found: one of them is a I-like β-LG with three aminoacid substitutions, while the other two are similar to β-LG with two aminoacid exchanges (Herrouin et al., 2000).

Lysozyme is known to be a natural antimicrobial agent since it catalyses the hydrolysis of glycosidic bonds of mucopolysaccharides in bacterial cell walls (Chiavari, Coloretti, Nanni, Sorrentino, & Grazia, 2005). This enzyme, together with other factors including immunoglobulins, lactoferrin and lactoperoxidase, may function in the infant's digestive tract to reduce the incidence of gastrointestinal infections (Businco et al., 2000). With regard to donkey's milk, previous studies revealed a high lysozyme content compared to mare's and bovine milk; that may be responsible for the low bacterial concentration noted (Salimei et al., 2004). Other authors reported the presence in donkey's milk of two variants of lysozyme: LYS A and LYS B, that differ in three aminoacid substitutions at positions 48, 52 and 61 (Herrouin et al., 2000).

Table 1
Comparison of the concentration of total caseins, total whey proteins and of the relative amounts of non-casein proteins in human, mare's, and donkey's milk

	Human[a] (mg/ml)	Mare (mg/ml)	Donkey (mg/ml)
Caseins	5.8	10.3[b]–14.0[a]	6.60
Whey proteins	2.1	8.03[a]–7.40[a]	7.50
Lysozyme	0.50[a]	1.10[a]	1.00
β-lactoglobulin	–	3.00[a]	3.75
α-lactalbumin	1.60[a]	3.30[a]	1.80

[a] Miranda et al. (2004).
[b] Malacarne et al. (2002).

In the present work, the content of α-lactalbumin, β-lactoglobulin and lysozyme, (expressed as mg/ml) in donkey's milk was determined at different stages of lactation by using a high performance liquid chromatography (HPLC) method. With this in view, skimmed donkey's milk at different stages of lactation (60, 90, 120, 160, 190 days after parturition) was subjected to reversed phase-high performance liquid chromatography (RP-HPLC) followed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The whey proteins obtained after these separations were identified by N-terminal sequencing and their content in mg/ml was determined by a quantitative method.

Despite the large amount of information on mare's milk casein characterization (Ochirkhuyag, Chobert, Dalgalarrondo, & Haertlé, 2000), the casein fraction of donkey's milk has not yet been investigated in depth. In the present work we describe three different techniques for casein separation, based on their different isoelectric points, hydrophobicity and molecular mass. The data obtained in this study could provide further information about the possibility of using donkey's milk as a replacement for human milk.

2. Materials and methods

2.1. Chemicals

Bis-tris, Tris (hydroxymethyl) aminomethane (trizma base), dithiotreitol (DTT), ammonium acetate, sodium dodecyl sulphate (SDS), lysozyme (from egg white), α-lactalbumin, β-lactoglobulin (from bovine milk) and other reagents were from Sigma Chemical Co. (St. Louis, MO). Superdex 75 HR 10/30, MonoQ HR5/5, Mono S HR 5/5, Mono P HR 5/5, HiTrap Desalting, Polybuffer 96 and 74, Blue Dextran 2000, the LMW Gel Filtration Calibration Kit and the HPLC system Äkta Purifier were produced by Amersham Biosciences (Uppsala, Sweden). Reversed-phase column C4 Prosphere, 150 × 4.6 mm, 300-Å pore size, 5-μ particle size, was from Alltech (Waukegan Rd Deerfield, IL). Mini Protean III, Trans-blot cell apparatus, polyvinylidene difluoride membrane (PVDF) and SDS-PAGE molecular weight standard low range were from Bio-Rad Laboratories (Hercules, CA). PM10 membranes were from Amicon Corporation.

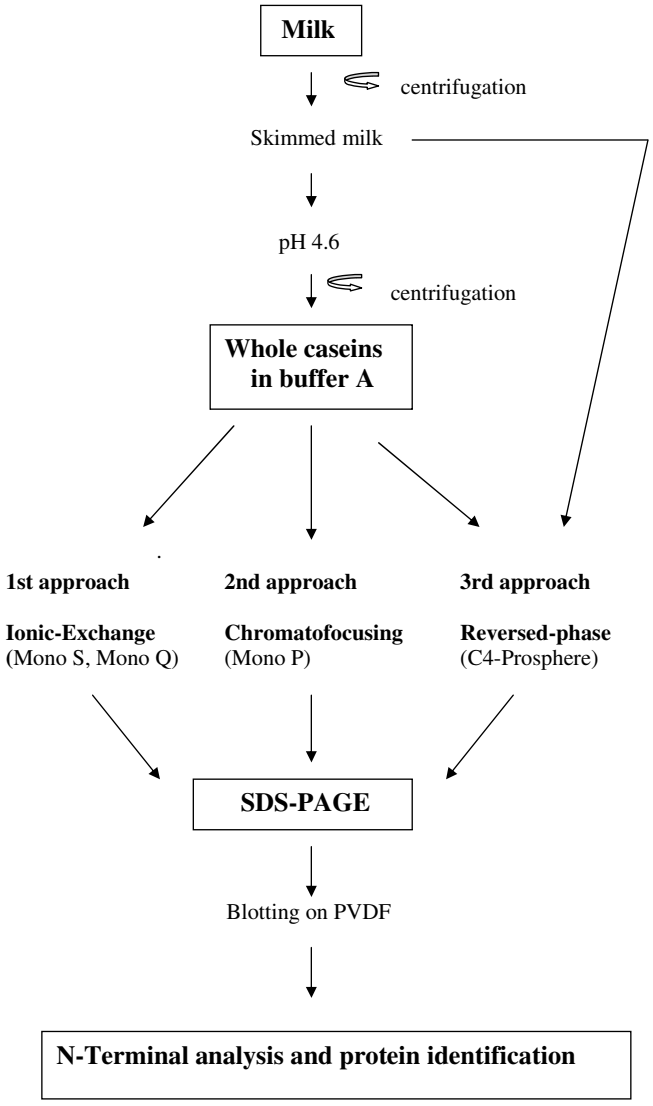
2.2. Preparation of milk samples

Bulk milk obtained from 10 Ragusana breed pluriparous asses was used. The animals were machine milked following the protocol previously described by Salimei et al. (2004). Skimmed milk was prepared from 20 ml of fresh milk by centrifugation at 3000*g* for 30 min at 15 °C. Whole casein was obtained from skimmed milk by adjust-

ing the pH to 4.6 with 10% (v/v) acetic acid and centrifuged at 3000*g* for 10 min in order to obtain a supernatant of whey proteins and the isoelectrically precipitated caseins that were resuspended in buffer A (50 mM ammonium acetate containing 8 M urea, pH 5.5 or pH 7.0). The protein concentration was determined following the method of Bradford (1976).

2.3. Chromatographic approaches

Three different chromatographic approaches were used to characterize the casein fraction in donkey's milk by using an Äkta Purifier HPLC system: ion-exchange chromatography (first approach), chromatofocusing (second approach) or reversed-phase HPLC (third approach). Each peak obtained after chromatography was subjected to dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The purified caseins were identified by N-terminal sequencing. This procedure is resumed in the following scheme:



2.4. Ion-exchange chromatography (first approach)

One aliquot of whole caseins was subjected to cationic-exchange chromatography on HPLC (Mono S HR 5/5 column, 1.0 ml bed volume). The column was equilibrated in buffer A at pH 5.5 or pH 7.0 at a flow rate of 0.5 ml/min and eluted by a linear gradient between buffer A and buffer B (1 M buffer A). The gradient used was: %B = 0, time = 10 min; %B = 100, time = 100 min; %B = 100, time = 110 min. Another aliquot of whole casein was desalted by a gel filtration chromatography on HPLC by using the HiTrap Desalting column (Sephadex G-25 Superfine), equilibrated with 50 mM NH_4HCO_3 and eluted with the same buffer. After this step the caseins were subjected to anionic-exchange chromatography on HPLC by a Mono Q HR 5/5 column (1.0 ml bed volume) equilibrated with 50 mM NH_4HCO_3 pH 7.9 at a flow rate of 0.5 ml/min, and eluted by a linear gradient between 50 mM NH_4HCO_3 and 1 M NH_4HCO_3 pH 7.9 (gradient used: %B = 0, time = 20 min; %B = 100, time = 140 min; %B = 100, time = 150 min). In all cases the proteins eluted from the columns (0.5 ml fractions) were detected at 280 nm by a UV 900 Monitor included in the HPLC system.

The chromatographic peaks obtained after Mono S analysis were desalted by the HiTrap Desalting column as described above, before being further characterized.

2.5. Chromatofocusing on HPLC (second approach)

A third aliquot of whole casein was subjected to gel filtration analysis on HPLC using a Superdex 75 HR 10/30 column equilibrated and eluted in buffer A, pH 5.5, at a flow rate of 0.5 ml/min. The void volume of the column (V_0) was determined using Blue Dextran 2000 (2000 kDa), and the column was calibrated by the LMW Gel Filtration Calibration Kit (ribonuclease A, 13.7 kDa; chymotrypsinogen A, 25.0 kDa; ovalbumin, 43.0 kDa; bovine serum albumin, 67.0 kDa). The effluent was monitored at 280 nm and collected in 0.5 ml fractions. The calibration curve was obtained relating the logarithm of the molecular weight of each standard protein to the K_{av} ($K_{av} = V_e - V_0/V_t - V_0$) corresponding to each standard. Pools 1 and 2 from gel filtration were subjected to a chromatofocusing analysis on HPLC performed in two pH ranges, 6.0–4.0 and 7.0–5.0, using a Mono P HR 5/5 column (1.0 ml bed volume). In the pH range of 6.0–4.0, the chromatofocusing column was equilibrated with starting buffer at pH 6.3 (Bis-tris 25 mM, pH 6.3 containing 8 M urea) and eluted by a linear gradient with the eluent buffer at pH 4.0 (100 ml containing 10 ml Polybuffer 74, 8 M urea, adjusted to pH 4.0 with 1 M HCl). The flow rate was 0.5 ml/min; the gradient used was: %B = 0, time = 0 min; %B = 100, time = 50 min. In the case of the pH range 7.0–5.0, the starting buffer was at pH 7.0 and the eluent at pH 5.0.

2.6. Reversed-phase chromatography on HPLC (third approach)

Whole caseins obtained after the isoelectric precipitation at pH 4.6, were resuspended in CL buffer (0.1 M bis-tris, pH 8.0 containing 8 M urea, 1.3% trisodium citrate, 0.3% DTT), and 100 μl was loaded into the reversed-phase column.

The samples for reversed-phase HPLC (RP-HPLC) from skimmed donkey's milk were clarified by the addition of two volumes of CL buffer. One hundred microliters of clarified samples were loaded into the RP-HPLC column (Miranda et al., 2004).

The reversed-phase column was a 300 Å C4 Prosphere (5 μm, 4.6 mm I.D., 150 mm. Alltech). The column was equilibrated in trifluoroacetic acid (TFA)/H_2O 1:1000 v/v (buffer A) and elution was achieved by the following step gradient with TFA/H_2O/acetonitrile 1:100:900 v/v (buffer B): %B = 0, time = 10 min; %B = 20, time = 10 min; %B = 40, time = 0.1 min; %B = 60, time = 40 min. The flow rate was 1 ml/min and fractions of 0.5 ml were collected. The proteins eluted from RP-HPLC columns were monitored at 280 nm by a UV 900 Monitor included in the HPLC system.

Each chromatographic peak eluted from each chromatographic course was collected and subsequently analyzed by SDS-PAGE.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Laemmli (1970), under reducing conditions using a 15% or 13% acrylamide-bis acrylamide solution and the Mini Protean III apparatus (Bio-Rad, gel size 7 × 8 cm × 0.75 mm). The markers used were Bio-Rad molecular weight standards, low range (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa). Five to ten micrograms of each sample were incubated with the denaturing loading solution (12% glycerol; 1.2% SDS; 5.4% β-mercaptoethanol; saturated bromophenol blue) at 100 °C for 5 min and then loaded onto the gel. Electrophoresis was performed at 4 °C with a constant voltage of 200 V. The proteins were visualized on the gel by Coomassie Blue staining (0.1% Coomassie Brilliant Blue R250 in 50% methanol and 10% acetic acid). The fractions obtained from the various chromatographic methods were analyzed by 13% (the casein proteins fraction) or 15% SDS-PAGE (the whey proteins fraction).

2.8. N-terminal analysis and identification of proteins

Following electrophoresis, performed as described above, the polyacrylamide gel was equilibrated for 15 min in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11.0, containing 10% (v/v) methanol, and blotted onto a PVDF membrane (Sequi-blot, Bio-Rad) treated as described by Egito et al. (2002). N-terminal aminoacid sequence was determined by Edman degradation using an automatic protein sequencer (Protein Sequencer G1000A, Hewlett Packard) on passively transferred electrophoretic bands on PVDF membrane.

The identification of proteins and peptides was performed by consulting the Swiss-Prot and TrEMBL protein data-bases available on the ExPASy (Expert Protein Analysis System) proteomic server (http://us.expasy.org/) at the Swiss Institute of Bioinformatics. The α_{S1}- and β-caseins from donkey's milk were identified by comparing the sequence obtained after N-terminal analysis with those of the α_{S1}- and β-caseins from mare's milk. The accession numbers were: Q9GKK3 (β-casein precursor, *Equus caballus*); Q8SPR1 (α_{S1}-casein, *E. caballus*). The accession numbers for donkey's milk lysozyme, α-lactalbumin and β-lactoglobulin, consulted to compare the obtained N-terminal sequences, were P11375, P28546, P13613, respectively. In all cases, the sequence homology for the first 10 aminoacids was 100%.

2.9. Lysozyme and whey proteins content determination

For the quantitative determination of lysozyme, α-lactalbumin and β-lactoglobulin milk samples were taken at different stages of lactation: 60, 90, 120, 160, 190 days after parturition. In this study we used the bulk milk obtained from 10 multiparous asses, as explained in Section 2.2.

Donkey's milk lysozyme and whey proteins content was determined by an HPLC method by using a reversed-phase column; sample milk preparation, column equilibration and elution were performed as described under Section 2.6. Each standard solution of egg white lysozyme (2.0, 1.0, 0.5, 0.25, 0.15 mg/ml), bovine milk β-lactoglobulin (0.17, 0.30, 0.75, 1.0, 1.5 mg/ml) and bovine milk α-lactalbumin (0.25, 0.35, 0.50, 0.75, 1.0, 1.5 mg/ml) was prepared in CL buffer. A 100-μl solution of each standard was separately loaded on the RP-HPLC column. The area of each standard peak was measured using the valley-to-valley integration mode and quantification was achieved by a calibration curve obtained relating the concentration in micrograms of each standard loaded in the column to the peak area corresponding to each concentration. From each milk sample the quantity of lysozyme, β-lactoglobulin, and α-lactalbumin was determined by using the calibration curve.

3. Results and discussion

3.1. Ion-exchange chromatography followed by SDS-PAGE (first approach)

The total protein content determined on the whole casein by the method of Bradford (1976) was 6.6 mg/ml.

The whole casein was separated into five peaks named A, B, C, D, E, and in another five named F, G, H, I, J by cationic-exchange chromatography (Mono S) performed at pH 5.5 and pH 7.0, respectively (Fig. 1a and b). Each peak was subjected to 13% SDS-PAGE (Fig. 1c and d, respectively) to choose which one was suitable to N-terminal sequence; this analysis revealed mainly β-caseins (sequence: REKEELNVSS) and α_{S1}-caseins (sequence: RPKLPHRQPE), which presented marked homology with α_{S1}- and β-caseins from mare's milk (Egito et al., 2001, 2002).

In particular, peaks A and F were identified as β-casein, having a molecular weight of about 35.4 and 33.3 kDa, respectively; peaks D and E showed the same electrophoretic pattern with two bands of 33.0 and 30.7 kDa both identified as α_{S1}-caseins, as well as peak I that proved to be a mixture of two α_{S1}-caseins with a molecular weight of 31.3 and 29.4 kDa. Peak J, with a molecular weight of 29.4 kDa, was identified as α_{S1}-casein. The amount of peaks B and C was not sufficient for N-terminal analysis; however, peak C could be a mixture of two α_{S1}-casein since it showed the same electrophoretic pattern of peaks D and E. After the electrophoretic course, peaks G and H proved not homogeneous: both of them showed one diffuse band of 36.1 kDa and three neighboring bands with a molecular weight of 35.4 (probable β-casein), 31.3, and 29.4 (probable α_{S1}-casein).

The anionic-exchange chromatography (Mono Q) separated the whole donkey's milk casein into two peaks consisting mainly of β-caseins (data not shown).

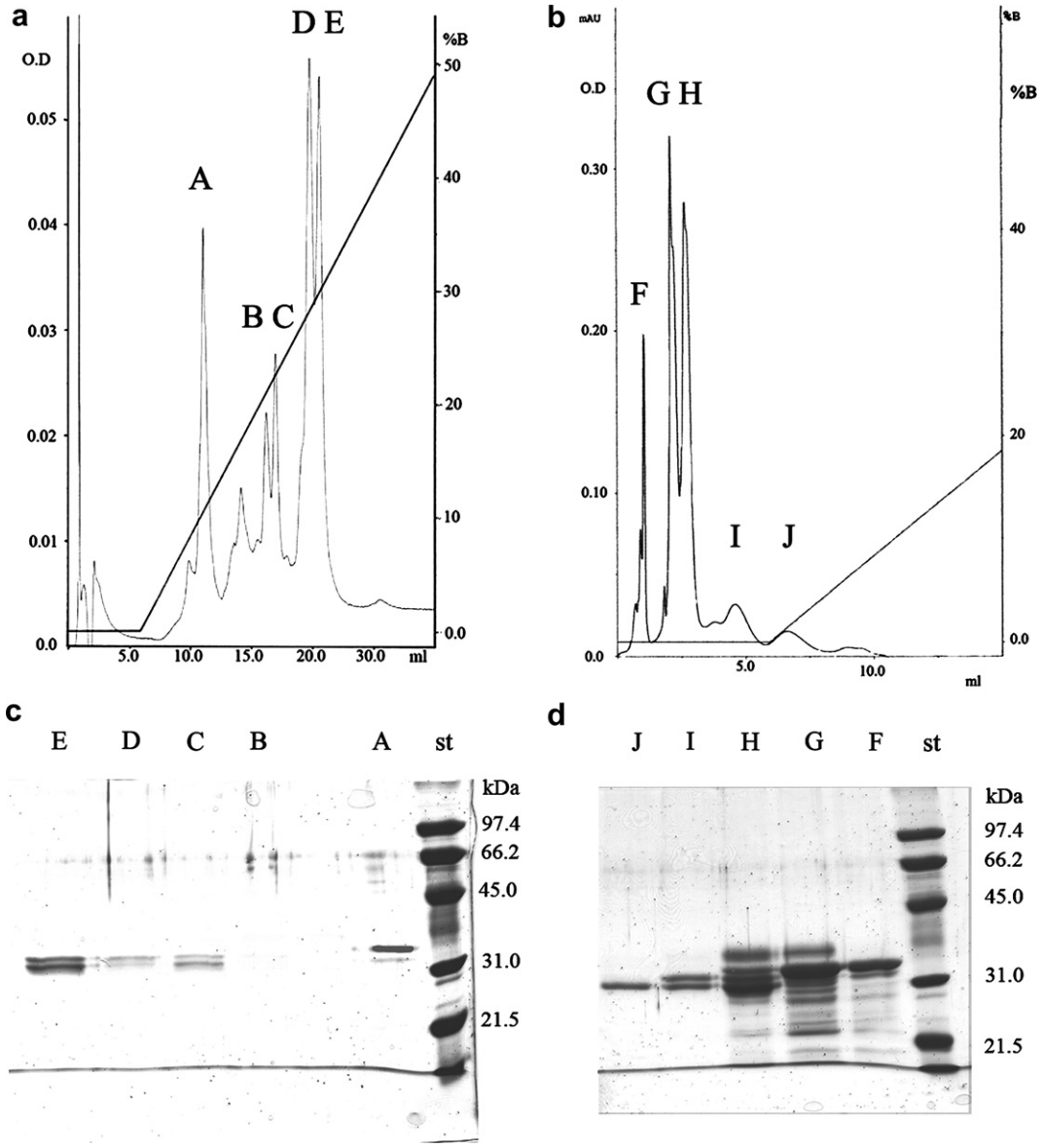


Fig. 1. Cationic-exchange chromatography on HPLC (Mono S HR 5/5) analysis on whole casein performed at pH 5.5 (a) and at pH 7.0 (b). 13% SDS-PAGE of the peaks separated by Mono S analysis at pH 5.5 (c) and pH 7.0 (d). (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa ovalbumin; 31.0 kDa carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

3.2. Chromatofocusing followed by SDS-PAGE (second approach)

Another purification, a protocol based on the different isoelectric points and the different molecular weight of caseins was set up. From the gel filtration in HPLC three peak proteins (1, 2, 3) were obtained, as shown in Fig. 2. The molecular weight of the proteins corresponding to these peaks were estimated using the calibration curve (see Fig. 2, inset). Peaks 1, 2 and 3 showed a molecular weight of 48.9, 32.3, and 7.0 kDa, respectively. The retention time of the void volume was 15.1 min corresponding to a volume of 7.55 ml. The high molecular weight of peak 1 may be due to the anomalous behaviour of the caseins in this separation condition. Peaks 1 and 2 from gel filtration were subjected to further studies (chromatophocusing analysis), whereas the amount (total protein content) of peak 3 was not enough to perform other chromatographic approaches.

The chromatofocusing of peak 1, in the pH range 6.0–4.0, resulted in three peaks named K, L and M (Fig. 3a) while chromatofocusing of peak 1, in the pH range of 7.0–5.0 resulted only in one peak named N (data not shown). After a 13% SDS-PAGE analysis (Fig. 3b), peak K proved not homogeneous with a diffuse band having a molecular weight ranging from 34.5 to 28.7 kDa; peak L showed one band of 29.9 kDa not identified by N-terminal sequence, and peak M showed one band of 35.4 kDa. The peak N showed one intense band with a molecular weight of 35.4 kDa that after N-terminal analysis proved to be β-casein (sequence: REKEELNVSS) and another unidentified weak band of 31.2 kDa. Chromatofocusing of peak 2 from gel filtration in the pH range 6.0–4.0 resulted in two peaks named O and P (Fig. 4a). After a 13% SDS-PAGE analysis, the amount of protein in peak O proved too low for sequencing, whereas peak P showed one band of 35.4 kDa, that after N-terminal analysis proved to be a β-casein, and another band of 31.2 kDa (Fig. 4b). Since peaks 1 and 2 from gel filtration were not completely resolved, it may be possible that the peaks M, N and P (β-caseins showing the same molecular weight) may come from the same gel filtration peak.

The separation of caseins based on their different isoelectric point did not result in a good separation of the casein fractions; therefore a reversed-phase chromatography was undertaken.

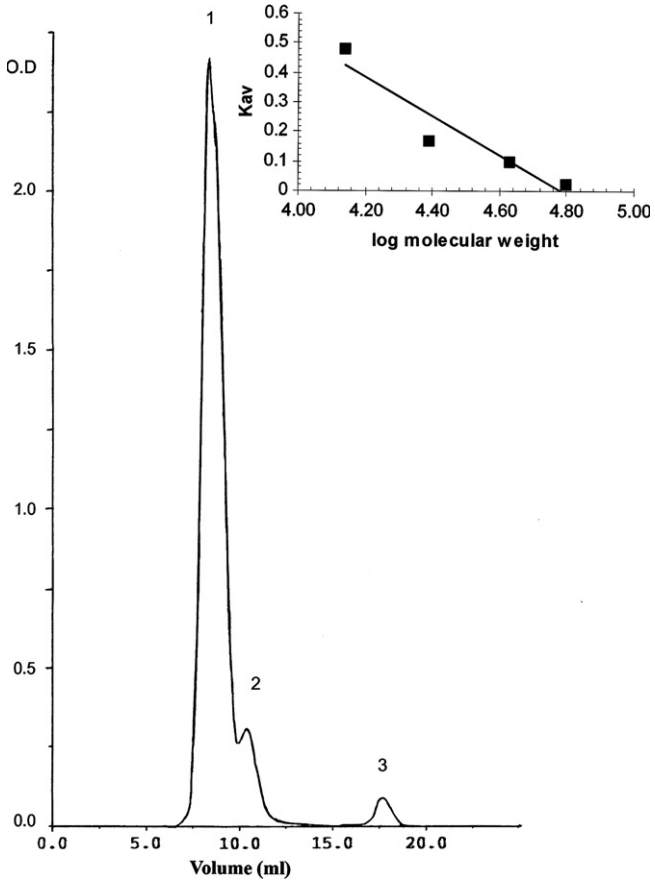


Fig. 2. Gel filtration analysis of whole caseins performed as described under Section 2. (Inset) Calibration curve of the Superdex 75 obtained by LMW Gel Filtration Calibration Kit (ribonuclease A, 13.7 kDa; chymotrypsinogen A, 25.0 kDa; ovalbumin, 43.0 kDa; bovine serum albumin, 67.0 kDa).

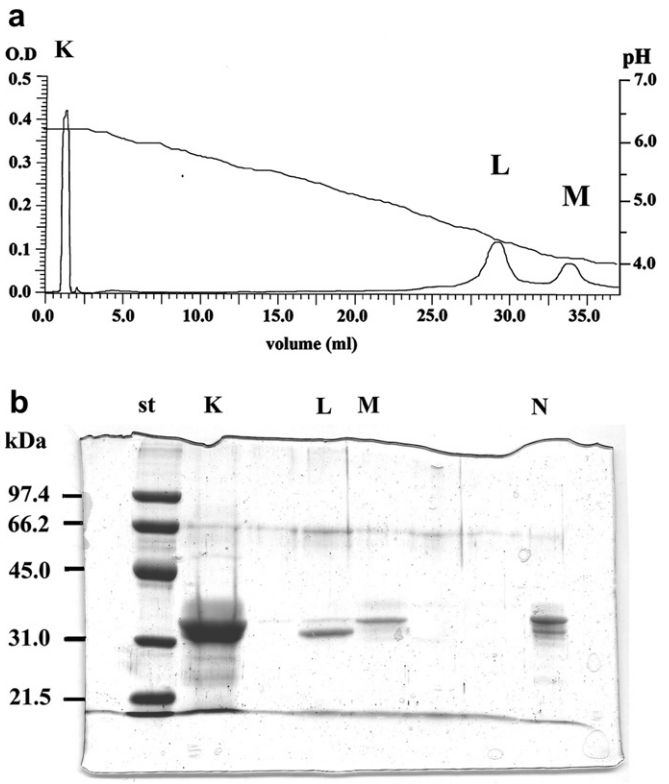


Fig. 3. (a) Chromatofocusing analysis (Mono P H/R 5/5) in the pH range 6.0–4.0 of peak 1 eluted from the gel filtration. (b) 13% SDS-PAGE of peaks eluted from chromatofocusing. Peaks K, L, M, were obtained from chromatofocusing analysis in the pH range of 6.0–4.0. Peak N was from chromatofocusing analysis in the pH range of 7.0–5.0. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

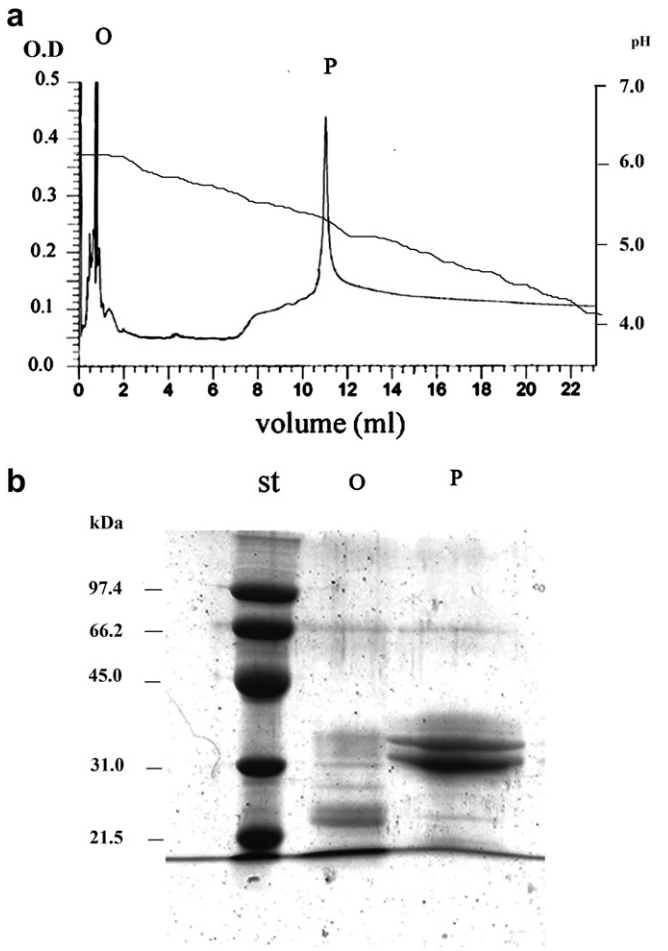


Fig. 4. (a) Chromatofocusing analysis (Mono P H/R 5/5) in the pH range 6.0–4.0 of peak 2 eluted from gel filtration. (b) 13% SDS-PAGE of peaks O and P eluted from chromatofocusing analysis in the pH range of 6.0–4.0. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

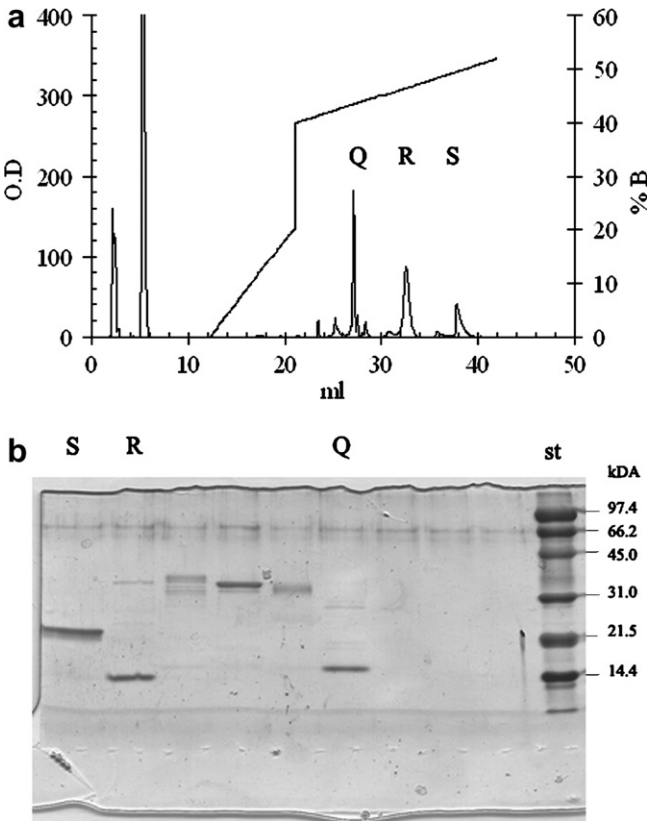


Fig. 5. (a) Reversed-phase HPLC of skimmed donkey's milk performed as described under Section 2. (b) 13% SDS-PAGE of peaks Q, R and S eluted from RP-HPLC. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme). The arrows indicate the casein fraction.

3.3. Reversed-phase chromatography followed by SDS-PAGE (third approach)

Reversed-phase chromatography on HPLC (RP-HPLC) followed by 15% SDS-PAGE was performed on the skimmed donkey's milk and on the whole caseins obtained after the isoelectric precipitation at pH 4.6.

Three main peaks were recovered from RP-HPLC of skimmed donkey's milk (Fig. 5a) namely Q, R and S, identified after 15% SDS-PAGE and N-terminal sequence as whey proteins, as well as other peaks identified as caseins. The electrophoretic separation of the peaks eluted by RP-HPLC is shown in Fig. 5b: peak Q with a molecular weight of 14 kDa proved to be lysozyme (sequence, KVFSKXELA), peak R was identified as α-lactalbumin, molecular weight 14.1 kDA (sequence, KQFTKXELSQVLXSM), and peak S with a molecular weight of 22.4 kDa was β-lactoglobulin (sequence TNIPQTMQ). The molecular weights calculated in this work for lysozyme, α-lactalbumin and β-lactoglobulin are in good agreement with those reported for donkey's milk by Herrouin et al. (2000).

In order to obtain a better separation of the caseins fraction, a RP-HPLC was performed on the donkey's milk precipitated caseins. Five peaks were recovered (from T to X, Fig. 6a) each of them submitted to 13% SDS-PAGE (Fig. 6b) The protein amount of peak T (a widespread band of 35.8 kDa) and peak U (the main band of 30.1 kDa) was not sufficient for their identification by N-terminal sequence; peak V showed a more intense band with molecular weight of 33.3 kDa, identified after sequencing as α_{S1}-casein (RPKLPHRQPE) and an unidentified weaker band of 35.1 kDa. Both peak W and peak X, with a molecular weight of 37.5 kDa, were identified as β-caseins. Furthermore, the β-casein sequence of peak X (REKEALNV) showed an $E \rightarrow A$ substitution in the fifth aminoacid. The minor absorbance (measured at 280 nm) of the casein fraction observed after RP-HPLC of skimmed milk with respect to the whey protein fraction may be due to the very low percentage of aromatic residue present in the caseins with respect to the whey proteins.

3.4. Lysozyme and whey protein content determination

The concentration of the total whey proteins (determined by the method of Bradford) was 7.5 mg/ml. The

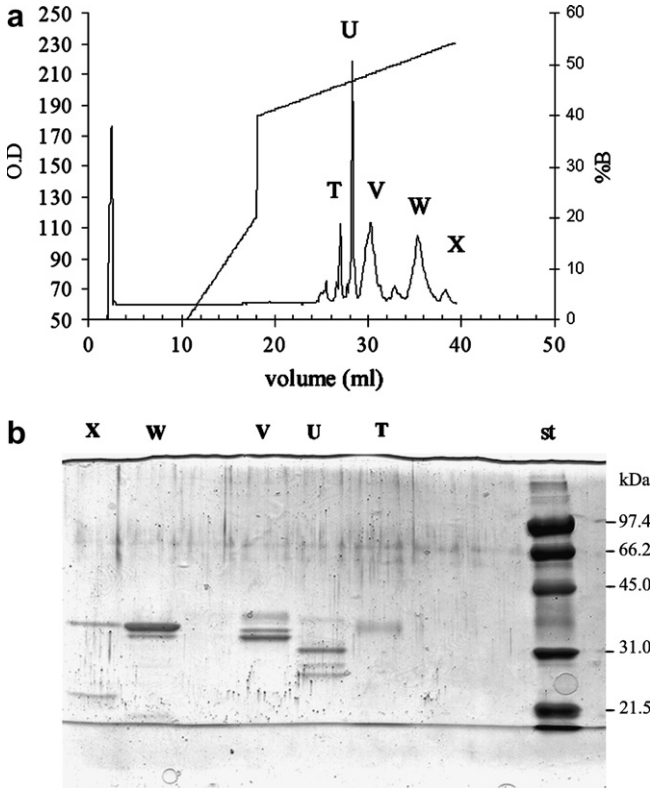


Fig. 6. (a) Reversed-phase HPLC of whole caseins precipitated at pH 4.6. (b) 13% SDS-PAGE of peaks T, U, V, W and X eluted from RP-HPLC. (st) Bio-Rad low molecular weight standard (97.4 kDa, phosphorylase b; 66.2 kDa, bovine serum albumin; 45.0 kDa, ovalbumin; 31.0 kDa, carbonic anhydrase; 21.5 kDa, soybean trypsin inhibitor; 14.4 lysozyme).

Table 2
Quantitative determination of lysozyme, β-lactoglobulin, α-lactalbumin in different stages of lactation

Days after parturition	Lysozyme (mg/ml)	β-lactoglobulin (mg/ml)	α-lactalbumin (mg/ml)
60	1.34	Not determined	0.81
90	0.94	4.13	1.97
120	1.03	3.60	1.87
160	0.82	3.69	1.74
190	0.76	3.60	1.63

lysozyme, β-lactoglubulin and α-lactalbumin concentrations (in mg/ml) calculated quantitatively by the RP-HPLC method at different stages of lactation (60, 90, 120, 160 and 190 days after parturition) are shown in Table 2. The lysozyme content was variable during the different stages of lactation with a marked decrease 160 days after parturition. The mean content of lysozyme in donkey's milk was about 1.0 mg/ml. In human milk, a quantitative determination of lysozyme, performed during the entire period of lactation showing a considerable variability of the lysozyme content (Lewis-Jones, Lewis-Jones, Connoly, Lloyd, & West, 1985; Peitersen, Bohn, & Andersen, 1975). The β-lactoglubulin content in mg/ml proved to be stable during the different stages of lactation with a mean value of about 3.75 mg/ml. Finally, the donkey's milk α-lactalbumin content 60 days after parturition was 0.81 mg/ml and increased two-fold 90 days after parturition, remaining relatively stable with a mean of about 1.8 mg/ml until the last stage of lactation.

4. Conclusions

The present study has increased our knowledge about the "donkey milk" product. Through three different chromatographic approaches, followed by SDS-PAGE under reducing condition, it was possible to separate and identify α_{S1}-caseins having N-terminal sequence RPKLPHRXPE and variable molecular weights: 30.0, 31.3, 33.0 kDa, and β-caseins, having N-terminal sequence REKEELNVS and molecular weights varying from 33.0 to 37.5 kDa. All the donkey's milk proteins identified in this study are summarized in Table 3.

The molecular mass of each class of caseins in equids is in the range of 19.0–25.0 kDa (Miranda et al., 2004), but in the present study they showed a molecular mass ranging from 30.0 to 37.5 kDa. Nevertheless, an abnormal behaviour of monomeric caseins in Laemmli gels was observed in earlier reports (Basch, Douglas, Procino, Holsinger, & Farrel, 1985). Actually, it was not possible to determine in donkey's milk the presence of other types of caseins, such as α_{S2}-, γ- and κ- that were found, although in a small amount, in mare's milk. It may be that these caseins in donkey's milk are present in minute, and therefore undetectable quantities, making their characterization difficult. On the other hand, in the separation approach described here, the caseins were purified by a series of chromatographies followed by SDS-PAGE (under reducing condition). It is possible that α_{S2}- and κ-caseins, not identified here, could be separated by different approaches (see Egito et al., 2001, 2002). However, the presence of α_{S2} casein in human milk has not been demonstrated (Miranda et al., 2004). Studies involving other chromatographic procedure of separation are in progress in order to further define the composition and characterization of donkey's milk caseins.

The amount of lysozyme in donkey's milk varied considerably during the different stages of lactation, with a mean value of 1.0 mg/ml, and proved to be higher with respect to that in bovine (traces), human (0.12 mg/ml) and goat's milk (traces), whereas, it was very close to mare's milk (0.79 mg/ml), as observed by Stelwagen (2003). The mean β-LG content in donkey's milk (3.75 mg/ml) was very close to that of bovine milk (3.3 mg/ml) and pony mare's milk (3.0 mg/ml), whereas in human milk the β-LG is absent (Chatterton et al., 2004; de Wit, 1998; Miranda et al., 2004). During the different stages of lactation, the β-lactoglobulin content remained invariable.

The α-lactalbumin content showed a marked increase three months after parturition to reach the value of 1.8 mg/ml, that remained almost stable during the remaining period of lactation. This result obtained for donkey's milk is in good agreement with the α-lactalbumin content

Table 3
Donkey's milk proteins identified by three proteomic approaches

Chromatographic peak	Protein	Molecular weight (kDa)	N-terminal sequence
A (cationic exchange, pH 5.5)	β-Casein	35.4	REKEELNVS
B (cationic exchange, pH 5.5)	–	–	–
C (cationic exchange, pH 5.5)	Probable α_{S1}-casein	33.0	–
	Probable α_{S1}-Casein	30.7	–
D (cationic exchange, pH 5.5)	α_{S1}-Casein	33.0	RPKLPHRQPE
	α_{S1}-Casein	30.7	RPKLPHRQPE
E (cationic exchange, pH 5.5)	α_{S1}-Casein	33.0	RPKLPHRQPE
	α_{S1}-Casein	30.7	RPKLPHRQPE
F (cationic exchange, pH 7.0)	β-Casein	33.3	REKEELNVS
G–H (cationic exchange, pH 7.0)	–	36.1	–
	Probable β-Casein	35.4	–
	Probable α_{S1}-Casein	31.3	–
	Probable α_{S1}-Casein	29.4	–
I (cationic exchange, pH 7.0)	α_{S1}-Casein	31.3	RPKLPHRQPE
	α_{S1}-Casein	29.4	RPKLPHRQPE
J (cationic exchange, pH 7.0)	α_{S1}-Casein	29.4	RPKLPHQPE
K (chromatofocusing, pH 6–4)	–	34.5 ÷ 28.7	–
L (chromatofocusing, pH 6–4)	–	29.9	–
M (chromatofocusing, pH 6–4)	Probable β-Casein	35.4	–
N (chromatofocusing pH 7–5)	β-Casein	35.4	REKEELNVS
	–	31.2	–
O (chromatofocusing, pH 6–4)	–	–	–
P (chromatofocusing, pH 6–4)	β-Casein	35.4	REKEELNVS
	–	31.2	–
Q (reversed phase)	lysozyme	14.6	KVFSKXELA
R (reversed phase)	α-Lactalbumin	14.1	KQFTKXELSQVLXSM
S (reversed phase)	β-Lactoglobulin	22.4	TNIPQTMQ
T (reversed phase)	–	35.8	–
U (reversed phase)	–	30.1	–
V (reversed phase)	α_{S1}-Casein	33.3	RPKLPHQPE
	–	35.1	–
W (reversed phase)	β-Casein	37.5	REKEELNVS
X (reversed phase)	β-Casein	37.5	REKEALNVS

in human milk (1.6 mg/ml) but is very low compared to the pony mare's α-lactalbumin content (3.3 mg/ml). Table 3 gives a comparison of the relative amounts of the whey proteins and of the total caseins in human, donkey's and mare's milk.

On the basis of the results obtained in our studies, donkey's milk, as well as mare's milk as indicated by other authors (Doreau, 1994; Doreau & Boulot, 1989), could be considered suitable for feeding young children affected by severe Ig-E mediated cow's milk allergy.

Both mare's and donkey's milk have been widely used to replace human milk in the past, because chemical composition and especially protein content are close to that of human milk; the high lysozyme content found in donkey's milk may be responsible for the low bacterial count reported by Salimei et al. (2004) and could be useful to prevent intestine infections in infants.

However, further clinical challenges by using donkey's milk in children affected by cow's milk protein allergy (CMPA) should be performed in order to better evaluate the effects of donkey's milk in children with confirmed CMPA, and to compare in these patients the effects of donkey's milk with the effects of dairy cow's milk or infant formulae, normally based on dairy cow's milk.

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