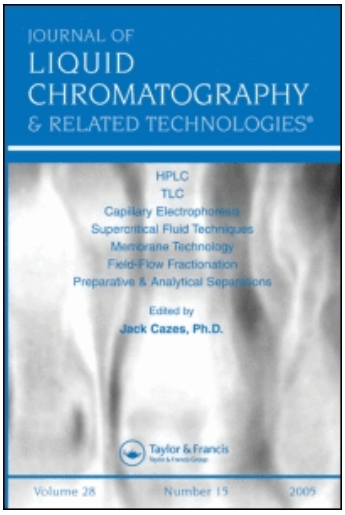


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Chromatographic Separation and Quantification of Major Human Milk Proteins

Isabel M. P. L. V. O. Ferreira[a]
[a] REQUIMTE-Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal

**To cite this Article** Ferreira, Isabel M. P. L. V. O.(2007) 'Chromatographic Separation and Quantification of Major Human Milk Proteins', Journal of Liquid Chromatography & Related Technologies, 30: 4, 499 — 507
**To link to this Article: DOI:** 10.1080/10826070601093796
**URL:** http://dx.doi.org/10.1080/10826070601093796

*Journal of Liquid Chromatography & Related Technologies*®, 30: 499–507, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601093796

# Chromatographic Separation and Quantification of Major Human Milk Proteins

**Isabel M. P. L. V. O. Ferreira**
REQUI*M*TE-Serviço de Bromatologia, Faculdade de Farmácia
da Universidade do Porto, Porto, Portugal



**Abstract:** High Performance liquid chromatography using a reverse phase column Chrompack P 300 RP, which contains a polystyrene-divinylbenzene copolymer based packing (8 μm, 300 Å, 150 × 4.6 mm i.d.) was applied to separate human casein and α-lactalbumin. The chromatographic separation was performed rapidly with this HPLC/UV method.

Calibration curves were constructed using human casein and α-lactalbumin. Linearity between protein concentrations and UV absorbance at 280 nm was maintained over the concentration ranges of 0.09–1.5 and 0.45–3.6 g/L, respectively. Caseins were collected, after preparative HPLC separation, concentrated and lyophilised for further use as standards. The concentration of α-lactalbumin was estimated by comparison with a commercial standard. The correlation coefficient for each standard curve invariably exceeded 0.99 for the two proteins.

The validity of the method was verified. Sample dilution with water proved to be a simple and adequate method for sample preparation. Repeatability was evaluated by ten consecutive injections and human milk. The RSD values for concentrations were all below 3.11%. Recovery studies were carried out to determine the accuracy of the method. Recoveries ranged between 85 and 97%.

**Keywords:** Human milk, Proteins, Caseins, α-Lactalbumin, HPLC

Address correspondence to Isabel M. P. L. V. O. Ferreira, REQUI*M*TE-Serviço de Bromatologia, Faculdade de Farmácia da Universidade do Porto, Rua Anibal Cunha 164, 4099-030, Porto, Portugal. E-mail: isabel.ferreira@ff.up.pt

## INTRODUCTION

Human milk proteins are of nutritional and physiological significance to the newborn infant. The protein composition of human milk is different from that of bovine milk.[1,2] One major difference is the level of protein, with mature milk containing 9–11 g/L in contrast to bovine milk which contains around 33 g/L. Although some of the protein components of human and bovine milk are similar, sequence homology and concentrations are often different.

Human milk $\alpha$-lactalbumin has a high nutritional value with an amino acid composition adapted to the requirements of newborns.[3] It is not identical to bovine milk $\alpha$-lactalbumin, although there is a 72% sequence homology. The complete amino acid sequences for bovine and human $\alpha$-lactalbumin have been published.[4,5]

Human milk casein, another important nutritionally available protein, is present in casein micelles. It represents the least understood class of proteins in human milk, in spite of several studies that have been carried out.[6–11] It is known that, the function of human milk micelles of caseins is to deliver nutrients, particularly insoluble minerals such as the necessary calcium phosphate, and essential amino acids. Important differences exist between human and bovine caseins. $\beta$-casein is the predominant casein in human milk, at an average concentration of 5 g/L of milk, whereas cow's milk contains a large proportion of $\alpha$-caseins.[2,7,12,13]

The amino acid sequence of human $\beta$-casein has been established by protein sequencing using recurring Edman degradation and by deduction from nucleotide sequence.[14] Comparison of the sequences of bovine and human $\beta$-caseins requires alignment of the N-terminal extremity of the human protein with residue 10 of bovine $\beta$-casein. Homology is then observed between the two proteins, which share 47% of identical residues.[15] Human casein also differs from cow's casein in its physicochemical properties, i.e., the precipitate formed by human casein at pH 4.6 is looser and softer than that formed by bovine casein.[16] The marked differences in the content and in the casein subunits between human and bovine species makes it unlikely that methods for analyzing bovine casein, such as adjustment of milk to pH 4.6 followed by centrifugation, can easily be used for human milk. It is known that, acid precipitation of human milk casein leads to various amounts of coprecipitating whey proteins.[17,18]

The evolution of the proportions of $\alpha$-lactalbumin and $\beta$-casein through the lactating period is still unknown, although several studies have shown dramatic changes in their amounts.[6,19] Thus, $\alpha$-lactalbumin and $\beta$-casein contents during lactation is still a point of controversy. The lack of a thorough and dynamic study might come from the multiple techniques reported for assaying the levels of these proteins in the complex medium that is human milk.

$\alpha$-Lactalbumin is frequently quantified by chromatographic methods such as fast protein liquid chromatography, gel filtration, anion-exchange, and

reversed phase chromatography with UV detection.[20–22] Methods currently used to separate and quantify human caseins, especially for β-casein, include FPLC, isoelectric precipitation, and microparticle enhanced nephelometric immunoassays.[8,23–25] Most of these methods were developed primarily to analyze bovine casein.

The development of rapid and reliable analytical methods to study human milk composition during lactation is useful. Thus, the goal of the present work was to develop and validate an HPLC/UV method for separation and quantification of human α-lactalbumin and β-casein.

## EXPERIMENTAL

### Sampling and Sample Preparation



A total of 10 milk samples were collected from healthy lactating mothers randomly chosen after delivery of full term infants. Milk was collected in a sterile polypropylene tube (10 mL), half at the beginning of a feeding and half at the end of feeding. The first five samples (numbered from 1 to 5) were collected from five different mothers when the infants were an age of one and a half months, the other five samples (numbered from 6 to 10) were collected from the same mother of sample 1, after 2, 3, 4, 5, and 6 months of lactation. All milk samples were frozen immediately after collection and stored at −20°C until use. They were thawed at 40°C in a water bath and vigorously homogenized immediately before their analysis. After that, the samples were skimmed by centrifugation at 700 g, at 4°C, for 10 min, the lipid layer was removed by siphoning with a pipette, and a portion of the aqueous phase was diluted 1/5 with deionised water. The protein fraction profile was performed by HPLC with the method described previously. Samples were not filtered prior to analysis because previous data suggested that α-lactalbumin could adhere to the filter.

### Instrumentation

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco) equipped with two type PU-980 pumps, a type UV-970 detector, and an auto sampler model AS-950. The column was a reversed phase Chrompack P 300 RP column that contains a polystyrene-divinylbenzene copolymer based packing (8 μm, 300 Å, 150 × 4.6 i.d.). The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used.

Lyophilization was carried out using a Labconco 4.5 apparatus (Kansas City, Mo).

## Separation Conditions

Gradient elution was carried out with a mixture of two solvents.[26] Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in 95% acetonitrile-5% water. Proteins were eluted with the following gradient: 0–5 min, 36–43% B; increasing to 44% during 3 min; 8–13 min, 44–50% B; 13–15 min returning to initial conditions (50–36% B), in a total run time of 15 min. The flow rate was 1.0 mL/min. The column was used at ambient temperature, detection was at 280 nm, and 100 μL were injected.

## Reagents and Proteins Standards

All reagents used were of analytical grade purity. Solvents for HPLC were filtered through 0.22 μm NL 17 filters (Teknokroma, Madrid, Spain) and degassed under vacuum for at least 15 min before use. Human α-lactalbumin was supplied by Sigma Chemical Co., it had a minimum purity of 90% (according to Sigma).

β-Casein separated from human milk was collected after HPLC separation, lyophilised, and used as standard for preparing calibration curves. Protein standards were dissolved in a mixture of 70% water and 30% of acetonitrile (v/v).

## Purification of Human Milk Casein

Human β-casein was prepared from breast milk collected during the first month of lactation from a female volunteer. β-casein was prepared according to the method of Kunz and Lonnerdal.[7,8] Milk was defatted and acidified to pH 4.3 using 1 M HCl. Calcium chloride was then progressively added to a final concentration of 60 mM Ca2+ in acidified milk. After ultracentrifugation at 189.000 × g for 1 h at 4°C, whole casein was recovered in the supernatant and further purified by RP-HPLC. Two major peaks with retention time around 6 and 8 min were collected and freeze dried. The first peak was identified as β-casein by amino acid analysis using an automatic analyzer (Applied Biosystem LC 491 Protein Sequencer, Foster City, U.S.A.) after acid hydrolysis (110°C, 24 h, 5–7 N HCl, under vacuum), and by sequencing of the five N-terminal amino acid residues by recurring Edman degradation. The second peak was identified as α-lactalbumin by comparison with the retention time of the standard solution.

## Statistical Analysis

Data are presented as the mean ± standard deviation. The results were statistically analyzed by analysis of variance (ANOVA). Differences were considered significant for $p < 0.05$. Statistical analyses were all performed with SPSS for Windows version 14 (SPSS Inc, Chicago, IL).

## RESULTS AND DISCUSSION

### Validation of the Method

Figures 1 and 2 show chromatograms of a standard mixture of $\alpha$-lactalbumin and $\beta$-casein, and a human milk sample, respectively.

#### Linearity

The external standard method was used to calibrate the chromatographic system for the quantification of $\beta$-casein and $\alpha$-lactalbumin. Calibration curves were constructed using human casein obtained by the procedure described above, and a commercial standard of human $\alpha$-lactalbumin. Linearity between the concentration of human $\beta$-casein and $\alpha$-lactalbumin and the UV absorbance at 280 nm was maintained over the concentration ranges of 0.09–1.5 and 0.45–3.6 g/L, respectively. The values of the slope, intercept, and correlation coefficient are given in Table 1.

#### Limits of Detection

The value of the detection limit was calculated as the concentration corresponding to three times the standard deviation of the background noise and was 0.02 g/L for human casein, and 0.009 g/L for $\alpha$-lactalbumin.

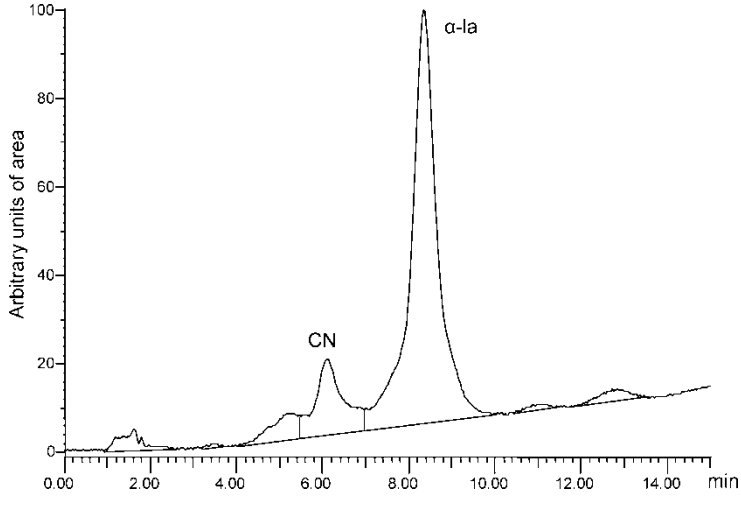


***Figure 1.*** Typical chromatographic profile of a mixture of human milk protein standards (chromatographic conditions described in the text): CN – casein (0.2 g/L); $\alpha$-la – $\alpha$-lactalbumin (1.0 g/L).

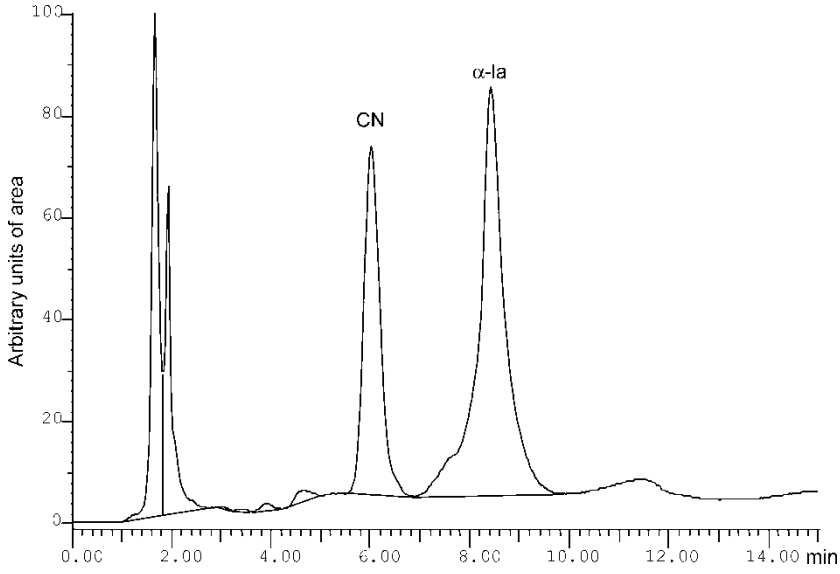


***Figure 2.*** Typical chromatographic profile of human milk proteins (chromatographic conditions described in the text): CN – casein; $\alpha$-la – $\alpha$-lactalbumin.



## Precision

Sample dilution with water proved to be a simple and adequate method for sample preparation. Repeatability was evaluated by ten consecutive injections of human milk. The RSD (relative standard deviation) values for concentrations were all below 3.11 and 1.68%, respectively, for human casein and $\alpha$-lactalbumin.

## Recovery

The reliability of the method was confirmed by three recovery experiments. Recovery studies were carried out to determine the accuracy of the method. Recoveries ranged between 89 and 97% (Table 2).

No loss in performance of the method or the column due to matrix contamination (e.g. sugars) was observed during this study. Also, the use of

***Table 1.*** Calibration curves determined by external standard method (n = 5)

| Protein | Concentration range (g/L) | Slope (area counts/mg) | Intercept (area counts) | $r^2$ |
|---|---|---|---|---|
| Human casein | 0.09–1.5 | $3.0\ (\pm 0.2) \cdot 10^4$ | $1.2\ (\pm 0.16) \cdot 10^3$ | 0.9988 |
| $\alpha$-Lactalbumin | 0.45–3.6 | $7.4\ (\pm 0.4) \cdot 10^5$ | $2.7\ (\pm 0.17) \cdot 10^4$ | 0.9999 |

***Table 2.*** Results for the recoveries obtained by the standard additions method performed on sample 1

| Human protein | Conc[a] (g/L; n = 3) | Recovery (%) |
|---|---|---|
| Casein | 0.5 | 95 |
| Casein | 0.8 | 97 |
| $\alpha$-Lactalbumin | 1.0 | 89 |
| $\alpha$-Lactalbumin | 2.5 | 95 |

[a]Concentration of protein added to the samples before analysis.

***Table 3.*** Mean casein and $\alpha$-lactalbumin contents of human milk (5 samples from different mothers with one and half months lactation, and 5 samples from the same mother from 2 to 6 months lactation)

| Samples | CN (g/L) | $\alpha$-la (g/L) |
|---|---|---|
| 1 | 3.68 $\pm$ 0.02 | 4.09 $\pm$ 0.03 |
| 2 | 3.54 $\pm$ 0.01 | 3.70 $\pm$ 0.02 |
| 3 | 2.99 $\pm$ 0.02 | 2.28 $\pm$ 0.01 |
| 4 | 3.12 $\pm$ 0.04 | 3.81 $\pm$ 0.02 |
| 5 | 4.37 $\pm$ 0.02 | 6.33 $\pm$ 0.03 |
| 6 | 3.52 $\pm$ 0.02 | 3.79 $\pm$ 0.05 |
| 7 | 3.02 $\pm$ 0.01 | 3.54 $\pm$ 0.03 |
| 8 | 2.99 $\pm$ 0.02 | 2.53 $\pm$ 0.02 |
| 9 | 2.41 $\pm$ 0.04 | 1.95 $\pm$ 0.01 |
| 10 | 2.21 $\pm$ 0.01 | 1.60 $\pm$ 0.01 |



TFA in mobile phase did not have adverse effects on the long term stability of the method.

Table 3 presents the mean contents of major proteins in ten human milk samples (five samples with one and a half months of lactation and five samples from the same mother at different lactation times). ANOVA analysis showed significant differences ($p < 0.05$) between protein content in milk from different mothers at the same lactation time (one and a half months). A significant decrease ($p < 0.05$) was observed for the casein and $\alpha$-lactalbumin concentrations during the first six months of lactation.

## CONCLUSIONS

The method, herein validated, proved to be adequate to separate and characterize $\beta$-casein and $\alpha$-lactalbumin fractions of human milk and to quantify milk protein contents. In the future, this method will be applied by our research group to study the evolution of human milk casein and $\alpha$-lactalbumin during lactation.

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Received October 13, 2006
Accepted November 13, 2006
Manuscript 6967