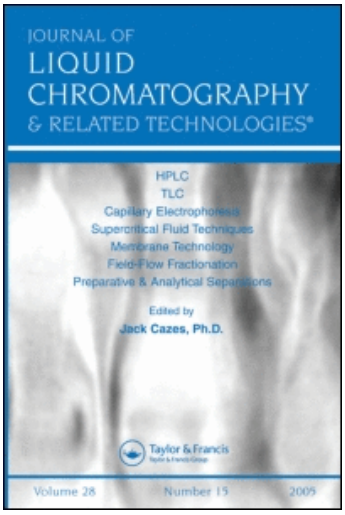

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DEVELOPMENT, VALIDATION, AND APPLICATION OF AN HPLC/UV METHOD FOR QUANTIFICATION OF CASEIN IN INFANT FORMULAE AND FOLLOW-UP MILKS

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ABSTRACT

A simple, rapid, and accurate HPLC method was used to determine the casein content in infant formulae and follow-up milks. The proposed methodology used a Chrompack 300 RP chromatographic column and an UV detector. Gradient elution was carried out with a mixture of two solvents. Solvent A was trifluoroacetic acid in water and solvent B was acetonitrile-water-trifluoroacetic acid.

A linear relationship between the concentration of casein and the UV absorbance at 280 nm was obtained. This linearity was maintained over the concentration range 0.04-2.0 mg/mL. Preliminary studies made on processed milks showed that casein content in those products was not affected by heat treatment when compared with raw milk casein content. Hence, the quantification of casein in other milk products could be possible using this chromatographic column.

The detection limit value was 0.02 mg/mL. The validity of the method was verified. For recovery studies several determinations were carried out. Four samples were analysed before and after the addition of known amounts of casein. Recovery values ranged

between 80 and 98%. The precision of the method was also evaluated, and the %CV found was less than 2%.

The developed methodology was applied to the monitorization of casein in twelve samples of infant formulae and follow-up milks. The mean values obtained were 7.5 ± 2.8 (n=14) and 16.6 ± 3.7 (n= 10), respectively.

INTRODUCTION

The protein content of human milk presents qualitative and quantitative differences when compared with that in cow's milk. Therefore, reduction of the protein content and alteration of the casein/whey protein ratio are the first important modifications that industry has to make to cow's milk to prepare infant formulae with a composition closer to that of human milk.[1] This reduction of protein content is due mainly to decrease in casein content. Follow-up milks are given to infants after 4-6 months of age to make the transition from human milk or infant formulae to cow's milk. Their protein content is greater than that of the infant formulae, but lower when compared to cow's milk protein content. Quality control of infant formulae and follow-up milks and the respective labelling, urges the need for economic, time-saving, and accurate methods to be developed. Over the past few years, milk proteins have been separated by gel electrophoresis,[2] ion-exchange chromatography,[3] gel permeation chromatography,[4] reverse-phase chromatography,[5] and, more recently, by capillary electrophoresis.[6] Total protein content in milk can be determined by a variety of methods, including Kjeldahl,[7] dye-binding colorimetry,[8] and infrared spectrophotometry.[9] Each method has its own merits, but the use of HPLC, enable good separations and very high resolutions.

The aim of this work was to develop and validate a rapid and accurate HPLC method for the determination of casein in infant formulae and follow-up milks. Caseins are phosphoproteins that consist of subspecies -, -, -, and - casein and are assembled in an aggregate of micelles.[10] The proposed methodology employs a Chrompack 300 RP chromatographic column. In this type of column the separation is based on hydrophobic interactions, a phenomenon of great biological significance because they are one of the main forces that stabilize the three-dimensional structure of proteins.

EXPERIMENTAL

Chromatography

The chromatographic analysis was carried out on a Gilson, high performance liquid Chromatograph (Gilson Medical Electronics, Villiers le Bel,

France) equipped with a type 305 pump, a type 302 pump and a type 7125 Rheodyne Injector with a 20 μL loop. A Gilson 118, variable long wave ultraviolet detector was also used. The chromatographic separation was achieved with a Chrompack 300 RP Chromatographic column, 10 μm. The integrator used was a Gilson 712 HPLC System Controller Software. Gradient elution was carried out with a mixture of two solvents. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 95% acetonitrile-5% water- 0.1% trifluoroacetic acid. Elution was performed at a solvent flow rate of 1.0 mL/min with linear gradient from 36% of B to 47% of B within 5 min, keeping these conditions for 2 min., followed by another linear gradient from 47% of B to 52% of B within 5 min and returning to the initial conditions within 2 min. The effluent was monitored at 280 nm.

Materials

Bovine milk casein and purified α-, β- and κ-casein were obtained from Sigma, Chemical Co. All reagents used for the preparation of solvents were p.a. grade (Merck). The solvents were prepared, freshly, with double deionized water and filtered through a 0.45 μm filter by means of an all glass Millipore filtration unit.

Standards containing 0.04; 0.06; 0.08; 1.00; 1.50; 2.00; 2.50 mg of casein/mL were prepared by dissolution of bovine casein in a mixture of 50% solvent A and 50% solvent B.

Sampling

Three samples of raw milk were obtained directly from the producer.

Nine samples of processed cow's milk which included brands of pasteurised, UHT-treated, and powdered milks, were randomly purchased from the market and used in the preliminary trials.

Monitorization of casein content in adapted milk formulae was performed on twelve brands, which included seven brands of infant formulae and five brands of follow-up milks. The respective powdered milk samples were reconstituted with deionized water according to the manufacturers instructions.

All samples were skimmed by centrifugation (5 min, 5000 r.p.m.), filtered using Whatman paper nº52, and subsequently diluted with deionized water to obtain a casein content within the linearity of the method.

Statistical Analysis

Data are represented as the mean ± standard deviation.

Analysis of variance was used to determine the effects of heat treatment on protein "native" conformation and was also used to determine the effects of type of brand, on the one hand, and type of formulation on the other, on the casein content. Fisher's protected least significant difference *t*-test (PLSD), at the 5% significance level, was applied to perform all possible pair-wise comparisons. All statistical analyses were done with the Statview™ 4.0 statistical package (Abacus Concepts, Berkeley, CA 94704-1038, USA).

RESULTS AND DISCUSSION

Standard Calibration Curves

Typical chromatograms for casein separation are shown in Figure 1a, which depicts the separation of an aqueous standard solution of casein and Figure 1b which illustrates the separation of casein in an infant formula sample.

Two peaks were obtained when casein standard was injected as apparent from inspection of Figure 1. The first peak was a mixture of α and κ caseins and the second peak was owed to β-casein. This identification was based on the comparison of these retention times with those of the corresponding standards of different purified casein fractions. Since our principal objective was the determination of the casein content of milk and adapted milk formulae the linearity and validation of the method was applied only to the total casein content, namely, the sum of the two peaks.

A linear relationship between concentration of casein and UV absorbance at 280 nm was obtained. This linearity was maintained over the concentration range of 0.04-2.5 mg/mL of casein (r=0.9994). The value of the detection limit, calculated as the concentration corresponding to three times the standard deviation of the background noise, was 0.02 mg/mL.

Preliminary Studies

Analysis made on raw and processed milks (including powdered milk) suggested that casein quantification in those products was not affected by heat treatment. Similar concentrations of casein were obtained between raw milk samples and raw cow's milk samples, heated up to boiling point during 2 min. and immediately cooled down to room temperature by placing in ice until moment of analysis (Table 1).

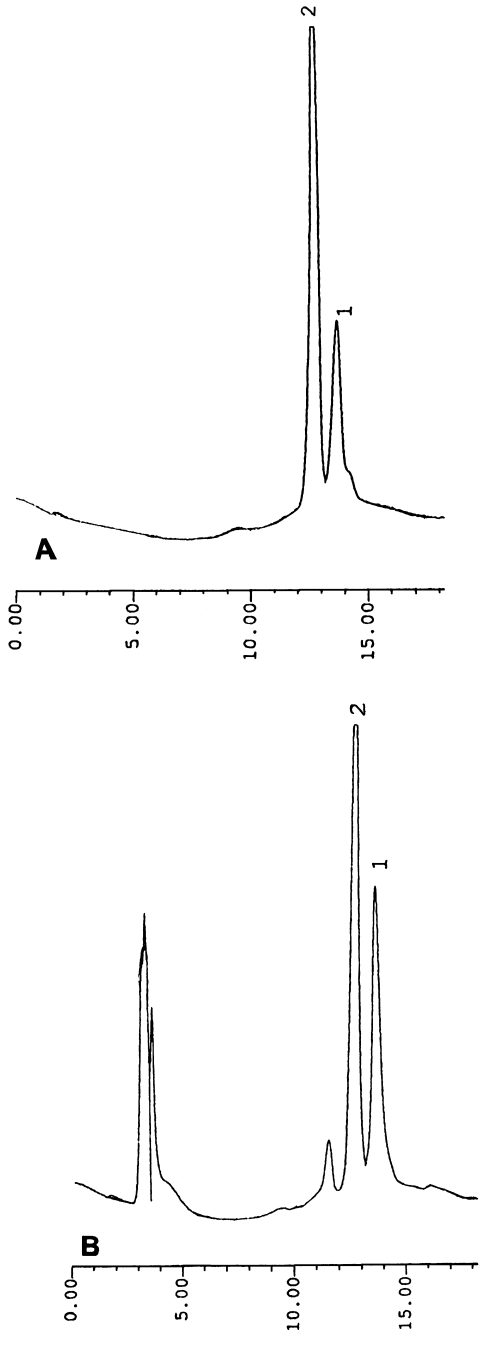

Figure 1. Typical chromatograms for (a) separation of an aqueous standard solution of casein (concentration 1.5 mg/mL) and for (b) separation of casein in an infant formula sample. 1- β-casein; 2 – sum of other casein fractions.

Table 1

Casein Composition of Raw and Differently Processed Cow's Milk[a]

Type of Milk	Casein Content (mg/mL)
Raw milk	27.9 ± 2.7
Boiled raw milk	29.7 ± 3.3
Pasteurised	27.2 ± 1.9
UHT treated	27.6 ± 3.1
Powdered (after reconsitution	28.8 ± 2.9

[a] Values are expressed as mean ± standard deviation of three samples analyzed in duplicate (n=6).

No significant differences were observed between the chromatographic behaviour of "native" and "denatured" states of casein when analysed by ANOVA methodology ($p>0.05$). Thus, the quantification of casein in those milk products could be possible using this chromatographic column.

Validation of the Method

Recovery studies which were carried out to determine the accuracy of the method used one infant formula and one follow-up milk; these were each spiked with 3 different concentrations of casein, namely, 2.0, 5.0, and 10.0 mg/mL. The results obtained thereof, are listed in Table 2. Recoveries ranged between 80.0 and 98.0% as apparent from inspection of Table 2.

The precision of the analytical method was evaluated by measuring the peak chromatographic area 10 times on the same brand of infant formula. The CV% was less than 2%.

This method features a rapid and convenient separation, together with a high degree of precision and accuracy and seems to be suitable for routine analysis. The short analysis time of 18 min is a good advantage relative to other HPLC methods.[5]

Table 2

Recovery of Casein from an Infant Formula Sample[a] and from a Follow-up Milk[b]

Present (mg/mL)	Added (mg/mL)	Found[c] (mg/mL)	Standard Deviation	CV (%)	Recovery (%)
	2.00	10.03	0.21	2.09	92.0
8.19[a]	5.00	12.69	0.29	2.28	90.0
	10.0	17.99	0.30	1.67	98.0
	2.0	17.20	0.41	2.38	80.0
15.6[b]	5.0	20.49	0.26	1.27	97.8
	10.0	24.71	0.34	1.50	91.1

[a] Mean value of triplicate analysis of each studied concentration; [b] follow-up milk; [c] Mean value of triplicate analysis of each studied concentration.

Table 3

Results Obtained in the Monitorization of Casein Content In Infant Formulae[a]

Samples	Casein Content (mg/mL) Found	Labelled
1	9.72 ± 0.2	9.0
2	5.10 ± 0.08	6.0
3	8.19 ± 0.11	7.8
4	4.26 ± 0.08	Not mentioned
5	11.6 ± 0.10	13.0
6	4.43 ± 0.06	5.00
7	8.89 ± 0.12	Not mentioned

[a] Values are expressed as mean ± standard deviation of duplicate determinations.

Table 4

Monitorization of Casein Content in Follow-up Milks[a]

Samples	Casein Content (mg/mL) Found	Labelled
8	13.3 ± 0.15	14.0
9	22.7 ± 0.27	20.2
10	13.9 ± 0.21	14.0
11	15.6 ± 0.10	Not mentioned
12	17.2 ± 0.35	17.5

[a] Values are expressed as mean ± standard deviation of duplicate determinations.

Monitorization of Casein Content in Twelve Samples of Infant Formulae and Follow-Up Milks

Mean results and standard deviations of duplicate determinations of casein content for a variety of commercial infant formulae and follow-up milks are listed in Tables 3 and 4, respectively.

As apparent from Tables 1, 3, and 4 the casein content of cow's milks, infant formulae, and follow-up milks was significantly different among each other. As expected cow's milk presented greater casein concentrations, 27.9 ± 3.9 mg/mL (n= 6) when compared with adapted milk formulae. Infant formulae presented lower casein content 7.5 2.8 mg/mL [although the 7 brands assayed, except sample 4 and 6, showed significant differences ($p<0.05$) between each other] than those of follow-up milks, 16.6 ± 3.7 mg/mL (irrespective of the significant variation between samples, $p<0.05$).

Despite the between brand significant variation, it should be noted that the casein contents of the adapted milk formulae assayed were similar to the levels in the respective label, when mentioned.

CONCLUSIONS

The described procedure is suitable for routine determination of casein content in infant formulae and follow-up milks. Easy handling sample preparation, appropriate accuracy, precision, and rapidity are characteristics of the method. This procedure can possibly be extended to monitoring of the levels of casein in other dairy products.

As expected, the adapted milk formulae contain lower amounts of casein when compared to cows milk composition, especially infant formulae, the composition of which, is intended to be similar to that of human milk.

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