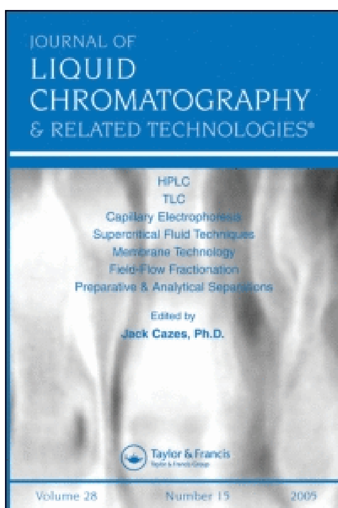
This article was downloaded by:
On: *23 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



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

Determination of Caseinomacropeptide by an RP-HPLC Method and Monitoring of the Addition of Rennet Whey to Powdered Milk

Isabel M. P. L. V. O. Ferreira[a]; M. B. P. P. Oliveira[a]
[a] CEQUP/Serviço de Bromatologia, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

Online publication date: 30 January 2003

To cite this Article Ferreira, Isabel M. P. L. V. O. and Oliveira, M. B. P. P.(2003) 'Determination of Caseinomacropeptide by an RP-HPLC Method and Monitoring of the Addition of Rennet Whey to Powdered Milk', Journal of Liquid Chromatography & Related Technologies, 26: 1, 99 — 107

To link to this Article: DOI: 10.1081/JLC-120017155

URL: http://dx.doi.org/10.1081/JLC-120017155

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 1, pp. 99–107, 2003

Determination of Caseinomacropeptide by an RP-HPLC Method and Monitoring of the Addition of Rennet Whey to Powdered Milk

Isabel M. P. L. V. O. Ferreira* and M. B. P. P. Oliveira

CEQUP/Serviço de Bromatologia, Faculdade de Farmácia,
Universidade do Porto, Porto, Portugal

Downloaded At: 20:27 23 January 2011

ABSTRACT

A precise, sensitive, and reliable RP-HPLC method was developed and validated to enable the separation and quantification of caseinomacropeptide. The optimized method used a polystyrene-divinylbenzene column and gradient elution with two solvents. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 95% acetonitrile-5% water-0.1% trifluoroacetic acid. The flow rate used was 1 mL/min. The effluent was monitored by a UV detector at 214 nm and enabled the separation of two peaks corresponding to the aglyco components of the two principal genetic variants (A and B), eluted after the less well resolved glyco caseinomacropeptide components. The determinations were performed in

*Correspondence: Isabel M. P. L. V. O. Ferreira, CEQUP/Serviço de Bromatologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 4050-047 Porto, Portugal; E-mail: isabel.ferreira@ff.up.pt.

DOI: 10.1081/JLC-120017155
1082-6076 (Print); 1520-572X (Online)
Copyright © 2003 by Marcel Dekker, Inc.
www.dekker.com

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

the linear range of 15–200 μg/mL. Detection limit was 2 μg/mL. The validity of the method was verified. The recoveries were 93 and 98%. The precision of the method was also evaluated being the %CV less than 4.87%.

The method was applied to the analysis of rennet and acid wheys and whey protein concentrates produced by the dairy industry. It was also applied to the detection of rennet whey in powdered milks.

Key Words: Caseinomacropeptide; HPLC/UV; Whey; Powdered milk.

Downloaded At: 20:27 23 January 2011

INTRODUCTION

The caseinomacropeptide (CMP) frequently referred to as the glycomacropeptide (GMP) is found in cheese whey (or rennet whey). It is derived from the action of chymosin on k-casein. It is the more hydrophilic C-terminal portion of the molecule, containing the oligosaccaharides O-linked to threonine and serine residues. CMP has been the subject of growing interest in recent years.[1,2] Because of its several biological activities, and unique aminoacid composition having no phenylalanine, CMP is thought to be a potential ingredient for dietetic foods and pharmaceuticals.

Single-strength whey powders contain about 12 percent protein and 75 percent lactose.[2,3] CMP can potentially account for more than 16% of the protein content. Different new techniques can be used to produce whey protein concentrates (WPC's) with 35, 55, or 80% protein in the dry powder, which can be used as food ingredients in a great variety of whey-based foods. However, its addition must be declared in the label.[4]

On the other hand, dairy products, such as milk powder, should be prepared exclusively from milk and not contain solids from whey. The absence of rennet whey solids from milk powder is required according to legislation.[5] Considering the lower price of rennet whey, it is financially attractive to adulterate milk powder. Thus, the presence of CMP can be a good chemical marker to evaluate milk powder authenticity.[6]

HPLC using ion-exchange, gel permeation, hydrophobic interaction, and reverse-phase separations have become important techniques for separation and quantification of milk proteins as they combine versatility, short analysis time, and high resolution.[7–9] Earlier work had established the use of a polystyrene-divinylbenzene chromatographic column as an analytical tool for milk proteins analyses, as it has excellent chemical and pH stability and is quite robust,[10–12] which is in agreement with other authors.[8]

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:27 23 January 2011

This paper deals with an RP-HPLC procedure, using a polystyrene-divinylbenzene column, optimized and validated to separate and quantify CMP in whey and WPC's. The method can also be used to detect rennet whey in milk powder by CMP measurement.

EXPERIMENTAL

Sampling

Whey protein powders with 35% and 80% protein contents (sample 1—WPC35 and sample 2—WPC80, respectively), produced by the dairy industry were obtained directly from a cheese manufacture.

Raw milk was obtained directly from the producer. Skim milk was prepared by separating the fat from the whole milk by centrifugation at 700 g, at 4°C, during 10 min. Mineral and rennet wheys were made in the laboratory from fresh raw skimmed milk. Mineral acid whey (sample 3) was prepared by isoelectric precipitation of casein at pH 4.6 using hydrochloric acid 1 M. Rennet whey (sample 4) was prepared by chymosin treatment using commercial rennet (60 RU/mL, 25 μL per 100 mL milk) to coagulate the caseins.

Four dry milk powder samples were obtained from commercial sources (numbered as samples 5 and 6) and from a dairy industry (numbered as 7 and 8).

Sample Preparation

Powered milks were reconstituted with deionized water according to the manufacturer's instructions.

CMP and other whey protein fractions were extracted after precipitation of caseins with hydrochloric acid 1 M until pH 4.6, centrifugation (700 g, 10 min) and filtration of supernatant.

Reagents and Protein Standards

All reagents used were of analytical grade purity. Solvents for HPLC were filtered through a 0.22 μm NL 17 filter and degassed under vacuum for at least 15 min before use. Caseinmacropeptide, α-lactalbumin and β-lactoglobulin standards were supplied by Sigma Chemical Co.

The protein standards and whey protein concentrates were dissolved in a mixture of 50% of solvent A and 50% of solvent B (v/v).

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:27 23 January 2011

Reversed-Phase HPLC Separation

The HPLC equipment consisted of a Gilson chromatograph (Gilson Medical Electronics) equipped with a type 302 pump, a type 305 pump, and a type 7125 Rheodyne Injector with a 20 μL loop. A Gilson 118 variable longwave ultra-violet detector was also used. The equipment was controlled by software Gilson 712 that controlled the solvent gradient, data acquisition, and data processing. The column was a reversed-phase column *Chrompack* P 300 RP that contains a polystyrene-divinylbenzene copolymer-based packing (8 μm, 300 Å, 250 × 4.6 i.d.). The column was equilibrated in 80% solvent A (0.1%, v/v, TFA in water) and, after sample injection, a series of linear gradients was applied to 100% solvent B (0.09%, v/v, TFA, 80%, v/v, acetonitrile in water) as follows: 1–6 min, 20–40 B; 6–16 min, 40–45% B, 16–19 min, 45 50% B; 19–20 min, 50% B; 20–23 min, 50–70% B; 23–24 min, 70 100% B. The column was re-equilibrated after a 1 min hold at 100% B by a 2 min linear gradient to 20% B, followed by an isocratic period of 3 min. The flow-rate was 1 mL/min at room temperature. Detection was performed by absorbance measure at 214 nm and total run time was 30 min.

Validation of the Method

The validation of the described method for the determination and quantification of CMP was accomplished by testing the linearity, the precision, and the accuracy.

The recovery of added internal standard to rennet whey was evaluated to determine the accuracy of the test. The whey was analysed in duplicate before and after the addition of CMP in two different concentration levels (0.10 and 0.15 mg/mL).

RESULTS AND DISCUSSION

Separation and Quantification of Caseinomacropeptide in Whey and WPC's

Two peaks corresponding to the aglyco components of the two principal genetic variants (A and B) of CMP (Fig. 1, peaks 1 and 2, respectively) eluted after less resolved glyco-CMP components. A similar chromatographic profile was obtained by other authors.[8]

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

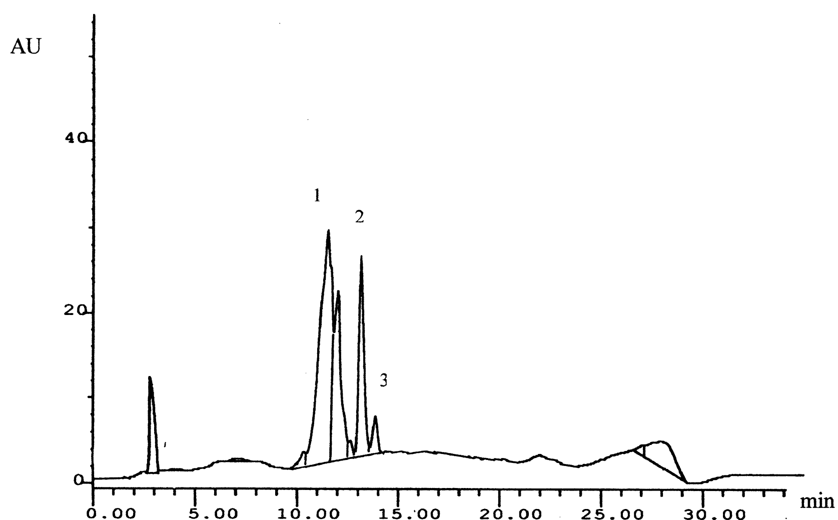


Figure 1. Typical HPLC chromatogram of CMP standard solution. Peak 1: glyco CMP. Peaks 2 and 3: aglyco CMP, A and B variants, respectively.

Downloaded At: 20:27 23 January 2011

The external standard method was used to calibrate the chromatographic system for caseinomacropeptide quantification. For this purpose, standard solutions with concentrations ranging from 15 to 200 μg/mL were used. Each solution was analysed in triplicate. The linearity of the method was checked through the calibration curves and obtained by linear regression of the peak area (sum of peaks 1 and 2) vs. concentration. Coefficient of correlation was 0.9997. The response factor, or slope of standard curve was highly reproducible between runs.

The detection limit value was calculated as the concentration corresponding to three times the standard deviation of the background noise and was 2 μg/mL.

Generally, procedures for CMP determination require trichloroacetic acid precipitation of the other whey proteins, leaving only the CMP in solution.[13] However, it is known that the different non-glycosylated and glycosylated forms of the caseinomacropeptide have different sensitivities to trichloroacetic acid precipitation.[7] This limitation pointed out the need for a method that would be quantitative and suitable for application of analysis of milk samples. The pretreatment of samples with hydrochloric acid 1 M until pH 4.6 was used with success. Recovery studies were carried out to

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

determine the accuracy of the method. Recoveries of 93% and 97% were obtained for two concentration levels, 0.10 and 0.20 mg/mL, respectively.

The precision of this method was evaluated, taking into account its repeatability; the %CV was less than 4.87% ($n = 10$).

CMP in WPC's and whey samples was calculated from integrated peak areas (sum of peaks 1 and 2) using the response factor determined with the standard solutions. Peak identification was carried out by comparison with commercially purified bovine whey protein standards. The results are given in Table 1 and are presented either on an individual concentration basis (whey) or as % powder mass (WPC-35, WPC 80).

Detection of Rennet Whey in Powdered Milks

Downloaded At: 20:27 23 January 2011

Figure 2 shows the RP-HPLC elution patterns of soluble proteins in genuine and adulterated powdered milks, using the conditions and gradient described above. Using the reference proteins, α-lactalbumin and β-lactoglobulin were identified as eluted near 22 and 25 RT, respectively. Peaks eluted at 6 and 10 RT were unidentified, but might correspond to proteose-peptone derived from degradation of β-casein in milk and recovered in whey. Only the powdered milk numbered as sample 7 presented the peaks characteristic of CMP, which indicate adulteration by the addition of rennet whey. When injecting the same sample with detection set at 280 nm, CMP peaks were not present. The absence of absorption at 280 nm is a characteristic of CMP,[6] and confirmed the adulteration by rennet whey addition.

Table 1. RP-HPLC determination of CMP in acid whey, rennet whey WPC35 and WPC80 samples.

Sample	Description	CMP content
1	WPC35	5.61[a]
2	WPC80	12.9[a]
3	Acid whey	0.20[b]
4	Rennet whey	1.42[b]

[a]Mean values expressed as % powder mass.
[b]Mean values expressed as mg/mL.

Marcel Dekker, Inc. • 270 Madison Avenue • New York, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

Downloaded At: 20:27 23 January 2011

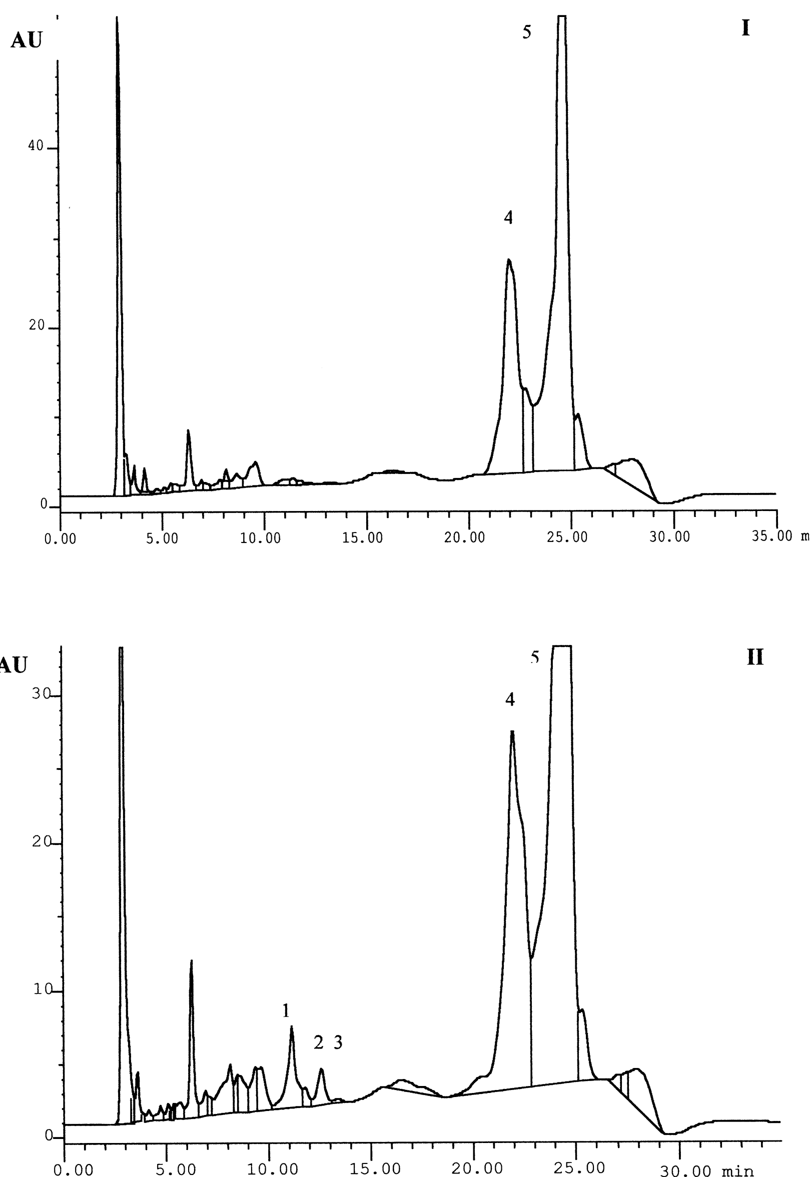


Figure 2. Typical HPLC chromatograms obtained for a genuine (I) and an adulterated milk sample (II). Peak 1: glyco CMP. Peaks 2 and 3 refer to aglyco CMP, A and B variants, respectively. Peak 4 refers to α-lactalbumin and peak 5 is from β-lactoglobulin.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016
©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

CONCLUSIONS

The described HPLC/UV procedure is suitable for routine separation and quantification of caseinomacropeptide in whey and WPC's powders. Appropriate accuracy, precision, and rapidity are characteristics of the optimised method. It can also be a useful tool to detect the adulteration of powdered milk with rennet whey.

ACKNOWLEDGMENT

The authors gratefully acknowledge financial support from FCT through Project POCTI/36452/QUI/2000.

Downloaded At: 20:27 23 January 2011

REFERENCES

1. Saito, T.; Yamaji, A.; Itoh, T. A new isolation method of caseinoglycopeptide from sweet cheese whey. J. Dairy Sci. **1991**, *74*, 2831–2837.
2. Brody, E.P. Biological activities of bovine glycomacropeptide. British J. Nut. **2000**, *84*, S39–S46.
3. Huffman, L.M.; Harper, W.J. Maximizing the value of milk through separation technologies. J. Dairy Sci. **1999**, *82* (10), 2238–2245.
4. Official Journal of the EU L-276, Directive no 90/0496 of the Conseil of 6 October 1990.
5. Official Journal of the EU L-142, Regulation no 1898/87 of the Conseil of 2 July 1987.
6. Olieman, C.; van den Bedem, J.W.; Neth. A sensitive HPLC method of detecting and estimiating rennet whey total solids in skim milk powder. Milk Dairy **1983**, *37*, 27–36.
7. Léonil, J.; Mollé, D. A method for determination of macropeptide by cation-exchange fast protein liquid chromatography and its use for following the action of chymosin in milk. J. Dairy Res. **1991**, *58*, 321–328.
8. Elgar, D.F.; Norris, C.S.; Ayers, J.S.; Pritchard, M.; Otter, D.E., Palmano, K.P. J. Simultaneous separation and quantification of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. Chrom. A **2000**, *878*, 183–196.
9. Strange, E.D.; Malin, D.; Van Hekken, D.L.; Basch, J.J. Chromatographic and electrophoretic methods used for analysis of milk proteins. J. Chromatogr. **1992**, *624*, 81–90.

MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016

©2002 Marcel Dekker, Inc. All rights reserved. This material may not be used or reproduced in any form without the express written permission of Marcel Dekker, Inc.

10. Ferreira, I.M.P.L.V.O.; Marques, J.M.G.L.; Mendes, E.; Ferreira, M.A. Development, validation and application of an HPLC/UV method for quantification of casein in infant formulae and follow-up milks. J. Liq Chrom & Rel.Technol. **2000**, *23* (13), 2057–2065.
11. Ferreira, I.M.P.L.V.O.; Mendes, E.; Ferreira, M.A. HPLC/UV analysis of proteins in dairy products using a hydrophobic interation chromatographic column. Anal. Sci. **2001**, *17*, 499–501.
12. Veloso, A.C.A.; Teixeira, N.; Ferreira I.M.P.L.V.O. Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea-polyacrylamide gel electrophoresis. Detection of milk adulterations. J. Chromatogr. **2002**, *in press.*
13. Sharma, S.K.; Hill, A.R.; Mittal, G.S. An improved method to measure glycomacropeptides (GMP) in renneted milk. Milchwissenschaft **1993**, *48*, 71–73.

Received July 7, 2002
Accepted August 12, 2002
Manuscript 5904

Downloaded At: 20:27 23 January 2011