ELSEVIER

Journal of Chromatography A, 790 (1997) 195–205

JOURNAL OF CHROMATOGRAPHY A

Development of a capillary zone electrophoresis method for caseinoglycomacropeptide determination

Samir Cherkaoui[a], Nathalie Doumenc[b], Pierre Tachon[b], Jean-Richard Neeser[b], Jean-Luc Veuthey[a,*]

[a]*Laboratory of Pharmaceutical Analytical Chemistry, University of Geneva, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland*
[b]*Nestlé Research Center, Vers-Chez-Les Blanc, P.O. Box 44, 1000 Lausanne 26, Switzerland*

Received 31 December 1996; received in revised form 18 June 1997; accepted 3 July 1997

Abstract

Caseinoglycomacropeptide (CGMP) is a polypeptide of 64 amino acid residues, derived from the C-terminal part of bovine κ-casein. A sensitive and selective capillary zone electrophoresis method has been developed and validated for the analysis and quantitation of CGMP. Separation is carried out at 30 kV, using an uncoated fused-silica capillary and 20 m*M* sodium citrate buffer at acidic pH 3.5. The described method allows the separation of various CGMP subcomponents. The validation data proves that the method has the requisite selectivity, sensitivity, reproducibility and linearity for CGMP assay and for quality control during CGMP manufacturing (batch-to-batch reproducibility). © 1997 Elsevier Science B.V.

Keywords: Caseinoglycomacropeptide; Peptides; Validation

1. Introduction

Among the different caseins existing in milk, κ-casein is the primary substrate of chymosin (Rennin, EC 3.4.23.4), a proteinase that coagulates the milk casein complex. This enzyme cleaves the peptide bond Phe105–Met106, yielding an N-terminal fragment (*para* κ-casein; residues 1–105), which remains with the coagulated caseins, and a C-terminal fragment [a soluble caseinoglycomacropeptide (CGMP); residues 106–169, $M_r \cong 7000$] which is recovered in the whey [1].

Several physiological and biological functions of CGMP have been reported: inhibition of gastric secretion [2,3], growth promoting effect on bifidobacteria [4], depression of platlet aggregation [5], inhibition of oral *Actinomyces* adhesion to red blood cell membranes [6], inhibition of adhesion of oral *Streptococci* to saliva-coated hydroxyapatite beads [7], inhibition of adhesion of *Streptococcus sanguis* to human buccal epithelial cells [8], inhibition of cholera toxin binding to its receptor [9].

Two main genetic variants are known and designated as A and B. The κ-casein-A differs from the B variant in the substitution of a threonine residue for alanine at position 136 and of an aspartic acid residue for isoleucine at position 148.

The glycomacropeptide contains all of the carbohydrate originally present in κ-CN. So far, the threonines at positions 131, 133, 135, 136, 142 and the serine at position 141 have been identified as points of attachment of oligosaccharide chains. To these five glycosylation sites, carbohydrate chains made of one or more of the residues N-acetyl-

*Corresponding author.

0021-9673/97/$17.00
PII S0021-9673(97)00756-5

neuraminic acid (NANA), galactose (Gal), N-acetylgalactosamine (GalNAc) may be O-glycosidically linked [10]. The majority of the carbohydrate chains contains NANA as a terminal residue α2–3 or α2–6 linked respectively to GalNAc or Gal.

As a result, all of the macropeptide components have a very similar peptide backbone (except for the amino acid replacement between the genetic variants), the main difference being the attachment of different total amounts of carbohydrate and/or the variable phosphate content.

Various methods have already been reported to follow the action of chymosin in cheese making or to determine the content of CGMP in dairy products:

(i) Methods based on the determination of the N-acetylneuraminic acid, a specific indicator of the macropeptide [11]. The main problem with determining this acid is that it is not found in the same proportion in all macropeptide molecules and that it does not appear at all in some macropeptides released by chymosin.

(ii) Another alternative is to use chromatographic methods such as size-exclusion [12,13], reversed-phase [14–16] and ion-exchange chromatography [17]. However, these methods are time consuming and limited by a poor chromatographic resolution obtained.

Today, high-performance capillary electrophoresis (HPCE) is a well established analytical method in the fields of chemistry and biochemistry. Very high efficiency and resolution, broad range of applications, short analysis time and low solvent consumption are the main reasons for this success. HPCE has proven to be particularly useful in the analysis of proteins, peptides and their glycoforms [18–20]. Recently, several reports have shown capillary electrophoresis (CE) to be a powerful method for milk proteins [21–27]. Some of these methods deal with CGMP analysis [25–27]. In the first paper [25], a CE method was developed for the separation of the major whey proteins. Under acidic conditions (70 m*M* phosphate pH 2.5), only one CMP peak was separated from the whey proteins. In a second paper [27], the same authors described preliminary studies on the performance of CE in an untreated fused-silica capillary for the analysis of CGMP. The analysis was investigated under the same acidic conditions as above (70 m*M* phosphate buffer, pH 2.5) and basic conditions (phosphate and borate buffers at pH 6–9). At pH 2.5, the isolated CGMP appeared as one main peak followed by three other just distinguishable peaks, the last of which was CGMP containing NANA. The absence of other CGMP minor forms was attributed to the sensitivity of the method. For both papers, no quantitative aspect was approached by the authors. In the third paper [26], a capillary zone electrophoresis (CZE) method is used to estimate rennet whey solids in skim milk and acid buttermilk powder, based on the non glycosylated A and B genetic variants of CGMP.

However, only the last report [26] has considered the quantitative aspect of the analysis and none has reported a successful method validation.

The aim of this work has been to set up and validate a rapid and selective CE procedure for the analysis and quantitative determination of a caseinoglycomacropeptide mixture. This method has been used to compare different CGMPs obtained through different procedures, as well as different batches yielded by the same method.

2. Experimental

2.1. Chemicals and reagents

CGMP samples were obtained from Nestec (Verschez-les Blancs, Switzerland). The CGMP protein content is about 90% as determined by total nitrogen measurement and carbohydrate content is 10%. Bovine serum albumin (BSA) was supplied by Fluka (Buchs, Switzerland). β-Lactoglobulin and α-lactalbumin were purchased from Sigma (St. Louis, MO, USA). The β-lactoglobulin sample contains β-lactoglobulins A and B and is 90% pure. Deionized water was used for standard and sample preparation. Citrate buffer solutions and 1 *M* sodium hydroxide were obtained from Fluka. The electrolyte solutions were filtered through a 0.45 μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Instrumentation and electrophoretic procedure

Experiments were performed on a HP ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany) with a diode array detection system and a power supply able to deliver up to 30 kV. A CE Chemstation (Hewlett-Packard) was used for instrument

control, data acquisition and data analysis. A Hewlett-Packard fused-silica capillary of 64.5 cm (56 cm to the detector window)×50 μm I.D., was used for all experiments. An alignment interface containing optical slit matched to the capillary I.D. was used. Capillaries and interface were obtained from Hewlett-Packard.

All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample introduction was achieved by pressure injection for 20 s at 50 mbar (30 nl). During sample analysis, a constant voltage was applied and the temperature of the separation system was kept at 40°C. For all experiments, detection was carried out at 200 nm with a bandwidth of 10 nm.

CE separations were carried out with 20 m*M* sodium citrate buffer at pH 3.5 (Fluka). Each morning, the capillary was rinsed with 1 *M* sodium hydroxide for 15 min, followed by water for 10 min. To achieve high migration time reproducibility and to avoid solute adsorption, a washing method was performed. It included flushing the capillary with 1 *M* NaOH for 2 min, followed by water for 2 min and then buffer for 3.5 min. As the electrolysis of a solution can alter the running buffer pH and subsequently change the electroosmotic flow (EOF), a replenishment system was also used to maintain high reproducibility.

Prior to each sequence, two blank injections were performed in order to stabilize the capillary wall surface, and to allow the buffer and sample solutions to reach a constant temperature on the autosampler tray. Triplicate injections of the solutions were made.

2.3. Standard solutions

Standard solutions were prepared by weighing accurately CGMP, dissolving and diluting in water solution. The use of water as a dissolving solvent allows sample stacking which is effective in enhancing sensitivity (increasing peak shape) by on-column preconcentration of sample within the capillary.

3. Results and discussion

The following experiments were carried out: (i) optimization of running conditions and assessment of method selectivity. (ii) Measurement of within-day precision (repeatability) and between-day precision (reproducibility) by repeated injections of CGMP samples (target concentration 1 mg ml^{-1}). (iii) Measurement of detector linearity over a range of (0.1 and 2 mg ml^{-1}).

3.1. Method development

The influence of the pH on the CGMP analysis was investigated using 20 m*M* sodium citrate buffer at different acidic pH values. The operating voltage and temperature were fixed at 30 kV and 40°C, respectively.

This acidic buffer system was selected in order to produce a pH value around the isoelectric point (p*I*) of CGMP (around 4). At low pH values, CGMP migration is mainly controlled by its electrophoretic velocity, since the EOF is generally small. In this case, detection is performed at the cathode. In addition, a buffer solution with low ionic strength is very suitable to achieve shorter separation by applying a high voltage (30 kV) which generates a current of only ca. 18 μA.

At a fixed sodium citrate concentration (20 m*M*), Fig. 1 shows that the pH strongly influences both the electrophoretic profile and the peak efficiency. This pH sensitivity might be related to the presence of several glycoforms with closer p*I* values in the studied pH range. This variation between glycoforms p*I* values is probably due to the nature and content of the attached sialylated carbohydrate chains and/or to the degree of phosphorylation.

Using an uncoated fused-silica capillary, pH of 3.5 yielded the best compromise in terms of analysis time, selectivity and separation efficiency. At this pH, all CGMP forms are anionic. Therefore, they are eluted after the EOF (t_{EOF}=8 min) and pass the detector with migrations times higher than 8 min. Thus, pH 3.5 was used in subsequent stages of the method development. Other analysis conditions are summarized in Table 1.

Under the optimized conditions, we were able to achieve a good baseline separation of several CGMP subcomponents. The electrophoretic profiles of two different CGMP batches manufactured by the same preparation procedure (501 and 502) are shown in Fig. 2. The sharpest peak, which is supposed to be the carbohydrate free fraction of CGMP, is flanked

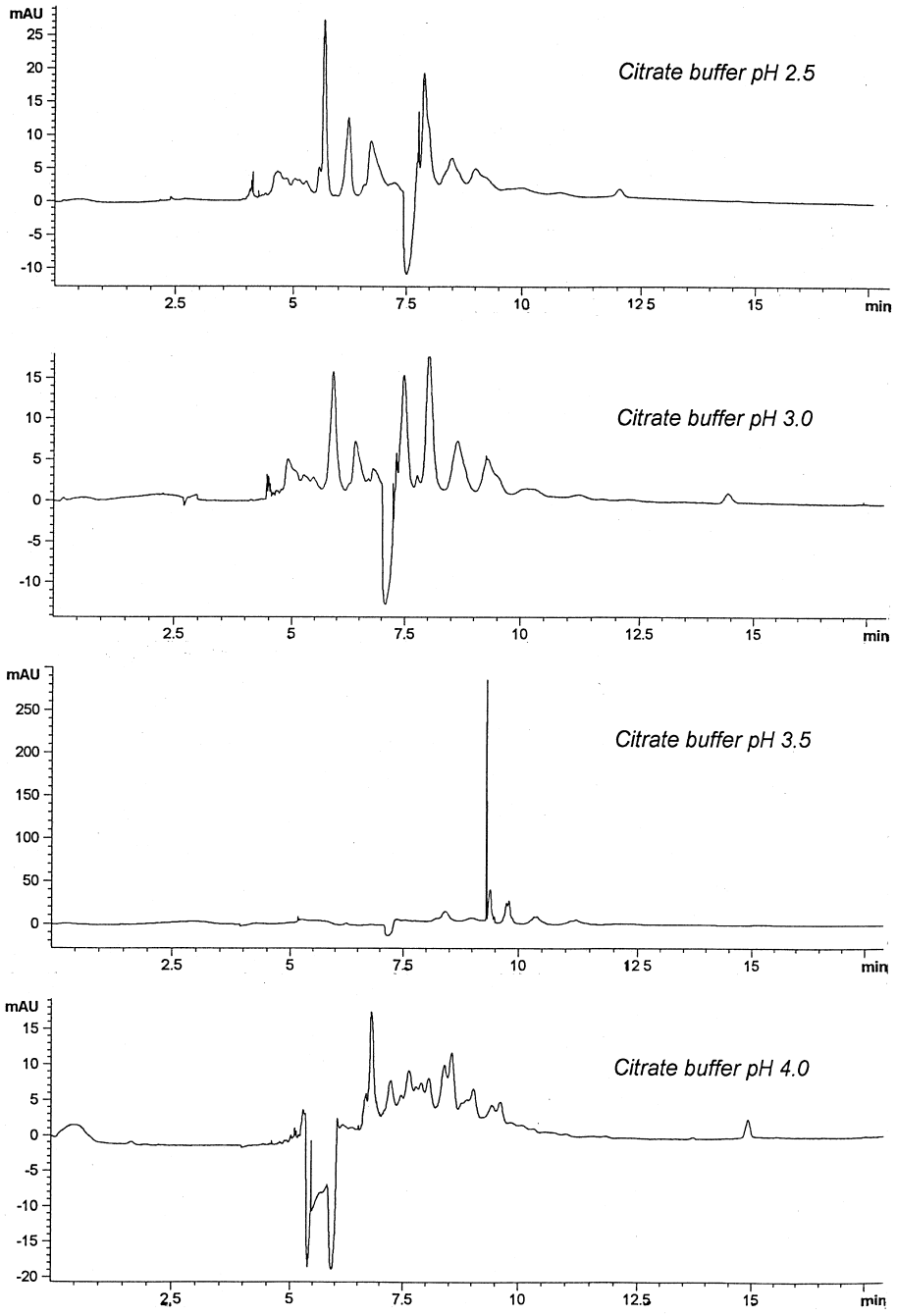

Fig. 1. Electropherograms of CGMP obtained at different sodium citrate buffer (20 m*M*) pH. (A) pH 2.5, (B) pH 3.0, (C) pH 3.5 and (D) pH 4.0. Other operating conditions: capillary: uncoated fused-silica, L=64.5 cm, l=56 cm, I.D.=50 μm; applied voltage: 30 kV, temperature 40°C. Other conditions are given in Section 2.2.

Table 1
Experimental conditions

Pre-conditioning	
Rinse 1	3.5 min with the selected buffer
Post-conditioning	
Rinse 1	2 min with 1 *M* NaOH
Rinse 2	2 min with water
Temperature	40°C
Injection	Pressure 1000 mbar s^{-1}
Voltage	30 kV (voltage ramp: 1 min)
Sample	Desired concentration with dilution in water
Buffer	20 m*M* sodium citrate, pH 3.5 (Fluka)
Detection	200 nm
Capillary dimensions	64.5 cm/56 cm×50 μm
Instrument	Hewlett-Packard, HP^{3D} capillary electrophoresis

on both sides by other peaks with a slightly different charge-to-mass ratio.

Peaks eluting after this subcomponent were not identified but might correspond to various glycoforms with an increasing degree of sialylation. This would be in accordance with the separation mechanism based on the charge-to-mass ratio. As a matter of fact, because of the ionization of the NANA fixed on the carbohydrate chains, the glycosylated forms of the CGMP have an increased negative charge. It has been reported that CGMP can contain up to nine or ten NANA residues [28]. Peaks eluting before the sharpest one might correspond to the glycosylated non sialylated CGMP fractions. Work is in progress in order to identify the different fractions by mass spectrometry.

The A and B variants of the CGMP could not be separated because these genetic variants only differ by one acidic amino acid residue which is almost not ionized at the selected electrophoresis pH, thus inducing no charge difference.

3.2. Method validation

The optimized method was validated for CGMP quantification. The validation approach is similar to that generally adopted for high-performance liquid chromatography (HPLC) methods [29]. The procedure requires the assessment of the specificity, migration time and peak area reproducibility and detector response linearity with sample concentration and sensitivity.

3.2.1. Selectivity

To explore further quantitative determination of CGMP, we had to check if there was no interference between the major milk proteins and the CGMP. Therefore, the analysis of BSA, β-Lg and α-Lac was carried out under the optimized conditions. The whey proteins, α-lactalbumin, BSA and β-lactoglobulin having p*I*s of 4.4, 4.8 and 5.2, respectively, are positively charged at pH 3.5 and will migrate before the EOF. As our quantitation is based on peaks eluting after the EOF, the whey protein will never interfere with the glycomacropeptide fractions, which proves the selectivity of the method. β-Lactoglobulin and α-lactalbumin broad peaks observed in Fig. 3 are essentially due to protein adsorption. BSA, which is known to be a very hydrophobic protein, is adsorbed on the capillary and is not recovered during the analysis. BSA is eluted only after the washing procedure (1 *M* NaOH).

3.2.2. Precision

In order to determine the repeatability (within-day precision) of the method, replicate injections ($n=8$) of 1 mg ml^{-1} CGMP solution were made. In Table 2, the average values and relative standard deviations (R.S.D.s) are given for the migration time, peak area and normalized peak area. In all cases, the precision was less than 1% for the migration time and 3% for the peak area. The good peak area precision was achieved without adding any internal standard to circumvent the injection variation.

In CE, peak areas are related to both sample

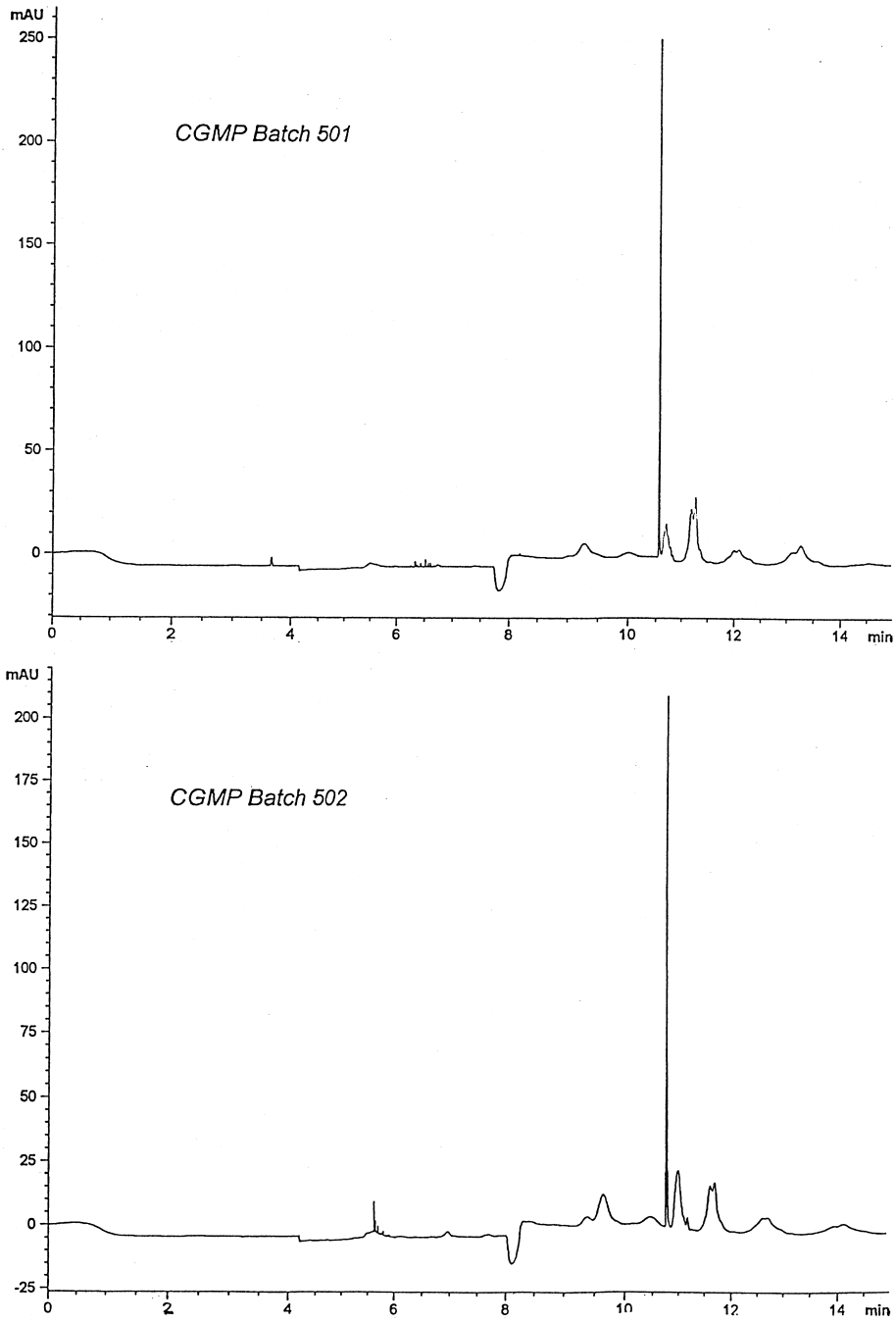

Fig. 2. Typical electropherograms of CGMP obtained with sodium citrate buffer (20 m*M*) at pH 3.5. (A) batch 501 and (B) batch 502. Other operating conditions as in Fig. 1.

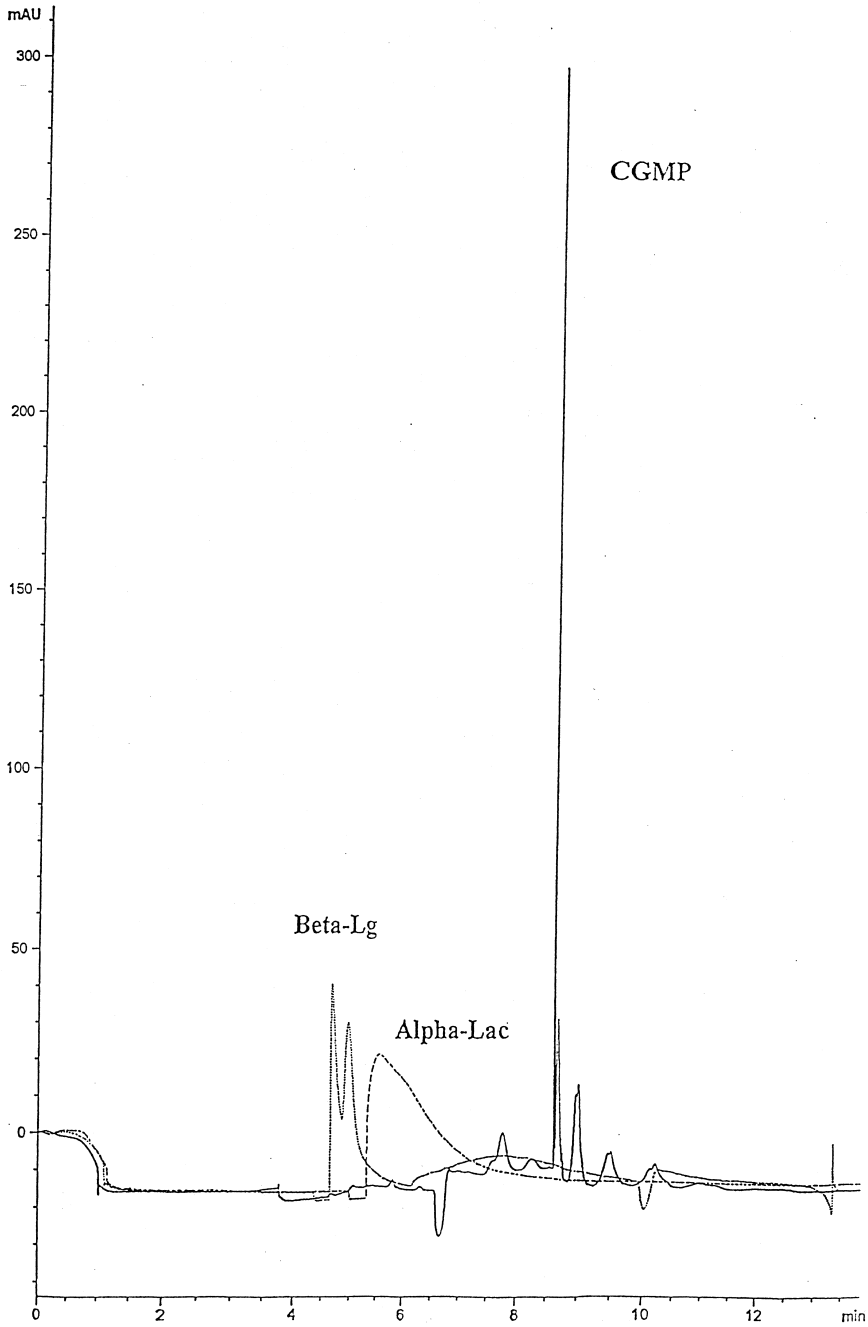

Fig. 3. Electropherogram of CGMP and other whey proteins obtained with sodium citrate buffer (20 m*M*) at pH 3.5. Other operating conditions as in Fig. 1.

Table 2
Within-day precision of CE migration times, peak areas and normalized peak areas for eight replicate injections of CGMP standards prepared at 1 mg ml^{-1}

	Migration time (min)			Total area[b]			Normalized area		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
NGF[a]	10.870	0.104	0.95	235.18	7.25	3.08	21.63	0.58	2.69
CGMP	–	–	–	1267.63	25.62	2.02	112.40	1.56	1.39

[a] NGF refers to the putative non glycosylated fraction of CGMP.
[b] CGMP peak area is obtained by adding all the subcomponent peak areas.

concentration and migration time. This effect can be compensated by dividing the main peak area by its corresponding migration time. This normalization resulted in the peak area R.S.D. improvement (1.39% vs. 2% unnormalized).

The reproducibility (between-days precision) was also evaluated over three days by performing six successive injections daily. The results (Table 3) show that the reproducibility of migration time, peak area and normalized peak area were satisfactory.

3.2.3. Linearity

The linearity of the method was evaluated over a concentration range from 0.1 to 2 mg ml^{-1}. Good linearity in terms of peak area response was obtained [slope 1269.04 (standard error, 20.03); y-intercept −30.34 (standard error, 23.30); correlation coefficient $r^2=0.9963$]. Table 4 reports the regression analysis data for the total CGMP and for its sharpest peak calibration curves.

3.2.4. Freedom from interference

A solution of the dissolving solvent, water in this case, was injected into the system in duplicate. No interfering peaks were observed.

3.2.5. Limit of detection (LOD)

The LOD is defined as the lowest concentration of analyte that can be clearly detected above the baseline signal ($3\times S/N$). The LOD was established by injecting test solutions at various concentrations of CGMP. The estimated limit of detection was determined as 30 μg ml^{-1}, giving a limit of quantitation (LOQ) value of 90 μg ml^{-1}.

Table 3
Between-day precision of CE migration times, peak areas and normalized peak areas for replicate injections of CGMP standards prepared at 1 mg ml^{-1}

	Migration time (min)[a]			Total area[b]			Normalized area		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
Day 1 ($n=8$)	10.870	0.104	0.95	1267.63	25.62	2.02	112.40	1.56	1.39
Day 2 ($n=6$)	9.901	0.153	1.55	1162.16	25.91	2.23	115.01	2.099	1.82
Day 3 ($n=6$)	10.553	0.181	1.71	1230.53	54.56	4.43	113.9	3.843	3.37
	10.48	0.44	4.16	1224.8	56.79	4.64	113.64	2.68	2.36

[a] Migration time refers to the sharpest peak in the electropherogram.
[b] Peak area is obtained by adding all CGMP subcomponent peak areas.

Table 4
Regression data for the calibration curves

	Range of linearity (mg ml^{-1})	Regression data	
		Line	r^2
Total peak	0.1–2	$y=-30.01+1269.04c$	0.9963
Sharpest peak	0.1–2	$y=-30.34+267.77c$	0.9976

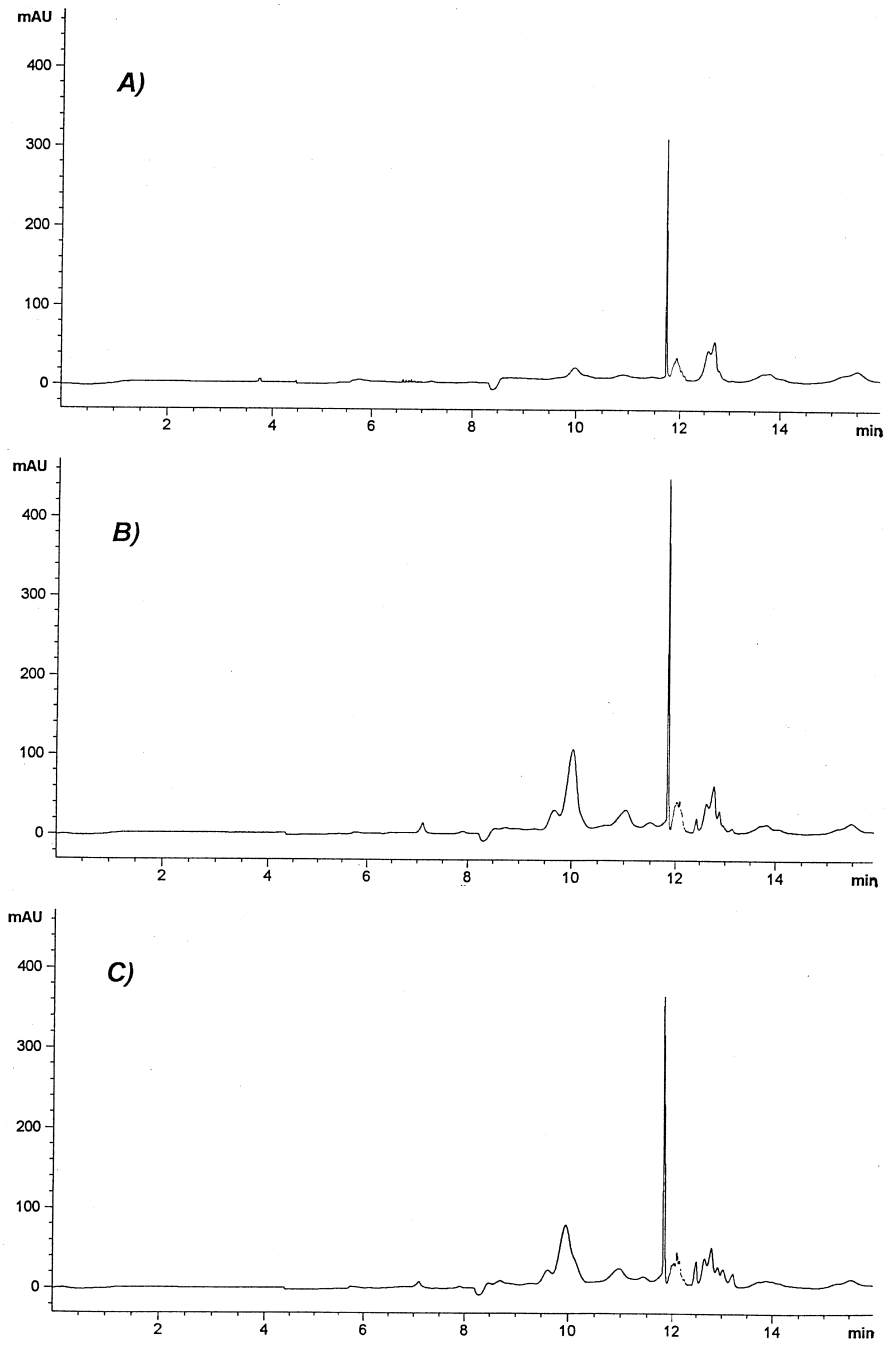

Fig. 4. Electropherograms of CGMP manufactured by two different preparation procedures. (A) Procedure 1 (501), (B) procedure 2 (batch 1) and (C) procedure 2 (batch 2). Other operating conditions as in Fig. 2.

4. Applications

4.1. Batch-to-batch reproducibility

Optimized electrophoretic conditions were used to compare the CGMP purity from different batches. As can be seen in Fig. 2, batches 501 and 502 show the same electrophoretic profiles with a very good baseline. As shown in Table 5, the described method can also estimate the percentage of the putative non glycosylated fraction (main fraction in terms of peak height), by dividing its peak area by the total peak area. The highest peak represents 17.37 and 18.55% (areas ratios) for batches 501 and 502, respectively.

4.2. CGMP from different preparation procedures

As the CGMP composition depends on the preparation procedure, the validated method could be used to compare CGMP samples from different methods.

Three CGMP samples were then injected under the optimized conditions. As can be seen from Fig. 4, the different samples show the same electrophoretic profiles but peaks, eluting before the sharpest one, are more important in the case of procedure II. The putative glycosylated non sialylated, non glycosylated and the highly sialylated fractions represent, respectively, 18.09%, 15.59% and 66.3% for procedure I and 46.7%, 14.26% and 39.02% for procedure II (see Table 6). This difference may have a crucial importance, as it is known that CGMP bioactivity may be associated with the nature and content of the attached carbohydrate moieties.

5. Conclusions

CZE, at acidic pH and in the presence of sodium citrate buffer, offers a suitable and rapid method for the qualitative and quantitative determination of CGMP and its several subcomponents. Selectivity was demonstrated for all others major whey protein and no interfering peaks from the dissolving solvent were observed. This method has been validated and has shown good performance in terms of selectivity, sensitivity, reproducibility and an adequate detection linearity over the required concentration range. The validated method may also be used to assess the component identity (percentage of the various glycoforms) and to check the CGMP purity when yielded by different methods or batches.

The major advantages of the described method are: (1) its very high efficiency and selectivity, (2) the speed of analysis time, (3) the low solvent consumption and (4) the separation mechanisms

Table 5
Batch-to-batch reproducibility

	% NGF	Migration time (min)			Total area			Normalized area		
		Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
CGMP 501 (n=8)	17.37	10.502	0.045	0.43	1194.5	23.04	1.93	107.88	1.985	1.84
CGMP 502 (n=6)	18.55	10.870	0.104	0.95	1267.6	25.62	2.02	112.40	1.56	1.39

Evaluation of the sharpest peak fraction percentage.

Table 6
Evaluation of main CGMP fractions manufactured by two different preparation procedures

	Putative fraction identities		
	Before the sharpest peak	Sharpest peak	After the sharpest peak
Procedure I (501)	18.09%	15.59%	66.30%
Procedure II (batch 1)	48.31%	14.22%	37.42%
Procedure II (batch 2)	45.03%	14.30%	40.62%

Percentages are given as peak areas ratios.

which make it an attractive complement/alternative to conventional techniques.

References

[1] A. Kobata, in M.I. Horowitz and W. Pigman (Editors), Glycoconjugates, Vol. II, Academic Press, New York, 1978, pp. 423–440.
[2] L.S. Vasilevskaya, Fiziol. Zh. SSSR imemi I.M.Sechenova 64 (1978) 871.
[3] E.Y. Stan, M.P. Chernikov, Vopr. Med. Khim 25 (1979) 348.
[4] N. Azuma, K. Yamauchi, T. Mitsuoka, Agric. Biol. Chem. 48 (1984) 2159.
[5] P. Jolles, S. Levy-Toledano, A.-M. Fait, C. Soria, D. Gillessen, A. Thomaidis, F.W. Dunn, J.P. Caen, Eur. J. Biochem. 158 (1986) 379.
[6] J.R. Neeser, A. Chambaz, S.D. Vedovo, M.-J. Prigent, B. Guggenheim, Infect. Immun. 56 (1988) 3201.
[7] J.R. Neeser, M. Golliard, A. Woltz, M. Rouvet, M.-L. Dillman, B. Guggenheim, Oral Microbiol. Immunol. 9 (1994) 193.
[8] J.R. Neeser, R.C. Grafstrom, A. Woltz, D. Brassart, V. Fryder, B. Guggenheim, Glycobiology 5 (1995) 97.
[9] Y. Kawasaki, H. Isoda, M. Tanimoto, S. Dosako, T. Idota, K. Ahiko, Biosci. Biotech. Biochem. 56 (1992) 195.
[10] H.E. Swaisgood, in P.F. Fox (Editor), Advanced Dairy Chemistry, Vol. I, Elsevier Applied Science, New York, 1992, p. 63.
[11] G. Robitaille, K.-F. Ng-Kwai-Hang, H.G. Monardes, J. Dairy Res. 58 (1991) 107.
[12] A.C.M. Van Hooydonk, C. Olieman, Neth. Milk Dairy J. 36 (1982) 153.
[13] S.K. Sharma, A.R. Hill, G.S. Mittal, Milchwissenschaft 48 (1993) 71.
[14] C. Carles, B. Ribadeau-Dumas, FEBS Lett. 185 (1985) 282.
[15] D. Molle, J. Leonil, J. Chromatogr. A 708 (1995) 223.
[16] P. Minkiewicz, C.J. Slangen, F.M. Lagerwerf, J. Haverkamp, H.S. Rollema, S. Visser, J. Chromatogr. A 743 (1995) 123.
[17] J. Leonil, D. Molle, J. Dairy Res. 58 (1991) 321.
[18] T.F. Chen, J. Chromatogr. 559 (1991) 445.
[19] K. Kakehi, S. Honda, J. Chromatogr. A 720 (1996) 377.
[20] P.G. Righetti, Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996.
[21] M. Kanning, M. Casella, C. Olieman, LC·GC Int. 34 (1993) 701.
[22] T.M.P. Cattaneo, F. Nigro, P.M. Toppino, V. Denti, J. Chromatogr. A 721 (1996) 345.
[23] N. De Jong, S. Visser, C. Olieman, J. Chromatogr. A 652 (1993) 207.
[24] F.-T.A. Chen, A. Tusaka, J. Chromatogr. A 685 (1994) 331.
[25] J. Otte, K.R. Kristiansen, M. Zakora, K.B. Qvist, Neth. Milk Dairy J. 48 (1994) 81.
[26] J. Van Riel, C. Olieman, Electrophoresis 16 (1995) 529.
[27] J. Otte, L. Midtgaard, K.B. Qvist, Milchwissenschaft 50 (1995) 75.
[28] H.J. Vreeman, S. Visser, K.J. Slangen, J.A.M. van Rel, Biochem. J. 240 (1986) 87.
[29] K.D. Altria, D.R. Ruud, Chromatographia 41 (1995) 325.