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Routine *o*-glycan characterization in nutritional supplements — a comparison of analytical methods for the monitoring of the bovine kappa-casein macropeptide glycosylation

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Abstract

Analytical procedures, including capillary isoelectric focusing (CIEF), high-performance anion-exchange chromatography coupled to amperometric detection (HPAEC-PAD) and normal-phase chromatography with fluorescence detection are presented for the characterization of a highly *O*-glycosylated caseinomacropeptide (CGMP) and the detection of subtle glycosylation differences between CGMP Batches obtained with two different preparation procedures. Modified two-step CIEF allowed monitoring of glycopeptide heterogeneity and determination of the isoelectric points of acidic glycoforms. The mixture of wide and narrow pH range ampholytes was optimized to improve glycoform resolution. The p*I* of the different CGMP glycoforms was evaluated with p*I* internal standards and found to range between 3.08 and 3.58, which indicates a very acidic glycopeptide. Moreover, the monosaccharide composition was determined with HPAEC-PAD after neutral and amino sugars release by using adequate acidic hydrolysis of CGMP. Results indicated a similar composition for Batches I and II, but the monosaccharide percentages were 3–4 fold higher in Batch I, particularly for galactose and glucose. This likely reflects a higher content in lactose in the case of Batch I. Finally, *O*-linked oligosaccharides were released with an automated hydrazinolysis and derivatized with a sensitive labelling reagent, 2-aminobenzamide. The derivatives were then analyzed by normal-phase HPLC coupled with fluorescence detection, and separated on the basis of hydrophilic interaction, which allowed oligosaccharide mapping of the two CGMP. It appeared that the two CGMP preparations had an almost identical *O*-glycan population, but CGMP Batch I was more glycosylated than Batch II. Additionally, the sizes of the separated glycans, expressed as the number of glucose units, were tentatively assigned using calibration with a partial hydrolysate of dextran. In conclusion, a combination of electrophoretic and chromatographic techniques was found powerful in studying glycoprotein heterogeneity and assessing batch-to-batch consistency.

Keywords: Oligosaccharide mapping; Isoelectric focusing; Casein; Glycomacropeptides

1. Introduction

Recent progress in protein separation and purification techniques, as well as in biotechnology have enabled industrial production of bioactive glycopro-

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teins. However glycosylation of recombinant proteins varies according to cell culture conditions, the nature of the host cells used, and the downstream processing techniques used for isolating the protein [1,2]. It is now clear that the glycans attached to glycoproteins can affect various properties of the protein including its biological half life and immunogenicity and they should, therefore, be monitored in different production batches. Several techniques have previously been reported to provide convenient and reliable *N*-glycan analysis but well-validated techniques for *O*- are not available. Among the different caseins present in bovine milk, κ-casein is the primary substrate of chymosin (Rennin, EC 3.4.23.4). This enzyme cleaves the peptide bond Phe105-Met106, yielding an N-terminal fragment (para κ-casein; residues 1–105) that remains with coagulated caseins, and a C-terminal fragment (a soluble caseinoglycomacropeptide (CGMP); residues 106–169, $M_r \cong 7000$) which is recovered in the whey [3]. CGMP is a heterogeneous compound, which in fact consists of a number of glycoforms and phosphorylated forms with an identical peptidic backbone (except for a number of amino acids between genetic variants), but it can differ with respect to the structure, location and incidence of individual oligosaccharides [4]. This glycomacropeptide contains all the carbohydrates originally present in kappa casein. Five glycosylation sites at threonine 131,133, 135, 136, 142, and serine 141 have been identified. *O*-Glycans may contain one or more *N*-acetylneuraminic acid α2–3 or α2–6 linked to *N*-acetylgalactosamine (GalNAc) or galactose (Gal) residues, respectively. Several physiological and biological functions of CGMP have already been reported such as inhibition of gastric secretion [5,6], growth promoting effect on bifidobacteria in the case of human CGMP [7], depression of platelet aggregation [8], inhibition of adhesion of several oral micro-organisms to either blood cell membranes, saliva coated hydroxyapatite beads or epithelial cells [9–11]. Antithrombic and antihypertensive activities together with a regulation potential of the digestive tract render this molecule of particular interest in the fields of nutrition, cosmetic and pharmaceuticals. In several cases, its bioactivity has been associated with the nature and content of its carbohydrate moiety [12].

Glycosylation studies may be carried out at different levels: (i) starting from the intact molecule has the advantage of being a straightforward method that does not require extensive sample preparation; (ii) analysing the monosaccharide composition may also be useful, although small changes in monosaccharide composition or variation in the type of linkage between monosaccharides may not be detected; (iii) mapping of the isolated oligosaccharides released either chemically or enzymatically from the glycoprotein gives additional information on glycosylation consistency.

The emergence of capillary electrophoresis with the various possibilities for separation have brought about new possibilities in the field of glycoform separations [13–19]. In our previous investigations, capillary zone electrophoresis (CZE) using an uncoated fused-silica capillary [20] or a coated poly-(vinyl alcohol) (PVA) capillary [21] was applied to the separation of CGMP glycoforms. The baseline separation of different CGMP subcomponents was achieved with a citrate buffer at pH 3.5. This validated method aimed at assessing component identity (percentage of the various glycoforms) and checking the purity of CGMP obtained by different methods. Besides CZE, capillary isoelectrofocusing (CIEF) represents an interesting alternative for glycoform separation. Glycoforms which vary in their degree of sialylation, phosphorylation and/or sulfation may be resolved by this method. Schwer [22] demonstrated that CIEF can be applied for routine analysis of protein samples in quality and purity controls.

For monosaccharide analysis, high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) has been established as the method of choice because of its high selectivity and sensitivity [23]. Indeed, this method does not require derivatization of released monosaccharides, since the amperometric detector directly monitors these molecules. In a previous paper, HPAEC-PAD was successfully applied to determine sialic acid percentage in various CGMP Batches [24].

Oligosaccharide mapping after the chemical or enzymatic release from the glycoprotein is a particularly useful approach to assess batch-to-batch consistency of the protein or peptide glycosylation.

A relative amount of each separated structure may indicate subtle changes occurring in the glycosylation pattern of a glycoprotein. Oligosaccharide mapping is generally performed using either liquid chromatography or capillary electrophoresis. Several chromatographic techniques have also been developed for the analysis of various glycoprotein-derived oligosaccharides [25]. Derivatization of glycans with numerous labelling reagents has been proposed to allow either UV or fluorescence detection for a quantitative detection of glycans at sub-picomolar level. Among the different fluorophores available, 2-aminobenzamide (2-AB) has been demonstrated to be a non-selective, efficient and sensitive labelling agent [26] for the analysis of reductive carbohydrates. Several authors have described separations of 2-AB glycans by HPLC [27–38]. Kopp et al. [39] demonstrated that RP-HPLC of desialylated glycans derivatized with 2-AB is a sensitive method with which batch-to-batch consistency of recombinant glycoproteins can be monitored. More recently, we reported the extremely high reproducibility of normal-phase HPLC for the oligosaccharide mapping of *N*-glycans derivatized with 2AB [40].

Therefore, three analytical methods providing information at different levels on the CGMP glycosylation were compared: glycoform separation using CIEF, simple monosaccharide analysis by HPAEC-PAD and oligosaccharide mapping by normal-phase HPLC. The aim of the present study was to evaluate the potential of these approaches in detecting subtle glycosylation differences between two CGMP Batches obtained from two different preparation procedures.

2. Experimental

2.1. Chemicals, reagents and proteins

CGMP samples from two preparation procedures were kindly donated by Nestec (Vers-chez-les-Blancs, Switzerland). CGMP protein content was ca. 90%, as determined by total nitrogen measurement, and carbohydrate content was 10%.

Hydrochloric acid and 2.5–4 ampholytes were supplied by Fluka (Buchs, Switzerland). Sodium hydroxide (50%, w/w) and acetonitrile were obtained from Fisher (Pittsburgh, PA). Formic acid of HPLC grade, phosphoric acid 1 *M*, ampholines pH 3.5–5 and standard monosaccharides were purchased from Sigma (St. Louis, USA). Ammonium hydroxide and sodium hydroxide 1 N were supplied by Prolabo (Fontenay-sous-Bois, France). Polyacrylamide (PAA) coated fused-silica capillaries, CIEF gel, carrier ampholyte pH 3–10 and the protein markers Ribonuclease A (RNase A, p*I* 9.45), Carbonic Anhydrase II (CAH II, p*I* 5.9), β-lactoglobulin A (β-LGA, p*I* 5.1) and cholecystokinin flanking peptide (CCK, p*I* 2.75), were obtained from Beckman (Beckman Instruments, Fullerton, CA, USA). All other chemicals were analytical grade reagents.

For monosaccharide analyses and CE experiments, ultrapure water, obtained by a Milli-Q RG purification unit from Millipore (Bedford, MA, USA), was used for standard and sample preparation. All solutions and samples were filtered through a 0.45-μm microfilter (Supelco, Bellefonte, PA, USA) before use.

2.2. Glycoforms analysis by CIEF

CIEF was performed using a P/ACE 5500 with a UV detector and equipped with a capillary cartridge of 50-μm I.D. and 375-μm O.D. (Beckman). The absorbance of the focused proteins was detected at 280 nm. The separation temperature was set at 20°C. Before each injection the capillary was rinsed for 1 min with water at 20 p.s.i., and between runs it was flushed with water, followed with 10 m*M* H_3PO_4 for 2 min each.

Traditional two-step CIEF method experiments were performed with 91 m*M* H_3PO_4 as anolyte at the capillary inlet and 20 m*M* NaOH as catholyte at the outlet in normal polarity mode. A focusing step takes place in the capillary section located between the inlet end of the capillary and the detection window. The modified two-step method consisted of focusing the glycoforms in the 7-cm capillary section located between the detection window and the outlet end of the capillary. Experiments were performed with 20 m*M* NaOH at the inlet and 91 m*M* H_3PO_4 at the outlet in reversed polarity mode.

For the modified two-step CIEF, a polyacrylamide (PAA) coated capillary (50 μm I.D.) with an effec-

tive length of 20 cm (total length 27 cm) was filled, at low pressure (0.5 p.s.i.) for 1 min at the capillary outlet, with 0.2–2.2 μg of CGMP per μl, 2% (v/v) pH 3.5–5 and pH 3–10 ampholytes in the ratio (25/75, v/v). This sample/ampholyte mixture was prepared in CIEF gel (Beckman). In order to focus the glycoforms, the electric field was optimised at 500 V/cm for 2 min. Finally, a low pressure (0.5 p.s.i.) combined with some field strength was applied to mobilize the glycoforms.

2.3. Monosaccharide composition determination by HPAEC-PAD

2.3.1. Standard and sample preparation

Stock standard solutions of monosaccharides (fucose, mannose, glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine) and mannitol used as internal standard (1 mg/ml) were prepared in water. Working standard solutions were obtained by diluting stock standard solutions with water. Calibration curves reporting peak area ratio as a function of monosaccharide concentrations were established in the range of 0.5–10 μg/ml, in the presence of 5 μg/ml of mannitol as internal standard. Within-day method precision was determined by performing 6 injections of a 2.5 μg/ml solution containing a monosaccharide mixture and internal standard. Between-day precision was also evaluated over 3 days by performing six successive injections each day.

2.3.2. Hydrolysis of CGMP samples

For neutral monosaccharide analysis (fucose, mannose, glucose, galactose), CGMP was dissolved in 1 ml HCl 2 *M*, heated at 100°C for 4 h, evaporated to dryness and diluted in 1 ml water. The solution was deionized by passing it through a column containing 3 ml each of Dowex 50W X8-400 (H^+ form) and Amberlite IRA-400 (Cl^- form), and the column was washed with water. The combined eluate and washing fluids were evaporated to dryness and dissolved in 1 ml water. Mannitol, used as the internal standard and was added at a concentration of 5 μg/ml. For the analysis of hexosamines (*N*-acetylglucosamine and *N*-acetylgalactosamine), CGMP (2 mg/ml) was dissolved in 1 ml HCl 4 *M*, heated at 100°C for 4 h, and evaporated to dryness. Since the *N*-acetyl group is removed under such extremely acidic conditions, re-*N*-acetylation is performed by treating the residue with a mixture of 1.5 ml of a saturated aqueous solution of sodium bicarbonate and 0.5 ml of acetic anhydride. The mixture was kept overnight in the refrigerator and was deionized in a manner similar to that described for neutral monosaccharide analysis. Before injection, solutions were filtered through a 0.2-μm filter.

2.4. HPAEC-PAD of monosaccharides

HPAEC data were generated on a Dionex DX 500 chromatography system (Sunnyvale, CA, USA) consisting of a GP50 gradient pump and an ED40 Electrochemical detector. A Dionex cell outfitted with a gold working electrode was used for all experiments. Injections were performed by a Waters 717 plus autosampler (Milford, MA, USA). Detection output was interfaced to a software Chrom-Card program (Fisons instruments, Milan, Italy) on AST Bravo LC 4/33 computer for data handling and chromatogram generation. The HPAEC CarboPac MA1 column (250×4 mm I.D.), associated with a guard column containing the same stationary phase, was supplied by Dionex. Separation was achieved at a concentration of 460 m*M* NaOH and a flow rate of 0.4 ml/min. Detection was performed by standard carbohydrate waveform (E_1=0.05 V, t_1=400 ms, E_2=0.75 V, t_2=200 ms, E_3=−0.15 V, t_3=400 ms) [41].

2.5. Oligosaccharide mapping by HPLC

2.5.1. Hydrazinolysis release and preparation of glycans

Exhaustive dialysis of the glycoprotein was performed against aqueous TFA solution (0.1%) for 4 days at 4°C, followed by extensive lyophilisation for a minimum of 5 days to ensure complete removal of water. CGMP oligosaccharides were then released by hydrazinolysis [42] using automation [43] on the GlycoPrep 1000 (Oxford GlycoSciences, Abingdon, UK) operating in *O*-mode. Samples were evaporated to dryness before labelling.

2.5.2. Fluorescent labelling of O-glycans

The pools of recovered *O*-glycans were evapo-

rated and labelled with 2-AB [26] using a signal kit from Glyko (Novato, CA) with an incubated time of 2 h at 65°C. The glycans were separated from excess 2-AB by adsorption onto a hydrophilic filter in the presence of acetonitrile from which they were subsequently eluted with water.

2.5.3. Oligosaccharide mapping by HPLC

The HPLC system consisted of a P1000 XR gradient pump (Thermo Separation Products, Les Ullis, France), and a fluorescence detector FP 920 (JASCO, Nantes, France) (λ_{exc}=330 nm and λ_{emiss}= 420 nm). A WO Industrial Electronics temperature control module (Jetstream 2) was employed.

The 2-AB glycans were separated by normal-phase chromatography on a GlycoSep N column (4.6×250 mm) (Glyko, Novato, CA) in the following gradient conditions: solvent A was acetonitrile, solvent B was ammonium formate (50 m*M*, pH 4.4). Linear gradient starts at 65% A and then %B increases to 0.21%/min at a flow rate of 0.4 ml/min. The column was washed in 100% B for 9 min before decreasing the concentration to 65% A. Column temperature was 30°C.

3. Results and discussion

3.1. Glycoform analyses by CIEF

It has been demonstrated that the two-step CIEF method using capillaries with a reduced electroosmotic flow and pH stable coatings provides better reproducibility of peak migration times than the one-step method. Therefore, this technique was selected for the analysis of CGMP glycoforms. First attempts to separate the CGMP glycoforms using the traditional ''two-step'' CIEF method was not satisfactory since it afforded broad peaks and long analysis time. Indeed, acidic proteins tend to remain close to the inlet end of the capillary and have therefore to cross a long segment of the capillary before reaching the detection window.

In order to shorten the distance of the most acidic forms, a modified ''two-step'' CIEF technique was developed in which the glycoforms are focused in the shorter section of the capillary located between the detection window and the outlet end of the capillary, by using a reversed polarity configuration. Thus, a capillary section of 7 cm only was crossed by the solute before its detection. This markedly decreased the analysis time by factor 10, and improved the mobilisation of acidic glycoforms maintaining a flat profile (Fig. 1).

Comparing the migration times of p*I* internal standards, the first separation with a large 3–10 ampholyte pH range indicated that CGMP was very acidic (p*I* ranging from 2.74 to 3.3); the most acidic glycoforms were probably not detected because of a p*I* outside the pH gradient formed within the capillary. These results are in good agreement with previous studies reporting a high sialic acid content determined by HPAEC-PAD [24]. A mixture of wide (3–10) and narrow (3.5–5) pH range ampholytes significantly improved the separation. The ratio giving optimal resolution and separation was a mixture of 25/75 of pH 3.5–5 and pH 3–10 ampholytes, and CGMP consisted of a mixture of at least 14 glycoforms (Fig. 2). Attempts to use other acidic ampholytes (2.5–4/3–10) did not significantly improve this profile.

Method precision was evaluated through six replicate injections of four p*I* standards with a p*I* ranging from 2.75 to 9.45. Migration times appeared very reproducible and exhibited less than 0.6% relative standard deviation (RSD). In contrast, relative peak areas were not reproducible, this was attributed in part to the close p*I* values of the different glycoforms (differing less than 0.02 pH unit) leading to several unresolved peaks which hamper accurate peak integration.

A calibration curve was constructed with a linear relationship between p*I* and the migration times (t_m) of p*I* markers, and the equation ($pI=-2.176t_m+14.876$) was calculated with a coefficient of determination $r^2=0.989$. The p*I* range of the different CGMP glycoforms was evaluated from six consecutive analyses of each CGMP Batch: p*I* of CGMP Batch I glycoforms ranged from 3.08 to 3.58 (RSD< 0.8%) while those of Batch II were between 3.17 and 3.57 (RSD<0.4%). These results showed that the glycoforms of the two CGMP Batches had almost the same p*I* range, although the profiles of the two Batches differed in terms of number of peaks suggesting a higher glycosylation heterogeneity for Batch II (Fig. 3). However, the method could not be

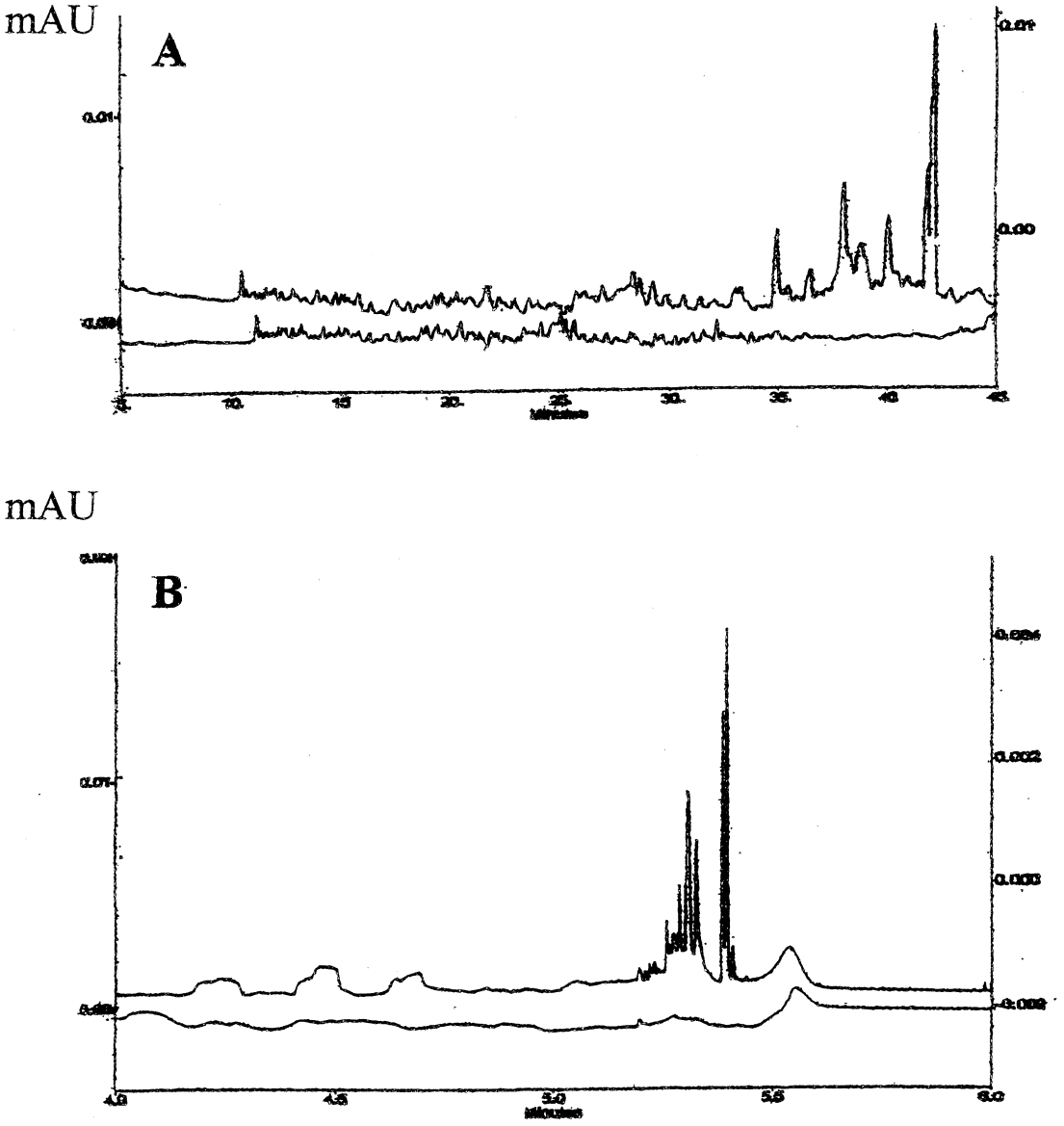

Fig. 1. Separation of CGMP glycoforms from Batch I using (A) traditional two-step CIEF, (B) modified two-step CIEF. For each condition, CGMP and background electropherograms are superimposed Conditions: PAA coated capillary (50 μm I.D.×27 cm total length), anolyte 91 m*M* H_3PO_4, catholyte 20 m*M* NaOH. Carrier ampholytes: 2% of pH 3–10 ampholytes in CIEF gel from Beckman. Focusing (2 min) and mobilisation at 13.5 kV at (A) normal polarity, (B) reversed polarity. Concentration of CGMP Batch I: 2 μg/μl.

employed to quantify these differences as peak areas could not be accurately integrated.

The modified two-step CIEF method allowed determination of the CGMP glycoforms p*I*, but this technique did not appear appropriate to point out differences in the glycoform pattern of the two CGMP Batches. We subsequently determined the CGMP monosaccharide composition by using

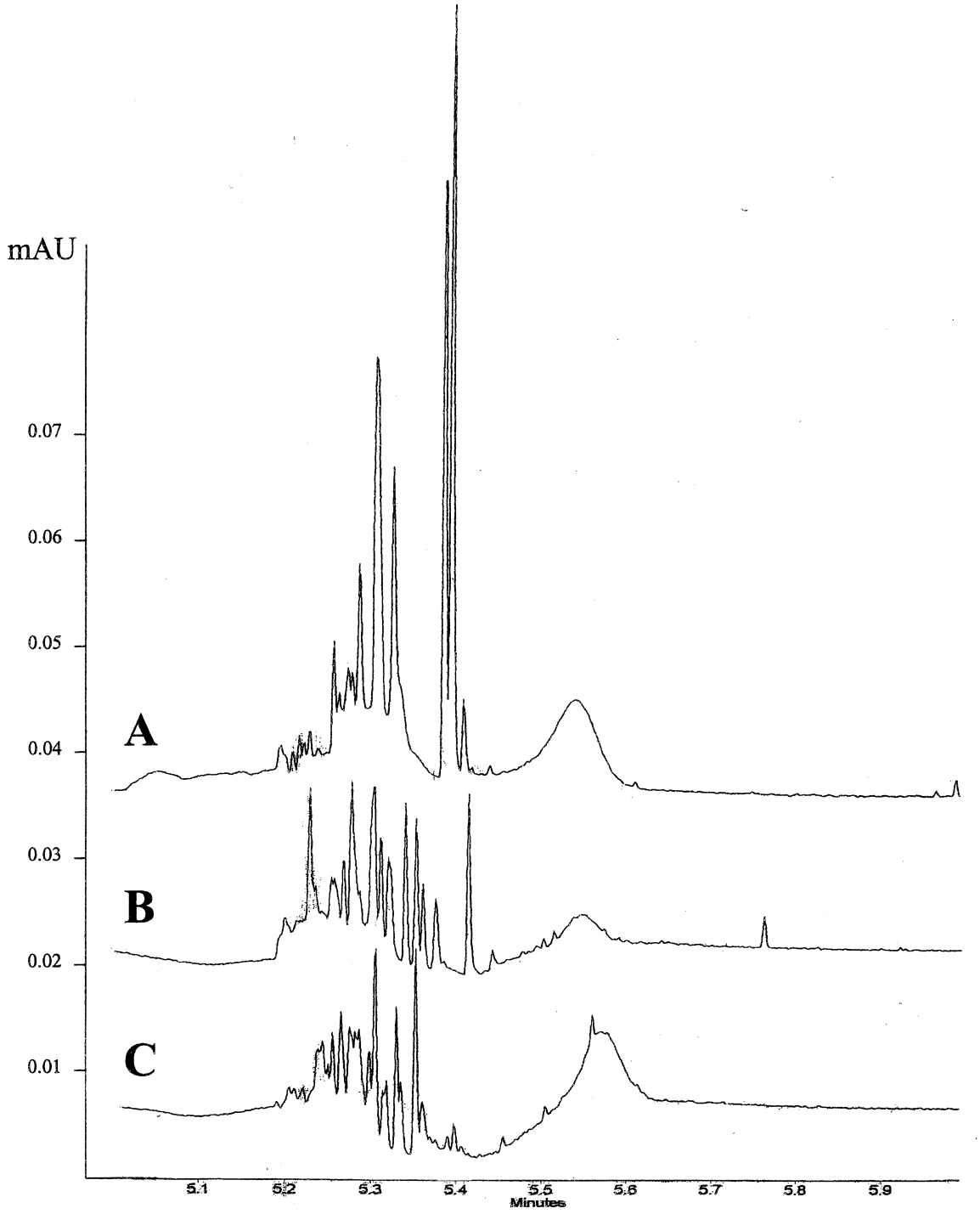

Fig. 2. Effect of the ratio pH 3.5–5 ampholines and pH 3–10 ampholytes on the separation of CGMP glycoforms using the modified two-step CIEF method: (A) pH 3–10 ampholytes, (B) 25/75 mixture of pH 3.5–5 ampholines and pH 3–10 ampholytes, (C) 50/50 mixture of pH 3.5–5 ampholines and pH 3–10 ampholytes. Other conditions as in Fig. 1.

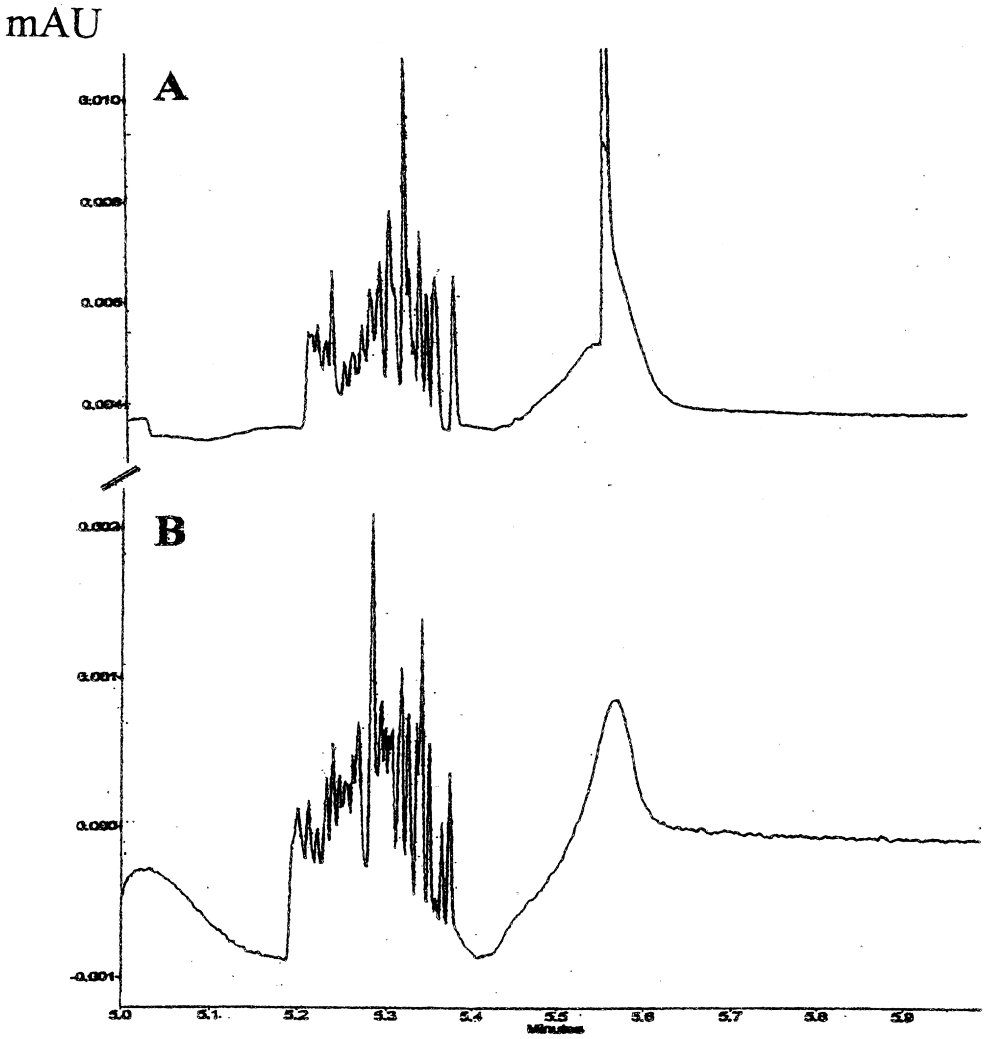

Fig. 3. Comparison of CGMP glycoform profiles from two Batches: (A) Batch I, (B) Batch II. Carrier ampholytes: 2% of 25/75 mixture of pH 3.5–5 ampholines and pH 3–10 ampholytes in CIEF gel from Beckman. Other conditions as in Fig. 1.

HPAEC-PAD in order to evaluate the technique potential in detecting glycosylation differences between two CGMP Batches.

3.2. Determination of monosaccharide composition by HPAEC-PAD

The method is based on the release of monosaccharides after acidic hydrolysis of CGMP followed by their separation by HPAEC-PAD. For an accurate quantification of monosaccharides (no interference of neutral sugar degradation products or incomplete release of aminosugars), two hydrolysis conditions were performed. Mild acidic conditions (2 *M* HCl) were used for the analysis of neutral monosaccharides, and stronger conditions (4 *M* HCl) for *N*-acetylhexosamines since aminosugars can only be released under extremely acidic conditions. Fig. 4 shows the separation of standard (neutral and amino) monosaccharides, and the profiles of the neutral sugar pools, and neutral and amino sugar pools released from CGMP Batch I. The HPAEC-PAD

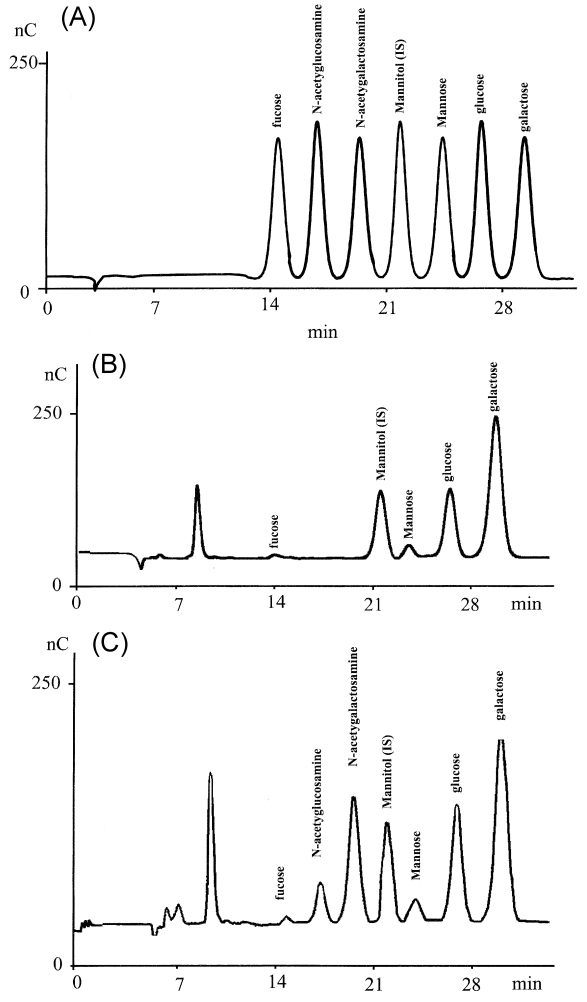

Fig. 4. HPAEC-PAD separation of (A) standard monosaccharides in the presence of internal standard (mannitol), (B) neutral monosaccharides released from CGMP Batch I, (C) neutral and amino-sugars released from CGMP Batch I. Acidic hydrolysis was performed with (B) 2 *M* HCl, and (C) 4 *M* HCl. Other experimental conditions are described in Section 2.4.

method was validated for routine determination of monosaccharides released from glycoproteins and to detect differences of glycosylation between two CGMP Batches. The linearity of the detector response was evaluated for a concentration ranging from 0.5 to 10 μg/ml by plotting the peak area ratio (the analyte peak area divided by the internal standard peak area) versus concentration. Each sample was injected in triplicate with mannitol as internal standard. Linear regression correlation coefficients (r^2) were, in all cases, higher than 0.999. The limit of detection (LOD) (estimated as a signal-to-noise ratio equal to 3) was determined as less than 0.02 μg/ml for all the studied monosaccharides, giving a limit of quantitation (LOQ) value less than 0.06 μg/ml. These values indicate the high sensitivity of the described method. Within-day ($n=6$) and intra-day (3 days) method precision was evaluated for retention times and peak area ratios. For all monosaccharides, repeatability was better than 0.9% for retention time and 2.5% for peak area ratio, respectively. Results showed that the reproducibility of retention time (RSD<1.1%) and of peak area (RSD<3.8%) was satisfactory.

The validated method was applied to determine the monosaccharide composition of the two CGMP samples produced by different manufacturing procedures. As shown in Table 1, the monosaccharide percentages are 3–4 fold higher in the case of CGMP Batch I (7.99% against 2.34% for CGMP Batch II), particularly for galactose and glucose. In fact, the glucose and galactose contents represent 1.68 and 3.66% for CGMP Batch I, whereas they are only 0.15 and 0.58% for CGMP Batch II. This likely reflects a higher content of contaminating lactose in the case of Batch I. Moreover, small amounts of fucose and mannose were only detected in Batch I. This probably reflects the presence of *N*-type sugar chains from other contaminating whey glycopeptides.

The developed HPAEC-PAD method appears to be a valuable tool for the quantitation of neutral and amino-monosaccharides from glycomacropeptide, and particularly for the detection of contaminating whey glycopeptides.

Table 1
Monosaccharide composition of the two CGMP Batches ($n=6$)

	CGMP Batch I		CGMP Batch II	
	%	RSD	%	RSD
Fucose	<0.03	n.d.	n.d.	n.d.
N-Acetylglucosamine	0.48	0.59	0.27	2.94
N-Acetylgalactosamine	1.94	2.56	1.34	2.19
Mannose	0.21	1.17	n.d.	n.d.
Glucose	1.68	1.44	0.15	2.63
Galactose	3.66	1.54	0.58	3.39

n.d.=not detected.

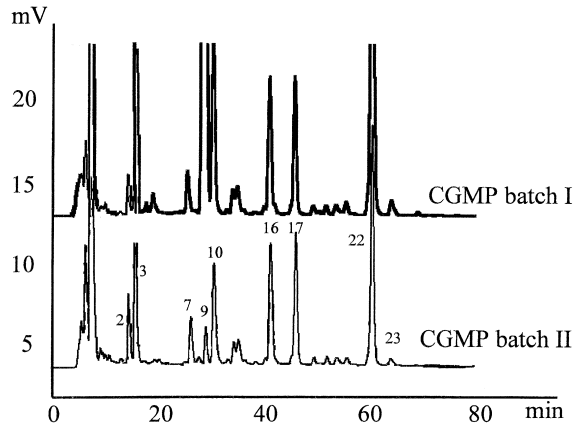

Fig. 5. Normal phase HPLC separation of 2-AB *O*-glycans released from CGMP Batch I (Top) and from CGMP Batch II (Bottom). Injected quantities: 0.9 and 2.1 μg of starting CGMP for Batches I and II, respectively. Experimental conditions are described in Section 2.5.

3.3. Oligosaccharide mapping by HPLC

For supplementary information on CGMP glycosylation and in order to detect minor differences that a simple monosaccharide analysis cannot afford, oligosaccharide mapping of the two CGMP Batches was performed by normal-phase HPLC coupled with fluorescence detection. This method relies on the liberation of all *O*-linked oligosaccharides from the glycomacropeptide by automated hydrazinolysis, followed by a sensitive labelling of the released glycans with 2-aminobenzamide and subsequent separation by HPLC using an amide stationary phase. In such a system, the separation of oligosaccharides is based on a hydrophilic interaction which reflects predominantly the size (i.e. number of monosaccharide residues) of each glycan. Fig. 5 shows a comparison of the two profiles of the glycan pools released from the two CGMP Batches. As

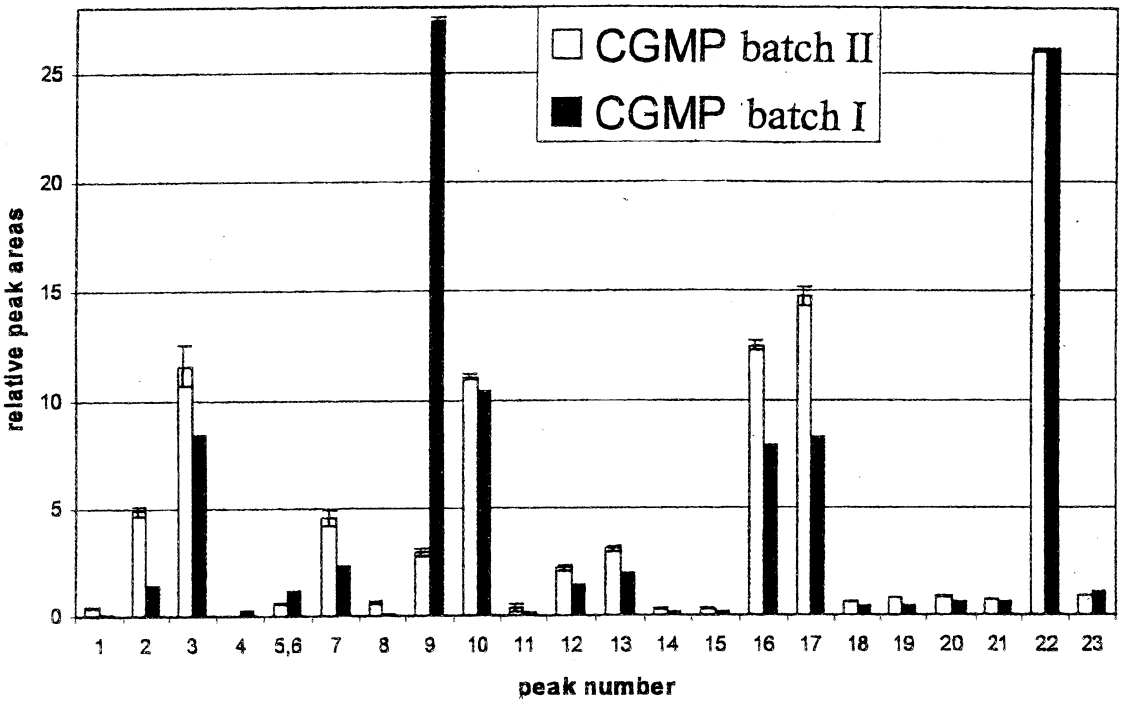

Fig. 6. Relative proportion of the major peaks obtained for the CGMP Batch I and CGMP Batch II derived oligosaccharides, separated by normal-phase chromatography. Values represent the mean of three analyses and bars indicate the corresponding standard deviations.

illustrated in Fig. 5, six major and several minor peaks could be observed in both chromatograms. These results indicate that the two CGMP preparations present an almost identical population of *O*-glycans. Derivatization with the 2-AB fluorescent dye provides a non-selective labelling independent of the glycan structure [26]. Therefore, we can assume that all the released glycans exhibit the same response which allow a quantitative comparison of the glycan pools: the peak intensities in the two profiles suggest that CGMP Batch I is more glycosylated than CGMP Batch II. Indeed, 0.9 and 2.1 μg were injected to produce the glycan map of CGMP Batch I and II, respectively, while most of the peaks in the CGMP Batch I exhibit at least a two-fold higher intensity. In addition, differences in the relative proportion of each structure were observed in the two glycan pools as demonstrated in Fig. 6. A Student *t*-test showed that the relative amount of several structures was significantly different in the two glycan pools. The most marked difference was observed for the relative amount of peak 9 which was 10 fold higher than CGMP Batch I. Furthermore, this Batch carries two predominant structures (peaks 9 and 22), each of which represents more than 25% of the total glycans found. In contrast, the CGMP Batch II glycosylation is more heterogeneous with five structures (peaks 3, 10, 16, 17 and 22) exhibiting a relative abundance over 10%. This result correlates the higher glycosylation heterogeneity found with the CIEF as a higher number of peaks was observed in the CGMP Batch II profile. To provide more information on the structural differences of the glycans attached to the two studied

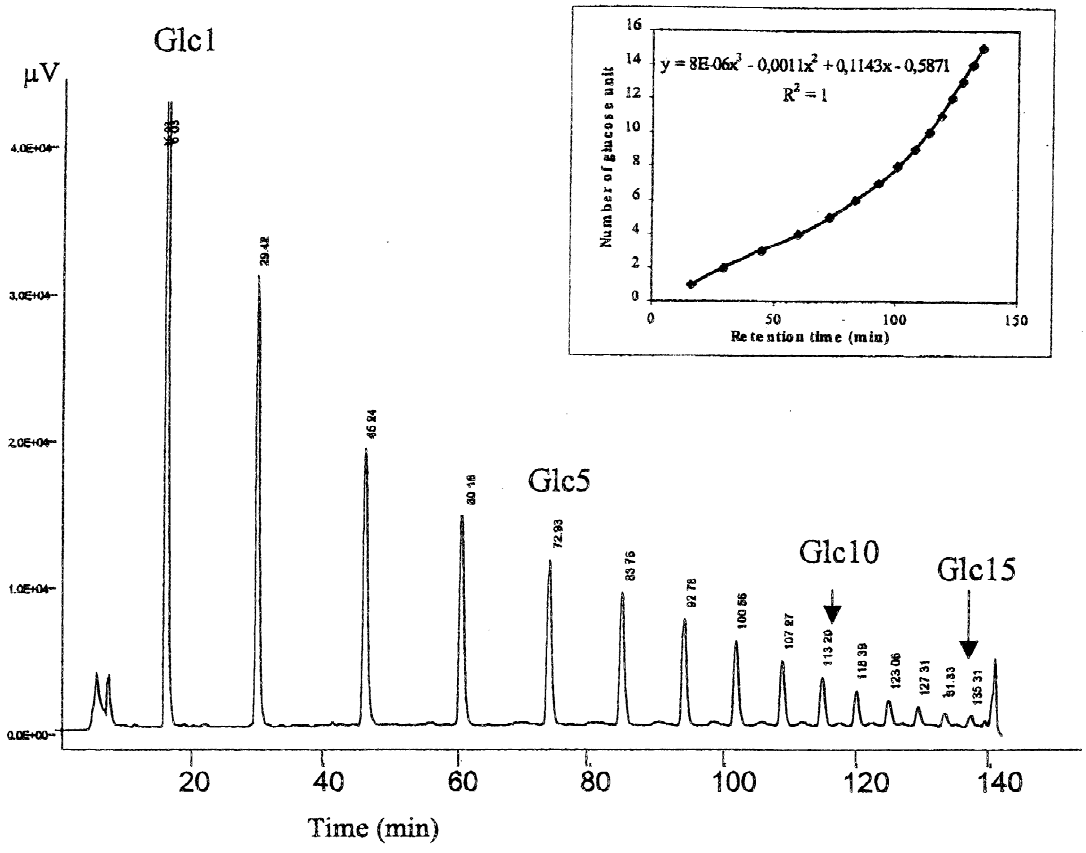

Fig. 7. Normal phase HPLC separation of 2-AB standard dextran hydrolysate and calibration curve yielded by plotting the glucose units in the standard dextran hydrolysate against their retention times.

glycomacropeptides, the size of the separated glycans, expressed as the number of glucose units, was determined by calibration with a partial dextran hydrolysate (Fig. 7). The glucose units of the different oligomers in this dextran hydrolysate were therefore plotted against their retention time. Many standard structures have previously been analysed in the same manner (Royle et al., manuscript submitted), making it possible to correlate the calculated glucose unit of each structure to its size. We could therefore deduce that structures 2 and 3 in Fig. 5 correspond to monosaccharides while structures 7–10 mainly consist of disaccharide structures; peaks 11–18 correspond to trisaccharide structures and peaks 18–23 are attributed to longer glycans (typically composed of 3–4 monosaccharide units).

4. Conclusion

In this paper, three analytical methods have been investigated for the characterization of a highly *O*-glycosylated bovine κ-casein macropeptide. A modified ''two-step'' CIEF has been shown to be a powerful and rapid technique for monitoring glycopeptide heterogeneity. Both CGMP Batches were found to be very acidic with p*I* values ranging from 2.74 to 3.3. Moreover, the results suggested a higher glycosylation heterogeneity for Batch II. However, this method did not allow quantitation of CGMP glycoforms, but provided information on the electrophoretic pattern. Subsequently, HPAEC-PAD has been successfully applied to determine neutral and amino-monosaccharides from CGMP and point out differences in the carbohydrate composition, as well as in the degree of glycosylation, of the two CGMP Batches. Results indicated a similar monosaccharide composition for Batches I and II, but the former exhibited a higher content in glucose and galactose, likely released from hydrolysis of contaminating lactose. Finally, normal-phase HPLC for oligosaccharide mapping was successfully applied to compare the glycosylation of CGMP Batches. Both showed identical oligosaccharide profiles. Nevertheless, comparing peak intensities revealed that Batch I was more glycosylated, which is in agreement with the data from monosaccharide analysis. In conclusion, combining these analytical methods provided complementary information and the overall results highlighted the lower degree of glycosylation of CGMP Batch II associated, in contrast, to a higher heterogeneity of *O*-glycan structures. Such procedures should be applicable to the analysis of other mucin-like proteins or glycopeptides and should allow a sensitive and reliable comparative analysis of *O*-glycosylation on a routine basis.

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