Quantitative analysis of bovine whey glycoproteins using the overall N-linked whey glycoprofile

Valk-Weeber, Rivca L.; Eshuis-de Ruiter, Talitha; Dijkhuizen, Lubbert; van Leeuwen, Sander S.

Published in:
International Dairy Journal

DOI:
10.1016/j.idairyj.2020.104814

International Dairy Journal 110 (2020) 104814

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Quantitative analysis of bovine whey glycoproteins using the overall *N*-linked whey glycoprofile

Rivca L. Valk-Weeber [a], Talitha Eshuis-de Ruiter [b], Lubbert Dijkhuizen [a, 1], Sander S. van Leeuwen [a, *, 2]

[a] *Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG, Groningen, the Netherlands*

[b] *Life Sciences, FrieslandCampina, Stationsplein 4, 3818 LE, Amersfoort, the Netherlands*

ARTICLE INFO

Article history:
Received 30 April 2020
Received in revised form
6 July 2020
Accepted 13 July 2020
Available online 29 July 2020

ABSTRACT

Bovine whey is an important ingredient in human nutrition and contains many biofunctional, glycosylated proteins. Knowledge on the glycoprotein composition of whey and whey products is valuable for the dairy industry. This paper describes a method for the characterisation of whey, or whey powders, by *N*-linked glycoprofile analysis. Application of the method for analysis of whey protein products showed clear differences in glycoprotein composition between concentrate, isolate and demineralised whey powders. The quantitative potential was explored by screening 100 pooled farm milk samples. IgG and lactoferrin protein concentrations determined by *N*-glycoprofile analysis matched well with ELISA results. The protein concentration of GlyCAM-1 was determined to be $\geq$1 mg mL^{-1}. The approaches presented in this work allow simultaneous concentration estimation of the three major whey glycoproteins, lactoferrin, IgG and GlyCAM-1 on the basis of their *N*-linked glycoprofiles, also in highly processed samples where conventional methods of detection (ELISA) are less suitable.

1. Introduction

Bovine milk is a complex mixture of water, lipids, carbohydrates (e.g., lactose), vitamins, minerals and proteins, which can be further divided into caseins and whey proteins (Korhonen, 2009). The whey proteins are the proteins present in the milk serum fraction, which remains after removal of the caseins. Acid whey is the fraction that remains in solution at 20 °C when defatted milk is acidified to pH 4.6, resulting in disruption of the casein micelle structure and precipitation of casein proteins (Farrell et al., 2004). When making cheese via rennet treatment of milk, a sweet whey is produced that contains casein fragments (Thomä-Worringer, Sørensen, & López-Fandiño, 2006).

Many whey proteins are bioactive glycoproteins occurring in varying quantities, such as lactoferrin (0.1–0.3 mg mL^{-1}), immunoglobulins G, A and M (IgG, IgA, IgM; 0.1–0.5 mg mL^{-1}), lactoperoxidase (0.03 mg mL^{-1}) and glycosylation dependent cellular adhesion molecule 1 (GlyCAM-1; PP3; lactophorin, 0.3–0.4 mg mL^{-1}) (Kussendrager & van Hooijdonk, 2000; Larson & Rolleri, 1955; O'Riordan, Kane, Joshi, & Hickey, 2014). The whey glycoproteins contain glycans of the *N*- or *O*-linked class defined by the amino acid side-chain to which they are linked. *N*-linked glycans can be further divided into three main classes, oligomannose, hybrid- or complex-type, depending on the monosaccharide composition of the glycan antennae present on the common core structure of two *N*-acetylglucosamine and three mannoses ($GlcNAc_2Man_3$) (Moremen, Tiemeyer, & Nairn, 2012). Some proteins, such as lactoferrin, are exclusively *N*-glycosylated, while the casein fragment casein glycomacropeptide (GMP) is exclusively *O*-glycosylated (Moore, Anderson, Groom, Haridas, & Baker, 1997; Vreeman, Visser, Slangen, & Van Riel, 1986). The protein GlyCAM-1 carries both *N*- and *O*-linked glycans (Kjeldsen, Haselmann, Budnik, Sørensen, & Zubarev, 2003).

The *N*-linked glycans can be selectively released by peptide-*N*-glycosidase F. A chromatographic profile generated from the *N*-linked structures of whey (whey *N*-linked glycoprofile), is a complex mixture of structures (O'Riordan et al., 2014; van Leeuwen,

* Corresponding author. Tel.: +31(0)503611576.
E-mail address: s.s.van.leeuwen@umcg.nl (S.S. van Leeuwen).
[1] Current address: CarbExplore Research BV, Zernikepark 12, 9747 AN, Groningen, The Netherlands.
[2] Current address: Laboratory Medicine, University Medical Centre Groningen (UMCG), Hanzeplein 1, 9713 GZ, Groningen, The Netherlands.

https://doi.org/10.1016/j.idairyj.2020.104814

Schoemaker, Timmer, Kamerling, & Dijkhuizen, 2012). Previously, we have shown that a glycoprofile obtained from acid whey is mostly the result of three main *N*-glycan contributing proteins: IgG, lactoferrin and GlyCAM-1 (Valk-Weeber, Dijkhuizen, & van Leeuwen, 2019). Of these three main proteins, GlyCAM-1 contributes the highest total number of glycan structures to the whey *N*-glycan pool (Valk-Weeber, Deelman-Driessen, Dijkhuizen, Eshuis-de Ruiter, & van Leeuwen, 2020a). The *N*-glycans of GlyCAM-1 are highly sialylated and fucosylated (Valk-Weeber et al., 2020a), both of which are important functional epitopes recognised as being involved in immune system modulation (Falconer, Subedi, Marcella, & Barb, 2018; Johnson, Jones, Ryan, & Cobb, 2013; Kaneko, Nimmerjahn, & Ravetch, 2006). The other main proteins, lactoferrin and IgG also have known antimicrobial and immune regulatory functions, mediated in part by their glycan structures (Figueroa-Lozano, Valk-Weeber, van Leeuwen, Dijkhuizen, & de Vos, 2018; Shields et al., 2002). Upon ingestion of milk and whey-based products, these proteins play an active role in the innate immune defence, as well as acting as decoys for bacteria in the intestine (Li et al., 2019; Sauer et al., 2019; Ulfman, Leusen, Savelkoul, Warner, & van Neerven, 2018).

The components of whey, most notably lactose and the protein fractions, are commonly utilised in other products. Commercial whey powders, such as whey protein concentrates (WPC), and isolates (WPI) are used as emulsifiers or texture enhancers (Fox & Mulvihill, 1982). Demineralised whey is often applied as a base ingredient of infant formula (Mettler, 1980). The protein composition and functional characteristics of these whey powders depends on the conditions used during their production, such as heat treatments or filtration steps (Morr & Ha, 1993; van Lieshout, Lambers, Bragt, & Hettinga, 2019). Whey powders and the bioactive glycan structures contained therein are applied in various food products. The composition and glycoprofiles of proteins in bovine milk whey and whey powders are thus important to consider.

While multiple methods are available for the accurate determination of lactoferrin and IgG in milk or milk whey (Le, Zhao, & Larsen, 2019; Palmano & Elgar, 2002), the options for GlyCAM-1 are much more limited. GlyCAM-1 can be determined by chromatography after separation from the other proteins (Paquet, Nejjar, & Linden, 1988), but GlyCAM-1 often coelutes with other proteose proteins (Elgar et al., 2000; Innocente, Biasutti, & Blecker, 2011). Currently, no chromatographic technique is available for the determination of lactoferrin, IgG and GlyCAM-1 in a single analysis. In addition, regular protein chromatography does not generate additional information about the functional glycans. By analysis of the *N*-linked glycoprofile of (acid) whey proteins, or whey protein powders, the three main glycoproteins can be recognised in the glycoprofile. It was hypothesised that the glycoprofile allows protein concentration quantitation for lactoferrin, IgG and GlyCAM-1 by careful evaluation of key glycan structures in the obtained chromatograms. The concentrations of lactoferrin and IgG in milk are well established, yet the concentration of GlyCAM-1 is relatively unknown. By combining the quantitative and qualitative evaluation of single whey glycoprofiles, a large amount of data was generated concerning bovine whey glycoprotein composition and the glycan structures present.

2. Materials and methods

2.1. Materials

Reference bovine lactoferrin, and α-lactalbumin were provided by FrieslandCampina Domo (Amersfoort, The Netherlands). Bovine gamma globulin (purity >98%) was from Serva (Heidelberg, Germany). For comparison, bovine IgG was isolated from mature milk using an 1 mL HiTrap Protein G HP antibody purification column (GE Healthcare, UK), according to manufacturer's specifications. GlyCAM-1 was obtained via heat-treatment of milk as described by Valk-Weeber et al. (2020a). PNGase F (*Flavobacterium meningosepticum*) was from New England Biolabs (Ipswich, UK). Aliquots of pooled tank milk (quality control samples of pasture or regular milk), originating from 100 different individual farms in the Netherlands were obtained from FrieslandCampina Domo. Anonymised powders of whey protein concentrate (WPC), whey protein isolate (WPI) and demineralised whey products with different processing backgrounds were obtained from FrieslandCampina Domo.

2.2. Whey preparation and protein isolation

Samples were prepared according to the procedure of Valk-Weeber et al. (2020a). Milk samples were thawed in a water bath of 37 °C and homogenised. An aliquot of 1 mL was defatted by centrifugation at 4000×*g* for 10 min. An amount of 400 μL defatted milk was transferred into a new tube. Caseins were removed by addition of 125 mM ammonium acetate at pH 4.6 at a ratio of 1:1. The samples were vortexed and left at room temperature for 5 min before centrifugation at 11,000×*g* for 5 min to precipitate the caseins. An aliquot of 100 μL of the supernatant (acid whey) was transferred into a new tube and 400 μL 100 mM ammonium acetate in methanol (MeOH + NH_4Ac) was added and mixed by vortexing. Whey protein precipitation was facilitated by centrifugation for 5 min at 11,000×*g*. The solvent (containing lactose) was carefully pipetted from the protein pellets. The protein pellets were re-dissolved in 75 μL of 2% SDS and 2% β-mercaptoethanol in 80 mM phosphate buffer, pH 7.5. After addition of the solvent, the samples were incubated at 37 °C for 10 min, after which they were vortexed vigorously and further incubated for an additional 10 min, followed by a final vortex mixing. The proteins were denatured by heating for 15 min at 85 °C and cooled to room temperature. An aliquot of 25 μL 10% NP-40 (NP-40 substitute, Sigma) was added to each sample and vortex-mixed. Finally, 2 μL diluted PNGase F (100 units per experiment) was added to the samples and mixed. Glycans were released overnight at 37 °C.

2.3. Whey protein powder analysis

Samples of whey protein powders (WPC, WPI or demineralised whey) were dissolved in MilliQ water to give 20 mg mL^{-1} protein solutions, adjusted by their initial % protein (Table 1). An aliquot of 50 μL (corresponding to 1 mg of protein) was mixed with 50 μL 125 mM ammonium acetate solution, pH 4.6. Further processing, including lactose removal by MeOH + NH_4Ac and glycan release, was performed as described above for the whey preparation method.

2.4. Labelling and clean-up

Isolated glycans were labelled with anthranilic acid (2-AA, Sigma) or 2-aminobenzamide (2-AB). The 2-AA label was chosen in combination with fluorescent detection in view of its higher sensitivity in these applications. The 2-AB label was chosen for applications that required mass spectrometry analysis. Direct in-solution labelling of whey digests was performed as follows. Whey protein digests of a total volume of 102 μL were mixed 1:1 with labelling solution [0.7 M 2-AA or 2-AB and 2 M 2-picoline borane or sodium cyanoborohydride in dimethylsulphoxide (DMSO, Sigma):glacial acetic acid (7:3, v/v)]. Incubations were performed for 2 h at 65 °C (Bigge et al., 1995). Labelling reagents were removed by 96-well microcrystalline cellulose SPE as

Table 1
Whey powder types, processing methods, and protein content of the powders analysed.[a]

Powder type	Code	Whey type	Process	Protein (%)	NSI (%)	Heat treatment
Concentrate	C1	Acid	UF	35	68	Pasteurised
Concentrate	C2	Cheese	UF	35	83	Pasteurised
Concentrate	C3	Cheese	UF	35	93	Pasteurised
Concentrate	C4	Cheese	UF	35	87	Pasteurised
Concentrate	C5	Cheese	UF	35	93	Pasteurised
Concentrate	C6	Cheese	UF	80	NA	Unknown
Concentrate	C7	Cheese	UF	80	NA	Unknown
Concentrate	C8	Cheese	UF	80	NA	Unknown
Concentrate	C9	Cheese	Unknown	80	NA	Unknown
Concentrate	C10	Acid	UF	80	NA	Pasteurised
Concentrate	C11	Cheese	UF	80	NA	Pasteurised
Concentrate	C12	Cheese	UF	80	NA	Unknown
Concentrate	C13	Acid	UF	80	NA	Pasteurised
Isolate	I1	Cheese	UF + MF	90	NA	Unknown
Isolate	I2	Cheese	Unknown	90	NA	Unknown
Isolate	I3	Cheese	UF + MF	90	NA.	Unknown
Isolate	I4	Cheese	Unknown	90	NA	Unknown
Isolate	I5	Cheese	Unknown	90	NA	Unknown
Isolate	I6	Unknown	Unknown	90	NA	Unknown
Isolate	I7	Cheese	Unknown	90	NA	Unknown
Demineralised	D1	Cheese	Unknown	13	72	Pasteurised
Demineralised	D2	Cheese	Unknown	13	65	Pasteurised
Demineralised	D3	Cheese	IEX/NF/SF	13	82	Pasteurised
Demineralised	D4	Cheese	IEX/ED/NF	13	58	Pasteurised
Demineralised	D5	Cheese	IEX/NF	13	66	Pasteurised
Demineralised	D6	Cheese	ED/IEX	13	70	Pasteurised
Demineralised	D7	Cheese	ED/IEX	13	81	Pasteurised
Demineralised	D8	Cheese	ED/IEX/SF	13	74	Pasteurised
Demineralised	D9	Cheese	IEX	13	80	Pasteurised

[a] Abbreviations are: NSI, nitrogen solubility index; UF, ultrafiltration; MF, microfiltration; IEX, ion exchange; NF, nanofiltration; ED, electrodialysis; SF, softening; NA, not available.

described (Ruhaak et al., 2008). Samples were diluted with 612 μL acetonitrile (ACN) (final concentration 75%, v/v) prior to application to the cellulose SPE.

2.5. Lactose determination

To quantitate lactose in supernatant and pellet after precipitation with MeOH containing 100 mM ammonium acetate, three milk samples were each processed in triplicate. In the final precipitation stage, the supernatant was carefully removed from the protein pellet and collected. The pellets were resuspended in 1 mL MilliQ water. The supernatant fraction, containing MeOH, ammonium acetate and lactose, was evaporated under nitrogen flow, and the dry residue was resuspended in 1 mL MilliQ water. To verify the initial concentration of lactose in the selected samples, the defatted milk was centrifuged over a 10-kDa molecular mass cut-off filter (Amicon Ultra, Merck Millipore, Tullagreen, Cork, IRL) and collected. The concentration of lactose was determined against an 8-point calibration curve of lactose (lactose monohydrate, Sigma), from 2 μM to 2 mM. Samples were diluted to approximate 175 μM before analysis. High-performance anion-exchange chromatography (HPAEC) was performed on an ICS-3000 ion chromatography system (Thermo Fisher Scientific, Waltham, MA, USA), equipped with a CarboPac PA-1 column (250 mm × 2 mm, Thermo Fisher Scientific). Detection was performed with a pulsed-amperometric detector (PAD) with a gold working electrode (four-step potential waveform) (Altunata, Earley, Mossman, & Welch, 1995). Lactose was eluted with a linear gradient of 10 mM NaOH + 2.5 mM sodium acetate to 40 mM NaOH + 25 mM NaOH in 25 min at a flow rate of 0.25 mL min^{-1}. The gradient was followed by a washing step with 100 mM NaOH and 600 mM sodium acetate for 5 min (0.25 mL min^{-1}) and reconditioning to initial conditions.

2.6. Protein precipitation efficiency assay

To evaluate the efficiency of protein isolation *via* precipitation (see above), a comparison was made with protein recovery by desalting using molecular mass cutoff filters. The experiments were also performed on ultracentrifuged whey (100,000×*g*, 1 h, 4 °C, ~pH 6.7), prepared using a Sorvall RCM120GX micro-ultracentrifuge (Thermo Fisher Scientific). To test the effect of pH on the precipitation process, in addition to acid whey and ultracentrifuged whey, precipitation of neutralised acid whey (pH 7) was performed. Desalted whey was obtained by filtration of (acid or ultracentrifuged whey) using a molecular mass cutoff (MWCO) filter (10-kDa Amicon Ultra, Merck Millipore, Tullagreen, Cork, IRL). Protein precipitation of the different whey samples was carried out with MeOH + NH_4Ac according to the whey preparation procedure as described above. The normally discarded supernatant (containing lactose) was collected to check for the presence of any protein. The supernatant was concentrated to approximately 20% of the original volume, by evaporation of the MeOH under a stream of nitrogen. A dilution was made to approximate 1 mL with MilliQ water and any protein present was recovered by centrifuging over a 10 kDa cutoff filter, as described above.

2.7. HPLC analysis

Fluorescently labeled glycans were separated on an Acquity UPLC Glycan BEH Amide column (2.1 mm × 100 mm, 1.7 μm, Waters Chromatography BV, Etten-Leur, the Netherlands), using an UltiMate 3000 SD HPLC system (Thermo Fisher Scientific, Waltham, MA) equipped with a Jasco FP-920 fluorescence detector (λ_{ex} 330 nm, λ_{em} 420 nm, Jasco Inc, Easton, MD). An injection volume of 3 μL was used. Ternary gradients were run using MilliQ water, acetonitrile and a buffer solution consisting of 250 mM formic acid

in MilliQ water, adjusted to pH 3.0 using ammonia. The buffer solution was maintained at a constant 20% throughout the run. Elution was performed by a slow sloping gradient of 22%–40% MilliQ water (total concentration, including buffer) from 0 to 67.5 min. The remaining percentage of the solvent composition comprised of acetonitrile. Final gradient conditions were maintained for 9 min after completion of the gradient and the column reconditioned back to initial conditions for 13 min.

2.8. Determination of lactoferrin and immunoglobulin concentration by ELISA

The concentrations of bovine immunoglobulins G, A and M and lactoferrin in the milk samples were quantified by the bovine ELISA quantitation kits (E10-118, E10-131, E10-101 and E10-126, respectively; Bethyl Laboratories, Montgomery, TX, USA). Manufacturer's protocols were strictly followed, with the additional use of the Roche ELISA Blocking reagent as described below. ELISA plates (Costar, #3590) were coated with the capture antibody followed by a blocking step using blocking reagent for ELISA from Roche Diagnostics (Mannheim, Germany). Milk samples were diluted within the calibration curve range (7.8–500 ng mL^{-1} for bovine IgG and lactoferrin and 15.6–1000 ng mL^{-1} for bovine IgA and IgM) using Blocking reagent for ELISA. As a last step, horseradish peroxidase (HRP)-labelled detection antibody was diluted in ELISA blocking reagent (Roche). TMB substrate solution (SB02, Thermo Fisher) was added to the wells. After 15 min, colour development was stopped using 1 M HCl (Sigma). Absorbance was measured at 450 nm using a Bio-Rad iMark ELISA plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Data were analysed using a four-parameter logistic (4-PL) curve-fit.

2.9. Lactoferrin calibration curve for glycoprofile-based determination of glycoprotein concentration

A calibration curve of lactoferrin was prepared in the range of 25–500 μg mL^{-1} in MilliQ. Samples for the calibration curve were diluted 1:1 with 125 mM ammonium acetate solution at pH 4.6; proteins in 100 μL aliquots were precipitated with 400 μL MeOH + NH_4Ac. For resuspension of protein pellets, glycan release, labelling and analysis, the whey preparation procedure was followed as described above.

3. Results and discussion

3.1. Sample preparation optimisation

To ensure efficient and repeatable analysis, the sample processing conditions were optimised. Acid whey contains glycoproteins of interest, as well as salts and a large quantity of lactose. To prevent competition between lactose and released glycans in the fluorescent labelling reaction, most lactose had to be removed from the samples. An effective approach for removal of lactose was a protein precipitation step of acid whey with 4:1 (v/v) MeOH containing 100 mM ammonium acetate (MeOH + NH_4Ac). After removing MeOH + NH_4Ac from the protein pellets obtained by centrifugation, analysis of the pellet and solvent fractions showed that lactose was >95% reduced in the pellet fractions (Supplementary Material, Table S1). The protein precipitation of lactoferrin, IgG and GlyCAM-1 appeared complete with acidified (pH 4.6) whey, while a minimal loss occurred when using neutralised or ultracentrifuged (pH 6.7) whey (Supplementary Material, Fig. S1).

This pH-related precipitation efficiency of proteins, especially in the isoelectric point range of many proteins (pH 4–6), has been well described (Feist & Hummon, 2015). While sample loss appears minimal for neutralised or ultracentrifuged whey, it is recommended to acidify the whey (or whey powder solutions) to pH 4.6 prior to precipitation with MeOH + NH_4Ac. Protein recovery was similar after precipitation or use of 10 kDa cut off filters for desalting whey (Supplementary Material, Fig. S2). The obtained protein pellets were redissolved and digested by PNGase F, followed by direct in-solution reducing end labelling and subsequent cellulose clean-up in 96-wells plate format, adapted from a procedure described by Ruhaak et al. (2008). In the final protocol, 2-methylpyridine borane complex was preferred as catalyst over cyanoborohydride in view of its lower toxicity and lower sensitivity to water (Ruhaak, Steenvoorden, Koeleman, Deelder, & Wuhrer, 2010). Evaluation of both catalysts resulted in identical results under the optimised conditions (Supplementary Material, Fig. S3), fitting previous reports (Ruhaak et al., 2010). With the optimised protocol, a sample set could be analysed in just a few days, from whey preparation to overall glycoprofile analysis (Supplementary Material, Fig. S3).

3.2. Protocol repeatability

Ideally, an internal standard should be added to the raw milk samples to correct for any bias in the procedure. In view of the difference in degree of polymerisation (DP) of an internal standard and the large DP range of the glycans obtained from whey, an added oligosaccharide is unlikely to be representative for the whole DP range and thus will not guarantee a good correction for all glycans present in the whey. In addition, any added oligosaccharide added to the milk prior to sample processing is lost during the protein precipitation step. Therefore, we opted to evaluate the method without use of any internal standard. The repeatability of the protein precipitation step was evaluated by a six-fold processing of three different samples of pooled milk. HPLC chromatograms of overall whey glycoprofiles were divided into 32 different complex-peak clusters and integrated (Fig. 1). The coefficient of variation (CV) was calculated for each peak cluster, over a total of 18 replicates between 3 samples. Variation was less than 6% for all integrated peak clusters (Supplementary Material, Table S2), indicating that the method was highly repeatable and sufficient for quantitative applications.

3.3. Qualitative whey protein powder analysis

The main applications for the methods outlined here are glycoprofile analysis of acid whey prepared directly from milk. The methodology can be easily adapted for analysis of other sources of whey proteins. Instead of using acid whey, an aqueous protein solution can be mixed 1:1 with buffer at pH 4.6 to obtain a suitable solution for further processing. One such application is the analysis of commercial whey-derived protein powders. The major whey protein powder products are demineralised whey, whey protein concentrate (WPC) and whey protein isolate (WPI) (Table 1). WPCs are obtained by processing the whey with ultrafiltration, ion exchange, or a combination thereof, reducing the lactose, water and mineral contents and increasing the protein content to 35–90% (Carter & Drake, 2018). WPIs are obtained by performing ultrafiltration after an initial microfiltration step. This microfiltration step removes lipids from the whey, and subsequent ultrafiltration yields a more pure and higher concentration WPI (Hanemaaijer, 1985). For infant formulas and products where a lower mineral content is preferred, demineralised whey is used, obtained by nanofiltration or ion-exchange (Jost, Maire, Maynard, & Secretin, 1999; Mettler, 1980).

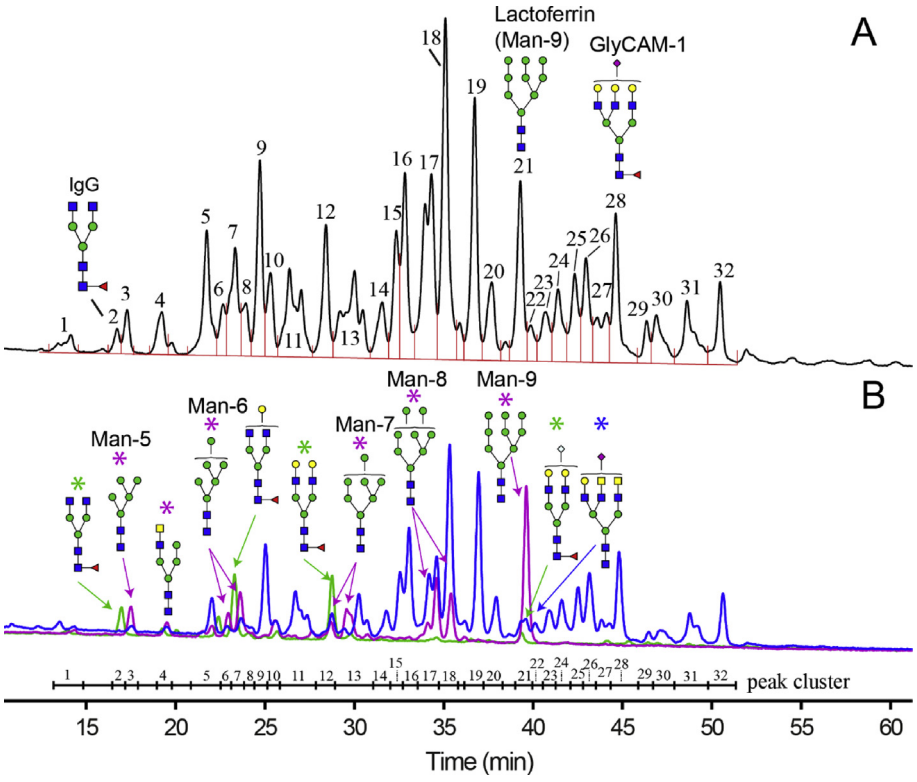

Fig. 1. HPLC chromatogram of the overall bovine acid whey glycoprofile (A), divided into 32 separate integrated peak clusters. A selection of glycans (denoted with asterisks) from IgG (——; green asterisks), lactoferrin (——; pink asterisks) and GlyCAM-1 (——; blue asterisks) is annotated in the overlay chromatogram (B). The symbols used to represent the monosaccharides of a typical glycan structure are: ●, mannose; ●, galactose; ◆, *N*-acetylneuraminic acid; ▲, fucose; ■, *N*-acetylglucosamine; ■, *N*-acetylgalactosamine. The oligomannose glycans $GlcNAc_2Man_{5-9}$ are marked with the abbreviations Man-5 to Man-9. The three signature glycans of IgG, lactoferrin and GlyCAM-1, used for quantitation of their respective protein concentration, are shown above the whey chromatogram (A).

The glycoproteins present in whey determine its overall glycoprofile. The full whey glycoprofile contains a large number of *N*-glycans, which have been identified previously and attributed to lactoferrin, lactoperoxidase, α-lactalbumin, IgG and GlyCAM-1 (Valk-Weeber et al., 2020a). Of these five proteins lactoferrin, IgG and GlyCAM-1 contribute the majority of the structures found in the whey glycoprofile, as summarised in Fig. 1. Each protein contains unique signature glycans by which they can be identified in the overall profile (Valk-Weeber et al., 2020a) (Fig. 1).

The glycoprofiles of a selection of commercial WPCs, WPIs and demineralised powders were analysed and compared (Fig. 2). The WPC powders differed in their initial protein content (35 and 80%, respectively) (Table 1). Of each powder, a normalised amount of 1 mg of whey protein was analysed. The glycoprofiles obtained for the 35% and 80% protein concentrates were generally very comparable, with some differences. Of the 35% WPC, the C1 glycoprofile displayed lower peak intensities than the rest (Fig. 2A). This is also the powder with the lowest (68%) nitrogen solubility index (NSI), which is indicative of the soluble protein present in the whey powder. Since the powders were dissolved prior to analysis, any undissolved glycoprotein will not be taken into the analysis, resulting in a lower overall glycoprofile. Of the 80% WPC, the C10 and C13 (acid whey) glycoprofiles were most intense (Fig. 2B), indicating higher glycan (and thus glycoprotein) quantities.

The glycoprofiles of demineralised whey samples (Fig. 2C) were, overall, similar to those of WPC. Highest glycan peak intensities were observed for samples D2, D5, D6 and D7, while the overall lowest were observed for D3. In D3 the signature glycans of GlyCAM-1 were present in markedly lower intensity, indicating a lower concentration for this protein. The peak height of Man-9 (marked LF, Fig. 2C) in this powder, however, was similar to the other demineralised powders tested. This suggests that some glycoprotein (most notably GlyCAM-1) was lost during processing of this powder, but that lactoferrin was not affected.

WPC and demineralised whey thus appeared relatively similar in composition. Despite the highest protein content (90%), the glycoprofiles of WPI were markedly lower than the other two whey powders (Fig. 2D). The WPI glycans observed were primarily signature structures of GlyCAM-1. The WPI signature structures of IgG and lactoferrin were observed to have a much lower intensity compared with the other protein powders (Fig. 2). These proteins are thus likely to be removed from whey during the production process of WPI. The main difference between WPI and WPC production is the application of a microfiltration step for WPI production, suggesting that this step may result in glycoprotein loss.

Qualitative evaluations of the commercial whey powder glycoprofiles, clearly demonstrated that differences in glycoprotein composition can occur due to processing conditions and protein concentration. For an accurate determination of glycoprotein concentrations, quantitation by integration of the obtained whey profiles was explored.

3.4. Peak cluster area and protein concentration correlation

Glycoprofiles of pooled bovine (tank) milk quality control samples from 100 individual farms in the Netherlands were analysed and integrated according to Fig. 1. Concentrations of lactoferrin, IgG, IgA and IgM were determined by ELISA (Supplementary Material, Table S1). Pearson correlations were made between the

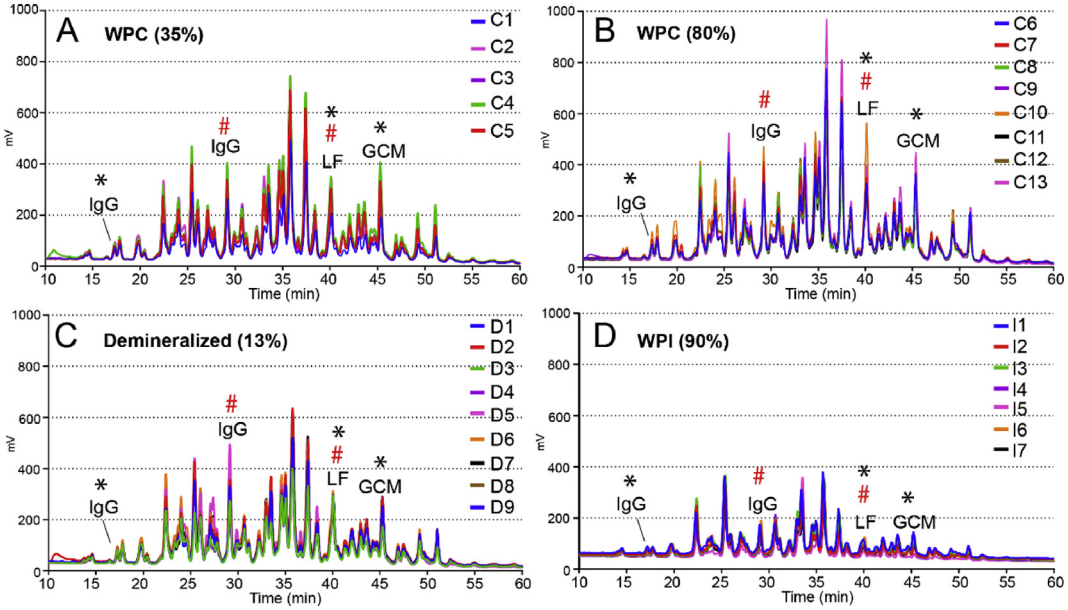

Fig. 2. Overlays of the HPLC chromatograms obtained by glycoprofile analysis of WPC (A and B), demineralised whey powders (C), and WPI (D). Glycoprofiles were determined for samples corresponding to 1 mg of protein. The peak clusters containing signature glycans (Fig. 1) of immunoglobulin G (IgG), lactoferrin (LF) and GlyCAM-1 (GCM) in the chromatogram are marked with (*). Peak clusters with the strongest Pearson correlation (Table 2) for the protein concentrations of IgG and LF are marked with (#).

ELISA determined protein concentrations and the integrated glycan peak clusters (Table 2).

Several of the glycan peak clusters had a significant correlation with the ELISA determined protein concentration of lactoferrin, IgG, IgA and IgM (Table 2). The peak clusters showing significant correlations could be traced back to their protein of origin. For example, the oligomannose structures ($GlcNAc_2$-Man_{5-9}) of lactoferrin eluted under peak clusters 3 (Man-5), 6, 7 (Man-6), 12, 13 (Man-7), 17, 18 (Man-8) and 21 (Man-9), and peak cluster 4 containing a hybrid-type glycan of lactoferrin (Table 2; Fig. 1). Similar comparisons could be made for IgG, with the most notable structure being a fucosylated di-antennary structure, $GlcNAc_2$-Fuc-Man_3-$GlcNAc_2$-Gal_2, eluting under peak cluster 12 (Fig. 1.). A large number of glycan peak clusters (peak clusters 1, 5, 8, 9, 11, 14–16, 20, 22, 23, 25, 26, 30–32) did not correlate significantly with lactoferrin, IgG, IgA or IgM. These peak clusters have been associated with GlyCAM-1 (Table 2; Fig. 1) (Valk-Weeber et al., 2020a), a protein in whey of which the concentration has not been determined by ELISA.

Table 2
Correlations between ELISA determined protein concentrations and glycan peak clusters.[a]

Protein peak cluster	Pearsson correlations (r)			
	LF	IgG	IgA	IgM
2^{IgG}	0.56	0.57	ns	0.43
$3^{LF,\ GCM}$	0.78	0.48	0.55	0.56
$4^{LF,\ GCM,\ IgG}$	0.60	0.45	ns	0.42
$6^{LF,\ GCM,\ IgG}$	0.77	0.39	0.48	0.56
$7^{IgG,\ LF,\ GCM}$	0.70	0.47	0.50	ns
$10^{GCM,\ LF,\ IgG}$	0.49	0.55	ns	0.46
11^{GCM}	ns	ns	ns	ns
$12^{IgG,\ GCM,\ LF}$	0.59	0.78	0.53	0.59
$13^{LF,\ GCM}$	0.54	ns	ns	ns
16^{GCM}	ns	ns	ns	ns
$17^{GCM,\ LF}$	0.40	ns	0.47	ns
$18^{GCM,\ LF}$	0.62	ns	ns	ns
19^{GCM}	ns	0.41	ns	
$21^{LF,\ GCM,\ IgG}$	0.81	ns	0.48	ns
24^{GCM}	ns	ns	ns	ns
$27^{GCM,\ IgG}$	0.43	ns	ns	ns
$28^{GCM,\ IgG}$	ns	0.42	ns	ns
$29^{GCM,\ IgG}$	0.42	0.14	ns	ns

[a] Two-tailed Pearsson correlations between protein concentration and peak cluster areas (alpha = 0.01) were calculated in GraphPad Prism 7.0. Correlations were deemed significant when a P value of <0.0001 was obtained (ns, not significant). Peak clusters were integrated according to Fig 1. Peak clusters containing N-glycans of lactoferrin (LF), immunoglobulin G (IgG) or GlyCAM-1 (GCM) according to Fig. 1B are marked with a superscript (highest contributor first). Peak clusters without any significant correlation for any of the tested parameters were excluded from the table.

The majority of the significant peak cluster to protein correlations found for lactoferrin and IgG ranged between r 0.40 and 0.60, yet for some clusters a higher correlation was found ($r \geq 0.70$; Table 2). The intensity of peak clusters showing this higher correlation may to a large extent be determined by the eluting glycans of the corresponding glycoprotein. The strongest correlation ($r = 0.81$, $P < 0.0001$) was found for peak cluster 21 (Table 2), which corresponds to the Man-9 structure of lactoferrin (Fig. 1). This structure was selected for further exploration of lactoferrin quantitation by glycoprofile analysis.

3.5. Protein concentration determination: lactoferrin

In previous work, it was determined that the glycoprofile of lactoferrin in mature milk is highly constant over time and independent of inter-cow and breed differences (Valk-Weeber, Eshuis-de Ruiter, Dijkhuizen, & van Leeuwen, 2020b). Of the total amount of glycans present on lactoferrin, 37.2% is the oligomannose Man-9 (Valk-Weeber et al., 2020b). Man-9 elutes in a relatively isolated section of the chromatogram (Fig. 1, peak 21), thereby making it a suitable target for integration and quantification.

A random selection of 12 individual samples was made out of the total of 100 pooled tank milk samples (Supplementary Material, Table S3). Quantification of the Man-9 peak was performed against a calibration curve prepared of a lactoferrin standard (Fig. 3). To estimate the concentration of the Man-9 peak, the whey glycoprofile peak area (Fig. 1, peak 21) had to be corrected for contributions by co-eluting structures from proteins other than lactoferrin (Fig. 1). Man-9 is also present on lactoperoxidase,

however, its low protein concentration in combination with a relatively low percentage of Man-9 on this protein minimises the contribution of lactoperoxidase to the Man-9 peak in the whey glycoprofile (Valk-Weeber et al., 2020a).

One of the co-eluting structures with Man-9 is a mono-sialylated structure from IgG (Fig. 1). The glycoprofiles of isolated IgG from milk in this study and commercial IgG from bovine blood serum were highly similar (Supplementary Material, Fig. S4). In addition, the IgG glycoprofile found in our study showed a high similarity with that in earlier work (Adamczyk et al., 2014; Fujii, Nishiura, Nishikawa, Miura, & Taniguchi, 1990). It is thus likely that the IgG glycoprofile is conserved between cow populations. In this study, the use of pooled tank milk homogenised the IgG profile and the glycoprofile was assumed to be stable. We analysed the glycan distribution of IgG and calculated the percentages of its individual glycans (Supplementary Material, Table S4, Fig. S5). Using these percentages, by integration of a single IgG glycan, the area of the other glycan peaks in the profile can be calculated. To estimate the area contribution of co-eluting glycans of IgG with Man-9 (Fig. 1, peak 21), the area of the IgG signature glycan was integrated (Fig. 1, peak 2), and converted into the area of the interfering glycan under peak 21, allowing for an area correction.

Finally, the co-eluting structures of GlyCAM-1 were considered, applying the same procedure as for IgG. The individual glycans of GlyCAM-1 were expressed as percentages of its total profile (Supplementary Material, Table S5 and Fig. S6). The signature glycan chosen for GlyCAM-1 was a tri-antennary glycan exclusive to GlyCAM-1 (Fig. 1, peak 28). The contribution to Man-9 (Fig. 1, peak 21), was estimated by calculating the co-eluting peak area of GlyCAM-1 at this position.

The average contributions of IgG and GlyCAM-1 to the total uncorrected concentration of lactoferrin were estimated as 0.02 mg mL^{-1} and 0.07 mg mL^{-1}, respectively (Table 3). The corrected lactoferrin concentration ranged from 0.14 to 0.28 mg mL^{-1}, which is within its normal reported range (Cheng et al., 2008). These values are, however, higher than our ELISA-determined values (Table 3).

The different methods used for lactoferrin detection may explain this discrepancy. The antibodies used for the ELISA detection of lactoferrin interact with conformational structures, which are lost upon denaturation of the lactoferrin protein (Van Berkel, Van Veen, Geerts, & Nuijens, 2002). Commercial ELISA kits for bovine lactoferrin determination are often used before and after treatments, to determine only the non-denatured fraction (Mazri, Sánchez, Ramos, Calvo, & Pérez, 2012; Teixeira et al., 2013). To quantify the full lactoferrin (native and denatured) pool, chromatographic tools are often used (Billakanti, McRae, Mayr, & Johnson, 2019). Different methods (ELISA or chromatographic) are known to yield different concentrations of lactoferrin in the same sample (Indyk & Filonzi, 2005). Finally, the ELISA detectable concentration of (human) lactoferrin is known to decrease significantly during storage, i.e., only 46% was detected after 6 months of frozen storage (Rollo, Radmacher, Turcu, Myers, & Adamkin, 2014). Considering that the bovine milk samples used in this study were stored frozen for >6 months prior to lactoferrin determination by ELISA, a significant portion is likely denatured and thus remained undetected by ELISA. In contrast, lactoferrin quantitation by the glycoprofile analysis method operates independently of the folding of this protein. The method was not applied yet on fresh milk, which is an interesting target for future investigation of this hypothesis. For previously frozen milk, the protein precipitation and glycan extraction protocols described resulted in isolation of glycans from both folded and denatured protein molecules, allowing determination of the total lactoferrin concentration.

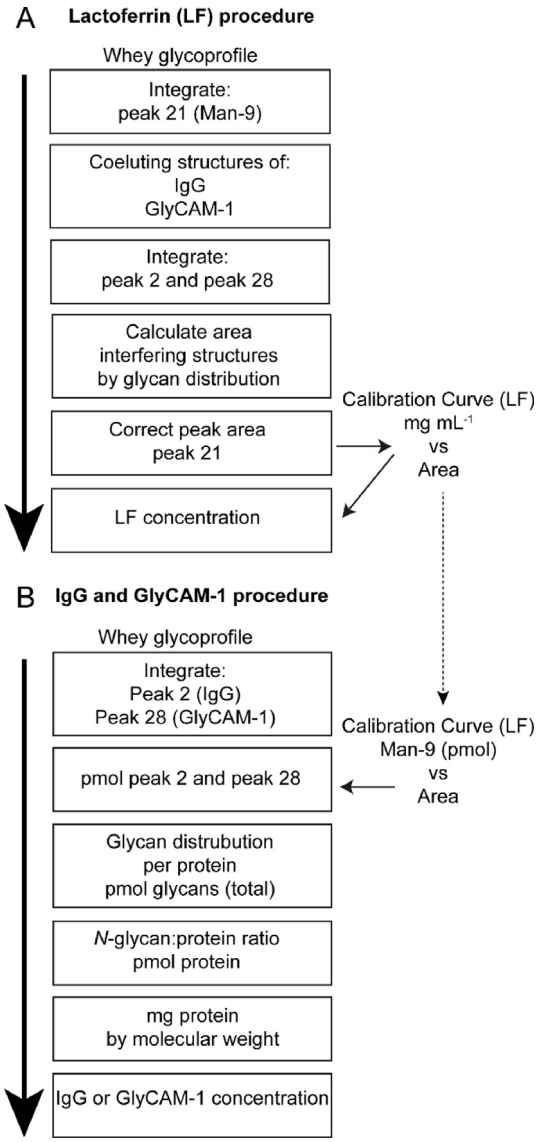

Fig. 3. Flowchart for the determination of the concentration of lactoferrin, IgG and GlyCAM-1 by whey glycoprofile analysis. For lactoferrin, procedure A was followed, using peak cluster 21 (Fig. 1) and area correction for minor interfering glycans from IgG and GlyCAM-1 as calculated from peak 2 and 28, using the glycan distribution (Supplementary Material Tables S4, S5 and Figs. S5, S6). For IgG and GlyCAM-1, concentration determination was performed by procedure B, calculating molar quantities of signature glycan peaks 2 and 28 (Fig. 1) and converting to the total glycan amount present per protein using their individual glycan distribution (Supplementary Material Tables S4, S5 and Figs. S5, S6). The molar quantities obtained were converted to protein amounts by the *N*-glycan:protein ratio and molecular weight. The full procedure is detailed in the main text, Sections 3.5 and 3.6.

3.6. Protein concentration determination: IgG and GlyCAM-1

While lactoferrin is commercially available, this is not the case for GlyCAM-1. However, the concentrations of the IgG and GlyCAM-1 proteins in the whey can be estimated based on a calibration curve for lactoferrin (Fig. 3). For this purpose, the peak areas obtained were converted into molar quantities, using Man-9 of lactoferrin as a reference. Based on the carbohydrate content of lactoferrin (7.7%) (Valk-Weeber et al., 2019), and the Man-9 content (37.2%), it was calculated that of the total carbohydrate weight, 2.87% originates from Man-9. For each gram of lactoferrin, 28.7 mg of Man-9 (1882.6 g mol^{-1}) is present, which corresponds to 15.2 nmol.

Table 3
Concentrations of lactoferrin, IgG and GlyCAM-1 determined in a selection of 12 pooled milk samples by whey profile analysis and ELISA.[a]

Method sample	Interference		Concentration				
	IgG	GlyCAM-1	Lactoferrin		IgG		GlyCAM-1
	Profile	Profile	Profile	ELISA	Profile	ELISA	Profile
1	0.02	0.08	0.24	0.15	0.43	0.36	1.41
2	0.02	0.08	0.21	0.18	0.43	0.50	1.42
3	0.02	0.06	0.28	0.21	0.39	0.38	1.09
4	0.02	0.06	0.21	0.16	0.48	0.45	1.13
5	0.01	0.07	0.19	0.14	0.36	0.44	1.22
6	0.01	0.06	0.15	0.11	0.37	0.37	1.10
7	0.01	0.07	0.25	0.17	0.26	0.40	1.23
8	0.02	0.07	0.25	0.17	0.39	0.39	1.23
9	0.02	0.06	0.19	0.11	0.43	0.40	1.09
10	0.02	0.06	0.20	0.16	0.40	0.41	1.01
11	0.01	0.07	0.14	0.10	0.38	0.38	1.30
12	0.01	0.07	0.16	0.10	0.35	0.33	1.17
Average	0.02	0.07	0.21	0.15	0.39	0.40	1.20

[a] Quantification of lactoferrin, IgG and GlyCAM-1 was performed by glycoprofile analysis (profile) according to Fig. 3, or by ELISA. The following peak areas and structures were used for area correction; for IgG (Fig. 1: peak 2) for GlyCAM-1 (Fig. 1: peak 28). Using the percentual distribution of the glycans for both IgG and GlyCAM-1 (Supplementary Material Tables S4, S5 and Figs. S5, S6), the area contribution of co-eluting structures with Man-9 of lactoferrin (Fig. 1: peak cluster 21) was calculated and converted to a concentration (mg mL^{-1}) using a calibration curve of lactoferrin.

The glycans are fluorescently labelled at their reducing end; therefore, a single label is present on each glycan molecule. The detected fluorescent signal is a linear function of the number of fluorophores present. Using this information, a response ratio between the molar amounts of the detected fluorescent signal and the glycan structures present could be calculated. By interpolating the area of unknown peaks onto the calibration curve of the Man-9 peak, the molar amounts of the unknown peaks (and corresponding glycan structures) could be determined. This approach was used to calculate the molar amounts of the signature glycans of IgG (Fig. 1, peak 2) and GlyCAM-1 (Fig. 1, peak 28).

For IgG (Fig. 1, peak 2), this is a fucosylated di-antennary structure with a relative abundance of 10.7% (Supplementary Material, Table S4). For GlyCAM-1 (Fig. 1, peak 28), a tri-antennary structure was selected with a relative abundance of 5.5% (Supplementary Material, Table S5). Using the percentage of the individual signature glycans relative to the total amount of glycans present on a protein, the total molar amount of glycans for their respective proteins was calculated.

The amount of GlyCAM-1 protein has a direct 1:1 relation with its glycan quantity, since it contains a single 100% occupied *N*-glycosylation site (Sørensen & Petersen, 1993). For IgG, the ratio of glycan to protein is 2:1, given the established 100% occupancy on both heavy chains (Takimori et al., 2011). To calculate the amount of protein, the molecular weight of the protein was used. For IgG, its molecular mass of 150 kDa is well established (Kickhöfen, Hammer, & Scheel, 1968). For GlyCAM-1 however, the literature is inconsistent. On SDS-PAGE the PP3 (GlyCAM-1) fraction elutes as a doublet of 28 kDa and 17 kDa, while the gene sequence codes for a protein with a mass of 15.3 kDa (Girardet & Linden, 1996; Sørensen & Petersen, 1993). Its molecular mass, including post-translational modifications by phosphorylation and both *O*- and *N*-glycosylation, has been determined as 19.4 kDa (Kjeldsen et al., 2003). For the protein concentrations calculated in this work, the molecular mass of 19.4 kDa was used.

The IgG and GlyCAM-1 concentrations determined by analysis of the whey glycoprofiles of 12 milk samples are displayed in Table 3. The concentrations for IgG ranged from 0.26 to 0.48 mg mL^{-1} when determined from the whey glycoprofile and from 0.33 to 0.50 mg mL^{-1} when determined by ELISA. Highly similar results thus were obtained with both these methods; estimation of the IgG concentration from the whey glycoprofile seems a valid approach.

For GlyCAM-1 with a 19.4 kDa mass, concentrations determined by glycoprofile analysis ranged from 1.09 to 1.42 mg mL^{-1}. This GlyCAM-1 concentration range is significantly higher than its commonly accepted bovine milk concentration of 0.30–0.40 mg mL^{-1} (Bär et al., 2019; Larson & Rolleri, 1955). This lower concentration originates from analysis of heated milk, as a percentage of the total protein content, by proxy using protein nitrogen determination and electrophoretic separation of protein fractions (Larson & Rolleri, 1955). Larson and Rolleri (1955) stated that the determined concentration does not account for losses occurring during heating. Comparison of (un)heated whey glycoprofiles showed clear loss of GlyCAM-1 protein by heat treatment (Valk-Weeber et al., 2020a). Bär et al. (2019) recently quantified GlyCAM-1 by mass spectrometry analysis using reaction monitoring with isotopically labelled peptides and reported concentrations of 0.37 mg mL^{-1} in raw bovine milk and 4.2 g 100 g^{-1}) in whey isolated from raw milk. The concentrations of other proteins determined in the same study (bovine serum albumin, α-lactalbumin, lactoferrin) were far below (in the case of BSA) or on the lower end of the concentration range commonly reported for these proteins. It is therefore possible that this method also underestimated the GlyCAM-1 concentration. These results show that GlyCAM-1 glycans dominate the glycoprofile of bovine whey, and that its protein concentration may in fact be much higher than previously reported.

4. Conclusions

Approaches for qualitative and quantitative characterisation of whey, or whey powders, by *N*-linked glycoprofile analysis are presented. From a single analysis, the concentrations of the three main *N*-glycosylated proteins (lactoferrin, IgG and GlyCAM-1) can be estimated, as well as simultaneously generating data on the glycan structures present. The protocol is easily modifiable to facilitate analysis of protein powders, expanding the range to commercial whey products. The concentrations of IgG determined by this protocol were identical to its concentration determined by ELISA, while the lactoferrin concentration was slightly higher, but within the normal range reported for this protein. Particularly for (partially) denatured lactoferrin that cannot reliably be determined by ELISA, glycoprofile analysis may be a suitable proxy for its quantitation as it does not discriminate between native and denatured protein. Based on the concentrations of *N*-glycosylated

proteins determined in this study, GlyCAM-1 has the highest concentration in bovine whey, followed by IgG and lactoferrin. This observation fits our previous result, showing that GlyCAM-1 glycans dominate the overall glycoprofile, despite its small size and single glycosylation site (Valk-Weeber et al., 2020a,b). The contrasting data regarding GlyCAM-1, both in terms of the protein properties, size and concentration, illustrates the need for further research towards this protein.

Acknowledgements

This work was financially supported by FrieslandCampina, the University of Groningen/Campus Fryslân, and the University of Groningen.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2020.104814.

References

Adamczyk, B., Tharmalingam-Jaikaran, T., Schomberg, M., Szekrényes, Á., Kelly, R. M., Karlsson, N. G., et al. (2014). Comparison of separation techniques for the elucidation of IgG N-glycans pooled from healthy mammalian species. *Carbohydrate Research, 389*, 174–185.

Altunata, S., Earley, R. L., Mossman, D. M., & Welch, L. E. (1995). Pulsed electrochemical detection of penicillins using three and four step waveforms. *Talanta, 42*, 17–25.

Bär, C., Mathis, D., Neuhaus, P., Dürr, D., Bisig, W., Egger, L., et al. (2019). Protein profile of dairy products: Simultaneous quantification of twenty bovine milk proteins. *International Dairy Journal, 97*, 167–175.

Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M., & Parekh, R. B. (1995). Nonselective and efficient fluorescent labeling of glycans using 2-amino benzamide and anthranilic acid. *Analytical Biochemistry, 230*, 229–238.

Billakanti, J., McRae, J., Mayr, M., & Johnson, K. (2019). Advanced analytical tools for bovine lactoferrin identification and quantification in raw skim milk to finished lactoferrin powders. *International Dairy Journal, 99*, Article 104546.

Carter, B. G., & Drake, M. A. (2018). Invited review: The effects of processing parameters on the flavor of whey protein ingredients. *Journal of Dairy Science, 101*, 6691–6702.

Cheng, J. B., Wang, J. Q., Bu, D. P., Liu, G. L., Zhang, C. G., Wei, H. Y., et al. (2008). Factors affecting the lactoferrin concentration in bovine milk. *Journal of Dairy Science, 91*, 970–976.

Elgar, D. F., Norris, C. S., Ayers, J. S., Pritchard, M., Otter, D. E., & Palmano, K. P. (2000). Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *Journal of Chromatography A, 878*, 183–196.

Falconer, D. J., Subedi, G. P., Marcella, A. M., & Barb, A. W. (2018). Antibody fucosylation lowers the FcγRIIIa/CD16a affinity by limiting the conformations sampled by the N162-glycan. *ACS Chemical Biology, 13*, 2179–2189.

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., et al. (2004). Nomenclature of the proteins of cows' milk — Sixth revision. *Journal of Dairy Science, 87*, 1641–1674.

Feist, P., & Hummon, A. B. (2015). Proteomic challenges: Sample preparation techniques for Microgram-Quantity protein analysis from biological samples. *International Journal of Molecular Sciences, 16*, 3537–3563.

Figueroa-Lozano, S., Valk-Weeber, R. L., van Leeuwen, S. S., Dijkhuizen, L., & de Vos, P. (2018). Dietary N-glycans from bovine lactoferrin and TLR modulation. *Molecular Nutrition & Food Research, 62*, 1700389.

Fox, P. F., & Mulvihill, D. M. (1982). Milk proteins: Molecular, colloidal and functional properties. *Journal of Dairy Research, 49*, 679–693.

Fujii, S., Nishiura, T., Nishikawa, A., Miura, R., & Taniguchi, N. (1990). Structural heterogeneity of sugar chains in immunoglobulin G. Conformation of immunoglobulin G molecule and substrate specificities of glycosyltransferases. *Journal of Biological Chemistry, 265*, 6009–6018.

Girardet, J. M., & Linden, G. (1996). PP3 component of bovine milk: A phosphorylated whey glycoprotein. *Journal of Dairy Research, 63*, 333–350.

Hanemaaijer, J. H. (1985). Microfiltration in whey processing. *Desalination, 53*, 143–155.

Indyk, H. E., & Filonzi, E. L. (2005). Determination of lactoferrin in bovine milk, colostrum and infant formulas by optical biosensor analysis. *International Dairy Journal, 15*, 429–438.

Innocente, N., Biasutti, M., & Blecker, C. (2011). HPLC profile and dynamic surface properties of the proteose-peptone fraction from bovine milk and from whey protein concentrate. *International Dairy Journal, 21*, 222–228.

Johnson, J. L., Jones, M. B., Ryan, S. O., & Cobb, B. A. (2013). The regulatory power of glycans and their binding partners in immunity. *Trends in Immunology, 34*, 290–298.

Jost, R., Maire, J. C., Maynard, F., & Secretin, M. C. (1999). Aspects of whey protein usage in infant nutrition, a brief review. *International Journal of Food Science and Technology, 34*, 533–542.

Kaneko, Y., Nimmerjahn, F., & Ravetch, J. V. (2006). Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science, 313*, 670–673.

Kickhöfen, B., Hammer, D. K., & Scheel, D. (1968). Isolation and characterisation of yG type immunoglobulins from bovine serum and colostrum. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie, 349*, 1755–1773.

Kjeldsen, F., Haselmann, K. F., Budnik, B. A., Sørensen, E. S., & Zubarev, R. A. (2003). Complete characterization of posttranslational modification sites in the bovine milk protein PP3 by tandem mass spectrometry with electron capture dissociation as the last stage. *Analytical Chemistry, 75*, 2355–2361.

Korhonen, H. J. (2009). Bioactive components in bovine milk. In Y. W. Park (Ed.), *Bioactive components in milk and dairy products* (pp. 15–42). Ames, IO, USA: Wiley Blackwell.

Kussendrager, K. D., & van Hooijdonk, A. C. M. (2000). Lactoperoxidase: Physico-chemical properties, occurrence, mechanism of action and applications. *British Journal of Nutrition, 84*, 19–25.

Larson, B. L., & Rolleri, G. D. (1955). Heat denaturation of the specific serum proteins in milk. *Journal of Dairy Science, 38*, 351–360.

Le, T. T., Zhao, D., & Larsen, L. B. (2019). Analytical methods for measuring or detecting whey proteins. In H. C. Deeth, & N. Bansal (Eds.), *Whey proteins* (pp. 155–184). Academic Press.

Li, M., Bai, Y., Zhou, J., Huang, W., Yan, J., Tao, J., et al. (2019). Core fucosylation of maternal milk N-Glycan evokes B cell activation by selectively promoting the l-fucose metabolism of gut *Bifidobacterium* spp. and *Lactobacillus* spp. *mBio, 10*, 1–19.

Mazri, C., Sánchez, L., Ramos, S. J., Calvo, M., & Pérez, M. D. (2012). Effect of high-pressure treatment on denaturation of bovine lactoferrin and lactoperoxidase. *Journal of Dairy Science, 95*, 549–557.

Mettler, A. E. (1980). Utilization of whey by-products for infant feeding. *International Journal of Dairy Technology, 33*, 67–72.

Moore, S. A., Anderson, B. F., Groom, C. R., Haridas, M., & Baker, E. N. (1997). Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *Journal of Molecular Biology, 274*, 222–236.

Moremen, K. W., Tiemeyer, M., & Nairn, A. V. (2012). Vertebrate protein glycosylation: Diversity, synthesis and function. *Nature Reviews Molecular Cell Biology, 13*, 448–462.

Morr, C. V., & Ha, E. Y. W. (1993). Whey protein concentrates and isolates: Processing and functional properties. *Critical Reviews in Food Science and Nutrition, 33*, 431–476.

O'Riordan, N., Kane, M., Joshi, L., & Hickey, R. M. (2014). Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology, 24*, 220–236.

Palmano, K. P., & Elgar, D. F. (2002). Detection and quantitation of lactoferrin in bovine whey samples by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *Journal of Chromatography A, 947*, 307–311.

Paquet, D., Nejjar, Y., & Linden, G. (1988). Study of a hydrophobic protein fraction isolated from milk proteose-peptone. *Journal of Dairy Science, 71*, 1464–1471.

Rollo, D. E., Radmacher, P. G., Turcu, R. M., Myers, S. R., & Adamkin, D. H. (2014). Stability of lactoferrin in stored human milk. *Journal of Perinatology, 34*, 284–286.

Ruhaak, L. R., Huhn, C., Waterreus, W. J., De Boer, A. R., Neusüss, C., Hokke, C. H., et al. (2008). Hydrophilic interaction chromatography-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins. *Analytical Chemistry, 80*, 6119–6126.

Ruhaak, L. R., Steenvoorden, E., Koeleman, C. A. M., Deelder, A. M., & Wuhrer, M. (2010). 2-picoline-borane: A non-toxic reducing agent for oligosaccharide labeling by reductive amination. *Proteomics, 10*, 2330–2336.

Sauer, M. M., Jakob, R. P., Luber, T., Canonica, F., Navarra, G., Ernst, B., et al. (2019). Binding of the bacterial adhesin FimH to its natural, multivalent high-mannose type glycan targets. *Journal of the American Chemical Society, 141*, 936–944.

Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., et al. (2002). Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *Journal of Biological Chemistry, 277*, 26733–26740.

Sørensen, E. S., & Petersen, T. E. (1993). Phosphorylation, glycosylation and amino acid sequence of component PP3 from the proteose peptone fraction of bovine milk. *Journal of Dairy Research, 60*, 535–542.

Takimori, S., Shimaoka, H., Furukawa, J. I., Yamashita, T., Amano, M., Fujitani, N., et al. (2011). Alteration of the N-glycome of bovine milk glycoproteins during early lactation. *FEBS Journal, 278*, 3769–3781.

Teixeira, A. G. V., Bicalho, M. L. S., Machado, V. S., Oikonomou, G., Kacar, C., Foditsch, C., et al. (2013). Heat and ultraviolet light treatment of colostrum and hospital milk: Effects on colostrum and hospital milk characteristics and calf health and growth parameters. *The Veterinary Journal, 197*, 175–181.

Thomä-Worringer, C., Sørensen, J., & López-Fandiño, R. (2006). Health effects and technological features of caseinomacropeptide. *International Dairy Journal, 16*, 1324–1333.

Ulfman, L. H., Leusen, J. H. W., Savelkoul, H. F. J., Warner, J. O., & van Neerven, R. J. J. (2018). Effects of bovine immunoglobulins on immune function, allergy, and infection. *Frontiers in Nutrition, 5*, 1–20.

Valk-Weeber, R. L., Deelman-Driessen, C., Dijkhuizen, L., Eshuis-de Ruiter, T., & van Leeuwen, S. S. (2020a). In depth analysis of the contribution of specific glycoproteins to the overall bovine whey N-linked glycoprofile. *Journal of Agricultural and Food Chemistry, 68*, 6544–6553.

Valk-Weeber, R. L., Dijkhuizen, L., & van Leeuwen, S. S. (2019). Large-scale quantitative isolation of pure protein N-linked glycans. *Carbohydrate Research, 479*, 13–22.

Valk-Weeber, R. L., Eshuis-de Ruiter, T., Dijkhuizen, L., & van Leeuwen, S. S. (2020b). Dynamic temporal variations in bovine lactoferrin glycan structures. *Journal of Agricultural and Food Chemistry, 68*, 549–560.

Van Berkel, P. H. C., Van Veen, H. A., Geerts, M. E. J., & Nuijens, J. H. (2002). Characterization of monoclonal antibodies against human lactoferrin. *Journal of Immunological Methods, 267*, 139–150.

van Leeuwen, S. S., Schoemaker, R. J. W., Timmer, C. J. a M., Kamerling, J. P., & Dijkhuizen, L. (2012). N- and O-Glycosylation of a commercial bovine whey protein product. *Journal of Agricultural and Food Chemistry, 60*, 12553–12564.

van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E., & Hettinga, K. A. (2019). How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical Reviews in Food Science and Nutrition*, 1–24.

Vreeman, H. J., Visser, S., Slangen, C. J., & Van Riel, J. A. (1986). Characterization of bovine kappa-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. *Biochemical Journal, 240*, 87–97.