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Bioactive Constituents, Metabolites, and Functions

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In depth analysis of the contribution of specific glycoproteins to the overall bovine whey *N*-linked glycoprofile

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ABSTRACT:

The *N*-linked glycoprofile of bovine whey is the combined result of individual protein glycoprofiles. In this work, we provide in-depth structural information on the glycan structures of known whey glycoproteins, namely lactoferrin, lactoperoxidase, α-lactalbumin, immunoglobulin-G (IgG) and glycosylation dependent cellular adhesion molecule 1 (GlyCAM-1, PP3). The majority (~95%) of *N*-glycans present in the overall whey glycoprofile were attributed to three proteins; Lactoferrin, IgG and GlyCAM-1. We identified specific signature glycans for these main proteins; Lactoferrin contributes oligomannose-type glycans, while IgG carries fucosylated di-antennary glycans with Gal-β(1,4)GlcNAc (LacNAc) motifs. GlyCAM-1 is the sole whey glycoprotein carrying tri- and tetra-antennary structures, with a high degree of fucosylation and sialylation. Signature glycans can be used to recognize individual proteins in the overall whey glycoprofile, as well as for protein concentration estimations. Application of the whey glycoprofile analysis to colostrum samples revealed dynamic protein concentration changes for IgG, lactoferrin and GlyCAM-1 over time.

Keywords: whey, protein glycosylation, PP3, GlyCAM-1, milk, colostrum

INTRODUCTION

Milk is classically considered to be composed of three fractions: (butter)fat, casein and serum. The serum fraction, frequently called whey, contains the proteins that remain after removal of the caseins [1].

Main high-abundance proteins of the whey fractions are α-lactalbumin, β-lactoglobulin, serum albumin, immunoglobulin G (IgG), Glycosylation dependent cellular adhesion molecule 1 (GlyCAM-1; also known as proteose peptone 3, PP3, lactophorin) and lactoferrin. Proteins present in medium abundance include the immunoglobulins IgA, IgM, lactoperoxidase and osteopontin [2]. Minor abundance proteins include lysozyme and folate binding protein, but also many others. In total over 900 minor abundance proteins have been identified, most of which have not been extensively studied [3].

While some proteins are critical for milk stability (β-lactoglobulin) or for solubilizing calcium phosphate (caseins) [4], others are known to have specific biological functions. These bioactive proteins are often glycosylated, e.g. lactoferrin, immunoglobulins, lysozyme and lactoperoxidase. Decoration of proteins with carbohydrate moieties occurs either *N*- or *O*-linked, based on the location of the glycans. Mucin-type *O*-linked glycans, initiating with *N*-acetyl-galactosamine (GalNAc) bound to a serine or threonine residue, differ greatly from *N*-linked glycans, initiating with a tri-mannosyl-chitobiose core bound to an asparagine residue [5].

Some proteins carry exclusively *N*-linked (e.g. lactoferrin) or *O*-linked structures (e.g. osteopontin), while others may carry both (e.g. immunoglobulins, GlyCAM-1). Analysis of *O*- and *N*-linked structures requires different approaches. While most *N*-linked glycans can be released by peptide:*N*-Glycosidase F (PNGase F), no such universal enzyme is available for

O-linked structures; their release typically involves chemical treatment, e.g. alkali β-elimination. Here we focus on isolation and structural analysis of *N*-linked glycans.

A number of studies have focused on individual bovine whey glycoproteins [6,7]. These glycoproteins each have their own glycosylation fingerprints (arising from the glycan structures present). All glycoproteins in a milk sample contribute to its overall whey glycoprofile. Glycan structures of similar size and monosaccharide composition tend to co-elute in chromatographic analysis. This complexity makes identification of individual glycan structures a challenge. The types of glycans present on most bioactive whey glycoproteins have been annotated [2]. It is unknown, however, what the contribution is of individual glycoproteins to the overall whey glycoprofile. Changes in whey protein glycans over the course of lactation have been reported, but these studies focused mostly on IgG and lactoferrin, leaving GlyCAM-1 unstudied [8].

Whey protein powders, containing IgG, lactoferrin and GlyCAM-1, are processed into different food products, including infant formulas [9]. Lactoferrin is known to have antimicrobial and immunostimulatory functions. The latter function is mediated by Toll-like receptors, and depends on the composition of the lactoferrin glycoprofile [10]. Similarly, core-fucosylation as present on the glycans of IgG is crucial for receptor interaction [11]. Unique functions for GlyCAM-1 and its glycans remain to be identified, although evidence exists for antimicrobial and mucin-like lubricating properties of this protein [12,13]. Efficient methods for the unraveling of the overall glycoprofile of whey are crucial for predicting the functional properties of whey, and the products they are processed into.

Here, we used UPLC-FLD to identify unique signature *N*-glycans of the whey proteins lactoferrin, lactoperoxidase, α-lactalbumin, IgG and GlyCAM-1. In addition, an overview of the *N*-glycan contribution of each protein to the overall whey glycoprofile is provided. We

applied the overall whey glycoprofile analysis method towards milk and colostrum samples. Information on the *N*-glycans of lactoferrin and IgG and their protein concentration in colostrum already was available [8,14], but this information was lacking for GlyCAM-1. Here we show that the concentrations of lactoferrin, IgG and GlyCAM-1 in whey can be followed over time by analysis of their unique glycan structures in the whey glycoprofile.

MATERIALS AND METHODS

Materials.

Bovine lactoferrin, lactoperoxidase and α-lactalbumin samples were provided by FrieslandCampina Domo (Amersfoort, the Netherlands). Bovine gamma globulin fraction 2 (purity >98%) was from Serva (Heidelberg, Germany). PNGase F (*Flavobacterium meningosepticum*) was from New England Biolabs (Ipswich, UK). Jack bean α-mannosidase (75 U/mL in 3.0 M $(NH_4)_2SO_4$, 0.1 mM zinc acetate, pH 7.5) was purchased from Sigma-Aldrich Chemie N.V. (Zwijndrecht, the Netherlands). Green coffee bean α-galactosidase (25 U/mL 100 mM sodium phosphate pH 6.5, containing 0.25 mg/ml bovine serum albumin), bovine testis β-galactosidase (5 U/mL in 20 mM sodium citrate phosphate, 150 mM NaCl, pH 4.0), *Streptococcus pneumoniae* sialidase, (4 U/mL 20 mM Tris-HCl, 25 mM NaCl, pH 7.5) and *Arthrobacter ureafaciens* α-sialidase (5 U/mL in 20 mM Tris-HCl pH 7.5, containing 25 mM NaCl) were from Prozyme (Ballerup, Denmark). *Streptococcus pneumoniae* β-*N*-acetylhexosaminidase (40 U/mL in 20 mM Tris-HCl, 50 mM NaCl pH 7.5), jack bean β-*N*-acetylhexosaminidase (50 U/mL in 20 mM sodium citrate phosphate pH 6.0), bovine kidney α-fucosidase (2 U/mL in 20 mM sodium citrate phosphate, 0.25 mg/ml BSA pH 6.0 were from Prozyme (Ballerup, Denmark). Pooled tank milk of Holstein-Friesian cows was obtained from FrieslandCampina Domo. Colostrum and milk samples were collected from 8 cows from a local organic farm (Rietveldhoeve farm, Aduard, Groningen, the Netherlands). Colostrum

was collected directly after calving, and then at approximately 12, 24, 36, 48, 60 h *post-partum* (Supporting Information, Table S1). Milk samples were collected at 1, 2 and 3 months.

Whey Preparation and Protein Isolation.

Milk samples were thawed in a water bath at 37 °C and homogenized. An aliquot of 1 mL was defatted by centrifuging at 4000 x *g* for 10 min. An amount of 400 µL defatted milk was transferred into a new tube. Of colostrum samples, an amount of 50 µL was transferred and mixed with 350 µL of MilliQ water. For GlyCAM-1 analysis, defatted milk was heated to 95°C for 30 min prior to subsequent processing. Caseins were removed by addition of 400 µL 125 mM of ammonium acetate at pH 4.6 (ratio of 1:1). The samples were vortexed and left at room temperature for 5 min before centrifuging at 11000 x *g* for 5 min to precipitate the caseins. An aliquot of 100 µL of the supernatant (acid whey) was transferred into a new tube, 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 11000 x *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 at 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 for 15 min at 85 °C and cooled to room temperature. An aliquot of 25 µL of 10% NP-40 (NP-40 substitute, Sigma) was added to each sample and mixed by vortexing. Finally, 2 µL of diluted PNGase F (100 units/experiment) was added to the samples and mixed. Glycans were released overnight at 37 °C.

Labeling and Cleanup.

Isolated glycans were labeled with anthranilic acid (2-AA, Sigma) or 2-aminobenzamide (2-AB). The 2-AA label was chosen for applications with fluorescent detection due to the higher sensitivity in these applications. 2-AB was chosen for applications that required mass spectrometry analysis. Direct in solution labeling of whey digests was performed as follows. Whey protein digests of a total volume of 102 µL were mixed 1:1 with labeling solution (0.7 M 2-AA or 2-AB and 2 M of 2-picoline borane or sodium cyanoborohydride in dimethylsulfoxide (DMSO, Sigma): glacial acetic acid (7:3, v/v)). Incubations were performed for 2 h at 65 °C [15]. Labeling reagents were removed by 96-well microcrystalline cellulose SPE as described [16]. Samples were diluted with 612 µL acetonitrile (final concentration 75% v/v) prior to application to the cellulose SPE.

Anion-exchange SPE Fractionation.

For fractionation of the glycans into sialylated and neutral fractions, 4 aliquots of 100 µL of acid whey were processed and labeled with 2-AB as described above. The labeled aliquots were pooled and fractionated by anion exchange solid phase extraction (IRIS MAX, 1 mL, Screening Devices). The cartridge was conditioned with 1 mL of acetonitrile and 1 mL of MilliQ water. The labeled whey was passed over the column and collected (neutral glycan fraction). After washing with 1 mL of MilliQ, the acidic glycans were eluted with 1 mL of 25% acetonitrile+0.1% TFA. The fractions were lyophilized and redissolved in 400 µL of MilliQ before HPLC analysis.

HPLC Analysis.

Fluorescently labeled glycans were separated on an Acquity UPLC Glycan BEH Amide column (2.1 mm x 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 (λex 330 nm, λem 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. A constant 20% of the buffer was maintained throughout the run. Elution was performed by a slow sloping gradient of 22% to 40% MilliQ water (total concentration, including buffer) from 0 to 67.5 min. The remaining percentage of the solvent composition comprised of acetonitrile. After completion of the gradient, final gradient conditions were maintained for 9 min and the column reconditioned back to initial conditions for 13 min.

Exoglycosidase Assays.

Sequential digestions with glycosidases (Supporting Information) were performed in 50 mM sodium acetate buffer at pH 5.5 overnight. After each digestion step, the enzymes were removed by 10 kDa cut-off centrifugal filters (Millipore, Tullagreen, Cork, IRL). The 2-AA labeled dextran calibration ladder was from Waters Chromatography BV (Etten-Leur, the Netherlands).

Lactoferrin Concentration Determination.

The concentration of lactoferrin in the colostrum samples was quantified by a bovine lactoferrin ELISA quantitation set (E10-126, Bethyl Laboratories, Montgomery, TX, USA), as described previously [14].

Mass Spectrometry Analysis.

Mass spectrometry analysis was performed using identical slope and solvent composition as used for the HPLC-fluorescent detection. Settings used for the mass spectrometry analysis were as described earlier [14]. Glycans were identified by their (derivatized) monoisotopic molecular mass, using the GlycoMod tool [17] (https://web.expasy.org/glycomod/) and a 0.2 Dalton mass tolerance.

RESULTS AND DISCUSSION

Overall Whey Glycoprofile.

The overall *N*-glycan profile of bovine acid whey showed a complex pattern of peaks (Figure 1). There are multiple glycosylated proteins present in acid whey that contribute *N*-glycans to the overall chromatogram. Glycans with similar degree of polymerization and monosaccharide composition tend to elute at the same time. Due to the high number of structures present in the chromatogram, multiple structures can overlap and form combined peaks (peak clusters). Structures, including their isomers were identified by LC-MS. It should be noted that for optimal fluorescent detection, glycans were labeled with 2-AA, while for LC-MS analysis glycans were labeled with 2-AB for improved positive ion mode sensitivity. Identical chromatographic conditions were used for both detection methods. While glycans labeled with 2-AB have a higher retention in the chromatography setup used, the chromatographic patterns are the same [14]. Using the structures identified in the 2-AB labeled glycoprofile, the structures in the 2-AA labeled whey glycoprofile were appointed (Figure 1). Structures were further confirmed by sequential exoglycosidase treatment (Supporting Information, Figures S1 and S2). In our study, we were able to identify at least 69 individual glycan structures, not including isomers (see overview in Supporting Information, Table S2).

Sialylated and neutral glycans were separated by anion-exchange SPE and profiled (Figure 2). The neutral glycans (Figure 2, black line, 15-33 min) dominated the first half of the chromatogram, while the sialylated glycans eluted in the second half (Figure 2, red line, 33-55 min). Shorter sialylated structures overlap with the larger neutral structures between 33- and 47-min retention.

Previous publications have reported the absence of α(2,3)-linked sialic acid on bovine whey glycans [8,18], while others did not specify the linkage type [19]. Recently, we reported the

presence of α(2,3) sialic acid on bovine lactoferrin isolated from colostrum, but not on the mature milk-derived protein [14]. Exoglycosidase treatment of the (mature) whey glycoprofile, with sialidase from *Streptococcus pneumoniae*, with strong preference for α(2,3)-linked sialic acid, confirmed the presence of α(2,3) linkages in trace amounts (Supporting Information, Figure S3). This sialic acid was present on multiply sialylated di-, tri- and tetra-antennary structures, not only on lactoferrin-derived structures. This indicates that the presence of α(2,3)-linked sialic acid is a general feature of whey glycoproteins, and not only of lactoferrin in the colostrum phase.

The bovine milk glycoprofile has been investigated previously, either with glycans isolated from commercial whey powders [18], colostrum whey [20] or mature milk (Holstein and Jersey cows) [21].

In the analysis of bovine whey glycosylation, the *N*-acetylgalactosamine-*N*-acetylglucosamine (LacdiNAc) motif is important to consider, especially when mass spectrometry is used for structural identification. Since *N*-acetyl-glucosamine (GlcNAc) and *N*-acetylgalactosamine (GalNAc) are isomers and cannot be distinguished by mass spectrometry, MS-based annotation is often difficult and leads to ambiguous results. However, considering the high amount of LacdiNAc motifs reported on bovine milk proteins in earlier studies [18,19], the presence of LacdiNAc motifs in high abundance was expected. Karav *et al* and Nwosu *et al*, made no distinction between GlcNAc and GalNAc and instead, the shared identifier HexNAc was used. These papers do not report LacdiNAc structures, but refer to non-galactosylated tri- and tetra-antenna structures instead [21,22]. Moreover, whereas some studies report complex-type structures up to tetra-antennary with up to three sialic acid residues [8,18], other studies report a more limited glycoprofile, mainly oligomannose and di-antennary complex-type structures. Sriwilaijaroen *et al.* used PNGase A (instead of PNGase F), which has affinity for oligomannose-, hybrid- and short complex-type (up to di-antennary) glycans, possibly

234 explaining the more limited glycoprofile obtained [19]. Karav *et al* used the bifidobacterial
235 enzyme EndoBI-1 instead of PNGase F [22]. This enzyme cleaves between the two GlcNAc
236 residues of the chitobiose core, thereby information on core-fucosylation is lost. Previous
237 reports have shown that a significant number of structures carry core-fucosylation [8,18,21]. Loss
238 of this highly relevant information thus is a significant disadvantage of the use of the EndoBI-
239 1 enzyme for this type of analysis. In our study, multiply sialylated di- and tetra-antennary
240 structures were detected, but not multiply sialylated tri-antennary structures. Van Leeuwen *et*
241 *al.* detected trace amounts of multiply sialylated tri-antennary structures, but only in
242 concentrated fraction; these structures may have remained below the limit of detection in our
243 study [18]. Conversely, we detected a number of doubly sialylated tetra-antennary structures, not
244 reported by van Leeuwen *et al*. Therefore, the data found in our study and in earlier work
245 complement each other. Overall, the level of complexity observed in our study is comparable
246 with that in Takimori *et al.* and Van Leeuwen *et al.* [8,18].

247 **Individual Whey Glycoproteins.**

248 Glycoproteins in bovine whey each have a signature fingerprint of glycans. The concentration
249 of these proteins in bovine whey varies, highest concentrations were reported for IgG (0.3-0.6
250 mg/mL), GlyCAM-1 (0.3-0.5 mg/mL) and lactoferrin (0.1-0.3 mg/mL) [4,23]. The other
251 immunoglobulins, IgA and IgM are present at approximately 5 to 10 times lower
252 concentrations than IgG [24]. While α-lactalbumin is present in higher protein concentrations
253 (1.5 mg/mL), only ~10% of this protein is glycosylated [25]. Lactoperoxidase is typically
254 present in concentrations around 0.03 mg/mL [26]. Together, these proteins are most likely the
255 main contributors to the overall whey *N*-linked glycoprofile.

256 GlyCAM-1 is currently not commercially available and therefore had to be isolated from milk
257 samples. When milk is heated, only the heat-stable proteins remain in solution. Acid whey
258 prepared of this heated milk is known as the proteose peptone (PP) fraction, with GlyCAM-1

initially labeled as PP3 [23]. PP3 reportedly is the main contributor to the *N*-linked glycoprofile [27]; the second most abundant protein is a casein proteolytic fragment (PP5) that is not *N*-glycosylated [28]. The 60 kDa protein osteopontin can also be recovered from the PP fraction. Osteopontin, like casein, is solely *O*-glycosylated and will therefore not interfere in our *N*-glycoprofile analysis [29]. SDS-PAGE analysis confirmed that other glycoproteins (IgG/lactoferrin/α-lactalbumin) were sufficiently removed by heating the whey (Supporting Information, Figure S4). Glycan fingerprints were analyzed for the selected main glycoproteins as well as for heated whey (GlyCAM-1) (Figure 3).

The glycan fingerprint of GlyCAM-1 is dominated by sialylated complex-type glycan structures, existing in di-, tri-, and tetra-antennary configurations (Figure 3). The observed high levels of sialylated and core-fucosylated glycans for GlyCAM-1 fit previous reports [6]. A few structures were observed here that have not been previously described for GlyCAM-1. For example, we observed multiply sialylated structures, in some cases with a combination of Neu5Ac and Neu5Gc. These include a tetra-antennary structure with two Neu5Ac and one Neu5Gc moiety (Supporting Information, Table S2, nr 70). In addition, tri-sialylated di-antennary structures were found (Supporting Information, Table S2, nr 63 and 64), indicating the addition of a third sialic acid on a GlcNAc, instead of on the terminal Gal(NAc). It should be noted that sialylation on GlcNAc only occurred via an α(2,6) linkage, and only when the Gal(NAc) was α(2,3) sialylated.

The glycans on α-lactalbumin showed significant overlap with the glycans found on GlyCAM-1 (Figure 3). The glycan fingerprint of α-lactalbumin was characterized by the presence of di-antennary glycans, of which the majority bears the GalNAc-β(1,4)-GlcNAc (LacdiNAc) motif. Both fucosylation and sialylation was abundantly present on the glycans from α-lactalbumin. A minor amount of tri-antennary structures was also identified. The major structures found in the glycosylation fingerprint of α-lactalbumin in this study

corresponded with those in an earlier report [25]. Minor additional peaks were also observed in the glycan fingerprint, indicating the presence of additional glycan structures. Based on their positions in the chromatogram, these other glycan structures are hypothesized to be variations of the identified di-antennary and tri-antennary structures.

The glycan fingerprint of lactoferrin was dominated by the oligomannose structures Man-5 to Man-9, with Man-8 and Man-9 being the most abundant of the set (Figure 3), fitting previous results on mature milk derived lactoferrin [7,14]. In addition, di-antennary structures were present, decorated with either galactose or *N*-acetylgalactosamine. Hybrid type structures were also found on lactoferrin. The complete profile of glycan structures of bovine lactoferrin fits to previous reports [7,14,30].

In earlier work, Wolf *et al.* identified the glycan structures on lactoperoxidase [31]. Our study visualized the same glycans (Figure 3), and also confirmed the presence of hybrid structures, which were hypothesized by Wolf *et al.* In addition, the relative quantities of the individual glycans of lactoperoxidase could be calculated. Lactoperoxidase carries a mixture of oligomannose, di-antennary complex and hybrid structures. The relative abundance of the oligomannoses on lactoperoxidase differs from that observed on lactoferrin. On lactoperoxidase, the oligomannoses Man-5 to Man-7 were most abundant. Hybrid- and complex-type structures are decorated with galactose or GalNAc, with the doubly GalNAc decorated structure present in the highest amounts. A small quantity of sialylated structures was also detected on lactoperoxidase, decorated with Neu5Ac or Neu5Gc, or a combination of these sialic acids.

Bovine IgG contains di-antennary glycan structures, of which the majority was core-fucosylated (Figure 3). The antennae were decorated with galactose (LacNAc), which is unique as all other bovine milk glycoproteins contain significant amounts of LacdiNAc

epitopes. A number of structures was sialylated; Neu5Gc is the predominant sialic acid on this protein.

Signature Glycans and Contribution of Individual Proteins to the Overall Whey Glycoprofile.

The individual protein glycan fingerprints gave valuable information on the glycan heterogeneity of each protein. Next, we attempted to visualize the contribution of each protein to the overall whey glycoprofile. An overlay of the glycan fingerprints of the individual whey proteins was prepared, reflecting their reported concentrations in a bovine whey sample (Figure 4). The concentrations were chosen according to the literature established concentration range for IgG, lactoferrin, α-lactalbumin and lactoperoxidase. GlyCAM-1 was analyzed directly from a heated defatted milk sample (pooled tank milk) and therefore represents a typical milk concentration. In the resulting overall glycoprofile, many glycan structures overlap. However, various individual glycans could be identified as signature structures for a single glycoprotein.

The majority of the glycan structures in the overall whey glycoprofile appeared to originate from GlyCAM-1 (Figure 4, blue line). A large portion of the glycans from GlyCAM-1 was sialylated, and the majority of the sialylated structures in the whey glycoprofile are likely to originate from GlyCAM-1. The acidic (sialylated) glycan fraction of the glycan pool indeed showed striking similarities to the glycan fingerprint of GlyCAM-1 (compare Figures 2, 4). Tri- and tetra-antennary structures were not observed (or in very minor quantities) on the other glycoproteins. Therefore, tri- and particularly tetra-antennary structures (with and without sialylation) are signature glycan structures for GlyCAM-1. While sialylated di-antennary structures were also observed on the other glycoproteins, the majority of these glycans originate from GlyCAM-1. LacdiNAc motifs are very common on α-lactalbumin and

GlyCAM-1, however the contribution of α-lactalbumin to the overall glycoprofile is limited (see Figure 4 and below).

The second highest contributor to the overall bovine whey glycoprofile is lactoferrin. The oligomannose type glycans that were found in the overall glycoprofile are almost exclusively from lactoferrin (Figure 4). While this glycan class was also found on lactoperoxidase (Figure 3), the contribution of lactoperoxidase to the overall glycoprofile was minimal (see Figure 4 and below). Man-9 in particular eluted in a relatively isolated part of the overall glycoprofile (Figure 4, starred structure), with little overlap or contribution from other co-eluting glycan structures. Therefore, the Man-9 peak was identified as signature glycan for lactoferrin in milk and whey (Figure 4).

The third highest contributor to the overall glycoprofile is IgG. Core fucosylated di-antennary glycans with LacNAc motifs are signature glycans for IgG. The glycans from IgG eluted in the first half of the chromatogram (Figure 4). The three most abundant glycan structures of IgG were readily identifiable in the overall whey glycoprofile, although there was some overlap with other co-eluting species (Figure 4).

As described earlier, only ~10% of the total amount of α-lactalbumin is glycosylated, therefore, the contribution of this protein, although present at a relatively high concentration, to the overall glycoprofile remained very low (Figure 4). The glycan structures found on α-lactalbumin were also present on GlyCAM-1 (Figure 3). Therefore, no unique signature structures were identified for α-lactalbumin.

Although lactoperoxidase is clearly glycosylated (Figure 3), it does not possess any unique identifier glycan structures. Especially lactoperoxidase and lactoferrin showed similarities in glycan structures, albeit in different relative quantities (Figure 3). In view of the low

concentration of lactoperoxidase in bovine whey, its contribution to the overall glycoprofile of whey is nihil (Figure 4). No signature glycans were identified for lactoperoxidase.

Other Immunoglobulins.

While the protein concentrations of IgA and IgM are ~10 times lower than IgG protein, their percentages of carbohydrate are higher than for IgG. For IgG, the carbohydrate content was estimated at 2-4%, consisting entirely of *N*-glycans [32,33]. For IgA the carbohydrate content was estimated at 7-10%, while IgM contains 10-12% carbohydrate per weight [32]. Although present in much lower protein concentrations, IgA and IgM may still contribute significantly to the overall whey glycoprofile. To the best of our knowledge, no glycoprofiling for bovine IgA and IgM has been performed. Human IgA contains *O*-glycans in addition to *N*-glycans, which contribute to the overall glycan weight, but not to the *N*-glycoprofile. The *N*-linked glycans of human IgA are of the di-antennary type [34]. Human IgM contains 5 *N*-linked glycosites on each heavy chain, occupied with di-antennary (77% of total) and oligomannose (23% of total) type glycans [35]. Assuming that the glycosylation of bovine IgA and IgM is similar to that of their human variants, a low to moderate contribution to the di-antennary pool can be expected. But no unique glycan signature structures are evident. Further analysis of the glycoprofiles of bovine IgA and IgM is needed to draw solid conclusions.

Whey Glycoprofiles of Colostrum.

In previous work, we have shown that the glycoprofile of lactoferrin undergoes significant alterations during the short colostrum period [14]. Here, the whey glycoprofiles of colostrum and mature milk samples from eight different cows (Supporting Information, Table S1) were analyzed and compared (Figure 5). The protein content (both caseins and whey proteins) of colostrum is significantly higher than of mature milk [36]. To allow for efficient casein coagulation and whey protein analysis, the colostrum was diluted 8 times prior to casein

precipitation. To compare late colostrum with mature milk, the last colostrum sample was also analyzed without additional dilution. Two cows were selected for a comparative analysis, cow 1 and cow 3. Cow 1 had low concentrations (<0.1 mg/mL) of lactoferrin during the colostrum phase, as determined by ELISA analysis (Supporting Information, Table S1). In contrast, lactoferrin concentrations were very high (>20 mg/mL) in the day 1 sample of cow 3.

In colostrum, large increases in the concentrations of lactoferrin and IgG were expected [14,36]. The difference in protein concentration of the major proteins (lactoferrin, IgG and GlyCAM-1) between these two cows also was reflected in their glycoprofiles: the colostrum glycoprofile for cow 3 was much more intense than the one obtained for cow 1 (Figure 5). Based on the signature structures defined above, the galactosylated (LacNAc) di-antennary glycans mostly belonged to IgG, while oligomannoses mostly originated from lactoferrin. Sialylated di-antennary glycans with LacdiNAc motifs, tri- and tetra-antennary glycans are signature structures for GlyCAM-1. A selection of the signature structures was annotated in Figure 5. Using these signature glycan structures, it is notable that in cow 3 the structures of lactoferrin were present in higher concentrations than in cow 1, which is in agreement with the higher concentration of lactoferrin found by ELISA (Supporting Information, Table S1). Moreover, the levels of IgG related glycans in cow 3 were significantly higher in the early colostrum phase than in cow 1. Based on the intensity of their signature glycan structures, both cows showed very rapid decreases in lactoferrin and IgG concentrations between the colostrum at day 1 and day 3. From the intensity of the signature structures of IgG, lactoferrin and GlyCAM-1, as well as from the relative proportion of the signature structures of these proteins, their relative protein concentrations were assessed. In early colostrum it appeared that the whey protein balance heavily shifted towards lactoferrin and IgG. Considering GlyCAM-1, a higher concentration of this protein was observed in colostrum, with a rapid

403 decrease in concentration over the colostrum period. However, the concentration of GlyCAM-
404 1 did not increase as extensively as that of IgG and lactoferrin.

405 The glycoprofiles obtained in early colostrum were different from those obtained from late
406 colostrum and mature milk. Altered glycoprofiles, most notable by an increased degree of
407 sialylation and fucosylation in early colostrum, have been reported for both IgG and
408 lactoferrin [8,14,37]. This was clearly reflected by the presence of high levels of sialylated IgG
409 structures (Figure 5, peaks 7, 8, 9, 11), which were absent, or severely decreased on mature
410 IgG (Figure 3). Care has to be taken to identify the tri-antennary glycan structures of
411 GlyCAM-1: some of the upregulated sialylated di-antennary structures of IgG co-elute with
412 the tri-antennary structures of GlyCAM-1 in colostrum (Figure 5, area 10). The number of
413 multiply sialylated tetra-antennary structures of GlyCAM-1 appeared higher in early
414 colostrum, indicating that an increased sialylation was also occurring on GlyCAM-1 (Figure
415 5, area 12).

416 Maturation of GlyCAM-1 appeared relatively slow in 50% of the analyzed cows. In cow 3, an
417 increase in GlyCAM-1 signature structures was observed from month 1 to 3. Similar changes
418 were observed for cows 2, 6 and 7 (Supporting Information, Figures S5, 8, 9). This may
419 indicate that the concentration of GlyCAM-1 increased during the first three months of
420 lactation, or that the glycosylation pattern had not stabilized completely, leading to an
421 observed increase of less complex glycan structures later in the lactation cycle.

422 To our knowledge, GlyCAM-1 has not yet been quantified in bovine colostrum. In koala, a
423 significantly higher GlyCAM-1 concentration was observed in colostrum versus mature milk
424 [38]. In camel milk, GlyCAM-1 (PP3) was only detected in colostrum after 48 h post-partum [39].
425 Our results suggest that the GlyCAM-1 concentration in cows is increased in colostrum,
426 followed by an immediate decrease and finally a slow stabilization over the first months of

lactation. However, further research towards colostrum GlyCAM-1, both in concentration and glycosylation, is needed.

The Contribution of GlyCAM-1.

The reported concentration of GlyCAM-1 in bovine milk (0.3-0.4 mg/mL) [23], was in the same range as that of the biologically relevant proteins IgG (0.5 mg/mL) and lactoferrin (0.1-0.3 mg/mL). Based on the generated whey glycoprofile, GlyCAM-1 was the highest contributor of (sialylated) glycans. GlyCAM-1 is not only present in the milk of typical dairy livestock (i.e. bovine, ovine and caprine [40], but also has been reported in murine milk [12]. While a GlyCAM-1 gene homologue is found in humans, no functional GlyCAM-1 proteins are secreted into the milk [41]. In contrast, Gustafsson *et al.* tentatively reported GlyCAM-1 (lactophorin) in human milk, based on the SDS-PAGE derived molecular weight analysis of major glycoproteins [42]. Trace amounts of GlyCAM-1 were detected by Hettinga *et al.*, also suggesting that a homologue in fact is present in human milk [43].

Regarding the origin and function of GlyCAM-1, there is limited and also contradicting information. Originally, GlyCAM-1 was described as PP3, a protein originated from the milk fat globule membrane (MFGM). This conclusion was supported by the cross-reactivity of GlyCAM-1 with an antibody to soluble glycoprotein (SGP), an MFGM protein [44]. The conclusion that PP3 is a MFGM protein was questioned in detail by Girardet *et al.*, who instead suggested that the cross reactivity of the anti-SGP antibodies detected GalNAc, an epitope that is common on MFGM proteins, as well as on GlyCAM-1 [45]. Here, we report that GalNAc, in LacdiNAc motifs, is very abundant on most whey glycoproteins, which may explain the observed cross-reactivity. Contrarily, Bak *et al* described that a C-terminal peptide of PP3 acted as a membrane anchor [46], supporting membrane association. Another publication suggested that GlyCAM-1 (PP3) exists in a membrane bound and a secreted form in mice [12].

451 This also fits the observations of Sørenson *et al* and Hettinga *et al,* showing significant levels
452 of GlyCAM-1 in both serum, as well as the MFGM fraction [40,43].

453 GlyCAM-1 is not solely expressed in the mammary glands, it is also detected in the epithelial
454 cells of lymph nodes, lungs, uterus and cochlea [47]. In lymph nodes, GlyCAM-1 mediates
455 lymphocyte trafficking, while in other tissues the function remains unknown. In the mammary
456 gland, GlyCAM-1 expression is regulated by progesterone and prolactin [47]. Suggested
457 functions for GlyCAM-1 included the inhibition of lipases [48], acting as a lubricant [12], or
458 protection against mastitis [49] possibly by its anti-bacterial properties [13]. In the immune system,
459 GlyCAM-1 has been indicated in L-selectin mediated leukocyte rolling and trafficking [50].
460 Changes in GlyCAM-1 expression levels have been implicated in inflammation response [51,52].

461 Our data shows that GlyCAM-1 is the major contributor to the mature bovine whey *N*-linked
462 glycoprofile. This novel insight comes as a surprise, as GlyCAM-1 has remained a
463 significantly under-studied protein. Literature concerning this particular protein is relatively
464 scarce and is further complicated due to the different synonyms used for GlyCAM-1, such as
465 lactophorin and PP3. Furthermore, information is contradictory with regards to GlyCAM-1
466 nomenclature, size, location in the milk, concentration and functionality. GlyCAM-1 is the
467 dominant protein in heated milk; therefore, it is also likely to remain intact in processed whey
468 powders. The proteins lactoferrin and IgG have both been identified as proteins with
469 important immune stimulatory functions, which are mediated by their glycans. The highly
470 glycosylated GlyCAM-1 potentially also has significant effects on the functional properties of
471 the products it is processed into. Lactoferrin *N*-glycans were previously shown to influence
472 TLR-mediated response in THP-1 and HEK293 reporter cell lines [10]. Considering the high
473 levels of GlyCAM-1 glycans in bovine milk, this protein is an interesting target for further
474 studies. In conclusion, the approaches reported in this paper for bovine whey glycoprofile
475 analysis allow a rapid screening and interpretation of milk and whey (product) samples from

various sources, visualizing variations in individual whey protein concentrations based on their signature glycans. In this work we explored (methods for) the qualitative analysis of the overall bovine milk glycoprofiles. The quantitative potential of this approach remains to be explored.

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Supporting information

Full description of the exoglycosidase assay procedure. Collection schedule and lactoferrin concentrations of the colostrum and milk samples. *N*-glycan structure overview and m/z values of the detected structures in bovine whey. Exoglycosidase assay chromatograms of whey *N*-glycans, separated into a neutral and acidic fraction. Chromatogram of a whey profile digestion with a α2,3-specific sialidase. SDS-PAGE analysis of whey and glycoproteins on a 12.5% polyacrylamide gel. Colostrum and milk whey glycoprofiles obtained from cows 2, 4, 5, 6, 7 and 8.

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672 **Figures**

673 **Figure 1.** Overall acid whey HPLC glycoprofile obtained for pooled milk from Holstein-
674 Friesian cows. Glycan structures detected and identified by mass spectrometry were added to
675 the spectrum; for a full overview of all structures, see Supporting Information, Table S2.
676 Structures with main contributions to the peak intensity are marked with *.

677

678 **Figure 2.** Overlay of the neutral (black line) and acidic (sialylated, red line) glycan fractions
679 of the overall whey HPLC glycoprofile, obtained from pooled milk of Holstein-Friesian cows.
680 The 2-AB labeled glycans were divided into neutral and acidic fractions by anion exchange
681 chromatography fractionation.

682

683 **Figure 3.** HPLC chromatograms of the glycan fingerprints of individual glycoproteins present
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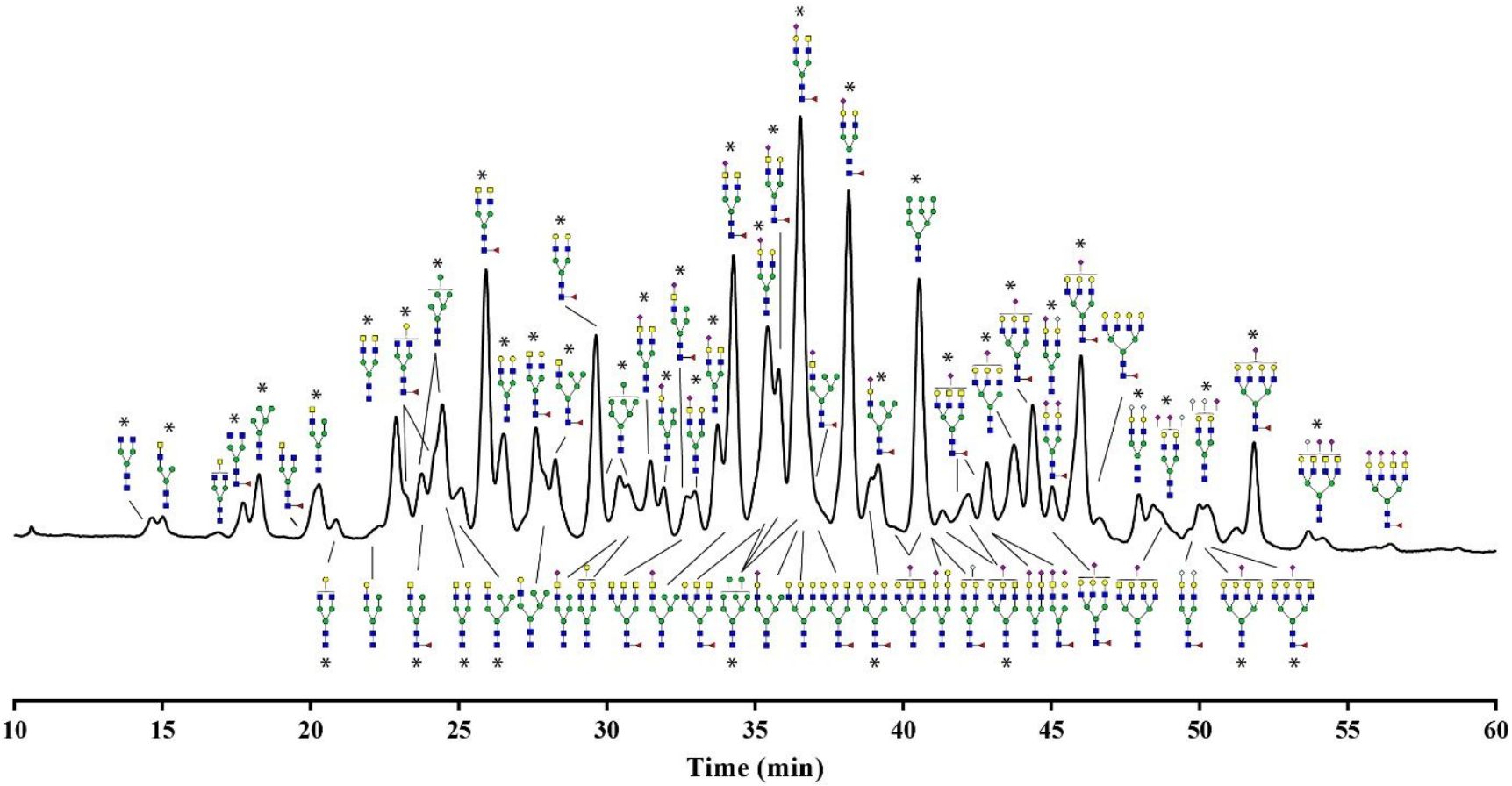

Figure 1. Overall acid whey HPLC glycoprofile obtained for pooled milk from Holstein-Friesian cows. Glycan structures detected and identified by mass spectrometry were added to the spectrum; for a full overview of all structures, see Supporting Information, Table S2. Structures with main contributions to the peak intensity are marked with *.

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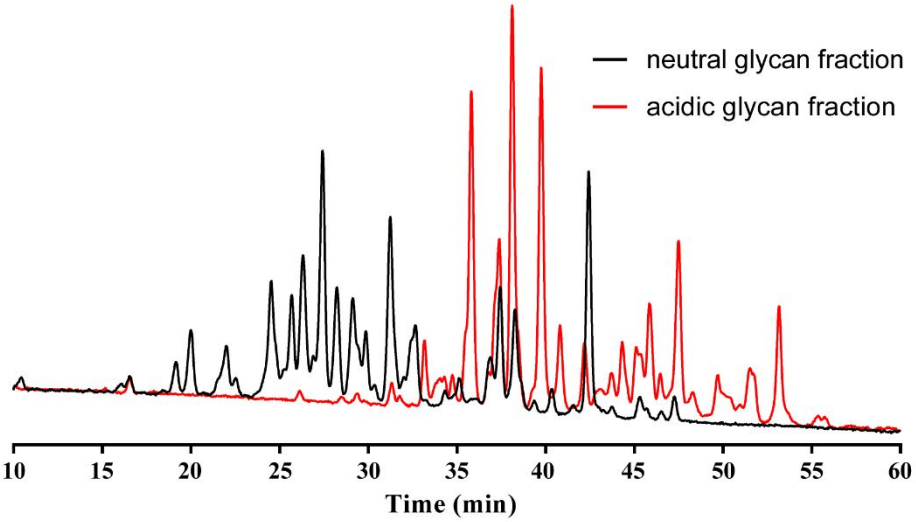

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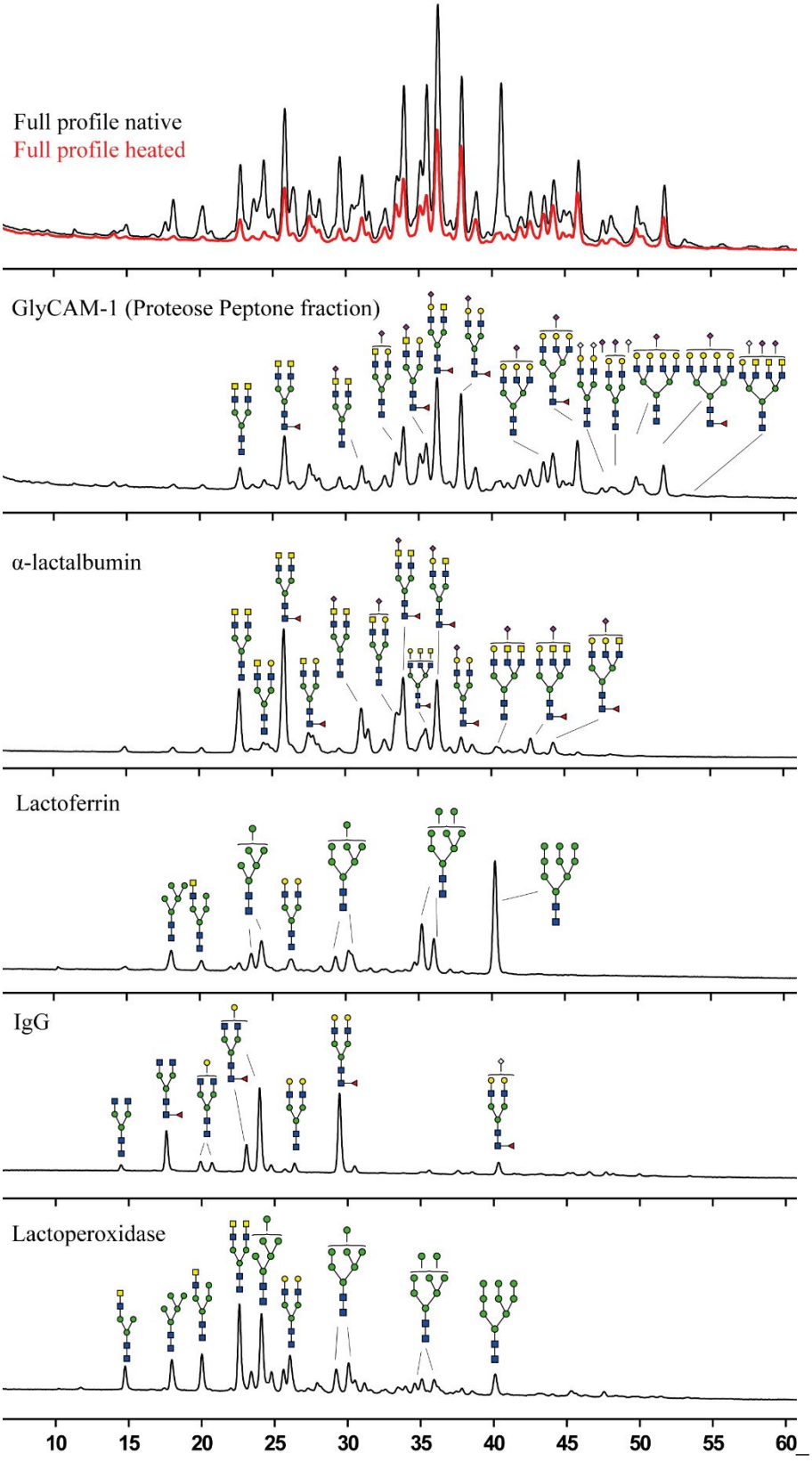

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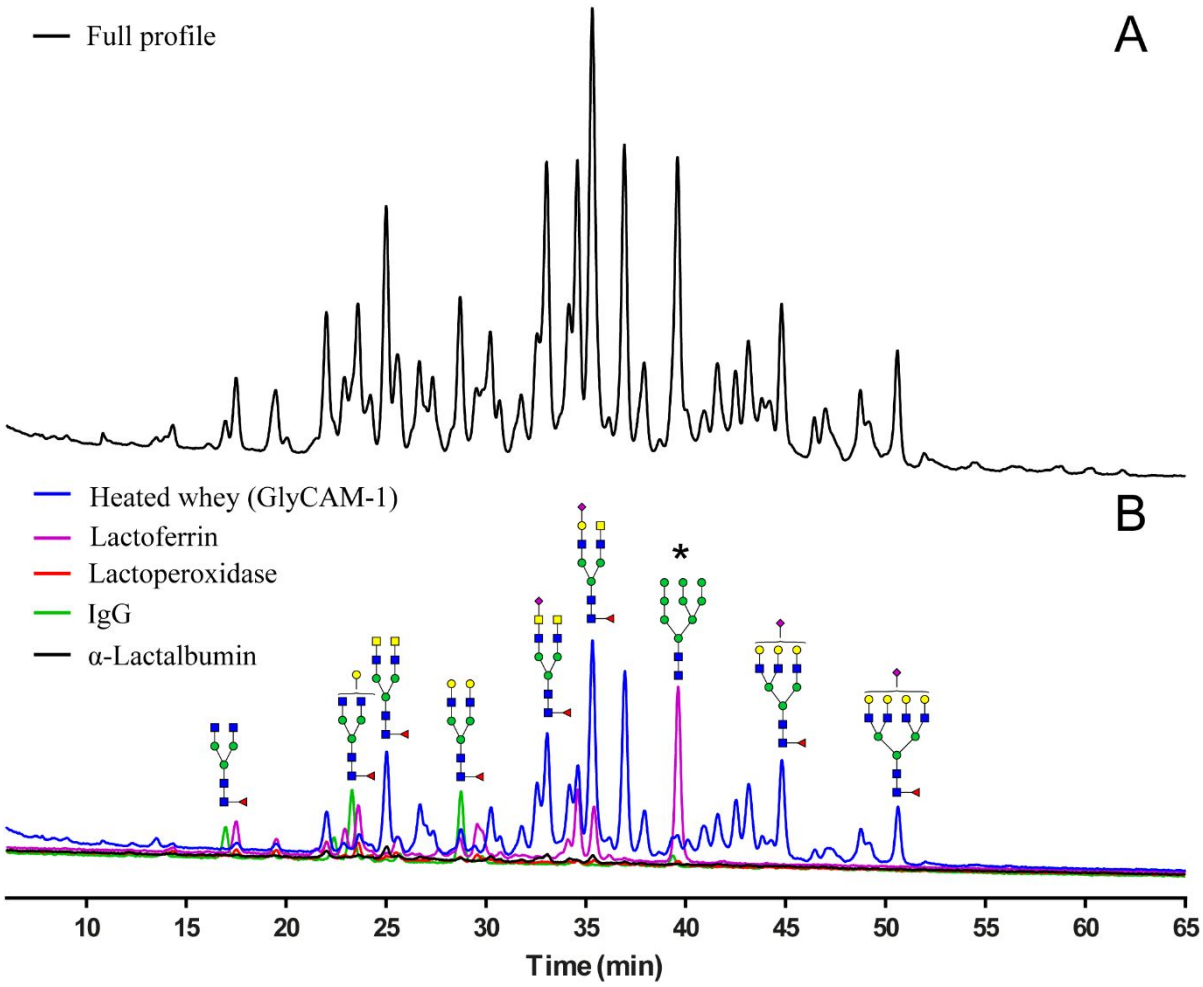

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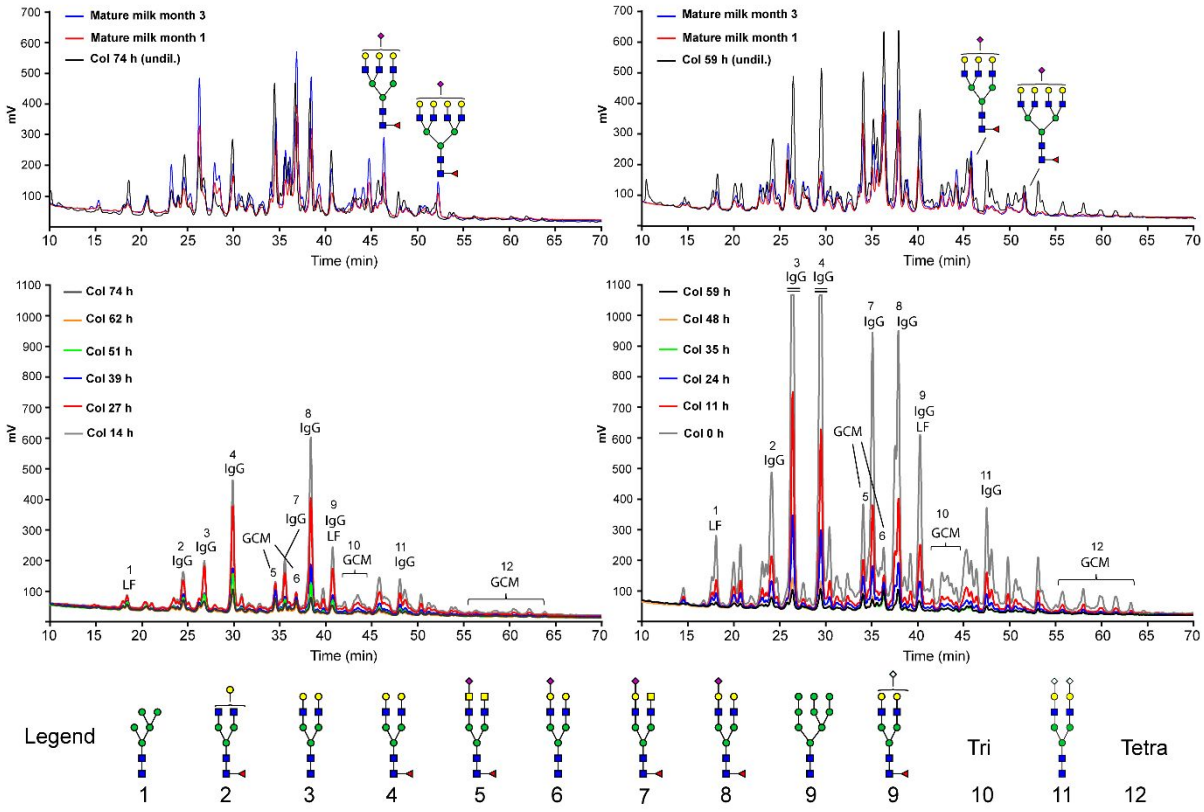
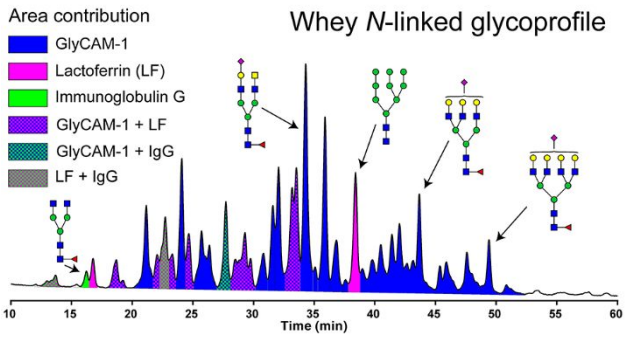


Figure 5. HPLC chromatograms of whey glycoprofiles of 8-times diluted colostrum (bottom) and undiluted (undil.) colostrum versus mature milk (top). Glycoprofiles displayed are from (left) cow 1 and (right) cow 3 (Supporting Information, Table S1). A selection of structures from lactoferrin (LF), immunoglobulin G (IgG), and GlyCAM-1 (GCM) was annotated. Sections with multiple co-eluting tri-antennary (Tri) and tetra-antennary (Tetra) glycans are bracketed. Additional glycoprofiles from cows 2, 4, 6, 7 and 8 are provided in the supporting information (Supporting Information, Figures S5-10).

738 **For Table of Contents only:**



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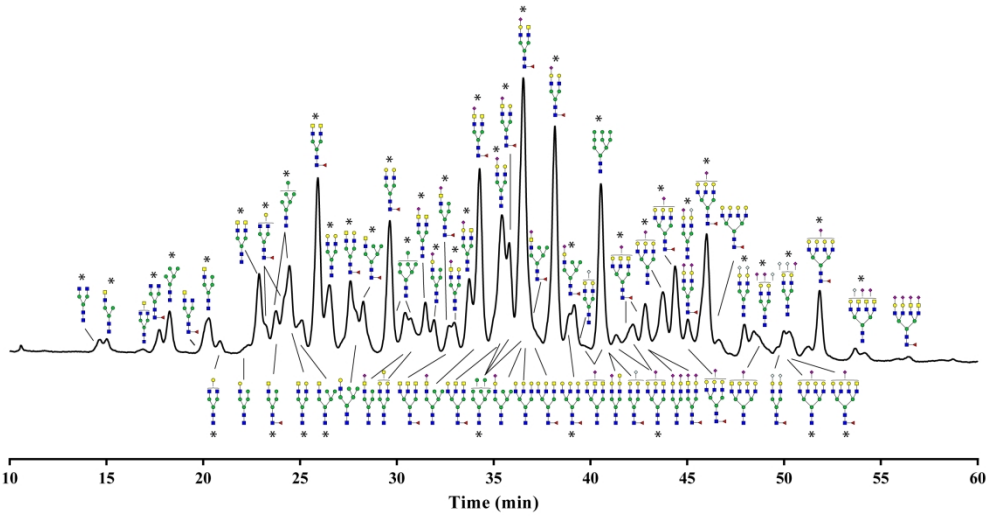

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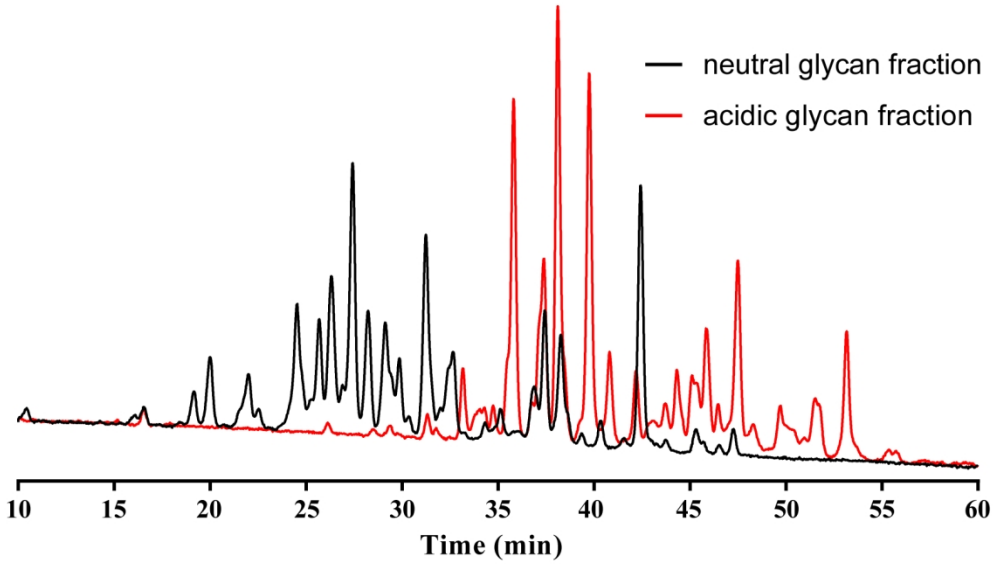

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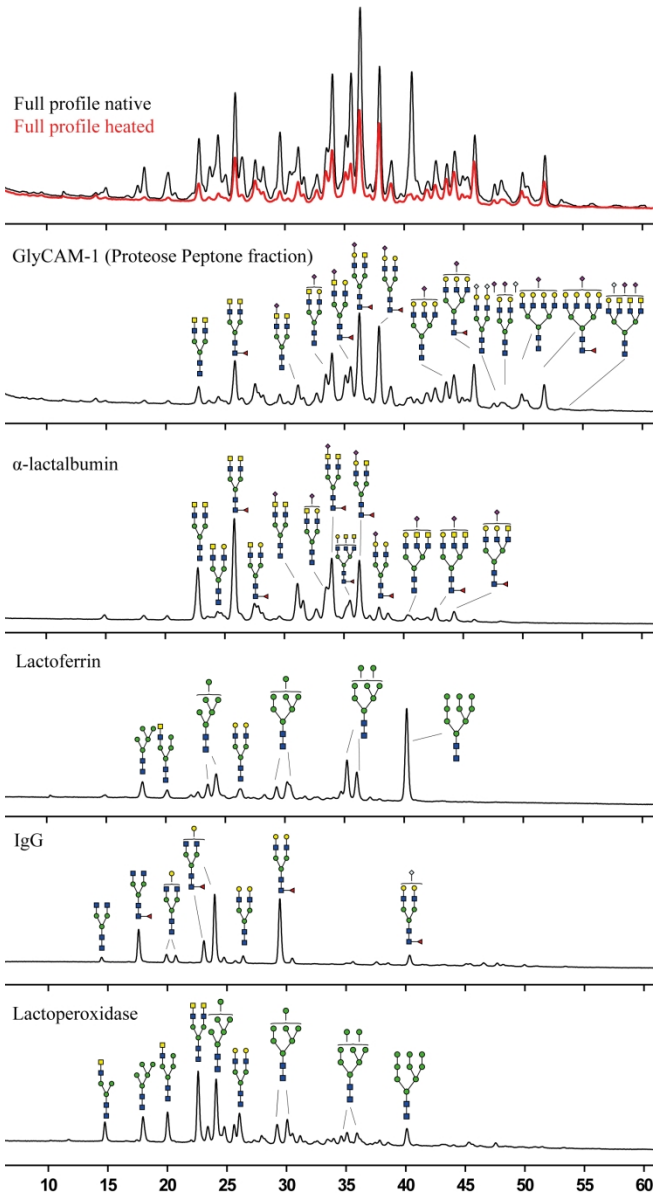

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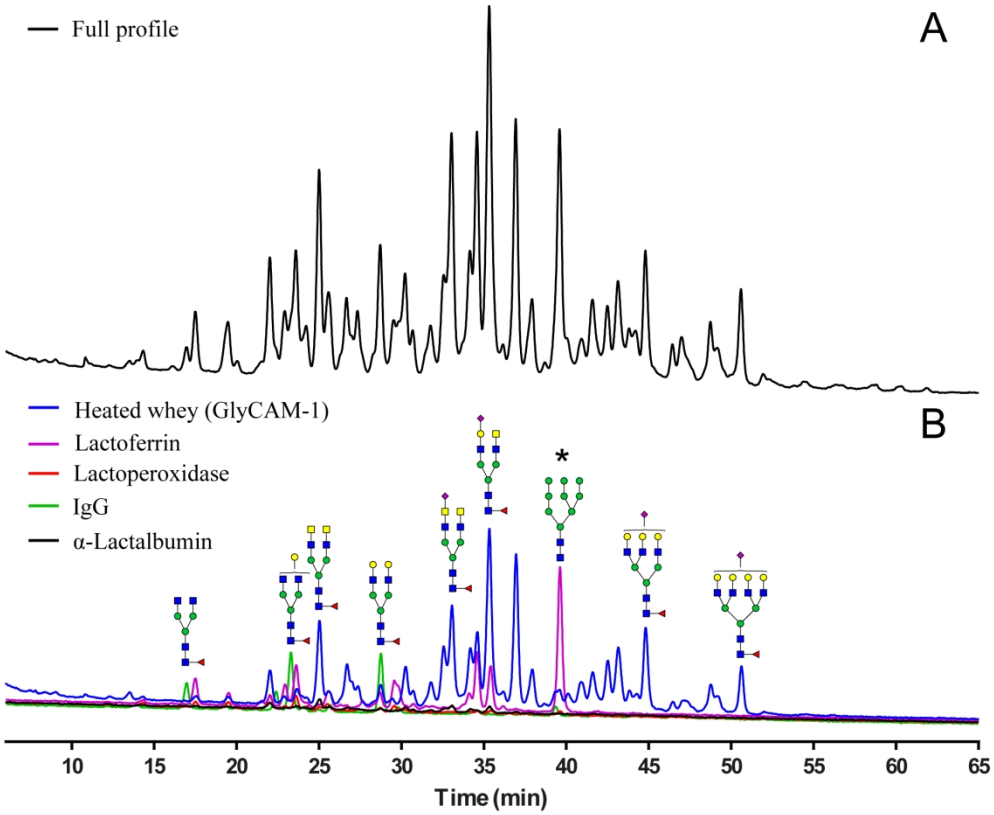

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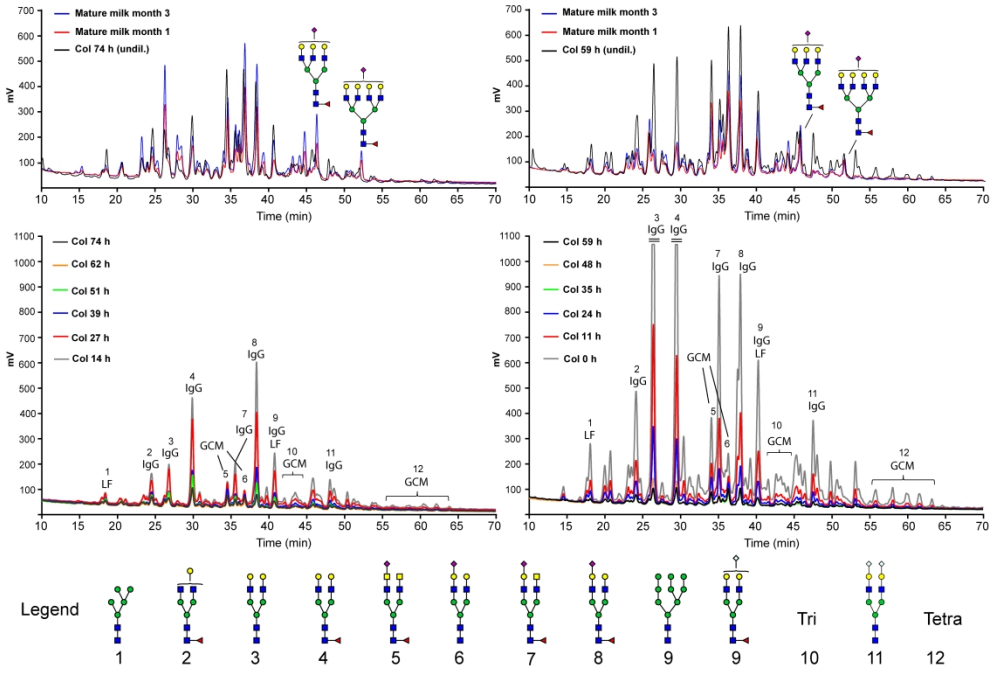

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