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Determination of Soluble Immunoglobulin G in Bovine Colostrum Products by Protein G Affinity Chromatography−Turbidity Correction and Method Validation

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ABSTRACT: Immunoglobulin-containing food products and nutraceuticals such as bovine colostrum are of interest to consumers as they may provide health benefits. Commercial scale colostrum products are valued for their immunoglobulin G (IgG) content and therefore require accurate analysis. One of the most commonly used methods for determining total soluble IgG in colostrum products is based on affinity chromatography using a Protein G column and UV detection. This paper documents improvements to the accuracy of the Protein G analysis of IgG in colostrum products, especially those containing aggregated forms of IgG. Capillary electrophoresis−sodium dodecyl sulfate (CE-SDS) analysis confirmed that aggregated IgG measured by Protein G does not contain significant amounts of casein or other milk proteins. Size exclusion chromatography identified the content of soluble IgG as mainly monomeric IgG and aggregated material MW > 450 kDa with small amounts of dimer and trimer. The turbidity of the eluting IgG, mainly associated with aggregated IgG, had a significant effect on the quantitative results. Practical techniques were developed to correct affinity LC data for turbidity on an accurate, consistent, and efficient basis. The method was validated in two laboratories using a variety of colostrum powders. Precision for IgG was 2−3% (RSD_r) and 3−12% (RSD_R). Recovery was 100.2 ± 2.4% (mean ± RSD, $n = 10$). Greater amounts of aggregated IgG were solubilized by a higher solution:sample ratio and extended times of mixing or sonication, especially for freeze-dried material. It is concluded that the method without acid precipitation and with turbidity correction provides accurate, precise, and robust data for total soluble IgG and is suitable for product specification and quality control of colostrum products.

KEYWORDS: Bovine milk, colostrum, IgG, IgG aggregates, affinity chromatography, turbidity, size exclusion chromatography, capillary electrophoresis

INTRODUCTION

Freeze- and especially spray-dried bovine colostrum powders are important commercial products with applications in health and nutraceutical supplements and other formulations.[1−7] The immunoglobulin G (IgG) content of such products is used as an indicator of authenticity and quality—The IgG content of cow's milk falls rapidly with each successive milking after calving as the secretion becomes rapidly more milklike.[8] The soluble IgG content of colostrum powder is also taken as an indication of the harshness or otherwise of the manufacturing process and the stability of the product in storage. IgG largely survives pasteurization but may become aggregated, denatured, and rendered insoluble by harsher treatments or unsuitable storage conditions.[9−19]

IgG assay methods were recently reviewed by Gapper et al.[20] and earlier, more briefly, by Indyk and Filonzi.[21] Two of the most commonly used types of assays for commercial quality control of IgG in colostrum products are immunological-based assays and affinity chromatography assays. One limitation of immunoassays is that their accuracy is highly dependent upon the reference standard used.[22] Currently, the only commercially available bovine IgG reference standard is serum derived. Use of a serum-derived IgG reference standard for immunoassays results in an underestimation of IgG content in milk products due to differences in the proportions of the IgG-1 and IgG-2 subtypes between bovine serum and milk.[22,23] Thus, for best accuracy, immunological assays for colostrum products should use a colostrum-derived IgG reference standard. Affinity chromatography is based on a different mechanism of binding that is not expected to be as sensitive to the differences between serum-derived and colostrum-derived IgG.

Affinity chromatography based on a commercial column containing Protein G bound to Sepharose beads has provided the basis for very effective analytical methods for IgG determination. Protein G, a cell surface protein of Group G streptococci, binds to the Fc region of IgG by a nonimmune mechanism. The recombinant Protein G produced in *Escherichia coli* contains two IgG-binding regions, and the albumin-binding region of native Protein G has been genetically deleted.[24] This Protein G has equivalent affinities for the IgG-1 and IgG-2 subtypes present in bovine milk products.[25] Protein G columns were primarily developed for isolation and purification of IgG; however, they are also suitable for use in quantitative liquid chromatography (LC) assays. Protein G affinity liquid chromatography with UV detection (affinity LC) was first used for bovine IgG

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determination in whey proteins,[26] and a modified protocol was subsequently adopted by the New Zealand dairy industry and others for routine determination of total soluble IgG in bovine milk and colostrum products. A further modification of this protocol has been published with analysis of the IgG in whey following acid precipitation of solubilized colostrum,[27] and recently, this method was subjected to an AOAC collaborative study.[28] The reported IgG values for acid whey from colostrum are generally much lower than for total soluble IgG, especially in products containing appreciable highly aggregated forms of IgG. Whether total soluble IgG, or acid-precipitated whey IgG, is the most appropriate measure of colostrum quality is a separate and complex issue. The bioavailability in the gut of IgG from soluble aggregates may not be greatly different to that for monomeric IgG, especially if the binding is not covalent.[29]

The present paper studies the precision and accuracy of the method for quantitative determination of soluble IgG in colostrum products based on affinity LC using a turbidity correction calculation. This method does not use acid precipitation as a sample preparation step prior to analysis. The lack of an acid precipitation step allows for quantitation of the total soluble IgG content in colostrum products, including aggregates of IgG, and an evaluation of the degree of aggregation present. Information concerning the degree of aggregation can be useful in solving manufacturing issues and comparing the quality and storage conditions of colostrum products. The importance of both sample extraction and correction of turbidity contributions to apparent protein absorbance is highlighted for improved accuracy of results. Several calculation techniques for turbidity correction are evaluated for their accuracy, consistency, and efficiency.

Because aggregation can be caused by association with other proteins as well as self-association, the nature of the turbidity and the composition of the colostrum proteins retained by Protein G affinity chromatography are investigated using size exclusion high performance chromatography (SE-HPLC) and CE-SDS.

MATERIALS AND METHODS

Materials. All reagent chemicals were AR grade unless otherwise stated. Water was deionized and Milli-Q-purified (Waters Corp., Hartford, MA). Bovine blood serum IgG for calibration was reagent grade, ≥95% [sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE)] (no. I-5506, Sigma-Aldrich, St. Louis, MO). Bovine serum IgG for fortification was laboratory grade (no. IGGL, ICP Biotech, Auckland, New Zealand). Lactoferrin and casein were production grades (>95% protein purity) (Westland Milk Products Hokitika, New Zealand). Buffer A (loading buffer): 25 mM sodium dihydrogen orthophosphate plus 0.3 M sodium chloride, pH 6.50. Buffer B (eluting buffer): 50 mM glycine, pH 2.5. The buffers were filtered through 0.45 μm pore nylon filters and used within 4 days with storage at 4 °C. Bovine colostrum powders were obtained from commercial production runs at several dairy factories in Australia and New Zealand and represented a range of different feedstocks and processing conditions. The powder samples were stored in sealed containers at 4 °C until analyzed.

Standard Solutions for LC Calibration. IgG solid material was accurately weighed into a glass tube, and 0.15 M sodium chloride was added by weight to obtain a concentration of approximately 6.25 mg/g. The IgG was dissolved by thorough vortexing and then centrifuged at 3000*g* for 10 min. Aliquots were diluted by weight with 0.15 M sodium chloride to a final concentration of 0.6 mg/g. After vortexing, the absorbances at 280 and 320 nm were recorded on a Shimadzu UV-1700 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The concentration of the stock solution (mg/g) and the estimated % purity of the IgG material were calculated from A_{280} using an $E_{1\%}$ value for IgG at 280 nm of 13.8 and correcting for scattering by subtraction of 1.7 × A_{320}. Additional aliquots were diluted by weight with loading buffer to provide standard solutions over a concentration range of 0.5−2.5 mg/g.

Liquid Chromatography. Two instruments with similar specifications were used as follows: (A) Shimadzu LC-10 system (Shimadzu Corp.) with LC-10AD*VP* pump and low pressure gradient mixer, SIL-10AD*VP* autosampler, column oven, SPD-M10A*VP* diode array detector (DAD), and Class *VP* chromatography data system. (B) Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) with G1311A pump and low pressure gradient mixer, G1329A autosampler, column oven, G1315 DAD, and ChemStation for LC 3D rev. A10.02 chromatography data system. The affinity columns used were HiTrap Protein G HP 1 mL column (no. 17-0404-01, Amersham Biosciences/GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Extraction. Subsamples of colostrum powder were weighed into polypropylene tubes, and loading buffer was added by weight. Two proportions of sample:solution were tested at 1:160 and 1:80 w/w. After thorough vortex mixing, the suspensions were either end-over-end mixed in a closed tube for 2 h at room temperature or sonicated in a Soniclean 160HT Sonicator (Soniclean Pty, Thebarton SA, Australia) for 10 min. A subsample of each suspension was centrifuged at 16000*g* for 10 min, and the supernatant was transferred to an autosampler vial for affinity LC analysis. A second set of sample extracts were acid treated prior to analysis to precipitate caseins, IgG aggregates, and denatured whey proteins using the addition of 2 mL of 10% acetic acid and filtration.[27]

Affinity LC Determination of IgG. Injections were made onto the affinity column and DAD spectra acquired at 1 Hz, 240−494 nm, during elution with the following gradient: 0−6 min, 100% buffer A, 1 mL/min; 6.0−7.5 min, from 100% buffer A to 100% buffer B, 2 mL/min; 7.5−10 min, 100% buffer B, 2 mL/min (linear gradients). The acquired raw DAD data for the IgG peak were handled in three different ways for correction of turbidity contributions. First, the full DAD spectra accumulated across the peak were exported to a Microsoft Excel spreadsheet. The turbidity contribution was calculated by a custom macro, TCORRECT, developed to extrapolate to 280 nm the linear fitted log−log absorbance data 340−494 nm (155 points) at each time point across the peak. The macro then subtracted this turbidity contribution from the raw 280 nm absorbance at each time point and integrated the corrected IgG peak. This calculation method was referred to as point to point. Second, in a simplification of the above procedure, the IgG peaks at 280, 340, 360, 380, 400, 420, and 440 nm were extracted and integrated using the data system software. The IgG peak area at 280 nm was corrected for turbidity by using a spreadsheet to extrapolate the six-point log−log fit of area vs wavelength 340−440 to 280 nm. This calculation method was referred to as the averaged correction. The third method involved using the point to point data to calculate a numerical single factor that could be used in measuring turbidity for a well-known and predictable colostrum product. The IgG concentration in colostrum extracts was calculated from the corrected peak area using the linear calibration for the areas of standard solutions of IgG that had also been corrected for turbidity.

Extractability of IgG from Colostrum. End-over-end mixing was compared with sonication for extraction of IgG from both a spray-dried and a freeze-dried colostrum powder. Two solvent/sample ratios (80:1 or 160:1 w/w) and four extraction time periods were used (mild sonication: 5, 10, 20, and 40 min; or end-over-end: 0.5, 1, 2, and 4 h). All samples were prepared in duplicate. Extracts were analyzed for total soluble IgG by the affinity LC method using the averaged turbidity correction factor calculation.

Method Validation. A range of experiments were carried out in two laboratories to validate the method for accuracy, precision, and

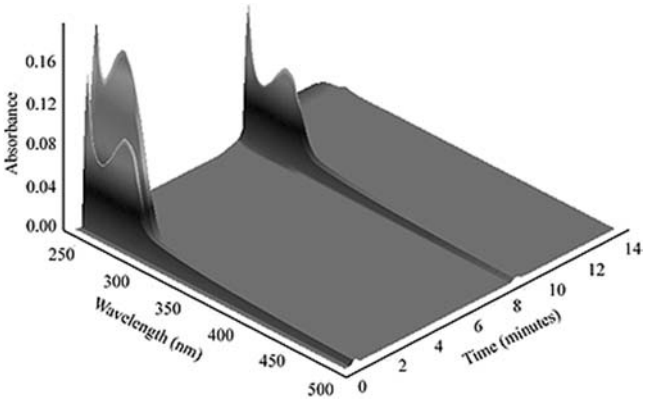

Figure 1. Typical 3D DAD elution pattern for reconstituted colostrum powder eluted off a Protein G column.

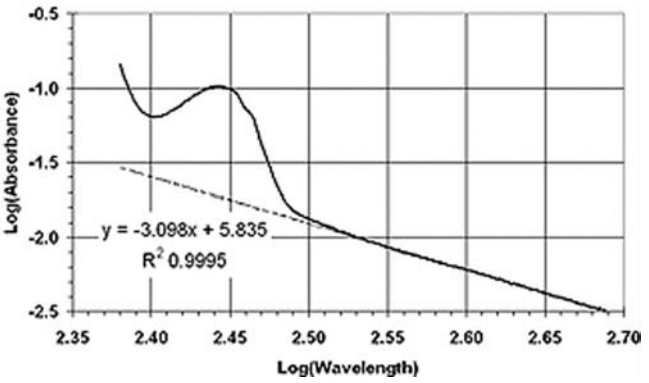

Figure 2. DAD absorbance spectrum for IgG in a colostrum extract eluting from a Protein G column. Log−log plot. Raw spectrum, —; extrapolation of the log−log regression at 340−480 nm, − − −.

robustness using different types of colostrum powders. This included linearity and range, replicate analyses on the same colostrum samples on different days and in different laboratories, interference from two milk proteins, and recovery of added IgG. Subsamples of skim milk powder or colostrum powder were fortified with casein, lactoferrin, or IgG using weighed aliquots of stock standard solutions in 0.15 M NaCl (typically 5−10 mg/g).

Nature of IgG Aggregates and Turbidity. Isolation of soluble IgG from colostrum was performed by affinity LC with a 5 mL Protein G column (no. 17-0405-01, GE Healthcare Bio-Sciences AB) using conditions similar to those used for analytical determinations. The IgG fraction was concentrated and buffer exchanged using an Amicon Ultracel 3K centrifugal ultrafiltration unit (Millipore, Cork, Ireland). The protein purity of IgG standards and compositions of IgG peaks from affinity LC was checked using size exclusion high-performance liquid chromatography (SE-HPLC), SDS-PAGE, and capillary electrophoresis−sodium dodecyl sulfate (CE-SDS). SE-HPLC was performed using a TSK-Gel G3000SW column (no. 05147, Tosoh Biosciences LLC, Tokyo, Japan) with 25 mM $NaH_2PO_4 \cdot H_2O$ and 100 mM Na_2SO_4, pH 7, buffer as the mobile phase. CE-SDS was performed under reducing conditions using an SDS molecular weight kit (no. 390953, Beckman Coulter, California) and an Agilent G1600 capillary electrophoresis system.

RESULTS AND DISCUSSION

Figure 1 shows a typical 3D DAD chromatogram for reconstituted colostrum powder analyzed by Protein G affinity chromatography using a pH gradient as described above. Casein, other proteins, and other UV-absorbing colostrum components are not retained and elute near the void volume. A_{240} values begin to rise from about 7 min due to elution of glycine buffer B. The IgG protein A_{max} is at about 280 nm with the peak at ca. 8.8 min. The IgG spectrum shows a tail extending well into the visible region typical of light scattering by larger particles, which we attribute to soluble IgG aggregates. This turbidity is exemplified by the A_{340} peak, which overlaps the A_{280} peak, although the two are not completely coincident. Generally, the A_{340} peak is narrower and elutes slightly earlier, indicating weaker binding of the turbid components to the Protein G substrate.

Correction for Turbidity. The significant elevation of absorbance at 280 nm due to light scattering by soluble IgG aggregates must be taken into account if the Protein G affinity LC method is to provide accurate data for the concentration of IgG in colostrum products. All dispersed particles, including soluble aggregates of IgG, scatter light depending upon their size (relative to the wavelength of light), refractive index (relative to that of the surrounding medium), and shape. Often, in the absence of chromophores in the wavelength range and for dilute solutions with minimal multiple light scattering, log−log plots of turbidity versus wavelength yield straight lines, as has been demonstrated for IgG by spectrophotometry.[30] This allows the turbidity contributions to be accurately calculated by extrapolation to lower wavelengths where the analyte chromophores absorb. The equation has the form

$$\text{Log (turbidity)} = A + B \times \text{Log (wavelength)}$$

where A and B are empirical parameters. This equation can be used to correct the protein absorbance signal at 280 nm: $A_{280,corr} = A_{280} - (10^A \times 280^B)$.

Such turbidity corrections are relatively common when protein concentrations are being estimated using spectrophotometry at 280 nm.[31] The corrections are not always applied or appropriately executed. For example, the original affinity LC assay for IgG in whey[26] and a more recent modification for colostrum[27,28] used absorbance measurements at 280 nm for the estimation of IgG concentrations. However, only the IgG concentrations for standard solutions were corrected for turbidity using the absorbance at 320 nm and assuming Rayleigh scattering (very small particles relative to the wavelength of the scattered light) with fourth-power wavelength dependence:

$$\begin{aligned}\text{turbidity-corrected absorbance} &= A_{280} - (320/280)^4 \times A_{320} \\ &= A_{280} - 1.706 \times A_{320}\end{aligned}$$

Figures 2 and 3 show the observed spectrum of IgG from a sample of reconstituted colostrum powder eluted from a Protein G column plotted on log−log and linear scales, respectively. The long tail toward the visible arising from scattering is very evident. The slope of the regression of the log−log plot 340−480 nm is −3.10, and the plot is highly linear (R^2 = 0.9995, n = 155). In Figure 2, the extrapolation of this turbid baseline into the UV, the turbidity-corrected IgG spectrum, and a reference spectrum for pure bovine plasma IgG are also shown.

Three turbidity correction techniques were evaluated. The first called point−point corrected the absorbance at each time point across the IgG peak, a second called averaged used a simplified averaged correlation for the IgG peak areas measured

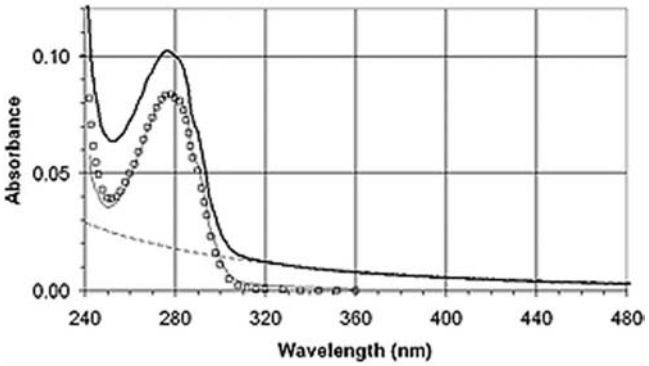

Figure 3. DAD absorbance spectrum for IgG in a colostrum extract eluting from a Protein G column. Raw spectrum, ——; extrapolation of the log−log fit at 340−480 nm, _ _ _; spectrum with turbid baseline correction, □; reference spectrum for bovine plasma IgG without turbidity correction (peak absorbance normalized to corrected colostrum spectrum), ____.

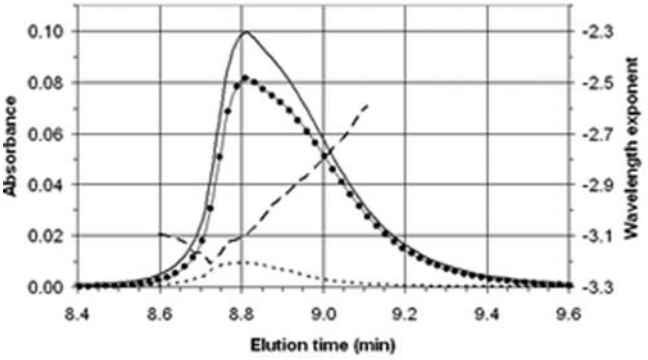

Figure 4. Elution pattern for IgG in a colostrum extract using a Protein G column. A280, ____; A340, - - -; log−log wavelength exponent, _ _ _; A280 with turbidity correction at every time point, ____; and A280 with turbidity correction factor 1.849 × A340 (mean wavelength exponent −3.166), ●.

over a range of wavelengths, and the third called single used a single numeric correction factor.

The point to point technique most accurately determined turbidity under chromatographic conditions, using a Microsoft Excel macro, TCORRECT. The macro was developed to calculate the turbid contribution to A_{280} from log−log regressions across the IgG elution peak. The wavelength range collected by the DAD (240−494 nm) was chosen to allow convenient use of a spreadsheet while preserving maximum resolution. The regression equations were calculated for the logs of the absorption data versus wavelength over the range 340−494 nm at 1 s time increments across the IgG peak, and the equations were extrapolated to estimate the contribution at A_{280} from turbidity. Data below 340 nm were not used for the regression to minimize absorbance contributions from chromophores. The IgG peak with corrected A_{280} absorbances was integrated for determination of IgG concentrations in colostrum sample extracts. Figure 4 shows the measured signals at 280 and 340 nm for the IgG peak from a colostrum sample under our affinity LC conditions and the corrected peak at 280 nm after subtraction of the turbid signal at each point by the above procedure. The corrected IgG peak area was 81.3% of the uncorrected peak area (18.7% turbid contribution). The wavelength exponents, from log−log slopes, across the peak varied from −3.2 to −2.6, consistent with the light scattering particles being much larger than the Rayleigh limit (diameter < wavelength/20). This data verified that appropriate sample turbidity corrections are needed to accurately report the IgG content of aggregated, turbid solutions. Without appropriate correction models, the IgG content will be overestimated for aggregated colostrum products.

The averaged technique simplified the calculations for affinity LC use and involved the use of chromatography integrator outputs to provide an averaged correction across the whole IgG peak. The accuracy of a turbidity correction based on log−log extrapolation of the set of six extracted IgG peak areas for each sample over the range 340−440 at 20 nm intervals (see data below) was studied.

For the third technique, the multiplication factor for the IgG peak area at 340 nm was calculated to correspond to the true (point to point) turbid area at 280 nm for a range of colostrum samples to establish what potential errors would arise from the use of a single correction factor, Y, for unknown samples:

$$\text{turbidity-corrected absorbance} = \text{area}_{280} - Y \times \text{area}_{340}$$

The mean exponent of −3.166 for the IgG in the colostrum extract in Figure 4 corresponds to a correction factor Y of 1.849, and this provides an 18.7% turbid correction, the same as from point to point. The across the peak application of this factor gave a corrected peak shape that also closely corresponded to that accurately calculated by point to point (Figure 4). Applying the Rayleigh limit correction factor of 1.7 (log−log slope of −4) to the IgG peak area at 320 nm provides a turbid correction of 18.2% for this extract. As the log−log slopes near the peak apex were ca. −3.1 for this sample, the Rayleigh limit correction will underestimate turbidity, but protein chromophore effects at 320 nm could be providing some cancellation of errors. The UV spectra of IgG from affinity LC of milk products have not been well characterized, but we have noted a small but significantly elevated absorbance at 320 nm not accounted for by turbidity (Figure 3). Because of the elevated absorbance observed at 320 nm, it is recommended that any single correction factors be based on A_{340}.

Calibration. The calibrations with IgG standards were highly linear ($R^2 > 0.9995$) in the range 0.05−1 mg IgG injected for both of the LC instruments. Standard solutions of reagent grade IgG from bovine plasma proved satisfactory, provided great care was taken with the preparation of the stock standard solution, its calibration for protein content by spectrophotometry, and the storage of aliquots for future use (−20 °C, single thaw). Typical purities found for different batches of Sigma IgG ranged from 80 to 90% protein with 1−2% unretained material from the affinity LC monitored at 280 nm. The turbid peak at 340 nm was <1%. The unaccounted for weight was presumably comprised of mainly salts and water.

Precision and Accuracy for IgG Content of Colostrum. Five spray-dried bovine colostrum powders from different batches of raw colostrum using varied manufacturing conditions were analyzed by the affinity LC method with correction for turbidity and by the method of Copestake et al.[27] with acid precipitation prior to affinity LC (Table 1). Total soluble IgG levels were in the range 41−132 mg/g. The size of the turbid peak (18−32% of raw IgG peak area at 280 nm) indicated the presence of significant amounts of soluble IgG aggregates in colostrum extracts prepared without acid precipitation. In contrast, acid

Table 1. IgG Content of Five Spray-Dried Colostrum Powders and a Skim Milk Powder Determined by Affinity LC[a]

	total soluble		acid whey	
	IgG (mg/g)	turbidity 280 nm area (%)	IgG (mg/g)	turbidity 280 nm area (%)
colostrum #262	83.5	26	41.2	3.9
colostrum #263	221	21	132	2.6
colostrum #264	161	18	99.4	2.7
colostrum #265	132	32	69.1	2.6
colostrum #266	183	22	112	2.6
skim milk powder	1.40	<5	<1	

[a] Samples extracted using both the current method (total soluble IgG) and the acid-precipitated method.[27] Turbidity was calculated by averaged correction calculation. Mean of duplicate subsamples.

Table 2. Analysis of Soluble IgG by Affinity LC in a Skim Milk Powder and a Colostrum Powder Fortified with Lactoferrin or Casein[a]

	IgG (mg/g powder)		turbidity (% raw peak area at 280 nm)	factor (A_{340})
turbidity correction	averaged correction	point to point correction	averaged correction	single factor
skim milk powder (SMP)	1.4	1.1	NC	NC
SMP + 100 mg/g lactoferrin	1.5	1.0	NC	NC
SMP + 200 mg/g lactoferrin	1.7	1.2	NC	NC
SMP + 100 mg/g casein	1.1	1.0	NC	NC
SMP + 200 mg/g casein	1.1	1.0	NC	NC
colostrum	80	79	29	1.79
colostrum + 100 mg/g lactoferrin	82	81	32	1.73
colostrum + 200 mg/g lactoferrin	83	82	31	1.76
colostrum + 100 mg/g casein	82	82	33	1.74
colostrum + 200 mg/g casein	82	82	35	1.74
colostrum mean ± RSD (n = 10)	82 ± 1.4%	81 ± 1.7%	32 ± 6.9%	1.75 ± 1.4%

[a] Turbidity correction reported by averaged and point to point calculation. Factor is the multiplier of peak area at 340 nm to give the measured turbidity at 280 nm. Means of duplicate subsamples. NC, not calculated.

Table 3. Recovery of Soluble IgG from a Colostrum Powder Containing 28 mg/g IgG and Fortified with IgG at ca. 100 mg/g[a]

	sample	% recovery
analyst 1	1	100
	2	97.3
	3	98.4
	4	100
analyst 2	5	99.0
	6	99.3
	7	105
	8	103
	mean ± RSD (n = 8)	100 ± 2.4

[a] Four replicates by different analysts on different days. Correction for turbidity by averaged technique.

precipitation resulted in 50−60% lower IgG levels and only small amounts of turbidity, consistent with the extracts containing reduced levels of IgG aggregates. The skim milk powder contained only low levels of IgG (<2 mg/g) and turbidity. The skim milk powder and one of the colostrum samples were also analyzed after fortification with casein or lactoferrin at levels of 100 or 200 mg/g with no effect on the measured IgG levels or turbidity (Table 2). The precision for IgG in the colostrum sample across all of the treatments was excellent (RSD 1.4−1.7%, n = 10). A single factor of 1.76 × area$_{340}$ provided a precise turbidity correction for the colostrum samples. Replicate fortification experiments with IgG added to a colostrum powder provided data on the accuracy and intermediate precision of the method (Table 3). The experiments were carried out on 2 days by different analysts and using different Protein G columns. Recoveries for IgG at 100 mg/g were 100.2 ± 2.4% (mean ± RSD, n = 8).

Further data on the performance of the method and the relative accuracy of the turbidity correction and calibration techniques were provided by the analysis of three colostrum samples with five replicates (Table 4). The precision for soluble IgG in these samples (RSD 0.2−2.5%) confirmed the earlier data (Table 2). Overall, there was no difference in the size of the turbid peak or level of IgG between using point to point or averaged correction techniques for these four samples (Tables 2 and 4). Soluble IgG aggregates could be more concentrated near the leading edge of the IgG elution peak[27] due to weaker binding to the affinity column, but the averaged correction did not lead to errors as compared to the point to point correction. The nature of the IgG aggregates appeared similar for these colostrum samples with levels of turbidity, log−log slopes, and the single factor to estimate turbidity from the area at 340 nm all being similar (mean 1.76).

Table 4. Precision for Soluble IgG Determination in Colostrum by Affinity LC[a]

	averaged correction				point to point correction	
	IgG	turbidity	factor	Log−log	IgG	turbidity
	mg/g ± RSD (%)	raw peak area (%)	× area 340 nm	slope	mg/g ± RSD (%)	raw peak area (%)
colostrum #262	78.2 ± 2.0	29	1.75	−2.95	77.4 ± 2.5	31
colostrum #263	219 ± 1.4	24	1.76	−2.97	219 ± 1.5	23
colostrum #266	181 ± 0.2	23	1.77	−3.00	181 ± 0.2	23

[a] Correction for turbidity by averaged or point to point correction techniques. Means for five replicate subsamples.

Table 5. Affinity LC Analysis of Soluble IgG in Five Colostrum Samples by Two Laboratories with Five Replicates[a]

	turbid area [raw peak area 280 nm (%)]			log−log slope		turbid factor × area 340 nm		IgG [mg/g powder (mean ± RSD, n = 5) (%)]		
sample	lab. one point to point	lab. one averaged	lab. two averaged	lab. one averaged	lab. two averaged	lab. one averaged	lab. two averaged	lab. one point to point	lab. one averaged	lab. two averaged
1073	7.20	7.20	12.1	−3.44	−3.94	1.91	1.81	214 ± 1.5	220 ± 3.0	259 ± 2.9
1074	34.2	34.3	24.3	−2.64	−3.07	1.81	1.48	33.2 ± 5.7	33.5 ± 13.6	40.8 ± 1.7
1075	18.4	18.8	16.8	−3.01	−3.13	1.84	1.68	143 ± 1.5	147 ± 3.5	149 ± 2.3
1076	17.3	20.3	17.9	−3.04	−3.31	2.10	1.70	151 ± 1.1	150 ± 4.1	156 ± 0.2
1077	24.2	27.4	20.9	−3.09	−3.10	2.32	1.69	84.9 ± 1.8	83.8 ± 3.2	92.2 ± 1.3

[a] Correction for turbidity by point to point or averaged techniques.

A comparison between two laboratories using five colostrum powders from a wider range of factories representing different feedstocks, manufacturing processes, and conditions was completed (Table 5). Laboratory 1 used an end-over-end extraction procedure, while laboratory 2 used a sonication procedure. The broader range of sample types was confirmed by the IgG data with sample #1073, a freeze-dried powder, having the highest soluble IgG (average 230 mg/g), lowest % turbidity (average 9%), and lowest log−log slope (average −3.7). Samples #1075, 1076, and 1077 were typical spray-dried products with IgG contents of 85−150 mg/g, turbidities of 17−21%, and log−log slopes of −3.1 to −3.0. Sample #1074 was an experimental spray-dried material with low soluble IgG (average 36 mg/g) and high turbidity (24%, log−log slope −2.6). The precision for IgG content calculated by point to point correction for laboratory 1 and by averaged correction for laboratory 2 was similar. The poorer precision observed for laboratory 1 using averaged correction was attributed to integration software baseline setting difficulties for the small, non-Gaussian turbid peaks. The laboratory 1 mean IgG levels by both calculation procedures are the same for each sample, confirming the equivalence of the turbidity corrections. However, a trend of higher levels of IgG was observed from laboratory 2 in comparison to laboratory 1 (Table 5), especially for samples #1073 and #1074 at +19 and +22%, respectively. The three, more standard, spray-dried samples averaged approximately +5% higher IgG content measured by laboratory 2 as compared to laboratory 1. Because the sample extraction technique used in each laboratory was different, the extraction method was identified as a potential source of interlaboratory variability, and extractability was therefore evaluated in later experiments.

Turbidity correction was essential for accuracy of IgG determination in colostrum extracts. Point to point as well as averaged correction techniques gave equivalent results for total soluble IgG in a range of colostrums (Tables 2, 4, and 5). The application of a single factor of 1.76 to the peak area at 340 nm gave a comparable accuracy of correction for several spray-dried colostrums (Tables 2 and 4), but this factor may be less applicable to other colostrums from different feedstocks and manufacturing processes, especially freeze-dried products (Table 5). Because of efficiency and accuracy, the preferred method for correction is the averaged technique. In situations where the colostrum feedstock is well studied and the IgG compositions more predictable, the simplified single factor approach may be suitable.

Extractability of IgG from Colostrum. End-over-end mixing was compared with sonication for extraction of IgG from colostrum powders at two solvent/sample ratios and four extraction times. Figure 5A compares the levels of soluble IgG extracted from a spray-dried colostrum sample using sonication or end-over-end shaking for different time periods. The quantity of IgG extracted was greatest with 6−24 min sonication or 1−4 h shaking. The slopes of the log−log plots and the size of the turbid peak were also relatively constant over these periods. The higher solution/sample ratio resulted in extraction of approximately 3% more IgG. For a freeze-dried powder (Figure 5B), there were stronger influences of solution ratio and extraction time on the measured IgG. The higher solution/sample ratio, sonication treatments, and longer extraction times all led to more soluble IgG being extracted. Sonication for 5 min was equivalent to 1−2 h of end-over-end mixing. The increases in the relative size of the turbid peak for this sample (5−11%, data not shown) closely matched those for soluble IgG indicating that stronger extraction conditions led to solubilization of more aggregated IgG of larger molecular size. This was confirmed by the log−log slopes (averaged correction, 340−440 nm), which were −3.9 to −3.7 for the short extraction periods and low solution:sample ratio, increasing to −3.4 to −3.5 for stronger extraction conditions. The nature of the aggregated IgG in the spray-dried product appeared different to that in the freeze-dried product with the latter exhibiting no plateau in the amount of IgG being

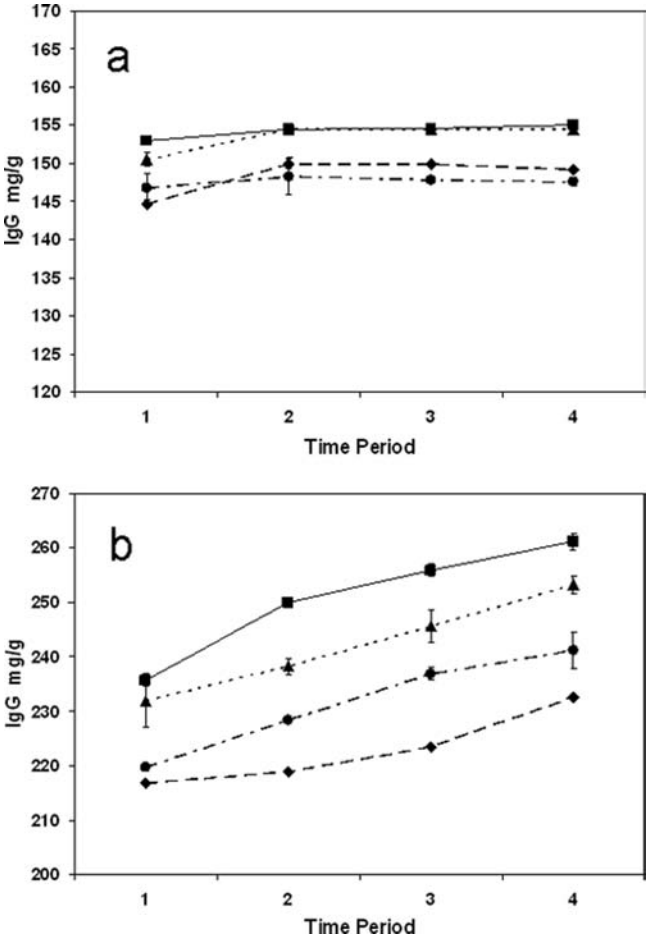

Figure 5. Extraction of soluble IgG with 0.15 M NaCl from two colostrum samples. (a) Powder manufactured by spray-drying. (b) Powder manufactured by freeze-drying. Four extraction time periods; end-over-end mixing (0.5, 1, 2, and 4 h) vs sonication (5, 10, 20, and 40 min). Key: 0.25 g/40 mL, sonication (■); 0.25 g/40 mL, end-over-end (▲); 0.5 g/40 mL, sonication (●); and 0.5 g/40 mL, end-over-end (♦). Mean of duplicates ± range.

solubilized with extraction time (Figure 4B). Sonication of a solution of laboratory grade IgG in loading buffer did not result in any change in IgG with time (3−24 min): concentration 0.979 ± 0.035 mg/g; turbid peak 5.0 ± 0.57% (mean ± SD, n = 8). This confirmed that sonication did not directly cause formation of IgG aggregates. The extractability data showed that differences in IgG levels determined by the two laboratories (Table 5) were mainly attributable to variations in extraction conditions and that this step needs to be standardized for a more extensive interlaboratory study of the method.

A solution:sample ratio of 160:1 was the most effective for determining total soluble IgG in colostrum powders. Sonication in a low power bath for short periods (5−10 min) was as effective as end-over-end mixing for longer periods. Sonication for longer periods continues to solubilize some IgG, especially from freeze-dried products. This may be partially a result of the rise in temperature, which may accelerate the solubilization. However, extended extraction periods are less practical and, along with elevated temperatures, may lead to denaturing of IgG.[32] Therefore, a solution:sample ratio of 160:1 and either sonication for 10 min or end-over-end shaking for 2 h is recommended.

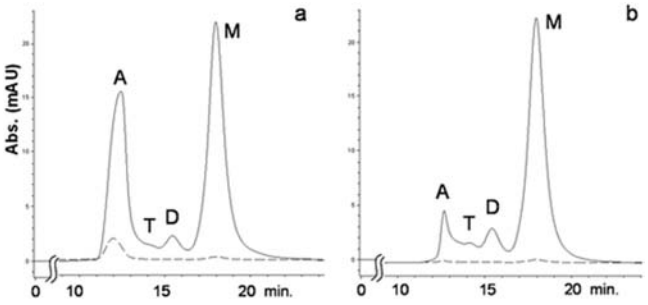

Figure 6. Size exclusion chromatography of IgG from affinity LC of a colostrum extract. (a) Total soluble. (b) Acid precipitated whey. 280 nm, ____; 340 nm, _ _ _. M, monomeric IgG 150 kDa; D, IgG dimer 300 kDa; T, IgG trimer 450 kDa; and A, aggregate >450 kDa.

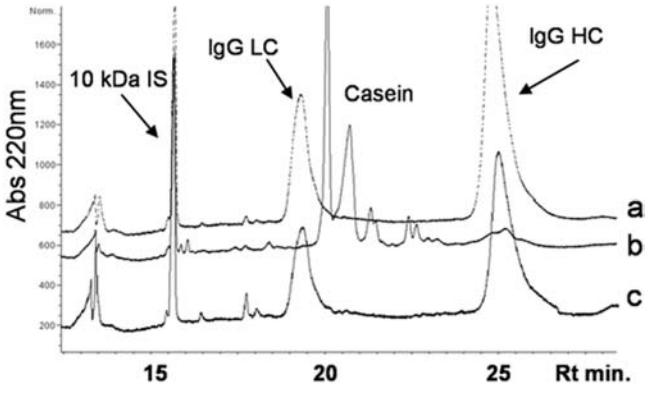

Figure 7. CE-SDS size distribution of reduced proteins. (a) IgG from colostrum isolated by affinity LC, (b) casein, and (c) IgG aggregate from SE-HPLC of fraction a above.

Nature of IgG Aggregates and Turbidity. SE-HPLC provided additional information about the molecular weights of the soluble IgG analyzed by Protein G affinity chromatography. The SE column had an exclusion volume corresponding to approximately 500 kDa molecular weight (MW). Typically soluble IgG isolated from spray dried colostrum using affinity LC (Figure 6a) contained monomeric IgG as the major peak (approximately 57%), minor amounts of IgG dimer (6%) and trimer (4%), and then another large peak (33%) MW > 450 kDa corresponding to aggregates of IgG. The turbidity of the IgG was mainly associated with the aggregate peak (Figure 6a, trace at 340 nm) as expected. The corresponding SE-HPLC chromatogram for the IgG from acid whey of colostrum (Figure 6b) contained approximately 73% monomer, 10% dimer, 6% trimer, and 11% aggregates, demonstrating that the acid precipitation method[27] not only determines monomeric IgG but also measures some dimer, trimer, and larger aggregates.

Although the fortification experiments (Table 2) showed no direct interferences with the method from casein or lactoferrin, the possibility remained that IgG bound to the affinity LC column included aggregates incorporating other milk proteins, which could lead to inflated levels in the measurement of soluble IgG. Experiments using SDS-PAGE under reducing conditions similar to those previously reported[27] and using Coomassie Blue staining failed to detect other major milk proteins, including casein, in the IgG fractions of colostrum products purified by affinity chromatography (data not shown). However, SDS-PAGE

was inadequate to definitively eliminate the presence of casein at levels below about 10% of the IgG. Therefore, further analyses were carried out using CE-SDS, which can provide similar separations to PAGE but of higher resolution and dynamic range.[26,33]

The IgG peaks from affinity LC of colostrum extracts (total and acid precipitated) and an IgG standard were collected and examined by CE-SDS (reducing conditions). Fractions of the colostrum-derived IgG were also collected from the SE-HPLC column and assayed by CE-SDS. The reducing conditions cleaved any aggregate bonds present, enabling identification of IgG fragments such as heavy chain and light chain as well as any non-IgG proteins that may have been incorporated in aggregates. The CE peaks were relatively broad due to the polyclonal nature of colostrum IgG (Figure 7a). However, casein was adequately resolved to be detectable down to ca. 0.3% of IgG (sum of light and heavy chain peaks) (Figure 7b).

The soluble IgG eluted from the affinity LC column for acidified and nonacidified colostrum was 99.5 and 98.9% IgG, respectively, by CE-SDS. IgG dimer, trimer, and aggregate fractions from SE-HPLC of IgG from both acidified and non-acidified colostrum were found to be predominantly IgG (≥94%). The aggregate peak (>450 kDa) from SE-HPLC of nonacidified colostrum was 94% IgG . In comparison, the aggregate peak from SE-HPLC of nonacidified colostrum was 96% IgG. Both highly aggregated fractions contained approximately 4% nonidentified peaks, mainly between 12 and 20 kDa, which may represent IgG degradation products. Of all of the SE-HPLC fractions tested, casein was detected solely in the IgG aggregate from nonacidified colostrum (Figure 7c). The level of casein at 1.8% equates to a barely detectable 0.4% of the total soluble IgG for that colostrum sample (Figure 7a). It was concluded that soluble IgG measured using Protein G affinity chromatography is predominantly IgG and contains less than 2% other milk proteins and IgG degradation products. The minimal amount of casein present in colostrum-derived IgG aggregates supports the hypothesis that affinity LC without acid precipitation and with turbidity correction provides an accurate assessment of the soluble IgG content of aggregated colostrum samples. Acid precipitation is useful for affinity chromatography analysis of monomeric IgG in colostrum samples;[27] however, the IgG content measured also includes perhaps 25% of dimer, trimer, and aggregated forms of IgG. The colostrum products examined in the present work were processed under mild conditions. It is possible that harsh processing conditions might increase the association of casein with IgG aggregates.

The SE-HPLC data showed that soluble IgG aggregates can bind to and be eluted from a Protein G column in much the same way as monomeric IgG. For analysis of colostrum products, use of an acid precipitation sample preparation step was found to be unnecessary, as CE-SDS data showed that IgG aggregates retained by Protein G were minimally associated with the casein and other milk proteins. Casein and lactoferrin are also neither bound to Protein G nor affected quantitation of IgG from colostrum. In addition, acid precipitation was found to reduce the amount of soluble aggregated IgG, resulting in lower values for IgG content. Acid precipitation of colostrum extracts is useful in assays where only monomeric IgG is of interest,[27] but we have shown that IgG measured by this method also includes some dimer, trimer, and aggregated material. Not including the acid precipitation step provides useful information for assessment of IgG aggregation in colostrum samples. However, the aggregated IgG scatters light, which inflates the A_{280} value and thus the apparent soluble IgG content. Turbidity correction is thus crucial in accurately measuring total soluble IgG in colostrum products. Without turbidity correction, determination of IgG content can be overestimated by up to 30% due to the presence of aggregates as seen in Table 1, leading to inaccurate comparisons among products.

This research has confirmed that the affinity LC method without acid precipitation and using a valid correction for turbidity provides both accurate and precise data for total soluble IgG in colostrum extracts. The improved accuracy of the method with turbidity correction provides a sound, objective, and efficient basis for the quality control and evaluation of colostrum and colostrum-containing products, especially those containing aggregated IgG.

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