FOOD COMPOSITION AND ADDITIVES

Determination of Immunoglobulin G in Bovine Colostrum and Milk by Direct Biosensor SPR-Immunoassay

HARVEY E. INDYK
NZMP-Fonterra, PO Box 7, Waitoa, New Zealand
ENRICO L. FILONZI
Biacore AB, Box Hill, Melbourne, Victoria 3128, Australia

An automated biosensor surface-plasmon resonance-based assay was developed for the determination of immunoglobulin G (IgG) in bovine milk and colostrum with either goat or rabbit antibovine IgG or protein G used as detecting molecule. The method is configured as a direct and nonlabeled immunoassay, with quantitation against an authentic IgG calibrant. Whole colostrum or milk is prepared for analysis by dilution into buffer. Analysis conditions, including ligand immobilization, flowrate, contact time, and regeneration, were optimized, and nonspecific binding was evaluated. Performance parameters included working range of 15–10 000 ng/mL, method detection limit of 0.08 mg/mL, overall instrument response reproducibility relative standard deviation (RSD_R) of 0.47%, mean between-run RSD_R of 10.5% for colostrum, and surface stability over 200 analyses. The proposed method was compared with independent alternative methods. The technique was applied to the measurement of IgG content during early lactation transition from colostrum to milk, as well as in consumer milk, colostrum, and hyperimmune milk powders.

In contrast to human neonates, calves are agammaglobulinemic at birth, and passive immunity depends entirely on the absorption of maternal colostral immunoglobulin (Ig) antibodies during the early neonatal period (1–6). The principal Ig glycoprotein in bovine colostrum and milk is IgG (>80%), of which serum IgG_1 dominates, with a minor contribution (ca 10%) from an IgG_2 isotype (1, 2, 5, 7–9). The relatively high levels of IgG in early bovine colostrum thus provide an essential source of this nutrient to the calf immediately following parturition and until it can establish immunosufficiency. Several factors, including individual variation, breed, parity, and lactation, influence the concentration of IgG in bovine colostrum (1, 2, 5), although pathologies may also have a profound effect (6). Although the commercial milk supply should be colostrum-free (9), there has been significant interest in colostrum-supplemented products based on the alleged prophylactic and immunotherapeutic benefits of absorbed IgG. Thus, colostral milks from both immunized and nonimmunized herds have been processed into hyperimmune products designed for pharmaceutical and nutraceutical usage (10).

Whether intended for protection of the commercial milk supply, physiological studies of ruminant colostrogenesis, lactation and neonatal absorption, or in support of claims made for colostrum-derived products, reliable analytical techniques are required for the measurement of IgG. Immunochemical techniques have generally been used for analysis of IgG in colostrum and milk, including immunonephelometry (11–13); microparticle-enhanced immunonephelometry (14); single-radial immunodiffusion (sRID; 1, 3, 4, 7–9, 12, 15–21); and enzyme-linked immunosorbent assay (ELISA; 8, 10, 22–24). Alternatively, nonbiospecific electrophoretic (PAGE, IEF, CE, CZE) and high-performance chromatographic (RPLC, HIC, IC, FPLC, SEC) techniques have been used for determination of bovine whey proteins, including IgG (16, 25–30). Although immunoaffinity chromatography (IAC), using either immobilized antibodies or Fc-binding proteins such as protein G has been used predominantly for the purification of IgG fractions, biospecific analytical applications are also emerging (10, 27, 30–32).

Recent developments in affinity-based immunosensor techniques exploit the potential for label-free analyte detection and quantitation through coupling of the antibody–antigen interaction via an optical, piezoelectric, or electrochemical signal transducer (33). Optical biosensor-based techniques using surface-plasmon resonance (SPR) detection of binding events in real-time are increasingly prevalent, and general principles have been reviewed (34, 35). Although applied predominantly for analysis of binding kinetics and specificity, the technique has also been used for concentration assays by either end-point or rate-based techniques. The prerequisite covalent immobilization of acceptor ligand to the extended carboxymethylated dextran transducer surface is commonly achieved by carbodiimide-amine coupling chemistry, yielding a random orientation of ligand (36). Although there are several applications for milk systems targeting residues and vitamins, the technique has not generally

Received August 20, 2002. Accepted by SG December 11, 2002.
Corresponding author's e-mail: harvey.indyk@nzmp.com.

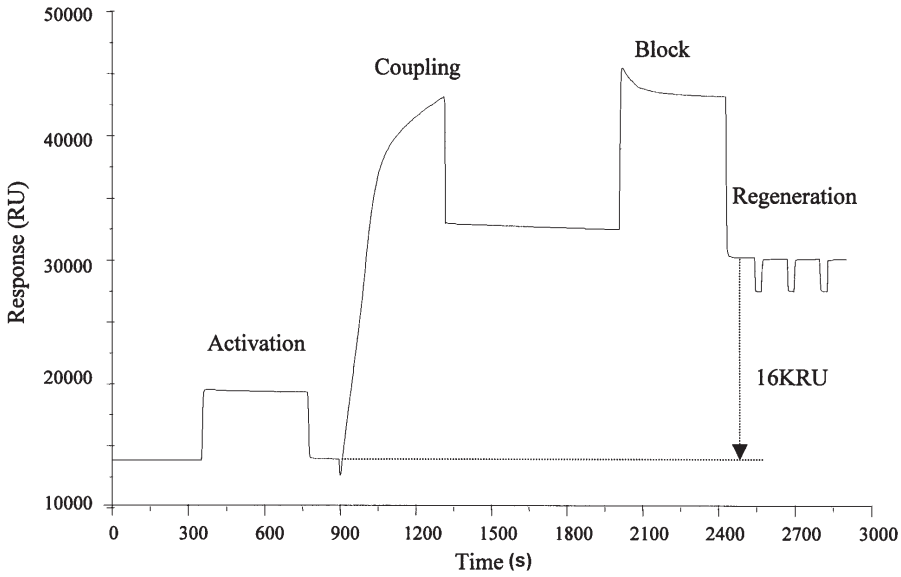

Figure 1. Sensorgram of goat antibovine IgG immobilization over CM5 sensor chip. Amount immobilized ca 16 ng/mm^2.

been applied to the quantitation of bovine Ig. Nevertheless, it has been used in probing neonatal Fc-receptor:IgG interactions (37), kinetics of glutathione S-transferase (GST):anti-GST IgG binding (38), and serum IgG antibody activity (39). A recent study has reported the application of SPR-immunoassay for quantitative detection of potential plant protein adulterants in milk products (40).

We describe a biosensor SPR-immunoassay format for determination of bovine IgG and its application to early lactation colostrum, milk, and commercially available products containing elevated IgG content.

Experimental

Apparatus

(**a**) *Biacore® Q*.—Biacore AB (Uppsala, Sweden).

(**b**) *Variable volume micropipets*.—10–100 and 100–1000 μL, 1–10 mL.

(**c**) *Graduated tubes*.—10.0 mL polystyrene, disposable.

(**d**) *Volumetric flasks*.—5, 10, 50, 100 mL.

(**e**) *Microtiter plates*.—96-well, polystyrene (No. 650160; Greiner, Frickenhausen, Germany).

(**f**) *Vials*.—1.5 mL, Eppendorf, polystyrene (Biolab Sci, Auckland, New Zealand).

Reagents

(**a**) *Antibodies*.—Goat antibovine IgG (H+L; No. 6030-01; Southern Biotechnology, Birmingham, AL); rabbit antibovine IgG (whole molecule; B5645; Sigma, St. Louis, MO); chicken egg-yolk antibovine IgG (AgResearch, Hamilton, New Zealand).

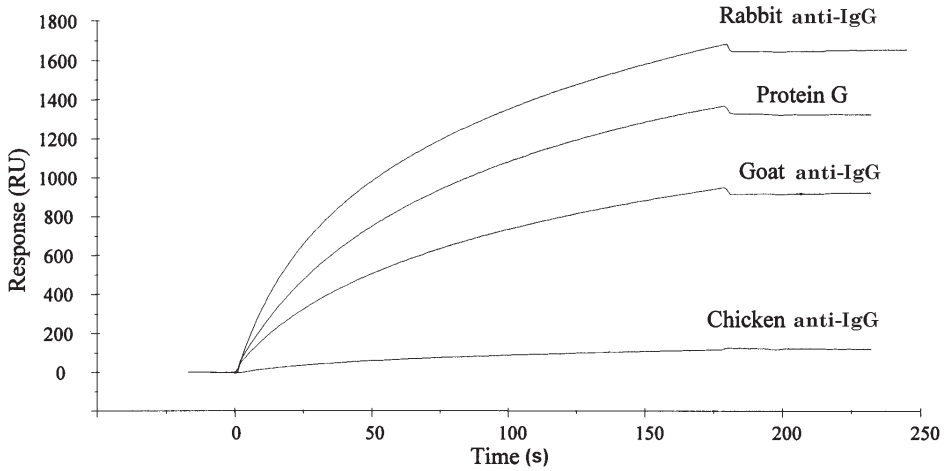

Figure 2. Sensorgrams of bovine IgG standard (10 μg/mL) over immobilized ligands.

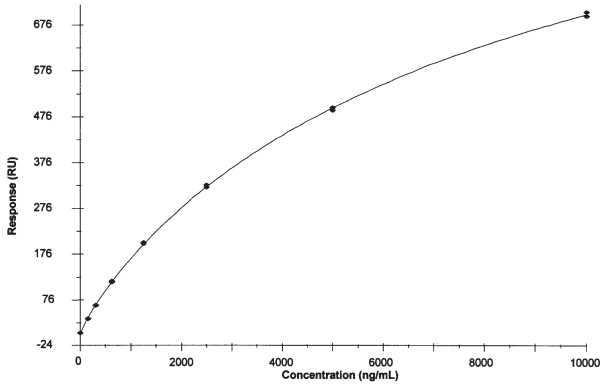

Figure 3. Typical calibration curve of SPR response vs bovine IgG (duplicate standards).

(**b**) *Binding protein*.—Protein G, lyophilized (No. 17-0619-01; Amersham Biosciences, Uppsala, Sweden).

(**c**) *IgG*.—Bovine IgG (No. G5009; Sigma).

(**d**) *Amine coupling kit*.—(Biacore AB).

(**e**) *Sensor chip*.—CM5 (Biacore AB).

(**f**) *Running buffer*.—10mM HEPES, 150mM NaCl, 3.4mM EDTA, 0.005% surfactant P20, pH 7.4 (HBS-EP; Biacore AB).

(**g**) *Immobilization buffers*.—Sodium acetate (10mM, pH 4.7 and 5.0).

(**h**) *Regeneration reagent*.—Phosphoric acid (10 and 100mM).

(**i**) *Water*.—Purified to ≥18MΩ.

Immobilization

Antibody or binding protein immobilization to a CM5 4-flow cell sensor surface was achieved by amine-coupling under instrument control at 25°C. Briefly, the designated flow cell was activated with an N-ethyl-N′-(dimethylaminopropyl) carbodiimide and N-hydroxysuccinamide (EDC-NHS) reagent (5 μL/min, 7 min). Goat antibovine IgG (25 μg/mL in immobilization buffer, pH 5.0), rabbit antibovine IgG (35 μg/mL in immobilization buffer, pH 5.0), chicken antibovine IgG (25 μg/mL in immobilization buffer, pH 5.0), or protein G (300 μg/mL in immobilization buffer, pH 4.7) were separately immobilized to the appropriate activated flow cell (5 μL/min, 7–10 min). Finally, unreacted ester functionalities were deactivated with ethanolamine (1M, pH 8.5, 5 μL/min, 8 min). Final immobilization levels in resonance units (RU, where 1 RU = 1 pg/mm^2) were determined from the sensorgram. Following immobilization, the chip was stored between analyses over dessicant at 4°C in a sealed container.

Standards

The bovine serum γ-globulin standard was used without spectral correction for protein (Abs_{280nm}), based on the supplier claim of ca 99% IgG electrophoretic purity.

(**a**) *IgG stock*.—40 mg/mL; 400 mg lyophilized IgG was dissolved in 10.0 mL phosphate buffer (50mM $NaH_2PO_4.H_2O$, pH 6.5). 250 μL aliquots were stored at –20°C until required.

(**b**) *IgG intermediate I*.—400 μg/mL. 100 μL of stock (**a**) was diluted to 10.0 mL in HBS-EP buffer.

(**c**) *IgG intermediate II*.—100 μg/mL. Intermediate I (**b**) was diluted 1 + 3 with HBS-EP buffer.

(**d**) *IgG calibrants*.—10 000, 5000, 2500, 1250, 625, 312.5, and 156.0 ng/mL. Intermediate II was serially diluted with HBS-EP buffer.

Sample Preparation

Raw bovine milk was collected from a single 4-year-old Jersey (2nd calving) between days –1 prepartum and +20 postpartum. Aliquots (1.0 mL) were diluted 1:100 in HBS-EP buffer, divided into 1.0 mL aliquots, and frozen (–18°C) until analyzed by SPR-immunoassay. In preparation for analysis, aliquots were thawed at 37°C and further diluted to a final 1:10 000 (<day 1 postpartum) or 1:1000 (>day 1 postpartum) with HBS-EP buffer.

Consumer whole (3.3% fat) and skim bovine milks (0.1% fat) were retail purchased and directly diluted 1:1000 in HBS-EP buffer. Caprine, ovine, and equine milk samples were diluted 1:500; human milk 1:100, in HBS-EP buffer. Spray-dried, skimmed colostrum powders were prepared 1:10 in phosphate buffer (50mM $NaH_2PO_4 \cdot H_2O$, pH 6.5) with incubation for 2 h at 37°C, and aliquots were frozen (–18°C) until analyzed by SPR-immunoassay. In preparation for analysis, aliquots were thawed at 37°C and further diluted with HBS-EP buffer to a final 1:50 000 dilution. A typical hyperimmune powder was dissolved directly in HBS-EP buffer to a final 1:5000 dilution.

Analysis

Reagents and immobilized sensor chip were allowed to equilibrate to ambient temperature before use. Calibration standards and sample extracts (200 μL) were dispensed (in duplicate) into the appropriate wells of a 96-well microtiter plate and sealed with adhesive foil. The instrument system was equilibrated with HBS-EP buffer and analysis was initiated under previously optimized conditions (flow rate: 20 μL/min, contact time: 180 s). Regeneration was typically achieved with 10mM phosphoric acid (flow rate, 50 μL/min; contact time, 44–70 s). The response at 40 s after commencement of the dissociation phase, relative to the baseline sampled 10 s before sample injection, was used for quantitation.

Each injection cycle required ca 8 min, with a complete 96-well microtiter plate completed in ca 18 h, including system equilibration and duplicate calibration.

Alternative Analysis Techniques

Affinity-LC was performed on a Hi-Trap Protein G column (Amersham Biosciences) as described previously (27, 30). RID using a commercial kit (RN 200.3) was used as prescribed by the manufacturer (The Binding Site, San

Diego, CA), and an automated nephelometric technique used as recently described (13).

Results and Discussion

IgG specific surfaces were prepared by amine coupling of ligand, following preliminary experiments to establish optimal electrostatic ligand adsorption conditions. Surface immobilization levels attained were ca 12 000 RU (ca 80 fmole) for goat anti-IgG, 10 000 RU (66 fmole) for rabbit anti-IgG, 8000 RU (53 fmole) for hen egg anti-IgG, and 2600 RU (86 fmole) for protein G. A typical immobilization procedure is illustrated in Figure 1 for the generation of a goat antibovine IgG specific surface.

All 4 immobilized ligands bound bovine IgG, as illustrated with a standard in Figure 2. Each sensorgram illustrates the SPR response due to mass concentration changes during both association and dissociation phases of the interaction between immobilized ligand and bovine IgG.

Significant binding responses (800–1600 RU) were obtained with goat and rabbit antibovine IgG as well as protein G. However, despite its comparable immobilization level, chicken antibovine IgG yielded a relatively low binding response (ca 100 RU). Although a concentration assay was feasible, it was decided not to proceed with this ligand.

A calibration curve of response against linear IgG concentration is shown for goat antibovine IgG in Figure 3, illustrating an assay range typically dependent on both concentration and affinity for ligand. With the high ligand immobilization levels achieved, analytical sensitivity (slope) was greatest at lower IgG levels (<1 μg/mL), while increasing saturation of binding sites at higher analyte levels led to a characteristic reduction in sensitivity.

The extent of nonspecific binding of sample components to the carboxymethylated dextran (CMD) surface was established with colostrum at various dilution levels injected over a nonimmobilized reference flow cell. Although nonspecific binding was detected (20–50 RU) at low dilutions, binding at analytical dilution levels was <10 RU and considered negligible. The ionic strength of HBS-EP buffer (150mM NaCl) is known to minimize the predominantly electrostatic adsorption of sample-matrix proteins binding nonspecifically to the hydrophilic CMD surface. The potential for nonspecific binding was further evaluated with serial dilutions of colostrum (1:20 000–1:320 000) in buffer. The normalized dilution responses for both colostral and standard bovine IgG are illustrated in Figure 4 over a goat antibovine IgG immobilized surface. Drift due to potential sample-matrix interferences is minimal, indicative of negligible nonspecific binding as well as verifying ligand specificity.

To further confirm binding specificity, competition experiments were performed for each binder. After establishment of solution equilibrium of colostral IgG and ligand, inhibition of surface association was monitored over the same immobilized ligand and was >95% for both protein G and goat antibovine IgG.

Instrument limit of detection [mean response plus 3 × standard deviation (SD)] was determined from the replicate, between-run response of buffer and measured 16.8 ng/mL. The method detection limit ($SD \times t_{n-1,\ 0.01}$) was estimated from the replicate analysis of a commercial milk sample containing a low IgG content (0.81 mg/mL) and measured 0.08 mg/mL ($n = 10$).

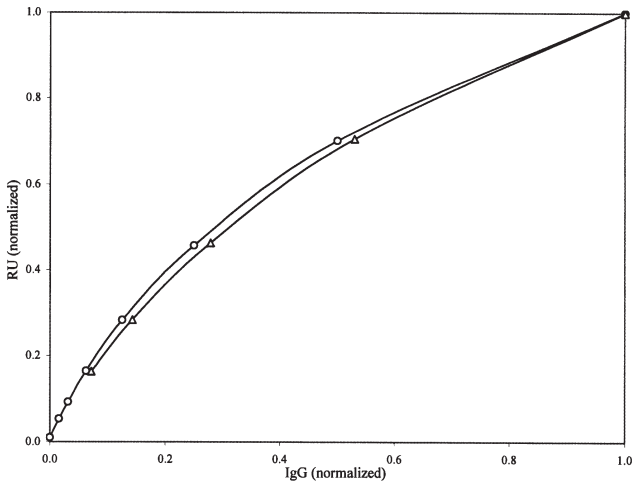

Figure 4. Dilution series for bovine IgG standards (o) and colostrum (Δ) over goat antibovine IgG surface. Response (RU) and IgG concentration are normalized.

Instrument precision was estimated from aggregate duplicate-well analyses over the 3 immobilized ligands and measured 0.47% relative standard deviation (RSD). Within-run assay precision (RSD_r) for high IgG-level colostrum, colostrum powder, and low IgG-level mature milk over a goat antibovine IgG surface measured 1.49, 2.68, and 3.54%, respectively. Overall between-run assay precision (RSD_R) over goat antibovine IgG, rabbit antibovine IgG, and protein G surfaces for these samples was 12.60, 10.52, and 12.73%, respectively.

The active surfaces were durable over at least 200 injections, with a typical binding capacity decrease of ca 1 RU/cycle, which illustrates a stable immobilized ligand and an effective regeneration protocol.

Changes in IgG content during early bovine lactation were investigated in the milk of an individual animal from precolostrum (–6 h) to mature milk (+20 days) postparturition. Table 1 compares the data obtained from analyses over the 3 immobilized ligands, and Figure 5 illustrates the comparative results of affinity-LC and RID analyses against the overall mean SPR-immunoassay data.

Based on 2-way analysis of variance (ANOVA), biosensor analysis over the 3 ligands yielded statistically equivalent data ($F = 1.84$, $P = 0.188$), while the overall mean data did not differ significantly from affinity-LC or RID ($F = 0.45$, $P = 0.645$), despite these methods using different recognition molecules, standards, and calibration techniques. The equivalence of data from such independent analytical methods indicates an unbiased estimate of IgG level in fluid milk and colostrum. Figure 5 illustrates the significant physiological response to

Table 1. Comparison of lactation IgG content over 3 ligands[a], mg/mL

Day	Goat anti-IgG	Protein G	Rabbit anti-IgG
–0.25	60.10	54.85	52.20
0.5	9.95	8.66	9.25
1	2.76	2.40	2.33
2	1.70	1.42	1.48
3	1.35	1.09	1.15
5	0.93	0.73	0.79
7	0.88	0.68	0.76
10	0.61	0.47	0.52
15	0.54	0.41	0.45
20	0.57	0.44	0.51

[a] Data are means of replicate determinations over each surface ligand (n = 2).

parturition, with ca 100-fold decrease between early colostrum and mature milk.

Skim colostrum and whole hyperimmune milk powders, as well as retail fluid milks, were analyzed by the described SPR-immunoassay technique. The data appear in Table 2, along with comparative data against affinity-LC, RID, and nephelometry.

Despite fundamentally different analytical principles, the 4 techniques were generally comparable, although Tukey's honestly significantly different (HSD) post-hoc comparison revealed that SPR-immunoassay yields results lower than affinity-LC and higher than RID, but equivalent to nephelometry.

Pooled raw bovine, ovine, and caprine milk, as well as equine and human milk from individual donors, were evaluated for IgG content by the described biosensor method over both goat antibovine IgG and protein G. Results are listed in Table 3.

Although the goat antiserum is immunospecific for bovine IgG and displayed minimal binding of IgG from other species milks, the class-specific protein G ligand provided estimates of milk IgG content in the 5 species surveyed.

The sensitivity, specificity, and quantitation range of biospecific methods are generally determined by ligand–analyte affinity, and an optimized SPR-immunoassay assay therefore relies predominantly on appropriate ligand selection and immobilization chemistry, as well as buffer conditions, contact time, and regeneration protocol. The approach is unique among biospecific techniques in its reliance on multiple regeneration, effected via disruption of noncovalent ligand–analyte interactions at the sensor surface. Three bovine-IgG ligands, evaluated as potential detection systems, provided comparable performance parameters, stable surfaces under multiple regeneration conditions, low detection limits, and minimal nonspecific binding at the high sample dilution levels facilitated by the inherent sensitivity of SPR detection.

Polyclonal goat and rabbit antibovine IgG antibodies and protein G exhibited comparable specificity for analyte, even though, as an Fc-binding protein, protein G is unable to discriminate between IgG sub-classes (41, 42). Despite a high immobilization level, avian Ig (IgY) unexpectedly failed to bind effectively with bovine IgG when compared with other ligands, since this antibody has been found useful in other biospecific bovine IgG applications (13, 24, 42).

Of potential significance is that reported IgG levels in bovine milk have generally been based on techniques that rely on different serum IgG standards and antisera raised against se-

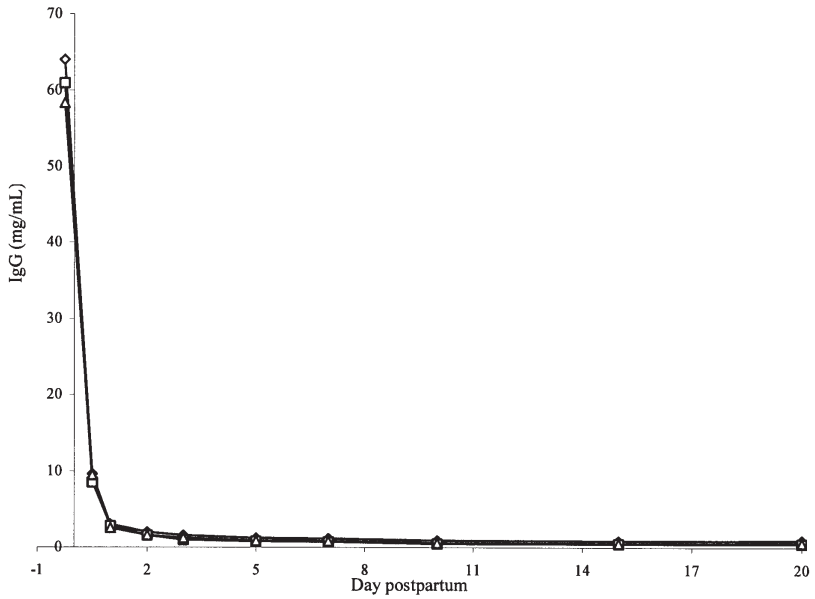

Figure 5. Variation of IgG content during early lactation of a single cow by SPR-immunoassay (Δ), affinity-LC (◊), and RID (□).

Table 2. Method comparison of IgG content[a,b]

Sample	SPR	Affinity-LC	RID	Nephelometry
WM	0.48	1.20	0.30	ND
WM	0.54	0.49	0.50	ND
SM	0.46	ND	0.40	ND
WMP	2.73	4.00	2.00	ND
CP	112.1	158.4	95.0	120.0
CP	118.8	161.1	110.0	129.0
CP	111.4	161.1	90.0	114.0
HIMP	4.00	ND	4.30	ND

[a] Data are means of replicate determinations ($n \geq 2$).

[b] WM = Liquid whole milk (mg/mL); SM = liquid skim milk (mg/mL); WMP = whole milk powder (mg/g); CP = colostrum powder (mg/g); HIMP = hyperimmune milk powder (mg/g); ND = no data.

rum rather than milk IgG. The influence of these factors has previously been studied with RID and ELISA. Such findings lead to the suggestion that serum IgG may contribute to an underestimation of milk IgG as a direct consequence of the different IgG_1:IgG_2 ratios in bovine serum and colostrum or milk, regardless of antibody source (8, 9). Nevertheless, consistent with most analytical methods reported, the presently described biosensor-SPR technique has used commercially available polyclonal goat and rabbit antibovine, affinity-purified serum IgG immunogen, and recombinant protein G (minus albumin-binding region), together with purified bovine serum IgG as assay calibrant.

Recent studies comparing analytical methods for the determination of milk IgG have revealed evidence of method variability between nephelometry, ELISA, and RID (13). It has also been reported that the estimation of bovine IgG content of human breast milk by RIA, competition, or sandwich ELISA techniques is method-dependent, despite the consistent use of rabbit antibovine IgG (43). ELISA and RID have been compared with different immunoreagents (8), whereas RID was found comparable with electrophoretic techniques for IgG determination in equine colostrum (16). Affinity-LC was reportedly biased high compared with ELISA during the determination of IgG content of commercial colostrum products (10). Quantitation of bovine IgG by reversed phase-LC has been compared with affinity-LC, SDS-PAGE, and RID (30). Peak asymmetry under affinity-LC conditions revealed a structural integrity of IgG dependent on product processing conditions (27).

Despite the fundamental differences among the 4 independent analytical techniques used here (SPR-immunoassay, RID, nephelometry, and affinity-LC), the present study has shown reasonable intermethod agreement, suggesting that each can yield a reliable estimate of IgG content in milk and colostrum. Nevertheless, it is imperative for any immunoassay that the extent of potential nonspecific binding interferences be revealed, especially for label-free detection systems such as SPR-immunoassay or affinity-LC. The high sample dilutions facilitated by the inherent sensitivity of SPR detection, and the ability to perform comparative binding experiments over a reference surface, provide the described biosensor technique with the capability of both evaluating and minimizing nonspecific binding.

It has generally been reported that colostrum and milk may be assayed whole, rather than fat-free or as whey during IgG determination (1, 7, 15, 16, 18–20), and this protocol has been adopted during the evaluation of the SPR-immunoassay. In this study, IgG content in mature bovine milk ranged from 0.35 to 0.80 mg/mL, which compares with literature values of 0.2–1.8 mg/mL obtained by a variety of analytical techniques (9, 13, 14, 18, 20, 44). The concentration of IgG has been consistently demonstrated to be high in mammalian colostrum, with a rapid decline through the transition to mature milk. In the case of bovine colostrum, IgG levels at Day 1 postpartum have reportedly ranged widely from 30 to 110 mg/mL, thereafter reducing to mature milk levels of 0.5–1.4 mg/mL after ca 15 days (8, 9, 13, 18, 21, 44). The present biosensor-SPR-based study of a single lactating cow provides data consistent with this significant temporal relationship, with IgG levels of 55–65 mg/mL immediately prior to parturition declining to ca 0.5 mg/mL in mature milk. The colostrogenic mechanisms of prepartum IgG transfer into mammary secretions have been reviewed and describe a process whereby lactogenic hormones mediate epithelial IgG_1 receptor activity (44).

There are limited comparative data regarding the IgG content of mature milk from mammalian species. Estimates based on the described SPR-immunoassay technique using the class-specific protein G ligand are, however, comparable with reported values for ovine (0.3–0.7 mg/mL), caprine (0.2–0.5 mg/mL), equine (0.3–0.6 mg/mL), and human (0.03–0.10 mg/mL).

A feature of SPR-immunoassay is the biospecific recognition of analyte by ligand, which in the present application facilitates a measurement of functionally active IgG. The retained IgG content of both colostrum powder from nonimmunized and hyperimmune milk powder from immunized cows therefore supports the known general resistance toward thermal denaturation of colostral IgG during commercial spray-drying, although susceptibility of bovine IgG

Table 3. Comparison of IgG content of species milks[a] by SPR-immunoassay (mg/mL)

	IgG[b]	
	Goat anti-IgG	Protein G
Cow	0.72	0.50
Sheep	0.16	1.64
Goat	0.06	0.61
Horse	0.08	0.18
Human	0.01	0.07

[a] Milks were raw and unprocessed.

[b] Data are means of replicate determinations over each surface ligand ($n = 2$).

at temperatures >95°C has previously been reported (23, 45, 46). Milk concentrates derived from both nonimmunized and immunized herds are currently used as immunotherapeutic components, and international trade will increasingly rely on traceability of analytical methods for IgG content. In the absence of an internationally recognized reference method, the present study indicates that the SPR-immunoassay may fulfill this need.

As with all immunoassay techniques, the described method provides an estimate of IgG based on binding to a specific detecting molecule, which may not always correspond exactly with total concentration. Hence, discrepancies between generic biospecific methods will generally occur because of differences in their specific interaction chemistries. It is also apparent that several common factors critically influence the analysis of IgG in milk and colostrum, irrespective of the end-point measurement technique. Thus, internationally standardized protocols advocating the use of purified colostral IgG, as both immunogen and standard, are clearly required to facilitate a more realistic comparison of the different analytical methods currently available.

Conclusions

A concentration assay based on a biosensor SPR-immunoassay is described, which relies on the specific, label-free, and real-time interaction at the sensor surface between bovine IgG in milk and colostrum and either of 3 ligands. The optimized ligand immobilization and regeneration protocol provides a stable and multiple-use IgG-specific interaction surface for quantitation by SPR optics. The described biosensor-immunoassay is rapid, sensitive, precise, and accurate and provides analytical information comparable with alternative methods in current use, while offering the benefits of automation.

Acknowledgments

We thank David Copestake (Fonterra Research Centre, Palmerston North, New Zealand) for affinity-LC and RID, and Roger Collin (AgResearch, Hamilton, New Zealand) for nephelometric analyses. We are also grateful for valuable discussions with Don Otter and Colin Hughes (Fonterra Research Centre) and acknowledge the support of this project from the Specialist Ingredient Group (NZMP, Hautapu, New Zealand), Biacore AB (Uppsala, Sweden), and NZMP-Fonterra (Hamilton, New Zealand).

References

(1) Muller, L.D., & Ellinger, D.K. (1981) *J. Dairy Sci*. **64**, 1727–1730

(2) Weaver, D.M., Tyler, J.W., VanMetre, D.C., Hostetler, D.E., & Barrington, G.M. (2000) *J. Vet. Intern. Med*. **14**, 569–577

(3) Arthington, J.D., Cattell, M.B., & Quigley, J.D. (2000) *J. Dairy Sci*. **83**, 1463–1467

(4) Pedersen, R.E., Paulrud, C.O., & Tucker, W.B. (2000) *J. Dairy Sci*. **83**, 2829–2833

(5) Korhonen, H., Marnila, P., & Gill, H.S. (2000) *Br. J. Nutr*. **84**, S75–S80

(6) Lona, V.D., & Romero, C.R. (2001) *J. Dairy Sci*. **84**, 389–391

(7) Pritchett, L.C., Gay, C.C., Besser, T.E., & Hancock, D.D. (1991) *J. Dairy Sci*. **74**, 2336–2341

(8) Li-Chan, E.C.Y., & Kummer, A. (1997) *J. Dairy Sci*. **80**, 1038–1046

(9) Levieux, D., & Ollier, A. (1999) *J. Dairy Res*. **66**, 421–430

(10) McConnell, M.A., Buchan, G., Borissenko, M.V., & Brooks, H.J.L. (2001) *Food Res. Int*. **34**, 255–261

(11) Hills, L.P., & Tiffany, T.O. (1980) *Clin. Chem*. **26**, 1459–1466

(12) Lebreton, J.P., Joisel, F., Boutleux, S., Lannuzel, B., & Sauger, F. (1981) *Lait* **61**, 465–480

(13) Collin, R., Prosser, C., McLaren, R., Thomson, M., & Malcolm, D. (2002) *J. Dairy Res*. **69**, 27–35

(14) Montagne, P., Gavriloff, C., Humbert, G., Cuilliere, M.L., Duheille, J., & Linden, G. (1991) *Lait* **71**, 493–499

(15) Fleenor, W.A., & Stott, G.H. (1981) *J. Dairy Sci*. **64**, 740–747

(16) Curadi, M.C., Orlandi, M., Grepp, G.F., Toppino, P.M., Barzaghi, S., & Cattaneo, T.M.P. (2000) *Milchwissenschaft* **55**, 446–449

(17) Davenport, D.F., Quigley, J.D., Martin, J.E., Holt, J.A., & Arthington, J.D. (2000) *J. Dairy Res*. **83**, 2813–2819

(18) Mainer, G., Perez, M.D., Sanchez, L., Puyol, P., Millan, M.A., Ena, J.M., Dominguez, E., & Calvo, M. (2000) *Milchwissenschaft* **55**, 613–617

(19) Tyler, J.W., Steevens, B.J., Hostetler, D.E., Holle, J.M., & Denbigh, J.M. (1999) *Am. J. Vet. Res*. **60**, 1136–1139

(20) Levieux, D. (1991) *Lait* **71**, 327–338

(21) Lacy-Hulbert, S.J., Malcolm, D.B., Copeman, P.J.A., Woolford, M.W., & Franks, R. (1996) *Proc. New Zeal. Soc. Anim. Prod*. **56**, 263–265

(22) Varley, M.A., Rucklidge, G.J., Wilkinson, R.J., & Maitland, A. (1985) *Res. Vet. Sci*. **38**, 279–281

(23) Rosenthal, I., Bernstein, S., & Merin, U. (1999) *Milchwissenschaft* **54**, 367–368

(24) Erhard, M.H., Amon, P., Nuske, S., & Stangassinger, M. (1999) *J. Anim. Physiol. Anim. Nutr*. **81**, 239–248

(25) Gonzalez-Llano, D., Polo, C., & Ramos, M. (1990) *Lait* **70**, 255–277

(26) Strange, E.D., Malin, E.L., Van Hekken, D.L., & Basch, J.J. (1992) *J. Chromatogr*. **624**, 81–102

(27) Kinghorn, N.M., Norris, C.S., Paterson, G.R., & Otter, D.E. (1995) *J. Chromatogr*. *A* **700**, 111–123

(28) Bennett, L.E., Charman, W.N., Williams, D.B., & Charman, S.A. (1994) *J. Pharm. Biomed. Anal*. **12**, 1103–1108

(29) Merin, U., Bernstein, S., Van Creveld, C., Yagil, R., & Gollop, N. (2001) *Milchwissenschaft* **56**, 70–73

(30) Elgar, D.F., Norris, C.S., Ayers, J.S., Pritchard, M., Otter, D.E., & Palmano, K.P. (2000) *J. Chromatogr. A* **878**, 183–196

(31) Hage, D.S., & Nelson, M.A. (2001) *Anal. Chem*. **73**, 199A–205A

(32) Hage, D.S. (1998) *J. Chromatogr. B* **715**, 3–28

(33) Bilitewski, U. (2000) *Anal. Chem*. **72**, 692A–701A

(34) Malmqvist, M. (1993) *Nature* **361**, 186–187

(35) Homola, J., Yee, S.S., & Gauglitz, G. (1999) *Sensors Actuators B* **54**, 3–15

(36) Johnsson, B., & Lofas, S. (1991) *Anal. Biochem.* **198**, 268–277

(37) Raghavan, M., Bonagura, V.R., Morrison, S.L., & Bjorkman, P.J. (1995) *Biochemistry* **34**, 14649–14657

(38) Quinn, J.G., & O'Kennedy, R. (2001) *Anal. Biochem.* **290**, 36–46

(39) Pei, R., Cui, X., Yang, X., & Wang, E. (2000) *Talanta* **53**, 481–488

(40) Haasnoot, W., Olieman, K., Cazemier, G., & Verheijen, R. (2001) *J. Agric. Food Chem.* **49**, 5201–5206

(41) Bjorck, L., & Kronvall, G. (1984) *J. Immunol*. **133**, 969–974

(42) Akita, E.M., & Li-Chan, E.C.Y. (1998) *J. Dairy Sci*. **81**, 54–63

(43) Maeda, S., Morikawa, A., Tokuyama, K., & Kuroume, T. (1993) *Acta Paediatr*. **82**, 1012–1016

(44) Barrington, G.M., McFadden, T.B., Huyler, M.T., & Besser, T.E. (2001) *Livest. Prod. Sci.* **70**, 95–104

(45) Chen, C.-C., & Chang, H.-M. (1998) *J. Agric. Food Chem.* **46**, 3570–3576

(46) Dominguez, E., Perez, M.D., Puyol, P., Sanchez, L., & Calvo, M. (2001) *J. Dairy Res*. **68**, 511–518