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REVIEW

Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: a review

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Abstract The immunoprotective properties of bovine milk immunoglobulin G (IgG) have led to a recent proliferation of nutritional products incorporating this protein. It has therefore become critical that reliable analytical techniques for the measurement of the IgG content in such products are available. This literature review surveys current methods of analysis for IgG, including separation-based or immuno-based concentration analysis. The review also discusses nutraceutical applications, regulatory issues, stability of IgG and the significance of primary reference material in IgG analysis.

Keywords Immunoglobulin G · IgG · Bovine · Milk · Colostrum · Protein · Analysis · Dietary supplements

Abbreviations

AC	Affinity chromatography
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CZE	Capillary zone electrophoresis
ELISA	Enzyme-linked immunosorbent assay
IA	Immunoassay
IEC	Ion exchange chromatography
Ig	Immunoglobulin
IgG	Immunoglobulin G
LC	Liquid chromatography
RID	Radial immunodiffusion
RP-LC	Reversed-phase liquid chromatography
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SPR	Surface plasmon resonance

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Introduction

Immunoglobulins (Igs) are a family of globular proteins with antimicrobial and other protective bioactivities. They exist at different concentrations in blood serum, milk and colostrum, as shown in Table 1. Qualitative and quantitative differences are dependent on species, and are found in various isotypes, with immunological activities that are dependent on the Ig class [1]. Igs derived from bovine lacteal secretions (i.e. colostrum and milk) have been utilised in the immunological supplementation of infant formulae and other foods, yielding sales of approximately US$100 million in 2004 [2–4]. In the highly competitive and valuable international market for IgG-containing products, the products are often priced on the basis of IgG content, and it is of importance that these claimed values are accurate and precise.

The Igs are the principal agents that protect the gut mucosa against pathogenic microorganisms, and in colostrum they confer passive immunity to the ruminant neonate until its own immune system is developed [5, 6]. IgG antibodies express multifunctional activities, including complement activation, bacterial opsonisation and agglutination, and act by binding to specific sites on the surfaces of most infectious agents or products, either inactivating them or reducing infection [7].

In bovine colostrum and milk, immunoglobulin G (IgG; subclasses IgG1 and IgG2) is the major immune component, although low levels of IgA and IgM are also present [8, 9]. The levels of the different Ig classes are shown in Table 1. In the transition from colostrum to mature milk, Ig levels decrease sharply during the first five days post partum [10, 11].

In ruminants, the predominant milk immunoglobulin IgG1 is derived mainly from blood, with transport across mammary alveolar cells mediated by an active receptor mechanism. IgG2 either derives from blood, or is synthesised by the plasma or epithelial cells of the mammary gland and is transferred to the mammary secretory cells. IgA and IgM are also synthesised by the plasma or epithelial cells of the mammary gland [12]. The Igs are accumulated in the mammary gland during the prepartum dry period, and are then secreted in colostrum and milk. IgG1 constitutes approximately 80% of the total Ig content of bovine milk, whereas the predominant Ig in most other nonruminant mammalian milks, including human, is IgA. This distribution generally reflects the route by which passive immunity is conferred from mother to infant.

IgG is a monomeric glycoprotein consisting of two heavy (long) and two light (short) polypeptide chains that are linked by disulfide bonds [12] (Fig. 1). The polypeptide chains contain both constant (Fc) and variable (Fab) regions of amino acid sequence, with the antigen-binding sites located in the Fab N-terminal region [13]. Genes encoding the Fc domain are the primary determinants characterising Ig class, with subclasses IgG1 and IgG2 differing primarily in the Fc domain of their heavy chains [10].

Enrichment of bovine Igs in infant formulae and other foods may help to reduce viral and microbial infections [14], and may provide consumers with improved immune activity, although some researchers suggest that certain bovine Ig subclasses may display detrimental effects [15]. The immunological activity of bovine IgG in milk from cows immunised against human pathogens is reported to be similar to that of IgG in human milk, demonstrating the benefit of hyperimmune bovine milk in the human diet [3, 4].

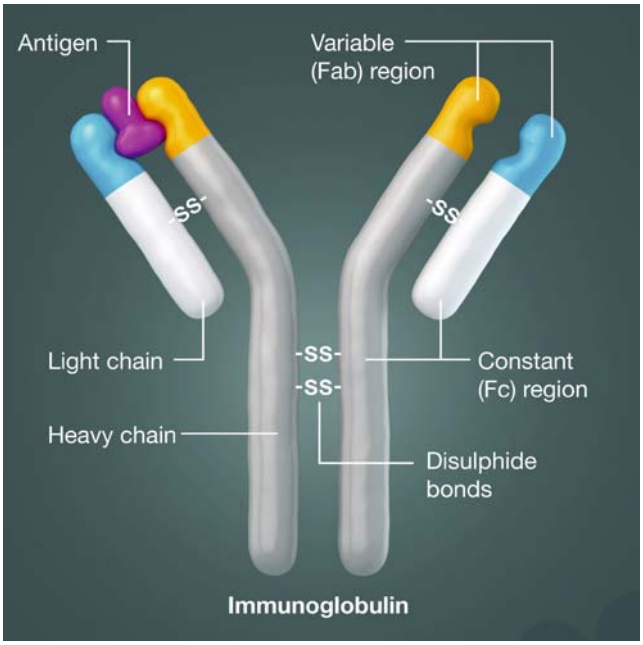

Fig. 1 Schematic of an immunoglobulin G (IgG) molecule

A prerequisite for any potential application of bovine IgG in the infant or adult human diet is its reliable quantitative determination in milk and colostrum, or indeed any foods or dietary supplements incorporating IgG derived from bovine sources. This review discusses applications of bovine IgG and the current analytical techniques used for its quantification.

Dietary applications

Bovine milk and colostrum contain abundant bioactive components, including growth factors, Igs, lactoperoxidase, lysozyme, lactoferrin, cytokines, nucleosides, vitamins, peptides and oligosaccharides, that are of increasing relevance to human health [16–18]. IgG, the principal Ig of bovine colostrum, provides the major component of passive immunity to the newborn calf, and, indeed, represents the majority of total colostral protein. The prophylactic or therapeutic use of bovine colostrum derived from non-

Table 1 Immunoglobulin (Ig) concentration in bovine and human serum and mammary secretions (adapted from [1])

Species	Immunoglobulin	Concentration (mg mL^{-1})		
		Blood serum	Colostrum	Milk
Bovine	Total IgG	25.0	32–212	0.72
	IgG1	14.0	20–200	0.60
	IgG2	11.0	12.0	0.12
	IgA	0.4	3.5	0.13
	IgM	3.1	8.7	0.04
Human	Total IgG	12.1	0.4	0.04
	IgA	2.5	17.4	1.00
	IgM	0.9	1.6	0.10

immunised cows in human health is not new, and dates back several decades [19]. In fact, the benefits of cow's milk consumption in preventing infection have been recognised for many centuries in human history. However, the large-scale enrichment or isolation of active IgG that is achievable with modern membrane separation and processing technologies has recently stimulated a dramatic growth in the nutraceutical food and dietary supplements market. Thus, despite taxonomic issues, development of ruminant-derived antibodies for commercial applications in human health has dominated, compared with the potential use of human-derived preparations [6].

For these reasons, bovine colostrum- and milk-based whey products targeting the immune sector are generally synonymous with commercially available fractionated IgG products when health claims are based on stimulation of gastrointestinal or oral health. In contrast, bovine colostrum supplementation has also been promoted for other potential non-immunologically mediated benefits, particularly in sports nutrition, where its positive effect on performance and serum insulin-like growth factor (IGF) in training athletes has been reported [20]. The public's concern regarding their state of immunity is now a major global trend and is reflected in the sales of immune support products, of which bovine milk and colostrum provide the basis for many. This trend is overwhelmingly founded on the belief that oral consumption of IgG-enhanced products is safe and may provide consumers improved protection from gastrointestinal tract infection. Consumer acceptance of such value-added whey protein products began in Asia and has now migrated to Europe and the US, targeting sports nutrition, infant formulae, dietary supplements and physiologically functional foods.

Although normal bovine colostrum contains IgG active against specific enteric pathogens, this specificity is dictated by previous systemic challenge and often the concentration is too low to afford optimal protection for humans [21]. One strategy used to overcome this possible limitation of normal colostrum is to limit its collection to the first 6 h, when the IgG content is very high, or to concentrate the Ig by fractionation techniques. A further refinement of the clinical efficacy of normal bovine colostrum and milk has been the production and availability of hyperimmune milks. The IgG-enhanced fraction of milk from cows immunised with human enteropathogenic microorganisms has been demonstrated to provide protection to humans, generally accepted to be via mediation of microbial adherence to intestinal epithelial cells [3, 22]. Recent reviews of the human clinical applications of immune milks or IgG concentrates for bacterial gastrointestinal tract, oral, parasitic and viral infections provide in vitro, in vivo or animal model evidence for provisional efficacy, while emphasising that consideration needs to be given to the potential for allergenic sensitivity to bovine lacteal proteins, including IgG itself [6, 23–26].

Regulatory issues

The increasing acceptance of bovine lacteal products as functional foods and dietary supplements is consistent with a growing consumer preference for perceived natural "wellness" products as viable alternatives or complements to synthetic drug therapies. This is inevitably leading to a convergence of food, pharmaceutical and nutritional science, with regulatory consequences that are currently difficult to resolve, particularly with the developing international trade in such products. Internationally, bovine milk and colostrum products that exploit IgG content have become valuable niche products in a highly competitive market [4, 6, 17, 26]. Therefore, although it is critical that reliable analytical techniques are available for the measurement of IgG content, there are currently no such internationally accepted reference methods, despite a plethora of published protocols. Neither are there specific regulatory standards that define colostrum-based dietary supplements, given the dependence of IgG content on animal health, feeding practice, collection period and processing conditions. Although IgG measurement has traditionally been required to establish the absence of colostrum in the domestic consumer milk supply, the recent development of the nutraceutical and therapeutic market for IgG-based milk products has mandated a much higher regulatory level of international traceability with respect to label claim. Indeed, the dietary supplement industry has an urgent need for standardised analytical methods to support label claims and international trade, and, in the US, the Association of Analytical Communities (AOAC International) is currently undertaking the collaborative study of two methods for this purpose.

Currently, the US Food and Drug Administration (FDA) has accepted the safety of hyperimmune milks on the basis of clinical studies that show no adverse health effects. Further, provided no health claims are made, such products are regulated as foods rather than biologics, the latter referring to drugs of natural origin, which are subject to more rigorous international regulations. From a commercial perspective, the entry of IgG products into the health market is more facile than that of pharmaceutical interventions (e.g. vaccination and antibiotic treatment) because of less stringent regulatory issues applied to dietary supplements and specialist functional foods. Indeed, such products are a relatively new and largely unregulated area of the consumer market, leading to difficulties for manufacturers who attempt to be regulatory compliant. In the future these immunoproducts are likely to be increasingly regulated as physiologically functional foods.

Stability

For any protein, the expression of biological activity is critically dependent on its three-dimensional folded structure, with the native state considered to be a time-average of closely related, thermodynamically most stable conformations [27]. In general, protein denaturation may be partial, where the native conformation may be disturbed yet retain functionality, or extensive, leading ultimately to loss of physiological function. Thus, denaturation initiates the potentially reversible and first-order unfolding of a native protein via partially unfolded "molten-globule" intermediate states that can finally result irreversibly in higher order aggregation and precipitation [28–30].

The conformational integrity of a biologically active protein, and its resistance to denaturation, is of increasing significance with the trend towards utilising individual protein isolates therapeutically in foods, and this consideration clearly applies to the physiological efficacy of IgG-enriched foods and supplements. Indeed, whether the source of bovine IgG is intact colostrum, hyperimmune milk, whey protein or the fractionated Ig, it is critical that the integrity of both Fab and Fc domains is maintained during production, storage and gastrointestinal passage. Unlike caseins, which are essentially devoid of higher order structure, whey Igs are relatively heat-labile [31]. In order to maintain conformational integrity, functional properties and biological activity, it is essential to minimise exposure of milk proteins to heat during dairy processing. In the case of IgG, it should also be noted that its immunoactivity, as measured by an immunoassay, may not necessarily be equivalent to its antigen-binding activity, as measured by its ability to neutralise a specific pathogen, and both characteristics may be differently affected by conditions that can facilitate denaturation [14, 31, 32].

Several recent studies have reported specifically on the stability of bovine IgG, either in model systems or in intact milk or colostrum, to various stresses, including storage, isolation and processing variables such as heat, pH and pressure treatments [14, 31–39]. These studies have utilised various analytical techniques based on different detection principles and have inevitably yielded a range of reported stability information. Generally however, they suggest that the structure and the function of IgG are labile to thermal treatment above about 65 °C and are therefore somewhat vulnerable to conventional pasteurisation conditions. Selective immunoassays have revealed that there may be some slight domain differentiation within IgG with respect to structural stability [32, 34, 35]. Thus, irrespective of the source of IgG, there is evidence that Fab and Fc regions have characteristic, but essentially satisfactory, structural stabilities to moderate heat, pH and proteinase action. Therefore, it seems that, on current evidence, the immunoprophylactic or therapeutic properties of bovine IgG preparations may be considered to be secure following oral consumption by humans.

Methods of analysis

Structure and activity

Although the primary focus of this review is to summarise analytical techniques for quantitative analysis, brief mention should also be made of techniques utilised for the determination of both inherent molecular structure and biological activity. These characteristics are significant, because the principal physiological roles of IgG are antigen binding and effector functionality, both of which are critically dependent on the native structure of the Ig.

For all biologically active proteins, protein structure elucidation is an essential component in understanding function, and is generally considered at four levels: primary (amino acid sequence), secondary (local structural elements), tertiary (three-dimensional folded monomeric structure) and quaternary (assembled multimeric structure). Various techniques are available for probing the spatial and conformational structure of proteins in their native state. Methods include mass spectrometry and spectroscopic techniques, including absorption (ultraviolet and infrared), circular dichroism, fluorescence, light scattering, nuclear magnetic resonance, Raman, and neutron, electron or X-ray diffraction, each of which is particularly appropriate for specific features at the four levels of protein structure [40, 41]. Glycosylation of IgG within both the Fc domain and the Fab domain is also an important determinant of function, and analytical techniques to characterise the glycosylation profiles of IgG include electrophoretic, chromatographic and mass spectrometric approaches [42].

The determination of specific bioactivity is clearly a critical complement when characterising the sample with respect to its IgG contribution, especially as activity and concentration may not necessarily concur. While beyond the scope of this review, the quantification of the specific biological activity of IgG contained in milk, colostrum, hyperimmune milk or a protein fraction is generally achieved by titration, whereby serial dilutions of sample are tested for activity against a specific pathogen. Most commonly, enzyme-linked immunosorbent assay (ELISA), agglutination and neutralisation assay formats are utilised [4, 14, 25, 43, 44].

Quantitative analysis

Analytical techniques used in estimating IgG concentration are, in general, common to those used in estimating other

proteins present in mammalian milk, and such methods have been reviewed recently [41]. Thus, in pure and dilute solution, IgG may be quantified by simple ultraviolet absorptivity or fluorescence measurements. Such measurements are typically applied to determine concentration in standard calibrants, the former related by the Beer–Lambert law and the latter based on a linear relationship between the intensity of aromatic residue fluorescence and protein concentration. Further, colorimetric methods (e.g. Biuret) that target the amide peptide bonds may be used in such simple systems. However, such approaches are inadequate when estimates of IgG concentration in more complex matrices are required because of their inherent inabilities to differentiate individual protein species.

Two fundamentally different analytical approaches based on either separation or immunoassay principles are generally utilised for the determination of the IgG content in milk-based matrices. An approach based on alternative physical principles is also discussed. This section overviews the principles of these techniques and, while it is inappropriate to discuss the details of all citations individually here, a summary of the published literature is given in Table 2.

Separation-based techniques

Both liquid chromatography and electrophoresis techniques are suitable for the identification and quantification of IgG in complex matrices including bovine milk and colostrum. It is common for caseins to be removed prior to analytical separation, although this is not always described, with some reporting measurement of IgG simultaneously with measurement of both casein and whey proteins.

Liquid chromatography (LC)

A variety of high-performance liquid chromatographic techniques are available, most of which have been evaluated for the analysis of proteins, and many of which have been used specifically for IgG estimation (see reviews [106, 107]. The method of choice is usually dependent on the individual proteins of interest, as generally no single method has been demonstrated to be suitable for the accurate simultaneous quantification of all milk proteins within an intact sample.

Reversed-phase liquid chromatography (RP-LC)

RP-LC has increasingly become one of the most prevalent of the LC techniques. In this mode, the polarity of the stationary phase is less than that of the mobile phase, such that sample components interact reversibly with the hydrophobic surface of the stationary phase. Despite some uncertainties in retention mechanism, gradient elution is achieved by decreasing the polarity of the eluent, resulting in analyte elution predominantly in order of increasing hydrophobicity.

RP-LC has been used extensively for the analysis of proteins, although variable success in the separation of certain whey proteins has been reported [84, 108, 109]. A method for the accurate simultaneous quantification of the major whey proteins, including IgG, utilising a hydrophobic underivatised polystyrene-divinyl benzene support has been described [86].

Size exclusion chromatography (SEC)

Exclusion chromatography is a technique for separating molecules based on their size and shape in solution. The technique is commonly referred to as gel permeation chromatography when used with organic solvents or, alternatively, gel filtration when used with aqueous solvents. In SEC, the stationary phase consists of uniformly porous particles, and, unlike other chromatographic techniques, there is no interaction between the mobile phase and the stationary phase and therefore gradient elution is not required. Proteins are separated based on molecular size and are mediated by their ability to enter the pores of the stationary phase, hindering their passage through the column. SEC has been reasonably successful for the separation of whey proteins, with IgG quantified on a Sepharose 12 column [82]. However, problems with IgG estimation by SEC have also been reported, particularly in the presence of co-eluting caseins [92]. Quantification of IgG in species other than bovine has also been demonstrated [83].

Ion exchange chromatography (IEC)

The term IEC is used to describe the separation of ionic solutes based on their charge-based competitive interaction with ionisable sites of the stationary phase. Ion exchange columns are packed with resin comprising an insoluble rigid matrix, such as polystyrene crosslinked with divinylbenzene, and with a surface of ionisable sites that can carry positive or negative charge. An ionic solvent gradient is typically required for separation and, because the experimental conditions include many variables, including the type and concentration of other ions in solution, as well as changes in sample composition, IEC can be relatively unpredictable in comparison with other chromatographic modes.

Although IEC may be suitable for many whey proteins, it has also been shown that, depending on column choice, IgG may not bind to the column sufficiently to allow reliable quantification [92]. Both IEC and SEC are notably more suited to the purification of IgG and other proteins than as a quantitative technique [54].

Table 2 Survey of methods published for the determination of mammalian immunoglobulin G (IgG) for lacteal secretions

Sample matrix examined	Technique	Details of study	Approximate IgG levels (% w/w)[a]	Ref.
Bovine colostrum + whey	RID[b]	Comparison of procedures for colostral preparation in RID analysis	1–9	[45]
Bovine colostrum	RID	Comparison of IgG concentration in the colostrum of different cattle breeds	4–7	[46]
Human milk	RID	Study of the levels of IgG in human colostrum and milk at various times post partum	0.01–0.2	[47]
Equine colostrum	RID	Examination of the relationship between colostral IgG concentration, colostral specific gravity and foal serum IgG at 24 h post partum	4–6	[48]
Caprine milk	RID	Characterisation of the IgG change from colostrum to milk in native Korean goats of various ages	0.05–15	[49]
Bovine milk	RID	Discussion of parameters of a semi-automated RID as applicable to quantification of IgG in milk	0.02–0.15	[50]
Bovine colostrum	RID	Examination using RID of influence of dairy management and production variables on IgG levels in colostrum	2–8	[51]
Bovine milk	RID	Characterisation of the reduction in IgG and somatic cell count of healthy cows at each milking for 10 days post partum	0.1–4	[52]
Bovine, ovine and caprine milk + colostrum	RID	Discussion of correlation between serum protein and colostral IgG for three species	0.2–14	[53]
Bovine milk + colostrum	RID	Examination of variation in IgG, β-lactoglobulin, α-lactalbumin and bovine serum albumin concentration over successive milkings post partum	0.1–9	[54]
Bovine colostrum	RID	Comparison of colostral IgG concentration between Holstein and Guernsey cows	2–12	[55]
Bovine colostrum	RID	Examination of whether addition of non-Ig proteins as whey protein concentrate affects IgG absorption from maternal colostrum or colostrum supplements in neonatal calves	N/A[n]	[56]
Bovine colostrum	RID	Investigation of skimming temperature effects (to obtain whey fraction) and IgG concentration throughout colostral period	0.1–4	[57]
Bovine colostrum	RID	Determination of the IgG content of colostrum and milk replacer diets using RID in a study of the influence of bovine antiserum injections in calves	0.5–9	[58]
Bovine colostrum	RID	Study of the effect of short-term frozen storage of colostrum on serum IgG concentrations in neonatal calves	0.08	[59]
Caprine colostrum	RID	Determination of IgG concentration of colostrum refrigerated over time and evaluation of thawing and pasteurisation effects on IgG concentration	1–3.2	[60]
Bovine colostrum	RID	Study of the effect of on-farm batch pasteurisation on IgG concentration and comparison of serum IgG concentration in calves fed fresh vs pasteurised colostrum	3–8	[61]
Bovine colostrum	RID	Study of the concentration variation in IgG at different times during the first milking	0.5–9	[62]
Porcine plasma	RID	Description of the use of RID to detect bovine IgG in porcine plasma	N/A	[63]
Bovine colostrum	RID	Study of serum IgG levels of Jersey dairy calves when fed colostrum of high or low IgG concentrations	3.1–8.4 (colostrum)	[64]
Bovine colostrum	RID	Examination of the effect of timing of first milking colostrum collection on IgG concentration	7.5–11	[65]

Table 2 (continued)

Sample matrix examined	Technique	Details of study	Approximate IgG levels (% w/w)[a]	Ref.
Cameline colostrum and milk	RID	Study using RID of the variation of IgG, α-lactalbumin, serum albumin and lactoferrin in colostrum and milk samples from Tunisian camels throughout the first 14 milkings post partum	0.2–20	[66]
Cameline milk	RID	Effect of heat denaturation on IgG and other whey proteins estimated using RID	0.03–1.3	[37]
Cameline milk	RID	Determination using RID of lactoferrin and IgG in raw and fermented cameline milk with seasonal and geographical variation	0.05–13	[67]
Caprine colostrum	RID	Evaluation of temperature, time and high pressure treatment effects on IgG stability in caprine colostrum	1.7–3.7	[68]
Bovine milk	ELISA[c] + RID	Evaluation of two commercial immunoassay kits, normally used for detecting bovine adulteration of sheep and goat's milk, with possibility of using for classification of bovine milk according to heat treatment	N/A	[69]
Bovine milk + whey	ELISA + RID	Standardisation of an ELISA method for IgG in milks and whey and comparison with RID	0–0.02	[70]
Human milk	ELISA + RID	Comparison of two ELISA methods and one RID method for analysis of bovine IgG in human breast milk	Up to 0.0003	[71]
Bovine milk	ELISA + RID	Investigation of IgG stability in milk following various thermal treatments	0.02–0.04	[31]
Various	ELISA + RID	Comparison of antigen and antibody sources suitable for immunochemical assays of IgG in milk and dairy products	No products analysed	[72]
Bovine milk + colostrum	ELISA + RID	Study of effect of pH, heat, homogenisation and ultrasonic treatments on IgG stability. Also, investigation of the protective effects of sugars, sugar alcohols and amino acids during heat denaturation of IgG	0.02	[73]
Porcine colostrum	ELISA	Description of an ELISA assay for IgG estimation in porcine plasma and colostrum samples	Colostrum ~4–7	[74]
Bovine colostrum	ELISA	Study using competitive ELISA of denaturation of the antigen-binding region of IgG after heat treatment	0.06	[32]
Bovine colostrum	ELISA	Examination using ELISA of the effect of pH/heat combinations on the binding activity of IgG	0.08	[34]
Equine colostrum + milk	ELISA	Study of the intestinal uptake of equine colostral IgG in newborn foals	0.1–5.5	[75]
Bovine milk concentrate	ELISA	Comparison of binding ability of IgG to various human microbial pathogens	Up to 10	[44]
Enriched soymilk	ELISA	Investigation of IgG activity (after "pulsed electric field" treatment) of soymilk enriched with hyperimmunised dairy milk protein	15	[76]
Ruminant milks	ELISA	Description of a competition ELISA method for detecting bovine milk in caprine, ovine and bubaline (buffalo) milks	LOD° of 0.0001	[77]
Bovine milk	ELISA	Study of IgG concentration in milk from various regions of Spain throughout 1 year	0.025	[78]
Bovine IgG standard	ELISA	Investigation of IgG activity using ELISA after "pulsed electric field" treatment	0.0001–0.01	[79]
Ruminant milks + cheeses	ELISA	Presentation of a sandwich ELISA method for detecting bovine IgG in caprine, ovine and bubaline milk cheese	N/A	[80]
Enriched soymilk	ELISA	Investigation of the effect of high pressure processing on the immunoactivity of IgG in enriched soymilk	Up to 0.01	[14]
Bovine milk	ELISA + AC[d]	Investigation of the Fc region binding ability in IgG following various production treatments	Skim milk powder ~0.5 powder	[35]

Table 2 (continued)

Sample matrix examined	Technique	Details of study	Approximate IgG levels (% w/w)[a]	Ref.
Bovine milk + colostrum powders	AC	Presentation of an AC method for analysis of bovine colostrum-based products and comparison of this method with RID, SPR immunoassay and RP-LC	8.5	[81]
Bovine milk	IEC[e] + SEC[f]	Investigation of bovine milk protein separation using IEC and SEC. Best separation of whey proteins reported by SEC	0.05	[82]
Cameline colostrum	SEC	Study of camel colostrum from parturition to 5.5 months post partum	Up to 1.7	[83]
Bovine milk	RP-LC[g]	Examination of pasteurised milk for a range of whey proteins including IgG in order to estimate degree of denaturation following heat treatment	Actual IgG concentration not reported	[84]
Bovine milk	RP-LC	Examination of raw and pasteurised milk for a range of whey proteins including IgG	~9	[85]
Bovine whey	RP-LC + SDS-PAGE[h]	Description of simultaneous quantification of the major whey proteins including IgG	~0.4–5.6	[86]
Bovine milk	SDS-PAGE	Description of preparation of immunoaffinity columns to isolate IgG from milk colostrum and cheese whey and subsequent IgG analysis by SDS-PAGE	N/A	[87]
Bovine colostral whey	SDS-PAGE	Estimation using SDS-PAGE of IgG concentration of samples after reversed micellar extraction	1	[88]
Equine colostrum + milk	SDS-PAGE, CZE[i], SDS-CE[j] and RID	Comparison of techniques to study their ability to separate and quantify proteins, including IgG, from mare's colostrum and milk	0.3–5	[89]
Whey protein standards	CGE[k]	Discussion of CE method for separation of β-lactoglobulin, α-lactalbumin, bovine serum albumin and IgG. Presentation of quantification of only β-lactoglobulin and α-lactalbumin	N/A	[90]
Purified IgG	SDS-CGE	Presentation of SDS-CGE method for the qualitative and quantitative analysis of bovine IgG	0.05–0.2	[91]
Bovine whey + whey protein concentrate	CZE, CGE, SEC, IEC, AC, SDS-PAGE and Native-PAGE	Comparison of CE methods with various liquid chromatography and PAGE methods for the analysis of whey proteins in liquid whey and whey protein concentrate	0.015–2	[92]
Bovine whey protein	CZE	CE method for the detection of the main components of bovine whey protein	0.3–0.6	[93]
Tablets, powders and capsules	CZE	Description of CE method for analysis of IgG in tablets, powders and capsules and correlation of results with label claims	3–26	[94]
Bovine milk	Nephelometric IA[l] + RID	Assessment of nephelometric methodology for quantification of IgG in milk and comparison with RID	0.01–0.14	[95]
Bovine milk	Nephelometric IA	Description of a microparticle-based nephelometric assay used to estimate IgG in bovine milk	0.035–10	[96]
Bovine milk	Nephelometric IA	Description of a nephelometry method for analysis of IgG in milk and comparison of results with ELISA and RID	0.05–0.175	[97]
Bovine colostrum	Nephelometric IA	Description of the effects of heat treatment on IgG concentration and activity while investigating the necessary heating required to eliminate important pathogens	6	[39]
Bovine colostrum	Nephelometric IA	Study to identify the temperature at or below which heat treatment of colostrum would have no significant effect on IgG activity	2–16	[38]
Bovine milk + colostrum	SPR[m] IA	Description of a biosensor SPR-based assay developed for quantification of IgG in colostrum, milk and products containing elevated IgG levels. Comparison of the method against AC, RID and nephelometry	0.05–12	[98]

Table 2 (continued)

Sample matrix examined	Technique	Details of study	Approximate IgG levels (% w/w)[a]	Ref.
Bovine milk + colostrum	SPR IA	Description of the quantification of a number of minor milk proteins, including IgG, in a variety of bovine milk products	0.05–12	[99]
Porcine plasma	Lateral flow IA	Description of a novel lateral flow immunoassay device for detection of bovine IgG in porcine plasma. Qualitative only	Down to 0.1	[100]
Various	Monolithic AC	Presentation of a novel monolithic chromatographic technique for quantitative IgG analysis	No products analysed	[101]
Bovine colostrum	Hydrometer	Presentation of a method for estimating IgG in colostrum by measurement of colostral specific gravity	1–14	[102]
Bovine colostrum	Hydrometer	Study examining the effect of temperature on hydrometer (colostrometer) readings for estimating IgG in fresh colostrum	0–14	[103]
Bovine colostrum	Hydrometer + RID	Discussion of hydrometer readings as an estimate of IgG in fresh colostrum and the effect of temperature and additional colostral components	~4	[104]
Bovine colostrum	Hydrometer + RID	Comparison of hydrometer readings of colostrum on-farm with RID to assess the sensitivity, specificity and predictive value of hydrometer readings for measuring IgG in colostrum	2–14	[105]

[a] Approximate IgG levels have all been converted to % w/w from their published figures for ease of comparison
[b] RID, radial immunodiffusion
[c] ELISA, enzyme-linked immunosorbent assay
[d] AC, affinity chromatography
[e] IEC, ion exchange chromatography
[f] SEC, size exclusion chromatography
[g] RP-LC, reversed-phase liquid chromatography
[h] SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis
[i] CZE, capillary zone electrophoresis
[j] CE, capillary electrophoresis
[k] CGE, capillary gel electrophoresis
[l] IA, immunoassay
[m] SPR, surface plasmon resonance
[n] N/A, not available
[o] LOD, limit of detection

Affinity chromatography (AC)

One of the most promising LC techniques for quantitative IgG analysis is AC, which combines both separation and immunoassay attributes. This approach exploits the specific binding of IgG to a receptor such as Protein G or Protein A covalently coupled to the column support. Protein G is a *streptococcal* cell surface protein that specifically binds IgG via Fc domains with species specificity [110]. As the receptor binds IgG specifically, all other milk proteins will pass through the column when a loading buffer of neutral pH is used. Bound IgG is eluted with a buffer of low pH, which cleaves the interaction binding site between immobilised receptor and protein. A typical affinity chromatogram is shown in Fig. 2. Although the technique is a common tool for IgG purification [87], quantitative analysis of IgG may be accomplished by interpolation against a reference standard calibration curve [81, 92].

It is important to note that, as absorbance detection is at 280 nm, the identity of the protein species present in any chromatographic peak cannot be confirmed, and so it is assumed that the bound material present in the eluted peak is, by definition, IgG alone. However, it has been shown that sample preparation has a major influence on the observed result, with overestimation of IgG if caseins are not removed from solution prior to injection [81].

A variation of AC is perfusion affinity chromatography. In this technique, the traditional column support material, such as crosslinked agarose, is replaced by highly porous polystyrene-divinylbenzene, "flow through" particles facilitating a far greater surface area available for coupling with recombinant Protein G or A and thereby allowing much

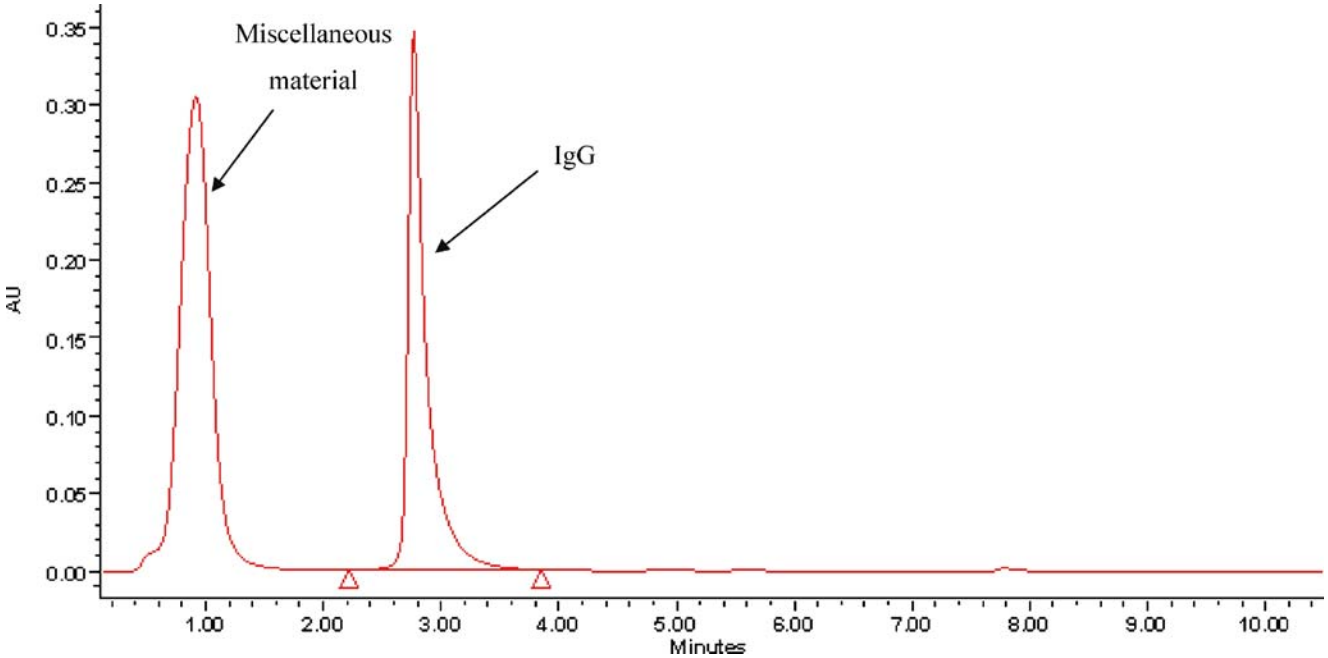

Fig. 2 Affinity chromatogram of a high protein spray-dried colostrum powder. Column: Pharmacia Protein G HiTrap 1 mL; loading buffer: 50 mM $NaH_2PO_4.H_2O$, 0.3 M NaCl pH 6.5; elution buffer: 20 mM glycine pH 2.5; flow rate 0.8–2.0 mL min^{-1}; ambient temperature; injection volume: 50 μL; detection: 280 nm

higher flow rates than for conventional AC columns. Binding and elution mechanisms are the same as for AC, with the limiting step reportedly being the rate of dissociation of IgG from Protein G at higher flow rates [111]. A further format of AC is high-performance monolithic affinity chromatography. This exploits the same antigen-receptor mechanism common to IgG affinity techniques, but employs a solid monolithic support in the form of a disc as the stationary phase. Because of this macroporous support, flow rates can be greatly increased, making the technique promising as a very fast analytical or screening tool. Other than proprietary application notes, limited evidence is available, but one study describing bovine IgG isolation is discussed [101].

Electrophoresis

Gel electrophoresis

Electrophoresis is a powerful and common technique for the separation of proteins. Samples in buffer are added to lanes in a gel and an electrical potential is applied across the gel. The potential drives molecules from one electrode to the other, with the gel material acting as a molecular sieve and separating the molecules by size. During electrophoresis, macromolecules migrate through the gel, depending on the ionic strength and temperature of the buffer, the electrical field strength and molecular hydro-

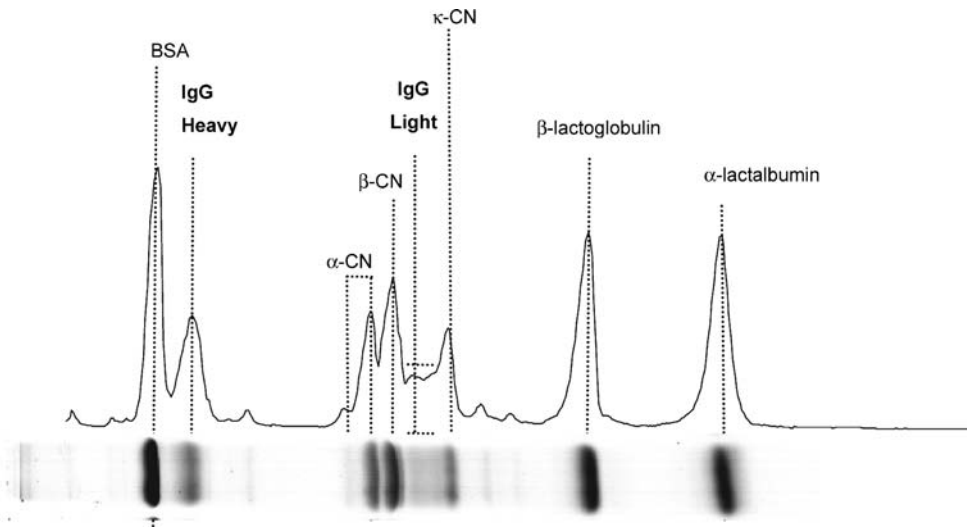

Fig. 3 A single sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel lane with corresponding densitometer trace. Gel: 4% stacking gel and a 12% separating gel; reservoir buffer: Tris-HCl/glycine, pH 8.6; gel stained using Amido black; protein bands were scanned using a laser scanning computing densitometer; sample was composed of individual bovine milk protein standards (0.1 mg mL^{-1})

phobicity, size and shape. Once separation is complete, the protein bands can be visualised by treating the gel with a stain such as Coomassie blue, which specifically binds to the proteins. Each band represents a different protein or protein subunit. Quantification is performed by scanning the gel using a densitometer and interpolating against a calibration curve prepared from authentic protein standards [88, 112, 113]. An example of a densitometer trace and a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) lane is shown in Fig. 3.

Both reduced and nonreduced variants of electrophoresis are possible approaches for IgG quantification. In SDS-PAGE, the proteins are denatured by SDS and IgG is cleaved into light and heavy chains, which appear as separate bands. SDS-PAGE is therefore unable to distinguish whether the IgG present was available in the initial sample in its native state or denatured. Preparation of native gels is also possible (without SDS treatment), but separation of the proteins is insufficient for accurate identification and quantification.

SDS-PAGE is not commonly used in isolation for IgG quantification, but is usually reported alongside other techniques or processes [86–88]. Two studies comparing SDS-PAGE with other techniques for the analysis of equine and bovine samples are discussed [89, 92].

Capillary electrophoresis (CE)

CE has been used to quantify dairy proteins for a number of applications [114]. The technique has the ability to separate proteins of different genetic variants and is often utilised to simultaneously separate and quantify multiple components. Because of significant differences in the concentration of casein compared with whey protein in intact milk, caseins are often analysed separately. Nevertheless, β-lactoglobulin and α-lactalbumin can be detected in such a casein electropherogram [115]. Whey proteins are usually initially separated from casein by a number of available methods prior to analysis by one of two different modes of CE.

Bovine IgG has been determined by capillary zone electrophoresis (CZE) [93] and capillary gel electrophoresis (CGE) [90–92]. A method for the analysis of IgG in tablets, powders and capsules has also recently been described [94]. CZE separates proteins based on the electrophoretic mobility of the proteins in a sample solution. A sample aliquot is injected into a capillary column and an electrical potential is applied across the column to gain separation of proteins in solution, yielding an electropherogram. CGE is the adaptation of traditional gel electrophoresis to a capillary format using polymers in solution to create a molecular sieve. This allows proteins with similar mass to charge ratios to be separated by size. The determination of IgG using CGE has been achieved for samples following prior removal of casein and major whey components [91]. The applicability of this method is relatively poor for the quantification of IgG in complex samples such as milk, although the method was suitable for the detection of light and heavy chain subunits, as well as dimeric IgG. Although neither CZE nor CGE was completely adequate for the quantification of all constituents of whey, CZE was superior for the quantification of IgG in samples of whey following casein removal [93].

Immuno-based techniques

Immunoassay is based on a specific interaction between antigen and antibody (or binding protein), and is a powerful analytical technique that complements alternative physicochemical techniques based on molecular separation principles. Conventional immunoassay relies on labelling one component of the interaction with an enzyme, fluorophore or chemiluminescent probe, whereas more recent developments have extended immunoassay through direct monitoring of the interaction by various transducer techniques, including surface plasmon resonance, acoustic waveguide, atomic force microscopy and quartz crystal microbalance. In general, immunoassay methods are particularly useful for detecting conformational changes in proteins, including IgG, due to denaturation, as well as adulteration of dairy products.

Radial immunodiffusion (RID)

To date, this has been an extremely popular technique because of its widespread availability and simplicity, although it can be time-consuming and has been shown to suffer from poor quantitative precision [45]. Samples are typically applied to wells cut in an agarose gel set with antibody raised to bovine IgG. During incubation, the sample diffuses through the gel, with the IgG forming a precipitin complex with the antibody. The ring diameter is proportional to the amount of IgG present. A calibration curve is constructed using standards of known concentration, and unknowns are read against this curve.

Many different kits are commercially available, and it is routine for RID kit manufacturers to supply a proprietary standard with certified reference values. However, it is important to consider that the method of primary standard quantification can vary between manufacturers, leading to significant variation in estimated IgG results when using kits from different manufacturers. This aspect is discussed further under "Primary reference material".

The technique is widely adaptable, having been reported for quantitative IgG analysis in human, ovine, caprine, equine and porcine samples in addition to bovine colostrum and milk [47–49, 53, 60, 63, 67, 68].

Nephelometry

Nephelometry is best described as a turbidity technique that, in this context, exploits anti-IgG1 antibodies detecting target analyte in diluted colostrum or milk [97]. An antibody–antigen complex that scatters light to a greater degree than the unbound compounds is produced, with the extent of light scattering proportionally related to the concentration of IgG1 in the sample.

A variant of this immunoassay principle relies on microparticle-supported nephelometry, typically based on the ability of antigen-coated microparticles to agglutinate in the presence of corresponding antibodies, producing measurable solution turbidity. Free antigen solution inhibits the immunoagglutination of microparticles, and the measurement of scattered light during inhibition allows the determination of antigen concentration [116]. The technique has been applied to the determination of α_s-casein and κ-casein [117], α-lactalbumin and β-lactoglobulin [118] and IgG [96, 97] in bovine milk. Microparticle-enhanced nephelometric immunoassay was demonstrated to be facile and rapid, and yielded acceptable precision parameters and correlation against values obtained by alternative methods, including both RID and conventional immunonephelometry [97].

Enzyme-linked immunosorbent assay (ELISA)

In general, ELISA techniques are widely used in immunology for either the qualitative or quantitative detection of antigens. In this context, they rely on the interaction between the antigen (bovine IgG) and antibodies raised against the antigen. Many formats of ELISA are available (direct, sandwich and inhibition modes), depending on the sequence of antigen and antibody addition. For the quantification of bovine IgG, antibodies raised against bovine IgG (typically polyclonal) are bound to the plastic surface of an ELISA 96-well microtitre plate and samples are applied directly to the surface, resulting in IgG binding specifically to the antibody. Detection and quantification are based on colorimetric measurement of bound antibody-enzyme conjugate and interpolation with a standard curve. Quantitative measurement of IgG using ELISA techniques is common in stability studies of IgG where the effects of various processing conditions such as thermal or pH treatments are examined [31, 32, 34, 73].

In addition to quantitative IgG measurement, ELISA has been utilised for the detection of bovine IgG in ovine, caprine and buffalo milk and cheeses [77, 80]. The technique is also easily adapted to show specific activity against selected pathogens [76].

Lateral flow immunoassay

A recent novel technique for IgG detection is lateral flow immunoassay. This consists of a patented lateral flow device for the detection of bovine IgG based on an antibody–gold conjugate incorporated into a glass fibre–nitrocellulose assembly [100]. Although this may be a useful qualitative tool, the accurate quantification of IgG cannot be achieved as described without further developments.

Surface plasmon resonance (SPR)

The optical phenomenon of SPR is sensitive to mass-dependent changes in refractive index associated with surface-binding events, and can be exploited to directly monitor biomolecular interactions in a real-time, label-free and automated platform [119]. A biosensor surface modified by a covalently attached ligand is used to support a binding interaction, and, for quantitative measurements of protein concentration, the immobilised ligand is commonly a specific antibody. The sample flows over the surface to allow association of target antigen over a set time, before a regeneration step is performed to remove the analyte from the surface ready for the next sample. This procedure can be repeated for known standards, and hence samples of unknown concentration can be quantified by interpolation. Typical sensorgrams of bovine IgG calibration standards are illustrated in Fig. 4. In common with other immunoassay approaches, SPR-based techniques are inherently specific, and are particularly useful for determining proteins at low concentrations and where more dominant milk proteins can make chromatographic and electrophoretic techniques challenging. This makes SPR-based immunoassay particularly useful for the analysis of minor dairy proteins.

An automated biosensor immunoassay utilising SPR optics has been used for the quantification of minor bovine milk proteins including IgG [98, 99]. A polyclonal goat anti-bovine IgG was applied to the estimation of the IgG content in liquid colostrum, milk, and colostrum and hyperimmune milk powders. The described SPR method was compared with independent AC, RID and nephelometry methods and gave comparable data. Utilising the *staphylococcal* receptor Protein A as ligand, mouse IgG has been determined using an alternative automated SPR system [120].

Physical techniques

Physical techniques are based on the correlation of a measurable physical parameter (e.g. density, specific gravity, viscosity, hardness and turbidity) with the concentration of a specific component. As such, they are based on different principles from the previously

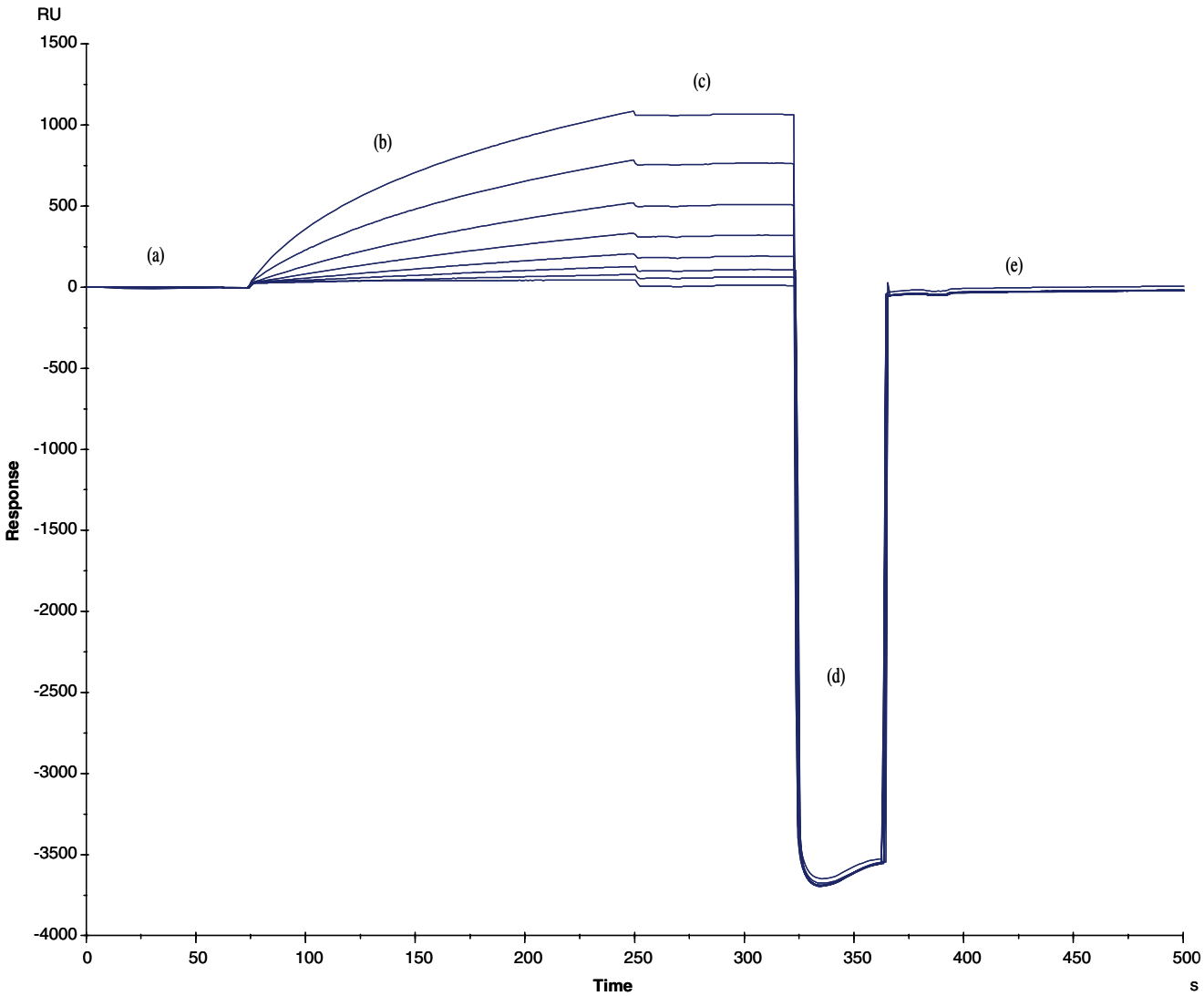

Fig. 4 Superimposed surface plasmon resonance (SPR)-derived sensorgrams monitoring response (RU) vs. time (s) of immunoglobulin G (IgG) calibration standards (0, 156, 312.5, 625, 1250, 2500, 5000, 10,000 ng mL^{-1}) over goat anti-bovine IgG immobilised sensorchip. Run conditions: flow rate, 20 μL min^{-1}; contact time, 3 min; regeneration: 35 μL of 10 mM glycine pH 1.75. (*a*) baseline; (*b*) association; (*c*) dissociation; (*d*) regeneration; (*e*) baseline

described techniques. Although uncommon for protein analysis because of the complex matrices involved, the estimation of IgG in fresh colostrum via specific gravity has been reported.

This method is based on the correlation between the specific gravity of fresh colostrum and IgG concentration [102], and is a physical measurement that is influenced by factors including temperature and sample composition [103, 104].

Limited information describing the precision of this technique has been published [104, 105], and, although it may remain a useful analysis for field estimations (e.g. on-farm), its inherent variability makes it unreliable for commercial, manufacturing or regulatory applications.

Method summary

The analysis of bovine IgG content in foods and supplements may be required to satisfy several purposes, including food safety, nutritional database information, regulatory compliance, quality control, quality assurance, as well as clinical or structure–activity studies. The different functions of academic, commercial and regulatory laboratories will therefore influence method selection, and each of the analytical techniques available has attributes that suggest their use, depending on the intended purpose of the analysis. Thus, the "fitness-for-purpose" of each approach is a function of the context of the analysis. Whilst this review has described a diverse range of reported

Table 3 Comparison of techniques for bovine IgG quantification

Technique	Capital cost	Consumables cost	Multi-analyte capability[a]	Binding protein/ antibody required[a]	Prevalence of technique	Automation	Operator skill	Throughput	Conformational state capability	Sensitivity	Precision
RP-LC	++	+	+	−	+++	+++	++	++	+	+	+
SEC	++	+	+	−	+++	+++	++	++	+	+	+
IEC	++	+	+	−	+++	+++	++	++	+	+	+
AC	++	+	−	+	+++	+++	++	++	+++	++	++
PAGE	+	++	+	−	+++	−	++	+	+++	+	+
CE	++	+	+	−	+	++	++	++	+	+	++
RID	+	+	−	+	++	−	+	+	+	+	+
Nephlometry	++	+	−	+	+	+	++	+++	+	+	++
ELISA	+	+	−	+	+++	+	++	++	+++	++	++
LFI	+	+	−	+	+	++	++	++	+	+	+
SPR	++	+	−	+	+	++	++	++	++	++	++
Physical	+	−	−	−	+	−	+	+	−	−	−

− n/a ("no)
+ low ("yes)
++ moderate
+++ high

analytical techniques for bovine IgG estimation in milk, colostrum and dietary supplements over a wide concentration range (as summarised in Table 2), any attempt to establish an independent ranking of preferred methods would clearly be inappropriate.

A generalised comparison of the principal characteristics of the various techniques is shown in Table 3.

In general, methods relying on analyte separation (by various mechanisms) can be used to quantify IgG simultaneously with other milk proteins present and therefore feature a multi-analyte capability, while immunoassays are usually specific for the individual target protein. This characteristic feature will frequently influence the selection of a generic technique, although other factors may also be significant. In addition, all described techniques are variously influenced by the conformational state of the protein and may therefore estimate either native or total bovine IgG content. As bovine IgG is a multi-unit protein polymer, processing stresses can compromise its conformational integrity, leading to potential loss of biological function. Where such concerns are the focus, immunobased techniques are favoured, since antibody selection facilitates the detection of specific conformational moieties of IgG consequent to protein unfolding.

Currently, many investigations are research-driven in an academic setting. However, the increasing requirement for routine regulatory and compliance testing of IgG-supplemented foods will undoubtedly modify method selection, since assay cost, ease of use and automation are strategically significant factors in this context. Thus, in any food quality program targeting bovine IgG, there will continue to be a need to distinguish between rapid, inexpensive methods and highly sophisticated reference methods.

Primary reference material

Analytical issues associated with the selection of protein standards are generic, particularly in the application of immunoassays, and this concern has recently been discussed in the clinical context [121]. As described in this review, there are a large number of diverse analytical methods available that rely on either separation technologies or immunochemical principles for quantifying bovine milk or colostrum IgG. In all these assays, a prerequisite for the accurate quantification of bovine IgG is the availability of a robust, well-characterised bovine milk or colostrum IgG reference material for use as a calibrant, and ideally a validated method should be used to characterise all such reference materials. Although each of the different analytical techniques should ideally yield an equivalent response to both standard and measurand, the preparation of a pure IgG reference used for quantification is critical, because its

higher level structural status may influence the measurement. Thus, methods that measure the total amount of bovine IgG, such as SDS-PAGE under reduced conditions or RP-LC, should ideally produce similar results to immuno- and affinity-based assays that are inherently dependent on the structural integrity of the protein. Both sample preparation and calibration standard preparation are therefore very important.

Purified bovine IgG calibration standards derived from milk or colostrum are presently unavailable from commercial sources. The most commonly cited commercially available purified standard is bovine IgG isolated from serum [122–124]. Commercial standards have stated purities of IgG usually >95% by either SDS-PAGE or LC, although this value does not account for non-proteinaceous material also present. In view of this, it is recommended that the protein concentration be determined after reconstitution, using ultraviolet absorbance at 280 nm. As there are contradictory values ranging from 12.0 to 14.0 for the molar extinction coefficient for bovine IgG [125–129], this will affect the final calculated concentration value. It is also important to ensure that the final matrix of the calibration standard resembles that of the sample as much as practicable. Thus, a commercial standard can be used provided it is well characterised and any possible constraints are understood.

Commercial kits for the estimation of bovine IgG based on ELISA, RID or other immunoassays are usually supplied with their own proprietary reference material. These calibration standards are typically derived from bovine serum characterised for IgA, IgG and IgM. Although these standards may be acceptable for use in assay systems where IgG characterisation is based on physical parameters, such as SEC, RP-LC and SDS-PAGE, their use in immuno- or affinity-based assays may result in erroneous IgG quantification because of subclass differences between IgG from bovine serum and bovine milk or colostrum [72].

A number of researchers have produced their own in-house reference materials [32, 54, 57, 130, 131] and, in these instances, their accurate characterisation is paramount. This requires the determination of both total protein (using either spectrophotometric analysis at 280 nm or dye-binding methods) and the purity of the bovine IgG; it is not sufficient to specify just one of these parameters [57, 131]. A more absolute method of determining milk protein composition would be amino acid analysis [132]. Other factors for consideration when characterising bovine milk or colostrum IgG standards include sample matrix or buffer compatibility, IgG1: IgG2 subclass ratio, and relative structural integrity of the IgG.

Even when the same calibration standard is used in different assay systems, there remains the possibility that variable IgG values will be obtained for the same sample, e.g. an ELISA assay produced lower IgG values than a similar RID method [70]. In this example, a thorough examination of the variability of the RID and ELISA immunoassays attributed the differences largely to both the standard IgG (from bovine serum) and the antibody specificity, where the authors stated that "the most accurate quantification of IgG in milk or milk products would require standard curves that were based on IgG that had been specifically purified from these sources" [72].

Conclusions

It is apparent that there is an abundance of published methods for determining the IgG content in bovine milk- and colostrum-based products. Such applications are generally either to support commercial production, or to provide nutritional information to the consumer. Instrumentation and analytical principles are varied, and several can be used to investigate specific facets of IgG, either in isolation or in complement to each other. Despite a relative plethora of such applications, very few peer-reviewed methods have been described for IgG analysis in the rapidly developing dietary supplements market.

The developing international trade of nutraceutical products based on declared IgG content will require increasing traceability of analytical data, and hence the equivalence of alternative methods utilised for labelling will be under scrutiny in order to achieve regulatory compliance. Several factors can compromise the traceability of IgG claims, including the inherently low content of IgG in milk relative to the major milk proteins, potential matrix interferences, variations in sample preparation, the IgG reference material used as primary calibrant and antibody specificity.

There is clearly a need for internationally accepted analytical methods for the determination of bovine IgG in such products, and any such methods will need to further address the requirement for a characterised and commercially available primary bovine lacteal IgG reference material that accounts for IgG subclass variations.

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