Determination of Caprine Serum Albumin in Milk using Bromocresol Green Dye

By

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ABSTRACT

The objective of this study was to develop and validate a rapid and sensitive analytical technique to measure caprine serum albumin (CSA) concentrations in milk, demonstrate its utility and investigate the relationship between CSA and milk constituents. The principle of the technique is based on the binding of albumin to bromocresol green (BCG) dye at pH 4.0 and the absorption of the dye-albumin complex was determined spectrophotometrically at 640nm. A linear relationship existed between CSA concentrations (0.01 to 8.0 mg/ml) and absorbance values. Intra-assay and inter-assay coefficients of variation (CV) for the proposed BCG dye-binding assay were 2.6% and 12% respectively. Recovery from milk samples spiked with albumins was 89.15% ± 7.6. Individual milk samples (n=126) were collected from lactating Saanen goats at various stages of lactation in a local dairy herd in Quebec and the CSA concentrations in milk were assayed using the proposed BCG method. Caprine serum albumin concentrations in milk ranged between 0.31 and 0.52 mg/ml. The concentrations of CSA were significantly higher ($P < 0.05$) at the beginning of lactation, declined rapidly and remained relatively stable in mature milk. Caprine serum albumin correlated positively ($P < 0.05$) with somatic cell counts (SCC), total solids (TS), protein, fat and whey and correlated negatively ($P < 0.05$) with lactose concentration and casein. The proposed BCG-dye binding method is easy to perform, rapid, sensitive, reliable and can be used to determine CSA concentrations in milk collected from goats under different physiological conditions.

RÉSUMÉ

L'objectif de cette étude était de développer et valider une technique analytique sensible et rapide pour mesurer la concentration de serum-albumine dans le lait chèvre (CSA) , démontrer son utilité et enquêter sur le rapport entre le composition de lait et CSA. Le principe de la technique est fondé sur le fait d'attacher d'albumine au colorant vert de bromocresol (BCG) à pH 4.0 et l'absorption du complexe d'albumine de colorant a été déterminé spectrophotometric à 640nm. Un rapport linéaire a existé entre le logarithme de concentrations CSA (0.01 à 8.0 mg/ml) et absorbance. Intra-analyse et inter-analyse coefficients de variation étaient 2.6% et 12% respectivement. Le récupération des échantillons de lait avec des albumines était (89.15% ± 7.6). Les échantillons de lait individuels (n=126) ont été recueillis de chèvres Saanen produisant du lait aux stades différents de lactation dans un troupeau laitier local à Québec et les contenus de CSA dans le lait étaient analyse en utilisant de la nouvelle méthode BCG. La concentration de CSA dans les laits s'étendait entre 0.33 et 0.52 mg/ml. Le niveau de CSA dans le lait était significativement plus haut ($P < 0.05$) au début du lactation, diminuer rapidement et était relativement stable dans le lait mature. Serum-albumine dans le lait chevre était faiblement corrèle ($P < 0.05$) avec les comptes de cellule somatiques (SCC), le total solides (TS), la protéine, le gras et le petit lait mais corrélé ($P < 0.05$) négativement avec les concentrations de lactose et le caséine. Cette méthode analyse de BCG est simple, rapide, et fiable et peut être utilisé pour déterminer des concentrations de

CSA dans le lait provenant des chèvres sous des différentes conditions physiologiques.

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Olaniyan for their love and support. My foremost appreciation goes to our Lord, Jesus Christ, the giver of wisdom and the creator of knowledge and mankind.

Dedication

This work is dedicated to the blessed memory of my late mother, Mrs. Serah Olaniyan. I miss you Mum but I take solace in God and believe that we shall meet again to depart no more at the feet of our Lord, Jesus Christ. May your gentle soul rest in perfect peace (Amen).

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CONTRIBUTIONS TO KNOWLEDGE

We developed and validated a rapid and sensitive bromocresol green dye (BCG) dye-binding method for the first time to determine caprine serum albumin (CSA) concentrations in milk and applied this technique to study changes in CSA concentrations in milk during established lactation. We observed that CSA tend to be high in colostrum, declined gradually as lactation progressed and remained stable in mature milk. The positive correlation between CSA and SCC suggested that CSA can be used as a more specific marker of inflammations in the mammary gland of goats.

ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
BCG	Bromocresol green
BCP	Bromocresol purple
BSA	Bovine serum albumin
CMT	California Mastitis Test
CN	Casein
CSA	Caprine serum albumin
CORR	Correlation
DIM	Day in milk
ELISA	Enzyme-linked immunosorbent assay
Ig	Immunoglobulin
IMI	Intramammary infection
NAGase	N-acetyl glucosaminidase
NCN	Non-casein-nitrogen
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PMN	Polymorphonuclear neutrophils
SAS	Statistical Analysis Software
SCC	Somatic cell count
SRID	Single radial immunodiffusion
VALACTA	Quebec dairy production centre of expertise

1. INTRODUCTION

Although the physiological function of serum albumin in milk is unclear, increased serum albumin levels in milk can be used as an indicator of mammary gland health status. Bovine serum albumin-immunoglobulin (BSA/Ig) complex in milk has been linked to immune defense reactions occurring during early stages of udder infections (Lieske et al., 2005).

Leitner et al. (2004a) studied the milk composition of goats as affected by subclinical mastitis and found that serum albumin were significantly higher in milk from infected (0.47±0.05 mg/ml) than healthy (0.28±0.02 mg/ml) glands. Higher concentrations (2.97 ± 2.46 mg/ml) of caprine serum albumin (CSA) have been reported in colostrums (Levieux et al., 2002).

Accurate determination of serum albumin in milk from healthy udder is affected by its low concentration and interference from other milk constituents. Indicator dyes such as methyl orange, coomasie blue, Cu (II)-Arsenazo K, bromophenol blue, bromocresol purple (BCP) and bromocresol green (BCG) have been previously used in determining serum protein concentrations in domestic animals and humans (Keay et al., 1983; Affonso et al., 1985; Akram, 2005). However, different dyes have shown varied degree of specificity, sensitivity and suitability for specific serum proteins (Barber et al., 1992). Rodkey (1965) established

the direct spectrophotometric determination of albumin in human serum using the BCG dye-binding technique. Other researchers have utilized the same approach in determining BSA in milk (Guzman et al., 1986; Bouchard et al., 1999; Lieske et al., 2005). To date, limited studies have reported the use of anionic dye in quantifying CSA concentrations in milk.

In principle, albumin binds quantitatively with BCG at pH 4.0 resulting in the formation of a green color that can be measured at 640nm. The dye-binding technique is characterized by an ionic interaction between positively charged albumin and negatively charged dye molecules at acidic pH (4.0 to 4.2). Factors such as optimal pH, ionic strength of buffer, sample preparation, dilution rate, incubation time and interfering proteins affects the accuracy of the technique (Gustafsson, 1976; Lieske et al., 2005).

Serum albumin enters the milk from the blood or systemic fluids by leaking through the epithelial tight junctions (Kerhli and Shuster, 1994; De Wit, 1998; Nguyen and Neville, 1998) and its concentrations in milk is significantly influenced by stage of lactation (Sheldrake et al., 1983), inflammations in the mammary glands (Levieux et al., 2002) and physiological transitions from lactation to involution and from involution to lactogenesis (Sordillo et al., 1987).

Positive correlation between BSA and SCC had been reported in bovine milk (Lindmark-Mansson et al., 2000). Somatic cell counts in bovine milk have been established as an indicator of mammary gland

health and milk quality (Kitchen et al., 1980, 1981; Kehrli et al., 1994; Rogers et al., 1994; Jayarao et al., 2003). However, relationship between SCC and intramammary infections in lactating goats has been questioned because cytoplasmic particles and epithelial cells apart from leukocytes are shed into milk during milk secretion which in turn results in high SCC even in uninfected udders (Manser, 1986; Park and Humphrey, 1986; Paape et al., 2001). Therefore, an accurate measurement of CSA concentrations in milk may also be considered as an alternative index of the health status of mammary glands of goats.

Caprine serum albumin in milk had been previously quantified using single radial immunodiffusion (SRID) assay (Levieux et al., 2002), and enzyme-linked immunosorbent (ELISA) assay (Leitner et al., 2004a). However, the measurement of the ring-shaped precipitates using a video camera system and the 20 to 22 h incubation of plates in a moist box makes the SRID procedure tedious, time consuming and cumbersome for quick screening of milk samples (Levieux et al., 2002). Furthermore, the production of antibodies limits the use of ELISA for determining serum albumin in large number of samples. The dye-binding method is commonly used in determining serum proteins because of its simplicity and rapidity but often times criticized for overestimating albumin.

Bovine and caprine serum albumins show similarity in their structures and conformations but only share comparable binding properties (Trivedi et al., 1997). Therefore, it will be very pertinent to

investigate the biophysical properties of CSA in milk to further understand its molecular-structural changes and interactions with natural milk proteins. This work intends to describe a new colorimetric method for measuring CSA in milk.

The objectives of the study are:

1. To establish a rapid and sensitive analytical technique to quantify caprine serum albumin concentrations in milk
2. To validate the analytical procedure and demonstrate its utility.
3. To investigate the relationship between caprine serum albumin concentrations and other milk constituents.

2. LITERATURE REVIEW

2.1 MILK SYNTHESIS AND SECRETION

The epithelial cells of the alveoli are the basic milk secretion unit in the mammary gland. Milk precursors derived from blood or interstitial fluids are transformed into milk constituents by different synchronized biochemical processes (Neville, 1998) and then the secreted milk is stored within the lumen of the alveoli, ducts and teat cisterns until removed by milking or suckling (Akers, 2003). The major milk constituents are water, fat, proteins, carbohydrates and minerals.

Lactose is synthesized in the golgi apparatus of mammary epithelial cells from glucose and galactose by the action of lactose synthetase (Akers, 2003). Proteins in milk are synthesized mainly in the rough endoplasmic reticulum from free amino acids or peptides absorbed from the bloodstream. The basolateral cell membrane serves to regulate the uptake of milk protein precursors from the interstitial fluids. Caseins, α-lactalbumin and β-lactoglobulin are formed from free amino acids derived from blood. However, immunoglobulins and serum albumin are absorbed from the blood intact and passed into the milk (Tyler and Ensminger, 2006). This is believed to result from passive transport in secretion vesicles and or escape through the tight junctions of the capillary endothelium of actively secreting glands (Klobassa et al., 1987).

Milk fat triglycerides are synthesized in the cytoplasmic surface of the smooth endoplasmic reticulum of mammary epithelial cells from fatty

acids and glycerol through the glycerol phosphate pathway (Sutton, 1989). Milk fatty acids are either taken up from plasma lipids (Chilliard et al., 2000) or they are synthesized de novo within the mammary epithelial cells from acetate and 3-hydroxybutyrate derived from rumen fiber digestion (Neville and Picarno, 1997).

2.1.1. Integrity of the tight junctions of the mammary gland

Tight junctions are located between adjacent mammary epithelial cells and control the passage of blood solutes into the secreted fluid in the mammalian udder (Nguyen and Neville, 1998). During lactation, when the ducts and alveoli are filled with milk, the epithelium is positioned between two very different milieus, the milk containing lactose, milk proteins and low concentrations of sodium and chloride, and the interstitial fluid containing plasma proteins and high concentrations of sodium and chloride (Nguyen and Neville, 1998). Tight junctions therefore form a barrier between the systemic (basolateral) and the milk (apical sides). In mammary epithelium, intact tight junctions prevent paracellular leakage of blood serum components into milk and prevent milk components from crossing into the blood. Tight junctions also maintain a small transepithelial potential difference between blood and milk (Peaker et al., 1977).

During lactation, the tight junctions are impermeable, allowing milk to be stored between nursing or milking periods while restricting the

leakage of milk components from the lumen or leakage of interstitial fluid components into the milk. However, tight junctions may become leaky during late lactation (drying-off) and undergo closure around parturition (Nguyen and Neville, 1998). In dry glands, high intramammary pressure induced by the cessation of milking ruptures the tight junctions and therefore becomes leaky (Anderson et al., 1985). Disrupted tight junctions in mammary epithelial cells allow the movement of ions and interstitial fluid into milk, thereby increasing sodium and chloride concentrations in milk (Nguyen and Neville, 1998).

During colostrogenesis, tight junctions are leaky, thereby increasing the movement of serum proteins and antibodies from blood into mammary secretions. Serum albumin and immunoglobulin concentrations are generally higher in colostrums than in mature milk of goats and cows (Anderson et al., 1985).

Tight junctions can readily be opened during leukocyte transmigration and reseal quickly to re-establish permeability barrier (Nash et al., 1987).

Lactating goats producing recombinant proteins in their milk have been suspected to have elevated serum albumin concentrations in milk due to excessive demand on mammary secretory cells resulting in disrupted or leaky tight junctions and movement of serum components into milk (Baldassarre, 2007). The integrity of the tight junctions therefore plays

a significant role in determining the serum albumin concentration of secreted milk.

Decline in milk secretion is often associated with disruption in mammary tight junctions (Stelwagen et al., 1994). Leaky tight junctions reduce milk secretion in response to a reduction in lactose synthesis which may be mediated by the cytoskeleton (Olivier and Smith, 1983; Noble and Hurley, 1999).

The mechanism by which disruption in tight junctions affects milk secretion has not been established. However, Nguyen and Neville, (1998) suggested that the stretching associated with milk stasis has been implicated in a mechano-transduction-signaling pathway which in turn could alter both milk synthesis and tight junction permeability. The tensile nature of the tight junctions can be seen during mechanical stretching where the intramembrane strands move laterally to re-arrange from a compact network to an elongated network (Pitelka et al, 1983). Therefore, the assembly of the tight junctions is a complex process which is influenced by multiple factors including vesicular trafficking and extracellular proteases (Kohler et al., 2005).

2.2. GROSS COMPOSITION OF CAPRINE MILK

Milk is a colloidal suspension of casein micelles and fat globules in a serum phase containing whey proteins, vitamins, minerals and lactose. Caprine milk contains 3.0 to 4.4% fat, 4.13 to 4.73% lactose, 3.19 to 3.86

% proteins, 11.17 to 13.44% total solids and 0.79 to 0.89% ash (Guo et al., 2001).

Milk composition varies according to breeds and is influenced by stage of lactation, age, diet, length of interval between milking, ambient temperature, disease especially mastitis and season of year (Herrington et al., 1972; Walstra et al., 1984).

Caprine milk has almost the same composition as bovine milk (Table1). However, caprine milk contains predominantly smaller fat globules than bovine milk (Fevrier et al., 1993). Mehaia (1995) reported average fat globule sizes in this decreasing order: bovine > ovine > caprine which is inconsistent with other reports (Anifantakis,1986; Juarez and Ramos, 1986).

Casein contents in caprine milk range between 16 and 26g/L, the proportion of non-protein-nitrogen (NPN) of the total nitrogen content is between 3% and 13%, the ionized calcium levels between 0.07 and 0.19g/L and the total inorganic phosphorus between 0.45 and 1.0g/L (Remeuf and Lenoir, 1986).

2.2.1. Caprine milk proteins

Protein content in caprine milk range between 3.19 and 3.86% and its composition is influenced by breed, stage of lactation, feeding, climate, parity, season and udder health status (Juarez and Ramos, 1986; Park, 1991, 2006). Caprine milk protein is distributed in various fractions and

has a higher level of non-protein-nitrogen (NPN) and lower casein-N than ovine and bovine milk (Park et al., 2007). The two major protein fractions in milk are caseins and whey proteins (β-lactoglobulin, α-lactalbumin, lactoferrin, transferrin and serum albumin).

The principal caseins in caprine milk are α-s1-CN, α-s2-CN, β-CN and κ-casein and their composition is greatly influenced by genetic polymorphisms on α-s1- , α-s2-, β- and κ-casein loci (Martin et al., 2003). Caprine casein micelles contain more calcium and inorganic phosphorus than the bovine casein micelles (Jennes, 1980; Remeuf and Lenoir, 1986).

β-lactoglobulin is the major whey protein in ruminant milk and it accounts for more than 50% of the whey protein and has the ability to bind large amounts of vitamins (Mepham,1987).

α-Lactalbumin, a calcium binding protein is the second most abundant whey protein and it accounts for approximately 13% of the total whey protein and it is essential for lactose biosynthesis in mammary cells (Akers, 2003).

Lactoferrin and globulins are minor whey proteins associated with the immune system while SCC, N-acetyl-β-D-glucosaminidase (NAGase) and serum albumin are related to inflammation (Piccinini et al., 2007). Lactoferrin is mainly synthesized by glandular epithelial cells and secreted into mucosal fluid (Ward et al., 2005; Rainard and Riollet, 2006). Caprine milk contains about 20 to 200 µg/ml of lactoferrin (Park et al., 2007) and

positive correlations between lactoferrin and SCC in bovine milk has been reported (Lindmark-Mansson et al., 2006).

Serum albumin and immunoglobulins are not specific to milk and are considered to be the same as those found in blood (Park et al., 2007)

Table 1. Caprine, ovine and bovine milk compositions

Constituents	Caprine	Ovine	Bovine
Total solids (%)	11.17-13.44	16.19 – 18.89	13.00 – 13.40
Fat (%)	3.00 - 4.40	5.28 - 7.94	4.18 - 4.50
Protein (%)	3.19 - 3.86	5.21 - 6.15	3.31 - 3.43
Lactose (%)	4.13 - 4.73	4.07 - 4.61	4.50 - 4.62
Casein (%)	2.34 - 2.86	4.46 - 4.74	2.49 - 2.63
Whey (%)	0.58 - 0.64	1.16 - 1.24	0.76 - 0.86
Serum albumin (mg/ml)	0.26 - 0.30	0.49 - 0.55	0.10 - 0.40
Lactoferin (µg/ml)	20 - 200	-	20 - 200
Ash (%)	0.79 - 0.87	0.88 - 0.90	0.72 - 0.74
Calcium (g/100g)	0.12 - 0.17	0.39 - 0.403	0.11 - 0.118
Phosphorus(g/100g)	0.10 - 0.16	0.14 - 0.141	0.087 - 0.093
Sodium (g/100g)	0.04 - 0.10	0.062 - 0.086	0.038- 0.042
Magnesium(g/100g)	0.01 - 0.02	0.02 - 0.021	0.011 - 0.013
Potassium (g/100g)	0.083 -0.19	0.08 - 0.16	0.155 - 0.165

Sources : (Guo et al., 2001; Lindmark- Mansson et al., 2003; Leitner et al., 2004a, 2004b; Sahan et al., 2005; Garcia et al., 2006)

2.2.2. Serum albumin in milk

Serum albumin in milk is a blood-derived protein and its concentration is significantly influenced by the infection status of mammary glands, stage of lactation and blood-milk passive transport (Poultrel et al.,1983; Sheldrake et al.,1983; Kehrli et al., 1994). Albumin is the most abundant protein in the circulatory system and its concentrations in mammalian blood range between 3 and 5g /100ml and has a half-life of approximately 20 days (Waldmann, 1977). Albumin in plasma plays a significant role in the transport, metabolism and distribution of free fatty acids, bile acids, bilirubin, calcium and magnesium, maintains blood osmotic pressure and impacts free radical protection (Carter and Ho, 1994).

Serum albumin in milk is a non-specific protein to the mammary gland and it enters the milk from the systemic fluids by leaking through the epithelial tight junctions (De Wit, 1998). In mammals, albumin is synthesized largely in the liver initially as preproalbumin which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum. The product, proalbumin, is in turn cleaved in the golgi vesicles to produce the secreted albumin (Friedli, 1996). Non-hepatic synthesis of albumin has been reported in mouse retina (Dodson et al., 2001), mouse skeletal tissue (Wagatsuma et al., 2002), human ovarian epithelial cells (Varricchio and Stromberg, 1994) and bovine tracheal gland serous cells (Jacquot et al., 1988). De novo

synthesis of albumin in the mammary gland of goats had been suggested (Phillippy and McCarthy, 1979; Leitner et al., 2004a).

Milk of healthy goats contains (0.26 to 0.30 mg/ml) serum albumin while mastitic caprine milk contains (0.42 to 0.52 mg/ml) serum albumin (Leitner et al., 2004a). Serum albumin concentrations (2.97± 2.46 mg/ml) in goats' colostrums are higher than in mature milk (Levieux et al., 2002). Milk of healthy sheep contains 0.50 to 0.60 mg/ml serum albumin (Nudda et al., 2003). Bovine milk in established lactation contains 0.1 to 0.4 mg/ml BSA representing approximately 1.5% of total milk protein and about 8% of total whey protein (Fox et al., 1988; Farrell Jr et al., 2004).

2.3. METHODS USED FOR MEASURING SERUM ALBUMIN IN MILK

Several methods have been used to quantify serum albumin in bovine, ovine and caprine milk (Table 2). These include radial immunodiffusion (RID) technique (Levieux et al., 2002), ELISA (Shuster and Harmon, 1990), electroimmunodiffusion assay (Mangino and Weissler, 1981), polyacrylamide gel electrophoresis (PAGE) (Sheffield, 1997), size exclusion chromatography (Lieske et al., 2005), digital imaging technology (Casper et al., 1998) and dye-binding technique (Guzman et al., 1986).

2.3.1. Single radial immunodiffusion assay

Single radial immunodiffusion (SRID) is one of the most commonly used immunological techniques utilized in quantifying serum protein concentrations. The technique measures antigen-antibody precipitate in suspension photometrically or in a gel (Mancini et al., 1965). Usually, whey diluted in veronal buffer is applied to well of a plate of agarose gel containing antibodies specific for caprine albumin. Plates are incubated for 20 to 22h in a moist box at 37°C after sample addition. Following diffusion into the agarose, the diameters of the formed precipitate rings are measured automatically using a magnifying video camera system (Levieux et al., 2002). The diameter of the precipitating ring is directly proportional to the square root of the protein concentration.

This method is more sensitive compared to electrophoretic and dye-binding methods due to the specificity of the antibodies. However, the production of antibodies, long time of incubation and the measurement of the ring-shaped precipitate makes the technique tedious, time consuming and cumbersome for quick screening of milk samples (Lieske et al., 2005). The sensitivity of the SRID technique depends on the ability to detect an immunoprecipitate formed at the antigen/antibody equivalence point (Mancini et al., 1965).This technique has been criticized for lack of accuracy and repeatability compared to ELISA. However, electroimmunodiffusion combines immunodiffusion and electrophoretic separation and therefore tend to be a more rapid, accurate and sensitive

method and requires minimal amounts of reagents compared to SRID (Mangino and Weissler, 1981). Single radial immunodiffusion assay has been reported to give higher CSA values (0.35 to 0.45 mg/ml) than that obtained by ELISA (0.26 to 0.30 mg/ml) (Levieux et al., 2002; Leitner et al., 2004a). Different methods used for determining serum albumin often give divergent results. However, each technique offers its strengths and weaknesses.

2.3.2. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay is a highly sensitive immunological technique and has been recently utilized in determining serum protein concentrations. The technique involves the conjugation of a secondary antibody to an enzyme (Shuster and Harmon, 1990). Enzyme-linked immunosorbent assay is very sensitive and accurate compared to immunodiffusion, electrophoretic and dye-binding methods because the technique combines the specificity of antibodies with the sensitivity of the conjugate enzyme. However, the production of antibodies is a major limitation. Reports have shown that CSA values measured by ELISA (0.26 to 0.30 mg/ml) were lower than those measured by SRID (0.35 to 0.45 mg/ml) assay (Levieux et al., 2002; Leitner et al., 2004a).

2.3.3. Dye-binding technique

Bromocresol green dye-binding method is widely used in serum protein determination. Bovine serum albumin in milk has been previously determined colorimetrically using this technique (Guzman et al., 1986; Bouchard et al., 1999; Lieske et al., 2005). However, this technique has not been used for measuring CSA in milk. The principle of the method is that albumin binds anionic dye at pH 4.2, and absorption of the dye-albumin complex can be measured spectrophotometrically at 640nm (Lieske et al., 2005).

In comparison with other techniques, the dye-binding method is easy to perform, fast and reliable and can be utilized in screening large number of milk samples. However, it has been shown that BCG method gives slightly higher BSA results (0.50 to 1.10 mg/ml) probably due to the binding of dye with globulin (Guzman et al., 1986; Lieske et al., 2005) and therefore do not compare well with more specific immunological methods (0.15 to 0.28 mg/ml) for serum albumin determination (Sheldrake et al., 1983).

2.3.4. Electrophoresis

Polyacrylamide gel electrophoresis is commonly used as a qualitative technique for the separation and identification of whey proteins. However, the quantification of the separated protein bands or peaks is subjected to errors which make the method less accurate compared to immunological and dye-binding techniques (Darling and Butcher, 1975). This technique is based on the migration of different proteins in semi-solid media. Whey samples are loaded in the gel and then stained with Coomasie blue to visualize the protein bands.

Quantification of the separated proteins is done by scanning the stained protein-containing gels using a densitometer followed by peak area determination (Basch et al., 1985). The area under each peak represents a measure of protein in the gel and individual protein concentrations in samples are determined by comparison with the standard peak areas.

Polyacrylamide gel electrophoresis of whey proteins is too time consuming for rapid screening of large numbers of samples because it involves multi-steps of separation, staining, destaining, gel preparation and densitometric scanning. On the other hand, the technique is semi-quantitative and not very reproducible compared to ELISA and dye-binding techniques. The reliability of serum proteins concentrations obtained using PAGE depends on the accuracy with which the peak areas are measured and the accuracy with which the standards are calibrated

(Darling and Butcher, 1976). Reports in literature regarding CSA and BSA show that electrophoretic and ELISA methods are in good agreement (Stewalgen et al., 1994; Nudda et al., 2003; Leitner et al., 2004a; Piccinini et al., 2007). However, BCG method gives slightly higher BSA (0.50 to 1.10 mg/ml) values than those obtained by electrophoresis (0.25 to 0.60 mg/ml) or ELISA (0.12 to 0.48 mg/ml) (Guzman et al., 1986; Bouchard et al., 1999).

Table 2. Analytical techniques used in measuring serum albumin in milk

Methods	Principle	Sensitivity	Bovine serum albumin (mg/ml)	Caprine serum albumin (mg/ml)	Ovine serum albumin (mg/ml)	References
Single radial immunodiffusion	Immunoprecipitation	Sensitive	0.15 -0.28	0.35 - 0.45	-	Sheldrake et al.(1983), Levieux et al. (2002)
Electroimmuno diffusion	Immunodiffusion / Electrophoretic separation	Sensitive	0.18 -0.31	0.23 - 0.40	-	Babajimopoulos and Mikolajcik, (1977) , Sordillo et al.(1984)
Gel electrophoresis	Relative mobility of protein in media	Less sensitive	0.25 - 0.60	-	0.55 - 0.72	Nudda et al.(2003) Piccinini et al. (2007)
Dye-binding	Protein binding	Less sensitive	0.50-1.10	-	-	Guzman et al. (1986) , Bouchard et al.(1999)
Enzyme-linked immunosorbent assay	Antigen-anitody	Very sensitive	0.12 - 0.48	0.26 - 0.30	0.49 -0.55	Stewalgen et al. (1994) , Leitner et al. (2004a, 2004b)

2.3. Somatic cell counts in dairy goats

Milk somatic cells are primarily leukocytes (white blood cells) and some epithelial cells shed from the lining of the mammary gland. Leukocytes are derived from blood and consist of macrophages, lymphocytes, and polymorphonuclear (PMN) cells, primarily neutrophils (Harmon, 1994). Leukocytes provide defense mechanism used to fight infection and assist in repairing damaged tissues. Somatic cells are simply animal body cells present at low levels in normal milk. Leukocytes (in particular neutrophils) are massively recruited from blood into the gland as a result of an inflammatory response (Kehrli and Shuster, 1994). Somatic cell counts in cow's milk is widely used as a tool for indirect diagnosis of intramammary infections (IMI) and used as a milk quality indicator (Ostensoon et al., 1988; Leitner et al., 2000; Haenlein, 2002).

In Canada and the United States of America (USA), the legal milk SCC limit established for cows is 750,000 cells/ml and for goats and sheep is 1,000,000 cells/ml. In the European Union (EU) countries, the legal limit for cows is 400,000 cells/ml and there is no legal limit for sheep and goats (Paape et al., 2007). However, there has been considerable controversy on the relationship between SCC and IMI in goats (Smith and Roguinsky., 1977; Sheldrake et al., 1981; Manser, 1986; Paape et al., 2001). In general, SCC in milk of healthy goats (270 to 2,000 x 10^3 /ml) is higher than that found in milk of healthy cows (10 to 200 x 10^3 /ml cell) (Paape et al., 2001). This can be attributed to physiological differences in

milk secretion between the two species. Milk secretion in goats is an apocrine process resulting into the shedding of cytoplasmic particles and epithelial cells, apart from the leukocytes, into milk resulting into high SCC in goats compared to cows. Other non-infectious factors such as management practices, stage of lactation and parity contributes to elevated SCC in goats and need to be considered when establishing legal limits for goats (Dulin et al., 1983; Park and Humphrey, 1986; Paape et al., 2001, 2007). Somatic cell counts in bovine milk is indicative of the number of leukocytes in milk which increase in numbers with mastitis but caprine milk in contrast contains many non-leukocytic cell particles that are not related to mastitis (Haenlein, 2002).

Somatic cell counts ranging from 200,000 to 500,000 cells/ml of milk has been reported in cows with subclinical mastitis (Urech et al., 1999). However, mean SCC of 1,750,000 cells/ml and 2,358,000 cells/ml has been reported in mastitic goats and sheep respectively (Leitner et al., 2004a, 2004b). The composition of SCC in milk also differs between goats and cows. In healthy animals, PMN constitute 45 to 74% of SCC in caprine milk but only 5 to 20% in bovine milk. This is because neutrophils migrate at a faster rate into caprine milk than in bovine milk which in turn contributes to higher SCC in caprine milk (Contreras et al., 1997).

An increase in milk SCC is usually attributed to IMI at parturition (Moroni et al., 2005a, 2005b; Raynal-Ljutovac et al., 2007). However, breed, parity, stage of lactation, estrus and seasonal variations contribute

significantly to changes of SCC in milk of sheep and goats (Wilson et al., 1995; Gonzalo et al., 2002, 2005; Moroni et al., 2007). Paape et al. (2007) assessed the effects of stage of lactation and parity on milk SCC in 26,607 goats over a period of 5 years and found that SCC were lowest at first parity (200,000 cells/ml) at 15 days of lactation and reached maximum counts (500,000 cells/ml) at 285 days of lactation. However, by the fifth parity, mean SCC was (250,000 cells/ml) at 15 days of lactation and increased to a maximum of (1,150,000 cells/ml) at 285 days of lactation. Increase in milk SCC during lactation can be attributed to dilution effect, because milk production decreases as lactation progresses and milk SCC follows a linear increase throughout lactation (Paape et al., 2007). Wilson et al. (1995) reported that more than 90% of the variation in milk SCC in goats was not due to IMI. However, increasing days in milk (DIM) and month of the year contributes significantly to increased cell counts in the absence of IMI. Studies on the influence of estrus on SCC in Saanen goats revealed that maximum levels of SCC (7,000, 000 cells/ml) were observed at day of estrus and then decreased sharply to (2,000,000 cells/ml) at day 10 after estrus (Moroni et al., 2007). The increase in SCC at estrus was attributed to the estrogenic action that characterizes estrus period.

California Mastitis test (CMT) scores, Coulter Counter and Fossomatic machines, Pyronin Y-methyl green stain direct microscopic method, NAGase and antitrypsin values have been successfully used to

estimate SCC (Haenlein, 2002). Poultrel and Lerondelle (1983) reported that 219 normal caprine milk samples had 58% negative CMT scores despite being bacteriologically negative. However, Coulter Counter tests gave 1.4 million SCC and with Fossomatic, 614,000 SCC for the same negative samples. This shows that the prediction of goat udder infection using total SCC may be unreliable unless the SCC testing equipment is calibrated with caprine milk (Zeng et al., 1999).

2.3.1. Effects of Somatic cell counts on milk yield and composition

Changes occur in milk yield and composition with increasing SCC in goats and cows due to injury of udder cells which reduces the synthesis of milk constituents and cause changes in permeability of membranes and interstitial spaces that increases the passage of components from blood to milk (Raynal-Ljutovac et al., 2007). Baudrey et al. (1997) reported that goats' milk with high SCC (> 1,750,000 cells/ml) had reduced milk (641 kg) and fat yields (31.6 g/kg) and increased total protein yield (30.1 g/kg) compared to low cell counts milk (< 750,000 cells/ml) with milk (771 kg), fat (31.9 g/kg) and protein (28.6 g/kg) yields respectively. The increase in protein can be attributed to breakdown of the blood-milk barrier caused by inflammation of the udder resulting into increased movement of serum components into milk (Paape et al., 2001). Other authors showed that SCC (1,750,000 cells/ml) did not affect fat content of goat milk (Pasquini et al., 1996; Ying et al., 2002; Leitner et al., 2004a). Pisoni et al. (2004)

reported lower fat content in goat milk infected with *Staphylococcus aureus* (4.65 million cells/ml milk) compared to non-infected milk (1.03 million cells/ml milk).

Increase in the concentration of proteins from blood during mastitis leads to an increase in the concentration of soluble whey proteins (Nudda et al., 2003; Albenzio et al., 2004). Jaubert et al. (1999b) collected goats' milk samples from 18 herds during one lactation and divided them into two groups having on average, SCC of 710,000 and 2,332,000 cells ml and observed an increase of whey proteins with an increase of SCC (0.41 g/l for low SCC and 0.55 g/l for high SCC). In bulk tank goats' milk collected at three lactation periods over two years, milk with high SCC (> 1,800, 000 cells/ml) had high concentrations of soluble proteins (8.2 g/kg) compared to low SCC (< 700,000 cells/ml; 6.5 g/kg soluble proteins) but there was no change in casein content (Morgan and Gaspard,1999).

Studies on subclinical mastitis in goats' revealed that milk with high SCC (1,750,000 cells/ml) had reduced lactose concentrations (4.17%) compared to low SCC milk (417,000 cells/ml; 4.7% lactose concentration) (Leitner et al., 2004a). The decrease in lactose concentrations in goats' milk as a result of increased SCC can be attributed to decrease in the synthetic function of the mammary gland and consequent flow of minerals from blood to milk to maintain osmotic equilibrium (Zeng and Escobar., 1996a; Jaubert et al., 1996b). An increase in the concentrations of sodium, chloride in milk and decrease in potassium concentrations in milk

has been associated with an increase of SCC in goats. Sodium concentrations (0.538 g/l and 0.351g/l) have been reported for high and low SCC goats' milk respectively (Morgan and Gaspard, 1999). Potassium is believed to leak out of milk while sodium present in blood in high levels leaks into milk through ruptured mammary epithelial during mastitis (Pirisi et al., 2000).

Table 3. Effects of somatic cell counts on caprine milk composition

Milk constituents	Effect	References
Protein	↑	Ying et al. (2002)
	↓	Pisoni et al. (2004)
Lactose	↓	Jaubert et al. (1996)
Fat	↓	Pisoni et al.(2004)
Total solids	-	Jaubert et al. (2004)
Casein	-	Pizzilo et al.(1996)
Whey protein	↑	Leitner et al. (2004)
Immunoglobulin	↑	Jaubert et al. (1996)
Sodium	↑	Morgan and Gaspard (1999)
Chloride	↑	Morgan and Gaspard (1999)
Potassium	↓	Ying et al. (2002)

3. MATERIALS AND METHODS

3.1. REAGENTS

All reagents were of analytical grades and purified water from Milli-Q system was used throughout the experiment.

3.1.1. Bromocresol green dye stock solution

The BCG stock solution was prepared by mixing 4ml of 10.4% NaOH, 0.50g of BCG dye (Sigma Chemical Co. St. Louis, MO. USA), 10ml of Tween 20, 30ml of 85% lactic acid and then completed to 1l with de-ionized water and the pH was adjusted to 4.0 by drop-wise addition of 0.1N NaOH. Bromocresol green stock solution stock solution was stored in closed amble bottle at 4°C and remained stable for 3 months. The BCG working solution was obtained by further dilution with de-ionized water (1:5) before addition to whey.

3.1.2. Citric acid - Phosphate buffer

An equal portion of 0.05% citric acid and 0.09% bisodium hydrogen phosphate was mixed to constitute a buffer at pH 6.5, to simulate the serum phase of milk and accommodates adjustment in pH.

3.2. INSTRUMENTATION

ELx800™ Absorbance Microplate Reader (Bio-Tek Instruments Inc, Vermont, and USA) equipped with tungsten halogen bulb as light source

and four standard filters and Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments Inc, Vermont, USA) were used in the study. The readers have wavelength range of 380 to 900nm, dynamic range of 0.1 to 4.0 optical density (OD) and a reading speed of 12 seconds regular mode, 8 seconds single wavelength rapid mode and 13 seconds dual wavelength rapid mode.

3.3. SAMPLE PREPARATION

Whey was prepared from milk by acid precipitation using acetic acid and sodium acetate followed by centrifugation at 2,000 x g, for 15 min at 21°C according to AOAC (1999). Supernatant (whey) were further defatted by centrifugation for another 15 min at the same speed and stored at - 20°C until analyzed for serum albumin. Skim milk was prepared by centrifugation at 10,000 rpm for 30 min at 4°C.

3.4. CAPRINE SERUM ALBUMIN STANDARD CURVE

Purified lyophilized caprine albumin (Sigma Chemical Co. St. Louis, MO.USA) was dissolved in citric acid buffer to prepare CSA standard. The CSA standard curve was daily prepared by diluting 8.0mg/ml CSA standards to 6.0, 4.0, 2.0, and 1.0 mg/ml with de-ionized water). One ml of BCG working solution was then added to 200µl of each diluted standard in a 48-clear flat bottom well, incubated for 3min at room temperature and absorbance was read at 640nm against a blank (deionized water). The

optical density of blank was subtracted from standards values to obtain real sample optical density. CSA standard curve was obtained by plotting absorbance values of standards against logarithm of their respective albumin concentrations and the CSA concentrations in milk samples were calculated from the linear equation of the curve.

To assess the stability of the developed BCG method, standard curves were consecutively generated 10 times within 2 weeks and the relative standard deviation (RSD) of the measured absorbance in 4 replicates at each calibration point on 10 curves (range of 0.1 to 8 mg/ml) was determined.

3.5. VALIDATION OF THE PROCEDURE

3.5.1. Intra-assay and Inter-assay precisions

The repeatability and reliability of the BCG method was evaluated by performing repeated measurements on milk samples (n=8) within one day at different dilutions using same reagents and assay performed under same analytical conditions. Hundred micro liter of acid-precipitated whey (1:4 dilutions with deionized water) was used for all samples. The variability of the method was further tested by repeating assay on 10 duplicates of milk samples (n=4) for 4 successive days under the same conditions to assess between-batch variation. In a parallel approach, assay was performed on milk samples (n=4) at different dilution rates to assess how parallel the responses were to the standard curve.

3.5.2. Spiking and Recovery

Caprine milk samples (n=8) were spiked with albumin standards at concentrations of 1.0, 2.0, 3.0, and 4.0 mg/ml. The spiked milk samples were analyzed by the modified BCG method. The quantitative recovery of added CSA were calculated and expressed as percentage CSA recovered divided by amount added.

3.6. APPLICATION OF THE DYE-BINDING METHOD

Individual milk samples (n=53) from 6 healthy Saanen goats (same age and parity) in early lactation were collected weekly over a period of 70 days and assayed for CSA to test the utility of the developed BCG method.

To further test the utility of the proposed BCG method and to correlate CSA with milk constituents, individual milk samples (n=74) were collected from lactating Saanen goats at three different stages of lactation and were tested for serum albumin in milk, SCC, fat, protein, lactose, total solids, casein, and whey. Animals were 1 to 9 years old with an average milk production of 1000 liters per goat (300 days of lactation). Goats were milked less frequently to induce drying-off of their glands before we collected milk samples for this study.

Milk samples were analyzed for fat, protein, lactose concentrations and SCC using a Milko Scan Infrared analyzer (Model: Foss 4000, Foss Food Technology, Denmark) calibrated for caprine milk at the Quebec

dairy production centre of expertise (Valacta), Sainte Anne de Bellevue, Quebec, Canada . Total solids, caseins and whey in milk were analyzed according to the procedures of AOAC (1999) with the exception that N in milk and whey was determined using TruSpec Nitrogen Determinator (Model: 630-100-200 Leco Corporation, USA).

3.7. STATISTICAL ANALYSIS

All validation studies were repeated two times and all measurements were carried out at least in duplicates. Repeatability, reproducibility, averages, standard deviations, coefficients of variation and correlation coefficients were determined using Microsoft Excel 2003.

Caprine serum albumin data were analyzed as repeated measures using PROC MIXED procedure of SAS (2003) with the following model:

$$Y_{ij} = \mu + D_i + G_j + D_i * G_j + eij$$

where Y_{ij} = the observations for the dependent variables,

μ= overall mean

D_i = the effect of i^{th} days in milk

G_j = the random effect of j^{th} goat

$D_i * G_j$ = the effect of interaction between days in milk and goat

eij = the residual error

Normality of distribution for serum albumin was assessed using the Shapiro-Wilk test. Multiple comparisons of the serum albumin concentrations were made using Scheffe's Test.

Effects of sample pre-treatments and incubation time were tested using the Student's t-test. Differences were considered significant at $P < 0.05$. Values of SCC were log transformed before statistical analysis to enhance linearity.

Pearson correlation coefficients between CSA and other milk components and their associated probabilities were determined using PROC CORR of SAS (2003).

4. RESULTS AND DISCUSSION

4.1. METHOD DEVELOPMENT AND VALIDATION

4.1.1. Dye-binding assay

The quantitative parameters for the determination of CSA in milk using the proposed BCG method are summarized in Table 4. The CSA concentrations (0.31 to 0.52 mg/ml) in our study agree with those reported in literatures for CSA using SRID (0.35 to 0.45 mg/ml: Levieux et al., 2002). However, our values are higher than those obtained using ELISA (0.26 to 0.30 mg/ml .Leitner et al., 2004a).

The proposed BCG method showed acceptable linearity, accuracy and precision for the determination of CSA in milk. For routine screening of milk samples for serum albumin concentrations, the BCG method can be a simple and rapid alternative. However, immunological methods may be more suitable for confirmatory serum albumin test owing to their sensitivity and specificity. The preparation of antibodies and long incubation time makes SRID and ELISA time consuming and technically demanding. Using the BCG method, the run time for a sample was less than 5 minutes after sample preparation.

Table 4. Quantitative parameters for the determination of caprine serum albumin using bromocresol green dye

Parameter	BCG
pH	4.0
Color	Yellow – Blue / Green
Detergent	Tween-20
Specificity	Non-specific
Wavelength	640nm
Incubation time	3 min
Assay temperature	Ambient
Storage temperature	4°C
Buffer	Citric acid-Phosphate
Response function	Linear
Shelf-life	3 months
Signal stability	> 60min
Reagent purity	Over 95%
Dilution factor	1: 4
Regression equation	y = mx + c
Correlation coefficient	0.99

y = mx + c where x is the caprine serum albumin concentrations in mg/ml

4.1.2. Caprine serum albumin standard curve

A typical standard curve utilized in the method development is presented in Figure 1. A linear relationship was obtained when absorbance values were plotted against the logarithm of CSA concentration (0.1 to 8.0 mg/ml) and the correlation coefficient for the linear equations exceeded 0.99. Parallel results were obtained when diluted whey samples were assayed using the BCG method (Figure 2). The RSD of measured absorbance in 10 standard curves at each calibration point was within 10%. Purified lyophilized caprine albumin was used as a standard to prevent overestimation of the results. In our study, a combination of citric acid and sodium phosphate provided a buffer system suitable for use as a diluent for CSA standard. Other studies have used the Jennes and Koops buffer, pH 6.7, in preparing BSA standard for BCG method (Lieske et al., 2005). However, it involves the addition of six chemicals (Macasek et al., 1996). The calibration of the BCG method is therefore best performed in a buffer at natural pH of milk (i.e. 6.3 to 6.7: Lieske et al., 2005). The use of dairy-based diluents for caprine albumin standard in this study resulted in incomplete recovery of albumin. In line with our results, the dispersion of bovine albumin standards in dairy-based diluents reduced analytical recovery of BSA from spiked milk and overestimated BSA concentrations in milk (Lieske et al., 2005). This confirms a high degree of similarity in the biophysical properties of BSA and CSA in milk.

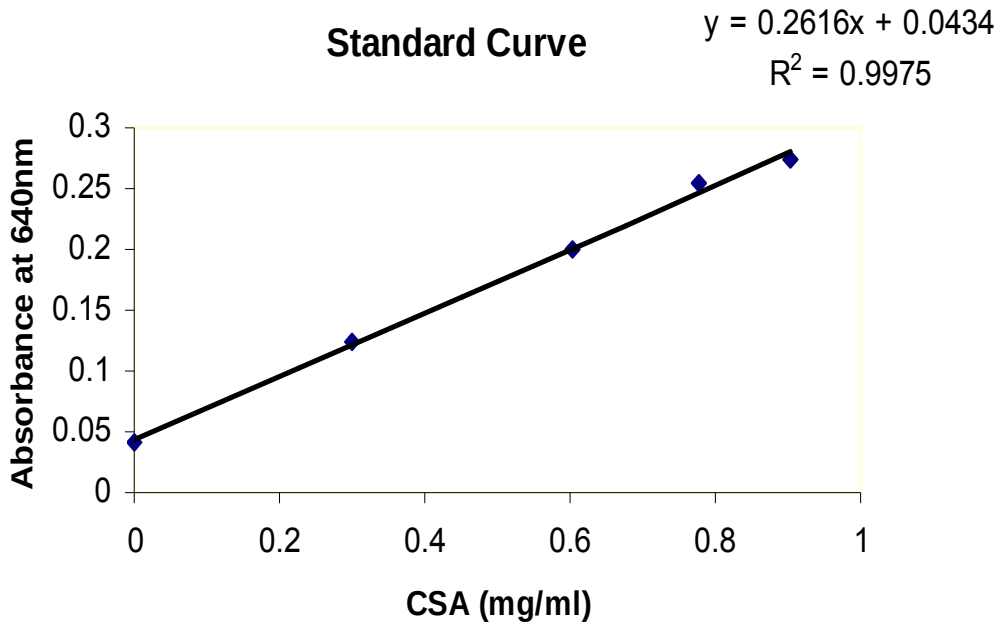

Figure 1. Caprine serum albumin standard curve

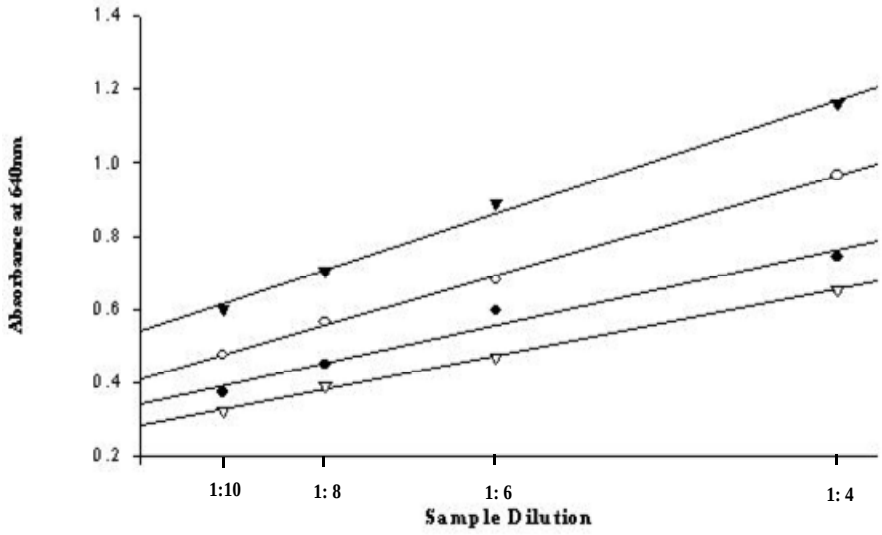

Figure 2. Curves for dilutions of whey samples parallel to the standard curve

4.1.3. Effects of sample treatments on the determination of Caprine serum albumin in milk

Effects of sample preparation on the accuracy of the BCG method are presented in Table 5. Validation of the BCG method showed that CSA is best estimated from whey substrate after acid-precipitation and double centrifugation to remove fat and cells. The CSA values in our study were found to be significantly higher ($P < 0.05$) in skimmed milk substrate than in whey. In agreement with our findings, Lieske et al. (2005) obtained higher BSA values in skimmed milk than in whey. Higher CSA values in skimmed milk may be due to interference from natural milk proteins. Serum albumin may bind with fatty acids undermining the accuracy of the assay when whey substrate is used (Perez et al., 1993; Friedli, 1996).

There was no detectable color change when BCG assay was performed on undiluted skim milk substrate. It appeared that the analytical availability of CSA was best in whey substrate but different CSA values in diluted and undiluted whey substrates indicated the need for further investigations. Following repeated measurements of CSA at different dilutions and pH adjustment, a dilution factor of 1:4 proved sufficient to obtain reliable CSA estimates. Pre-dilution of whey with de-ionized water did not produce conformational re-arrangement of whey proteins. However, dilution effects were accounted for in the calculation of CSA values.

Further results in our study showed no evidence of co-precipitation or co-sedimentation of CSA with casein during acidification. However, previous studies with bovine milk revealed that BSA may partially precipitate with casein during acidification due to the proximity between the isoelectric points of serum albumin and caseins (Walstra et al., 1984). However, whey samples prepared by acid precipitation or ultra-centrifugation have been reported to contain similar amounts of BSA when enzyme immunoassay was used (Shuster and Harmon, 1990). In general, the opaque nature of milk and its turbidity makes treatment necessary before analysis.

Table 5. Effects of sample treatments on the accuracy of the determination of serum albumin in caprine milk (n=8).

Substrate	Mode of preparation	CSA (mg/ml)
Whey (undiluted)	33% Acetic acid, 2,000 x g, 15min at 21°C	0.286 ± 0.034
Whey (diluted)	33% Acetic acid, 2,000 x g, 15min at 21°C	0.176 ±0.008*
Skimmed milk(undiluted)	10,000 rpm, 30 min at 4°C	No color change
Skimmed milk (diluted)	10,000 rpm, 30 min at 4°C	0.675 ±0.161*

Values are presented as mean ± standard deviation. *$P < 0.05$.

4.1.4. Effects of incubation time on bromocresol green dye- binding assay

There was no significant difference ($P > 0.05$) in CSA when absorbance was read 3 or 60 min after incubation. However, the non-specific binding of the BCG dye to immunoglobulins in milk was minimized by shortening the incubation time to 3 min. Webster (1977) showed that the binding of BCG to albumin occurred in less than 30 seconds after the dye addition. However, color intensity increased with time due to the binding of BCG to serum globulin. Overall, serum albumin reacts faster with BCG dye than globulins.

There is the possibility of species difference in reactivity of BCG dye to serum albumin in milk. The dye-binding method of measuring serum albumin has been suggested to be non-specific as evident in the varied reaction of BCG dye with albumin-depleted sera from ovine and bovine (Keay and Doxey, 1984).

The formation of CSA/dye complex (color development) over time depends on the immune defense reactions in the mammary gland from which milk is collected, the concentration of immunoglobulins in milk and on the protein-protein interactions between CSA and immunoglobulins (Lieske et al., 2005). However, these interferences may be minimized by the separation procedures and thermal treatments applied to milk before the actual analysis.

4.1.5. Spiking and recovery

The analytical recovery of added CSA was 89.15 ± 7.6% (Table 6) and was lower than the recovery reported for BSA using BCG method (98.8 ± 1.5%: Lieske et al., 2005). However, the mean recovery in our study fall within the range (80 to 110%) recommended by (AOAC, 1993) for substances greater than 0.1mg/ml. The RSD for all measurements (n=8) was within 10%. Response to the additions of CSA was linear and the correlation coefficient between the CSA recovered and added was 0.97± 0.02.

The good recovery of albumin suggests minimal interference from other serum or milk proteins and confirms the colorimetric method as a reliable technique to quantify CSA. This also suggests minimal molecular and structural changes of serum albumin when binding with dye and further proves that serum albumin is completely dispersed in the dye and ready to react with the BCG reagent. On the other hand, the incomplete recovery of added CSA at certain concentrations may be due to the aggregation of CSA with the natural immunoglobulin in milk (Lieske et al., 2005).

Table 6. Accuracy and precision of the dye-binding method

Method	Intra-assay Precision	Inter-assay Precision	Parallelism	Recovery
BCG	2.16%	12%	Yes	89.15 ± 7.6%

4.1.6. Intra and inter-assay precisions

Intra-assay (2.6%) and inter-assay (12%) coefficients of variations (CV) in our study (Table 6) were within acceptable limits required to validate a bioanalytical method (AOAC, 1993) and demonstrates that the proposed BCG method can generate reproducible results and shows quantitative accuracy and precision. The relatively high inter-assay precision is likely due to molecular-structural changes of CSA in the dye (Lieske et al., 2005). After re-validation, the proposed BCG method was used to measure serum albumin in milk samples collected from lactating goats.

4.2. CAPRINE SERUM ALBUMIN CONCENTRATIONS IN MILK DURING EARLY LACTATION

Concentrations of CSA in milk collected from goats in early lactation (7 to 70 days in milk) ranged between 0.31 and 0.52 mg/ml (Figure 3). Concentrations of CSA declined ($P < 0.05$) rapidly between day 7 and day 17 in lactation and remained stable between days 17 and 66. The CSA values in our study agree with those of Sordillo et al. (1984), Belewu and Adewusi (2001) and Leitner et al. (2004a) who reported values ranging between 0.26 and 0.56 mg/ml for healthy goats.

Serum albumin concentration in milk of healthy animals is usually very low and it enters the milk from the systemic fluid by leaking through the epithelial tight junctions (De Wit, 1998). During lactation, the tight junctions do not allow blood constituents other than small ions to pass

between the epithelial cells (Anderson et al., 1985). However, tight junctions may become leaky during colostrogenesis when the gland transit from involution to lactogenesis and at late lactation during physiological change from lactation to involution (Sordillo et al., 1984, 1987) resulting in increased paracellular transport. High intramammary pressure induced by cessation of milking at late lactation may also ruptures blood-milk barrier, thereby increasing paracellular permeability (Anderson et al., 1985).

Serum albumin concentration in milk also increases during inflammations in the mammary gland (Kehrli et al., 1994). The enhanced influx of leukocytes through the epithelium due to inflammatory response of the mammary gland to infection causes reduced tight junction integrity (Stewalgen et al., 1999) and hence exchange of constituents between blood and milk.

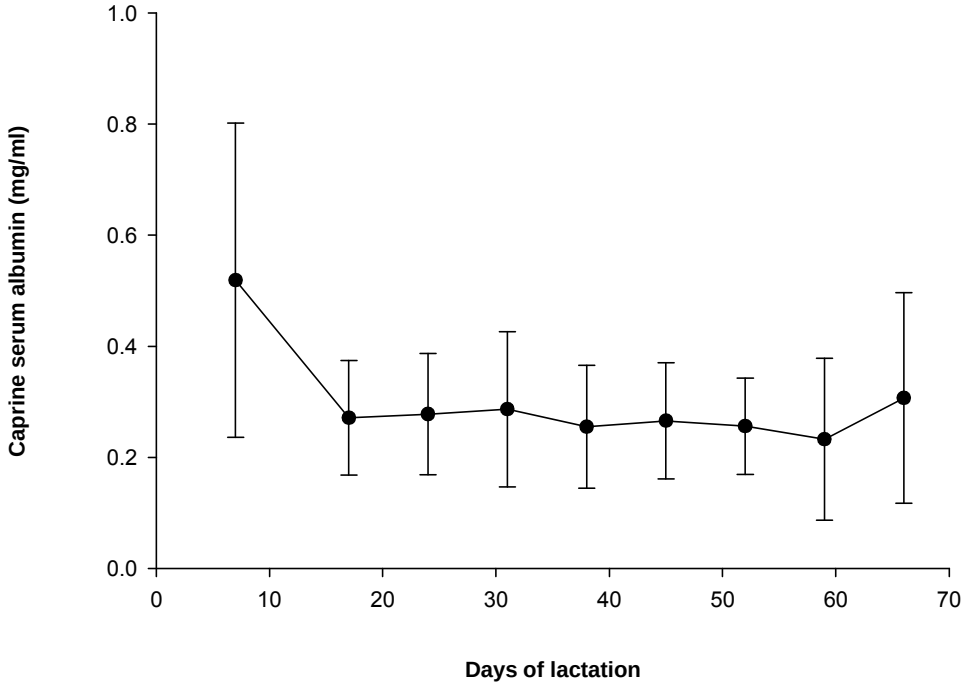

Figure 3. Caprine serum albumin concentrations in lactating Saanen goats' milk. Values are means ± standard deviation (n=6 goats at each time).

The relatively high CSA concentration at day 7 in our study may be due to the residual effect from parturition during which the tight junction is leaky allowing the movement of blood solutes and leukocytes into the gland and closes back at the beginning of lactation (Neville and Nguyen, 1988). This is consistent with the findings of Sordilo et al. (1984) in which CSA gradually declined from 0.64 mg/ml at day 7 to 0.23 mg/ml at day 30 of lactation. Bovine serum albumin in milk shows same pattern, high at parturition (1.21 ± 0.44 mg/ml) and then gradually decreased to (0.20 mg/ml) at day 8 of lactation (Sordillo et al., 1987; Levieux and Ollier,

1999). In general, protein concentrations is highest in colostrum during the first few days post partum and decrease thereafter until mature milk is secreted (Kunz et al., 1999).

Apart from physiological and pathological factors, the discrepancy in CSA values reported may also be due to differences in analytical techniques owing to the fact that immunodiffusion technique and ELISA were used in the compared studies. The CSA trend detected by the dye over the 70 days of lactation proves that the BCG method is valid and adequate to determine serum albumin concentrations in caprine milk during established lactation.

4.3. RELATIONSHIP BETWEEN CAPRINE SERUM ALBUMIN AND MILK CONSTITUENTS.

Caprine serum albumin concentrations in milk, SCC and milk composition of Saanen goats (n=73) are presented in Table 7. The mean CSA concentration was 0.32 ± 0.16 mg/ml, the lowest value was 0.18 mg/ml and the highest value was 1.17 mg/ml. The mean value agree with those reported for healthy goats (0.279 ± 0.022 mg/ml: Leitner et al., 2004a). Caprine serum albumin correlated positively ($P < 0.05$) with SCC, protein, fat, whey and total solids. However, lactose and casein correlated negatively ($P < 0.05$) with CSA. The correlation coefficients between CSA and milk constituents are presented in Table 8.

The positive correlation between CSA and SCC (r = 0.402, $P < 0.05$) is in agreement with those in literatures which report increased serum albumin concentrations (0.47 ± 0.05 mg/ml, 0.76 ± 0.06 mg/ml) in subclinically mastitic milk (high SCC: 1,750,000 cells/ml, 2,358,000 cells/ml) for goats and sheep respectively (Leitner et al., 2004a, 2004b). However, in the same study, low SCC milk (417,000 cells/ml, 270,000 cells/ml) had lower serum albumin concentrations (0.28 ± 0.02 mg/ml, 0.52 ± 0.03 mg/ml) in goats and sheep respectively. Similar findings have also been reported for bovine milk (Nagamoto et al., 1996).

The positive association between CSA and SCC could be attributed to the leakage of serum protein from the interstitial fluid into milk during leukocyte transmigration (Fernandes et al., 2004). Milk accumulation in the udders due to the drying-off process practiced in the herd may have contributed to the elevated SCC and CSA (Raynal-Ljutovac et al., 2007). During drying-off, the feed back inhibition of lactation and the absorption of water in the gland attract more leukocytes into the milk. High Intra-alveolar pressure due to excessive accumulation of milk may disrupt the tight junctions by stretching, thereby causing leakage of serum proteins into milk (Mepham, 1987). This hypothesis is strengthened by the positive correlation between CSA and protein (r = 0.84, $P< 0.05$); SCC and protein (r = 0.34, $P< 0.05$). In the absence of established mammary inflammation, milk constituents related to inflammations and innate immune defenses may be poorly related to SCC (Piccinini et al., 2007). The integrity of the

epithelial tight junction therefore determines the movement of leukocytes and serum components from blood into milk (Stelwagen et al., 1999).

Somatic cell counts in caprine milk in our study averaged 1,414,750 cells/ml (Table 7) which is within the values reported for healthy goats (De Cremoux, 1995; Haenlein, 2002; Byeng et al., 2007). A high SCC is considered normal in goat. However, milk with SCC above 2,000,000 cells/ml may be considered subclinically mastitic (Leitner et al., 2004a). Somatic cell count in healthy goats (270,000 to 2,000,000 cells/ml) is much higher than in healthy cows (10,000 to 200,000 cells/ml) and ewes (270,000 cells/ml) (Paape et al., 2001). This is likely due to the shedding of cytoplasmic particles and epithelial cells into milk during milk secretion (Byeng et al., 2007).

In our study, protein, fat, total solids and whey positively correlated ($P < 0.05$) with CSA. No previous data regarding direct relationship between caprine serum albumin and milk constituents were found in literature. However, Leitner et al. (2004a) reported higher protein(3.5 ± 0.05%) and whey (0.68 ± 0.04 %) contents in milk with high CSA (0.47± 0.05 mg/ml) compared to milk with low CSA (0.28 ± 0.02 mg/ml, protein 3.4± 0.05%; whey 0.61 ± 0.03%) which agrees with our results. This can be attributed to the leakage of serum proteins from blood into milk (De Wit, 1998).

Table 7. Statistical description of variables used in the correlation study (n = 73).

Variables	Mean	SD	Minimum	Maximum
CSA (mg/ml)	0.32176	0.15786	0.18434	1.17393
SCC (Log/ml)	6.15068	0.63838	5.0000	7.0000
SCC (10^3)	1,415	4.34891	100	10,000
Protein (%)	3.26973	0.77282	2.04000	6.4900
Fat (%)	2.67822	0.64704	1.62000	4.42000
Lactose (%)	3.79370	0.75846	0.35000	4.97000
Total solids (%)	10.32494	1.19849	6.38965	14.23292
Casein (%)	2.82439	0.64573	1.65180	5.44659
Whey (%)	0.44534	0.24842	0.11752	1.17915

SD- Standard Deviation

The negative correlation (r= -0.582, $P < 0.05$) between lactose concentration and CSA in our study is consistent with reports in goats (Leitner et al., 2004a; Byeng et al.,2007) , sheep (Leitner et al., 2003) and cows (Rogers et al., 1989; Klei et al.,1998; Fernandes et al., 2004). This can be attributed to decrease in the synthesis function of the goats' mammary gland (Raynal-Ljutovac et al., 2007) and probably due to the passage of lactose from milk into blood via leaky tight junctions (Fernandes et al., 2004).

Table 8. Correlation coefficients between Caprine serum albumin and milk constituents.

n=73	CSA (mg/ml)	SCC (log/ml)	Protein (%)	Fat (%)	Lactose (%)	TS (%)	Casein (%)	Whey (%)
CSA	1.00							
SCC	0.40**	1.00						
Protein	0.84**	0.34*	1.00					
Fat	0.31*	0.22	0.32**	1.00				
Lactose	-0.58**	-0.48**	-0.46**	0.16	1.00			
TS	0.50**	0.01	0.68**	0.66**	0.20	1.00		
Casein	-0.25*	0.03	-0.17	-0.15	0.12	-0.17	1.00	
Whey	0.25**	-0.03	0.17	0.15	-0.12	0.17	-1.00	1.00

*(*P< 0.05, **P <0.01)*

The negative association between casein concentrations and CSA in our study may be due to reduced secretion and increased proteolysis of caseins by blood-borne protease such as plasmin (Politis et al., 1992).

Somatic cell count correlated positively (r= 0.34, *P*< 0.05) with protein and negatively (r = - 0.48, *P* < 0.05) with lactose which is in agreement with previous reports in goats (Ying et al., 2002; Byeng et al., 2007). Increase in the flow of systemic fluids into milk during leukocyte transmigration may be responsible for the positive association between SCC and protein (Fernandes et al., 2004).

The decreased lactose concentration associated with high SCC can be attributed to decreased lactose synthesis in the mammary gland (Raynal-Ljutovac et al., 2007).

Correlations between SCC and fat, total solids, casein and whey were not significant ($P > 0.05$). In contrast, high SCC in caprine milk has been associated with decreased fat percent (Pisoni et al., 2004), increased whey (Leitner et al., 2004a) and no significant effect on total solids (Jaubert et al.,1996) and casein (Pizzilo et al., 1996).

5. CONCLUSION

The described BCG assay shows a high degree of accuracy and precision and can be reliably used as a routine technique for the determination of serum albumin concentrations in milk of goats under any physiological conditions. However, sample pre-treatment, pH of dye, ionic strength of buffer, incubation time and dilution rate are the critical factors in the procedure. The proposed BCG method is easy to perform, rapid, sensitive, inexpensive and provides reproducible results. Application of the technique in determining CSA in milk revealed that healthy caprine milk contains approximately 0.31 to 0.52 mg/ml CSA depending on the physiological status of goats. Serum albumin concentrations in milk tend to be high at the beginning of lactation, declines gradually and remain relatively stable in mature milk. The positive association between CSA and SCC can be attributed to the leakage of serum protein from the interstitial fluid into milk during leukocyte transmigration.

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