Journal of Dairy Research (2012) **79** 495–501. doi:10.1017/S0022029912000441

Direct estimation of sialic acid in milk and milk products by fluorimetry and its application in detection of sweet whey adulteration in milk

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Received 24 November 2011; accepted for publication 10 July 2012

Sialic acid, being a biologically active compound, is recognised as an important component of milk and milk products. Almost all the sialic acid estimation protocols in milk require prior hydrolysis step to release the bound sialic acid followed by its estimation. The objective of this work was to estimate sialic acid in milk and milk products by fluorimetric assay which does not require a prior hydrolysis step thus decreasing the estimation time. The recovery of added sialic acid in milk was 91·6 to 95·8%. Sialic acid in milk was found to be dependent on cattle breed and was in the range of 1·68–3·93 g/kg (dry matter basis). The assay was further extended to detect adulteration of milk with sweet whey which is based on the detection of glycomacropeptide (GMP) bound sialic acid in adulterated milk. GMP is the C-terminal part of κ-casein which is released into the whey during cheese making. For detection of adulteration, selective precipitation of GMP was done using trichloroacetic acid (TCA). TCA concentration in milk was first raised to 5% to precipitate milk proteins, especially κ-casein, followed by raising the TCA concentration to 14% to precipitate out GMP. In the precipitates GMP bound sialic acid was estimated using fluorimetric method and the fluorescence intensity was found to be directly proportional to the level of sweet whey in adulterated milk samples. The method was found to detect the presence of 5% sweet whey in milk.

Keywords: Sialic acid, Fluorimetry, Milk, adulteration, Sweet whey, Glycomacropeptide.

Sialic acids are substituted neuraminic acid derivatives and to date more than 50 members of sialic acid family have been identified (Spichtig et al. 2010). The main representative forms of sialic acid are *N*-acetylneuraminic acid (NeuNAc) and *N*-glycolylneuraminic acid (Salcedo et al. 2011). In milk sialic acid is found in four different forms; lipid-bound, protein-bound, oligosaccharide-bound and as free sialic acid (Wang et al. 2001). Sialic acids are bioactive compounds and their role in infant nutrition for brain development has been emphasised (Wang & Brand-Miller, 2003) as large amounts of this carbohydrate are found in the brain and in the central nervous system in the form of gangliosides and glycoproteins contributing to the functioning of cell membranes and membrane receptors. There are many methods available for the analysis of sialic acids, and they have been reviewed recently (Lacomba et al. 2010). Classically, sialic acid determination in biological samples has been carried out by spectrophotometry involving the nonspecific reaction between sialic acid and the various reagents like resorcinol, thiobarbituric acid (Warren, 1959; Aminoff, 1961; Romero et al. 1997) and the enzyme coupled assay (Sugahara et al. 1980; Teshima et al. 1988). HPLC based methods have also been employed for the estimation of sialic acid in biological samples (Martin et al. 2007; Spichtig et al. 2010; Lacomba et al. 2011). All of these methods require the release of sialic acids before analysis either by acid or enzymatic hydrolysis. Recently, Matsuno & Suzuki (2008) described a simple fluorimetric method (FluA) for quantification of sialic acid in glycoproteins in which sialic acid can be estimated even in the bound form. In present study, the method has been applied for the estimation of sialic acid in skim milk, commercial GMP, whey protein concentrate (WPC), whey protein isolate (WPI), and milk based infant formula.

Industry has shown increasing interest in the use of dried cheese whey as a partial substitute for non-fat milk solids in ice-cream and related desserts (Josephson et al. 1980). Being a cheap dairy by-product, adulteration of milk with whey

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becomes economically attractive. Such practice makes market competition unfair and defrauds the consumer (de Carvalho et al. 2007). The most frequently used method to detect cheese whey adulteration is the identification of GMP which is present in the cheese whey but not in milk (Chavez et al. 2008). Various techniques used for the detection of GMP are colorimetric (Koning et al. 1966; Josephson et al. 1980), chromatographic (Saito et al. 1991; Thoma et al. 2006; Fernandez et al. 2011), immunological (Bremer et al. 2008; Chavez et al. 2008; Martin-Hernandez et al. 2009), electrophoretic (Cherkaoui et al. 1997; Nakano et al. 2007) and by use of biosensors (Spreeta, Taxas Instruments, Attleboro, MA). As GMP contains bound sialic acid in its structure, the fluorescence based sialic acid estimation method was further applied for the detection of GMP in milk with a view to detect adulteration of milk with sweet whey.

Materials and Methods

Unless otherwise specified cow milk samples were used. Pooled raw cow and buffalo milk samples were collected from Institute's cattle yard in pre-cleaned glass bottles. Rennet (meito rennet-*Mucor meihei*, type II) and NeuNAc were purchased from Sigma Aldrich Inc. St. Louis, USA. Other chemicals were of analytical grade and were purchased from Merck Specialties Pvt. Ltd, Mumbai, India. A sample of milk-based infant formula was obtained from the local market. The WPC and WPI were obtained as gift from Modern Dairies, Karnal. Two commercial GMP samples (designated as GMP I and GMP II) were obtained as gift from Davisco Food International, USA and Arla Foods, Denmark.

Fluorimetric method of sialic acid in milk and milk products

The FluA method described by Matsuno & Suzuki (2008) for the estimation of sialic acid in glycoprotein was adapted for milk with slight modifications. Milk samples were skimmed (4000 ***g***, 10 min, 4 °C) and then diluted 8 times with distilled water and used directly for further analysis, while other milk products (commercial GMP preparations, WPC, WPI, and infant formula) were dissolved in distilled water to the final concentration of 200 µg/ml (w/v). All the reagent solutions were precooled in an ice bath before use. Sixty microlitres of freshly prepared sodium periodate (10 mM) was added to 600 µl of prepared sample taken in a 10 ml polypropylene test tube. The tube was placed in ice-bath for 45 min and then 300 µl sodium thiosulphate (50 mM) was added to terminate the reaction. Then, 1·5 ml ammonium acetate (4·0 M, pH 7·5) and 1·2 ml 50% ethanolic solution of acetoacetanilide (100 mM) were added and the tubes were incubated at 25 °C for 10 min. The fluorescence intensities of the content were measured in fluorimeter (Cary Eclipse, Varian) using excitation and emission wavelength at 372 and 471 nm, respectively. The above mentioned protocol was followed for the estimation of total sialic acid in skim milk samples. For the estimation of protein bound sialic acid, the milk samples were dialysed with 3500 Da cut off cellulose dialysis membrane (Fisherbrand) using Milli Q water at 4 °C for 24 h with frequent changes of water. Protein bound sialic acid in milk sample was estimated by measuring sialic acid in the retentate. The other forms of sialic acid i.e. free and oligosaccharide bound sialic acid (F + O) were estimated together by difference.

Standard curve for sialic acid

Appropriate volumes of aqueous stock solution (0·16 mM) of NeuNAc were diluted with distilled water to obtain a series of standard solutions of sialic acid (8·3 to 66·6 nmol) in 600 µl. These standard solutions were treated as described above. The fluorescence intensity obtained was plotted against concentration of NeuNAc expressed as sialic acid.

Recovery experiment

NeuNAc levels in milk were raised by 8·3, 16·6 and 24·9 nmol by addition of calculated amounts of sialic acid. Sialic acid levels were then measured as explained above ($n = 5$) using the regression equation obtained from the standard curve and then recovery was calculated.

NeuNAc determination by thiobarbituric acid (TBA) method

NeuNAc content of two commercial GMP samples was also estimated by TBA assay (Matsuno & Suzuki, 2008). Briefly, GMP was dissolved in water up to 1 mg/ml. 200 µl of this solution was mixed with 250 µl 90 mM H_2SO_4 in a test tube. The test tube was kept at 80 °C/1 h in a water bath. The contents of the tubes were neutralised by adding 45 µl 1 M NaOH followed by addition of 250 µl periodic acid (25 mM in 62·5 mM H_2SO_4). The contents were incubated at 37 °C/30 min followed by termination of reaction by addition of 200 µl sodium arsenite (2% in 0·5 M HCl). The contents were allowed to stand at room temperature for 3 min and subsequently 2 ml TBA solution (0·1 M, aqueous) was added. The test tube was heated in boiling water bath for 7·5 min and subsequently cooled in ice water bath. Then, 5 ml *n*-butanol/HCl solution (95:5, v/v) was added and content was shaken vigorously. Absorbance of butanol layer (upper layer) was measured at 550 nm in a UV-Visible spectrophotometer (Shimadzu UV 2550). For standard preparation, 194 µl 0·16 mM stock NeuNAc solution was mixed with 259 µl 88 mM H_2SO_4. The mixture was hydrolysed and treated as mentioned above and used as reference for sialic acid estimation.

The estimated sialic acid content in commercial GMP samples obtained by both the methods (FluA and TBA) was compared using paired *t*-test (Medndham et al. 2000).

Preparation of sweet whey

Milk samples were skimmed (4000 ***g***, 10 min, 4 °C) and then brought to 32 °C. Rennet was added to final

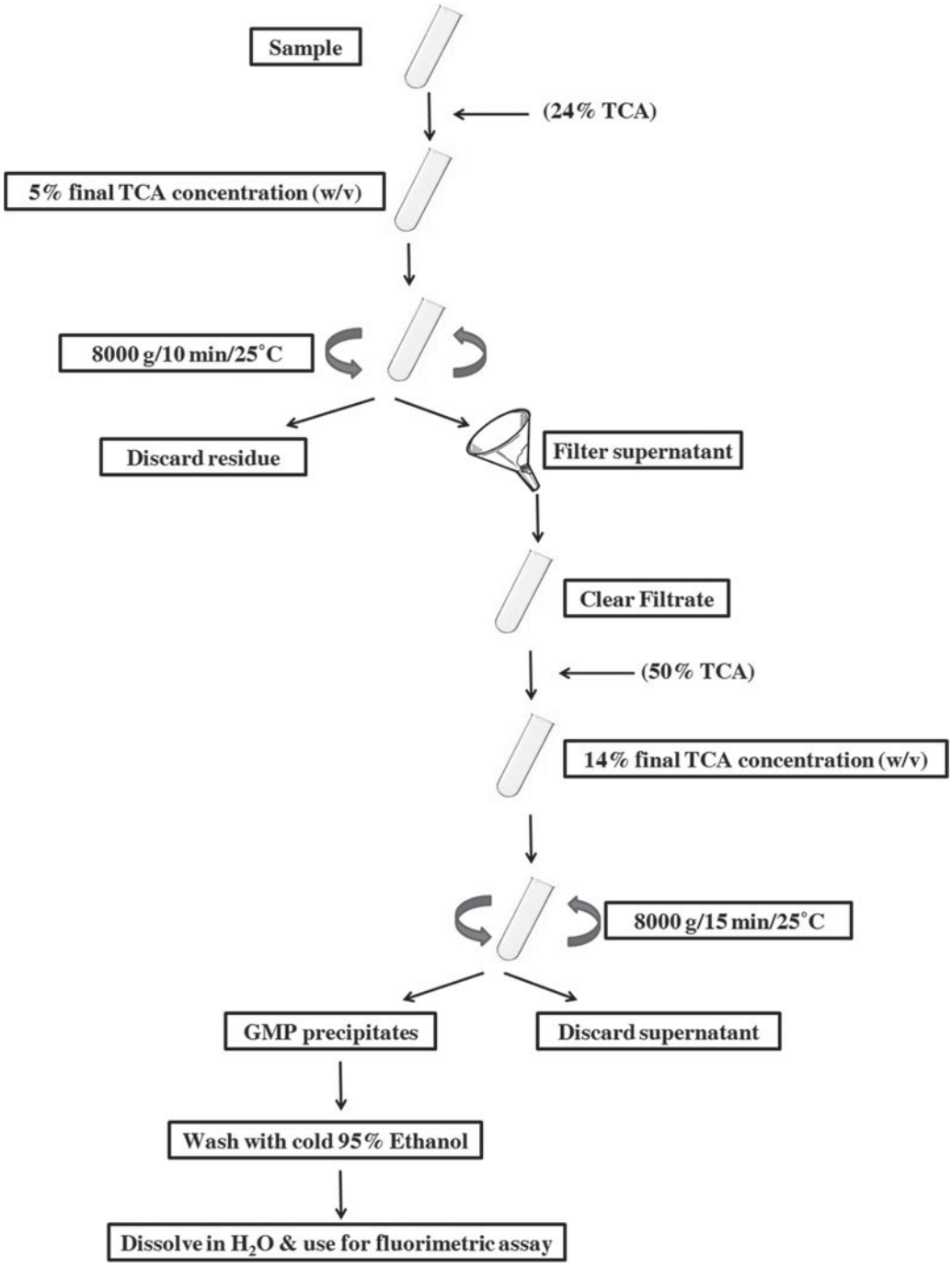

Fig. 1. Isolation of GMP from adulterated milk samples for the detection of sweet whey in milk

concentration of 0·02% (w/v) and stirred for 1 min over a magnetic stirrer and then incubated for 45 min at 32 °C. Set curd was cut and centrifuged (6000 ***g***, 10 min, 4 °C) to get clear whey. Whey was heated at 80 °C/5 min in the water bath, quickly brought to room temperature and then stored at 4 °C. The sweet whey obtained was used on the same day.

Preparation of adulterated milk

Adulterated milk samples were prepared by adding sweet whey to cow milk. For this, binary milk mixtures (v/v) of sweet whey in cow milk containing 5, 10, 20 and 50% sweet whey were prepared. The samples were stored at 4 °C and analysed within 24 h.

Table 1. Estimated values of sialic acid (means ± SE) in skim milk spiked with different levels of sialic acid and recovery of added sialic acid ($n = 5$)

Added NeuNAc (nmol) (A)	Estimated sialic acid (nmol)		Recovery of spiked sialic acid	
	Unspiked sample (B)	Spiked sample (C)	nmol (C–B)	In percent $\frac{C-B}{A} \times 100$
8·3	31·80 ± 0·56	39·41 ± 0·57	7·56 ± 0·17	91·6
16·6	31·80 ± 0·56	47·36 ± 0·58	15·51 ± 0·66	93·7
24·9	31·80 ± 0·56	55·66 ± 0·64	23·81 ± 0·72	95·8

Detection of sweet whey in milk using FluA method

The procedure followed to isolate GMP from adulterated milk samples for the detection of sweet whey in milk is outlined in Fig. 1. Three millilitres of authentic milk sample, adulterated milk sample and sweet whey were mixed with 24% (w/v) trichloroacetic acid (TCA) solution to get a final TCA concentration of 5% (w/v) in a 15 ml polypropylene conical centrifuge tube. After incubating the mixture for 30 min at 35 ± 2 °C, the contents were centrifuged (8000 ***g***, 10 min, 25 °C) and supernatant was filtered through Sartorius 292 filter paper. The clear filtrate obtained was again mixed with 50% TCA (w/v) to a final concentration of 14% (w/v) followed by centrifugation (8000 ***g***, 15 min, 25 °C). The supernatant was discarded and precipitates obtained were washed with cold (10–15 °C) 95% ethanol. The precipitates were then resuspended in 4 ml distilled water. 600 µl of this solution was used for the estimation of sialic acid by above mentioned FluA method.

Results and Discussion

Sialic acid residues are often present at the terminal position of numerous carbohydrate chains of glycoconjugates (glycoproteins, glycolipids and lipopolysaccharides) (Lacomba et al. 2010). Typically, the analysis of sialic acids start with their release either by enzymatic or acid hydrolysis followed by determination by different analytical techniques. The FluA method for the sialic acid estimation is based on the periodate oxidation of sialic acid with the generation of formaldehyde which is then estimated by cyclisation reaction between acetoacetanilide and formaldehyde (Hantzsch reaction) in the presence of ammonia (Matsuno & Suzuki, 2008). The resulting dihydropyridine derivative is fluorescent and its intensity depends on the initial amount of sialic acid in the reaction mixture. Since in the *O*-glycosylation binding of sialic acid, the C2 is involved (Thoma-Worringer et al. 2006), the C8 and C9 are free and available for oxidation by periodate, therefore, this method is also applicable to bound sialic acid. Almost all other widely used methods of sialic acid estimation require release of bound sialic acid either by acidic or enzymatic hydrolysis. Major milk carbohydrate i.e. lactose as observed did not show any fluorescence as it is oxidised more slowly by periodate at 0 °C, therefore the oxidation conducted at such a low temperature is highly specific to sialic acid in milk. For the estimation of sialic acid in milk and milk products, a standard curve of NeuNAc in Milli Q water was prepared and the fluorescence responses were found to be linear between 8·3 and 66·6 nmol ($r^2 = 0{\cdot}999$).

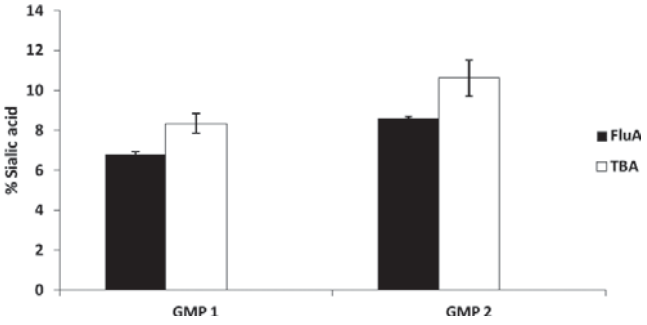

Fig. 2. Sialic acid content in two commercial GMP preparations using fluorimetric method (FluA) and thiobarbituric acid (TBA assay). Error bars represent the standard error ($n = 5$).

Recovery of sialic acid

The recovery was estimated by spiking milk samples with known concentrations of NeuNAc (8·3, 16·6 and 24·9 nmol). Estimated values of sialic acid in unspiked samples, spiked samples and recovery calculations are shown in Table 1. The recovery of added sialic acid in milk was in the range of 91·6–95·8%. These results confirm the applicability of the method to milk. Further, the specificity of the method was established by adding glucose (1 g/l) and lactose (30 g/l) to milk samples which did not change the fluorescence intensity of such samples.

Analysis of NeuNAc in GMP by TBA and FluA method

FluA method was compared with TBA method. NeuNAc content was determined in two commercial GMP samples both by TBA and FluA. Content of NeuNAc was found to be lower in GMP I (FluA 6·8% ± 0·1; TBA 8·35% ± 0·52) than in GMP II (FluA 8·6% ± 0·11; TBA 10·63% ± 0·97) (Fig. 2). Using the *t*-test, the mean results from both the assays were not significantly different ($P < 0{\cdot}05$) from each other. According to manufacturer's specification, GMP I contains 7% and GMP II contains 9% sialic acid. The estimated TBA values were found to be higher than those obtained by FluA method. The higher estimated values of sialic acid obtained by TBA method vis-à-vis FluA method are perhaps due to the

Table 2. Total sialic acid, protein bound and other forms of sialic acid (F + O) in skim milk samples (dry matter basis) of different cow and buffalo breeds ($n = 3$) estimated by fluorimetric method (means ± SE).

	Sialic acid (g/kg)		
Milk samples†	Total	Protein bound	(F + O)‡
Cow breeds			
Sahiwal	1·68 ± 0·06	1·07 ± 0·06	0·61 ± 0·06
Tharparkar	3·60 ± 0·07	1·99 ± 0·08	1·61 ± 0·13
Karn Fries	3·60 ± 0·09	2·19 ± 0·06	1·42 ± 0·15
Karn Swiss	3·93 ± 0·07	1·83 ± 0·08	2·09 ± 0·15
Buffalo breed			
Murrah	3·14 ± 0·05	1·70 ± 0·05	1·44 ± 0·01

†Pooled milk samples collected on different days.
‡Other forms of sialic acid i.e. free and oligosaccharide bound (F + O) sialic acid were estimated by difference.

low specificity of TBA reagent (Lacomba et al. 2010). In a similar comparative study done by other workers (Fernando & Woonton, 2010) for the estimation of NeuNAc in GMP, higher values were obtained using TBA assay compared with HPLC based method.

Estimation of sialic acid in milk and milk products

The level and distribution of various forms of sialic acid in skim milk i.e. total sialic acid, protein bound sialic acid and other forms of sialic acid (F + O) in the milk of different Indian cow breeds and one buffalo breed estimated by FluA method is given in Table 2. The sialic acid content in milk on dry mass basis was dependent on breed and was in the range of 1·68–3·93 g/kg. Sialic acid in Sahiwal, an indigenous cattle breed, was less than 50% of that of Tharparkar, another indigenous cattle breed. Tharparkar and Karan Fries (crossbred Holstein Friesian and Tharparkar) have similar levels of sialic acid. The sialic acid content of Karan Swiss (crossbred Brown Swiss and Sahiwal) was found to be highest of all the breeds studied. Sialic acid in Tharparkar cattle and Murrah buffalo breeds are significantly higher than the reported value of 2·30 g/kg (Koliwer-Brandl et al. 2011) and 0·19 g/kg (Spichtig et al. 2010) for bovine milk. These differences are possibly due to differences in breed, nutrition and climate. Dietary uptake of sialic acid has been correlated with the brain development. The dietary sources of sialic acid may play a role in determining the final concentration of sialic acid in brain and may possibly affect the learning ability of human infants (Wang et al. 2001). As sialic acid is an attractive constituent, manufacturers may claim their milk products have higher sialic acid content as a market strategy. Therefore, to check such claims, the determination of sialic acid in commercial products, which declare the presence of sialic acid e.g. GMP powders, would be useful and this method can be easily used for this purpose. Table 3 represents the percentage of sialic acid in the commercial GMP samples as calculated by this method compared with those mentioned on the labels. For comparison, the other products such as infant formula, WPC, WPI have also been included in the study. The estimated values by FluA method indicate that in commercial GMP samples, the values were in close agreement with those mentioned on the label. Thus, the FluA method can be used for ascertaining the quality of GMP with respect to its sialic acid content.

Table 3. Sialic acid content (mean ± SE) of different milk products as estimated by fluorimetric method.

	% Sialic acid	
Milk products†	Estimated	As mentioned on label
GMP I	6·8 ± 0·10	7·0
GMP II	8·6 ± 0·11	9·0
Milk based infant formula	0·97 ± 0·06	–‡
WPI	0·94 ± 0·04	–‡
WPC-70	1·2 ± 0·06	–‡

†Same sample was analysed five times.
‡Not mentioned on the label.

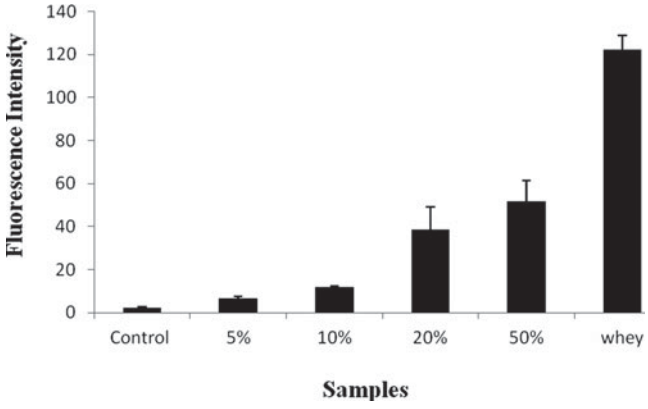

Fig. 3. Relative fluorescence intensity of control (pure milk), milk samples adulterated with sweet whey (5, 10, 20 and 50%) and whey. Error bars represent standard error in estimation ($n = 6$).

Application of FluA method for detection of adulteration of milk with sweet whey

GMP is the major source of sialic acid in sweet whey (Nakano & Ozimek, 1999) and is also a marker peptide of sweet whey adulteration in milk. For the detection of adulteration of milk with sweet whey, GMP in adulterated milk sample was selectively precipitated using TCA. For this, TCA concentration in milk sample was first raised to 5% in order to precipitate caseins and whey proteins. In the supernatant thus obtained, TCA concentration was increased to 14% to precipitate GMP. Since GMP contains bound sialic acid, the precipitated GMP was subjected to fluorescence measurement. As the concentration of whey

was increased in the samples, amount of GMP or sialic acid increased with corresponding increase in fluorescence intensity (Fig. 3). It was observed that the control milk samples also showed low fluorescence which may be due to unreacted acetoacetanilide (Matsuno & Suzuki, 2008). The limit of detection (LOD) of the method as described by AOAC (Lynch, 2005) was found to be 5%. LOD was calculated as the blank value plus 3 times the standard deviation. Although there was increase in fluorescence with increase in the level of sweet whey in milk, the increase was not linear, probably due to partial coprecipitation of GMP with casein by TCA (Fernandez et al. 2011). We have tried different levels of TCA (3, 5 and 8%) for precipitation of milk proteins and it was found that 5% TCA was best to achieve optimum results for detection of adulteration of milk. Although 5% TCA can partially precipitate GMP, the described method can be used for detection of adulteration in milk with cheese whey qualitatively. When sialic acid in GMP is required to be measured for quality control purpose, direct FluA method described here can be used.

Conclusions

The results obtained in the present study reveal that the FluA method following Hantzsch reaction can be employed for the estimation of sialic acid in milk and milk products. The method is rapid in comparison with existing methods available for the estimation of sialic acid in skim milk and milk products as it does not require prior hydrolysis. The method was further applied for the qualitative detection of sweet whey adulteration in milk and can detect presence of 5% sweet whey in milk. Since, many biological properties of GMP (cholera toxin binding, brain development etc.) have been attributed to its sialic acid content, the FluA method can also find application in ascertaining the quality of commercial GMP preparations with respect to their sialic acid content.

This work was supported by National Agricultural Innovation Project, Indian Council of Agricultural Research, India and authors sincerely acknowledge for the financial support.

References

Aminoff D 1961 Methods for the quantitative estimation of *N*-acetylneuraminic acid and their application to hydrolysates of sialomucoids. *Biochemical Journal* **81** 384–392

Bremer MGEG, Kemmers-Voncken AEM, Boers EAM, Frankhuizen R & Haasnoot W 2008 Enzyme-linked immunosorbent assay for the detection of rennet whey powder in milk and buttermilk powder. *International Dairy Journal* **18** 294–302

Chavez NA, Salinas E, Jauregui J, Palomares LA & Macias K 2008 Detection of bovine milk adulterated with cheese whey by western blot immunoassay. *Food and Agricultural Immunology* **19** 265–272

Cherkaoui S, Doumenc N, Tachon P, Neeser JR & Veuthey JL 1997 Development of a capillary zone electrophoresis method for caseinoglycomacropeptide determination. *Journal of Chromatography A* **790** 195–205

de Carvalho BMA, de Carvalho LM, Alcântra LAP & Bonomo RCF 2007 Métodos de detecção de fraude em leite por adição de soro de queijo. *REDVET. Revista electrónica de Veterinaria* **8** 1–7

Fernandez A, Menendez V, Riera FA & Alvarez R 2011 Caseinomacropeptide behaviour in a whey protein fractionation process based on α-lactalbumin precipitation. *Journal of Dairy Research* **78** 196–202

Fernando SF & Woonton BW 2010 Quantitation of *N*-acetylneuraminic (sialic) acid in bovine glycomacropeptide (GMP). *Journal of Food Composition and Analysis* **23** 359–366

Josephson R, Holloway-Thomas DJ & Warthesen J 1980 Cheese whey detection in frozen desserts. *Journal of Dairy Science* **63** 1356–1360

Koliwer-Brandl H, Siegert N, Umnus K, Kelm A, Tolkach A, Kulozik U, Kuballa J, Cartellieri S & Kelm S 2011 Lectin inhibition assays for the analysis of bioactive milk sialoglycoconjugates. *International Dairy Journal* **21** 413–420

Koning P, Elisses J & De Vries H 1966 A method for the detection of small percentages of whey powder in milk powder. *Netherlands Milk and Dairy Journal* **20** 203–212

Lacomba R, Salcedo J, Alegria A, Barbera R, Hueso P, Matencio E & Lagarda MJ 2011 Sialic acid (*N*-acetyl and *N*-glycolylneuraminic acid) and ganglioside in whey protein concentrates and infant formulae. *International Dairy Journal* **21** 887–895

Lacomba R, Salcedo J, Alegria A, Lagarda MJ, Barbera R & Matencio E 2010 Determination of sialic acid and gangliosides in biological samples and dairy products: A review. *Journal of Pharmaceutical and Biomedical Analysis* **51** 346–357

Lynch J 2005 Appendix E: laboratory quality assurance In *Official Method of Analysis of AOAC International*, (Eds W Horwitz & GW JrLatimer). Geneva, Switzerland: AOAC International

Martin MJ, Vázquez E & Rueda R 2007 Application of a sensitive fluorometric HPLC assay to determine the sialic acid content of infant formulas. *Analytical and Bioanalytical Chemistry* **387** 2943–2949

Martin-Hernandez C, Munoz M, Daury C, Weymuth H, Kemmers-Voncken AEM, Corbation V, Toribio T & Bremer MGEG 2009 Immunochromatographic lateral-flow test strip for the rapid detection of added bovine rennet whey in milk and milk powder. *International Dairy Journal* **19** 205–208

Matsuno K & Suzuki S 2008 Simple fluorimetric method for quantification of sialic acids in glycoproteins. *Analytical Biochemistry* **375** 53–59

Medndham J, Denney RC, Barnes JD & Thomas MJK 2000 *Vogels's Textbook of Quantitative Chemical Analysis*. 6th edition. New Delhi, India: Pearson Education (Singapore) Pte. Ltd.

Nakano T, Ikawa N & Ozimek L 2007 Detection of sialylated phosphorylated k-casein glycomacropeptide electrophoresed on polyacrylamide gels and cellulose acetate strips by the thiobarbituric acid and malachite green dye reactions. *Journal of Agricultural and Food Chemistry* **55** 2714–2726

Nakano T & Ozimek L 1999 Determination of sialic acid by the thiobarbituric acid reaction in sweet whey and its fractions. *Journal of Agriculture and Food Chemistry* **47** 2613–2616

Romero EL, Pardo MF, Porro S & Alonso S 1997 Sialic acid measurement by a modified Aminoff method: a time-saving reduction in 2-thiobarbituric acid concentration. *Journal of Biochemical and Biophysical Methods* **35** 129–134

Saito T, Yamaji A & Itoh T 1991 A new isolation method of caseinoglycopeptide from sweet cheese whey. *Journal of Dairy Science* **74** 2831–2837

Salcedo J, Lacomba R, Alegria A, Barbera R, Matencio E & Lagarda MJ 2011 Comparison of spectrophotometric and HPLC methods for determining sialic acid in infant formulas. *Food Chemistry* **127** 1905–1910

Spichtig V, Michaud J & Austin S 2010 Determination of sialic acids in milk and milk based products. *Analytical Biochemistry* **405** 28–40

Sugahara K, Sugimoto K, Nomura O & Usui T 1980 Enzymatic assay of serum sialic acid. *Clinica Chimica Acta* **108** 493–498

Teshima S, Tamal K, Hayashi Y & Emi S 1988 New enzymatic determination of sialic acid in serum. *Clinical Chemistry* **34** 2291–2294

Thoma C, Krause I & Kulozik U 2006 Precipitation behaviour of caseinomacropeptide and their simultaneous determination with whey proteins by RP-HPLC. *International Dairy Journal* **16** 285–293

Thoma-Worringer C, Sorensen J & Lopez-Fandino R 2006 Health effects and technological features of caseinomacropeptide. *International Dairy Journal* **16** 1324–1333

Wang B & Brand-Miller J 2003 The role and potential of sialic acid in human nutrition. *European Journal of Clinical Nutrition* **57** 1351–1369

Wang B, Brand-Miller J, McVeagh P & Petocz P 2001 Concentration and distribution of sialic acid in human milk and infant formulas. *American Journal of Clinical Nutrition* **74** 510–515

Warren L 1959 The thiobarbituric acid assay of sialic acids. *Journal of Biological Chemistry* **234** 1971–1974