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***Research article***

# QUANTITATIVE DETERMINATION OF SIALIC ACID IN INDIAN MILK AND MILK PRODUCTS

D.Karunanithi[1,2], A.Radhakrishna[2] and V.M.Biju[1*]

[1] Department of Chemistry, National Institute of Technology, Tiruchirappalli, Tamilnadu - 620015, India.
[2]Shriram Institute for Industrial Research, Bangalore, Karnataka – 560 048, India.
*****Correspondence mail id: vmbiju@ymail.com

**ABSTRACT**

**Background:** Sialic acids are acidic sugars with a 9-carbon backbone, expressed as terminal residue on mammalian glycoconjugates. Sialic acid was found in two forms as N-acetylneuraminic acid (Neu5Ac) and N-Glycolylneuraminic acid (Neu5Gc) in meat and milk products. Neu5Ac is important for the brain and neuro development while Neu5Gc is suspected as human carcinogen. There is no data reported on sialic acid content in Indian milk and milk based products.
**Objective:** To determine sialic acid (Neu5Ac and Neu5Gc) content in Indian milk and milk products and thermal stability of non-human sialic acid (Neu5Gc) evaluation.
**Design:** Milk products like chocolate, infant food products, milk based energy drinks, butter etc were examined for the sialic acid content. For Neu5Gc thermal stability studies cow, buffalo, cross cows and processed milk were used.
**Conclusion:** The study indicates the level of sialic acid (Neu5Ac and Neu5Gc) in Indian milk and milk based products. Further the stability studies shows degradation (20 to 22 %) of non-human sialic acid (Neu5Gc) to the total sialic acid content.
**Key words:** Sialic acid, Milk, Milk products, Neu5Ac, Neu5Gc

## INTRODUCTION

Sialic acids are acidic sugars with a 9-carbon backbone, expressed as terminal residue on mammalian glycoconjucates. N-Acetylneuraminic acid (Neu5Ac) and its hydroxylated form, N-Glycolylneuraminic acid (Neu5Gc), are the two major forms of sialic acid in most mammals. Sialic acid is endogenously synthesized in human and other mammalian tissues starting with glucose. Neu5Gc was absent in humans, due to deletion in the human gene coding for CMP-Neu5Ac hydroxylase (Chou et al., 1998; Irie et al., 1998) the enzyme responsible for Neu5Gc. But accumulation of Neu5Gc is possible in humans through dietary source like meat and milk based products. The incorporation of Neu5Gc into human glycoconjucates could potentially lead to health risk because Neu5Gc can be antigenic when linked to glycoconjucates. (Wang, 2009). Neu5Ac is one of the promising components for the rapid neural growth, development of brain and its supplementation shows improvements in learning and memory power in animal model experiments (Wang et al., 2007; Morgan et al., 1980; Wang et al., 2007). In the early years of infant development, the endogenous synthesis of sialic acid is inadequate but the demand for sialic acid is high. So the exogenous supplies of sialic acid through mother milk, infant food formulas or milk based products are crucial to the neural development. Non- human sialic acid (Neu5Gc) that differs by one oxygen atom from the human sialic acid (Neu5Ac) and the incorporation of Neu5Gc may generate chronic inflammation and contribute to the diet related carcinomas ( Hedlund et al., 2008; Padler-karavani et al., 2008; Kawai et al., 1991; Tangvoranuntakul et al., 2003; Malykh et al., 2001) and other diseases in human (Lofling et al., 2009).

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The level of sialic acid (Neu5Ac and Neu5Gc) in milk and milk based products depends on genetics, nutrition of the animal, geographical region, and milk from different breeds of cattle, lactation period or combination of these factors. The concentration of sialic acid (Neu5Ac and Neu5Gc) in Indian milk and milk based products is unknown. As plants and microbes are unable to produce sialic acid; incorporation of Neu5Gc in human body is due to the intake of Neu5Gc rich foods like meat, milk and milk based products which may leads to chronic inflammation and carcinogen. Hence, present study was aimed to determine sialic acid (Neu5Ac and Neu5Gc) content in milk and milk based products and also assess the thermal stability under boiling condition of Neu5Gc.

## EXPERIMENTS

### Sample Collection and preparation

Milk based products were purchased from supermarkets in Bangalore, Karnataka, India. Cow (Kangayam), buffalo (*Bubalus bubalis*) , cross cow ( Holstein Friesian cross) and goat milks were collected from Krishnagiri, Hosur and Trichy of Tamilnadu state, India. The collected milk & milk based products were stored at - 20°C. Milk and milk based products were homogenized and taken for analysis.

### Quantitation of Sialic acid

Sialic acid in milk and milk products were determined by LC-MS/MS method and the method was previously reported by our research group (Karunanithi et al., 2012). Major amount of sialic acid are bound to glycoprotein, oligosaccharides and glycolipids in milk and milk based products. Samples were hydrolyzed with dilute hydrochloric acid (HCl) to liberate the free form of sialic acid from their conjugates.

Milk based products were hydrolyzed using 45 mM of HCl acid at 80°C for 75 minute while the raw milk was hydrolyzed using 30 mM HCl acid. Agilent 6460 ESI (-Ve) LC-MS/MS with mass hunter workstation software version B.02.1 was used for data acquisition. Thermo hypersilgold C-18 (150 x 4.6 mm ID, 3 µ) column was used. A gradient pump programme was used 0.3 percentage of formic acid in water and 5 mM of ammonium formate in 90 percentage methanol and 10 percentage water. The optimized MRM transition for Neu5Ac was 308.1 to 87 *m/z*, fragmentor at 62 V, collision energy was set as 8V, for Neu5Gc the transition was 324.0 to 116.0 *m/z*, fragmentor and collision energy was 74 and 12 V respectively.

## RESULTS AND DISCUSSIONS

### Milk and Milk products

Both milk and milk based products contains sialic acid (Neu5Ac and Neu5Gc) were present in both forms. These sialic acid levels varied in all milk products based on the added level of milk and milk solids to the products. Different types of energy drinks were analyzed for sialic acid content. All energy drinks showed the presence of sialic acid in both forms in the range of 1058.8 $\pm$ 138 mg/Kg (Neu5Ac) and 309 $\pm$ 46 mg/Kg (Neu5Gc). In energy drinks 1.3 to 4.4 percentage of Neu5Gc contributed to the total sialic acid content. High protein mix samples showed higher level of Neu5Ac and Neu5Gc as 4978 $\pm$ 125 and 149.5 $\pm$ 4 mg/Kg respectively. Infant food products and milk products found to be different levels of sialic acid and are shown in Fig 1. The mean levels of Neu5Ac and Neu5Gc in milk based products are shown in Table 1.

Different varieties of milk from Indian cow, buffalo, cross cow and goat milk were included in this study. Three day continuous secretion of milk was studied from each varieties and the sialic acid level was evaluated. All the milk samples shows the different range of sialic acid concentration, but the consistency of sialic acid concentration with three different day secretion was satisfactorily, the % RSD was below 5. Cow milk shows lowest Neu5Gc content, and it was below 1% to the total sialic acid. Buffalo and cross cow milk show the percentage of Neu5Gc in the range of 1 to 4. Sialic acid content in goat milk was 131.2 $\pm$ 5 mg/L, in this Neu5Gc contributed 52.5 $\pm$ 1.5 percentage. It was also observed that among the different milk analyzed the Neu5Gc percent was very high to the total sialic acid in goat milk. The level of Neu5Ac and Neu5Gc were tabulated in Table 2.

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**Table. 1: Sialic acid concentration in Indian milk based products (n=6)**

| S.No | Product | Mean Neu5Ac, mg/Kg | Mean Neu5Gc, mg/Kg | Mean Total sialic acid, mg/Kg |
|---|---|---|---|---|
| 1 | Butter | 55.00 | 1.56 | 56.56 |
| 2 | Milk Peda | 522.00 | 15.60 | 537.60 |
| 3 | Paneer | 595.20 | 10.50 | 605.70 |
| 4 | Yoghurt | 68.80 | 1.51 | 70.31 |
| 5 | Flavoured milk | 45.60 | 0.90 | 46.50 |
| 6 | Cheese | 76.70 | 2.30 | 79.00 |
| 7 | Fresh cream | 121.90 | 1.40 | 123.30 |

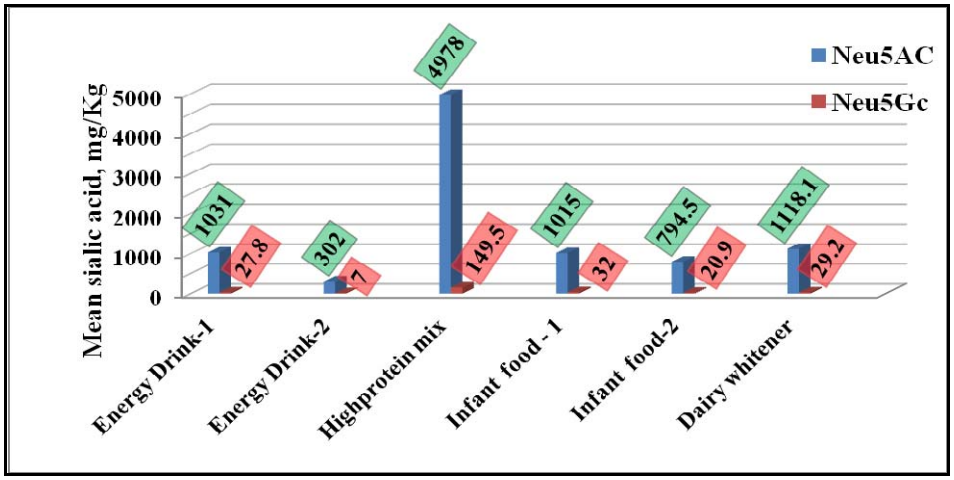


**Fig 1 Level of sialic acid in infant food products and milk products (n=6)**

**Table 2. Sialic acid secretion of three consecutive days in bovine and goat milk**

| Milking Day | Analyte, mg/L | Cow | Buffalo | Cross cow | Goat milk |
|---|---|---|---|---|---|
| Day 1 | Neu5Ac | 222.0 | 261.0 | 304.0 | 65.2 |
| | Neu5Gc | 2.0 | 6.2 | 5.2 | 68.3 |
| Day 2 | Neu5Ac | 215.1 | 255.0 | 297.0 | 59.5 |
| | Neu5Gc | 1.95 | 6.0 | 5.2 | 67.3 |
| Day 3 | Neu5Ac | 217.8 | 264.0 | 289.0 | 62.3 |
| | Neu5Gc | 2.1 | 6.3 | 5.0 | 71.2 |
| Mean | Neu5Ac | 218.3 | 260 | 296.7 | 62.3 |
| | Neu5Gc | 2.02 | 6.17 | 5.13 | 68.9 |

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## Infant food products

Human breast milk contains sialic acid exclusively in the form of N-acteylneuraminic acid (Neu5Ac), while the infant food formula contains the both form of sialic acid because infant formulas are prepared by using bovine milk. So understanding the level of Neu5Gc in the infant food products is very important. Infant food products have variable level of sialic acid in the range of 250 to 1200 mg/Kg and it has 1.5 to 4.8 percentages of Neu5Gc of total sialic acid. (Fig. 1)

## Milk based products

Paneer is one of the important products from milk which is commonly used in Indian dishes. Paneer has around 605.7 $\pm$ 88 mg/Kg of total sialic acid, among which Neu5Gc was 1.5 to 3 percentages. Yoghurt is another milk product, produced by bacterial fermentation of milk, has 68.2 $\pm$ 10.5 mg/Kg of total sialic acid. Fresh cream contains 123.3 $\pm$ 15 mg/Kg of total sialic acid and it is used to prepare fruit salad, ice creams, sweet meals etc. Indian cheese had shown 79 $\pm$ 14 mg/Kg of total sialic acid content. In cheese maximum of 97.5 $\pm$ 1 percentage Neu5Ac, 2.5 $\pm$ 1 percentage of Neu5Gc was recorded. Milk pedas are another delicious and tastiest milk products prepared by using condensed milk and milk powders. Milk peda contains 550 $\pm$ 55 mg/Kg of total sialic acid (TSA) and the Neu5Gc was contributed 2.9 $\pm$ 0.3 % of TSA.

## Chocolates

Ten different types of commonly available milk based chocolates were evaluated for the determination of sialic acid. All the brands were shown the presence of sialic acid in the range of 30 to 450 mg/Kg. The levels of sialic acid in different types of chocolates are shown in Fig. 2. In chocolates were shown 1.7 to 4 percentage of Neu5Gc to the total sialic acid concentration.

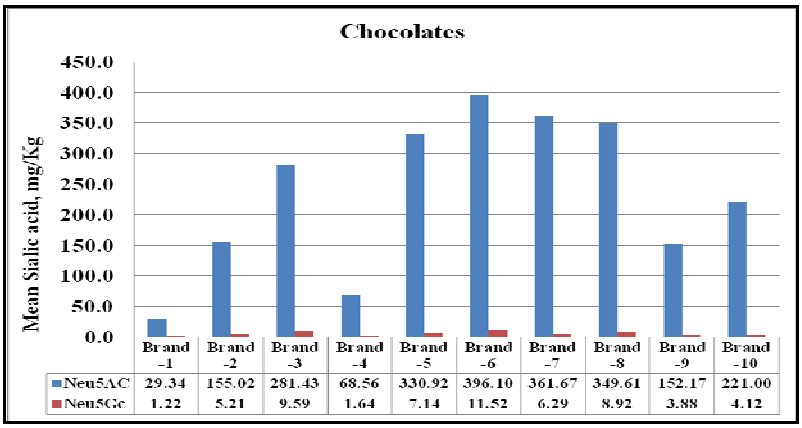


**Fig 2 Distribution of sialic acid content in various chocolates**

Different types of milk and milk based products were analyzed by LC-MS/MS and MRM were shown in Fig 3.

## THERMAL STABILITY OF NEU5GC

The thermal stability of Neu5Gc in milk was assessed by boiling the milk at different times. Raw milk Neu5Gc content was taken as initial concentration and 500 mL of milk was boiled. The initial first boiling was occurred in 5 minutes and the Neu5Gc concentration was estimated. The milk was continuously boiled upto 40 minutes and the every 5 min Neu5Gc concentration was quantified. The initial raw milk Neu5Gc level was taken as reference, the boiled milk Neu5Gc contents were compared. It was observed that there was a significant reduction of Neu5Gc level in boiled milk and the reduction may due to Neu5Gc getting degraded.

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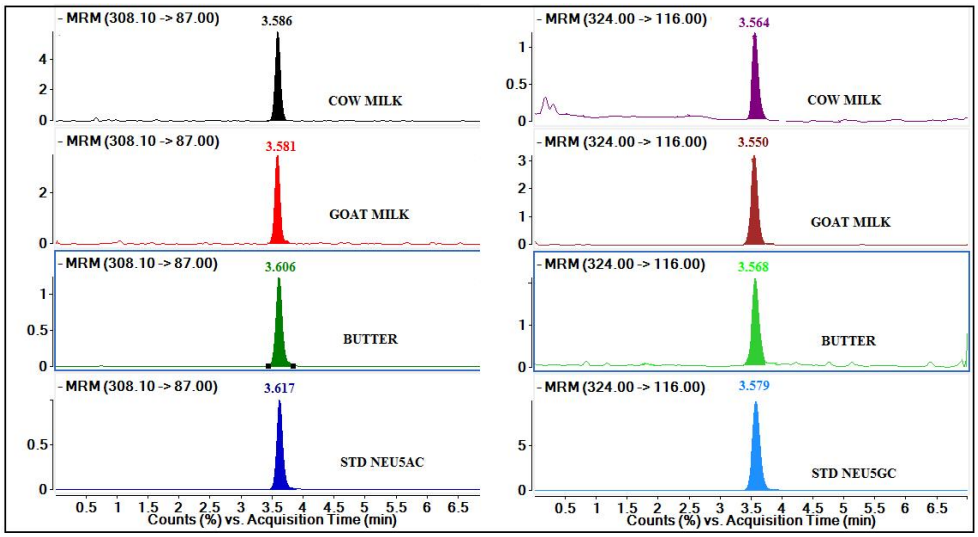


**Fig 3 MRM of Neu5Ac and Neu5Gc for Standard and sample**

In first boiling of milk (5 minute), 7 to 8 percentage of Neu5Gc degradation was observed. Degradation was gradually increased and it reached 22 to 25 percentages in 40 minutes of boiling. The boiling time increase the rate of degradation of Neu5Gc increased significantly compare to Neu5Ac, this may be due to the low level of Neu5Gc present in milk compare to Neu5Ac. The degradation of with respect to boiling time was shown in Fig 4.

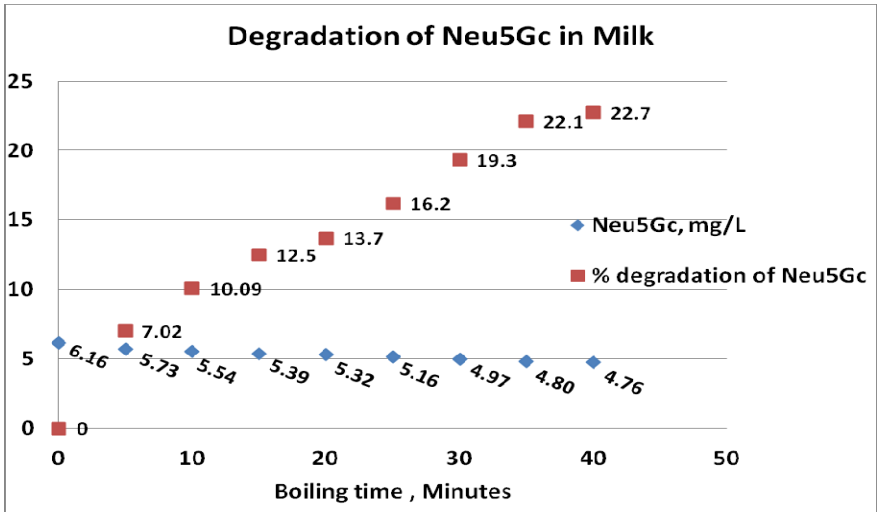


**Fig 4 Degradation of Neu5Gc when boiling the milk**

## CONCLUSION

This study has established the level of sialic acid in Indian milk (cow, buffalo and cross cows) and milk products like paneer, cheese, butter, fresh cream etc. The results also demonstrate the percentage of Neu5Ac and Neu5Gc in total sialic acid in the different Indian milk and milk products. In milk and milk products, the Neu5Gc was less than 5 percentages to the total sialic acid level where as goat milk has shown more than 50 percentage of Neu5Gc. When boiling the milk there is a significant loses of non-human sialic acid (Neu5Gc) shows the thermal degradation. This study outlined the nutritional importance of Indian milk products with respect to the sialic acid concentration.

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