

## Research Article

**NMR and Mass Characterization of Crisose - A Novel Tetrasaccharide from Goat Milk**

PUSHPRAJ SINGH\*

Department of Chemistry, Govt. Girls Degree College, Chhibramau, Kannauj-209721, (Affiliated to CSJM University, Kanpur), Uttar Pradesh, India

**ARTICLE DETAILS***Article history:*

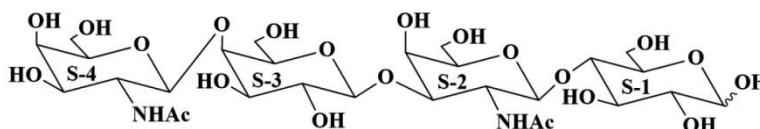
Received on 23 February 2022

Modified on 7 March 2022

Accepted on 12 March 2022

*Keywords:*Carbohydrates,  
Oligosaccharides,  
Tetrasaccharide,  
Goat Milk,  
NMR,  
Crisos.**ABSTRACT**

Milk is a gift of nature to mankind that is necessary for the nutrition, growth, and development of newborn infants. Besides the other regular constituents it has oligosaccharides in it which are indigestible by the infant yet are consumed by the microbial populations in the developing intestine. These oligosaccharides are believed to facilitate enrichment of a healthy infant gastrointestinal micro biota, often associated with bifidobacteria. Goat milk is a rich source of oligosaccharides that have enormous biological activities such as anti-bacterial, immunological, anti-inflammatory, hypoallergenic, and therapeutic properties. It is used against tuberculosis in folk medicine and helps in the enhancement of platelet count during dengue fever. Goat milk oligosaccharides have anti-inflammatory properties and are involved in the repairing process after a dextran sodium sulphate-induced colitis. Keeping in mind the biological importance of goat milk and the role of oligosaccharides, in the present studies, goat milk was analyzed for its oligosaccharide content which led to the isolation of a novel tetrasaccharide, Crisose,  $C_{28}H_{48}O_{21}N_2$ . The structure of the isolated oligosaccharide was elucidated by chemical transformation, chemical degradation,  $^1H$ ,  $^{13}C$ , 2D-NMR (COSY, TOCSY, HMBC and HSQC) and mass spectrometry as under.



The geometry of compound Crisose was optimized by B3LYP method and 6-31 G (d,p) basis set.

© KESS All rights reserved

**INTRODUCTION**

Oligosaccharides are the complex carbohydrates present in milk or colostrum of mammalian species that play a key role in various physiological, pathological and biological activities such as biological recognition, anti-complementary, anti-coagulant, anti-inflammatory, anti-viral, anti-bacterial, anti-tumor, anti-oxidant, lipid lowering, immunological, prebiotic and hypoglycemic activities [1-4]. Oligosaccharides play a critical role in preventing inflammatory processes, reducing diabetes, obesity and cardiovascular risks, modulating the gut flora and affecting different gastrointestinal activities [5-8].

Goat milk oligosaccharides have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid-induced colitis and may be useful in the management of inflammatory bowel disease [9, 10]. Goat milk oligosaccharides play an important role in intestinal protection and repair after damage caused by DSS (dextran sodium sulphate)-induced colitis and their implication in human intestinal inflammation [11, 12]. Goat milk possesses anti-hypertensive and immunomodulatory properties [13]. In view of the above facts, we have chosen to investigate goat milk for its oligosaccharide constituents and their structure elucidation. For this purpose, goat milk was collected in bulk and was processed by the modified method of Kobata and Ginsburg involving deproteination, filtration,

\*Author for Correspondence:

Email: pushpraj1509@gmail.com

lyophilization followed by gel filtration, HPLC and column chromatography. We are describing the structure elucidation of one novel goat milk oligosaccharide, Crisose and its Density Functional Theory (DFT) studies.

### Theoretical Study

The quantum chemical calculations have been performed on B3LYP functional and 6-31G (d,p) basis set employing DFT. Geometry of compound Crisose was first optimized and the presence of positive wave number values for all the optimized geometry indicated the stability of the compound. All computations were performed using the Gaussian 09 program package [14].

### Experimental

#### General Procedures

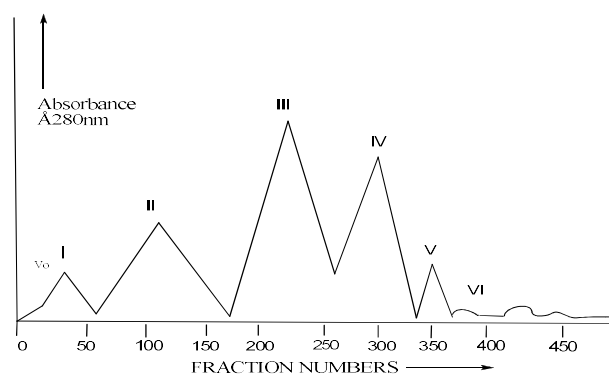
The sugars were visualized on TLC with 50% aqueous  $H_2SO_4$  reagent and on paper chromatography (PC) with acetyl acetone and *p*-dimethylaminobenzaldehyde reagents. The adsorbent for TLC was silica gel G (SRL) and CC silica gel (SRL, 60-120 mesh). PC was performed on Whatman No.1 filter paper using solvent system of toluene, butanol and water in varied proportions. Sephadex G-25 (PHARMACIA) was used in gel permeation chromatography. Freeze drying of the oligosaccharide mixture was done with the help of CT 60e (HETO) lyophilizer and centrifuged by a cooling centrifuge Remi instrument C-23 JJRCI 763. To check the homogeneity of the compounds reverse phase HPLC system was used equipped with Perkin Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotter. Authentic samples of *N*-acetylgalactosamine (GalNAc), galactose (Gal), glucose (Glc), were purchased from Aldrich Chemicals. Optical rotations were measured with an AA-5 series automatic polarimeter in 1cm tube.  $^1H$  and  $^{13}C$  NMR spectra of the oligosaccharide were recorded in  $D_2O$  and the spectra of the acetylated oligosaccharide was recorded in  $CDCl_3$  at 25°C on a Bruker AM 300 FT NMR spectrometer. The electrospray mass spectra were recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer. The samples (dissolved in suitable solvents such as methanol/acetonitrile/water) were introduced into the ESI source through a syringe pump at the rate 5 $\mu$ L per min. The ESI capillary was set at 3.5 KV and the cone voltage was 40V. The spectra were collected in 6s scans and the print outs were averaged spectra of 6-8 scans. The C, H and N analyses were recorded on CARLO-ELBA 1108 an elemental analyzer.

### Isolation of Goat Milk Oligosaccharides by Modified Method of Kobata and Ginsburg [15]

11 Litres of goat milk was collected in 15 days with normal milking condition from a single domestic goat (Battisi) from Nagla Seth village, Shamsabad, Farrukhabad District, Uttar Pradesh, India. The milk was fixed by addition of equal amount of ethanol (11 L), the preserved milk was taken to laboratory and then it was centrifuged for 15 min at 5000 rpm at 4 °C. The solidified lipid layer was removed by filtration through glass wool column under cold atmospheric condition. More ethanol was added to clear filtrate to a final concentration of 68% and the resulting solution was left over night at 0 °C. The white precipitate formed mainly of lactose and protein was removed by centrifugation and washed twice with 68% ethanol at 0 °C. The supernatant and the washings were combined and filtered through a micro filter and lyophilized affording 215 g of crude oligosaccharide mixture.

### Sephadex G-25 Gel Filtration of Goat Milk Oligosaccharide Mixture

13.20 g of lyophilized material (mixture of oligosaccharides) of goat milk was purified on Sephadex G-25 column chromatography for the separation of oligosaccharides from other constituents of milk by using glass triple distilled water as eluent at a flow rate of 3 ml/min. Goat milk oligosaccharide mixture was packed in a column (1.6x40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h to settle down.



**Graph 1:** Sephadex G-25 chromatography of goat milk oligosaccharides detected by phenol-sulphuric acid method. Elution was made with TDW.

Presence of neutral sugars was monitored in all the eluted fractions by phenol-sulphuric acid test.

**Table 1:** Goat milk oligosaccharide mixture (13.2 g) chromatographed over Sephadex G-25 column chromatography – Details

Fraction No.	Solvent	Compound (in gm)	Phenol-H <sub>2</sub> SO <sub>4</sub> Test for Sugar	Further Investigation
1-60	Glass triple Distilled H <sub>2</sub> O	0.73	-ve [ I ]	-
61-171	"	2.45	+ve [ II ]	] Purified by Column Chromatography
172-275	"	2.95	+++ve [ III ]	
276-343	"	2.86	+++ve [ IV ]	
344-374	"	0.96	++ve [ V ]	-
375-389	"	0.51	-ve [ VI ]	-

In this experiment the UV-monitored Sephadex G-25 chromatography of goat milk oligosaccharide mixture showed seven peaks *i.e.* I, II, III, IV, V, VI and VII. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume that was confirmed by positive coloration with *p*-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent [16]. Fractions under peaks II, III and IV gave a positive phenol-sulphuric acid test for sugars, which showed the presence of oligosaccharide mixture in the goat milk. These fractions under peaks II, III and IV were pooled and lyophilized together affording 8.6 g of oligosaccharide mixture.

The amount of oligosaccharide mixture of the pooled fractions (peaks II, III and IV) obtained from Sephadex G-25 column chromatography was 8.6 g. This process was repeated further, which resulted into a total of 14.35 g of the oligosaccharide mixture.

#### Confirmation of Homogeneity of Goat Milk Oligosaccharides by Reverse Phase HPLC

Pooled fractions (peaks II, III and IV) obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analyzed by reverse phase HPLC. The HPLC system was equipped with Shimadzu CLASS-VP V6.13 solvent delivering system, 235-diode array detector and G.P. 100 printer plotter. The column used for this purpose was C18 Purosphere 25 cm × 0.4 cm × 5-μ m (from E. Merck). A binary gradient system of acetonitrile: 0.5% trifluoroacetic acid (5:95) in triple distilled water (TDW) to CH<sub>3</sub>CN: 0.5 % TFA (60:40) within 25 min at a flow rate of 1 ml/min was used. The eluents were detected at 220 nm. Twelve peaks were noticed in the sample (pooled fraction II and III) at the varied retention times from 1.092 min to 20.675 in, for convenience the peaks were numbered in their increasing order of retention time *i.e.* [299(R<sub>1</sub>), 1.323(R<sub>2</sub>), 2.272(R<sub>3</sub>),

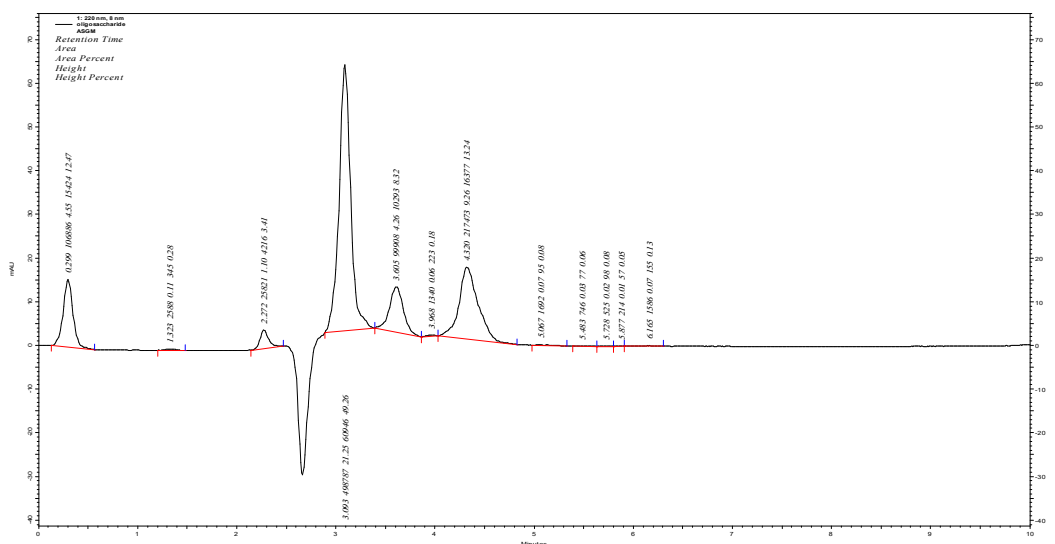
3.093(R<sub>4</sub>), 3.605(R<sub>5</sub>), 3.968(R<sub>6</sub>), 4.320(R<sub>7</sub>), 5.067(R<sub>8</sub>), 5.483(R<sub>9</sub>), 5.728(R<sub>10</sub>), 5.877(R<sub>11</sub>) and 6.165(R<sub>12</sub>)].

**Table 2:** HPLC Table of crude goat milk oligosaccharides

S.No.	Retention Time(min)	Area	Area %	Height	Height %
1	0.299	106886	4.55	15424	12.47
2	1.323	2588	0.11	345	0.28
3	2.272	25821	1.10	4216	3.41
4	3.093	498787	21.25	60946	49.26
5	3.605	99908	4.26	10293	8.32
6	3.968	1340	0.06	223	0.18
7	4.320	217473	9.26	16377	13.24
8	5.067	1692	0.07	95	0.08
9	5.483	746	0.03	77	0.06
10	5.728	525	0.02	98	0.08
11	5.877	214	0.01	57	0.05
12	6.165	1586	0.07	155	0.13

#### Acetylation of the Oligosaccharide Mixture

11 g Oligosaccharide mixture obtained from Sephadex chromatography was acetylated with pyridine (11 ml) and acetic anhydride (11 ml) at 60 °C and the solution was stirred overnight. Further in order to remove the excess reagents, the reaction mixture was evaporated under reduced pressure and the viscous residue was taken up in CHCl<sub>3</sub> (200 ml) and washed twice with ice-cold water, evaporated to dryness yielding acetylated mixture (12.30 g). The acetylation converted the free sugars into their non-polar acetyl derivatives which were resolved nicely on TLC giving 10 spots *i.e.* a, b, c, d, e, f, g, h, i and j. Detection of the spots on TLC was done by spraying with 50% H<sub>2</sub>SO<sub>4</sub> in distilled water and heating. Varied proportions of CHCl<sub>3</sub>: MeOH was used as chromatography solvent.



**Graph 2:** Reverse phase HPLC of goat milk oligosaccharides (For the chromatographic conditions, see Tables 1 and 2)

### Purification of Acetylated Milk Oligosaccharide on Silica Gel Column

Purification of the acetylated oligosaccharide mixture (11.0 g) was carried out over silica gel (500 g) using varied proportion of Hex:CHCl<sub>3</sub>, CHCl<sub>3</sub>, CHCl<sub>3</sub>:MeOH as eluents, collecting fraction of 500 ml each. All these fractions were checked on TLC and the fractions showing similar spots (R<sub>f</sub> values) were collected together for further investigations. Repeated column chromatography of fractions I and II, led to the isolation of one chromatographically pure compound 'b' Crisose (308 mg).

### Deacetylation of Compound 'b'

Acetylated compound 'b' (50 mg) was dissolved in acetone (3 ml) and 3 ml of NH<sub>3</sub> was added to it and was left overnight in a stoppered hydrolysis flask. After 24 h ammonia was removed under reduced pressure and the compound was washed with (3 x 5 ml) CHCl<sub>3</sub> (to remove acetamide) and the water layer was finally freeze dried giving the natural oligosaccharide 'B' Crisose (39 mg).

### Methyl glycosidation/acid hydrolysis of compound 'B', Crisose

Compound 'B' Crisose (8 mg) was refluxed with absolute MeOH (2 ml) at 70 °C for 18 h in the presence of cation exchange IR-120 (H<sup>+</sup>) resin. The reaction mixture was filtered while hot and filtrate was concentrated giving methyl glycoside of compound 'B'. In the solution of methyl glycoside of compound 'B', 1, 4-dioxane (1 ml), and 0.1N H<sub>2</sub>SO<sub>4</sub> (1 ml) were added and the solution was warmed for 30 min at 50°C. The hydrolysis was complete after 24 h. The

hydrolysate was neutralized with freshly prepared BaCO<sub>3</sub>, filtered and concentrated under reduced pressure to afford α- and β-methyl glycosides along with Gal and GalNAc. Their identification was confirmed by comparison with authentic samples of α- and β-methyl glycosides, Gal and GalNAc (TLC, PC).

### Kiliani hydrolysis of compound 'B', Crisose [17]

Compound 'B' Crisose (5 mg) was dissolved in 2 ml Kiliani mixture (AcOH-H<sub>2</sub>O-HCl, 7:11:2) and heated at 100°C for 1 h followed by evaporation under reduced pressure. It was dissolved in 2 ml of H<sub>2</sub>O and extracted twice with 3 ml CHCl<sub>3</sub>. The aqueous residual solution was made neutral by addition of 1-2 drops of 2N NaOH and was evaporated under reduced pressure to afford Glc, Gal and GalNAc which were compared by authentic samples of Glc, Gal and GalNAc, respectively.

### Description of Compound 'B' Crisose

Acetylated Crisose 'b' (308 mg) was obtained from column chromatography. On deacetylation of 50 mg of compound Crisose acetate 'b' with NH<sub>3</sub>/acetone, it afforded compound 'B' (39 mg) [α]<sub>D</sub><sup>25</sup> = -17° (c 1% H<sub>2</sub>O). For experimental analysis, this compound was dried over P<sub>2</sub>O<sub>5</sub> at 100°C and 0.1 mm pressure for 8 hr. It gave positive Phenol-sulphuric acid test, Feigl test and Morgan-Elson test.

C <sub>28</sub> H <sub>48</sub> O <sub>21</sub> N <sub>2</sub> :	%C	%H	%N
Calculated:	44.91	6.41	3.74
Found:	44.92	6.41	3.74

**<sup>1</sup>H NMR of Acetylated Compound 'b', Crisose Acetate in CDCl<sub>3</sub> at 300 MHz**

6.24 [d, 1H, J=3.3 Hz,  $\alpha$ -Glc(S-1) H-1], 5.68 [d, 1H, J=8.1Hz,  $\beta$ -Glc(S-1) H-1], 4.51 [d, 2H, J=7.5 Hz,  $\beta$ -GalNAc(S-2) H-1 &  $\beta$ -Gal(S-3) H-1], 4.48 [d, 1H, J=8.7 Hz,  $\beta$ -GalNAc(S-4) H-1], 3.86 [m, 1H,  $\beta$ -Glc(S-1) H-4], 3.88 [m, 1H,  $\beta$ -GalNAc(S-2) H-3] and 3.85 [m, 1H,  $\beta$ -Gal(S-3) H-4].

**<sup>13</sup>C NMR of Acetylated Compound 'b', Crisose Acetate in CDCl<sub>3</sub> at 300 MHz**

88.83 [1C,  $\alpha$ -Glc(S-1) C-1], 91.41 [1C,  $\beta$ -Glc(S-1) C-1], 100.77 [2C,  $\beta$ -GalNAc(S-2) C-1 &  $\beta$ -Gal(S-3) C-1] and 101.01 [1C,  $\beta$ -GalNAc(S-4) C-1].

**<sup>1</sup>H NMR of Compound 'B', Crisose in D<sub>2</sub>O at 300 MHz**

5.59 [d, 1H, J= 3.9 Hz,  $\alpha$ -Glc(S-1) H-1], 4.53 [d, 1H, J= 8.4 Hz,  $\beta$ -Glc(S-1) H-1], 4.38 [d, 2H, J= 7.8 Hz,  $\beta$ -GalNAc(S-2) H-1 &  $\beta$ -Gal(S-3) H-1], 4.31 [d, 1H, J= 7.2 Hz,  $\beta$ -GalNAc(S-4) H-1], 2.08 [s, 3H, NHC(=O)CH<sub>3</sub>,  $\beta$ -GalNAc(S-4)] and 1.95 [s, 3H, NHC(=O)CH<sub>3</sub>,  $\beta$ -GalNAc (S-2)].

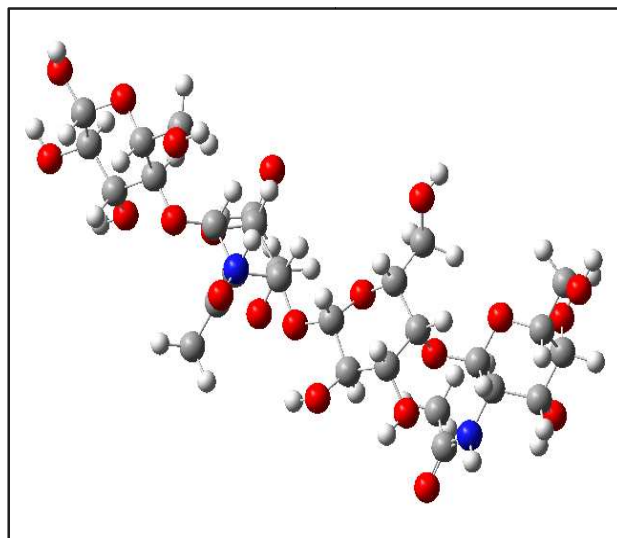
**ES Mass of Compound 'B', Crisose**

787[M+K]<sup>+</sup>, 771[M+Na]<sup>+</sup>, 748[M]<sup>+</sup>, 690[748-NHC(=O)CH<sub>3</sub>], 652[690-2H<sub>3</sub>O<sup>+</sup>], 651[652-H<sup>+</sup>], 545[748-S<sub>4</sub>], 503[545-CH<sub>2</sub>CO], 481[545-HCHO-2OH], 465[503-2H<sub>3</sub>O<sup>+</sup>], 421[465-CH<sub>3</sub>CHO], 406[481-CH<sub>3</sub>CHO-CH<sub>2</sub>OH], 383[545-S<sub>3</sub>], 325[383-NHC(=O)CH<sub>3</sub>], 319[383-HCHO-2OH], 301[319-H<sub>2</sub>O], 259[325-HCHO-2H<sub>2</sub>O], 223[259-2H<sub>2</sub>O] and 180[383-S<sub>2</sub>].

**RESULT AND DISCUSSION****Stability of Molecular Geometry of Isolated Compound 'B' Crisose**

Density functional theory (DFT), a computational method, was employed to evaluate the structure-activity relationship. In this study, the geometry of compound Crisose was optimized at B3LYP method and 6-31 G (d,p) basis set using Gaussian 09 program package [14]. The molecular geometries can be determined by the quantum mechanical behavior of the electrons and computed by ab-initio quantum chemistry methods to high accuracy. Molecular geometry represents the three-dimensional arrangement of the atoms that determines several properties of a substance including its reactivity, polarity, phase of matter, color, magnetism, and biological activity. The compound 'B' was found to be highly polar in nature with the total dipole moment of 11.3017 Debye. The total energy of compound was -2785.0912 a. u., which represents the stability of molecule. All the rings

were present in the most stable chair form. The compound 'B' Crisose possesses C<sub>1</sub> symmetry. The optimized structure of compound is given below (Fig. 1).



**Figure 1:** Optimized geometry of compound 'B', Crisose

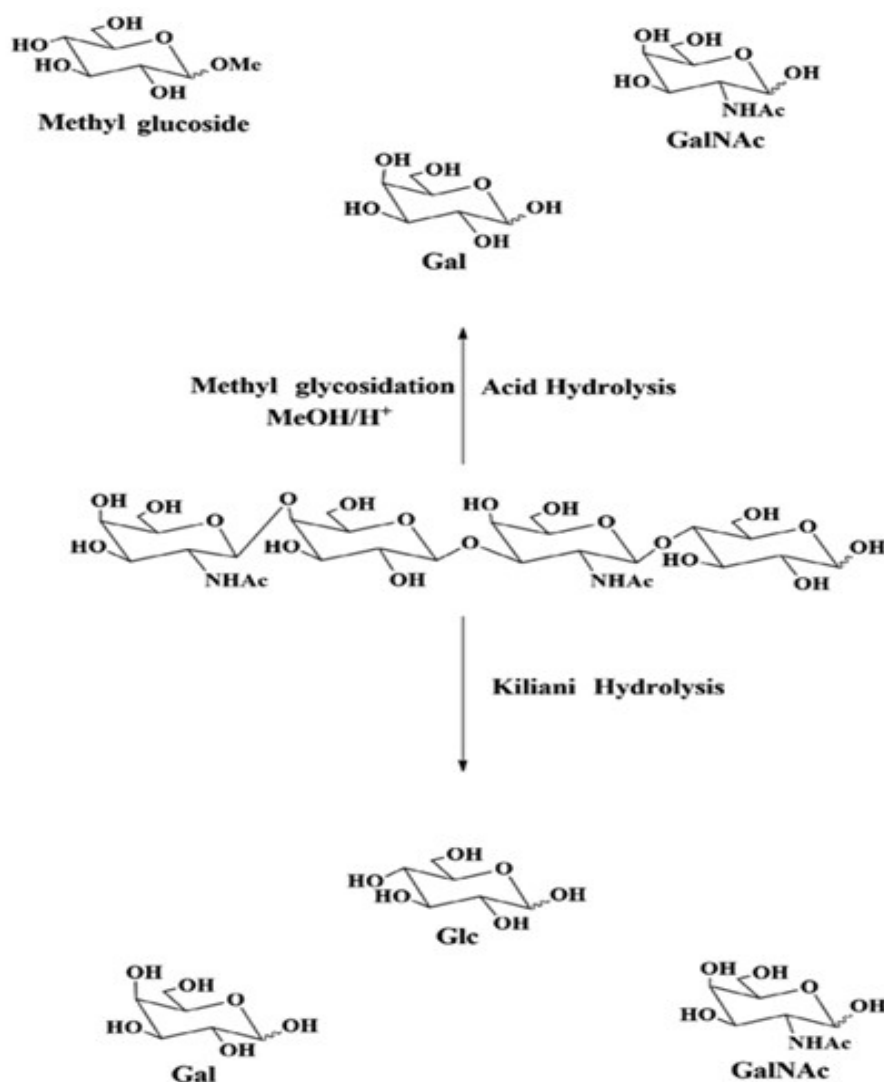
**Structure Elucidation of Isolated Goat Milk Oligosaccharide Crisose ('B')****NMR Spectroscopy**

The structure of the novel milk oligosaccharide, compound 'B' Crisose was elucidated with the help of chemical degradation, chemical transformation, spectroscopic techniques like NMR (<sup>1</sup>H, <sup>13</sup>C and 2D-NMR), structure reporter group theory and mass spectrometry. In the present study, analogies between chemical shift of certain 'structural reporter group resonances' were used to make proton resonance assignments as well as structural assignments of the oligosaccharides by comparing the <sup>1</sup>H NMR data of acetylated oligosaccharides and natural oligosaccharides. The <sup>1</sup>H NMR assignments were made by interpretation of data of COSY, TOCSY, HSQC and HMBC experiments.

**Compound 'B' Crisose**, C<sub>28</sub>H<sub>48</sub>O<sub>21</sub>N<sub>2</sub>, [ $\alpha$ ]<sub>D</sub><sup>25</sup> = -17°, isolated by repeated column chromatography of the oligosaccharide mixture, obtained from Goat milk, gave positive Phenol-sulphuric acid test, Feigl test, Morgon-Elson test showing the presence of normal and amino sugar(s) in the compound. The <sup>1</sup>H NMR spectrum of acetylated Crisose 'b' in CDCl<sub>3</sub> at 300 MHz exhibited four doublets for five anomeric protons at  $\delta$ 6.24(1H),  $\delta$ 5.68(1H),  $\delta$ 4.51(2H) and  $\delta$ 4.48(1H) indicating that Crisose 'B' may be a tetrasaccharide in its reducing form giving signals for  $\alpha$  and  $\beta$ -anomers at the reducing end. The tetrasaccharide nature

of Crisose acetate 'b' was further confirmed by the presence of four signals for five anomeric carbons at  $\delta 88.83(1C)$ ,  $\delta 91.41(1C)$ ,  $\delta 100.77(2C)$  and  $\delta 101.01(1C)$  in  $^{13}C$  NMR of acetylated Crisose 'b' at 300 MHz in  $CDCl_3$ . Moreover the tetrasaccharide nature of Crisose 'B' was supported by the presence of four anomeric proton doublets for five anomeric protons at  $\delta 5.59(1H)$ ,  $\delta 4.53(1H)$ ,  $\delta 4.38(2H)$ , and  $\delta 4.31(1H)$  in  $^1H$  NMR spectrum of compound Crisose 'B' in  $D_2O$  at 300 MHz. The reducing nature of compound Crisose 'B' was again confirmed by its methyl glycosylation followed by its acid hydrolysis ( $MeOH/H^+$ ), which led to the isolation of  $\alpha$  and  $\beta$ -methyl glucosides, along with Gal and GalNAc suggesting the presence of glucose at the reducing end and presence of Gal and GalNAc in the Crisose 'B'. The HSQC spectrum of acetylated compound Crisose 'b' at 300 MHz in  $CDCl_3$  showed the presence of four cross peaks of five anomeric protons and carbons in their respective

region at  $\delta 6.24 \times 88.83$ ,  $\delta 5.68 \times 91.41$ ,  $\delta 4.51 \times 100.77$  and  $\delta 4.48 \times 101.01$  suggesting that compound Crisose 'B' must be a tetrasaccharide in its reducing form. Thus  $^1H$  and  $^{13}C$  NMR spectra of acetylated Crisose 'b' justify the five anomeric signals for tetrasaccharide with total integral intensity of four anomeric protons/carbons. For convenience, starting from reducing end, the monosaccharides present in compound Crisose 'B' have been designated as S-1, S-2, S-3 and S-4. To confirm the monosaccharide constituents in compound Crisose 'B', it was hydrolyzed under strong acidic conditions of Kiliani hydrolysis which gave three monosaccharides i.e. Glc, Gal and GalNAc which were found identical with the authentic samples of Glc, Gal and GalNAc by co-chromatography (TLC and PC), confirming that the tetrasaccharide compound Crisose 'B' was consist of three types of monosaccharide units i.e. Glc, Gal and GalNAc.



**Scheme 1:** Acid hydrolysis/methyl glycosidation and Kiliani hydrolysis of Crisose ('B')

The molecular formula  $C_{28}H_{48}O_{21}N_2$  was in agreement with mass ion peak obtained from ES-MS spectrum of compound Crisose 'B' which showed the highest mass ion peak at  $m/z$  748  $[M]^+$  for a tetrasaccharide. The  $^1H$  NMR spectrum of compound Crisose 'B' in  $D_2O$  at 300 MHz contain two anomeric proton doublets at  $\delta$ 5.59 (d,  $J=3.9$  Hz) and  $\delta$ 4.53 (d,  $J=8.4$ Hz) for  $\alpha$  and  $\beta$ -anomers of reducing monosaccharides (S-1) i.e. Glc. The  $^1H$  NMR spectrum of Crisose acetate 'b' in  $CDCl_3$  at 300 MHz contain two anomeric proton doublets at  $\delta$ 6.24 (d,  $J=3.3$  Hz) and  $\delta$ 5.68 (d,  $J=8.1$  Hz) for  $\alpha$  and  $\beta$ -anomers of reducing monosaccharides (S-1) i.e. Glc. The anomeric protons signal present at  $\delta$ 5.68 in TOCSY Spectrum of Crisose acetate 'b' assigned to  $\beta$ -Glc (S-1) gave three cross peaks at  $\delta$ 5.68x5.03,  $\delta$ 5.68x5.27 and  $\delta$ 5.68x3.83, which was later identified as H-2, H-3 and H-4 of reducing Glc respectively by COSY spectrum of acetylated Crisose 'b' at 300 MHz in  $CDCl_3$ . The chemical shift of H-4 of S-1 at  $\delta$ 3.83 suggested that H-4 of S-1 was available for glycosidic linkage by next monosaccharide unit. Further the  $^1H$  NMR signal present at  $\delta$ 3.83 assigned to H-4 of reducing Glc (S-1) gave a cross peak at  $\delta$ 3.83x100.77 in HMBC spectrum of Crisose acetate 'b' which was

between H-4 of reducing Glc and C-1 of S-2, confirmed a (1 $\rightarrow$ 4) linkage between Glc (S-1) and S-2. The anomeric carbon of S-2 at  $\delta$ 100.77 gave its complimentary anomeric proton signal at  $\delta$ 4.51 (7.5 Hz) in the HSQC spectrum of Crisose acetate 'b'. The chemical shift values of anomeric carbon at  $\delta$ 100.77 and anomeric proton at  $\delta$ 4.51 were having resemblance with literature value of anomeric chemical shift value of GalNAc hence S-2 was confirmed as GalNAc. Further the coupling constant of anomeric signal (S-2) at  $\delta$ 4.51 with larger  $J$  value of 7.5 Hz confirmed the  $\beta$ -configuration of the glycosidic linkage between (S2 $\rightarrow$ S1) in Crisose acetate 'b'. Moreover the presence of  $\beta$ -GalNAc as next monosaccharide in Crisose 'B' was confirmed by appearance of anomeric proton signal at  $\delta$ 4.38 (7.8 Hz) along with a singlet of three protons at  $\delta$ 1.95 in  $^1H$  NMR spectrum of Crisose 'B' in  $D_2O$  at 300 MHz. Further the anomeric proton signal at  $\delta$ 4.51 assigned to S-2( $\beta$ -GalNAc) showed three cross peaks at  $\delta$ 4.51x4.07,  $\delta$ 4.51x3.88 and  $\delta$ 4.51x5.21 in the TOCSY spectrum of Crisose acetate at 300 MHz which was later identified as H-2, H-3 and H-4 of  $\beta$ -GalNAc (S-2) respectively by COSY spectrum of acetylated Crisose 'b' in  $CDCl_3$  at 300 MHz.

**Table 3:** Anomeric Proton Values of Compound Crisose 'B' in  $D_2O$  and Crisose Acetate 'b' in  $CDCl_3$  at 300 MHz

Moieties	In $D_2O$		In $CDCl_3$	
	$^1HNMR$ ( $\delta$ )	Coupling constant (J) Hz	$^1HNMR$ ( $\delta$ )	Coupling constant (J)Hz
$\alpha$ -Glc(S-1)	5.59	3.9Hz	6.24	3.3Hz
$\beta$ -Glc(S-1)	4.53	8.4Hz	5.68	8.1Hz
$\beta$ -GalNAc(S-2)	4.38	7.8Hz	4.51	7.5Hz
$\beta$ -Gal(S-3)	4.38	7.8Hz	4.51	7.5Hz
$\beta$ -GalNAc(S-4)	4.31	7.2Hz	4.48	8.7Hz

The chemical shift of H-2 of S-2 at  $\delta$ 4.07 was due to presence of  $NHCOCH_3$  at C-2 of S-2 ( $\beta$ -GalNAc) and the chemical shift of H-3 of S-2 at  $\delta$ 3.88 showed the availability of -OH group for glycosidic linkage by next monosaccharide unit i.e. S-3. Further the  $^1H$  NMR signal present at  $\delta$ 3.88 assigned to H-3 of  $\beta$ -GalNAc (S-2) gave a cross peak at  $\delta$ 3.88x100.77 in HMBC spectrum of Crisose acetate 'b' which was between H-3 of  $\beta$ -GalNAc (S-2) and C-1 of S-3 confirmed a 1 $\rightarrow$ 3 linkage between S-2 and S-3. The anomeric carbon of S-3 at  $\delta$ 100.77 gave its complimentary anomeric proton signal at  $\delta$ 4.51 in the HSQC spectrum of Crisose acetate 'b'. The chemical shift values of anomeric carbon at  $\delta$ 100.77 and anomeric proton at  $\delta$ 4.51 were having

resemblance with literature value of anomeric chemical shift value of Gal, confirming that S-3 was Gal. Further the coupling constant of anomeric signal of S-3 at  $\delta$ 4.51 with larger  $J$  value of 7.5 Hz confirmed the  $\beta$ -configuration of the glycosidic linkage between (S3 $\rightarrow$ S2) in Crisose acetate 'b'.

Further the presence of  $\beta$ -Gal as next monosaccharide in Crisose 'B' was confirmed by appearance of anomeric proton doublet at  $\delta$ 4.38 (7.8 Hz) in  $^1H$  NMR spectrum of Crisose 'B' in  $D_2O$  at 300 MHz. Further the anomeric proton signal at  $\delta$ 4.51 (7.5 Hz) assigned for  $\beta$ -Gal (S-3) showed three cross peaks at  $\delta$ 4.51x5.13,  $\delta$ 4.51x5.41 and  $\delta$ 4.51x3.82 in the TOCSY spectrum of acetylated

Crisose 'b' which was later identified as H-2, H-3 and H-4 of  $\beta$ -Gal (S-3) respectively by COSY spectrum of acetylated Crisose 'b' in  $\text{CDCl}_3$  at 300 MHz.

The chemical shift at  $\delta 3.82$  assigned to H-4 of sugar S-3 showed the availability of OH group for glycosidic linkage by next monosaccharide unit i.e. S-4. Further the HMBC spectrum of Crisose acetate 'b' at 300 MHz showed a cross peak signal of H-4 of  $\beta$ -Gal (S-3) and anomeric carbon of next monosaccharide C-1 of S-4 at  $\delta 3.82 \times 101.01$  confirmed a (1 $\rightarrow$ 4) linkage between S-4 and S-3. The anomeric carbon at  $\delta 101.01$  gave its complimentary anomeric proton signal at  $\delta 4.48$  in the HSQC spectrum of acetylated Crisose 'b'. The chemical shift values of anomeric carbon at  $\delta 101.01$  and anomeric proton at  $\delta 4.48$  were having resemblance with literature value of anomeric chemical shift value of GalNAc, confirming that S-4 was GalNAc. The coupling constant of anomeric signal (S-4) at  $\delta 4.48$  with larger J value of 8.7 Hz confirmed the  $\beta$ -configuration of the glycosidic linkage between (S4 $\rightarrow$ S3) in Crisose acetate 'b'. Further the presence of  $\beta$ -GalNAc as next monosaccharide in Crisose 'B' was confirmed by appearance of anomeric proton signal at  $\delta 4.31$  (7.2 Hz) along with a singlet of three protons at  $\delta 2.08$  in  $^1\text{H}$  NMR spectrum of Crisose 'B' in  $\text{D}_2\text{O}$ . The anomeric proton signal at  $\delta 4.48$  (8.7 Hz) assigned to  $\beta$ -GalNAc (S-4) gave three cross peaks at  $\delta 4.48 \times 3.90$ ,  $\delta 4.48 \times 5.40$  and  $\delta 4.48 \times 5.02$  in the TOCSY spectrum of Crisose acetate 'b' which was later identified as H-2, H-3 and H-4 of  $\beta$ -GalNAc (S-4) respectively by COSY spectrum of acetylated Crisose 'b' in  $\text{CDCl}_3$  at 300 MHz.

The chemical shift of H-2 of S-4 at  $\delta 3.90$  was due to  $\text{NHCOCH}_3$  at C-2 of S-4. Since The chemical shift values of ring protons of S-4 at  $\delta 5.40$  and  $\delta 5.02$  does not reside in the linkage region and hence they did not show any cross peak in the linkage region i.e.  $\delta 3.5$ - $4.2$  ppm confirming that  $\beta$ -GalNAc (S-4) was present at non-reducing end and none of its -OH group were available for glycosidic linkage, which was confirmed by the TOCSY and COSY spectra of acetylated Crisose 'b' in  $\text{CDCl}_3$  at 300 MHz. All the  $^1\text{H}$  NMR assignments for ring protons of monosaccharide units of Crisose 'B' were confirmed by COSY and TOCSY experiments. The positions of glycosidation in the oligosaccharide were confirmed by position of anomeric signals, Structure reporter groups (SRG) and comparing the signals in  $^1\text{H}$  and  $^{13}\text{C}$  NMR of acetylated and deacetylated

oligosaccharide. The glycosidic linkages in Crisose 'B' were assigned by the cross peaks for glycosidically linked carbons with their protons in the HSQC and HMBC spectra of Crisose acetate 'b'. All signals obtained in  $^1\text{H}$  and  $^{13}\text{C}$  NMR of compound Crisose 'B' were in conformity with the assigned structure and their positions were confirmed by 2D NMR viz. COSY, TOCSY, HSQC and HMBC experiments of Crisose acetate 'b'. Thus based on the pattern of chemical shifts of  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, COSY, TOCSY, HSQC and HMBC experiments, it was interpreted that the compound Crisose 'B', was a tetrasaccharide having the following structure:

**$\beta$ -GalNAc(1 $\rightarrow$ 4)- $\beta$ -Gal(1 $\rightarrow$ 3)- $\beta$ -GalNAc(1 $\rightarrow$ 4)Glc**

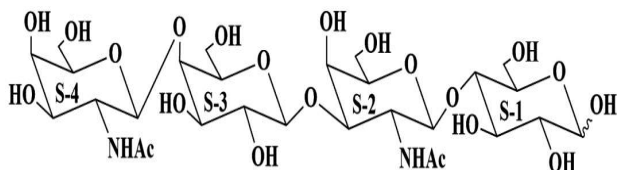
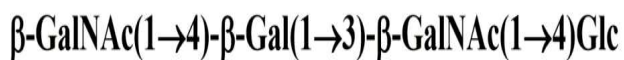
The Electrospray Mass Spectrometry data of compound Crisose 'B' not only confirmed the derived structure of Crisose 'B' but also supported the sequences of monosaccharides in this compound. The highest mass ion peak were recorded at m/z 787 and at m/z 771 which were due to  $[\text{M}+\text{K}]^+$  and  $[\text{M}+\text{Na}]^+$ . It also contains the molecular ion peak at m/z 748 confirming the molecular weight of Crisose 'B' as  $748[\text{M}]^+$  and was in agreement with its molecular formula  $\text{C}_{28}\text{H}_{48}\text{O}_{21}\text{N}_2$ . Further the mass fragments were formed by repeated  $\text{H}^+$  transfer in the oligosaccharide and was accompanied by the elimination of terminal sugar less water. The tetrasaccharide Crisose 'B' m/z 748(I) fragmented to give mass ion peak at m/z 545(II) [748-S<sub>4</sub>], this fragment was arised due to the loss of terminal  $\beta$ -GalNAc (S<sub>4</sub>) (203) moiety from tetrasaccharide indicating the presence of  $\beta$ -GalNAc (S<sub>4</sub>) at the non-reducing end. It was further fragmented to give mass ion peak at m/z 383(III) [545-S<sub>3</sub>] which was due to loss of  $\beta$ -Gal (S<sub>3</sub>) (162) moiety from trisaccharide.

This fragment of 383 was further fragmented to give mass ion peak at m/z 180(IV) [383-S<sub>2</sub>] which was due to loss of  $\beta$ -GalNAc (S<sub>2</sub>) (203) moiety from the disaccharide. The other fragmentation pathway in ES Mass spectrum of Crisose 'B', m/z 748 shows the mass ion peak at 690[748-NHCOCH<sub>3</sub>], 652[690-2H<sub>3</sub>O<sup>+</sup>], 651[652-H<sup>+</sup>], 545[748-S<sub>4</sub>], 503[545-CH<sub>2</sub>CO], 481[545-HCHO-2OH], 465[503-2H<sub>3</sub>O<sup>+</sup>], 421[465-CH<sub>3</sub>CHO], 406[481-CH<sub>3</sub>CHO-CH<sub>2</sub>OH], 383[545-S<sub>3</sub>], 325[383-NHCOCH<sub>3</sub>], 319[383-HCHO-2OH], 301[319-H<sub>2</sub>O], 259[325-HCHO-2H<sub>2</sub>O], 223[259-2H<sub>2</sub>O] and 180[383-S<sub>2</sub>].





Based on result obtained from chemical degradation/acid hydrolysis, Chemical transformation, Electro spray mass spectrometry and 1D-NMR viz.  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, Structure Reporter Groups (SRGs) and 2D-NMR viz. COSY, TOCSY, HMBC and HSQC spectra of Crisose acetate 'b' and Crisose 'B', the structure and sequence of isolated a novel oligosaccharide was deduced as:



### Structure of the Isolated Compound 'B', Crisose

### CONCLUSION

In summary, we conclude that the structure of the isolated goat milk novel oligosaccharide, compound 'B', Crisose is being reported for the first time from any natural source/any milk and was elucidated with the help of  $^1\text{H}$ ,  $^{13}\text{C}$ , 2D NMR spectroscopy and mass spectrometry. Using Gaussian 09 program at B3LYP method and 6-31 G basis set, the geometry of compound 'B' Crisose has been optimized.

### ACKNOWLEDGMENTS

Authors are thankful to the Head, Department of Chemistry, Lucknow University for providing the NMR facilities, Council of Scientific and Industrial Research (CSIR) New Delhi for their research grant and CDRI (SAIF) Lucknow for successfully running Mass experiments.

### CONFLICTS OF INTEREST

Authors have no conflict of interest to declare.

### REFERENCES

- [1] Singh P., Gupta N.K. and Deepak D. NMR, mass and DFT Studies of Ariose: A novel oligosaccharide from donkey (*Equus asinus*) milk. *Journal of Drug Delivery and Therapeutics*, **2019**, 9(4), 104-111.
- [2] Srivastava A.K., Singh P. and Deepak D. Isolation and NMR Studies of novel oligosaccharide from Goat milk. *J. Biol. Chem. Res.*, **2016**, 33, 901-908.
- [3] Catherine J.F. The Immunological Components of Human Milk and Their Effect on Immune Development in Infants, *J. Nutr.*, **2005**, 135, 1-4.
- [4] Goldman A.S. The immune system of human milk: antimicrobial, anti-inflammatory, and immunomodulating properties. *Pediatr. Infect. Dis. J.*, **1993**, 12, 664-672.
- [5] Urishima T., Taufic E., Fukuda K. and Asakuma S. Recent Advances in Studies on Milk Oligosaccharides of Cows and Other Domestic Farm Animals. *Biosci Biotechnol. Biochem.*, **2013**, 77, 455-466.
- [6] Singh P., Shahi S. and Deepak D. Isolation and NMR Characterization of Riesose- A Novel Oligosaccharide from Gaddi Sheep's Milk. *J. Biol. Chem. Res.*, **2018**, 35, 752-760.
- [7] Srivastava A.K., Singh P. and Deepak D. Isolation and NMR Studies of Novel Oligosaccharide from Goat Milk. *J. Biol. Chem. Research*, **2016**, 33(2), 901-908.
- [8] Singh P., Srivastava A.K. and Deepak D. Isolation and Structure Elucidation of Caprose (novel oligosaccharide) from Goat Milk. *J. Biol. Chem. Research*, **2017**, 34(1), 14-20.
- [9] Singh P., Shahi S. and Deepak D. Isolation and NMR Characterization of Riesose- A Novel Oligosaccharide from Gaddi Sheep's Milk. *J. Biol. Chem. Res.*, **2018**, 35, 752-760.
- [10] Hakkarainen J., Toivanen M., Leinonen A., Frangsmyr L., Stromberg N., Lapinjoki S., Nassif X. and Tikkanen-Kaukanen C. Human and Bovine Milk Oligosaccharides Inhibit *Neisseria meningitidis* Pili Attachment In Vitro1, *J. Nutr.*, **2005**, 135, 2445-2448.
- [11] Villoslada F.L., Debras E., Nieto A., Concha A., Gálvez J., López-Huertas E., Boza J., Obled C. and Xausa J. Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis, *Clin. Nutr.*, **2006**, 25, 477-488.
- [12] Kumar S., Tandon H.K.L. and Kapila S. Antihypertensive and immunomodulatory property of enzyme hydrolysates derived from goat casein. *Milk Science International*, **2011**, 66, 40-42.
- [13] Singh P., Srivastava A.K. and Deepak D. Isolation and Structure Elucidation of Caprose (Novel Oligosaccharide) from Goat Milk. *J. Biol. Chem. Res.*, **2017**, 34(1), 14-20.
- [14] Frisch, M.J., et al., **1995**. GAUSSIAN 94 Users Manual (Gaussian Inc., Pittsburgh, PA).
- [15] Gunjan, Kumar K. and Deepak D. Structural characterization of novel milk

oligosaccharide Aurose from cow colostrum, *J. Mol. Struct.*, **2019**, 1176, 394-401.

- [16] Dubois M., Gilles K.A., Hamilton J.K., Rebers P.A. and Smith F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **1956**, 28, 350-356.
- [17] Singh A.K., Ranjan A.K., Srivastava G., and Deepak D. Structure elucidation of two novel yak milk oligosaccharides and their DFT studies, *J. Mol. Struct.*, **2016**, 1108, 87-91.