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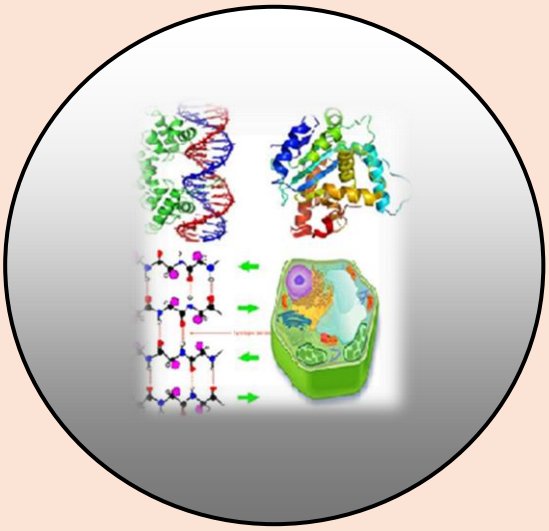

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Structural Characterization of Kankrej Cow Milk Oligosaccharides by 2D NMR Techniques

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ABSTRACT

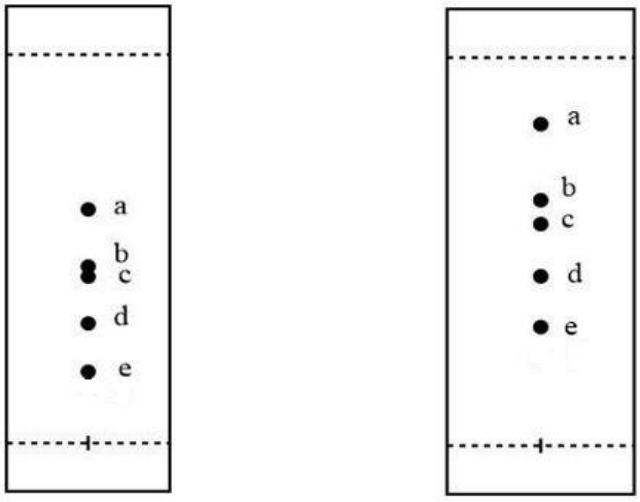
Cow milk is one of the most common available milk worldover. The importance of cow milk is well defined and describe in Ayurveda and other ancient medicinal system. Different varieties of cow are present in India and the milk constituent of every cow is different. The biological activity in the cow milk depends on the fodder they take and the flora and fauna around them. In our research group we have worked upon different variety of cow species and reviewed the oligosaccharides content of this species. It was found that the biological activity present in the cow milk is due to its oligosaccharide contents while researches on the milk oligosaccharide contents of cows belonging to Rajasthan state of India, which is an arid zone. Three varieties of cow are most abundantly available in Rajasthan i.e. Rathi, Kankrej and Tharparkar. While characterizing the milk oligosaccharides of the cows belonging to Rajasthan, a special and novel variation was found in the oligosaccharides structures present in their milk. The oligosaccharides present were not in their reducing form rather they were present as their glycosides. The same observations were also found in the oligosaccharide content of the Kankrej cow, as the oligosaccharide present therein was present as their methyl glycoside. In this present article we have described the general procedure of isolation (filteration, evaporation and lyophilisation), purification (gel filteration, Column chromatography and HPLC) and structures elucidation (^{1}H, ^{13}C, HMBC, HSQC, TOCSY and COSY) of the oligosaccharide present in the Kankrej cow milk.

Keywords: Milk oligosaccharides, Kobata and Ginsburg, Kankrej cow milk, Structure elucidation and Purification.

INTRODUCTION

Amongst all the various mammalian milks, cow's milk has shown importance, which is well described in Ayurveda and Charak Samhita. The availability of cow milk is common world over. Recent research on cow's milk has shown the benefit of cow milk. In India, number of varieties and species are available that produce a quantitative output of cow milk. example, Jersey, Sahiwal, Gir, Kankrej, Rathi, Tharprkar, etc., among the three cow breeds, Kankrej, Rathi, and Tharprkar, are native to Rajasthan State of India. As the major part of Rajasthan is made up of deserts and is an arid zone, and it is a well-defined fact that the constituents of any milk depend on the food and fodder of the animal, the flora and fauna, and the geographical conditions around them. In milk, the main constituents are protein, fats, and carbohydrates (lactose and oligosaccharides). Oligosaccharides are important constituents of any milk responsible for their biological activity. Generally, these oligosaccharides are present as reducing oligosaccharides and are made up of monosaccharide building blocks, i.e., glucose (Glc), galactose (Gal), N- acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), L-fucosamine (FucNAc), N-acetyl neuraminic acid or sialic acid (NeuGc)[1], with variable configurations, i.e., α and β and linked at different positions of monosaccharides, resulting in (1→2), (1→3), (1→4) and (1→6) linkages constituting straight and branched chain oligosaccharides. The oligosaccharides isolated from the milk of cow species found in Rajasthan showed a rare structural diversity. As oligosaccharides isolated from these cow species, were not present as free sugars; instead, they were present as their methyl glycosides. Furthermore, in some cases, aromatic substitution was also seen in the monosaccharide rings present in the oligosaccharides. The isolation and identification of these oligosaccharides is not an easy task. For that purpose, standard methods are being used for obtaining the crude oligosaccharide mixture of particular milk. The oligosaccharide content of milk varies from two to three percent. The most standard method for the isolation of milk oligosaccharide was the method of Kobata and Ginsburg, which wasused for isolation of milk oligosaccharides [Kobata and Ginsburg et al 2010]. In this process the collected milk samples were freeze for their preservation at -20^0 C and after the collection of appropriate quantities, they were processed by the above method. There were some other methods that were also developed by different scientists in different parts of the world, for example, Egge et al 1983, Smith et al 1978, Urashima et al 1997, Weiruszeski et al 1985, Kobata and Ginsburget al 2010, and the Modified Kobata and Ginsburg method and Deepak et. al 2018. Further in the early nineties, we concentrated on this particular project and made some modifications, and we named this process the modified method of Kobata and Ginsburg. The major change in this process was that we never freeze the sample; rather, after the collection of milk, we mix an equal amount of ethyl alcohol to preserve it. After preservation, the process was the same as adopted by Kobata and Ginsburg: deprotination, microfiltration, and lyophilization to obtain the crude milk oligosaccharide mixture of particular milk. Further, we have again made some modifications to the methods of Kobata and Ginsburg. For the purification of the crude oligosaccharide mixture, which still contained glycoprotein and lactose, we performed gel filtration or sephadex chromatography [Giddings JC. 1994] to this crude oligosaccharide mixture to separate the higher molecular weight compound, like glycoprotein, and protein with the lower molecular weight compound, lactose, from the crude oligosaccharide mixture.

The sephadex chromatography fractionated and separated the glycoprotein and lactose from the oligosaccharide mixture. After obtaining the oligosaccharide mixture, the major challenge was to isolate these oligosaccharides in their purified form. Further due to the presence of hydroxyl groups present in the oligosaccharides; they were highly polar compounds. It was a difficult task to purify them due to their highly polar nature because it was not possible to isolate them in their purified form by silica or alumina column chromatography. To avoid this problem, we have again modified the process of Kobata and Ginsburg by converting these polar oligosaccharides into their non-polar acetyl derivatives by their acetylation in the presence of acetic anhydride and pyridine. Which was monitored andvisualized easily by thin layer chromatography and were isolated and purified comfortably on silica column chromatography by using a gradient system of appropriate solvents, i.e., hexane, chloroform, and methanol, in their various proportions. The conversions of oligosaccharides into their acetyl derivatives were helpful in the structure elucidation of the oligosaccharides also. As such, these acetylated oligosaccharides were easily soluble in $CDCl_3$, providing good resolution in their NMR spectra. Moreover, the ^{1}H and ^{13}C NMR, along with COSY, TOCSY, HSQC, and HMBC experiments, were also helpful in assigning the position of glycosidic linkages in the oligosaccharides. For this purpose, we have collected 10 liters of Kankrej cow milk from Hanuman Garhi district in Rajasthan state, India. This was processed by the modified method of Kobata and Ginsburg involving deprotination, microfiltration, gel chromatography, and lyophilization. This resulted in obtaining 252 g of oligosaccharide mixture out of this mixture. 11 g of the oligosaccharide mixture was acetylated with 11 ml of acetic anhydride and 11 ml of pyridine at 60^{0} C with continuous stirring for 24 hours. Further, it was dried under reduced pressure at room temperature, and it was extracted with 250 ml of chloroform and dried over anhydrous sodium sulphate, resulting in the isolation of a 10.68 g of acetylated oligosaccharide mixture which was purified on silica column chromatography. This acetylated oligosaccharide mixture was visualized on thin layer chromatography, which gave five spots, i.e., a, b, c, d, and e.



2:98 (MeOH:$CHCl_3$) **5 : 95 (MeOH:$CHCl_3$)**

Figure 1.TLC of Acetylated Oligosaccharides Mixture.

ACETYLATION OF OLIGOSACCHARIDE MIXTURE

11g oligosaccharide mixture was acetylated with pyridine (11 ml) and acetic anhydride (11 ml) at 60^{o}C and the solution was stirred overnight. The mixture was evaporated in a water bath under reduced pressure, and viscous residue was taken in $CHCl_3$ (500 ml) and washed twice with cold water. It was then evaporated to dryness, yielding the acetylated mixture (10.68g).

The acetylation converted the free sugars into their non-polar acetyl derivatives, which were resolved nicely on thin layer chromatography (TLC) [Ghebregzabher M et al 1976], giving eight spots, i.e., **a, b, c, d,** and **e,** of which four compounds were finally separated by column chromatography over silica gel using varying proportions of hexane, chloroform, and methanol as eluents. Detection of the spots was done by spraying it with 50% H_2SO_4.

PURIFICATION OF ACETYLATED MILK OLIGOSACCHARIDES ON SILICA GEL COLUMN [Kriz J et al 1994]

Column Chromatography-1

Purification of the acetylated oligosaccharide mixture (10.68g) was carried over silica gel (250g) using various proportions of $CHCl_3$ and $CHCl_3$: MeOH as eluents, collecting fractions of 250 ml each. All these fractions were checked on TLC, and those showing similar spots were taken together for further investigations. The chromatography was controlled on TLC, and details are given in the table below as follows:

Table 1.10.68g mixture chromatographed over 250g SiO_2(CC-1).

Fraction No.	Solvent	Eluted Residue Amorphous	Spots on TLC	Further Investigation
1-10	CHCl3	853mg	No Spots	-
11-20	CHCl3	95mg	No Spots	-
21-30	CHCl3	283mg	a with streaking	-
31-33	CHCl3	138mg	a	Physico-chemicalinvestigation
34-44	CHCl3	122mg	a with streaking	-
45-54	MeOH: CHCl3 (0.1: 99.9)	870mg	No Spots	-
55-60	MeOH: CHCl3 (0.2: 99.8)	235mg	b with streaking	-
61-65	MeOH: CHCl3 (0.2: 99.8)	109mg	b c	CC-2
66-69	MeOH: CHCl3 (0.2: 99.8)	140mg	No Spots	-
70-79	MeOH: CHCl3 (0.5:99.5)	211mg	d with Streaking	CC-3
80-89	MeOH: CHCl3 (1: 99)	577mg	Streaking	-

90-94	MeOH: $CHCl_3$ (1: 99)	122mg	e with streaking	-
95-98	MeOH: $CHCl_3$ (2: 98)	68mg	e with streaking	-
99	MeOH: $CHCl_3$ (2: 98)	168 mg	e	Physico-chemicalinvestigation
100-107	MeOH: $CHCl_3$ (2: 98)	1.033 g	Streaking	-
108-119	MeOH: $CHCl_3$ (5: 95)	1.145 g	Streaking	-
120-132	MeOH: $CHCl_3$ (8: 92)	1.239 g	Streaking	-
133-140	MeOH: $CHCl_3$ (10: 90)	1.112 g	Washing	-

Substance **"a"**(138mg) was obtained from fraction (31-33) of chromatography [1] and **"e"** (168 mg) chromatography [1] was obtained from fraction (99).

Column Chromatography-2

Combined fractions 61-65 (109 mg) from Chromotography [1] containing b and c was chromatographed over 10g silica gel. The elution was carried out using $CHCl_3$ and $CHCl_3$:MeOH as eluents, collecting fractions of 10ml each and discussed in table given below:

Table 2.Fractions No. 61-65 (109 mg) Mixture Chromatographed over 10 gm silica gel.

Fraction no.	Solvent	Eluted Residue Amorphous (mg)	Spots on TLC	Further Investigation
1-10	$CHCl_3$	3mg	No Spots	_
11-12	MeOH:$CHCl_3$ (0.1:99.9)	13mg	b with streaking	_
13	MeOH:$CHCl_3$ (0.1:99.9)	40mg	b	Physio-chemical investigation
14-20	MeOH:$CHCl_3$ (0.1:99.9)	_	No Spots	_
21-30	MeOH:$CHCl_3$ (0.2:99.8)	2mg	No Spots	_
31-34	MeOH:$CHCl_3$ (0.5:99.5)	8mg	c with streaking	_
35	MeOH:$CHCl_3$ (0.5:99.5)	36mg	c	Physio-chemical investigation
36-40	MeOH:$CHCl_3$ (0.5:99.5)	2mg	Streaking	_
41-45	MeOH:$CHCl_3$ (0.5:99.5)	5mg	Streaking	_
46-50	MeOH:$CHCl_3$ (1:99)	3mg	Washing	_

Substance "b" (40mg) and "c" (36mg) were obtained from fraction (61-65) of chromatography.

Column Chromatography-3

A fraction 70-79 (211 mg) from Chromatography 1 containing d was chromatographed over 20 g silica gel. The elution was carried out with $CHCl_3$ and $CHCl_3$: MeOH in different proportions collecting 20ml each and discussed in table given below:

Table 3.Fractions No. 70-79 (211 mg) Mixture Chromatographed over 20 g silica gel.

Fraction no.	Solvent	Eluted Residue Amorphous (mg)	Spots on TLC	Further Investigation
1-5	$CHCl_3$	27mg	Streaking	_
6-12	MeOH:$CHCl_3$ (0.1:99.9)	14mg	Streaking	_
13-18	MeOH:$CHCl_3$ (0.2:99.8)	62mg	d with streaking	_
19-30	MeOH:$CHCl_3$ (0.5:99.5)	52mg	d	Physio-chemical investigation
31-37	MeOH:$CHCl_3$ (1:99)	6mg	d with streaking	_
38-42	MeOH:$CHCl_3$ (1:99)	21mg	Streaking	_
43-50	MeOH:$CHCl_3$ (2:98)	16mg	Washing	_

Substance **"d"** (52mg) was obtained from fraction (19-30) of chromatography [3].

Table 4. Acetylated and Deacetylated oligosaccharides obtained from Kankrej Cow Milk.

Acetylated & Deacetylated oligosaccharides of Kankrej Cow Milk						
Acetylated Compound				Deacetylated Compound		
Alphabetical name	Analytical notation	Quantity (mg)		Alphabetical name	Analytical notation	Quantity (mg)
		Obtained by column	taken for deacetylation			
a	SKCM-a	138	54	A	SKCM-A	42
b	SKCM-b	40	40	B	SKCM-B	34
c	SKCM-c	36	36	C	SKCM-C	31
d	SKCM-d	52	42	D	SKCM-D	39

Table 5. Description of Isolated Oligoglycosides from Kankrej Cow Milk.

	A	B	C	D
Analytical Notation	SKCM-A	SKCM-B	SKCM-C	SKCM–D
Physical State	Syrupy	Syrupy	Syrupy	Syrupy
Mol. Formula	$C_{31}H_{54}O_{26}$	-	-	$C_{33}H_{57}O_{26}N$
ES mass(m/z)	842	-	-	883
Phenol-sulphuric test	+ve	+ve	+ve	+ve
Morgon-Elson test	+ve	+ve	+ve	+ve
Thiobarbituric acid test	-ve	-ve	-ve	-ve
Bromocresol green test	-ve	-ve	-ve	-ve

DEACETYLATION OF ISOLATED COMPOUNDS [Singh AK et al 2016]

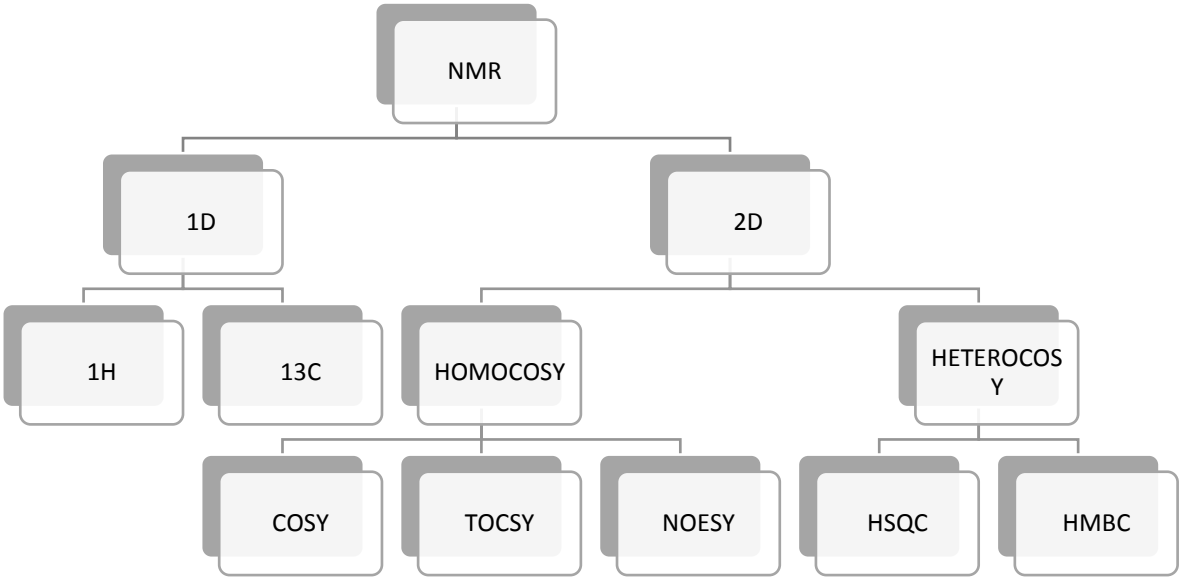
The compounds **(a, b, c, and d)** obtained from column chromatography of the acetylated oligosaccharide mixture were dissolved in acetone, and NH_4OH was added and left overnight in a stoppered hydrolysis flask. Ammonia was removed under reduced pressure, and the compounds were washed three times with $CHCl_3$ (to remove acetamide), and the water layer was finally freeze dried, giving the deacetylated oligosaccharides, which were finally freeze dried, giving the deacetylated native oligosaccharides **(A, B, C, and D)**. Details are given in Table No. 11.

1. Test for normal sugar for milk oligosaccharide.
2. Test for amino sugar for milk oligosaccharide.
3. Test for sialic acid for milk oligosaccharide.
4. Test for carboxylic acid for milk oligosaccharide.

Structure Elucidation of Milk Oligosaccharide

Milk oligosaccharides are a complex class of compounds that have varied biological activity. Milk is composed of protein, fat, and carbohydrates. The major carbohydrate content of the milk is lactose; along with, some other oligosaccharides ranging from trisaccharide to undecasaccharide which have been reported in the milk. These oligosaccharides are referred as milk oligosaccharides. The first study of these oligosaccharides showed their prebiotic and probiotic activities. Further studies on the biocompatibility of these oligosaccharides showed their involvement in various biological processes; they showed immunosuppressive [Xiao et al 2019], antibacterial [Craft et al 2019], cancer resistance [Fang et al 1985], brain-developing [Fan Y et al 2023] and anti-inflammatory activities [Maslowski et al 2009]. These oligosaccharides are made up of glucose, galactose, glcNHAc, galNHAc, fucose, and silaic acid as their building blocks. While looking into their structures, it was found that in most of the oligosaccharides, glucose was present at their reducing end, followed by galactose linked to reducing glucose by β (1→4) glycosidic linkage. In other words, we can also say that the lactose was β-gal (1→4) β-glc (1→4). It has always been seen that fucose and sialic acid were mostly present at the non-reducing end if they are part of the oligosaccharide chain. Later, it was also found that there was some change at the reducing end of the oligosaccharide. Although glucose was present at the reducing end, the second monosaccharide was not galactose; instead, galNHAc was present as a second monosaccharide with the same β (1→4) glycosidic linkage. In some of the oligosaccharides, it was found that the reducing glucose was followed by another glucose linked by β (1→4) glycosidic linkage. Recently, in some sheep milk oligosaccharides, galactose was present at the reducing monosaccharide and again linked by another galactose by (1→2) glycosidic linkage, making it a rare oligosaccharide. Very recently, it was reported that the milk oligosaccharides present in cow milk were not in their reducing form; instead, they were present as their methyl glycoside. The specialty of the cow milk oligosaccharides may also be referred to as very special as the oligosaccharides present had an aromatic substitution in them. We have come to the conclusion that these oligosaccharides were very rare in nature and have a complex and complicated structure, which makes it difficult to elucidate their stereoscopic structure. There are a number of methods by which we can interpret the structure of milk oligosaccharide.

The traditional method in which the structure was elucidated by chemical degradation and chemical transformation, including acid hydrolysis for the identification of monosaccharide units in the oligosaccharide, while in another methodology, i.e., methylglycosidation [Gangwar et al 2018], followed by acid hydrolysis. The nature-reducing monosaccharide present in the oligosaccharide along with the monosaccharide constituent of the oligosaccharide, these methodologies become successful to a certain extent, but their limitation was that they are unable to explain the position of interglycosidic linkages, the configuration of glycosidic linkages, and the conformation of the monosaccharide present therein. To overcome these problems, the structure elucidation of these rare oligosaccharides was performed by using the physicochemical technique in general and nuclear magnetic resonance in particular. Since the isolation of these rare oligosaccharides was very difficult, and it is also very difficult to interpret the structure of these oligosaccharides due to their stereoscopic constraints. Keeping in mind all these problems, we came to the conclusion that NMR is the only non-invasive physicochemical tool by which we can interpret the structure of these oligosaccharides. Since the quantities of these oligosaccharides isolated from natural sources are very meagre, this further created more complications. This problem was solved by incorporating the fourier transformation techniques into the NMR machine, by which we can elucidate the structure of any quantity, even if it is present in microgram quantities. NMR is the only non-invasive tool made by physicists to help chemists and biologists. This technique is used for the structure elucidation of synthetic as well as naturally occurring compounds. It is also used as a diagnostic tool for the identification of various diseases inside the human body, which is called MRI (magnetic resonance imaging). In recent times, high-resolution NMR machines (400-800 MHz) loaded with the Fourier transformation technique (FT NMR) are capable of providing the data for the structure elucidation of organic molecules, even if they are present in micrograms. The combination of 1D and 2D NMR experiments is capable of providing complete data for the stereoscopic structure, configuration, and conformation of any organic molecule.



The NMR experiment may be classified into two classes of experiments, i.e., 1D and 2D. 1D NMR is further classified into ^{1}H and ^{13}C NMR, while the 2D NMR experiments are diversified into two classes, i.e., HOMOCOSY and HETEROCOSY. As comes from their name, the HOMOCOSY experiments are those experiments in which the correlation between proton and proton is generated. The example of these experiments is 1H-1H COSY. ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H NOESY experiments. The HETEROCOSY experiments are those in which the proton-carbon correlation is studied. The examples of HETEROCOSY experiments are HSQC and HMBC.

For structure elucidation of these sugars, we combine the results obtained from different experiments of NMR, which are ^{1}H NMR, ^{13}C NMR, and 2D experiments of NMR, i.e., correlated spectroscopy COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), HSQC (homoneuclear single quantum correlation spectroscopy), and HMBC (heteroneuclear multiple bond coherence spectroscopy).

^{1}H NMR OF MILK OLIGOSACCHARIDES: It is the primary and most informative NMR experiment. It tells us about the number and nature of hydrogens present in the molecules [Buda et al 2016]. The range of ^{1}H NMR is δ0- 10 ppm. Basically, there are three types of hydrogens in any natural or synthetic structure, i.e., CH_3, CH_2, and CH. If we look into the chemical shift of these protons with reference to tetramethylsilane, the methyl (CH_3) protons are located from δ1.0–2.2, the methylene (CH_2) protons reside between δ2.2–3.2, and the CH protons are found between δ3.2–4.2, provided no electronegative element is added to these groups, which creates a lot of difference in their chemical shifts, making a downfield shift from 1 to 2 ppm. While looking into the structure elucidation of an oligosaccharide, the most important centre is the anomeric proton or carbon, which is the starting point of the structure elucidation of oligosaccharides.

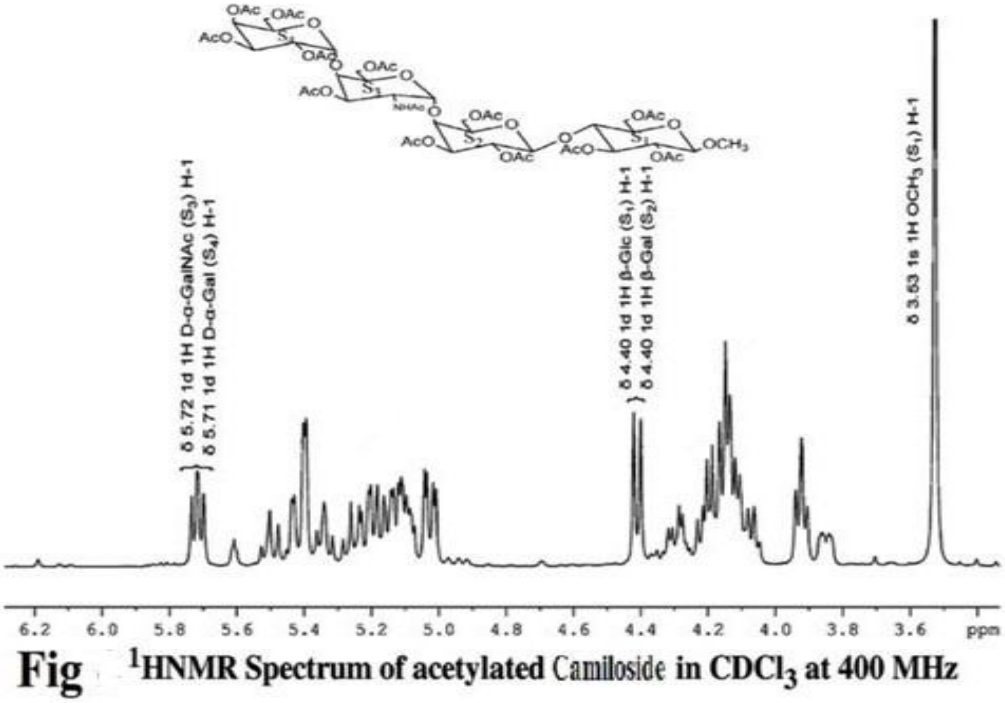


Fig ^{1}HNMR Spectrum of acetylated Camiloside in $CDCl_3$ at 400 MHz

The chemical shift value of an anomeric proton varies from δ4–δ5.5, provided no functional group like acetoxy or benzoxy is added, which further causes a downfield shift of δ1–δ1.2 ppm [Agrawal PK et al 1992]. The chemical shift of any anomeric proton depends on the configuration and conformation of a particular monosaccharide, i.e., glucose, galactose, galNHAc, glcNHAc, and fucose. The chemical shift values of different monosaccharides are given in the following table:

Table 6.^{1}H Chemical Shifts and Coupling Constant of D-Aldohexoses[Bock Ket al 1983].

Compound	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
D-Hexopyranoses							
α-glucose	5.09 (J=3.6)	3.41 (J=9.5)	3.61 (J=9.5)	3.29 (J=9.5)	3.72	3.72 (J=2.8)	3.63 (J=5.7, 12.8)
β-glucose	4.51 (J=7.8)	3.13 (J=9.5)	3.37 (J=9.5)	3.30 (J=9.5)	3.35	3.75 (J=2.8)	3.60 (J=5.7, 12.8)
α-galactose	5.16 (J=3.8)	3.72 (J=10.0)	3.77 (J=3.8)	3.90 (J=1.0)	4.00	3.70 (J=6.4)	3.62 (J=6.4)
β-galactose	4.48 (J=8.0)	3.41 (J=10.0)	3.56 (J=3.8)	3.84 (J=1.0)	3.61	3.70 (J=3.8)	3.62 (J=7.8)
α-mannose	5.05 (J=1.8)	3.79 (J=3.8)	3.72 (J=10.0)	3.52 (J=9.8)	3.70	3.74 (J=2.8)	3.63 (J=6.8, 12.2)
β-mannose	4.77 (J=1.5)	3.85 (J=3.8)	3.53 (J=10.0)	3.44 (J=9.8)	3.25	3.74 (J=2.8)	3.60 (J=6.8, 12.2)
β-allose	4.76 (J=8.5)	3.30 (J=3.3)	4.05 (J=3.2)	3.51 (J=9.5)	3.66	3.76 (J=2.4)	3.57 (J=6.0, 12.8)
β-gulose	4.76 (J=8.3)	3.52 (J=3.6)	3.95 (J=3.6)	3.70 (J=0.8)	3.92	3.62 (J=6.0)	3.58 (J=6.0)

Table 7.^{1}H Chemical Shifts and Coupling Constant of D-Aldopentoses[Bock Ket al 1983].

Compound	H-1	H-2	H-3	H-4	H-5a	H-5e
D- Pentopyranoses						
β-xylose	4.47 (J=7.8)	3.14 (J=9.2)	3.33 (J=9.0)	3.51	3.82 (J=5.6)	3.22 (J=10.5, 11.4)
α-xylose	5.09 (J=3.6)	3.42 (J=9.0)	3.48 (J=9.0)	3.52	3.58 (J=7.5)	3.57 (J=7.5)
β-arabinose	5.12 (J=3.6)	3.70 (J=9.3)	3.77 (J=9.8)	3.89	3.54 (J=2.5)	3.91 (J=1.7, 13.5)
α-arabinose	4.40 (J=7.8)	3.40 (J=9.8)	3.55 (J=3.6)	3.83	3.78 (J=1.8)	3.57 (J=1.3, 13.0)
β-ribose	4.75 (J=2.1)	3.71 (J=3.0)	3.83 (J=3.0)	3.77	3.82 (J=5.3)	3.50 (J=2.6, 12.4)

α-ribose	4.81 (J=6.5)	3.41 (J=3.3)	3.98 (J=3.2)	3.77	3.72 (J=4.4)	3.57 (J=8.8, 11.4)
β-lyxose	4.89 (J=4.9)	3.69 (J=3.6)	3.78 (J=7.8)	3.73	3.71 (J=3.8)	3.58 (J=7.2, 12.1)
α-lyxose	4.74 (J=1.1)	3.81 (J=2.7)	3.53 (J=8.5)		3.84 (J=5.1)	3.15 (J=9.1, 11.7)

Table 8.^{1}H Chemical Shifts and Coupling Constant for Methyl-D-Hexosides [Bock K et al 1983].

Compound	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	OMe
D-hexopyranosides								
α-glucose	4.70 (J=4.0)	3.46 (J=10.0)	3.56 (J=10.0)	3.29 (J=10.0)	3.54	3.77 (J=2.8, 12.8)	3.66 (J=5.8)	3.31
β-glucose	4.27 (J=8.2)	3.15 (J=9.6)	3.38 (J=9.6)	3.27 (J=9.6)	3.36	3.82 (J=2.4, 12.8)	3.62 (J=6.4)	3.46
α-galactose	4.73 (J=3.0)	3.72 (J=9.8)	3.68 (J=2.3)	3.86 (J=1.0)	3.78	3.67 (J=8.2, 12.0)	3.61 (J=4.6)	3.31
β-galactose	4.20 (J=8.0)	3.39 (J=10.0)	3.53 (J=3.8)	3.81 (J=0.8)	3.57	3.69 (J=7.6, 11.2)	3.64 (J=4.4)	3.45
α-mannose	4.66 (J=1.6)	3.82 (J=3.5)	3.65 (J=10.0)	3.53 (J=10.0)	3.51	3.79 (J=1.9, 12.0)	3.65 (J=5.8)	3.30
β-mannose	4.47 (J=0.9)	3.88 (J=3.2)	3.53 (J=10.0)	3.46 (J=10.0)	3.27	3.83 (J=2.2, 12.2)	3.63 (J=6.7)	3.44

Table 9.^{1}H Chemical Shifts and Coupling Constant for Methyl-D Pentosides [Bock Ket al 1983].

Compound	H-1	H-2	H-3	H-4	H-5e	H-5a	OMe
D-pentopyranosides							
α-arabinose	4.16 (J=8.0)	3.43 (J=10.0)	3.57 (J=3.9)	3.85	3.82 (J=2.8, 13.8)	3.57 (J=1.0)	3.44
β-arabinose	4.72 (J=2.8)	3.74 (J=10.0)	3.72 (J=3.0)	3.89	3.55 (J=2.3, 13.0)	3.77 (J=1.0)	3.30
α-lyxose	4.58 (J=3.2)	3.77 (J=3.8)	3.68 (J=4.0)	3.76	3.69 (J=4.8, 12.0)	3.42 (J=9.0)	3.32
β-lyxose	4.51 (J=2.2)	3.14 (J=3.8)	3.60 (J=7.5)	3.75	3.89 (J=4.0, 12.5)	3.23 (J=7.5)	3.37

α-ribose	4.51 (J=3.0)	3.70 (J=3.2)	3.86 (J=3.2)	3.72	3.47	3.68	3.35
β-ribose	4.52 (J=5.1)	3.51 (J=3.4)	3.91 (J=3.4)	3.79	3.74 (J=3.5, 12.5)	3.61 (J=7.0)	3.37
α-xylose	4.67 (J=3.4)	3.44 (J=10.0)	3.53	3.47	3.59 (J=5.0, 11.0)	3.39 (J=11.0)	3.30
β-xylose	4.21 (J=7.9)	3.14 (J=9.5)	3.33 (J=9.5)	3.51	3.88 (J=5.5, 12.3)	3.21 (J=11.0)	3.44

After the assignment of anomeric proton signals, which are determined by the chemical shifts obtained from the HSQC experiment, which provide the chemical shift of anomerric carbon and anomeric proton of a particular monosaccharide, the values so obtained are compared with the anomeric values of proton/carbons and the anomeric/proton values of a particular monosaccharide with the literature values, which are given in the above table No. 6,7,8, and 9. Further, the comparison of the observed value with the reference value confirms the nature of monosaccharides, i.e., glucose, galactose, fucose, etc. This whole process is known as Structure Reporter Group (SRG). The next stage in the interpretation of an oligosaccharide is the assignment of glycosidic linkages, which is not an easy task and is the limitation of the 1D and ^{1}H NMR experiments. Although in the traditional system of structure elucidation, the comparative study of the ^{1}H NMR spectrum of natural compounds and their derivatized compounds, such as acetate, gives an idea regarding the position of glycosidiclinkages, it does not give the perfect result, which could be resolved by doing the 2D NMR experiments.

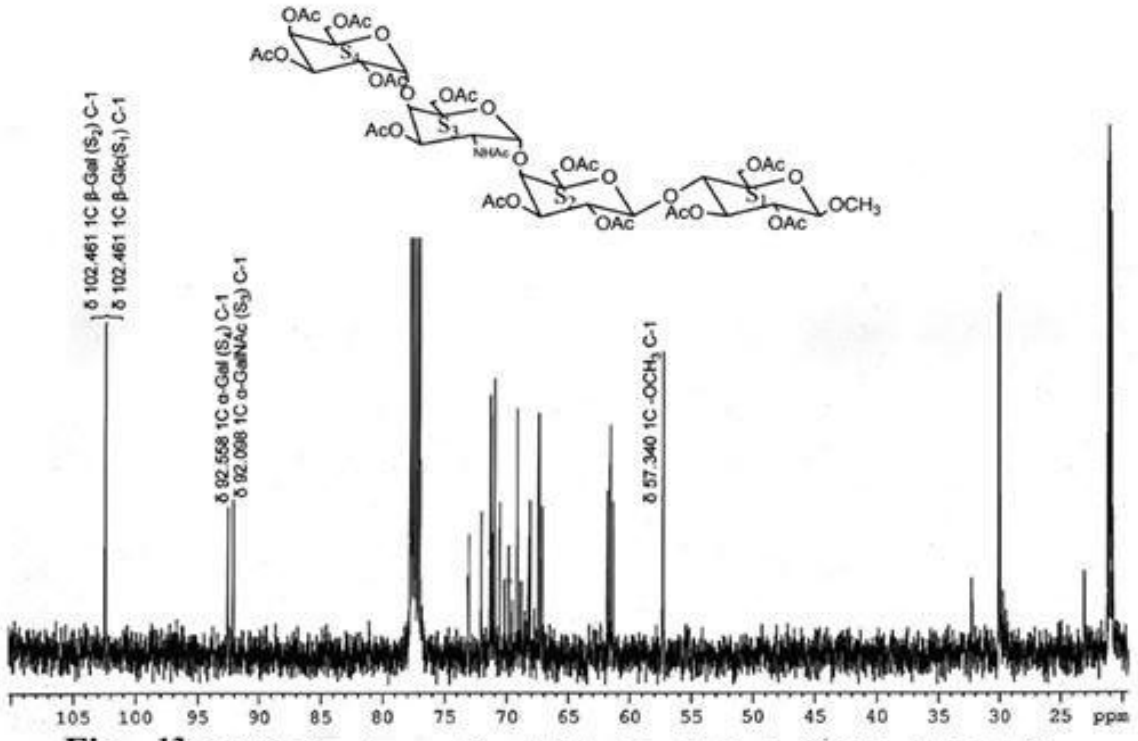


Fig ^{13}C NMR Spectrum of acetylated Camiloside in $CDCl_3$ at 100 MHz

^{13}C NMR OF MILK OLIGOSACCHARIDE: It is the next experiment in the series of structure elucidation of oligosaccharides, which is comparatively easier than ^{1}H NMR to interpret because there is no splitting in ^{13}C NMR, and by counting the ^{13}C NMR signal in the ^{13}C spectrum, we can count the number of carbons present in a particular structure [Bush et al 1983]. Further, the range of ^{13}C NMR is from δ0-200 ppm, which has a 20-time wider range than the ^{1}H NMR, which gives a better resolution than the ^{1}H NMR and also resolves the crowding of signals that overcrowded in the ^{1}H NMR spectrum. Like the ^{1}HNMR, the molecule of the carbohydrate has also three types of signals, i.e., CH_3, CH_2, and CH, and the chemical shift sequence of these signal are the same as in case of proton NMR i.e. the CH_3 signal comes first when we start from the TMS signal and range of methyl carbon are generally δ0-35ppm followed by the CH_2 signal which range is δ35-60ppm further the most important CH signal resides in the region δ65-80, here again the most important signals are anomeric carbons, the range of anomeric carbon are from δ90-110ppm[Agrawal et al 1992].

Table 10.^{13}C Chemical Shifts for Aldoses [Bock et al 1983].

D-Hexopyranoses						
Compound	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**
α-Allose	93.7	67.9	72.0	66.9	67.7	61.6
β-Allose	94.3	72.2	72.0	67.7	74.4	62.1
α-Altrose	94.7	71.2	71.1	66.0	72.0	61.6
β-Altrose	92.6	71.6	71.3	65.2	75.0	62.5
α-Galactose	93.2	69.4	70.2	70.3	71.4	62.2
β-Glactose	97.3	72.9	73.8	69.7	76.0	62.0
α-Glucose	92.9	72.5	73.8	70.6	72.3	61.6
β-Glucose	96.7	75.1	76.7	70.6	76.8	61.7
α-Gulose	93.6	65.5	71.6	70.2	67.2	61.7
β-Gulose	94.6	69.9	72.0	70.2	74.6	61.8
α-Idose	93.2	73.6	72.7	72.6	73.6	59.4
β-Idose	93.9	71.1	68.8	70.6	75.6	62.1
α-Mannose	95.0	71.7	71.3	68.0	73.4	62.1
β-Mannose	94.6	72.3	74.1	67.8	77.2	62.1
α-Talose	95.5	71.7	70.6	66.0	72.0	62.4
β-Talose	95.0	72.5	69.6	69.4	76.5	62.2

D-Pentoopyranoses	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**
α-Arabinose	97.6	72.9	73.5	69.6	67.2
β-Arabinose	93.4	69.5	69.5	69.5	63.4
α-Lyxose	94.9	71.0	71.4	68.4	63.9
β-Lyxose	95.0	70.9	73.5	67.4	65.0
α-Ribose	94.3	70.8	70.1	68.1	63.8
β-Ribose	94.7	71.8	69.7	68.2	63.8
α-Xylose	93.1	72.5	73.9	70.4	61.9
β-Xylose	97.5	75.1	76.8	70.2	66.1

Although the anomeric carbons are of CH nature, but due to the anomeric effect and dipole present in the monosaccharide molecule, they give this variation and are located downfield, showing a difference from other CH Carbons. The ^{13}C NMR signal is also helpful for giving the idea of the glycosidically linked methine proton, but as such, it also does not provide the exact position of glycosidic linkages, which is only covered by the results obtained from 2D NMR experiments.

Table 11. ^{13}C Chemical Shifts for Some Methyl Aldosides [Bock et al 1983].

D-Hexopyranosides							
Compound	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**	**OMe**
α-Allose	100.0	68.3	72.1	68.0	67.3	61.7	56.3
β-Allose	101.9	72.2	71.4	68.0	74.8	62.2	58.0
α-Altrose	101.1	70.0	70.0	64.8	70.0	61.3	55.4
β-Altrose	100.4	70.7	70.2	65.6	75.6	61.7	57.7
α-Galactose	100.1	69.2	70.5	70.2	71.6	62.2	56.0
β-Galactose	104.5	71.7	73.8	69.7	76.0	62.0	58.1
α-Glucose	100.0	72.7	74.1	70.6	72.5	61.6	55.9
β-Glucose	104.0	74.1	76.8	70.6	76.8	61.8	58.1
α-Gulose	100.4	65.5	71.4	70.4	67.3	62.0	56.3
β-Gulose	102.6	69.1	72.3	70.5	74.9	62.1	58.1
α-Idose	101.5	70.9	71.8	70.3	70.8	60.2	55.8
α-Mannose	101.9	71.2	71.8	68.0	73.7	62.1	55.9
β-Mannose	101.3	70.6	73.3	67.1	76.6	61.4	56.9
α-Talose	102.2	70.7	66.2	70.3	72.1	62.3	55.6

D-Pentopyranosides	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**OMe**
α-Arabinose	107.0	73.9	75.6	71.5	69.3	60.0
β-Arabinose	103.0	72.1	70.1	71.4	65.7	58.1
α-Lyxose	102.0	70.4	71.6	67.4	63.3	55.9
α -Ribose	100.4	69.2	70.4	67.4	60.8	56.7
β -Ribose	103.1	71.0	68.6	68.6	63.9	57.0
α -Xylose	100.6	72.3	74.3	70.4	62.0	56.0
β -Xylose	105.1	74.0	76.9	70.4	66.3	58.3

2D NMR OF MILK OLIGOSACCHARIDE (OS): these experiments become helpful in assigning the correlations of proton-proton and proton-carbon inspite the molecule, and they in pin-pointing the exact location of glycosidic linkages and ring proton/carbons. As defined earlier, there are two types of correlations, i.e., proton proton and proton carbon, which are described as HOMOCOSY and HETEROCOSY respectively [Bush et al 1983]. Since the oligosaccharides are very complex structures with a limited area of chemical shift, there is a lot of overcrowding of protons making complcation. This crowding occurs into the range of δ3-5 ppm, which are due to ring proton and anomeric protons as well. To resolve and overcome these problems, the most necessary action is the assignment of anomeric protons carbons, which tells us the number of monosaccharides in the molecule.

For the assignment of anomeric signals, the HSQC experiment plays a definite role by assigning cross peaks of anomeric proton carbons in the region of δ4.2–5.8 ppm (the chemical shift region of anomeric protons) into δ90–110 (the chemical shift of anomeric carbons). These cross peaks verify and justify, rather confirm, the number of anomeric signals, further defining the number of monosaccharides in the particular oligosaccharides.

HETERO NUCLEAR SINGLE QUANTUM COHRENCE (HSQC) EXPERIMENT ON MILK OLIGOSACCHARIDE (OS) [Farooq H et al 2013]: It is an example of the correlation of proton and carbon chemical shifts of each and every carbon and proton present in the molecule. It is a very important experiment for the assignment of the correlation of proton and carbon present in the oligosaccharide. The most important information obtained from this experiment is the assignment of anomeric proton and carbon signals, which come as cross peaks in the HSQC spectrum on two axes, in which one axis is for the chemical shift of proton signals from δ0 to δ10 pmm, while the other axis gives information regarding the ^{13}C chemical shift, which ranges from δ0 to δ200 ppm. In the cross peaks for each and every carbon obtained in their respective region. The cross peaks of anomeric proton carbon are obtained in the region δ4 toδ5.6 ×δ90 toδ110 ppm. It also provides information regarding the position of glycosidic linkages, which could be seen in the region δ3.2 –δ4.2 ×δ60-δ75ppm.

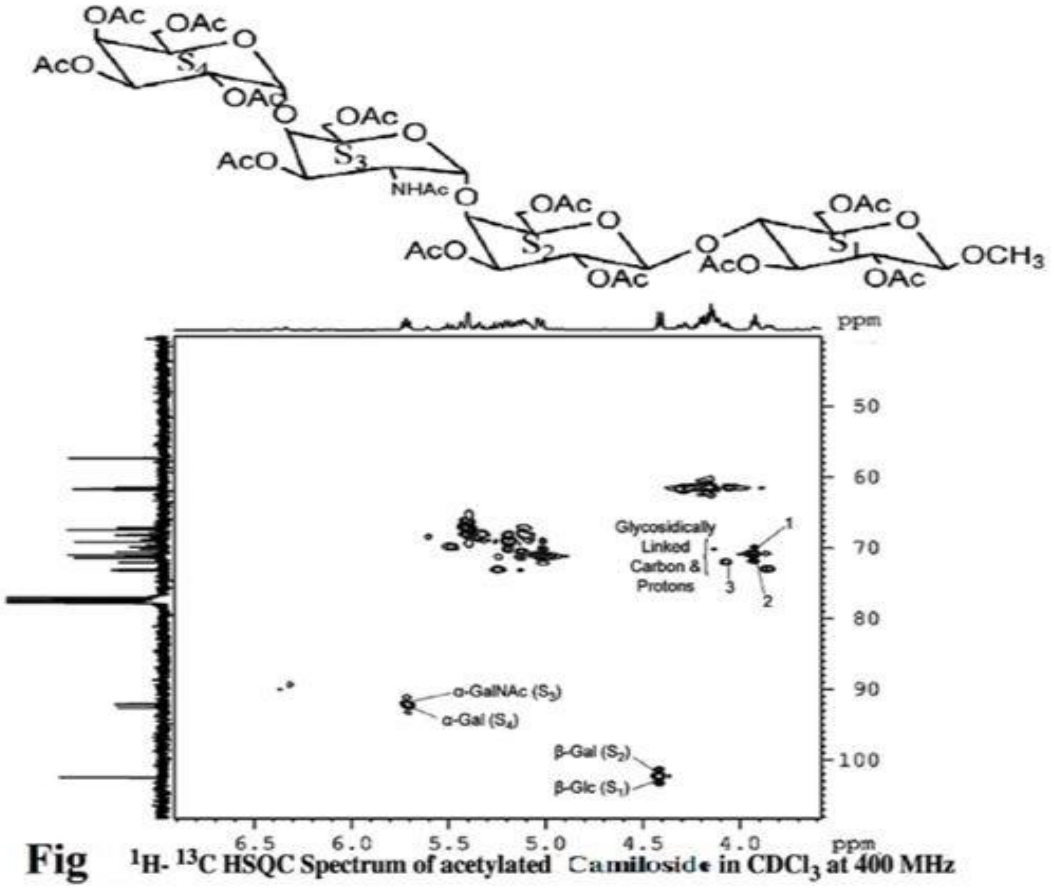


Fig ^{1}H- ^{13}C HSQC Spectrum of acetylated Camiloside in $CDCl_3$ at 400 MHz

2D TOCSY EXPERIMENTS OF MILK OLIGOSACCHARIDES [Davis et al 1985]: It is an important experiment of HOMOCOSY experiments that defines the total correlation of proton proton in each monosaccharide unit in a particular oligosaccharide.

This experiment sieves and saggregates the total methine protons of the oligosaccharides with respect to every monosaccharide present in the oligosaccharides. For example, an oligosaccharide contains five monosaccharides. For inconvenience, we rename them S1, S2, S3, S4, and S5. From the reducing end, we start our experiments with the reducing sugar S1 and select the chemical shift of the reducing monosaccharide S1 from the HSQC experiments. Further, this anomeric chemical shift is examined under the TOCSY experiment, which defines the total number of correlations of S1 and provides a cross peak for H1×H2, H1×H3, H×H4, and H1×H5. It is important to note that the TOCSY experiment examines each anomeric proton signal vertically with respect to the cross peak, the excess peak we obtain does not follow the order of 2, 3, or 4 of the ring proton but may arise according to their chemical shift, and they don't follow any order. Its order may be (4,3,2), (3,2,4), or (2,3,4); the order of the correlation is confirmed by COSY experiments. The information obtained from the TOCSY experiment is very important. Besides the segregation of the ring proton of a particular anomeric proton, it also gives an idea for the position of glycosidic linkages, i.e., the cross peaks residing in the chemical shift region of 3–4 on the x-axis suggest this position is available for glycosidic linkage by the next monosaccharide.

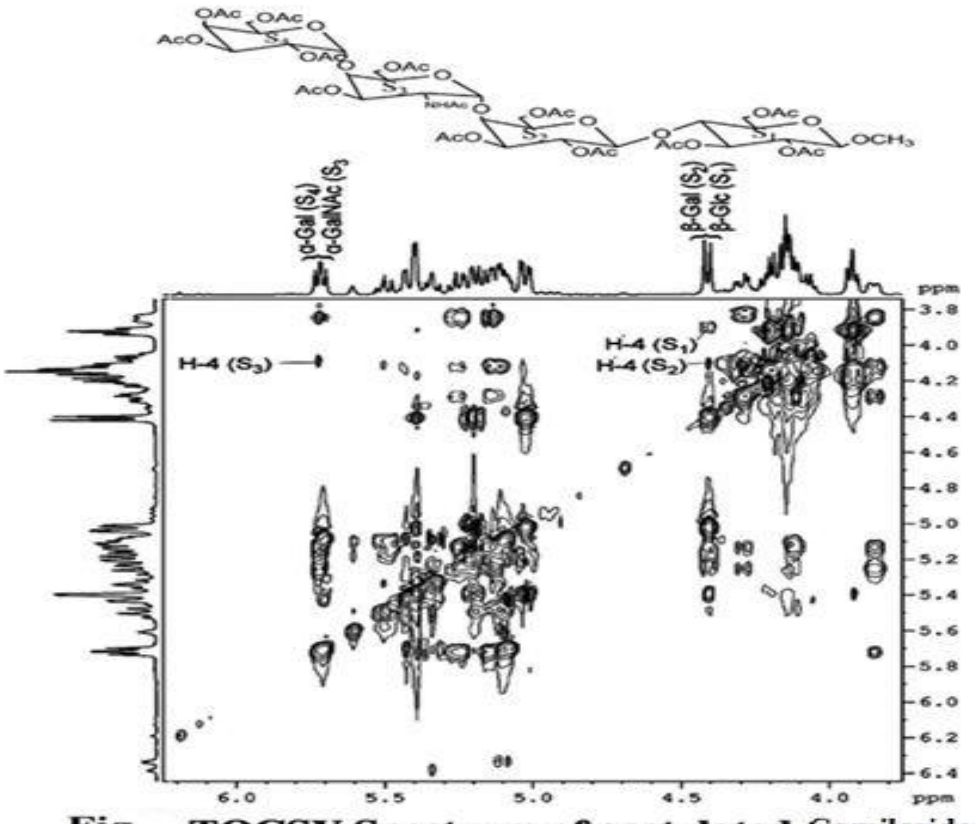


Fig TOCSY Spectrum of acetylated Camiloside in $CDCl_3$ at 400 MHz

2D COSY EXPERIMENT OF MILK OLIGOSACCHARIDES [Davis et al 1985]: Correlative spectroscopy may be classified into two categories, i.e., HOMOCOSY and HETEROCOSY which provide correlations between the same type of nuclei, i.e., proton-proton correlation, and connections between different nuclei, i.e., proton-carbon, respectively.

In HOMOCOSY 1H-1H experiments, we get the correlations between adjacent or vicinal protons. The information obtained from the COSY experiment is very important. Before the advent of the COSY experiment, these correlations were obtained by decoupling or double resonance experiments, which were also limited to one carbon length, but in those experiments, the correlations obtained were very limited, and the correlation of a full molecule was not possible. After the invention of HOMOCOSY experiments, a series of correlations may be obtained in the form of cross-peaks. In case of milk oligosaccharides, these correlations not only provide important information but also fix the position of glycosidic linkages in the oligosaccharide.

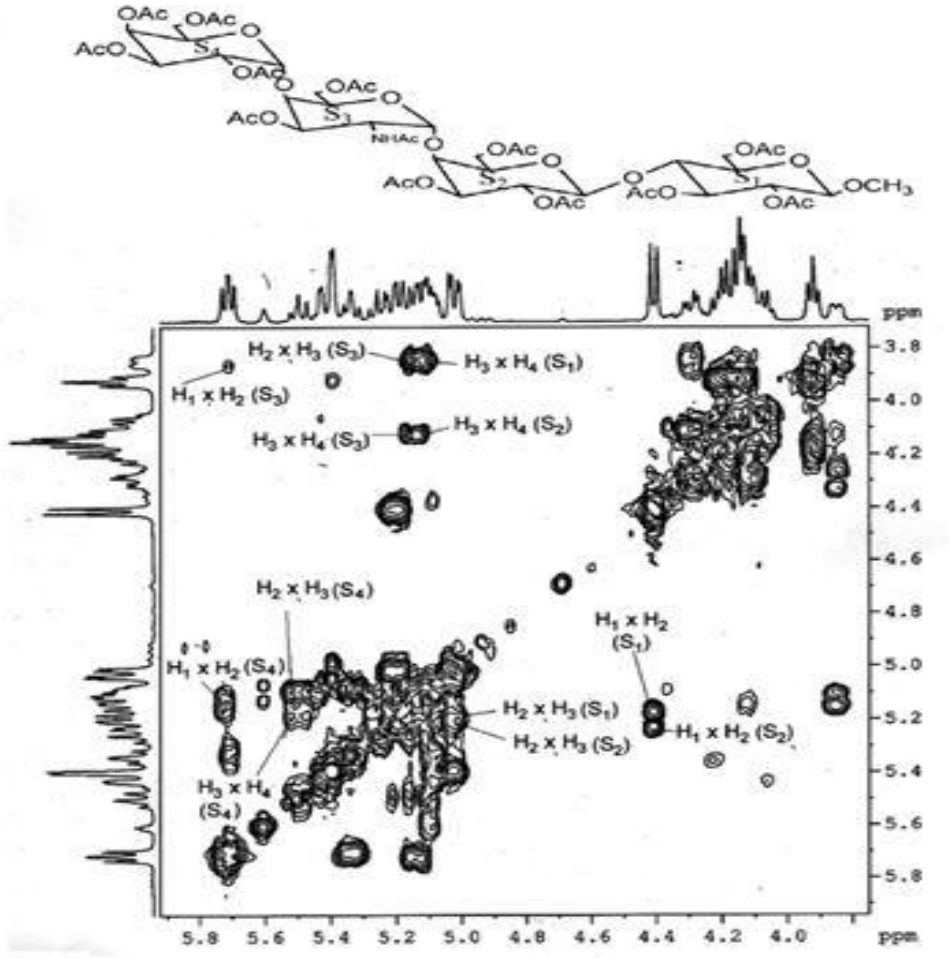


Fig COSY Spectrum of acetylated Camiloside in $CDCl_3$ at 400 MHz

For these purposes, the identification of anomeric signals is very important, which is procured and confirmed by HSQC experiments. After obtaining the chemical shift of a particular anomeric proton, we further examine it in the TOCSY experiment, which sieves and saggregates the ring proton of a particular anomeric proton but also the cross peak, so we obtain the sequence for the assignment of H2, H3, H4 and H5 of a particular anomeric proton. This sequencing of H2 to H5 is confirmed by analysing the cross peak obtained from the COSY spectrum, which confirms the position of each methine proton along with the position of glycosidic linkage.

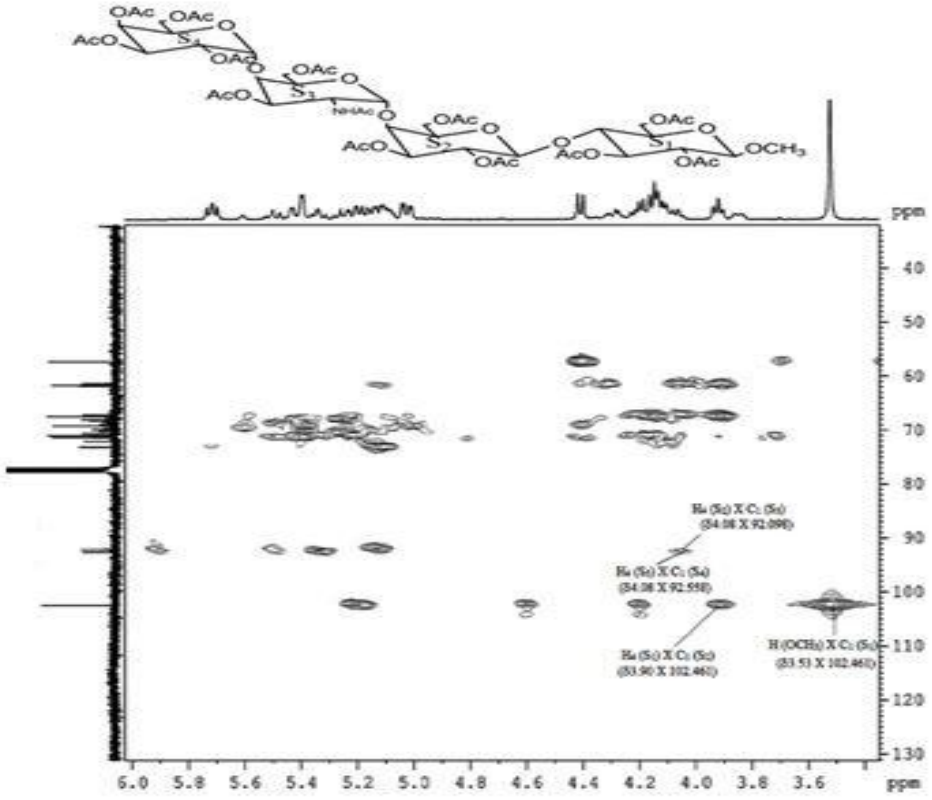


Fig ^{1}H-^{1}C HMBC Spectrum of acetylated Camiloside in $CDCl_3$ at 400 MHz

HETERONUCLEAR MULTIPLE BOND CORRELATIONS SPECTROSCOPY (HMBC) [Davis et al 1985]: This experiment is an example of the correlation of heteronuclei and is used for the assignment of glycosidic linkages and the sequence of monosaccharides in the particular oligosaccharide. At this stage, it is necessary to define the process of sequencing in an oligosaccharide. For convenience, the monosaccharides present in an oligosaccharide are conventionally identified as S1, S2, S3, S4, etc., starting from the reducing end. After the assignment of the reducing anomeric proton signal, i.e., S1, and assigning it's a ring proton by the TOCSY experiment and confirming the correlation and sequencing of ring protons, the next problem is the assignment of the next monosaccharide in sequence, i.e., S2. Since the structure elucidation of milk oligosaccharide is performed on acetylated oligosaccharide, we look into the chemical shift of ring protons, and the chemical shift of ring proton, which recides at δ3.4 to δ4.1 ppm, reflects that this particular ring proton is involved in glycosidic linkage with the next monosaccharide. For this purpose, the chemical shift so obtained is examined in the HMBC spectrum, which gives the cross peak between the methine signal of S1 (H-2, H-3, H-4) with the chemical shift between δ3.4 to δ4.1 ppm and the anomeric carbon of the next monosaccharide, i.e., S2. This process is continued until the non-reducing end of the oligosaccharide is obtained. The methine proton signals of the non-reducing end are obtained in the chemical shift region between δ4.3 to δ5.3 ppm. This whole process confirms the sequence of monosaccharides in an oligosaccharide, which may have a straight or branched chain oligosaccharide.

While in the process of sequencing monosaccharide by HMBC experiment, due to some steric hinderence and obstacle, these HMBC cross peaks are lost sometimes. Hence, restricting the process of sequencing monosaccharides into oligosaccharides to overcome this problem, another experiment of 2D HMBC, called reverse HMBC, is incorporated.

In this experiment, the lost cross peaks between the ring anomeric proton of S1 and the anomeric carbon signal of S2 are reexamined for the continuation of the sequencing process. For this purpose, the chemical shift of the ring proton involved in glycosidic linkages is further considered in the HSQC experiment, from where the ^{13}C NMR chemical shift of this particular ring proton is obtained, which is again looked for in the HMBC experiments, which provide the cross peak between the ^{13}C chemical shift of the ring proton and the anomeric proton chemical shift of the next sugar in sequence. This whole process is called reverse HMBC. That helps for continuing a sequence of the monosaccharide in a particular oligosaccharide.

CONCLUSION

In this article we have described in detail the methodology for isolation and structure elucidation of oligosaccharide isolated from Kankrej cow milk. We have given the methodology for isolation of milk oligosaccharide incorporating deprotination, microfiltration, lyophylisation, and purification involving gel filteration, column chromatography and HPLC, spectroscopic techniques like 1D (^{1}H and ^{13}C), 2D (HSQC, TOCSY, COSY and HMBC) has also been defined for structure elucidation of milk oligosaccharides.

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