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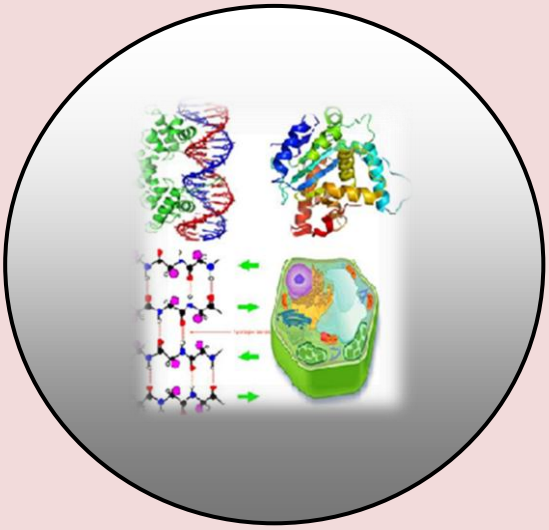

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Horse/Mare Milk Oligosaccharides: Isolation, Structure Elucidation and Biological Activities

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ABSTRACT

Oligosaccharides are biologically active components of any mammalian milk. Due to various compositions of its monosaccharide contents i.e. Glucose, Galactose, GlcNAc, GalNAc, Fucose and Sialic acid they show varied biological activities. The configuration of glycosidic linkages and conformation of monosaccharides also play a decisive role in the biological activity of these naturally occurring oligosaccharides. The position of glycosidic linkages between different monosaccharides, in a straight or branched chain also plays a definite role in the biological activity of oligosaccharides isolated from particular milk. Besides the common milks like cow, buffalo, goat and sheep, some other rare milks i.e. mare, yak, camel and elephant has also been investigated for their oligosaccharide contents. In the present article we are describing the oligosaccharide contents of mare milk. Horse/mare is a robust mammal used for transport and other purposes. Its milk shows prebiotic, anti-adhesive and anti-inflammatory, lowers cholesterol intake as well as to control cardiovascular diseases, cures Crohn's disease, ulcerative colitis as well as hepatitis and chronic gastric ulcers. In view of its biological activity a number of milk oligosaccharides have been isolated from mare milk and their structures elucidated by chemical degradation, chemical transformation and spectroscopic techniques like NMR (1D and 2D) 1H, 13C, HSQC, HMBC, COSY, TOCSY and Mass spectrometry. This paper deals in detail with the techniques used in isolation, purification, structure elucidation and biological activity of Mare milk oligosaccharides.

Keywords: Horse/Mare, Milk oligosaccharides, Structure elucidation, NMR, Biological activity.

INTRODUCTION

Milk is a complete and highly nutritional food with well-known nutritional and dietary characteristics, especially for children. Milk has very low allergenicity, better digestibility and least side effects. Milk has received increasing attention as potential ingredients of health-promoting functional foods targeted at diet-related chronic diseases, such as cardiovascular disease, diabetes type-2 and obesity. Many of these beneficial properties of milk for human health are related with oligosaccharides, glycoprotiens, peptides, fatty acids. Milk is considered as a rich source of many bioactive components such as oligosaccharides, Proteins, Lipids, Lactose, Vitamins and Immunoglobulins etc. These components not only help in meeting the human nutritional requirements, but also play a relevant role in preventing various disorders such as hypertension and cardiovascular diseases, osteoporosis, dental caries, poor gastrointestinal health, colorectal cancer, and others. Various Factors present in milk are known to modulate the developing microbiota within the infant gastrointestinal tract (GIT), immunoglobulin, lactoferrin, lysozyme, bioactive lipids, luecocytes and various milk glycans (glycolipids, glycoprotiens and free oligosaccharides) (Newburg et al. 2005). Milk oligosaccharides, which are one of the most important components of milk, have recently been recognized as significant anti-infectional compounds against pathogenic viruses and bacteria as well as being an energy source for the newborn. Milk oligosaccharides exhibit complex structures which are capable of modulating intestinal microbiota by exerting a prebiotic, anti-adhesive and anti-inflammatory effect and of improving the immune system of newborn (Daddaoua et al., 2006; Mills, Ross, Hill, Fitzgerald, & Stanton, 2011; Underwood et al., 2015). Oligosaccharides are present in the form of glycoconjugates (glycoproteins and glycolipids) in all cell walls mediating a variety of events such as inflammation, cell–cell recognition, immunological response, metastasis, and fertilization. These oligosaccharides have a monosaccharide chain and are responsible for many of the beneficial effects of milk on human health. These compounds may be associated with the development of the central nervous system and have therapeutic applications because of their prebiotic, anti-adhesive, and anti-pathogenic effects. Milk oligosaccharide is one of the most important components of milk which considered as “nutraceutical” that is, the food that conveys immunological and other health benefits together with nutritional contribution. Milk oligosaccharides seem to serve as an analog of epithelial receptor in intestine, acting as protectors of mucosa (Newburg, Ruizpalacios, & Morow 2005). The consumption of functional oligosaccharides can reduce the risk of lifestyle related disease such as cardiovascular disease, cancer, obesity and diabetes, (Mussato & Mancilha, 2007). Amongst the important source of oligosaccharides, Milk of different origin is one of the richest natural sources and a large number of biologically active oligosaccharides have been isolated in our laboratory from donkey, buffalo, goat, camel, dog, sheep, reindeer, polar bear, kangaroo, lal mua cow and mare etc. Various developments on medicinal and pharmaceutical researches have revealed a lot of nutritional and medicinal properties of these isolated oligosaccharides. A broad range of milk oligosaccharides and their derivatives act as an effective drug against most of acute and chronic diseases, and play an essential role in many molecular processes impacting eukaryotic biology and diseases. Human milk oligosaccharides have been known to have many biological functions including as prebiotics for stimulating the growth of beneficial intestinal bacteria, as receptor analogs to inhibit the binding of pathogens and as

components involved in modulating immune system. One of the most striking feature of human milk is its diversity of oligosaccharides, with numerically more than 200 till date. It was postulated that a mixture of oligosaccharides are more beneficial than a single structure. For this reason, the milk of domestic animal has become focal point in the recent years as the source of complex oligosaccharides with associated biological activities. More than 500 milk oligosaccharides have been isolated from milk of cow, buffalo, donkey, horse, sheep, goat, bear and human. These Oligosaccharides are divided into two broad classes, neutral and acidic. Neutral oligosaccharides do not contain any charged carbohydrate residues. However, acidic oligosaccharides contain one or more residues of N-acetylneuraminic acid (Sialic acid) which are negatively charged (hence these are termed as acidic oliogosaccharides). These oligosaccharides are the complex mixture of six monosaccharides namely, D-glucose, D-galactose, N-acetyl-glucosamine, N-acetyl-galactosamine, L-fucose and N-acetyl-neuraminic acid. These oligosaccharides isolated from various milk sources are categorized in two classes i.e. sialylated oligosaccharide and non-sialylated oligosaccharide. The composition and the level of these two classes of oligosaccharides are influenced by factors related to the animal, their lactation, and their milk production in the same way as other milk components. Studies indicate that these variables can also induce differences between the level of acidic and neutral oligosaccharide during lactation (Mehra and Kelly 2006). Both these class of oligosaccharides have been tested for their varied biological activities and exhibited a wide-range of applications such as anti-diabetic, antioxidant, reduction of oxidative stress, cardioprotective, prevention of urogenital infections, exert immunomodulatory properties, used in treatment of hyperammonimia and protosystemic encephalopathy, reduce gastrointestinal diseases, increasing capacity of absorping minerals of calcium, magnesium and iron, used in the treatment of arteriosclerosis and colon cancer, anti-hypersensitivity, hypocholesterolemic effects, enhancement of brain development and monocytes to produce tumor necrosis factor alpha, anti-hyperlipidemic and anti-microbial effects etc.

Mare milk and its derivatives (koumiss and cheese) (Urbisinov and Servetnick-Chalaya, 1983; Koroleva, 1988) are widely used in the Euroasiatic region (Mongolia, Russia, etc.), has good nutritional value with protein, lipid, lactose, oligosaccharides and therapeutic food source because of high content of lactoferrin. Mare milk has been played an important role in various human deseases from ancient time .Use of horse milk to treat the suffering of patients from tuberculosis has been practiced for a long time in Russia and Mongolia. It has been reported that drinking mare milk increases the number of erythrocytes and lymphocytes. Mare's milk constituents have a modulatory effect on inflammatory processes through their influence on the chemotaxis process and lowering of the respiratory burst; hence, the milk may be helpful when curing illnesses with recurring inflammations (Ellinger et al., 2002) Mare's milk has a composition similar to human milk and is well digested, so it is a perfect alternative to cow's milk in the feeding of children who are allergic to cow's milk. Mare milk's low content of α-lactalbumin and β-lactoglobulin makes it appropriate for such children. Its high concentration of lactose allows for a better growth of intestinal microflora. Mare's milk is characterised by a high content of lysozyme, lactoferrin and lactadherin and has an inhibiting effect on the development of pathogenic bacteria for such children**.** Due to its lower content of fat and cholesterol it can be used to lower cholesterol intake as well as to control cardiovascular diseases.

Mare's milk has been proved to play a role in curing Crohn's disease, ulcerative colitis as well as hepatitis and chronic gastric ulcers. The qualities of mare's milk make it appropriate for use in children's and elderly people's nutrition in prophylaxis and as an aid to the process of curing various diseases. Mare milk is similar to human milk, with particular reference to its low nitrogen content, its low ratio of casein to whey protein, and its high content of lactose (Bonomi *et al*., 1994). Mare milk contains a lower content of protein and higher lactose content than cow and sheep milk. Mare milk contains calcium, phosphorus, magnesium, zinc, iron, copper, and manganese, which are beneficial to human beings. Mare milk contains vitamins A, C, E, B_1, B_2, B_{12} and pantothenic acid. West European countries (in particular Germany and France), have recently begun selling mare milk powder products in chemist's and health food stores (to be directly ingested or to be rehydrated in water). Mare milk has hinteresting nutritional characteristics, especially with regard to diets for the elderly, convalescents, and infants (Stoyanova *et al.,* 1988). Mare milk contains more essential fatty acids, especially linoleic and linolenic acid, than cow milk (Huo et al. 2002). Mare's milk is plentiful of the fat-resembling substances that contribute in the transfer of certain nerve impulses and the regulation of blood pressure.

BIOLOCICAL ACTIVITIES OF OLIGOSACCHARIDES OF SOME IMPORTANT ANIMALS

Milk oligosaccharides isolated from different animal species have shown various biological activies which are depend upon genetic composition and their natural inhabitants of particular animal species. In laboratory various milk oligosaccharide have been isolated from various animal species such as donkey, camel, dog, elephant, goat, sheep and mare etc. the biological activities shown by these milk oligosaccharids are as-

Mare Milk Oligosaccharides

Number of oliogosaccharides has been isolated from Mare milk. (Srivastava et. al. 2019, Deepak et. al. 2019, Maurya et. al. 2017) Mare milk has shown antioxidant, lipid lowering and post heparin lipid lowering properties. Mare milk has anti tuberculosis, immunostimulant, antioxidant (Srivastava et. al. 2014) and anti-diabetic activities. Mare's milk has high content of lysozyme, lactoferrin, oligosaccharides (Maurya et. al. 2017) and lactadherin which increases its inhibiting effect on the development of pathogenic bacteria (Srivastava et. al. 2015) for such children. Mare milk can be used in the treatment of gynecological, cardiovascular diseases, pneumonitis, tuberculosis, and asthma. It helps in the treatment of increasing weight gain in underweight people, robustness, and energy (Kinik et al., 2000; Ozden, 2008). Mare's milk has shown anti-oxidant, lipid lowering and post heparin lipolytic activity as well as they promote cellular immune response as observed in vitro both in terms of cellular proliferation and reactive oxidative burst and also interpreted as an activation of innate immune defense mechanism.

Buffalo Milk Oligosaccharides- Buffalo milk oligosaccharides have also been evaluated for their ability to stimulate non-specific immunological resistance of the host against parasitic infections.

Dog Milk Oligosaccharides- These milk oligosaachharides have a dominant N-acetylneuraminlactose sulphate oligosaccharide which plays an important role in the nutrition of the rat pups[j].

Camel Milk Oligosaccharides- Camel milk oligosaccahrides contain sialyl oligosaccharides and this sialic acid may exhibit a number of health benefits for human infants, including the promotion of infant brain development.

Elephant Milk Oligosaccharides- Elephant milk oligosaccharide fractions contained a high ratio of sialyl oligosaccharides; this may be significant with respect to the formation of brain components, such as gangliosides of the suckling calves.

Bovine Milk Oligosaccharide- It is an inhibitor of the binding of this toxin to the intestinal mucosa in the suckling young of these species. It is microbiotic modulators involved to preventing the pathogen binding to the intestinal epithelium and serving as nutrients for a selected class of beneficial bacteria.

Goat Milk Oligosaccharides- 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[81]. Its oligosaccharides play important roles in intestinal protection and repair after damage caused by DSS (Dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation.

Human Milk oligosaccharides are metabolized by specific strains of bifidobacteria and thereby contribute to the establishment of a unique beneficial gut microbiota in infants during breast-feeding, so can be interpreted as prebiotics.

STRUCTURES OF NEUTRAL & ACIDIC MILK OLIGOSACCHARIDES OF SOME DOMESTIC ANIMALS

NEUTRAL ANIMAL MILK OLIGOSACCHARIDES	
1- GalNAc-*β*-(1→4-)Glc	Cow Colostrum Saito et al. (1984)
2- Gal-*β*-(1→4)-GlcNAc	Cow Colostrum Saito et al. (1984)
3-Fuc-α-(1→2)-Gal(*β*1→4)-Glc	Goat, Cow Colostrum Urashima et al. (1994a), Saito et al. (1987)
4- Gal-α-(1→3)-Gal-*β*-(1→4)-Glc	Goat, Sheep, Horse Colostrum Urashima et al. (1994a) (1989a,b)
5- Gal-*β*-(1→3)-Gal-*β*-(1→4)-Glc	Goat, Sheep, Horse, Colostrum Urashima et al. (1994a, 1989a, 1989b)
6- Gal-*β*-(1→6)-Gal-*β*-(1→4)-Glc	Goat, Sheep, Horse Colostrum Urashima et al.(1994a) (1989a) (1989b)
7- Gal-*β*-(1→4)-GlcNAc Fuc-α-(1→3)	Cow Colostrum Saito et al. (1987)
8- GalNAc-α-(1→3)-Gal-*β*-(1→4)-Glc	Cow Colostrum Urashima et al. (1991b)
9- GlcNAc-*β*-(1→6)-Gal-*β*-(1→4)-Glc	Goat milk Chaturvedi and Sharma (1988)
10- Gal-*β*(1→4)GlcNAc-*β*(1→6)Gal-*β*(1→4)Glc	Goat milk Chaturvedi and Sharma (1988)
11-Gal-*β*(1→4)-GlcNAc-*β*-(1→3)Gal*β*(1→4)Glc	Horse Colostrum Urashima et al. (1991b)
12 – Gal-*β*(1→4)GlcNAc(*β*1→6)Gal(*β*1→4)Glc Fuc (α1→3)	Goat milk Chaturvedi and Sharma (1990)

13- Gal(*β*1→3)GlcNAc(*β*1→6)Gal(*β*1→4)Glc Fuc (α1→3)	Goat milk Chaturvedi and Sharma (1990)
14 -Gal(*β*1→4)GlcNAc(*β*1→6) Gal(*β*1→4)Glc Gal(*β*1→3)	Cow, Horse Colostrum Urashima et al. (1991b) (1989b)
15- Gal(*β*1→4) GlcNAc(*β*1→3)Gal(*β*1→4)Glc Gal(*β*1→3)	Goat milk Chaturvedi and Sharma (1988)
16- Gal (*β*1→4)GlcNAc(*β*1→6) Gal(*β*1→4)Glc Gal(*β*1→4)GlcNAc(*β*1→3)	Horse colostrum Urashima et al. (1991a)

Acidic animal milk oligosaccharides

17 - Neu5Ac(α2→3)Gal	Cow colostrum Urashima et al. (1997c) Cow colostrum Schneir and Rafelson (1966)
18- Neu5Ac(α2→3)Gal(*β*1→4)Glc	Goat colostrum Urashima et al. (1997b) Sheep colostrum Nakamura et al. (1998)
19- Neu5Ac(α2→6)Gal(*β*1→4)Glc	Cow colostrum Schneir and Rafelson (1966) Goat colostrum Urashima et al. (1997b)
20 - Neu5Gc(α2→3)Gal(*β*1→4)Glc	Cow colostrum Kuhn and Gauhe (1965) Goat colostrum Urashima et al. (1997b)
21 - Neu5Gc(α2→6)Gal(*β*1→4)Glc	Goat,Sheep colostrum Veh et al. (1981), Urashima et al(1997b)
22 - O-Acetyl-Neu5Ac(α2→3)Gal(*β*1→4)Glc	Cow colostrum Urashima et al. (1997c)
23- Neu5Ac(α2→6)Gal(*β*1→4)GlcNAc	Cow colostrum Kuhn and Gauhe (1965) Goat colostrum Urashima et al. (1997b)
24 - Neu5Gc(α2→6)Gal(*β*1→4)GlcNAc	Cow colostrum Kuhn and Gauhe (1965)
25- Neu5Ac(α2→6)Gal(*β*1→4)GlcNAc-1-PO_4	Cow colostrum Kuhn and Gauhe (1965)
26- Neu5Ac(α2→6)Gal(*β*1→4)GlcNAc-6-PO_4	Cow colostrum Kuhn and Gauhe (1965)
27- Neu5Ac(α2→3)Gal(*β*1→3)Gal(*β*1→4)Glc	Cow colostrum Parkinnen and Finne (1987)
28 - Gal(*β*1→3)Gal(*β*1→4)Glc Neu5Ac(α2→6)	Goat milk Viverge et al. (1997)
29 - Gal(*β*1→6)Gal(*β*1→4)Glc Neu5Ac(α2→6)	Goat milk Viverge et al. (1997)
30- Neu5Ac(α2→8)Neu5Ac(α2→3)Gal(*β*1→4)-Glc	Cow colostrum Kuhn and Gauhe (1965)

STRUCTURES OF NEUTRAL & ACIDIC MILK OLIGOSACCHARIDES OF JAPANESSE POLAR BEAR. Ursus thibetanus japonicus (1999)

Structure of oligosaccharide	Name of oligosaccharides
1.Gal(α1-3)Gal(β1-4)Glc	α3'-Galactocyl lactose
2. Fuc(α1-2)Gal(β1-4)Glc	2'-fucosyl lactose
3. Gal(α1-3)Fuc(α1-2Gal(β1-4)Glc	B-tetrasaccharide
4. Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)]Glc .	B-Pentasaccharide
5. Gal(α1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Glc(β1-4)Glc	Monofucosyl hexasaccharide
6. Gal(α1-3[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Difucosylheptasaccharide
7. Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3){Gal(α1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc .	Trifucosylundecasaccharide

STRUCTURES OF NEUTRAL & ACIDIC MILK OLIGOSACCHARIDES OF OLIGOSACCHARIDES IN HOODED SEAL AND AUSTRALIAN FUR SEAL (T. URASHIMA et al. 2000)

1. Gal(β1-4)Glc	Lactose
2. Fuc(α1-2)Gal(β1-4)Glc .	2'-Fucosyllactose
3. Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-neopentose
4. Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-fucopentose
5. Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc	Lacto –N-neohexaose
6. Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc	Monofuc Mono fucosyl lacto N-neo hexose-a
7. Gal(β1-4)GlcNAc(β1-3)[Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc	(monofu Mono fucosyl lacto N neo hexose- b

STRUCTURES OF NEUTRAL & ACIDIC MILK OLIGOSACCHARIDES OF JAPANESSE POLAR BEAR. Ursus thibetanus japonicus (1999)

Structure of oligosaccharide	Name of oligosaccharides
1.Gal(α1-3)Gal(β1-4)Glc	α3'-Galactocyl lactose
2. Fuc(α1-2)Gal(β1-4)Glc	2'-fucosyl lactose
3. Gal(α1-3)Fuc(α1-2Gal(β1-4)Glc	B-tetrasaccharide
4. Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)]Glc .	B-Pentasaccharide
5. Gal(α1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Glc(β1-4)Glc	Monofucosyl hexasaccharide
6.Gal(α1-3[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)GlcNAc(β1-3)Gal(β1-4)Glc	Difucosylheptasaccharide
7 . Gal(α1-3)[Fuc(α1-2)]Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3){Gal(α1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)}Gal(β1-4)Glc .	Trifucosylundecasaccharide

STRUCTURES OF NEUTRAL & ACIDIC MILK OLIGOSACCHARIDES OF OLIGOSACCHARIDES IN HOODED SEAL AND AUSTRALIAN FUR SEAL (T. URASHIMA et al. 2000)

1. Gal(β1-4)Glc	Lactose
2. Fuc(α1-2)Gal(β1-4)Glc .	2'-Fucosyllactose
3. Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-neopentose
4. Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	Lacto-N-fucopentose
5. Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc	Lacto –N-neohexaose
6. Fuc(α1-2)Gal(β1-4)GlcNAc(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc	Monofuc Mono fucosyl lacto N-neo hexose-a
7.Gal(β1-4)GlcNAc(β1-3)[Fuc(α1-2)Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc	(monofu Mono fucosyl lacto N neo hexose- b

METHOD AND PROCEDURE FOR ISOLATION OF MILK OLIGOSACCHARIDES

Isolation of oligosaccharides is very difficult task because of low concentration in their natural resources but technological advances in equipment and analytical method made it possible to isolate these compounds in a considerable amount. Presently more than 550 milk oligosaccharides have been isolated and characterized by the various glycobiologist, out of which more than 100 oligosaccharides were isolated by our research group. Furthermore new oligosaccharides being isolated from the milk of different species due to their qualitative variation in genetic factors, that's lead to their biosynthesis. Some very conventional methods to be used for the isolation of oligosaccharides from the milk of different origins are-

1. KOBATA and GINSBURG (Kobata et. al. 1970)
2. URASHIMA (Urashima, T. et.al. 1997)
3. SMITH (Smith, D.F. et.al. 1978)
4. EGGE (Egge,H et.al. 1983)
5. WEIRUSZESKI (Weiruszeski, J.M. et.al. 1985)
6. MODIFIED METHOD OF KOBATA AND GINSBERG (Jamal et. al. 2023)

Out of the above method, our research group has been used the modified method of Kobata and Ginsburg. In the present study, we worked on the isolation and structure elucidation of four novel milk oligosaccharides from Mare milk.

Isolation of Milk Oligosaccharides by Modified Method of Kobata and Ginsberg

In this method, the samples of milk were collected and equal amount of alcohol was madded into it and it was centrifuged for 15 min at 5000 rpm at -4 ^{0}C. The solidified lipid layer was removed by filtration through glass wool column in cold atmospheric condition. Ethanol was further added to the clear filtrate (supernatant) upto a final concentration of 68% for precipitating out the lactose and proteins and the resulting solution was left overnight at 0 ^{0}C. The white precipitate of lactose and protein was formed; and removed by centrifugation for 15 min at 5000 rpm at -4 ^{0}C and washed twice with 68% ethanol. Further for complete removal of remaining lactose, the supernatant was passed through a micro-filter (0.24 mm) and lyophilized to get the crude oligosaccharide mixture.

The lyophilized material responded positively to Morgane-Elson test and Thiobarbituric acid assay suggesting the presence of N-acetyl sugars in oligosaccharide mixture. This lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluant at a flow rate of 3 ml/min. Each fraction was analyzed by Phenol sulphuric acid reagent for the presence of neutral sugar containing oligosaccharides and these fractions were used for purification and isolation of milk oligosaccharides.

Acetylation Analysis of Milk Oligosaccharide (Jamal L. et. al. 2023)

Acetylation is the main chemical transformation of the carbohydrates which is performed to enhance certain analytical objectives. Acetylation is generally carried out by using the Ac_2O/pyridine or sodium acetate or zinc chloride with Ac_2O for converting more polar sugar into less polar, so that they could be conventionally isolated by column chromatography. Further acetylation studies were also found to be helpful in determining the position of glycosidic linkages and good resolution of ^1H NMR.

Chromatographic Techniques Required For Purification of Milk Oligosaccharides

A number of purification methods were used during the isolation and purification of milk oligosaccharides and they have been discussed as under:

Thin Layer Chromatography (Wilson, K. et. al. (1986) & Kobata, A. et. al. (1969)

Thin layer chromatography is an important technique for the analysis monosaccharide, oligosaccharide and carbohydrate polymers. The adsorbents used in TLC are magnesium silicate, alumina, silica gel and the resolution of mixture of compounds depends on the choice of suitable polar and non- polar solvent system. Although the thin layer chromatography is limited to less polar compounds and it is not very effective for the isolation of highly polar compounds like oligosaccharides etc, however it could be useful after the chemical transformation or acetylation of oligosaccharides. Urashima et al used a combination of gel filtration chromatography, preparative TLC and ^1H-NMR to purify and determine the composition of Oligosaccharides from milk. TLC is also useful in monitoring the progress of various chemical transformations in course of oligosaccharides isolation.

Paper Chromatography (Synder et. al. 1988 & Gareil et. al. 1988)

Paper chromatography is the oldest method of separation and qualitative determination of oligosaccharides. Paper chromatography operates by a liquid-liquid partitioning process and the purification milk oligosaccharides was performed by the descending paper chromatography with the following solvents on Whatman filter paper-

1. Upper layer of ethyl acetate-pyridine-H_2O (2:1: 2)
2. Ethyl acetate-pyridine-acetic acid-H_2O(5:5: 1: 3)
3. Propanol-H_2O (4:1)
4. 1-butanol-pyridine-H_2O (6:4:3)
5. Lower layer of phenol-formic acid-2-propanol·.H_20O(80:5:100)
6. Upper layer of pyridine-ethyl acetate-H2O (.0:3.6:1.15)
7. Phenol-H_2O-conc.NH_4OH (150:40:1)
8. Upper layer of ethyl acetate-acetic acid-H_2O (3:1: 3)
9. Ethanol- 1M ammonium acetate, P^H 7.8 (5:2)
10. Butanol- acetic acid- ammonium hydroxide (2:3:1)

Oligosaccharides were visualized by $AgNO_3$ reagent, aniline oxalate reagent or riodate-benzidine reagent; oligosaccharides containing N-acetyl amino sugars were visualized with Morgan-Elson reagent, while oligosaccharides containing sialic acid were developed with Thiobarbituaric acid (TBA) reagent. Separation time varies with the size of the oligosaccharides: approximately 3 days for tetra and pentasaccharides and 10 days for hexa, hepta, and octasaccharides and the reducible oligosaccharides move slightly slower than the corresponding non-reducible oligosaccharides.

Column Chromatography (Gunnar et. al. 1990)

This is the most frequently applied method in the isolation of oligosaccharides, in which a column is packed with particulate material such as silica or alumina and then solvent is passed through it at atmospheric pressure. It works on the basis of differences in the molecular polarity compounds. The sample is dissolved in solvent and adsorbed at the top of the column. Since the oligosaccharides are highly polar in nature hence the isolation of oligosaccharides by column chromatography are not easy to perform, and therefore acetylation of oligosaccharides were performed to convert polar oligosaccharides to non-polar acetyl derivative of oligosaccharide. The acetylated oligosaccharide mixtures were purified by repeated column chromatography on silica. Column chromatography is advantageous over other chromatographic techniques because it can be used in both analytical and preparative applications. The polarity of solvent is usually changed stepwise and fractions are collected according to the separation required, with the eluted solvent usually monitored by TLC.

High Performance Liquid Chromatography (Kupiec et. al. 2004 & Verzele et. al. 1990)

HPLC is a technique by which molecules in solution are separated according to differences in their ionic properties or affinity for column packing material. The three main techniques used in HPLC are ion exchange chromatography (IEC), reversed phase chromatography (RPC) and affinity chromatography. This separation mechanism is based on the ability of the solid-phase to interact with the OH-groups of the analytes and separates them according to the amount of OH-groups they carry. This leads to a size dependent separation of oligosaccharides, which elute in inverse relationship to their polymerization degree, while monomers are separated individually. RPC has become a widely used HPLC separation mode, in RPC the mobile phase is polar (aqueous solutions) and stationary phase is nonpolar. The use of fully acetylated Oligosaccharides overcomes problems of solubility and has resulted in the fractionation of malto-oligosaccharides up to DP30 in about 150 min using RPC. By far the most frequently used systems for separation of Oligosaccharides are those using chemically bonded phase which fractionate materials on the basis of their relative affinities for mobile phase and bonded phase. IEC is also one of the most important separation processes in chemistry. Bonded silica and bonded glass with ionic groups on their surface are used in such HPLC techniques as the stationary phase separation media. Ionic solute molecules are attracted to the stationary phase ionic groups of the opposite charge and during elution the retarded substances are reversibly charged for ions of the same charge. Anion exchange HPLC has been recently developed and has exceptional resolving power for complex oligosaccharides. Such analysis is carried out at high P^H coupled with pulsed amperometric detection (PAD), allowing separation of Oligosaccharides and polysaccharides up to DP$\geq$50. Peaks in HPLC analysis are simultaneously detected with an RI-detector and with an UV-detector.

However, since all peaks for which standard compounds are available can be conveniently quantified with the RI-detector, therefore an additional dilution and quantification is only optional. As the peaks for the added acceptor sugars are baseline-separated and no problem with the recovery of the baseline is observable, quantification of the acceptor sugars is rendered from a grievous ordeal using the overaged pA1-column to a triviality. Using the UV lamp to monitor N-Acetyl-group containing oligosaccharides showed two peaks for all experiments when N-Acetyl-group containing acceptor sugars were used, one for at least one dissacharide and one for at least one trisaccharide. The comparison of the respective peak areas suggests a strong prevalence of new disaccharides over new trisaccharides, but an exact quantification of the particular new reaction products is impossible. The characteristics of the column allow quantification of lactose only at the beginning of the experiment, when no other disaccharides are present in the reaction mixture, but quantification of the monomers is possible throughout the experiment. Another advantage of HPLC is that less time is need per analysis in comparison of other techniques. Typically, a sample clean-up step prior to derivatisation with ion-exchange chromatography is required to remove neutral monosaccharides and amino acids. In addition, after derivatisation a solid phase extraction with a Sep-Pack C18 is used to remove excess reagents and non-tosylated or un-benzoylated derivatives. Both Neu5Ac and Neu5Gc Tosyl and per-O-benzoylated derivatives can be separated on a Supelcosil LC18 column by isocratic elution using a mobile phase of water-acetonitrile and UV detection. The per-O-benzoylated derivative of Neu5Gc elutes later than that of Neu5Ac due to the extra benzoyl group on the Nglycolyl moiety. HPLC has been emerged as a most popular alternative method to other conventional methods for the isolation and purification of oligosaccharides because of its speed of performance, wide applicability, sensitivity and high resolution. The following sequences are applied for better resolution and yield.

1. **Choice of Solvent System**: The separation of different compounds in mixture depends on different chemical and physical properties of the solvent. In certain cases, TLC analysis of the sample is used as a first indication of the correct operating conditions, silica gel plates for normal phase column and silylated silica gel plates for reversed-phase columns.
2. **Optimization of Analytical Columns of Small Quantities**: A preliminary analytical search is necessary for the right choice of conditions, which saves time, sample and solvent, required in a HPLC system.
3. **Optimization of Analytical HPLC Separation Aiming For Small Capacity Factors**: A good analytical HPLC separation is usually a prerequisite for a successful preparative operation. Relative intention (selectively, a) is a very important parameter in determining possible sample size and it is necessary to maximize this value.
4. **Scaling of Preparative HPLC Apparatus:** In many preparative HPLC examples, the column is actually overloaded, nonlinear adsorption isotherms are obtained and peaks are not symmetrical. Scaling-up a successful analytical separation may because problems associated with the solubility often sample. This is especially true for reversed phase HPLC, if the compound under investigation does not dissolve in aqueous solvents. Diluting the sample may help but if the volume injected is too great, separation efficiency decreases. If on the other hand the sample is too concentrated, precipitation on the column may occur.

Normal phase HPLC (Shirley et. al. 1996)

Highly hydrophilic nature of Oligosaccharides makes them suitable for normal phase chromatography. However the most commonly used normal phase matrix, silica, has not proven to be very useful for oligosaccharide separations, but much success has been achieved by using hydrophilic bonded phases, especially amino propyl silica. Oligosaccharides injected onto the column in a high organic (typically acetonitrile) aqueous solvent, are eluted from the column by an increasing aqueous concentration gradient. Since aminopropyl group are positively charged at neutral P^H, Sialylated and phosphorylated Oligosaccharides will be bound to column in the absence of salt, but this can be overcome to some extent by use of low P^H buffers. Recently, new type of hydrophilic bonded phase have been developed (e.g. diol and amide) that dot have this limitation.

Reverse phase HPLC (RP-HPLC) (Marie-Isabel et. al. 2004)

Although RP-HPLC is an excellent technique for organic compounds due to high chromatographic efficiency and selectivity, it has not been successful in the separation of Oligosaccharides due to their hydrophilic nature, which leads to very weak interactions with the column matrix. Thus very few exceptions, RP-HPLC have not been used to separate native Oligosaccharides of glycoprotein origin. However RP-HPLC can give good separation of Oligosaccharides that have been made more hydrophobic by derivatization. Most of the reducing end tags that are available were developed specifically to make use of high resolving power of RP-HPLC. Per-O- methylated and per-O-acetylated Oligosaccharides are hydrophobic and give good RP-HPLC separation. Both of these derivatizations are best performed on reduced oligosaccharides due to the potential for up to four products from each oligosaccharide. Acetylation has the advantages of increased UV detection sensitivity and the option to remove the acetyl groups at later stage. Although these two derivatives have been successfully used for the separation of polysaccharide derived Oligosaccharides, they have rarely been used for the separation of glycoprotein derived oligosaccharides. This is probably due to the difficulty in quantitative derivatization for the acetyl derivatives and lack of detection sensitivity for methyl derivatives. However, since methylated oligosaccharides can be detected at high sensitivity by ESI-MS (electronspary ionization mass spectrometry), it is likely that RP-HPLC of methylated oligosaccharides with ESI-MS detection will increase in popularity. Reverse phase chromatography is capable of separating closely related oligosaccharides and this apparently occurs on the basis of their three-dimentional conformations. By RP-HPLC it is possible to separate lacto-N-tetraose from lacto-N-neotetraose and lacto-N-fucopentaose from its isomer. Sumiyoshi W et al analyzed major neutral oligosaccharides in the milk of sixteen Japanese women by RP-HPLC and found in colostrum and mature milk (30 d lactation), lacto-N-fucopentaose (LNFP) I was the most abundant oligosaccharide, followed by 2'-fucosyllactose (2'-FL) + lacto-N-difucotetraose (LNDFT), LNFP II + lacto-N-difucohexaose II (LNDFH II), and 3-fucosyllactose (3-FL).

STRUCTURE DETERMINATION OF MILK OLIGOSACCHARIDES

The structural determination of oligosaccharides is highly complex task due to the presence of many monosaccharides with multiple and diverse substitution in composition, stereochemistry (configuration) and the pattern of inter residue linkage, but advancement in analytical tools and technology made it possible to elucidate this complex structure. Mass spectrometry (MS) and nuclear magnetic resonance (^1H, ^{13}C and 2D i.e. HSQC, HMBC, COSY

and TOCSY) spectroscopy are some of the most appropriate tools which help in structure elucidation of milk oligosaccharide and give the information regarding composition, sequence, branching, linkage analysis and structural information of the oligosaccharide molecule. The various chemical and physiochemical techniques used in the structural determination of oligosaccharides are as follows:

Acid Hydrolysis

Traditionally, hydrolysis of oligosaccharide has an important role in the determination, identification and confirmation of constituent monosaccharide units of the oligosaccharides in their structure. In practical, different condition of acid hydrolysis i.e. mild to strong is generally used depending on the nature of monosaccharide present in the oligosaccharide. Kiliani acid hydrolysis (Killiani et. al. 1930) (AcOH: H_2O: HCl::7:11:2) is commonly used for the hydrolysis of oligosaccharides comprised of normal sugars monosaccharide. Mannich and Siewart hydrolysis (Mannich and Siewart et. al. 1942) (conc. HCl /Acetone) is employed for determination of the sugar sequence in the oligosaccharide. During hydrolysis, aliquot are taken at different time intervals to obtain intermediate products. The monosaccharides are identified either by direct comparison with authentic sample ($[\alpha]^D$, TLC and PC) followed by their chemical transformation.

PerMethylation / Acetylation Analysis (Townsend et. al. 1988)

Permethylation/peracetylation is the main chemical transformation of the carbohydrates which is performed to enhance certain analytical objectives i.e. increasing the volatility and hydrophobicity, of carbohydrates. Permethylation is generally carried out by using the sodium hydroxide (NaOH)/methyl iodide or (DMF)/methyl iodide or using Hakomori's procedure and peracetylation by Ac_2O/pyridine or sodium acetate or zinc chloride with Ac_2O. The sequence in oligosaccharides and the site of glycosidic linkage can also be deduced by permethylation studies followed by acid hydrolysis. Further acetylation studies are also found to be helpful in fixing the position of glycosidic linkages, good resolution of ^1H NMR and better insight of the molecule.

Methylglycosidation / Acid Hydrolysis (Jamal et. al. 2023)

Methylglycosidation of the compound is done by refluxing the compound with MeOH at 70 ^0C for 12-18 hrs in the presence of cation exchange IR-120 (H+) resin followed by its acid hydrolysis. This results into the isolation of α and β-methylglycosides of the reducing monosaccharide along with constituent monosaccharide which could then be identified with co- chromatography with authentic sample.

Nuclear Magnetic Resonace (NMR) Spectroscopy in the Analysis of Milk Oligosaccharide (Pawan et. al. 1992)

Oligosaccharides are a group of monosaccharides with vast structural and chemical diversity; and limited chemical shift dispersion in NMR spectra makes their study by NMR challenging. The assignments of ^1H and ^{13}C resonances to their corresponding protons and carbon atoms are usually achieved using a combination of 1D and 2D-NMR experiments such as homonuclear ^1H-^1H COSY and ^1H-^1H TOCSY experiments and heteronuclear ^1H-^{13}C HSQC and ^1H-^{13}C HMBC. To elucidate the structure of oligosaccharides following information must be required.

1. **Number of Sugar Residues:** In structural analysis of oligosaccharides, the assignment of anomeric proton chemical shift is of highest importance. The anomeric proton resonances are found in the shift range of δ4.2-5.5 ppm in ^1H NMR.

Additionally, the number of anomeric C-1 resonances present in a ^{13}C NMR spectrum confirms the number of monosaccharide unit in oligosaccharide molecule. The range of anomeric carbon varies from δ90 – 110 ppm.

2. **Constituents Monosaccharides:** The identification of monosaccharide residue is very important in the structure determination of oligosaccharides. The chemical shift data of ^{1}H and ^{13}C NMR together becomes very useful in the identification of monosaccharide unit present in oligosaccharide.

3. **Anomeric Configuration:** In carbohydrate chemistry, it has been established that "Half of sugar chemistry rotates around the anomeric centre". In oligosaccharide molecule normally a α-anomer resonates downfield compared to the β-anomer in D- pyranoses in $^{4}C_{1}$ conformation. If H-1 and the H-2 are both are in an axial configuration in pyranose structure, a large coupling constant (8-10 Hz) is observed, whereas if they have equatorial-axial configuration, this is smaller ($J_{1,2}$ ~4 Hz), and for equatorial-equatorial oriented protons, even smaller coupling constants are observed (<2 Hz). The ^{13}C chemical shift for anomeric configuration of sugar residue appears in δ90-110 ppm, but most importantly the one bond ^{13}C-^{1}H coupling constants in pyranose can be used to determine the anomeric configuration clearly.

4. **Linkages and Sequence:** The ^{1}H and the ^{13}C chemical shift may give an indication for the linkage of complete oligosaccharide moiety. The effect of glycosylation shift depends on the linkage type and the changes in the chemical shift are in general larger at the glycosylation site than at neighboring positions. A HMBC and Inter residue NOEs experiment may give information about the glycosidic linkages. Position of linkage could be well defined by a comparable study of ^{1}H assignment of natural and acetylated oligosaccharide. Moreover 2D experiments of Homo and Hetero nuclear experiments like COSY, TOCSY and HMBC gives relevant information.

5. **Position of Appended Groups:** The attachment of a non-carbohydrate group like a methyl, acetyl, sulfate or a phosphate group could be pointed by the proton and carbon NMR chemical shifts. Attachment of these groups affects the proton and carbon resonance where the group is located. Normally downfield shifts of ~δ0.2-0.5 ppm are observed for protons and δ5-10 values for ^{13}C. This places these resonances in a less crowded area of the spectra and helps the identification of novel residues.

6. **Structure Reporter Group:** Since the NMR data of oligosaccharide are highly complex, Vligenthart et.al. introduced the "structural reporter group" (SRG) concept, which was based on signals outside the bulk region (δ 3-4) in the ^{1}H NMR spectra of the oligosaccharide. This structural reporter group concept helped in the identification of novel residues and characterization of oligosaccharides. Moreover they are NMR fingerprints of a particular linkage or group.

^{1}H and ^{13}C NMR Experiments

^{1}H-NMR spectroscopy is often the first step in structural studies of oligosaccharides by NMR. The number of sugar residues can be estimated by integration of the signals in the region of the anomeric protons. The number of anomeric resonances in the ^{13}C-NMR spectrum will further confirm these results. ^{1}H and ^{13}C experiments can also give some indication about the linkage and sequence of the sugar residues through changes in chemical shifts, but in general both homo- and heteronuclear 2D-NMR experiments are required for complete assignment of oligosaccharides.

If the anomeric signals are well resolved they appear as doublets from which the $^{3}J_{H1,H2}$ coupling constants can be obtained, and give information about the anomeric configuration. ^{13}C-NMR is much less sensitive than ^{1}H NMR due to the low natural abundance of the ^{13}C nucleus and the fact that the gyromagnetic ratio is only ¼ of that of ^{1}H, but the ^{13}C spectra show a greater dispersion of chemical shifts.

^{1}H NMR Spectroscopy (Kobata et. al. 1972 & Waard et. al. 1992), A high resolution ^{1}H-NMR spectra gives valuable information about milk oligosaccharide's structure. The chemical shift of a particular anomeric proton and its splitting pattern gives an idea of the monosaccharide units present; simultaneously it also fixes the configuration of sugar linkage and conformation of that monosaccharide unit. The proton NMR spectroscopy of oligosaccharides suffers from severe spectral overlap, because most of the monomeric residues differ only in their stereochemistry and their magnetic properties are only little influenced by their position in chain. The chemical shift of anomeric protons and methine protons of different sugars are confined to the region δ 4.3-5.5 and δ 3.0-4.2 respectively hence it requires expert interpretation of spectra for monosaccharide identification. The analysis of reducing oligosaccharides showed that the anomeric configuration of the reducing end sugar also exerts its influence on the spectral parameters of residues in its spatial neighborhood, being sometimes even the non-reducing end sugar. In D-pyranoses $^{4}C_{1}$ conformation the α-anomer resonates downfield in comparison to β-anomer. The chemical shift value for α-anomer lied in the range 4.9-5.4 ppm and for β-anomer it lied in the region 4.4-4.8 ppm. The α-anomeric doublet showed coupling constant J=3-4 Hz whereas the β-anomeric doublet showed J value of 6-9 Hz. All these values were correlated with known structures to yield relevant information in terms of monosaccharides units and their relative abundance. The structure of different linkages can be defined in terms of NMR parameters of their structural reporter groups. In case of milk oligosaccharides the anomeric proton resonances are found in the chemical shift range 4.3-5.5 ppm and the remaining ring proton resonance are found in the range 3.0-4.2 ppm. But in case of acetylated oligosaccharides acetyl groups induce a strong downfield shift of proton which directly linked to acetylated carbons. Hence the signals of methine protons and methylene protons occur downfield in the region of 4.0-4.8 ppm. The resonances of protons linked to the non-acetylated carbons at the site of glycosidic linkage and at the ring C-5 occur in the chemical shift range between 3.5 and 3.9 ppm. To resolve the spectral complexities of oligosaccharides, Vligenthart et. al. introduced the "structural reporter group" concept, which was based on signals outside the bulk region (δ 3-4) in the ^{1}H-NMR spectra of the oligosaccharide. This approach is used to identify individual sugars or sequence of residues. These structural reporter groups include anomeric proton, equatorial protons, deoxy protons and that distinct functional group such as amide group. ^{1}H-NMR gives anomeric protons at 4.3-5.9 ppm, methyl doublets of 6-deoxy sugars at 1.1-1.3 ppm, methyl singlet of acetamido groups at 2.0-2.2 ppm and various others with distinctive chemical shift. Some of the common spectral feature of the ^{1}H-NMR structural reporter groups of milk oligosaccharides are summarized below (Kobata et. al. 1972, Waard et. al. 1992 & Dua. et. al. 1986).

1. In the ^{1}H-NMR spectra the reducing Glc residue is characterized by the H-1 signals for its α and β anomers at δ5.221 ($J_{1,2}$ 3.7 Hz) and δ 4.688 ($J_{1,2}$ 8.0 Hz) respectively with ratio of 7:10.

2. The 4-substituted reducing Glc shows anomeric signals for both the α- and the β-anomeric at δ 5.22 and 4.66 ppm, with H-2 of the β-from in the range of δ3.2-3.3 ppm as triplet.

3. The 3,4-disubstituted reducing Glc shows anomeric signals from both the α- and the β- anomeric at δ 5.22 and 4.66 ppm, with H-2 of the β-from at a typical downfield shift above δ3.35 ppm.

4. The 3-substituted β-linked Gal shows signal for H-1 at 4.4 ppm and H-4 of β-linked Gal showed at a typical downfield shift around δ 4.13-4.15 ppm due to substitution at the 3-position.

5. The H-4 of (1→6) linked β-Gal appeared at δ 3.8-3.9 ppm and H-4 of (1→3) linked β-Gal at δ 3.9-4.2 ppm.

6. Signal for H-1 of the unsubstituted Gal residue appears around 4.44-4.47 ppm.

7. β-linked GlcNAc residues with anomeric signals appear at δ 4.6-4.7 ppm and CH_3 signals in the range of δ 2.02-2.08 ppm. H-1 of the (1→6) linked GlcNAc appears at lower chemical shift value (δ 4.6 ppm.) than the (1→3) linked GlcNAc residue (4.7ppm). A splitting of the anomeric doublets is due to the anomerization of the reducing terminal.

8. The H-2 of β-GlcNAc appeared at 3.6-3.8 ppm and H-2 of β-GalNAc appeared at 3.8-4.2ppm.

9. Presence of anomeic signal with a integration of two proton at 4.44-4.6 ppm suggest a LNT structure in which one β-Gal is attached to Glc by (1→4) linkage while another β-Gal unit is attached to β-GlcNAc or β-Glc by (1→3) linkage i.e. β-Gal(1→3) β-GlcNAc(1→3/6) β-Gal (1→4) Glc or β-Gal(1→3) β-Glc (1→3/6) β-Gal(1→4) Glc moieties is present.

10. α-linked Gal residue appeared at δ4.94-5.2 ppm. The (1→4) linked α-Gal residues showed anomeric signal at δ 5.02 ppm, (1→2) linked α-Gal residues showed anomeric signal at δ 5.20 ppm and (1→3) linked α-Gal residues showed anomeric signal between δ 5.02-5.20 ppm.

11. α-linked Fuc residues anomeric signals appeared at δ5.02-5.43 ppm. The presence of fucose subunit could be inferred by the presence of CH_3 doublet at δ 1.1-1.3, H-5 at δ 4.2-4.9 and the anomeric doublet at δ5.02-5.4 ppm.

12. Generally (1→4) linked fucose occur near δ4.98ppm, (1→2) linked fucose occur near δ5.38ppm and (1→3) linked fucose occur between the two.

13. The presence of sialic acid residue could be ascertained by the characteristic resonances of H-3 axial and equatorial protons at 1.78 and 2.75 ppm respectively. The location of Neu5Ac residue can be deduced as follows. (a) the signal for H-3a and H-3e of Neu5Ac residue can be used to discriminate between (2-3) and (2-6)-α-linkage to Gal. (b) for an α-Neu5Ac(2-3)-β-Gal-(1- sequence, the signal for H-3 of Gal residue is shifted downfield by 0.6 ppm of the ring protons.

^{13}C NMR SPECTROSCOPY

^{13}C NMR and ^{1}H NMR spectroscopy are closely related techniques but give sufficiently different data, which complement each other. ^{13}C NMR spectra not only give information of the anomeric configuration of the carbohydrate residues but also provide information of the composition of component monosaccharide (Bush et. al. 1985), their sequence and the overall conformation.

Proton decoupled ^{13}C NMR spectra are well resolved and usually provide an unambiguous identification of a monosaccharide, especially when anomeric chemical shift is compared with a collection of ^{13}C NMR data. This spectroscopy is also useful for the structure elucidation of oligosaccharides because of greater chemical shift dispersion in lack of complexities arising from spin-spin coupling overlap of resonances The number, sequence and linkage of a sugar could be assigned by the ^{13}C NMR data and identify of monosaccharide has also been established by the comparison of chemical shift of anomeric carbon with the reported values.

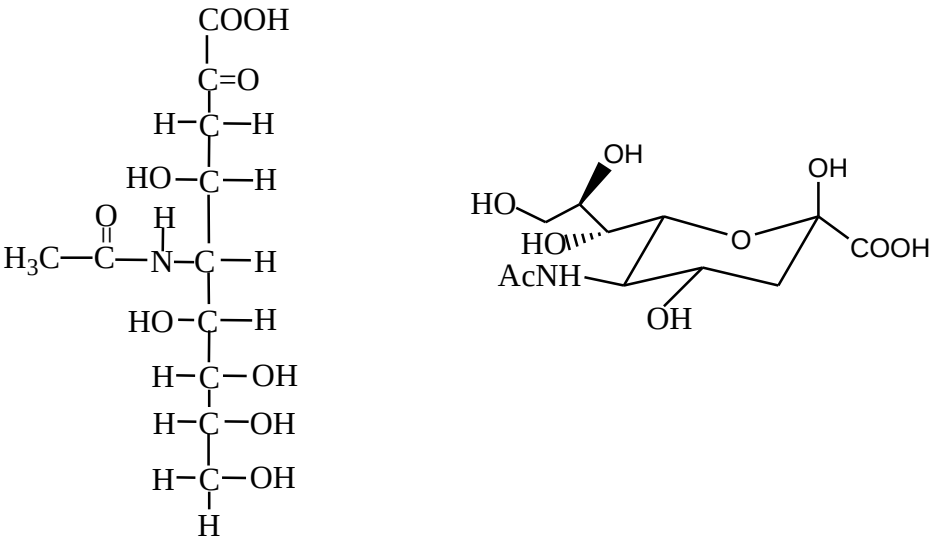


Figure 1. N- ACETYL NEURAMINIC ACID.

(5-amino3, 5 dideoxy-D-glycero-D-galacto-2-nonulosonic acid)

- Other important carbon decoupled signals of sialic acid are given in following table.

Table 1:

Sialic acid residue	^{1}H Chemical Shift	^{13}C Chemical Shift
α- Neuraminic- 5Ac 1	-	174.0-174.6
2	-	100.2-101.0
3 ax	1.693-1.801	40.5-41.0
3 eq	2.668-2.762	-
4	3.56-3.68	69.0-69.3
5	3.79-3.85	52.5-52.7
6	3.63-3.71	73.3-73.7
7	3.55-3.65	69.0-69.3
8	3.86-3.90	72.5-72.7
9	3.64	63.3-63.9
9’	3.87-3.88	-
C=O	-	175.7-175.8
CH_3	22.8-22.9	2.025-2.038

The total number of monosaccharide units presents in milk oligosaccharide can be fixed by counting the numeric carbons presents in the ^{13}C NMR of that particular compound. These signals generally appear between the range of δ95-110.the chemical shift of the anomeric gives perfect information regarding the nature of the monosaccharides. It also provides information regarding the nature of glycosidic linkages. The singles due to β-linkages usually appear 2.0-6.0 ppm downfield from their α-counterpart. The resonances of $–CH_3$ of 6-deoxy sugars, methoxy function, $-CH_2OH$ of normal hexoses and ring carbons generally appear in the region δ 16-199, δ 55-62, δ 60-64 and δ 65-85 respectively(Duus et. al. 2000). The presence of sialic acid residue could also be well determined by ^{13}C NMR spectroscopy. The anomeric signals (C-2) appear at δ 100-101 ppm while signal for –COOH group appears at δ 174 ppm.

In the chemical shift analogy method that the chemical shifts of carbon atoms in identical residues of similar oligosaccharide structure will be influenced only by glycosylation shifts, primarily by the δ shift (approximately 8 ppm downfield) for a substituted carbon atom and secondarily by the β shift (1-2 ppm upfield) for those carbon atoms adjacent to the linkage position.

For carbohydrates, most of whose carbons which are of the methane type, a proton decoupled experiments like 'attached proton test' (APT), 'distortionless enhancement by polarization transfer' (DEPT) and related 'insensitive nuclei enhanceced by polarization transfer' (INEPT) pulse sequence spectroscopy are less useful, but it serving mainly to provide rigorous identification of C-6 in hexapyranosides.

One dimensional ^{1}H and ^{13}C NMR Spectrum of sugar residue shows only some recognizable signals as listed in Table No 2.

^{1}H	**ppm**	**^{13}C**	**ppm**
C-C**H**$_3$	1.1-1.3	C-**C**H3	16-18
(C**H**$_3$)COO	1.8-2.2	(**C**H$_3$)COO	18-22
(C**H**$_3$)CON	2.0-2.2	(**C**H$_3$)CON	22-23.5
C**H**(NH)	3.0-3.8	**C**H(NH)	52-58
O-C**H**$_3$	3.3-3.5	O-**C**H$_3$	55-61
H**2**-H**6**	**3.2-4.5**	**C**H$_2$OH	57.7-64.7
		CH$_2$OR	66-70
		C2-**C**5	65-87
		C1 (ax-o, red)	90-95
		C1 (ketose)	98-100
H1 (ax)	4.3-4.8	**C**1 (ax-O,glyc)	98-103
		C1 (eq-O,red)	95-98
		C1 (eq-O,glyc)	103-106
		C1 (fur)	103-112
H1 (eq)	5.1-5.8	**C**OO	170-180

TWO-DIMEMSIONAL NMR SPECTROSCOPY

One-dimensional NMR methods provide limited information for the determination of the complete structure and stereochemistry of oligosaccharides due to the substantial overlapping of multiplets and other overlapped signals (Kover et. al. 2000).

The structure elucidation of oligosaccharides is the unambiguous assignment of the ^{1}H resonances of individual sugar residues, is poorly resolved in the region of ring protons i.e. from 3.2-4.0 ppm. For the structure determination, it is important to observe the individual components of the overlapping multiplets. In the recognition of NMR signals belonging to closed spin system i.e. to individual sugar residues, is always the first stage of structural analysis. The general approach is to assign an isolated resonance often an anomeric proton (4.3-5.4 ppm) or the methyl resonance (1.2-1.4 ppm) in 6-deoxy sugars, then to correlate spins in a step-wise manner around the spin system of the ring. However, spin correlation can be done by one-dimensional difference decoupling if only few assignments are needed. These difficulties could be overcome by the use of modem two-dimensional NMR experiments because they are more efficient for the simultaneous determination of a large number of spin correlations. 2D NMR spectroscopy provides actual, high quality and well interpretable data of the sugar molecule (Pozsgay et.al 1998).

There are two fundamental types of 2D NMR spectroscopy are use in structure determination of sugar residues.

1. Correlated spectroscopy in which both frequency axes contain chemical shift (δ) information.
2. *J*-resolved spectroscopy in which one frequency axis contains spin coupling (*J*) and other chemical shift (δ) information.

CORRELATED SPECTROSCOPY (COSY): (Christian et. al. 1999)

This technique was invented by Belgian Jeener and is also known as JEENER experiment (Jeener et. al. 1971) and is one of the important 2D NMR spectroscopic method for structure determination. ^{1}H-^{1}H COSY (correlated Spectroscopy) is useful for determining which signals arise from neighboring protons, especially when the multiplets overlap or there is extensive second order coupling. A COSY spectrum yields through bond correlation via spin-spin coupling (Aue et. al. 1975). There are two types of correlation spectroscopy i.e. homo COSY and hetero COSY. In homonuclear shift correlation 2D experiments, the correlation is between similar nuclei i.e. either ^{1}H-^{1}H or ^{13}C-^{13}C. The normal NMR spectra are plotted on a two frequency axes and the conventional 1D spectrum appears along the diagonal. The clear representation of 2D NMR spectrum is obtained as contour plots of mutual coupling which exists between two nuclei (^{1}H-^{1}H, ^{13}C-^{13}C), cross peak appears at the chemical shift coordinates (X, Y) and (Y, X) Identification of monosaccharide units is the first approached by analyzing the 1H homonuclear shift-correlation spectra. COSY spectra contain information on spin coupling networks within the constituent's residues of the oligosaccharide through the observation of cross peaks. Assignment of this spectrum by coupling-correlation requires an initial point for the identification of the individual spin system of sugar rings. The most downfield ^{1}H signals (anomeric) are always a convenient starting point for the assignment. Within typical aldohexopyranosyl ring, the coupling network is unidirectional i.e. H-1 couples to H-2; H-2 couples to H-1 and H-3, H-3 to H-2 andH-4 and so on. However, the presence of no or small coupling between H-4 and H-5 ($J_{4,5}$=2-3 Hz) of galactopyranosyl residue and coupling between H-1 and H-2 in mannopyranosyl residue prevents detection of cross peaks. COSY experiments and its RELAY extensions give coupling pattern along with shift information, which allow each monosaccharide residue to be identified and designated as α or β and also provide information about sugar identity and substitution pattern.

Sugar analysis indicated the presence of 3Gal, 1Glc, 2GlcNAc and 3NeuAc residues, in the sialyl oligosaccharide present in milk. Further, on the basis of the upfield resonances of the easily distinguished the H-1 signals of Gal and GlcNAc. The H-1 signal of Glc-β was correlated with H-2 signal at δ 3.320 ppm. The structure was also deduced by correlating the characteristic chemical shift of H-3ax and NeuAc residue α-2, 6-linked to GlcNAc III (Fievre et. al. 1989) and the low field shifted value of Gal IV H-3 at 4.078 ppm with H-1 and H-2 resonance on the COSY spectrum.

There are different types of 2D COSY NMR experiments are found to be extremely useful in the identification of each monosaccharide unit of oligosaccharides moiety.

Relay Correlation Spectroscopy (RELAY-COSY): (Bax et. al. 1985) In this type of 2D technique, anomeric proton not only correlates with H-2 proton, but also with other intra residue protons (H-3, H-4, H-5and H-6) in a well resolved region of the 2D spectrum. But this technique is less successful in the assignment of the H-6 proton.

Phase Sensitive Correlation Spectroscopy (PS-COSY): This technique display the entire coupling information of concerning protons. In this method remote connectivities can easily be traced along the shape of cross peaks displaying coupling of identical magnitude, with active becoming passive (vice-versa) and passing through the point of degeneracy. So gluco and galacto and manno configuration can be distinguished by this type of 2D method.

Double-quantum filtered Correlation Spectroscopy (DQF-COSY): (Paul et. al. 1996)

This Technique was introduced by Rance *et al*. For Better visualization of cross peaks, which are close to diagonal axis, can be achieved by the introduction of a double quantum filter, which generates a COSY spectrum having both crosspeaks and a diagonal multiplet antiphase structure. It provides a clear and accurate way of obtaining chemical shift values coupled protons. It not only provides characteristic multiplicity within the crosspeak, enabling identification of particular sugar units but also provides semiquantitative information of the coupling constants of protons involved in the crosspeak.

Triple quantum Filtered Correlation Spectroscopy (TQF-COSY)

In this method all the spin system that contain less than three or more mutually coupled spins are eliminated by the use of a triple quantum filtered. This technique is useful in making assignments of mutually couple H-5, H-6 in hexopyranosides system.

IN ADEQUATE Spectroscopy (IN ADEQUATE): (Derome et. al. 1988)

The two dimensional 'incredible natural abundance double quantum transfer experiment' provides direct information on carbon bounding, Relayed techniques generally combine two coherence transfer steps, one of which is used to modulate the signals and generate the t_1 dimension, while the other remains fixed and in series to pass on the signal to a more interesting destination. For instance, the most common path is to transfer magnetization from one proton to another and then on to a heteronucleus coupled to the second proton. Thus, the original proton can be correlated not only with the heteronucleus to which it is directly attached, but also with another one nearby providing the required information about the molecular skeleton.

Total Correlation Spectroscopy (TOCSY): (Braunschweiler et.al. 1994)

A more recent 2D NMR experiment for identifying extended couplings is TOCSY (**TO**tal **C**orrelation **S**pectroscop**Y**) which is also known as HOHAHA (**HO**monuclear **HA**rtman **HA**hn Spectroscopy). In this technique an isotropic mixing is added after the evolution time by applying a sequence of pulse which effectively averages out chemical shifts.

This can be thought of as a sequence of pulses, each of which refocuses the chemical shifts. In effect, all coupled spins will have the same precession frequency, so they will be strongly coupled and their transitions will be thoroughly mixed. As the mixing period gets longer, correlation with more distant protons can be observed (e.g. mix=80-100 msec, can correlate H1 to H6 in carbohydrate), So this method mainly use for the structural assignments of complex carbohydrates. The 2D HOHAHA technique is helpful in determination of the subspectrum of each monosaccharide unit of the oligosaccharides. Milk oligosaccharides give cross peak in the region of the anomeric proton and other sugar proton in the TOCSY spectrum. This experiment is especially helpful in sugar for which similar chemical shifts of methine protons leads to many instances of strong coupling or intermediate coupling. TOCSY /HOHAHA can give total correlation of all protons in a chain with each other and it serves for the identification of single residue.

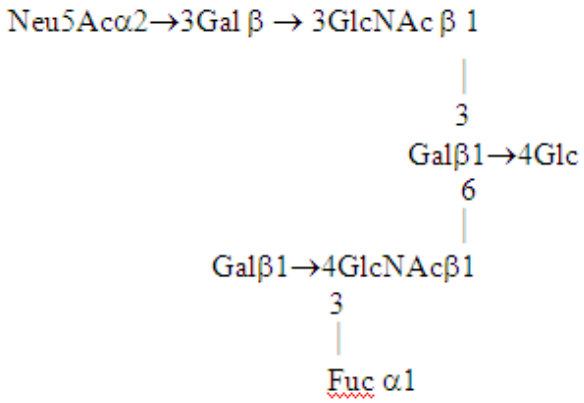
Nuclear Overhauser Effect Spectroscopy (NOESY): (Do Tsch et. al. 1994)

The Nuclear Overhauser effect (NOE) holds a position of great importance in organic structure elucidation as it enables us to define the three dimensional stereochemistry of sugar molecules of interest. ^{1}H-^{1}H NOESY is useful for determining which signals arise from protons, which are close in space (distance smaller than $5A^{0}$) but not closely connected by chemical bonds. By measuring crosspeak intensity, distance information can be extracted. A NOESY spectrum yields through space correlation via the Nuclear Overhauser Effect Spectroscopy. Inter residue NOEs are the principle assignment tool for determination of the sequence of sugar residue and also in determining their linkage positions. In practice semi selective excitation of one carbohydrate proton, combined with multistep-relayed coherence transfer and a terminal NOE transfer has been used for the sequential analysis of oligosaccharides. Assignment of the anomeric and some of the other protons resonance may be made with the help of data from decoupling and NOE experiments. In a glycoside (G-O-S_1-O-S_2), because the proton on C-1 of S_1 is close enough to the proton bonded to C-4 of S_1, it is possible to demonstrate coupling between the two rings from a ROESY experiment. The ^{PK} 1,3–diaxial and eq-ax proton pairs in pyranosyl ring produce intra NOESY crosspeak i.e. for β-glycopyranosyl residue crosspeaks are observed between H-1and H-3 (and H-5) whereas strong crosspeak observed between H-1 and H-2 in an α-glucopyranosyl configuration. In the structural determination of Lacto-N-hexaose, selective decoupling irradiation at each of the three doublets assigned to the β- Galactose H1 at 4.424, 4.454 and 4.504 ppm identified the resonances of the corresponding H2 at 3.546, 3.489 and 3.496 ppm, respectively. Selective decoupling irradiation of the narrow doublet at 4.145 ppm, which is assigned to H4 of a β-galactose, which is C-3, substituted. Decoupling at the gal H2 resonance at 3.546 ppm identifies this same gal H3 resonance this completing the assignment of H1, H2 H3 and H4 of the branching galactosyl residue. Confirmation of β-GlcNAc H1 is found in NOE observed at that doublet on irradiation of β-GlcNAc H3 at 4.077 ppm. The appearance of β-GlcNAc H3 as a structural reporter group result from fucosylation at C-4 was observed in case of LNF-II. Irradiation at 4.077 ppm also gives NOE at the doublet at 4.504 ppm allowing its assignment to β-gal H1. Irradiation at α-Fuc H1 NOE at β-GlcNAc H4 (3.749 ppm) due to interring NOE and at α-Fuc H2 (3.683ppm) due to intra-ring NOE. Selective decoupling at β-GlcNAc H3 and decoupling at H1 identifies H2 completing the assignment of the resonances of β-GlcNAc.

An effective way of connecting the monosaccharide residue is by monitoring the nuclear Overhauser effect from signal for an anomeric reporter group to the hydrogen of the substituted position in the adjacent ring.

Rotating Frame Overhauser Enhancement Spectroscopy (ROESY): (Jim´enez Blanco et. al. 2000 and Breg et.al. 1988)**:** The NOESY technique has the disadvantage that for molecules with a molecular mass in the order of 1000 to 3000 the signal may disappear, since the NOE effect change its sign depending on the molecular correlation time. Due to the well know problems involved with NOE measurements at medium field strength form medium-sized molecules, a 2D NOE in a rotating frame (ROESY) can be of importance. In cases where attempts to obtain reliable NOE crosspeaks are unsuccessful, a ROESY spectrum can show all NOE crosspeaks defining interglycosidic linkage. Because NOE is a function of molecular rotation time, which itself depends on the size and shape of the molecule. Viscosity of the medium and temperature are important in this type of experiment.

Heteronuclear Multiple Quantum Coherence (HMQC) or HSQC Spectroscopy: (Willer et. al. 1985)**:** Sometimes the proton resonances of oligosaccharides are too overlapping to be disentangled by homonuclear correlation alone. In such cases heteronuclear correlation maps may enable the assignment of ^{1}H resonances, because in such a spectrum one observes connectivities between ^{1}H and ^{13}C chemical shifts. This method spreads the ^{1}H NMR spectrum in the ^{13}C dimension, thus greatly improving the resolution and eliminating the effects of strong 1H couplings. Usually ^{1}H-^{13}C crosspeaks do not superimpose until the ^{1}H and ^{13}C chemical shifts are identical due to the presence of a very similar chemical environment. The ^{1}H-^{13}C one-bond correlations through a ^{13}C-decoupled ^{1}H detected heteronuclear multiple quantum coherence spectrum at a higher contour level led to the assignment of all ^{13}C resonances of the α anomer. Correlation of H-2 and H-3 resonances at 4.05 and 4.36 ppm with ^{13}C resonances at 53.5 and 51.5 ppm led to the assignment of these amido substituted carbons to C-2 and C-3, respectively. The chemical shift for NeuAc with H-3 at 1.786/2.756 ppm (ax/eq) and a β- linked Gal with H-1 at 4.503 ppm is linked $\alpha2\rightarrow3$ to a Gal residue. Further via a NOE effect, this Gal residue could also be connected to H-3 of one of the GlcNAc residue with H-1 at 4.736/4.732 ppm (α/β) and H-3 at 3.803 ppm. C-1 of this GlcNAc found at 103.2 ppm locates the sialylated branch of the 3- position of the 3,6-disubstituted Gal residue. The presence of α- linked Fuc is verified by signals of H-1 at 5.099 ppm, H-5 at 4.825 ppm, and CH3 at 1.170 ppm. Chemical shifts for C-1 at 101.6 ppm of GlcNAc residue showed that this sequence is connected to the 6-position of the 3,6 disubstituted Gal residue. Thus the structure was detected as –



^{1}H-^{13}C one-bond dipolar coupling values were measured for samples of the human milk oligosaccharides "lacto-*N*-fucopentaose" (LNF-1, LNF-2, and LNF-3), "lacto-*N* difucohexaose" (LND-1), "lacto-*N*-tetraose" (LNT), and "lacto-*N*-*neo*-tetraose" (LNnT), four of which have Lewis blood group epitopes, and extracted the complete information about the sugar residue (Martin et. al. 2000).

MASS SPECTROSCOPY

In the discovery of novel compounds in natural product research, it is customary to quickly identify the presence of known bioactive compounds prior to embarking on expensive isolation, purification, and characterization work. The characterization and analysis of glycoconjugates by mass spectrometry (McCloskey et. al. 1990) has undergone a number of improvements in the last 10 years, especially with the development of methods capable of ionizing and analyzing these compounds in their native states. Mass spectrometry is probably the most broadly applicable analytical tool in the chemical sciences in biological research as capabilities to address large molecules. In the field of Natural product chemistry, the separated milk oligosaccharides can be identified with greater certainty by mass spectroscopic technique. Mass spectroscopy is an important analytical tool for structure elucidation of complex oligosaccharides (Khare et. al. 1987) comprising composition, sequence, branching, and linkage analysis, including anomericity and finally also rings sizes and absolute configuration, i.e., identity of the subunits. Because most oligosaccharides are composed of five unique monosaccharide units with different incremental masses (fucose, 146 Da; hexose, 162 Da; *N* –acetylhexosamine, 203 Da; *N*-aceylneuraminic acid, 291 Da; *N*-glycolylneuraminic acid, 307 Da), knowledge of the molecular weight can be used to determine the potential composition of the oligosaccharide (Barr et. al. 1991). In general, mass spectrometry provides the possibility of structural elucidation based on characteristic fragmentations of the molecules under investigation. A nomenclature for the possible fragment ions of oligosaccharides has been proposed by Domon and Costello, i.e., B and C for fragment ions containing the non-reducing side, Y and Z for those containing the reducing sugar unit, as well as A and X type fragment ions for those arising from cross-ring cleavages (Domon et. al. 1988). Which subsequently can give sequence and to some extent linkage information along with knowledge on whether the monosaccharides are *e.g.* pentoses or hexoses.

In general, most of the human milk oligosaccharides consist of building blocks, i.e., lactose at the reducing end linked to multiple units of *N*-acetyl lactosamines, which differ in size, branching, and linkage, with additional fucose or sialic acid residues linked to the core oligosaccharides. The structural analysis of milk oligosaccharides must be addressed to the following aspects: (Kunz et. al. 1993)

- Composition analysis.
- Determination of branching positions.
- Differentiation of the two core-constituents *N*-acetyllactosamine, i.e., Gal β(1-3) GlcNAc (LacNAc, lacto-series or "type I") and Galβ(1-4) GlcNAc (Lac-*neo*-NAc, lacto-neo-series or "type II").
- Determination of the position of fucose and / or sialic acid residues.
- Linkage of the *N*-acetyllactosamine subunits (and the lactose to the first *N*-acetyllactosamine) is β(1-3).

• Linkage of the fucose residues to *N*-acetylglucosamines depends on the linkage of the corresponding *N*-acetyllactosamine subunits: in the lacto-series, the fucose is (α 1-4) linked and in the lacto-neoseries the linkage is (α1-3).

With the concentration of these points in own mind, all carbohydrate researchers trace the Mass spectroscopic data and elucidate an exact and precise mass of the complex milk oligosaccharide. This spectrometric technique offers the possibility of structural investigations of each purified milk oligosaccharides; it has been demonstrated by using of "soft" ionization techniques (FAB-MS, LC-MS, thermospray and electrospray MS) has even expanded the utility of MS for the analysis of large biopolymers. Fast-atom bombardment (FAB) and electrospray-ionization (ESI), mass spectrometry have both been utilized successfully to this end. Matrix-assisted laser desorption /ionization (MALDI) is the most suitable ionization method for the analysis of carbohydrates collected after HPAEC, because MALDI is 10-100 times more sensitive than FAB for detection of underivatized oligosaccharides and is more tolerant of salts than either FAB or ESI. The molecular ion was fragmented into the fragment units which were formed by the decomposition pathways in which repeated H transfer in the oligosaccharide is accompanied by the elimination of terminal sugars less water, (Brown et.al. 1971) such fragmentation goes on until the monosaccharide is left (**Scheme1**).

ELECTROSPRAY IONIZATION- MASS SPECTROSCOPY (ESI-MS) -

In recent years Electrospray Ionization Mass Spectroscopy (Kogelberg et. al. 2004) (ESI MS) has become an indispensable tool in the modern analytical laboratories as a research instrument, developed by Feen and co-workers. In this technique Electrospray ionization occurs during the electrostatic nebulization of a solution of charged analyte ions by a large electrostatic field gradient. In this technique, a stream of liquid containing sample is injected directly in to the ES source where the sample molecules are stripped of solvent, leaving them as multiple charged species whose charge reflect the number of functional groups that can be protonated (positive ion mode) or deprotonated (negative ion mode) at the high pH of carrier liquid.

The most widely used mass spectrometric approach for structural elucidation of oligosaccharides (Finke et. al. 1999) is ESI- tandem- MS^n, typically performed on triple-quadrupole instruments using precursor-ion selection in a first MS step, collision-induced dissociation and mass analysis of fragment ions in a second MS step. This is mainly carried out with sodiated derivatized (permethylated or peracetylated) oligosaccharides for two major reasons: (1) To reach the sensitivity level required and then to enhance the yield of significant fragment ions, (2) sequence, branching, and linkage analysis can be performed based on the identification of non-reducing terminal fragment ions due to cleavage of glycosidic bonds and linkage-specific additive mass increments due to cross-ring cleavages. Both derivatized and underivatized oligosaccharides have also been investigated in MS experiments using doubly-charged metal-cation attached precursor ions, i.e., $[M+Cat]^{2+}$ (Cat: Mg^{2+}, Ca^{2+}, Ni^{2+}, or Co^{2+}). MS investigations of protonated, permethylated oligosaccharides in a quadrupole ion-trap mass spectrometer were reported to give simpler and more predictable mass spectra based on the predominant formation of B-type fragment ions. For nonderivatized oligosaccharides, linkage-specific cross-ring fragments are observed, this has been shown for small oligosaccharides accessible by

electrospray/ionization Mass spectroscopy (ESI-MS); cross-ring fragment ions are found both in the positive and negative ion mode and are believed to originate from a ring opening at the reducing end sugar in a pericyclic hydrogen rearrangement of the retro-aldol reaction type. This may be followed by cleavages after enolization, resulting in loss of linkage-specific neutral fragments. However, it could be proven by 180 exchange of the reducing end oxygen that for underivatized oligosaccharides in the positive ion mode predominantly glycosidic bond cleavages of B/Y-type occur and only the linkage of the reducing end monosaccharide is accessible. Molecule ion species were subjected to fragmentation in positive and negative ion mode in order to check whether a differentiation between isomers is possible.

Positive Ion Mode- In positive-ion mass spectra (Kennedy et. al. 1988) resulting from the fragmentation of the sodiated molecules of Lacto-N tetraose (LNT), and Lacto-N-neo-Tetraose (LNnT) at *m/z* 730 are compared. The relative intensities of fragments originating from cleavage of the glycosidic bond between Lac and Lac-NAc, B_2 (*m/z* 388) and C_2 (*m/z* 406), are different. The intensities of B_2 and C_2 are equal in the spectrum of LNT, whereas for LNnT the intensity of B_2 is much higher than C_2. Although there is no difference in the nature of the bond cleaved, the resulting fragment disaccharides differ in linkage of the monosaccharide subunits. Since the remaining *N*-acetyllactosamine sub-unit is (1-3)-linked in the case of LNT, the signal intensity of the B_2 fragment ion is reduced by an additional loss of the galactose (Δ m = 180 Da), which is suggested to occur via a β-elimination.

Negative Ion Mode - Mass Spectroscopic investigations in the negative-ion mode (Bahr et. al. 1998) yield unambiguous structural information for oligosaccharides, even for larger ones, as shown below. Under the chosen experimental conditions exclusively deprotonated molecules $[M - H]^-$ are detected, which are most probably formed by the loss of acetic acid from initial acetate attached ions $[M - CH_3COO]^-$ or HCl from $[M-Cl]^-$ ions, respectively.

The ESI-Mass spectra analysis of human milk oligosaccharide structures based on iso-lacto-N-octaose core are nonfucosylated iso-lacto-N-octaose, Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6 [Galβ1-3GlcNAcβ1-3] Galβ1-4Glc, mono fucosylated and trifucosylated iso-lacto-N-octaose, Galβ1-3GlcNAcβ1-3Galβ1-4 (Fucα1-3) GlcNAcβ1-6[Galβ1-3GlcNAc β1-]Galβ1-4Glc and Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4(Fucα1-)GlcNAcβ1-6[Galβ1-3(Fucα1-4)GlcNAcβ1-3]Galβ1-4Glc,both containing an internal Le**x** epitope, characterized easily.

In Glycoconjugates, Electro spray MS (ES-MS) (Carr et. al. 1991) has become a popular method for analysis of recombinant glycoprotein's. Since the electrospray interface requires a continuous infusion of solvent, it is compatible with on-line LC-MS or CE-MS, thus enhancing the data obtained from a chromatographic **"fingerprint"** of a tryptic digest or a pool of released oligosaccharides. Since electrospray ionization produces families of multiply-charged ions, mass spectrometers with relatively modest mass ranges can be employed to analyze even large glycoproteins such as immunoglobulins with excellent intrinsic mass resolution. Thus, electrospray methods can, and have, been used to look at the glycosylation heterogeneity of intact glycoproteins such as ribonuclease. On the other hand, the very complex envelope of multiply- charged ions requires sophisticated deconvolution analysis to extract information on the original molecular weight(s) of the species present. Indeed, very large and heterogeneous glycoproteins can yield "a bell shaped hump" of completely unresolved ions in the critical 1000-2000 Da mass region. In some cases, the best approach may be tying a high-resolution separation method (capillary HPLC or CE) to a relatively low-resolution mass spectrometer via an electrospray interface.

FAST ATOM BOMBARDMENT MASS SPECTROSCOPY (FAB-MS)

FAB-MS are mild ionizing mass spectrometric technique (Ahmad et. al. 1988) (also known as Secondary Ion Mass Spectrometric) has been used to determine the polar molecules such as oligosaccharide, peptide etc. In FAB-MS experiments, an accelerated beam of atom is fired from the gun towards the largest, which has been preloaded with a viscous matrix. This technique is very helpful in the mass spectrometric determination of milk oligosaccharides. Purified milk oligosaccharides may be analyzed directly by FAB-MS but derivatization of the oligosaccharides to enhance their surface activity markedly improves the sensitivity of the method. A first insight into the composition of milk oligosaccharides can be obtained by the determination of the molecular ions either using negative ion desorption of native compounds positive ion desorption with peracetylated or permethylated compounds. The spectra of the peracetylated oligosaccharides show intense pseudomolecular ion $M+Na^+$ and $M+H^+$ together with the daughter ion formed by the elimination of one or two $CH_2=C=O$ units, i.e., 42 or 84 a.m.u. in comparison to the FAB spectra could be obtained with 1-mercapto-2,3-propanediol and the addition of sodium acetate to the target. Besides the very intense pseudomolecular ion $M+Na^+$ and $M+K^+$, often constituting the base peak, fragments ions characteristic for the carbohydrate constituent are also present in the reduced and peracetylated neutral oligosaccharides glucose as glucitol, fucose, Galactose and N-acetylglucosamine each contributes specific mass increments to the molecular weight.

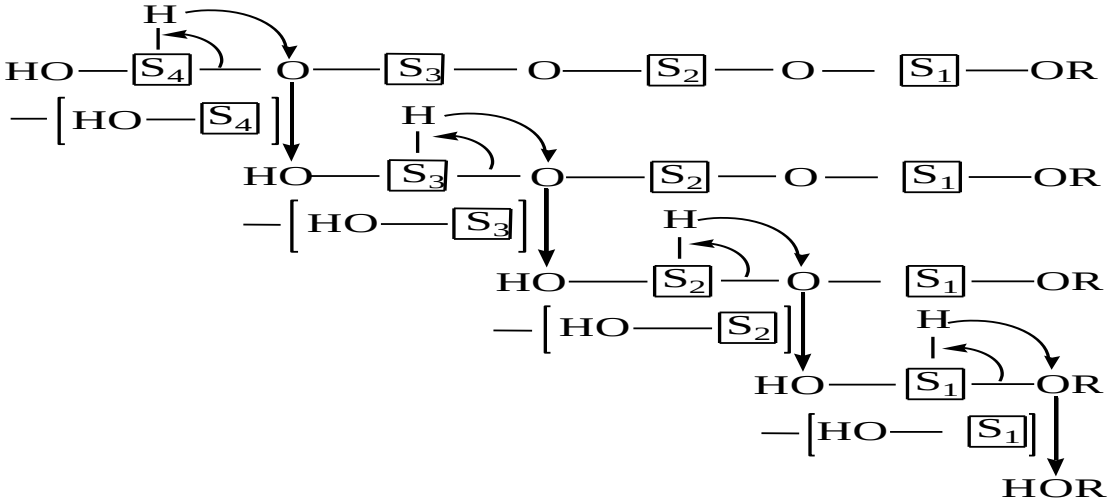
MATRIX ASSISTED LASER DESORPTION/IONISATION MASS SPECTROSCOPY (MALI-MS)

One of the most intriguing of the recent MS technologies is matrix-assisted laser desorption MS (Tsengl et. al. 2000) (known as LD-MS or MALDI-MS), this spectrometric technique has become an important tool for the analysis of milk oligosaccharides. Matrix-assisted laser desorption/ionization coupled with Fourier-transform mass spectrometry (MALDI-FTMS) offers high resolving power and high sensitivity and makes it especially suited for analyzation of large bimolecules. With this mass spectrometric method, neutral and anionic milk oligosaccharides are analyzed simply in positive and negative mode, respectively and derivatization of compound is not required in this technique for structure determination.

Matrix-assisted laser desorption -PSD (MALDI-PSD) mass spectrometry is now widely used in the molecular mass determination of underivatized oligosaccharides. An analysis of the relative ion intensities in the MALDI-PSD mass spectra oligosaccharides was very useful for distinguishing the linkage isomers and for the characterizing the type of glycosyl linkage (Ferguson et. al. 1983). The native oligosaccharides of lacto-N-neo tetraose (Gal β 1-4 GlcNAc β 1-3Galβ1-4 Glc; LNnT) and lacto-N-tetraose (Gal β 1-3 GlcNAc β 1-3Galβ1-4 Glc; LNT) were analyzed by using curved field reflection matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOFMS).' (Klen et. al. 1998). Since a curved-field reflection TOFS enables a simultaneous focusing of a wide mass range of metastable fragment ions, the relative ion intensities in the PSD mass spectra can be discussed. The PSD mass spectra of LNnT and LNT were distinguish in the relative ion intensities. In case of LNT, β-elimination could occur in the GlcNAc at the C-3 position, which was bonded by Gal; however, it did not occur in LNnT. The 3-0 elimination caused a difference in the relative ion intensities in the PSD mass spectra of LNnT and LNT.

The β1-3 glycosyl linkage cleaved more easily than the β1-4 glycosyl linkage in the MALDI-PSD fragmentation. In the PSD-mass spectra of the oligosaccharides, the PSD ions produced by cleavage of the α1-6 had higher intensities than those of the β1-4 glucosyl linkage, indicating that the α1-6 linkages cleaved much more readily than the β1-4 glycosyl linkage.

H-TRANSFER IN OLIGOSACCHARIDE AND ELIMINATION OF NONREDUCING SUGAR RESIDUE



COMPUTATIONAL METHOD (Imberty et. al. 2000)

Computational science is the application of computational and mathematical techniques to the solution of large and complex problems (Gallopoulos et. al. 1994). Theoretical or computational chemistry research includes studies like geometrical studies including computation of geometrical parameters for a new molecule, simulation of spectra related to this molecule with computation of some or more properties for these molecules. Computational chemistry program allow scientist to generate and present molecular data including geometries, energy, electronic properties and bulk properties (Ramachandran et. al. 2008). Computation chemistry deals with computer simulation to assist in solving complex problems related with compound modelling, geometry of organic molecules, optimized energy etc. (Lemmen, et. al. 2000) computation chemistry applies varied approach as MEP (Molecular electrostatics potential), empirical and semi empirical approach, MOT(molecular orbital theory) and DFT (density functional theory) to solve these problems. These approaches are being used in performing the following studies-

1. Electronic structure determinations
2. Geometry optimizations
3. Frequency calculations
4. Internal interaction through AIM approach.
5. Transition state determinations.
6. Protein calculation. i.e. Docking.
7. Funding of electron and charge distributions.
8. Potential energy surfaces (PES) detection.
9. Rate constants determination for chemical reaction (Kinetics).
10. Thermodynamic calculation- heat of reaction, energy of activation.

Currently, there are two way to approach chemistry problems: computational quantum chemistry and non-computational quantum chemistry.

Computational quantum chemistry is primarily concerned with the numerical computational of molecular electronic structure by ab initio and semi-empirical techniques and non-computational quantum chemistry deals with the formulation of analytical expression for the properties of molecules and their reactions. Scientists mainly use three different methods to make calculations:

I. Ab-initio calculation is called a Hartree Fock calculation (abbreviated HF), in which the primary approximation is called the central field approximation. This means that the Columbic electron-electron repulsion is not specifically taken into account. However, its net effect is included in the calculation. This is a variational calculation, meaning that the approximate energies calculated are all equal to or greater than the exact energy. The second approximation in HF calculations is that the wave function must be described by some functional form, which is only known exactly for a few one electron systems. The functions used most often are linear combinations of Slater type orbitals or Gaussian type orbitals. An alternative ab initio method is Density Functional Theory (DFT), in which the total energy is expressed in terms of the total electron density, rather than the wave function. In this type of calculation, there is an approximate Hamiltonian and an approximate expression for the total electron density.

II. Semi-empirical calculations are set up with the same general structure as a HF calculation. Within this framework, certain pieces of information, such as two electron integrals, are approximated or completely omitted. In order to correct for the errors introduced by omitting part of the calculation, the method is parameterized, by curve fitting in a few parameters or numbers, in order to give the best possible agreement with experimental data. Semi-empirical calculations have been very successful in the description of organic chemistry, where there are only a few elements used extensively and the molecules are of moderate size. However, semi-empirical methods have been devised specifically for the description of inorganic chemistry as well.

III. Molecular mechanics method, the data base of compounds used to parameterize the method (a set of parameters and functions is called a force field) is crucial to its success [235]. Whereas a semi-empirical method may be parameterized against a set of organic molecules, a molecular mechanics method may be parameterized against a specific class of molecules, such as proteins. Such a force field would only be expected to have any relevance to describing other proteins.

Hence, computational chemistry is a branch of chemistry that generates data which complements experimental data on the structures, properties and reaction of substances. The calculations are based primarily on Schrödinger's equation and particularly useful for the determination of properties that are inaccessible experimentally and for the interpretation of experimental data.

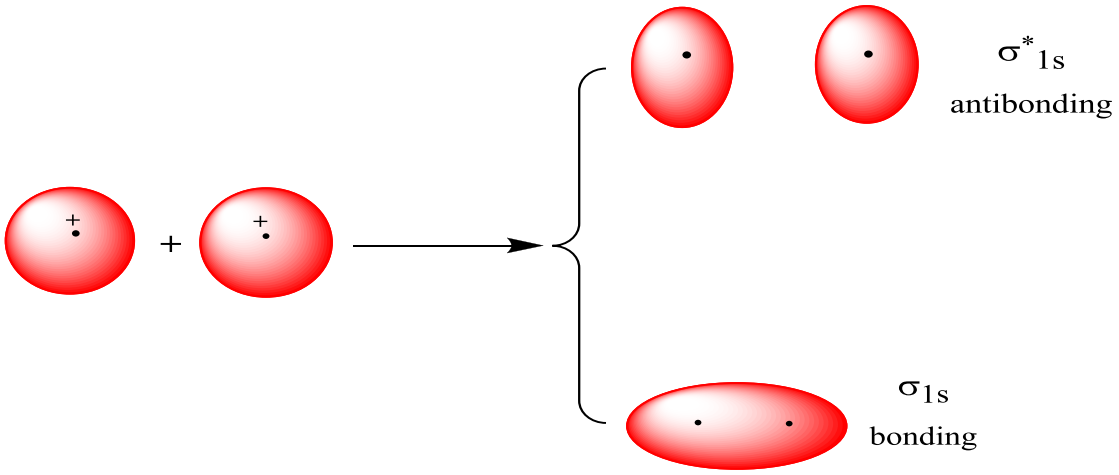
DENSITY FUNCTIONAL THEORY (DFT) (Bagayoko et. al. 2014)

In the past 30 years, Density Functional Theory has emerged as the most popular electronic structure method in computational chemistry. Density functional theory (DFT) has become ubiquitous in science, including quantum chemistry, life science, solid-state physics and material science, because of its low cost and excellent performance. Density functional theory is a functional i.e. function of another function; here functional is the electron density which is function of space and time. The electron density used in DFT as the fundamental property unlike Hartee- fock theory which deals directly with the multi-body

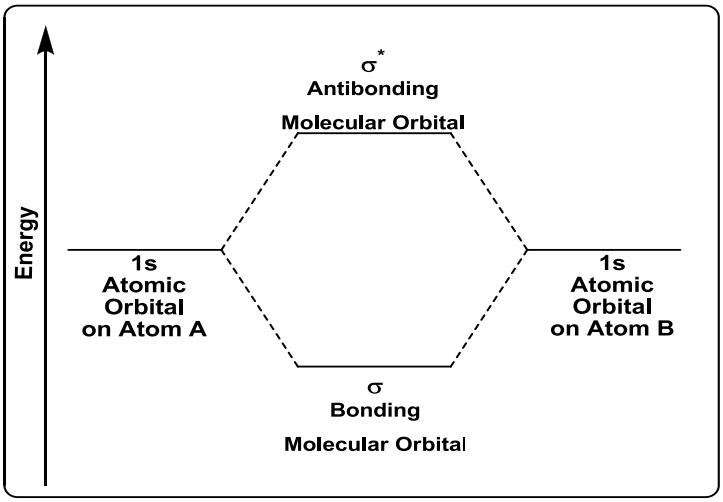
wave function. Density functional theory provides a powerful tool for computations of the quantum state of atoms, molecules and solids, and of ab-initio molecular dynamics. The theory was first developed in 1964 by Hohenberg and Kohn (Hogenberg & Kohn, et al,. 1964) who determined that all ground-state properties can be cast as a functional of the charge density that must be minimized in energy. In the past few decades, the use of DFT technique to interpret and predict the properties of organic molecules has grown tremendously because of its low computational efficient and less time consuming properties. DFT is among the most popular and versatile methods available in condensed-matter physics, computational physics, and computational chemistry. DFT is a widely used computational quantum mechanics model for chemical and biological systems and works on the basis of various exchange-correlation functionals. Among all the functionals, B3LYP is a common hybrid exchange-correlation functional which is based on exact exchange from Hartree-Fock theory and other functional sources such as generalized gradient approximations from Becke88 and Lee-Yang-Parr.

Molecular orbitals theory (Jeffrey et. al. 1974)

Molecular orbital (MO) theory is a method for determining molecular structure in which electrons are not assigned to individual bonds between atoms, but are treated as moving under the influence of the nuclei in the whole molecule. The spatial and energetic properties of electrons within atoms are fixed by quantum mechanics to form orbitals that contain these electrons. While atomic orbitals contain electrons ascribed to a single atom, molecular orbitals, which surround a number of atoms in a molecule, contain valence electrons between atoms. Molecular orbital theory, which was proposed in the early twentieth century, revolutionized the study of bonding by approximating the positions of bonded electrons the molecular orbitalsas Linear Combinations of Atomic Orbitals (LCAO). These approximations are made by applying the Density Functional Theory (DFT) and Hartree Fock (HF) models to the Schrödinger equation. Molecular orbital (MO) theory uses a linear combination of atomic orbitals (LCAO) to represent molecular orbitals resulting from bonds between atoms. These are often divided into bonding orbitals, anti-bonding orbitals, and non-bonding orbitals. Consider the H_2 molecule, for example. One of the molecular orbitals in this molecule is constructed by adding the mathematical functions for the two 1*s* atomic orbitals that come together to form this molecule. Another orbital is formed by subtracting one of these functions from the other, as shown in the figure below.



One of these orbitals is called a bonding molecular orbital because electrons in this orbital spend most of their time in the region directly between the two nuclei. It is called a sigma (σ) molecular orbital because it looks like an *s* orbital when viewed along the H-H bond.



Electrons placed in the other orbital spend most of their time away from the region between the two nuclei. This orbital is therefore anti bonding, or sigma star (σ*) molecular orbital. The bonding molecular orbital concentrates electrons in the region directly between the two nuclei. Placing an electron in this orbital therefore stabilizes the H_2 molecule. Since the σ* anti-bonding molecular orbital forces the electron to spend most of its time away from the area between the nuclei, placing an electron in this orbital makes the molecule less stable.

Lewis Theory uses curly arrows to denote electron migration during a chemical reaction and has led to a greater understanding of the factors controlling chemical reactions. Pauling with others developed Resonance Theory, which provided the rationale to an all-embracing orbital theory. The use of "canonical forms" and "resonance hybrids", along with extensive use of curvy arrows has provided the fundamental background to modern organic theory, but for *e.g*. Diels-Alder and pericyclic reactions, the curly arrow format is not very clear and in some instances the reactions are described as no-mechanism reactions. Woodward and Hoffmann showed that by examining the interaction of the frontier molecular orbitals (i.e. the Highest Occupied, HOMO and Lowest Unoccupied, LUMO) both the region and stereo specificity could be accounted for Woodward and Hoffmann work was assimilated into general organic reaction theory (Hirofumi et. al. 2013).

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