Goat Milk Oligosaccharides and Their Biological Activities

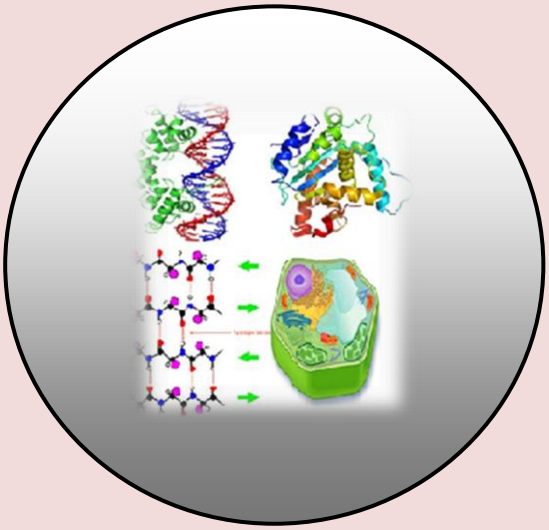
By

Pushpraj Singh, Unnati Singh, Manisha Shukla and Desh Deepak

ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 40 (2), 2023 Pages No. 66-109



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 40, No. 2, 66-109, 2023
Ms 40/02/49/2023

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

Dr. Manisha Shukla
http:// www.sasjournals.com
http:// www.jbcr.co.in
jbiolchemres@gmail.com

RESEARCH PAPER

Received: 20/10/2023 Revised: 28/12/2023 Accepted: 29/12/2023

Goat Milk Oligosaccharides and Their Biological Activities

Pushpraj Singh, Unnati Singh, Manisha Shukla and Desh Deepak
Department of Chemistry University of Lucknow, Lucknow-226007(UP), India

ABSTRACT

The biological activities of any milk are due to its oligosaccharide contents. These oligosaccharides are present as straight and branched chains, comprised of varied combinations of glucose, galactose, GlcNAc, GalNAc, fucose and sialic acid which are the linked together by O-glycosidic linkages at different positions of these monosaccharides with the variations in configurations of glycosidic linkages (α or β). Due to the varied structures of these oligosaccharides they exhibit different biological activities. Indian geographical terrains have a large variety of goat species. Goats are inhabitants of Himalayan region upto the Ran of Kutch. They are also found in southern part of the country. The constituents of their milk vary depending on their fodder, resulting in variation in the structure of the constituentoligosaccharides. The most important monosaccharides present in the goat milk oligosaccharides are fucose and sialic acid which are mostly present at their non-reducing end. These two carbohydrate moieties are responsible for important biological activities of these oligosaccharides. The stereoscopic structure of these oligosaccharides is elucidated by 2D-NMR techniques and Mass spectrometry. This article emphasizes on isolation, structure elucidation, stereoscopic structures of isolated compounds and their biological activities.

Keywords: Goat, Milk oligosaccharides, Structure elucidation, NMR and Biological activities.

INTRODUCTION

Carbohydrates are a huge family of naturally occurring compounds which include sugars, starch and cellulose as well as materials found in bacterial cell walls and insect exoskeletons. They are present in the form of oligosaccharides, polysaccharides, glycoconjugates i.e. glycosaminoglycans, glycoproteins, glycopeptides, glycolipids and proteoglycans; and being the main source of energy for living organisms and the central pathway of energy storage and supply for most cells (Singh et.al. 2018, Lager et.al. 2006, Tozzi et.al. 2006, Burke et.al. 2006).

They play an important role in drug design and disease treatment which include embryogenesis, fertilization, hormonal activities, cell- proliferation, and blood group specificity (Sears et.al. 1996, Heide et.al. 2004). The ever-increasing role of carbohydrates in biological process related to immunology, virology, cancer, antibiotic action and host of the life threatening disease has heightened the interest in the accessibility of specific sugar based molecule (Hanessian et.al. 2000) They are frequently required component of antibiotic agents that bind to either protein or R.N.A. target structures to form glycoconjugates having medicinal properties (Koeller et.al. 2000). Glycolipids and glycoproteins present in carbohydrate play a major role in inflammation, immune response, metastasis, hormone function regulation and fertilization and many other biomedical important processes (Varki et.al. 1993). A better understanding of biological capacity of oligosaccharides present in carbohydrate leads to the development of novel therapeutics and nutritional supplements targeting these interactions (Alper et.al. 2001). Oligosaccharides have received interest because of their role as prebiotics and able to act as important analogs for microbes and prevent mucosal attachment including pathogens such as Pneumococci, Haemophilus influenza and Neisseria meningitides pili (Kim et.al. 2010). Numerous oligosaccharides have been isolated from the milk or colostrums of many mammalian species like domestic herbivorous animals, non-human primates, marsupials, carnivores, caprines, monotremes, equines, bovine and marine mammals. Milk oligosaccharides (MOs) have been categorized in two classes i.e. sialylated & nonsialylated oligosaccharides (Polonovski et.al. 1933, Urashima et.al. 2003) and both classes of oligosaccharides have been tested for their varied biological activities. It has been found that sialyl and fucosyl lactsomines such as sialyl Lewis^x and sialyl Lewis^y are critical epitopes during lectin-ligand binding steps (Wang et.al. 1992). MOs play a pivotal role in medicinal chemistry in immunostimulation, cancer and allergic responses (Schwonzen et.al. 1992, Abe et.al. 1993, Srivastava et.al. 1989). They are abundant in breast milk influence adhesive events in vivo and in addition to their possible functions in neonatal host, defense and inflammatory events. Oligosaccharides, along with lactose, play a role in postnatal brain development (Oddy et.al. 2002). MOs function as soluble receptors for intestinal bacteria and viruses (Coppa et.al. 2006, Zivkovic et.al. 2011). Sialic acid present in milk oligosaccharides also contribute to the increased concentration of NeuAc, present in cerebral and cerebellar glycoconjugates of breast fed and thus play an important role in the development of the infant brain (Boehm et.al. 2003, Nakamura et.al. 2003). Sialylated milk oligosaccharides can neutralize influenza virus through binding to hemagglutinin (Weis et.al. 1988). Similarly, fucosylated milk oligosaccharides also bind to several strains of norovirus, thereby preventing the docking of the virus to cellular glycan receptors (Jiang et.al. 2004). MOs also inhibit the adhesion of bacteria such as *Campylobacter jejuni*, *Listeria monocytogenes*, and *Streptococcus pneumonia* to the intestinal epithelium (Andersson et.al. 1986, Simon et.al. 1997). Furthermore, milk oligosaccharides protect against bacterial enterotoxins such as cholera toxin by competing with cell-bound glycan receptors (Otnaess et.al. 1983).

Milk Oligosaccharides (MOs)

Besides the protein, fat and lipid, one of the major components of milk is carbohydrate which contains lactose and oligosaccharides. Carbohydrates are one of the most dominant solid fractions of both human and bovine milk.

Besides the main milk sugar i.e. lactose which is an energy source for the neonate, number of more complex free oligosaccharides are also present in milk (Ulrik et.al. 2012). However, the oligosaccharides are indigestible by the enzymes in the gastrointestinal tract, thus MOs does not participate in milk for the purpose of nourishment (Joy et.al. 1997). Oligosaccharides have been isolated from various mammalian milk of different origin like buffalo, equine, caprine, ezo brown bear, Japanese black bear, elephant, donkey, rat, dog, echidna, platypus, kangaroo, cow, sheep, goat, yak, mare, camel and human etc. (Singh et.al. 2017, Villoslada et.al. 2006, Osthoff et.al. 2007, Saksena et.al. 1999, Kumar et.al. 2013, Boehm et.al. 2007). A broad range of 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 and exhibit varied biological activities such as immunostimulant, hypoglycemic, anti-tumor, antiviral, anticancer, anticoagulant, anti-complementary, immunological and anti-inflammatory activities. They are made up of monosaccharides linked through α or β-glycosidic bonds and most of the oligosaccharides are made up of a lactose core and additional hexoses, N-acetylhexosamines, neuraminic acid and fucose (Sundekilde et.al. 2012). The monosaccharides that make up milk oligosaccharides (MOs) include glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), N-acetylneuraminic acid (NeuAc), and N-glycolylneuraminic acid (NeuGc) (Chai et.al. 2005, Mehra et.al. 2006, Wu et.al. 2010). Two classes of oligosaccharides have been identified in milk; acidic oligosaccharides containing sialic acid (in the form of N-acetyl neuraminic acid or N-acetyl glycolyl neuraminic acid) and neutral oligosaccharides containing N-acetylhexosamine (Tao et.al. 2008). The highest concentration of oligosaccharides is found in early postparturition milk which is known as colostrum. Oligosaccharides in bovine milk are 20 times less concentrated than human milk. However, in bovine colostrum, the concentration of sialylated oligosaccharides is exceptionally high (Veh et.al. 1981). Furthermore, Human milk oligosaccharides (HMOs) are highly fucosylated with as much as 70% fucosylation, whereas bovine milk oligosaccharides (BMOs) do not contain fucosylation at any appreciable levels (Tao et.al. 2009, Gopal et.al. 2000); conversely, BMOs and HMOs have as much as sialylated oligosaccharides (Ninonuevo et.al. 2006). Human milk does not contain the NeuGc monosaccharide residue that is found in bovine colostrum and other mammalian milks. 6'-sialyllactose (6'-SL) is effective in inhibiting the *Pseudomonas aeruginosa* invasion of pneumocytes, and 3'-SL and 3'-sialyl-3-fucosyl-lactose were identified to inhibit leukocyte rolling and adhesion to endothelial cells (Mariarosaria et.al. 2014, Bode et.al. 2004). Comprehensive studies characterizing these oligosaccharides support the idea that their structural diversity is the basis for a multitude of biological functions including stimulation of selected beneficial Bifidobacteria in vitro studies, participation in the innate immune system, preventing adhesion of pathogenic bacteria like Helicobacterpylori and certain viruses. Lower abundant oligosaccharides from bovine milk were shown to have complex structures closely related to those from human milk oligosaccharides, so bovine colostrum is currently being used in a variety of health promoting supplements worldwide. Furthermore, sialic acid, a component of bovine milk oligosaccharides, is essential for brain development and cognitive function (Singh et.al. 2016). HMOs containing α 1, 2-linked fucose inhibits the stable toxin-producing *E. coli* in vitro and its toxin-induced secretary diarrhea in vitro and in vivo, and the content of 2-linked

fucosyl oligosaccharides in human milk is significantly associated with lower risk of diarrhea in breastfed infants, suggesting a major role for these oligosaccharides in immunity. It was also observed that the oligosaccharides detected in milk can vary with ABO or Lewis blood type of donor as the enzymes involved in their synthesis are also responsible for the formation of the structural determinants of these blood types (Dennis et.al. 2012). The sialylated oligosaccharides isolated from pooled human milk recognize cancer associated antigens expressed by most human adenocarcinomas of the breast. The S Le^x tetrasaccharide sequence reacts specifically to monoclonal antibody CSLEX1, obtained after immunization of mouse with human gastric adenocarcinoma membrane proteins (Kiyoyasu et.al. 1984). The human milk derived acidic oligosaccharides fraction wasfound to enhance the production of certain cytokines after long term exposure(20 d) in vitro in CD4+ as well as in CD8+ T-Cell subfraction (Thomas et.al. 2004). Significantly oligosaccharides isolated from donkey milk possess high degree of immunostimulant activity and proposed to be very helpful in cure of atherosclerosis (Tafaro et.al. 2007). Buffalo milk oligosaccharides have been examined for immunostimulant activity by the well-established methods using mouse/SRBC model & found promising during preliminary screening. The data on HA titre, PFC count and DTH response to SRBC of mice treated with milk oligosaccharides showed that the treatment induced a marked enhancement of antibody & DTH response to SRBC. Goat milk oligosaccharides (GMOs) play an important role in intestinal protection and repair after a damage caused by DSS (dextron sodium sulphate) induced colitis and their implication in human intestinal inflammation (Villosladaa et.al. 2006). Human milk oligosaccharides (HMOs) participate in several protective and physiological roles, including immunoregulation and inhibition of pathogen adhesion in the gastrointestinal tract of infants (Klein et.al. 2000) and it is a well-established source of prebiotic oligosaccharides that plays a critical role in establishing the intestinal flora of infants by stimulating growth of beneficial bacteria (Pearl et.al. 2015). Malaria caused by single celled parasites of Plasmodium genus of which *P. falcipuram* is the most pathogenic, that expresses a large amount of glycosylphatidyl-linositol (GPI) containing an oligosaccharide which is responsible for malaria's morbidity and mortality (Ranjan et.al. 2012). Glycosphingolipids in the Globo-Ganglio and Lacto- series have been investigated as components of potential tumor vaccines. Glycosylated MUC-2-KLH and Globo H-KLH vaccines play a cardinal role in the treatment of Prostate cancer (Tatiana et.al. 2017). Monomeric and multivalent oligosaccharides that bind to bacterial and viral receptors have been recognized to abrogate infection by agents such as Helicobacter pilori, influenza virus and HIV (Yarema et.al. 1998).

Bovine milk oligosaccharides (BMOs) have several cardinal biological activities including the prevention of pathogen binding to the intestinal epithelial and as nutrients for beneficial bacteria. Number of oligosaccharides are present in bovine milk containing not only lactose core (as in human and other animal milk), but also contains lactose amines as basic core units in it. Human milk oligosaccharide binds to a wide range of lectins on the surface of epithelial cells living the mouth, oesophagus and stomach and throughout the gastrointestinal system in the new born baby, thus it prevents opportunistic infection whilst the baby's immune system was developing (Rhodes et.al. 2008, Geralyn et.al. 2014). Oligosaccharides lectin binding has also been used to target therapeutic agents to diseased cells to express high densities of specific lectins on their surface e.g. GalNAc clusters have been used to target antisensenucleotides to hepatocytes to potentially allow treatment of

hepatitis A (Prakash et.al. 2014). A fraction of oligosaccharides isolated from goat milk reduces intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis and contribute to the recovery of damaged colonic mucosa. The elephant milk oligosaccharide fraction contained a high ratio of sialyl oligosaccharide that is significant with respect to the formation of brain components such as gangliosides of suckling calves (Yusuke et.al. 2006). Donkey milk oligosaccharides have ability to stimulate non-specific and specific immunological resistance (Tafaro et.al. 2007) and the oligosaccharide mixture of Donkey's milk has also shown significant stimulation of antibody, delayed type hypersensitivity response to sheep red blood cells in BALB/C mice (Saksena et.al. 1999). The orally treated animals were recognized for a six time increase in HA titre, two times increase in PFC and DTH response. Cow milk oligosaccharides were able to reduce the adhesion of enterotoxic *E. coli* strains of the calf (Maria et.al. 2002). The milk oligosaccharides isolated from camel milk expresses the anti-tuberculosis activity (Fukuda et.al. 2010). The N-acetylneuraminlactose sulphate may play an important role in the nutrition of rat pups, which was the dominant oligosaccharide in the dog milk (Cailin et.al. 2014). Goat milk containing galacto-oligosaccharides could be recommended to decrease most of infant allergy and diseases (Mervat et.al. 2009); it also shows therapeutic virtues for individuals with certain dietetic problems. Fucosylated human milk oligosaccharide and related glycoconjugates can be used for several specific disease by inhibition of enteric pathogens such as stable toxin of *Escherichia coli* (in vitro and its toxin induced secretory diarrhea in vitro and in vivo), noroviruses and Campylobacter (Crane et.al. 1994); and also show inhibitory effect on certain virulence-related abilities of monocyte, lymphocyte and neutrophil adhesion to endothelial cells and act as anti-inflammatory agents.

Goat Milk Oligosaccharides (GMOS)

Goat milk play a very important role in traditional Indian medicinal system for treatment of mal-absorption syndromes, intestinal disorder, coronary disease, premature infant nutrition, cystic fibrosis, infant allergy, inhibiting and dissolving cholesterol deposits and is a rich source of protein, fat, lactose, minerals, amino acid, calcium and vitamin A. In Naturopathic medicine, goats are referred to as bioorganic sodium animals. They are also associated with vigour, flexibility and vitality. Bioorganic sodium is an important element in keeping joints mobile and limber. It has traditionally been used in medicinal cultures to nourish and regenerate an over-taxed nervous system. It is used against tuberculosis in folk medicine and also helps in the enhancement of platelets count during dengue fever. It has almost 35% of daily needs for calcium in one cup. It is extremely high in riboflavin, phosphorous, vitamin B-12, protein and potassium. Fresh goat milk is sometimes substituted for breast milk. This reduces the risk of the child developing electrolyte imbalances, metabolic acidosis, megaloblastic anaemia, and a host of allergic reactions. It has many advantages in terms of percentage of over-sized fat droplets smaller than cow milk because the goat milk fat globule has a greater surface area, and lipases in the gut are sup-posedly able to attack the lipids more rapidly. Almost 20% of the fatty acids of goat milk fall into the short-chain fatty acids category (C4:O to C12:O) compared with l0-20% for cow milk. Lipases attack the ester linkages of the shorter-chain fatty acids more readily, so these differences may contribute to more rapid digestion and absorption of goat milk fat. Some physicochemical properties of goat milk such as smaller fat globules, higher percentage of short and medium chain fatty acids, and softer curd formation of its proteins are

advantageous for higher digestibility and healthier lipid metabolism relative to cow milk. Thus goat milk has been recommended for either infants, old, convalescent people, and goat milk-based diet (animal fat) has a beneficial effect and thus its consumption may be recommended especially in cases of mal-absorption syndromes. Goat milk oligosaccharides (GMOs) play an important role in intestinal protection and repair after damage caused by DSS (Dextron Sodium Sulphate) induced colitis and their implication in human intestinal inflammation. GMOs have anti-inflammatory effects in rats with trinitrobenzenesulfonic (T) acid induced colitis and may be useful in the management of inflammatory bowel disease. Goat milk containing galacto-oligosaccharides could be recommended to decrease most of infant allergy and diseases. In our lab, we have isolated some goat milk oligosaccharides which are Apriose, Caprose, Agriose, Boviose, Hircose, Aprose and Gaurose. In the present study, we have worked on the oligosaccharide contents of goat milk and structure elucidation of four novel milk oligosaccharides.

Physiological and Biological Effects of Milk Oligosaccharides

A number of oligosaccharides isolated from milk of different origin like human, cow, goat, buffalo, sheep, mare, yak, donkey etc. contains high concentration of bioactive oligosaccharides and these bioactive oligosaccharides have shown antitumor, anticancer, antigenic, immunostimulant activities etc. In general milk oligosaccharides can be used as following-

1. Milk Oligosaccharides as Pre-biotic (Kunz et.al. 2000).
2. Milk Oligosaccharides as Immunomodulatries (Mills et.al. 2011, Wang et.al. 2003, Von et.al. 1997).
3. Milk Oligosaccharides in Brain Development.
4. Milk Oligosaccharides in Mineral Absorption (Chonan et.al. 1996, Scholz et.al. 2001).
5. Milk Oligosaccharides as Tumor Markers (Boudry et.al. 2017).
6. Milk Oligosaccharides as Anti-infective (Zopf et.al. 1996).
7. Milk Oligosaccharides as Anti-adhesive agents (Hakkarainen et.al. 2005, Zivkovic et.al. 2011).

Biological Activities of Milk Oligosaccharides Based on Different Constituent Monosaccharides and Linkages

There are number of factors which are responsible for various biological activities of different milk oligosaccharides. Some important factors that affect the biological activities of milk oligosaccharides are summarized below.

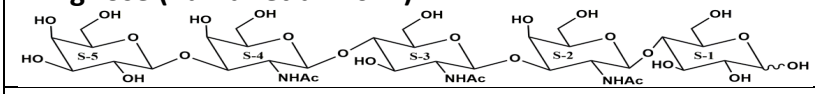
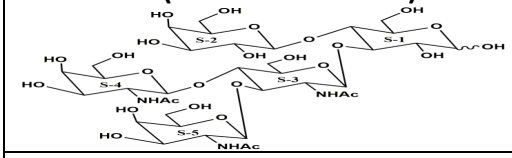
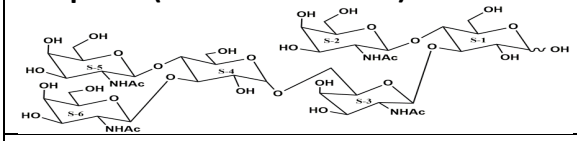
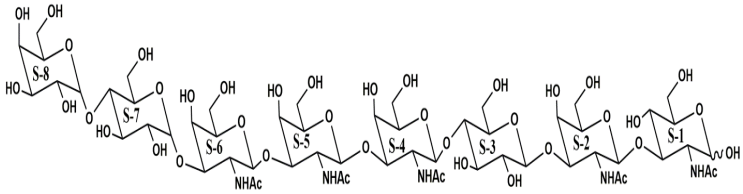
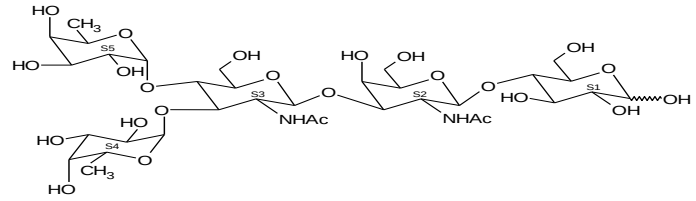
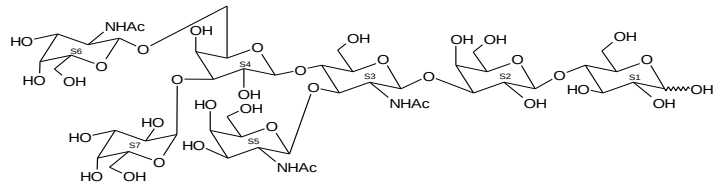
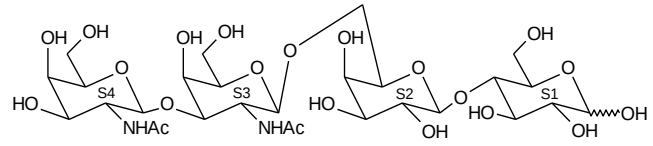
1. Galactose and fucose containing oligosaccharides label tumor cell surfaces and inhibit cell adhesion to fibronectin (Boehm et.al. 2003).
2. Human milk galactose and sialic acid also contribute to the increased concentration of NeuAc, present in cerebral and cerebellar glycoconjugates of breast fed and thus play an important role in the development of the infant brain (Kim et.al. 2005).
3. Galacto-oligosaccharidesin milk improve intestinal micro-flora and fermentation in newborn (Quaggin et.al. 2007).
4. Fucosylated and related glycoconjugates of human milk oligosaccharide can be used for several specific diseases by inhibition of enteric pathogens such as stable toxin of *Escherichia coli* (in vitro and its toxin induced secretory diarrhea in vitro and in vivo), noroviruses and Campylobacter (Starnes et.al. 1988, Svenson et.al. 1992).

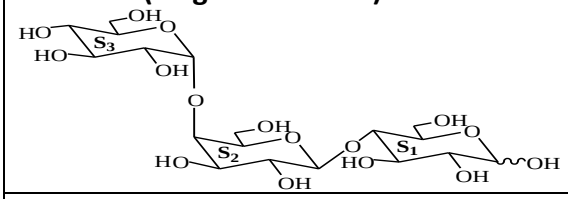
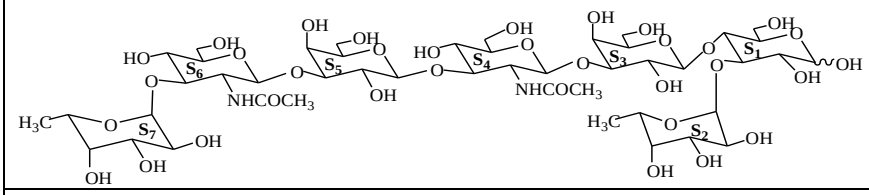
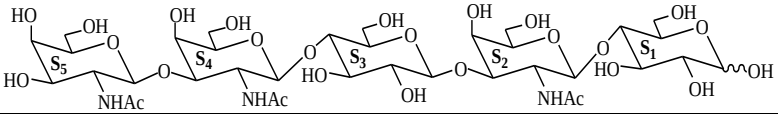
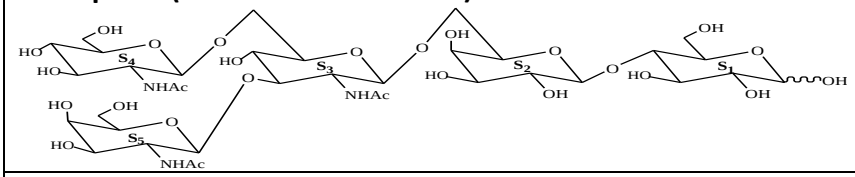
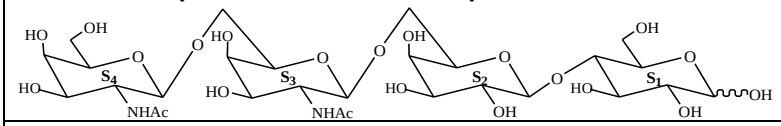
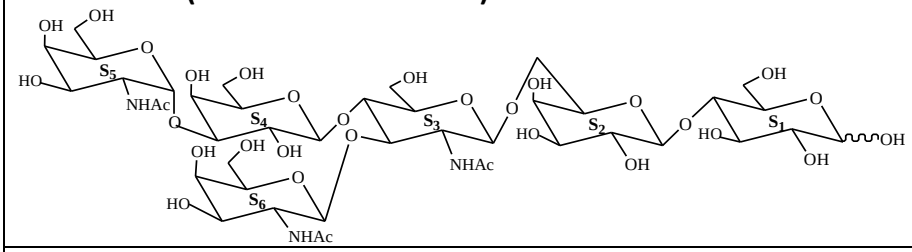
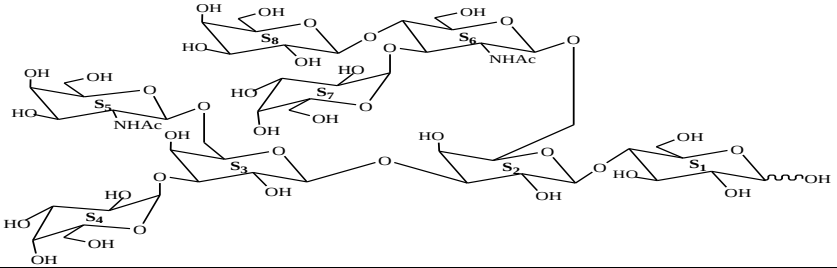
5. A non-digestible milk oligosaccharide due to the presence of β-glycosidic linkage ingredient allow specific changes both in the composition and activity in the gastrointestinal micro-flora, and confer benefits upon host well-being and health as its pre-biotic activity (Ben et.al. 2004, Guillermo et.al. 2003).
6. MOs can directly interact with immune cells to have cell cycle and immunomodulatory effects, that have shown both neutral and acidic oligosaccharides, reduce proliferation in the HIEC, Caco-2 and HT 29 cell lines (Lim et.al. 2007).
7. N-and O-linked oligosaccharide releases the histamine and becomes the mediator of other allergenic response and allergenic symptoms (Johansson et.al. 2005).
8. Some important enteric pathogens, for example- rotavirus, are inhibited by human milk oligosaccharide, α-1,2-linked fucosylated oligosaccharide, in conjugation with other families of oligosaccharide, constitute a powerful innate immune system of human milk (Newburg et.al. 2004).
9. Sialylated human milk oligosaccharide inhibits binding of pathogenic strains of Escherichia; and lewis antigen-containing HMOs could prevent HIV from binding to DC-SIGN in the breastfed infant, as they do in vitro (Lars et.al. 2012).
10. The presence of sialic acid inhuman milk serves as anti-inflammatory components and reduces platelet-neutrophill complex formation leading to a decrease in neutrophill B2 integrin expression (Zhu et.al.2005, Brito et.al. 1995).
11. The α-1,2-linked fucose of human milk oligosaccharides inhibits the toxin-producing Escherichia coli in vitro and its toxin induced secretary diarrhea in vitro and in vivo (Mehra et.al. 2014). The Glycoconjugates present in human milk also inhibit the binding of Campylobacter jejuni in vitro and in vivo and also inhibit binding by calciviruses in vitro (Ruiz-Palacios et.al. 2003). The specific fucosylated oligosaccharides of human milk have been observed to inhibit specific pathogens and conclude the family of α-1,2-linked fucosylated oligosaccharides that constitute a powerful innate system of human milk (Newburg et.al. 2004).
12. The prebiotic is non-digestible oligosaccharide due to the presence of β-glycosidic linkage and found intact at high concentrations in the small intestine and colon, thus meeting the criteria that constitute a prebiotic: resistance to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption. Human milk components, including oligosaccharides, affect the gastrointestinal flora of infants and it has been demonstrated that the prebiotic mixture modulates the intestinal flora and modulates the immune system as human milk does. Oligosaccharides present in human milk changes the microbiota towards a predominant population of grampositive nonsporulating bacilli, Bifidobacteria, and lactic acid-producing organisms (Lactobacilli), so the population of bifid bacterium increased in breast fed infants (Ben et.al. 2004, Lars et.al. 2012).
13. Sialylated milk oligosaccharides can neutralize influenza virus through binding to hemagglutinin (Tao et.al. 2016). Similarly, fucosylated milk oligosaccharides also bind to several strains of norovirus, thereby preventing the docking of the virus to cellular glycan receptors (Eunus et.al. 2016).
14. Rotavirus infection is responsible for the diarrhea in infants and the ability of rotavirus to infect MA-104 cells in culture is inhibited by human milk, and this inhibition is due to mucin-associated 46kDa milk glycoprotein named lactadherin (Hans et.al. 2017).

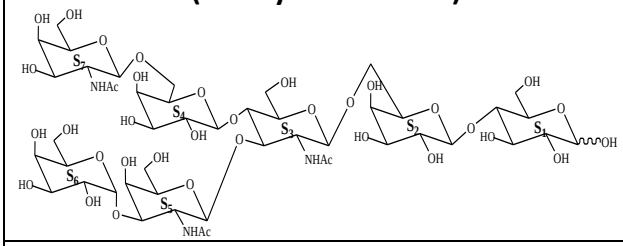
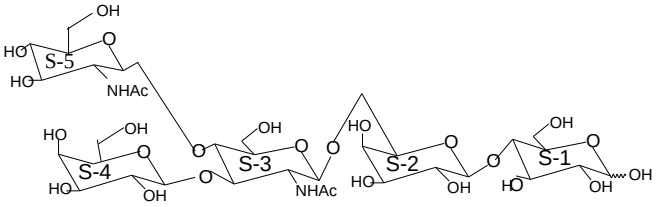
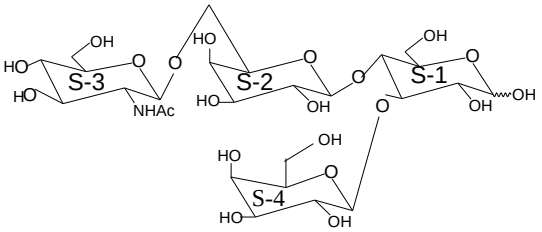
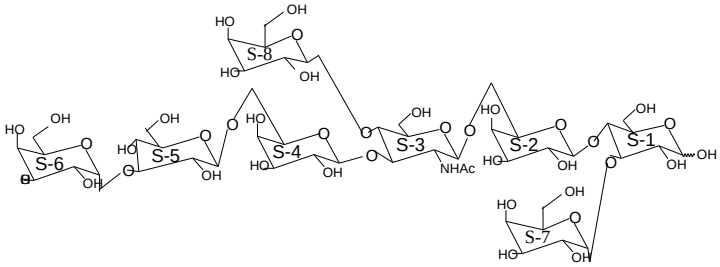
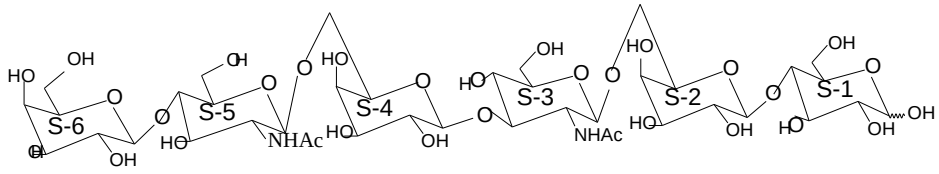
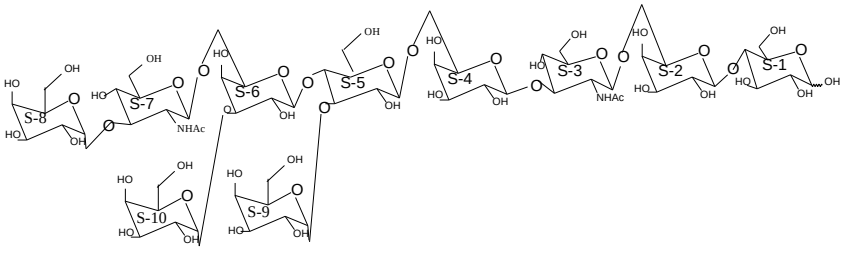
Furthermore after sialic acid is removed from lactadherin and its ability to inhibit rotavirus is essentially lost, which suggests that the glycon portion of the molecule is responsible for inhibition and specific terminal sialic acid is required for inhibition.

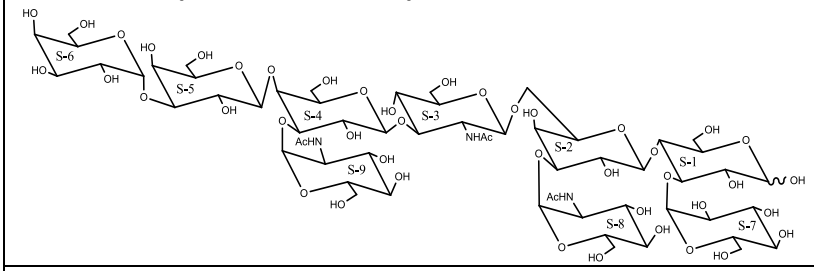
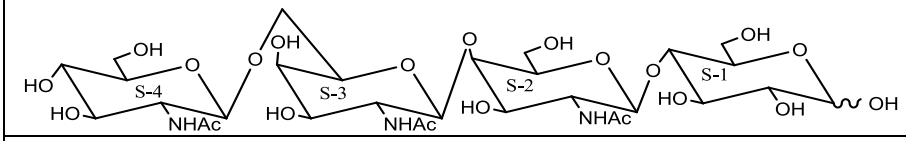
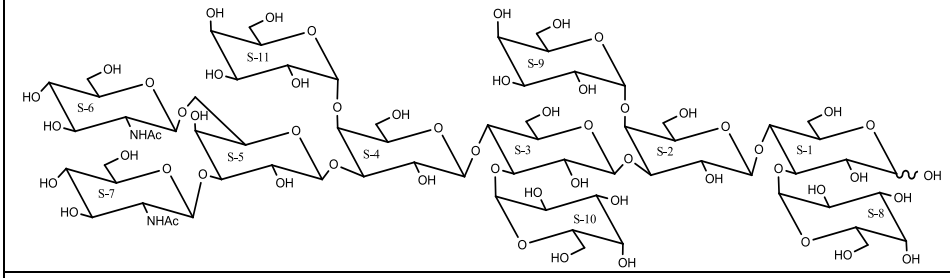
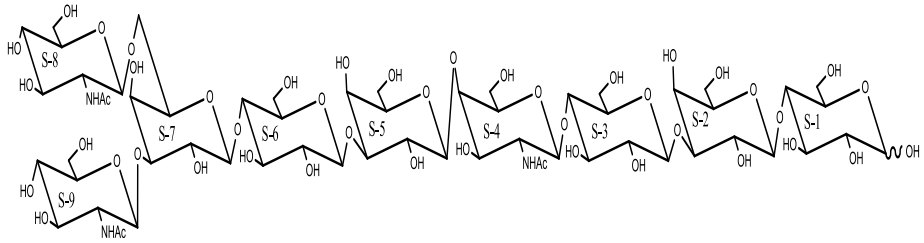
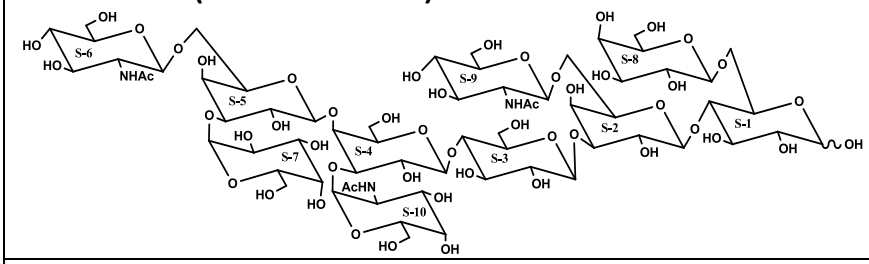
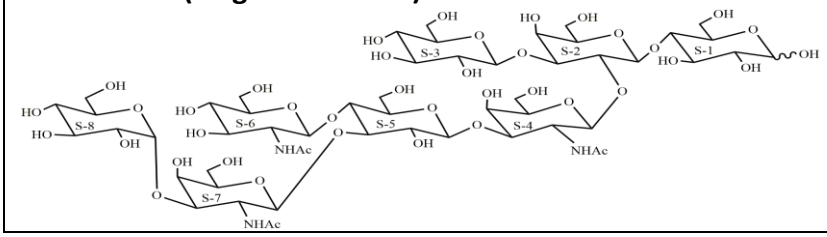
STRUCTURES OF MILK OLIGOSACCHARIDES OF SOME DOMESTIC ANIMALS ISOLATED IN OUR LABORATORY

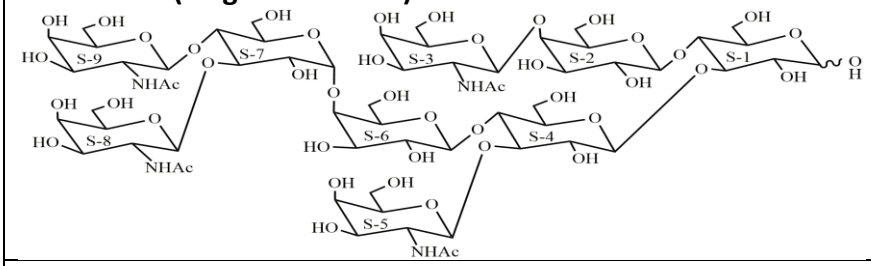
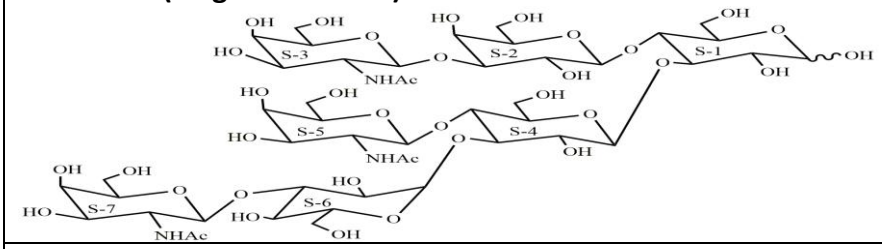
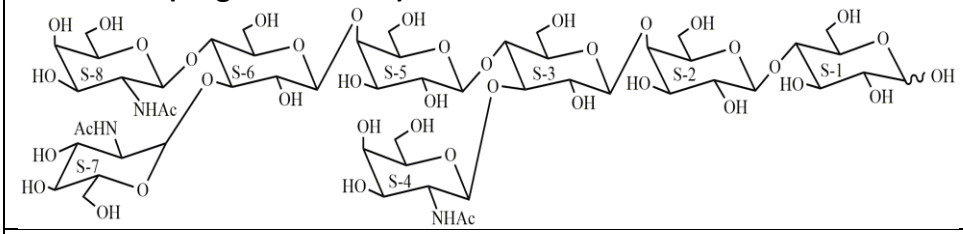
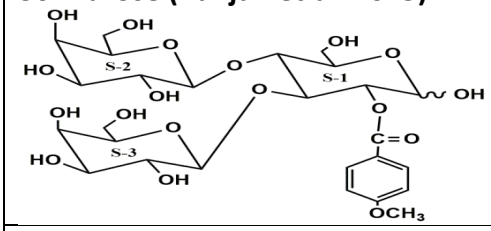
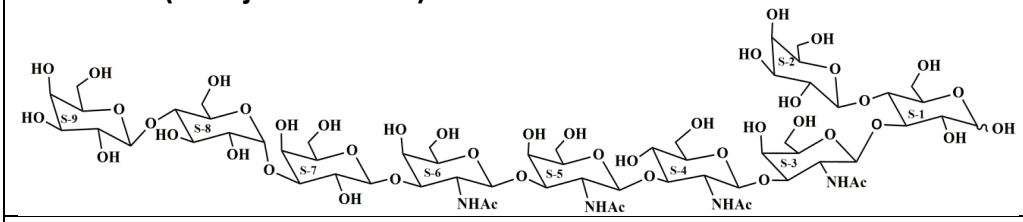
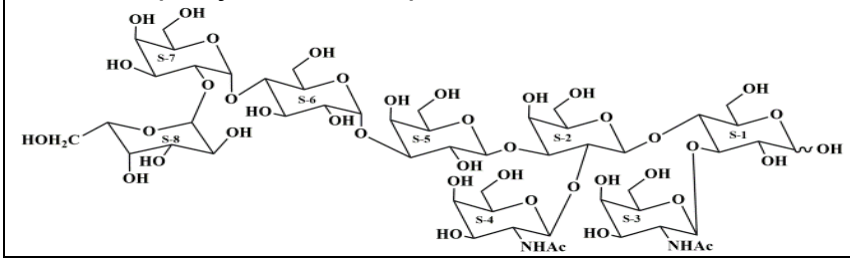
A number of structurally diverse milk oligosaccharides which have been isolated from milk of different origin by our **'Carbohydrate Research Group'**, are given below:

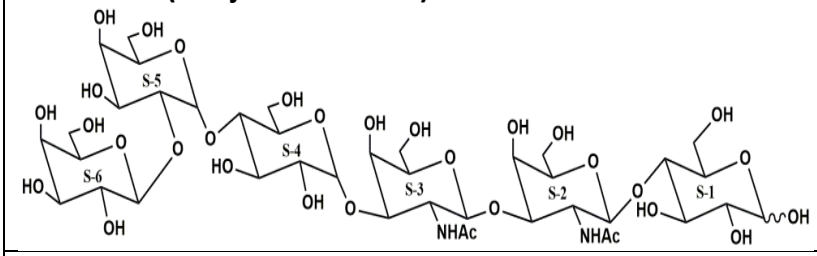
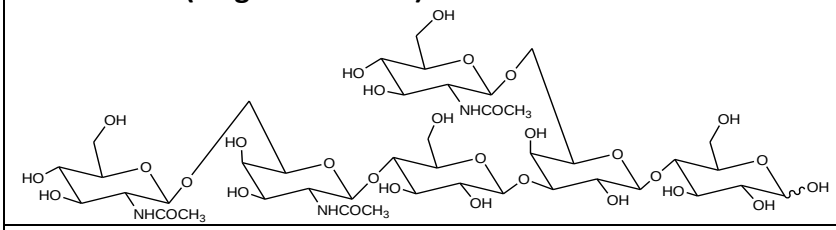
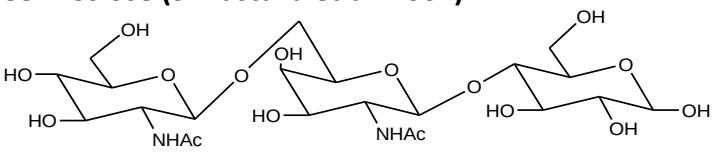
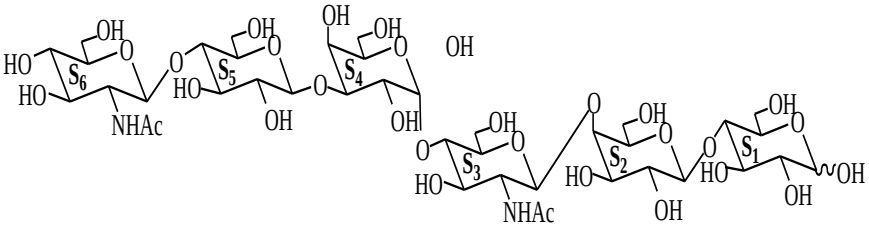
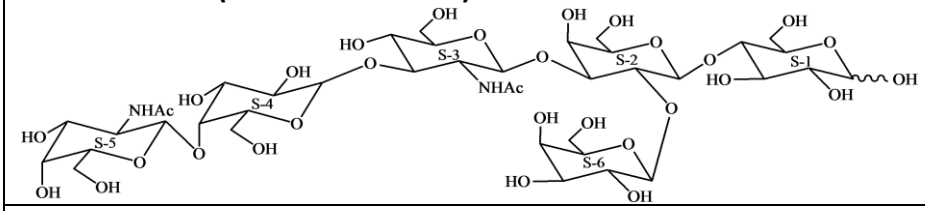
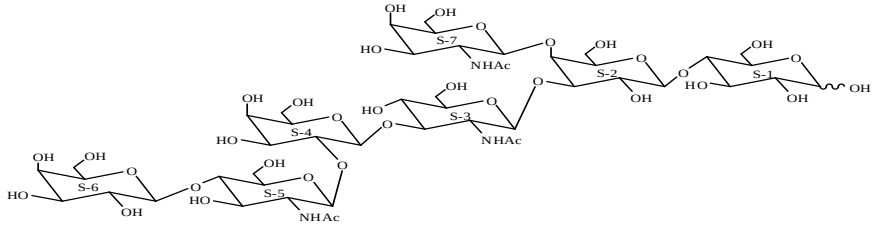
Name of Milk Oligosaccharide and its Structure	**Animal**
1. Agriose (Kumar et.al. 2017) 	Goat Milk
2. Boviose (Kumar et.al. 2017) 	Goat Milk
3. Aprose (Kumar et.al. 2017) 	Goat Milk
4. Hircose (Kumar et.al. 2017) 	Goat Milk
5. Gaurose (Srivastava et.al. 2008) 	Goat Milk
6. Apriose (Srivastava et.al. 2008) 	Goat Milk
7. Caprose (Singh et.al. 2019) 	Goat Milk

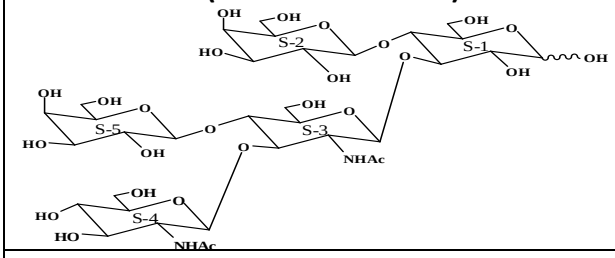
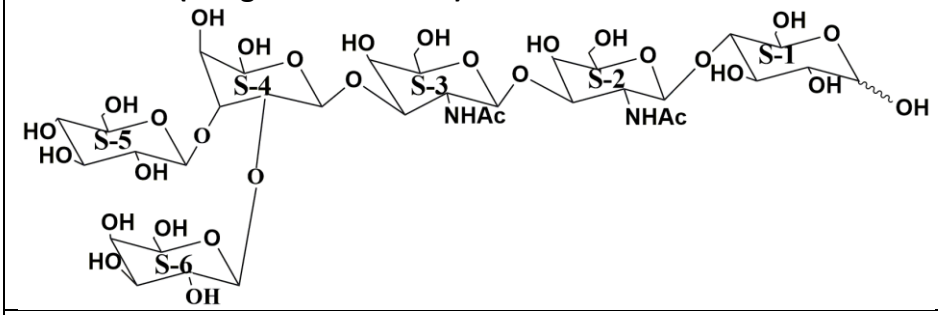
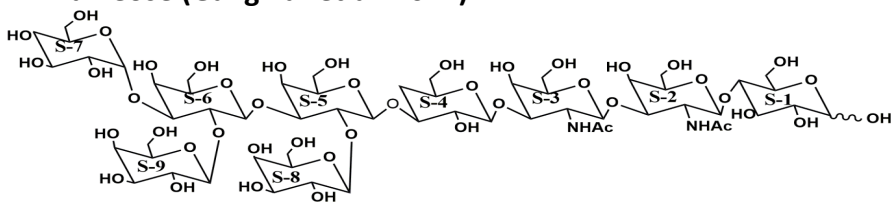
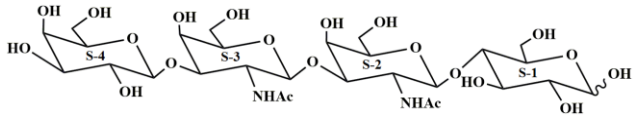
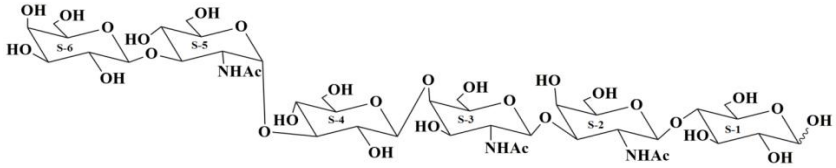
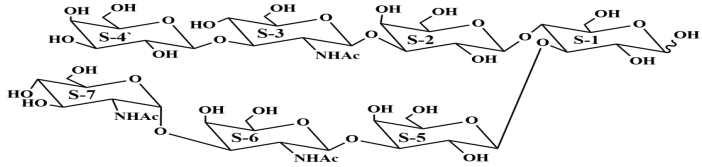
8. Risose (Singh et.al. 2019) 	Donkey Milk
9. Quinose (Gupta et.al. 2007) 	Donkey Milk
10. Ariose (Gupta et.al. 2007) 	Donkey Milk
11. Equose (Srivastava et.al. 2007) 	Mare Milk
12. Marose (Srivastava et.al. 2007) 	Mare Milk
13. Calibose (Srivastava et.al. 2007) 	Mare Milk
14. Harose (Srivastava et.al. 2007) 	Mare Milk

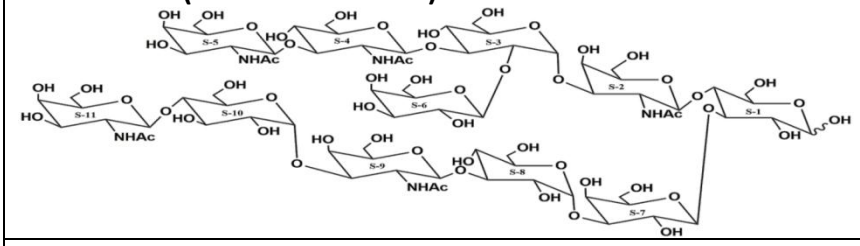
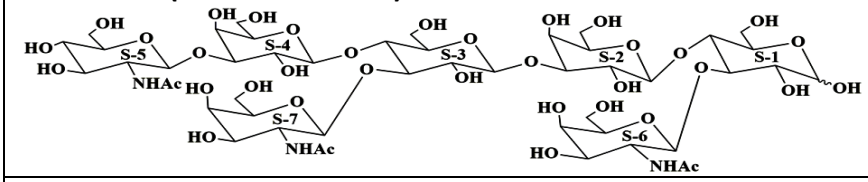
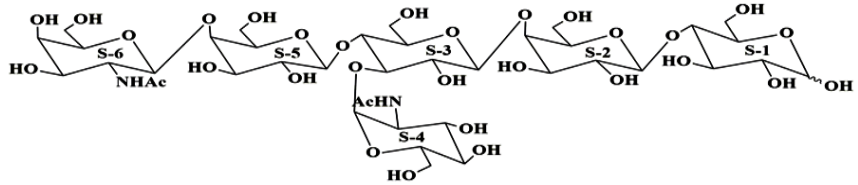
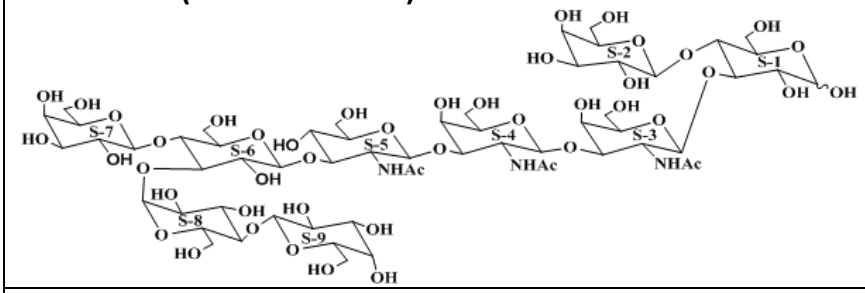
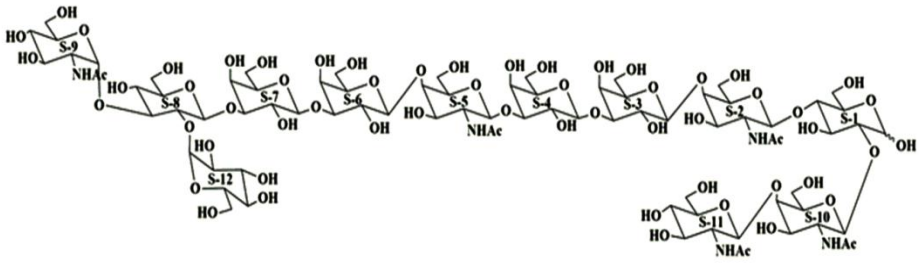
15. Aminose (Maurya et.al. 2017) 	Mare Milk
16. Medalose (Gangwar et.al. 2018) 	Camel Milk
17. Camelose (Singh et.al. 2007) 	Camel Milk
18. Amelose (Singh et.al. 2007) 	Camel Milk
19. Dramelose (Singh et.al. 2007) 	Camel Milk
20. Ramelose (Singh et.al. 2007) 	Camel Milk

21. Areiose (Shahi et.al. 2008) 	Sheep Milk
22. Isose (Shahi et.al. 2008) 	Sheep Milk
23. Riesose (Singh et.al. 2018) 	Sheep Milk
24. Rieose (Shahi et.al. 2008) 	Sheep Milk
25. Oviasose (Shahi et.al. 2008) 	Sheep Milk
26. Nienose (Singh et.al. 2018) 	Yak Milk

27. Runose (Singh et.al. 2018) 	**Yak Milk**
28. Ienose (Singh et.al. 2018) 	**Yak Milk**
29. Uniose (Singh et.al. 2018) 	**Yak Milk**
30. Aurose (Ranjan et.al. 2015) 	**Cow Colostrum**
31. Tarose (Gunajn et.al. 2017) 	**Cow Colostrum**
32. Osose (Gunjan et.al. 2017) 	**Cow Colostrum**

33. Tososе (Gunjan et.al. 2017) 	**Cow Colostrum**
34. Famiose (Singh et.al. 2019) 	**Donkey Milk**
35. Bosiose (Srivastava et.al. 2007) 	**Yak Milk**
36. Gariose (Singh et.al. 2007) 	**Donkey Milk**
37. Eutheriose (Kumar et.al. 2017) 	**Buffalo Milk**
38. Theriose (Kumar et.al. 2017) 	**Buffalo Milk**

39. Orientose (Kumar et.al. 2017) 	Buffalo Milk
40. Mesose (Gangwar et.al. 2017) 	Camel Milk
41. Rameose (Gangwar et.al. 2017) 	Camel Milk
42. Asose (Verma et.al. 2018) 	Black Cow Milk
43. Urose (Verma et.al. 2018) 	Black Cow Milk
44. Ausose (Verma et.al. 2018) 	Black Cow Milk

45. Tausose (Verma et.al. 2018) 	Black Cow Milk
46. Rusose (Khan et.al. 2018) 	Lal Muha Cow milk
47. Usose (Khan et.al. 2018) 	Lal Muha Cow milk
48. Taurose (Khan et.al. 2018) 	Lal Muha Cow milk
49. Uruose (Khan et.al. 2018) 	Lal Muha Cow milk

METHOD AND PROCEDURE FOR ISOLATION OF MILK OLIGOSACCHARIDES

The physiological significance of oligosaccharides in biological systems is now widely appreciated and studies concerning the structure and function of carbohydrates require sufficient quantities of defined oligosaccharides for the assignment of their three dimensional stereoscopic structures for better understanding of their structure activity relationships. Besides the fact that these molecules often found only in low concentrations in nature, the identification and isolation of complex oligosaccharides from the natural sources is further complicated by their micro heterogeneity. The isolation and identification of these oligosaccharides is a difficult task due to low resolution on chromatography but

thanks to modern chromatographic and physicochemical techniques by which we can isolate oligosaccharides in their purest form. Although more than 350 milk oligosaccharides have been isolated and characterized by the various researchers, out of which more than 100 oligosaccharides were isolated in 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. Generally, oligosaccharides from the milk of different origins are extensively fractionated and isolated by most conventional processing techniques which are:

1. KOBATA and GINSBURG (Kobata et.al. 1972)
2. URASHIMA et.al. (Urashima et.al. 1997)
3. SMITH et.al. (Smith et.al. 1978)
4. EGGE et.al. (Egge et.al. 1983)
5. WEIRUSZESKI et.al. (Horton et.al. 2015)
6. Modified Method of KOBATA and GINSBURG (Gangwar et.al. 2018)

Finally the method which has been used in our laboratory is modified method of Kobata and Ginsburg which is described as under:

Isolation of Milk Oligosaccharides by Modified Method of Kobata and Ginsburg (Gangwar et.al. 2018)

According to this method, about 11 litres of milk was collected from one animal and equal amount of alcohol (11 litres) was added into it and was kept in deep freezer. Further, 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 added to the clear filtrate (supernatant) to 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 presence of carbohydrates was checked in lyophilized material by positive phenol-sulphuric acid test and Morgon-Elson testsuggesting the presence of normal sugars and N-acetyl sugars in oligosaccharide mixture.

Purification of Oligosaccharide Mixture by Sephadex G-25 Column Chromatography

The lyophilized material (mixture of oligosaccharide) was further purified by fractionating it on Sephadex G-25 chromatography using glass triple distilled water as eluent 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 of Milk Oligosaccharide

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 at 60^{0}C. Acetylations of milk oligosaccharide mixture make the oligosaccharide less polar in nature, so that they could be isolated conventionally by column chromatography.

Further acetylation studies were also found to be helpful in fixing the position of glycosidic linkages, good resolution of ^{1}H NMR and better insight of the molecule.

CHROMATOGRAPHICTECHNIQUES REQUIRED FOR PURIFICATION OF MILK OLIGOSACCHARIDES

Various purification methods were used during the isolation and purification of milk oligosaccharides which have been discussed as under:

Thin Layer Chromatography (Robyt et.al. 2000, Jain et.al. 2000, Reiffova et.al. 2006)

Thin layer chromatography is an important established method for the analysis of monosaccharide, oligosaccharide and carbohydrate polymer in their acetylated form. The main adsorbent used in TLC for separation of oligosaccharides is silica gel that is fixed to an aluminum or glass plate and the resolution of mixture of compounds depends on the choice of suitable polar and non-polar solvent system. The mixture of acetylated oligosaccharides may be separated through TLC that involves a stationary phase and mobile phase, carrying the samples with it. 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 ^{1}H-NMR to purify and determine the composition of Oligosaccharides from milk. Moreover, it is also useful during the monitoring of various reactions used in structure elucidation.

Paper Chromatography (Wilson et.al. 1987, Kobata et.al. 1969)

Paper chromatography is the simplest and oldest method for separation and qualitative determination of oligosaccharides because of different migration velocities of oligosaccharides through the sorbent layer in a fixed separation time. 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_2O (80:5:100)
6. Upper layer of pyridine-ethyl acetate-rhO (J.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 Morgon-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. It is also helpful in recognizing the oligosaccharide contents obtained after the acid hydrolysis of milk oligosaccharides.

Column Chromatography (Gronberg et.al. 1992)

Column chromatography is the most frequently applied method in the isolation of oligosaccharides, in which a column is packed with particulate material such as silica and then solvent is passed through it at atmospheric pressure. In this technique compounds in column are separated (fractionated) according to differences in their molecular polarity. 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 (Marston et.al. 1991)

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography. All chromatographic separations, including HPLC is based on mechanisms of adsorption, partition and ion exchange, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless-steel column, and a liquid mobile phase. Separation of the components of a solution results from the difference in the relative distribution ratios of the solutes between the two phases. For HPLC purification, a judicious selection of operating parameters is required for achieving the desired purity and yield. The following sequence is followed for better resolution and yield-

1. **Choice of solvent system**-The separation of compounds depends upon the different chemical and physical properties of the solvent. So the firstly the appropriate solvent system was choosed for the separation of different compounds from a mixture. TLC analysis of the sample is used as a first indication of the correct operating conditions. Silica gel plates for normal-phase column and sialylated silica gel plates for reversed-phase columns are commonly used.

2. Optimization of analytical columns of small quantities (Synder et.al. 1988)- In the HPLC system, to save time, sample and solvent required a preliminary analytical search is necessary for the right choice of conditions.

3. Optimization of analytical HPLC separation aiming for small capacity factor-A good analytical HPLC separation is usually a prerequisite for a successful preparative operation. Relative intention (selectively**,** α) is a very important parameter in determining possible sample size and it is necessary to maximize this value.

4. Scaling of preparative HPLC apparatus- Scaling-up a successful analytical separation may cause problems associated with the solubility of the sample because the column is actually overloaded, nonlinear absorption isotherms are obtained and peaks are not symmetrical.

Normal phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase.

Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether. This method is sensitive to chain length, showing limited selectivity among oligosaccharides of similar size but different stereochemistry. Normal phase HPLC using amino bonded silica gel has been the two most common methods to fractionate oligosaccharides utilizing either ultraviolet (UV) or refractive index (RI) for detection.

Reverse phase HPLC (RP-HPLC) (Kennedy et.al. 1985, Daniel et.al. 1981)

Reverse phase chromatography is a powerful analytical tool and involves a hydrophobic, low polarity stationary phase, which is chemically bonded to an inert solid such as silica. The separation is essentially an extraction operation and is useful for separating non-volatile components. It works on the principle of hydrophobic interactions hence the more nonpolar the material is, the longer it will be retained.

STRUCTURE DETERMINATION OF MILK OLIGOSACCHARIDES

The structural determination of oligosaccharide is extremely complex due to the presence of many monosaccharides with multiple and diverse substitution in composition, stereochemistry (configuration) and the pattern of inter residue linkage. The elucidation of such complex structures poses a substantial challenge. Traditionally, chemical degradation or transformations which include acid hydrolysis and methylation or acetylation analysis were used for structure elucidation of milk oligosaccharide. However, analytical methods, such as mass spectrometry (MS) and nuclear magnetic resonance (^{1}H, ^{13}C and 2D i.e. HSQC, HMBC, COSY and TOCSY) spectroscopy are the most appropriate for structure elucidation of milk oligosaccharide which 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 plays 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 there in the oligosaccharide. Kiliani acid hydrolysis (Kiliani 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 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.

Acetylation of Milk Oligosaccharides

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. Peracetylation is generally carried out by Ac_2O/pyridine. The sequence in oligosaccharides and the site of glycosidic linkage can also be deduced by Acetylation studies followed by acid hydrolysis. Further acetylation studies are also found to be helpful in fixing the position of glycosidic linkages, good resolution of ^{1}H NMR and better insight of the molecule.

Methylglycosidation / Acid Hydrolysis

Methylglycosidation of the compound is done by refluxing the compound with MeOH at 70 ^{0}C 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. This is used for confirming the reducing monosaccharide present in the oligosaccharide.

Nuclear Magnetic Resonace (NMR) Spectroscopy in the Analysis of Milk Oligosaccharide (Bush et.al. 1988, Dabrowski et.al. 1989, Jain et.al. 1991, Pabst et.al. 1991)

NMR spectroscopy is a sophisticated and powerful analytical technology that has a variety of applications in scientific research. 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 and intriguing. The assignments of ^{1}H 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 ^{1}H-^{1}H COSY and ^{1}H-^{1}H TOCSYexperiments and heteronuclear ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC. To elucidate the structure of oligosaccharides following information must be required.

1. **Number of Sugar Residues** (Duus et.al. 2000): In structural analysis of an oligosaccharide, the assignment of anomeric proton chemical shift is of utmost important. The anomeric proton resonances are found in the shift range of δ4.2-5.5 ppm in ^{1}H 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 (Bock et.al. 1974):** 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 Dpyranoses 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 unequivocally.
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** (Van et.al. 1996, Vinogradov et.al. 1998): 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 (SRG)** (Fournet et.al. 1978, Vliegenthart et.al. 1983)**:** 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 assignmentof 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.

In the ^{1}HNMR Spectroscopy (Waard et.al. 1992, Agrawal et.al. 1992, Fales et.al. 1972, Dua et.al. 1986), 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 features of the 1 H-NMR structural reporter groups (SRGs) of milk oligosaccharides are summarized below (Kobata et.al. 1972, Waard et.al. 1992, Agrawal et.al. 1992).

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 δ4.688 ($J_{1,2}$ 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 δ5.22 and δ4.66 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 δ5.22 and δ4.66 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 δ4.4 ppm and H-4 of β-linked Gal showed at a typical downfield shift around δ4.13-4.15 ppm due to substitution at the 3-position.
5. The H-4 of (1→6) linked β-Gal appeared at δ3.8-3.9 ppm and H-4 of (1→3) linked β-Gal at δ3.9-4.2 ppm.
6. Signal for H-1 of the unsubstituted Gal residue appears around δ4.44-4.47 ppm.
7. β-linked GlcNAc residues with anomeric signals appear at δ4.6-4.7 ppm and CH_3 signals in the range of δ2.02-2.08 ppm. H-1 of the (1→6) linked GlcNAc appears at lower chemical shift value (δ4.6 ppm) than the (1→3) linked GlcNAc residue (δ4.7ppm). A splitting of the anomeric doublets is due to the anomerization of the reducing terminal.
8. The H-2 of β-GlcNAc appeared at δ3.6-3.8 ppm and H-2 of β-GalNAc appeared at δ3.8-4.2ppm.
9. Presence of anomeic signal with a integration of two proton at δ4.44-4.6 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 δ5.02 ppm, (1→2) linked α-Gal residues showed anomeric signal at δ5.20 ppm and (1→3) linked α-Gal residues showed anomeric signal between δ5.02-5.20 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 δ4.2-4.9 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 δ1.78 and δ2.75 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.

^{1}H-NMR Data of Common Glycopyranoses Found in Milk Oligosaccharides (Agrawal et. al. 1989)

Sugar	H-1	H-2	H-3	H-4	H-5	H-6	$NHCOCH_3$
β-D-Glc	4.64	3.25	3.50	3.42	3.46	3.72,3.90	-
α-D-Glc	5.23	3.54	3.72	3.42	3.84	3.76,3.84	-
β-D-Gal	4.53	3.45	3.59	3.89	3.65	3.64,3.72	-
α-D-Gal	5.22	3.78	3.81	3.95	4.03	3.69,3.69	-
β-L-Fuc	4.55	3.46	3.63	3.74	3.79	1.26	-
α-L-Fuc	5.20	3.77	3.86	3.81	4.20	1.28	-
β-D-GlcNAc	4.72	3.65	3.56	3.46	3.46	3.75,3.91	2.06
α-D-GlcNAc	5.21	3.88	3.75	3.49	3.86	3.77,3.85	2.06
β-D-GalNAc	4.68	3.90	3.77	3.98	3.72	3.82,3.84	2.06
α-D-GalNAc	5.28	4.19	3.95	4.05	4.13	3.79,3.79	2.06

^{13}C-NMR spectroscopy (Bock et.al. 1982, Agrawal et.al. 1989, Gorin et.al. 1981) has been extensively used to assign the conformation and type of anomeric linkages in Oligosaccharides. The ^{13}C-NMR spectroscopy also has enormous potential in oligosaccharides structure determination because its greater chemical shift dispersion and lack of complexities arise from spin-spin coupling and overlapping resonances, also with those arising from solvents. In contrast to the rather crowded and poorly resolved ^{1}H-NMR spectrum, the proton noise decoupled ^{13}C-NMR is usually well resolved and has few overlapping lines and therefore is inherently easy to interpret but difficult to assign chemical shifts to specific carbon due to small differences in chemical shift among ring carbons of each monomeric residue as well as from different monomeric residue. The appearance of anomeric resonances in a well separated chemical shift range of 90-110 ppm help greatly in determining the number of O-linked monosaccharides. The C-1 of reducing end residue appears in the region 90-98 ppm and other non-reducing monosaccharide units appear at 98-110 ppm. The rest of methine and methylene resonances absorb between 51-86 ppm.

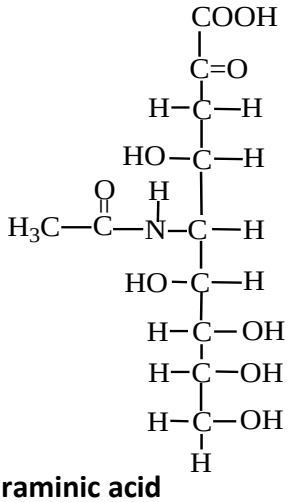
The appearance of methine resonances between 52-57 ppm (Bush et.al. 1985) is generally associated with amino substituted carbon signals at an amino sugar residue. The low field absorption in the region 170-176 ppm reflects the presence of a carboxylic group of hexapyranoic acids or the carbonyl group of acetamido sugars. The presence of an acetaamido sugar may further be complemented by the appearance of methyl resonances in the region 20-24 ppm. The spectral region between 57-64 ppm contains signals for all the unsubstituted hydroxy methylene resonances C-6, whereas methyl resonances of 6-deoxy sugars generally appear in the region 16-19 ppm.

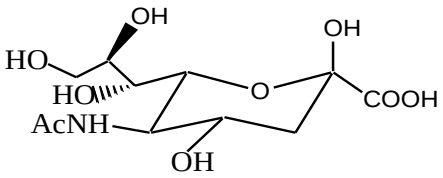
Since naturally occurring monosaccharides are generally hexoses or pentoses therefore, each hexose and pentose unit introduces either six or five resonances, respectively. Accordingly in a well-resolved ^{13}C-NMR spectrum, in most cases the number of monosaccharide residues can be easily ascertained by simply dividing the total number of signal absorbing between 60-85 ppm either by five or four or by combination of both. In a hexose monosaccharide besides the anomeric signal it give rise to five resonances whereas in case of 6-deoxy hexose and pentose it give rise to four resonances in the above mentioned chemical shift range. The coupling pattern for GalNAc and GlcNAc in ^{1}H-NMR is similar to Gal and Glc respectively but in ^{13}C NMR an upfield shift of $\delta_{C2\alpha}$~55.4, $\delta_{C2\beta}$~58ppm for GlcNAc and an upfield shift of $\delta_{C2\alpha}$~51.4, $\delta_{C2\beta}$~54.9ppm for GalNAc has been reported. In the chemical shift analogy method 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. The ^{13}C chemical shift reveals the anomeric configuration in a manner similar to the proton chemical shift but most importantly the one bond ^{13}C-^{1}H coupling constant in pyranoses can be used to determine the anomeric configuration. For D sugars in the $^{4}C_1$ conformation a $J_{C1,H1}$~170 indicates an α-anomeric sugar whereas $J_{C1,H1}$~160 indicates an β-anomeric sugar configuration.

^{13}C-NMR Data of Common Glycopyranoses of Oligosaccharide Found in Milk (Agrawal et. al. 1992)

Sugar	C-1	C-2	C-3	C-4	C-5	C-6	MeCONH
β-D-Glc	96.8	75.2	76.7	70.7	76.7	61.8	-
α-D-Glc	93.0	72.4	73.7	70.7	72.3	61.8	-
β-D-Gal	97.4	72.9	73.8	69.7	75.9	61.8	-
α-D-Gal	93.2	69.3	70.1	70.3	71.3	62.0	-
β-L-Fuc	97.2	72.7	73.9	72.4	71.6	16.3	-
α-L-Fuc	93.1	69.1	70.3	72.8	67.1	16.3	-
β-D-GlcNAc	95.9	57.9	74.8	71.1	76.8	61.9	23.1,175.5
α-D-GlcNAc	91.8	55.0	71.7	71.3	72.5	61.8	22.9,175.1
β-D-GalNAc	96.3	54.8	72.0	68.9	76.0	61.9	23.1,175.8
α-D-GalNAc	92.0	51.2	68.4	69.6	71.4	62.1	22.9,175.4

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 (Vliegenthart et.al. 1982). The other characteristic signals of sialic acid are as below-





N-acetyl Neuraminic acid

(5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulosonic acid)

^{13}C and ^{1}H-NMR values of Sialic acid residue found in Milk (Agrawal et.al. 1992)

Sialic acid residue	**^{1}H Chemical Shift**	**^{13}C Chemical Shift**
α- Neuraminic- 5Ac 1	-	174.0-174.6
2	-	100.2-101.0
3ax	1.693-1.801	40.5-41.0
3eq	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

Two Dimensional NMR Spectroscopy

2D-NMR spectroscopy provides actual, high quality and well interpretable data of the sugar molecule. Chemical shift correlation maps obtained by 2D-NMR experiments are found to be extremely useful in the identification of components of oligosaccharides, without relying on the analogy with any reference data. There are two fundamental types of 2D-NMR spectroscopy. The first is correlated spectroscopy, in which both frequency axis contain chemical shift information and the other is J resolved spectroscopy in which one frequency axis contains spin coupling (J) and other chemical shift (δ) information. Correlated 2D-NMR spectroscopy may be divided into two groups based on the mechanism of interaction producing the observed signals. The first group includes COSY and TOCSY, HETCOR and HSQC based on scalar coupling through coherent transfer of transverse magnetization, and reveals through bond connectivities. The second group, based on dipole couplings through incoherent transfer of magnetization and provide information through space connectivities, the NOESY experiment is representative of this group.

The salient features of two-dimensional NMR spectroscopy applied to oligosaccharides are described as below-

Correlated Spectroscopy (COSY) (Burum et.al. 1980, Morris et.al. 1986, Kessler et.al. 1988, Derome et.al. 1989)

A Homonuclear through-bond correlation, 1H-1H COSY is used to establish the direct neighboring connectivity of protons. The anomeric protons are often used as starting point in the assignment of the ring protons due to the fact that they have well separated chemical shifts and are generally only coupled to one proton. The connectivity between protons within a sugar residue can form this be mapped out via a series of cross-peaks. There can be difficulties in assigning all protons, due to overlapping signals or lack of cross-peaks due to small 3J coupling constants. 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). Therefore, COSY spectra contain information on spin coupling networks within the constituent residues of the oligosaccharide through the observation of cross peaks (Homans et.al. 1986, Gronberg et.al. 1992, Bodenhausen et.al. 1971).

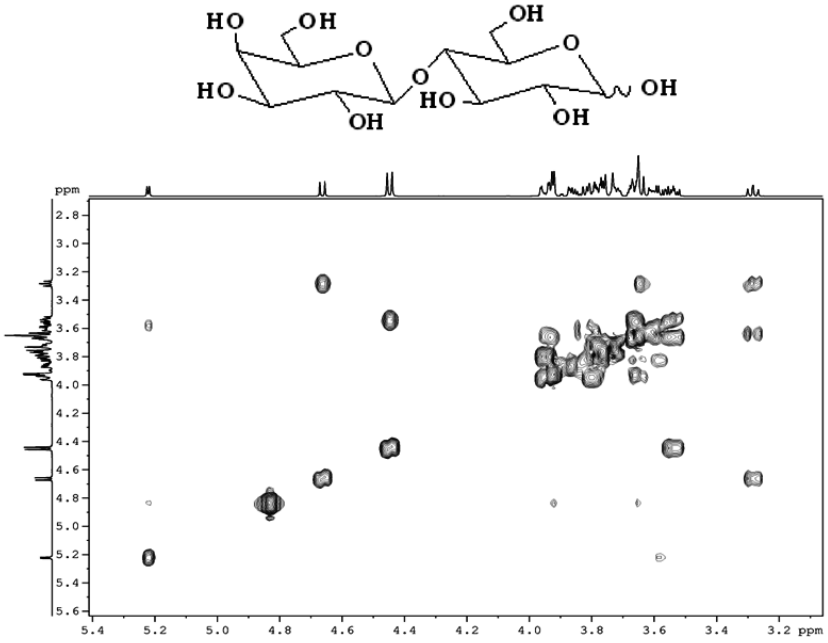


Figure: COSY Spectrum of Lactose.

Assignment of this spectrum by coupling-correlation requires an initial point for the identification of the individual spin systems 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 and H-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 galactosyl residue and coupling between H-1 and H-2 in mannopyranosyl and H-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 galactosyl residue and coupling between H-1 and H-2 in mannopyranosyl residue prevents detection of cross peaks.

Thus COSY experiments and its RELAY extensions give coupling patterns 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.

Total Correlation Spectroscopy (TOCSY) (Bax et.al. 1985, Braunschweiler et.al. 1983, Dunkel et.al. 1992)

A Homonuclear through-bond correlation, 1H-1H TOCSY is related to COSY in the way that cross-peaks of coupled protons are detected. The additional information from the TOCSY spectrum is that there are cross-peaks between (almost) all spins in the spin system. The magnetization is transferred, during the mixing time, to the vicinal coupling partner, and can be further transferred throughout the entire spin system. The magnetization transfer can be interrupted by small 3J coupling constants.

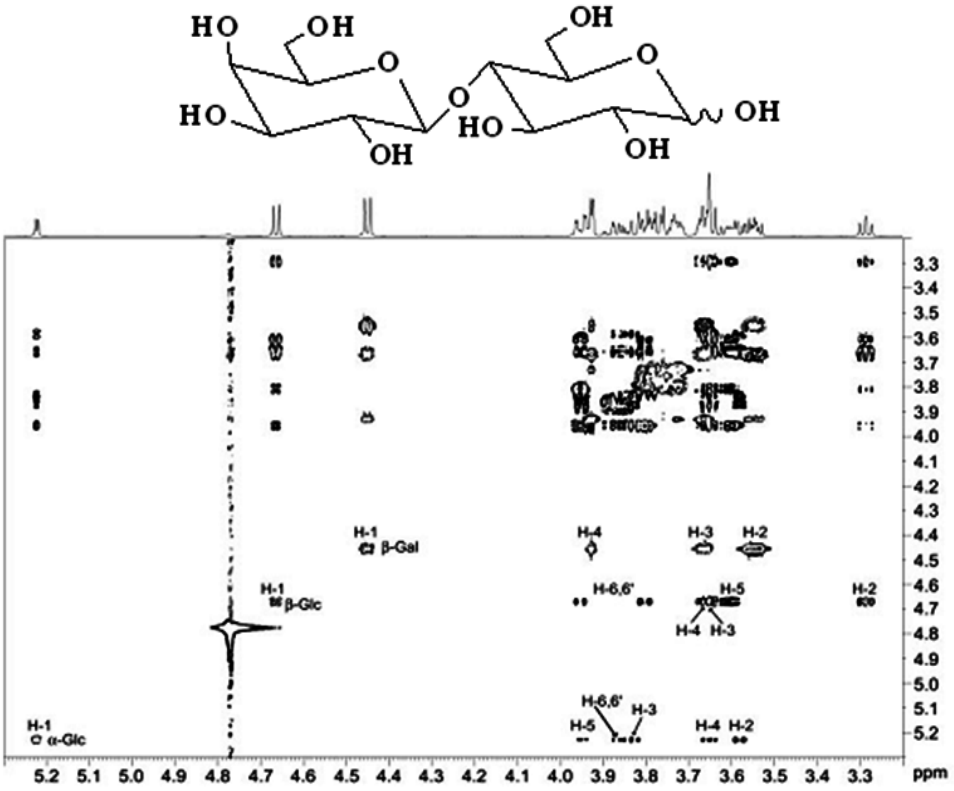


Figure: TOCSY Spectrum of Lactose

This property is advantageous when deducing the configuration to be manno, galacto or gluco. The number of transfer steps can be adjusted by changing the mixing time, a mixing time of 20 ms (one step transfer) will give essentially the same information as the COSY experiment, whereas a mixing time of 80-120 ms will give a five to six steps transfer. Thus TOCSY can give total correlation of all protons in a chain with each other and serve for the identification of single residue in oligosaccharides. H. Kogelberg et.al. used the TOCSY technique in structural elucidation of octasaccharide isolated from human milk. Thus TOCSY spectrum of acetylated sugar gives complete information regarding the position of glycosidic linkage in a particular monosaccharide unit.

However the sequence of cross peaks in TOCSY spectrum could be confirmed by COSY spectrum of same sample. It also provides the information for Carbon and protons involved in the glycosidic linkages. The anomeric proton/ carbon cross peaks lies in the range of δ4.2-5.5 ppm and δ90-112 ppm respectively. However, Cross peak of proton and carbon for the position involve in the glycosidic linkage lies between δ70-80 ppm for carbon and δ3.5-4.2ppm for proton.

Nuclear Overhauser Effect Spectroscopy (NOESY) (Jeener et.al. 1979, Kumar et.al. 1980)
The principal use of NOSEY in oligosaccharide structure determination has been in the assessment of molecular conformation (i.e. 3D structure). Cross peaks are observed in 2D-NOESY spectra between proton pairs that are close in space (typically less than 5Å). The intranuclear distance between H-1 and H-5 (and H-3) of β-D-galactopyranosyl residue is about 2.5 Å, and thus a strong cross peak is observed between these protons in NOESY spectrum. In the α configuration, only a nuclear overhauser effect between H-1 and H-2 is observed. In general, 1,3 diaxial and 1,2-eq-ax proton pairs in pyranosyl rings will produce intraresidue NOESY crosspeaks. However NOESY cross peaks may be observed between linkage sites and O-glycoside conformation in oligosaccharides. Vicinal proton coupling, which is very useful in proton assignments for individual pyranoside rings, is less valuable for correlating monosaccharides residue to each other. Since the protons across the glycosidic linkage are four bonds apart they do not show scalar coupling and thus no correlation between individual spin systems could be observed by COSY or HOHAHA. The presence of an intraresidue NOE from the anomeric proton of a particular sugar residue to protons of other sugar residue in case of oligosaccharides, defines the glycosidic linkage between the two residues. This effect depends on the local conformation about the glycosidic linkage. NOE depends not only on the proximities of the protons but also on the correlation time of the molecule.
Since NOE also depends on the distances between protons, it is possible to determine inter proton distances directly from NOE data. In practice semi selective excitation of one carbohydrate proton, combined with multistep-relayed coherence transfer and the terminal NOE transefer has been used for the sequential analysis of oligosaccharides. Assignment of the anomeric and some other protons resonance may be made with the help of data from decoupling and NOE experiments. In the structural determination of Lacto-N-hexose, selective decoupling irradiation at each of three doublets assigned to the β-galactose H-1 at 4.424, 4.453 and 4.504 ppm identified the resonance of the corresponding H-2 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 H-4 of a β-galactose which is glycosylated at C-3 identifies the H-3 resonance of β-galactose at 3.695 ppm. Decoupling at the Gal H-2 resonance at 3.546 ppm identifies the same Gal H-3 resonance and thus completing the assignment of H-1, H-2, H-3, and H-4 of the branching galactosyl residue. Thus an effective way of connecting two monosaccharides residue is by monitoring the nuclear overhauser effect for an anomeric reporter group to the hydrogen of the substituted position in the adjacent ring.
Heteronuclear Single Quantum Coherence (HSQC) (Key et.al. 1992)
Heteronuclear through-bond correlations, ^{1}H-^{13}C HSQC provides proton carbon coupling across a single bond and correlates the protons with the directly bonded carbons, through one bond couplings.

The cross-peaks contain information about the chemical shifts of the corresponding protons and carbons. The number of monosaccharides can more easily be determined in an HSQC spectrum than in a ^{1}H spectrum due to the added dispersion of chemical shifts also in the carbon dimension. The HSQC spectrum gives information about cross peak of anomeric and ring proton to their directly attached carbon atom with linkages between monosaccharides in oligosaccharide moiety.

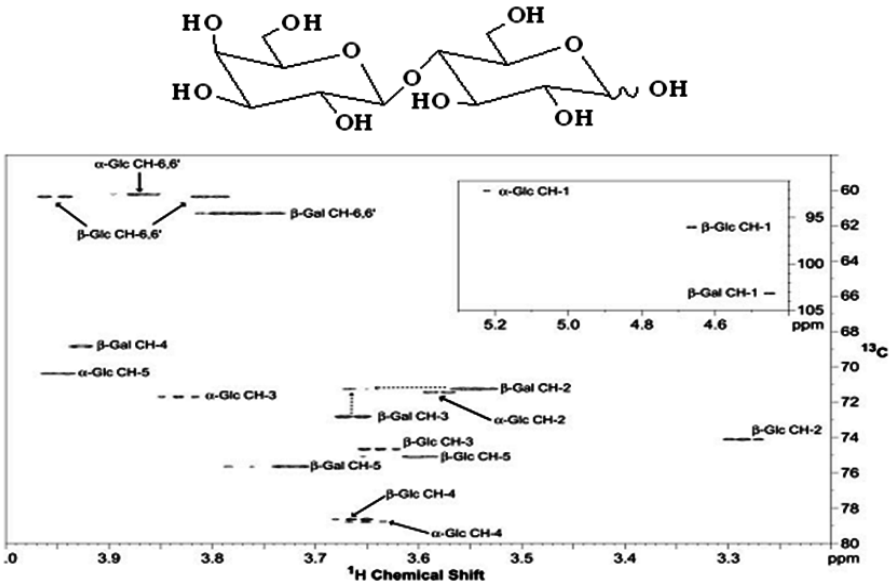


Figure: HSQC Spectrum of Lactose

Heteronuclear Multiple Bond Correlation (HMBC)

^{1}H-^{13}C HMBC shows cross-peaks between protons and carbons that are two or three bonds away. With this experiment it is possible to study quaternary carbons that were not visible in the HSQC experiment. The HMBC experiment is used to establish the linkage between monosaccharide units via the glycosidic bond. The protons at acetyl-protected position now show a three bond ^{13}C-^{1}H coupling and can be easily detected by HMBC experiment and thus position of glycosidic linkage was confirmed. The sensitivity of heteronuclear multiple bond correlation (HMBC) is increased by the use of ^{13}C labeled acetic anhydride, and the assignments can be readily identified. Thus with the help of HMBC experiment, the correlation of proton with adjacent carbon can be achieved and this information is very useful in structural elucidation of oligosaccharides.

Mass Spectrometry (MS) (Barr et.al. 1991)

Mass spectrometry is an important tool for the structural analysis of carbohydrates comprising composition, sequence, branching, and linkage analysis and offers precise results, analytical versatility and very high sensitivity. Recent advances in MS techniques enable us to obtain meaningful information on the molecular details of oligosaccharides. The inherent attributes of MS in terms of sensitivity, specificity and robustness are ideal for profiling and quantifying oligosaccharides in milk.

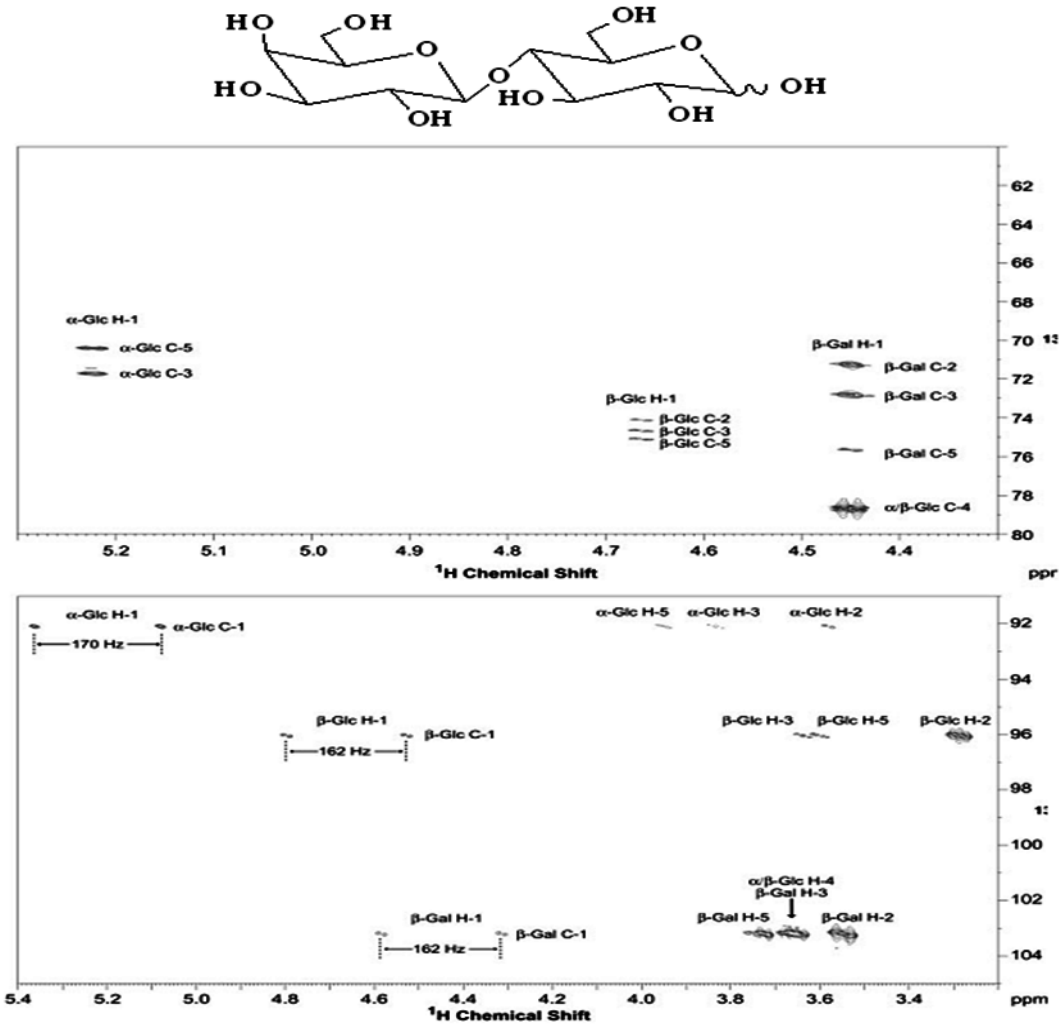


Figure: HMBC Spectrum of Lactose

It can give accurate mass of molecular or fragment ions, which provide peripheral structural information of the oligosaccharide molecule. The characterization and analysis of glycoconjugates and oligosaccharides by mass spectrometry has undergone a number of improvements in the last ten years, especially with the development of methods capable of ionizing and analyzing these compounds in their native states. Most of the oligosaccharides are composed of five unique monosaccharide units with different incremental masses and knowledge of the molecular weight can be used to determine the potential composition of the oligosaccharide. In general, mass spectrometry provides the possibility of structural elucidation based on characteristic fragmentations of the molecules under investigation. 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 and Gal, 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:

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

There are basic components common to all MS methods the sample inlet, ionization source, a mass analyzer (with associated data system) and an ion detector. Both the mass analyzer and the ion detector are maintained under high vacuum. The sample can be introduced into the mass spectrometer as a gas, a solid or a liquid. Gaseous analytes are introduced directly into the ion source and are ionized by electron impact or chemical ionization. Solids are placed on a sample probe and irradiated by laser in matrix-assisted laser desorption/ ionization. Liquids are introduced directly into the ionization source as in electrospray ionization (ESI). ESI is typically used to couple liquid chromatography to mass spectrometry. Sample introduction into the instrument is performed while maintaining the vacuum inside the system, typically below 10^{-6} torr. The sample molecules are converted to ions in the ionization source before being extracted electrostatically into the mass analyzer and separated according to their mass-to-charge ratio (*m/z*). Ions are then detected as electrical signals and transmitted to a data system. 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, such fragmentation goes on until the monosaccharide is left.

Fast Atom Bombardment Mass Spectrometry (FAB-MS) (Barber et.al. 1982, Antonio et.al. 2006, Daniele et.al. 1997, Bruno et.al. 1988):

The development of fast particle desorption culminate with the development of FAB by Michael Barber in the early 1980's. The techniques of FAB and LSIMS are very similar in concept and design as they both involve the bombardment of a solid spot of the analyte/matrix mixture on the end of a sample probe by a fast particle beam. Fast atom bombardment (FAB) ionisation has been shown to be a very useful method in the mass spectrometric analysis of a wide range of natural compounds, such as glycosides, saccharides, antibiotics and other highly polar compounds. However, although FAB itself can provide relative molecular mass information of glycosides and saccharides, the low abundance of $[M+H]^+$ ions and chemical noise from the background of the FAB matrix greatly limit the molecular mass information. 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. In FAB-MSan abundant molecular ion or its protonated species $(M+H)^+$ or a cationic species $(M+Na)^+$, $(M+K)^+$ is obtained. It play decisive role in the structure elucidation of milk oligosaccharides.

Recently it has been seen that the FAB-MS not only fixed the molecular weight of the oligosaccharide but also ascertain the sequence of the monosaccharide units. The molecular ion (M^+) fragments into the fragment units which were formed by the decomposition pathway in which repeated H transfer in the oligosaccharide is accompanied by the elimination of terminal sugars less water, such fragmentation goes on until the monosaccharide is left **(**figure).

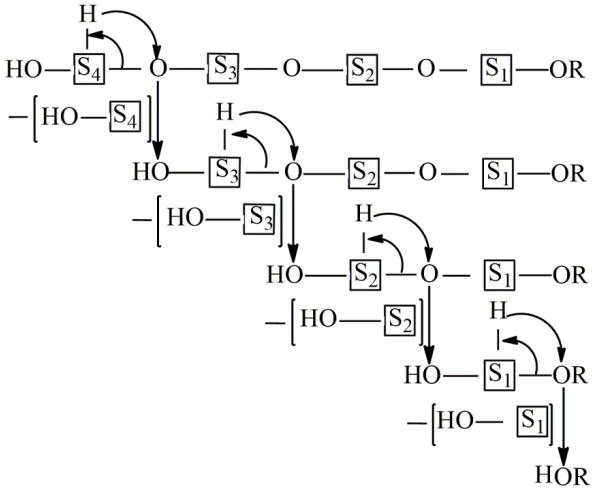


Figure: H-Transfer in oligosaccharide and elimination of monosaccharide from non-reducing end

Negative ion fast-atom bombardment mass spectrometry has been important tool in the structure elucidation of milk oligosaccharides and the result have also been found to be comparable with the proposed structure, based on the results obtained from high resolution NMR spectroscopy. By FAB-MSwe can identify the presence of acetamido monosaccharides, fucosylated and sialyalated branching. Besides the routine losses of H_2O, OH, CH_2OH etc. was also observed.

Electron Spray Ionization- Mass Spectrometry (ESI-MS) (Bathori et.al. 2000, Taki et.al. 1994, Kriz et.al. 1994, David et.al. 1999)

In recent years Electrospray Ionization Mass Spectrometry (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 oligosaccharidesis 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.

CONCLUSION

Goat milk has varied biological activities which are due to their oligosaccharide contents in general and presence of fucose and sialic acid present therein, in specific structures.

ACKNOWLEDGEMENTS

Authors are thankful to CSIR-New Delhi and UP State Higher Education Research and Development Department for financial support.

REFERENCES

Singh, P., Maurya, R.K., Rizvi A.H. and Deepak D. (2018). Isolation and 2D-NMR Studies of Aliose- A Novel Hexasaccharide from Donkey's Milk, *Journal of Biological and Chemical Research*; 35(2): 378-385.

Lager, I., Looger, L.L., Hilpert, M., Lalonde, S. and Frommer, W.B. (2006). Conversion of a Putative Agrobacterium Sugar-binding Protein into a FRET Sensor with high Selectivity for Sucrose, *J. Biol. Chem.*; 281(41): 30875-83.

Tozzi, M.G., Camici, M., Mascia, L., Sgarrella, F. and Ipata P.L. (2006). Pentose phosphates in Nucleoside Interconversion and Catabolism, *FEBS J.*; 273(6): 1089-101.

Burke, L.M. and Hawley, J.A. (2006). Fat and Carbohydrate for Exercise, *Curr Opin Clin Nutr Metab Care.*; 9(4): 476-81.

Sears, P. and Wong, C.H. (1996). Intervention of carbohydrate recognition by proteins and nucleic acids, *Proc. Natl. Acad. Sci*. USA; 93(22): 12086-12093.

Heide, K., Vladimir, E.P., Yibing Z., Alexander, M.L. and Wengang, C. (2004). Determination by electrospray mass spectrometry and 1H-NMR spectroscopy of primary structures of variously fucosylated neutral oligosaccharides based on the iso-lacto-N-octaose core, *Eur. J. Biochem.*; 271(6): 1172-1186.

Hanessian, S. and Lou, B. (2000). Stereocontrolled Glycosyl Transfer Reaction with Unprotected Glycosyl Donors, *Chem. Rev*.; 100(12): 4443-4463.

Koeller, K.M. and Wong, C.H. (2000). Synthesis of Complex Carbohydrates and Glycoconjugates: Enzyme Based and Programmable One-Pot Strategies, *Chem. Rev.*; 100(12): 4465-94.

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct, *Glycobiology*; 3(2): 97-130.

Alper, J. (2001). Searching for Medicine's Sweet Spot, *Science*; 291(5512): 2338-2343.

Kim, K.S. (2010). Acute Bacterial Meningitis in Infants and Children, *Lancet Infect Dis.*; 10(1): 32-42.

Polonovski, M. and Kespagonal, A. (1933). Nouvelles Acquisition sur les composes glucidiques du lai de femme, Bull, *Soc. Chem. Biol*.; 15: 320-49.

Urashima, T., Nagata, H., Nakamura, T., Arai, I., Saito, T., Imazu, K., Hayashi, T., Derocher, A.E. and Wiig, O. (2003). Differences in oligosaccharide pattern of a sample of polar bear colostrum and mild-lactation milk, *Comp Biochem Physiol B Biochem Mol Biol*.; 136(4): 887-96.

Wang, W., Lundgren, T., Lindh, F., Nilsson, B., Gronberg, G., Brown, J.P., Mentzer-Dibert, H. and Zopf, D. (1992). Isolation of two novel Sialyl-Lewis X-active oligosaccharides by high-performance liquid affinity chromatography using monoclonal antibody Onc-M26, *Arch. Biochem. Biophys*; 292: 433-441.

Schwonzen, M., Schmits, R., Baldus, S.E., Vierbuchen, M., Hanish, F.G., Pfreundschuh, M., Diehl, V., Bara, J. and Uhlenbruck, G. (1992). Monoclonal antibody FW6 generated against a mucin-carbohydrate of human amniotic fluid recognizes a colonic tumour-associated epitope, *Br. J. Cancer*.; 65(4): 559-565.

Abe, K., Mckibbin, J.M.J. and Hakomori, S. (1993). The monoclonal antibody directed to difucosylated type 2 chain (Fuc alpha 1 leads to 2Gal beta 1 leads to 4[Fuc alpha 1 leads to 3] GlcNAc; Y Determinant), *Eur. J. Biochem. Biol. Chem.*; 258: 11793-11797.

Srivastava, R. and Kulshretha, D.K. (1989). Bioactive polysaccharides from plants, *Phytochemistry*; 28: 2877-2883.

Oddy, W.H. (2002). The Impact of Breast Milk on Infant and Child Health, *Breastfeed Rev.* 10(3): 5-18.

Coppa, G.V., Zampim, L., Galeazzi, T. and Gabrielli, O. (2006). Prebiotics in human milk: a review, *Dig. Liver.* Dis.; 38(2): 291-294.

Zivkovic, A.M., German, J.B., Lebrilla, C.B. and Mills, D.A. (2011). Human milk glycobiome and its impact on the infant gastrointestinal microbiota, *Proc. Natl. Acad. Sci. USA*.; 108(1): 4653-4658.

Boehm, G. and Stahl, B. (2003). Oligosaccharides, Functional Dairy Product, *Woodhead Publ. Ltd., Cambridge, England.*; 203-243.

Nakamura, T., Kawase, H., Kimura, K., Watanabe, Y., Ohtani, M., Arai, I. and Urashima, T. (2003). Concentrations of sialyloligosaccharides in bovine colostrum and milk during the prepartum and early lactation. *J. Dairy Sci*. 86: 1315-1320.

Weis, W., Brown, J.H., Cusack, S., Paulson, J.C., Skehel J.J. and Wiley, D.C. (1988). Structure of the Influenza Virus Haemagglutinin complexed with its receptor, sialic acid, *Nature Publishing Group*.; 333(6172): 426-431.

Jiang, X., Huang, P., Zhong, W., Tan, M., Farkas, T. and Morrow, A.L. (2004). Human milk contains elements that block binding of noro-viruses to human histo-blood group antigens in saliva, *J. Infect Dis*. 190(10): 1850-9.

Andersson, B., Porras, O., Hanson, L.A., Lagergard, T. and Svanborg, E.C. (1986). Inhibition of attachment of Streptococcus pneumoniae and *Haemophilus influenza* by human milk and receptor oligosaccharides, *J. Infect Dis*.; 153: 232-237.

Simon, P.M., Goode, P.L., Mobasseri, A. and Zopf, D. (1997). Inhibition of Helicobacter pyloribinding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides, *Infect Immun.,* 65: 750-757.

Otnaess, A.B. and Laegreid, A. (1983). Inhibition of enterotoxin from *Escherichia coli* and *Vibrio cholera* by gangliosides from human milk, *Infect Immun*. 40: 563-9.

Ulrik, K.S. and Daniela, B. (2012). Natural Variability in Bovine Milk Oligosaccharides from Danish Jersey and Holstein-Friesian Breeds, *J. Agric. Food Chem.*; 60(24): 6188-6196.

Joy, M.C. and Bryan, W.W. (1997). Selected Indigestible Oligosaccharides Affect Large Bowel Mass, Cecal and Fecal Short-Chain Fatty Acids, pH and Microflora in Rats, *J. Nutr*., 127(1): 130-136.

Singh, P., Srivastava, A.K. and Deepak, D. (2017). Isolation and structure elucidation of Caprose (novel oligosaccharide) from Goat milk. *J. Biol. Chem. Research.*; 34(1):14-20.

Villoslada, F.L., Debras, E., Nieto, A., Concha, A., Galvez, J. Huertas, E.L., Boza, J. Obled, C. and Xaus, J. (2006). Oligosaccharides isolated from goat milk reduce intestinal inflammation in a rat model of dextran sodium sulfate-induced colitis, *Clin. Nutr*. 25(3): 477-488.

Osthoff, G., Wit, M.D., Hugo, A. and Kamara, B.I. (2007). Milk composition of three free-ranging African elephant (*Loxodonta africana africana*) cows during mid-lactation, *Comp. Biochem. Physiol. B.*; 148: 1-5.

Saksena, R., Deepak, D., Khare, A., Sahai, R., Tripathi, L.M. and Srivastava, V.M.L. (1999). A novel pentasaccharide from immunostimulant oligosaccharide fraction of buffalo milk, *Biochimica Biophysica Acta (BBA)* General Subj.; 1428: 433-445.

Kumar, S., Teotia, U.V.S. and Aswal, A.P.S. (2013). Antihypertensive property of yak milk caseinates hydrolyzed with different proteases. *Int. J. Livest. Res*.; 3: 130-134.

Boehm, G. and Stahl, B. (2007). Oligosaccharides from milk, *J. Nutr.*; 137 (3): 847-849.

Sundekilde, U.K., Barile, D., Meyrand, M., Poulsen, N.A., Larsen, L.B., Lebrilla, C. B., German, J.B. and Bertram, H.C. (2012). Natural Variability in Bovine Milk Oligosaccharides from Danish Jersey and Holstein-Friesian Breeds. *Journal of Agricultural and Food Chemistry*; 60(24): 6188-6196.

Chai, W., Piskarev, V.E., Zhang, Y., Lawson, A.M. and Kogelberg, H. (2005). Structural Determination of Novel Lacto-N-decaose and its Monofucosylated Analogue from Human Milk by Electrospray tandem Mass Spectrometry and ^{1}H NMR Spectroscopy, *Arch Biochem Biophys*.; 434: 116-127.

Mehra, R. and Kelly, P. (2006). Milk Oligosaccharides: Structural and Technological Aspects, *Int. Dairy J*.; 16: 1334-1340.

Wu, S., Tao, N., German, J.B., Grimm, R. and Lebrilla, C.B. (2010). Development of an Annotated Library of Neutral Human Milk Oligosaccharides, *J. Proteome Res*.; 9: 4138-4151.

Tao, N., Peters, E.J., Freeman, S., German, J.B., Grimm, R. and Lebrilla, C.B. (2008). Bovine Milk Glycome, *J. Dairy Sci*.; 91: 3768-3778.

Veh, R.W., Michalski, J.C., Corfield, A.P., Sander, W.M., Gies, D. and Schauer, R. (1981). New Chromatographic System for the Rapid Analysis and Preparation of Colostrum Sialyl Oligosaccharides, *J. Chromatogr A*.; 212: 313-322.

Tao, N., Peters, E.J., German, J.B., Grimm, R. and Lebrilla, C.B. (2009). Variations in Bovine Milk Oligosaccharides during early and middle Lactation stages Analyzed by High Performance Liquid Chromatography-chip/mass spectrometry, *J. Dairy Sci*.; 92: 2991-3001.

Gopal, P.K. and Gill, H.S. (2000). Oligosaccharides and Glycoconjugates in Bovine Milk and Colostrum, *Br J. Nutr*.; 84(Suppl 1): S69-S74.

Ninonuevo, M.R., Park, Y., Yin, H., Zhang, J., Ward, R.E., Clowers, B.H., German, J.B., Freeman, S.L., Killeen, K. and Grimm, R. (2006). A Strategy for Annotating the Human Milk Glycome, *J. Agric Food Chem*.; 54: 7471-7480.

Mariarosaria, M. (2014). The Predominant Milk Oligosaccharide 6'-sialyllactose reduces the internalisation of *Pseudomonas aeruginosa* in Human Pneumocytes, *Journal of Functional Foods*.,; 6: 367-373.

Bode, L., Kunz, C., Muhly, R.M., Mayer, K., Seeger, W. and Rudloff, S. (2004). Inhibition of Monocyte, Lymphocyte and Neutrophil adhesion to endothelial cells by Human Milk Oligosaccharides, *Thromb Haemost*.; 92(6): 1402-10.

Singh, A.K., Ranjan, A.K., Srivastava, G. and Deepak, D. (2016). Structure Elucidation of two Novel Yak Milk Oligosaccharides and their DFT studies, *Journal of Molecular Structure*.; 1108: 87-91.

Dennis, B.V.D. and Rudolf, G. (2012). Human Milk Oligosaccharides and Lewis Blood Group: Individual High-Throughput Sample Profiling to Enhance Conclusions from Functional Studies,*Adv Nutr,* 3(3): 440S-449S.

Kiyoyasu, F., Masaki, H. and Terasaki, P.I. (1984). Characterization of Sialosylated Lewis as a New Tumor-associated Antigen, *Cancer Res*; 44: 5279-5285.

Thomas, E. and Stahl, B. (2004). Human Milk-derived Oligosaccharides and Plant-derived Oligosaccharides Stimulate Cytokine Production of Cord Blood T-Cells in Vitro. *Pediatric Research*; 56: 4.

Tafaro (2007). Immunological properties of donkey's milk: its potential use in the prevention of Atherosclerosis, *Curr. Pharm.*; 13(36): 3711.

Villosladaa, F.L. (2006). Oligosaccharides isolated from Goat Milk reduce Intestinal Inflammation in a Rat model of Dextran Sodium Sulfate-induced colitis, *Clinical Nutrition*.; 25: 477-488.

Klein, N., Schwertmann, A., Peters, M., Kunz, C. and Strobe, S. (2000). Immunomodulatory Effects of Breast Milk Oligosaccharides, *Adv Exp Med Bio.*; 478: 251-259.

Pearl, D.H. and Walker, W.A. (2015). Why is initial bacterial colonization of the intestine important to the infant's and child's health?, *J. Pediatr Gastroenterol Nutr*.; 60(3): 294-307.

Ranjan, R. and Mark, C.F. (2012). Terminal Galactosylation of Glycoconjugates in Plasmodium falciparum asexual blood stages and *Trypanosoma brucei* bloodstream trypomastigotes, *Experimental Parasitology*.; 130: 314-320.

Tatiana, P. (2017). Naturally occurring anti-glycan antibodies binding to Globo H-expressing cells identify ovarian cancer patients, *J. Ovarian Res*.; 10: 8.

Yarema, K.J. and Bertozzi, C.R. (1998). Chemical approaches to Glycobiology and emerging Carbohydrate-based Therapeutic Agents, *Curr Opin Chem Biol*.; 2(1): 49-61.

Rhodes, J.M., Campbell, B.J. and Yu, L.G. (2008). Lectin-epithelial interactions in the Human Colon, *Biochem Soc Trans.*; 36(6): 1482-6.

Geralyn, D.M. (2014). Human Milk Oligosaccharides Enhance Innate Immunity to Respiratory Syncytial Virus and Influenza in Vitro, *Food and Nutrition Sciences*; 5(14): 13.

Prakash, T.P. (2014). Targeted delivery of Antisense Oligonucleotides to Hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice, *Nucleic Acids Res.*; 42(13): 8796-807.

Yusuke, U. (2006). Structural determination of the oligosaccharides in the milk of an Asian elephant (Elephas maximus), *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*; 145(4): 468-478.

Maria, J.M., Samuel, M.S. and Pablo, H. (2002). Binding of Milk Oligosaccharides by several Enterotoxigenic Escherichia coli strains isolated from Calves, *Glycoconjugate Journal*; 19: 5-11.

Fukuda, K., Yamamoto, A., Ganzorig, K., Khuukhenbaatar, J., Senda, A., Saito, T. and Urashima, T. (2010). Chemical Characterization of the Oligosaccharides in Bactrian camel (*Camelus bactrianus*) milk and colostrums, *J. Dairy Sci*. ; 93(12): 5572-87.

Cailin, R.H. (2014). Comparison of the nutrient composition of commercial dog milk replacers with that of dog milk, *J Am Vet Med Assoc*.; 244(12): 1413-1422.

Mervat, I.F., Kholif, S.F. and **Kholif, A.M. (2009).** Evaluation of Goat Milk Containing Galacto-oligosaccharides After Supplementing the Ration with Amino Acids, *International Journal of Dairy Science*.; 4: 27-33.

Crane, J.K., Azar, S.S., Stam, A. and Newburg, D.S. (1994). Oligosaccharides from human milk block binding and activity of the *Escherichia coli* heat-stable enterotoxin (STa) in T84 intestinal cells, *J Nutr.*; 124(12): 2358-64.

Kunz, C., Rudloff, S., Baier, W., Klein, N. and Strobel, S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects, *Ann Rev Nutr*.; 20(1): 699-722.

Mills, S., Ross, R.P., Hill, C., Fitzgerald, G.F. and Stanton, C. (2011). Milk intelligence: Mining milk for bioactive substances associated with human health, *Int. Dairy J*.; 21(6): 377-401.

Wang, B. and Miller, B. (2003). The role and potential of sialic acid in human nutrition, *J. Eur. J. Clin. Nutr*.; 57(11): 1351-1369.

Von, I.M. and Thomson, R.J. (1997). Sialic acids and sialic acid-recognizing proteins: Drug discovery targets and potential glycopharmaceuticals, *J. Curr. Med*. Chem.; 4: 185-210.

Chonan, O. and Watanuki, M. (1996). The effect of 6′-galactooligosaccharides on bone mineralization of rats adapted to different levels of dietary calcium. *Int J Vitam Nutr Res*; 66: 244-249.

Scholz, A.K., Schsafsma, G., Van, D.H.E. and Schrezenmeir, J. (2001). Effects of prebiotics on mineral metabolism. *Am. J. Clin. Nutr.* 73: 459S-464S.

Boudry, G. (2017). Bovine Milk Oligosaccharides decrease gut permeability and improve inflammation and microbial dysbiosis in diet-induced obese mice, *J. Dairy Sci*.; 100 (4): 2471-2481.

Zopf, D. (1996). Oligosaccharide anti-infective agent, *The Lancet*; 347(9007): 1017-1021.

Hakkarainen, J., Toivanen, M., Leinonen, A., Frangsmyr, L., Stromberg, N., Lapinjoki, S., Nasssif, X. and Tikkanen, K.C. (2005). Human and Bovine Milk Oligosaccharides Inhibit *Neisseria meningitidis* Pili Attachment in Vitro, *J. Nutr*.; 135: 2445-2448.

Zivkovic, A.M. and Barile, D. (2011). Bovine Milk as a Source of Functional Oligosaccharides for Improving Human Health, *Adv. Nutr*.; 2(3): 284-288.

Urashima, T. and Taufik, T. (2011). Oligosaccharides in milk: Their benefits and future utilization, *J. Anim. Sci. Technol*.; 33: 189-197.

Kim, E.Y., Gronewold, C., Chatterjee, A., Lieth, C.W., Kliem, C., Schmauser, B., Wiessler, M. and Frei, E. (2005). Oligosaccharide mimics containing galactose and fucose specifically label tumour cell surfaces and inhibit cell adhesion to fibronectin, *Chembiochem*.; 6(2): 422-31.

Quaggin, S.E. (2007). Sizing up sialic acid in glomerular disease, *J. Clin. Invest.*; 117: 1480-1483.

Starnes, H.F., Warren, R.S., Jeevanandam, M., Gabrilove, J.L., Larchian, W., Oettgen, H.F. and Brennan, M.F. (1988). Tumor necrosis factor and the acute metabolic response to tissue injury in man. *J. Clin. Investig*.; 82:1321-1325.

Svensoon, L. (1992). Group C Rotavirus Requires Sialic Acid for Erythrocyte and Cell Receptor Binding, *J. Virol*., 66(9): 5582-5585.

Ben, X.M., Zhou, X.Y., Zhao, W.H., Yu, W.L., Pan, W., Zhang, W.L., Wu, S.M., Van, Beusekom, C.M. and Schaafsma, A. (2004). Supplementation of milk formula with galacto-oligosaccharides improves intestinal micro-flora and fermentation in term infants, *Chin Med J (Engl)*.; 117(6): 927-31.

Guillermo, M., Ruiz, P., Luz, E.C., Pilar, R., Bibiana, C.M. and David, S.N. (2003). *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection, *J. Biol. Chem*.; 278(16): 14112-14120.

Lim, D.Y., Jeong, Y., Tyner, A.L. and Park, J.H. (2007). Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin, *J Physiol Gastrointest Liver Physiol*.; 292(1): 66-75.

Johansson, P., Nilsson, J., Angstrom, J. and Meller, P.H. (2005). Interaction of Helicobacter pylori with sialylated carbohydrates: The dependence on different parts of the binding trisaccharide Neu5Ac{alpha}3Gal{beta}4GlcNAc, *Glycobiology*.; 15: 625-636.

Newburg D.S., Ruiz-Palacios G.M., Altaye M., Chaturvedi P., Meinzen-Derr J., Guerrero M. F. and Morrow A.L. (2004). Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants, *Glycobiology*; 14(3): 253-63.

Lars, B. (2012). American Society for Nutrition Human milk oligosaccharide concentration and risk of postnatal transmission of HIV through breastfeeding, *Am. J. Clin Nutr*.; 96: 831-839.

Zhu L., Cao X., Chen W., Zhang G., Sun D. and Wang P.G. (2005). Syntheses and Biological Activities of Daunorubicin Analogs with Uncommon Sugars, *Bioorg, Med Chem*.; 13 (23): 6381-7.

Brito, B.E., Romano E.L. and Grunfeld C. (1995). Increased lipopolysaccharide-induced tumour necrosis factor levels and death in hypercholesterolaemic rabbits. *Clin. Exp. Immunol.*; 101: 357-361.

Mehra R. (2014). Novel High-Molecular Weight Fucosylated Milk Oligosaccharides Identified in Dairy Streams, *PLoS One*; 9(5): e96040.

Ruiz-Palacios, G.M., Cervantes, L.E., Ramos, P., Chavez-Munguia, B. and Newburg, D.S. (2003). Campylobacter jejuni binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc) and fucosyloligosaccharides of human milk inhibit its binding and infection, *J Biol Chem*.; 278(16): 14112-20.

Newburg, D.S., Ruiz-Palacios, G.M., Mekibib, A. and Chaturvedi, P. (2004). Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants, *Glycobiology*; 14: 253-263.

Tao, H. (2016). Sialylated immunoglobulin G can neutralize influenza virus infection through receptor mimicry, *Oncotarget*; 7(13): 15606-15617.

Eunus, S.A. (2016). Norovirus Drug Candidates that inhibit viral capsid attachment to human histo-blood group antigens, *Antiviral Res*.; 133: 14-22.

Hans, D. and Prell, C. (2017). Benefits of Lactoferrin, Osteopontin and Milk Fat Globule Membranes for Infants, *Nutrients*; 9(8): 817.

Kumar, K. (2017). "Conformational & Configurational Studies of Goat Milk Oligosaccharides by NMR", Ph.D. dissertation, Lucknow University.

Srivastava, A.K. (2008). "Isolation of Oligosaccharides from Goat's Milk as Bioactive agent", Ph.D. dissertation, Lucknow University.

Srivastava, A.K., Singh, P. and Deepak, D. (2016). Isolation and NMR Studies of novel oligosaccharide from Goat milk, *J. Biol. Chem. Research*; 33(2): 901-908.

Singh, P., Srivastava, A.K. and Deepak, D. (2017). Isolation and structure elucidation of Caprose (novel oligosaccharide) from Goat milk, *J. Biol. Chem. Research.*; 34(1): 14-20.

Singh, P., Gupta, N.K. and Deepak, D. (2019). Structure Elucidation of Novel Trisaccharide Isolated from Donkey's milk, *J. Biol. Chem. Research.*; 36(1): 157-163.

Gupta, N.K. (2007). "Isolation of Milk Oligosaccharides as Immunostimulants", Ph.D. dissertation, Lucknow University.

Srivastava, A. (2007). "Isolation of Oligosaccharides from Mare's Milk as an Immunostimulant and Antibacterial Agent", Ph.D. dissertation, Lucknow University.

Maurya, R.K., Srivastava, A. and Deepak, D. (2017). Isolation and structure elucidation of novel oligosaccharide Aminose from Mare Milk, *J. Biol. Chem. Research*; 34(1): 231-237.

Gangwar, L., Singh, R. and Deepak, D. (2018). Structure Elucidation of a Novel Oligosaccharide (Medalose) from Camel Milk, *Journal of Molecular Structure*; 1153: 157-161.

Singh, R. (2007). "Isolation of Milk Oligosaccharides from Camel Milk as Antibacterial and Immunomodulators", Ph.D. dissertation, Lucknow University.

Shahi, S. (2008). "Milk Oligosaccharides as Immunostimulants", Ph.D. dissertation, Lucknow University.

Singh, A.K. (2018). "Structure Elucidation of Milk oligosaccharides by 2D-NMR", Ph.D. dissertation, Lucknow University.

Ranjan, A.K. (2015). "Isolation of Biologically active Oligosaccharides from Milk of Ovies aries and Their Structure Elucidation", Ph.D. dissertation, Lucknow University.

Gunjan (2017). "Stereoscopic Structure elucidation of Cow Colostrum Oligosaccharides by NMR", Ph.D. dissertation, Lucknow University.

Singh, P., Maurya, R.K., Rizvi, A.H. and Deepak, D. (2017). Famiose: A novel hexasaccharide from Donkey's milk, *J. Biol. Chem. Research*; 34(2): 548-556.

Srivastava, G. (2008). "Isolation of Yak Milk Oligosaccharides as therapeutic agents", Ph.D. dissertation, Lucknow University.

Singh, P., Gupta, N.K. and Deepak, D. (2019). NMR and Mass Characterization of Novel Oligosaccharide (Gariose) from Donkey Milk, *Universal Review*; VIII(V), 361-374.

Kumar, A. (2012). "Isolation and Structure Elucidation of Milk Oligosaccharides as Therapeutic agents", Ph.D. dissertation, Lucknow University.

Gangwar, L., Kumar, A. and Deepak, D. (2017). Isolation and Structure Elucidation of Biologically Active Novel Pentasaccharide from the Milk of *Bubalus bubalis*, *International Journal of Carbohydrate Research*; 7(1), 9-13.

Verma, P. (2018). Ph.D. dissertation, Lucknow University.

Khan, M. (2018). "Configurational, Conformational and Computational Analysis of Milk Oligosaccharides", Ph.D. dissertation, Lucknow University.

Kobata, A. and Ginsburg, V. (1972). Oligosaccharides of human milk. III. Structure of a new hexasaccharide, lacto-*N*-hexaose, *Journal of Biological Chemistry*; 247: 1525-1529.

Urashima, T., Kusaka, Y., Nakamura, T., Saito, T., Maeda, N. and Messer, M. (1997). Chemical Characterization of milk oligosaccharides of the brown Bear, Ursus arctos yesoensis, *Biochim. Biophys. Acta*; 1334: 247-255.

Smith, D.F., Zopf, D.A. and Ginsburg, V. (1978). Fractionation of sialyl oligosaccharides of human milk by ion-exchange chromatography, *Analytical Biochemistry*.; 85: 602-608.

Egge, H., Dell, A. and Von Nicoli, H. (1983). Fucose containing oligosaccharides from human milk. I. Separation and identification of new constituents, *Arch. Biochem. Biophys*.; 224(1): 235-53.

Horton, D. (2015). Foreword: Carbohydrate Research: a half century of carbohydrate science, *Carbohydrate Research*; 403: 3-7.

Gangwar, L., Singh, R. and Deepak, D. (2018). Structure Elucidation of a Novel Oligosaccharide (Medalose) from Camel Milk, *Journal of Molecular Structure*; 1153: 157-151.

Robyt, J.F. (2000). Thin-layer Chromatography of Carbohydrates,*Laboratory of Carbohydrate Chemistry and Enzymology*, Iowa State University, Ames, IA, USA; 2235-2244.

Jain, R. and Sherma, J. (2000). Encyclopedia of Analytical Chemistry, R. A. Meyer (Ed.), *John Wiley and sons Ltd. Chichester*; 1583-1603.

Reiffova, K. and Nemcova, R. (2006). Thin-layer chromatography analysis of fructooligosaccharides in biological samples, *J. Chromatogr A*.; 1110 (1-2): 214-21.

Wilson, K. and Goulding, K.H. (1987). Immunochemical techniques. In: A biologist guide to principles and techniques of practical biochemistry, *Edward Amold, Landon*; 116-134.

Kobata, A. and Ginsburg, V. (1969). Oligosaccharides of human milk. II. Isolation and characterization of a new pentasaccharide, lacto-*N*-fucopentaose III, *Journal of Biological Chemistry*; 244: 5496-5502.

Gronberg, G., Lipniunas, P., Lundgren, T., Lindh, F. and Nilsson, B. (1992). Structural analysis of five new monosialylated oligosaccharides from human milk, *Archives of Biochemistry and Biophysics*; 296: 597-610.

Marston, A. and Hostettman, K. (1991). Modern Separation Methods, *Natural Product Reports*; 8: 391-413.

Synder, L.R., Glajch, J.L. and Kirland, J. (1988). *Practical HPLC Method Development* Wiley-Interscience, New York.

Kennedy, J.F. and Cabral, J.M.S. (1985). Immobilization of biocatalyst by metal-link/chelation processes, in Immobilized Cells and Enzymes (Woodward, J., ed.), IRL, Oxford, UK, pp. 19-37.

Daniel, P.F., DeFeudis, D.F., Lott, I.T. and McCluer, R.H. (1981). Quantitative microanalysis of oligosaccharides by high-performance liquid chromatography, *Carbohydr. Res.*; 97: 161-180.

Kiliani, H. (1930). Über Digitalinum verum, *Ber. Deutsch. Chem. Ges*.; 63: 2866.

Bush, C.A. (1988). High Resolution NMR in the Determination of Structure in Complex Carbohydrates, *Bull. Magn. Reson.*; 10:73-96.

Dabrowski, J. (1989). Two-dimensional proton magnetic resonance spectroscopy, *Meth. Enzymo L.*; 179: 122-156.

Jain, D.C., Agrawal, P.K. and Thakur, R.S. (1991). ^{13}C NMR spectroscopy of oleanane triterpenoids, *Planta Med.*; 57: 94.

Pabst, A., Barron, D., Semon, E. and Schreier, P. (1991). Isolation of a novel linalool disaccharide glycoside from raspberry fruit, *Tetrahedron Letters.*; 32(37): 4885-4888.

Duus, J.O., Gotfredsen, C.H. and Bock, K. (2000). Carbohydrate Structural Determination by NMR Spectroscopy: Modern Methods and Limitations, *Chem. Rev.*; 100: 4589-4614.

Bock, K. and Pedersen, C. (1974). A study of ^{13}CH coupling constants in hexopyranoses, *J. Chem. Soc., Perkin Trans.*; 3: 293-297.

Van, H. (1996). *Encyclopedia of Nuclear Magnetic Resonance*, John Wiley & Sons Ltd: Chichester; 1107-1137.

Vinogradov, E.V., Peterson, B.O., Thomas-Oates, J.E., Duus, J.O., Brade, H. and Holst, O. (1998). Characterization of a Novel Branched Tetrasaccharide of 3-Deoxy-D-*manno*-oct-2-ulopyranosonic Acid: The Structure of the Carbohydrate backbone of the lipopolysaccharide from *Acinetobacter Baumannii* strain Nctc 10303 (Atcc 17904), *Journal of Biological Chemistry*.; 273(43): 28122-28131.

Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vliegenthart, F. G., Binette, J.P. and Schmid, K. (1978). Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma alpha 1-acid glycoprotein by 360-MHZ 1H NMR spectroscopy and permethylation analysis, *Biochemistry.*; 17(24): 5206-14.

Vliegenthart, J.F.G., Dorland, L. and Halbeek, H.V. (1983). *High Resolution H-1 Nuclear Magnetic Resonance Spectroscopy as a Tool in the Structural Analysis of Carbohydrates Related to Glycoproteins, Advan. Carbohyd. Chem. Biochem.*; 41: 209-374.

Kobata, A. and Ginsburg, V. (1972). Isolation and characterization of a new hexasaccharide, lacto-n-hexaose, *Journal of Biological Chemistry*; 247: 1525-1529.

Waard, P., Leefang, B.R., Vliegenthart, J.F.G., Boelens, R., Vuisiter, G.W. and Kaptein, R. (1992). Application of 2D and 3D NMR experiments to the conformational study of diantennary oligosaccharide, *J. Biomol. NMR.*; 2(3): 211-226.

Agrawal, P.K. (1992). NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides, *Phytochemistry*; 31(10): 3307-3330.

Fales, H.M., Milne, G.W., Pisano, J.J., Brewer, H.B., Blum, M.S., MacConnell, J.G., Brand, J. and Law, N. (1972). Biological applications of electron ionization and chemical ionization mass spectrometry, *Recent Prog. Horm. Res*.; 28: 591-626.

Dua, V.K., Rao, B.N., Wu, S.S., Dube, V.E. and Bush, C.A. (1986). Characterization of the oligosaccharide alditols from ovarian cyst mucin glycoproteins of blood group A using high pressure liquid chromatography (HPLC) and high field 1H NMR spectroscopy, *J. Biol Chem.*; 261: 1599-608.

Bock, K. and Thoegersen, H. (1982). Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides, *Ann. Rep. NMR Spectrosc.*; 13: 1.

Bock, K., Pedesen, C. and Pedersen, H. (1984). Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides, *Adv. Carbohydr. Chem. Biochem*; 42: 193.

Agrawal, P.K. and Bansal, M.C. (1989). The Carbon-13 NMR of Flavonoids, Elsevier, Amsterdam; 96-116.

Agrawal, P.K., Jain, D.C., Gupta, R.K. and Thakur, R.S. (1985). Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins, *Phytochemistry*; 24: 2479-2496.

Gorin, P.A. (1981). Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Polysaccharides, *J. Adv. Carbohydr. Chem. Biochem*.; 38: 13-104.

Bush, C.A., Panitch, M.M., Dua, V.K. and Rohr, T.E. (1985). Carbon nuclear magnetic resonance spectra of oligosaccharides isolated from human milk and ovarian cyst mucin, *Anal. Biochem*; 145: 124-136.

Vliegenthart, J.F.G., Dorland, L., Halbeek, H.V. and Haverkamp, J. (1982). NMR Spectroscopy of Sialic Acids. In Sialic Acids-Chemistry, Metabolism and Function, *Cell Biology Monographs*; 10: 127-172.

Burum, D.P. and Ernst, R.R. (1980). Net polarization transfer via a J-ordered state for signal enhancement of low-sensitivity nuclei, *J. Magn. Reson*.; 39: 163.

Morris, G.A. (1986). Modern NMR techniques for structure elucidation, *Magn. Reson. Chem.*; 24: 371-403.

Kessler, H., Gehrke, M. and Griesinger, C. (1988). Two-Dimensional NMR Spectroscopy: Background and Overview of the Experiments, *Angew. Chem. Int.*; 27: 490.

Derome, A.E. (1989). The use of NMR spectroscopy in the structure determination of natural products: two-dimensional methods, *Nat. Prod. Rep.*; 6: 111.

Homans, S.W., Dwek, R.A., Boyd, J., Mahmoudian, M., Richards, W.G. and Rademacher, T.W. (1986). Conformational transitions in N-linked oligosaccharides, *Biochemistry*; 25: 6342-6350.

Homans, S.W., Dwek, R.A., Fernandes, D.L. and Rademacher, T.W. (1983). The use of two-dimensional correlated spectroscopy to obtain new assignments in the high-resolution 1H nuclear magnetic resonance spectrum of the biantennary complex oligosaccharide isolated from human serum transferrin by hydrazinolysis, *Biochim. Biophy. Acta*; 760: 256-261.

Homans, S.W., Dwek, R.A., Fernandes, D.L. and Rademacher, T.W. (1984). The analysis of coupling networks in a complex oligosaccharide mixture derived from the Fc region of rabbit immunoglobulin G using 1H-1H correlated NMR spectroscopy combined with double quantum NMR spectroscopy, *Biochim. Biophys. Acta.*; 798: 78-83.

Homans, S.W., Dwek, R.A., Fernandes, D.L. and Rademacher, T.W. (1983). Solution conformation of biantennary complex type oligosaccharides: Determination of major conformers about the glycosidic linkages, *FEBS Lett.*; 164: 231-235.

Homans, S.W., Dwek, R.A. and Rademacher, T.W. (1987). Tertiary structure in N-linked oligosaccharides, *Biochemistry.*; 26: 6553-6560.

Gronberg, G., Lipniunas, P., Lundgren, T., Lindh, F. and Nilsson, B. (1992). Structural analysis of five new monosialylated oligosaccharides from human milk, *Archives of Biochem. Biophys*.; 296(2): 597-610.

Bodenhausen, G. and Freeman, R. (1971). Two-dimensional nuclear magnetic resonance spectroscopy, *J. Magnetic Resonance.*; 28: 471.

Bax, A. and Davis, D.G. (1985). MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy, *J. Magn. Reson.*; 65: 335-360.

Braunschweiler, L. and Ernst, R. (1983). Coherence transfer by isotropic mixing: Application to proton correlation spectroscopy, *J. Magn. Reson.* 53: 521-528.

Dunkel, R., Mayne, C.L., Pugmire, R.J. and Grant, D.M. (1992). Improvements in the computerized analysis of 2D INADEQUATE spectra, *Analytical Chemistry*; 64: 3133-49.

Jeener, J., Meier, B.H., Bachmann, P. and Ernst, R. (1979). Investigation of Exchange Process by Two-Dimensional NMR Spectroscopy, *J. Chem. Phys*.; 71(11): 4546-4553.

Kumar, A., Ernst, R. and Wutrich, K. (1980). A two-dimensional nuclear overhauser enhancement (2D NOE) experiment for the elucidation of complete proton-proton cross-relaxation networks in biological macromolecules, *Biochem. Biophys. Res. Commun.*; 95: 1-6.

Key, L.E., Keifer, P. and Saarinen, T. (1992). Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity, *J. Am. Chem. Soc.*; 114: 10663-10665.

Barr, J.R., Anumula, K.R., Vettese, M.B., Taylor, P.B. and Carr, S.A. (1991). *Anal. Biochem.*; 192: 181-192.

Barber, M. (1982). Fast atom bombardment mass spectrometry, *Analytical Chemistry*; 54 (4): 645-647.

Antonio, M.F., Silvia, R., Antonio, G., Cordula, A.H., Gottfried, P., Julio, J.B., Emilia, M.G. and Kunz, C. (2006). Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology, *International Dairy Journal*; 16: 173-181.

Daniele, V., Grimmonprez, L. and Maryse, S. (1997). Chemical characterization of sialyl oligosaccharides isolated from goat (*Capra hircus*) milk, *Biochimica et Biophysica Acta*.; 1336: 157-164.

Bruno, D. and Catherine, E.C. (1988). Isolation and Identification of Flavonoids, *Glycoconjugate J.*; 5: 397-409.

Bathori, M., Blunden, G. and Kalasz, H. (2000). Two-dimensional thin-layer chromatography of plant ecdysteroids, *Chromathographia*; 52: 815-817.

Taki, T., Kasama, T., Handa, S. and Ishikawa, D. (1994). A simple and quantitative purification of glycosphingolipids and phospholipids by thin-layer chromatography blotting. *Anal. Biochem*; 223: 232-238.

Kriz, J., Adamcova, E., Knox, J.H. and Hora, J. (1994). Characterization of adsorbents by high-performance liquid chromatography using aromatic hydrocarbons Porous graphite and its comparison with silica gel, alumina, octadecylsilica and phenylsilica, *J. Chromatogr. A.*; 663: 151-161.

David, S.H. (1999). Affinity Chromatography: A Review of Clinical Applications, *Clinical Chemistry*; 45: 593-615.

Corresponding author: Dr. Desh Deepak and Dr. Manisha Shukla, Department of Chemistry University of Lucknow, Lucknow-226007(UP), India.
Email: shuklam321@gmail.com, deshdeepakraju@rediffmail.com