Chromatographic Techniques used in Separation of Milk Oligosaccharides

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Abstract

Chromatography plays a major role in purification and analysis of milk oligosaccharides. Chromatographic separations can be carried out by using a variety of supports, including immobilized silica on glass plates (TLC), volatile gas (Gas Chromatography), paper (paper chromatography) and liquids which may incorporate hydrophilic, insoluble molecules(liquid chromatography). Apart from ion exchange chromatography, affinity chromatography and Gel Permeation Chromatography (GPC), High Performance Liquid Chromatography (HPLC) is the most frequently used technique.

Keywords: Thin Layer Chromatography, High Performance Liquid Chromatography**,** Gel Permeation Chromatography

Introduction

Milk oligosaccharides are important components in various animal milk because of their enormous health benefit as anti cancer, antioxidant, immunomodulator and various other therapeutic properties. To better understand the relationship between oligosaccharide structures and biofunctions, efficient qualitative and quantitative methods are essential prerequisites. However, mostly these oligosaccharides are highly complex in nature and difficult to analyze because of their high polarity, lack of chromophores, and the presence of multiple isomeric structures. The objective of this review is to describe and discuss the recent advances in the analysis of milk oligosaccharides with the application of different chromatographic techniques, including gel permeation, high performance anion-exchange, porous graphitized carbon, reverse-phase high performance liquid chromatography etc. These methods have been used to characterize the structures of milk oligosaccharides.

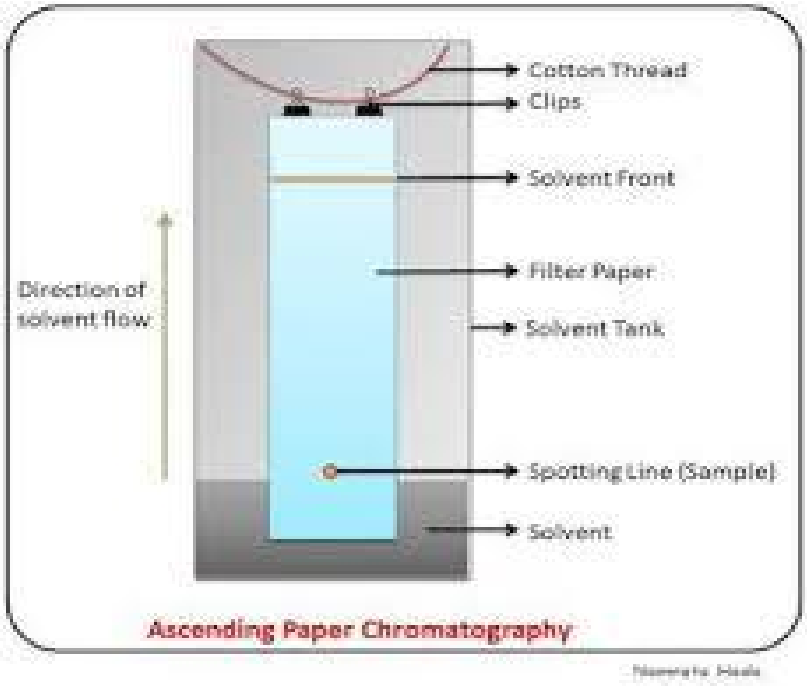
Paper Chromatography

Separation through Paper Chromatography technique is due to differential migration velocities through the sorbent layer in a fixed separation time. Paper chromatography uses a strip of paper as the stationary phase and is based on liquid-liquid partition process [1]. Capillary action is used to pull the solvents through the paper and separate the solutes. In this, chemical interaction with the paper makes compound travel at different rates [2]. Two-way paper chromatography, also called two-dimensional chromatography, involves using two solvents and rotating the paper 90° in between. This is useful for

separating complex mixtures of similar compounds. For milk oligosaccharides the descending paper chromatography was performed with the following solvents on Whatman filter paper:

Upper layer of ethyl acetate-pyridine-H_2O (2:1:2)
Ethyl acetate-pyridine-acetic acid-H_2O (5:5:1:3)
Propanol-H_2O (4:1)
1-butanol-pyridine-H_2O (6:4:3)
Lower layer of phenol-formic acid-2-propanol: H_2O (80:5:100)
Upper layer of pyridine-ethyl acetate- H_2O (6:4:3)
Phenol-H_2O-conc.NH_4OH (150:40:1)
Upper layer of ethyl acetate-acetic acid-H_2O (3:1:3)
Ethanol- 1M ammonium acetate, pH 7.8 (5:2)
Butanol- acetic acid- ammonium hydroxide (2:3:1) [3]

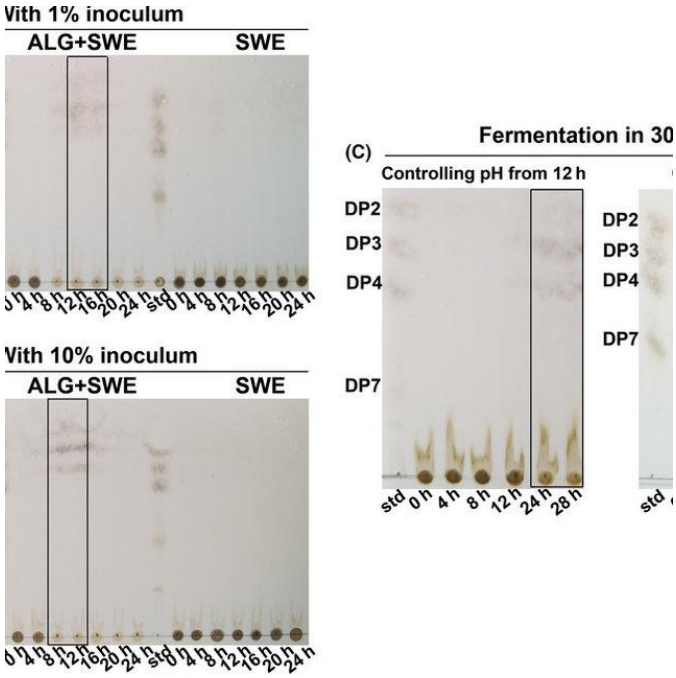
Oligosaccharides were visualized with $AgNO_3$ reagent, aniline oxalate reagent or iodate-benzidine reagent, oligosaccharides containing N-acetyl amino sugars were located with Morgan-Elson reagent, while oligosaccharides containing sialic acid were developed with Thiobarbituric acid (TBA) reagent [4]. Separation time varies with the size of the oligosaccharides: approximately 3 days for tetra and pentasaccharides and 10 days for hexa, hepta, and octasaccharide [5]. The reduced oligosaccharides move slightly slower than the corresponding unreduced oligosaccharides. In Paper Chromatography, Lacto-N-neohexaose migrates 5% faster than lacto-N-hexaose so Lacto-N-hexaose and Lacto-N-neohexaose can easily be distinguished by paper chromatography after reduction by sodium borohydride [6].



Thin Layer Chromatography (TLC)

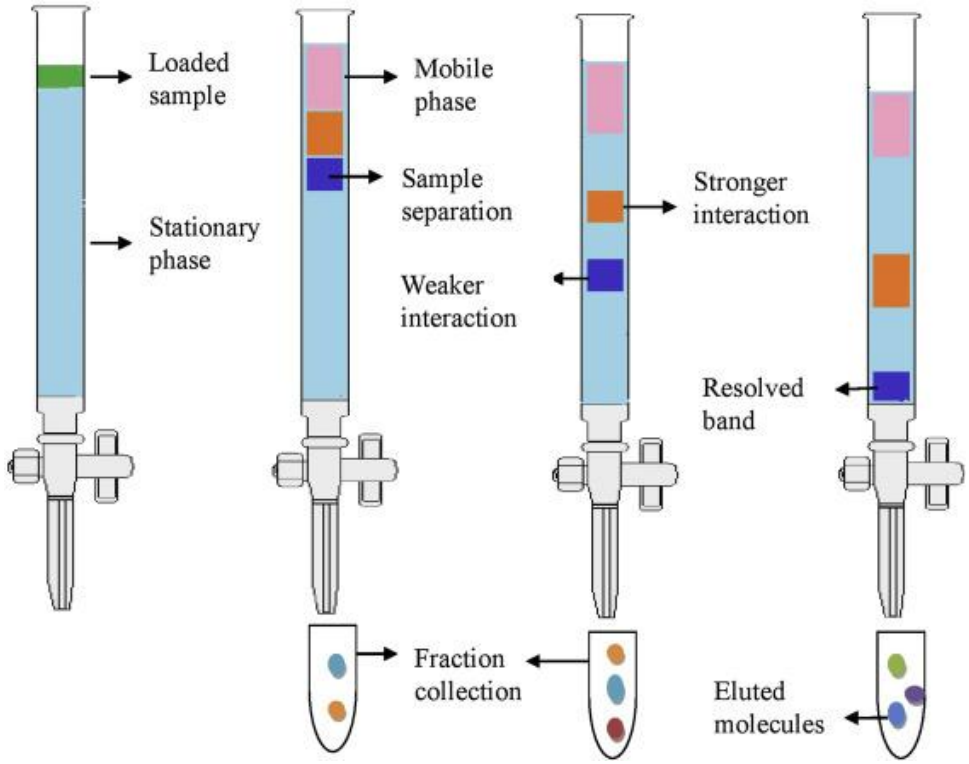
Thin layer chromatography is the technique which requires less time and small quantity of material used in isolation and purification of the given sample. Separation through TLC involves distribution of mixture of two or more substances between a stationary phase, carrying the samples with it. The mobile

phase is liquid which travels up the stationary phase, carrying the samples with it. Components of the samples will separate on the stationary phase according to how much they adsorb on the stationary phase versus how much they dissolve in the mobile phase. The resolution of mixture of compounds depends on the choice of suitable solvent system. Starting from non polar single solvent system to highly polar three solvent systems were available for the thin layer chromatography. Availability of various quality of silica gel including the high retention times plays an important and decisive role for purification of natural products. It has been reported that acetylated oligosaccharides could be detected and purified by TLC [7].



Column Chromatography

Column chromatography is a technique of separation of various derivatized oligosaccharides by means of a column which is packed with particulate material such as silica or alumina and then solvent is passed through it at atmosphere or low pressure. The basis of separation can be liquid/solid (adsorption) or liquid/liquid (partition). The sample is dissolved in solvent and adsorbed at the top of the column. The solvent is usually changed stepwise, and fractions are collected according to the separation required, with the eluted solvent usually monitored by thin layer chromatography (TLC). The major advantage is that no pumps or expensive equipment are required, and the technique can be scalped up to handle sample sizes approaching a gram in the laboratory. Column chromatography is advantageous over other chromatographic techniques because it can be used in both analytical and preparative applications. It can be used to determine the number of components of a mixture, but it can also be used to separate and purify substantial quantities of those components for subsequent analysis [8]. The derivatized oligosaccharides are purified in quantitative yields by column chromatography.



High Performance Liquid Chromatography (HPLC)

HPLC is a universal separation technique that is capable of separating compounds using their relative interaction with the stationary phase and the solvent eluting through the column. HPLC is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify and quantify compounds. Three main techniques used in HPLC are ion exchange chromatography (IEC), reversed phase chromatography (RPC) and affinity chromatography. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase (liquid) through the column and a detector that shows the retention times of analytes [9]. The sample to be analyzed is introduced in small volume to the stream of liquid mobile phase. The analyte's motion through the column is slowed by specific chemical or physical interactions with the stationary phase as it transverses the length of the column [10]. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes is called the retention time; the retention time under particular conditions is considered a reasonably unique identifying characteristic of a given analyte. The use of smaller particle size column packing (which creates higher backpressure) increases the linear velocity giving the components less time to diffuse within the column, improved resolution in the chromatogram [11]. Bonded silica and bonded glass with ionic groups on their surface are used in such HPLC techniques as the stationary phase separation media. Such analysis is carried out at high pH coupled with pulsed amperometric detection (PAD), allowing separation of oligosaccharides and polysaccharides up to particle diameter, i.e. DP >= 50 mm. The separation depends on the molecular size, sugar composition and type of linkages between the monosaccharide units. The use of fully acetylated oligosaccharides overcome problems of solubility, and has resulted in the fractionation of malto-oligosaccharides up to DP30 in about 150 min, using systems for separation of oligosaccharides are those using chemically bonded phase which fractionate materials on the basis of their relative affinities for mobile phase and bonded phase [12]. For HPLC purification and achieving pure compound, the following sequence is followed for better yield and resolution:

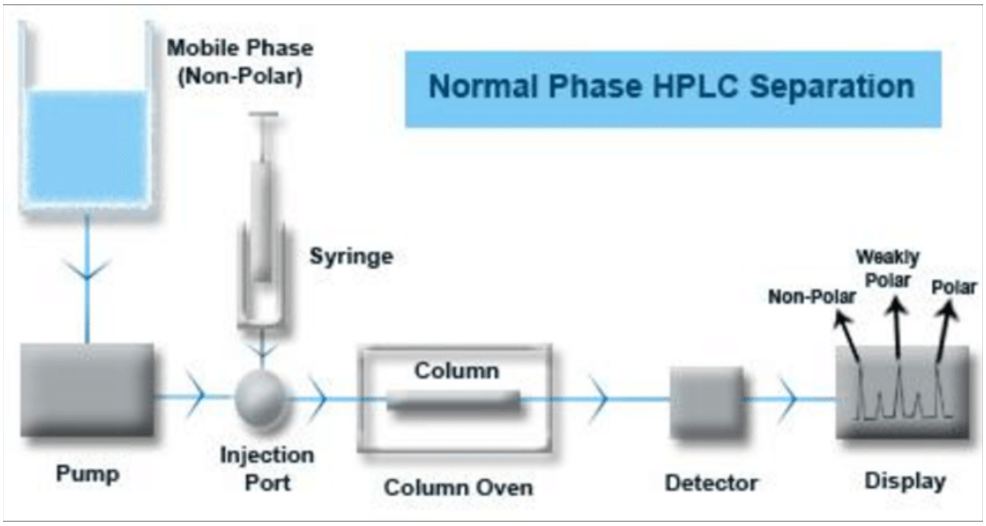
1. **Choice of solvent system:** Choice of solvent system for separation of different compounds is the important step for purification of HPLC method. Silica gel plates are commonly used for normal phase column and sialylated silica gel plates are used for reversed-phase column.
2. **Optimization of analytical columns of small quantities:** A preliminary analytical search is necessary for the right choice of conditions, which saves time, sample and solvent, required in a HPLC system.
3. **Scaling of preparative HPLC apparatus:** Scaling-up a successful HPLC column for analytical separation is an important factor for purification and better yield because overloading of column may lead to non-linear adsorption with the solubility of sample. This is especially true for reversed phase HPLC, if the compound under investigation does not dissolve in aqueous solvents.

Normal Phase HPLC

Normal-phase HPLC is carried out on a waters alliance 2640 separations module using a Glycosep N column (oxford Glycosciences, 25 mm × 3.9 mm internal diameter i.e., i.d.). The mobile phases are acetonitrile (solvent A) and ammonium formate, pH 4.4 (solvent B). The following elution conditions are used:

Step 1: 80% A, 20% B equilibration conditions, 0.4 ml/min.
Step 2: 20 to 58% B over 152 min, linear gradient.
Step 3: 58 to 100% B for 3 min, linear gradient.
Step 4: Equilibration of column at 80% A , 20% B at 0.4 ml/min for 15 min.

2-AMAC- derivatized oligosaccharides are detected on a waters 474 fluorescence detector at an excitation of 428 nm and emission wavelength of 525 nm. Methyl-4-aminobenzoate-labeled dextran ladders are detected on kratos spectroflow 783 absorbance detectors at a wavelength of 305 nm. Glucose unit equivalent values for each glycan moiety are determined by internal calibration of the system by co injection of an M4-AB-labeled dextran ladder and a 2-AMAC- labeled dextran ladder. For collection of glycans from the HPLC run only 2-AMAC- labeled glycans are injected [13].



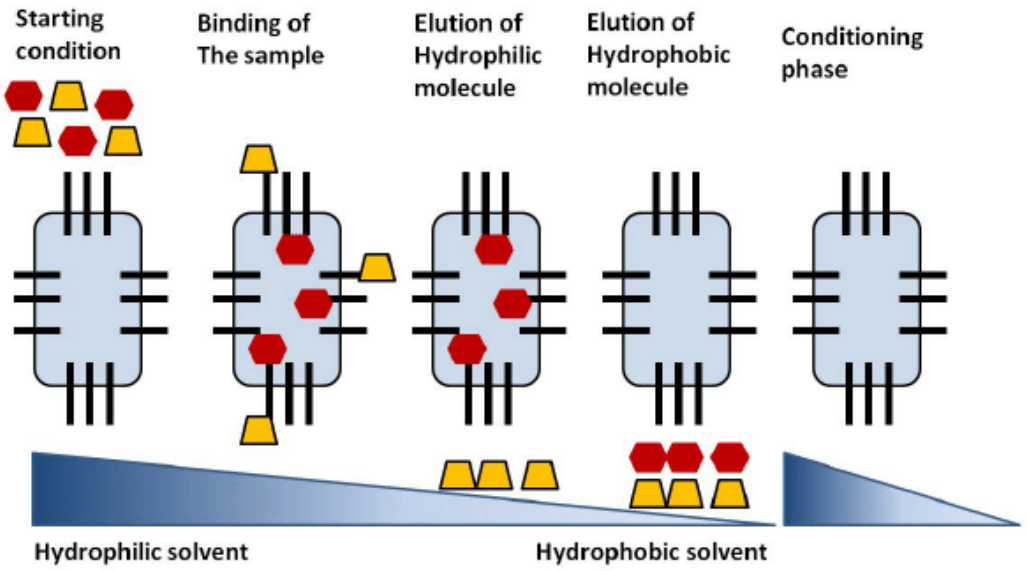
Reverse Phase HPLC (RP-HPLC)

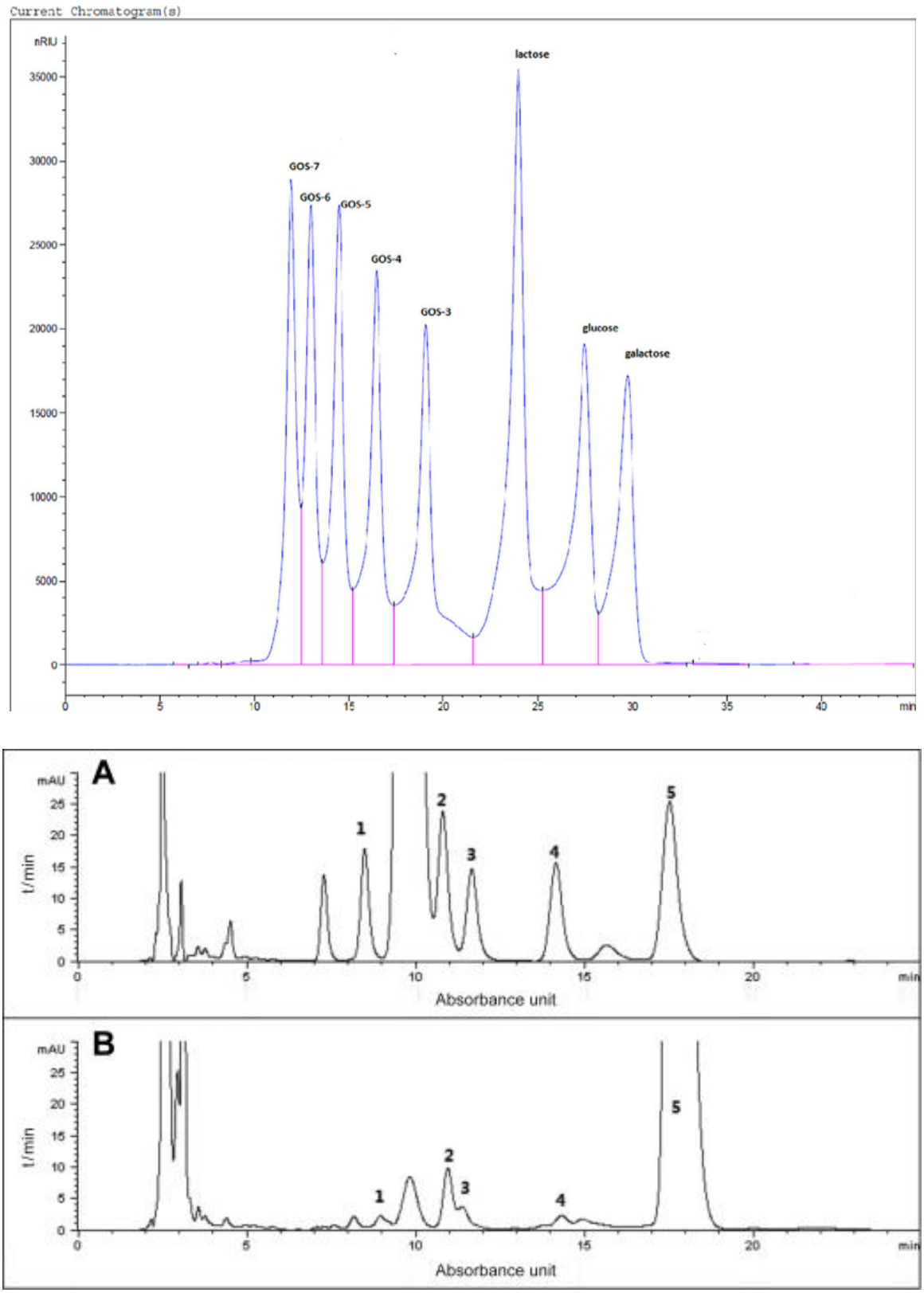
RP-HPLC is the most widely used HPLC technique for organic compounds due to high chromatographic efficiency and selectivity, it has not been successful in the separation of Oligosaccharides due to their hydrophilic nature, which leads to very weak interactions with the column matrix, such as silica.

Reverse-phase HPLC was carried out on a Hewlett-Packard series 1050 using a C18 symmetry column (25 cm × 4.6 mm i.d.). The mobile phases are 100 m ammonium acetate, pH 6.6 (solvent A), and acetonitrile (solvent B). The following elution conditions are used:

Step 1: 90% A, 100% B equilibration conditions, 1.0 ml/min.
Step 2: 10 to 21% B over 35 min, linear gradient.
Step 3: Wash with 20% water/ acetonitrile for 5 min.
Step 4: Re-equilibration for 15 min.

2-AMAC derivatized glycans are detected on a Perkin-Elmer LC 240 fluorescence detector at an excitation of 428 nm and emission wavelength of 525 nm. The peaks, collected after injecting 25 ml of derivatized glycans, are lyophilized in 1.5 ml Eppendorf tubes prior to analysis by MALDI-TOF.



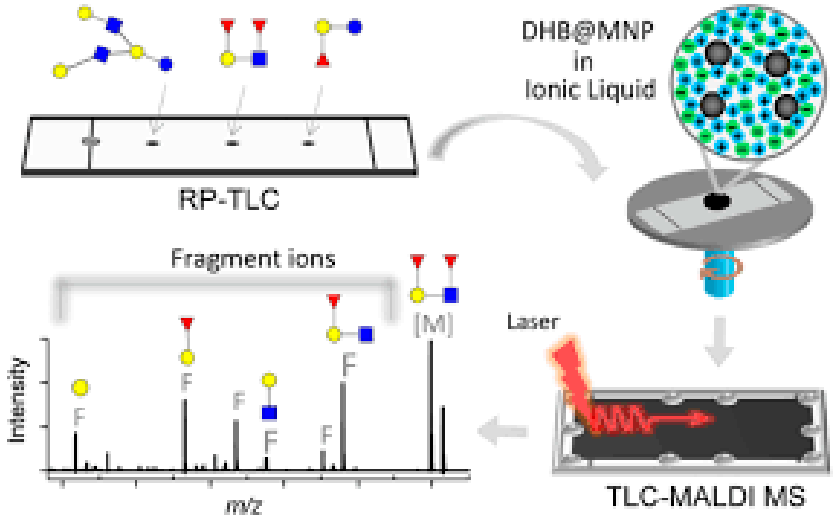


Graphitized Carbon Solid phase extraction (GC-SPE)

Oligosaccharides are purified by graphitized carbon (GC) solid-phase extraction. GC cartridges are washed with 0.10% (v/v) trifluoroacetic acid in 80% acetonitrile/water (v/v) followed by conditioning with water. The oligosaccharide fractions are located onto each cartridge and washed with water at a flow rate of 1 ml/min to remove salts and small mono/disaccharides. Oligosaccharides were eluted with 20% acetonitrile/water (v/v) and dried prior to mass spectrometry (MS) analysis [14].

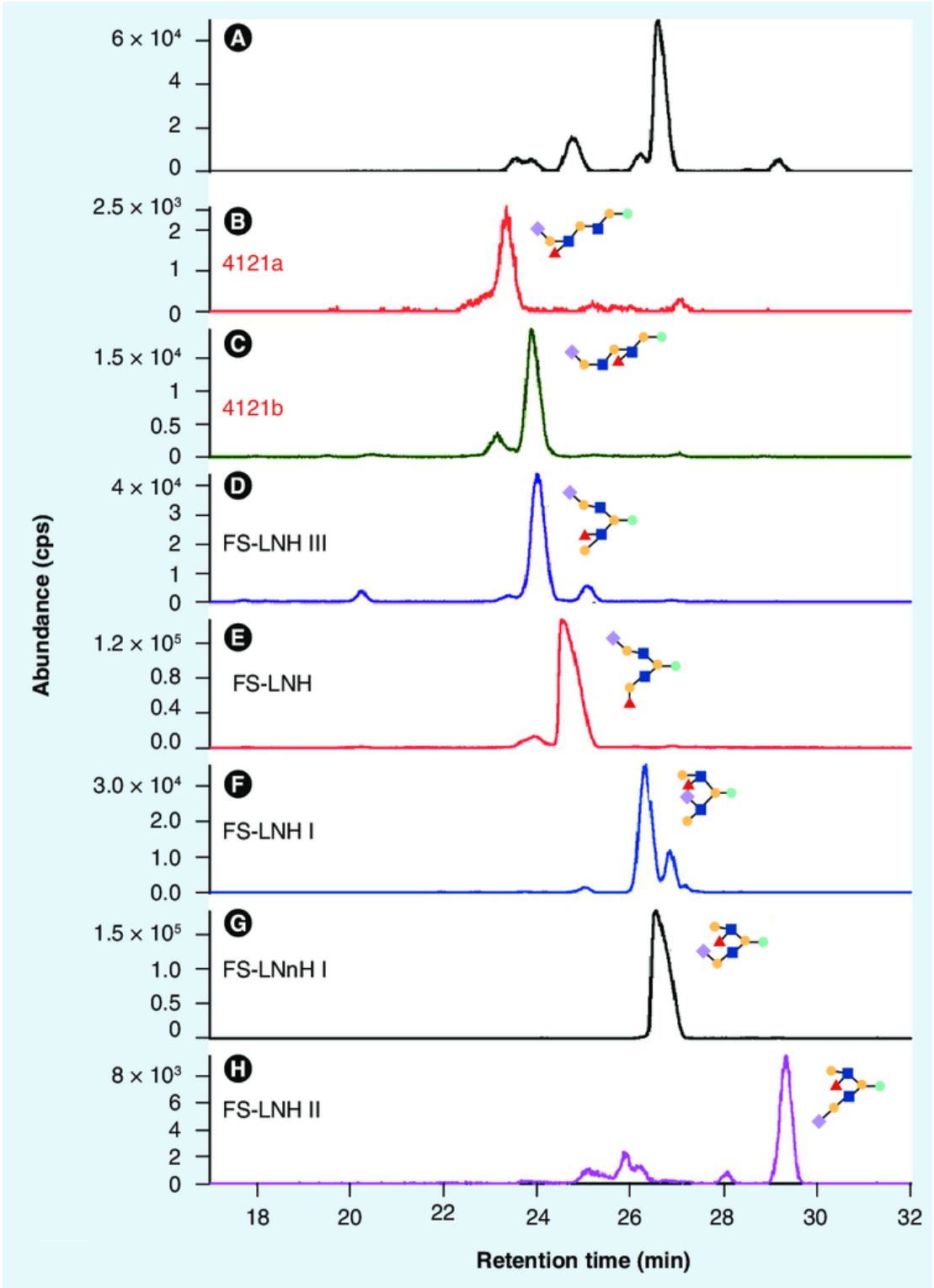
High Performance Liquid Chromatography Mass Spectrometry

Milk oligosaccharides are reconstituted in water and analyzed using an Aligent HPLC-chip Time-of Flight (Chip TOF) Mass Spectrometry (MS) system equipped with a microwell-plate autosampler loading pump, nano pump, HPLC-Chip/ MS interface, and the Agilent 6210 TOF MS detector. The chip consisted of a 9 × 0.075 mm i.d. enrichment column and a 43 × 0.075 mm i.d. analytical column, both packed with 5 um porous graphitized carbon (PGC) as the stationary phase. For sample loading, the capillary pump delivered 0.1% formic acid in 3.0% acetonitrile/water (v/v) isocratically at 4.0 ul/min. The drying gas temperature was set at 325° C with a flow rate of 4 l/min (2 litre of filtered nitrogen gas and 2 litre of filtered dry grade compressed air). MS spectra are acquired in the positive ionization mode over a mass range of m/z 44-2500 with an acquisition time of 1.5 seconds per spectrum [15].



Anion Exchange Chromatography

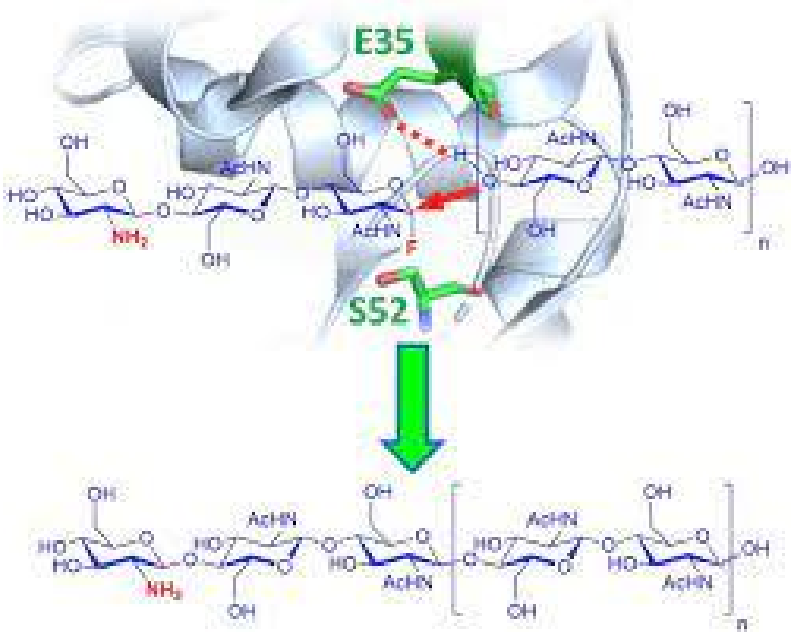
The ionic nature of some types of oligosaccharides make them good candidates for separation using methods based on anion exchange chromatography. Native neutral and acidic oligosaccharides have often been analyzed by HPAEC-PAD, as originally described by Townsend et al. This method takes advantage of the presence of reducing termini of sugars and their ability to become oxidized easily. This transformation causes a current change at the detection electrodes and is recorded as a quantitative signal, reflecting the amount of material being oxidized [6]. The high pH values are used to help deprotonation of the oligosaccharides according to their respective pKa values and thus help to control separation. This method has been applied to the separation of oligosaccharides of different origins into subgroups of neutral, acidic, sulfated, phosphorylated sugars. For example, acidic sugars from glycoproteins have been separated into mono sialylated, tri-, disialylated using HPAEC-PAD. With this method, labeling cannot be used, because the reducing end of carbohydrates must not be blocked. This is in most cases an advantage, and if needed for further analyzes fractions can be dialyzed and labeled after separation [16].



Affinity Chromatography

Some compounds have affinities for carbohydrates and can be useful in the development of separation methods based on affinity chromatography. A very simple example is graphitized carbon which Packer et al. showed is useful for desalting glycans samples before chromatography and/or MS. More on the biological side, lectin-affinity columns such as concanavalin A, lectin, *Phaseolus vulgaris*, erythro agglutin. *Ricinus communis* agglutinin I, *Triticum vulgaris* agglutinin. Glycine max agglutinin and Ulex europaeus agglutinin have been used in a sequential scheme by Hoja-Lukowiez et al. to study branched oligosaccharides in rat liver beta-glucuronidase [17]. Affinity chromatography allowed first proteolytic

glycopeptides into subclasses. Hirabayashi et al. studied the oligosaccharide specificity of galectins by frontal affinity. Galectins have been described as widely distributed sugar-binding proteins whose basic specificity for beta-galactosidase is conserved by evolutionary preserved carbohydrate-recognition domains (CRDs) [18,19].



Size Exclusion Chromatography (SEC)

Monosaccharide and low molecular–weight oligosaccharides have relatively small molecular dimensions and therefore macroporous chromatographic supports currently used for the SEC of higher molecular weight polymers are only seldom used for the GPC of oligosaccharide. This technique involves the transport of a liquid mobile phase through a column containing the separation medium, a porous material. The pores have a specifically controlled range of sizes, and the matrix is chosen for its chemical and physical stability and inertness (lack of adsorptive properties). Gels may be formed from polymers by cross linking to form a three dimensional matrix. For example, a stationary phase for exclusion chromatography is xerogels of polyacrylamide (Bio-Gel) and cross-linked dextran (Sephadex) type. The pores in the gel matrix, which are filled with the liquid phase, are usually comparable in size to the molecules of interest for separation [20, 21].

Gas Chromatography (GC)

Gas chromatography is a separation technique in which the mobile phase is a carrier gas, usually an inert gas such as helium or nitrogen and the stationary phase is a microscopic layer of lipid on an inert solid support, inside glass or metal tubing, called a column. In combination with mass spectrometry (MS), gas chromatography is a sensitive analytical technique for monosaccharide composition analysis, allowing detection of small amounts of carbohydrate. The method provides information on both identity and quantity of the component monosaccharides [22]. The most frequently used GC columns are fused silica wall coated open tubular columns which allow high resolution of peaks even when derivatization results in more than one chromatographic peak per monosaccharide. Although identification of the monosaccharide derivatives is made on the basis of GC retention time, GC-MS provides a further refinement in identification. The mass spectra from these derivatives give fragmentation patterns that can be compared with known spectra obtained from standards. In gas chromatography of milk

oligosaccharides, the glass columns packed with 3.8% of SE-30 (2.0 m × 4.0 mm i.d.; for sialic acid derivatives) or 3% of OY-255 (2.0 m × 2.0 mm i.e.; for partially methylated alditol acetates) on Chromosorb W-HP (100-120 mesh) are used [23].

Capillary Electrophoresis

Capillary electrophoresis offers high mass sensitivity and rapid analysis times. Capillary electrophoresis was employed to separate three sets of structural isomers of sialylated oligosaccharides from human milk and bovine colostrums. Various buffers are used to achieve optimal baseline resolution. To resolve 3'- and 6'- sialyl lactoses, 0.2 M aqueous sodium phosphate containing 40% methanol as an organic modifier was used as a running buffer. To resolve 3' and 6'- sialyl lactosamine, 0.4 M Tris HCL buffer containing 250 Mm sodium dodecyl sulfate and 10% methanol as the organic modifier. For different sets of structural isomers of sialylated oligosaccharides different running buffers with respect to buffer type, concentration, pH, presence of organic modifiers and surfactants are used. Similar electrophoresis condition may be useful for resolving and analyzing other structural isomers of acidic oligosaccharides by capillary electrophoresis. Oligosaccharides could be detected by intrinsic absorbance, absorbance or fluorescence of end-labeled oligosaccharides, or by Direct Current (DC) or pulsed amperometry [24].

Gel Electrophoresis

Gel electrophoresis methods are very user friendly and are fast, it need less equipment and the basic approach uses high-percentage polyacrylamide slab gels to separate fluorescently tagged oligosaccharides by electrophoresis. The resulting gel banding patterns are visualized by sophisticated CCD imagers and fluorography. FACE (Fluorophore Assisted Carbohydrate Electrophoresis) offers a rapid and sensitive method to separate and quantify both monosaccharide and oligosaccharides. Demonstrations of the resolving power of FACE for complex mixtures of oligosaccharides and oligosaccharide isomers are very limited [3, 25].

Paper Electrophoresis

Paper electrophoresis is a time-honored approach for separation of neutral as well as charged oligosaccharides. The norms for it are long run times, high voltages and unpleasant buffer systems. High voltage paper electrophoresis are performed in pyridine-acetate buffer, pH 5.4 (pyridine-acetic acid-water, 3:1:387), or in 0.06 M borate buffer, pH 9.5. Sugars are located either with alkaline AgNO3 or in a Packard radio chromatogram scanner [25].

Conclusion

Above mentioned separation techniques are some of the important and appropriate methods included in chromatography which help in separation and identification of different oligosaccharides present in various sources of milk.

Abbreviations

TLC: Thin Layer Chromatography
GPC: Gel Permeation Chromatography
HPLC: High Performance Liquid Chromatography
SEC: Size Exclusion Chromatography
GC: Gas Chromatography
IEC: Ion Exchange Chromatography

RP-HPLC: Reversed Phase High Performance Liquid Chromatography
GC: Graphitized Carbon
PAD: Pulsed Amperometric Detection
DP: Particle Diameter
MS: Mass Spectrometer
TOF-MS: Time-Of-Flight Mass Spectrometry
HPAEC-PAD: High Performance Anion Exchange Chromatography/Pulsed Amperometric Detection

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