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Proceeding Paper

Optimized Purification Processes for Isolation and Modification of Oligosaccharides from Rathi Cow's Milk †

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Abstract: Purification and characterization of milk oligosaccharides is a challenging process due to the complexity of the constituent oligosaccharides, which behave differently under various chemical treatment procedures and often lose their structural properties in the process. Rathi cow's milk is widely used in the Rajasthan region of India for its nutritional and medicinal benefits. Here, we aim to present an optimized method for the purification and analysis of oligosaccharides present in Rathi cow milk. Contrary to the freeze transport methods used earlier, we treated the collected milk with ethanol for preservation, followed by microfiltration, lyophilization, and fractionation on silica gel (60–120 mesh size) column chromatography (CC) coupled with chloroform/methanol-mediated gradient elution. Fractions 31–45 (1.78 g), 71–80 (470 mg), and 106–120 (498 mg) from CC-1 and fractions 26–49 (1.14 g) from CC-2 were analysed for sugar content via the phenol–sulfuric acid method. Fraction homogeneity was confirmed using high-performance liquid chromatography. Isolated analytes were treated with acetic anhydride/pyridine (1:1, *v/v*) to form less polar oligosaccharide derivatives, which could then be easily visualized and semi-quantitated using partition chromatography (thin later and paper) with chloroform/methanol. Structural identities of the purified oligosaccharides were determined using a combination of mass spectrometry and NMR (^{1}H, ^{13}C, HSQC, TOCSY, COSY, HMBC) techniques. Our results clearly demonstrate that the ethanol-based preservation, transport, and purification of oligosaccharides is a simple and robust method for the analysis of Rathi cow's milk oligosaccharides. Furthermore, using the acetylation, purified oligosaccharides allow for rapid analysis on thin-layer chromatography, which is quite cost effective compared with other analytical methods.

Keywords: CMOs; oligosaccharides; TLC; separation of sugars; NMR

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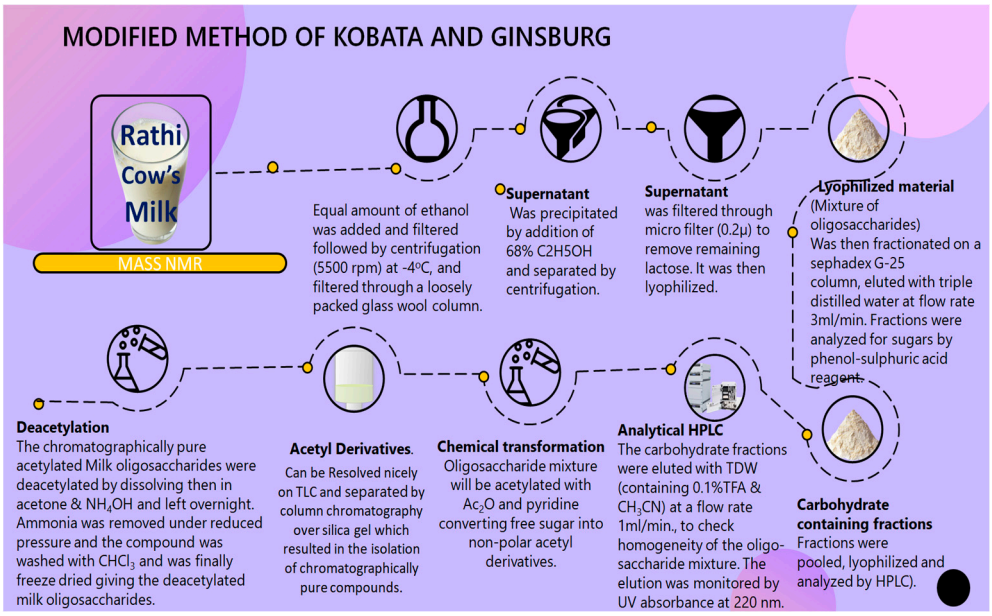
1. Introduction

Various methods for isolating milk oligosaccharides illustrate the development of techniques to improve yield, purity, and specificity in the extraction of oligosaccharides in carbohydrate fractions from milk. Each method uses unique steps to isolate oligosaccharides from other milk components, emphasizing different separation principles such as solvent extraction, ion exchange, gel filtration, and membrane-based filtration. The Weiruszeski technique primarily uses ion-exchange chromatography to isolate sialylated oligosaccharides. By using a pyridine acetate buffer gradient, sialyl-oligosaccharides are successfully eluted using the different charges of the molecules. This method is suitable for the precise isolation of charged oligosaccharides, especially sialylated forms. However, the use of ethanol in this procedure may affect sensitivity to lower abundance oligosaccharides,

which may be a limitation with limited sample quantities. Urashima's method focuses on extraction with organic solvents (chloroform/methanol) to remove proteins, followed by Bio-Gel P-2 column chromatography for fractionation. Its focus on distilled water fractionation minimizes contamination and provides an accurate carbohydrate profile. Urashima's method is particularly characterized by the gentle handling of oligosaccharides, which is of crucial importance for the subsequent analysis. However, organic solvents pose handling risks and potential sample loss, and additional steps are required for specific quantification of sialic acid and hexose levels. The Smith's method uses ethanol precipitation and gel permeation chromatography using Sephadex G-25 to partially isolate oligosaccharides. By applying the Somogyi method of deproteinization, non-carbohydrate impurities are effectively removed, and a crude oligosaccharide fraction is obtained. Although this method is efficient, there may be problems with yield consistency, particularly since it relies heavily on solvent precipitation and cold centrifugation, which could result in partial loss of oligosaccharides during separation. Membrane technology is a sophisticated, two-stage filtration method that utilizes ultrafiltration [1] (50 kDa) followed by nanofiltration [2] (1 kDa) to selectively filter oligosaccharides. Its continuous diafiltration approach effectively separates smaller molecules and retains larger oligosaccharides, ideal for optimized isolation with minimized sample contamination [3]. However, the use of tangential flow requires complex operational controls (e.g., specific transmembrane pressures) and the required equipment and temperature stability (300 °C) make it more demanding and resource intensive than other methods. Egge's method combines centrifugation, acetone precipitation, rotary evaporation, and ion-exchange chromatography [4] for multi-step isolation that includes crystallization to remove lactose. It is able to distinguish sialic acid-containing compounds from neutral oligosaccharides and produce high-purity oligosaccharides suitable for detailed structural analysis. Despite its precision, this approach is time-consuming and can result in losses at each centrifugation and filtration step, potentially reducing the overall yield of oligosaccharides. While each of these methods offer specific advantages such as the simplicity of Urashima [5] and the scalability of membrane techniques, each of these methods comes with inherent trade-offs. Techniques such as Weiruszeski and Egge et al. provide high specificity in oligosaccharide isolation but can require significant time and resource investment [6]. Membrane filtration is characterized by continuous separation and minimization of processing steps but requires precise conditions and equipment. In summary, the choice of method depends on the available volume of the sample, the desired purity of the oligosaccharide, and the resources. Future approaches could combine aspects of these methods to achieve high yield, purity, and practicality in a single optimized protocol.

2. Methods

An adequate quantity, which was 10 L [7], of Rathi cow milk was obtained from a domestic cow in the Panch-Mukhi district of Rajasthan, preserved with ethanol, and centrifuged [8]. The clear filtrate was mixed with additional ethanol until a concentration of 68% was reached [9–11]. The white precipitate, mainly lactose and protein, was purified twice with 68% ethanol. Supernatant and wash water were combined to produce a crude oligosaccharide mixture (283 g). It was then filtered with a micro-filter (0.2 µ) to remove any lactose residues (residue discarded). This oligosaccharide mixture (10 g) was acetylated with Ac_2O and pyridine (1:1, *v/v*) to obtain acetyl derivatives that were less polar and could be easily loaded and run on a TLC plate and chromatography column for analysis and isolation, respectively. Individual fractions from column chromatography were examined by TLC and combined fractions were subjected to analysis for sugars using the phenol–sulfuric acid method (Scheme 1).



Scheme 1. Modified method of Kobata and Ginsburg.

3. Isolation Methods of Milk Oligosaccharides

To characterize oligosaccharides, various kinds of isolation, purification, and separation procedures were used. Researchers including Victor Ginsburg, Akira Kobata, Tadasu Urashima, Stephen Thrul, D.F. Smith, Richard Kuhn, Heinz Egge, and Desh Deepak [9,12–18] have developed a variety of methods for isolation of milk oligosaccharides and worked on their structure elucidation. Thanks to advancements in contemporary chromatographic and physicochemical techniques, such as HPLC [19], gel electrophoresis [20], ion-exchange chromatography [21], and graphitized carbon-based solid-phase extraction (S.P.E.) [22], we can now readily isolate oligosaccharides in their purest form. In our investigation of isolating and revealing the structure of milk oligosaccharides from Rathi cow's milk, we employed a modified variant of the Kobata and Ginsburg method, which had been developed in our laboratory. The procedure devised in our lab is detailed below.

4. Isolation of Cow Milk Oligosaccharides (CMOs)

In this method by Deepak et al., milk samples of Rathi cow were collected and an equal amount of alcohol was added (for preservation); then, the mixture underwent centrifugation for 15 min at 5000 rpm at −4 °C. The solidified lipid layer was eliminated via filtration through a glass wool column under cold atmospheric conditions. Additional ethanol was added to the clear filtrate (supernatant) to achieve a final concentration of 68% to precipitate the lactose and proteins. The solution was left overnight at 0 °C. A white precipitate formed from lactose and protein was removed by centrifugation for 15 min at 5000 rpm at −4 °C. The 68% ethanol was used twice to wash this precipitate. To ensure complete removal of remaining lactose, after passing through a micro-filter (0–24 mm), the supernatant was lyophilized to extract the crude oligosaccharide mixture (283 g). This lyophilized material (crude oligosaccharide mixture) was analysed using the phenol–sulfuric acid reagent to detect presence of the neutral sugar-containing oligosaccharides. These fractions were subsequently utilized to isolate and purify CMOs using gel filtering (Sephadex chromatography).

5. Gel Filtration or Sephadex Chromatography [23]

The fundamental principle of gel-permeation chromatography is the use of dextran-containing materials to separate macromolecules according to the sizes of their molecules. This technique is primarily employed to determine the protein molecules' molecular weights and to reduce the salt concentration in protein solutions. In a gel-permeation

column, the stationary phase comprises inert molecules with small pores. When a solution containing molecules of different sizes is passed through the column at a constant flow rate, larger molecules, which cannot permeate into the gel particles, are retained between the particles in a restricted area. These larger molecules move rapidly through the spaces between the porous particles and quickly traverse the column. Conversely, smaller molecules diffuse into the pores, and, as their size decreases, they possess greater retention time within the column. The most widely used column material is Sephadex G type, although other materials like dextran, agarose, and polyacrylamide are also used.

6. Acetylation of Cow Milk Oligosaccharides

The process of acetylating oligosaccharides in milk has traditionally used acetic anhydride. Essentially, acetylation confirms the existence of -OH groups in a specific moiety. In this context, acetylation is generally performed using Ac_2O with pyridine. This modification makes the milk oligosaccharides less polar, enabling their conventional isolation through column chromatography. Additionally, acetylation helps in determining the positions of glycosidic linkages, improves the resolution and removes the spectral degeneracy of ^1H NMR, and provides better insights into the molecular structure. Non-acetylated oligosaccharides were removed from the mixture of acetylated oligosaccharide (O.S.) mixture via biphasic separation; optimization is provided below. (Figure 1, Table 1)

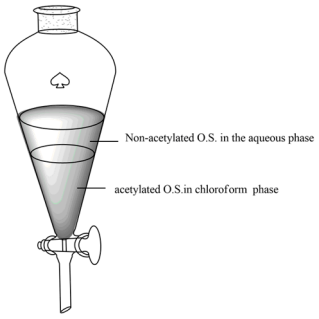


Figure 1. Separation of acetylated CMOs. (OS= Oligosaccharides).

Table 1. Separation efficiency values for various solvent ratios utilized in biphasic separation processes.

Bi-Phasic Solvent Composition	Separation Efficiency for Acetylated Oligosaccharides #	Impurity of Non-Acetylated Oligosaccharides Expected
Chloroform (100%)	N/D	+++++
Chloroform/Water (90:10)	1.57	+
Chloroform/Water (80:20)	1.34	-
Chloroform/Water (70:30)	0.08	-

The symbol "-" indicates a negligible degree, "+" indicates a lower degree, whereas "+++++" signifies the highest degree. # Separation efficiency was calculated by taking the ratio of chloroform solubilized acetylated oligosaccharides to the volume of chloroform taken in the extraction buffer. N/D: value not determined as 100% chloroform contained both acetylated and non-acetylated oligosaccharides.

Following acetylation, we proceeded to purify the milk oligosaccharides using chromatographic techniques, as outlined below.

7. Purification of Cow Milk Oligosaccharides

For qualitative and quantitative analysis, chromatography is an essential biophysical method that makes it easier to separate, identify, and purify the constituents of a mixture. This technique is fundamental in the purification and analysis of milk oligosaccharides. Applying a mixture to a surface or into a solid and liquid stationary phase (stable phase) is the basic idea of chromatography. With the help of the mobile phase, the molecules are separated as they move. Molecular characteristics associated with adsorption (liquid–solid), distribution (liquid–solid), affinity, and fluctuations in molecular weights are key factors

that affect this separation process. Because of these variations, certain mixture components stay in the stationary phase longer and pass through the chromatography system more slowly, while others enter the mobile phase more rapidly and leave the system sooner.

Chromatography involves three main components:

(1) ***Stationary Phase:*** A solid phase or a liquid layer adsorbed on the surface of a solid support makes up this phase;
(2) ***Mobile Phase:*** A liquid or gaseous component makes up this phase;
(3) ***Purified compounds.***

The nature of the interactions among the stationary phase, mobile phase, and the components of the mixture is a critical determinant in the successful separation of molecules. Partition-based chromatographic methods are particularly helpful for separating and identifying small compounds, such as fatty acids, carbohydrates, and amino acids. Below is a description of the different chromatographic methods we used in our laboratory to separate oligosaccharides.

8. Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is a widely used analytical technique for separating non-volatile mixtures. The process is easy, quick, and affordable and allows chemists to quickly gain insight into the nature of a sample. TLC is extensively employed for qualitative analysis and monitoring the progress of chemical reactions in the fields of chemistry, biochemistry, and pharmacology. TLC operates on the principle of the solid–liquid adsorption chromatography technique. In this method, the stationary phase is a solid adsorbent substance, such as alumina, silica gel, or cellulose, coated onto glass plates. The sample mixture is carried by the mobile phase as it ascends through the stationary phase due to capillary action. The mixture is first applied as small dots to the lower part of the plate using a micropipette tip or a fine capillary tube and it separates the analytes at different speeds as the solvent moves upwards. The solvent, solid phase, and material polarity all affect the upward movement rate. Fluorescence, radioactivity, or certain chemicals (visualizers) can be used to produce a visible coloured spots when the sample molecules are colourless, making them easier to locate in the chromatogram. This colour can be observed in both regular and UV light. The position of each molecule in the mixture is measured by calculating the ratio between the distance travelled by the molecule and the solvent. This measurement is called the relative mobility and is expressed with the symbol Rf; molecules are qualitatively described using the Rf value. The Rf values usually differ for different molecules, so their analysis or separation is possible using this technique. If the spots appear streaky or not well-separated, gradually increase the proportion of acetic acid, for example, an optimum ratio of chloroform, methanol, and acetic acid is 96:4:3.

9. Column Chromatography (CC)

Column chromatography is a widely utilized technique for the purification of various substances, including fatty acids, carbohydrates, and amino acids. These compounds exhibit distinct characteristics, such as binding affinity, molecular size, geometric configuration, interactions with the stationary phase, and overall charge. The effectiveness of this method lies in its ability to accommodate the diverse properties of these biomolecules. In the process of column chromatography, a glass column is filled with a stationary phase. Initially, the sample intended for separation is introduced into this stationary phase, followed by the application of a wash buffer that serves as the mobile phase. As the sample components traverse the glass column, they are separated based on their specific interactions with the stationary phase and their varying affinities for the mobile phase. This chromatographic approach facilitates the efficient purification of a range of biomolecules by capitalizing on their unique chemical and physical attributes.

10. Acetylated Milk Oligosaccharide Purification from Rathi Cow Milk on Silica Gel Column [24]

The acetylated cow milk oligosaccharides (CMOs) were purified by packing a silica gel column and eluting with a solvent system of chloroform and methanol in gradient. The oligosaccharide mixture was loaded to the column, and fractions were collected as the solvents eluted the components based on their polarity. The purity of collected fractions was monitored using TLC and then the fractions were pooled, followed by drying under reduced pressure for further analysis. An average ratio of ~1:77 of OS mixture with silica gel (Figure 2) was found useful in chromatography 1–5 (results/tables are provided in the Supplementary File).

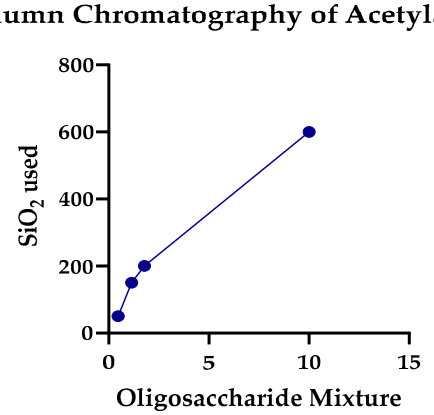


Figure 2. Optimized amount of silica corresponding to the amount of applied material.

11. Conclusions

This research successfully refined the purification and modification techniques for extracting oligosaccharides from Rathi cow's milk, establishing a straightforward, reliable, and cost-efficient method for their analysis. The ethanol-based preservation method addresses the challenges associated with sample collection in remote areas, where immediate access to laboratory facilities is limited, ensuring the integrity of oligosaccharide content during long-distance transportation, combined with a sequence of microfiltration, lyophilization, and silica gel column chromatography. The implementation of gradient elution using chloroform and methanol enabled efficient fractionation, ensuring optimized use of both solvents and silica gel. This approach not only minimized wastage but also enhanced time efficiency by yielding optimal results in the separation process, while the phenol–sulfuric acid method confirmed the sugar content. Additionally, the acetylation process yields less polar acetyl derivatives, which prevent analytes from adhering to the silica gel column or TLC, thereby facilitating efficient separation and analysis, respectively. These advancements not only improve analytical accuracy but also carry significant implications for nutritional and medicinal research on cow's milk. Furthermore, this contributes to a deeper understanding of cow milk's bioactive components and their potential applications in health and wellness.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/blsf2024040021/s1, Table CC1-CC5.

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