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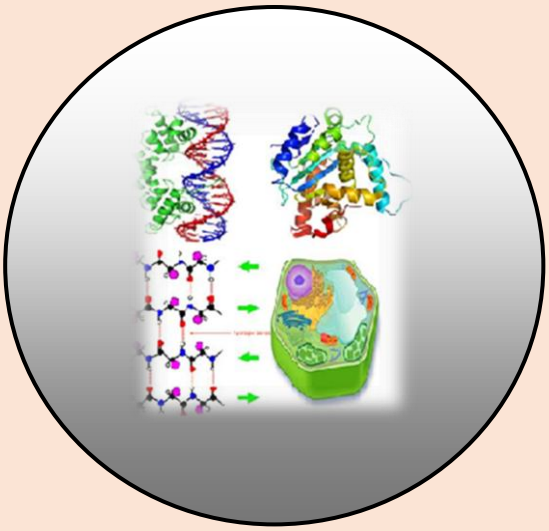

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Lubna Jamal
http:// www.sasjournals.com
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Isolation and Purification of Milk Oligosaccharides by Sephadex Chromatography and High Performance Liquid Chromatography

Lubna Jamal, Anil Mishra and Desh Deepak
Department of Chemistry, University of Lucknow, Lucknow, U.P., India

ABSTRACT

Milk is a natural and rich source of many novel oligosaccharides. For the study of milk oligosaccharides it is important to isolate them from the milk. A variety of Isolation, Separation and Purification techniques are reported for the isolation of milk oligosaccharides. Among different techniques we have used the modified method of Kobata and Ginsburg for the isolation of milk oligosaccharides and for their purification we have used column chromatography, sephadex chromatography and High Performance Liquid Chromatography (HPLC) etc. Sephadex chromatography is used to separate molecules by molecular weight or molecular size. HPLC is a recent technique in analytical chemistry used to separate each component of a mixture. Both sephadex and high performance liquid chromatography are very useful for researchers and widely used for the separation and purification of natural products from their mixture. In our study, we used these techniques for the isolation and purification of milk oligosaccharides.
Keywords: Oligosaccharide, Column chromatography, Sephadex and HPLC.

INTRODUCTION

Sephadex and HPLC are advanced analytical techniques which are extremely valuable in the separation of naturally occurring compounds from their sources. In our study, we worked on milk oligosaccharides. The high polarity of milk oligosaccharides and the presence of other organic compounds in milk with high molecular weights such as- glycoprotein, protein and lactose etc. are main problems in the separation of oligosaccharides. Sephadex and HPLC techniques were employed to overcome these problems. Sephadex sieved out the compounds according to their molecular weights i.e., glycoprotein, protein, etc. are separated from oligosaccharides mixture which are identified by positive phenol-sulphuric acid test. Moreover, HPLC tells us about the homogeneity of oligosaccharide mixture means it tells us about the number of oligosaccharides and their percentage in the oligosaccharide mixture.

SEPHADEX CHROMATOGRAPHY [Wikipedia, Giddings and Hunt, 1989]

Sephadex chromatography is a technique by which molecules in solution are fractionated according to differences in their molecular sizes (or more specifically their hydrodynamic volume) as they pass through a column packed with one of the many chromatographic media available. This technique involves the transport of a liquid mobile phase through a column containing the separation medium, a porous material. A gel is a heterogeneous phase system in which a continuous liquid phase, normally aqueous in nature is contained within the pores of a continuous solid phase, the gel matrix. 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 structure. For example BioGel is formed by cross linking polyacrylamide and sephadex by cross linking dextrans. 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. In this chromatography the molecules are reported on the basis of their molecular size. In case of milk oligosaccharide, they are separated in order of glycoproteins, proteins, oligosaccharides and finally lactose and monosaccharides.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY [Hasan Huda et al., 2018, Marston & Hostettman, 1991, Mellis et al., 1981]

It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules. High performance liquid chromatography (formerly known as High pressure liquid chromatography, HPLC) is principally a highly improved form of column liquid chromatography. It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules. HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention time of the molecules. It operates under the same basic principle and separation of a sample into its constituent parts is done according to the difference in their molecular size, ionic properties and affinity for column packing material. The amount of resolution is important and is dependent upon the extent of interaction between the solute components and the stationary phase. The interaction of the solute with mobile and stationary phase can be manipulated through different choices of both solvents and stationary phases. Many techniques are used in HPLC; the best among them are reversed phase chromatography (RPC), ion exchange chromatography (IEC) and affinity chromatography (AC). The stationary phase used in such HPLC techniques is silica bonded with ionic groups on its surface and glass bonded with ionic groups on their surface. Such analysis is carried out at high pH coupled with pulsed amperometric detection (PAD), allowing separation of oligosaccharides and polysaccharides up to DP>=50. The separation depends on the molecular size, sugar composition and type of linkages between the monosaccharide units. The recent advancement of HPLC over the last decade has enabled higher resolution through both the column efficiency and the ever-expanding range of new sorption materials. 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 [Verzele et al., 1990]

The most important step in the separation of different compounds from a mixture is choosing an appropriate solvent system.

The separation of compounds is the function of the physical and chemical properties of the solvent. In certain cases, first indication of the correct operating conditions is done using TLC analysis, sialylated silica gel plates are used for reversed-phase columns and silica gel plates for normal-phase column.

2. Optimization of analytical columns of small quantities [Synder et al., 1988]

A preliminary analytical search is necessarily required in a HPLC system for the right choice of conditions which saves solvent, time and sample, etc.

3. Optimization of analytical HPLC separation: small capacity factors

For a successful preparative operation, a good analytical HPLC separation is usually required. Relative retention time (selectively, α) plays a very important role for determining possible sample size. It is advisable for one to maximize this value.

4. Scaling of preparative HPLC apparatus [Gareil and Rosset, 1988]

In a large number of HPLC examples, the column is overloaded, nonlinear absorption isotherms are obtained and the peaks are usually not symmetrical in nature.

NORMAL PHASE HPLC [Shirley Churms, 1996]

In this method, analytes are separated on the basis of polarity. NP-HPLC uses two phases for separation, one of which is a polar stationary phase and another is non-polar mobile phase. Silica is the most preferred stationary phase and typical mobile phases are hexane, methanol, chloroform and even mixtures of these. Polar samples are retained on the polar surface of the column packing for longer period than less polar materials. This method is particularly found sensitive to chain lengths which show limited selectivity among similar sized oligosaccharides but having varied stereochemistry. Oligosaccharides which are injected in a high organic (typically acetonitrile) aqueous solvent onto the column are eluted from it by increasing the gradient of aqueous concentration.

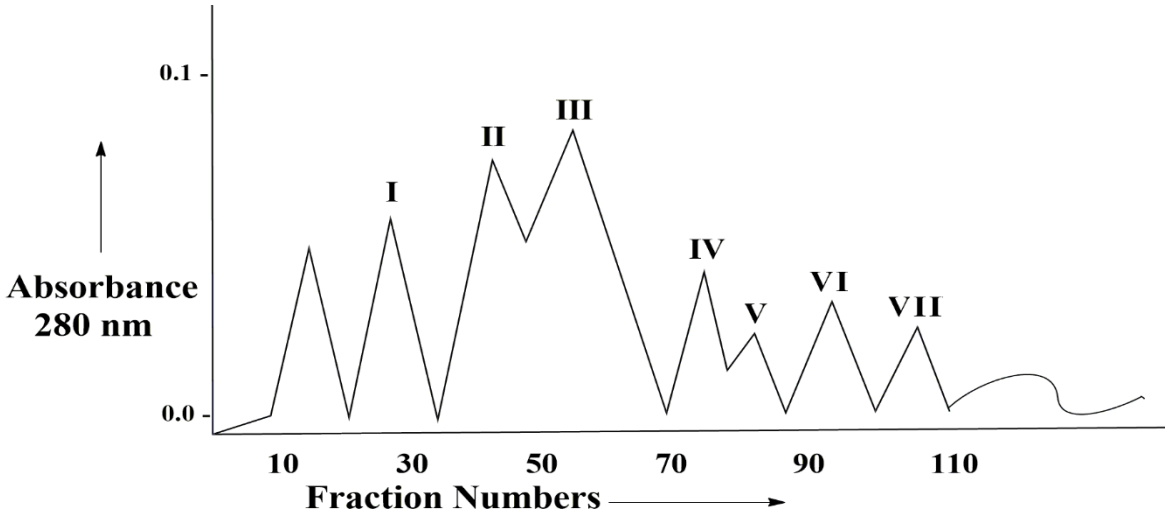
REVERSE PHASE HPLC [Dey et al., 2017, White and Kennedy, 1986, Marie-Isabel et al., 2004, Daniel et al., 1981, Tomiya et al., 1988]

It is an important analytical technique used for separation and determination of milk oligosaccharides. The basis on which the separation is done is the hydrophobicity of the molecule, low polarity stationary phase. The separation is fundamentally an extraction process particularly useful for separating non-volatile components. The process of separation reckons on the hydrophobic binding of the solute molecule (from the mobile phase) to the immobilized hydrophobic ligands (attached to the stationary phase i.e. the sorbent). Only weak interactions are noticed with typical reversed phase packing i.e. octadecyl substituted siliceous packings due to the polarity of underivatized form of oligosaccharides. The use of water as the mobile phase elutes more polar oligosaccharides even before the less polar molecule. Reverse phase chromatography is also found useful for the separation of derivatized mono and oligosaccharide. For this purpose acetonitrile – water [Jaroslava et al., 2017] is used as an eluant for benzoyl derivatives. Similarly, acetonitrile –ammonium acetate pH 7.0 serves as the eluant for acetyl derivatives and acetonitrile 0.01 M potassium phosphate 7.0 as eluant for phenylisocynate derivatives. Another reversed-phase column is Dextropak, essentially a C_{18}, optimized for separation of milk oligosaccharides. Since oligosaccharides suffer a serious drawback that they are very hydrophilic and lack a specific chromophore, almost all of the RP-HPLC methods employ the use of chemical derivatization for the introduction of a hydrophobic chromophore or fluorophore.

EXPERIMENTAL PROCEDURE

PROCEDURE FOR SEPHADEX G-25 GEL FILTRATION OF DIFFERENT MILK OLIGOSACCHARIDE MIXTURE

The repeated gel filtration was performed by Sephadex G-25 chromatography for different Milk oligosaccharide mixture (Single milk is used for Single experiment). The Milk oligosaccharide mixture was packed in a column (1.6 x 40 cm) (void volume = 25 ml) equilibrated with glass triple distilled water (TDW) and it was left for 10-12 h to settle down. The material was applied on a Sephadex G-25 column and was eluted for separation of protein and glycoprotein from oligosaccharide (low molecular weight component). Presence of neutral sugars was monitored in all eluted fractions by phenol-sulphuric acid test. In this U.V. monitored Sephadex G-25 chromatography of Milk oligosaccharide mixture showed different peaks. A substantial amount of proteins, glycoproteins and serum albumin were eluted in the void volume which was confirmed by positive coloration with p-dimethylaminobenzaldehyde reagent and phenol-sulphuric acid reagent. Those fractions which gave a positive phenol-sulphuric acid test for sugars showed the presence of oligosaccharide mixture in the milk. These fractions were pooled and lyophilized.



Sephadex G-25 chromatography of Lal Muha Cow's milk Oligosaccharides

PROCEDURE FOR HPLC FOR THE CONFIRMATION OF HOMOGENITY OF MILK OLIGOSACCHARIDE

Oligosaccharide mixture were quantitatively analysed by reverse phase HPLC. The HPLC system was equipped with Perkin-Elmer 250 solvent delivering system, 235 diode array detector and G.P. 100 printer plotters. The cyano column used for this purpose was a binary gradient system. The eluents were detected at 240 nm. Different peaks were noticed in the sample at the varied retention times.

COMPARATIVE STUDY OF SEPHADEX G-25 GEL FILTRATION AND HPLC OF MILK OLIGOSACCHARIDE MIXTURES OBTAINED FROM VARIOUS MAMMALIAN MILK

After completion of sephadex chromatography and HPLC experiments we got different graphs for different milk oligosaccharide mixtures. The comparative study of all these experiments is as under-

1. LAL MUHA COW MILK OLIGOSACCHARIDE

a. Sephadex G-25 Gel filtration of Lal Muha Cow's Milk Oligosaccharide Mixture

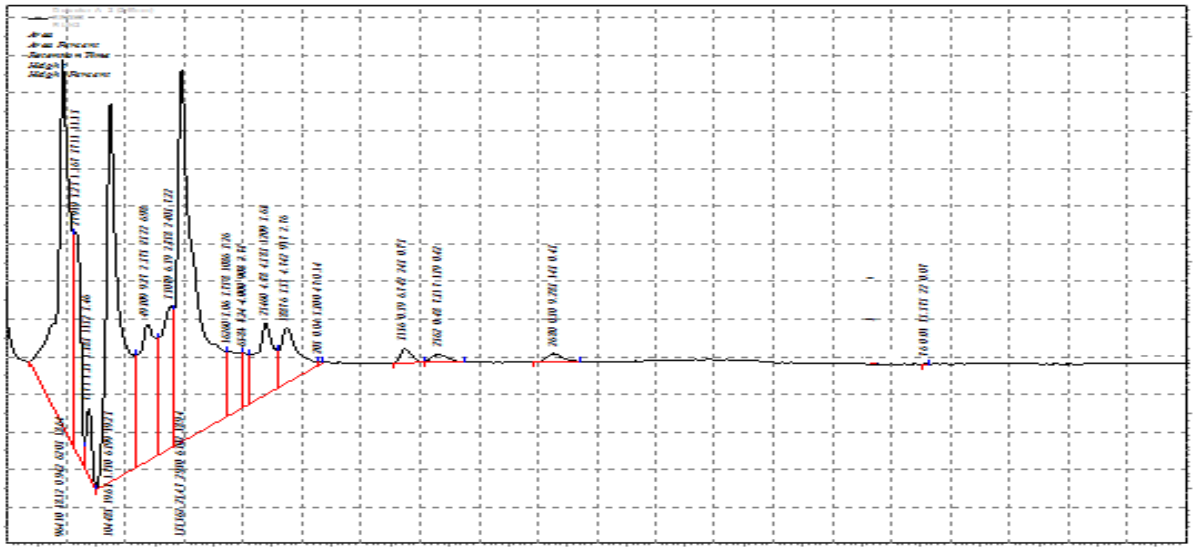
U.V. monitored Sephadex G-25 chromatography of Lal-Muha Cow Milk oligosaccharide mixture showed seven peaks i.e. I, II, III, IV, V, VI and VII. Fractions (6.8 g) under peaks II, III and IV gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in Lal Muha cow milk. These fractions (peak II and III) were pooled and lyophilized.

b) Result of 11.4 gm of Lal Muha Cow's milk oligosaccharide mixture Chromatographed over Sephadex G-25 chromatography

FRACTION NO.	SOLVENT	COMPOUND (gm)	PHENOL H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-30	Glass triple distilled	1.15	-ve	-
				-
31-48	water	2.80	+ve	Fractions under
49-72	,,	2.95	+ve	peaks II+III + IV, 6.8
73-85	,,	1.05	+ve	g, purified by
86-115	,,	0.72	-ve	column
116-150	,,	1.25	-ve	chromatography
151-185	,,	0.85	-ve	after acetylation

c. Confirmation of Homogeneity of Lal Muha Cow milk Oligosaccharide by RP HPLC

Sixteen peaks were noticed in the sample at the varied retention times from 00.942 min. to 15.575 min. for convenience the peaks were numbered in their increasing order of retention time i.e. 15.575 min. (R_1), 09.283 min. (R_2),07.317 min. (R_3), 06.742 min. (R_4), 05.300 min. (R_5), 04.742 min. (R_6), 04.383 min. (R_7), 04.000 min. (R_8), 03.758 min. (R_9), 02.950 min. (R_{10}), 02.758 min. (R_{11}), 02.375 min. (R_{12}), 01.750 min. (R_{13}), 01.383 min. (R_{14}), 01.167 min. (R_{15}) & 00.942 min. (R_{16}).



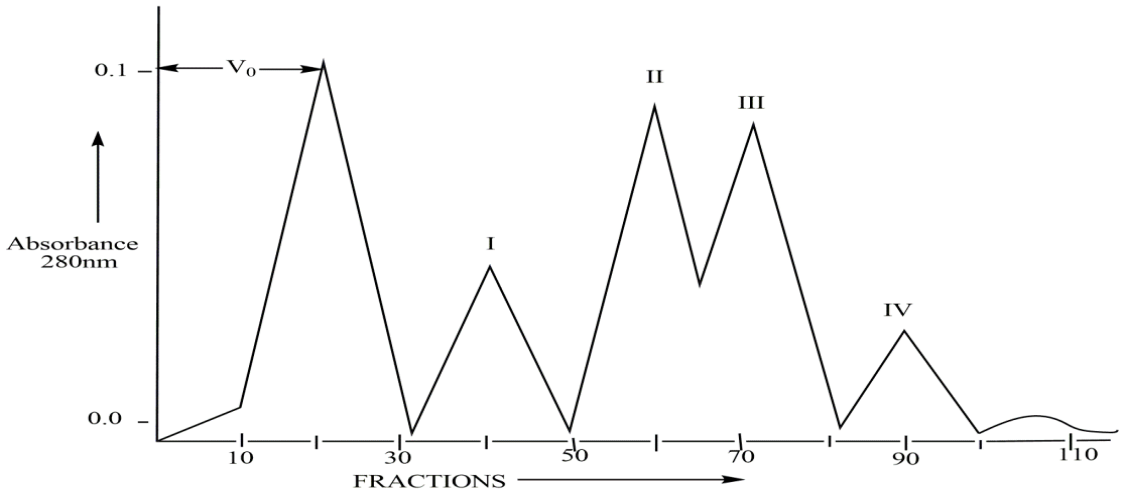
d. HPLC Table of Lal Muha Cow Milk Oligosaccharide

PK	Retention time	Area	Area %	Height	Height%
1	15.575	0022	00.07	000076	00.01
2	09.283	0143	00.43	002680	00.50
3	07.317	0139	00.42	002562	00.48
4	06.742	0243	00.73	003156	00.59
5	05.300	0047	00.14	000205	00.04
6	04.742	0917	02.76	018876	03.55
7	04.383	1209	03.63	025460	04.78
8	04.000	0908	02.73	006581	01.24
9	03.758	1086	03.26	016260	03.06
10	02.950	6302	18.94	135562	25.47
11	02.758	2403	07.22	035079	06.59
12	02.375	2322	06.98	049309	09.27
13	01.750	6399	19.23	104485	19.63
14	01.383	1152	03.46	007171	01.35
15	01.167	3711	11.15	027919	05.25
16	00.942	6204	18.64	096410	18.12

2. COW COLOSTRUM OLIGOSACCHARIDES

a. Sephadex G-25 Gel filtration of Cow Colostrum Oligosaccharide Mixture

U.V. monitored Sephadex G-25 chromatography of Cow colostrum oligosaccharide mixture showed four peaks i.e. I, II, III and IV. Fractions (11.0 g) under peaks II and III gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in cow colostrum. These fractions (peak II and III) were pooled and lyophilized i.e. taken for further investigation.



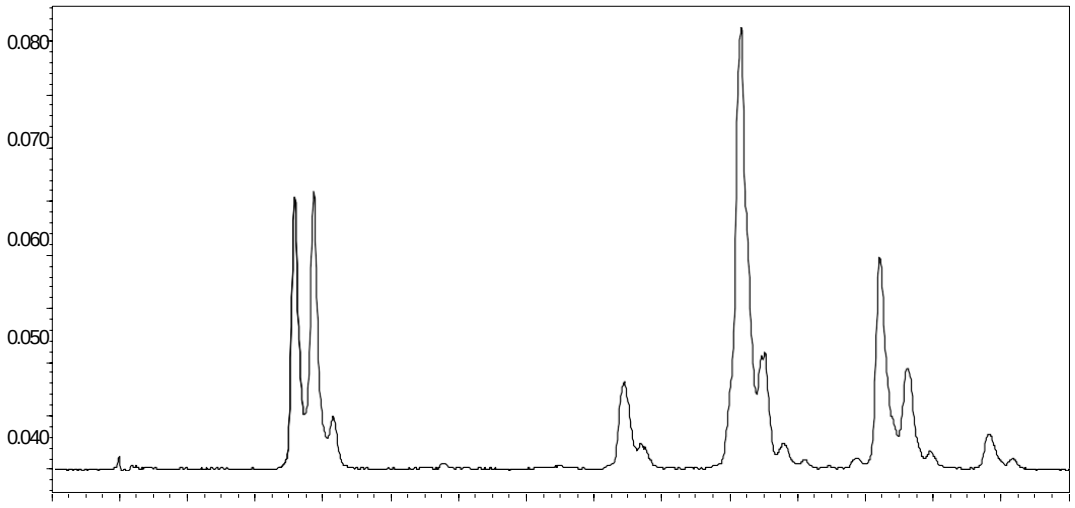
Sephadex G-25 chromatography of Cow Colostrum Oligosaccharides

b) Result of 18.2 g Cow Colostrum oligosaccharide mixture Chromatographed over Sephadex G-25 chromatography

Fraction No.	Solvent	Compound (in gm)	Phenol-H_2SO_4 test for sugar	Further investigation
1-31	Glass triple Distilled	3.53	-ve (I)	
32-46	water	1.47	+ve (II)	Fractions under peak III
47-64	,,	4.50	+++ve (III)	and IV, 11.0 g, purified by
65-78	,,	6.50	+++ve (IV)	column chromatography
79-100	,,	0.52	-ve (V)	after acetylation
	,,			

c. Confirmation of homogeneity of cow colostrum oligosaccharide by reverse phase HPLC

Twelve peaks were noticed in the sample (pooled fraction under peaks III and IV) at the varied retention times from 1.925 min to 19.892min. For convenience the peaks were numbered in their increasing order of retention time *i.e.* [1.925min (R_1), 3.250(R_2), 4.225(R_3), 4.950(R_4), 5.450(R_5), 6.725(R_6), 8.200(R_7), 10.233(R_8), 13.250(R_9), 15.433(R_{10}), 19.000(R_{11}) and 19.892(R_{12})



d. HPLC table of cow colostrum oligosaccharide

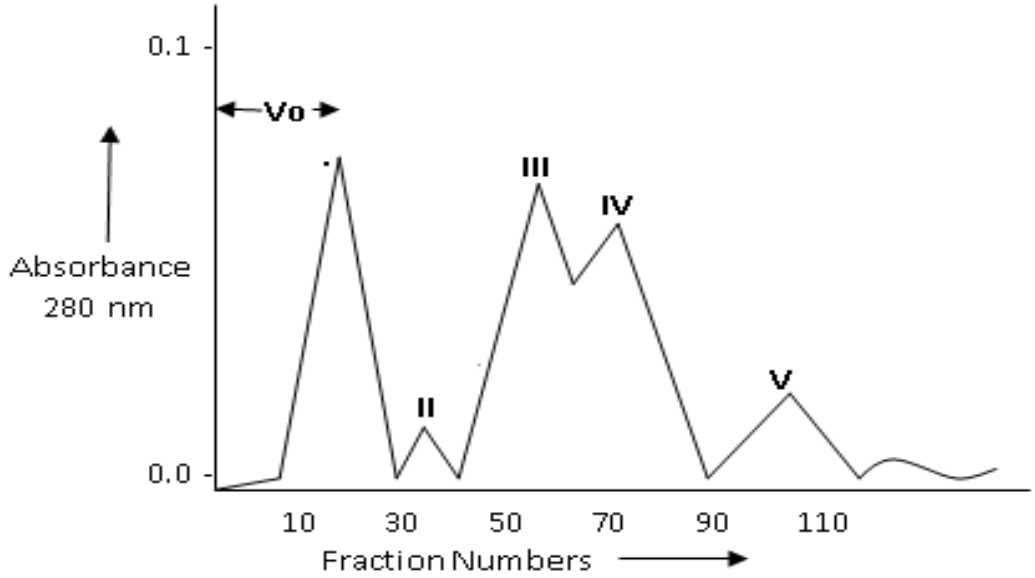
Peak	Retention time	Area	Area%	Height	Height%
1	1.925	2510836	7.210	25646	2.492
2	3.250	5392454	15.484	222236	21.594
3	4.225	3791632	10.887	83985	8.161
4	4.950	1942657	5.578	96702	9.396
5	5.450	2557166	7.343	50398	4.897
6	6.725	5147728	14.781	86464	8.402

7	8.200	3526955	10.127	39140	3.803
8	10.233	1908336	5.480	44421	4.316
9	13.250	1941528	5.575	92688	9.006
10	15.433	683106	1.961	16513	1.605
11	19.000	1899121	5.455	171444	16.659
12	19.892	197146	0.566	11938	1.160

3. BUFFALO MILK OLIGOSACCHARIDE

a. Sephadex G-25 Gel filtration of Buffalo milk Oligosaccharide Mixture

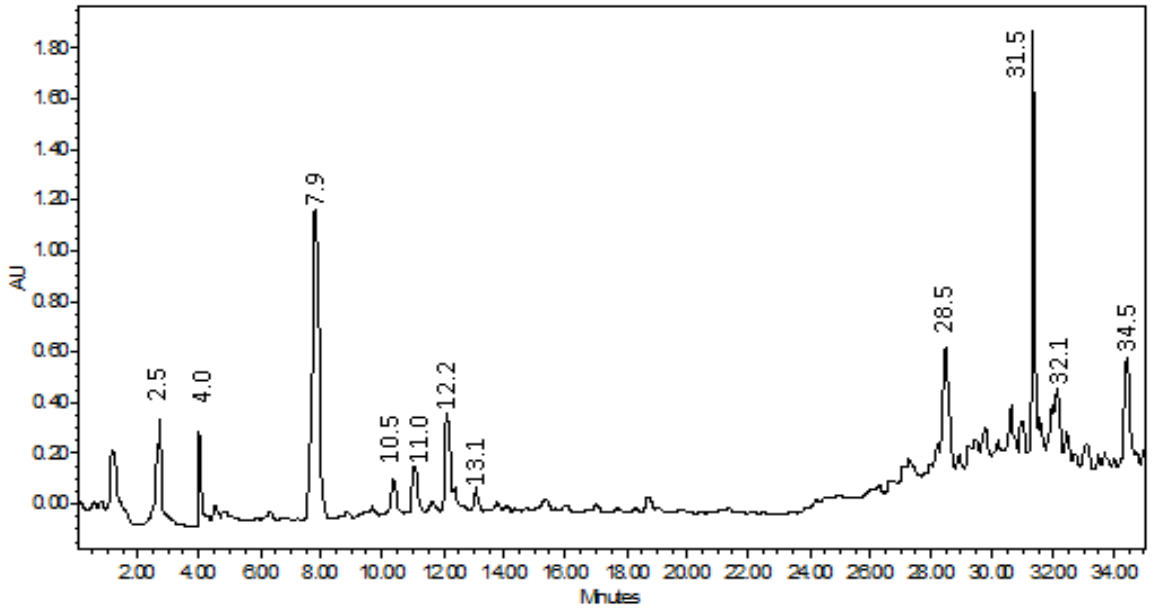
U.V. monitored Sephadex G-25 chromatography of Buffalo milk oligosaccharide mixture showed five peaks i.e. I, II, III, IV and V. Fractions under peaks III and IV gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in Buffalo. These fractions (peak III and IV) were pooled and lyophilized.



Sephadex G-25 Chromatogram of Buffalo Milk Oligosaccharides

b. 22.5 gm of Buffalo Milk Oligosaccharide Mixutre Chromatographed Over Sephadex G-25 Chromatography

FRACTION NO.	SOLVENT	COMPOUND (grams)	PHENOL-H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-30	Glass triple distilled water	3.2	-ve (I)	-
31-42	,,	1.8	-ve (II)	Fractions under peak III and IV, 14.7 g, purified by column chromatography after acetylation
42-68	,,	10.0	+ve (III)	
69-92	,,	4.7	+ve (IV)	
93-120	,,	1.4	-ve (V)	
	,,			



HPLC chromatogram of Buffalo Milk Oligosaccharides

c. Confirmation of Homogeneity of Buffalo milk oligosaccharide by RP-HPLC

Eleven peaks were noticed in the sample (pooled fraction III and IV). For convenience the peaks were numbered in their increasing order of retention time i.e. 2.5 (R_1), 4.0 (R_2), 7.9 (R_3), 10.5 (R_4), 11.0 (R_5), 12.2 (R_6), 13.1 (R_7), 28.5 (R_8), 31.5 (R_9), 32.1 (R_{10}), 34.5 (R_{11}).

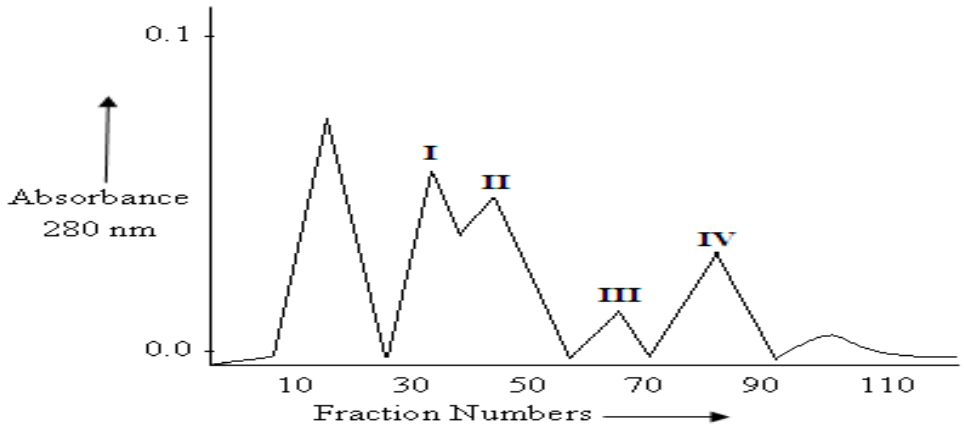
d. HPLC Table of Buffalo Milk Oligosaccharides

S. No.	Retention Time	Percentage of Peak Area (Analyte)
1.	2.5	8.0
2.	4.0	7.0
3.	7.9	18.0
4.	10.5	4.5
5.	11.0	6.5
6.	12.2	7.5
7.	13.1	3.5
8.	28.5	9.3
9.	31.5	19.0
10.	32.1	8.2
11.	34.5	8.5

4. CAMEL MILK OLIGOSACCHARIDE

a. Sephadex G-25 Gel filtration of Camel milk Oligosaccharide Mixture

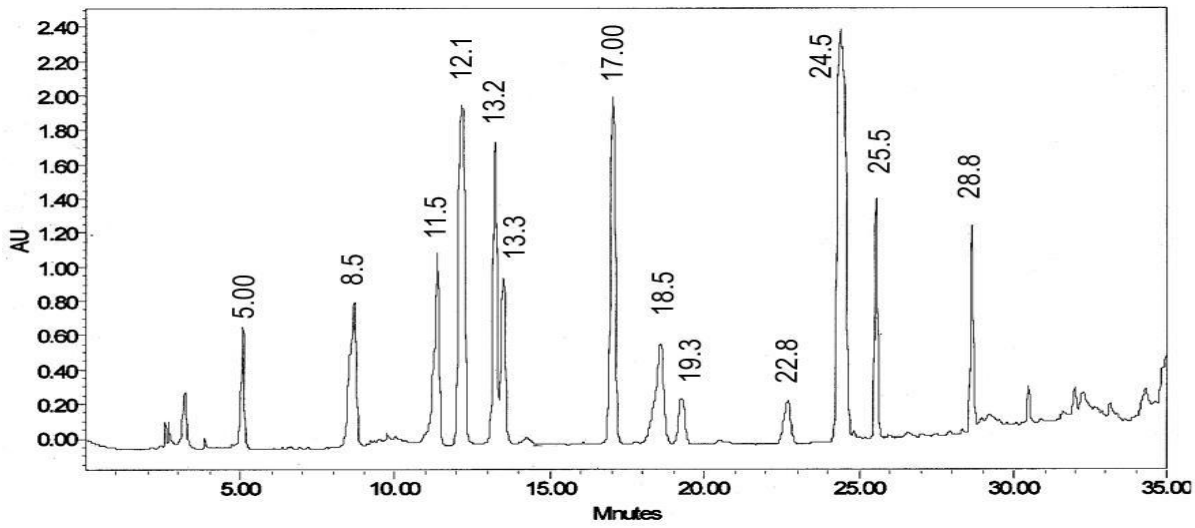
U.V. monitored Sephadex G-25 chromatography of Camel milk oligosaccharide mixture showed four peaks i.e. I, II, III and IV. Fractions under peaks III and IV gave a positive phenol-sulphuric acid test for sugars which showed the presence of oligosaccharide mixture in Camel milk. These fractions (peak III and IV) were pooled and lyophilized.



Sephadex G-25 Chromatogram of Camel Milk Oligosaccharides

b. Result of 11.99 g Camel Milk Oligosaccharide Mixture Chromatographed Over Sephadex G-25 Chromatography

FRACTION NO.	SOLVENT	COMPOUND (grams)	PHENOL-H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATION
1-25	Glass triple distilled water	1.79	-ve (I)	-
26-41	,,	1.87	-ve (II)	-
42-58	,,	2.27	+ve (III)	Fractions under peaks III and IV, 6.13 g, purified by column chromatography after acetylation
59-84	,,	3.86	+ve (IV)	
85-110	,,	2.20	-ve (V)	



c. Confirmation of Homogeneity of Camel Milk Oligosaccharide by RP-HPLC

Thirteen peaks were noticed in the sample at the varied retention times from 5.00min. to 28.8 min. for convenience the peaks were numbered in their increasing order of retention time i.e. 5.00 min. (R1), 8.5 min. (R2), 11.3 min. (R3), 12.1 min. (R4), 13.2 min. (R5), 13.3 min. (R6), 17.00 min. (R7), 18.5 min. (R8), 19.3 min. (R9), 22.8 min. (R10), 24.5 min. (R11), 25.5 min. (R12) and 28.8 min. (R13).

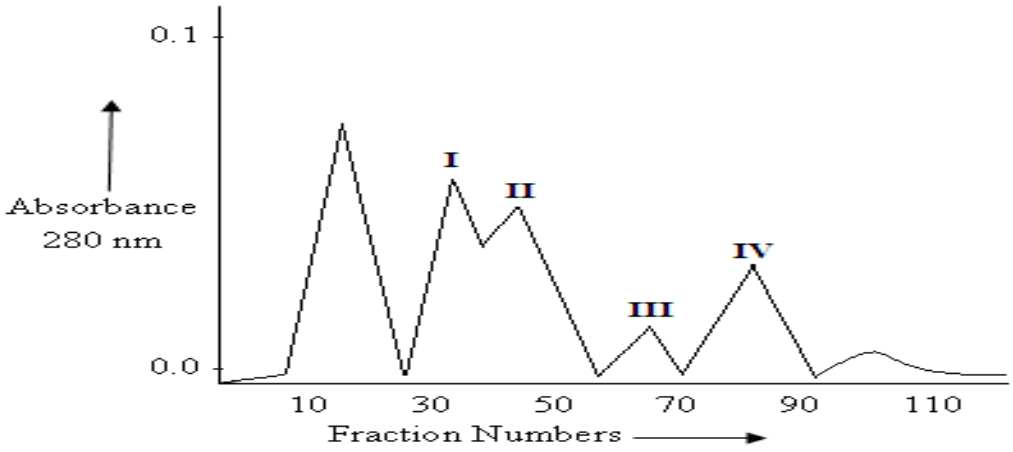
d. HPLC Table of Camel Milk Oligosaccharides

Peak Number	Retention Time	% Area of each peak
1	5.00	4.2
2	8.50	5.3
3	11.3	6.5
4	12.1	7.0
5	13.2	12
6	13.3	6
7	17.00	11
8	18.5	5
9	19.3	4
10	22.8	4
11	24.5	15
12	25.5	7
13	28.5	9

5. YAK MILK OLIGOSACCHARIDE

a. Sephadex G-25 Gel filtration of Yak milk Oligosaccharide Mixture

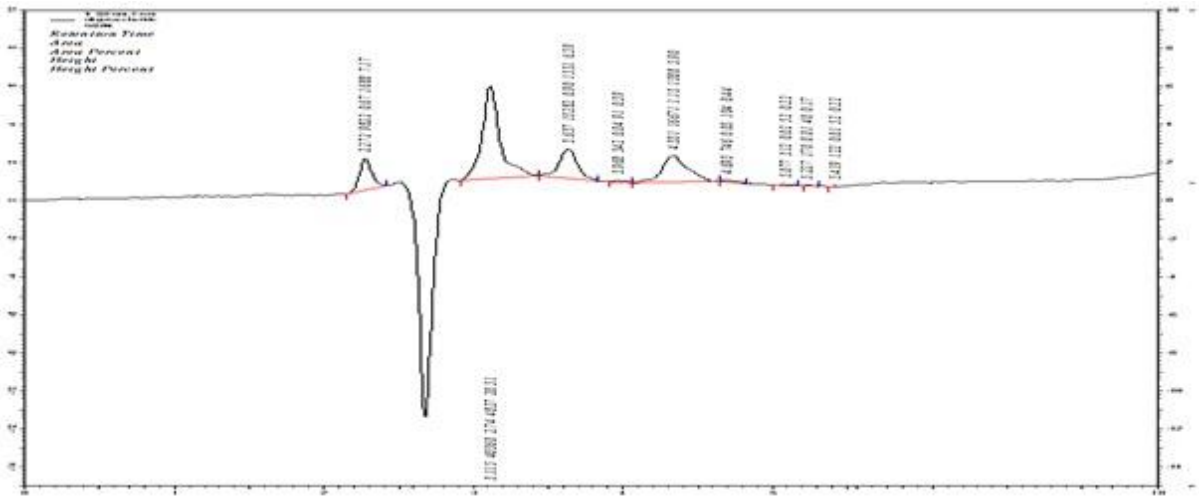
The sephadex G-25 chromatography of yak milk oligosaccharide mixture which was monitored by UV spectrophotometry showed four peaks i.e. I, II, III and IV. Fractions under peaks I and II gave a positive phenol-sulphuric acid test which showed the presence of oligosaccharide mixture in yak milk. They were pooled together and lyophilized. Repeated sephadex chromatography was performed to obtain 6.5 gm of oligosaccharide mixture.



Sephadex G-25 chromatography of Yak's milk oligosaccharides

b. Result of 10 gm of Yak milk oligosaccharide mixture chromatographed over Sephadex G-25 chromatography

FRACTION NO.	SOLVENT	COMPOUND (grams)	PHENOL-H_2SO_4 TEST FOR SUGAR	FURTHER INVESTIGATI-ON
1-27	Glass triple distilled water	1.97	-ve (I)	-
28-42	,,	0.92	-ve (II)	Fractions under peaks III, IV and V, 4.33g, purified by column chromatography after acetylation
43-56	,,	1.27	+ve (III)	
57-87	,,	1.86	+ve (IV)	
88-106	,,	1.20	+ve (V)	



c. Confirmation of Homogeneity of Yak Milk Oligosaccharide by Reverse Phase HPLC

Fraction III and IV obtained from Sephadex G-25 column, containing oligosaccharide mixture were qualitatively analyzed by reverse phase HPLC. The eluants were detected at 220 nm. Nine peaks were noticed in the sample (pooled fractions I and II) at the varied retention times from 2.272 min. to 5.419 min. The peaks were numbered in increasing order of retention time i.e. 2.272 (R_1), 3.115 (R_2), 3.637 (R_3), 3.968 (R_4), 4.331 (R_5), 4.693 (R_6), 5.077 (R_7), 5.227 (R_8) and 5.419 (R_9).

HPLC chromatogram of Yak Milk Oligosaccharides

d. HPLC Table of Yak Milk Oligosaccharides

Pk#	Retention time	Area	Area (%)	Height	Height (%)
1	2.272	9822	0.67	1688	7.17
2	3.115	40360	2.74	4827	20.51
3	3.637	13282	0.90	1551	6.57
4	3.968	542	0.04	91	0.39

5	4.331	16671	1.13	1388	5.90
6	4.693	746	0.05	104	0.44
7	5.077	312	0.02	52	0.22
8	5.227	170	0.01	40	0.17
9	5.491	122	0.01	52	0.22

RESULT AND DISCUSSION

In this study, the graphs and data obtained by sephadex chromatography and HPLC for different milk oligosaccharide mixture were taken and their comparative study was performed. Sephadex G-25 chromatography of Lal-Muha Cow Milk oligosaccharide mixture showed seven peaks while its HPLC showed sixteen peaks. These numbers are higher than other milks which were taken for the study such as-cow colostrum, buffalo milk, camel milk and yak milk. Cow colostrum, buffalo milk, camel milk and yak milk showed four, five, four and four peaks in sephadex chromatography respectively whereas their HPLC spectrum showed twelve, eleven, thirteen and nine peaks respectively. The comparison of these milks is explained with the help of following tables-

TABLE 1. FOR SEPHADEX CHROMATOGRAPHY EXPERIMENTS.

S. NO.	MILK OLIGOSACCHARIDE TAKEN	QUANTITY CHARGED (gm)	QUANTITY OBTAINED (gm)	QUANTITY OF OLIGOSACCHARIDE MIXTURE OBTAINED (gm)
1.	Lal-Muha cow milk	11.4	10.67	6.80
2.	Cow colostrum	18.2	16.52	11.0
3.	Buffalo milk	22.5	21.10	14.7
4.	Camel milk	11.9	11.79	6.13
5.	Yak milk	10.0	7.22	4.33

Table 2. A COMPARATIVE TABLE OF RESULTS OBTAINED BY SEPHADEX CHROMATOGRAPHY AND HPLC FOR DIFFERENT MILK OLIGOSACCHARIDES

NAME OF THE MILK	NO. OF PEAKS OBTAINED AFTER SEPHADEX CHROMATOGRAPHY	LYOPHILIZED FRACTIONS	NO. OF PEAKS OBTAINED BY HPLC	RETENTION TIME RANGE (MIN.)
Lal-Muha cow milk	Seven (I, II, III, IV, V, VI and VII)	Peak II and III	Sixteen	0.942-15.595
Cow Colostrum	Four (I, II, III and IV)	Peak II and III	Twelve	1.925-19.892
Buffalo milk	Five (I, II, III, IV and V)	Peak III and IV	Eleven	2.50-34.50
Camel milk	Four (I, II, III and IV)	Peak III and IV	Thirteen	5.00-28.50
Yak milk	Four (I, II, III and IV)	Peak I and II	Nine	2.272-5.491

CONCLUSION

After comparing all the graphs and data obtained for different oligosaccharide mixtures from sephadex G-25 chromatography we can conclude that among all the fractions only those fractions which have oligosaccharide mixture shows the positive phenol sulphuric acid test (generally middle fractions).

After that the chromatogram obtained from HPLC shows various peaks with different retention times for different milk oligosaccharide mixture.

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Corresponding author: Prof. Desh Deepak, Department of Chemistry, University of Lucknow, Lucknow, U.P., India
Email: deshdeepakraju@rediffmail.com