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A novel method for quantification of lactose in different mammalian milk through HPTLC & determination by Mass Spectrometric technique

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Abstract

Lactose molecules are chemically disaccharide and basic core unit of sugars (oligosaccharides) which is enrich in mammalian milk. Lactose plays multiple roles in the health of the neonates, by stimulating growth of selected beneficial bacteria in the gut, participating in development and growth of new born. All mammalians produces lactose with oligosaccharides (LOS) in different concentration during their lactation period. In this study we presenting a novel high-performance thin-layer chromatography (HPTLC) method for estimation of lactose concentration present in milk of different mammalian *viz:* Indian mare (*Equus caballus*), Cow (*Bos primigenius* indicus), Buffalo (*Bubalus bubalis*), Camel (*Camelus dromedarius*) and Donkey (*Equss asinus*). Separation and quantification were achieved by using ternary mobile phase of n-butanol: glacial acetic acid: water (75:10:15 *v/v/v*) on precoated silica gel $60F_{254}$ aluminium plates; lactose standard in milk samples were also confirmed by mass spectrometric determination and densitometric determination of HPTLC plate were carried out after derivatization with methanol-sulphuric acid reagent. After HPTLC densitometric scanning, it was analyzed that concentration of free lactose in milk of Mare, Cow, Camel, Buffalo & Donkey were 2.16 %, 5.70 %, 6.70%, 7.17 % & 6.38 % respectively.

Keywords: Mammalian Milk, Lactose with oligosaccharides (LOS), Lactose, HPTLC, Quantification

1. Introduction

Lactose is most abundant solid components in mammalian milk. The relatively high concentration of lactose in milk obscured until recently the presence of the oligosaccharide fraction, which was initially described with the name "gynolactose" and was later analysed and characterised by Grimmonprez and Montreuil[1]. In the same account researchers hypothesised that lactose induces the biosynthesis of oligosaccharides and act as a basic key molecule. We know that milk of different mammals containing variable concentrations of lactose but contain a limited repertoire of oligosaccharides[2,3].

Lactose is disaccharide molecule (**Fig.1)** catabolised into glucose and galactose by enzyme lactase. Lactose-intolerant individuals have a lactase deficiency; therefore, lactose is not completely catabolised in its monomers. While lactose intolerance is not a dangerous condition, its global prevalence has created a large market for lactose-free products. Commercially available lactose free products are produced by breaking down lactose into glucose and galactose by enzymatic hydrolysis[4-7]. However, the milk of mammalian species and its products containing varying amounts of residual lactose which is unknown. So this has created the need for simple, reliable, and accurate analytical method to quantify lactose concentration in milk and milk made products of different mammalians. This type of study may helpful for population specially suffering from lactose intolerance to choose the milk and its products having less quantity of lactose compound.

Currently available analytical methods for the detection of lactose include mid-infrared detection, fluorometry[8], online dialysis[9], polarimetry, colorimetric method, spectroscopic, gravimetric detection, spectrophotometry [10-12], amperometric biosensor[13], HPLC[14], differential pH techniques and enzymatic assays[15-19]. These methods are time-consuming because of extensive sample preparation and cannot differentiate individual carbohydrates.

To resolve these problems for accurate quantification of lactose, we develop the cost effective, rapid and reliable high performance thin layer chromatographic (HPTLC) method. To the authors' best knowledge, this is the first report on the simultaneous quantification of lactose in milk samples of different mammals by HPTLC and EI-MS technique.

2. Experimental

2.1 Chemicals and Materials

All chemicals were laboratory grade and solvents were analytical grade, lactose was purchased from Central Drug House, HPTLC plates Silica higlachrosep Nano UV 60 F254 [20 cm x 10 cm] were procured from S D fine-chem Limited, Mumbai, India.

2.2 Milk collection

Milk samples were collected from Indian mare (*Equus caballus*) body weight 430 kg & age about 41 months, Cow (*Bos primigenius* indicus) body weight 393 kg & age about 24 months, Camel (*Camelus dromedarius*) body weight 771 kg & age about 38 months, Buffalo (*Bubalus bubalis*) body weight 450 kg & age about 26 months and Donkey (*Equss asinus*) body weight 238 kg & age about 32 months mammalians [20, 21] at Badshahbagh Animal Hospital, Lucknow. All animals were kept in a controlled room temperature at 25$\pm$ 2^0C, humidity was 50-70 % and 12:12 hrs light / dark cycle, light from 06:00 to 18:00, under hygienic conditions; animals were acclimatized for one week before starting the experiment. The animals had free access to normal diet and water. The study was approved by the Animal Ethics Committee of Directorate, Department of Animal Husbandry, Badshahbagh, Lucknow, Uttar Pradesh (India).

2.2.1 Isolation of Lactose with oligosaccharide (LOS) from milk

For quantification of lactose in milk samples of different mammalian species, 100 mL milk samples were collected from each mammal at Animal house, Directorate of Animal Husbandry, Lucknow, U.P. (India). Diet and atmospheric condition of mammals was normal, milking process was hand made from each teat of animals and collected milk samples were kept at -20 °C temperature. To isolate LOS from milk sample, all samples to keep at normal temp and add 20 % ethanol and centrifuged at 1500 g for 20 min at -4^0C. The upper solidified lipid layer was removed by filtration through glass wool column in cold atmospheric condition and proteins were removed by centrifugation of remaining filtrate at 6500 x g, proteins are settle down in bottom of centrifuge tube and filtrate was lyophilized to get crude LOS mixture which was further purified over Sephadex G-25 column.

2.2.2 Sephadex G-25 gel filtration of Lactose with oligosaccharides (LOS)

This lyophilized material LOS was further purified on Sephadex G-25 chromatography [22] for separation of micro and macro molecules from LOS fraction. Glass triple distilled water (TDW) was used as mobile phase, LOS mixture was packed in column (1.6 ×40 cm) (void volume =25 mL) and the flow rate of mobile phase was 1 mL in 3 minute. A substantial amount of remaining proteins; glycoproteins and casein were eluted in very small amount in some fractions, was confirmed by positive colouration with p-dimethylaminobenzaldehyde and phenol-sulphuric acid reagent. LOS fractions gave a positive phenol-sulphuric acid test showed the presence of lactose and oligosaccharides **(Table 1)**. These fractions were polled & lyophilized together and used for HPTLC analysis.

2.3 Preparation of crude extract

Accurately weighed 1.0 mg of the coarse powder of LOS samples was dissolve in HPLC grade water to prepare 1 mg / mL solution for HPTLC analysis.

2.4 Preparation of standard and working solutions

Stock solution of lactose standard was prepared separately by dissolving 1 mg lactose in 1 mL HPLC grade water. A stock solution of 1000 µg /1000 µL of standard were further diluted with same solvent to obtain working solutions of concentration of 100µg/ mL for further analysis.

2.5 Chromatographic Instrumentation

HPTLC was performed on 20 cm × 10 cm TLC aluminum plates pre-coated with 200 µm layer thickness of silica gel 60F_{254} (sd. Fine chem. Ltd, Mumbai, India). Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland) was used for sample and standards application on HPTLC plate. TLC developing chamber was a twin-trough made up from Camag (Muttenz, Switzerland), detection was achieved by a Camag TLC Scanner 3 densitometer (Muttenz, Switzerland) with user-friendly winCATS Software (Version 3.2.1) installed on a computer. The statistical was achieved by using Microsoft Excel Windows software.

2.6 High Performance Thin Layer Chromatographic Procedure

Lactose with oligosaccharides samples (LOS) of all five mammalian species and six analytical standards of lactose was applied on HPTLC glass plates with 6 mm band width using Camag 100

microlitre sample syringe (Hamilton, Switzerland) with a Linomat 5 applicator (Camag, Switzerland) under a flow of N_2 gas. The Linear ascending development was carried out with n-butanol: glacial acetic acid: water (75:10:15 *v/v/v*) as a mobile phase in two Camag glass twin trough chamber (20 x 10 cm). Both chambers were previously saturated with mobile phase vapour for 30 minutes and total run time were about 45 minutes for both TLC plates at room temperature (27 ± 2°C) relative humidity was 50 % $\pm$ 2 %. After run, plate no. (I) was dried through hair drier and derivatized with phenol sulphuric acid reagent and stand until plate was dried and kept it on hot plate to revel the bands of all tracks of standards & samples. After this, scanning of HPTLC plate were performed by using Camag TLC Scanner 3 at λ_{600} nm in visible absorbance mode for all tracks, TLC plate were developed at distance of approximately 80 mm from the point of application and slit dimensions were 5 mm × 0.45 mm and the scanning speed was100 mm/s and winCATS Software [Version 3.2.1] was used for scanning of HPTLC. Another plate (II) was used for mass spectrometric studies of marker compound Lactose in milk samples.

2.8 Confirmation of markers compounds by Mass Spectrometric analysis

After scanning of derivatized plate (HPTLC plate no. I) and underivatized preparative glass plate (HPTLC plate no. II) put together on equal surface and the exact positions of Lactose compound as appear in plate (I) were marked with a pencil on preparative plate (II) in all tracks of milk samples and after this, scratch out the marked area of lactose standard from plate no (II) with the help of spatula and needle further it were dissolve in 50% ethanol in water and allow to stand for 60 minutes and supernatant was purified with 0.22 microne filter paper. Mass spectrometric measurements for standard lactose and lactose obtained from TLC plate were recorded on JEOL JMS-D-300 spectrometer with the ionization potential of 70 eV, Electron ionization (EI) mass spectra on Quantro-II mass at 800 amu / sec scanning in positive ion mode. After analysis of Mass spectra of compounds, it was observed that lactose standard present with molecular mass of m/z 343 (M+H), m/z 360 (M+ H_2O) in all milk samples.

2.9 Quantification of lactose in milk oligosaccharide samples

10 µL samples of each LOS samples were applied through Linomat 5 applicator on a TLC plate, developed & scanned at λ_{600} visible mode. Peak area was recorded in each sample & standard tracks and the amount of lactose in all samples were calculated by using the calibration plot.

3. Results

3.1 Method optimization

For quantification of Lactose standard in milk samples, one binary and two ternary mobile phase were tried; n-butanol: acetic acid: water (11:4.5: 4.5 *v/v/v*), n-butanol: glacial acetic acid: water (75:10:15 *v/v/v*), *v/v*) and chloroform: methanol: water (85:10:05, *v/v/v*) to avoid interference from the other constituents. The best chromatogram of lactose standard was obtained with second mobile phase system i.e. n-butanol: glacial acetic acid: water (75:10:15 *v/v/v*) after derivatization H_2So_4-methanol reagent. In preliminary examination, it appeared that an immersion in the dipping solution for more than 3 seconds led to diffusion of spots. As no better derivatization was obtained by increasing the dipping time, a shorter dipping time *i.e.* 2 seconds was selected. Coloring reaction was checked by scanning the plate after 12, 24, 36 and 48 hrs to see the stability of the standard. In comparison with the measured values at 0 h, no significant variation was observed in the area which indicates the standard compound is stable for at least for two days (results are described in intra-day & inter-day study)[23].

3.2 Method validation of HPTLC method

On the basis of SFSTP protocol and regulatory guidelines and the ICH documents [24,25] some suggestions about experimental design and data evaluation are proposed by the SFSTP Commission, which has tried to elaborate a rational, practical and statistically reliable strategy in order to guarantee the quality of analytical results generated. However, a minimum of three series and three replicates at each concentration level using at least three concentration levels: a level close to the lowest range value, a medium level and a level close to the highest range value must always be envisaged for the validation phase. Some examples of possible experimental designs giving the number of calibration and validation standards to be prepared in the validation phase are presented in references [23].

In this study, HPTLC method for lactose was validated in terms of precision, accuracy, recovery, robustness; LOD and LOQ were checked as per ICH guidelines. Intra & inter-day precision results were expressed as % RSD, Accuracy of the method was tested by performing the recovery studies of the pre-analyzed sample with standard at five different levels in each samples and result were expressed as % recovery and % RSD [23, 26, 27].

3.3 Validation for lactose standard solutions

Two micro liters of six calibration solutions: 50, 100, 200, 400, 600 & 800 ng (m = 6) of lactose standard were applied on the HPTLC plates. Peak area data and peak height of corresponding amounts were considered for regression analysis. Five independent series (n = 5) of validation standards were prepared daily and this operation was repeated on three different days (k = 3). Peak area data and the corresponding amounts were treated by linear least square regression analysis. In accordance with ICH Q2B norms for the assay of a finished product, validation solutions were prepared in a concentration range covering a minimum 80–120 % of the expected content [23].

3.4 Response function

The response function of an analytical method is, within the range selected, the existing relationship between the response (signal) and the amount (quantity) of the analyte in the sample system.

3.5 Calibration for Lactose

A stock solution of 1000 µg/ mL of lactose were prepared in water and dilution was done to obtain a solution of 100 µg/ mL which was used for further analysis. Different volumes of diluted solution [0.5,1.0, 2.0, 4.0, 6.0 and 8.0 µL] were applied on TLC plate to furnish 50-800 ng / spot of lactose standard as shown in **Fig. 2a** and simultaneous presences of lactose in samples were assessed by 3 D plot as shown in **Fig. 2b**. Peak area data of corresponding amounts of standard were treated by linear least square regression analysis **(Table 2).**

3.6 Linearity and Detection Limit

Linearity was checked by applying standard solutions of lactose at six different concentrations. The calibration curve was drawn in the concentration range of 50–800 ng / spot. The equation for the calibration curve of lactose was Y= 726.20+5.49x and correlation coefficient of the

calibration plot was 0.995 **(Fig 3)**. Results of regression analysis on the calibration curve and quantification range are described in **Table 2**.

3.7 Precision Studies

Instrumental precision were checked by repeated scanning of the same spots (100, 400 and 800 ng / spot) of lactose standards five times and the RSD values were found 0.6895, 0.9725, and 0.9391 respectively. To determine the precision of the developed assay; 200, 400 and 800 ng / spot of the standards were applied on TLC plate and analyzed five times within the same day to determine the intra-day variability and the % RSD values for standards were 1.0332, 1.3035, 1.1328. Similarly the inter-day precision was tested on the same concentration levels on consecutive days and the % RSD values were 2.1564, 2.0253, 2.8007 (**Table 3**). Peak area data and peak height of both standards biomarkers amounts were considered for regression analysis **(Table 4)**.

3.8 Quantification of lactose in milk samples & recovery studies

HPTLC method was subsequently applied for the analysis and quantification of lactose in milk samples bands of lactose was observed at R_f 0.51 in standard track (**Fig. 4a)** as well as at same R_f in all sample tracks of lactose as shown in **Fig. 4b,c,d,e &f**. It was analyzed that free lactose content in Indian Mare, Cow, Camel, Buffalo & Donkey milk samples are 2.16 %, 5.70 %, 6.70%, 7.17 % & 6.38 % respectively (**Fig. 5)**. The recovery ranges for lactose was obtained 98.04 % to 102.84 % were obtained in all milk samples **(Table 5)**.

3.9 Mass spectrometric analysis confirmation

In present studies, the qualitative studies of lactose standard milk samples were done with mass spectrometric determination and it were observed that the m/z 343 (M+H), m/z 360 (M+ H_2O) in all milk samples and it further fragmented to give mass ion peak at m/z 325[343-H_2O], 276 [325-CH_3OH, OH]. Further fragmentation of m/z 343 from another way it gives m/z at 278[343-CH_3COOH, OH], 260[278-H_2O], 242[260-H_2O], 175[242-CH_3OH,H_2O,OH], 116[175-CH_2CO, OH], 143[175- CH_3OH], 117[242-CH_2OH, CH_3COOH,2OH], 99[117-H_2O], and in other fragmentation path 187[260-OH, H_2O],118[187-H_2O,3OH] fragment obtained as shown in **Fig 6**.

In this study, a simple, an original, cost-effective and easily adaptable HPTLC method is developed and validated for the quantitative determination of lactose concentration in milk sample of five mammalian species. After HPTLC densitometric scanning and mass spectrometric

confirmation, it was observed that lowest concentration of lactose found in mare milk (2.16 %) and highest concentration of lactose found in buffalo milk (7.17 %) sample.

4. Conclusions

In conclusion, the proposed HPTLC method would also be helpful those infants suffering from early lactose intolerance and depending upon infant milk formulations. The developed HPTLC method may be utilized by researcher those are working in sugar chemistry and this method will be helpful in quantification of isolated different types of sugar molecules from milk, honey, blood, fruits and other natural products / combinations / formulations where lactose sugars are used as an ingredient.

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Dedications

First Author Dr. Amit Srivastava dedicated this article to his father Late Dr. R.B. Srivastava, M.V.Sc, Lucknow (India).

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Tables

Table 1. Sephadex G-25 (1.6 x 40 cm) chromatography of mare milk sugar fraction

Fraction No.	Mobile Phase	Compound (in grams)	Phenol-H_2SO_4 test For sugar	Milk oligosaccharide obtained (in grams)
1-20	Glass T D H2O	0.10	-ve [I]	
21-35	"	0.20	**+ve** [II]	0.90 (Mare MOS)
36-57	"	0.61	**++ve** [III]	
58-64	"	0.09	**+ve** [IV]	
1-27	Glass T D H2O	0.09	-ve [I]	
28-51	"	0.05	-ve [II]	
52-57	"	0.76	**+ve** [III]	0.86 (Cow MOS)
58-87	"	0.10	**+ve** [IV]	
1-19	Glass T D H2O	0.20	-ve [I]	
20-30	"	0.15	**+ve** [II]	
31-63	"	0.30	**+ve** [III]	0.65 (Camel MOS)
64-91	"	0.35	**+ve**[IV]	
1-23	Glass T D H2O	0.10	-ve [I]	
24-59	"	0.20	**+ve [II]**	
60-87	"	0.24	**++ve [III]**	0.90 (Buffalo MOS)
88-114	"	0.46	**+ve [IV]**	
1-47	Glass T D H2O	0.25	-ve [I]	
48-91	"	0.32	**+ve [II]**	
92-157	"	0.35	**+ve [III]**	0.75 (DonkeyMOS)
158-224	"	0.08	-ve [IV]	

Table 2. Method validation data for quantification of Lactose standard by using proposed HPTLC densitometric method using under visible mode.

Sl no.	Parameters	Lactose
1	R_f	0.51
2	Dynamic range (ng /spot)	50-800 ng
3	Equation	*Y*= 728.206 +5.493x
4	Slope	5.493
5	Intercept	728.206
6	Limit of detection	25
7	Limit of quantification	50
8	Linearity (correlation coefficient)	0.9957

Table 3. Precision studies for Lactose by using proposed HPTLC densitometric method using under visible mode.

Concentration (ng /spot) Amount of Lactose	Instrumental precision (% RSD)	Method precision (% RSD)	
		Intra-day	Inter-day
100	0.6895	1.0332	2.1564
400	0.9725	1.3035	2.0253
800	0.9391	1.1328	2.8007

Table 4. HPTLC Peak and Height response of lactose by using densitometric scanning

Concentration	Lactose (R_f 0.51)	
	Peak Area	Peak Height
50 ng	824.99	44.57
100 ng	1256.62	71.36
200 ng	1954.60	111.10
400 ng	3030.86	172.75
600 ng	4169.98	215.30
800 ng	4942.27	250.69

Table 5. Result and statistical data for recovery studies for Lactose in oligosaccharide fraction of different mammalian species

Sl. no.	Lactose present in *Mare milk* (ng)	Lactose added (ng)	Calculated amount of Lactose (ng)	Analyzed amount of Lactose (ng)	Recovery (%)	% RSD
1	216	100	316	325	102.84	
2	216	150	366	370	101.09	
3	216	200	416	408	98.07	2.367
4	216	300	516	509	98.04	
5	216	350	566	580	98.64	
	Lactose present in *Cow milk* (ng)	Lactose added (ng)	Calculated amount of Lactose (ng)	Analyzed amount of Lactose (ng)	Recovery (%)	% RSD
1	570	300	870	884	102.60	
2	570	400	970	980	100.92	
3	570	500	1070	1086	101.49	1.735
4	570	600	1170	1152	98.46	
5	570	800	1370	1367	99.78	
	Lactose present in*Camel milk* (ng)	Lactose added (ng)	Calculated amount of Lactose (ng)	Analyzed amount of Lactose (ng)	Recovery (%)	% RSD
1	670	400	1070	1089	101.77	
2	670	450	1120	1121	100.08	
3	670	500	1170	1163	99.40	1.090
4	670	600	1270	1264	99.52	
5	670	800	1470	1462	99.45	
	Lactose present in *Buffalo milk* (ng)	Lactose added (ng)	Calculated amount of Lactose (ng)	Analyzed amount of Lactose (ng)	Recovery (%)	% RSD
1	717	500	1217	1221	100.32	
2	717	600	1317	1328	100.83	
3	717	700	1417	1409	99.43	0.689
4	717	800	1517	1529	100.79	
5	717	1000	1717	1709	99.53	
	Lactose present in *Donkey milk* (ng)	Amount of Lactose added (ng)	Calculated amount of Lactose (ng)	Analyzed amount of Lactose (ng)	Recovery (%)	% RSD
1	638	200	838	827	98.68	
2	638	400	1038	1031	99.32	
3	638	600	1238	1243	100.40	0.648
4	638	800	1438	1449	100.76	
5	638	1000	1638	1627	99.32	

Figures

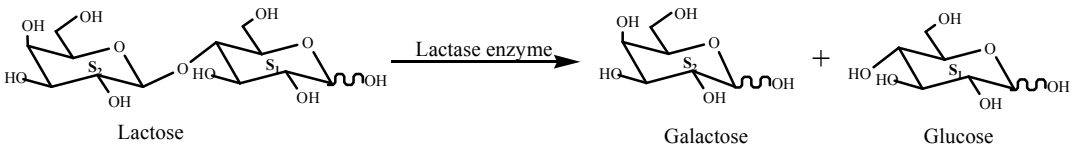

Fig.1. Lactose molecule and its enzymatic hydrolysis

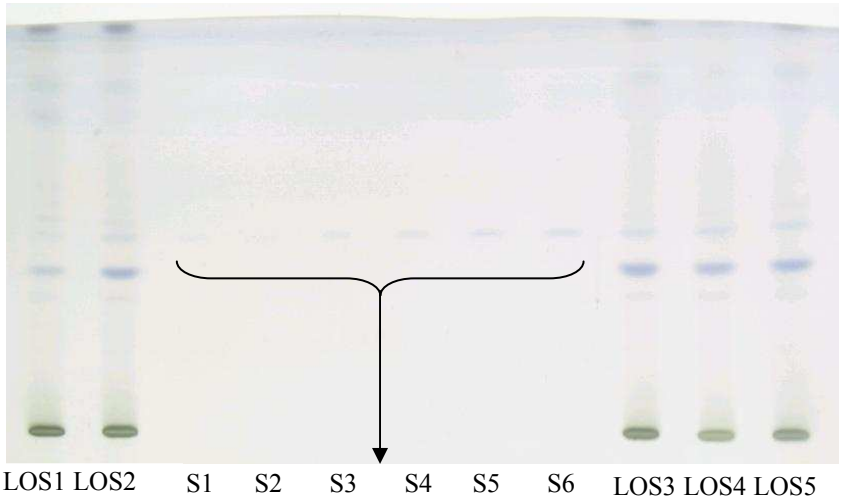

Fig.2a. HPTLC image of milk samples with lactose standard derivatized with methanol-sulphuric acid reagent
(LOS1= Mare milk, LOS 2= Cow milk, LOS3=Camel milk, LOS4=Buffalo milk, LOS5=Donkey milk), S1 - S5= Lactose standards with different concentrations

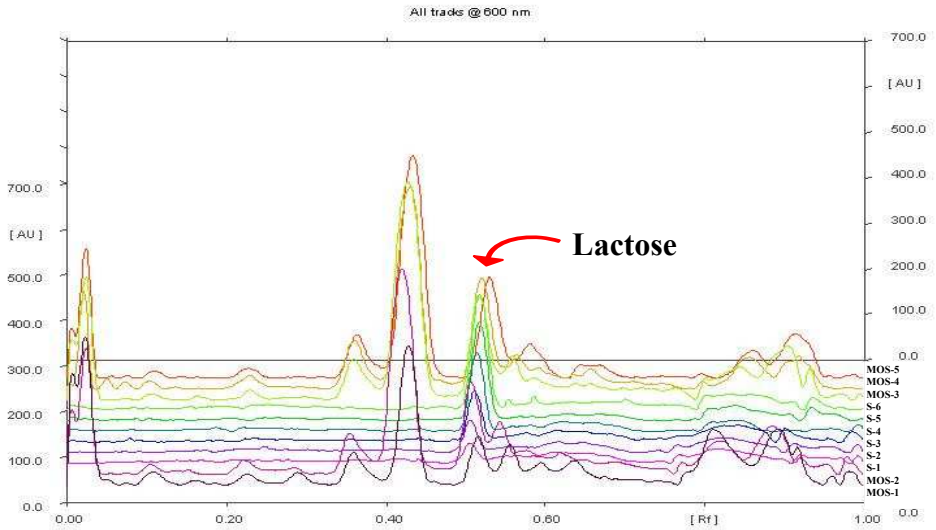

Fig.2b. 3D HPTLC chromatogram of mammalian milk samples with lactose standard

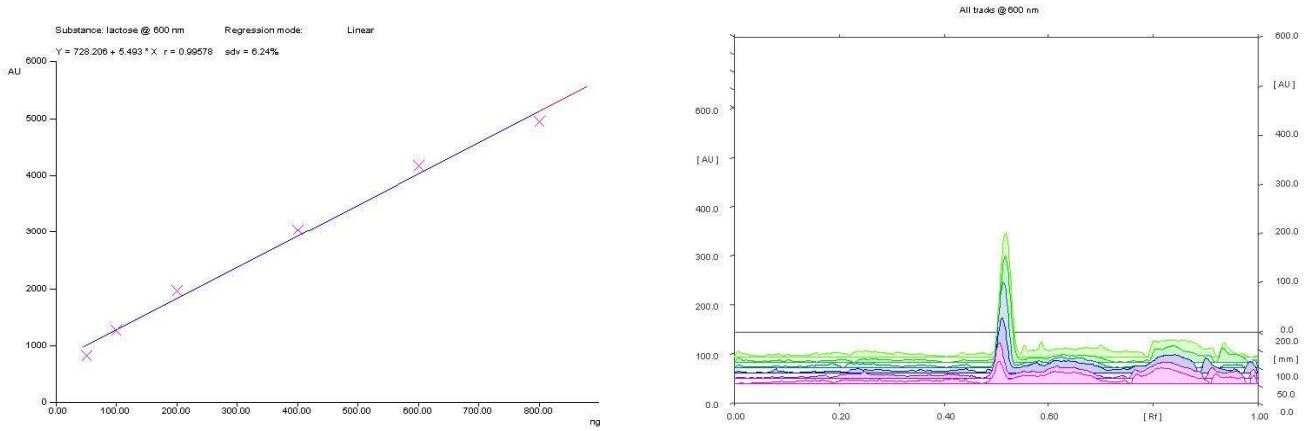

Fig.3. Correlation coefficient and HPTLC chromatogram of lactose standard

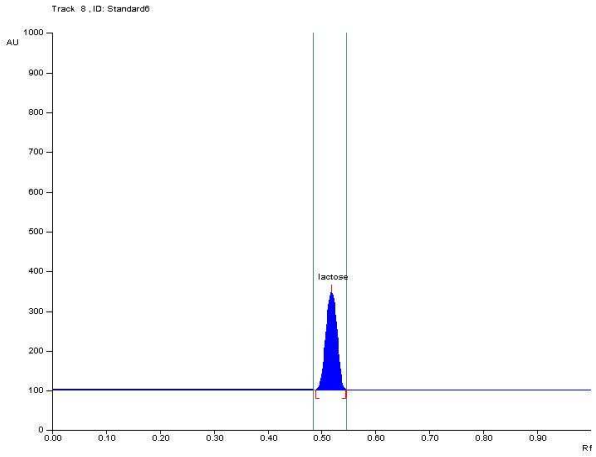

Fig.4a. HPTLC chromatogram of lactose standard

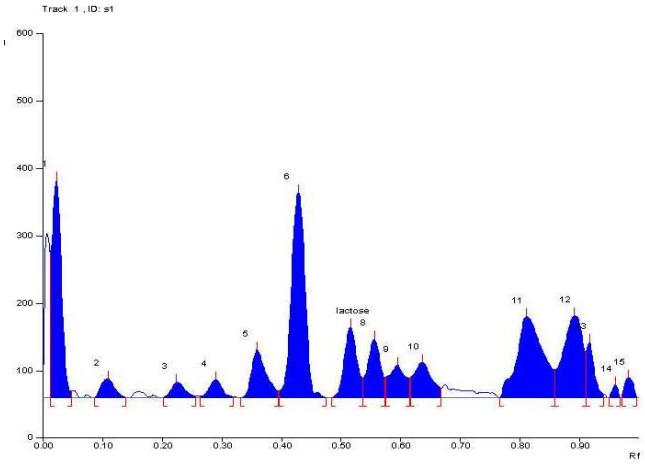

Fig.4b. HPTLC chromatogram of Mare milk LOS

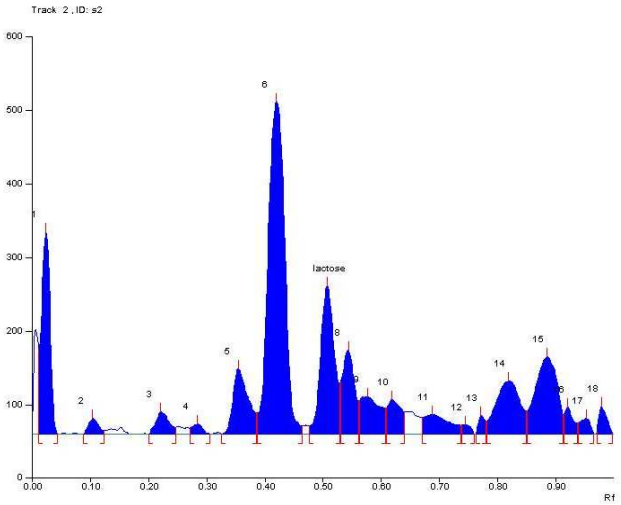

Fig.4c. HPTLC chromatogram of Cow milk LOS

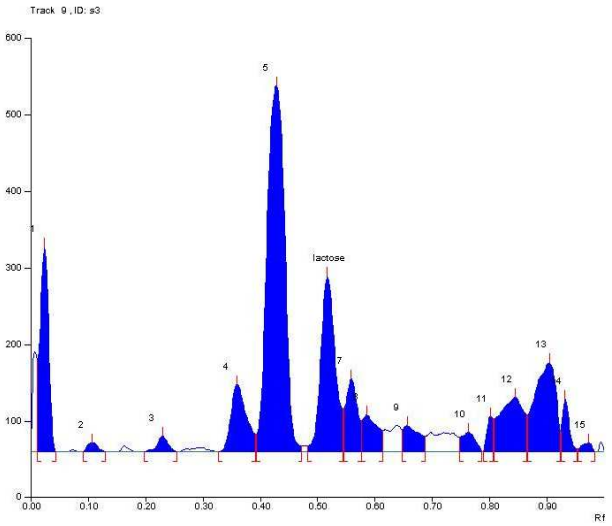

Fig.4d. HPTLC chromatogram of Camel milk LOS

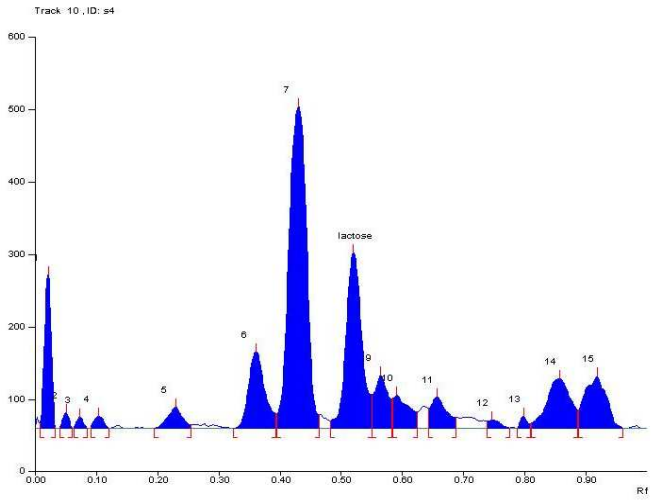

Fig.4e. HPTLC chromatogram of Buffalo milk LOS

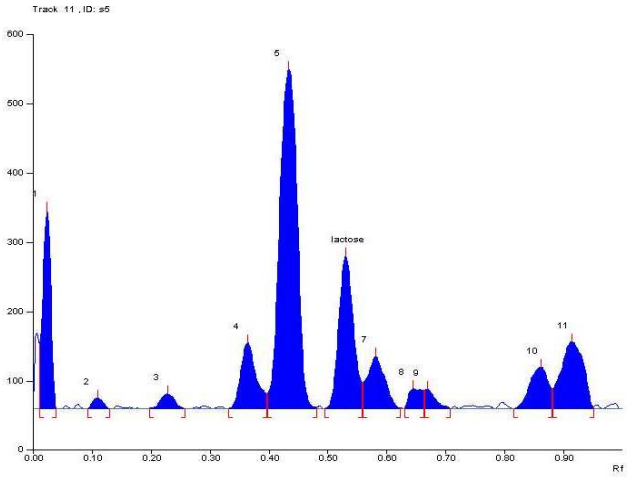

Fig.4f. HPTLC chromatogram of Donkey milk LOS

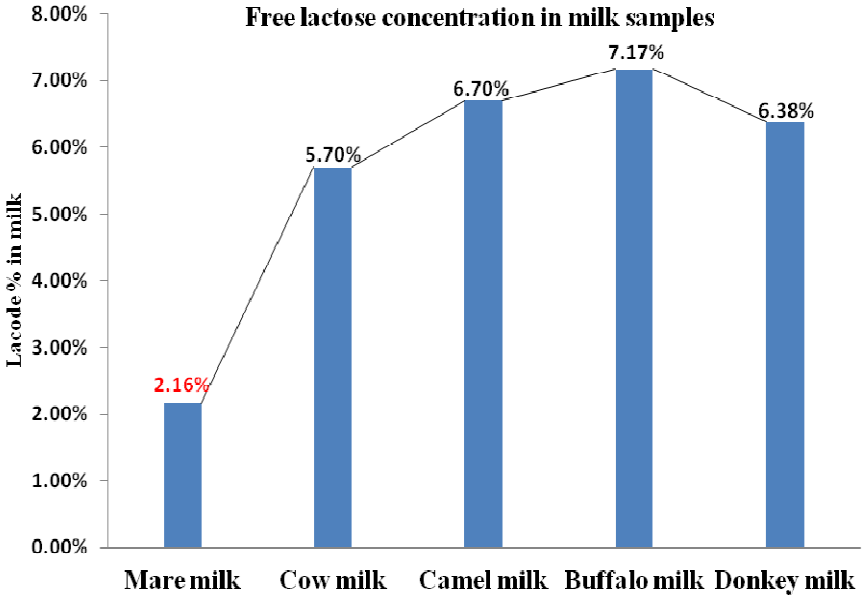

Fig.5. Lactose percentage in milk samples of different mammals

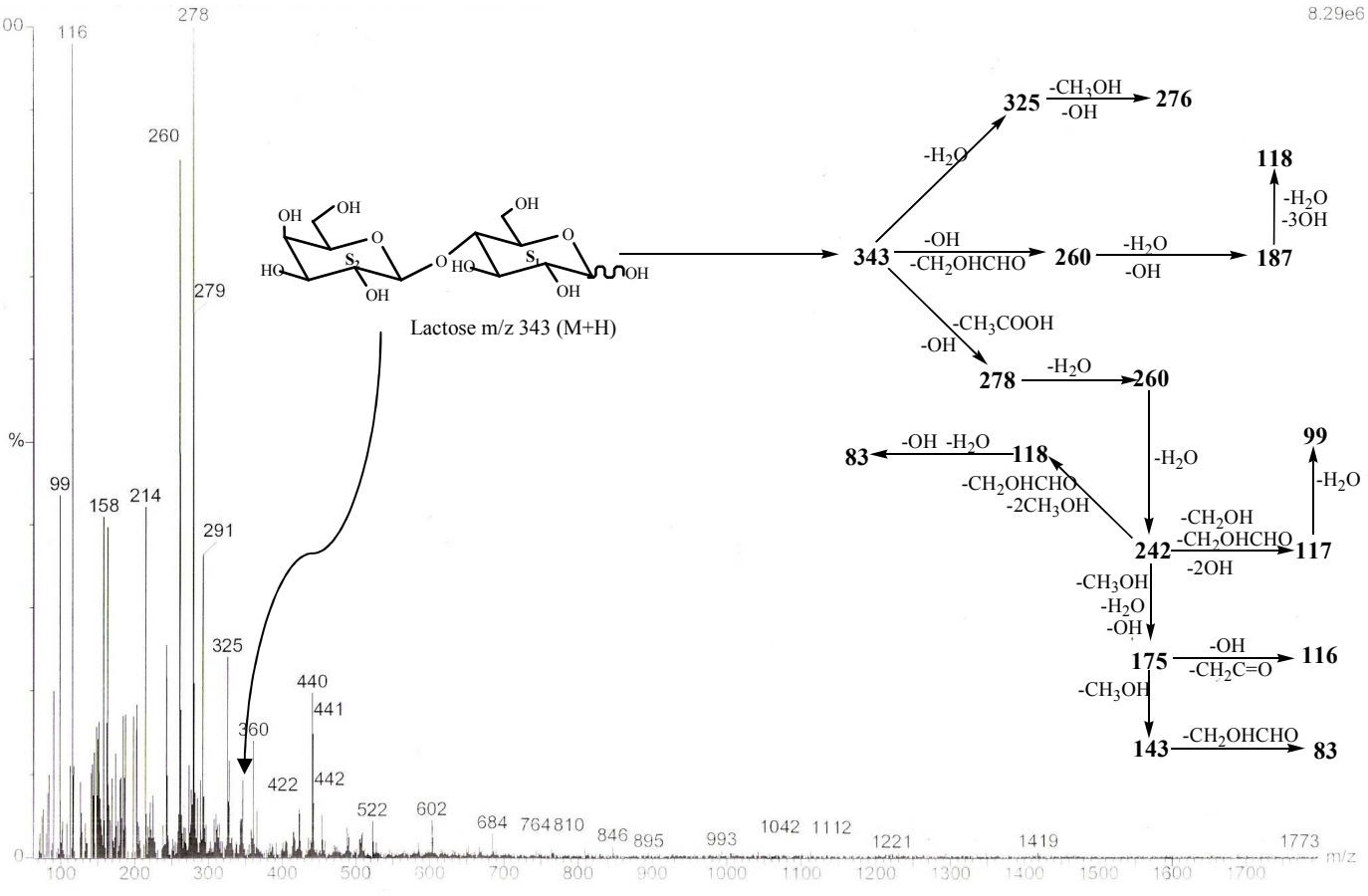

Fig.6. Electron ionization-Mass spectrometry fragmentation of Lactose standard in positive ion mode