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Colorimetric method development and Validation for estimation of lactose in milk

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Abstract

Lactose is one of the major carbohydrate found in milk. Several spectrophotometry methods were carried out for the determination of lactose in milk samples. Here we validated a simple, accurate, specific and precise visible spectroscopy method according to the ICH guidelines for the estimation of lactose in milk (buffalo milk and packet samples). It is a simple and rapid technique for the estimation of lactose in fluid milk and whey. In this method Trichloroacetic acid (TCA) as a precipitating agent. The color development is based on the reaction of picric acid with lactose in the presence of phenol, sodium hydroxide and sodium bisulfate. The λmax of standard lactose was found to be 482 nm. The exhibited the linearity in the concentration range of 10-0µg/ml with a correlation coefficient of 0.999. The precision studies of the method revealed results of % R.S.D values less than 2% indicating that the developed method is precise. The percentage recovery for the proposed method was found to be 99%.

Keywords: Lactose analysis, milk, colorimetric method, validation.

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1. Introduction

Lactose is the major carbohydrate in the milk of most species. Lactose is a disaccharide composed of the monosaccharides D-glucose and D-galactose, joined in a ß-1, 4-glycosidic linkage. The chemical name for lactose is 4-0-ß-D-galactopyranosyl-D-glucopyranose. It is essentially unique to milk, although it has been identified in the fruit of certain plants [1] Carbohydrate is made up of molecules called saccharides. Simple saccharides contain 1 or 2 molecules and are called monosaccharides or disaccharides, or, more commonly, sugars. Oligosaccharides and polysaccharides are chains that contain a few too many sugar molecules and may be referred to as starches [2].

Lactose is the principal sugar (or carbohydrate) naturally found in milk and dairy. Lactose is composed of glucose and galactose, two simpler sugars used as energy directly by our body. Lactase, an enzyme, splits lactose into glucose and galactose. According to more recent studies, lactose may play a role in the absorption of calcium and other minerals such as copper and zinc, especially during infancy. Moreover, if it is not digested in the small intestine, lactose may be used by the intestinal microbiota (the microorganism population that lives in the digestive tract) as a nutrient (prebiotic). Lactose and other milk sugars also promote the growth of bifidobacteria in the gut and may play a life-long role in countering the aging-associated decline of some immune functions [3]. Lactose, the naturally-occurring sugar in milk, is a pretty unique sugar in nature. There are many natural sugars like glucose, fructose and sucrose and these sugars are found widely in nature; from fruits, vegetables and grains to honey and maple sap. But lactose It's almost non-existent in nature outside the milk of mammals – humans, cows, goats, buffalo and sheep alike [4].

Several spectrophotometry methods were carried out for the determination of lactose in milk samples [5-8]. The present investigation deals with the development and validation of a simple colorimetric method for the estimation of lactose in fluid milk and whey. In this method Trichloroacetic acid (TCA) as a precipitating agent [9]. The color development is based on the reaction of picric acid with lactose in the presence of phenol, sodium hydroxide and sodium bisulfate.

2. MATERIALS AND METHODS

All spectral measurements made on ELICO SL 244 double beam UV-VIS spectrophotometer using 1.00 cm quartz curettes. Digital weighing balance (SHIMADZU AUX 220) used for weighing.

Milk Samples And Solutions

a. Milk samples: The sample of milk purchased from the local market.
b. Milk solutions: The milk sample was prepared in the following concentration 1000µg/ml and make serial dilutions 10-50µg/ml
c. Extractor solution by using Trichloroacetic acid: 5ml of 24%TCA was added to 5ml of milk and mixed thoroughly used for extraction of lactose in milk samples.

Reagents

The following stock solutions were prepared in distilled water: 1% phenol, 5% sodium hydroxide, 1% picric acid and 1% sodium disulfite (Na2S205).

A working solution (two days shelf life) was prepared by mixing one volume of phenol, two volumes of sodium hydroxide, two volumes of picric acid and one volume of fresh sodium disulfite solution thoroughly 9 (Teles et al., 1978). The working solution was diluted 1:1 with distilled water.

24% trichloroacetic acid (TCA) was prepared in distilled water. The proposed method will depend mainly on TCA as a total protein precipitating agent as well as modified Teles' reagent by replacing sodium bisulfite (NaHSO3) with sodium disulfite.

Standard Solutions

Lactose: lactose solutions were prepared in a working solution and used as the standard in the following concentration, 1000 µg/ml for the visible-482.

METHODS

1. Extraction of lactose: A 5ml of sample milk, 5ml of 24% TCA were added mixed thoroughly. The sample was then filtered through Whatman no. 40 filter paper and 1ml of the clear filtrate was diluted to 10 ml with a working solution.
2. Spectrophotometric methods: The Spectrophotometric methods of visible-482 nm, were used.
3. Selection of Analytical Wavelength

10-50 µg/ml solutions of LACTOSE were prepared in working solutions by appropriate dilution and the spectrum was recorded between 400-800 nm. The maximum wavelength of casein was found to be nm.

Selection Of Analytical Wavelength

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METHOD VALIDATION

The proposed method has been extensively validated as per ICH guidelines in terms of specificity, linearity, accuracy, precision, robustness, ruggedness, limits of detection (LOD) and quantification (LOQ)10.

Specificity

Specificity is the ability to assess the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). This definition has the following implications: Identification, Purity Tests, and Assay.

Acceptance criteria: The spectra of the standard drug should not show any peak at their respective λmax.

Linearity

Accurately weigh individually 10mg of casein transfer to 10ml of volumetric flask. And add 2 ml of NaOH and sonicate it for 10min and then make up to 10 ml with NaOH (100µg /ml). Than above stock solutions are diluted to prepare 10-50 µg /ml solutions of casein. Check the absorbance for each solution for 6 times. A graph of concentration Vs absorbance was plotted and the correlation coefficient was calculated.

Acceptance criteria

1. Correlation coefficient should not less than 0.999
2. %RSD values of 10-50 µg /ml solutions should not be more than 2.0%

Accuracy (% Recovery)

The accuracy of the analytical method was assessed by determination of recovery for three concentrations corresponding to 50,100 and 150% of test solution concentration. For each concentration, three sets were prepared. The mean recovery of lactose was reported.

Preparation of standard stock solution: About 10 mg of valsartan and hydrochlorothiazide was taken into 10ml volumetric flask, and diluted up to 10 ml with diluent (1000 µg /ml) from these stock solution prepare 10-50µg /ml solution.

A) Preparation of 50% (15µg /ml) solution:

From the above standard solution (100 µg /ml) take 1ml and 0.5 ml from sample solution into 10 ml volumetric flask, make up to the mark with diluent and check the absorbance.

B) Preparation of 100% (20µg /ml) solution:

From the above standard solution (100 µg /ml) take 1.5 ml and 0.5 ml from sample solution into 10 ml

volumetric flask, make up to the mark with diluent and check the absorbance.

C) Preparation of 150% (25µg /ml) solution:

From the above standard solution (100 µg /ml) take 2 ml and 0.5 ml from sample solution into 10 ml volumetric flask, make up to the mark with diluent and check the absorbance.

Acceptance criteria: The mean % recovery of casein should not be less than 98.0% and not more than 102.0%

Precision

The degree of reproducible results produced by a sample at different conditions

Repeatability- The study was performed morning and evening for all concentrations (10-50 µg /ml solution) 6 times and % RSD was calculated.

Intermediate precision- The study was performed different analyst (analyst-1 and analyst-2) for all concentrations (10-50 µg /ml solution) 6 times and % RSD was calculated

Reproducibility- The study was performed today and tomorrow for all concentrations (10-50 µg /ml solution) 6 times and % RSD was calculated.

Robustness

The robustness study was carried out by using 20 (µg/ml) concentrations. The estimation of casein was performed at different environmental conditions (room temp at 29˚C and elevated temp 35˚C).

Ruggedness

The ruggedness is to expresses within-laboratories variations: different days, different analysts, different equipment, etc. I have carried out the ruggedness by using 20 (µg/ml) concentrations.

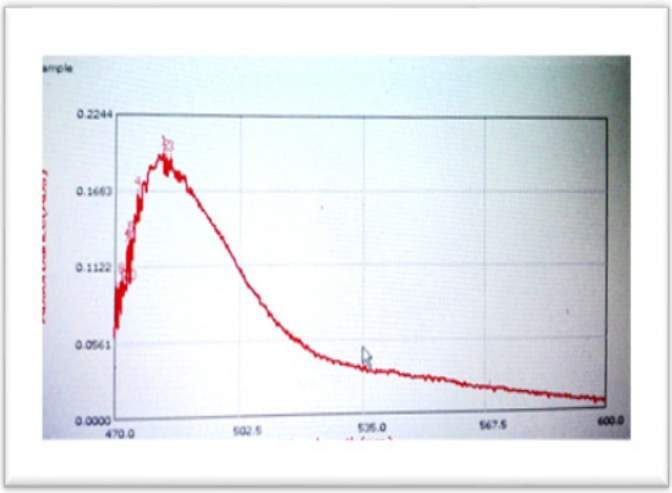

Figure 1: UV spectrum of lactose at 482nm

RESULTS AND DISCUSSION

Table1: Standard graph values of Lactose

S.no	Concentration (µg/ml)	Absorbance
1	10	0.0423
2	20	0.0791
3	30	0.1121
4	40	0.1472
5	50	0.1818

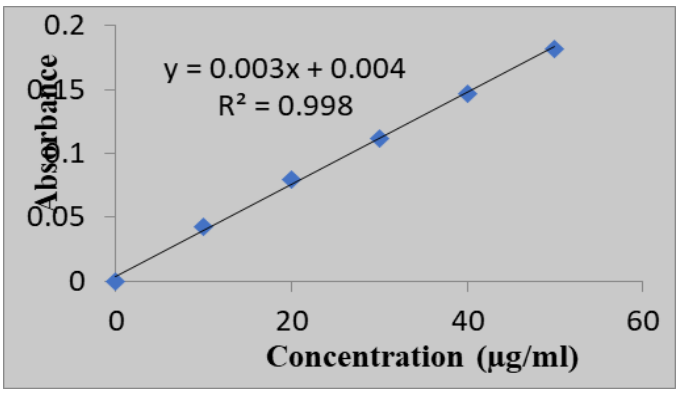

Figure 2: Standard graph of lactose

Table 2: Results of the Assay of Lactose in Milk

S.no	Conc.	Standard Absorbance (nm)	Test Absorbance (nm)	Amount Found (µg/ml)	(µg/ml) %Purity
1	20	0.0791	0.0765	19.62	98.1
2	20	0.0791	0.0771	19.80	99
3	20	0.0791	0.0761	19.51	97.5
4	20	0.0791	0.0769	19.74	98.7
5	20	0.0791	0.0766	19.65	98.2
Mean of assay values		Standard deviation		% RSD	
0.07664		0.000385		0.5019	

Table 3: Linearity study of lactose

S.no	Conc. ((µg/ml)	Absorbance (Mean, N=6)	Standard Deviation	%RSD
1	10	0.04270	0.000654	1.532124
2	20	0.07763	0.001335	1.719835
3	30	0.11291	0.001597	1.414111
4	40	0.14656	0.000779	0.531422
5	50	0.18230	0.004319	0.431926

Table 4: The Precision study of lactose

Precision		Repeatability		Reproducibility		Intermediate Precision	
		Morning	Evening	Day 1	Day 2	Analyst 1	Analyst 2
Absorbance at 20µg/ml	S1	0.0791	0.0792	0.0791	0.0812	0.0791	0.0795
	S2	0.0765	0.0769	0.0765	0.0845	0.0765	0.0769
	S3	0.0785	0.0789	0.0785	0.0805	0.0785	0.0783
	S4	0.0798	0.0799	0.0798	0.0825	0.0798	0.0794
	S5	0.0785	0.0782	0.0785	0.0810	0.0785	0.0781
	S6	0.0789	0.0787	0.0789	0.0808	0.0789	0.0782
Mean	0.07855	0.07863	0.07855	0.08175	0.07855	0.0784	
Standard Deviation (SD)		0.00111	0.00102	0.00111	0.00151	0.00111	0.00096
Relative Standard Deviation(RSD)		0.01417	0.01296	0.01417	0.01852	0.01417	0.01223
%RSD		1.4170	1.29608	1.41706	1.8515	1.41706	1.22343

Table 5: Accuracy study of lactose

Recovery Level	Standard Conc. 20(µg/ml)	Test Conc. 20(µg/ml)	Absorbance			Mean	% Recovery
			S1	S2	S3		
50%	1ml	0.5ml	0.0507	0.0491	0.0921	0.0639	99%
100%	1ml	1ml	0.0716	0.0705	0.0911	0.0777	98%
150%	1ml	1.5ml	0.0981	0.0978	0.0919	0.0859	97%

Table 6: Robustness study of lactose

Robustness		At Room Temperature (29°C)	Elevated Temperature (35°C)
Absorbance at 20µg/ml	S1	0.0821	0.0845
	S2	0.0795	0.0892
	S3	0.0805	0.0838
	S4	0.0819	0.0846
	S5	0.0815	0.0852
	S6	0.0816	0.0862
Mean		0.08118	0.08558
Standard Deviation (SD)		0.00099	0.00195
Relative Standard Deviation(RSD)		0.01223	0.02273
%RSD		1.22292	2.06984

Table 7: Ruggedness study of lactose

Ruggedness		Analyst 1	Analyst 2
Absorbance at 20µg/ml	S1	0.0791	0.0795
	S2	0.0765	0.0769
	S3	0.0785	0.0783
	S4	0.0798	0.0794
	S5	0.0785	0.0781
	S6	0.0789	0.0782
Mean		0.07855	0.0784
Standard Deviation (SD)		0.00111	0.00096
Relative Standard Deviation(RSD)		0.01417	0.01223
%RSD		1.41706	1.22343

Table 8: LOD and LOQ

Limit of Detection	Limit of Quantification
0.234 µg/mL	0.542 µg/mL

Table 9: Summary of Validation Parameters for the Proposed Visible Spectroscopy Method

S.no	Parameters	Results
1.	Absorption maximum (λmax)	482nm
2.	Beer's Law limit (µg/mL)	10-50 µg/mL
3.	Slope	0.003379
4.	Intercept	0.008370
5.	Coefficient of correlation	0.999
6.	Accuracy (%recovery)	99%
7.	Precision (% RSD)	1.23343%
8.	Robustness (% RSD)	1.2229%
9.	Ruggedness (% RSD)	1.23343
10.	Limit Of Detection (LOD)	0.234 µg/mL
11.	Limit Of Quantification (LOQ)	0.542 µg/mL

Detection Limit & Quantitation Limit

ICH guideline describes several approaches to determine the detection and quantitation limits. These include visual evaluation, signal-to-noise ratio and the use of standard deviation of the response and the slope of the calibration curve. In the present study, the LOD and LOQ were based on the third approach and were calculated according to the 3.3σ/S and 10σ/S criterions, respectively; where σ is the standard deviation of y-intercepts of regression lines and s is the slope of the calibration curve.

CONCLUSION:

The proposed method development provides simple, specific, precise, accurate and reproducible results. The method was validated as per ICH guidelines in terms of specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), robustness and ruggedness. The proposed method can be used for routine analysis of lactose present in milk.

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