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Development of spectroscopic method for quantification of starch in milk

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Abstract
A spectroscopic technique was utilized to develop method for quantification of starch in milk. For application of spectroscopy, colour was developed by reaction between starch and iodine, absorption of the colour was measured at 549 nm and standard curve was constructed for concentration of starch versus absorbance obtained. Recovery factor was developed from amount of starch spiked and amount of starch obtained from standard curve. After developing the method, it was validated for performance by true (known) sample approach. The concentration of starch obtained by application of the developed method was compared with the concentration of the starch actually added for preparation of the sample. For statistical analysis t-test: two sample assuming equal variances was applied. The validation of method developed for quantification of starch suggested that the difference between the estimated amount of starch and amount of starch actually added to milk was statistically non-significant.

Keywords: Milk adulteration, starch, quantification, spectroscopy

Introduction
Adulteration of milk is a serious problem to be tackled by the dairy industry. Among various adulterants used in form of carbohydrates, starch is used mostly though it is not permitted legally. Starch or cereal flours, may be added to makeup the density after water addition and mask its detection. Starch is seldom added in the pure form to adulterate the milk. Usually poor quality starch or starchy materials such as cereal flours, arrowroot, wheat flours, mashed potato etc. are added. Starch is a common adulterant in milk products as it gets easily mixed up with products like khoa, burfi because of the similarity in the colour. Starch adds to the weight of the products, and is, therefore, a cheap source of adulteration [1]. If test for starch is positive, quantitative determination of starch should be carried out for determination of SNF content in milk sample.

To detect adulteration of milk with starch, use of this iodine test is reported [2-6]. In this test first milk is acidified with acetic acid and filtrate is collected. The filtrate obtained is treated with a few drops of a dilute solution of iodine [7].

For quantification of starch in milk a Lane and Eynon method is given [8]. A method for starch estimation in ice cream is described [9]. A polarimetric method is reported for quantitative estimation of added starch in milk [10]. This method is also confirmed for its applicability in ice cream, gulabjamun mix and gulabjamun [11]. It appears that no work is reported for development of spectroscopic method for quantification of starch in milk.

There is very limited work reported in the area of quantification of starch in milk using chemical methods in conjunction with spectroscopy. Available methods are tedious, lengthy, involve the use of highly sophisticated instruments and are not applicable for routine purpose. Moreover, dairy industry is also in need of simple and inexpensive methods for quantification of starch in milk. Because of these reasons there is a long standing need to develop simple methods for quantification of starch in milk which can be used for routine purpose with minimum requirement of time, labour and instrumentation.

Materials and Methods
Milk samples
The genuine cow milk was brought from Livestock Research Station and the genuine buffalo milk was brought from Reproductive Biology Research Unit, Anand Agricultural University,

Anand. The ratio of cow milk to buffalo milk was taken as 40:60 to prepare mixed milk sample. Milk Samples were prepared by spiking the raw milk with starch at the suitable level.

Chemicals and Reagents

Iodine (Loba Chemie Pvt. Ltd.), Potassium ferrocyanide (AR, S.D. Fine Chemicals Ltd., India), Starch (Qualigens Fine chemicals), Trichloroacetic acid (Loba Chemie Pvt. Ltd.), Zinc acetate (ExcelaR, Qualigens Fine Chemicals, Glaxo India Ltd.)

Instrument

Spectrophotometer (Micro Controller Based UV-Vis Spectrophotometer CL-1320, Chemiline)

Test procedure developed for determination of starch in milk

A) Preparation of standard curve

1. In a beaker, 100 ml milk was taken and 0.03 g of starch was added to it and mixed well.
2. It was heated in boiling water bath for 10 min to dissolve starch properly and cooled to room temperature.
3. An aliquot of 20 ml milk was taken from the above mixture.
4. 28 ml of hot distilled water, 6 ml zinc acetate and 6 ml potassium ferrocynide were added to it and mixed thoroughly.
5. The content was filtered through Whatman filter paper No. 1.
6. Steps 1 to 5 were performed for pure milk (Control) simultaneously.
7. Filtrates of sample (with starch) and control (without starch) were collected in clean and dry conical flasks.
8. For preparation of standard curve, different volumes of filtrate (1, 2, 3, 4, 5 ml) containing starch were taken in different test tubes so as to provide a concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mg starch per test tube. Total volume was made up to 5 ml in each test tube with control filtrate. Test tube containing 5 ml control filtrate (obtained from pure milk) served as blank.
9. In each test tube 1 ml of 15 per cent TCA was added and the content was mixed.
10. 50µl of iodine solution (1 %) was added to each test tube using micropipette and mixed well to develop the blue colour.
11. The absorbance was measured at 549 nm against blank in a spectrophotometer.
12. A standard curve was plotted using absorbance (along y-axis) and starch concentration (in mg) (along x-axis).

B) Estimation of starch in milk samples

1. Adulterated milk samples added with starch were prepared by mixing different amounts of starch (in the range of 0.0025 to 0.027 g per 100 ml of milk).
2. To obtain the filtrate, steps 1 to 5 of the procedure used in Section A were followed.
3. Aliquots of 5 ml filtrate were collected in test tubes and steps 9 to 10 of Section A were followed.
4. The absorbance was measured at 549 nm against blank in a spectrophotometer.
5. The standard curve prepared as above was used for determination of added starch in milk.

Results and Discussion

For quantitative determination of added starch in milk, qualitative test based on reaction with iodine is used. In this test, iodine reacts with the amylose component of starch and gives blue coloured complex. The acidic condition in the reagent mixture accentuates the blue colour [12]. Attempt was made to use this reaction for developing a spectrophotometric method to estimate amount of starch mixed in the milk.

This study was divided into 4 parts: to develop colour and find out the maxima of the coloured complex formed, construction of standard curve, estimation of the recovery in method and validation of the method.

Spectral Characteristics

In order to get spectra for starch mixed in milk, starch was added to milk to make adulterated sample. 20 ml portion of milk was heated for 10 min to dissolve the starch properly in milk. 28 ml of hot distilled water was added in milk and was coagulated by addition of 6 ml zinc acetate and 6 ml potassium ferrocynide followed by filtration through Whatman filter paper grade 1. To 5 ml of filtrate, 1 ml of 15 per cent TCA was added to reduce the pH (as starch-iodine complex is more stable at low pH). 50 µl of 1 per cent iodine solution was added to develop the colour and the content was scanned for absorption maxima in visible region (400-800 nm) in spectrophotometer. The spectrum obtained from the scan is presented in Fig. 1.

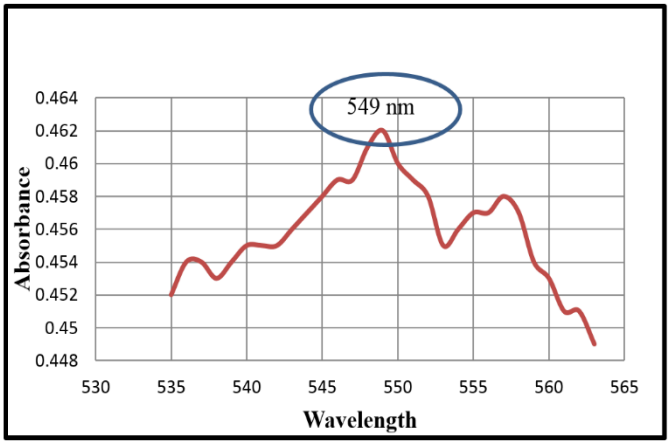

Fig 1: Absorption maxima (λ_{max}) of starch iodine complex

Different peaks were obtained during scanning, e.g. 536, 540, 549, 557 nm, among which the most prominent and highest peak was obtained at 549 nm, therefore this maxima (549 nm) was selected for further study.

Construction of standard curve

After finding the maxima, in the second part, standard curve was prepared. For preparation of standard curve, 0.03 g starch was added in 100 ml milk and filtrate was prepared according to the procedure described in Section A. Different aliquots (0, 1, 2, 3, 4, 5 ml of filtrate from sample mixed with starch containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg starch) were taken and total volume was made to 5 ml with the filtrate of control milk sample, wherein, no starch was added. To these test tubes, colour was developed by following the procedure as described in Section A. The intensity of the colour obtained with varying amount of starch is presented in Fig. 2.

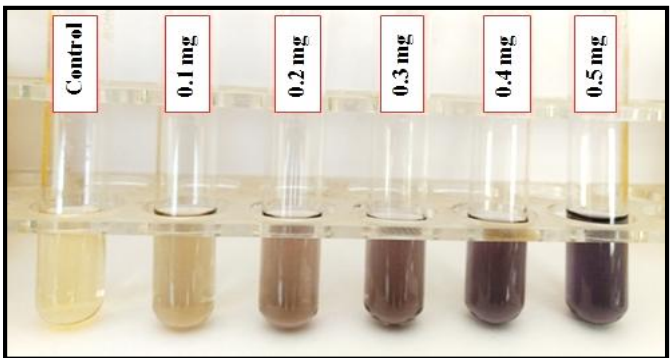

Fig 2: Intensity of the colour obtained with varying amounts of starch

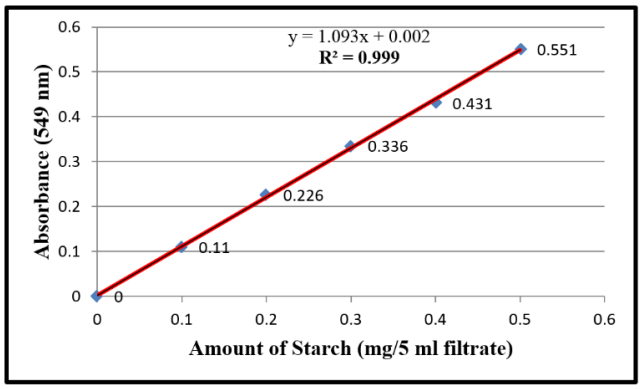

Fig 3: Standard curve for starch

It is evident from the Fig. 2 that intensity of the blue colour formed gradually increased with the increase in concentration of the starch mixed in the milk.

The resulted intensity of the colour was determined by measuring the absorbance of the each test tube at 549 nm. Three replications were conducted and average values of this exercise are given in the Table 1 below. The graph (standard curve) of absorbance versus concentration of starch was plotted, the standard curve so obtained is presented in Fig. 3.

Table 1: Observations taken for preparation of standard curve (starch)

Sr. No.	Starch (mg/5 ml filtrate)	Absorbance (549 nm)
1	0.0	0.000
2	0.1	0.110
3	0.2	0.226
4	0.3	0.336
5	0.4	0.431
6	0.5	0.551
The values are average of three replications		

It was evident from data given in Table 1 and Fig. 3 that absorbance values of the reaction mixture were linearly increasing with the increase in concentration of the starch mixed in the milk and coinciding very well with increasing intensity of the colour. The slope (R^2 = 0.999) indicated that the regression line was very well fitted in the data.

Estimation of Recovery

After developing the standard curve, experiments were conducted to estimate amount of starch obtained from standard curve after mixing known amount of starch in the milk. The starch was mixed in milk in the range of 0.0025 to 0.027 g per 100 ml of milk. Different amounts of starch were selected to cover the entire range of standard curve. The amount of starch in each case was estimated from the standard curve. The per cent recovery of starch obtained in the experiment was derived from amount of starch expected in the 5 ml of filtrate and the amount of starch estimated in 5 ml of filtrate from standard curve. Three replications were conducted. The data obtained for the recovery of starch are presented in Table 2.

Table 2: Estimation for recovery of starch in the experiment

Replication	Amount of starch expected (mg/5 ml filtrate)	Amount of starch estimated (mg/5 ml filtrate)	Recovery (%)
R1	0.041	0.040	97.56
	0.150	0.147	98.00
	0.290	0.288	99.31
	0.350	0.334	95.42
	0.450	0.435	96.66
R2	0.067	0.060	89.55
	0.183	0.172	93.98
	0.267	0.242	90.63
	0.367	0.326	88.82
	0.450	0.412	91.55
R3	0.050	0.047	94.00
	0.167	0.167	100.0
	0.217	0.215	99.07
	0.317	0.316	99.68
	0.417	0.415	99.50
Average			95.58

It appeared from the results that the recovery of starch in the experiments varied from 88.82 to 100 per cent, with an average of 95.58 per cent. Therefore, to estimate the actual amount of starch mixed in the milk, results obtained from the experiment need to be multiplied by the recovery factor. This recovery factor was obtained through dividing 100 by average per cent recovery observed in the experiment. Hence, the recovery factor for the starch in the present study was 1.046 (*i.e.* 100/95.58).

The amount of starch recovered in the filtrate was lower than the amount of starch mixed in the milk. This might be attributed to some losses of starch during preparation of the filtrate. The losses may occur due to retention of starch in the coagulum. The starch may retain in the coagulum due to two possible reasons. One is interaction of the starch with casein. Another reason may be retention of starch in the serum portion remained in the coagulum as moisture. The finding of the present study is in the line with views expressed by the author [13]. These authors stated that both protein and starch

are typical polyelectrolytes and that the interaction in acidic condition must be caused by the attraction of opposite charges which are caused by the dissociation of some ionizable groups of the components.

Validation of the Method

After developing the recovery factor, the amount (g per 100 ml milk) of starch derived from standard curve was multiplied by recovery factor to get amount of starch mixed in 100 ml of milk by this experiment. The data obtained for amount of starch actually mixed in the milk and corresponding amount of starch practically estimated in the experiment, along with their statistical analysis are presented in Table 3.

Table 3: Amount of starch mixed and estimated in 100 ml of milk

S. No	Starch from std. curve (A) (g/100 ml milk)	Starch estimated (A x 1.046*) (g/100 ml milk)	Starch mixed (g/100 ml milk)
1	0.0024	0.0025	0.0025
2	0.0088	0.0091	0.0090
3	0.0172	0.0180	0.0174
4	0.0200	0.0210	0.0210
5	0.0261	0.0273	0.0270
6	0.0036	0.0038	0.0040
7	0.0103	0.0110	0.0110
8	0.0145	0.0152	0.0160
9	0.0195	0.0200	0.0220
10	0.0247	0.0260	0.0270
11	0.0028	0.0030	0.0030
12	0.0100	0.0110	0.0100
13	0.0129	0.0134	0.0130
14	0.0189	0.0198	0.0190
15	0.0249	0.0260	0.0250
Average	0.0144	0.0151	0.0151
t Stat		0.0043	
P(T<=t) two-tail		0.9965	
Test		NS	

* Recovery factor
NS= Non-significant

The starch was mixed in the milk in the range of 0.0025 to 0.027 g per 100 ml of milk, with an average of 0.0151 g per 100 ml of milk. The amount of starch estimated in the milk by the method developed in this study ranged from 0.0025 to 0.0273 g per 100 ml of milk, with an average of 0.0151 g per 100 ml of milk. The comparison between actual amount of starch mixed per 100 ml milk and amount of starch estimated per 100 ml milk by the method of the study revealed that the difference between the two was statistically non-significant. The results suggested that method worked out in the study was suitable for practical purpose.

From review of literature it appears that method (based on Lane and Eynon method) is reported for quantification of starch in milk [8]. In this method, milk is coagulated by ethanol; while in the present study milk was coagulated by combination of zinc acetate and potassium ferrocyanide. In this reported method, analysis is conducted on precipitates after washing with ethanol to make them free from lactose/sugar; while in the present study filtrate is used for analysis. In this method, starch is hydrolyzed to glucose by hydrochloric acid with heating for 2.5 hours and the reducing sugars before and after hydrolysis are measured by Lane and Eynon method; while in the present study the filtrate was acidified with TCA and colour was developed by addition of 1 per cent iodine solution, which was measured spectrophotometrically at 549 nm.

Conclusion

For quantification of starch, spectroscopic method based on iodine test used to detect starch in milk was used. After developing the blue colour by reaction between starch and iodine reagent in milk serum, the maxima of colour absorption was obtained in visible region and it was found to be 549 nm. Then standard curve was constructed by developing the colour with starch at the rate of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg per 5 ml milk serum. After constructing standard curve work was carried out to know actual recovery obtained from the standard curve. From the average per cent recovery obtained in the experiment (95.58 %), the recovery factor was derived (1.046). The amount of starch obtained from standard curve was converted into amount of starch in 100 ml of milk by back calculation. The amount of starch thus obtained per 100 ml of milk was multiplied by the recovery factor to compensate the losses of starch occurring during the procedure followed in the experiment. Finally, performance of the method was tested by comparison of amount of starch obtained in milk by the developed method with actual amount of starch mixed in the milk. The difference between the two was statistically non-significant. The results suggested that method worked out in the study was simple, relatively faster and suitable for practical purpose.

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