Colorimetric Measurement of Lactose in Dairy Products

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Sugars in dairy products are generally determined by either of two basic techniques, the Munson-Walker method or polarimetry. The modification of the Munson-Walker method reported is based on measuring colorimetrically the amount of cupric ion that is not reduced in the reaction between Fehling solution and lactose. The protein and fat (chocolate when present) of dairy products are removed simultaneously by precipitation with Rivanol at neutral pH. After reaction with lactose, the unreacted cupric ion is determined as a cupric-ammonium complex having maximum absorbance at 625 mμ. A linear inverse correlation exists between absorbance of the cupric-NH_3 complex from the unreacted Fehling solution and the lactose concentration. The method can be used for products containing sucrose, such as sweetened condensed milk and ice cream.

Sugars in dairy products are generally determined by either of two basic techniques, the Munson-Walker method (*1*) or polarimetry. In the Munson-Walker method, or modifications thereof, the quantity of cuprous oxide precipitated from Fehling solution is empirically related to the amount of reducing or invert sugar present. The amount of lactose equivalent to the cuprous oxide precipitated is determined by reference to published tables. In polarimetric methods the optical rotatory power of a solution is measured by a polarimeter and related to the sugar concentration by mathematical equations.

The method reported is a colorimetric modification of the Munson-Walker method (*1*), based on measuring colorimetrically the amount of cupric ion which is not reduced in the reaction between Fehling solution and lactose. In the Munson-Walker method reduction of cupric ion by sugar fragments continues after the precipitated cuprous oxide is removed by filtration. This reduction by sugar fragments could be stopped by the addition of borate ion. The amount of cupric ion which had not been reduced by lactose could be measured colorimetrically after conversion to a cupric-ammonium complex. A linear inverse correlation was found between the absorbance of the ammoniacal complex of the cupric ion from the unreacted Fehling solution and the lactose concentration.

Before the lactose content of a dairy product can be determined, the protein and fat must first be removed without inverting sucrose, which is present in some dairy products. It was found that protein and fat could be simultaneously precipitated at a neutral pH by the use of Rivanol. Chocolate in ice cream or milk is precipitated with the protein by Rivanol, allowing lactose determinations to be made on these products if no other reducing sugars are present in the mixture. Emulsifying and stabilizing agents do not interfere with the action of Rivanol at the level found in ice cream.

Reagents and Apparatus

Reagents. All solutions were made from reagent grade chemicals.

A copper sulfate solution was prepared by dissolving 34.639 grams of copper sulfate ($CuSO_4 . 5H_2O$) in distilled water, diluting to 500 ml., and filtering through an asbestos mat.

An alkaline tartrate solution was prepared by dissolving 173.0 grams of Rochelle salt ($NaKC_4H_4O_6 . 4H_2O$) and 50 grams of sodium hydroxide (NaOH) in distilled water and diluting to 500 ml.

These solutions are the Soxhlet modification of Fehling solution.

A 4% Rivanol solution was prepared by dissolving 10 grams of Rivanol (2-ethoxy-6,9-diaminoacridine lactate, obtained from Winthrop Laboratories under the name Ethodin) in 240 ml. of distilled water and warming on a steam bath until solution was complete.

A 4% boric acid solution was prepared by dissolving 20 grams of boric acid (H_3BO_3) in 480 ml. of distilled water and warming to secure solution.

A solution 4*M* with respect to both ammonium chloride and ammonium hydroxide was prepared by dissolving 214.0 grams of NH_4Cl and 250 ml. of concentrated NH_4OH in distilled water and diluting to 1 liter.

Apparatus. The absorbance measurements were made with a Bausch and Lomb Spectronic 20 colorimeter. The reactions between sugars and Fehling solution were carried out in borosilicate test tubes, Kimax 177 × 22 mm., in a boiling water bath. The hot reaction solutions were filtered through Gooch crucibles containing free-flowing asbestos mats.

Size of Sample. For the data reported a 25-gram sample of milk was used or 25 grams of a dilution of concentrated product which had been diluted so that its protein content approximated that of milk. This sample could be readily handled without special glassware or apparatus.

Action of Rivanol. The determination of lactose in the presence of sucrose, as in ice cream or sweetened condensed milk, requires that the protein and fat be removed at a neutral pH to avoid inversion of the sucrose. Rivanol simultaneously precipitates the fat and all proteins except the immune globulins (*3*). The immune globulin content of milk ranges from 0.05 to 0.11% (*2*). In the determinations of lactose by the method described, the solution remaining after removal of the aggregated proteins is diluted by a factor of 10. The concentration of the immune globulins of milk is reduced to the order of 0.005 to 0.011%. On the basis of total solids determination, Rivanol at the concentration used reduced the total solids concentration of skim milk by 37%, which is the order of the protein concentration of milk solids-not-fat.

The amount of Rivanol used is in excess of that required to cause maximum protein precipitation. After the precipitated protein has been filtered off, the excess Rivanol is removed from solution by addition of a drop of 10% NaOH. This raises the pH of the solution to about 8.5, where the Rivanol is precipitated. The aggregated Rivanol is removed by filtering through paper.

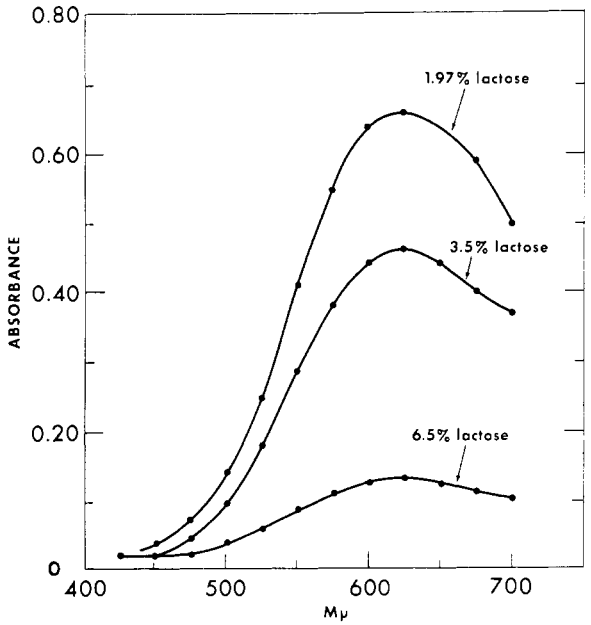

Figure 1. Absorbance curve of cupric- NH_3 complex

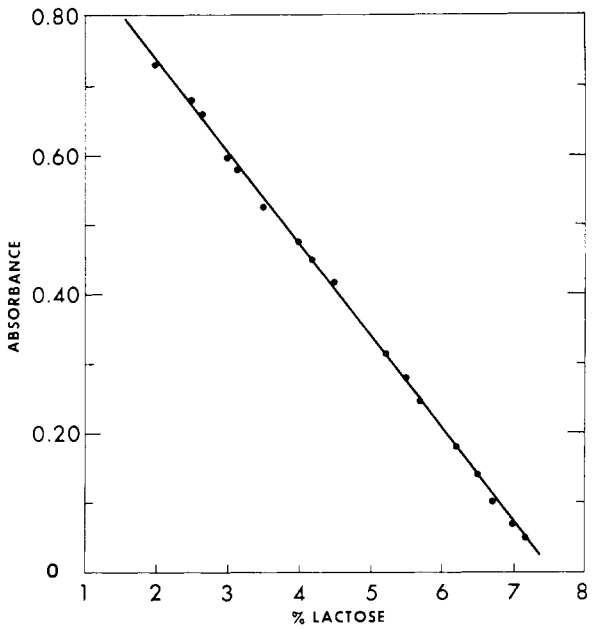

Figure 2. Calibration curve

Color Reaction. The blue color of Fehling solution is reduced by lactose or by invert sugars. The amount of blue color remaining, stabilized by the action of borate on the sugar fragments, is the basis for the measurement of the amount of lactose which reacted with the original Fehling solutions. Spectrophotometrically, the cupric tartrate complex has no absorbance maximum in the visible region. By conversion of the Cu^{+2} ion to a cupric-ammonium complex, an absorbance maximum is found to exist at 625 mμ, as shown in Figure 1.

Effect of Variables on Color Development

Complexing of Sugar Fragments by Borate. The most important variable involved in color development was the continuous reduction of cupric ions by sugar fragments. To stop this action, the reaction mixture of Fehling solution and lactose was filtered into a solution of boric acid. Complexing between borates and sugar fragments has been reported (*4*).

Time of Heating. In either the Munson-Walker or the Lane-Eynon method (*1*) the time of boiling is critical; therefore the best heating conditions for the colorimetric procedure were determined. To have uniform "coming up" and heating time, the reaction mixture was heated in a boiling water bath of such size that the temperature of the water was not reduced below boiling by the insertion of the tube containing the sample.

Table I shows the effect of time of heating on the absorbance of the resulting solution. As no change occurs after the first 4 minutes, the time of heating selected on the basis of this and similar experiments at other concentrations was 6 minutes, since the longer time was easier to measure with accuracy.

Concentration of Lactose. The amount and concentrations of Fehling solution used are determined by the concentration of lactose in the solutions examined. The Munson-Walker technique of using a volume of lactose solution equal to that of the Fehling solution was followed. The volume of each solution was scaled down from 50 ml. to 10 ml. The concentration of lactose in the solution to be analyzed is limited to the range of approximately 0.2 to 0.7% or absorbance values of 0.735 to 0.075.

General Procedure

Preparation of Calibration Curve. Prepare a calibration curve by transferring 25.0 grams of solutions containing 2, 3, 4, 5, 6, and 7% lactose to 250-ml. beakers. Add 5 ml. of 4% Rivanol and 2 drops of 10% NaOH to each solution and mix the solutions by shaking. Remove the precipitated Rivanol after about 5 minutes by filtering through folded filter paper (Reeve Angel, No. 812). Transfer 10 ml. of each filtered solution to 100-ml. volumetric flasks and make to volume with distilled water. Transfer 5 ml. of Fehling solutions A and B to a test tube (177 × 22 mm.) and add 10.0 ml. of the diluted solution of lactose. Mix the reaction solution immediately with a glass stirring rod which is left in the tube, and place the tube and its contents in a boiling water bath for 6 minutes with occasional stirring.

At the end of heating filter the hot mixture immediately through an asbestos mat in a porcelain Gooch crucible into a clean 250-ml. suction flask containing 2 ml. of 4% boric acid solution. Transfer 5 ml. of this solution to a 25-ml. volumetric flask, add 15.0 ml. of a solution of 4*M* NH_4Cl and 4*M* NH_4OH from a buret, and make the solution to volume with distilled water. Measure the absorbances of the solutions at 625 mμ. Then plot the absorbances against the respective per cent lactose.

The calibration curve for lactose using 0.5-inch (diameter) cuvettes in a Bausch

Table I. Effect of Time of Heating on Reduction of Fehling Solution by 0.3% Lactose Solution at 100° C.

Time, Min.	Absorbance, 625 Mμ	Time, Min.	Absorbance, 625 Mμ
2	0.560	5	0.510
3	0.540	6	0.520
4	0.520		

Table II. Determination of Lactose Added to Skim Milk

Added, %	Recovered, %	Difference, %
0	5.08	...
1.07	6.13	0.02
2.00	6.96	0.12
2.80	8.25	0.37
3.81	8.96	0.07
4.70	9.96	0.16
5.62	10.88	0.18
	Av.	0.15
	Std. dev.	0.19

Table III. Determination of Lactose in Presence of Sucrose in Skim Milk

Sucrose Added, %	Lactose, % Detd.	Calcd.	Diff.
0	5.17	...	...
4.96	5.00	4.91	0.09
9.84	4.57	4.66	0.09
15.10	4.54	4.39	0.15
20.00	4.15	4.12	0.03

and Lomb Spectronic 20 colorimeter is shown in Figure 2. The slope of the graph is −0.1323.

Sample Analysis. Weigh into a 250-ml. beaker 25.0 grams of milk or other dairy product, diluted until its protein concentration is equivalent to that of milk (about 3%). Add 5 ml. of 4% Rivanol and allow the mixture to stand 5 minutes for maximum protein precipitation. Remove the aggregated protein by filtration through folded filter paper. To the filtrate add 1 drop of 10% sodium hydroxide and proceed as directed under "Preparation of Calibration Curve." Determine the concentration of lactose from the calibration curve.

Testing of Method. The method was tested for accuracy and precision by increasing the lactose concentration of a skim milk sample by from 1 to 6% with added lactose. The concentration of lactose was then determined by the described procedure. A single determination was made on each concentration shown in Table II. On the basis of these data the accuracy of the method in the concentration range investigated is within ±0.2%. The largest deviation was 4.7% greater than the true value.

Effect of Sucrose. In some dairy products sucrose is used as a sweetening agent. To determine the effect of added sucrose on the measurement of lactose, 5, 10, 15, and 20% commercial sucrose was added to a skim milk sample and the lactose determined. Allowing for the dilution due to the added sucrose, the amount of lactose found and the amount calculated to be present at each concentration of sucrose are shown in Table III.

The amounts of lactose determined are in good agreement with the calculated amounts. Therefore, the method can be applied to the analyses of dairy products containing sucrose.

Discussion

The linear relationship between the amount of unreacted Fehling solution and the concentration of lactose points to the existence of a similar relationship between the amount of reacted Fehling solution and the concentration of lactose. By the Munson-Walker method, the amount of precipitated cuprous oxide retained on the asbestos mat is used as a measure of the extent of the reaction. That the amount of cuprous oxide retained on the filter from the hot reacting solution is not stoichiometrically related to sugar content was observed in these analyses by the appearance of precipitated cuprous oxide in the cold filtrate. This filtrate has been in contact with borate ion and the reducing action stopped, as found by the measurement of identical absorbance of samples of filtrate taken before and after the appearance of precipitated cuprous oxide. On the basis of these observations, cuprous oxide in a strong alkaline medium in the presence of tartrate has a definite solubility when hot. This fact may explain the need for such careful reproduction of all operations in successfully using the Munson-Walker procedure.

Procedural difficulties which gave technicians the most trouble were in the removal of excess Rivanol after precipitation of the proteins. The amount of Rivanol remaining depends upon the protein content of the product being examined. This Rivanol is precipitated by the addition of a small amount of base. If too much base is added, a cloudy suspension is produced which is not removed by filtration and causes low results. Its nature suggests that it contains some proteins. It is obtained only when dealing with dairy products. If the pH is raised too far, the precipitate reverts to a suspension which reacts with the cupric ion of Fehling solution. The usual appearance of a slight yellow color does not affect the results, as long as the filtrate is clear of suspended material.

This method has the same limitations as the Munson-Walker method, in that it is not specific for lactose. All reducing sugars present in the material analyzed will react with the reagent used.

Literature Cited

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FORAGE ESTROGENS

Relative Potencies of Several Estrogen-Like Compounds Found in Forages

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ALFALFA, RED CLOVER, and subterranean clover have been shown to contain at least five compounds (*2, 3, 13, 15*) which can produce an estrogen-like response in the mouse—coumestrol, genistein, biochanin A, formononetin, and daidzein. All, with the exception of biochanin A, have also been found in ladino clover. Each compound has been evaluated at various times by different workers (*5–10, 17, 18*). However, since the bioassay techniques varied considerably between laboratories, it may be difficult or impossible to correlate results.

Bioassays of plants normally measure only total activity. To provide data which will enable us to determine the relative contribution of each compound to the total uterine stimulating activity of a forage, it is necessary to know the relative amounts of each present as well as their relative potencies. The objective of the research reported herein was to provide data on the relative potencies of each of the five estrogen-like compounds, employing diethylstilbestrol and estrone as standards.

Experimental

Preparation of Compounds. The coumestrol and coumestrol acetate were prepared synthetically by a previously published method (*12*). Formononetin was isolated from red clover, and its identity confirmed by comparison of its physical properties with those of an authentic sample. Daidzein was obtained from formononetin by demethylation. Genistein (m.p. 301° C. decomp.) was isolated from subterranean clover. Biochanin A was synthesized according