

as the present study is concerned, they illustrate the importance of knowing ahead of time something about the various market parameters, in order that collections of vegetable samples may be made at the proper seasons.

Information obtained from this study indicates the feasibility of estimating the inorganic element content of various vegetables in an urban market. Because many consumer groups obtain their food from rather well-defined urban markets, the extension of this approach to additional markets would seem desirable. This suggests that a market analysis could be made by sampling lots from different producing areas according to delivery volume at different seasons. The number of lots to be collected, and the number of spectrographic plates for each lot, would depend on the vegetable and the producing area sampled.

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SEPARATION OF MILK COMPONENTS

Chromatographic Isolation of Citric Acid and Lactose from Skim Milk

A chromatographic procedure for separating citric acid and lactose from skim milk in pure form and sufficient amounts for carbon-14 measurements is described. Lactose in 70% yield was separated from citric acid by ion exchange chromatography. The purity of the lactose was determined. Citric acid was recovered in 60% yield and was identified and its purity was determined.

IN THE COURSE OF METABOLIC studies in the intact dairy cow using carbon-14 labeled metabolites, a method to obtain pure citric acid and lactose from skim milk for carbon-14 measurements was desired. Present methods for separating lactose (6) and citric acid (5) from milk are inadequate for obtaining pure compounds. Accordingly, an ion exchange chromatographic technique described by Busch *et al.* (3) has been modified to per-

mit the isolation of citric acid and lactose from skim milk.

Apparatus and Reagents

The anion exchange resin employed was Dowex AG 2-X8 (Bio-Rad Laboratories, Richmond, Calif.) analytical grade, 50 to 100 mesh, with a capacity of 1.4 meq. per ml. of resin in water. Prior to use, the resin was converted to the formate form by eluting with 3*N* sodium formate until the eluate contained no chloride by the silver nitrate test. Excess formic acid was washed from the resin by distilled water

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until the eluate was above pH 4. A 280-ml. resin bed was prepared by slurring with water and adding it to a chromatographic column 3×50 cm.

The cation exchange resin was Dowex AG 50 W-X8 (Bio-Rad Laboratories) analytical grade 20 to 50 mesh, with a capacity of 1.7 meq. per ml. of resin bed in water. The resin was converted to the hydrogen form by eluting with 3*N* hydrochloric acid until the effluent from the resin gave a negative calcium test with ammonium oxalate. Excess acid was washed from the resin with distilled water

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until the effluent was above pH 4. The size of the chromatographic column was the same as that described above.

The apparatus for elution of the citric acid from the anion exchange resin was similar to that employed by Busch, Hurlbert, and Potter (3). An 8-liter reservoir containing 6*N* formic acid was connected to a 1-liter round-bottomed flask containing 1 liter of water. This flask was fitted with an inlet and outlet. The outlet was connected to the top of the chromatographic column. The contents of the flask were stirred by a magnetic stirrer (Precision Scientific Co., Chicago, Ill.). The apparatus was so arranged that the flow, by gravity, from the reservoir through the chromatographic column was 3 to 4 ml. per minute (7). Fractions of 40 ml. of the eluate were collected by an automatic fraction collector (Micro-Chemical Specialties Co., Berkeley 4, Calif.).

All reagents used were of reagent grade. Norit A is a decolorizing charcoal (Pfanstiehl Laboratories, Waukegan, Ill.).

Procedure

Precipitate the casein fraction from 1 liter of skim milk by adjusting the pH to 4.5 with 3*N* hydrochloric acid, and remove the fraction from the whey by filtering through Reeve-Angel No. 230 filter paper. Wash the precipitate with three 150-ml. portions of water; add these washings to the whey. Adjust the combined solution with 3*N* ammonium hydroxide to pH 5.5 and heat to 85° C. in a water bath. Maintain the temperature of the solution at 85° C. for 1 hour. Separate the whey proteins which have precipitated by filtration while hot through a Reeve-Angel No. 230 filter paper. Wash the precipitate with three 150-ml. portions of water. Combine the filtrate and washings and adjust the pH of the solution to 6.8 with 3*N* ammonium hydroxide. Heat the solution to, and keep it at, 80° C. for one-half hour. Remove the precipitate formed by the second heating by filtering it through a Reeve-Angel N 230 filter paper. Wash the precipitate with three 150-ml. portions of water. Combine the filtrate and washings and heat (to a rolling boil) in the presence of 20 to 30 grams of Norit A. If foaming occurs, caused by the protein, add 3 grams of Norit A and heat the solution again to a boil. Filter the solution immediately and run 200 ml. of boiling water through the filter paper. The filtrate containing lactose and citrate is clear, colorless, and protein-free.

Prior to use, arrange the ion exchange resin columns in series with the cation resin column above the anion resin column. Wash the resin beds with 500 ml. of 70% ethyl alcohol followed by 1 liter of distilled water. Pass the citrate-lactose solution through these resin beds at the flow rate of 3 to 4 ml. per minute. Collect the eluate containing lactose, concentrate it to about 50 ml. in a vacuum distil-

lation apparatus (2), and crystallize it by the addition of 500 ml. of 95% ethyl alcohol. Recrystallize the lactose from water and alcohol.

Elute the citric acid, which has been absorbed by the anion exchange resin, with 6*N* formic acid using the elution apparatus. Elute the citric acid from the resin after about 1 liter of formic acid has passed through the column. The acid is detected by a simple modification of the quantitative technique of Babad and Shtrikman (7). To three drops of the eluate add 4 ml. of acetic anhydride and 1 ml. of pyridine; a dark brown to red color which develops without heating indicates the presence of citric acid. Combine the fractions containing the citric acid and concentrate to 5 ml. in the vacuum distillation apparatus. Remove the formic acid by repeating the concentrations twice, each time adding 100 ml. of water. Neutralize the concentrated citric acid with 30% of sodium hydroxide and crystallize it as the sodium salt by adding about 100 ml. of absolute methanol. Wash the collected crystals with methanol and dry them overnight.

Discussion

The purity of the lactose recovered in this procedure was determined by carbon and hydrogen analysis and by optical rotation. In addition, carbon-14-labeled lactose isolated from skim milk by this procedure was shown by isotope dilution using reagent grade lactose, to be of sufficient purity for carbon-14 measurements.

Calculated for C₁₂H₂₂O₁₁: C = 39.97%; H = 6.71%. Analysis values: Found: C = 41.16%, H = 6.79%. for C₁₂H₂₂O₁₁·H₂O: (6). [α]²⁰_D = +52.6° [H₂O; (8)]. Found: [α]²⁰_D = +52.0° [H₂O; (5)].

The lactose was recovered in 70 to 80% yield, based upon the determination of lactose in milk as described by Erickson and Richardson (4). The greatest loss of lactose in the procedure occurred in the final clarification with Norit A.

To obtain free citric acid for analysis, a second liter of skim milk was processed.

The concentrated citric acid solution, obtained by concentration of the citric acid fraction eluted from the anion exchange resin, was further reduced to a very thick sirup, in vacuum, over sodium hydroxide. The citric acid crystals were identified as citric acid by determining the melting points of derivatives and the neutralization equivalent as shown in Table I.

The purity of the citric acid crystals was determined by carbon and hydrogen analysis. Calculated for C₆H₈O₇·H₂O: C = 34.26%; H = 4.80%. Analysis values: Found: C = 34.87%; H = 4.69%.

The recovery of citric acid, based upon the method of Babad and Shtrikman (7) was about 60% of the total in skim milk. Citric acid was lost in each step of the protein removal, but the greatest loss oc-

Table I. Melting Points of Derivatives and Neutralization Equivalent of Isolated Citric Acid

	Derivatives °C. ^a	Melting Point,	Neutralization Equivalent of C ₆ H ₈ O ₇ ·H ₂ O
	<i>p</i> -Nitrobenzyl	<i>p</i> -Bromophenacyl	
Citric acid (8)	102	148	70.0
Isolated citric acid ^a	100-102	147-149	69.5

^a All temperatures uncorrected.

curred upon the precipitation of the whey protein by heating.

Other methods of removing the protein without heating the whey were unsatisfactory. Citric acid precipitated as the salt when the protein was removed by using barium or lead ions. Dialysis of the whey to separate the protein resulted in low yields of citric acid and lactose.

The ion exchange resin was pretreated with 70% ethyl alcohol to reduce the microbial action to a minimum while the citrate and lactose solution was being passed through the resin. Without first treating the resin with alcohol, lactic acid could be eluted by formic acid from the anion exchange resin.

At present, the procedure is being used routinely in the author's laboratory to separate citric acid and lactose from the milk of cows, sows, and dogs.

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