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ABSTRACT

Quantitative determination of various protein constituents in processed dairy products such as ice cream is a problem to manufacturers and regulatory agencies. A method for determining casein and whey protein was developed. Because for most products fat content also must be known, fat was quantitated first by the standard Mojonnier test, and the aqueous layer from this test was retained for protein analysis. Ratios of casein to whey protein were determined from ratios of phosphorus to nitrogen with .85% phosphorus for casein. Although the above method appeared satisfactory, further flexibility can be built into it by use of radial immunodiffusion so that products can be assayed specifically for casein, whey protein, or any suspected protein adulterant. Casein, strawberry, chocolate, and vanilla ice creams, three control ice creams, vanilla ice milk, coffee creamer, cheese kisses, whole milk, and nonfat dry milk were tested. The radial immunodiffusion and phosphorus analysis gave sample caseins in reasonable agreement.

INTRODUCTION

The frozen desert standard of identity requires a minimum fat content of 10% and milk solids content of 20% (7). The whey solids content cannot exceed 25% of the nonfat milk solids. Thus, a method is needed to distinguish between casein and whey protein contribution to solids in a given ice cream formulation. Furthermore, extension of this method to other dairy products would be desirable. Dye binding methods are suitable for protein determinations in ice cream, but results are slightly different for total milk proteins, caseins, and whey proteins, depending upon which protein has been used as the calibration standard (12). Total nitrogen determinations of casein or noncasein protein fraction would not be reliable, because in processed products whey protein complexes with casein when subjected to heat (4, 5, 11). Thus, for determining how much whey protein or casein is in a processed dairy product, either this complex must be broken, or some other unique property of proteins must be utilized. This report describes a method for determination of casein by its ratio of phosphorus to nitrogen and suggests an approach to extend the method to other proteins.

MATERIALS AND METHODS

Materials

All reagents were analytical grade. New glassware was acid cleaned, washed with phosphate-free detergent, and then rinsed with deionized water. Distilled water was passed through a mixed ion exchange column and then stored in polyethylene bottles. All wash solutions were delivered from polyethylene squeeze bottles.

Phosphorus Reagents

We weighed 50.0 mg of recrystallized 1-amino-2-naphthol-4-sulfonic acid (ANS) [recrystallized according to the method of Fiske and Subbarow (6)], 100 mg of anhydrous sodium sulfite, and 300 mg of sodium bisulfite into a 100-ml beaker. We ground the ANS into solution with a glass stirring rod using a minimal volume of H_2O. When dissolved, it was

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diluted to 50.0 ml. This solution will keep about 10 days when protected from light.

Ammonium molybdate (.28% wt/vol) was prepared by dissolving 2.8 g of ammonium molybdate tetrahydrate in 1 liter of H_2O. It is stored in a polyethylene bottle.

The phosphorus standard was prepared with .3510 g monopotassium phosphate (dried in a convection oven at 105°C and cooled in a desiccator) and 10 ml of 10 N H_2SO_4 diluted to 1,000 ml with H_2O. This solution, containing 80 μg P/ml, was used to prepare standards of 2 and 4 μg P, which were run in duplicate along with duplicate 0 μg P blanks with each set of unknowns.

Sample Preparation

Casein was precipitated from raw skim milk at 40°C and pH 4.6 with the slow addition of .5 N HCl (4). After the casein settled, the whey was decanted, and the casein was filtered, then washed four times with acetate buffered water at pH 4.6 and dried in a shelf dryer under high vacuum (<5 mm/Hg). The casein was defatted by extraction for 2 h with ethyl ether; residual ether was removed by drawing a stream of nitrogen across the product.

Three ice cream mixes with varying quantities of sodium caseinate added were prepared with compositions in Table 1; ice cream was prepared from these mixes under controlled conditions (10) in our laboratory.

Four ice creams of known composition were obtained through the cooperation of a commercial ice cream manufacturer. Other ice creams and samples were purchased from commercial firms.

Sodium caseinate[4] was purchased from National Casein of New Jersey, Riverton, NJ 08077. Kontrol stabilizer was donated by the Germantown Manufacturing Company, Broomall, PA 19008. Antifoam B Silicone emulsion was purchased from J. T. Baker Chemical Company.

[4] Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

TABLE 1. Composition of test ice cream mixes.

Mix	Ingredients	(%)[a]
#1	Butteroil	10.0
	Sodium caseinate	2.0
	Sweet whey protein	.5
	Sucrose	15.0
	Stabilizer-CMC	.20
	Water	72.3
#2	Butteroil	10.0
	Skimmilk powder	7.5
	Sweet whey powder	2.5
	Sucrose	15.0
	Stabilizer-Kontrol[b]	.35
	Water	64.65
#3	Mix #1	50.0
	Mix #2	50.0

[a] Percent by weight.

[b] Ingredients: mono- and diglycerides, cellulose gum, guar gum, polysorbate 80, and carrageenan. Sodium silico-aluminate added for anticaking purposes.

Methods

Fat Analysis. The AOAC procedure (1) was used for fat analysis.

Phosphorus. The procedure of Meun and Smith (14) was used for phosphorus determinations.

Nitrogen. Total nitrogen was determined by a modification of the AOAC procedure (2). Duplicate or triplicate 25.0-ml aliquots of samples were pipetted into separate 100-ml Kjeldahl flasks. To each flask, 2 g of mixed catalyst (10 parts K_2SO_4 and 1 part $CuSO_4$), 2 boiling chips, and 2 ml of concentrated H_2SO_4 were added. Samples were digested until clear with the addition of antifoam B as needed to retard foaming. The samples were cooled, distilled, titrated, and total nitrogen was calculated according to standard AOAC procedure. Total protein was calculated by multiplying total nitrogen by 6.38.

Radial Immunodiffusion. Radial immunodiffusion (RID) assays were by the general method described by Guidry and Pearson (9) with the protein solvent described (4). The standard protein in the assay was sodium caseinate which was dissolved and diluted in

Buffer A (50 mM tris, 4 mM citrate pH 9.0, 5 M urea, and 10 mM dithiothreitol). Total protein was determined by the method of Bradford (3). The antibody was raised against the α-casein complex (15); however, antibodies against whole casein were equally effective. Test samples were prepared from the same aqueous layer used in the phosphorus determination. Equal volumes of this latter layer and Buffer 2A (Buffer A prepared at 2× the concentration given above) were mixed, and a 3 μl aliquot was withdrawn for RID assay.

Casein Determination. Duplicate samples of casein (1 g) were weighed into beakers, dissolved with .1 N NaOH (∿pH 8), transferred to 100-ml volumetric flasks, and made to volume with water. Duplicate samples of nonfat dry milk (2 to 4 g) were made up to volume in 100-ml volumetric flasks. Ten milliliter aliquots of casein and nonfat dry milk solutions were pipetted into separate Mojonnier fat extraction flasks. Duplicate 5 to 6-g samples of melted ice cream were weighed directly into individual Mojonnier flasks. To each flask, .12 to .13 g of NaCl was added to increase the polarity and salt out phospholipids (16). Blanks of 10.0 ml of water were run. All samples and blanks were extracted two times by the AOAC (1) fat extraction procedure.

The aqueous layer from each Mojonnier extraction flask was transferred quantitatively to a 150-ml beaker with 30 to 40 ml of water; alcohol was removed by bubbling a gentle stream of nitrogen through the sample in a water bath at 41° ± 1°C for 2 h. The residue was transferred quantitatively to a 100-ml volumetric flask, aided by a glass rod, and made up to volume. A 10.0-ml aliquot of this solution was transferred quantitatively to a 100 ml beaker; 10 ml of H_2O and 40 ml of 18% TCA were added. The mixture was stirred 5 to 10 min until the particles agglomerated. The entire contents were filtered through a #934 AH Reeves Angel glass fiber filter, with the smooth side up. The filter was inserted into a Büchner funnel, and filtration was facilitated by application of light suction when needed. The protein precipitate first appeared as a general cloudiness of small particle size. After being stirred, the particles agglomerated in 5 to 10 min, settled out, and left a clear supernatant. The fine and coarse precipitates appeared and were held by the filtering system. The precipitate, beaker, and stirring rod were washed with 12% trichloroacetic acid (TCA) from a polyethylene squeeze bottle. The TCA was removed from the beaker, stirring rod, and precipitate with washes with ethyl ether. The stem of the funnel was inserted into a 100-ml volumetric flask through which the precipitate as well as the beaker and stirring rod were washed with warmed .1 N NaOH (no higher than 50°C) to dissolve all precipitate. The flask contents were cooled to 25°C and made up to volume with .1 N NaOH. Total nitrogen determinations (2) were on aliquots of the .1 N NaOH solution of the TCA precipitate when made up to volume. Aliquots were transferred immediately to the Kjeldahl flask and made acid with H_2SO_4 to avoid loss of nitrogen on standing. This may be from decomposition of the protein and loss of NH_3 at the high pH of the TCA precipitate in NaOH solution.

For phosphorus determinations (14), 2.0 ml of the redissolved protein solution was pipetted into a 30 ml micro-Kjeldahl flask; 1.0 ml of 7.2 N H_2SO_4 and 2 to 3 acid-washed carborundum boiling chips were added. The sample was evaporated on a Kjeldahl digestion rack until white H_2SO_4 fumes appeared. Each flask was removed from the heat and cooled until all flasks reached this stage. Flasks were placed again on the digestion rack at a lower heat until fumes reappeared and heated for 5 min (loss of H_2SO_4 fumes must be avoided as the heteropoly color is affected by the amount of acid). One drop of 30% H_2O_2 was added directly into the boiling liquid and digestion continued for 2 min; then a second drop of H_2O_2 was added, and the sample was digested until bubbling stopped. Flasks were cooled in tap water; 4.5 ml of ammonium molybdate solution and .5 ml of ANS were added. Each flask was heated carefully in a boiling water bath for 10 min, removed, and cooled in tap water for several minutes. Absorbance was read at 830 nm in a spectrophotometer against a water blank. The absorbance of reagent blanks, treated in the same manner, also was determined. The micrograms of phosphorus for the unknowns were calculated from the standard curve with .85% phosphorus in casein. The percentage of casein in unknown samples was calculated from the following formula:

$$\% \text{ casein} = \frac{\dfrac{\mu g\ P \times 100}{.0085}}{\dfrac{\text{wt of sample}}{1000} \times \text{ml of sample in digest} \times 10^6}$$

where μgP = μgP calculated from standard curve, and wt of sample = 1 g casein or 2 to 4 g NFD or 10 g milk or 5 to 6 g ice cream divided by the dilution factor (1000)

ml of sample in digest = number of ml of digest used for phosphorus determination

.0085 = phosphorus content in casein for sample

The formula calculates out to be:

$$\% \text{ casein} = \frac{11.764705 \times \mu gP}{\text{sample wt} \times \text{ml of sample in P digest}}$$

RESULTS AND DISCUSSION

When casein is precipitated isoelectrically from processed dairy products, a coprecipitation of whey protein occurs (4, 5, 11). This coprecipitation is thought to result from a sulfhydryl-disulfide interchange among the milk proteins. Several approaches were attempted to break the disulfide complex in the processed dairy products so that casein alone could be precipitated totally. Reducing agents such as dithiothreitol and 2-mercaptoethanol in combination with pH manipulation from 4.5 to 9.0 were not successful. Rennet, calcium chloride, and ultracentrifugation were not successful nor were Sephadex or DEAE-cellulose chromatography. Therefore, from the standpoint of a routine test of protein contents of milk solids-not-fat in processed dairy products, a different approach had to be tried. Among milk proteins, caseins account for nearly all of the bound phosphate (8); thus, if the casein phosphorus could be quantitated, this could be used to calculate the amount of casein present, and when this is subtracted from the total protein content, it would give the whey or noncasein protein content.

Precipitation of proteins from ice cream, either by titrating to pH 4.6 with sulfuric acid or by adding a predetermined quantity of acetate buffer, worked well for caseins except when an ice cream was stabilized with carboxymethyl cellulose (CMC). In this case, the pH had to be lowered to 4.3 before all cloudiness disappeared. Since many commercial products contain some CMC, the question could be raised whether all of the casein had been precipitated. For this reason as well as coprecipitation of whey protein, other precipitating agents were sought that would settle all of the milk proteins. Zinc-copper solutions, saturated ammonium sulfate or magnesium sulfate, and trichloroacetic acid (TCA) were used. All the reagents quantitatively precipitated the proteins with the exception of the zinc-copper solution. However, the saturated solutions of ammonium and magnesium sulfate were difficult to store and maintain in a saturated condition during pH adjustments. The TCA in a final concentration of 12% worked well as it precipitates the carbohydrate containing caseins (13) which account for some phosphorus that otherwise would remain in solution as well as two-thirds of any possible macropeptide. Direct isoelectric or TCA precipitation from products brings down some neutral fat as well as phospholipids which must be removed prior to casein phosphorus determination.

A test for protein alone does not determine the legality of an ice cream's composition. The official fat test as published in AOAC (1) is a modification of the Rose-Gottleib procedure

TABLE 2. Time effect of sodium hydroxide on total protein determination.

Sample	% Protein[a] Immediately	% Protein[a] 10 Days
Cheese kisses	2.17	1.63
Coffee whitener	4.52	.15
Nonfat dry milk	32.39	28.80
Vanilla ice cream – A	2.70	2.60
Vanilla ice cream – B	4.22	3.61
Vanilla ice milk	3.15	2.64
Chocolate ice cream	2.52	2.23
Strawberry ice cream	2.41	2.16

[a]Protein analyses were in triplicate.

with Mojonnier equipment and is commonly called the Mojonnier test. If the fat-free residue from this test contains most of the milk proteins, it would be an ideal starting solution for protein determinations. The aqueous layer from the Mojonnier extract, properly diluted, is ideal for protein precipitation with TCA, if the alcohol first is removed by bubbling nitrogen through the sample.

The use of TCA as a precipitating agent yielded a further advantage in that virtually all of the proteins were precipitated. Thus, when the protein is dissolved in NaOH, a solution is obtained which is free of fat and inorganic material yet contains all of the proteins in the samples. The total protein content can be determined after nitrogen analysis is on an aliquot of this solution. Total protein minus the casein as determined by phosphorus content would give the whey protein. The total protein content was determined on a variety of products (Table 2). It is imperative to take an aliquot for total protein analysis immediately after dissolving the precipitate. Data in Table 2 shows the effect of protein remaining in a basic solution on total protein determination. Aliquots taken immediately for protein analyses all have higher results than those aliquots taken after 10 days. This indicates that some nitrogen is lost on standing.

Because phosphorus is the entity measured, various steps in this procedure were evaluated with solutions of casein and sodium caseinate (Table 3). Little or no effect on casein by exposure to ammonia from the Mojonnier extraction procedure could be demonstrated. Essentially the same phosphorus was obtained whether the casein was precipitated with TCA directly from the solution or taken through the entire Mojonnier extraction procedure and then precipitated. Acid and buffer precipitation had no effect on casein phosphorus determination for either the casein solution or sodium caseinate solution. Evaluation of a sodium caseinate solution gave high readings as might be expected, because some phospholipids and inorganic phosphorus still remained in the commercial sample. When subjected to the Mojonnier extraction procedure, which removes the lipids, the solution showed no great difference among results of the various treatments. Only a trace of phospholipids escaped the Mojonnier extraction. The quantity was too small to be visualized on TLC plates by a phosphorus sensitive spray but appeared as barely visible spots after the plates were charred. Hence, conditions of the Mojonnier process do not affect adversely the determination of casein phosphorus content.

Effects of processing of milk on phosphorus determination were studied in samples of raw, pasteurized, and pasteurized-homogenized

TABLE 3. Validation of phosphorus determination with casein and sodium caseinate solutions.

	(μgP/mg of casein)
Casein solution	
No treatment	7.48
TCA precipitated (no other treatment)	7.18
Buffer[a] precipitated (no other treatment)	7.28
Mojonnier extracted and TCA precipitated	7.18
Mojonnier extracted and buffer precipitated	7.05
NH_4OH added and held 2 h	7.58
NH_4OH and alcohol	7.50
Sodium caseinate solution	
No treatment	8.38
TCA precipitated (no other treatment)	6.23
Buffer precipitated (no other treatment)	6.23
Mojonnier extracted and TCA precipitated	6.13
Mojonnier extracted and buffer precipitated	6.05

TABLE 4. Casein phosphorus determination on whole milk samples precipitated with TCA or H_2SO_4.

Samples	Number of determinations	(μg Casein P/ml of milk[a])
TCA[b]	4	228 ± 6
H_2SO_4[c]	3	221 ± 6
TCA-cm[d]	2	222 ± 6
		Mean = 225 μg P/ml of milk or 2.59% casein

[a]Milk was obtained from an individual Jersey herd.

[b]Samples were Mojonnier extracted and TCA precipitated.

[c]Samples were Mojonnier extracted and precipitated with sulfuric acid.

[d]Samples were precipitated with TCA and fat extracted on Büchner funnel with chloroform:methanol.

whole milk carried through the complete casein method for determination of casein phosphorus. Processing did not affect determination as all three treatments gave nearly identical results: 178, 174, and 178 μg/ml of milk.

When it is not desirable to do a Mojonnier-type extraction, samples after precipitation with TCA could be extracted with 2:1 chloroform:methanol on a Büchner funnel fitted with a glass fiber filter. For this study, fresh whole pasteurized-homogenized milk was used. Aliquots of the milk were TCA precipitated and extracted with chloroform:methanol. These were compared (Table 4) with samples of the same milk which were subjected to a Mojonnier extraction and then precipitated with TCA or H_2SO_4. All for casein phosphorus compared favorably. A mean of 225 μg of phosphorus per milliliter of milk was found ($\sigma = \pm 6$); this yielded 2.59% casein in the milk.

TABLE 5. Comparison of milk solids-not-fat content calculated from phosphorus test against formulation contents.

Sample	% Milk solids-not-fat: Estimated from experimental[a] μg P	% Milk solids-not-fat: Intended
Vanilla ice milk	10.06	12.25
Vanilla ice cream	7.88	7.88
Chocolate ice cream	7.27	6.92
Premium ice cream	11.85	11.50

[a]The complete test was run on the samples and the μg P/g of product determined. Assuming .85% μg P/g casein, casein is 80% of total protein, and protein comprises 36% of the milk solids-not-fat, the entry given in the table can be calculated.

The casein by phosphorus method was evaluated further with products other than casein, sodium caseinate, and processed whole milk, that is, with three control ice creams. There was no significant difference between the calculated and experimental percentages of casein in Sample 1 (1.80 and 1.80) and Sample 2 (2.22 and 2.28). Sample 3, formulated from 50% of each Sample 1 and 2 had a calculated 2.01% casein The experimental 2.30 theoretically should have been between the experimental results of Samples 1 and 2. The percentage of milk solids not-fat content of commercial samples of ice milk, vanilla, chocolate, and a premium ice cream, with intended formulations were compared with the milk solids-not-fats estimated from experimental micrograms of phosphorus (Table 5). The experimental quantities for vanilla and premium ice creams were close, whereas experimental measure for vanilla ice milk was lower and slightly higher for chocolate ice cream than the intended formulation. These were intended compositions subject to human error in making up the samples.

Although the above casein test appeared entirely satisfactory for most dairy products, further flexibility was built into the procedure by use of a separate back-up-method, radial immunodiffusion (RID) (9). With RID, samples can be assayed specifically for casein, whey protein, or any other suspected protein adulter-

TABLE 6. Comparison of casein by phosphorus and radial immunodiffusion determination.

Product	% Casein	
	RID	Phosphorus
Coffee creamer	2.63	2.98
Nonfat dry milk	25.85	26.56
Vanilla ice cream – 1	2.50	2.33
Vanilla ice cream – 2	3.27	3.51
Vanilla ice milk	2.41	2.98
Chocolate ice cream	3.46	2.15
Strawberry ice cream	1.94	2.07

ant with specific antibodies. The defatted, redissolved protein from the Mojonnier extraction was used for RID determinations. A comparison of the percentages of casein from the phosphorus determination and RID is in Table 6. The RID and phosphorus casein were in reasonable agreement and certainly close enough to prove the presence of casein rather than some other protein in the sample. Chocolate ice cream, as tested by the RID method, gave high results because of an interfering ingredient in the cocoa.

The method described above can be utilized to distinguish between casein and whey protein in ice creams and other dairy products. The method is relatively straightforward and can be used routinely. The degree of sophistication built into the method by use of RID as a backup procedure permits quantitative and qualitative determination of adulterant proteins that may have been added to a product.

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REFERENCES

1 Association of Official Analytical Chemists. 1980. Page 16 *in* Official method of analysis. AOAC, Washington, DC.

2 Association of Official Analytical Chemists. 1980. Page 47 *in* Official method of analysis. AOAC, Washington, DC.

3 Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248.

4 Douglas, F. W., Jr., R. Greenberg, H. M. Farrell, Jr., and L. F. Edmondson. 1981. Effects of ultra-high-temperature pasteurization on milk proteins. J. Agric. Food Chem. 29:11.

5 Elfagm, A. A., and J. V. Wheelock. 1978. Heat interaction between α-lactalbumin, β-lactoglobulin, and casein in bovine milk. J. Dairy Sci. 61:159.

6 Fiske, C. H., and Y. Subarrow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375.

7 Frozen desserts. 1978. Fed. Reg. 43:19384.

8 Gordon, W. G., and E. B. Kalan. 1974. Pages 92–93 *in* Proteins of milk in fundamentals of dairy chemistry. 2nd ed. B. H. Webb, A. H. Johnson, and J. A. Alford, ed. Avi Publ. Co., Inc., Westport, CT.

9 Guidry, A., and R. E. Pearson. 1979. Improved methodology for quantitative determination of serum and milk protein by single radial immunodiffusion. J. Dairy Sci. 62:1252.

10 Guy, E. J. 1980. Partial replacement of non-fat milk solids and cane sugar in ice cream with lactose hydrolyzed sweet whey solids. J. Food Sci. 45:129.

11 Kannon, A., and R. Jenness. 1961. Relation of milk serum protein and milk salts on the effects of heat treatment on rennet clotting. J. Dairy Sci. 44:808.

12 Kroger, M., E. E. Katz, and J. C. Weaver. 1978. Determining protein content of ice cream and frozen desserts. J. Dairy Sci. 61:274.

13 Mackinlay, A. G., and R. G. Wake. 1971. k-Casein and its attack by rennin (chymosin). Page 200 *in* Milk proteins chemistry and molecular biology. Vol. II. H. A. McKenzie, ed. Academic Press, Inc., New York, NY.

14 Meun, H. C., and K. C. Smith. 1968. A micro phosphate method. Anal. Biochem. 26:364.

15 Thompson, M. P., N. P. Tarassuk, R. Jenness, H. A. Lillenik, U. S. Ashworth, and D. Rose. 1965. Nomenclature of proteins of cow's milk. 2nd revision. J. Dairy Sci. 48:159.

16 Walstra, P., and J. J. DeGraaf. 1962. Notes on the determination of the phospholipid content of milk products. Neth. Milk Dairy J. 16:283.