

Indirect and Direct Determination of the Casein Content of Milk by Kjeldahl Nitrogen Analysis: Collaborative Study

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The classic method for determination of milk casein is based on precipitation of casein at pH 4.6. Precipitated milk casein is removed by filtration and the nitrogen content of either the precipitate (direct casein method) or filtrate (noncasein nitrogen; NCN) is determined by Kjeldahl analysis. For the indirect casein method, milk total nitrogen (TN; Method 991.20) is also determined and casein is calculated as TN minus NCN. Ten laboratories tested 9 pairs of blind duplicate raw milk materials with a casein range of 2.42–3.05% by both the direct and indirect casein methods. Statistical performance expressed in protein equivalents (nitrogen \times 6.38) with invalid and outlier data removed was as follows: NCN method (wt%), mean = 0.762, s_r = 0.010, s_R = 0.016, repeatability relative standard deviation (RSD_r) = 1.287%, reproducibility relative standard deviation (RSD_R) = 2.146%; indirect casein method (wt%), mean = 2.585, repeatability = 0.015, reproducibility = 0.022, RSD_r = 0.560%, RSD_R = 0.841; direct casein method (wt%), mean = 2.575, s_r = 0.015, s_R = 0.025, RSD_r = 0.597%, RSD_R = 0.988%. Method performance was acceptable and comparable to similar Kjeldahl methods for determining nitrogen content of milk (Methods 991.20, 991.21, 991.22, 991.23). The direct casein, indirect casein, and noncasein nitrogen methods have been adopted by AOAC INTERNATIONAL.

Milk casein determined by the Rowland protein fractionation scheme is defined as proteins that precipitate at pH 4.6 (1, 2). The amount of intact casein is a function of both casein synthesis in the mammary gland and the breakdown of casein that can occur as a result of the activity of native milk proteases (primarily plasmin) and microbial proteases. The extent to which casein breakdown products precipitate at pH 4.6 or remain soluble is a function of fragment solubility (3). Casein as percentage of total protein (based on total

nitrogen determination) have been reported between 73–79% for raw, commingled milk from dairy processing plant storage tanks (4–6). The range for milk from farm bulk tanks and individual cows is wider (7, 8).

The dairy industry has a strong economic interest in casein, because both the amount of intact casein and the degree of casein breakdown have a significant impact on the quality of dairy products and the processing characteristics of milk. For example, casein quality influences the heat stability of milk, gelation of UHT products, and the yield, flavor and texture of dairy products such as cheese and yogurt (9). The somatic cell component of many payment programs is based on the relationship between somatic cell counts and casein (10). At elevated somatic cell counts, there is an increase in milk proteolytic activity and a corresponding decrease in the amount of intact casein (11, 12).

Automated methods make the determination of somatic cell counts convenient, fast, and relatively inexpensive. However, somatic cell counts remain only an indirect indicator of protein quality. Other factors such as stage of lactation, breed, parity, season, region, and health history can effect both plasmin activity and the casein content of milk (3, 13–16). Widespread adoption of casein testing would be expected if a reliable "quick and easy" casein test was developed. A number of methods utilizing mid-infrared milk analyzers have been described (17–19), although they are not routinely used because of various methodological limitations.

The classic method for determination of milk casein is based on the Rowland milk protein fractionation scheme (1). The total nitrogen (TN) content of milk and the nitrogen in the filtrate (noncasein nitrogen; NCN) after casein precipitation at pH 4.6 are determined by Kjeldahl analysis. Casein is calculated as the difference between TN and NCN (multiplying nitrogen by 6.38 to express the results on a protein basis). There is academic controversy as to what exactly constitutes "casein," as some casein proteolysis products may be soluble at pH 4.6 and some noncasein nitrogen components may be trapped with the precipitate. However, the Rowland definition of casein is generally accepted as the reference method in the commercial dairy industry and has been reliably used since the mid 1900's as a variable in cheese yield prediction formulas (13). The current International Dairy Federation (IDF) procedure (2) for determination of milk casein is based on the Rowland procedure (1).

Submitted for publication September 22, 1997.

The recommendation was approved by the Methods Committee on Commodity Foods and Commodity Products and was adopted by the Official Methods Board of AOAC.

EXHIBIT

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98R 4-12-07

The AOAC describes 2 procedures for casein determination loosely based on the Rowland procedure, but they suffer from methodological problems and we are unaware of any laboratories that currently use either method successfully. Both methods are similar to the Rowland procedure, in that they involve precipitation of casein followed by Kjeldahl nitrogen analysis. Method I (927.03 A; 20) determines the nitrogen content of the casein directly by instructing the operator to transfer all the precipitate onto filter paper and then place it into a Kjeldahl flask. However, it is virtually impossible to quantitatively transfer precipitated casein this way, due to its stickiness and adhesion to glass. Thus, the results from this method are variable and low. Method II (927.03 B-C; 20) measures the nitrogen content of the filtrate, but it requires a warm temperature incubation (50°–60°C) which is less than optimum for the precipitation of casein, and no correction for the volume of precipitate is used.

The inclusion of an updated version of the Rowland procedure as an official AOAC method would be of value given the relative disuse of the current AOAC casein methods and the general acceptance of the Rowland procedure as a reference method for casein determination by both the IDF and the U.S. dairy industry. With these goals in mind, the primary objective of the present study was to describe the method performance of an improved and modified version of AOAC Method 927.03 B-C (method II; 20) for the indirect determination of milk casein by Kjeldahl analysis, based on the classic Rowland procedure (1) and the current IDF method (2). Additional objectives were to modify and improve upon AOAC Method 927.03 A (method I; 20) for the direct determination of milk casein by Kjeldahl analysis, and compare both the test results and method performance to those determined for the indirect casein method. The direct casein method has the advantage of requiring 1 rather than 2 Kjeldahl analyses compared with the indirect casein method.

Preliminary Work: Indirect Casein Method

The proposed indirect casein method in this study is based on the classic Rowland procedure (1) and was designed to harmonize procedural details with the current IDF method (2). In this procedure, casein is precipitated at pH 4.6 using acetic acid and sodium acetate. The NCN in the filtrate is measured by Kjeldahl nitrogen analysis. The TN of the test material is also determined and casein ($N \times 6.38$) is calculated as the difference between the TN and NCN. The performance of the TN Method 991.20 has been described previously (21, 22). Thus, the preliminary work for the indirect casein method focused on the NCN method.

Differences between the proposed NCN method and the IDF method (2) include temperatures of sampling and incubation. The proposed NCN procedure specifies that the test sample be tempered to $38^\circ \pm 1^\circ\text{C}$ before weighing the test portion, instead of 20°C used in the IDF procedure. The temperature of 38°C is the same as that used for most milk testing procedures (including TN), and is necessary to melt the fat and obtain a representative sample. The temperature specified in the IDF method is historically based because milk testing in the early and mid 1900's was all conducted at 20°C . Practical experience and in-house testing indicated that analytical results for NCN are simi-

lar if milk is weighed at either 20° or 38°C (assuming the fat is in solution). Thus, for consistency with the other AOAC Kjeldahl methods for milk (22–25), the proposed NCN procedure specifies that test samples be mixed and weighed at $38^\circ \pm 1^\circ\text{C}$.

Both the IDF (2) and Rowland procedure (1) specify that approximately 75 mL of 40°C water be added to the test portion, and that the flask be incubated at 40°C for 10 min after the addition of 1 mL 10% acetic acid. However, this is very inconvenient because virtually all water baths for tempering in a milk testing laboratory are set at 38°C . A number of experiments were conducted to investigate the effect of water and incubation temperature ranging from room temperature to 40°C . Test results were similar regardless of the temperature used. Use of room temperature was rejected because it represented a significant departure from the IDF and Rowland procedure. However, we considered it practical and desirable to specify 38°C instead of 40°C in the proposed NCN procedure because the 38°C temperature is more consistent with current water bath temperatures.

The principle behind the NCN procedure is the precipitation of casein at pH 4.6. However, when acetic acid is first added to the milk and water, the pH decreases to below 4.6. The pH approximates 4.6 once the sodium acetate is added. Thus, precipitation is technically conducted at a pH lower than prescribed. Furthermore, pH will vary according to the conditions of precipitation (e.g., temperature, milk composition) so precise pH control cannot be expected when fixed amounts of acetic acid and sodium acetate are added. Recognizing this, some researchers perform casein precipitation by direct addition of acetic acid until a pH of 4.6 is achieved, as indicated with an electrode. This approach yields casein test values that may differ from those achieved by separate additions of acetic acid and sodium acetate (Paul Paquin, University Laval, Quebec, Canada, personal communication). We believe this approach is valid and encourage further method development in this direction. However, our goal is to describe and characterize the performance of the traditional Rowland approach as currently used in AOAC Method 927.03 B-C (20) and IDF (2). In this case the results of the method define casein for commercial purposes. Which method is scientifically correct (fixed pH or fixed acid) is a matter for future research, as is the question of which protein and protein fragments actually fit the definition of casein.

The IDF NCN procedure (2) instructs the user to add 1 mL 10% acetic acid, wait 10 min, and then add 1 mL 1N sodium acetate. We attempted combining the acetic acid and sodium acetate and adding it as a single 2 mL reagent addition. The consistency of the precipitate was different than that obtained using the standard procedure and initial trials indicated the casein results with the combined reagent were slightly different than those obtained using the standard procedure. This approach was not pursued further.

Volume Correction Factor

A volume correction factor is used to correct the final measured nitrogen value for the decrease in volume that occurs when the pH 4.6 precipitate (casein and fat) is removed from the filtrate.

The IDF method uses a factor of 0.994 in the NCN equation to correct for the volume of the pH 4.6 precipitate in the 100 mL volumetric flask (2). Rowland (1) determined this factor experimentally by measuring the precipitate volume of actual samples, and derived the equation:

$$\text{Factor} = 1 - (0.11 \times F/100) + (0.07 \times C/100)$$

where F and C are the percentages of fat and casein respectively.

The factor 0.994 was calculated assuming milk with an average 3.7% fat and 2.6% casein content. Historically this single factor has been applied to the analysis of all raw, whole milk materials regardless of composition because differences in the factor due to normal variation in milk composition has a relatively insignificant impact on NCN determination. For example, in the extreme (and rather improbable) cases where a factor of 0.996 (assuming 2.7% fat and 2.2% casein) or 0.991 (assuming 6% fat and 3% casein) might be used, the difference in calculated NCN ($\times 6.38$) would be only 0.004% protein (calculated based on a 10 g test portion and titration volumes of 4.2 and 0.1 mL 0.1N HCL for the filtrate and blank, respectively).

Approaches other than the Rowland factor are possible. One is a direct calculation assuming the precipitate volume equals the weight of the precipitated protein and fat, as is done in the non-protein nitrogen Kjeldahl method (AOAC 991.21; 23). A second approach is based on the steric exclusion of serum proteins with respect to casein micelles which takes into account that not all the solvent present is available to serum proteins (26). However, if the assumptions are the same as with the Rowland factor, then the results using the direct calculation are similar to the Rowland approach, while the steric approach yields NCN values that average approximately 0.005% protein lower. Given the small magnitude of difference between the calculated results using the different approaches, the Rowland factor (e.g., 0.994) was selected for the proposed NCN method because it has the historical advantage and is currently accepted by the IDF.

Preliminary Work: Direct Casein Method

The direct casein method is based on the same principle as the indirect method, except that the nitrogen content of the casein is directly determined. This is done by precipitating the casein directly in the Kjeldahl flask, followed by Kjeldahl nitrogen analysis. The AOAC describes a similar direct casein method (927.03 A, method I; 20), but that procedure instructs the user to transfer the casein precipitate from a beaker onto filter paper, and finally into a Kjeldahl flask. This is virtually impossible to accomplish quantitatively because precipitated casein is very sticky and adheres to glass. The mechanics of precipitation in the proposed procedure were modeled after those successfully developed and described for the direct protein nitrogen Kjeldahl method (AOAC 991.22; 24, 27).

A 5 g test portion is used in the direct procedure, rather than 10 g as in the NCN procedure. A 5 g test portion yields a precipitate with a nitrogen content within the working range of the method. Unlike the NCN procedure where casein precipitation

is conducted at 38°C, no temperature control is utilized in the direct casein procedure because temperature control is impractical with Kjeldahl flasks and tubes. Furthermore, cooling to 20°C is unnecessary in the direct procedure because it is not a volumetric determination.

In the NCN procedure, casein is precipitated from 10 g milk after dilution with approximately 75 mL water followed by the addition of 1 mL 10% acetic acid and 1 mL 1N sodium acetate. When all volumes were reduced proportionally for the direct procedure (accommodating the 5 g milk), casein values were consistently higher than for the indirect method. Subsequent experiments determined that some NCN was "trapped" in the precipitate. The amount of NCN in the precipitate could be manipulated by controlling the volume of the solution in which precipitation was conducted—the greater the dilution volume the lower the apparent casein content by the direct method. The indirect casein test results agreed with the direct casein test results when the precipitation volume for the direct method was approximately 75 mL. The final volume proportions for the direct method were designed to be practical (round numbers, single water addition), yield similar results to those achieved using the indirect procedure, and maintain the same acetic acid and sodium acetate concentration during precipitation as used in the NCN procedure.

Casein Stability

The measured casein content of milk is affected by both milk handling and storage (time and temperature). There is normally very little casein breakdown in good quality raw milk. However, preliminary work has indicated that the measured casein content of milk decreases with time, even in good quality milk with refrigerated storage.

In order to determine the magnitude of this decrease, the casein content was immediately determined on 5 fresh raw bulk milk tank materials with low somatic cell counts ($<200 \times 10^3$). The unpreserved milks were then stored at 4°C over a 4-day period. Test samples of the 5 materials were tested daily for casein. The results are shown in Figure 1. Compared with the fresh milks (Day 0), the casein content decreased an average of 1.6% by Day 4 for these low somatic cell count milks. The magnitude of the time-dependent decrease may be larger for high somatic cell count or high bacterial count milks. Senyk et al. (12) observed that proteolysis, as indicated by tyrosine value, increased over time even in pasteurized milk stored at 6.7°C and preserved with potassium dichromate, and the amount of proteolysis increased with increasing somatic cell count (12).

While the determination of casein breakdown over time was done on a limited number of samples, the results were consistent with the observations of Senyk et al. (12) and it seems reasonable to conclude that changes in the casein content of milk may occur even under refrigerated storage. A note to this effect is included in both the direct and indirect casein procedures.

Effect of Heat Treatment on Measured Casein

Heat treatment of milk, such as might occur during pasteurization or drying, results in an interaction between whey and casein proteins (28). As a result, the casein content of milk appears to increase with the severity of heat treatment because

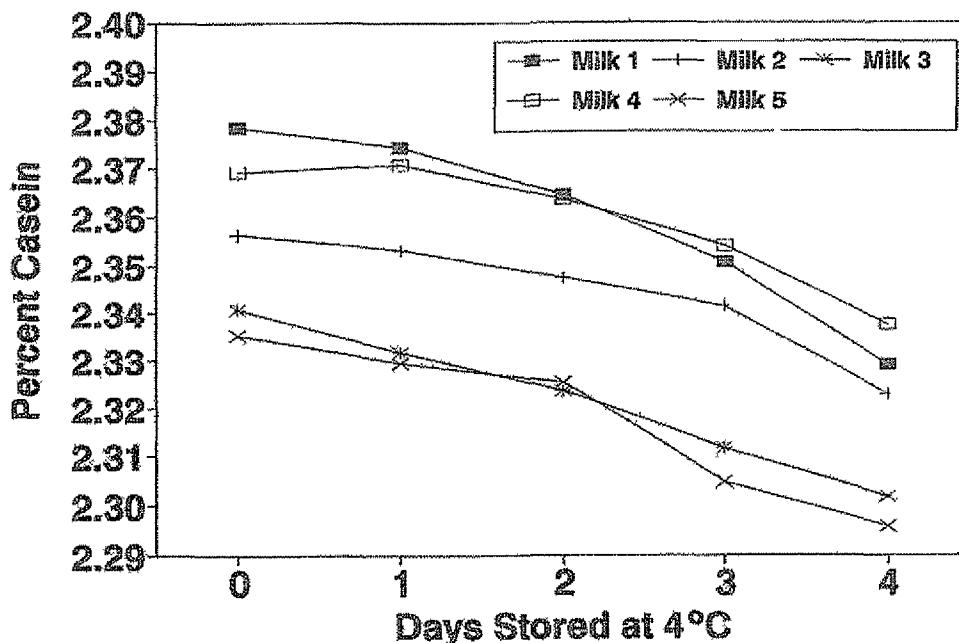


Figure 1. Changes in percent casein in raw milk stored at 4°C over 4 days.

why protein precipitates along with casein. Thus, neither the direct nor indirect casein methods are suitable for accurately determining the casein content of heat-treated milk.

Collaborative Study

Each of 10 laboratories received 2 sets of 18 raw milk test samples (9 pairs of blind duplicates per set). One set was used for the indirect casein test and the other for the direct casein test. The test samples were coded with random 3-digit numbers. Codes were different between and within sets, and analysis order was randomized for all laboratories and methods.

The 9 test materials were fresh, raw milks collected on a single day (Sunday) from individual farms and commingled herds. Each test material was cold split (4°–6°C) on Monday by mixing, pouring into a large plastic container, and agitating continuously with a motor driven stirrer while the milk (approximately 35 mL) was drawn from a spout directly into precoded plastic vials (Capitol Vial, Inc., Fultonville, NY). The test samples were refrigerated immediately after splitting. Splitting uniformity was verified by checking the fat and protein content of the first, middle, and last vials of each material using a Dairy Lab 2 mid-infrared milk analyzer (Foss Electric, Hillerød, Denmark).

The test sample vials, wrapped in plastic bags and sealed with tape to protect them from water contamination, were packed on wet ice and shipped to individual laboratories by overnight air delivery. Test samples arrived at the laboratories on Tuesday. Arrival temperature of the milk was verified as $\leq 4^\circ\text{C}$ by each laboratory.

Test sample preparation was specified so that all laboratories prepared the same materials by both methods on the same day. Test samples were prepared to the point where they were in the

Kjeldahl flask (or tube) and the copper sulfate, potassium sulfate, and sulfuric acid had been added. The same 5 materials (in blind duplicate) were prepared by all laboratories on Day 1 (Wednesday) and the remaining 4 materials were prepared on Day 2 (Thursday). This avoided the confounding effect of casein degradation over time. Kjeldahl analysis was conducted within 5 days of preparation.

Forms were provided for recording raw data, test results, and comments about each individual test sample. Additionally, a questionnaire was provided. Information, such as test material arrival time, apparatus and reagents used, actual testing conditions during analysis, and verification of Kjeldahl recovery, was requested to help assure that analysts followed all details of the procedures.

998.05 Noncasein Nitrogen Content of Milk—Kjeldahl Method

First Action 1998

(Applicable to fresh raw milk. Noncasein nitrogen content will increase with milk storage time due to casein breakdown even at 4°C. Noncasein nitrogen content of heat treated milk will be artificially low because of whey protein denaturation.)

Caution: See Appendix B, safety notes on H_2SO_4 , HCl, fuming acids, NaOH, ethanol, acetic acid and use of electrical equipment.

Method Performance:

(calculated on a protein equivalent basis: nitrogen $\times 6.38$)

$s_r = 0.010$; $s_R = 0.016$; $\text{RSD}_r = 1.287\%$; $\text{RSD}_R = 2.146\%$; $r = 0.028$; $R = 0.046$

(calculated on a percent nitrogen basis)

$s_r = 0.0015$; $s_R = 0.0026$, $RSD_r = 1.287\%$; $RSD_R = 2.146\%$;
 $r = 0.0043$, $R = 0.0072$

A. Principle

Casein is precipitated from milk at pH 4.6 using acetic acid and sodium acetate solutions. Precipitated milk casein is removed by filtration. Filtrate contains the noncasein nitrogen components of milk. Nitrogen content of filtrate is determined as in 991.20.

B. Apparatus

See 991.20B or 991.20I and in addition:

- (a) Volumetric flask.—100 mL.
- (b) Volumetric pipet.—50 mL.

C. Reagents

See 991.20C or 991.20J and in addition:

- (a) Sodium acetate solution.—1N (w/v), using analytical grade sodium acetate or sodium acetate trihydrate. Prepare 1N solution on anhydrous basis. Prepare fresh weekly.
- (b) Acetic acid solution.—10% (v/v), use analytical grade glacial acetic acid.

D. Preparation of Samples

Mix $38 \pm 1^\circ\text{C}$ milk as in 925.21. Immediately pipet 10 \pm 0.1 mL milk into preweighed 100 mL volumetric flask. Weigh. Record to nearest 0.0001 g. (Additional samples may be weighed at this point, but additions of water, acetic acid, and sodium acetate plus filtration should occur within 15 min of weighing milk. This minimizes proteolytic degradation of casein during sample preparation.) Add 75 ± 0.5 mL of 38°C water to flask. Add 1 ± 0.02 mL 10% acetic acid, C(b), to flask, stopper, and swirl gently. Incubate flask 10 min in a 38°C water

bath. Add 1 ± 0.02 mL 1N sodium acetate, C(a), and swirl to mix. Cool contents to 20°C . Bring to volume with 20°C water, stopper, and invert to mix. Let precipitate settle. Filter through pleated filter paper (Whatman No. 1, 15 cm, N-free; or equivalent) and collect entire filtrate. Filtrate should be clear and free of particulate matter; if not, repeat sample preparation using a fresh milk sample. Using a volumetric pipet, pipet 50 ± 0.05 mL filtrate into a Kjeldahl digestion flask (or tube) containing potassium sulfate and copper (II) sulfate pentahydrate catalyst solution as in 991.20D or 991.20K. Add sulfuric acid as in 991.20D or 991.20K. Flask may be stoppered and digested later. Digest and distill a blank solution (50 ± 1 mL room temperature water, 0.5 ± 0.02 mL 10% acetic acid, and 0.5 ± 0.02 mL 1N sodium acetate) each day samples are analyzed. Keep record of blank values. If blank values change, identify cause.

E. Determination

Proceed as in 991.20E or 991.20L.

F. Nitrogen Recovery Verification

Proceed as in 991.20F or 991.20M.

G. Calculation

Calculate results as follows:

$$\text{Noncasein nitrogen, \%} = [1.4007 \times (V_s - V_b) \times N \times 2 \times 0.994] / W_m$$

where V_s and V_b = mL titrant used for sample and blank, respectively; N = normality of HCl solution; W_m = weight, g, of milk. To calculate noncasein nitrogen on a protein equivalent basis:

Table 1. Percent total protein in milk determined by the Kjeldahl total nitrogen method

| Material | Laboratory | | | | | | | | | |
|----------|------------|--------|--------|--------|--------|--------|--------|---------------------|--------|--------|
| | A | B | C | D | E | F | G | H | I | J |
| 1 | 3.3125 | 3.3112 | 3.3274 | 3.3422 | 3.3040 | 3.3474 | 3.3400 | 3.3297 | 3.2948 | 3.3386 |
| | 3.3242 | 3.3358 | 3.3336 | 3.3380 | 3.3022 | 3.3536 | 3.3338 | 3.3325 | 3.2936 | 3.3309 |
| 2 | 3.9393 | 3.9407 | 3.9608 | 3.9566 | 3.9039 | 3.9508 | 3.9449 | 3.8716 ^a | 3.9083 | 3.9453 |
| | 3.9328 | 3.9268 | 3.9748 | 3.9423 | 3.9130 | 3.9396 | 3.9329 | 3.9540 ^a | 3.9302 | 3.9388 |
| 3 | 3.2939 | 3.2749 | 3.3221 | 3.3242 | 3.2905 | 3.3201 | 3.3216 | 3.3231 | 3.2980 | 3.3223 |
| | 3.3089 | 3.2936 | 3.3143 | 3.3289 | 3.3055 | 3.3074 | 3.3197 | 3.3185 | 3.3014 | 3.3037 |
| 4 | 3.3714 | 3.3442 | 3.3995 | 3.3606 | 3.3357 | 3.3634 | 3.3231 | 3.3544 | 3.3163 | 3.3752 |
| | 3.3750 | 3.3517 | 3.3729 | 3.3679 | 3.3426 | 3.3635 | 3.3668 | 3.3582 | 3.3305 | 3.3770 |
| 5 | 3.2190 | 3.2143 | 3.2448 | 3.2031 | 3.1917 | 3.2152 | 3.2145 | 3.2453 | 3.1937 | 3.2231 |
| | 3.2045 | 3.2002 | 3.2369 | 3.2235 | 3.1946 | 3.2284 | 3.2139 | 3.2459 | 3.1896 | 3.2315 |
| 6 | 3.2989 | 3.3275 | 3.3244 | 3.3221 | 3.2624 | 3.3046 | 3.2946 | 3.2982 | 3.2802 | 3.3182 |
| | 3.2761 | 3.2948 | 3.3212 | 3.3212 | 3.2576 | 3.3053 | 3.3042 | 3.3012 | 3.2904 | 3.3125 |
| 7 | 3.2925 | 3.3040 | 3.3155 | 3.2991 | 3.2462 | 3.2678 | 3.2312 | 3.2835 | 3.2553 | 3.3102 |
| | 3.2893 | 3.2682 | 3.3289 | 3.2967 | 3.2444 | 3.3149 | 3.2434 | 3.2952 | 3.2618 | 3.3016 |
| 8 | 3.1269 | 3.1432 | 3.1573 | 3.1490 | 3.0929 | 3.1296 | 3.1214 | 3.1262 | 3.1154 | 3.1364 |
| | 3.1332 | 3.1237 | 3.1279 | 3.1406 | 3.0871 | 3.1347 | 3.1250 | 3.1065 | 3.1108 | 3.1425 |
| 9 | 3.2464 | 3.2447 | 3.2973 | 3.2865 | 3.1919 | 3.2590 | 3.2244 | 3.2556 | 3.2292 | 3.2613 |
| | 3.2448 | 3.2446 | 3.2866 | 3.2706 | 3.1982 | 3.2628 | 3.2465 | 3.2433 | 3.2379 | 3.2623 |

^a Statistical outlier (Cochran test).

Noncasein protein, % = % noncasein nitrogen \times 6.38

Note: The factor 0.994 corrects for volume of precipitate, assuming raw, whole milk with ca 3.7% fat and 2.6% casein. The factor for precipitate volume may need to be adjusted (based on estimated fat and casein content) if liquid dairy products of significantly different composition are analyzed:

$$\text{Factor} = 1 - (0.11 \times \% \text{fat}/100) + (0.07 \times \% \text{casein}/100)$$

Ref.: *J. AOAC Int* 81, 763(1998).

998.07 Casein Nitrogen Content of Milk—Kjeldahl Method—Indirect Method

First Action 1998

(Applicable to fresh raw milk. Casein nitrogen content will decrease with milk storage time due to casein breakdown even at 4°C. Casein nitrogen content of heat treated milk will be artificially high because of whey protein denaturation.)

Method Performance:

(calculated on a protein equivalent basis: nitrogen \times 6.38)

$s_r = 0.015$; $s_R = 0.022$, $RSD_r = 0.560\%$; $RSD_R = 0.841\%$; $r = 0.041$; $R = 0.062$

(calculated on a percent nitrogen basis)

$s_r = 0.0023$; $s_R = 0.0034$, $RSD_r = 0.560\%$; $RSD_R = 0.841\%$; $r = 0.0064$; $R = 0.0096$

A. Principle

Total nitrogen and noncasein nitrogen contents of a milk sample are determined separately. Difference between these 2 determinations is casein nitrogen content of the milk.

B. Determination

(a) *Total nitrogen*.—Determine as in 991.20.

(b) *Noncasein nitrogen*.—Determine as in 998.05.

C. Calculation

Subtract noncasein nitrogen content from total nitrogen content of milk sample to obtain casein nitrogen. Multiply results by 6.38 to express casein on a protein basis.

Ref.: *J. AOAC Int* 81, 763(1998).

998.06 Casein Nitrogen Content of Milk—Kjeldahl Method—Direct Method

First Action 1998

(Applicable to fresh raw milk. Casein nitrogen content will decrease with milk storage time due to casein breakdown even at 4°C. Casein nitrogen content of heat treated milk will be artificially high because of whey protein denaturation.)

Caution: See Appendix B, safety notes on H₂SO₄, HCl, fuming acids, NaOH, ethanol, acetic acid, and use of electrical equipment.

Method Performance:

(calculated on a protein equivalent basis: nitrogen \times 6.38)

Table 2. Percent noncasein nitrogen (\times 6.38) in milk determined by the Kjeldahl noncasein nitrogen method

| Material | Laboratory | | | | | | | | | |
|----------|-----------------|--------|--------|--------|---------------------|--------|---------------------|---------------------|---------------------|---------------------|
| | A | B | C | D | E | F | G | H | I | J |
| 1 | 0.7609 | 0.7617 | 0.7810 | 0.7596 | 0.7732 | 0.7980 | 0.7833 | 0.7479 ^a | 0.7185 ^b | 0.7851 |
| | 0.7683 | 0.7513 | 0.7824 | 0.7547 | 0.7710 | 0.7883 | 0.7572 | 0.5532 ^a | 0.6896 ^b | 0.7858 |
| 2 | LA ^c | 0.8845 | 0.9116 | 0.8716 | 0.8628 | 0.9383 | 0.8945 ^d | 0.8976 ^a | 0.8760 | 0.8966 |
| | LA ^c | 0.8650 | 0.9115 | 0.8717 | 0.8801 | 0.9177 | 0.8233 ^d | 0.6180 ^a | 0.8776 | 0.9112 |
| 3 | 0.7179 | 0.7282 | 0.7474 | 0.7215 | 0.7556 | 0.7581 | 0.7147 | 0.4907 ^a | 0.7318 | 0.7454 |
| | 0.7341 | 0.7245 | 0.7506 | 0.7276 | 0.7215 | 0.7508 | 0.7539 | 0.7622 ^a | 0.7371 | 0.7446 |
| 4 | 0.7399 | 0.7424 | 0.7618 | 0.7408 | 0.7540 | 0.7712 | 0.7522 | 0.7096 ^a | 0.7365 | 0.8177 ^d |
| | 0.7438 | 0.7259 | 0.7838 | 0.7436 | 0.7520 | 0.7670 | 0.7567 | 0.7691 ^a | 0.7436 | 0.7681 ^d |
| 5 | 0.7321 | 0.7265 | 0.7520 | 0.7328 | 0.7423 | 0.7616 | 0.7498 ^d | 0.7415 ^a | 0.7489 | 0.7521 |
| | 0.7324 | 0.7563 | 0.7584 | 0.7247 | 0.7147 | 0.7609 | 0.6233 ^d | 0.6998 ^a | 0.7195 | 0.7513 |
| 6 | 0.7645 | 0.7716 | 0.7839 | 0.7534 | 0.7727 | 0.7843 | 0.6995 ^d | 0.7787 | 0.7592 | 0.7853 |
| | 0.7630 | 0.7695 | 0.7842 | 0.7521 | 0.7374 | 0.7701 | 0.7765 ^d | 0.7664 | 0.7524 | 0.7706 |
| 7 | 0.7299 | 0.7725 | 0.7428 | 0.7161 | 0.7323 | 0.7420 | 0.6709 ^d | 0.7540 | 0.7229 | 0.7425 |
| | 0.7355 | 0.7663 | 0.7377 | 0.7243 | 0.7556 | 0.7426 | 0.7353 ^d | 0.7247 | 0.7100 | 0.7303 |
| 8 | 0.6961 | 0.7422 | 0.7099 | 0.6829 | 0.6956 | 0.7121 | 0.6952 | 0.6993 | 0.6876 | 0.7150 |
| | 0.7262 | 0.7405 | 0.7082 | 0.6966 | 0.6994 | 0.7130 | 0.6982 | 0.7170 | 0.6907 | 0.7161 |
| 9 | 0.7376 | 0.7588 | 0.7579 | 0.7234 | 0.7457 ^d | 0.7553 | 0.7572 | 0.7457 | 0.7328 | 0.7524 |
| | 0.7231 | 0.7676 | 0.7583 | 0.7272 | 0.6997 ^d | 0.7541 | 0.7554 | 0.7467 | 0.7454 | 0.7674 |

^a Invalid data (did not bring volumetric flasks to volume).

^b Statistical outlier (Grubb's test).

^c Laboratory accident (no data available).

^d Statistical outlier (Cochran test).

$s_r = 0.015$, $s_R = 0.025$; $RSD_r = 0.597\%$, $RSD_R = 0.988\%$; $r = 0.044$, $R = 0.072$

(calculated on a percent nitrogen basis)

$s_r = 0.0024$; $s_R = 0.0040$; $RSD_r = 0.597\%$; $RSD_R = 0.988\%$; $r = 0.0068$; $R = 0.0113$

A. Principle

Casein is precipitated from milk at pH 4.6 using acetic acid and sodium acetate solutions. Precipitation must be done in Kjeldahl flask or tube. The acidified solution, which contains the noncasein nitrogen components of the sample, is separated from casein precipitate by filtration. Nitrogen content of casein precipitate is determined as in 991.20.

B. Apparatus

See 991.20B or 991.20F and in addition:

(a) *Pump dispenser*.—Capable of addition of 70 ± 0.5 mL H_2O .

(b) *Pump dispenser*.—Capable of addition of 30 ± 0.5 mL buffer solution.

C. Reagents

See 991.20C or 991.20J and in addition:

(a) *Sodium acetate solution*.—1N (w/v), using analytical grade sodium acetate or sodium acetate trihydrate. Prepare 1N solution on anhydrous basis. Prepare fresh weekly.

(b) *Acetic acid solution*.—10% (v/v), using analytical grade glacial acetic acid.

(c) *Buffer solution*.—Dilute 1 ± 0.1 mL 1N sodium acetate and 1 ± 0.1 mL 10% acetic acid to 100 mL with 20°C water. Prepare fresh weekly.

D. Preparation of Samples

Mix $38^\circ \pm 1^\circ C$ milk as in 925.21. Immediately place weighed sample (5 ± 0.1 mL) into Kjeldahl digestion flask (or digestion tube), either by weighing the sample directly into the flask or by determining the difference between the weight of a small container with the sample and the weight of the container after the sample has been poured into the flask or tube. Record to nearest 0.0001 g. Use pump dispenser to immediately add 70 ± 0.5 mL H_2O to the flask, rinsing any milk on neck of flask into bulb. (Additional samples can be weighed at this point, but the additions of acetic acid and sodium acetate plus filtration should occur within 15 min of weighing milk and adding water. This minimizes proteolytic degradation of casein during sample preparation). Add 0.75 ± 0.02 mL 10% acetic acid, C(b), to flask, and swirl gently. Ensure all acid is added to bulb of flask. Leave mixture at room temperature for 10 min. Add 0.75 ± 0.02 mL 1N sodium acetate, C(a), to Kjeldahl flask, and swirl gently. Pour mixture from Kjeldahl flask through pleated filter paper (Whatman No. 1, 15 cm, N-free; or equivalent) and collect filtrate. (Some casein precipitate will remain in flask and some will be collected on the filter paper. It is not necessary to remove the precipitate from the flask.) Filter completely before next pour.

Immediately after pouring mixture into filter paper (do not let precipitate dry on the inside of the neck of Kjeldahl flask), use pump dispenser to add 30 ± 0.5 mL buffer solution, C(c),

Table 3. Percent casein in milk determined by the Kjeldahl indirect casein nitrogen method

| Material | Laboratory | | | | | | | | | |
|----------|-----------------|--------|--------|--------|-----------------|--------|-----------------|-----------------|-----------------|-----------------|
| | A | B | C | D | E | F | G | H | I | J |
| 1 | 2.5516 | 2.5495 | 2.5464 | 2.5826 | 2.5308 | 2.5494 | 2.5567 | NA ^a | NA ^b | 2.5595 |
| | 2.5559 | 2.5845 | 2.5512 | 2.5833 | 2.5312 | 2.5653 | 2.5766 | NA ^a | NA ^b | 2.5451 |
| 2 | NA ^c | 3.0562 | 3.0492 | 3.0850 | 3.0411 | 3.0125 | NA ^b | NA ^d | 3.0323 | 3.0467 |
| | NA ^c | 3.0618 | 3.0633 | 3.0706 | 3.0329 | 3.0219 | NA ^b | NA ^d | 3.0526 | 3.0276 |
| 3 | 2.5760 | 2.5467 | 2.5747 | 2.6027 | 2.5349 | 2.5620 | 2.6069 | NA ^a | 2.5662 | 2.5769 |
| | 2.5748 | 2.5691 | 2.5637 | 2.6013 | 2.5840 | 2.5566 | 2.5658 | NA ^a | 2.5643 | 2.5591 |
| 4 | 2.6315 | 2.6018 | 2.6377 | 2.6198 | 2.5817 | 2.5922 | 2.5709 | NA ^a | 2.5798 | NA ^b |
| | 2.6312 | 2.6258 | 2.5891 | 2.6443 | 2.5906 | 2.5965 | 2.6071 | NA ^a | 2.5869 | NA ^b |
| 5 | 2.4869 | 2.4878 | 2.4928 | 2.4703 | 2.4494 | 2.4536 | NA ^b | NA ^a | 2.4448 | 2.4710 |
| | 2.4721 | 2.4439 | 2.4785 | 2.4988 | 2.4799 | 2.4675 | NA ^b | NA ^a | 2.4701 | 2.4802 |
| 6 | 2.5344 | 2.5559 | 2.5405 | 2.5687 | 2.4897 | 2.5203 | NA ^b | 2.5195 | 2.5210 | 2.5329 |
| | 2.5131 | 2.5253 | 2.5370 | 2.5691 | 2.5202 | 2.5352 | NA ^b | 2.5348 | 2.5380 | 2.5419 |
| 7 | 2.5626 | 2.5315 | 2.5727 | 2.5830 | 2.5139 | 2.5258 | NA ^b | 2.5295 | 2.5424 | 2.5677 |
| | 2.5538 | 2.5019 | 2.5912 | 2.5724 | 2.4888 | 2.5723 | NA ^b | 2.5605 | 2.5518 | 2.5713 |
| 8 | 2.4308 | 2.4010 | 2.4474 | 2.4661 | 2.3973 | 2.4175 | 2.4262 | 2.4269 | 2.4278 | 2.4214 |
| | 2.4070 | 2.3832 | 2.4197 | 2.4440 | 2.3877 | 2.4217 | 2.4268 | 2.3895 | 2.4201 | 2.4264 |
| 9 | 2.5088 | 2.4859 | 2.5394 | 2.5631 | NA ^b | 2.5037 | 2.4672 | 2.5099 | 2.4964 | 2.5089 |
| | 2.5217 | 2.4770 | 2.5283 | 2.5434 | NA ^b | 2.5087 | 2.4911 | 2.4966 | 2.4925 | 2.4949 |

^a Not available (invalid NCN data).
^b Not available (NCN statistical outlier).
^c Not available (NCN laboratory accident).
^d Not available (invalid NCN data and TN statistical outlier).

Table 4. Percent casein in milk determined by the Kjeldahl direct casein nitrogen method

| Material | Laboratory | | | | | | | | | |
|----------|------------|---------------------|--------|--------|---------------------|--------|-----------------|--------|---------------------|--------|
| | A | B | C | D | E | F | G | H | I | J |
| 1 | 2.5362 | 2.5500 | 2.5797 | 2.5775 | 2.4877 ^a | 2.5210 | LA ^b | 2.5473 | 2.5015 | 2.5657 |
| | 2.5320 | 2.5578 | 2.5723 | 2.5845 | 2.2157 ^a | 2.5245 | LA ^b | 2.5611 | 2.4887 | 2.5481 |
| 2 | 3.0291 | 3.0077 | 3.0398 | 3.0478 | 2.9884 ^a | 3.0190 | LA ^b | 3.0239 | 3.0035 | 3.0536 |
| | 3.0341 | 3.0259 | 3.0504 | 3.0510 | 1.2088 ^a | 3.0222 | LA ^b | 3.0138 | 2.9823 | 3.0537 |
| 3 | 2.5549 | 2.5646 ^c | 2.5771 | 2.5814 | 2.2907 ^a | 2.5472 | LA ^b | 2.5686 | 2.5396 | 2.5763 |
| | 2.5644 | 2.4608 ^a | 2.5368 | 2.5995 | 2.4247 ^a | 2.5455 | LA ^b | 2.5590 | 2.5375 | 2.5658 |
| 4 | 2.5952 | 2.4237 ^c | 2.6462 | 2.6220 | 2.5889 ^a | 2.5930 | LA ^b | 2.6277 | 2.5859 | 2.6152 |
| | 2.5937 | 2.5739 ^c | 2.6225 | 2.6495 | 2.5796 ^a | 2.5765 | LA ^b | 2.5926 | 2.5706 | 2.6108 |
| 5 | 2.4514 | 2.6194 ^c | 2.5026 | 2.4687 | 2.0890 ^a | 2.4418 | LA ^b | 2.4623 | 2.4233 | 2.4846 |
| | 2.4591 | 2.5600 ^a | 2.4859 | 2.5008 | 2.4724 ^a | 2.4464 | LA ^b | 2.4704 | 2.4319 | 2.4787 |
| 6 | 2.5222 | 2.5205 | 2.5380 | 2.5601 | 2.5493 ^a | 2.5053 | 2.5016 | 2.5230 | 2.3723 ^c | 2.5384 |
| | 2.5204 | 2.5371 | 2.5358 | 2.5670 | 2.5038 ^a | 2.5224 | 2.5448 | 2.5057 | 2.5094 ^d | 2.5158 |
| 7 | 2.5224 | 2.5200 | 2.5497 | 2.5764 | 1.0742 ^d | 2.5389 | 2.5573 | 2.5359 | 2.5288 | 2.5447 |
| | 2.5293 | 2.5068 | 2.5651 | 2.5827 | 2.2864 ^a | 2.5163 | 2.5903 | 2.5426 | 2.5134 | 2.5495 |
| 8 | 2.3568 | 2.3957 | 2.4211 | 2.4363 | 1.2502 ^a | 2.3957 | 2.4163 | 2.4131 | 2.3652 | 2.3959 |
| | 2.3770 | 2.4121 | 2.4305 | 2.4403 | 2.3604 ^a | 2.3943 | 2.4310 | 2.4066 | 2.4082 | 2.4250 |
| 9 | 2.4745 | 2.4840 | 2.5039 | 2.5249 | 2.0303 ^a | 2.4807 | 2.6058 | 2.4861 | 2.4718 | 2.4262 |
| | 2.4658 | 2.4843 | 2.4852 | 2.5308 | 1.0754 ^a | 2.4907 | 2.4973 | 2.4869 | 2.4629 | 2.4966 |

^a Invalid data (crystallization after digestion)^b Laboratory accident^c Statistical outlier (Cochran test)

to Kjeldahl flask, rinsing any precipitate on neck of flask down into bulb. Swirl to mix. Pour mixture onto same filter paper after first filtration is complete, and combine filtrates (use same side of flask neck for all pour overs to reduce area to which precipitate can adhere). Immediately rinse neck of Kjeldahl with additional 30 ± 0.5 mL aliquot of C(c), swirl to mix, and filter through same filter paper after second filtration is complete. Add filtrate to previous 2 filtrates. Filtrate should be clear and free of particulate matter. If particulates appear, re-

cycle filtrate through same filter paper or repeat test. Clear filtrate can be discarded.

Remove filter paper after filtration and filter paper has dried slightly. Ensure no precipitate on filter paper is lost. Pinch filter paper at top and twist sides and bottom to form oblong shape. If any precipitate remains on either inner or outer lip of Kjeldahl flask, wipe with filter paper so precipitate adheres to filter paper. Drop filter paper into Kjeldahl flask. Add potassium sulfate, copper (II) sulfate pentahydrate catalyst so-

Table 5. AOAC statistical parameters by test material for percent total protein in milk determined by the Kjeldahl total nitrogen method

| Material | Data | No of labs | No of tests | Mean, % | Statistic | | | | | |
|-------------------------|-----------------|------------|-------------|---------|-----------|--------|----------------------|----------------------|--------|--------|
| | | | | | s_r | s_R | RSD _r , % | RSD _R , % | r | R |
| 8 | all | 10 | 20 | 3.1265 | 0.0097 | 0.0180 | 0.3096 | 0.5743 | 0.0271 | 0.0503 |
| 5 | all | 10 | 20 | 3.2167 | 0.0076 | 0.0187 | 0.2366 | 0.5799 | 0.0213 | 0.0522 |
| 9 | all | 10 | 20 | 3.2497 | 0.0076 | 0.0272 | 0.2324 | 0.8358 | 0.0211 | 0.0760 |
| 7 | all | 10 | 20 | 3.2825 | 0.0140 | 0.0283 | 0.4274 | 0.8615 | 0.0393 | 0.0792 |
| 6 | all | 10 | 20 | 3.3008 | 0.0096 | 0.0204 | 0.2923 | 0.6191 | 0.0270 | 0.0572 |
| 3 | all | 10 | 20 | 3.3096 | 0.0084 | 0.0145 | 0.2552 | 0.4391 | 0.0236 | 0.0407 |
| 1 | all | 10 | 20 | 3.3263 | 0.0089 | 0.0178 | 0.2069 | 0.5353 | 0.0193 | 0.0499 |
| 4 | all | 10 | 20 | 3.3583 | 0.0131 | 0.0218 | 0.3908 | 0.6501 | 0.0368 | 0.0611 |
| 2 | all | 10 | 20 | 3.9354 | 0.0204 | 0.0230 | 0.5176 | 0.5849 | 0.0570 | 0.0644 |
| 2 | outlier removed | 9 | 18 | 3.9379 | 0.0092 | 0.0183 | 0.2326 | 0.4639 | 0.0256 | 0.0512 |
| Grand mean ^a | | | | 3.3454 | 0.0098 | 0.0210 | 0.2943 | 0.6276 | 0.0278 | 0.0594 |

^a Grand mean values were calculated using only material data where either no statistical outliers were identified (materials 1, 3, 4, 5, 6, 7, 8 and 9) or with outliers removed (material 2). Grand material mean was calculated by averaging. All other statistical means were calculated from the square root of the average of the squared deviations.

Table 6. AOAC statistical parameters by test material for percent noncasein nitrogen ($\times 6.38$) in milk determined by the Kjeldahl noncasein nitrogen method

| Material | Data | No. of labs | No. of tests | Mean, % | Statistic | | | | | |
|-------------------------|-----------------|-------------|--------------|---------|-----------|--------|----------------------|----------------------|--------|--------|
| | | | | | s_r | s_R | RSD _r , % | RSD _R , % | r | R |
| 8 | all | 10 | 20 | 0.7071 | 0.0085 | 0.0165 | 1.2033 | 2.3309 | 0.0238 | 0.0461 |
| 3 | all | 9 | 18 | 0.7367 | 0.0131 | 0.0142 | 1.7748 | 1.9329 | 0.0366 | 0.0399 |
| 7 | all | 9 | 18 | 0.7344 | 0.0174 | 0.0219 | 2.3638 | 2.9776 | 0.0486 | 0.0612 |
| 7 | outlier removed | 8 | 16 | 0.7379 | 0.0102 | 0.0169 | 1.3850 | 2.2860 | 0.0286 | 0.0472 |
| 5 | all | 9 | 18 | 0.7355 | 0.0322 | 0.0322 | 4.3732 | 4.3732 | 0.0901 | 0.0901 |
| 5 | outlier removed | 8 | 16 | 0.7417 | 0.0128 | 0.0157 | 1.7260 | 2.1170 | 0.0358 | 0.0440 |
| 9 | all | 10 | 20 | 0.7456 | 0.0118 | 0.0174 | 1.5892 | 2.3279 | 0.0332 | 0.0486 |
| 9 | outlier removed | 9 | 18 | 0.7481 | 0.0062 | 0.0144 | 0.8286 | 1.9204 | 0.0174 | 0.0402 |
| 4 | all | 9 | 18 | 0.7556 | 0.0136 | 0.0215 | 1.8005 | 2.8417 | 0.0381 | 0.0601 |
| 4 | outlier removed | 8 | 16 | 0.7510 | 0.0074 | 0.0150 | 0.9829 | 1.9971 | 0.0207 | 0.0420 |
| 6 | all | 10 | 20 | 0.7648 | 0.0197 | 0.0200 | 2.5821 | 2.6100 | 0.0553 | 0.0559 |
| 6 | outlier removed | 9 | 18 | 0.7677 | 0.0102 | 0.0185 | 1.3275 | 1.7539 | 0.0265 | 0.0377 |
| 1 | all | 9 | 18 | 0.7650 | 0.0100 | 0.0270 | 1.3089 | 3.5250 | 0.0280 | 0.0755 |
| 1 | outlier removed | 8 | 16 | 0.7726 | 0.0078 | 0.0144 | 1.0075 | 1.8628 | 0.0218 | 0.0403 |
| 2 | all | 8 | 16 | 0.8871 | 0.0200 | 0.0280 | 2.2600 | 3.1564 | 0.0561 | 0.0784 |
| 2 | outlier removed | 7 | 14 | 0.8912 | 0.0097 | 0.0240 | 1.0895 | 2.6957 | 0.0272 | 0.0673 |
| Grand mean ^a | | | | 0.7616 | 0.0098 | 0.0163 | 1.2865 | 2.1461 | 0.0277 | 0.0462 |

^a Grand mean values were calculated using only material data where either no statistical outliers were identified (materials 3 and 8) or with outliers removed (materials 1, 2, 4, 5, 6, 7 and 9). Grand material mean was calculated by averaging. All other statistical means were calculated from the square root of the average of the squared deviations.

lution, and sulfuric acid as in 991.20D or 991.20K. Flask may be stoppered and digested later. Digest and distill a blank (including filter paper) each day that samples are analyzed. Keep a record of blank values. If blank values change, identify cause.

E. Determination

Proceed as in 991.20E or 991.20L.

F. Nitrogen Recovery Verification

Proceed as in 991.20F or 991.20M, except add filter paper to all recovery flasks.

G. Calculation

Calculate casein nitrogen in milk as in 991.20G.

Ref.: *J. AOAC Int.* 81, 763(1998).

Table 7. AOAC statistical parameters by test material for percent casein in milk determined by the Kjeldahl indirect casein nitrogen method

| Material | Data | No. of labs | No. of tests | Mean, % | Statistic | | | | | |
|-------------------------|------|-------------|--------------|---------|-----------|--------|----------------------|----------------------|--------|--------|
| | | | | | s_r | s_R | RSD _r , % | RSD _R , % | r | R |
| 8 | all | 10 | 20 | 2.4194 | 0.0137 | 0.0211 | 0.5646 | 0.8710 | 0.0382 | 0.0590 |
| 5 | all | 8 | 16 | 2.4717 | 0.0177 | 0.0177 | 0.7160 | 0.7160 | 0.0496 | 0.0496 |
| 9 | all | 9 | 18 | 2.5076 | 0.0098 | 0.0248 | 0.3922 | 0.9907 | 0.0275 | 0.0696 |
| 6 | all | 9 | 18 | 2.5332 | 0.0132 | 0.0195 | 0.5229 | 0.7704 | 0.0371 | 0.0546 |
| 7 | all | 9 | 18 | 2.5496 | 0.0171 | 0.0294 | 0.6707 | 1.1530 | 0.0479 | 0.0823 |
| 1 | all | 8 | 16 | 2.5571 | 0.0111 | 0.0174 | 0.4358 | 0.6796 | 0.0312 | 0.0487 |
| 3 | all | 9 | 18 | 2.5714 | 0.0168 | 0.0188 | 0.6530 | 0.7814 | 0.0470 | 0.0527 |
| 4 | all | 8 | 16 | 2.6054 | 0.0177 | 0.0235 | 0.6783 | 0.9012 | 0.0495 | 0.0657 |
| 2 | all | 7 | 14 | 3.0468 | 0.0102 | 0.0207 | 0.3341 | 0.6800 | 0.0285 | 0.0580 |
| Grand mean ^a | | | | 2.5847 | 0.0145 | 0.0217 | 0.5601 | 0.8412 | 0.0409 | 0.0615 |

^a No statistical outliers were identified. Grand material mean was calculated by averaging. All other statistical means were calculated from the square root of the average of the squared deviations.

Table 8. AOAC statistical parameters by test material for percent casein in milk determined by the Kjeldahl direct casein nitrogen method

| Material | Data | No of labs | No of tests | Mean, % | Statistic | | | | | |
|-------------------------|------------------|------------|-------------|---------|-----------|--------|----------------------|----------------------|--------|--------|
| | | | | | s_r | s_R | RSD _r , % | RSD _R , % | r | R |
| 8 | all | 9 | 18 | 2.4067 | 0.0144 | 0.0241 | 0.5987 | 1.0000 | 0.0403 | 0.0674 |
| 5 | all | 6 | 16 | 2.4805 | 0.0178 | 0.0510 | 0.7192 | 2.0561 | 0.0500 | 0.1428 |
| 5 | outliers removed | 7 | 14 | 2.4648 | 0.0106 | 0.0251 | 0.4288 | 1.0197 | 0.0296 | 0.0704 |
| 9 | all | 9 | 18 | 2.4921 | 0.0311 | 0.0368 | 1.2464 | 1.4778 | 0.0870 | 0.1031 |
| 6 | all | 9 | 18 | 2.5189 | 0.0350 | 0.0412 | 1.3910 | 1.6342 | 0.0981 | 0.1153 |
| 6 | outliers removed | 8 | 16 | 2.5286 | 0.0144 | 0.0190 | 0.5679 | 0.7500 | 0.0402 | 0.0531 |
| 7 | all | 9 | 18 | 2.5428 | 0.0116 | 0.0249 | 0.4544 | 0.9776 | 0.0324 | 0.0696 |
| 1 | all | 8 | 16 | 2.5467 | 0.0073 | 0.0289 | 0.2876 | 1.1332 | 0.0205 | 0.0808 |
| 3 | all | 8 | 16 | 2.5549 | 0.0285 | 0.0307 | 1.1168 | 1.2019 | 0.0799 | 0.0860 |
| 3 | outliers removed | 7 | 14 | 2.5810 | 0.0127 | 0.0191 | 0.4952 | 0.7469 | 0.0355 | 0.0536 |
| 4 | all | 8 | 16 | 2.5937 | 0.0400 | 0.0521 | 1.5433 | 2.0085 | 0.1121 | 0.1459 |
| 4 | outliers removed | 7 | 14 | 2.6072 | 0.0148 | 0.0250 | 0.5687 | 0.9600 | 0.0415 | 0.0701 |
| 2 | all | 8 | 16 | 3.0286 | 0.0081 | 0.0212 | 0.2663 | 0.6995 | 0.0226 | 0.0593 |
| Grand mean ^a | | | | 2.5754 | 0.0154 | 0.0254 | 0.5971 | 0.9875 | 0.0435 | 0.0719 |

^a Grand mean values were calculated using only material data where either no statistical outliers were identified (materials 1, 2, 7, 8 and 9) or with outliers removed (materials 3, 4, 5, and 6). Grand material mean was calculated by averaging. All other statistical means were calculated from the square root of the average of the squared deviations.

Results and Discussion

Laboratories reported amino acid or ammonium sulfate recoveries between 98.3 and 100.9%, which are within method specifications of $\geq 98\%$ (22). Six laboratories (A, D, E, G, H, I) used block digestors with steam distillation and the remaining 4 laboratories used traditional Kjeldahl equipment.

The data for the determination of total, noncasein, indirect casein and direct casein nitrogen, expressed as protein equivalents ($N \times 6.38$) are presented in Tables 1–4, respectively. Method performance, with and without statistical outliers removed, is presented in Tables 5–8. The total amount of invalid and outlier data was within acceptable limits for collaborative studies (29).

Only one statistical outlier (Cochran, laboratory H, material 2) was identified for the TN method, reflecting that all of the laboratories have extensive experience and routinely run the TN method (Table 1 and 5). For the NCN method, data for material 2 from

laboratory A was unavailable due to an accident and the data for the first 5 materials from laboratory H were invalid because the volumetric flasks were not brought to volume (Table 2 and 6). Of the remaining data, 6 Cochran and 1 Grubb's outliers were identified. One of the Cochran outliers (laboratory J, material 4) occurred in a laboratory with traditional Kjeldahl equipment, while all other outliers were from laboratories using block digestors. Indirect casein was calculated only when the data from both the TN and NCN were acceptable (i.e., no laboratory accidents, invalid or outlier data), and no statistical outliers were identified when outlier tests were run (Table 3 and 7).

No data were available for the direct casein test for the first 5 materials from laboratory G due to a laboratory accident (Tables 4 and 8). All the direct casein data from laboratory E were identified as invalid because the data were clearly unreasonable and the laboratory reported problems with crystallization after digestion. Of the remaining data, 4 Cochran outliers were iden-

Table 9. Comparison of method performance statistics for determination of total, protein, casein, noncasein, and nonprotein nitrogen by Kjeldahl analysis

| AOAC method number | Method | Method performance statistics ^a | | | | | | |
|--------------------|-----------------------------------|--|-------|-------|----------------------|----------------------|-------|-------|
| | | Grand mean | s_r | s_R | RSD _r , % | RSD _R , % | r | R |
| 991.20 | Total nitrogen | 3.395 | 0.014 | 0.017 | 0.385 | 0.504 | 0.038 | 0.049 |
| 991.22 | Protein nitrogen, direct method | 2.995 | 0.008 | 0.021 | 0.285 | 0.702 | 0.024 | 0.059 |
| 991.23 | Protein nitrogen, indirect method | 2.976 | 0.014 | 0.031 | 0.483 | 1.051 | 0.040 | 0.088 |
| Current study | Casein nitrogen, indirect method | 2.585 | 0.015 | 0.022 | 0.560 | 0.841 | 0.041 | 0.062 |
| Current study | Casein nitrogen, direct method | 2.575 | 0.015 | 0.025 | 0.597 | 0.988 | 0.044 | 0.072 |
| Current study | Noncasein nitrogen | 0.762 | 0.010 | 0.016 | 1.287 | 2.146 | 0.028 | 0.046 |
| 991.21 | Nonprotein nitrogen | 0.204 | 0.006 | 0.012 | 2.817 | 5.707 | 0.016 | 0.033 |

^a Mean, s_r , s_R , r, and R expressed as protein equivalents, wt% (nitrogen $\times 6.38$).

Table 10. Mean percent protein by material determined by the total, noncasein, indirect casein, and direct casein nitrogen Kjeldahl methods

| Method | Material (% protein) ^a | | | | | | | | | Grand mean |
|---|-----------------------------------|--------|--------|---------|--------|--------|--------|--------|--------|------------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Total nitrogen | 3.3268 | 3.9379 | 3.3096 | 3.3583 | 3.2167 | 3.3008 | 3.2825 | 3.1265 | 3.2497 | 3.3454 |
| Noncasein nitrogen | 0.7726 | 0.8912 | 0.7367 | 0.7510 | 0.7417 | 0.7677 | 0.7379 | 0.7071 | 0.7481 | 0.7616 |
| Casein nitrogen, indirect method | 2.5571 | 3.0468 | 2.5714 | 2.6054 | 2.4717 | 2.5332 | 2.5496 | 2.4194 | 2.5076 | 2.5847 |
| Casein nitrogen, direct method | 2.5467 | 3.0286 | 2.5610 | 2.6072 | 2.4648 | 2.5286 | 2.5428 | 2.4067 | 2.4921 | 2.5754 |
| Difference between indirect and direct casein | | | | | | | | | | |
| | 0.0104 | 0.0182 | 0.0104 | -0.0018 | 0.0069 | 0.0046 | 0.0068 | 0.0127 | 0.0155 | 0.0093 |
| Indirect casein nitrogen as a % of total nitrogen | | | | | | | | | | |
| | 76.88 | 77.37 | 77.70 | 77.58 | 76.84 | 76.75 | 77.67 | 77.38 | 77.16 | 77.26 |

^a Material means determined from the collaborative studies of the individual methods.

tified. Three of these outliers were from Laboratory B (materials 3-5) and close inspection of the data suggests that the cause may have been test sample mixup rather than procedural error.

Linear regression indicated no significant ($p > 0.05$) relationship between protein concentration and method performance for any of the method statistics determined for the total, indirect casein or direct casein nitrogen methods (Tables 5, 7 and 8). There was a significant relationship between protein concentration and both s_R and R for the NCN method, primarily due to the slightly larger reproducibility standard deviation determined for material 2 (Table 6).

Table 9 compares the statistical performance of the indirect casein, direct casein, and NCN methods with the Kjeldahl nitrogen methods for raw milk that have previously been collaboratively studied: TN 991.20 (22), direct protein nitrogen 991.22 (24), indirect protein nitrogen 991.23 (25), nonprotein nitrogen 991.21 (23), ordered from high to low protein. The TN method theoretically represents maximum achievable precision because there is essentially no test sample manipulation other than warming and weighing. The one exception is the NPN method, which has the potential for better absolute repeatability because the procedure uses 0.01N HCl instead of the 0.10N HCl as the titrant.

The repeatability standard deviation and r value were very similar for the methods that use a 0.1N HCl titrant (e.g., all but NPN). Reproducibility standard deviation and R value were also very comparable, although the reproducibility of the protein, casein, and noncasein nitrogen methods are slightly greater than that for TN due to the additional preparation steps and/or multiple analyses. RSDs increased with decreasing protein level, as expected. Overall, performance of the indirect casein, direct casein, and noncasein nitrogen methods compared favorably with the previously studied methods and within expectations for the protein levels measured. The performance of the TN method in the present study (Table 5) was similar to that determined in the original collaborative study of the method (Table 9; 22).

Tables 5-8 present mean test results for each of the methods studied from low to high protein, but the order of the materials is not necessarily the same among methods. A comparison by material is shown in Table 10. With the exception of material 4, the indirect casein results were higher than the direct casein results, averaging 0.01% protein greater ($p < 0.05$). This small bias would be relatively insignificant in routine application considering other factors that can influence the casein test. It is possible that the bias between methods might be eliminated by further modification of the dilution volume during precipitation in the direct casein procedure. However, it is the authors' opinion that the potential for a small improvement in accuracy was not enough to justify pursuing this approach at this time. A similar bias has been observed for the indirect and direct protein nitrogen methods, except that the indirect protein method averaged 0.02% lower than the direct method (Table 9; 27).

The indirect casein nitrogen method is considered the "reference" method from a historical perspective. Casein nitrogen (indirect) as a percent of TN is calculated in Table 10, and averaged 77.26%, with a range of 76.75 to 77.70%. This range of casein nitrogen as a percent TN is typical for good quality commingled raw milk (4-6).

Collaborators' Comments

Problems noted by collaborative study participants occurred primarily in laboratories using block digestors. The NCN flask/tubes tend to foam during digestion. Foaming can be controlled by starting the digestion on cool burners and digesting at a low heat setting until white smoke appears (22). This extends digestion time, however, and crystallization may occur after digestion if there is not enough residual sulfuric acid left (resulting in nitrogen loss). This is especially problematic for block digestors because heating of the tubes cannot be individually controlled, excessive fume aspiration rates can result

in the removal of too much sulfuric acid, and the amount of sulfuric acid initially added is less than that used in the traditional Kjeldahl system (20 mL as opposed to 25 mL). Block digester conditions can be optimized for NCN digests but requires more effort and attention on the part of the operator compared with the traditional system.

Similarly, crystallization of the direct casein digests after digestion was observed by some laboratories using block digestors. Foaming was generally not a problem, but the extra organic matter (due to the filter paper) can result in an insufficient amount of sulfuric acid left at the end of digestion if digestion conditions and aspiration rate are not optimized. Again, block digestors require more effort and attention to obtain high quality results compared with a traditional Kjeldahl system.

Recommendations

The Associate Referee recommends the following:

- (1) The new method for determination of the noncasein nitrogen content of milk be adopted official first action.
- (2) The new indirect method for determination of milk casein content be adopted official first action to replace AOAC method II, 927.03 B-C.
- (3) The new direct method for determination of milk casein content be adopted official first action to replace AOAC method I, 927.03 A, as an alternative to the indirect casein method.

Acknowledgments

We thank the Cornell University and USDA Dallas Milk Market Administrator laboratories for their help with the preliminary work, and the following laboratories for their participation in the collaborative studies:

- Barbano Laboratory, Food Science Department, Cornell University, Ithaca, NY
- Dairy Quality Control Institute, Inc., St. Paul, MN
- Northeast Dairy Herd Improvement Association, Ithaca, NY
- State of Wisconsin Department of Agriculture, Trade and Consumer Protection, Bureau of Laboratory Services, Madison, WI
- USDA Chicago Milk Market Administrator Laboratory, Lisle, IL
- USDA Cleveland Milk Market Administrator Laboratory, Middleburg Heights, OH
- USDA Dallas Milk Market Administrator Laboratory, Carrollton, TX
- USDA Kansas City Milk Market Administrator Laboratory, Lenexa, KS
- USDA Louisville Milk Market Administrator Laboratory, Louisville, KY
- USDA Tulsa Milk Market Administrator Laboratory, Tulsa, OK

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