FOOD COMPOSITION AND ADDITIVES

Kjeldahl Nitrogen Analysis as a Reference Method for Protein Determination in Dairy Products

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Measurement of total nitrogen by Kjeldahl analysis is the historical reference method for determination of the protein content of dairy products and is used for both calibration and validation of alternative methods for protein determination. Accurate evaluation of alternative methods is not possible if there is large uncertainty regarding the reference values. When Kjeldahl analysis is used to establish reference values, the performance of the Kjeldahl testing must be verified and within established expectations. Advice is given for Kjeldahl system optimization, evaluation of test results, and troubleshooting. Techniques for successful Kjeldahl nitrogen analysis of dairy products other than milk are discussed.

The reference method for the determination of the protein content of dairy products is based on measurement of total nitrogen by Kjeldahl analysis (1, 2). Nitrogen is multiplied by a factor, typically 6.38, to express the results as total protein (also called "crude" protein). The total protein in milk is composed of approximately 77% caseins, 17% whey proteins (sometimes called serum proteins), and 6% nonprotein nitrogen-containing (NPN) substances (3–8). The NPN components are primarily urea, creatine, creatinine, amino acids, and other minor nitrogen containing compounds (9). The "true" protein (caseins and whey) content of milk, rather than total protein (caseins, whey, and NPN), can also be determined by Kjeldahl analysis and better reflects the value of milk protein to both the consumer and the processor (10). However, this paper will focus solely on measurement of total nitrogen because of its historical and continued wide-range use.

The validity of using the single factor, i.e. 6.38, to express nitrogen results on a protein basis has also been questioned, because of both the error associated with use of a single factor and the accuracy of the factor itself (11, 12). However, any single conversion factor will incorporate error because it assumes that nitrogen content is a fixed proportion of the "total" protein. In reality, milk contains a variety of proteins and nitrogen-containing nonprotein substances, all of which differ in nitrogen content, and the proportion of these substances differs among milk and dairy products. Use of different factors for different milk and dairy products might reduce this error but not eliminate it. Furthermore, the generation and use of different factors would more likely cause confusion rather than improved accuracy. For the purposes of the dairy and food industries, the consistent use of a single factor is more advantageous than the inconsistent use of multiple factors. The factor 6.38 will be used to express nitrogen on a protein basis in this paper, recognizing the inherent inadequacies.

Total nitrogen determined by Kjeldahl analysis is traditionally used as the reference to establish protein values for dairy materials. Recovery of nitrogen is greater than 98% when Kjeldahl analysis is conducted using ammonium sulfate and individual amino acids, although there are no definitive primary standards available that adequately mimic the varied sources of nitrogen in dairy products and the complexity of the various matrixes. The choice of Kjeldahl to determine reference values is based on historical use, general acceptance within the dairy industry, soundness of the analytical principle, precision and presumed accuracy, and lack of suitable alternatives such as a primary reference standard. Nitrogen, as determined by Kjeldahl analysis and expressed on a protein basis (6.38), empirically defines total protein for proximate analysis of dairy products.

The procedure and performance characteristics of the Kjeldahl method for determination of total nitrogen content of milk are described in both AOAC Method **991.20** (1) and International Dairy Federation Standard (IDF) 20B:1993, Parts 1 and 2 (13). The method is internationally recognized as an International Organization for Standardization–IDF–AOAC method on the basis of the current harmonized interlaboratory study guidelines. To briefly describe the method, a test portion is digested with sulfuric acid in the presence of potassium sulfate and a low concentration of copper catalyst, and nitrogen is liberated and retained as ammonium sulfate. Potassium sulfate is used to elevate the boiling point of sulfuric acid and to increase the oxidizing power of the digestion mixture. Ammonia is released from the acid digest by the addition of sodium hydroxide. The ammonia is distilled, collected in a boric acid solution, and titrated with standardized hydrochloric acid. The amount of hydrochloric acid used is proportional to the amount of nitrogen originally present in the test portion. Only macro-Kjeldahl equipment, either traditional system or block digestor with steam distillation, is de-

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scribed in the official method. Micro-Kjeldahl equipment is not suitable for Kjeldahl reference testing. This is because the accuracy and precision of the micro-Kjeldahl system are inferior compared with macro-Kjeldahl because of the use of small sample sizes, which magnify sampling and analytical errors (2).

Kjeldahl for Calibration and Validation

Kjeldahl nitrogen analysis is used for both calibration and validation of alternative methods for determining protein in dairy products. In either situation, success depends on the accuracy of the Kjeldahl-derived values.

Methods that require calibration include mid-infrared spectroscopy, near-infrared spectroscopy, Fourier transform infrared spectroscopy, and dye binding (4, 14–17). Calibration is performed by using representative sample materials with known protein contents. Validation involves testing sample materials with a known protein content that were not part of the calibration set. Thus, the accuracy of both calibration and validation fundamentally depends on the accuracy of the reference values. In the case of Kjeldahl-derived reference values, lack of agreement with a secondary testing method may be expected because of differences in the methodologies used and the actual chemical or physical properties being measured. Lack of agreement because of large uncertainty around the reference values, however, confounds both calibration and validation.

Like the Kjeldahl method, the combustion (Dumas) method is based on the determination of nitrogen. The combustion method can be calibrated, independent of Kjeldahl analysis, by using a nitrogen-containing standard such as EDTA. Determination is based on the combustion of the sample material to release nitrogen gas. The liberated nitrogen can be quantitated by a variety of detection systems, including gas chromatography or thermal detection. The combustion method is a fairly rapid test that requires little sample preparation or reagent use, the method is based on sound analytical principles, and the instrumentation is commercially available from a variety of suppliers. Kjeldahl analysis, on the other hand, is labor and reagent intensive, time consuming, and subject to numerous sources of errors if procedural details are not adhered to. As a result, there has been much interest in evaluating combustion-based instrumentation as an alternative to Kjeldahl analysis.

Studies designed to evaluate the combustion method generally rely on Kjeldahl analysis to establish reference values for the validation materials (18). Unfortunately, interpretation of the results is sometimes confounded by unreliable Kjeldahl values. Nitrogen values obtained by using combustion have been reported to be higher than those from Kjeldahl analysis in some studies but not in others, although any bias is difficult to accurately quantitate because of the imprecision in the testing methodology (18).

Kjeldahl as a Reference Method

One of the major difficulties in studies designed to evaluate analytical alternatives to Kjeldahl is the performance of the Kjeldahl method itself. The confusion lies in the purpose of the Kjeldahl testing: Is it being evaluated or is it being used as a reference test? Often a study sets out to evaluate an alternate method using Kjeldahl as the reference test, but instead it concludes that the alternative method is superior because the Kjeldahl performance is variable and imprecise.

Any study designed to compare analytical results with reference values must have a reasonable degree of confidence in the reference values. The analytical method in question is being evaluated, not the reference values. Thus, when Kjeldahl nitrogen analyses are used to derive reference values, the performance of the Kjeldahl testing must be verified and within established expectations. The quality of Kjeldahl-derived data from laboratories that do not meet quality control specifications for the Kjeldahl method is demonstrably inferior to that of laboratories where specifications are met (19, 20).

The criteria for evaluation of Kjeldahl nitrogen testing of milk are well established and documented, both in terms of system optimization and method performance (1, 2). The reasonableness of the original collaborative study method performance statistics have been consistently verified in subsequent collaborative studies and ongoing interlaboratory sample exchanges (6, 10, 19, 21). This is contrary to the notion that method performance statistics obtained in a collaborative study are unrealistically optimistic because laboratories are on their "best behavior" and that the performance of the method in routine practice will be inferior. Furthermore, the use of Kjeldahl analysis to obtain reference values for evaluation of another method is not "routine practice." If an interlaboratory evaluation or collaborative study is being undertaken, every effort should be made to ensure that the reference testing is precise, accurate, and reliable so that the method under question, not the reference test, can be evaluated.

Kjeldahl Analysis of Dairy Products Other than Milk

There are a number of AOAC methods for determining total nitrogen by Kjeldahl analysis for dairy products other than milk (22–27). Unfortunately, these rather old methods were adopted before the existence of a standardized protocol for method evaluation, so performance statistics for these methods have not been determined and formal collaborative studies have not been conducted. The reasons for this are very practical. Conducting a collaborative study requires time, money, interest, and the availability of willing and competent participants. Additionally, dairy products vary vastly in their homogeneity, perishability, stability, and component levels. Reasonable characterization of method performance would require separate attention to each product's individual properties and a thorough investigation of appropriate sampling, sample handling, and sample preparation.

Despite the absence of method performance statistics, nitrogen test results obtained by using Kjeldahl analyses are routinely used as reference values for estimation of protein in dairy products other than milk. This is an entirely valid approach if the properties of the dairy materials under study, as they pertain to Kjeldahl analysis, are understood by both the researcher and the analyst. Sample origin, homogeneity, stability, perishability, handling, and composition are of critical importance, as are the size of the test portion and normality of the titrant used in the Kjeldahl analysis.

Practical Advice

Successful Kjeldahl analysis of milk and other dairy products is indeed possible when the system is optimized and the characteristics of the test material are considered in the analyses. However, there is an obvious need for alternative methods that are quicker and less labor and reagent intensive. It is unlikely that Kjeldahl will be discarded or replaced as the reference method for nitrogen determination of dairy products any time in the immediate future, but the availability of alternative methods for routine use is practical, appropriate, and essential because for many laboratories and applications, the precision and authority of definitive reference testing are not necessary.

For those laboratories that conduct Kjeldahl analysis of dairy products, **Appendixes I–III** provide some advice. **Appendix I** discusses techniques for system optimization and evaluation, reiterating and elaborating on selected details described in Method **991.20** (1). With the exception of sample preparation, this method, although specific for milk, provides the basis for Kjeldahl analyses of most other dairy products.

Appendix II outlines a sequence of system evaluation and provides advice on troubleshooting when results of an initial evaluation are unsatisfactory. The steps are sequential, in that they are intended to be followed in the order presented and that the operator should not proceed to the next step until all prior steps have been optimized. This allows for a systematic evaluation to isolate and rectify potential sources of error.

Because collaborative studies have yet to be conducted, little guidance is available in the literature on techniques for Kjeldahl nitrogen analysis of dairy products other than milk. Laboratories generally rely on experience, expertise, and word-of-mouth. Two techniques that our laboratory uses to select the test portion size of materials other than milk are the calculation of residual sulfuric acid and choice of optimum titrant normality (28). These techniques are described in **Appendix III** and are designed to adjust the amount of a different sample material to the conditions optimized for the Kjeldahl analysis of milk. We offer these guidelines not as definitive rules but as techniques that have worked well for us and other laboratories where they have been used.

Another critical aspect in the analysis of dairy products are the issues surrounding sampling and sample handling (including homogeneity, stability, and perishability). This must be addressed individually by material and detailed advice is beyond the scope of this manuscript. Procedures for sampling and sample handling are described for many materials in the *Official Methods of Analysis of AOAC INTERNATIONAL* (29).

The last issue relates to the expected method performance. As previously noted, the method performance of the Kjeldahl total nitrogen method for milk is well documented. The results of Kjeldahl analyses in individual laboratories or in an interlaboratory study should be evaluated against the performance documented for the collaborative study of the official method (1, 2, 6). A discussion of how to interpret collaborative study statistics is given for those unfamiliar with the terminology (30). As the nitrogen level of the material decreases below that expected for the total nitrogen in milk, repeatability and reproducibility standard deviations are usually not adversely affected, whereas the relative standard deviations increase (6). At higher protein levels, our experience suggests that relative standard deviations tend to be similar to those observed for milk.

Conclusions

Kjeldahl nitrogen analysis can be successfully used to determine reference protein values for dairy products when the Kjeldahl system is optimized and method performance conforms to established expectations. A reference method can only be used to derive reference values when the method itself is in analytical control. In addition, proper sampling and sample handling are crucial to successful analysis and must be addressed individually by type of material.

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Appendix I: System Optimization (1, 2)

System Requirements

Macro-Kjeldahl equipment, either traditional system or digestion block (aluminum alloy or equivalent) with steam distillation, must be used. The traditional system should have adjustable controls for individual flasks, and the digestion block should have adjustable temperature control and a device for measuring block temperature.

Micro-Kjeldahl equipment is not described in AOAC Method **991.20** (1) and is not suitable for Kjeldahl reference testing. The accuracy and precision of the micro-Kjeldahl system are inferior to those of macro-Kjeldahl because the use of small sample sizes magnifies sampling and analytical errors.

Boiling Test

Traditional Equipment

In the case of traditional equipment, temperature settings for both the digestion and distillation burners must be optimized. The burners are first preheated (10 min for gas burners, 30 min for electronic burners). Flasks, containing 3–4 boiling chips and 250 mL 25°C water, are placed on each of the burners. The heat setting that brings the water from 25°C to a rolling boil in 5–6 min is determined for each burner. This is the maximum burner setting to be used.

This water boiling technique is not suitable for block digestor equipment. Nonetheless, boiling of the sulfuric acid should be visually observed after clearing during sample digestion in block digestion tubes.

Boil Time

Boil time refers to the appropriate amount of time the acid digest needs to boil after clearing. Too short a time results in inadequate digestion, and too long a time results in nitrogen loss. It is important to test all burner positions of the system.

For the traditional system, flasks for all burners are prepared by using a high-protein, high-fat milk material. Each burner is initially set at a low temperature to control foaming. Digestion at this setting continues until white fumes appear (this usually occurs within 20 min). Next, the burner setting is increased halfway to the maximum burner setting determined in the boiling test and held for 15 min. At the end of 15 min, the burner is adjusted to the maximum setting. After the digest clears (clear with light blue-green color), continue to boil.

For the block digestor, tubes are also prepared by using a high-protein, high-fat milk material. The block digestor is initially set at a low temperature to control foaming (about 180°–230°C). The tubes, with aspirator connected, are placed in the block digestor. Aspiration should be just enough to remove fumes. Excessive aspiration should be avoided because too much sulfuric acid will be removed. Digestion at this setting continues until white fumes appear (this usually occurs within 30 min). The temperature is increased to 410°–430°C, and digestion is continued until clear. After the digest clears, continue to boil.

For both the traditional and block digestor equipment, boiling should be visually observed during sample digestion and the flasks or tubes should be free of undigested material at the conclusion. The amount of time to boil after clearing is what needs to be determined. Select times between 1 and 1.5 h after clearing to test. The protein test will increase initially (0–1.5 h), become constant, and then decrease with extended boil times after clearing. Select a boil time for the group of burners that yields maximum average protein test.

Distillation

Ensure that the condenser tip leading into the receiving flask extends below the surface of the boric acid collecting solution. Collect at least 150 mL distillate. Because the receiving flask contains 50 mL boric acid solution at the beginning of

distillation, the total volume in the receiving flask should be greater than or equal to 200 mL at the conclusion.

Recovery

Detecting Nitrogen Loss

Nitrogen loss is determined by using ammonium sulfate standard (>99% pure). Ammonium sulfate should be predried in an oven (100°C overnight) and cooled to room temperature in a desiccator before use. Sucrose is added to the digestion flask to simulate the process of digestion by providing organic material that will consume sulfuric acid in an amount similar to that used during the digestion of a milk sample. If no sucrose is added, an excessive amount of sulfuric acid may remain at the end of digestion which could cause a violent reaction when sodium hydroxide is added. Blanks, containing sucrose but no ammonium sulfate, should also be run.

Recoveries should be ≥99% and ≤101% for all burners and positions, because problems can occur either with the whole system or just specific burner positions. Reasons for low recovery on specific burners include uneven heating, tubing leaks, or inadequate cooling during distillation.

The nitrogen loss test helps identify nitrogen loss in both the digestion and distillation steps. If nitrogen loss is detected, then a further test can be done to determine whether loss is occurring during the digestion or distillation.

To determine whether nitrogen loss is occurring during distillation, 0.1200 g dried, desiccated ammonium sulfate is weighed (to 4 decimal places) into the Kjeldahl flask or tube. This is followed by the addition of distilled water (300 mL for traditional system, 85 mL for block digestor), 10 mL 95–98% sulfuric acid, and (if using a traditional system) boiling chips. The contents of the flask or tube are mixed and cooled to room temperature. Distillation is then performed as usual, starting with the addition of sodium hydroxide.

If nitrogen loss is observed when the ammonium sulfate is digested and distilled, but is not observed when the distillation system is tested separately, then the loss is occurring during digestion. If loss is also observed when the distillation system is tested separately, then the loss may be occurring during distillation caused by leaks in the distillation system or may be because of errors in titration. Additional loss during digestion is not ruled out in this case.

Evaluating Digestion Efficiency

The nitrogen loss test helps identify nitrogen loss in both the digestion and distillation steps, but it does not evaluate the completeness of digestion. Digestion efficiency is determined by using amino acid standards (>99% pure). This test should be conducted only after first evaluating and controlling the system for nitrogen loss by using ammonium sulfate. Again, all burners and positions should be tested.

Lysine (in the form of lysine hydrochloride) and tryptophan are suggested as recovery standards because they are representative of the normal substrate in milk (i.e., protein). Both are easy to work with (stable and nonhydroscopic) and can be obtained in high purity. Tryptophan is considered a "hard to digest" amino acid because of its ring structure. Amino acid standards should not be predryed before use because decomposition may occur.

Amino acids can be added directly to Kjeldahl tubes (weigh a container with the amino acid, pour into tube, weigh empty container), but this may be difficult to do with traditional Kjeldahl flasks because of the relatively narrow necks. An alternative procedure is to weigh the amino acid directly onto a piece of ashless filter paper (Whatman No. 42 or equivalent), fold the filter paper containing the amino acid, and drop it down the neck of the flask. If this procedure is used, the amount of sucrose should be proportionally reduced by the weight of the filter paper (which is essentially cellulose). Do not use wax-based paper such as weigh paper.

Blanks should include filter paper, if used to add the amino acids, and sucrose.

Recoveries should be ≥98% and ≤101% for all burners and positions. When recoveries are adequate with one amino acid but not another, purity of the standard, calculations, or method of addition should be suspect.

The Kjeldahl nitrogen method for milk is optimized to digest milk protein but not nonprotein nitrogen-containing standards such as nicotinic acid or EDTA. Nonprotein nitrogen-containing standards are not suitable for determination of the adequacy of digestion for milk protein.

Crystallization

Sulfuric acid is both consumed and volatilized during the course of digestion. If there is not enough sulfuric acid remaining at the end of digestion, then nitrogen will be lost during digestion and recoveries will be low.

One very useful way to determine whether enough residual sulfuric acid is left is to look for crystallization after digestion. At the end of digestion, the digest should be cooled to room temperature (about 25 min). The cooled digest should be liquid or liquid with only a few small crystals. A noticeable amount of crystallization indicates that the amount of residual sulfuric acid is insufficient, and test results will be low.

After the digest is cooled, water is added to the flask or tube. This should be done only after the flask or tube is cooled and inspected for crystallization. Water added to a warm tube will prevent crystallization from being observed, but it will not prevent the nitrogen loss.

Blanks

Blanks (all reagents and no sample) should be run each day. Blanks should be similar between technicians, but each technician should run their own blanks for verification. AOAC Method **991.20** (1) does not specify the acceptable range for blanks, but our experience working in-house and with numerous different laboratories suggests that typical blanks using 0.1N HCl average 0.1 mL but may range between 0.05 and 0.2 mL.

Rounding

Weights should be recorded to 4 decimal places and titration volumes to 2 decimal places. Calculation of percent nitrogen should be carried out to at least 4 decimals before conversion to protein (N × 6.38). Report final protein values to 3 decimals and nitrogen values to 4 decimals.

Procedure

When all else fails, the procedure, apparatus, and reagents being used should be compared with the specifications in AOAC Method **991.20** (1). Any deviations should be suspect (such as not using a class A buret, not running blanks, using a different indicator or catalyst, inadequate or prolonged digestion times, and not knowing the normality of the HCl).

Note on Block Digestors

Block digestors may require more effort and attention on the part of the operator compared with the traditional system, especially when analyses are conducted on materials other than milk or Kjeldahl analysis is conducted on milk protein fractions. This is because heating of the tubes in the block digestor 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 (to control foaming) than that used in the traditional Kjeldahl system because of the size of the digestion tubes.

When foaming is encountered, it can be controlled by starting the digestion on cool burners and digesting at a low heat setting until white fumes appear (1). This extends digestion time, however, and crystallization may occur after digestion if there is not enough residual sulfuric acid left (resulting in nitrogen loss). Because the heating of the tubes in the block digestor cannot be individually controlled, it is strongly advised that only samples with similar digestion behavior be digested in the block at the same time. Laboratories with block digestors that try to run "mixed" sets inevitably encounter problems with the quality of their results.

Appendix II: Troubleshooting Guide

The following troubleshooting guide (Table 1) is designed to be used in conjunction with **Appendix I**, *System Optimization*, and assumes that AOAC Method **991.20** (1) is being followed. The steps are listed in the order they should be performed, and the operator should proceed to the next step only after the previous one is optimized and meets acceptable criteria.

Appendix III: Titrant Normality, Test Portion Size, and Measurement of Residual Sulfuric Acid

Two key factors when determining sample size for Kjeldahl analysis of dairy products other than milk are ensuring, first, that the normality of the titrant is appropriate for the amount of nitrogen in the test portion and, second, that there will be enough sulfuric acid remaining at the end of digestion (if the solids content of the test portion is too high, there will not be enough sulfuric acid remaining, resulting in low nitrogen recovery). Criteria for proper selection are discussed below.

Titrant Normality

If the level of nitrogen in the distillate is low, it is a simple matter to switch titrants from 0.1N HCl to 0.01N HCl. The distillate from the preparation of a 5 g milk sample will contain about 0.0250 g nitrogen. If the amount of nitrogen falls near or below 0.0040 g, titration with 0.01N HCl is advisable. When this is done, blanks should also be titrated with 0.01N HCl.

Test Portion Size

Adjustment of test portion size (for dairy products other than bovine milk) to ensure sufficient acid remaining at the end of digestion is more complex and depends on the amount and composition of the solids in the sample material. Our laboratory uses guidelines for calculating acid remaining after digestion for the traditional Kjeldahl system based on in-house experiments and the work of Bradstreet (28). These guidelines have worked well for us and for other laboratories to which they have been distributed, but they remain estimates only. Furthermore, our experience has been primarily with traditional Kjeldahl equipment. The block digestor method uses 20 mL sulfuric acid instead of 25 mL used in the traditional system. The lower amount of acid for the block digestor aids in the control of foaming. Thus, the amount of acid left will be less when a block digestor is used, and increasing the amount of sulfuric acid added is not advisable.

Regardless of the Kjeldahl system used, the amount of acid lost to volatilization is likely to vary among different equipment and digestion conditions. Laboratories should experiment with their own systems, but the general guidelines should give a reasonable starting point. It is also worthwhile noting that, when the amount of acid remaining is marginal, the cooled digest tends to be thick and syrupy and that extensive crystallization is often observed on the addition of water. At this point, nitrogen recovery is usually adequate. When the amount of remaining acid is insufficient, visible crystallization of the digest occurs during cooling but before the addition of water, and nitrogen loss is significant.

Guidelines for Selection of Test Portion Size

(**a**) *Assumptions.*—Sulfuric acid added (assuming 96% sulfuric acid): 44 g (25 mL) for traditional system or 35 g (20 mL) for block digestor; sulfuric acid consumption: 1 g fat = consumes 18 g H_2SO_4, 1 g protein = consumes 9 g H_2SO_4, 1 g lactose = consumes 7 g H_2SO_4, 1 g lactose = 1 g carbohydrate = 1 g ashless cellulose filter paper.

(**b**) *Calculations.*

Added H_2SO_4 = g H_2SO_4 initially added

Table 1. Sequential steps for system optimization and troubleshooting for Kjeldahl nitrogen analysis

Step	Test	Procedure	Causes of failure	Action
1	Boil test (traditional system only)	Determine burner setting (for both digestion and distillation) that brings 250 mL H_2O at 25°C to a rolling boil in 5–6 min	**Inadequate boiling =**	
			Insufficient heating	Increase burner setting; replace heating coil
			Excessive boiling =	
			Excessive heat	Lower burner setting
2	Blanks	Blank values should be similar over time and should titrate between 0.05 and 0.2 mL (applicable to 0.1N HCl titrant)	**High blanks =**	
			Titration error	Check for overtitration and correct reading of meniscus; check normality of HCl; check indicator preparation and addition to boric acid; prepare new indicator (color of solution may fade over time)
			Nitrogen contamination	Check glassware cleaning solutions, reagents, and atmosphere; compare blanks obtained using newly received reagents against reagents of known performance; check for nitrogen carryover from previous distillations by running blanks after distilling flasks or tubes containing only water and, if applicable, boiling chips
			Zero or negative blanks =	
			Indicator error	Check indicator preparation and addition to boric acid
			Acidic distillate	Check strength and delivery of NaOH; check preparation of boric acid solution and evidence of contamination
			Other	Check procedure for reading meniscus
3	Boil time	Select boil time that yields maximum protein test (1–1.5 h after clearing)	**Low test =**	
			Boiling not observed in some or all burner positions	*See* step 1; check voltage input (block)
			Inadequate digestion	Increase digestion time
			Nitrogen loss	Decrease digestion time
4A	Nitrogen loss (digestion/distillation); *see* also step 4B	By using conditions determined in steps 1 and 3, determine ammonium sulfate recovery for all burner positions; recoveries should be ≥99% and ≤101%	**Low recoveries =**	
			Nitrogen loss	Check for leaks, holes, glass erosion, and inadequate connections in tubing and glassware (if steam distillation unit is used, check for pinhole leaks in glass components); make certain distillation cooling water is cold and running through manifold; avoid foaming or splashing in tube or flask during digestions
			Crystallization of digest	Reduce fume aspiration rate (block digestor); ensure that correct amount of sucrose is added to flasks; confirm that conditions established in steps 1 and 3 were used
			Impure ammonium sulfate standard	Check purity and source; ensure adequate drying/cooling/desiccation of ammonium sulfate

Table 1. (*continued*)

Step	Test	Procedure	Causes of failure	Action
			Titration error	Use class A buret for titration; check normality of HCl; check endpoint (first trace of pink) and correct reading of meniscus; check indicator preparation
			Other	Check ammonium sulfate weights and balance calibration; verify calculations, molecular formula, and data entry; review type, source, and preparation of reagents
			High recoveries =	
			Nitrogen contamination	Check glassware cleaning solutions, reagents, and atmosphere; check for nitrogen carryover from previous distillations
			Titration error	Check drain time on buret (if faster than specified for class A, replace buret; also replace any burets with broken tips); *see* also **Low recoveries**, step 4A
			Other	*See* **Low recoveries**, step 4A
4B	Nitrogen loss (distillation only; this test is useful if nitrogen loss test step 4A fails)	Determine ammonium sulfate recovery for distillation only; recoveries should be ≥99% and ≤101%; adequate distillation recovery (step 4B) and inadequate distillation or digestion recovery (step 4A) indicates problem at digestion step	*See* step 4A	*See* step 4A
5	Digestion efficiency	By using conditions determined in steps 1 and 3, determine amino acid recovery for all burner positions; recoveries should be ≥98% and ≤101%	**Low recoveries =**	
			Impure amino acid standard	Check purity and source; ensure that amino acid standard was stored correctly and not predried before use; recheck with a different amino acid
			Digestion conditions	Look for evidence of crystallization after digestion (indicative of excessive digestion time or, for block, aspiration rate); look for black particles remaining on neck of flask, indicating presence of undigested material
			Other	Check amino acid weights and balance calibration; verify calculations, molecular formula, and data entry; blank titration values should be used in the calculations but abnormally high blanks must be investigated (*see* step 2)
			High recoveries =	
			Impure amino acid standard	*See* **Low recoveries**, step 5
			Nitrogen contamination	Check for carryover from previous distillations; verify that filter paper (if used) is low nitrogen

Table 1. (*continued*)

Step	Test	Procedure	Causes of failure	Action
6	Repeatability	Difference between duplicate determinations should be ≤0.038% protein (applicable to milk only)	Poor within-laboratory precision	Verify that criteria for steps 1–5 are met for all burners and distillation positions; check sample weighing procedure; review titration technique and reading of meniscus
			Nonhomogeneous or poor quality sample	Examine sample material, quality, and preparation
7	Reproducibility	Difference between single determinations of the same material in 2 laboratories should be ≤0.049% protein (applicable to milk only)	Poor between-laboratory precision	Verify that criteria for steps 1–6 are met in all laboratories
			Nonhomogeneous or poor quality sample	Examine material quality and splitting uniformity at place of origin, and the conditions of sample delivery, arrival, storage, quality, handling, and preparation at each receiving laboratory; look for evidence of adverse conditions during exchange of material between laboratories (e.g., vials leaking, elevated temperature)

$$\text{Consumed } H_2SO_4 = \text{g } H_2SO_4 \text{ consumed by digestion of organic matter: } [(\text{g fat} \times 18) + (\text{g protein} \times 9) + (\text{g lactose} \times 7)]$$

$$\text{Available } H_2SO_4 = \text{added } H_2SO_4 - \text{consumed } H_2SO_4$$

(**c**) *Goals.*—At least 20 g H_2SO_4 calculated as available.

(**d**) *Examples.*—(*1*) *Whole milk.*—3.8% fat, 3.3% protein, 5.0% lactose, 5 g test portion:

$$\text{Added } H_2SO_4 = 44.0 \text{ g (traditional system)}$$

$$\text{Consumed } H_2SO_4 = (0.19 \times 18) + (0.17 \times 9) + (0.25 \times 7) = 6.7 \text{ g}$$

$$\text{Available } H_2SO_4 = 44 - 6.7 = 37.3 \text{ g}$$

Conclude: 37.3 g sulfuric acid is calculated as available using a 5 g test portion of whole milk. This is greater than the minimum of 20 g and thus is adequate.

(*2*) *Cheddar cheese.*—33.0% fat, 25.0% protein, 1.3% carbohydrate, 1 g test portion added enclosed in 0.8 g ashless filter paper:

$$\text{Added } H_2SO_4 = 44 \text{ g (traditional system)}$$

$$\text{Consumed } H_2SO_4 = (0.33 \times 18) + (0.25 \times 9) + (0.01 \times 7) + (0.80 \times 7) = 13.9 \text{ g}$$

$$\text{Available } H_2SO_4 = 44 - 13.9 = 30.1 \text{ g}$$

Conclude: 30.1 g sulfuric acid is calculated as available by using a 1 g test portion. This is greater than the minimum of 20 g and thus is adequate.

(*3*) *Cream.*—40% fat, 2% protein, 3% lactose, 2 g test portion:

$$\text{Added } H_2SO_4 = 35 \text{ g (block digestor)}$$

$$\text{Consumed } H_2SO_4 = (0.80 \times 18) + (0.04 \times 9) + (0.06 \times 7) = 15.2 \text{ g}$$

$$\text{Available } H_2SO_4 = 35 - 15.2 = 19.8 \text{ g}$$

Conclude: 19.8 g sulfuric acid is calculated as available by using a 2 g test portion. This is marginally less than the minimum of 20 g desired. If the test is run by using a 2 g test portion, then the digest should be carefully inspected for evidence of crystallization after digestion (indicating insufficient residual acid and nitrogen loss). A better choice would be to use a 1.0 g test portion. Increasing the amount of sulfuric acid initially added is not recommended or advisable.

It should also be noted that the amount of nitrogen in the digest by using a 2 or 1 g test portion would be 0.0063 and 0.0031 g, respectively. In the latter case, and perhaps the former as well, a 0.01N HCl titrant should be used rather than 0.1N HCl.

(**e**) *Measurement of residual sulfuric acid.*—In reality, the issue of the minimum amount of acid that needs to remain at

the end of digestion is more complex than described previously. In addition to the amount of acid required to digest the solids in the test portion, a certain amount is lost to volatilization. The amount lost to volatilization is a function of time, temperature, and, particularly in the case of block digestors, aspiration rate. Aspiration rate, especially, has a strong influence on acid loss and can vary widely among and even within laboratories. A further consideration is the ratio of sulfuric acid to potassium sulfate and the amount of sulfuric acid required to convert potassium sulfate to acid sulfate.

The calculated minimum amount of acid left at the end of digestion (20 g) used previously as a benchmark for determining test portion size is actually an overestimation. It accounts for the acid consumed by the sample but only indirectly accounts for acid loss to volatilization. This is because in reality, the minimum amount of titratable residual acid is closer to 10–15 g (28). To come up with a figure for "available" acid, a liberal assumption was made that 5 g is lost to volatilization. Thus, the amount of available acid by calculation is approximately 20 g (15 g left as residual acid and 5 g for volatilization).

The actual amount of residual sulfuric acid remaining after digestion can be directly determined if the analyst feels adventuresome. After digestion, quantitatively transfer the contents of the Kjeldahl flask or tube to a 500 mL volumetric flask using distilled water. Cool to 20°C and bring to volume with 20°C distilled water. Mix well. Titrate a 25 mL aliquot with standardized 1N NaOH using a phenolphthalein indicator. Calculate the amount of residual sulfuric acid as follows:

$$\text{Residual } H_2SO_4, \text{ g} = 20 \times 49 \times N \times (V/1000)$$

where 20 is factor to convert to total volume, 49 is gram equivalent weight of H_2SO_4, N is normality of NaOH, V is mL of NaOH used for titration, and 1000 is factor to convert mL to liters.