METHODS FOR THE DETERMINATION OF RAW PROTEIN

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The nitrogen content of the organic materials is generally determined by the Kjeldahl process. Originally the Dumas process was a macro method, nowadays, however, it is used almost exclusively as a micro method, while the Kjeldahl method is applied both in macro and in micro form. The nitrogen content multiplied by 6.25 gives the raw protein content [1].

Demands of agriculture and the food industry for multiple rapid serial analyses has led to different variations of the two fundamental methods, to the development of automatic analysers, of a rapid method based on dye fixing and of neutron activation processes. Even now, official testing is done by the standard Kjeldahl process: digestion, distillation, titration. Samples analysed by this standard method are applied for verifying rapid methods, automatic analysers and for periodical checking. Besides, as it was reported in one of our earlier papers, the digestion step of the classical Kjeldahl method can be shortened significantly with an appropriate catalyst mixture and thus it is also suitable for rapid serial analyses [2].

Rapid method based on dye fixing

For the determination of the protein content in different protein products. in purified proteins and in animal tissue samples the P—R (phenol reagent) and BPS (bromsulfalein) methods are used. According to investigations by B. DIAMANT, D. REDLICH and D. GLICK the protein can be precipitated quantitatively in acidic solution with BSP, since the sulfonic acid groups of the dye stuff react with the basic groups of the proteins. The quantity of dye bound by the protein is determined by dyestuff extinction measurements before and after the reaction. The amount of the dye bound by unit protein (Kjeldahl nitrogen) is expressed by an empirical factor compensating the differences between the active groups in each proteins. This method is convenient for the determination of approximately 100 μg of protein [3].

** Hungarian Corn Trust

In the dairy production the Orange G dye fixing method is used for determining the protein content in the milk. This method has been applied by A. J. MacKENZIE and E. R. PERRIER for protein determination in feed and forage crops [4]. Accordingly 1 g of the dyestuff is dissolved in citric acid solution containing alcoholic thymol solution and diluted to 1000 mls. After drying at a temperature of 70 °C samples are ground to 40 mesh; 0,5 g of samples are mixed with 25 mls of Orange G dyestuff and shaken for 1 hour, then after 30 minutes of standstill, the solution is filtered through a filter paper. One ml of aliquot part is filled up with blank solution to the mark in a 100 mls measuring flask. The blank solution is a solution of citric acid containing ethyl alcoholic thymol. The extinction is measured at 475 nm, adjusting the 100% of transmittancy with the blank solution.

The calibration curves are to be taken with samples, the protein content of which have been determined by Kjeldahl process. The method was tried on cropping materials containing 0.3—5.4% of nitrogen. The different crops have different dye fixing capacity. No linear correlation is obtained but on the same crop, if the transmittancy of the unfixed dyestuff is plotted against the nitrogen content semi-logarithmically.

Besides the dye Orange G (G. I. Acid Orange 10) also the dyestuffs Orange 12 and C. I. Acid Black 1 (612 nm) were tried for protein determinations in the dairy industry.

The *Prometer* made by Foss Co. in Denmark is suitable for the rapid and automatic application of the dye fixing method. It is suggested first of all for the protein determination in corns, and crops [5].

Acceleration of the classical Kjeldahl method

The Kjeldahl method consists of three steps: digestion, distillation and titration. These steps were thoroughly examined with a view on a speedier analysis.

Digestion. The most complicated step of the determination is digestion, that is the quantitative transformation of the nitrogen content of the sample into ammonia. Much research was done concerning the optimum experimental conditions of the digestion, the effect of temperature, catalysts and oxidizers.

J. W. GUNNING was the first to apply potassium sulfate in order to increase the boiling point and to decrease thereby the time of digestion [6]. The acid/salt ratio that is the effect of the *temperature* has been dealt with among others by C. L. OGG and C. O. WILLITS, R. B. BRANDSTREET etc. [7, 8]. For each 10° of temperature increase the rate of digestion is about the double. Digestion is accompanied by acid loss. P. L. KIRK estimated the concentrated sulfuric acid demand of 1 g of carbohydrate to be 4 mls, while of 1 g of fat to be

nearly 10 mls [9]. During the digestion the salt/acid ratio is increasing and so is the temperature of digestion. Especially, in the macro procedure the acid loss must not be ignored. Even at the end of digestion the temperature must not exceed 410 °C, not to risk nitrogen loss. In spite of a recent report on digestion started with an acid/salt ratio of 0.8 : 1 [10], the most reliable and rapid processes are considered to be those with an acid/salt ratio providing for 370 to 380 °C boiling point. As the digestion could be accelerated by applying an appropriate catalytic mixture and oxidizing material, for safety's sake rather a relatively low boiling point was chosen: 3 gs of K_2SO_4 + 3 gs of $K_2S_2O_8$/25 mls of conc. H_2SO_4 are used for the digestion of 2 gs of samples.

Because of the great importance of the digesting temperature, even in our days many reports can be found in the literature on the Kjeldahl digesting devices [11—14]. To the digesting flasks such amount of heat must be transferred that their content should reach the real boiling point. Too much of heat may be disadvantageous, especially for high salt/acid ratio leading to overheating and thus resulting in loss of nitrogen.

One of the most debated and contradictory questions of the Kjeldahl method is that of the *catalyst*. Since H. WILFARTH [15] the first to suggest mercuric oxide as catalyst in 1885, the catalytic effect of some 40 elements has been studied. Although R. A. OSBORN and J. B. WILKIE found Hg, Se, Te, Ti, Mo, Fe, Cu, V, W and Ag [16] in this succession to be the most effective, the majority of the authors investigated Hg, Se and Cu or their mixture. Table 1 contains test results of J. MILBAUER relating on a few catalysts for their effect to reduce digesting time [17]. With catalyst-mixtures marked with * a digesting time of 50 mins, with those marked with ** a digesting time of 14 mins

Table 1

Comparison of the effectiveness of different catalysts
Literature data [17]

Digesting mixture		Digesting time in %
components	amounts in gs	
a b c	a b c	100
cc H_2SO_4	50 mls	
cc H_2SO_4	50 mls	
+	+	
*$HgSO_4$ + SeO_2 Hg/Se = 4 : 1	1.7 + 0.2	3.5
**$HgSO_4$ + SeO_2 + P_2O_5	1.7 + 0.2 + 20	0.8
HgO + P_2O_5	1.0 + 25	17
$CuSO_4$ + P_2O_5	1.25 + 10	3.6
V_2O_5	0.1	38
MnO_2	0.5	32

are given. In solution mixed with air the digesting time decreases to 40 mins, and 8 mins, respectively. It is to be noted, however, that the digesting time is depending, of course, also on the sample and on its grinding fineness. The mixtures containing phosphoric acid heavily attacked the Kjeldahl flasks.

Oxidizing agents acted like the catalysts and the appropriate digesting temperature namely they increased the reaction rate. Hydrogen peroxide of 30% conc., potassium permanganate and potassium peroxidisulfate are suggested by the literature. View differ about their efficiency, in general they decre ase the digesting time, but in an inproper quantity they may induce a loss of nitrogen. Using potassium peroxidisulfate, hydrogen peroxide and conc. sulfuric acid, may give rise to Caro's acid. The concentration of the acid and the amount of the reagent have to be chosen to minimize Caro's acid development to avoid nitrogen loss [18].

On the basis of our preliminary experiments, 2 gs of barley, peanut meal and fish meal were digested with different catalysts, sometimes with oxidizing agents recording the time needed for clearing the digested material. The following digesting mixture was found to be the most convenient for the digestion: 3 gs of the catalytic mixture 14 gs of Se+170 gs of HgO + 300 gs of K_2SO_4 + + 3 gs $K_2S_2O_8$/25 mls of conc. H_2SO_4; digesting time is about 1 hour. Many different samples have been tested to check the above digesting mixture for exemptness from loss of nitrogen. The results were compared to those got by Se and Cu-sulfate catalytic digestion and recapitulated in Table 2. Because the

Table 2

Protein content of vegetable and animal samples calculated for 100% dry substance

Sample	Protein %		
	0.2 g Se + 25 ml cc H_2SO_4	5 g ($CuSO_4$ + K_2SO_4) 25 ml cc H_2SO_4	3 gs of HgO cat. mixt. 3 g $K_2S_2O_8$ + 25 ml cc H_2SO_4
Cottonseed meal	48.7	48.7	48.8
Barley	12.7	12.7	11.9
Soya flour	51.8	52,5	53,0
Corn	10.8	10.8	10.8
Enriched food	39.8	40.0	38.7
Fish-meal	66.1	66.4	66.4
Methionine	50.5	50.5	50.5
Yeast	48.3	48.9	48.2
Sunflower	39.4	39.2	39.3
Wheaten groats	12.7	13.0	12.3
Lucerne	22.0	21.4	21.3
Peanut-meal	50.5	50.1	50.4

relative inhomogeneity 2 gs of samples were needed, therefore we had also to work in macro rather than in micro dimensions. Each value is averaged from

at least three parallel determinations. Standard deviations based on several determinations on fish-meal samples (F), corn sample (C) and peanut meal (P) are as follows: $S_F = 0.65$; $S_C = 0.26$; $S_P = 0.17$ respectively.

Distillation. The acidic digested material is alkalized with an appropriate quantity of NaOH. In case of mercury as catalyst the base-solution should contain sodium thiosulfate or sodium sulfide sufficient to precipitate mercury. Otherwise, the results obtained are lower than expected, as a part of the ammonia is retained in the alkalized digested material in form of a non-volatile complex $HgNH_2^+$.

Distillation can be performed either in a *Schulek-Vastag* apparatus or in a *Parnas—Wagner* device by applying steam distillation. In the first case, for 2 gs of samples, the distillation time is about 90 mins. In the second case, because of the high salt concentration, the distillation is troublefree only, if a stock solution of 200 mls is prepared and the ammonia is distilled from an aliquot part, e.g. 20 mls. The distillation time is about 10 mins. The *Parnas—Wagner* device is rather labour-consuming to apply. According to our experiments one person can not distil more than three samples per hour with one apparatus and cannot handle more than two devices simultaneously even doing his best. The *Schulek—Vastag* distillators operate simultaneously, their handling is not labour-consuming, thus they are suitable for more analyses in average than those of *Parnas—Wagner*. Steam distillation is suggested in those cases, when very urgent results are demanded.

Absorption of ammonia. For the absorption of ammonia either sulfuric acid (hydrochloric acid) of known normality — 0.1 N and 0.5 N in macro range — or boric acid can be used [19]. Boric acid as absorbent was firstly suggested by L. WINKLER [20]; saturated boric acid or its 4% solution is applied rather in micro- than in macro-analysis. Since the pH values of the boric acid solutions increase with dilution, the volumes of the distillates should be adjusted to about the same value prior to the titration. The advantages of the boric acid are as follows: the solution need not be pipetted and to the titration only one titrant of known value is necessary. The advantages of other acids: the volumes need not be adjusted to the same value prior to the titration, loss of nitrogen does not appear even if the distillation speed is high and the liquid distilled does not cool enough in the cooler, finally they are more efficient as ammonia absorbers.

According to literature data, the statistical evaluation of numerous results leads to the conclusion that application of boric acid yields somewhat lower nitrogen and protein percentages [21]. The same was proved by our experiments the results being compiled in Table 3.

Indicators. For end point indication a lot of colour indicators can be used, the most general being methyl red. Mixed indicators methyl red—methylene blue or methyl red—bromocresol green are especially advantageous.

Table 3

Comparison of absorption with sulfuric and boric acid

Catalyst	Protein percentage for 100% dry substance	
	0.1 N H_2SO_4	4% boric acid
Se + 25 mls cc H_2SO_4 Digestion time: 5 hours	60.3	58.1
Se + HgO + K_2SO_4 50 mls cc H_2SO_4 Digestion time: 2 hours	61.5 62.7	59.3 60.4

The sample is 62% meat meal

The end point observation is made easy by the grey transition colour formed by mixed indicators of appropriate ratio in a narrow pH interval.

Other methods. Several titration methods have been suggested in order to eliminate the distillation and acid-base titration. These are based on the principle of the oxidation of the ammonium ion to nitrogen by hypochlorite or hypobromite titrants. The process is as follows: the acidic digested material is neutralized by base and sodium hydrogen carbonate, then potassium bromide and sodium hypochlorite in known excess and arsenic acid titrant are added. The excess of the latter is titrated back with hypochlorite titrant e.g. in the presence of tartrasine or of Bordeaux B indicator [22].

A simpler way of titration is the following: the properly alkalized solution (NH_3 must not leave) is titrated directly with sodium hypobromite titrant applying dead-stop end point indication [23]. One of the drawbacks of the process is liability of the titrant to decomposition, its effectiveness should be checked day by day. The other drawback is that in macro range the metal salt catalysts would cause disturbance because of precipitate formation. This method suits for samples digestible by conc. hydrogen peroxide solution. In the micro range the hypobromite ion can be generated coulometrically, but this method suits only for amounts of 1—10 mgs of samples [24]. L. SARUDI and E. SISKA determine the ammonia absorbed in the boric acid by conductometry. Calibration is made by solutions containing ammonia prepared with saturated boric acid [25].

Automatic nitrogen and protein analyzers

Automation has gained ground also in the field of nitrogen determinations. The *Dumas-process* is combined with a furnace moved mechanically and an electronic process control. One of the commercial devices is the Merz's automatic

rapid micro nitrogen analyzer which burns the material explosively in pure oxygen flow. The combustion products are rinsed into the asotometer by carbon-dioxide through copper-oxide and copper-charge and the nitrogen is determined volumetrically. The results can also be obtained in digital form. According to the literature data two persons with one apparatus can complete 100 routine determinations per day. The samples are 3 to 30 mgs, so this analyzer is only suitable for examination of homogeneous organic materials or well-homogenized and pulverized samples. The applicability is demonstrated also by the results of such kind of samples. However, it is to be noted that the relative inhomogeneity of the basic materials used in animal feeds (fish-meal, peanut meal, corn, barley, soya flour, etc.) prevents the *Merz* apparatus from analyzing such small samples.

The Inc. Coleman Instruments developed an automatic nitrogen analyzer working on the Dumas principle, the 29A Nitrogen Analyzator II Model Coleman. Determination are made on 50—300 mgs depending on the protein content of vegetable samples (dried lucerne, rice, bran, oats, cotton seed flour, edible soybean protein, casein etc.) The samples are ground below 30 mesh. The still relatively small samples require a very thorough homogenization. For adjusing the Nitrogen Analyzator any kind of organic standards e.g. acetanilide may be used. According to literature data the values of the nitrogen content determined by the Kjeldahl method and the Nitrogen Analyzer show a good agreement, for a combustion at 900 °C with a catalytic powder of appropriate ratio of tungsten trioxide and copper oxide. Statistical evaluation of the standard deviation of experiments carried out on similar samples in several laboratories showed the Kjeldahl method to be somewhat more accurate. The capacity of the Nitrogen Analyzer is 20 to 40 determinations, per day, per head, what is less than that of a Kjeldahl digestion rack with several working units, however, its advantage is that it requires small place and only one operator [26—29].

Another possibility is automation of the *Kjeldahl method*. The Technicon Instruments Corp. realized a full automation from the separation of the aliquot part of the sample, through the digestion, to spectrophotometric ammonia determination; the distillation is eliminated. In addition to the base apparatus, Technicon AutoAnalyzer, a digesting unit was also developed what enables the analysis of solid materials.

The digestion of the samples is carried out in a rotating glass helix heated by an electric furnace with a measured amount of conc. sulfuric acid containing dissolved catalyst. If increased inhomogeneity requires to apply a greater sample, the material is suspended in conc. sulfuric acid, and from the suspension an aliquot part gets automatically into the digesting helix. The sample may range from 0.01—1.00 g. The sample and the digestant are continuously feed in on one end of the digestor helix, and the mixture proceeds continuously in the

tube while rotating. The temperature of the air under the tube is kept at 400 to 450 °C in general. The digestion time depends on the rotation speed, it is in generally 3- to 4 mins. At the other end of the digestor helix the sample is automatically diluted, to the spectrophotometric determination only aliquot part is necessary. The mostly recommended digestant is: sulfuric acid of 90% conc. containing selenium oxychloride and perchloric acid. Mercuric salt can also be used, but it has an interfering effect on the colour reaction. This effect is eliminated by adding EDTA to the sodium hydroxide solution used for the neutralization of the sulfuric acid. Generally the well-known indo-phenol-blue colour reaction is applied as spectrophotometric method, the ammonium ion reacting with sodium phenolate and sodium hypochlorite gives indo-phenol-blue colour in basic medium, at a pH $\sim$ 10.8. The extinction is measured at a wavelength of 600 nm.

Two questions arise concerning the digestion: how can the sample be digested so fast, and why there is no nitrogen loss at the relatively high temperature? The sample and the digesting mixture create a thin membrane and this is distributed on the hot spiral and thus the rate of digestion is much higher than in a digesting flask. The short digesting time inhibits nitrogen loss. As far as the rate of the analysis is concerned, according to the literature data, the highest value is 20 determinations/hour — however, with a loss of accuracy. The highest accuracy can be reached at a rate of 7 determinations/hour; the optimum value is 12 determinations/h. The AutoAnalyzer should be adjusted to a vegetable or animal control sample containing protein of a composition similar to that of the specimens. The nitrogen content of the control samples was previously determined by the classical Kjeldahl method. The optimum nitrogen content of the solutions is: 50—50 ppm. The standard deviation is: $\pm$ 1.5% [30—32].

Table 4

Comparison of protein content determinations by AutoAnalyzer and Kjeldahl Method

Sample	Protein percentage for 100% dry substance			
	Se		$CuSO_4$	
	A. A.	P. W.	A. A.	P. W.
Wheat	12.6	12.5	12.2	12.1
Soya	53.4	50.8	53.6	53.4
Fish-meal	65.0	65.4	63.6	63.0

A. A. = AutoAnalyzer determination
P. W. = Digestion by classical Kjeldahl method, distillation in Parnas-Wagner device
Digestion: 0.2 g Se + 25 mls cc H_2SO_4 (Se)
from $CuSO_4 \cdot 5H_2O$: K_2SO_4 1 : 4 mixture
4.4 gs + 25 mls cc H_2SO_4 ($CuSO_4$)

In Table 4 the results of some protein determinations carried out by the Technicon AutoAnalyzer of our Institute can be seen. The indo-phenol-blue colour reaction has been modified by M. MAHR and E. PUNGOR by replacing sodium phenolate by sodium salycilate, and measuring the extinction at 640 nm [33]. 0.5—2 gs sample was digested by selenium and copper sulfate catalyst, and the digested materials were filled in flasks of 200 mls to the mark. In the AutoAnalyzer method an appropriate aliquot part was used and the protein content was determined also by the classical method performing the distillation in a device type PARNAS—WAGNER. Results are mean values from three simultaneous determinations.

Also the original classical Kjeldahl method digestion, distillation, titration has been automated, see *Kjel-Foss Macro Automate* with six working units. The digestion with sulfuric acid is accelerated by concentrated hydrogen peroxide and an appropriate mixture of catalysts in the form of tablets. Concentrated sulfuric acid and hydrogen peroxide are fed automatically in an amount depending on the weight and on the fat content of the sample. The sample is put into the flask, then it is closed, and the further operations are automated. The content of the flasks is rapidly heated by gas burners up to the boiling point, when the majority of the substances are digested. In the second phase of digestion — at 410 °C — all substances are fully digested, the total nitrogen content is present in the form of ammonium sulfate. This is followed by rapid cooling, dilution, alkalization and distillation. The distilled and cooled ammonia is continuously titrated with a sulfuric acid titrant. The automated titration is controlled by the contrast colour change of the indicator. The result is read out in digital form. As the last phase of the operations, the flasks discharge automatically. According to the booklet issued by the producing firm, the Kjel—Foss Macro Automate is suitable for the investigation of all samples the nitrogen content of which can be determined by the standard Kjeldahl method. From the point of view of homogenization, preparation and grinding of the samples, it is advantageous to work with the macro method with sample weights of 500 or 1000 mgs depending on the protein content. 500 mgs are needed for protein and nitrogen contents higher than 45% and 7%, for lower values sample weight of 1000 mgs. The accuracy of the measurement agrees with that of the standard Kjeldahl method, since the digestion is complete in each case and no nitrogen loss occurs. The possible nitrogen content of the chemicals is corrected by the apparatus. The capacity is 20 samples/hour, the duration of the analysis is 12 min.

Neutron activation methods for the determination of protein content

For the determination of nitrogen and protein content of samples non-destructive neutron activation processes have also been elaborated.

Researchers from the USA developed the following method: the compressed sample (weight: 20 gs, protein content: 7—90%) is irradiated by fast neutrons for 2 mins; neutron flux is $\Phi \simeq 10^{10}$ n/cm^2 s. The effect of fast neutrons with nitrogen causes the following nuclear reaction.

$$^{14}\mathrm{N}(n, 2n)\ ^{13}\mathrm{N}\ .$$

After irradiation, the sample is cooled for 12 mins that the radionuclides of short half-life should disintegrate. The ^{13}N disintegrates with positron radiation, the half-life is $t_{1/2} = 10$ mins. The amount of nitrogen can be followed by measuring the 0.51 MeV annihilating radiation measured with NaI (Tl) scintillation detector. The presence of phosphorus and silicium causes interference, their effect should be compensated. According to the literature data the results show good agreement with those obtained by the Kjeldahl method. The time of a determination is 20 mins [34].

The Central Research Institute for Physics has developed an automatic apparatus operating with a radiation source (α, n) of long half-life, named *Autoprodet*, for the determination of the total protein content. The nitrogen content is followed by measuring the prompt radiation occuring as a result of the nuclear reaction. Some 1 kg of roughly ground samples should be irradiated. Having introduced the sample, the results are printed out after 10—15 mins or can be computerized. The p ercentage of the error expressed in protein percentage is less than 1—2%.

Summary

A short survey is given on different determinations of raw protein, with special respect to the rate of analysis and to the automation of determination.

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