international association of fish meal manufacturers

Hoval House, Orchard Parade, Mutton Lane, Potters Bar, Hertfordshire, EN6 3AR
Tel: (Potters Bar) 0707 42343/4/5

No. 15: January 1982

# RECOMMENDED METHOD OF ANALYSIS FOR AUTOMATIC DETERMINATION OF CRUDE PROTEIN IN FISH MEAL BY THE KJEL-FOSS PROCEDURE

## 1. General

The results of an international collaborative study (see appendix) have shown that the Kjel-Foss Automatic 16200 System is acceptable as an alternative to the standard Kjeldahl procedure (IAFMM Technical Bulletin No. 8) for determining the crude protein content of fish meal. The crude protein content of fish meal is calculated from the nitrogen figure by multiplying by the factor 6.25.

## 2. Principle

The method is based on the conversion of organic nitrogen to inorganic nitrogen (digestion according to Kjeldahl). The ammonium sulphate thus formed is diluted and made alkaline with sodium hydroxide and the ammonia distilled over and titrated by a dilute sulphuric acid solution.

## 3. Reagents

### A. Working chemicals

Technical grade reagents for the preparation of certain solutions will in most cases prove satisfactory. In some cases, however, where high levels of nitrogenous impurities may be present and difficulties are encountered, the appropriate analytical grade should be used. Deionised water should be used throughout.

Sulphuric acid concentrated, technical grade approx. 96% (v/v).
Hydrogen peroxide, technical grade approx. 35% (v/v).
Kjel-Tab M 5.25 g per tablet (5g potassium sulphate and 0.25g mercuric oxide).
Sodium hydroxide/sodium thiosulphate solution (prepared by dissolving 400g technical grade NaOH and 80g $Na_2S_2O_3$ $5H_2O$ per litre of water).
Indicator: Dissolve 1.000g methyl red and 0.250g methylene blue in ethanol (95% v/v) and dilute to 1*l* with ethanol. After filtration, 1ml of pure acetic acid (99%) is added. Dilute 10ml of this soln. to 1*l* with water. **Note** The indicator is commercially available in some countries.
Sulphuric acid dilute, (Dilute 30ml of concentrated sulphuric acid to 5*l* with water).

B. Calibration and test chemicals

Standard solution I (dissolve 0.750 + 0.001g analytical grade $(NH_4)_2SO_4$ in water and dilute to 1*l* with water).
Standard solution II (dissolve 30.000 + 0.030g analytical grade $(NH_4)_2SO_4$ in water and dilute to 1*l* with water).
Nicotinic acid, analytical grade.
Acetanilide, analytical grade.

**4. Apparatus**

Kjel-Foss Automatic 16200. The steam generator should be cleaned once a day and a kettle defurrer added in order to prevent the build-up of scale, especially in areas where hard water is used.

**5. Method**

In the Kjel-Foss Automatic, six specially designed Kjeldahl flasks are placed around a bucket. Every 3 minutes the whole flask system is turned through $60^{\circ}$, making it possible to complete an analysis within 12 minutes with a capacity of 20 per hour.

The basic functions in the 6 positions are as follows:

Pos. 1: The fish meal sample of approx. 0.5g is accurately weighed to the nearest 1mg and put in the Kjeldahl flask with 3 Kjel-Tab M tablets. Approximately 9ml of $H_2O_2$ are dispensed automatically into the flask and the chosen amount of $H_2SO_4$ (setting on the front panel: 40* equals 12.5ml) is dispensed from a syringe by depressing the starting handle. The lid is then closed by hand.

Pos. 2: 3 minutes of digestion. The flask is heated by a gas burner. The flame is lit automatically by electrical ignition.

Pos. 3: Further 3 minutes of digestion.

Pos. 4: The digest is cooled by a fan. At the end of the 3 minute period the lid is opened automatically, whereupon 140ml of water for dilution of the cooled digest is delivered into the flask through a sprinkler.

Pos. 5: $NaOH/Na_2S_2O_3$ solution is introduced into the flask in excess (50 ml) in order to neutralize the sulphuric acid and liberate the $NH_3$, whereupon the steam distillation is started.

The ammonia is distilled through a splash head and a water-cooled condenser into a glass beaker where the ammonia is immediately titrated by means of dilute sulphuric acid delivered from a syringe. The change in colour of the indicator previously introduced into the glass beaker regulates the syringe by means of a photocell with amplifier and a gear motor. The ammonia distilled over is continuously neutralized by dilute sulphuric acid, and the position of the syringe piston is measured by a potentiometer connected to an amplifier. The output given directly in digits is equal to the nitrogen content of the sample, on the assumption that the sample weight is exactly 500mg. For other sample sizes the result has to be changed accordingly. The results can be expressed in % of protein as well.

Pos. 6: The contents of the Kjeldahl flask are transferred into the waste container by means of air.

**6. Calibration of Equipment**

Calibration of the equipment will take account of impurities in the chemicals, thereby allowing the use of technical grade chemicals, and give correct results.

Check the instrument calibration daily by measuring three Standard Solution I samples and three Standard Solution II samples.

A sample of exactly 5ml Standard Solution I is used to check the read-out for low N-content samples.

A 10ml sample of Standard Solution II is used to check the read-out for high N-content samples.

A detailed calibration procedure is described in the Instruction Manual.

**7. Performance Check**

It is important to have optimum performance of the equipment on nicotinic acid as a guarantee of correct results on fish meal and other compounds difficult to digest.

Check tests should be done every working day after calibration of the equipment by determining the nitrogen content of known organic compounds, e.g. nicotinic acid and acetanilide. Because the organic compounds contain no fat, the acid setting at the front panel should be 25.

Nicotinic acid contains 11.4% N. A sample of exactly 500 mg is used to check the complete digestion of samples under "worst case conditions". When the range selector is in position 1000 mg-N-2 dec., the display should indicate 5.68.

A sample of exactly 500 mg acetanilide is used to check the digestion of samples. Acetanilide contains 10.36% N, and this figure (10.4) should be displayed when the range selector switch is in position 500 mg-N-1 dec.

Acetanilide and nicotinic acid complement each other. The acetanilide test checks that the temperature of digestion is not too high, the nicotinic acid test checks that it is not too low.

**8. Calculation**

Range selector "1000 mg-N-2 dec.". Multiply the read-out by (2 x 6.25) to give percentage crude protein. If an amount other than 500 mg has been used, multiply by $\frac{500}{x}$ where x is the weight of fish meal in mg.

**9. Repeatability**

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst should not exceed 0.40%.

*For calibration as well as performance check, the setting should be 25 only.

APPENDIX: *Reprinted from J. Sci. Food Agric. 1981,* **32**, *732–736*

# International Collaborative Study of an Automated Method for the Determination of Crude Protein in Fish-meals

S. M. Barlow, A. Bimbo[a], O. Bjørn Jensen[b] and G. L. Smith[c]

*International Association of Fish Meal Manufacturers, Hoval House, Orchard Parade, Mutton Lane, Potters Bar, Herts, [a] Zapata Haynie Corporation, PO Box 175, Reedville, Virginia 22539, USA, [b] Steins Laboratorium, Holsbjergvej 42, 2620 Albertslund, Denmark and [c] Torry Research Station, PO Box 31, 135 Abbey Road, Aberdeen AB9 8DG*

(*Manuscript received 3 July 1980*)

An automated macro-Kjeldahl instrument (Kjel-Foss) determines protein at the rate of 20 samples in 1 h. The methodology is similar to the Kjeldahl method officially recommended by the IAFMM for fish-meal. The two methods were compared in a collaborative study. Six fish-meals, tryptophan and acetanilide were analysed by 15 participating laboratories. The results showed good agreement between the two methods. There was little difference between the two methods in terms of repeatability in the laboratory, but variability between laboratories was significantly less when the automated method was used.

## 1. Introduction

An automated macro-Kjeldahl instrument has been introduced[1] which enables a laboratory to perform 20 protein analyses in 1 h. This instrumental method has been extensively compared with different protein methods including the AOAC procedure.[2,3] A collaborative study evaluated the automated method in comparison with the AOAC official method using various animal feeds, meats and a number of standards.[4] The results showed good agreement between the two methods.

Fish-meals containing high levels of protein (60–70%) can present difficulties in converting all the organic nitrogen to ammonium sulphate, unless particular attention is given to the details of the traditional Kjeldahl method. The International Association of Fish Meal Manufacturers (IAFMM) has published an officially recommended Kjeldahl method for the determination of crude protein in fish-meal.[5] An international collaborative study was undertaken to evaluate the automated method in comparison with the official IAFMM method.

## 2. Experimental

Samples of six fish-meals from different origins were sent to 15 participating laboratories (Table 1), along with two standards, acetanilide (99% purity) and tryptophan (99% purity). The standards were included to allow an assessment of the accuracy of the method (comparison of obtained value for nitrogen content with theoretical value). Each laboratory received seven fish-meal samples including a hidden duplicate and one sample of each standard, for each method to be tested. The samples of fish-meals and standards were ground to pass through an 18 mesh sieve, weighed out accurately to the third decimal place by the laboratory responsible for distribution of the samples, packaged in a nitrogen-free container and coded. The participating laboratories receiving the samples placed them directly into digestion flasks without preweighing. By this means determination of the moisture content in the meal proved unnecessary.

**Table 1.** List of participating laboratories

| Name of collaborator | Organisation | Address |
|---|---|---|
| — | Fiskernes Fiskeindustri | PO Box 60, DK-9990, Skagen, Denmark |
| O. Bjørn Jensen | Steins Laboratorium | Holsbjergvej 42, 2620 Albertslund, Denmark |
| — | Vestjysk Sildeolindustri | Ny Havn, 6700 Esbjerg, Denmark |
| E. V. Nielsen | Andelssildeolie-fabriken | AMBA, Ny Havn, 6700 Esbjerg, Denmark |
| Ms M. Kegl | National Inspectorate for Feedstuffs | H-1024 Budapest II, Keletl Karoly u.24, Hungary |
| P. Olafsson | Icelandic Fisheries Laboratories | Skulagata 4, Reykjavik Iceland |
| S. Olsen | The Fish Oil and Fishmeal Industries Research Institute | 5033 Fyllingsdalen, Bergen, Norway |
| Ing. A. Bellido | Pesca Peru | Av. Javier Prado Este 2465, PO Box 2881 (Lima 100), San Luis, Lima 30, Peru |
| P. Wajda | Polcargo | 13 Indyjska, Gydnia, Poland |
| Dr I. Moodie | Fishing Industry Research Institute | University of Cape Town, Rondebosch, Cape Province, South Africa |
| J. J. Dreyer | National Food Research Institute | CSIR, PO Box 395, Pretoria 0001, South Africa |
| Dr J. R. Burt | Ministry of Agriculture Fisheries and Food | Torry Research Station, Humber Laboratory, Wassand Street, Hull, HU3 4AR UK |
| A. Bimbo | Zapata Haynie Corporation | PO Box 175, Reedville, Virginia 22539, USA |
| D. Heinz | Seacoast Products Inc. | PO Box D, Port Monmouth, New Jersey 07758, USA |
| H. Arkeeda | USDA Commodity Inspection, Grain Division ARS East | Building 306, Room 2, Federal Grain Inspection Service, Beltsville, MD 20705, USA |

The samples and standards were analysed by each laboratory by either method or both making a single determination on each sample. Of the 15 laboratories, seven used both methods, three used only the automated method and five used only the official method.

The official method was performed according to the IAFMM recommended method[5] using dry potassium sulphate, mercuric oxide and concentrated sulphuric acid as the digestion medium, boiling the digest for at least 2 h as soon as the liquid had cleared. The automated method used the Kjel-Foss Automatic, Model 16200 using Kjel-Tabs (15 g $K_2SO_4$ and 0.75 g HgO) according to the manufacturers' instructions.[6] The digestion mixture consisted of sample, Kjel-Tabs, concentrated sulphuric acid and hydrogen peroxide solution. The instrument was calibrated each day with aliquots of standard ammonium sulphate solutions and occasionally the performance was checked by determining the nitrogen content of known organic compounds (nicotinic acid because of the difficulty of its digestion, and acetanilide to ensure no loss of nitrogen in samples easy to digest).

## 3. Results and discussion

Table 2 presents the individual results for the protein levels in the samples using either method. As there was no evidence of systematic biases affecting results by both methods from individual laboratories (the correlation between biases for the two methods was not significant—0.01, seven pairs), it was concluded that the laboratories represented a random sample and the data from all the laboratories were evaluated.

The duplicate values are presented in Table 3. The differences were subjected to Dixon's test[7] for outliers, and consequently the difference between the duplicate values obtained by laboratory 1 using the official method on sample C was discarded before calculation of repeatability. Each of these duplicate values was compared with the 'least squares' estimate which would have been obtained had that laboratory given no value for that sample (the estimated value was chosen which would minimise laboratory × sample interaction sum of squares in an analysis of variance).[8] As the estimate was very close to 70.04, the other duplicate value of 68.18 was discarded.

Comparing mean values for the two methods, the automated method values were on average 0.36 higher than those obtained using the official method, but this difference was not statistically significant (Student's $t = 1.56$, 20 degrees of freedom).

**Table 2.** Collaborative results for the determination of protein (%) by the official and automated methods

| | Sample | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | | E | | F | | Mean | |
| Laboratory | Off. | Auto. | Off. | Auto. | Off. | Auto. | Off. | Auto. | Off. | Auto. | Off. | Auto. | Off. | Auto. |
| 1 | 69.12 | 70.90 | 58.34 | 59.34 | 70.04 | 71.33 | 65.84 | 68.20 | 58.67 | 59.64[a] | 65.24 | 67.03 | 64.54 | 66.07 |
| 2 | 67.78 | 69.36[a] | 57.93[a] | 59.26 | (69.23) | 70.90 | 64.28 | 67.64 | 58.61 | 58.91 | 64.61 | 66.07 | 63.74 | 65.36 |
| 4 | | 69.38 | | 58.60 | | 70.72 | | 66.80 | | (58.50) | | 63.98 | | 64.66 |
| 5 | | 70.32 | | 59.77[a] | | 70.28 | | 65.52 | | 59.55 | | 66.61 | | 65.34 |
| 6 | | 69.44[a] | | 59.64 | | 70.83 | | 67.80 | | 59.25 | | 66.22 | | 65.53 |
| 7 | 68.64 | | 59.22 | | 71.15 | | 67.65 | | 59.86[a] | | 66.86 | | 65.56 | |
| 8 | 69.24 | 70.27 | 59.19 | 59.86 | 70.57 | 71.35 | 67.40[a] | 67.96[a] | 60.09 | 59.45 | 66.63 | 66.74 | 65.52 | 65.94 |
| 9 | 69.22[a] | 69.22[a] | 58.72 | 59.08 | 70.18 | 70.57 | 67.00 | 68.07 | 58.80 | 58.85 | 65.84 | 66.16 | 64.96 | 65.32 |
| 10 | 69.85 | 69.93 | 59.66[a] | 59.41 | 71.19 | 70.16[a] | 68.30 | 67.99 | 59.28 | 58.69 | 66.13 | 65.48 | 65.74 | 65.28 |
| 11 | 69.29 | 69.54 | 58.32 | 59.48 | 70.86 | 71.08[a] | 67.64 | 67.77 | 59.29[a] | 59.24 | 65.85 | 66.00 | 65.21 | 65.52 |
| 12 | 69.17[a] | | 58.96 | | 71.44 | | 66.18 | | 59.41 | | 65.96 | | 65.19 | |
| 13 | 70.53 | | 59.95 | | 71.28 | | 68.78[a] | | 60.23 | | 67.44 | | 66.37 | |
| 14 | 68.48 | | 55.98 | | 69.94 | | 66.98[a] | | 59.68 | | 66.15 | | 64.54 | |
| 15 | 69.74 | | 59.48[a] | | 70.97 | | 64.31 | | 59.82 | | 66.47 | | 65.13 | |
| 16 | 68.50 | 69.25[a] | 58.10 | 59.00 | 70.30[a] | 70.90 | 66.80 | 67.40 | 59.20 | 60.90 | 65.70 | 66.30 | 64.77 | 65.62 |
| *Mean* | 69.13 | 69.76 | 58.65 | 59.34 | 70.60 | 70.81 | 66.76 | 67.52 | 59.41 | 59.30 | 66.07 | 66.06 | 65.10 | 65.46 |

[a] Mean of the two values from duplicate samples; all other entries (except for those in parentheses) are single determinations. Those in parentheses are values estimated by 'least squares' where no determination was received.

**Table 3.** Results from duplicate samples

| Automated Method | | | | | Official Method | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Laboratory | Sample | Values | | Difference | Laboratory | Sample | Values | | Difference |
| 1 | E | 59.62 | 59.66 | 0.04 | 1[a] | C | 68.18 | 70.04 | 1.86 |
| 2 | A | 69.45 | 69.26 | 0.19 | 2 | B | 58.27 | 57.59 | 0.68 |
| 5 | B | 59.81 | 59.73 | 0.08 | 7 | E | 59.81 | 59.90 | 0.09 |
| 6 | A | 69.56 | 69.33 | 0.23 | 8 | D | 67.18 | 67.61 | 0.43 |
| 8 | D | 67.90 | 68.03 | 0.13 | 9 | A | 69.23 | 69.21 | 0.02 |
| 9 | A | 69.54 | 68.91 | 0.63 | 10 | B | 59.63 | 59.69 | 0.06 |
| 10 | C | 70.25 | 70.06 | 0.19 | 11 | E | 59.58 | 59.00 | 0.58 |
| 11 | C | 71.19 | 70.98 | 0.21 | 12 | A | 69.30 | 69.04 | 0.26 |
| 16 | A | 69.60 | 68.90 | 0.70 | 13 | D | 68.76 | 68.81 | 0.05 |
| | | | | | 14 | D | 67.31 | 66.64 | 0.67 |
| | | | | | 15 | B | 59.32 | 59.63 | 0.31 |
| | | | | | 16 | C | 70.40 | 70.20 | 0.20 |

[a] Outliers discarded—see text.

Because the sample was thoroughly mixed before sub-sampling and weighing prior to distribution to the participating laboratories, analysis of the 'hidden duplicates' (Table 3) permits an unbiased estimate of repeatability.

The between-duplicates variance ($s_0^2$), pooled over all pairs, was obtained for each of the two methods. For the official method the value was 0.075 (11 degrees of freedom) while for the automated method it was 0.060 (nine degrees of freedom), which is not significantly different from the former. Repeatability is defined[9] as: 2.83 $\sqrt{s_0^2}$ and the values obtained were 0.78 for the official method and 0.69 for the automated method.

The higher repeatability value for the official method obtained in this collaborative study compared with the recommended repeatability value (0.40) may be partly explained by the limited number of samples in this study and that the duplicates were hidden not allowing the analyst to carry out the determinations simultaneously or in rapid succession.

To obtain the between-laboratories variance ($s_L^2$), analysis of variance was performed on the data for each laboratory and meal using the mean of the two samples if duplicate samples were used, or the single value where there was one sample. The analysis was carried out after fitting 'least squares' estimates where no value had been given. The values for between-laboratory variance were 0.384 (11 degrees of freedom) for the official method and 0.097 (nine degrees of freedom) for the automatic. These two values were significantly different ($P < 0.05$).

Reproducibility is defined[9] as: $2.83 \sqrt{s_0^2 + s_L^2}$ and the values were 1.92 for the official method and 1.12 for the automated method.

The results for the standards, acetanilide and tryptophan, are presented in Table 4. Excluding the outliers which were identified by Dixon's test, there was no significant difference between the mean values for the two methods, considering the two standards separately.

**Table 4.** Results from standards, percentage nitrogen recovery

| Laboratory | Acetanilide | | Tryptophan | |
|---|---|---|---|---|
| | Automated | Official | Automated | Official |
| 1 | (114.2)[a] | 96.62 | — | (92.06) |
| 2 | 100.1 | 100.9 | 98.76 | 99.85 |
| 4 | 99.9 | | 98.47 | |
| 5 | 101.7 | | 99.56 | |
| 6 | 100.8 | | 98.98 | |
| 7 | | 99.52 | | 98.03 |
| 8 | 100.6 | 99.23 | 98.83 | 98.83 |
| 9 | 98.65 | 98.36 | 98.32 | 97.08 |
| 10 | 96.81 | 99.81 | 96.94 | 100.6 |
| 11 | 100.1 | 99.13 | 98.91 | 99.56 |
| 12 | | 98.46 | | (93.95) |
| 13 | | 100.1 | | 101.1 |
| 14 | | 98.55 | | — |
| 15 | | 99.42 | | 99.71 |
| 16 | 100.8 | 98.6 | 98.7 | 98.1 |
| *Mean*[a] | 99.94 | 99.06 | 98.61 | 99.21 |
| *Standard deviation*[a] | 1.44 | 1.07 | 0.72 | 1.30 |

[a] With outliers (in parentheses) discarded.

## 4. Conclusions

The protein values obtained by the automated technique were slightly higher than the IAFMM recommended official method for fish-meals, but this difference was not statistically significant. There was little difference between the methods in repeatability, but reproducibility was reduced when the automated method was used.

### Acknowledgement

Grateful thanks are expressed to all participating laboratories and to Mr Krogsgaard Madsen of Foss Electric for his helpful comments in the design of the trial.

### References

1. Oberreith, R.; Mermelstein, N. H. Instrument automates, accelerates nitrogen determinations. *Food Technol.* 1974, **28** (6), 40–43.
2. Montag, A. Nitrogen determination of KJEL-FOSS-AUTOMATIC analyser using the Kjeldahl method. *Gordian* 1974, **74**, 203–209

3. Wall, L. L., Sr.; Gehrke, C. W.; Neuner, T. E.; Cathey, R. D.; Rexroad, P. R. Total protein nitrogen; evaluation and composition of four different methods. *J.A.O.A.C.* 1975, **58**, 811–817.
4. Noel, R. J. Collaborative study of an Automated method for the determination of crude protein in animal feeds. *J.A.O.A.C.* 1976, **59**, 141–147.
5. International Association of Fish Meal Manufacturers. Recommended method of analysis for determination of crude protein in fish meal. *Technical Bulletin* 1979, **8**, 1–7.
6. *Kjel-Foss Automatic 16200 Operating Manual* A/S N. Foss Electric, 69 Slangerupgade, DK 3400 Hillerød, Denmark.
7. Dixon, W. J. Processing data for outliers. *Biometrics* 1953, **9**, 74–89.
8. Yates, F. *Experimental Design* Griffin & Co. Ltd, London 1970.
9. British Standards Institution. *Precision of test methods, Part 1* Guide for the determination of repeatability and reproducibility for a standard test method. BS 5479, 1979, 2 Park Street, London W1

## FISH MEAL SCIENTIFIC ADVISORY SERVICE

The International Association of Fish Meal Manufacturers (IAFMM) has established a permanent Scientific Advisory Service mainly for Feed Compounders and Concentrate Manufacturers and Agricultural Institutions. The staff of the IAFMM, in conjunction with its Scientific Committee, representing an international group of experts in nutrition, bacteriology, engineering and product development, will provide up-to-date information on any aspect of Fish Meal and its uses. All enquiries should be directed to:

Dr. S.M. Barlow
**International Association of Fish Meal Manufacturers.**

PD UCT