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Summary

A proposed new method to determine dilute pepsin digestibility, using a pepsin strength very much lower than the AOAC Official Method, was tested in 16 laboratories with 12 samples of fish meal . Results were calculated according to two different procedures in common use, i) according to AOAC Official method 971.09 or ii) according to a method first described by Lovern et al (1964) at the Torry Research Station, Aberdeen, Scotland. Variations in method of shaking and source of pepsin were also investigated. Pepsin digestibility values were lower and more variable when calculated by the Torry procedure. The Torry procedure required an additional N determination and was more subject to other analytical errors. The method of shaking affected the result when calculated according to the Torry but not the AOAC method. The source of pepsin had no significant effect. A suitable dilute pepsin method has been standardised and shown to have good repeatability and reproducibility when calculated according to that used in the AOAC method. The type of shaker and source of pepsin are recommended but need not be mandatory. It is recommended that this new method, with inclusion of a blank determination to the circulated method, be adopted as an IFOMA official method. The repeatability limit of this new method is 1.6 % units of digestibility. The reproducibility limit is 3.3 % units of digestibility. A draft of the new recommended method is given in Appendix 3.

Purpose

The purpose of the ring test was to establish a recommended IFOMA method for measuring pepsin digestibility in fish meals.

Introduction

Different methods are in use to measure the pepsin digestibility of fish meals. The AOAC Official Method 971.09 uses a strong solution of pepsin (0.2%) and gives high values of digestibility but does not distinguish between fish meals of different quality. The Torry method (Lovern et al 1964, Lovern 1965) uses a dilute pepsin solution (0.0002%). However, a survey of current practice revealed a number of modifications of the Torry method were in use (Bimbo, 1998). Early studies with the Torry method indicated there might be some correlation between pepsin digestibility and protein quality (specifically NPU) and digestibility in the rat. However, subsequent trials did not substantiate the use of dilute pepsin digestibility as an indicator of protein quality as measured in rat and chick growth assays (Barlow, 1976). Consequently, the method cannot be used as a measure of the nutritional quality of protein for specific species of animals. However, in certain circumstances fish meal traders have deemed it useful to specify minimum digestibility levels in contracts using pepsin concentrations more dilute than that specified by AOAC Official Method 971.09. Consequently there is a need for a standardised dilute pepsin method of known repeatability and reproducibility for use in contracts.

From a review of current practices (Bimbo, 1998) a number of potential factors affecting variability were identified and where possible (e.g. strength of pepsin, sample particle size) appropriate standard conditions were selected as the basis of the method to be tested. Three variables were selected for further study within the collaborative study.

(a) The AOAC method calculates pepsin digestibility as the portion of the total nitrogen in the sample that is soluble in acid pepsin solution. The Torry method calculates pepsin digestibility as the portion of the acid insoluble nitrogen that is soluble in acid pepsin solution. The acid insoluble N may be substantially less than the total N. Differences in method of calculation between laboratories would be a significant source of variation. The current study calculates the data in these two ways.

(b) The source of pepsin is not specified in AOAC. In the present study pepsin from Merck 1:10,000 activity (Product No 7190) was specified while laboratories not using this source were asked to standardise their source of pepsin according to ISO 6655 (1997). The effect of source of pepsin on the results was determined.

(c) Laboratories have different shaking equipment which can be classified into either end over end rotating as described in the AOAC method or the more readily available orbital or reciprocating shakers. The effect of type of shaker was determined.

Method

IFOMA collected ten fish meal samples from Denmark, Norway, Chile, Peru, USA and UK. These were the same samples as used in a ring test of determination of biogenic amines. The samples were coded 1 to 12 and circulated by Fish Industries to 16 participating laboratories. Samples were not reground but this was left to each participating laboratory as part of the whole procedure. Samples 1 and 5 were a pair of hidden duplicates and samples 3 and 10 were a second pair of hidden duplicates. Laboratories were asked to analyse each sample once only and to report three nitrogen determinations by the kjeldahl method (ISO FDIS 5983:1997(E) for each sample:
1. % kjeldahl nitrogen insoluble in acid pepsin solution (A)
2. % kjeldahl nitrogen insoluble in acid solution (B)
3. % kjeldahl nitrogen in the sample of fish meal (C).

The prescribed method of analysis is given in Appendix 1.
The laboratories participating are listed in Appendix 2.

In the course of conducting the ring test one laboratory (SSF) drew attention for the need to determine and correct for the contribution of N from filter paper used to collect the insoluble residue in determinations A and B. This was not specified in the circulated protocol and it was possible that some laboratories would automatically include a filter paper in their reagent blank determination of N while others might not. Participants were advised to report results without any adjustment if they had not already completed the analysis. In a subsequent questionnaire laboratories were asked whether or not a correction had been made and also the N supplied by a filter paper after washing with warm distilled water. The data was analysed first as received, in which a few had made corrections but the majority had not, and secondly after adjustment of the reported data for the determined contribution of nitrogen from the filter paper.

Results

The primary measurements (A, B, C), the dilute pepsin digestibility calculated according to the Torry method (Dig 1) and the AOAC method (Dig 2) averaged over the 12 samples are given for each of the 16 laboratories in Table 1, together with the standard deviation determined from the hidden duplicates (repeatability, s_r) for each laboratory. A consideration of these mean values indicates laboratories 8 and 14 returned low values for both Dig 1 and Dig 2 which can be traced to exceptionally high values for A, the acid and pepsin insoluble residue. Adjustment of this data for nitrogen contributed by filter paper did not account for the divergence of these two laboratories (Table 1F).

The data were subjected to a battery of tests to determine the presence of outlier laboratories. A Principal Component analysis was carried out using the five variable mean values displayed in Table 1F. The Principal Component plot is shown in Figure 1. This identifies Labs 8 and 14 as being different from the remaining group of laboratories. A Cluster Analysis examined the grouping of laboratories in more detail using the same data. The resulting dendrogram is shown in Figure 2. Starting with the entire set as single laboratories the laboratories are combined either singly or in groups so as to minimise the 'Within Group' sum of squares. This quantity starts with the value zero. The length of the horizontal line when new groupings are formed represents the increase in the sum of squares. Figure 2 clearly shows the increased variance when Labs 8 and 14 are added to the remainder. In addition, Labs 4, 9, 10, 15, 16 form a second group which differ little between themselves but are different to the remaining group of Labs 1, 2, 3, 5, 6, 7, 11 and 13. Finally the data were subjected to Grubbs test (Grubbs, 1969; Horwitz, 1993). Table 2 gives the standard deviation for each of the five variables between all laboratories and after excluding laboratory 8 and then labs 8 and 14. The Grubbs statistic, which is the percentage reduction in standard deviation on excluding the outlying values, is also shown. For measurement A and the derived dilute pepsin digestibilities the Grubbs statistics on removing lab 8 and labs 8 and 14 were significantly greater than the critical values at $P < 0.025$ (2-tail). Therefore, these two labs were regarded as outliers and the analyses are presented both for all laboratories and after exclusion of labs 8 and 14.

The primary measurements and calculated dilute pepsin digestibilities averaged across all laboratories are given for each meal in Table 3 and after correction for filter paper N in Table 3F. The amount of N in a washed filter paper varied considerably from zero to 0.82 mg N per paper used, mean 0.25 mg N SD ±0.245. Tables 4 and 4F give the corresponding values after excluding labs 8 and 14 as outliers. These tables also give the between laboratory standard deviations (reproducibility, s_R) for each sample. The between laboratory variability expressed as a percentage of the sample mean (Relative standard deviation, RSD_R) is large for measurement of % N insoluble in acid pepsin because the amount of residual N is very small. The amount of N insoluble in acid alone (B) was 60.4% (SD ± 9.64) of total N (A) resulting in a substantially lower estimate of digestibility by the Torry calculation with an accompanying greater between laboratory variability especially when expressed as a percentage of the lower mean value (RSD_R).

Table 5A gives the hidden duplicate standard deviation pooled across laboratories (repeatability, s_r). The filter paper correction makes no difference to the repeatability of the primary

measurements since both hidden duplicates are treated the same but this does have an effect on the repeatability of digestibility calculated by the Torry method but is insignificant in the modified AOAC calculation. Omitting labs 8 and 14 have no appreciable effect on the repeatability estimates.

Table 5B gives the between laboratory standard deviation pooled across all samples (reproducibility, s_R). Adjusting the data for filter paper N had very little effect but gave a numerical increase rather than the expected decrease in reproducibility. Omitting labs 8 and 14 substantially reduced the reproducibility estimates for measurement A (but not B or C) and the derived digestibility values.

Table 5C gives the variance component (s^2_r) due to repeatability within laboratories and the variance component purely due to between laboratories (s^2_L) estimated from the sub-set of pairs of hidden duplicate meals. If the laboratories do not contribute any variability, over and above the errors of determination, the laboratory component would be zero. Table 5C indicates the laboratory component is of the same order of magnitude as the basic error of determination within one laboratory. The reproducibility variance for a single determination made by a randomly selected laboratory is expected to be the sum of these two components ($s^2_R = s^2_r + s^2_L$). The estimate of s^2_R and hence of s_R in this subset of the data is generally a little less than that estimated from the full set shown in Table 5B.

The type of shaker used had a small effect (Table 6). Less N remained after acid pepsin (A value) when orbital shaking was used but the difference did not achieve significance ($P > 0.05$). In contrast less N remained after shaking in acid alone (B value) with the rotating shaker but again the difference was not significant. In the calculation of Torry digestibility from:

$$\text{Torry digestibility} = 100(B-A)/B$$

these two trends have a combined effect in giving a greater digestibility with orbital than with rotating shaking ($P < 0.05$).

Table 7 classifies the data according to the source of pepsin used, after excluding the two outlier laboratories. Eight laboratories used the specified Merck pepsin. Three laboratories used Sigma Pepsin A 1:10,000 Porcine. Two laboratories used Difco pepsin and one used US Biochemical 20015, 1:10494. A further lab used pepsin from Saarchem Unilab which was assayed and adjusted to the required activity. Those using Merck were compared with the average of all the other sources. There was no significant difference in any of the measured parameters or calculated digestibilities.

Discussion

Table 8 presents a summary of the digestibilities calculated by the two methods after the removal of the two outlying laboratories and correcting for filter paper nitrogen. All laboratories were capable of determining N in fish meal with acceptable repeatability (mean RSD_r 0.70%, range 0.2 to 1.4% for different labs) and reproducibility (mean RSD_R 1.14%, range 0.6 to 2.2% for different samples).

Since 0.4 of the fish meal N was soluble in acid alone, digestibility calculated by the Torry method reflects the effect of pepsin on only 0.6 of the whole N. This results in a substantial difference to the digestibility values as calculated by the Torry and AOAC methods. In the Torry method, the part (0.4) that is soluble in acid alone is ignored. When the Torry digestibility value

is used to describe a fish meal, it makes the assumption that the acid soluble material is of the same digestibility as the acid insoluble fraction. However, material that is soluble in dilute HCl alone in the laboratory is also likely to be readily solubilised and digested in the animal. In contrast, this fraction is included in the AOAC definition of pepsin digestible. Furthermore the determination of the acid insoluble N was more variable both within and between laboratories than either the acid pepsin insoluble or the total N. Consequently, the Torry method of calculation not only results in lower estimates of digestible nitrogen but also a greater variability both within and between laboratories when expressed in absolute standard deviations (Table 5B) and even more so when expressed as relative standard deviations (Table 8). When the same sample is analysed within one laboratory the difference in absolute value between the estimates should not exceed the repeatability limit (Table 8) in 95% of occasions. Thus while laboratories can distinguish between two fish meals differing in crude protein by 1.38 %units, pepsin digestibility needs to differ by 3.05 units calculated by the Torry method but only 1.57 units by the AOAC method. Similarly, the absolute difference between two single test results obtained using the same method on identical test material in different laboratories by different operators using different equipment should be less than the reproducibility limit (Table 8) in 95 % of cases. Analyses in different laboratories of the same sample could differ by as much as 5.23 units calculated by the Torry method and 3.38 units by the AOAC method. Equally different meals analysed in different laboratories would have to differ by at least these amounts before they could be considered different.

Figure 3 shows the relationship between the digestibilities calculated by the two methods for each sample averaged over the 14 laboratories. The Torry method gives a lower but extended scale of digestibility than the AOAC method with a good relationship between the two methods of calculation but with slope and intercept which are greatly different from unity and zero respectively. A good relationship is to be expected in this exercise because the same acid pepsin insoluble N determination is used in both calculations. This relationship does not apply to values determined by the original AOAC method (using 0.2% pepsin) compared with the usual Torry method. The greater scale of the Torry method (a range of 14 % units, 83 to 97 in the present samples compared with a range of 9 % units, 89 to 98) is offset by the 94% greater within laboratory variability and 60% greater between laboratory variability. Consequently, the Torry method of calculation is not more discriminatory between samples. In addition the Torry method requires an extra N determination of the acid insoluble N and is more affected by problems of filter paper N contamination of the filtered residues. Consequently, the AOAC method of calculation is preferred and is adopted for the future method.

The type of shaking equipment used affected the digestibility calculated according to the Torry method but not that by the modified AOAC method. Although the differences in the A and B values were not significant they suggest that the enzyme digest proceeded best under the gentle conditions of orbital shaking and that failure to digest sample through material being deposited on the vessel walls out of the solution was not a problem. In contrast solubility in dilute HCl alone seems to have been enhanced by the more vigorous shaking conditions in the end over end rotating shaker. The difference due to shaker type is a further reason for preferring the AOAC method of calculating results. No recommendation need be made to use a particular type of shaker but the preference would be for the more readily available modern orbital type of shaker.

The source of pepsin did not apparently affect the results. However, laboratory 12 reported the lowest amount of acid pepsin insoluble N and also used pepsin from Saarchem Unilab which required adjustment of the amount used to achieve the desired activity. It is conceivable that too

much activity was used. The two outlier laboratories reporting very high amounts of acid pepsin insoluble N both used Sigma pepsin but three other laboratories using the same pepsin source reported values within the range of the Merck enzyme. Consequently, it does seem prudent to specify the use of one source of enzyme.

Appendix 3 details the new method to be adopted by IFOMA as its official method. The type of shaker and source of pepsin are recommended but need not be mandatory. While the adoption of a common method may help reduce apparent discrepancies between laboratories apparently reporting the same analysis attention is drawn to the quite large repeatability and reproducibility limits (Table 8 and Appendix 3) relative to the range of values (88 – 98) likely in normal commerce. Thus, within one laboratory differences between fish meals of less than 1.6 % digestibility units cannot be distinguished. When the same fish meal is analysed in two different laboratories differences of up to 3.3 % digestibility units are to be expected.

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Table 1 Table giving the Laboratory Mean Values, and the 'hidden duplicate' Standard Deviation for each Laboratory, and variable. (No correction for filter paper N.)

	Mean Values					Hidden Duplicate Standard Deviation				
Lab.	A	B	C	Dig.1	Dig.2	A	B	C	Dig.1	Dig.2
1	0.587	6.98	11.14	91.38	94.63	0.059	0.022	0.099	1.025	0.484
2	0.569	6.92	11.17	91.63	94.81	0.036	0.074	0.141	0.585	0.276
3	0.584	7.01	11.18	91.51	94.68	0.125	0.032	0.018	2.047	1.068
4	0.415	6.65	11.26	93.65	96.24	0.010	0.250	0.021	0.150	0.083
5	0.531	6.01	11.10	91.09	95.11	0.029	0.406	0.092	0.822	0.276
6	0.592	6.54	11.31	90.84	94.65	0.100	0.175	0.025	1.567	0.861
7	0.543	6.42	11.30	91.28	95.10	0.043	0.086	0.040	0.867	0.375
8	1.007	6.85	11.19	84.92	90.89	0.043	0.040	0.055	0.843	0.339
9	0.378	6.57	11.13	94.14	96.54	0.000	0.032	0.057	0.038	0.011
10	0.427	6.98	11.06	93.61	96.04	0.021	0.074	0.050	0.442	0.172
11	0.538	6.27	11.12	91.06	95.09	0.150	0.525	0.039	2.416	1.316
12	0.350	6.91	11.21	94.73	96.84	0.011	0.101	0.160	0.298	0.132
13	0.573	7.06	11.19	91.68	94.77	0.049	0.084	0.073	0.729	0.434
14	0.814	7.01	11.08	88.13	92.53	0.057	0.110	0.010	1.191	0.506
15	0.508	6.84	11.12	92.38	95.33	0.010	0.059	0.046	0.161	0.100
16	0.477	6.88	11.22	92.89	95.65	0.021	0.025	0.066	0.370	0.158

Table 1F Table giving the Laboratory Mean Values, and the 'hidden duplicate' Standard Deviation for each Laboratory, and variable. (Corrected for filter paper N.)

	Mean Values					Hidden Duplicate Standard Deviation				
Lab.	A	B	C	Dig.1	Dig.2	A	B	C	Dig.1	Dig.2
1	0.535	6.92	11.14	92.09	95.10	0.059	0.022	0.099	1.037	0.488
2	0.562	6.92	11.17	91.72	94.87	0.036	0.074	0.141	0.587	0.277
3	0.584	7.01	11.18	91.51	94.68	0.125	0.032	0.018	2.047	1.068
4	0.415	6.65	11.26	93.65	96.24	0.010	0.250	0.021	0.150	0.083
5	0.526	6.01	11.10	91.16	95.15	0.029	0.406	0.092	0.817	0.276
6	0.592	6.54	11.31	90.84	94.65	0.100	0.175	0.025	1.567	0.861
7	0.504	6.39	11.30	91.85	95.45	0.043	0.086	0.040	0.865	0.374
8	1.007	6.85	11.19	84.92	90.89	0.043	0.040	0.055	0.843	0.339
9	0.365	6.56	11.13	94.32	96.64	0.000	0.032	0.057	0.037	0.010
10	0.427	6.98	11.06	93.61	96.04	0.021	0.074	0.050	0.442	0.172
11	0.482	6.22	11.12	91.92	95.60	0.150	0.525	0.039	2.477	1.317
12	0.303	6.86	11.21	95.40	97.26	0.011	0.101	0.160	0.286	0.128
13	0.543	7.03	11.19	92.08	95.04	0.049	0.084	0.073	0.739	0.434
14	0.810	7.01	11.08	88.18	92.57	0.057	0.110	0.010	1.191	0.506
15	0.494	6.82	11.12	92.57	95.46	0.010	0.059	0.046	0.161	0.100
16	0.464	6.87	11.22	93.07	95.76	0.021	0.025	0.066	0.371	0.158

Table 2. Standard deviation between laboratories for N soluble in acid pepsin (A), N soluble in acid alone (B), total N (C) and the dilute pepsin digestibilities calculated according to the Torry (Dig. 1) and AOAC (Dig. 2) procedures. The Grubbs statistic is also given (in brackets) where significant. The * denotes statistical significance at the P <0.025 level.

Measurement	A	B	C	Dig. 1	Dig. 2
All labs	0.0162	0.305	0.074	2.376	1.467
Excluding Lab 8	0.112 (30.9*)	0.315 -	0.076 -	1.641 (30.9*)	1.031 (29.7*)
Excluding Labs 8 & 14	0.082 (49.4*)	0.317 -	0.075 -	1.291 (45.7*)	0.747 (49.1*)

Table 3. Sample means, 'Between Laboratories' Standard Deviations, and Relative Standard Deviations. (No correction for filter paper N.)

Sample	1	2	3	4	5	6	7	8	9	10	11	12
% N Insoluble in Pepsin/Acid (A)												
Mean	0.340	0.378	0.527	0.520	0.343	0.301	0.557	0.451	0.861	0.515	1.135	0.731
S.D.	0.147	0.153	0.188	0.256	0.140	0.123	0.196	0.163	0.269	0.180	0.188	0.236
RSD_R	43.31	40.47	35.78	49.27	40.78	40.76	35.16	36.22	31.22	34.94	16.60	32.21
% N Insoluble in Acid Alone (B)												
Mean	6.036	7.661	5.388	7.619	6.006	8.110	5.347	7.761	7.351	5.426	6.500	7.718
S.D.	0.560	0.500	0.246	0.317	0.535	0.435	0.191	0.594	1.059	0.162	0.249	0.244
RSD_R	9.28	6.53	4.56	4.15	8.92	5.37	3.57	7.66	14.41	2.98	3.83	3.16
% N In original Sample (C)												
Mean	11.74	11.39	11.34	11.18	11.77	11.52	10.54	11.27	11.00	11.32	9.98	11.01
S.D.	0.110	0.137	0.087	0.117	0.110	0.087	0.222	0.113	0.073	0.128	0.155	0.077
RSD_R	0.93	1.20	0.77	1.04	0.93	0.76	2.10	1.01	0.67	1.13	1.56	0.70
Torry Pepsin Digestibility % (Dig.1)												
Mean	94.31	95.11	90.27	93.20	94.25	96.28	89.59	94.13	88.18	90.54	82.53	90.53
S.D.	2.527	1.839	3.279	3.244	2.368	1.515	3.584	2.267	3.349	3.141	2.843	2.985
RSD_R	2.68	1.93	3.63	3.48	2.51	1.57	4.00	2.41	3.80	3.47	3.45	3.30
AOAC Dilute Pepsin Digestibility % (Dig.2)												
Mean	97.11	96.68	95.36	95.36	97.09	97.38	94.70	96.00	92.18	95.45	88.61	93.35
S.D.	1.241	1.338	1.640	2.273	1.181	1.076	1.909	1.457	2.439	1.583	1.969	2.159
RSD_R	1.28	1.38	1.72	2.38	1.22	1.11	2.02	1.52	2.65	1.66	2.22	2.31

Table 3F. Sample means, 'Between Laboratories' Standard Deviations, and Relative Standard Deviations. (Corrected for filter paper N)

Sample	1	2	3	4	5	6	7	8	9	10	11	12
% N Insoluble in Pepsin/Acid (A)												
Mean	0.323	0.362	0.509	0.503	0.326	0.284	0.540	0.434	0.843	0.498	1.118	0.714
S.D.	0.149	0.157	0.189	0.262	0.147	0.124	0.203	0.169	0.276	0.187	0.198	0.241
RSD_R	46.32	43.25	37.02	52.20	45.12	43.55	37.55	38.99	32.68	37.49	17.74	33.83
% N Insoluble in Acid Alone (B)												
Mean	6.018	7.646	5.371	7.601	5.988	8.093	5.330	7.744	7.333	5.408	6.483	7.701
S.D.	0.560	0.497	0.241	0.314	0.542	0.433	0.187	0.592	1.069	0.164	0.252	0.250
RSD_R	9.31	6.50	4.48	4.14	9.04	5.35	3.51	7.64	14.58	3.04	3.89	3.24
% N In original Sample (C)												
Mean	11.74	11.39	11.34	11.18	11.77	11.52	10.54	11.27	11.00	11.32	9.98	11.01
S.D.	0.110	0.137	0.087	0.117	0.110	0.087	0.222	0.113	0.073	0.128	0.155	0.077
RSD_R	0.93	1.20	0.77	1.04	0.93	0.76	2.10	1.01	0.67	1.13	1.56	0.70
Torry Pepsin Digestibility % (Dig.1)												
Mean	94.59	95.31	90.57	93.42	94.53	96.49	89.88	94.34	88.43	90.84	82.75	90.74
S.D.	2.569	1.892	3.293	3.326	2.471	1.525	3.715	2.339	3.317	3.253	2.970	3.053
RSD_R	2.72	1.99	3.64	3.56	2.61	1.58	4.13	2.48	3.75	3.58	3.59	3.36
AOAC Dilute Pepsin Digestibility % (Dig.2)												
Mean	97.26	96.82	95.51	95.51	97.24	97.53	94.86	96.15	92.34	95.61	88.79	93.51
S.D.	1.258	1.368	1.640	2.328	1.242	1.083	1.972	1.507	2.502	1.642	2.064	2.212
RSD_R	1.29	1.41	1.72	2.44	1.28	1.11	2.08	1.57	2.71	1.72	2.33	2.37

Table 4. Sample means and 'Between Laboratories' Standard Deviations, and Relative Standard Deviations. (Excluding Labs. 8 & 14 as outliers). (No Correction for filter paper N.)

Sample	1	2	3	4	5	6	7	8	9	10	11	12
% N Insoluble in Pepsin/Acid (A)												
Mean	0.298	0.328	0.478	0.459	0.304	0.263	0.493	0.401	0.790	0.460	1.086	0.686
S. D.	0.086	0.075	0.095	0.208	0.093	0.066	0.091	0.092	0.158	0.076	0.143	0.215
RSD_R	28.71	22.98	19.94	45.34	30.71	24.99	18.36	22.91	20.04	16.49	13.16	31.33
% N Insoluble in Acid Alone (B)												
Mean	6.031	7.608	5.358	7.605	6.009	8.089	5.341	7.708	7.280	5.406	6.467	7.706
S. D.	0.595	0.514	0.246	0.335	0.573	0.450	0.186	0.618	1.119	0.163	0.235	0.252
RSD_R	9.87	6.75	4.60	4.40	9.53	5.57	3.48	8.02	15.37	3.02	3.64	3.27
% N In original Sample (C)												
Mean	11.74	11.40	11.34	11.17	11.78	11.53	10.56	11.28	11.01	11.32	10.00	11.02
S. D.	0.107	0.140	0.084	0.122	0.105	0.069	0.232	0.120	0.077	0.136	0.156	0.075
RSD_R	0.91	1.23	0.74	1.09	0.89	0.60	2.19	1.07	0.70	1.20	1.56	0.68
Torry Pepsin Digestibility % (Dig.1)												
Mean	95.01	95.70	91.12	93.99	94.90	96.74	90.77	94.70	88.96	91.50	83.16	91.09
S. D.	1.512	0.950	1.551	2.602	1.575	0.855	1.631	1.724	2.292	1.346	2.418	2.748
RSD_R	1.59	0.99	1.70	2.77	1.66	0.88	1.80	1.82	2.58	1.47	2.91	3.02
AOAC Dilute Pepsin Digestibility % (Dig.2)												
Mean	97.46	97.12	95.79	95.90	97.42	97.72	95.32	96.44	92.83	95.94	89.12	93.77
S. D.	0.723	0.663	0.840	1.848	0.781	0.574	0.897	0.813	1.424	0.658	1.484	1.960
RSD_R	0.74	0.68	0.88	1.93	0.80	0.59	0.94	0.84	1.53	0.69	1.67	2.09

Table 4F. Sample means and 'Between Laboratories' Standard Deviations, and Relative Standard Deviations. (Excluding Labs. 8 & 14 as outliers.) (Corrected for filter paper N.)

Sample	1	2	3	4	5	6	7	8	9	10	11	12
% N Insoluble in Pepsin/Acid (A)												
Mean	0.278	0.310	0.458	0.439	0.285	0.243	0.473	0.382	0.770	0.440	1.067	0.667
S.D.	0.080	0.072	0.084	0.211	0.097	0.055	0.094	0.093	0.163	0.079	0.151	0.219
RSD_R	28.59	23.14	18.33	48.06	34.22	22.57	19.83	24.37	21.10	17.99	14.20	32.79
% N Insoluble in Acid Alone (B)												
Mean	6.012	7.591	5.338	7.585	5.989	8.069	5.321	7.688	7.260	5.386	6.447	7.687
S.D.	0.595	0.508	0.239	0.331	0.579	0.447	0.180	0.614	1.128	0.163	0.237	0.258
RSD_R	9.90	6.69	4.47	4.37	9.67	5.54	3.39	7.98	15.54	3.03	3.67	3.35
% N in Original Sample (C)												
Mean	11.74	11.40	11.34	11.17	11.78	11.53	10.56	11.28	11.01	11.32	10.00	11.02
S.D.	0.107	0.140	0.084	0.122	0.105	0.069	0.232	0.120	0.077	0.136	0.156	0.075
RSD_R	0.91	1.23	0.74	1.09	0.89	0.60	2.19	1.07	0.70	1.20	1.56	0.68
Torry Pepsin Digestibility % (Dig.1)												
Mean	95.32	95.92	91.44	94.23	95.22	96.97	91.11	94.94	89.24	91.83	83.42	91.33
S.D	1.442	0.930	1.386	2.647	1.618	0.740	1.726	1.754	2.130	1.405	2.523	2.786
RSD_R	1.51	0.97	1.52	2.81	1.70	0.76	1.89	1.85	2.39	1.53	3.02	3.05
AOAC Dilute Pepsin Digestibility % (Dig.2)												
Mean	97.63	97.28	95.96	96.08	97.59	97.89	95.51	96.62	93.00	96.11	89.32	93.95
S.D.	0.671	0.627	0.739	1.876	0.816	0.479	0.926	0.821	1.464	0.690	1.566	1.992
RSD_R	0.69	0.64	0.77	1.95	0.84	0.49	0.97	0.85	1.57	0.72	1.75	2.12

Table 5A. Pooled 'Within Laboratory' standard deviations from the hidden duplicates, both for full data set, and after removing outliers (Labs 8 & 14), WITH and WITHOUT correction for N contributed by filter paper.

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	A	B	C	Dig.1	Dig.2
All Laboratories					
Without N Adj.	0.063	0.192	0.075	1.073	0.547
With N Adj.	0.063	0.192	0.075	1.083	0.547
Excluding Labs 8 & 14					
Without N Adj.	0.065	0.203	0.078	1.079	0.562
With N Adj.	0.065	0.203	0.078	1.090	0.562

Table 5B. Pooled 'Between Laboratory' standard deviations, both for full data set, and after removing outliers (Labs 8 & 14), WITH and WITHOUT correction for N contributed by filter paper.

	A	B	C	Dig.1	Dig.2
All Laboratories					
Without N Adj.	0.192	0.490	0.125	2.818	1.746
With N Adj.	0.197	0.491	0.125	2.884	1.795
Excluding Labs 8 & 14					
Without N Adj.	0.127	0.513	0.127	1.868	1.157
With N Adj.	0.128	0.514	0.127	1.869	1.171

Table 5C. Pooled estimates of the components of variance derived from the subset of data of the hidden duplicate samples of fish meals, after excluding outliers (Labs 8 and 14) and after correction for N contributed by filter paper.

Source of variance	A	B	C	Dig.1	Dig.2
Between laboratory s^2_L	0.00322	0.01752	0.00631	1.025	0.234
Within laboratory s^2_r	0.00423	0.04116	0.00613	1.178	0.315
Reproducibility standard deviation s_R	0.086	0.242	0.112	1.933	0.741

Table 6. Table displaying difference in variables due to Type of Shaker. Reduced Data set, excluding Labs 8 & 14, and after correction for N contributed by filter paper.

Shaker Type	A	B	C	Dig.1	Dig.2
Rotating(4)	0.54±0.019	6.47±0.19	11.21±0.05	91.5±0.29	95.1±0.17
Orbital(10)	0.46±0.028	6.79±0.08	11.17±0.02	93.0±0.40	95.8±0.26
	N.S.	N.S.	N.S.	P <0.05	N.S.

Table 7. Table displaying difference in variables due to Pepsin Source. Reduced Data set, excluding Labs 8 & 14, and after correction for filter paper N.

Pepsin Source	A	B	C	Dig.1	Dig.2
Merck (8)	0.49±0.026	6.80±0.10	11.15±0.02	92.6±0.35	95.5±0.23
Other (6)	0.48±0.042	6.56±0.14	11.22±0.04	92.5±0.70	95.6±0.39
	N.S.	N.S.	N.S.	N.S.	N.S.

Table 8. Mean overall samples of fish meal N and dilute pepsin digestibility calculated by two methods (Torry and modified AOAC) together with the relative repeatability and reproducibility standard deviations. Reduced Data set, excluding Labs 8 & 14, and after correction for filter paper N.

	Total N (%)	Torry digestibility (%)	Modified AOAC digestibility (%)
Mean	11.2	92.6	95.6
Relative standard deviations (%)			
Repeatability RSD_r	0.70	1.18	1.14
Reproducibility RSD_R	1.14	2.02	1.23
Critical differences (% units)			
Repeatability limit r	0.22	3.05	1.57
Reproducibility limit R	0.36	5.23	3.28

Figure 1 Plot of first two Principal Components. Labs 8 and 14 are seen to be separate from the remaining population of laboratories.

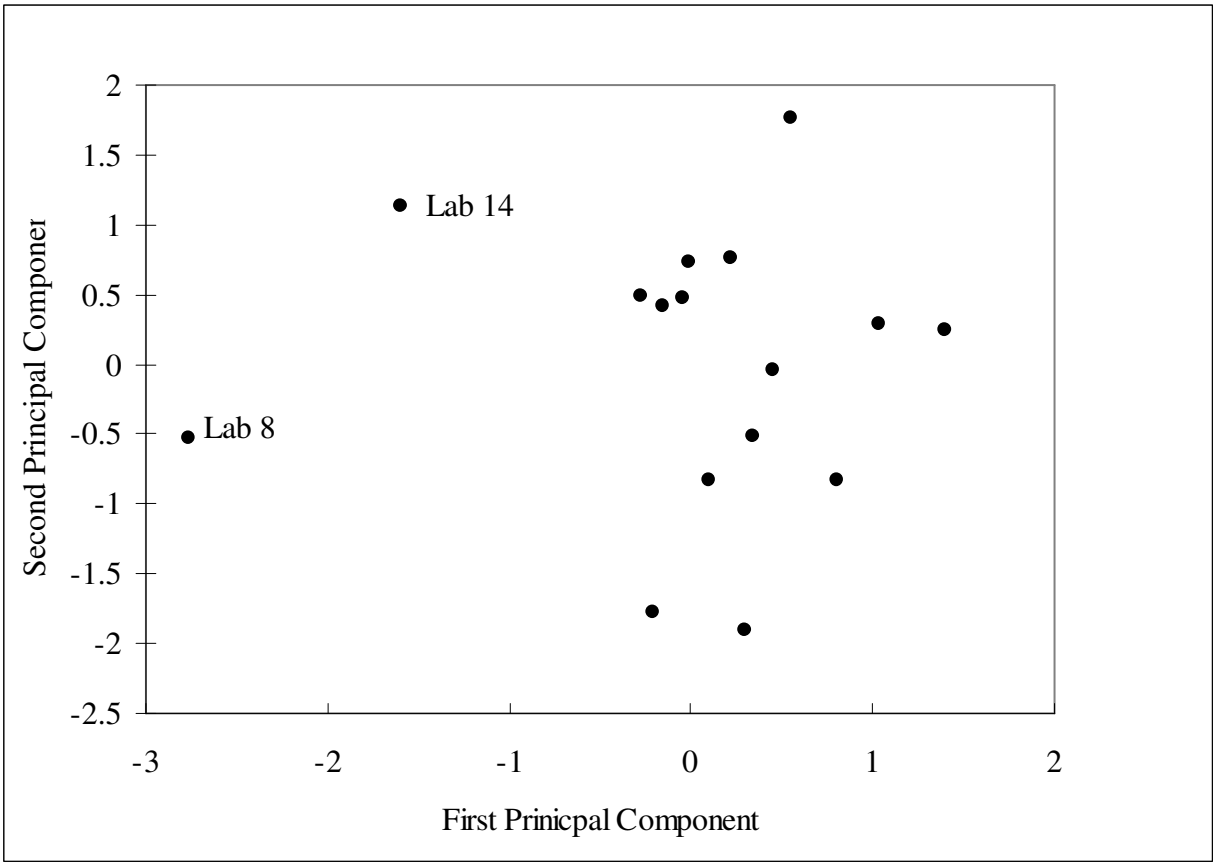


Figure 2.Dendrogram illustrating the grouping of laboratories. The length of the horizontal line joining groups is proportional to the increase in within group sums of squares when the lab or group of labs are combined.

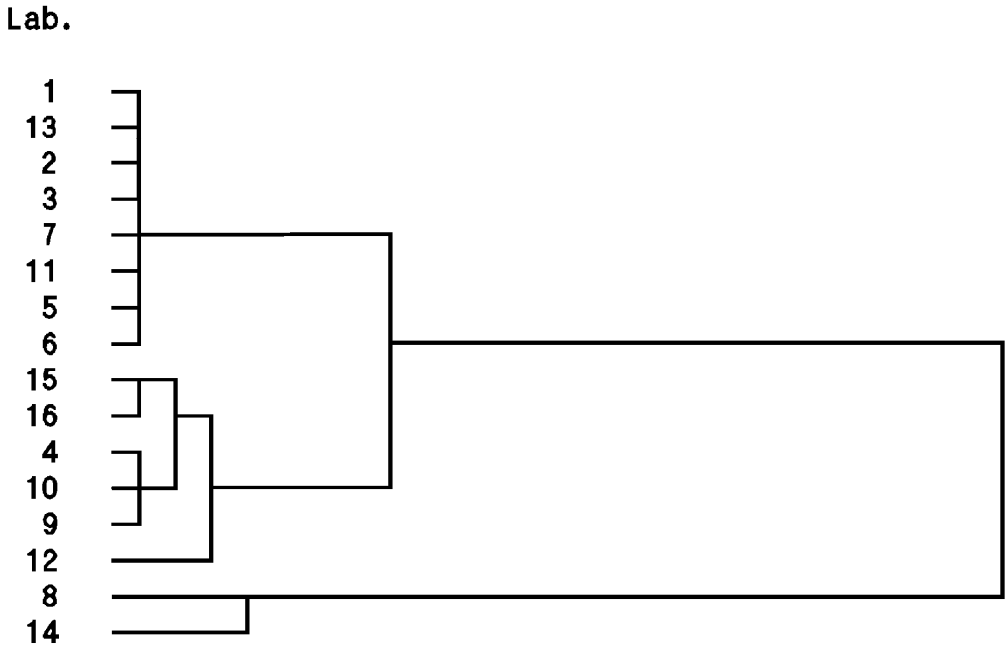
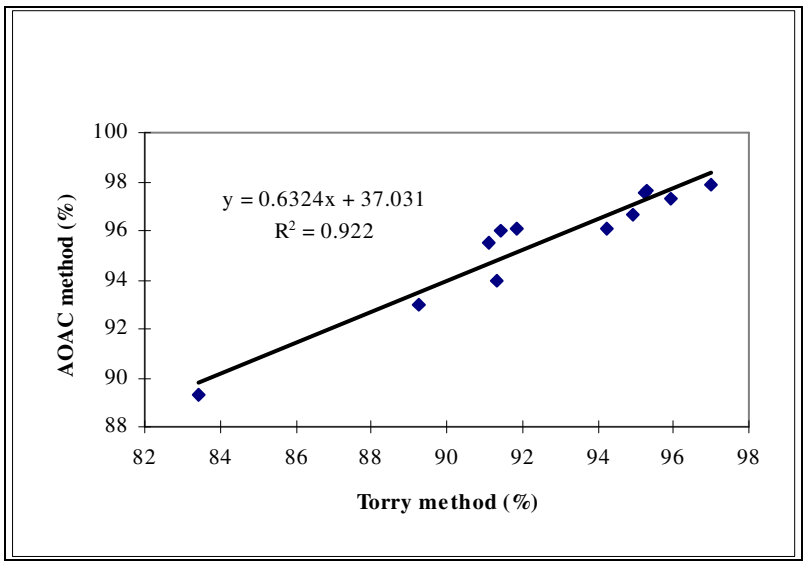


Figure 3. Relationship between dilute pepsin digestibility of N of 12 samples of fish meal calculated according to either the AOAC or Torry method from the same primary data.



Appendix 1. The circulated protocol.

PROPOSED IFOMA RECOMMENDED METHOD FOR DILUTE PEPSIN DIGESTIBILITY.

1.0 Applicable to fishmeal. This method cannot be used as a measure of the nutritional quality of protein for specific species of animals. However, in certain circumstances fish meal traders have deemed it useful to specify minimum digestibility levels in contracts using pepsin concentrations more dilute than that specified by AOAC Official Method 971.09. The purpose of this method is to harmonise the dilute pepsin digestibility procedure.

2.0 Reference: Modification of AOAC Official Method 971.09.

3.0 Summary: Protein is digested according to the conditions of the test. Determine the kjeldahl nitrogen insoluble in pepsin/acid solution, the kjeldahl nitrogen insoluble in the acid solution and the kjeldahl nitrogen in the original fishmeal sample.

The IFOMA pepsin digestibility of the protein is defined as:

The portion of the acid insoluble nitrogen that is soluble in pepsin/acid solution.

The AOAC dilute pepsin digestibility of the protein is defined as:

The portion of the total nitrogen in the sample that is acid/pepsin soluble.

4.0 Apparatus and Supplies

1. Analytical balance, capable of weighing to 0.10 mg.
2. Incubator capable of maintaining 45°C ±2.0
3. Rotating or Orbital Shaker.
4. Buchner Funnel, 150 mm.
5. Filter paper, S&S (Schleicher & Schuell) No. 589, Whatman 541 or equivalent.
6. Screw cap sample bottles or flasks capable of holding 200 ml.
7. Volumetric flask, 1000, 2000 ml.
8. Pipettes, 10 and 20 ml.
9. Graduated cylinder, 200 ml.

5.0 Reagents

1. Merck pepsin 1:10,000 activity (Product No. 7190) (See note 1). Store cool and dry.
2. Hydrochloric Acid, 37%.
3. Distilled water
4. 0.075 N Hydrochloric Acid. Dilute 6.3 ml of 37% hydrochloric acid to 1000 ml, or 12.6 ml to 2000 ml with distilled water.
5. 0.020% Pepsin/Acid Solution. Prepare fresh.

Dissolve 0.2000 grams of 1:10,000 activity pepsin in a litre of 0.075 N Hydrochloric Acid

6. 0.0002% Pepsin/Acid Solution. Prepare fresh.

Transfer 20 ml of the 0.020% pepsin solution to a 2 litre volumetric flask and dilute to

the mark with 0.075 N Hydrochloric Acid.
7. Also, apparatus and chemicals needed for the determination of kjeldahl protein. (ISO/FDIS 5983:1997(E).

6.0 Procedure

1. Grind the sample so that the particles pass through an ASTM No. 30-32 Screen (0.500-0.595mm). If it is difficult to grind to this particle size, then the fat content may be too high and the sample should be extracted with petroleum ether for 2 hours. Extraction should be done on the 1 gram weighed sample and the residue used for the rest of the test.
2. Weigh accurately 1.0 gram and record the weight to 0.1 mg, of the ground fishmeal into the 200 ml reaction bottle or flask. Mark this container as pepsin.
3. Weigh accurately 1.0 gram (and record weight to 0.1 mg) of the ground fishmeal into a second 200 ml reaction bottle or flask. Mark this container as acid.
4. Transfer 150 ml of the 0.0002% pepsin/acid solution (5.6) into the flask marked "pepsin".
5. Transfer 150 ml of the 0.075 N. Hydrochloric Acid Solution (5.4) into the flask marked "acid".
6. Incubate the flasks for 16 hours at 45°C±2.0. If an orbital shaker unit is used be sure there are no particles on the wall of the flask above the pepsin/acid solution. The orbital shaker should shake at 140-200 orbits/minute.
7. Filter the solutions through the filter paper using a gentle vacuum suction. Wash out any remaining particles from the flask or bottle with warm distilled water and transfer to the filter. Finally wash the filter with several portions of warm distilled water.
8. Determine the kjeldahl nitrogen content of the filtered residues according to ISO/FDIS 5983:1997(E).
9. Determine the kjeldahl nitrogen content of the original fishmeal sample according to ISO FDIS 5983:1997(E).

7.0 CALCULATIONS

1. Calculate the % nitrogen insoluble in pepsin/acid solution (A).
2. Calculate the % nitrogen insoluble in acid solution (B).
3. Calculate the % nitrogen in the Original Sample (C).
4. Calculate the IFOMA Pepsin Digestibility as follows:

$$\text{IFOMA Pepsin Digest.} = (B - A)/B \times 100$$

5. Calculate the % AOAC dilute pepsin digestibility as follows:

$$\text{AOAC Dilute Pepsin Digest.} = (C-A)/C \times 100$$

8.0 REPORT RESULTS
Report your results as:

% Nitrogen insoluble in pepsin/acid solution (A)

% Nitrogen insoluble in acid solution (B)

% Nitrogen in Original Sample (C)

Data is to be reported to 2 decimal places.

9.0 Report your data on the attached sheet.
Note 1:
If other brands of pepsin (see 5.1) are used it is essential to confirm their activity at 1388 units per mg of powder. Any variations should be adjusted when preparing the dilute pepsin solution (see 5.5 and 5.6).

The recommended method for determination of pepsin activity is given in Annex A (3 pages attached) taken from ISO 6655 (1997).

Appendix 2. Participants in dilute pepsin digestibility ring test.

Analyst	Laboratory
René Caquilpan / Bernada Hernández	Cesmec Ltda. Avda. Marathon 2595, Santiago, Chile
Joel Sieh	CN Labs, PO Box 117, Highway 14 West, Courtland, MN 56021, USA
Mario Guerrero Solar	Corpesca S.A. Huerfanos 863, 9th Floor, Santiago, Chile
Elizabeth Timme	CSIR, Lower Hope Street, Rosebank 7700, Cape Province, South Africa
Keith M. Batieste	Daybrook Fisheries Inc. Highway 11, PO Box 128, Empire, LA 70050, USA
Rosa Marrou	Inassa, Av La Marina 3035, San Miguel, Lima 32, Peru
Claudio Jofré Benios	Inspectorate Griffith, Avda Los Leones 1871, Providencia, Santiago, Chile
Carl W. Schulze	New Jersey Food Laboratory Inc. CN 06650, Trenton, New Jersey 08650, USA
Jorma Kinnari	Nutreco, Aquaculture Research Centre A/S PO Box 48, N 4001 Stavanger, Norway
Faith New	Omega Protein Corporation, PO Box 1799, Hammond, Louisiana 70404-1799, USA
G. Cotton	Salamon & Seaber, Britannia House, 68 Hanbury Street, London E1 5JL, UK
Irene Abarca / Marcelo Araos	SGS Chile, Agroindustrial Division, I. Valdivieso 2409 San Joaquin, Santiago, Chile
Bertha Sulca	SGS Del Peru S.A. Av Arequipa 3445,

	Lima 27, Peru
Arne Brodin	SSF Kjerreidviken 16, Fyllingsdalen N-5033, Norway
Gunnhildur Gísladóttir	The Icelandic Fisheries Laboratory, PO Box 1405, Skulagata 4, 121 Reykjavik, Iceland
Boyce H. Butler	Thionville Laboratories Inc. PO Box 23687, 5440 Pepsi Street, New Orleans, LA 70183, USA

APPENDIX 3

IFOMA METHOD FOR DETERMINATION OF PROTEIN SOLUBILITY IN DILUTE PEPSIN/ACID SOLUTION

1.0 Applicable to fishmeal. This method cannot be used as a measure of the nutritional quality of protein for specific species of animals. However, in certain circumstances fish meal traders have deemed it useful to specify minimum solubility levels in contracts using pepsin concentrations more dilute than that specified by AOAC Official Method 971.09. The purpose of this method is to harmonise the dilute pepsin solubility procedure.

2.0 Reference: Modification of AOAC Official Method 971.09.

3.0 Summary: Protein is digested according to the conditions of the test. Determine the Kjeldahl nitrogen insoluble in pepsin/acid solution and the Kjeldahl nitrogen in the original fishmeal sample.

The IFOMA dilute pepsin solubility of the protein is defined as:

The portion of the total nitrogen in the sample that is pepsin/acid soluble.

4.0. Apparatus and Supplies

4.1. Analytical Balance, capable of weighing to 0.1 mg.
4.2. Incubator capable of maintaining 45°C ±2.0
4.3. Orbital or rotating shaker.
4.4. Buchner funnel, 150 mm.
4.5. Filter Paper, S&S (Schleicher & Schuell) No. 589, Whatman 541 or equivalent.
4.6. Screw cap sample bottles or flasks capable of holding 200 ml.
4.7. Volumetric flask, 1000, 2000 ml.
4.8. Pipettes, 10 and 20 ml.
4.9. Graduated cylinder, 200 ml.

5.0. Reagents

5.1. Merck pepsin 2,000 FIP-U/g activity (Product No. 107190) (See note 1). Store cool and dry.
5.2. Hydrochloric acid, 37%.
5.3. Distilled water
5.4. 0.075 M Hydrochloric acid. Dilute 6.3 ml of 37% hydrochloric acid to 1000 ml, or 12.6 ml to 2000 ml with distilled water.
5.5. 0.020% Pepsin/acid solution. Prepare fresh.
Dissolve 0.2000 grams of 1:10,000 activity pepsin in a litre of 0.075 N. Hydrochloric acid
5.6. 0.0002% Pepsin/acid solution. Prepare fresh.
Transfer 20 ml of the 0.020% pepsin solution to a 2 litre volumetric flask and dilute to the mark with 0.075 M Hydrochloric acid.
5.7. Also, apparatus and chemicals needed for the determination of Kjeldahl nitrogen (ISO/FDIS 5983:1997(E).

6.0. Procedure

6.1. Grind the sample so that the particles pass through an ASTM No. 30-32 Screen (0.500-0.595mm). If it is difficult to grind to this particle size, then the fat content may be too high and the sample should be extracted with petroleum ether for 2 hours. Extraction should be done on the 1 gram weighed sample and the residue used for the rest of the test.
6.2. Weigh accurately 1.0 gram and record the weight to 0.1 mg, of the ground fishmeal into the 200 ml reaction bottle or flask.
6.3. Add 150 ml of the 0.0002% pepsin/acid solution (5.6).
6.4. Incubate the flask for 16 hours at 45°C±2.0. If an orbital shaker unit is used be sure there are no particles on the wall of the flask above the pepsin/acid solution. The orbital shaker should shake at 140-200 orbits/minute.
6.5. Filter the solution through the filter paper on the Buchner funnel using a gentle vacuum suction. Wash out any remaining particles from the flask or bottle with warm distilled water and transfer to the filter. Finally wash the filter and residue with several portions of warm distilled water until they are free from acid.
6.6. Determine the Kjeldahl nitrogen in the filtered residues plus filter paper (N_r) according to ISO/FDIS 5983:1997(E).
6.7. Determine the Kjeldahl nitrogen content of the original fishmeal sample according to ISO/FDIS 5983:1997(E).

7.0. Blank Test

7.1. Carry out a blank test using the same procedure (6.3 to 6.5) but omitting the test portion. Determine the Kjeldahl nitrogen in the washed blank filter paper (N_b) according to ISO/FDIS 5983:1997(E). (N.B. Correction is needed for significant contamination with N which may occur in filter paper. In addition the Kjeldahl method requires its own blank determination. Care should be taken to correct the determinations for the appropriate blank depending on the Kjeldahl procedure used.)

8.0. Calculations

1. Calculate the % nitrogen insoluble in pepsin/acid solution (A) as:

$A = 100(N_r - N_b/m)$

where
N_r is the nitrogen content in grams of the residue plus filter paper,
N_b is the nitrogen content in grams of the blank filter paper
m is the mass of sample in grams
2. Calculate the % nitrogen in the original sample (B).
3. Calculate the IFOMA Solubility in dilute pepsin/acid solution as follows:

IFOMA Solubility in dilute pepsin/acid solution = 100(B - A)/ B

Report the result to nearest 0.1% unit.

9.0. Precision

9.1. Inter-laboratory test

The results of an inter-laboratory test on the precision of the method are summarised below: Sixteen laboratories analysed 12 samples of fish meal. Two pairs of hidden duplicate meals were included from which repeatability was determined. After omitting two laboratories as outliers the sample mean solubilities ranged from 89.3 to 97.9, mean 95.6, standard deviation between sample means 2.46. The pooled variance component for within laboratory differences (s^2_r) was 0.315, the repeatability standard deviation (s_r) was determined as 0.56 % units and the relative repeatability standard deviation (RSD_r) as 0.59 %. The pooled variance component for between laboratory differences (s^2_R) was 0.234 (from the hidden pairs only) providing an estimate of the pure between laboratory standard deviation (s_L) of 0.484 % units. The reproducibility standard deviation (s_R, estimated from comparison between laboratories of all 12 samples) was 1.17 % units and the relative reproducibility standard deviation (RSD_R) as 1.23. Source of pepsin and type of shaker had no significant effect on the determination.

9.2. Repeatability limit

The absolute difference between two independent single test results obtained by the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time should be less than the repeatability limit ($r = 2.8\ s_r$) of 1.6 % units in 95% of cases.

9.3. Reproducibility limit

The absolute difference between two single test results obtained using the same method on identical test material in different laboratories by different operators using different equipment should be less than the reproducibility limit ($R = 2.8\ s_R$) of 3.3 % units in 95 % of cases.

Note 1:
If other brands of pepsin (see 5.1) are used it is essential to confirm their activity at 2,000 FIP u/g. Any variations should be adjusted when preparing the dilute pepsin solution (see 5.5 and 5.6).

The recommended method for determination of pepsin activity is given in Annex A of ISO 6655 (1997).