International Fishmeal & Oil Manufacturers Association

AN EVALUATION OF THE PEPSIN DIGESTIBILITY METHOD AS PRACTISED AND INTERPRETED BY IFOMA MEMBERS, ASSOCIATE MEMBERS AND ASSOCIATED LABORATORIES

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INTRODUCTION

The pepsin digestibility method has been around for many years, having been first published in some form or other as early as 1955. Published methods appear in the Journal of the American Association of Analytical Chemists (JAOAC) during the period 1957-1971 and in 1971 it was published as a First Action by AOAC. In 1973 it was published by AOAC as Final Action and there have been no official modifications to the method since then.

IAFMM (now IFOMA) has periodically evaluated the method over the years and several publications by Lovern 1964, Ambrose and Snyder 1964, Lovern, Olley and Pirie 1964, Dreosti, Wiechers and Conradie 1964 and 1966, Olley and Pirie 1966, Olley 1966, Olley and Payne 1967, Conterras and Romo 1965, March, Bieley, Goudie and Tarr 1966, Vargas and Bellido 1969, and Barlow 1976 among others have all addressed the pepsin test and it's applicability or not to fishmeal. Although early studies indicated that there might be some correlation between pepsin digestibility and the biological value of the protein (specifically NPU), these never worked out when collaborative trials were conducted in different countries with different types of fishmeal. In spite of this, over the last 43 years or so, the pepsin digestibility test in one form or another, under one modification or another has survived. In fact, today, even though the experts in nutrition caution against using this as an indicator of protein quality, many contracts incorporate some value for pepsin digestibility or one of its variations, within the guaranteed quality parameters. Many of the buyers and or users insist that even though it may not be an indicator of biological protein quality, it does give them some indication of how the fishmeal was processed and or handled, and thus the method is of value to them in evaluating different products and suppliers..

In view of this, the Scientific Committee, at their annual meeting in Rome in 1997 provisionally adopted the AOAC Method as the IFOMA recommended method for pepsin digestibility. The recommendation, which is subject to approval by the members during 1998, was formulated in an attempt to standardize on this test. At the Workshop on Quality Control held at the Annual Meeting, a lively discussion took place on the pepsin digestibility method, Torry, and Torry Modified methods. It became apparent that the method was open to individual interpretation and that it would be useful to collect the method as it is being performed in the member and associate member laboratories.

A circular letter was distributed on 24 November 1997 along with a questionnaire that could be filled out describing the details of the method. The letter asked that all replies be sent in no later than January 15, 1998.

As of this writing there were 18 replies with 4 other submissions of historical information on the original Torry Method. One submission included a valuable comparison of fishmeal ground to 30 and 60 mesh particle sizes. This information was of great value and I wish to thank the people who supplied the information.

SUMMARY OF RESPONSES

A summary of the responses appears in the following table . Tables comparing the individual coded responses are available if the Committee would like to see them but they are not included here.

DISCUSSION

Eighteen laboratories reported on their methodology for conducting the Pepsin Digestibility, Torry or Modified Torry Digestibility test. The name of the method and the way it is run is the subject of wide interpretation. In addition several laboratories reported on the GAFTA/EC Method which is somewhat similar to the ISO Standard Method. IFOMA has already rejected the ISO Method as being not practical for the type of work being done by the Association (too time consuming and it uses too much chemical volume per sample). The GAFTA/EC Method is similar to the ISO Method.

Since the provisional recommendation from the IFOMA Scientific Committee (unanimous vote of those members present) was that IFOMA adopt the AOAC Method, a "draft" IFOMA Recommended Method for "Pepsin Digestibility" is included with this report. Because of the wide variation in views of the members and those who participated in the Workshop on Analytical Methods and Quality Control, I have included several options within the method and a proposed procedure for identifying the option. An alternative and less practical procedure would be to write several individual methods. Hopefully the option method will satisfy all concerned parties.

There are then, 7 areas of concern:

Sample Grind or Particle Size- Some of the earlier work on this method by Olley and Pirie 1966 indicated that unground fishmeal appeared to give a good correlation to the NPU value of the protein. Subsequent work in Chile and Peru with anchovy meal indicated that grinding to at least a 20 mesh (0.841mm) particle size should be used to standardize the method. The AOAC Method calls for a 20 mesh particle size. Most of those who have responded are in the 20-35 mesh range (0.841 - 0.500 mm) particle size range. One of the submissions included a comparison of 20 samples of fishmeal ground to 30 and 60 mesh (0.595- 0.250 mm). A comparison of the data indicates an average difference of +0.76 absolute pepsin units if the finer grind is used. For purposes of this evaluation I have included a grind to 30 mesh or 0.595 mm.

Type of Apparatus- The AOAC Method specifically calls for the use of a rotating unit (revolving end over end) so that no fishmeal particles stick to the walls or inside the cap of the reaction container. It specifically mentions that rotating or shaker (orbital) type units are not satisfactory

for this test. However, the AOAC Method was first approved in 1971 so there could be new orbital shaking units that are satisfactory. The ISO Method and GAFTA Method, which run for 48 hours, require manual shaking of the reaction flask after 8, 24 and 32 hours. Of the replies received, 6 use rotating units as recommended by the AOAC Method and 12 use a shaker type system. If a collaborative trial is required, we should test the difference (if any) between these methods of agitating the reaction mixture.

Reaction Temperature- The AOAC Method calls for a reaction temperature of 45°±2.0. Most of the respondents appear to be in this range except for those running the ISO or GAFTA Methods. ISO and GAFTA call for a temperature of 40°±1.0. It was already reported, at the Workshop, that there was very little difference between 42 and 45° so I have retained the AOAC temperature designation in the method even though one suggestion from the Workshop wanted 45°±0.5. This problem might be more one of the type of incubator/bath and agitation equipment involved since the rotating units generate heat from the motor.

Pepsin Solution Strength- The AOAC Method calls for a pepsin strength of 0.20% pepsin in 0.075 N Hydrochloric Acid. Everyone reported using 0.075 N Hydrochloric Acid. Pepsin strength seems to vary by laboratory, country and what they are calling the method. Some commercial laboratories reported that they use 0.2, 0.02, 0.002 and 0.0002% pepsin depending upon what their customer has requested. The Torry or Modified Torry Method appears to require 0.0002% pepsin. The customers who purchase the fishmeal have their own requirements which is what we are trying to standardize. I have therefore entered 4 options for this part of the test identified as: 00, 10, 20, and 30. The reported method would then read IFOMA Recommended Method for Pepsin Digestibility Option 00 or 10 or 20 or 30 depending on the pepsin strength. 00 means no 0 after the decimal, 10 means one 0, 2 means 2 etc. Alternatively we could use the actual pepsin strength of 0.2%, 0.02%, 0.002% or 0.0002%.

Brand of Pepsin and Activity- AOAC does not specify the brand of pepsin it only specifies 1:10,000 activity. This is an important issue because some people claim that unless everyone is using the exact same brand, in fact the same bottle of pepsin, then the results will vary. This, of course is not practical and we must live with the brands that are available internationally, standardizing on the activity of 1:10,000. Many seem to be using Merck 7190 with an activity of 2000 FIB-U/g. The Merck pepsin in the Merck Index only has a 1:3000 activity. But they do mention that 1:10,000 activity is available on the market.

Blank Determination- The sixth area of difference concerns the determination of a blank. From the reports submitted, laboratories are running one or both of the following blanks but then reporting the result the same way. The AOAC Method does not call for running a blank determination within the pepsin digestibility test. In this way, any protein or nitrogen in the sample that is water or acid soluble becomes part of the digestible protein. You are then actually reporting the % of the original protein that is water, acid and pepsin digestible (soluble). The ISO and GAFTA Methods call for running a blank on the pepsin and acid but no sample. The Torry and Modified Torry Methods run a blank using the fishmeal sample but no pepsin. In this way, you determine the amount of protein that is digested by the acid solution and the amount of

protein that is digested by the pepsin acid solution. In other words, correcting or modifying the amount of protein that is digestible by eliminating the water/acid soluble protein. Twelve of the reporting laboratories make this modification while 7 run a blank determination as outlined in the ISO Method. One laboratory did it both ways, which is why this adds up to 19.

Calculation of the Results- The final major area of difference between the methods is then the way the digestible protein is calculated. In the AOAC Method, a protein determination is made on the original sample and on the filtered and wash residue after the digestion. If you then divide the % indigestible protein by the original sample protein you get the % of the original sample protein that is not digested. Five laboratories reported doing the calculation this way. The other laboratories run protein determinations on the residues from the acid and pepsin digestion. They then subtract the amount of protein not digested by the pepsin solution from the amount of protein not digested by the acid solution and divide by the amount of protein not digested by the acid solution. They are therefore reporting the amount of water/acid insoluble protein that is not digested by pepsin. They do not do a protein analysis on the original sample. Thirteen laboratories reported doing the calculation this way.

I look forward to the Committee's comments and for a lively discussion at the Spring Meeting.

Anthony P. Bimbo
January 23, 1998

	SUMMARY OF REPLIES	AOAC
Sample Grind, ASTM Mesh (mm)	1=No.18 (1.0), 1= No. 100 (0.149), 5= No. 35 (0.50), 1= No.25 (0.707), 4= No. 30 (0.595), 4= No. 20 (0.841), 2= No. 60 (0.25)	No.20 sieve (0.841 mm)
Pre-Extract the Fat	6 YES (0.5-6 HRS) 12 NO	YES
Mixing System	12= Shaker 6= Rotation (Revolving)	Revolving
Incubator/Bath	6= Water Bath 11= Incubator 1= Oven	Incubator
Reaction Temperature	4= 40° 13= 45° , 1=42-45°	45°
Temperature Range	±0.10= 2, ±0.50= 2, ±1.00= 2, ±2.00= 5, ±3.00= 1, No ± Given = 6	±2.00
Sample Size	1 Gram = 16 2 Gram = 1, 0.5 Gram = 1	1 Gram
Pepsin Brand	Merck 7190 = 7, Merck = 3, Difco = 2, Sigma = 2, US Biochemical = 1, Riedel de Haen 20895 = 2, Not Specif.= 1	Not Specific
Pepsin Activity	1:10,000 = 11, 2000 FIP-U/g = 7	1:10,000
Hydrochloric Acid	0.075 N = 18	0.075 N
Pepsin Strength	0.20% = 1, 0.02% = 1, 0.002% = 2, 0.0002% = 12, Not Specified = 1, 0.20%-0.0002% = 1	0.20%
Pepsin Solution Pre-Heated	YES = 7 NO = 11	YES
If Heated, to What Temp.	42-45°= 3, 40°= 1, 45°= 2 Not Reported= 1	42 - 45°
Volume of Pepsin-Acid Solution	150 ml = 14, 500 ml = 1, 225 ml = 1, 75 ml = 2	150 ml
Reaction Time	16 Hours = 16, 48 Hours = 2	16 Hours
Protein Analysis, Sample	YES = 9 NO = 9	YES
Protein Analysis, Residue	YES = 16 NO = 2	YES
Protein Analysis, Digest	YES = 1 NO = 17	NO
Blank Analysis	Sample + Acid, No Pepsin = 12 Pepsin + Acid, No Sample = 7	NO
Calculation	% Of Original Protein that is not digested = 5 Difference Between % Protein Digested with Acid and Digested with Pepsin = 13	% of Origin Protein that not digested
Method Name	AOAC = 3, GAFTA/EC = 2, AOAC MODIFIED TORRY = 1, TORRY = 6, MODIFIED TORRY = 6	AOAC
Regions Reporting	So. America = 9, USA = 3, Europe = 6	

DRAFT PROPOSED IFOMA RECOMMENDED METHOD FOR PROTEIN DIGESTIBLE IN PEPSIN/HYDROCHLORIC ACID SOLUTION.

1.0 Applicable to fishmeal [and feeds].

2.0 Reference: AOAC Official Method 971.09.

3.0 Summary: Protein is digested according to one of the options of the test. Determine the kjeldahl protein insoluble in pepsin/acid solution and the kjeldahl protein insoluble in the acid solution. Determine the kjeldahl protein in the original sample.

The pepsin digestibility of the protein is defined as:
1. The portion of the total protein that is insoluble in pepsin/acid solution. OR
2. The portion of the acid insoluble protein that is soluble in pepsin/acid solution.

4.0 Apparatus and Supplies

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

5.0 Reagents

1. Pepsin 1:10,000 activity. 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 with distilled water.
5. 0.20% Pepsin/Acid Solution. Prepare fresh.
   Dissolve 2 grams of 1:10,000 activity pepsin in 1 liter of 0.075 N. Hydrochloric Acid.
6. 0.02% Pepsin/Acid Solution. Prepare fresh.
   Transfer 100 ml of the 0.20% pepsin solution to a 1 liter volumetric flask and dilute to the mark.
7. 0.002% Pepsin/Acid Solution. Prepare fresh.
   Transfer 10 ml of the 0.20% pepsin solution to a 1 liter volumetric flask and dilute to the mark.
8. 0.0002% Pepsin/Acid Solution. Prepare fresh.
   Transfer 1 ml of the 0.20% pepsin solution to a l liter volumetric flask and dilute to

the mark.

9. Also, apparatus and chemicals needed for the determination of kjeldahl protein.

6. Procedure

1. Grind the sample so that the particles pass through an ASTM No. 30 Screen (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 1.0000 gram of the ground fishmeal into the 200 ml reaction bottle or flask. Mark this container as pepsin.
3. Weigh accurately 1.0000 gram of the ground fishmeal into a second 200 ml reaction bottle or flask. Mark this container as acid.
4. Transfer 150 ml of the required pepsin/acid solution into the flask marked "pepsin".
5. Transfer 150 ml of the 0.075 N. Hydrochloric Acid Solution into the flask marked "acid".
6. If you are running a reagent blank, add 150 ml of the pepsin/acid solution into a third flask marked "blank". Do not add fishmeal to the "blank" flask.
7. Incubate the flasks for 16 hours at 45°±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.
8. 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.
9. Determine the kjeldahl protein content of the filtered residues according to ISO/FDIS 5983:1997(E).
10. Determine the kjeldahl protein content of the original fishmeal sample according to ISO/FDIS 5983:1997(E).

7. CALCULATIONS

1. Calculate the % protein in the original sample (A). Perform a blank determination of the chemicals.
2. Calculate the % protein insoluble in pepsin/acid solution (B).
3. Calculate the % protein insoluble in acid solution (C).
4. Calculate the % of the protein that is pepsin digestible as follows:

$$\text{Pepsin Digestibility, \%} = \frac{\text{\% Protein in Original Sample - \% Protein in Pepsin Acid Residue}}{\text{\% Protein in Original Sample}} \quad \text{I}$$

OR

Pepsin Digestibility of
Acid Insoluble Residue = $\frac{\text{\% Protein in Acid Solution Residue - \% Protein in Pepsin Acid Residue II}}{\text{\% Protein in Acid Solution Residue}}$

Calculation I is the standard AOAC Pepsin Digestibility.

Calculation II is commonly referred to as the Torry or Modified Torry Pepsin Digestibility.

8. REPORT RESULTS

Report your results as:

% Pepsin Digestible Protein Option [00, 10, 20, 30] or [0.2, 0.02, 0.002, 0.0002].

OR

% Acid Insoluble Residue Pepsin Digestible Protein Option [00, 10, 20, 30] or [same as above]

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