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Protein quality of feeding-stuffs

7.* Collaborative studies on the microbiological assay of available amino acids

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1. Twenty-two samples of high-protein feeding-stuffs, sixteen of them fish meals, were used in a collaborative study of the precision and the limits of discrimination of the *Streptococcus zymogenes* assay procedure, as applied to the estimation of available methionine, tryptophan and isoleucine contents.

2. All the participating laboratories ranked the test samples in much the same sequence with respect to content for all three amino acids. There were apparently systematic differences between laboratories which impaired the precision of some of the estimates, and these were greatly reduced by including a common reference sample in the tests as an auxiliary standard.

3. Values for available methionine content for eleven test samples were highly correlated (r 0·86) and quantitatively similar to those obtained for chick growth assays, but those for available tryptophan content were markedly lower and were probably in error.

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In an earlier paper in this series, Carpenter & Woodham (1974) reported results of a study of between-laboratory reproducibility of chemical, microbiological and biological assay values for lysine, methionine, cystine and tryptophan contents for twelve samples of high-protein feeding-stuffs. An objective in their study was to evaluate selected laboratory techniques for predicting the biological availability of lysine, methionine and tryptophan in intact proteins. Statistical analysis was restricted to a comparison of over-all mean values obtained by the different analytical methods, together with estimates of their precision as assessed from inter-laboratory variability. The present paper gives a fuller analysis of microbiological assay results for available methionine and tryptophan, and also for isoleucine, in an extended range of test samples. It shows the precision and the limits of discrimination obtained in the estimation of these amino acids (1) within an assay, (2) between assays within a laboratory, (3) between laboratories.

The paper also reports microbiological assay values for 'total' methionine content, for comparison with values obtained for the same samples by the performic oxidation procedure (Moore, 1963) and with the values for 'available' methionine content.

EXPERIMENTAL

Collaborating laboratories

The composition of the Panel varied during the course of the study. In all, representatives of eleven laboratories contributed results to one or more of the collaborative tests, and generally six to eight laboratories took part in each test. All but two of the participating laboratories had had previous experience in the use of microbiological assay techniques.

Test materials

The test samples were collected by the Agricultural Research Council Group on Protein Quality. Most samples were of commercial origin and were taken as representing average samples of their particular types. The fish meals were designated as follows: FM 101, 103 and 104 were white fish meals manufactured in the UK; FM 105, 106, 118 and 119 were herring meals from Norway; FM 108 and 111 were herring meals from Iceland and Denmark respectively; FM 107 and 113 were anchovy meals from Peru; FM 109 and 110 were South African fish meals. FM 106, 113 and 118 were 'stabilized' by the addition of antioxidant during manufacture. The remaining fish meals and other test materials were described by Carpenter & Woodham (1974).

Preparation of samples for test

In the assays for available amino acids the test materials were ground to pass through a sieve with 0·42 mm apertures (40 mesh). Samples containing 100 mg

nitrogen were then taken, suspended in 20 ml buffer and digested with papain as described by Boyne, Price, Rosen & Stott (1967). All the participating laboratories used preparations of crude papain obtained from the same commercial source (crude papain, standardized to conform with the specification given in the British Pharmaceutical Codex (1954); British Drug Houses Ltd, Poole). No attempt was made to ensure that these belonged to the same batch.

For the assays of total methionine the test materials were hydrolysed with acid. Samples containing 100 mg N were suspended in 40 ml 3 M-HCl in 150 ml conical flasks covered with inverted beakers, and heated for 18 h at 121° using a steam autoclave. The hydrolysates were then neutralized with 4 M-NaOH, diluted to 1l and filtered.

Standard amino acids solution

Instead of using individual amino acid standards for the assays for different amino acids, a composite standard was prepared as recommended by Ford & Salter (1966). It was made by diluting a stock solution which contained (/ml): 2 mg each of L-lysine HCl, L-arginine HCl, L-leucine, L-isoleucine, L-threonine, L-serine; 1·5 mg L-valine; 1 mg L-methionine, L-histidine hydrochloride monohydrate; 5 mg sodium L-glutamate; 0·25 mg L-tryptophan. This stock solution was prepared in one laboratory and dispensed in 20 ml portions into McCartney bottles (28 ml capacity) which were then stoppered and sterilized by heating at 115° for 15 min, before distribution to the different laboratories. In assays for available amino acids the stock solution was diluted fifty times with water to give the working standard; in assays for total methionine the stock solution was similarly diluted, but with a solution of sodium chloride of the same concentration as was present in the test hydrolysates.

Basal medium

The composition of the basal medium was that described by Ford (1962), modified by increasing the K_2HPO_4 concentration from 60 to 90 g/l (Ford, 1964), and that of adenine, guanine, uracil and xanthine from 25 to 50 mg/l (Kennedy, 1965).

Inoculation of the test cultures

Each assay tube was inoculated with one drop of a 24 h culture (undiluted) of *Streptococcus zymogenes* grown in basal medium, supplemented with 1·5 g casein and 0·15 g sodium L-glutamate/l (see Boyne *et al.* 1967). This inoculum culture was grown at 37° and maintained by daily transfer. On Friday afternoons the culture was inoculated and kept in a cupboard at room temperature (about 20°) until the following Monday, when it was again transferred and incubated at 37°. This routine ensured that the test culture remained actively proteolytic and gave abundant growth in the casein-based inoculum medium.

Apart from the modifications mentioned above, the assay procedures and the method of calculation of the results were those described by Boyne *et al.* (1967).

Table 1. *Content of available methionine, isoleucine and tryptophan (g/kg crude protein (nitrogen × 6·25)) for thirteen fish meals, determined using the* Streptococcus zymogenes *assay procedure*

(Mean values for results from six to eight laboratories. For each test all fish meals were included in two independent assays at each laboratory)

	Amino acid		
	Methionine	Isoleucine	Tryptophan
Test 1*			
No. of laboratories ...	8	8	8
Fish meal			
White fish meal			
101	23·2	29·8	5·2
103	23·8	33·0	5·8
104	25·7	33·7	6·4
Herring meal			
105	23·8	35·2	6·1
108	23·6	35·0	5·7
111	25·1	38·7	6·9
106†	27·0	38·2	6·9
South African fish meal			
109	19·7	33·7	5·8
110	26·1	39·6	8·5
SEM‡	0·61	0·80	0·24
Least significant differences ($P = 0·05$)	1·7	2·3	0·7
Test 2*			
No. of laboratories ...	7	6	7
Fish meal			
White Fish meal			
104	24·4	29·6	5·1
Anchovy meal			
107	26·4	37·4	7·3
113†	25·9	38·2	7·7
Herring meal			
119	23·1	32·5	5·0
118†	23·5	36·1	6·1
SEM‡	0·46	0·91	0·26
Least significant differences ($P = 0·05$)	1·4	2·7	0·8

* For details, see p. 157.
† The fish meal was 'stabilized' by the addition of antioxidant during manufacture.
‡ Based on 'laboratory × fish meal' interactions; for details of statistical analysis, see below.

Statistical analysis

Analyses of variance were done for the results from the collaborative studies of available methionine, isoleucine and tryptophan contents for the fish meals. To test whether there were significant differences between the different samples of fish meal, the variation between fish meals was compared with that which remained after removing differences between fish meals and laboratories (that is the 'fish meals × laboratories' interaction). The corresponding coefficients of variation, which

Table 2. *Values for available methionine content (g/kg crude protein (nitrogen × 6·25)) for thirteen fish meals, determined using the* Streptococcus zymogenes *assay procedure at the six laboratories that took part in tests 1 and 2**

	Laboratories					
	A	B	C	D	E	F
		Test 1				
Fish meal						
White fish meal						
101	24·2	24·8	23·8	21·0	22·3	21·7
103	24·8	27·1	22·8	22·7	22·7	16·7
104	29·6	28·2	26·6	24·5	22·3	22·0
Herring meal						
105	30·4	24·9	23·4	21·4	20·2	21·4
108	27·2	26·1	22·1	21·8	20·0	22·6
111	27·1	28·6	22·5	24·0	21·4	24·0
106†	30·6	30·2	27·4	24·8	24·5	25·0
South African fish meal						
109	19·6	23·5	19·6	20·2	14·4	19·6
110	30·0	29·7	25·4	24·5	23·8	25·6
		Test 2				
White fish meal						
104	27·0	27·8	24·3	22·1	23·4	19·8
Anchovy meal						
107	28·6	30·2	25·8	25·0	21·4	22·9
113†	28·6	31·0	24·5	25·4	21·0	22·8
Herring meal						
119	26·2	28·1	19·8	22·4	18·8	19·6
118†	25·5	28·6	21·6	22·9	21·1	20·4

* For details, see below.
† The fish meal was 'stabilized' by the addition of an antioxidant during manufacture.

expressed the variation between laboratories, were calculated by pooling the variation between laboratories for each sample of fish meal. From the several sources of variation in the statistical analysis: (1) differences between laboratories, (2) interaction between laboratories and fish meals, (3) differences between assays within laboratories, (4) interaction between assays and fish meals within laboratories, the components of variance were estimated and used to calculate the standard errors that would apply to hypothetical results from one and three assays done at one and three laboratories.

RESULTS AND DISCUSSION

Assays for available methionine, isoleucine and tryptophan content for thirteen fish meals

Table 1 summarizes the results of two collaborative tests, in which the test samples were assayed twice at each of six to eight laboratories. Mean values are given with standard errors based on the 'fish meals × laboratories' interactions.

Statistical analysis of the results for available methionine showed that there was slightly greater variation between laboratories than between fish meals. In tests 1

Table 3. *Coefficients of variation for values obtained by six to eight different laboratories for available amino acid contents of thirteen fish meals, using the* Streptococcus zymogenes *assay procedure, in tests* 1 *and* 2 (*Table* 1)

(Values based on standard deviations derived from the pooled variation 'between laboratories within fish meals')

Amino acid	Test 1*	Test 2*	Average
Methionine	12·0	13·9	13·0
Isoleucine	8·1	11·5	9·8
Tryptophan	22·2	23·0	22·6

* For details, see p. 157.

and 2 some laboratories obtained consistently 'low' values and others 'high' values. Thus, in test 1, five of the eight participating laboratories obtained mean values for nine fish meals of 22·1, 22·8, 21·3, 23·7 and 22·9 g/kg crude protein (CP; N × 6·25), whereas the remaining three laboratories obtained values of 27·1, 27·0 and 27·0 g/kg CP. In test 2, four laboratories obtained mean values for five fish meals of 21·1, 23·5, 21·1 and 23·2 g/kg CP, and three laboratories obtained values of 27·2, 27·5 and 29·2 g/kg CP. The six laboratories that took part in both tests were consistent in obtaining 'high' or 'low' values for both tests, as is shown in Table 2. The values obtained by laboratories A and B were consistently higher than those obtained by the other four laboratories.

For available isoleucine the average values obtained in test 1 ranged from 31·9 to 37·6 g/kg CP and in test 2 from 30·0 to 40·3 g/kg CP. There was no indication that results from individual laboratories were consistently 'high' or 'low'. In both tests, variation between laboratories was slightly smaller than that between fish meals. It seemed possible that differences in the proteolytic activity of the preparations of papain used in the participating laboratories might contribute to the differences between laboratories. To check this, samples from a single batch of papain were distributed and compared with the laboratories' own papain in an assay for available methionine and tryptophan content for the five fish meals studied in test 2. Analysis of the results showed that the use of this common sample of papain had no effect in reducing the between-laboratories variation.

The tryptophan values showed substantially wider between-laboratory variation than those for methionine or isoleucine. Laboratory averages ranged from 4·6 to 8·0 g/kg CP in test 1 and from 4·4 to 8·1 g/kg CP in test 2. Laboratories that obtained 'high' values in test 1 also gave 'high' values in test 2. The coefficient of variation was considerably greater for the assay for tryptophan than for methionine and isoleucine (Table 3).

When calculating results for the assays a correction was applied for the amino acid content of the papain used in preparing the test digests. For methionine and isoleucine assays this correction was relatively small, but for the tryptophan assays it was proportionately much larger. Furthermore, the growth responses to graded concentrations of a papain 'blank' in the tryptophan assays did not parallel the responses

Table 4. *Estimates of the influence of replication within and between the laboratories on the precision of the results obtained by six to eight laboratories for available amino acid contents of thirteen fish meals, using the* Streptococcus zymogenes *assay procedure, in tests* 1 *and* 2

(Values are standard errors expressed as g/kg crude protein (nitrogen × 6·25))

		Combinations from which SE was calculated:			
		At one laboratory		At each of three laboratories	
No. of determinations ...		1	3	1	3
Amino acid	Test*				
Methionine	1	3·3	2·9	1·8	1·7
	2	3·6	3·4	2·1	2·0
Isoleucine	1	3·9	2·7	2·3	1·6
	2	4·7	3·7	2·7	2·1
Tryptophan	1	1·5	1·4	0·9	0·8
	2	1·6	1·4	0·9	0·9

* Tests 1 and 2 were carried out on different occasions: in test 1, eight laboratories took part; in test 2, seven laboratories contributed results for methionine and tryptophan, and six for isoleucine. Six laboratories were common to both tests.

to similarly graded concentrations of tryptophan estimated for the test digests, clearly indicating a failure in the assay procedure. The values for tryptophan given in Table 1 may therefore be subject to considerable error, and they probably understate the content of available tryptophan for the test samples. For tryptophan assays papain is not the enzyme of choice because a complicated assay procedure is required to make possible the simultaneous estimation of the response to the test sample and to the papain, where each amount of a test sample is assayed at more than one concentration of papain. Consequently, another enzyme must be sought which is as unimportant as a source of tryptophan as papain is of methionine and isoleucine.

In general, the laboratories ranked the fish meals in much the same sequence with respect to content, for all three amino acids. The apparently systematic differences between laboratories impaired the precision of the methionine estimates and the question was considered whether it might be permissible to discount results from the 'inexperienced' laboratories, but in the absence of objective criteria for selection all results submitted were included in the analysis. Table 3 gives the between-laboratory variation for the results for the three amino acids. It would clearly have been possible to reduce this variation by including a standard test sample for all the assays. Thus in test 1 (Table 1) if FM 104 was taken as the standard and assumed to contain 25·7 g methionine/kg CP (the average of values from the eight participating laboratories), and all results from each laboratory were multiplied by the factor needed to convert the result obtained for FM 104 to 25·7, the adjusted values gave a coefficient of variation between laboratories within fish meals of 8·4, compared with 12 before adjustment. The significance of the between-laboratories variation ($P < 0{\cdot}001$) was reduced but was still evident ($P < 0{\cdot}05$), indicating that differences

Table 5. *Comparisons of different estimates of total and available methionine (g/kg crude protein (nitrogen × 6·25)) for high-protein feeding-stuffs*

	Methionine				
	Total			Available	
Assay procedure* ...	Microbiological†		Chemical‡	Microbiological§	Chick‡
Laboratory ...	1	2			
Feeding-stuff*					
Fish meal					
FM 101	26	27	23	23	25
102	22	24	18	12	18
108	27	30	26	24	20
113	29	29	30	26	22
122	30	26	26	22	21
123	32	30	26	23	22
Meat meal					
MM 101	15	14	12	7·4	11
Decorticated groundnut meal					
GN 101	11	10	9·9	7·8	7·6
Soya-bean meal					
SB 101	18	16	14	13	—
Sunflower-seed meal					
SF 101	16	17	18	16	19
Yeast					
HY 101	15	16	17	14	9·9
104	15	15	14	13	9·9

* For more detail, see Carpenter & Woodham (1974).
† Each value is an average from two independent assays.
‡ From Carpenter & Woodham (1974).
§ Each value is the average of results obtained from six laboratories.

between fish meals were greater for some laboratories than for others. Again, if FM 104 was taken as the standard in test 2, the adjusted values gave a coefficient of variation of 8·4 compared with 14 before adjustment. With these results however, although the significance of the between-laboratories variation was again reduced, it was still high ($P < 0{\cdot}001$).

Results from test 1 showed that, for available methionine content, the standard deviations were: within one assay 1·6, between assays, within one laboratory 1·8, between laboratories 3·3. On this basis it should be possible, within one assay, to resolve differences of > 4·5 g/kg CP between fish-meal samples. It appears that greater accuracy was obtained by increasing the number of laboratories rather than by increasing number of replicates within one laboratory (Table 4). This conclusion should be treated with some reserve, however, as there is some doubt as to the laboratory component; in the methionine assays there seemed to be a partition into two distinct groups.

Total and available methionine content for high-protein feeding-stuffs

Carpenter & Woodham (1974) described a collaborative study of twelve food proteins – six fish meals, one meat meal, one soya-bean meal, one groundnut meal, one sunflower meal and two preparations of dried food yeast. Microbiological assay values for available methionine content for all these materials, contributed by six of the laboratories taking part in the present study, were compared with corresponding values obtained using chick growth assays and also with total methionine values obtained using chemical and microbiological assay methods. Standard errors calculated for the different estimates of methionine content in this selection of samples were all about 1·0. The results are fully discussed by Carpenter & Woodham (1974), but they are presented in Table 5 to facilitate comparison of 'microbiological' and 'chemical' values for total methionine content, and 'microbiological' values for available methionine content.

The 'microbiological' values for total methionine content were means of two independent assays carried out in each of two laboratories and, in general, the two sets of results were in close agreement. Comparison with the 'microbiological' values for available methionine content suggests that in the fish meals (excluding fish meal FM 102, which was an atypical material of low protein and high ash content) the availability ranged from 0·74 to 0·90, compared with only 0·51 for the meat meal and about 0·90 for the yeasts. A question arises whether the 'microbiological' values for total methionine content might tend to be too high (see Carpenter & Woodham, 1974) and thus give an erroneously low estimate of availability. The 'chemical' values for methionine content for the fish meals were on average marginally lower, but from the present results it is not possible to assess the significance of the apparently small differences.

The general pattern for the fish meals from this comparative study, again excluding FM 102, is that there were no large differences between them in their content of available methionine, whether judged from the biological or the microbiological assays. Certainly it would not be possible, from the evidence of a single test within a laboratory, to differentiate between these samples. The question whether such uniformity is typical of materials in commercial use is discussed by Carpenter & Woodham (1974), and will be the subject of a more extensive study of fish meals, meat meals and soya-bean meals (J. E. Ford, D. Hewitt & K. J. Scott, unpublished results). If we consider the additional fish meals used in the present study (compared with that of Carpenter & Woodham (1974)), it is again apparent that most of the variation would have been included within the limits of error associated with a single assay. Although there was a large between-laboratory variance in these tests, the different laboratories taking part generally ranked the meals in the same order.

From several published reports on comparative biological and microbiological procedures for the estimation of available methionine content, we may fairly conclude that for fish meals and meat meals and probably also for other classes of protein-rich feedstuffs, the *Strep. zymogenes* assay procedure is capable of grading samples in a similar order to that obtained using chick or rat assay procedures. There is

no similar information in the literature for isoleucine and tryptophan, although Carpenter & Woodham (1974) concluded that for the tryptophan tests the microbiological assay values were probably far too low, although there was a close correlation with the 'chick' values.

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