IAFMM

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. 20 SEPTEMBER 1984

CHEMICAL AND BIOLOGICAL ASSAY PROCEDURES FOR LYSINE IN FISH MEALS

STUART M. BARLOW, GRANT S. COLLIER, JUNE M. JURTIZ,
JAMES R. BURT, JOHANNES OPSTVEDT and ERIC L. MILLER

SUMMARY

Sixteen laboratories from different countries undertook chemical tests to determine the lysine content of 8 samples of fish meal. The chemical tests were total lysine (gas liquid chromatography or ion exchange chromatography), dye binding capacity (DBC), dye binding lysine (DBL), and reactive lysine (FDNB). The same samples of fish meal were analysed for biologically-available lysine in four laboratories by chick feeding experiments. Estimates of lysine by all methods except DBC differed significantly between laboratories, but generally meals were ranked similarly by each laboratory. The largest estimate of between laboratory variance, and also the poorest concordance of ranking of the meals by the different laboratories was shown by DBL. The chemical methods for measuring lysine gave means which were significantly different from the bioassay results. However, all the chemical methods gave values which correlated with the bioassay values. The DBC, DBL and FDNB methods gave similarly high correlation coefficients while total lysine was less well correlated. Use of the chemical methods to predict values for biologically-available lysine gave wide confidence limits which are likely to encompass the variability in the majority of commercial fish meal samples. Consequently none of the chemical methods can be regarded as satisfactory for the purpose of distinguishing between normal commercial samples of fish meals in terms of an absolute content of biologically-available lysine. The chemical methods may, however, be used in a more limited sense to detect differences in quality between meals of known history without indicating any absolute values for biologically-available lysine. The dye binding methods appear suitable for process control within a fish meal factory where the analyses are carried out in a single laboratory and the nature of the raw material is known.

Re-printed from the Journal of the Science of Food and Agriculture, volume 35, pages 154-164, 1984.

1. Introduction

Fish meal commands a higher price than other high protein feedstuffs. One reason for this, is that its amino acid composition is particularly suited to balance the relative deficiencies of cereal proteins in animal feeding. In particular, the high lysine content of fish meal compensates for the relatively low lysine in cereal proteins.

Carpenter *et al.*[1] suggested that the chemical measurement of lysine reactive to fluorodinitrobenzene (FDNB) provided a better, and more convenient, measure of the value of processed animal protein as a source of lysine than did total lysine, as measured by either column chromatography or microbiological assay, after hydrolysis. Since then, many procedures for measuring reactive lysine have been devised and applied to animal protein feedstuffs.[2]

In a previously conducted IAFMM collaborative study on lysine determination[3] using mainly experimental meals manufactured from one species of fish, the dye binding capacity (DBC), as measured by the Prometer (Foss Electric, Denmark), was shown to be a useful tool for predicting the content of available lysine as determined by chick results. The results with chicks also correlated closely with the results from a rat assay, with FDNB-reactive lysine, and with total lysine values. However, the range of protein quality was only moderate, and samples were not equally distributed throughout the range. Omission of one sample of high quality considerably reduced the degree of correlation between chemical test and bioassay values. It was felt desirable to extend the previous data by obtaining values from industrial samples of fish meal manufactured from a variety of species with an expected greater range of lysine contents, therefore a number of off-standard low quality meals were deliberately included as test samples. Meanwhile, a new DBC method, which was specific for lysine (Dye Binding Lysine-DBL), had been developed[4] and it was desirable to test this new method in comparison with the conventional DBC method and the FDNB-available lysine method on fish meals made from different species of fish and varying in quality.

In total, 16 laboratories from different countries undertook the chemical tests for lysine, and four laboratories evaluated the fish meals for lysine by chick bioassay.

2. Experimental

2.1. Laboratories and meals

Sixteen samples, consisting of blind duplicates of each of eight fish meals, were sent to 16 laboratories in various countries (Table 1) for a variety of chemical and biological tests for lysine content. Thus, the chemical results submitted by the laboratories represent two determinations on each of eight fish meals. The bioassay results, however, represent only one determination in each laboratory on each of the fish meals, with each meal included at two concentrations or more in test diets and each diet given to several replicate groups of chicks.

The fish meal samples were products of commercial production from Denmark, USA, Peru, Norway and South Africa, but some of the meals were deliberately chosen from off-standard produce with expected poor quality so as to ensure a wide variation in biological value for testing

Table 1. List of participants in the collaborative trial

	Names and addresses of participants
1.	Dr R. J. Nachenius, Fishing Industry Research Institute (FIRI), Rondebosch, Cape Province, South Africa
2.	Dr I. Mackie, Ministry of Agricuture, Fisheries and Food, Torry Research Station, P.O. Box 31, Aberdeen AB9 8DG
3.	Dr J. Opstvedt, Norwegian Herring Oil and Meal Industry Research Institute (SSF), 5033 Fyllingsdalen, Bergen, Norway
4.	Mr N. J. King, Colborn-Dawes Nutrition Ltd, Supplements Division, Heanor Gate, Heanor, Derbyshire DE7 7SG
5.	Dr P. J. Buttery, University of Nottingham, Department of Applied Biochemistry and Nutrition, School of Agriculture, Sutton Bonington, Loughborough LE12 5RD
6.	Dr J. M. McNab, Poultry Research Centre, Roslin, Midlothian EH25 9PS
7.	Dr J. A. Wakelam, George A. Palmer Ltd, Oxney Road, Peterborough PE1 5YZ
8.	Dr J. Ford and Dr D. Hewitt, National Institute for Research in Dairying, Shinfield, Reading RG2 9AT
9.	Dr R. Hurrell, Research Laboratory, Nestle Products Technical Assistance Co Ltd, CH 1814 La Tour de Peilz, Switzerland
10.	Dr J. Kvalvag and Dr J. A. Malcolmsen, Agricultural University of Norway, Chemical Research Laboratory, P.O. Box 31, 1432 AS-NLH, Norway
11.	Dr E. L. Miller, Department of Applied Biology, Universty of Cambridge, Pembroke Street, Cambridge CB2 3DX
12.	Mr E. H. Hansen, Technological Laboratory, Ministry of Fisheries, Building 221, Technical University, 2800 Lyngby, Denmark
13.	Mr C. J. Snowden, Grimsby Fish Meal Co, Pyewipe, Grimsby, Lincolnshire
14.	Mr C. Mathiesen, A/S N Foss Electric, 69 Slangerupgade, DK-3400 Hillerod, Denmark
15.	Mr G. Smith, Pauls and Whites Foods Ltd, Research and Advisory Department, New Cut West, Ipswich IP2 8HP
16.	Dr L. R. Njaa, Government Vitamin Institute, Directorate of Fisheries, P.O. Box 187, N5001 Bergen, Norway

purposes. The collection of poor quality meals was difficult because of their relative scarcity, and took over two years to complete. Thus, the variation in biological value of samples in this collaborative trial does not represent typical variation in normal commercial fish meals. Samples of each meal weighing 50 kg were sent to a UK laboratory for sub-sampling and distribution. From each of the eight samples, two representative sub-samples of 25 kg each were prepared, after grinding and homogenisation, making a total of 16 samples. These were further sub-sampled, ground more finely in a mechanical pestle and mortar, and each sample, weighing approximately 5 g, was randomly and separately coded by the laboratory, and packed in an airtight polypropylene container. Each laboratory conducting chemical analyses received one 5 g sample of each meal for each test to be conducted. The remainder of the fish meal samples were stored in sealed containers at −30°C by the distributing laboratory for the subsequent biological tests and reserves.

Laboratories performing the chemical tests stored the samples in a refrigerator until required. The container was thoroughly shaken and equilibrated to room temperature before opening immediately prior to weighing and testing. This avoided unacceptable changes in the moisture content of the sample.

Laboratories performing bioassays received the samples packed in screw top polypropylene containers. Because of the slight possibility of moisture change during storage, moisture determinations were performed on the meals immediately prior to mixing the diets. The means of the nitrogen values reported by the participating laboratories were used when formulating the diets.

2.2. Chemical analysis

All laboratories determined the protein content of the samples using the IAFMM recommended Kjeldahl method.[5]

Total lysine in the meals was determined by ion exchange and gas-liquid chromatography (g.l.c.) following acid hydrolysis of the fish meals. Each laboratory conducted the method routinely in use in the particular laboratory. The contents of lysine as determined by g.l.c. and ion exchange were not significantly different. These methods and results will be described elsewhere (Collier, G. S., private communication). Each sample was analysed only once, and the results reported as mg of lysine per g sample.

Participating laboratories were instructed to measure dye binding capacity (DBC) with the azo dye acid orange 12 in a pH 1.25 buffer containing oxalic acid. They were carried out in an automated commercial apparatus,[6] using 1.0 g of test material (or 0.8 g where the higher weight gave an off-scale reading) and 40 ml of buffered dye solution. However, laboratories 3 and 13 did not use the automated commercial apparatus, but used a laboratory shaker to mix the dye with the meal, and determined the optical density of the resulting solution with a spectrophotometer. The results were expressed as millimoles of dye bound per g of sample.

Dye binding lysine (DBL) measurements were performed with the azo dye acid orange 12 on samples of meal, each in duplicate, according to the method of Hurrell, Lerman and Carpenter,[4] and using the same automated commercial apparatus as used for the determination of DBC. One of the samples was treated with propionic anhydride in order to acylate the reactive lysine prior to treatment with dye. Thus, the quantity of reactive lysine could be calculated by difference. Laboratories 3 and 13 again used ordinary laboratory equipment instead of the commercial apparatus. The results were expressd as millimoles of dye bound per g of sample.

FDNB-reactive lysine was determined by the procedure of Carpenter[7] using the correction factor of 1.09. However, laboratory 21 used the modification published by Booth[16] and the correction factor of 1.064. The means of the two methods were not significantly different. Results were expressed in mg lysine per g sample.

2.3. Bioassays

Potencies of the test materials as sources of lysine wer· calculated from the growth or feed conversion efficiency response to increments of each test material compared with that obtained from increments of crystalline L-lysine added as L-lysine HCl to a basal diet deficient in lysine. The four

laboratories undertaking this test used different procedures, but using the same principle. The concentrations of fish meals included in the diets provided the same fish meal nitrogen level in corresponding diets in a particular laboratory.

Laboratory 7 used a basal diet of sesame meal (levels adjusted to take into account the crude protein of the meal), maize starch, maize oil, dried whey, vitamins and minerals, feeding 10 day old male broiler chicks for 8 days.[8] The response to two increments of meal or three increments of L-lysine HCl was measured in terms of feed conversion efficiency.

Laboratory 13 used a basal diet of maize meal, maize gluten, amino acids, maize starch, sucrose, maize oil and a vitamin mineral mix, feeding 7-day-old broiler-type chicks for 9 days.[9] The response to five increments of meal or five increments of L-lysine HCl was measured in terms of feed conversion efficiency.

Laboratory 14 used a basal diet of maize starch, maize gluten, amino acid mixture, ground oat hulls, fish solubles, groundnut oil and a vitamin-mineral mixture, feeding 7–10 day old male White Leghorn chickens for 14 days.[10,11] The response to two increments of meal or four increments of L-lysine HCl was measured in terms of weight gain.

Laboratory 18 used the general procedure described by Carpenter *et al.*[12] with the following modifications. Corn oil (20 g kg^{-1}) replaced an equal weight of hydrogenated vegetable shortening (Trex) to ensure an adequate supply of linoleic acid. Threonine (2 g kg^{-1}) replaced an equal weight of maize starch to bring the calculated supply of threonine in the basal diet to about 115–120% of requirement, instead of 90–100% of requirement. Basic zinc carbonate was included in the mineral pre-mix, and NaCl and $MnSo_4$ $4H_2O$ levels were corrected to 4690 mg kg^{-1} and 300 mg kg^{-1} respectively. DL-methionine (0.25%) was added to the basal diet. Additions of L-lysine HCl to the basal diet were at arithmetically rather than logarithmically spaced intervals to supply 1.6, 3.2 and 4.8 g lysine kg^{-1} diet. Test proteins were added to contribute 16.2 or 32.4 g crude protein kg^{-1} replacing an isonitrogenous amount of zein. Ten-day-old Shaver Starcross male chicks were fed for 12 days. Feed conversion efficiency values were used to estimate the response to lysine.

3. Results

3.1. Data

The chemical observations were expressed as the amount of lysine per sample (i.e. mg lysine g^{-1} sample), except for DBC and DBL results, which were expressed as millimoles of dye bound per g of meal. Assuming that propionic anhydride reduces dye binding directly in proportion to the lysine content of the meal, the dye binding lysine values were multiplied by the molecular weight of lysine (146) to give values in terms of mg lysine g^{-1} meal.

All values were transformed to a protein basis (g or millimole 16 g N^{-1}) using the mean nitrogen content of each fish meal calculated from analyses made by 16 laboratories. Outlying values[13] for nitrogen were removed (all the results from laboratory 8; and the results from laboratory 1 sample 14, laboratory 7 sample 16, laboratory 19 sample 12). The mean nitrogen values and standard deviations calculated from the between laboratory variation of the remaining results are given in Table 2. The transformed lysine values are given in Table 3. The statistical analysis was carried out using both the original (mg or mmol g^{-1} sample) and transformed (mg or mmol 16 g N^{-1}) data.

Table 2. Mean nitrogen content and standard deviation of samples, after removal of outliers

	Meal							
	A	B	C	D	E	F	G	H
	Sample no.							
	1 and 14	2 and 10	3 and 8	4 and 11	5 and 7	6 and 13	9 and 12	15 and 16
Mean nitrogen content (g N g^{-1} sample)	0.113	0.113	0.102	0.118	0.098	0.108	0.105	0.101
s.d.	0.0015	0.0015	0.0015	0.0017	0.0013	0.0017	0.0015	0.0015

Table 3. Transformed data for lysine determinations on 16 samples of fish meal

		Meal															
		A		B		C		D		E		F		G		H	
		Sample no.															
	Analysis laboratory no.	1	14	2	10	3	8	4	11	5	7	6	13	9	12	15	16
Total lysine (g lysine 16 g N^{-1})	1	9.17	8.75	8.23	7.52	6.60	5.66	9.25	7.89	8.30	8.14	9.52	9.81	7.13	6.22	6.80	7.59
	4[a]	9.62	6.78	9.02	7.34	7.21	7.12	8.86	8.72	8.36	8.43	9.13	7.92	7.52	7.20	5.87	5.58
	8	7.71	8.20	7.10	7.73	6.05	6.41	7.51	7.39	7.33	7.40	8.16	6.69	6.06	6.13	7.11	7.40
	13	8.72	7.97	7.62	7.92	6.21	6.04	7.73	7.77	8.17	7.00	7.88	8.33	6.00	6.01	6.70	6.32
	14	8.04	8.18	7.23	7.52	5.97	6.29	7.35	7.89	7.49	7.81	7.88	8.03	6.07	6.53	7.11	6.48
	18	7.44	7.87	6.75	7.32	6.07	5.93	7.31	7.10	6.97	7.05	8.00	7.69	6.30	6.24	6.56	6.83
	19	7.60	7.25	6.48	6.71	5.83	5.72	7.07	7.01	6.97	7.08	6.99	7.26	5.97	5.77	6.12	6.39
	20	8.42	8.08	7.79	7.40	(6.60)[b]	(6.41)[c]	9.07	7.05	7.85	7.89	7.63	7.81	6.42	6.41	6.74	6.69
Mean		8.16	8.04	7.31	7.45	6.19	6.07	7.90	7.44	7.58	7.48	8.01	7.95	6.28	6.19	6.73	6.81
Dye binding lysine (g lysine 16 g N^{-1})	2	7.21	7.56	6.34	6.86	4.45	4.59	6.67	6.95	6.56	6.70	6.92	7.14	5.79	5.50	5.98	6.16
	3[a]	8.30	8.18	8.32	8.28	9.02	8.65	8.22	7.93	9.41	9.22	8.68	8.47	8.62	8.51	9.07	9.16
	5	7.33	7.11	6.25	6.34	4.13	4.13	6.69	6.81	6.61	6.70	7.16	7.19	5.56	5.45	6.09	5.93
	7	7.77	7.44	6.30	6.05	3.33	3.74	6.42	6.20	5.75	6.08	6.43	7.19	5.34	5.34	5.70	6.12
	11	7.46	7.29	6.17	5.84	3.88	3.88	6.69	6.65	5.73	6.75	7.66	6.62	4.96	4.72	5.98	6.03
	13	8.57	7.87	7.68	7.10	4.73	5.53	7.89	8.01	8.25	8.34	8.88	8.79	7.11	6.87	8.01	7.41
	14	7.23	7.21	6.44	6.79	5.39	4.61	6.81	6.95	6.80	7.08	7.34	7.32	5.54	5.34	4.87	5.26
	18	7.19	6.12	5.41	5.22	3.28	3.37	6.30	5.22	5.75	5.77	6.30	5.64	3.70	4.17	5.22	5.15
	21	7.44	7.79	5.47	6.69	4.66	4.02	6.89	6.56	5.25	5.94	7.60	8.21	5.63	5.16	6.62	6.07
Mean		7.53	7.30	6.26	6.36	4.23	4.23	6.80	6.67	6.34	6.67	7.29	7.26	5.45	5.32	6.06	6.02
Dye binding capacity (mmoles dye 16 g N^{-1})	3[a]	100.4	102.3	100.3	102.2	112.9	113.8	96.8	97.1	118.2	116.6	107.1	108.2	109.3	109.6	112.7	112.7
	5	103.6	102.2	88.3	91.1	76.3	78.3	98.2	99.5	98.2	98.3	104.5	103.5	83.6	81.5	93.3	96.0
	11	106.5	105.7	92.7	91.9	79.4	77.7	100.7	98.5	99.3	96.7	104.1	105.1	85.5	85.3	95.9	93.5
	13	104.4	107.1	91.1	96.4	67.2	70.4	96.7	96.7	93.4	96.8	105.1	105.7	86.7	92.3	95.7	96.2
	14	106.1	102.1	92.2	94.0	80.8	80.0	99.6	98.6	97.2	97.5	106.6	(103.5)[d]	84.1	84.1	95.7	101.8
	21	106.2	107.5	92.3	92.5	72.3	84.1	99.3	99.3	99.5	97.0	103.2	104.8	84.4	83.5	96.1	96.3
Mean		105.4	104.9	91.3	93.2	75.2	78.1	98.9	98.5	97.5	97.3	104.7	104.5	84.9	85.3	95.3	96.8
FDNB lysine (g lysine 16 g N^{-1})	3	7.17	7.22	6.77	6.55	4.17	4.15	6.69	6.76	6.62	6.62	7.18	7.27	5.77	5.60	6.29	6.39
	10	6.36	6.46	5.82	6.00	4.01	3.94	5.81	5.93	5.99	5.78	6.54	6.48	5.13	4.96	5.72	5.34
	12	7.58	7.20	7.02	6.82	3.99	4.09	6.73	6.35	6.71	7.03	7.52	7.11	5.83	5.57	6.40	6.28
	21	7.41	7.60	7.35	6.62	3.52	4.35	6.45	7.31	7.19	6.36	7.58	7.84	5.40	6.38	7.08	7.15
Mean		7.13	7.12	6.74	6.50	3.92	4.13	6.42	6.59	6.63	6.45	7.21	7.18	5.53	5.63	6.37	6.29
Bioassay (g lysine 16 g N^{-1})	7	7.63		6.94		2.91		7.62		7.85		8.68		4.89		6.28	
	13	8.47		7.19		2.59		7.22		7.54		8.12		5.22		6.17	
	14	7.86		6.95		2.83		7.20		7.47		7.79		4.81		6.43	
	18	8.93		7.02		2.88		8.00		9.34		10.30		7.23		8.24	
Mean		8.22		7.03		2.80		7.51		8.05		8.72		5.54		6.78	

[a] Laboratories 3 and 4 results excluded from the analysis of these methods—replaced results are in parentheses. The original results were [b] 7.22 [c] 8.42 [d] 98.7.

The data were examined for outlying observations.[13] In both the DBC and DBL methods, laboratory 3 gave results markedly different from other laboratories, and effectively the same values for all the fish meals. The reason for this discrepancy was not apparent, but it was noted that laboratory 3 had not used the automated equipment, and that various procedures used differed considerably from those in the protocol. All the results from this laboratory, for these methods, were excluded from the analysis. The results from laboratory 13, which also used standard laboratory equipment, and not the automated equipment for DBC and DBL, produced results similar in value and variability to the other participating laboratories, and, therefore, were not excluded. Examination of the total lysine results from laboratory 4 showed poor reproducibility between some of the blind duplicates, not only for lysine but also other amino acids, particularly those with an interval of several days between the duplicate determinations. The cause was traced to a deterioration in the efficacy of the reagent, ninhydrin, during the period of analysis which was not compensated for by corresponding changes in reaction with the internal standard of nor-leucine. Therefore all the results for laboratory 4 were excluded. Laboratory 20 gave high total lysine values for samples 3 and 8; and laboratory 14 gave a low DBC value for sample 13. These values were replaced by the next highest or lowest values respectively.[13] This technique is a compromise between giving high estimates of variability through inclusion of chance erroneous results, and low estimates of variability through the exclusion of such values. The resulting transformed data set is given in Table 3 with the replaced results in parentheses.

3.2. Homogeneity of within-laboratory variation across fish meals

Before carrying out the two-way analysis of variance (anova) described in section 3.3, the data were analysed to check whether the within-laboratory variation was the same for all fish meals for a given method. For each method, a one-way anova was calculated using the laboratories and the duplicates to estimate the within- and between-laboratory variation for each fish meal.

For the DBL and total lysine methods, a tendency for the within-laboratory variation to increase as the fish meal mean increased was noted, both for the original and transformed observations, but this was not statistically significant at the 5% level. DBC and FDNB-reactive lysine did not show any such trend, and it was concluded that for all the methods the within laboratory variation did not differ significantly for any meal.

3.3. Differences between fish meals and estimation of the overall between- and within-laboratory variation for each method

For each method, the results for all the fish meals and laboratories were combined and used to calculate a two-way anova. The fish meals were considered to be a fixed factor and the laboratories to be a random effect.[14] Components of variance attributable to fish meals (F^2), laboratories (σ_L^2), fish meal × laboratory interaction (σ_{FL}^2 or σ_E^2) and to within laboratory error (σ_o^2) were estimated from the anovars given in table 4.[14]

The estimates of the within-laboratory, meal × laboratory interaction and between-laboratory

Table 4. Estimates of components of variance attributable to fish meals (F^2), laboratories (σ_L^2), fish meal × laboratory interaction (σ_{FL}^2 or σ_E^2) and within laboratory error (σ_o^2)

			Expected mean square	
Source of variation	d.f.	Mean square	Chemical methods	Bioassay
Fish meals	7	MS_F	$\sigma_o^2+2\sigma_{FL}^2+2LF^2$	σ^2+8F^2
Laboratories	L–1	MS_L	$\sigma_o^2+16\,\sigma_L^2$	$\sigma_E^2+8\sigma_L^2$
Meal × laboratory interaction	7(L-1)	MS_{FL}	$\sigma_o^2+2\sigma_{FL}^2$	σ_E^2
Within-laboratory error	8L	MS_E	σ_o^2	—

Table 5. Mean values, and components of variance expressed as percentages of the mean, for chemical and biological tests for lysine and dyebinding capacity (DBC)

		Component of variance (% of mean)		
Method	Mean	Within-laboratory σ_0	Meal × laboratory σ_{FL} or σ_E	Between-laboratory σ_L
Total lysine (g 16 g N^{-1})	7.22	5.43	1.29	5.24
DBC (mmol 16 g N^{-1})	94.5	2.24	1.73	0.56
DBL (g 16 g N^{-1})	6.24	5.27	4.13	10.25
FDNB (g 16 g N^{-1})	6.24	4.38	1.45	6.55
Bioassay (g 16 g N^{-1})	6.83	—	7.41	8.58

components of variance for each method are given in Table 5 expressed as a coefficient of variation (%) of the overall mean value. Since samples were not analysed in duplicate by the bioassay method, the estimates of components of variance are not exactly comparable for chemical and biological methods. The meal × laboratory component for the bioassay is inflated by within-laboratory variance. The between-laboratory component of variance is reduced by any contribution of σ_E^2 or σ_{FL}^2 which could not be measured. Correspondingly, σ_E^2 is an overestimate of the within-laboratory variance of the bioassay method if σ_{FL}^2 is not zero.

In all methods, highly significant differences existed in lysine value between the selected fish meals both in units per g of meal and per g of protein (Table 6). For total lysine, DBC and FDNB-reactive lysine, the meal × laboratory interaction was not significant, and, therefore, each laboratory ranked

Table 6. Mean values and standard errors for lysine determined by various procedures, and dyebinding capacity (DBC) of eight selected fish meals

	Meal								
	A	B	C	D	E	F	G	H	s.e. (mean)
Total lysine (g 16 g N^{-1})	8.10	7.38	6.13	7.67	7.53	7.98	6.23	6.77	0.111
DBC (mmole 16 g N^{-1})	105.1	92.3	76.7	98.7	97.4	104.6	85.1	96.1	0.99
DBL (g 16 g N^{-1})	7.41	6.31	4.23	6.73	6.50	7.27	5.39	6.04	0.123
FDNB (g 16 g N^{-1})	7.13	6.62	4.03	6.50	6.54	7.19	5.58	6.33	0.107
Bioassay (g 16 g N^{-1})	8.22	7.03	2.80	7.51	8.05	8.72	5.54	6.78	0.253

the meals in a similar fashion. For DBL the interaction term was just significant at $P<0.05$, mainly because of differences in the ranking of meal E in laboratories 13 and 21 (see Table 3). There were consistent differences between laboratories in all methods except for DBC (Table 7).

The components of variance attributable to laboratories (Table 5) is not well estimated because of the small number of laboratories undertaking each method. With the exception of DBC where the value was not significantly different from zero, the between-laboratory component of variance was of the same order of magnitude as the combined within laboratory and meal × laboratory interaction components. DBL had the largest estimate of the between-laboratory component of variance but this was not significantly different from the estimates for the other methods, excluding DBC. Much of this variation in DBL was attributable to laboratories 13 and 18 which consistently had very much higher or lower values respectively than the other six laboratories.

The results of the biological assays indicate that they were undertaken in a correct manner. Values from laboratory 18 were generally higher than those from the other three laboratories but the ranking of the meals was closely similar and the estimates of between-laboratory and combined within laboratory with meal × laboratory interaction components were not appreciably greater than those of the chemical methods other than DBC.

Table 7. Mean laboratory values for estimates of lysine by chemical and biological methods (mean values of determinations on eight fish meals)

Laboratory no.	Total lysine (g 16 g N^{-1})	DBC (mmol 16 g N^{-1})	DBL (g 16 g N^{-1})	FDNB (g 16 g N^{-1})	Bioassay (g 16 g N^{-1})
1	7.91	—	—	—	—
2	—	—	6.34	—	—
3	—	—	—	6.33	—
4	—	—	—	—	—
5	—	93.5	6.22	—	—
7	—	—	5.95	—	6.60
8	7.15	—	—	—	—
10	—	—	—	5.64	—
11	—	94.9	6.02	—	—
12	—	—	—	6.39	—
13	7.27	93.9	7.57	—	6.56
14	7.24	95.2	6.31	—	6.42
18	6.96	—	5.24	—	7.74
19	6.64	—	—	—	—
20	7.39	—	—	—	—
21	—	94.9	6.25	6.60	—
s.e.(mean)	0.098	0.53	0.082	0.068	0.179

3.4. Comparison of the methods: analysis of covariance

Ideally, in order to compare the methods, all of them should have been used in all the laboratories, but as can be seen from Table 7, no laboratory used all the methods, and most used only one or two. Consequently, the laboratories were treated as though they constituted replicate analyses in the methods, and were used to obtain mean values for each fish meal in each of the methods (Table 6). Analyses of covariance between each of the chemical methods and the bioassay values were calculated using these means. The correlation coefficients (Table 8) for both the original (units g^{-1} meal) and transformed (units 16 g N^{-1}) data were high and significant. Although transformation of the data altered the ranking of meals and reduced the range between best and poorest meals, correlation coefficients were slightly greater. Total lysine gave the lowest correlation and detailed examination indicated some deviation from a linear relationship, the total lysine content being considerably in excess of the biologically available lysine of low quality meals, especially meal C.

4. Discussion and conclusions

In the previous collaborative exercise,[3] the range of lysine values of mainly experimental meals manufactured from one species of fish was small. The aim of this collaboration of obtaining a variety of commercial meals covering different fish species and a wide range in biologically available lysine was achieved. However, the meals were not equally distributed over the whole range of values and the inclusion of one very poor quality meal markedly affected certain conclusions.

Table 8. Correlation coefficients between chemical and biological estimates of lysine in eight fish meals

	Bioassay (g g^{-1} meal)	Bioassay (g 16 g N^{-1})
Total lysine	0.87	0.88
DBC	0.94	0.96
DBL	0.96	0.97
FDNB	0.97	0.98

Because there were significant differences between laboratories for all the methods except DBC, the mean values given in Table 6, and the intercept of regression equations relating chemical with bioassay values, cannot be regarded as absolute values but are influenced by the particular laboratories participating in each method, therefore comparison of the values for different methods given in Table 6 must be made with caution.

The chemical methods for measuring lysine (total lysine, FDNB available lysine, and DBL) gave means which were significantly different from the lysine bioassay using chicks. It should be noted that FDNB and DBL (like DBC) do not estimate free lysine,[4] and therefore might underestimate the available lysine content in meals with a high proportion of soluble nitrogen, however, this effect was not apparent in the present study. Total lysine values, unfortunately , do not distinguish between the biologically available lysine and 'bound' lysine, the latter often occurring as a result of heat damage. This is clearly seen with the severely heat damaged sample of fish meal (C) giving a total lysine result of 6.13 g 16 g N^{-1}, whereas the bioassay result was 2.80 g 16 g N^{-1}. Also to a lesser extent the FDNB and DBL methods do not fully measure the nutritional damage to this meal, giving results of 4.03 and 4.23 g 16 g N^{-1} respectively. Furthermore, there are indications[3, 15] that nutritional damage caused by the excess use of formaldehyde (about 1%) on wet proteinaceous material may be considerably greater than can be predicted from chemical analysis for total or reactive lysine by any of the methods tested. None of the commercial samples of fish meal in this collaborative trial were treated with formaldehyde.

Since the meal × laboratory interaction was small or non-significant for the chemical methods, the correlation coefficient between results by chemical and bioassay methods is little affected by the selection of laboratories. All the chemical methods correlated well with the bioassay but did not achieve the same range in values as obtained with the bioassay. The correlation coefficients between chemical and biological methods found in this collaborative trial were similar to those reported by Carpenter and Opstvedt[3] for FDNB, total lysine, and DBC.

In order to facilitate the comparison of methods, DBC values were scaled from units of mmoles dye bound to an approximate estimate of g reactive lysine by assuming that lysine was 0.5 of the basic amino acids such that 1 mole dye bound was equivalent to 73 g lysine. The sensitivity of methods for detecting differences in biologically available lysine can then be compared using the slope of the

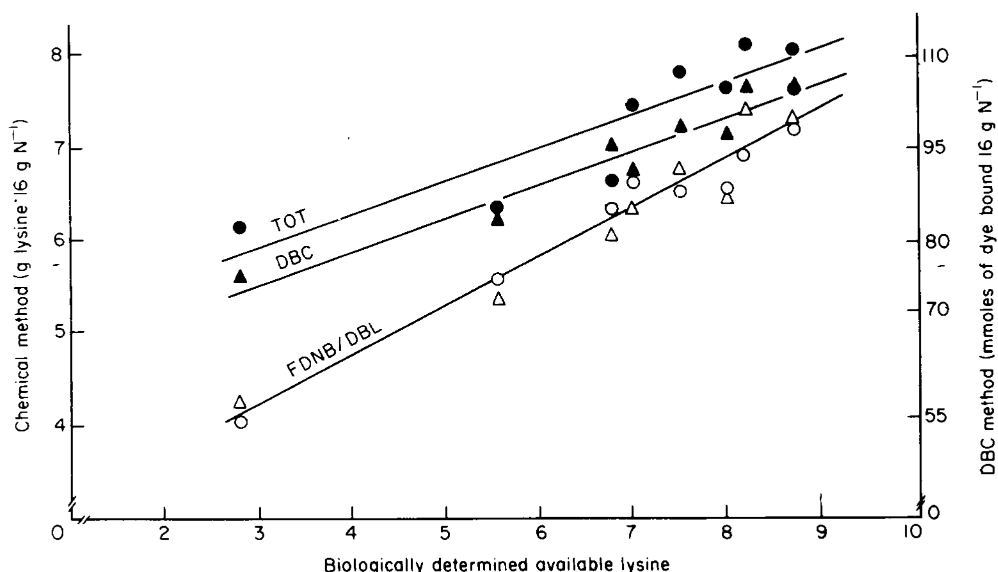

Figure 1. Least square regression lines relating (●) TOT (total lysine), (▲) DBC (dye binding capacity), (△) DBL (dye binding lysine), and (○) FDNB-reactive lysine to available lysine determined by bioassay with chicks. Values plotted are the means of several determinations in different laboratories for eight fish meals.

regression of the chemical value on the bioassay value. A value of 1.0 indicates a change in chemical value equal to a change in bioassay value. The relationships are plotted in Figure 1.

The regression coefficients obtained (g 16 g N^{-1} basis) were 0.36, 0.36, 0.53 and 0.53 for total lysine, DBC, DBL and FDNB-reactive lysine respectively. Omitting the very poor quality meal C which was known to have been severely overheated, the corresponding regression coefficients were 0.59, 0.44, 0.62 and 0.47. Thus, total lysine gave the poorest, and DBL and FDNB-reactive lysine the best indication of the low quality of meal C. For the remaining meals, which still encompassed a greater range of values than is likely to be found in normal commercial samples, total lysine and DBL gave the best indication of biologically available lysine in the protein, but even so the change in these values was only 0.6 of the change in bioassay value.

The lower correlation and regression coefficients for DBC compared with FDNB-reactive lysine or DBL is not surprising because the DBC test is not specific to lysine as dye is bound by histidine and arginine, and in addition there is some non-specific binding of dye.[4] Within this series of eight meals, total lysine and histidine plus arginine contents were correlated (r=0.78). This correlation enhances the apparent value of DBC as a measure of lysine, but clearly there is still variation in DBC that can be attributable to histidine and arginine and not to lysine. Examination of the extensive analyses of Kifer *et al.*[17,18] shows that similarly high correlation coefficients exist between lysine and the sum of histidine plus arginine content (g 16 g N^{-1}) of Menhaden (r=0.86) and Peruvian anchovy (r=0.89) meals. However, using mean values of the basic amino acid content of fish meals prepared from different species[19] there is no significant correlation between total lysine and the sum of histidine and arginine (r=0.62, 5 degrees of freedom).

One advantage of the DBC method appears to be its repeatability within, and reproducibility between, laboratories. However, only five laboratories made this determination. In a subsequent collaborative trial (Miller, E. L., private communication), values obtained in five laboratories, not all the same as in this collaborative trial, showed significant differences between laboratories with the component of variance attributable to laboratories being of the same order as obtained with total lysine and FDNB-reactive lysine in this trial.

The DBL method appears to be promising as a rapid predictor of chick bioassay values for lysine in fish meals. In terms of speed, safety and convenience it is to be preferred to the FDNB method. However, DBL had the largest estimate of between laboratory variation. It has previously been reported that the FDNB procedure gives considerably different absolute values in different laboratories,[3] and our results suggest that the DBL procedure might give even bigger problems. If this test is to be a useful tool in the quality control of fish meal, further modifications to the procedure will be required to improve the repeatability and reproducibility.

One aim of studies of protein quality is to develop laboratory methods which could be used to predict the biologically available lysine content of feedstuffs. Because of the variability in determination of both the biologically available lysine and of the chemical estimate, the accuracy of such prediction equations is generally poor. For example, if an average quality fish meal was shown by analysis to contain total lysine of 7.5 g 16 g N^{-1}, DBC of 100 mmol 16 g N^{-1}, DBL and FDNB-reactive lysine of 6.5 g 16 g N^{-1}, the confidence limits, derived from the relationships observed in this trial, would be ± 2.41, ± 1.54, ± 1.28 and ± 1.04 g 16 g N^{-1} about the predicted values for the biologically available lysine for each of the above chemical methods respectively.

In addition, the estimated value will vary according to which laboratory actually determined the chemical values. Such wide confidence limits are likely to encompass the variability in the majority of commercial samples. Consequently, none of the chemical methods can be regarded as satisfactory for the purpose of distinguishing between normal commercial samples of fish meals in terms of an absolute content of biologically available lysine.

The chemical methods may be used in a more limited sense to detect differences in quality between meals of known history without indicating any absolute value for the biologically active lysine. The dye binding methods appear suitable for process control within a fish meal factory where the analyses are carried out in a single laboratory, where the nature of the raw material is known and problems of variation in proportions of basic amino acids, and of free lysine, contributed by fish solubles, can be properly interpreted.

Acknowledgements

Gratitude is expressed to members of the Humber Laboratory, Torry Research Station, Ministry of Agriculture, Fisheries and Food UK for preparation and distribution of samples, and to all participating laboratories.

References

1 Carpenter, K. J.; Ellinger, G. M.; Munro, N. I; Rolfe, E. J. Fish products as protein supplements to cereals. *Br. J. Nutr.* 1979, **11**, 162–173.
2. Carpenter, K. J. Damage to lysine in food processing: its measurement and its significance. *Nutr. Abstr. Rev.* 1973, **43**, 423–451.
3. Carpenter, K. J.; Opstvedt, J. Applications of chemical and biological assay procedures for lysine to fish meals. *J. Agric. Food Chem.* 1976, **24**, 389–393.
4. Hurrell, R. F.; Lerman, P.; Carpenter, K. J. Reactive lysine in foodstuffs as measured by a rapid dye-binding procedure. *J. Food Science,* 1979, **44**, 1221–1231.
5. Anon. Recommended method of analysis for determination of crude protein in fish meal. *International Association of Fish Meal Manufacturers Technical Bulletin No. 8.* IAFMM, Potters Bar, 1979.
6. Jacobsen, E. E.; Moller, A.; Nielsen, J. J.; Schmidtsdorff, W.; Weidner, K. E. Evaluation of the dye binding method as a tool for practical check of fish meal quality. *International Association of Fish Meal Manufacturers, 12th Annual Conference, Rome.* IAFMM, Potters Bar, 1972.
7. Carpenter, K. J. The estimation of the available lysine in animal-protein foods. *Biochem. J.* 1960, **77**, 604–610.
8. Achinewhu, S. C.; Hewitt, D. Assessment of the nutritional quality of proteins: the use of 'ileal' digestibilities of amino acids as measures of their availabilities. *Br. J. Nutr.* 1979, **41**, 559–571.
9. Wessels. J. P. H.; Rossouw, L. C.; Hayes, J. P.; du Preez, J. J. Chicken available lysine in fish meals from six countries, *Annual Report of the Director of Fishing Industry Research Institute,* University of Cape Town 1976, No. 30, 43–45.
10. Opstvedt, J.; Mundheim, H. Determination of available lysine and methionine by a chick growth assay. *Vth International Symposium on Amino Acids.* 1977, Budapest, E3.
11. Opstvedt, J. Influence of residual lipids on the nutrition value of fish meal. VII. Effect of lipid oxidation on protein quality of fish meal. *Acta Agric. Scand.* 1975. **25**, 53–71.
12. Carpenter, K. J.; March, B. E; Milner, C. K; Campbell, R. C. Bioassay test for lysine. *Br. J. Nutr.* 1963, **17**, 309–323.
13. Grubbs, F. E. Procedures for detecting outlying observations in samples. *Technometrics* 1969, **11**, 1–21.
14. Scheffe, H. *The Analysis of Variance,* John Wiley and Sons, New York, 1959.
15. Hurrell, R. F.; Carpenter, K. J. Digestibility and lysine values of protein heated with formaldehyde or glucose. *J. Agric. Food Chem.* 1978, **26**, 796–802.
16. Booth, V. H. Problems in the Determination of FDNB-available lysine. *J. Sci. Food Agric.* 1971, **22**, 658–666.
17. Kifer, R. R.; Payne, W. L.; Miller, D.; Ambrose, M. E. The nutritive content of Menhaden (*Brevoortia tyrannus* and *B. patronus*) Fish meal evaluated by chemical methods. *Feedstuffs* (*Minneapolis*) 1968, **40** (20) p. 36.
18. Kifer, R. R.; Payne, W. L.; Bauersfeld, P. E.; Ambrose, M. E. The nutritive content of Peruvian anchovy fish meal evaluated by chemical methods. *Feedstuffs* (*Minneapolis*) 1968, **40**, (35) p. 32.
19. Miller, E. L. *Available amino acid content of fish meals. FAO Fisheries Report* No. 92, Rome, 1970.

Printed by Citadel Litho Plates, London