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Accuracy of Amino Acid Analysis of Fish Meals by Ion-exchange and Gas Chromatography

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ABSTRACT

Variations in amino acid content of fish meals can be due to differences between meals or to analytical shortcomings. The purpose of the cooperative study reported here was to compare the accuracy of the ion-exchange (IE) and gas chromatographic (GC) methods when applied to fish meal. The between-laboratory and within-laboratory variations were determined by analysing hidden duplicates of eight meals in four laboratories using IE and in a further three laboratories using GC.

The work exposed certain pitfalls and inconsistencies in replicating results. A major source of variation in the IE method was identified as the instability of the ninhydrin reagent leading to the consequent variation in the colour yield of some amino acids which was not compensated by the use of norleucine

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as an internal standard. Significant between-laboratory variations and fish meal × laboratory interactions for certain amino acids existed. The work confirmed a need for replicate analysis of samples, whether done by IE or GC.

Without discarding any outlying values, coefficients of variation for repeatability (within-laboratory variation) varied from 3·5 to 9·7% for IE and 3·5 to 8·4% (histidine at 20%) for GC. Coefficients of variation for reproducibility (between-laboratory variation) varied from 4·1 to 14·4% for IE and 4·5 to 11·9% (20% for histidine) for GC. The values for lysine reproducibility were 10·8% and 4·8% and for methionine were 13·6% and 8·2% respectively for IE and GC.

Neither of the methods was clearly superior for determining the amino acid content of fish meals. It would appear, however, that the GC method tended to result in lower within-laboratory variance, with arginine and histidine as exceptions.

An amino acid mixture, a centrally prepared hydrolysate and a protein of known composition were also studied.

Key words: Amino acid analysis, accuracy, fish meal, gas chromatography, ion-exchange, cooperative studies, reproducibility, repeatability.

1 INTRODUCTION

Variations in published amino acid contents for fish meals may be due to differences between meals or to analytical shortcomings. Although several collaborative trials aimed at establishing the accuracy of the ion-exchange (IE) and gas chromatographic (GC) methods for amino acid analysis have been reported (Bender *et al* 1959; Porter *et al* 1968; Knipfel *et al* 1971; Cavins *et al* 1972; Williams *et al* 1980; Kreienbring 1981; Sarwar *et al* 1983; Andersen *et al* 1984), none involved a comparison of the two different techniques. However, there are several reports on within-laboratory comparisons of the two techniques (Gehrke and Leimer 1971; Kirkman 1974; March 1975; Tajima *et al* 1978).

The work reported here was part of an international collaborative study organised by the International Association of Fish Meal Manufacturers to relate the results of chemical methods of lysine determination to those of biologically available lysine determined by chicken assay. The total lysine contents of the eight fish meals used in this study have been previously reported, together with other chemical and biological estimates of available lysine (Barlow *et al* 1984). The aim of the present study was to examine the between- and within-laboratory variations of the IE and the GC methods. Eight laboratories participated in a cooperative manner, using their own established methods to carry out the amino acid determinations. Five laboratories used IE and three used GC. The authors report the results on behalf of the participating laboratories listed below:

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2 EXPERIMENTAL

2.1 Fish meal samples

The eight fish meals were commercial products from Denmark, Norway, Peru, South Africa and the United States of America. 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 purposes. Thus the variation in amino acid content may not represent typical variation in normal commercial fish meals. However, for the present purpose of determining the variability of amino acid analysis and to facilitate comparison with published work on variability, the meals were regarded as a random sample. The collection and distribution of these meals as 16 samples consisting of duplicates of each meal to participating laboratories were described in detail in the earlier report (Barlow *et al* 1984). The samples for amino acid analysis were ground through a 50-mesh Tyler screen and thoroughly mixed at a central distribution point before being sent to the cooperating laboratories. Laboratories carried out a single analysis on each of the 16 coded samples.

2.2 Standard samples

Cooperating laboratories were also supplied with three standards. These were a solution in 0·1 M HCl of chemically pure amino acids in quantities similar to those expected in a hydrolysate of fish meal (Standard A), a sample of beef haemoglobin from BDH Chemicals Ltd that was hydrolysed by each laboratory (Standard B), and a hydrolysate of one of the eight fish meals, prepared by Laboratory 4 and distributed from the central point (Standard C). Each of the laboratories performed one analysis on each of Standards A, B and C.

2.3 Hydrolysis and analytical procedures

Hydrolysis and analytical procedures are described in Tables 1 and 2.

Of the IE laboratories, 4. 18 and 20 determined sulphur-containing amino acids as cysteic acid and methionine sulphone after pre-hydrolysis oxidation (Weidner

TABLE 1
Hydrolysis procedures used in the different laboratories

Laboratory code:	*1 (IE)*	*4 (IE)*	*18 (IE)*	*19 (IE)*	*20 (IE)*	*8 (GC)*	*13 (GC)*	*14 (GC)*
Hydrolysis conditions:								
Sample weight (mg)	*c* 25	1000	*c* 100	500	*c* 150–250	*c* 50	*c* 30	*c* 50
Volume of 6 M hydrochloric acid (ml)	60	250	420	300	800	60	20	60
Additional protecting reagents	3% aq. TiCl (2 ml) (internal standard added: Gawargious 1971)	None	None	None	None	Thioglycollic acid (50 μl)	None	Dithiothreitol (0·1 mM)
Duration (h)	10	24 (under N_2)	24 (under N_2)	22 (under N_2)	20 (under N_2)	22	22 (under N_2)	22 (under N_2)
Temperature (°C)	120 (pressure vessel)	Reflux	Reflux	Reflux	Reflux	110	110 (sealed tube)	110 (sealed flask)
Post-hydrolysis manipulation	Acid removed on rotary film evaporator prior to buffer addition	Acid removed on rotary film evaporator prior to buffer addition	Hydrolysate filtered and diluted to 1 litre. Internal standards added to aliquots. Acid removed on rotary film evaporator prior to addition of water (acidic and neutrals assay) or buffer (basics assay)	Hydrolysate filtered; internal standard added. Acid removed *in vacuo* at 37°C	Internal standard added to hydrolysate. Diluted to 1 litre. Aliquot filtered and acid removed on rotary film evaporator. Residue dissolved in 0·1 M HCl and filtered	Internal standard added to hydrolysate. Filtered. Acid removed on rotary film evaporator	Internal standard added to hydrolysate. Filtered. Acid removed *in vacuo* on freeze dryer	Internal standard added to hydrolysate. Filtered. Acid removed on rotary film evaporator
Hydrolysate clean-up step	No	No	No	No	No	Dowex 50 W × 12 cation exchanger	No	Dowex 50 W × 8 cation exchanger

TABLE 2
Methodologies for amino acid analysis of fish meal

Laboratory code:	*1 (IE)*	*4 (IE)*	*18 (IE)*	*19 (IE)*	*20 (IE)*	*8 (GC)*	*13 (GC)*	*14 (GC)*
Derivatisation	No	No	No	No	No	Residue derivatised. Trifluoroacetyl *n*-butyl ester amino acid derivatives formed	Residue derivatised. Heptafluorobutyryl *iso* butyl ester amino acid derivatives formed	Residue derivatised. Trifluoroacetyl *n*-butyl ester amino acid derivatives formed
Instrumentation	Single column Technicon amino acid analyser system	Single column Locarte amino acid analyser	Locarte amino acid analyser using two columns	EEL 294 Amino acid analyser and Locarte MK4A amino acid analyser	Single column NC 6 amino acid analyser	Packard Instruments 420 gas chromatograph, one column linked to Vidar 6300 integrator	Hewlett Packard 5710 gas chromatograph linked to HP 3353 data system	Carlo Erba 2350 gas chromatograph linked to Autolab IV data system
Calculation	Peak heights related to norleucine internal standard—added before hydrolysis	Peak areas related to standard mixture of amino acids (Hamilton Type H)	Peak areas related to norleucine and 6-amino-*n*-hexanoic acid internal standards	Peak areas related to norleucine and 6-amino-*n*-hexanoic acid internal standards	Peak areas related to norleucine internal standard	Peak areas related to tranexamic acid, internal standard—added after hydrolysis. Quantitative determination of arginine and histidine by comparison of peak heights with internal standard	Peak areas related to norleucine, internal standard—added after hydrolysis	Peak areas related to tranexamic acid, internal standard—added after hydrolysis

and Eggum 1966). Laboratory 19 did not determine cyst(e)ine and measured methionine in the usual acid hydrolysate; laboratory 1 did not determine cyst(e)ine and determined methionine by an iodoplatinate complex technique based on that described by Gawargious (1971). The GC laboratories either did not determine cyst(e)ine (laboratories 8 and 13) or used the method described by De Koning *et al* (1976) (laboratory 14); methionine was measured in the usual acid hydrolysates without pre-oxidation.

3 STATISTICAL METHOD

Analytical data relating to the fish meal samples were checked for outliers using the procedure described by Grubbs (1969). The test was applied separately to results by IE and GC.

For each amino acid and method the between- and within-laboratory variance components were estimated from a random effects analysis of variance table (Satterthwaite 1946; Scheffe 1959; Steiner 1974).

For a given method and amino acid, let Y_{ijk} be the observation on the ith meal in the jth laboratory on the kth duplicate. Then

$$Y_{ijk} = u + a_i^{\mathrm{F}} + b_j^{\mathrm{L}} + c_{ij}^{\mathrm{FL}} + e_{ijk}$$

where a_i, b_j, c_{ij} and e_{ijk} are independently distributed normal variables with zero mean and variances σ_{F}^2, σ_{L}^2, σ_{LF}^2, and σ_e^2. These latter quantities are the fish meal, laboratory, fish meal × laboratory interaction and within-laboratory variance components respectively. They are estimated from the ANOVA table (Table 3).

The variance components were estimated with

$$\sigma_e^2 = \mathrm{MS}_e \qquad \sigma_{\mathrm{LF}}^2 = (\mathrm{MS}_{\mathrm{LF}} - \mathrm{MS}_e)/\mathrm{K}$$

$$\sigma_{\mathrm{L}}^2 = (\mathrm{MS}_{\mathrm{L}} - \mathrm{MS}_{\mathrm{LF}})/\mathrm{IK} \qquad \sigma_{\mathrm{F}}^2 = (\mathrm{MS}_{\mathrm{F}} - \mathrm{MS}_{\mathrm{LF}})/\mathrm{JK}$$

The reproducibility standard deviation is defined (Steiner 1974) as

$$\sigma_x = \sqrt{\sigma_{\mathrm{L}}^2 + \sigma_{\mathrm{LF}}^2 + \sigma_e^2}$$

TABLE 3
Analysis of variance table

Source	*Degrees of freedom*	*Mean square*	*Expected mean square*
Mean	1		
Fish meals	I − 1	MS_{F}	$\sigma_e^2 + \mathrm{K}\sigma_{\mathrm{LF}}^2 + \mathrm{JK}\sigma_{\mathrm{F}}^2$
Laboratories	J − 1	MS_{L}	$\sigma_e^2 + \mathrm{K}\sigma_{\mathrm{LF}}^2 + \mathrm{IK}\sigma_{\mathrm{L}}^2$
L × F	(I − 1)(J − 1)	$\mathrm{MS}_{\mathrm{LF}}$	$\sigma_e^2 + \mathrm{K}\sigma_{\mathrm{LF}}^2$
Error	IJ(K − 1)	MS_e	σ_e^2

where in the final analysis I = 8, J = 4 for IE, J = 3 for GC, K = 2.

and the repeatability standard deviation as

$$\sigma_e = \sqrt{\mathrm{MS}_e}$$

For a given amino acid determination by either IE or GC, the reproducibility coefficient of variation is defined as $100\sigma_x/\bar{y}$, where $\bar{y}$ is the mean of the amino acid determinations, taken over all laboratories and meals. The repeatability coefficient of variation is defined as $100\sigma_e/\bar{y}$. The coefficient of variation of $\bar{y}$ is defined at $100\sigma_{\bar{y}}/\bar{y}$, where

$$\sigma_{\bar{y}}^2 = (\mathrm{MS_F} + \mathrm{MS_L} - \mathrm{MS_{LF}})/\mathrm{IJK}$$

is the variance of $\bar{y}$. These quantities are given in Table 4.

The approximate degrees of freedom for σ_x^2 and $\sigma_{\bar{y}}^2$ were found using Satterthwaite's (1946) approximation, and the reproducibilities for IE and GC were compared using an approximate F test with these degrees of freedom. The repeatabilities were compared using exact F tests.

The overall means of the two methods were compared using an approximate t test with $t = (\bar{y}_{\mathrm{IE}} - \bar{y}_{\mathrm{GC}})/\sqrt{\mathrm{Var}(\bar{y}_{\mathrm{IE}}) + \mathrm{Var}(\bar{y}_{\mathrm{GC}})}$ where the degrees of freedom were found using Satterthwaite's (1946) approximation. As no significant differences were found, these comparisons have not been tabulated.

4 RESULTS

4.1 Results of fish meal analyses

In the course of examining for outliers it became apparent that the results obtained by laboratory 4 indicated decreasing values with successive analyses of samples 1 to 16 such that samples 15 and 16, which were of the same meal and were analysed in close succession to one another, gave results that were in good agreement, whereas samples 1 and 14, for example, which were hidden duplicates of the same meal but were analysed several days apart, gave different results. This effect was very striking in the case of lysine. The average effect over all amino acids is shown in Fig 1. Laboratory 4 related these changes to deterioration of the ninhydrin reagent during the time course of the determinations, and consequently withdrew these results as being not representative of normal operation. Other outlying values were identified in the remaining data. These were scrutinised for errors of calculation; some were found and the results were corrected. There remained 17 outlying values (out of a total of 1744 determinations). Possible reasons for these values are discussed later but the values were retained in the analysis. Outlying values were reported by laboratories 1 and 18 (IE) and laboratories 8 and 13 (GC). Amino acids affected were alanine, glycine, threonine, serine, glutamic acid and aspartic acid by IE, and threonine, leucine, proline and methionine by GC.

For alanine and glycine, the reproducibility standard deviations were significantly greater for GC than for IE at the 5% level whereas for threonine, proline, glutamic acid, aspartic acid and lysine at the 1% level and for methionine at the 5% level they were greater for IE than GC (Table 4).

TABLE 4

Amino acid composition of fish meals (mg g^{-1} fish meal) as determined by ion-exchange (IE, laboratories 1, 18, 19, 20) or gas chromatography (GC, laboratories 8, 13, 14)

Amino acid	*Overall mean*[a]		*Repeatability standard deviation*		*Reproducibility standard deviation*		*Standard error of mean of two determinations*		*Repeatability coefficient of variation (%)*		*Reproducibility coefficient of variation (%)*		*Coefficient of variation of overall mean (%)*[b]	
	IE	*GC*	*IE*	*GC*	*IE*	*GC*	*IE*	*GC*	*IE*	*GC*	*IE*	*GC*	*IE*	*GC*
Alanine	41·35	42·26	1·64	2·28[c]	1·95	3·05[c]	1·57	2·59	3·9	5·3	4·7	7·2	2·1	3·3
Glycine	41·40	43·09	1·89	2·00	1·97	2·42[c]	1·44	1·96	4·4	4·6	4·8	5·6	2·7	3·1
Valine	31·74	33·51	1·73	1·71	2·69	2·29	2·40	1·95	5·2	5·1	8·5	6·8	5·0	4·5
Threonine	28·43	27·38	2·01[d]	1·26	2·10[d]	1·27	1·54	0·91	7·3	4·6	7·4	4·7	3·9	4·5
Serine	26·20	25·79	2·21[d]	1·08	3·01	1·92	2·58	1·76	8·6	4·2	11·5	7·5	5·2	5·6
Leucine	48·59	49·48	1·96	2·09	1·98	2·25	1·42	1·69	4·0	4·2	4·1	4·5	3·5	3·4
Isoleucine	27·47	29·33	1·66	1·56	1·66	1·83	1·17	1·46	5·7	5·4	6·0	6·2	4·6	4·3
Proline	28·28	27·59	2·06[d]	1·12	4·06[d]	1·26	3·79	0·98	7·5	4·1	14·4	4·6	5·6	0·9
Methionine	20·15	19·99	1·38[d]	0·91	2·73[d]	1·64	2·55	1·50	6·9	4·6	13·6	8·2	7·0	4·5
Glutamic acid	82·57	86·83	7·22[d]	3·08	10.93[d]	4·62	9·67	4·07	8·3	3·5	13·2	5·3	4·4	3·7
Aspartic acid	58·29	59·77	5·77[d]	2·54	7·35[d]	3·08	6·12	2·51	9·7	4·2	12·6	5·2	4·7	4·0
Phenylananine	25·19	24·65	1·34	0·99	1·47	1·32	1·12	1·11	5·4	4·0	5·8	5·3	3·8	3·8
Lysine	48·88	48·56	2·96	2·48	5·26[d]	2·33	4·83	1·54	6·1	5·1	10·8	4·8	6·3	5·2
Tyrosine	19·58	19·40	1·20	1·50	1·70	2·26	1·47	2·00	6·2	7·7	8·7	11·7	6·5	7·7
Arginine	37·96	38·41	1·38	3·21[c]	3·13	4·57	2·97	3·96	3·6	8·4	8·2	11·9	5·0	4·9
Histidine	14·36	12·40	1·20	2·48[c]	1·86	2·48	1·65	1·15	9·7	20·0	13·0	20·0	9·3	9·0

[a] No significant differences between the overall means for the two methods.
[b] The standard deviation of the overall mean can be found using (coefficient of variation × overall mean)/100.
[c] Standard deviation of GC method significantly greater than IE at 5% level.
[d] Standard deviation of IE method significantly greater than GC at 1% level except for methionine where reproducibility is significant at 4% only.

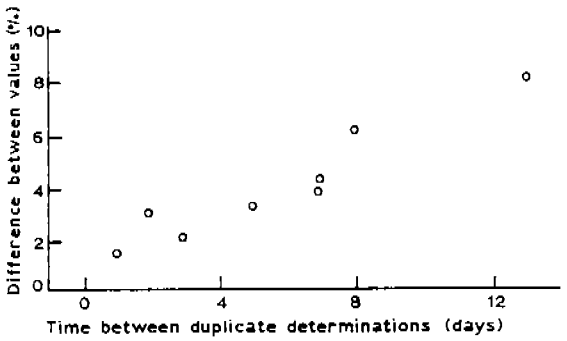

Fig 1. The difference in amino acid values for different samples of the same fish meal as related to the time delay between determinations in Laboratory 4. (Figures on the horizontal axis are sample numbers, but reflect time as the samples were analysed in numerical order.)

The repeatability standard deviations for the two methods (Table 4) differed ($P < 0{\cdot}05$) in nine out of 16 amino acids. In six out of these nine, the GC method had a smaller within-laboratory variance. The presence of outlying values in the IE method for two amino acids (glutamic and aspartic) may have caused significant differences to be found in these cases.

No significant differences were found between the overall means as estimated by the two methods.

In both methods the between-laboratory variance component (σ_L^2) was significantly different from zero ($P < 0{\cdot}05$) for all but six amino acids using IE and all but five using GC, indicating differences in values from one laboratory to another for most amino acids (see Table 5). Significant laboratory × meal interactions were found for proline, methionine, glutamic acid, lysine and arginine when determined by the IE method. There were no significant interactions in the results of the laboratories using the GC method. No attempt was made to identify which particular values caused these interactions.

4.2 Results with standard samples (Tables 6 to 8)

Considering the small number of laboratories involved and the lack of obvious and systematic differences in estimates by the two analytical methods, no attempt was made to differentiate between the two methods as regards statistical treatment of the amino acid compositions of the standards.

For Standard A, the means of estimates from the six laboratories differed significantly from the anticipated value for aspartic acid and isoleucine ($P < 0{\cdot}01$) and histidine ($P < 0{\cdot}05$). It is noteworthy that in the case of isoleucine and histidine all the analytical values, ie irrespective of which method of determination was used, were smaller than the theoretical value whereas in the case of aspartic acid all were greater.

For standard B, significant differences were found for serine ($P < 0{\cdot}01$) and for glycine, valine, threonine, methionine, aspartic acid, phenylalanine, tyrosine and histidine ($P < 0{\cdot}05$). With very few exceptions, the determined values were smaller than the theoretical values, irrespective of the method of analysis used.

TABLE 5
Estimates of the between- and within-laboratory components of variance in mg g^{-1} sample based on the entire data set

	σ_F^2	σ_L^2	σ_{LF}^2	σ_e^2	$\sigma_{L/\bar{y}}^2$ %
			IE		
Alanine	5·69	−0·124	1·243	2·679	—
Glycine	8·62	0·333	−0·042	3·574	1·4
Valine	10·07	5·067*	−0·829	2·996	7·1
Threonine	8·38	0·361	−0·004	4·051	2·1
Serine	8·20	2·905*	1·288	4·886	6·5
Leucine	22·53	0·285	−0·180	3·828	1·1
Isoleucine	11·37	0·639*	−0·660	2·764	2·9
Proline	−0·14	9·548*	2·697	4·253	10·4
Methionine	7·33	3·946*	1·621	1·904	9·9
Glutamic acid	39·81	24·680*	42·730	52·101	6·0
Aspartic acid	34·81	9·613*	11·113	33·330	5·3
Phenylalanine	6·81	0·180	0·188	1·785	1·7
Lysine	45·56	14·152*	4·779	8·780	7·7
Tyrosine	9·90	1·345*	0·089	1·452	5·5
Arginine	15·43	6·256*	1·634	1·890	6·6
Histidine	13·05	0·330	1·682	1·432	4·0
			GC		
Alanine	4·76	3·496*	0·610	5·204	4·4
Glycine	8·37	2·078*	−0·226	3·984	3·3
Valine	10·79	2·504*	−0·184	2·933	4·7
Threonine	11·28	0·167	−0·127	1·581	1·5
Serine	10·85	1·939*	0·582	1·177	5·4
Leucine	20·55	0·249	0·444	4·356	1·0
Isoleucine	9·54	0·980*	−0·050	2·420	3·4
Proline	0·43	−0·121	0·461	1·253	—
Methionine	6·24	−0·220	2·067	0·829	—
Glutamic acid	49·49	10·867*	0·993	9·490	3·8
Aspartic acid	36·77	3·120*	−0·041	6·434	3·0
Phenylalanine	5·90	0·362*	0·391	0·984	2·4
Lysine	50·59	−0·082	−0·633	6·155	—
Tyrosine	11·97	1·903*	0·987	2·236	7·1
Arginine	12·27	4·575*	5·978	10·333	5·6
Histidine	6·49	1·934*	−1·974	6·169	11·2

* Significantly different from zero.

Values obtained for Standard C, averaged over six laboratories, were very similar to those obtained for meal 4/11 of which Standard C was a centrally prepared hydrolysate. However, with the exception of serine, lysine and aspartic acid, they tended to be smaller.

5 DISCUSSION AND CONCLUSIONS

Consideration of the part of this study that deals with fish meals suggests that between-laboratory differences in estimates for certain amino acids are alarming.

TABLE 6
Amino acid composition of Standard A, an amino acid mixture, in mg g^{-1} sample

Amino acid	*Actual composition*	*Lab 1 (IE)*	*Lab 18 (IE)*	*Lab 19 (IE)*	*Lab 8 (GC)*	*Lab 13 (GC)*	*Lab 14 (GC)*	*Mean*	*Standard deviation*	*Outlier test*	t-*value*
Alanine	71·62	58·0	78·9	69·6	73·5	68·3	79·0	71·2	7·9	1·68	0·12
Glycine	66·56	56·0	73·5	63·9	67·8	63·3	75·0	66·6	7·1	1·50	0·01
Valine	61·35	50·0	65·2	61·9	61·2	58·7	64·0	60·2	5·5	1·86	0·53
Threonine	48·66	45·0	51·0	50·5	43·1	48·8	51·0	48·2	3·4	1·50	0·35
Serine	42·94	38·0	50·2	44·0	39·2	44·9	45·0	43·6	4·4	1·50	0·34
Leucine	87·03	77·0	87·0	86·7	85·8	84·8	88·0	84·9	4·0	1·97††	1·31
Isoleucine	49·73	41·0	46·5	41·3	43·6	45·8	46·0	44·0	2·5	1·24	5·70**
Proline	46·05	—	55·3	44·6	46·6	45·5	49·0	48·2	4·3	1·65	1·13
Methionine	30·70	28·0	—	31·4	25·2	30·5	34·0	29·9	3·3	1·34	0·56
Glutamic acid	143·28	88·0	144·0	149·0	148·7	147·4	146·0	137·2	24·2	2·04††	0·62
Aspartic acid	102·31	113·0	110·6	109·1	107·4	104·4	105·0	108·2	3·3	1·43	4·37**
Phenylalanine	46·08	40·0	49·6	47·0	48·6	47·0	45·0	46·2	3·4	1·81	0·09
Lysine	69·66	73·0	75·1	67·7	62·0	73·6	76·0	71·3	5·4	1·72	0·73
Tyrosine	35·86	30·0	35·7	36·6	28·2	38·5	36·0	34·2	4·1	1·46	1·02
Arginine	66·57	56·0	69·0	72·3	52·8	76·7	80·0	67·8	11·1	1·35	0·27
Histidine	21·57	19·0	20·6	19·8	15·8	14·9	—	18·0	2·5	1·24	3·15*

** Laboratory mean significantly different from actual composition, $P < 0·01$.
* Laboratory mean significantly different from actual composition, $P < 0·05$.
†† Significant, $P < 0·01$.

TABLE 7

Amino acid composition of Standard B (beef haemoglobin) in mg g^{-1} sample

Amino acid	*Theoretical composition*[a]	*Lab 1 (IE)*	*Lab 18 (IE)*	*Lab 19 (IE)*	*Lab 8 (GC)*	*Lab 13 (GC)*	*Lab 14 (GC)*	*Mean*	*Standard deviation*	*Outlier test*	*t-value*
Alanine	94·75	77·0	81·0	95·1	94·5	88·4	106·5	90·4	10·7	1·51	0·99
Glycine	42·14	41·0	45·5	45·6	44·1	42·9	51·5	45·1	3·6	1·79	2·03*
Valine	103·77	73·0	89·9	93·2	96·1	81·7	110·0	90·7	12·7	1·53	2·54*
Threonine	49·24	43·0	43·5	50·2	47·4	43·8	49·5	46·2	3·2	1·24	2·30*
Serine	58·98	51·0	43·7	53·6	51·4	50·0	51·0	50·1	3·4	1·91†	6·44**
Leucine	143·0	120·0	131·1	141·1	137·5	134·5	159·5	137·3	13·1	1·70	1·14
Proline	34·00	—	31·6	32·8	31·6	32·2	36·0	32·8	1·8	1·72	1·56
Methionine	17·63	—	12·3	13·0	10·1	13·5	18·0	13·4	2·9	1·13	3·62*
Glutamic acid	73·86	62·0	67·5	77·2	70·1	71·7	81·0	71·6	6·8	1·41	0·82
Aspartic acid	110·08	103·0	94·9	105·0	101·4	100·3	113·0	102·9	6·0	1·68	2·92*
Phenylalanine	82·95	73·0	73·5	83·5	74·3	77·3	82·0	77·3	4·5	1·38	3·08*
Lysine	95·01	95·0	85·5	35·3	101·6	96·0	120·5	89·0	28·8	1·87†	0·34
Tyrosine	26·77	21·0	21·3	28·0	18·1	24·7	23·5	22·8	3·4	1·53	2·87*
Arginine	36·02	32·0	34·6	14·7	38·3	34·8	47·5	33·7	10·8	1·76	0·54
Histidine	77·93	58·0	62·3	25·3	83·0	54·6	—	56·7	20·5	1·53	2·53*
Cysteine	3·58	—	4·1	—	—	—	—	4·1	—	—	—

[a] Ex: *Atlas of Protein Sequence and Structure*, Vol 4, ed Dayhoff M O. National Biomedical Research Foundation, Silver Spring, MD, 1969 (corrected for the fact that beef haemoglobin contains about 4% non-protein material).

** Laboratory mean significantly different from theoretical composition, $P<0·01$.

* Laboratory mean significantly different from theoretical composition, $P<0·05$.

† Significant, $P<0·05$.

TABLE 8

Total amino acid analysis of Standard C, a centrally prepared hydrolysate of Meal 4/11[a], in mg g^{-1} sample

Amino acid	*Lab 1 (IE)*	*Lab 18 (IE)*	*Lab 19 (IE)*	*Lab 8 (GC)*	*Lab 13 (GC)*	*Lab 14 (GC)*	*Mean*	*Standard deviation*	*Outlier test*
Alanine	36·7	49·9	44·9	46·6	45·0	53·0	46·0	5·5	1·68
Glycine	37·3	40·1	39·6	43·7	42·5	52·0	42·5	5·2	1·01
Valine	29·3	38·1	35·4	35·8	35·0	40·0	35·6	3·6	1·73
Threonine	29·3	31·7	32·8	29·9	32·3	36·0	32·0	2·4	1·13
Serine	24·7	29·4	30·1	29·6	31·4	33·0	29·7	2·8	1·80
Leucine	45·3	51·9	52·8	50·7	52·4	59·0	52·0	4·4	1·53
Isoleucine	27·3	30·0	29·7	28·2	30·1	32·0	29·5	1·6	1·36
Proline	—	28·0	27·3	26·1	28·2	30·0	27·9	1·4	1·28
Hydroxyproline	—	—	—	2·4	6·6	6·0	5·0	2·3	—
Methionine	15·3	—	19·5	17·9	18·7	21·0	18·5	2·1	—
Glutamic acid	66·7	89·1	100·0	96·2	100·3	106·0	93·1	14·1	1·87*
Aspartic acid	58·7	68·5	66·1	68·1	68·6	73·0	67·2	4·7	1·79
Phenylalanine	23·3	26·5	26·7	26·1	27·1	27·0	26·1	1·4	1·98**
Lysine	62·7	51·3	52·9	54·0	59·7	65·0	57·6	5·7	—
Tyrosine	17·3	21·2	21·2	17·0	22·4	20·0	19·9	2·2	1·28
Arginine	33·3	36·5	45·8	34·9	45·8	50·0	41·1	7·0	0·75
Histidine	14·0	14·0	14·9	12·0	13·1	—[b]	13·6	1·1	1·45

[a] See Table 5 for mean amino acid composition of this meal.
[b] Not determined in this laboratory.
* Significant, $P < 0{\cdot}05$.
** Significant, $P < 0{\cdot}01$.

Not only were between-laboratory variations statistically significant for most amino acids, but also significant fish meal × laboratory interaction existed in several cases showing the ranking of meals to be different in the various laboratories.

In an attempt to explain reasons for poor reproducibility, the chromatograms from three laboratories that supplied data containing outlying values (laboratories 1, 13 and 18) were scrutinised. The amino acid values of each IE analysis were calculated as a percentage of the mean values obtained by GC given in Table 4. The GC values were used as totally independent estimates of the true values for these meals. From this it became apparent that in laboratory 18 four runs done in close succession all had discrepant values for serine, aspartic acid and glutamic acid, even though the chromatograms gave no indication of anything amiss. If the ninhydrin reagent had in some way deteriorated, this did not affect the internal standard nor the other amino acids. The consistency of the threonine estimated values in these assays is particularly notable since the sequence of elution of the first four amino acids is aspartic acid, threonine, serine, glutamic acid. Laboratory 18 also obtained values for proline for meals 2, 3, 4 and 5 which were 127% to 140% of the corresponding GC means. These were the first runs, conducted immediately following a calibration run with the laboratory's own standard which gave a standard factor for the ratio internal standard:proline of 2·9 compared with values

of 4·0 and 4·3 for later standard runs. Results were calculated by the laboratory using the mean factor from the three standards, which for proline was 3·75. If the first standard was the correct one to use for the first four analyses, the use of the mean value instead would result in estimates which are 127% of the 'correct' values. It was noted that other amino acids in samples 2, 3, 4 and 5 were reasonably well estimated using mean colour factors. Again, one is forced to the conclusion that variation occurred in the colour yield of some amino acids to a greater extent than in others and that the use of an internal standard did not compensate for this variation. Similarly, in laboratory 1, marked differences were obtained in some runs for aspartic acid, serine and alanine while other amino acids in the same chromatogram were correctly estimated.

Clearly, in duplicate chromatograms where the majority of peaks show good agreement, large differences may be found for just one amino acid. It is unlikely that this is due to sampling, or (except for methionine and cystine) loss during hydrolysis. It cannot be due to errors in adding internal standard. The most likely explanation is temporary fluctuation in photometric response due to factors such as pumping rate of ninhydrin, temperature of heating bath, pH of solution or voltage of photometer. The need for vigilance against the occurrence of such variation is stressed.

No reason could be found for the outlier estimates supplied by laboratory 13, which used GC. The three values were 11% to 20% lower than the GC means for the amino acids in the particular meals. As there was a significant between-laboratory variance, with laboratory 14 giving consistently greater values than either laboratories 8 or 13, a relatively small downward deviation from a laboratory with a low average could appear as an outlier result.

For both methods of analysis it is clearly necessary that replicate analyses be performed to detect aberrant values, that such replicates should not run in close sequence but as far apart as possible, and that standards should be run at frequent intervals.

Hydrolysis procedures have already been shown to be a major source of variation in amino acid determinations (Porter *et al* 1968; Knipfel *et al* 1971; Bech-Andersen *et al* 1979; Rudemo *et al* 1979, 1980). This must be taken into consideration also when the repeatabilities of measurements with laboratories using the two different methods of determination are compared. On the face of it the laboratories using GC generally had better repeatability, ie smaller within-laboratory variation. However, the degree of confidence of these variance measurements is low and in only five instances, of which three involved outlying values in the IE results and one in the GC results, did the expected variances not overlap. Also, the methods of hydrolysis used by the three laboratories using GC were more similar than the methods used by the IE laboratories. Conclusions regarding the accuracy of the two measurement techniques *per se* should be reserved.

It was anticipated that comparison of estimates obtained for meal 4/11 with those for Standard C, which was a centrally prepared hydrolysate of that same meal, would illustrate the contribution of different hydrolysis procedures used at the participating laboratories to between-laboratory variation. Contrary to expectation, the between-laboratory variation was greater for the centrally

TABLE 9
Variances of total amino acid analysis of meal 4/11 and Standard C (bulk hydrolysate)

Amino acid	*Variance meal 4/11*	*Degrees of freedom*	*Variance bulk hydrolysate*	*Degrees of freedom*	F *ratio* $\frac{Bulk}{4/11}$
Alanine	7·15	12	30·84	5	4·31*
Glycine	3·07	12	26·56	5	8·65*
Valine	5·96	12	13·07	5	2·19
Threonine	4·00	12	5·65	5	1·41
Serine	5·47	12	7·86	5	1·44
Leucine	5·15	12	19·18	5	3·72*
Isoleucine	1·55	12	2·67	5	1·72
Proline	6·94	10	2·03	4	0·29
Hydroxyproline	2·32	6	5·29	2	2·28
Methionine	6·88	12	9·93	5	1·44
Glutamic acid	36·39	12	198·00	5	5·44*
Aspartic acid	11·03	12	22·45	5	2·04
Phenylalanine	2·03	12	1·98	5	0·98
Lysine	25·07	12	31·94	5	1·27
Tyrosine	4·25	12	4·96	5	1·67
Arginine	9·27	12	48·75	5	5·26*
Histidine	0·97	10	1·18	4	1·22
Cysteine	2·23	6	—	—	—

* Significant at $P < 0{\cdot}05$.

prepared hydrolysate (Table 9). This effect was, to a large extent, due to the smaller values obtained for Standard C at laboratory 1. However, the outlier test

$$\frac{\text{Mean over all laboratories} - \text{laboratory 1 value}}{\text{SE (all laboratories' results)}}$$

applied to the individual amino acids indicated the laboratory 1 values for phenylalanine ($P < 0{\cdot}01$) and glutamic acid ($P < 0{\cdot}05$) only to be outliers. It seems as if the observation made at one of the laboratories, viz that the hydrolysate on receipt from the central laboratory had a sediment, was a significant observation and that the distribution of hydrolysates to laboratories at ambient temperature over long distances was not to be recommended.

The analysis of beef haemoglobin for most amino acids gave values lower than those expected on the basis of amino acid sequence analysis even though these theoretical values were corrected to take account of the haem content of the beef haemoglobin. The moisture content of the beef haemoglobin test sample was not checked but might be responsible for the discrepancy.

5.1 Comparison with results of other collaborative studies

Coefficients of variation for repeatability (within-laboratory variation) derived from the estimated means and variances shown in Table 5 varied from 3·5% to 9·7% for IE and 3·5% to 8·4% with histidine at 20·0% for GC (Table 4). These values are of the same order as those recorded by others (Bender *et al* 1959; Cavins *et al* 1972; Kirkman 1974; Tajima *et al* 1978; Williams *et al* 1980; Kreienbring 1981; Andersen

et al 1984) but generally not as low as the values reported by Sarwar *et al* (1983) for IE analysis of protein concentrates. However, the latter authors (Sarwar *et al* 1983) have reduced their values by a factor of $\sqrt{2}$ to give the repeatability of a mean of duplicate determinations within a laboratory. Therefore, their values are not directly comparable.

Coefficients of variation for reproducibility (between-laboratory variation) varied from 4·1% to 14·4% for IE and 4·5% to 11·9%, again with histidine at 20·0%, for GC. The values for IE are greater for lysine, histidine, proline, aspartic acid and serine but similar or less for the remaining amino acids compared with those reported by Sarwar *et al* (1983). However, Sarwar *et al* (1983) also underestimated their coefficient of variation for reproducibility, which was calculated as $100\sigma_A/\sqrt{2}$ (mean) where σ_A^2 is the between-laboratories mean square, assuming duplicate determinations. Similarly, IE coefficients of variation for lysine, proline, aspartic acid, serine and methionine are greater whereas values for other amino acids are similar or less than those reported by Andersen *et al* (1984) for fish meal. Our coefficients of variation were obtained using the complete data set, except for the withdrawal of all results by laboratory 4, ie outliers were not discarded. In contrast, the values reported by Andersen *et al* (1984) were obtained after excluding 27% of the data.

5.2 IE vs GC

In only two (Kirkman 1974; Tajima *et al* 1978) of the four comparative studies (Gehrke and Leimer 1971; Kirkman 1974; March 1975; Tajima *et al* 1978) between the methods was statistical evaluation attempted. None of these was a collaborative exercise. In all four studies GC separation was performed of heptafluorobutyric *n*-propyl derivatives, among other derivatives (Gehrke and Leimer 1971). In one of the instances (March 1975), determined amino acid contents of enzymes were compared with theoretical composition figures and, with the exception of certain amino acids which could not then be determined by GC, the methods gave similar results. In the other instance, blood plasma and soya bean meal were found to be analysed equally well by both methods (Gehrke and Leimer 1971). Kirkman (1974) found that, apart from histidine, the composition of a mixture of amino acids was adequately determined by GC compared with IE. His values for alanine and arginine showed a greater spread, but in respect of the majority of the other amino acids (excluding methionine and cystine for which comparative figures were not available) GC determination gave lower relative standard deviations. IE gave the poorest reproducibility in respect of proline.

High coefficients of variation by both methods were a feature of the other study (Tajima *et al* 1978). For defatted soya bean powder, which most closely resembles fish meal, the IE results showed higher repeatability coefficients of variation in respect of 10 out of the 15 amino acids analysed by both methods. For IE the poorest coefficients of variation were in respect of methionine (51·1%), valine (33·7%), lysine (12·7%), aspartic acid (11·4%), arginine (10·1%), glutamic acid (10·0%) and serine (9·3%). For GC the poorest repeatability coefficients of variation were in respect of arginine (21·7%), lysine (16·6%), tyrosine (13·2%), glutamic acid (11·1%) and proline (7·6%).

In conclusion this work confirms the need for replicate analyses of samples by both IE and GC methods of amino acid analysis, and has exposed certain pitfalls, particularly in the IE methodology. Replicate analyses should not be run consecutively. Neither of the methods was demonstrated to be clearly superior for the purpose of the determination of the amino acid content of fish meals. On the basis of the results obtained from the small number of laboratories that took part in this cooperative study, it appears that the use of the GC method tended to result in lower within-laboratory variance, with arginine and histidine as exceptions.

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