Effect of Hydrolysis Time on the Determination of the Amino Acid Composition of Diet, Ileal Digesta, and Feces Samples and on the Determination of Dietary Amino Acid Digestibility Coefficients

Angela M. Rowan,† Paul J. Moughan,*,‡ and Margaret N. Wilson†

Department of Chemistry and Biochemistry and Department of Animal Science, Massey University, Palmerston North, New Zealand

A study was undertaken to determine the effect of hydrolysis time during amino acid analysis on individual amino acid yields from samples of a mixed diet, pig ileal digesta, and feces. Representative feed, digesta, and feces samples were hydrolyzed in duplicate in 6 M HCl in sealed evacuated tubes for 16, 24, 48, and 72 h, respectively, and then analyzed for their yields of alanine, arginine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine. There was a significant ($P < 0.05$) curvilinear effect of hydrolysis time on the yields of all amino acids except tyrosine. The changes in isoleucine, lysine, and serine yields with hydrolysis time were parallel for the three types of samples, but for the other amino acids (except tyrosine) there were significant ($P < 0.05$) hydrolysis time–source interactions. This would introduce error into the calculation of amino acid digestibility coefficients if samples were only hydrolyzed for a standard time. To minimize the error due to incomplete hydrolysis or partial loss of amino acids, correction factors for each type of sample were calculated and can be applied to other samples.

INTRODUCTION

Determination of the digestibility of amino acids in food, measured either at the terminal ileum (ileal) or over the entire digestive tract (fecal), is an important aspect of animal feed evaluation. Animals are fed the feedstuff to be evaluated, under standard assay conditions, and excreta are collected. The digestion coefficients for each amino acid are determined as the difference between feed amino acid intake and excreta amino acid output, expressed as a proportion of feed amino acid intake. In the calculation of the coefficient it is assumed that the amino acid contents of the food and excreta samples are known with complete accuracy. Unfortunately, however, this is seldom the case. It is now well established that the standard 24-h hydrolysis of protein in 6 M hydrochloric acid at 110 °C does not allow a quantitative determination of amino acid content to be made. There are partial losses of some amino acids (e.g., tyrosine, serine, and threonine) during the 24-h hydrolysis (Rees, 1946), while for other amino acids (e.g., valine, leucine, and isoleucine) a longer hydrolysis time may be required to obtain the maximum yield (Blackburn, 1968).

If the amounts of an amino acid in the food and excreta samples are underestimated to the same extent, then the calculated digestibility coefficient will be unaffected by the inaccuracy of the amino acid determination. However, if the amino acid contents of the food and excreta are underestimated by different proportions, this will lead to inaccuracy in the determination of digestibility. The extent of amino acid release or breakdown during the standard hydrolysis of protein is known to differ for different protein sources (Glazer et al., 1976).

The aim of the present study was to ascertain the relative effect of hydrolysis time on the amounts of amino acids in feed, ileal digesta, and feces samples. Where there was an effect of hydrolysis time on amino acid content of diet, feces, or ileal digesta samples, correction factors were determined.

MATERIALS AND METHODS

Procedure. Representative samples of ileal digesta and feces were obtained following standard procedures, from ileostomized and intact growing pigs (around 25–28 kg live weight) which had been fed a mixed, human-type diet comprising meat, vegetables, fruit, cereal, and dairy products (Rowan et al., 1992). Five separate 24-h collections of ileal digesta or feces were pooled to give composite samples of ileal digesta and feces, respectively. The composite ileal digesta and feces samples along with a representative sample of the diet were each weighed, frozen, freeze-dried, finely ground (1-mm sieve), and mixed for storage at −20 °C while awaiting chemical analysis.

Chemical Analysis. Duplicate samples (50 mg) of the diet, ileal digesta, and feces were accurately weighed into large (30 mL) hydrolysis tubes. To each tube was added 10 mL of glass-distilled 6 M HCl (containing 0.5 mg of phenol/mL), and the contents of the tubes were mixed. The tubes were evacuated and sealed, and hydrolysis was carried out at 110 ± 2 °C in a forced-air oven for 16, 24, 48, or 72 h for each type of sample. It was assumed that amino acid loss is minimal at 16 h and that by 72 h protein hydrolysis will be complete (Mahowald et al., 1962; Noltmann et al., 1962; Kohler and Palter, 1967; Tkachuk and Irvine, 1969). After hydrolysis, the contents of each tube were quantitatively transferred to 100-mL volumetric flasks and made up to volume with deionized water. The hydrolysates were filtered through Whatman No. 3 filter paper with the first 3–5 mL being discarded. Then 2 mL of the filtrate was evaporated to dryness under vacuum, and the dried samples were stored at −20 °C until analysis. Loading buffer (sodium citrate) was added to each dried sample immediately prior to loading onto a Beckman 119 BL amino acid analyzer. The amino acids were separated by ion-exchange chromatography and detected following reaction with ninhydrin. Methionine, cysteine, and tryptophan were not determined.

Data Analysis. The data for amino acid yield were analyzed statistically to determine whether there was an effect of time on amino acid yield and whether this effect differed with the source of material (interaction). A linear model that included terms for source, a linear effect of time, departure from linearity of the time effect (curvilinearity), linear time × source, and departure from linearity × source was fitted to the data for each amino acid

* Author to whom correspondence should be addressed.
† Department of Chemistry and Biochemistry.
‡ Department of Animal Science.

0021-8561/92/1440-0981$03.00/0

Table I. Statistical Significance[a] of a Linear Effect of Hydrolysis Time, Departure from a Linear Effect of Time, and Their Respective Interactions with Source of Material on Amino Acid Yield after the Hydrolysis (16, 24, 48, or 72 h) of a Mixed Diet, Ileal Digesta, and Feces

	significance[b] of time effect			
	main effect		interaction	
amino acid	linear	curvilinear	LT–S[c]	CT–S[d]
tyrosine	NS	NS	NS	NS
alanine				***
arginine				**
aspartate				***
glutamate				***
glycine				***
histidine				***
leucine				***
phenylalanine				***
proline				***
threonine				**
valine				*
lysine		***	***	NS
serine		*	***	NS
isoleucine		*	NS	NS

[a] Determined (reduction in sums of squares) by fitting a linear model which included terms for source, linear effect of time, departure from linearity (curvilinear effect), linear time × source, and departure from linear time × source. [b] NS, nonsignificant; *, significant $P < 0.05$; **, significant $P < 0.01$; ***, significant $P < 0.001$. [c] LT–S, linear time–source interaction. [d] CT–S, curvilinear time–source interaction.

separately and reduction in sums of squares used to determine levels of significance.

RESULTS

The difference in amino acid content (expressed as a proportion of the mean value) between duplicate samples was generally less than 5% units, regardless of the type of material analyzed. As shown in Table I, there was a statistically significant ($P < 0.05$) effect of hydrolysis time on amino acid yield for all amino acids examined except tyrosine. There was no change in tyrosine yield with hydrolysis time for all samples. For the amino acids alanine, arginine, aspartate, glutamate, glycine, histidine, leucine, phenylalanine, proline, threonine, and valine there was a significant ($P < 0.05$) curvilinear effect of time–source interaction. That is, amino acid contents changed over time in a curvilinear manner, but the changes were not the same for the diet, feces, and ileal digesta.

The curvilinear effect of time–source interaction was not significant ($P > 0.05$) for lysine and serine. The fitting of curvilinear and linear effect of time–source interaction terms in the statistical model, however, accounted for significant proportions of the total variance. A curvilinear relationship is more satisfactory, based on an understanding of the process of hydrolysis, and the curvilinear model was accepted as providing the best description of the lysine and serine data. For isoleucine, both interaction terms were nonsignificant ($P > 0.05$) but there was a significant ($P < 0.05$) curvilinear effect of time on amino acid yield.

The mean amino acid concentrations at each hydrolysis time and for each source of material are shown in Figures 1 and 2. For serine, there was a very small decrease in concentration over time of hydrolysis for the diet and ileal samples but a more marked decrease with the fecal material. The concentrations of arginine, glycine, valine, alanine, isoleucine, lysine, and phenylalanine generally increased over time of hydrolysis, but the increases were often small in magnitude. Threonine concentrations declined over time with the decrease being especially marked in the feces. There was also a gradual increase with time for glutamate concentration in the diet and ileal digesta samples, but for the feces samples glutamate decreased with time of hydrolysis. The determined leucine content of the diet sample increased gradually, although there was a more pronounced increase with the ileal digesta especially from 16 to 48 h of hydrolysis, but the fecal value gradually decreased with time of hydrolysis. There was little change in the determined concentration of aspartate for feces and diet but a more marked increase in the ileal digesta, especially over the first 16–24 h of hydrolysis. The determined histidine content of ileal digesta and diet samples increased with duration of hydrolysis, but for the feces there was an increase over the period from 16 to 24 h of hydrolysis followed by a decline to the levels at 16 h. Proline content of the ileal digesta and feces samples appeared to be largely unaffected by the length of hydrolysis, but in the diet sample the determined proline content tended to increase to 48 h and then decrease with a further extension of hydrolysis time.

For the amino acids showing a curvilinear effect of hydrolysis time on amino acid yield, factors were calculated (Table II) to allow correction of amino acid yield after a standard 24-h hydrolysis interval. The correction factor is the ratio of the maximum determined yield for the respective amino acid to the yield obtained after 24 h of hydrolysis.

DISCUSSION

It is well established (Rees, 1946; Blackburn, 1968; Kohler and Palter, 1967; Mawer and Nixon, 1969; Tkachuk and Irvine, 1969; Davies and Thomas, 1973; Finley, 1985; Garnett, 1985) that valine and isoleucine are released only slowly during acid hydrolysis while the labile amino acids, serine and threonine, are continuously destroyed. The slow release of valine and isoleucine was also observed in the present study, but this was more marked for isoleucine than for valine. For the diet sample there was little change in the determined valine content with time of hydrolysis. The determined level of threonine declined markedly over time for the feces sample but only very gradually for the diet and ileal digesta samples. Likewise, there was a sharp decrease in the serine content of the feces over time, as reported in other studies, but the decrease over time for serine in the diet and digesta was minimal. Although tyrosine is particularly susceptible to oxidation (Finley, 1985), there was no effect of hydrolysis time in the present study, which may have been due to the protective action of the added phenol (Glazer et al., 1976). In the present work some amino acids other than valine and isoleucine (e.g., glycine and glutamate) had markedly increased concentrations subsequent to the standard 24 h of hydrolysis. Also, histidine in the fecal material was progressively destroyed to a significant extent with hydrolysis beyond 24 h.

Of particular significance in this study was the finding that changes in amino acid concentration over time of hydrolysis were generally not of the same proportion for the three types of material examined. This has implications for the determination of amino acid digestibility coefficients. Only for the amino acids lysine, serine, and isoleucine were the curvilinear changes over time of similar proportions for the diet, digesta, and feces samples. For these three amino acids, values determined after a standard 24-h acid hydrolysis, although not quantitatively accurate, may be used to accurately determine coefficients of digestion. For the remaining amino acids, however, but with the exception of tyrosine where no effect of hydrolysis time was observed, use of compositional values after a standard 24-h hydrolysis, for calculation of digestibility

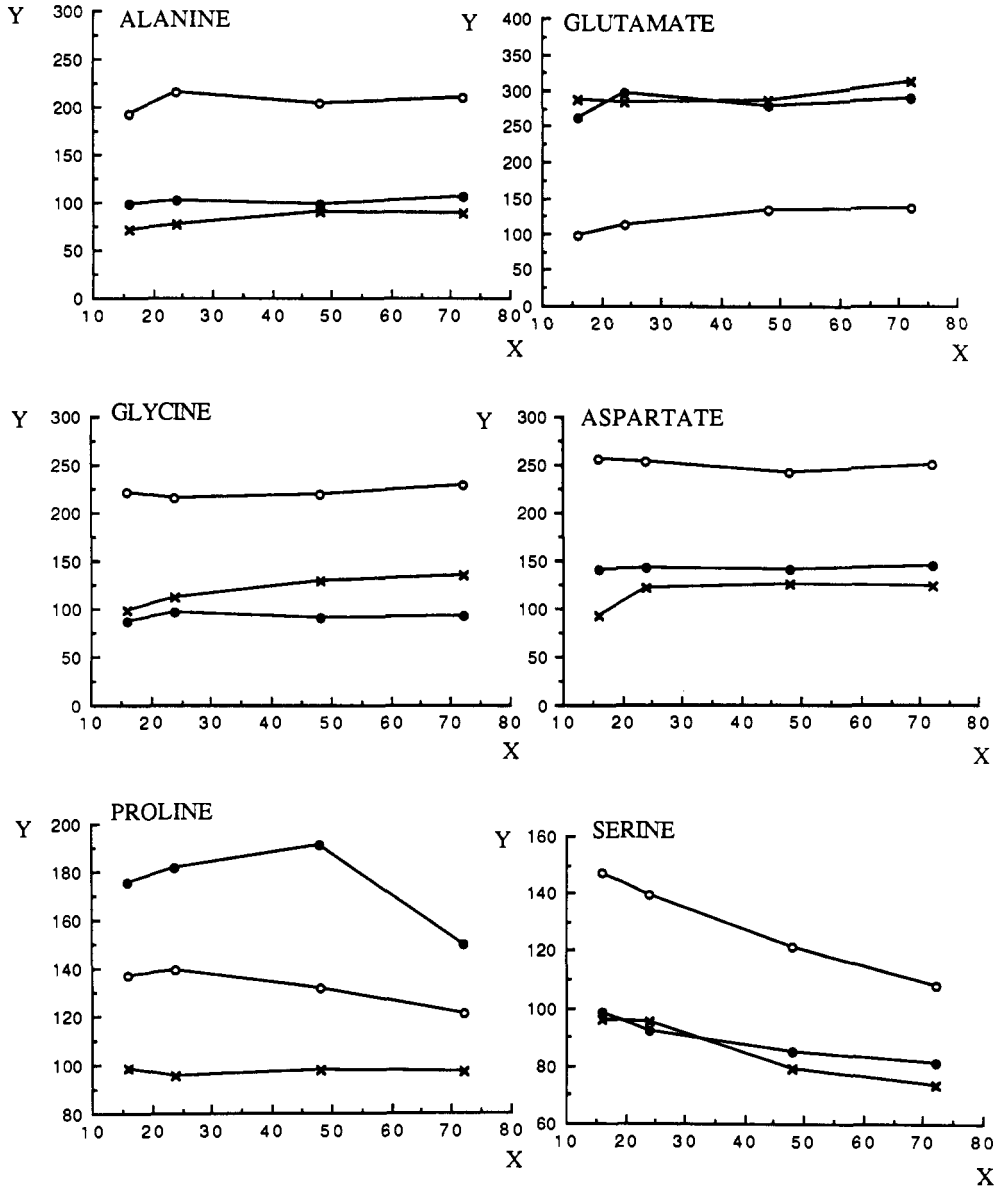

Figure 1. Effect of hydrolysis time (X axis, hours) during amino acid analysis on the mean yield of the nonessential amino acids (Y axis, mmol/g of freeze-dried sample) from diet (solid circles), ileal digesta (crosses), and feces (open circles) for those amino acids showing a curvilinear response.

coefficients, will lead to error. By way of example and in the most extreme case, the coefficient of apparent ileal digestibility for glycine using values obtained at 24 h was 0.734, whereas the coefficient determined using the maximal values was 0.661. Overall, however, the differences between the coefficients calculated using 24-h values and those calculated using maximal amino acid values were small (often less than 2% units). Because statistically significant curvilinear effects of time × source were found in the present work, however, and where a high level of accuracy is required in determining digestibility coefficients, correction of amino acid contents after the standard 24 h of hydrolysis is necessary. It should be noted that the maximum yield values during amino acid hydrolysis represent the net effect of release and loss and may not be the same as the total amount of amino acid originally present in the sample. Thus, the derived correction factors are not absolutes.

In view of the different effects of hydrolysis time on amino acid yield for the three types of material, ideally the optimum hydrolysis time should be determined for each type of material separately and maximal values used in calculating digestibility coefficients. Such an approach would be time-consuming and costly, however, and the use of simple generalized correction factors has been suggested as an alternative approach (Garnett, 1985). The correction factors determined for foods in the present study (Table II) were generally similar to values reported in the literature. Kohler and Palter (1967) gave factors for serine, isoleucine, and threonine of 1.08, 1.08, and 1.04, respectively, while Slump (1980) reported values of 1.05, 1.10, 1.08, and 1.07 for threonine, serine, valine, and isoleucine, respectively. Tkachuk and Irvine (1969) reported correction factors of 1.02–1.08, 1.02–1.15, and 1.06–1.14 for threonine, isoleucine, and serine, respectively. Comparable correction factors for the analysis of digesta or feces have not been reported in the literature. The approach of using generalized correction factors, however, has limitations. More accurate extrapolation could be obtained by deriving mathematical functions (Hirs et al., 1954; Robel and Crane, 1972) relating amino acid yield to hydrolysis time.

In conclusion, the presently reported significant hydrolysis time–source interactions indicate that, at least for the diet used here, error will be introduced when amino acid digestibility coefficients are determined on the basis

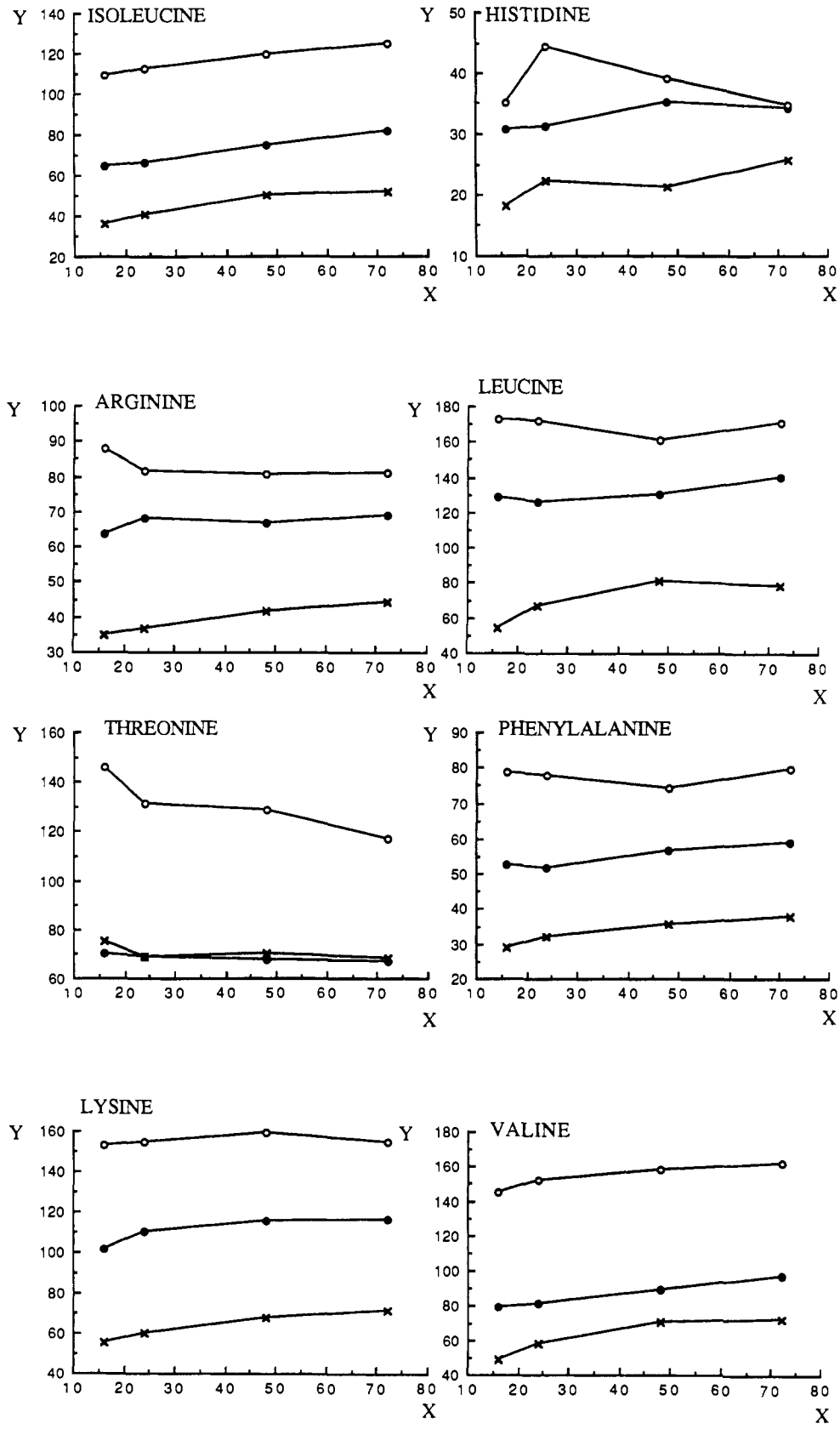

Figure 2. Effect of hydrolysis time (*X* axis, hours) during amino acid analysis on the mean yield of the essential amino acids (*Y* axis, mmol/g of freeze-dried sample) from diet (solid circles), ileal digesta (crosses), and feces (open circles) for those amino acids showing a curvilinear response.

of a single 24-h hydrolysis. Nevertheless, not correcting amino acid values after 24 h of hydrolysis for losses or incomplete release during hydrolysis would appear to generally result in only small differences in the determined digestibility coefficient. Where a high level of accuracy is required, however, multiple hydrolysis times should be used. As it is impractical to routinely submit samples for multiple hydrolyses for amino acid analysis, it is suggested that correction factors be obtained by hydrolyzing single representative samples for a range of times. These

Table II. Correction Factors[a] for the Amino Acids from Diet, Ileal Digesta, and Feces Samples Showing a Curvilinear Effect of Hydrolysis Time Independent of Source of Material (Overall) and Those where There Was a Significant Time–Source Interaction after Hydrolysis Time of 16, 24, 48, or 72 h

amino acid	overall correction factor
isoleucine	1.21
lysine	1.09
serine	1.04

	correction factor		
	diet	ileal digesta	feces
alanine	1.05	1.16	1.00
arginine	1.01	1.20	1.08
aspartate	1.02	1.03	1.00
glutamate	1.11	1.20	1.00
glycine	1.00	1.21	1.06
histidine	1.09	1.19	1.00
leucine	1.11	1.21	1.01
phenylalanine	1.17	1.18	1.03
proline	1.05	1.02	1.00
threonine	1.03	1.09	1.11
valine	1.20	1.25	1.06

[a] Correction factors determined by expressing the maximum amino acid yield as a proportion of the yield at 24 h.

correction factors could then be used to correct amino acid yields of similar samples hydrolyzed for only 24 h. Further work is required to look at a range of diets and to establish the accuracy of applying the same correction factors to all samples of the same type. The variation in correction factors reported in the literature indicates that estimation of correction factors for at least each type of sample (i.e., diet vs excreta) is a minimum requirement for the calculation of amino acid digestibility coefficients.

ACKNOWLEDGMENT

We acknowledge Dr. D. J. Garrick for advice on the statistical analysis.

LITERATURE CITED

Blackburn, S. *Amino acid determination*; Dekker: New York, 1968; p 271.

Davies, M. G.; Thomas, A. J. An investigation of hydrolytic techniques for the amino acid analysis of foodstuffs. *J. Sci. Food Agric.* **1973**, *24*, 1525–1540.

Finley, J. W. Reducing variability in amino acid analysis. In *Digestibility and amino acid availability in cereals and oilseeds*; Finley, J. W., Hopkins, D. T., Eds.; American Association of Cereal Chemists: St. Paul, MN, 1985; pp 15–30.

Garnett, N. K. The influence of amino acid hydrolysis times on the determination of amino acid requirements for poultry. In *Proceedings of the regional seminar on future developments in the poultry industry*; Ideris, A., Hamid, R., Eds.; World Poultry Science Association: Malaysia, 1985; pp 14–17.

Glazer, A. N.; Delange, R. J.; Sigman, D. S. Chemical modification of proteins. In *Laboratory Techniques in Biochemistry and Molecular Biology*; Work, T. S., Work, E., Eds.; North-Holland Publishing: Amsterdam, 1976; Vol. 4, pp 1–205.

Hirs, C. H. W.; Stein, W. H.; Moore, S. The amino acid composition of ribonuclease. *J. Biol. Chem.* **1954**, *211*, 941–950.

Kohler, G. O.; Palter, R. Studies on methods for amino acid analysis of wheat products. *Cereal Chem.* **1967**, *44*, 512–520.

Mahowald, T. A.; Noltmann, E. A.; Kuby, S. A. Studies on adenosine triphosphate transphosphorylase. I. Amino acid composition of adenosine triadenosine 5′-phosphate transphosphorylase (myokinase). *J. Biol. Chem.* **1962**, *237*, 1138–1145.

Mawer, G. E.; Nixon, E. The net absorption of the amino acid constituents of a protein meal in normal and cystinuric subjects. *Clin. Sci.* **1969**, *36*, 463–477.

Noltmann, E. A.; Mahowald, T. A.; Kuby, S. A. Studies on adenosine triphosphate transphosphorylase. II. Amino acid composition of adenosine triphosphate-creatine transphosphorylase. *J. Biol. Chem.* **1962**, *237*, 1146–1154.

Rees, M. W. The estimation of threonine and serine in proteins. *Biochem. J.* **1946**, *40*, 632–640.

Robel, E. J.; Crane, A. B. An accurate method for correcting unknown amino acid losses from protein hydrolysates. *Anal. Biochem.* **1972**, *48*, 233–246.

Rowan, A. M.; Moughan, P. J.; Wilson, M. N. The flows of deoxyribonucleic acid and diaminopimelic acid and the digestibility of dietary fibre components at the terminal ileum, as indicators of microbial activity in the upper digestive tract of ileostomised pigs. *Anim. Feed Sci. Technol.* **1992**, *36*, 129–161.

Slump, P. In *Symposium on protein metabolism and nutrition, Proceedings of the 3rd European Association for Animal Production*; European Association for Animal Production: Wageningen, The Netherlands, 1980; p 14.

Tkachuk, R.; Irvine, G. N. Amino acid compositions of cereals and oilseed meals. *Cereal Chem.* **1969**, *46*, 206–218.

Received for review July 25, 1991. Revised manuscript received December 16, 1991. Accepted February 13, 1992.