Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 89 (2005) 309–314
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Tryptophan determination in proteins and feedstuffs by ion exchange chromatography

G. Ravindran *, W.L. Bryden [1]

Department of Animal Science, University of Sydney, Camden, NSW 2570, Australia

Received 12 November 2003; revised 1 May 2004; accepted 1 May 2004

Abstract

A chromatographic method was developed for the determination of tryptophan content in food and feed proteins. The method involves separation and quantitation of tryptophan (released from protein by alkaline hydrolysis with NaOH) by isocratic ion-exchange chromatography with *O*-phthalaldehyde derivatization followed by fluorescence detection. In this procedure, chromatographic separation of the tryptophan and α-methyl tryptophan, the internal standard, was complete in 15 min, without any interference from other compounds. The precision of the method was 1–4% relative standard deviation. Accuracy was validated by agreement with the value for chicken egg white lysozyme, a sequenced protein, and by quantitative recoveries after spiking with lysozyme. The method allows determination in a range of feed proteins, containing varied concentrations of tryptophan, and is applicable to systems used for routine amino acid analysis by ion-exchange chromatography.

Keywords: Tryptophan; Proteins; Ion-exchange chromatography; Alkaline hydrolysis

1. Introduction

Tryptophan, an essential amino acid, plays an important role in brain function and related regulatory mechanisms. Some of its derivatives are potent drugs (Hussey, 1974). L-tryptophan has also been implicated with an outbreak of eosinophilia–myalgia syndrome in the United States of America and excessive dietary intake of tryptophan is reported to exert atherogenic effects (Doyle, Steinhart, & Cochrane, 1993). Its nutritional and toxicological importance emphasises the need for reliable analytical methods for the determination of tryptophan in food and feed proteins.

The determination of tryptophan presents several analytical problems due to the labile nature of tryptophan in the presence of light and hydrogen ions. Tryptophan, unlike most other amino acids, cannot be determined by routine ionexchange chromatography after acid hydrolysis with 6 M HCl, as it is oxidatively destroyed. It has to be analysed separately or using acid hydrolysis procedures modified to render tryptophan stable. Several procedures have been developed and evaluated over the years, but with variable success. The methods investigated include hydrolysis (acidic, alkaline and enzymatic) of the protein, a necessary prerequisite as a first step, followed by quantitation of tryptophan by different (chromatographic, colorimetric, fluorimetric) techniques (Friedman & Cuq, 1988). These methods are generally limited in accuracy and reproducibility, often as a result of degradation/precipitation losses during hydrolysis or interference by other amino acids or other compounds (lysinoalanine, for example) in the hydrolysate during quantitation.

* Corresponding author at: Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand.
E-mail address: g.ravindran@massey.ac.nz (G. Ravindran).
[1] Present address: School of Animal Studies, University of Queensland, Gatton, Qld 4343, Australia.

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.05.035

Modification of acid hydrolysis to render tryptophan stable is the most convenient approach, but to date no acid hydrolysis procedure capable of yielding full recoveries of tryptophan in feedstuffs has been reported. However, for purified proteins, classical acid hydrolysis will suffice if antioxidants such as thioglycolic acid are added (Yokote, Arai, & Akahane, 1986). The presence of carbohydrate, however, results in complete or near complete destruction of the amino acid (Friedman, Levin, Noma, Montague, & Zahnley, 1984). Hydrolysis using mercaptoethanesulfonic acid (Penke, Fereczi, & Kovacs, 1974) or *p*-toluenesulfonic acid containing 3-indole (Liu & Chang, 1971) or methanesulfonic acid (Simpson, Neuberger, & Liu, 1976) also suffers from this limitation. Reduction of tryptophan residues with pyridine–borane prior to HCl hydrolysis, as reported by Wong, Osuga, Burcham, and Feeney (1984), is an interesting and convenient approach, but has only been assessed using purified proteins. Enzymatic methods involve mild hydrolysis conditions compared to chemical hydrolysis, but the release of tryptophan is often reported to be incomplete (Delhaye and Landry, 1992).

Alkaline hydrolysis of protein-bound tryptophan in foods and feedstuffs (followed by reverse phase chromatography) has thus become the method of choice for tryptophan determination. The different alkalis used for protein hydrolysis are NaOH (Hugli & Moore, 1972), $Ba(OH)_2$ (Knox, Kohler, Palter, & Walker, 1970) or LiOH (Lucas & Sotelo, 1980; Nielsen & Hurrell, 1985). Use of 4.2 M NaOH (Hugli & Moore, 1972) appears to offer advantages over the use of $Ba(OH)_2$ or LiOH, as the precipitation/adsorption problems associated with $Ba(OH)_2$ and solubility problems with LiOH do not occur with the former.

The purpose of this investigation was to develop a method that involves effective use of alkaline hydrolysis, followed by quantitation of the released tryptophan by ion-exchange chromatography, employing the same system used for the analysis of other amino acids.

2. Materials and methods

2.1. Samples and reagents

Lysozyme (chicken egg white) was obtained from Seikagaku Co., Chuo-ku, Tokyo 103, Japan.

A standard stock solution (5 mM) of L-tryptophan (Calbiochem. Corp., La Jolla, CA) and α methyl-DL-tryptophan (Sigma Chemical Co., St.Louis, MO), both dried to constant mass under vacuum with P_2O_5, were prepared as follows: 51.06 mg tryptophan and 54.58 mg α-methyl tryptophan were dissolved in 50 ml of 0.05 M NaOH. An internal standard (5 mM) was prepared by dissolving 54.58 mg α-methyl tryptophan in 50 ml 0.05 M NaOH. A 5 M NaOH solution was prepared on the day of use. Ultrapure water was prepared for all solutions using a Milli-Q ultrapure water system (Millipore Corp., Bedford, MA).

All other chemicals and reagents were of the HPLC grade and all except *O*-phthalaldehyde (OPA; Sigma Chemical Co., St. Louis, MO) were purchased from BDH Chemicals (BDH Chemicals Australia Pty. Ltd, Kilsyth, Australia). The elution buffer for HPLC constituted 58.8 g tri-sodium citrate, 12.4 g boric acid and 13 ml 4 N NaOH, made up to 1 L with water and the pH adjusted to 9.3. The OPA buffer was prepared by dissolving 1.2 g OPA in 15–20 ml ethanol and mixing with 2.4 ml 3-mercaptopropionic acid and 10 ml of 10% brij-35, all then made up to 1 L with OPA stock buffer. The OPA stock buffer was made of 122.1 g sodium carbonate (anhydrous), 56.4 g potassium sulfate and 40.7 g boric acid, dissolved in the same order in water and made up to 3 L. All solutions used in HPLC analysis were filtered through 0.2 μm nylon-66 membrane filters (Rainin Instrument Co., Woburn, MA).

2.2. Equipment

A PASCAL type 2021 rotary high vacuum pump (John Morris Scientific Pty. Ltd., Sydney) was used for evacuation of air and a Labec autoclave (Laboratory Equipment Pty. Ltd., Sydney) for hydrolysis. Amino acid analysis was performed using a Shimadzu amino acid analysis system (Shimadzu Corp., Kyota, Japan). It was equipped with model LC-10AD pumps, an autoinjector (SIL-10A), a temperature control module (CTO-10A), a post column reaction system, a model RF-10A spectrofluorometric detector, a communication bus module (CBM-10A), a sodium cation exchange column (Shimpack Amino-Na, I.D. 6.0 mm×10 cm) and a data processing unit (Class - LC 10, version 1.4).

2.3. Hydrolysis procedure

Samples were hydrolysed with NaOH in Teflon containers under an atmosphere of nitrogen, according to the following procedure. Samples containing 20–30 mg protein were thoroughly dispersed in 10 ml of 4.2 M sodium hydroxide containing 0.15 mM α methyl tryptophan as internal standard, freshly prepared on the day of use. It is essential that the entire sample is wetted and that no material is encapsulated, as any caking traps air and makes evacuation difficult. Soluble starch (20 mg) was added to samples containing little or no starch. Soluble starch serves as a protective agent as it is preferentially oxidized. A drop or two of 2-octanol was added to prevent frothing (Spies, 1967). The internal standard was added to the standard tryptophan at the same time it was added to the sample and carried through the same procedure as for the sample, except that the hydrolysis step was omitted. The medium was homogenised in

Maxi Mix II (Thermolyne, Dubuque, Iowa) to ensure that the sample is dispersed well, flushed with nitrogen, and cooled in the freezer for at least an hour. The lids of the Teflon containers were then slightly opened, placed in a vacuum dessicator, and evacuated using a vacuum pump. The dessicator was then purged with nitrogen and the process repeated thrice for 30–45 min each time, to remove the air from the samples. This is a key step in the hydrolysis procedure, as any oxygen not removed would lead to oxidative losses of tryptophan. The containers were quickly removed from the dessicator under an atmosphere of nitrogen, lids closed tightly, and the samples hydrolysed in an autoclave at 120 °C for 15 h. The hydrolysates were cooled at 4 °C, acidified to a pH 6.5 with HCl (tryptophan is stable at pH 4–7), diluted to 50 ml (less for samples low in tryptophan) with sodium citrate buffer of pH 6.5 and filtered through 0.2 μm nylon 66 filter membrane into auto sampler vials. Samples, which are difficult to be filtered, were centrifuged at 25,000 rpm for 45 min.

To test the recovery and precision of tryptophan data, two samples of lysozyme were hydrolysed in quadruplicate on three different days. The performance of the method was also tested by spiking samples of blood meal, soyabean meal and wheat with lysozyme (8 mg) and hydrolysing in quadruplicate on each of three days. Further precision data was obtained on samples of two mixed diets, casein and soyabean meal, which were analysed in triplicate without spiking with lysozyme. The samples used in the latter two tests were included because they were involved in an Australian amino acid proficiency testing program (Rayner, 1997).

2.4. Chromatography and calculations

Tryptophan and α-methyl tryptophan were separated by ion exchange chromatography. Aliquots of the sample hydrolysates or standard mixture of tryptophan and α-methyl tryptophan were injected onto the column and eluted isocratically with sodium citrate buffer of pH 9.3 at a flow rate of 0.5 ml/min and a column temperature of 65 °C. *O*-Phthaldialdehyde (Sigma Chemicals Co., St. Louis, MO) was used for post-column derivatisation and fluorometric detection of amino acids.

The data processing unit provided the results for tryptophan (μg/mg) in the samples. This quantification was based on the internal standard technique, where the tryptphan contents were calculated by dividing the area of the component peak by the area of the internal standard and multiplying this value by the weight of the internal standard (μg/mg) and the response factor of tryptophan.

2.5. Estimation of tryptophan in feed samples

The method described above was used to determine the tryptophan contents in samples of seven common poultry feedstuffs containing varying concentrations of tryptophan. Representative samples of feedstuffs were obtained from commercial sources, ground in a laboratory mill to pass through a 1-mm screen and stored in airtight plastic containers until analysed. The samples were hydrolysed in duplicate. Lysozyme was used as the reference material in all analyses.

3. Results and discussion

The alkaline hydrolysis procedure employed in this study is similar to that of Hugli and Moore (1972), but with the modification that Teflon containers were used instead of pyrex glass tubes, thus avoiding the need for any sealing. In addition, the evacuation procedure employed in the present study is more convenient than the tap-water vacuum pump system or the high vacuum line equipped with a dry ice trap (Hugli & Moore, 1972), where samples have to be evacuated one by one.

The internal standard, α-methyl tryptophan, was included during hydrolysis and is another modification of Hugli & Moore's procedure. α-methyl tryptophan was chosen not only due to its chemical similarity in structure and hydrophobicity to tryptophan, but also because baseline resolution of the analytes was achieved by the column. In preliminary studies, α-methyl tryptophan was found to give consistently better recovery of tryptophan than 5-methyl tryptophan under the analytical conditions described herein. Nielsen and Hurrell (1985) reported that any losses of tryptophan during hydrolysis can be corrected from the recovery of either α-methyl tryptophan or 5-methyl tryptophan, used as the internal standard. The percentage recovery of free 5-methyl-DL-tryptophan added to the sample after alkaline hydrolysis is reported by these authors to be very close to that of protein-bound L-tryptophan.

The amino acid analysis system, column and one of the two buffers, that were employed in the present method, were the same as used for routine amino acid analysis (Siriwan, Bryden, Mollah, & Annison, 1993), except the pH of buffer, which was 10 in the routine analysis of amino acids. Fig. 1 shows the chromatography of the calibration standard where both tryptophan and 5-methyl tryptophan are resolved virtually to baseline within 15 min. No artifact peaks appeared on the chromatogram at the same retention time as these amino acids, as verified by the blank chromatogram. Peak purity checks were made by the elimination technique using gelatin, a protein that contains no tryptophan. The elution profiles for calibration standards and samples, where internal standard was added before as well as after hydrolysis, were also overlaid to compare the peak shape. The precision of the tryptophan analysis, achieved under the isocratic conditions, was assessed in terms of the reproducibility of retention time and

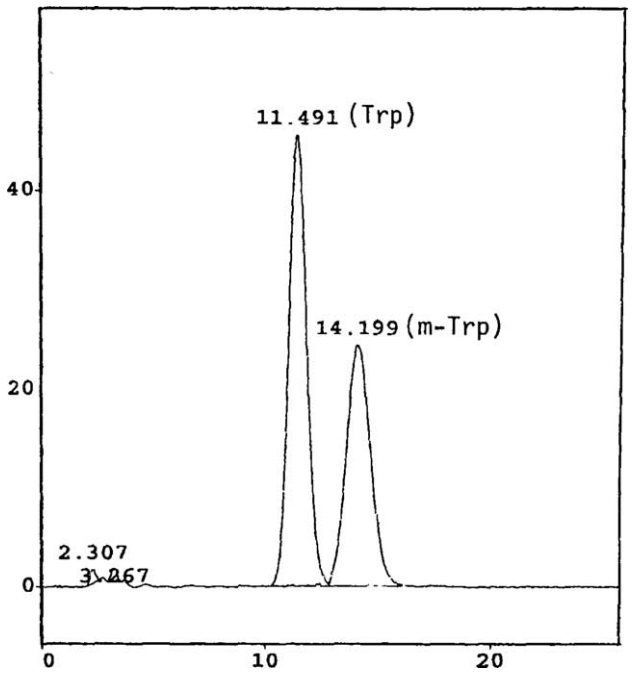

Fig. 1. Typical HPLC chromatogram using isocratic ion-exchange chromatography with *O*-phthalaldehyde derivatisation followed by fluorescence detection. Peaks for tryptophan and α-methyl tryptophan are shown.

Table 1
The recovery and precision data for tryptophan determinations in two samples of lysozyme (g/kg dry matter)

	Lysozyme[a]	
	Sample 1	Sample 2
Day 1		
Means ± SD[b]	73.2 ± 1.05	75.1 ± 3.27
RSD (%)	1.43	4.35
Recovery (%)	96.8	92.5
Day 2		
Means ± SD[b]	71.7 ± 0.822	77.3 ± 3.14
RSD (%)	1.15	4.06
Recovery (%)	94.9	94.3
Day 3		
Means ± SD[b]	72.8 ± 0.43	75.1 ± 2.83
RSD (%)	0.57	3.77
Recovery (%)	96.3	91.6
Overall		
Means ± SD[c]	72.7 ± 0.94	75.2 ± 2.83
RSD (%)	1.30	3.76
Recovery (%)	96.0	92.8

[a] Dry matter content of samples 1 and 2 were 96.8 and 95.0 g/kg, respectively.
[b] Means ± SD of four replicates.
[c] Means ± SD of 12 replicates.

peak area. Ten consecutive chromatographic runs of the same calibration standard resulted in coefficient variations of 0.08% and 1.14%, respectively, for retention time and peak area, indicating good reproducibility.

The accuracy of this method was first evaluated using lysozyme, a fully sequenced protein of known composition. Lysozyme has a theoretical value for pure tryptophan of 85.6 g/kg on the basis of sequence data (Canfield, 1963) and a protein content (w/w) of 1000 g/kg. The corresponding tryptophan and protein contents for the two lysozyme samples used in the present study were 75.6 and 883 g/kg and 82.0 and 958 g/kg, respectively. The tryptophan contents, quantitative recoveries for the two lysozyme samples and the precision data obtained are presented in Table 1. The recovery of tryptophan in lysozyme ranged from 91.6% to 96.8% in the six assays.

Spiking recovery studies were performed on three feed protein sources (blood meal, soyabean meal and wheat), with varying tryptophan concentrations, by adding around 8 mg lysozyme. Data presented in Table 2 show that the spike recoveries were high for whole wheat and soybean meal, ranging 90–95%. These data suggest that almost complete release as well as losses of tryptophan were reasonably accounted for by the present method. The relatively low spike recoveries (87–90%) in blood meal suggest that tryptophan in blood meal is more sensitive to degradation compared to that in other feedstuffs.

These three feed proteins were chosen for the recovery studies, because they have been tested previously in an amino acid proficiency program involving six Australian laboratories (Rayner, 1997). The mean tryptophan values from the inter-laboratory comparison were 13.68, 5.67 and 1.08 g/kg (air-dry weight basis) for blood meal, soybean meal and wheat, respectively. This compares well with the values of 13.23. 5.93 and 1.20 g/kg (air-dry weight basis) determined using the present methodology. The values presented in Table 2 are on a dry matter basis.

Further precision data was obtained on samples of two mixed diets, casein and soyabean meal (Table 3), which had been tested in an amino acid analysis proficiency program (Rayner, 1997). The mean tryptophan values from the inter-laboratory comparison were 1.80, 2.40, 10.80 and 6.40 g/kg (air-dry weight basis) for mixed diets I and II, casein, and soyabean meal, respectively. This compares reasonably well with the values of 1.68, 2.52, 10.96 and 6.37 g/kg (air-dry weight basis) determined using the present methodology.

The lysozyme samples and the three feed proteins were analysed on three nonconsecutive days to determine the precision of the method, which was calculated to be 1–4% relative standard deviation (Tables 1 and 2). The two lysozyme samples (75.6 and 82.0 g/kg tryptophan) exhibited a relative standard deviation that was comparable to these feed proteins containing 1.3–14.8 g/kg tryptophan, implying that a wide range of sample tryptophan concentrations can be assayed by this procedure.

Table 2
The recovery and precision data for tryptophan determination in three feed protein sources spiked with lysozyme

	Blood meal[a]		Soyabean meal[a]		Wheat[a]	
	Tryptophan content (g/kg DM)		Tryptophan content (g/kg DM)		Tryptophan content (g/kg DM)	
	Feedstuff	Determined[b]	Feedstuff	Determined[b]	Feedstuff	Determined[b]
Day 1						
Means ± SD[c]	14.56 ± 0.25	81.6 ± 1.09	6.45 ± 0.06	75.3 ± 0.81	1.34 ± 0.02	73.0 ± 1.12
RSD (%)	1.72	1.33	0.93	1.07	1.49	1.53
Mean recovery (%)		88.6		91.1		94.1
Day 2						
Means ± SD[c]	14.07 ± 0.24	82.2 ± 1.58	6.56 ± 0.11	74.7 ± 2.07	1.32 ± 0.01	73.2 ± 0.80
RSD (%)	1.71	1.92	1.68	2.78	0.76	1.09
Mean recovery (%)		90.1		90.0		95.1
Day 3						
Means ± SD[c]	14.2 ± 0.34	79.8 ± 1.11	6.49 ± 0.14	75.0 ± 1.86	1.32 ± 0.01	72.1 ± 1.40
RSD (%)	2.25	1.39	2.16	2.48	0.76	1.94
Mean recovery (%)		86.8		90.9		93.6
Overall						
Means ± SD[d]	14.3 ± 0.34	81.2 ± 1.58	6.50 ± 0.11	75.0 ± 1.54	1.33 ± 0.02	72.6 ± 1.15
RSD (%)	2.38	1.95	1.69	2.05	1.50	1.58
Mean recovery (%)		88.5		90.6		94.3

[a] Dry matter contents of blood meal, soyabean meal and wheat were 905, 918 and 906 g/kg, respectively.
[b] Amount in feedstuff + added tryptophan in the form of lysozyme (75.6 g/kg).
[c] Means ± SD of four replicates.
[d] Means ± SD of 12 replicates.

Table 3
Comparison of determined tryptophan content of mixed diets, casein and soyabean meal (g/kg air-dry basis) with the results of the Australian amino acid proficiency testing (AAPT) programme

	Mixed diet 1	Mixed diet 2	Casein	Soyabean meal
Replicate 1	1.66	2.58	11.25	6.38
Replicate 2	1.63	2.42	10.98	6.66
Replicate 3	1.75	2.55	11.07	6.07
Means ± SD	1.68 ± 0.06	2.52 ± 0.09	11.10 ± 0.14	6.37 ± 0.30
% RSD	3.57	3.57	1.26	4.71
AAPT results[a]	1.80	2.40	10.80	6.40

[a] Expressed as g/kg air-dry basis; from Rayner (1997).

Table 4
Comparison of determined tryptophan content (g/kg dry matter) of feedstuffs using the present procedure with literature values

Feedstuff	No of samples	Mean value (range)	Literature values, range[a]
Maize	5	0.62 (0.52-0.87)	0.53–0.96
Wheat	4	1.23 (1.21-1.45)	1.13–1.70
Sorghum	5	1.00 (0.89-1.13)	0.74–1.36
Soyabean meal	3	5.87 (5.36-6.05)	4.93–8.94
Canola meal	3	5.04 (4.72-5.60)	4.40–5.552
Cottonseed meal	2	4.87 (4.86-4.88)	4.60–5.20
Meat and bone meal	3	2.62 (2.14-3.26)	1.80–3.60

[a] *Sources*: Bolton and Blair (1974); Buttery and Soar (1975); Sato et al. (1984); Landry et al. (1988); Pinter-Szakacs and Molnar-Perl (1990); NRC (1994); (Zarkadas et al., 1995); Degussa (1996).

The present methodology was used to determine tryptophan contents of some commonly used feedstuffs and the results are presented in Table 4. The determined tryptophan contents in all feedstuffs were within the ranges reported in the literature.

In summary, the procedure reported in the present paper is dedicated, simple and precise, and a convenient application of an ion-exchange amino acid system that can be used for routine determination of tryptophan in a wide range of foods and feed proteins without any

interference either from other amino acids or any other compounds. The sample size and corresponding dilutions described in our procedure can be modified to accommodate limiting situations.

Acknowledgements

Financial support for this study was provided by the Australian Chicken Meat Research Council, Canberra and the Poultry Research Foundation, The University of Sydney, Camden. Drs. C.J. Rayner and Y. Mollah provided technical comment during the study.

References

Bolton, W., & Blair, R. (1974). *Poultry nutrition*. Bulletin 174, Ministry of Agriculture, Fisheries and Food. London: HSMO.

Buttery, P., & Soar, J. B. (1975). A spectrofluorimetric assay of the tryptophan content of feedstuffs. *Journal of the Science of Food and Agriculture, 26*, 1273–1277.

Canfield, R. E. (1963). The amino acid sequence of egg white lysozyme. *Journal of Biological Chemistry, 238*, 2698–2707.

Degussa (1996). *The amino acid composition of feedstuffs* (4th rev. ed.). Hanau, Germany: Degussa Feed Additives.

Delhaye, S., & Landry, J. (1992). Determination of tryptophan in pure proteins and plant material by three methods. *Analyst., 117*, 1875–1877.

Doyle, E. M., Steinhart, C. E., & Cochrane, B. A. (1993). Safety of food components. Naturally occurring toxicants. In *Food Safety 1993*. New York: Marcel Dekker, Inc.

Friedman, M., Levin, C. E., Noma, A. T., Montague, W. C., Jr., & Zahnley, J. C. (1984). Comparison of tryptophan assays for food proteins. In . In H. G. Schlossberger W. Kochen B. Linzen & H. Steinhart (Eds.), *Progress in tryptophan and serotonin research* (pp. 119–123). Berlin: de Gruyter.

Friedman, M., & Cuq, J. L. (1988). Chemistry, analysis, nutritional value, and toxicology of tryptophan in food: A review. *Journal of Agricultural and Food Chemistry, 36*, 1079–1093.

Hugli, T. E., & Moore, S. (1972). Determination of the tryptophan content of proteins by ion exchange chromatography of alkaline hydrolysates. *Journal of Biological Chemistry, 247*, 2828–2834.

Hussey, H. H. (1974). Sleep inducement by L-tryptophan. *Journal of American Chemical Society, 87*, 1126.

Knox, R., Kohler, G. O., Palter, R., & Walker, H. G. (1970). Determination of tryptophan in feeds. *Analytical Biochemistry, 36*, 136–143.

Landry, J., Delhaye, S., & Viroben, G. (1988). Tryptophan content of feedstuffs as determined from three procedures using chromatography of barytic hydrolysates. *Journal of Agricultural and Food Chemistry, 36*, 51–52.

Liu, T. Y., & Chang, Y. H. (1971). Hydrolysis of proteins with it p-toluenesulfonic acid. *Journal of Biological Chemistry, 246*, 2842–2848.

Lucas, B., & Sotelo, A. (1980). Effect of different alkalis, temperature and hydrolysis times on tryptophan determinations of pure proteins and of foods. *Analytical Biochemistry, 109*, 192–197.

Nielsen, H. K., & Hurrell, R. F. (1985). Tryptophan determination of food proteins by HPLC after alkaline hydrolysis. *Journal of the Science of Food and Agriculture, 36*, 893–907.

NRC. (1994). *Nutrient requirements of domestic animals. Nutrient requirements of poultry* (9th rev. ed.). Washington, DC: National Research Council, National Academy Press.

Penke, B., Fereczi, R., & Kovacs, K. (1974). A new acid hydrolysis method for determining tryptophan in peptides and proteins. *Analytical Biochemistry, 60*, 45–50.

Pinter-Szakacs, M., & Molnar-Perl, I. (1990). Determination of tryptophan in unhydrolyzed food and feedstuffs by the acid ninhydrin method. *Journal of Agricultural and Food Chemistry, 38*, 720–726.

Rayner, C.J. (1997). Report – Australian amino acid proficiency test. State Chemistry Laboratory & National Association of Testing Authorities, Melbourne, Australia.

Sato, H., Seino, T., Kobayashi, T., Murai, A., & Yugari, Y. (1984). Determination of the tryptophan content of food and feedstuffs by ion exchange liquid chromatography. *Agricultural and Biological Chemistry, 48*, 2961–2969.

Simpson, R. J., Neuberger, M. R., & Liu, T. Y. (1976). Complete amino acid analysis of proteins from a single hydrolysate. *Journal of Biological Chemistry, 251*, 1936–1940.

Siriwan, P., Bryden, W. L., Mollah, Y., & Annison, E. F. (1993). Measurement of endogenous amino acid losses in poultry. *British Poultry Science, 34*, 939–949.

Spies, J. R. (1967). Determination of tryptophan in proteins. *Analytical Chemistry, 39*, 1412–1416.

Yokote, Y., Arai, K. M., & Akahane, K. (1986). Recovery of tryptophan from 25-minute acid hydrolysates of protein. *Analytical Chemistry, 152*, 245.

Wong, W. S. D., Osuga, D. T., Burcham, T. S., & Feeney, R. E. (1984). Determination of tryptophan as the reduced derivative by acid hydrolysis and chromatography. *Analytical Biochemistry, 143*, 62–70.

Zarkadas, C. G., Yu, Z., Hamilton, R. I., Pattison, P. L., & Rose, N. G. W. (1995). Comparison between the protein quality of Northern adapted cultivars of common maize and quality protein maize. *Journal of Agricultural and Food Chemistry, 43*, 84–93.