ELSEVIER

Food Chemistry 85 (2004) 317–320

Food Chemistry

www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods Section

Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection

María M. Yust, Justo Pedroche, Julio Girón-Calle, Javier Vioque, Francisco Millán, Manuel Alaiz*

Instituto de la Grasa (Consejo Superior de Investigaciones Científicas), Avenida Padre García Tejero, 4, 41012 Sevilla, Spain

Received 28 January 2003; received in revised form 6 June 2003; accepted 1 July 2003

Abstract

A procedure for quantitation of tryptophan in feedstuff is described. It consist of hydrolysis in sodium hydroxide at 100 °C for 4 h, neutralization of the resulting hydrolysate to pH 7, dilution with sodium borate buffer (pH 9), and analysis by reverse-phase high-performance liquid chromatography with spectrophotometric determination of tryptophan at 280 nm. The recovery of tryptophan from lysozyme, added to some feedstuff before hydrolysis, ranges from 98.6 to 100%. A reduction of the time of analysis has been achieved as compared to previous methods for analysis of tryptophan.

Keywords: Tryptophan; Feedstuff; Sodium hydrolysis; High-performance liquid chromatography

1. Introduction

The oxidative degradation of tryptophan during acid hydrolysis of the parent protein precludes its analysis with the other essential amino acids in a single chromatographic run. The inconvenience of having to carry out a separate analytical procedure to analyse tryptophan has often resulted in tryptophan being omitted from the total amino acid data of proteins. However, accurate tryptophan data is essential because having a complete amino acid profile is necessary from a legislative and economic point of view. In particular, determination of tryptophan concentration in feedstuff is essential, because tryptophan is after lysine, cystine and methionine the amino acid most frequently found at limiting concentrations.

Numerous efforts have been made to find a generally applicable method for the analysis of tryptophan (Molnár-Perl, 1999). Some researchers have proposed direct reactions both with hydrolyzed and with intact proteins applying procedures such as second-derivative spectroscopy (Fletouris, Botsoglou, Papageorgiou, & Mantis, 1993; Servillo, Colonna, Balestrieri, Ragone, & Irace, 1982), *p*-dimethylaminobenzaldehyde (Votisky, 1984), *p*-phenylenediamine (Nagaraja, Yathirajan, & Vasantha, 2003) and ninhydrin in strong acid media (Pinter-Szakacs, & Molnár-Perl, 1990). Direct spectrophotometric analysis of the protein is rapid and does not require hydrolysis of the protein. However, direct determination is subject to error due to interferences and can only be considered as an approximate procedure. Colorimetric methods can be tedious and suffer from problems such as colour stability and interferences.

According to most suggestions tryptophan is to be measured in hydrolysates by chromatography under time-consuming and tedious special conditions. These include using (i) hydrochloric acid + additives, such as thioglycolic acid (Ashworth, 1987), mercaptoethanesulfonic acid (Gardner, 1984), mercaptoethanol (Ng, Pascaud, & Pascaud, 1987), phenol (Muramoto & Kamiya, 1990) or tryptamine (Fábián, Pintér-Szakács, & Molnár-Perl, 1990); (ii) organic acids, such as mercaptoethanesulfonic acid (Nakazawa & Manabe, 1992); (iii) enzyme (Garcia & Baxter, 1992); and (iv) bases

* Corresponding author. Tel.: +34-95-461-1550; fax: +34-95-461-6790.

E-mail address: alaiz@cica.es (M. Alaiz).

0308-8146/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2003.07.026

(Delhaye & Laundry, 1993; Huet & Pernollet, 1986; Laundry & Delhaye, 1992, 1997; Laundry, Delhaye, & Viroben, 1988; Slump, Flissebaalje, & Haaksman, 1991). The majority of these procedures involve the following basic steps: (a) alkaline hydrolysis of sample at 110–125 °C in air-deprived medium for 16–20 h; (b) dilution of hydrolysate, neutralized or not with cold concentrated HCl, with chromatographic buffer; (c) clarification of dilute hydrolysate; (d) high-performance liquid chromatography (HPLC) separation; (e) spectrophotometric or fluorimetric determination.

We have now explored some modifications of this basic procedure using sodium hydroxide to reduce the time of alkaline hydrolysis, in order to simplify the procedure and shorten duration of the analysis. The result is a method that allows simple and faster detection of tryptophan by alkaline hydrolysis followed by reverse-phase HPLC with spectrophotometric detection. This new method has been validated by analysing mixtures of amino acids, proteins, and samples of seed flours that are commonly used to feed livestock.

2. Experimental

2.1. Reagents and materials

Tryptophan, tyrosine, phenylalanine, lysozyme, trypsin, bovine serum albumin, and α-chymotrypsin were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. Samples of wheat, rapeseed, soybean, barley, and lupin seeds were analyzed as representatives of feedstuff. Seeds were grounded for preparation of flours. Deionized distilled water was used for the preparation of buffers. The buffers used for HPLC analysis were filtered through a 0.22-μm filter (Millipore, Bedford, MA USA). All the HPLC solvents were degassed with helium.

2.2. Instruments

The HPLC system (Waters, Milford, MA, USA) consisted of a Model 600E multi-solvent delivery system and a Model 484 UV-VIS detector. Recording and data processing were carried out using the MILLENNIUM 32 program. A 300 × 3.9 mm I. D. Nova-Pack C_{18} 4 μm column (Waters) was used. The column was maintained at 18 °C during analysis.

2.3. Hydrolysis

Samples (2–10 mg) were dissolved in 3 ml of 4 N sodium hydroxide, sealed in hydrolysis tubes under nitrogen, and incubated in an oven at 100 °C for 4 h. Hydrolysates were cooled down on ice, neutralized to pH 7 using 12 N HCl, and diluted to 25 ml with 1 M sodium borate buffer (pH 9). Aliquots of these solutions were filtered through a 0.45-μm Millex filters (Millipore) prior to injection.

Standard solutions of tryptophan were prepared by dilution of a stock solution (0.51 mg tryptophan/ml 4 N sodium hydroxide).They were diluted to 3 ml with 4 N sodium hydroxide and incubated as above.

2.4. Chromatography

20-μl samples were injected into the column. An isocratic elution system consisting of 25 mM sodium acetate, 0.02% sodium azide (pH 6)/acetonitrile (91:9) delivered at 0.9 ml/min was used.

3. Results and discussion

Besides tryptophan, the amino acids tyrosine and phenylalanine also absorb at 280 nm. A mixture of these three amino acids was resolved by reverse-phase HPLC chromatography as shown in Fig. 1a. As expected, tyrosine and phenylalanine were partially destroyed during alkaline hydrolysis (Fig. 1b).

The repeatability of the tryptophan quantitation was investigated by performing 16 determinations using 20-μl injections of the standard solutions. The coefficient of

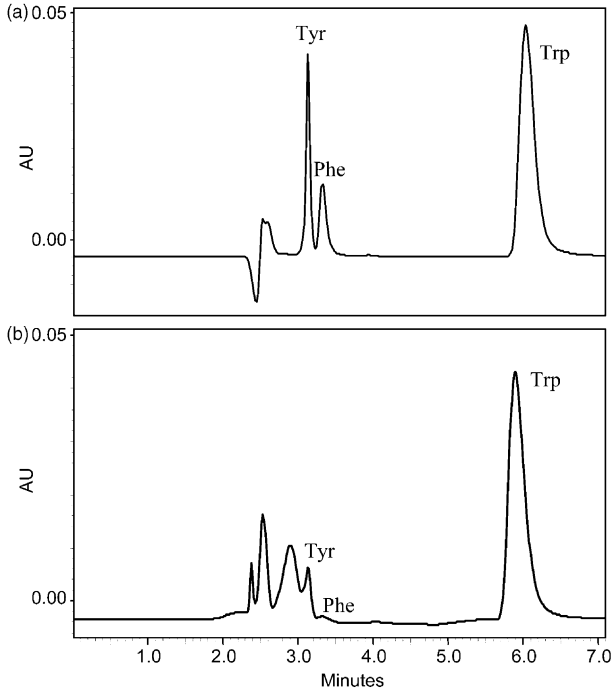

Fig. 1. Elution profile of a mixture of tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp) (same amount of each) analysed by reverse-phase chromatography with detection at 280 nm. (a) Sample was not subjected to hydrolysis before HPLC analysis. (b) Sample was subjected to hydrolysis with sodium hydroxide before HPLC analysis as described in Section 2.

Table 1
Determination of tryptophan in pure proteins

Proteins	Tryptophan content(residues/molecule)	
	Found[a]	Literature[b]
Lysozyme	5.98±0.06	6
Trypsin (bovine)	3.95±0.04	4
Bovine serum albumin	1.98±0.03	2
α-Chymotrypsin (bovine)	7.90±0.11	8

[a] Results are expressed as the mean±standard deviation of determinations made on four hydrolysates (4 h, 100 °C).
[b] Hugli and Moore (1972).

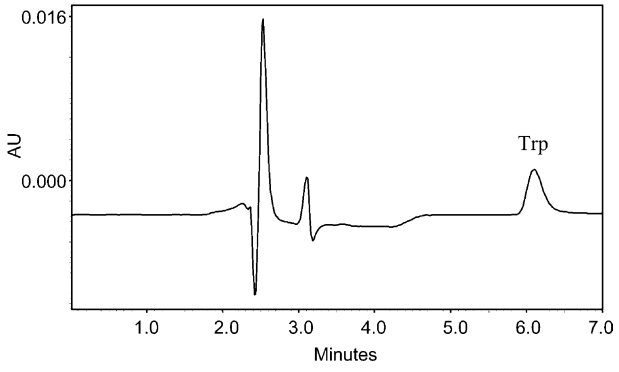

Fig. 2. Elution profile of lupin flour analysed by alkaline hydrolysis and reverse-phase chromatography with detection at 280 nm.

variation was found to be 1.6%. A study of the detector response (peak area versus mass of tryptophan injected) showed that the response was linear at least in the range 10–400 ng (correlation coefficient 0.998). The limit of detection was 10 pmol with a signal-to-noise ratio of 5.

The same method was used to determine the tryptophan content in four pure proteins (Table 1). The complete hydrolysis of proteins was achieved with 4 M sodium hydroxide at 100 °C for 4 h (Aitken & Learmonth, 1996). The results showed excellent agreement with published values determined by ion exchange chromatography (Hugli & Moore, 1972).

In order to validate the method for determination of tryptophan in more complex samples, tryptophan was determined in a series of seed flours. This type of samples are well known for containing molecules that absorb in the ultraviolet spectrum that are often a source of error for spectrophotometric analysis. As shown in Fig. 2, the new method proved to be adequate for analysing tryptophan in a complex sample such as lupin flour. Using a diode array detector, the whole ultraviolet spectrum in early-, medium-, and late-eluting portions of the tryptophan peak was recorded. This data (not shown) proved that the peak corresponding to tryptophan (elution time 6.1 min) contained tryptophan alone. Table 2 shows the data of tryptophan analysis of this series of flours. The values obtained were compared with data found in the literature. Results were similar to those previously reported for these materials.

Table 2
Content of tryptophan in some seed flours and recovery of exogenous (added) tryptophan

Sample	% Crude protein[a]	Trp[b] (g/100 g crude protein)	Literature[c]	Trp[d] (%)
Wheat	13.0	1.12±0.04	1.14	100.0
Soybean	53.2	1.51±0.02	1.43–1.71	99.2
Barley	13.3	1.11±0.03	1.13	99.7
Lupin	38.4	1.05±0.05	1.05	99.8
Rapessed	39.7	1.40±0.02	1.38	98.6

[a] Nitrogen content was determined by a Kjeldahl method on dry matter basis.
[b] Results are expressed as the mean±standard deviation of determinations made on four hydrolysates (4 h, 100 °C).
[c] Landry et al, (1988).
[d] A known amount of tryptophan was added as lysozyme to parallel samples before alkaline hydrolysis, and recovery of added tryptophan was calculated after analysis.

In order to further validate the method, a known amount of tryptophan was added in the form of lysozyme in parallel samples, and recovery of added tryptophan was calculated (Table 2). The results that were obtained compared very well with the values that had been previously published. Most importantly, recovery of tryptophan added before hydrolysis was very high (98.6–100%), even though hydrolysis time has been reduced to 4 h, as compared to other methods (Delhaye & Laundry, 1993; Huet & Pernollet, 1986; Laundry & Delhaye, 1992, 1997; Laundry, Delhaye, & Viroben, 1988; Slump, Flissebaalje, & Haaksman, 1991).

4. Conclusions

The procedure proposed in this paper for quantitation of tryptophan combines a high recovery of the amino acid from alkaline hydrolysates with fast separation by HPLC with spectrophotometric determination. This method is faster than others requiring longer times of hydrolysis and/or chromatography, is not subject to interference by other molecules absorbing in the ultraviolet region that can be found in complex samples such as seed flours, and can be carried out using standard HPLC equipment.

Acknowledgements

We thank M. Dolores García for technical assistence. This work was supported by CICYT Grants AGL2001-0526, and AGL2002-02836 and by a fellowship from the Ministerio de Educación y Ciencia (J.G.).

References

Aitken, A., & Learmonth, M. (1996). Quantitation of tryptophan in proteins. In J. Walker (Ed.), *The protein protocols handbook* (pp. 29–31). Totowa, NJ: Humana Press.

Ashworht, R. B. (1987). Amino acid analysis for meat protein evaluation. *Journal of the Association of Official Analytical Chemists*, *70*, 80–85.

Delhaye, S., & Laundry, J. (1993). Quantitative determination of tryptophan in food and feedstuffs: practical considerations on autoclaving samples for hydrolysis. *Journal of Agricultural and Food Chemistry*, *41*, 1633–1634.

Fábián, V., Pintér-Szakács, M., & Molnár-Perl, I. (1990). Gas chromatography of tryptophan together with other amino acids in hydrochloric acid hdrolysates. *Journal of Chromatography*, *520*, 193–199.

Fletouris, D. J., Botsoglou, N. A., Papageorgiou, G. E., & Mantis, A. J. (1993). Rapid determination of tryptophan in intact proteins by derivative spectrophotometry. *Journal of the AOAC International*, *76*, 1168–1173.

Garcia, S. E., & Baxter, J. (1992). H Determination of tryptophan content in infant formulas and medical nutritionals. *Journal of the AOAC International*, *75*, 1112–1119.

Gardner, M. L. G. (1984). Cysteine: A potential source of error in amino acid analysis of mercaptoethane sulfonic or hydrochloric acid hydrolysates of proteins and peptides. *Analytical Biochemistry*, *141*, 429–431.

Huet, J., & Pernollet, J. (1986). Chromatographic separation and determination of tryptophan in food-stuffs after barytic hydrolysis using Fractogel TSK W 40 S. *Journal of Chromatography*, *355*, 451–455.

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.

Laundry, J., & Delhaye, S. (1992). Simplified procedure for the determination of tryptophan of foods and feedstuffs from barytic hydrolysis. *Journal of Agricultural and Food Chemistry*, *40*, 76–779.

Laundry, J., & Delhaye, S. (1997). Tryptophan contents of pea and broad been seeds as a function of nitrogen content. *Journal of Agricultural and Food Chemistry*, *45*, 3855–3858.

Laundry, 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.

Molnár-Perl, I. (1999). Advances in the analysis of tryptophan and its related compounds by chromatography. *Advances in Experimental Medicine and Biology*, *467*, 801–816.

Muramoto, K., & Kamiya, H. (1990). Recovery of tryptophan in peptides and proteins by high-temperature and short-term acid hydrolysis in the presence of phenol. *Analytical Biochemistry*, *189*, 223–230.

Nagaraja, P., Yathirajan, H. S., & Vasantha, R. A. (2003). Highly sensitive reaction of tryptophan with p-phenylenediamine. *Analytical Biochemistry*, *312*, 157–161.

Nakazawa, M., & Manabe, K. (1992). The direct hydrolysis of proteins containing tryptophan on polyvinylidene difluoride membranes by mercaptoethanesulfonic acid in the vapour phase. *Analytical Biochemistry*, *206*, 105–108.

Ng, L. T., Pascaud, A., & Pascaud, M. (1987). Hydrochloric acid hydrolysis of proteins and determination of tryptophan by reversed-phase high-performance liquid chromatography. *Analytical Biochemistry*, *167*, 47–52.

Pinter-Szakacs, M., & Molnár-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.

Servillo, L., Colonna, G., Balestrieri, C., Ragone, R., & Irace, G. (1982). Simultaneous determination of tyrosine and tryptophan residues in proteins by second-derivative spectroscopy. *Analytical Biochemistry*, *126*, 251–257.

Slump, P., Flissebaalje, T. D., & Haaksman, I. K. (1991). Tryptophan in food proteins: A comparison of two hydrolytic procedures. *Journal of the Science of Food and Agriculture*, *55*, 493–496.

Votisky, E. (1984). In *Progress in tryptophan and serotonin research: proceedings fourth meeting of the international study group for Tryptophan research* (pp. 115–117). Berlin: Walter de Gruyter.