Simplified Procedure for the Determination of Tryptophan of Foods and Feedstuffs from Barytic Hydrolysis

J. Landry* and S. Delhaye

Laboratoire de Chimie Biologique, Centre de Grignon, INRA-CBAI, F-78850 Thiverval-Grignon, France

A procedure simplifying the treatment of barytic hydrolysate prior to chromatographic analysis of tryptophan was tested on eight samples of foods and feedstuffs. It involves the addition of 5-methyltryptophan as internal standard to the mixture subjected to hydrolysis, the dilution of a very small volume (3 μL) of liquid phase of cold (0 °C) hydrolysate with 1 mL of pH 4.5 buffer, and the chromatography of aliquots after dilution. Tryptophan was evaluated from 5-methyltryptophan. The simplified procedure compared with the conventional one, using the remainder of hydrolysate and requiring acidification, quantitative transfer, and clarification, gave identical results irrespective of samples. It is convenient and precise and leads to routine determination of tryptophan of a large number of samples.

INTRODUCTION

Numerous methods for the determination of tryptophan in complex samples such as foods or feedstuffs have been proposed (Friedman and Cuq, 1988). The majority of procedures recently developed involve the following basic steps: (1) alkaline hydrolysis of sample at 110–125 °C in air-deprived medium for 16–20 h; (2) dilution of hydrolysate, neutralized or not with cold concentrated HCl, with chromatographic buffer; (3) clarification of dilute hydrolysate; (4) HPLC separation; (5) detection and quantitation by fluorometry (Jones et al., 1981; Nielsen and Hurrell, 1985; Landry et al., 1988). Hydrolysis was performed in the presence of sodium, lithium, or barium hydroxide with or without carbohydrate or thiodiglycol as an antioxidant and with 5-methyltryptophan as an internal standard. Air-free atmosphere was drawn from the hydrolysis tube by repeated evacuations with or without flushing with nitrogen or argon or by allowing the water and the hydrolysis medium to boil when hydrolysis was carried out in an autoclave. Irrespective of specific conditions used for hydrolysis, tryptophan recovery was found to be quantitative after hydrolysis in the presence of barium hydroxide in a medium deprived of air (Delhaye and Landry, 1986; Landry et al., 1988, 1992) and incomplete after hydrolysis in the presence of sodium or lithium hydroxide (Nielsen and Hurrell, 1985; Landry et al., 1992). For quantitation, tryptophan losses during hydrolysis can be corrected from the recovery of the internal standard, 5-methyltryptophan (Nielsen and Hurrell, 1985; Landry et al., 1992).

From these facts, 5-methyltryptophan was thought to be as stable as tryptophan during barytic hydrolysis. If so, its use as internal standard together with barium hydroxide must allow the different handlings of hydrolysate prerequisite prior to HPLC to be minimized. The present paper examines such a possibility. So, two treatments of barytic hydrolysate prior to tryptophan isolation and quantification from a known amount of 5-methyltryptophan were compared. The first involved neutralization of hydrolysate with HCl followed by quantitative transfer, dilution to volume, and centrifugation. It corresponded to the conventional procedure. The second was limited to a dilution of a small volume of hydrolysate and constituted the simplified procedure. Both treatments yielded similar results for tryptophan originating from the same hydrolysate.

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Samples and Reagents. Samples represented an assortment of eight foods and feedstuffs. Tryptophan and 5-methyltryptophan (from Sigma) were used as 0.002 M solutions: 20.4 mg of tryptophan or 21.8 mg of 5-methyltryptophan was dissolved in 50 mL of water acidified with 100 μL of 6 N HCl [the precise concn. of tryptophan solution was checked by UV spectrophotometry using a molar extinction of 5630 at 280 nm, as reported by Mihalyi (1976)]. Standard mixture was prepared in two steps: (1) 150 μL each of stock solutions was mixed with 2.5 mL of $BaCl_2$ solution (obtained by acidifying 8.4 g of barium octahydrate with 6 N HCl to pH 3 and diluting to a final volume of 50 mL) and diluted to a final volume of 5 mL; (2) 25 μL of this solution was diluted with 1.5 mL of water (conventional procedure). With the simplified procedure two changes were made: in step 1, 1.5 mL of barium hydroxide solution (corresponding to liquid phase obtained after the heating of 8.4 g of barium octahydrate with 50 mL of water at 100 °C for 5 min following by cooling at 0 °C) was substituted for 2.5 mL of $BaCl_2$ solution; in step 2, dilution was made with chromatographic buffer. It is noteworthy that the salt concentrations in sample and standard solutions must be very close, if not identical, since the fluorescence intensities of tryptophan and 5-methyltryptophan varied with salt. Chromatographic mixture was made of 200 mL of methanol and 800 mL of buffer, which was 0.07 M sodium acetate adjusted to pH 4.5 with glacial acetic acid.

Autoclave. Hydrolysis was carried out using an autoclave (Certoclav, A-4050 Traun, Austria) equipped with an unconfined gasket ensuring the tightness between the cover and body of apparatus. This gasket is free to move laterally in both directions and apt to leak when it is subjected to temperature change, which allows one to find the autoclave at atmospheric pressure in the morning when the heating is stopped during the night.

Chromatographic Equipment. HPLC was performed using a system consisting of a SEDERE automatic sample injector (Touzart Matignon, France), a Waters M 510 pump, a Shimadzu RF 535 fluorescence monitor, and a Waters M 730 data module. The reversed-phase column (4 μm, 15 × 0.39 cm, i.d.) was a Nova-Pak C_{18} (Waters) maintained at 45 °C with a circulating bath.

Preparation of Sample. Preparation was performed according two procedures involving the following steps: (1) Weigh a sample containing 50 mg of protein into disposable 30-mL tubes in polypropylene. (2) Add 4.2 g of barium hydroxide octahydrate, 1.5 mL of 0.002 M 5-methyltryptophan (3 μmol), 2.5 mL of water, and 2 or 3 carborundum grains. Homogenize carefully with a vortex mix and add 4 mL of water to wash the tube wall. (3) Place tubes in a rack and cap with beakers. (4) Transfer rack to autoclave when water boils and bolt. When the pressure reaches 1 bar, allow the vapor to escape for 5 min to remove air from autoclave and from the mixture subjected to hydrolysis.

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Heat to raise the pressure to 1.4 bar (125 °C) and leave for 16 or 10 h (in the latter case the autoclave is allowed to cool until the use of hydrolysates). (5) Cool hydrolysates at 0 °C. (6a) Draw off 3 μL of liquid phase of hydrolysate and dilute with 1 mL of chromatographic buffer at pH 4.5 (simplified procedure). (6b) Acidify at 0 °C the remainder of the hydrolysate with 6 N HCl to pH 3, transfer quantitatively, and adjust to 50 mL. An aliquot of this dilution was centrifuged at 13000*g* for 5 min, and 25 μL of supernatant was mixed with 1.5 mL of water [conventional procedure according to Delhaye and Landry (1986) and Landry et al. (1988)].

Chromatography. A 10-μL pretreated sample or standard mixture of trypotphan and 5-methyltryptophan was injected into the column every 10 min and eluted isocratically at 0.7 mL min^{-1}. Tryptophan and 5-methyltryptophan were quantitated from their native fluorescence. The excitation and emission wavelengths were 285 and 345 nm, respectively. After about 50 runs, the column was washed with 50% methanol at 0.7 mL min^{-1} for 0.5 h and then equilibrated with buffer.

RESULTS

The procedures, as used in the present study, were adapted from that of Slump and Shreuder (1968). According to these authors an 8-h hydrolysis at 120 °C sufficed for obtaining complete release of tryptophan from maize and soybean. We confirmed the same length of time for wheat and soybean hydrolyzed at 125 °C. For convenience, the hydrolysis duration was of 16 h (overnight) at first, but with the aim of maximal simplification of the procedure it was reduced to 10 h. In the latter alternative, autoclave heating was started at the end of the working day and stopped after 10 h, the autoclave being left to cool for the rest of night, which avoided the time-consuming handling to bring the apparatus to a working temperature.

The elution profiles of tryptophan and 5-methyltryptophan as the result of the chromatography of the same wheat hydrolysate prepared according to the simplified (A) and conventional (B) procedures are depicted in Figure 1. The only differences detected between the chromatograms concerned the fluorescent material eluted between the tryptophan and 5-methyltryptophan and which appeared slightly higher for the sample treated through the conventional procedure.

The quantitative data on this similaritly are given in Table I, where the tryptophan contents of wheat and soybean determined through both procedures are reported. With the conventional procedure tryptophan was evaluated from a tryptophan or a 5-methyltryptophan standard. As seen in Table I, no significant differences were found between the procedures and between the standards taken for evaluation. This indicated that tryptophan and 5-methyltryptophan displayed the same stability under the hydrolysis conditions used in this experiment and the same behavior toward solid baryta and sedimenting debris in regard to adsorption, if any. As expected, the evaluation through 5-methyltryptophan leads to a decrease of the standard deviation.

Table II extends the data of Table I to eight samples. The results are expressed from the same hydrolysate as the ratio of the amount of tryptophan found with the simplified procedure to that found with the conventional procedure. No significant differences were noted among the diverse samples. Percentages run from 98.3 to 101.5% with a mean of 99.9%.

It is obvious that the simplified procedure is suitable only if the hydrolysis is performed under conditions which do not degrade tryptophan. This is the case with barium hydroxide in the absence of air, as shown previously (Delhaye and Landry, 1986; Landry et al., 1988). A purging

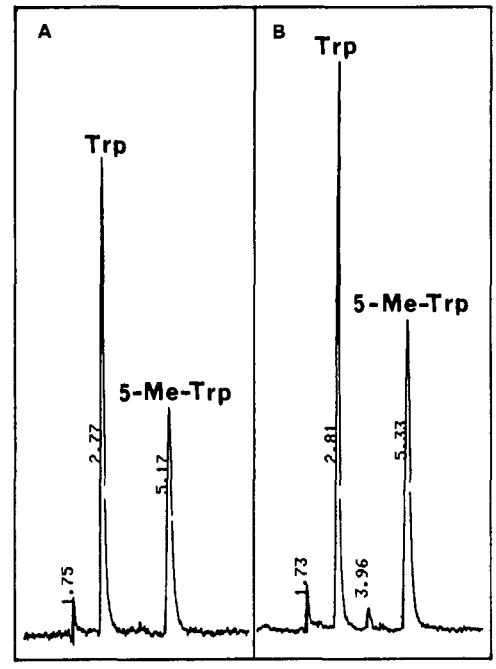

Figure 1. Chromatograms of sample of wheat hydrolysate containing 5-methyltryptophan (5-Me-Trp) obtained by HPLC and fluorometric detection. (A) Three microliters of liquid phase from hydrolysate at 0 °C was diluted with 1 mL of pH 4.5 buffer, and 10 μL was injected (simplified procedure); (B) the remainder of hydrolysate (about 8 mL) was acidified to pH 3 and diluted to 50 mL. A 25-μL sample of this solution was mixed with 1.5 mL of water; after clarification by centrifugation, 10 μL was injected (conventional procedure). Figures indicate retention times in minutes.

Table I. Tryptophan Content (Nanomoles per 10-mg Sample) of Wheat and Soybean As Evaluated from Simplified and Conventional Procedures[a]

	procedure		
	simplified	conventional	
sample	Std: 5-Me-Trp[b]	Std: 5-Me-Trp	Std: Trp
wheat	71.4 ± 0.7	70.8 ± 0.5	70.7 ● 1.0
soybean	327 ± 2	328 ± 2	325 ± 6

[a] Results are expressed as the mean ± standard deviation of determinations made on four replicate hydrolysates (10 h, 125 °C). [b] Standards (Std) are 5-methyltryptophan (5-Me-Trp) and tryptophan (Trp).

of long duration ensures the complete elimination of air from the autoclave and hydrolysis medium through boiling. To improve the efficiency, some grains of carborundum were added to the mixture subjected to hydrolysis. Their presence led dispersibility of data to be lowered.

Barium hydroxide octahydrate has a limited solubility in water (about 4 g in 100 g of water at room temperature) and must be added to the sample as a solid. This is a drawback in regard to sodium or lithium hydroxide. In the conventional procedure the amount of added alkali should be accurate as it dictates the amount of acid required for neutralization. This is not true with the simplified procedure since the hydrolysate aliquot for chromatography is taken from a solution saturated in barium hydroxide. Consequently, an approximate amount of baryta close to 4.2 g could be added to the sample provided that slight variations in alkali concentration do not adversely affect the tryptophan recovery. As expected, the tryptophan content of wheat and soya were found to be insensitive to alkali concentration when samples were hydrolyzed in the presence of 3.8, 4.2, and 4.6 g barium hydroxide octahydrate (data not shown). As a conse-

Table II. Comparison of the Tryptophan Content of Some Foods and Feedstuffs As Determined by Simplified and Conventional Procedures

sample	N/DM,[a] %	$(Trp)_{CP}$[b]/ [(N/DM) × 6.25], %	$(Trp)_{SP}$[c,d]/ $(Trp)_{CP}$, %
maize	1.66	0.78	99.7
			100.2
wheat	1.74	1.47	99.1
			99.9
sorghum	2.04	1.18	101.5
			101.1
whey	2.04	1.58	99.6
			100.1
bran	3.00	1.68	101.1
			101.5
pea	3.96	1.04	99.7
			99.1
soybean	7.75	1.53	100.0
			99.4
fishmeal	11.64	1.10	99.2
			98.3
av			99.9
SD			0.9

[a] Nitrogen on a dry matter basis. [b] CP, conventional procedure. [c] SP, simplified procedure. [d] Values obtained from two replicate hydrolysates.

quence, powdered barium hydroxide may be added to the sample with a calibrated scoop.

Finally, the rapidity of chromatographic separation of tryptophan and 5-methyltryptophan can be improved by increasing the flow rate for a given column. With the conditions described previously (Delhaye and Landry, 1986; Landry et al., 1988), tryptophan and 5-methyltryptophan were eluted at 4 ± 0.2 and 7.55 ± 0.5 min, respectively, and a sample was injected every 12 min. When the flow rate was increased from 0.5 to 0.7 mL min^{-1}, tryptophan and 5-methyltryptophan were eluted at 3.00 ± 0.3 and 5.5 ± 0.5 min, respectively (Figure 1), and a sample was injected every 10 min. In addition, the performance of the column was found to be unchanged after 50 consecutive injections, which is interesting for serial analyses.

DISCUSSION

The simplified protocol, as presented in this paper, combines the use of 5-methyltryptophan with the dilution of aliquot as treatment of hydrolysate prior to chromatography. Its offers more advantages than any other method. Among these advantages are the following.

(1) Great versatility is gained since alkali concentration, hydrolysis time, and treatment of hydrolysate can be varied in some limits without affecting the assay. Sampling hydrolysate at 0 °C marks the level of barium ions introduced into the chromatographic column independent of the amount of alkali used for hydrolysis and about 7 times lower than that brought with the conventional procedure for the same tryptophan amount. The coprecipitation of sample debris with baryta during cooling of hydrolysate led to better clarification. Therefore, the simplified procedure must increase the lifetime of the chromatographic column. On the other hand, it is possible from the same hydrolysate to perform several samplings which can be to treated in different ways if a particular sample reveals a problem.

(2) Handling is reduced since the treatment of sample consists of a 5-min purging of the autoclave prior to hydrolysis and a dilution of a hydrolysate aliquot taking less than 1 min prior to chromatography. Omitting the steps of neutralization, quantitative transfer, and clarification leads to a saving of from 10 to 15 min per sample. It is noteworthy that the procedure recently described by Slump et al. (1991) is far more time-consuming since it includes the boiling of barium hydroxide and water prior to their addition to the sample and two filtrations prior to chromatography due to the precipitation of barium ions from clarified hydrolysate. The same is true of procedures based on hydrolysis in test tubes which must be evacuated and sealed (Robel, 1967; Knox et al., 1970; Sato et al., 1984; Nielsen and Hurrell, 1985; Shibata et al., 1991). On the other hand, the use of disposable labware for all steps of the assay eliminates cleaning of expensive material such as tubes in TPX and glass volumetric flasks.

(3) Pollution is reduced as a very low amount of HCl is consumed for the acidification of the hydrolysate aliquot. The same is true for chromatographic solvent as 7 mL is consumed per run instead of about 20 mL (Shibata et al., 1991; Hagen and Augustin, 1989).

(4) The chromatographic analysis is faster, 10 min instead of 12 (Shibata et al., 1991) or 15 min (Hagen and Augustin, 1989) or more (Nielsen and Hurrell, 1985). Only Rogers and Pesti (1990) have reported the same duration for the isolation of tryptophan.

(5) Serial assays may be possible as a hydrolysate is ready for chromatography in 1 min. When an automatic sampler is used, dilution of the hydrolysate aliquot can be carried out in sample vials. In this case 50 ground samples can be hydrolyzed and assayed by one worker instead of 12–15 with the conventional procedure.

In a recent paper Pinter-Szakacs and Molnar-Perl (1990) have determined tryptophan of foods and feedstuffs from the differences between absorbances at 380 nm of solutions obtained by contacting the unhydrolyzed material with both ninhydrin-containing and nihydrin-free reagents (mixture of formic and hydrochloric acids) for 2 h. Such a procedure, although rapid, accurate, and without expensive specific equipment requirements, suffers from the disadvantage of too many handlings, namely two weighings, two filtrations, four volumetric adjustments, and two spectrophotometric readings with quartz cuvettes for a single determination.

In conclusion, the simplified procedure reported in the present paper is convenient and precise and leads to routine determination of tryptophan of a large number of samples.

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