Tryptophan Determination in Feedstuffs: A Critical Examination of Data from Two Collaborative Studies through the Evaluation of Tryptophan Recovery

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The tryptophan recovery from various procedures used by 23 laboratories having participated in two collaborative studies (French for the one, European for the other) on the tryptophan determination of several feedstuff samples was evaluated by taking the corresponding data obtained by our laboratory, applying an accurate method to the same samples, as reference. The percentages resulting from this normalization represented a set of values based on about 500 determinations. They emphasized that all of the procedures developed for tryptophan quantitation from alkaline hydrolysate and without the help of an internal standard led to a mean underestimation of about 15%, irrespective of the nature of alkali used for hydrolysis, the kind of analyzed sample, and the conditions used for assaying liberated tryptophan. A mean underestimation of the same extent can be seen in the literature data, originating from individual as well as collaborative studies and pertaining to the recovery of a known amount of tryptophan added to samples prior hydrolysis, which lends additional support to the validity of our procedure. The use of 5-methyltryptophan but not α-methyltryptophan as an internal standard results in the improvement of assay accuracy with tryptophan recoveries close to 100%.

Keywords: *Tryptophan; assay; feeds; recovery; collaborative study*

INTRODUCTION

Tryptophan is an essential amino acid for monogastric beings. The accurate determination of its content in foods and feeds therefore is of paramount importance. The majority of protocols elaborated for such a determination involve an alkaline hydrolysis of sample as the first analytical step, since tryptophan is destroyed by 6 M HCl under conditions currently used to release amino acids exhaustively from proteins (Friedman and Cuq, 1988) or, with some samples, only partially free after the proteolytic digestion (Delhaye and Landry, 1992). In alkaline medium tryptophan, although stable, is subject to degradation when heated in the presence of oxygen (Friedman and Cuq, 1988). Therefore, the accurate determination of tryptophan from alkaline hydrolysate requires a rigorous exclusion of air from the mixtures subjected to hydrolysis. If this condition is not fulfilled, significant oxidative losses occur, which have to be assessed so that they may be compensated for by the application of a suitable correction factor. The reliability of any analytical method developed for tryptophan assay from alkaline hydrolysate of sample is customarily estimated by evaluating the free tryptophan (Miller, 1967; Slump and Schreuder, 1969; Buttery and Soar, 1979; Nielsen and Hurrell, 1985) or an internal standard of a similar chemical structure, α- or 5-methyltryptophan (Wilkinson *et al.*, 1976; Nielsen and Hurrell, 1985; Bech-Andersen, 1991; Landry *et al.*, 1992; Landry and Delhaye, 1994), recovered after hydrolysis of samples spiked with known amounts of either of compound. It can be estimated from tryptophan recovered after hydrolysis of a protein whose amino acid sequence is known (Delhaye and Landry, 1986, 1992) or from radioactive tryptophan liberated from protein containing tritiated tryptophan (Nielsen and Hurrell, 1985).

An alternative method of assessing the reliability of a particular analytical procedure is to compare the tryptophan content of a selected sample, as obtained through this procedure, with the corresponding value determined from the same sample by using a method known to yield a quantitative recovery. In our laboratory we have devised such a method involving sample hydrolysis with 1.35 M barium hydroxide under specific conditions (Landry *et al.*, 1988; Landry and Delhaye, 1992; Delhaye and Landry, 1993). Among the observations made that account for its reliability are these: (1) Recovered tryptophan from pure proteins and from lysozyme added to a series of foods and feedstuffs ranges between 98 and 102% (Delhaye and Landry, 1986; Landry *et al.*, 1988). (2) The tryptophan contents of wheat, maize, and barley grains, as calculated from their nitrogen contents and the linear relationships connecting tryptophan and nitrogen content, reported in the literature, were 10% underestimated with respect to the corresponding values deduced from the linear relationships of the same type established from data obtained through our method of tryptophan assay (Landry and Delhaye, 1993). (3) Tryptophan values were 4–13% lower in average when 4 M NaOH or 4 M LiOH was substituted for 1.35 M $Ba(OH)_2$ as hydrolysis agent, all other conditions being equal (Delhaye and Landry, 1992; Landry and Delhaye, 1994a,b). (4) The tryptophan levels of various feedstuffs determined by our method were identical with or closely corresponded to the levels analyzed after hydrolysis in the presence of 4 M LiOH and correction to 100% recovery of 5-methyltryptophan (Landry *et al.*, 1992). The quantitative results obtained using our procedure were attributed to the transfer of mixtures to be hydrolyzed in the benchtop autoclave when water is boiling. Under these conditions the fast and regular heating of samples by water vapor allowed the efficient removal of atmospheric oxygen adsorbed by the solid particles of samples prior to their solvation by hydroxyl ions. Solvation prior to this removal would lead adsorbed air to be entrapped,

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impeding its pumping-off or its displacement through purging (Landry and Delhaye, 1994b).

The purpose of the present study was to assess the accuracy of various procedures tested for the determination of tryptophan in feedstuffs in terms of tryptophan recovery. This parameter was expressed, as percentage, by calculating the ratio of the tryptophan values found for a given sample by the numerous laboratories that participated in two collaborative studies to the corresponding value obtained with the same sample through our procedure. The resulting percentages were compared to appraise the influence of some factors involved in the assay on the recovery. They were also compared with the literature data to ascertain the validity of such an approach.

EXPERIMENTAL PROCEDURE

French Ringtest. Each of three studied samples was hydrolyzed in triplicate and analyzed according to the following procedure: Weigh an accurate quantity of sample containing about 100 mg of protein into a hydrolysis tube. Add 16.4 g of barium hydroxide octahydrate, 16 mL of water, and, alternatively, a suitable amount of 5-methyltryptophan as an internal standard. Remove air from the medium by sonication and then purging with nitrogen or argon. Heat at 120 °C for 16 h. Cool and acidify hydrolysate with 6 M HCl to pH 3.0–3.5. Dilute to a suitable volume with water. Filter a dilution aliquot through a 0.45-μm filter. Tryptophan and 5-methyltryptophan are isolated by high-performance liquid chromatography on a C_{18} reversed-phase column and quantified from their ultraviolet absorbance at 280 nm or their proper fluorescence (excitation 285 nm, λ emission 345 nm).

First European Ringtest. The operating conditions concerning this ringtest were reported by Ranfft and Faure (1993).

Reference Data. The data taken as reference were obtained by applying the conventional method of tryptophan assay, as reported by Landry and Delhaye (1992), to the samples analyzed by the French and European ringtests. The values obtained for a given sample without the use of an internal standard and with the help of 5-methyltryptophan were averaged since no significant differences were observed.

Estimation of the Tryptophan Recovery. The tryptophan content found for sample i (1, 2, ...) by the laboratory j (1, 2, ...) and by the reference laboratory (R) is given by $Trp_{i,j}$ and $Trp_{i,R}$, respectively. Under these conditions the estimated recovery of tryptophan is given by

$$(r\,\mathrm{Trp})_{ij} = 100 \times ([\mathrm{Trp}]_{ij}/[\mathrm{Trp}]_{i,\mathrm{R}})$$

The mean of tryptophan recoveries relative to all samples analyzed by laboratory j allows one to assess the impact of procedure used by that laboratory on tryptophan recovery.

RESULTS

The values of the tryptophan recovery, as calculated from the contents determined by the 12 laboratories that participated in the French ringtest, are set out in Table 1. Means range between 65.8 and 101.4%. Means of intralaboratory means, i.e., referring to the average percentage of tryptophan recovered from the three samples examined by each of laboratories, range from 74.7 to 100%, wich leads to an overall mean of 89.3 ± 7.7%. The set of values appears to be made up of two subsets. The first, A, includes the values of laboratories 2, 5, and 10, with a mean of 99.4 ± 2.0%, indicating a quantitative recovery. The second, B, comprises the values of other laboratories, with a mean of 85.5 ± 4.9%, revealing a partial recovery. This subset can be divided into three subsubsets, composed of the intralaboratory means corresponding to each of the three examined samples. Under these conditions the recovery of tryptophan from corn, soybean, and whey averages 87.9, 86.0, and 86.6%, respectively. It, therefore, appears to be independent of the nature of sample.

Table 1. French Ringtest on Determination of Tryptophan in Feedstuffs: Evaluation of Recovered Tryptophan[a]

	tryptophan recovered (%)					
lab no.	corn	soybean	whey	mean	SD[b]	CV%[c]
2	101.4	99.7	98.4	99.8	1.5	1.5
3	82.6	75.8	65.8	74.7	8.5	11.4
4	84.1	90.8	79.3	84.7	5.8	6.8
5	100.0	100.0	100.0	100.0		
6	87.0	81.7	82.9	83.9	2.8	3.3
7	91.3	84.0	87.6	87.6	3.7	4.2
8	88.4	86.1	86.5	87.0	1.3	1.5
9	92.8	90.0	90.7	91.2	1.5	1.6
10	101.4	98.7	94.8	98.3	3.3	3.4
11	89.9	89.2	82.4	87.2	4.1	4.7
12	87.0	90.4	85.0	87.5	2.7	2.9
overall means	89.3 ± 7.7	(all labs)				
	85.5 ± 4.9	(without labs 2, 5, and 10)				

[a] Calculated from data of laboratory 5 as described under Experimental Procedures. [b] Standard deviation. [c] Coefficient of variation.

The values of the tryptophan recovery, as calculated from the contents determined by the 14 laboratories that participated in the first European ringtest, are shown in Table 2. They vary from 73.5 to 116.3%. Three values are greater than 100%. They can be removed as outliers since the recoveries found for the other samples by each of the considered laboratories are far from to 100%. Under these conditions the set of values has an overall mean of 86.5 ± 5.6%. It can be divided into subsets A and B. Subset A contains the values from laboratory R. Subset B includes the other values, with a mean of 85.5 ± 4.2%, which is identical to that found for the subset B relative to the French ringtest. Subset B can be divided into several subsubsets according to the parameter taken into consideration. So, the recovery is found to average 84.2 ± 3.7, 91.5 ± 0.2, or 87.3% depending on whether data were obtained without using internal standard or with the help of 5- or α-methyltryptophan, respectively. Similarly, it averages 84.6 ± 4.5, 86.5 ± 4.3, or 88.7 ± 5.8% depending on whether the hydrolysis agent is lithium, sodium, or barium hydroxide; and 87.5, 90.2, 80.8, 83.2, or 86.3% when corn, soybean, fish meal, meat and bone meal, or mixed feed was analyzed, respectively. It is interesting to note that the tryptophan recovered from the protocol used by laboratory 5 averages 91.7%, in agreement with the estimation of 90–93% reported by Slump *et al.* (1991) from this laboratory, but the protocol used by laboratory 3 leads to a recovery averaging 91.4%, whereas it was found to give a quantitative recovery when it was compared with ours (Landry *et al.*, 1992).

DISCUSSION

Two subsets can be evidenced in the set incorporating the recovery values relative to the two ringtests examined in the present study. The first comprises the values originating from three laboratories that are equal or close to 100%. The second includes the values from 21 laboratories leading to an overall mean of 85%. The discrepancy observed between the two subsets could originate from a 15% underestimation of tryptophan in the standard solution, promoting an overestimation to the same extent in the evaluation of tryptophan content

Table 2. European Ringtest on Determination of Tryptophan in Feedstuffs: Evaluation of Recovered Tryptophan[a,b]

	tryptophan recovered (%)							
lab no.	corn	soybean	fish meal	meat and bone meal	mixed feed	mean	SD	CV%
1	84.3	94.4	85.5	83.7	78.1	85.2	5.9	6.9
2	93.5	87.7	77.3	85.1	88.8	86.5	6.0	6.9
3	92.1	94.6	87.8	89.8	92.9	91.4	2.7	3.0
4	91.0	90.9	83.9	82.7	90.7	87.8	4.2	4.8
5	96.6	95.6	88.2	86.9	91.4	91.7	4.3	4.7
6	[104.5]	90.6	75.4	79.5	87.0	83.1	6.9	8.3
7	77.5	76.5	76.0	70.7	74.3	75.0	2.7	3.6
8	86.5	89.6	77.1	84.8	88.8	85.4	5.0	5.9
9	78.7	95.1	82.9	84.8	81.8	84.7	6.2	7.3
11	[103.4]	84.0	82.9	92.2	83.6	85.7	4.4	5.1
12	91.0	91.5	80.8	84.5	88.8	87.3	4.6	5.3
13	92.1	[116.3]	80.2	81.3	85.6	84.8	5.4	6.4
14	78.9	91.9	73.5	76.0	90.7	82.4	8.5	10.3
R	100.0	100.0	100.0	100.0	100.0	100.0		
	overall means			86.5 ± 5.6		(all labs)		
				85.5 ± 4.2		(without lab R)		

[a] From the data reported by Ranfft and Faure (1993). Due to the lack of numbering related to a late participation in this ringtest, our laboratory was identified by R. Our data, not published in the paper of Ranfft and Faure (1993), were taken in reference. [b] Values in brackets were omitted for the evaluation of the mean.

of samples. Such an assumption is negated by the following: (1) The control of the standard solution of tryptophan by ultraviolet spectrophotometry using a molecular absorbance of 5660 at 278 nm (Mihalyi, 1976), as performed in the laboratory, the values of which are taken as reference, emphasizes a concentration between 97 and 98% of that expected from the weighed amount of tryptophan. (2) An incomplete dissolution of several milligrams of tryptophan, if it occurs when the stock solution of tryptophan is made, would be visible to the naked eye and would alert the worker to the inaccuracy of the procedure. (3) A 15% underestimation of tryptophan in the standard solution performed by three laboratories analyzing tryptophan independently from each other is highly unlikely. (4) An underestimation averaging 11%, close to that observed above, has been reported by the laboratory the values of which are taken as reference (Landry and Delhaye, 1994) when NaOH was substituted for $Ba(OH)_2$. Therefore, the discrepancy recorded between the two subsets is the reflection of tryptophan degradation during the alkaline hydrolysis prior to assay. In this context 85% must be close to the percentages reported in the literature when tryptophan recovery is determined by analyzing the tryptophan found after its addition to the sample in the free state or as engaged into a protein of known sequence or by evaluating the 5-methyltryptophan spiked into the sample.

Table 3 presents some data pertaining to individual as well as collaborative works relative to the added tryptophan recovered after hydrolysis in the presence of barium or sodium hydroxide. This set of values can be divided into two subsets. The first includes the values reported by Slump and Schreuder (1969), Knox *et al.* (1970), Buttery and Soar (1975), and Landry *et al.* (1988), indicating nearly quantitative recoveries. The second includes the other values showing recoveries ranging from 85 to 89%. Nevertheless, the high recoveries found by Buttery and Soar (1975) can be doubted on the basis of the data from Williams *et al.* (1982) and Nielsen and Hurrell (1985), whereas those found by Slump and Schreuder (1969), Knox *et al.* (1970), and Landry *et al.* (1988) agree with the similarity of hydrolysis procedures.

A comparison of the values set out in Tables 1–3 confirms or emphasizes the following.

Table 3. Literature Data for Recovery of Added Tryptophan to Complex Samples

ref	sample no.	recovery of added tryptophan (%)	
		range	mean ± SD
Miller (1967)	9	78–95	86.0 ± 5.6[a]
Slump and Schreuder (1969)	4	94–100	97.3 ± 3.2[a]
Knox *et al.* (1970)	4	90–96	93.5 ± 2.5[a]
Buttery and Soar (1975)	5	94–103	98.7 ± 3.1[a]
Nielsen and Hurrell (1985)			
procedure:			
Miller	6	76–99	88.6 ± 7.8[a]
Buttery and Soar	6	80–100	86.7 ± 10.3[a]
authors'	1	84–86	85.4 ± 1.0[b]
	1	87–89	88.3 ± 1.7[c]
Landry *et al.* (1988)	11	99–101	99.7 ± 0.7[d]
Williams *et al.* (1982)[e]			
procedure:			
Miller	5	83–91	87.2 ± 3.7[a]
Buttery and Soar	5	83–90	86.6 ± 2.5[a]

[a] Free tryptophan. [b] Protein-bound [^{3}H]tryptophan. [c] Free [^{14}C]tryptophan. [d] Tryptophan from lysozyme. [e] An English ringtest with the participation of seven laboratories.

(1) The quantitative character of our procedure is confirmed since similar percentages are found for tryptophan recovery whether it is evaluated from added tryptophan or from the data obtained by our procedure and taken as reference.

(2) Tryptophan recovery is nearly independent of the way in which the tryptophan liberated after sample hydrolysis is assayed, since Miller's procedure involves a colorimetry with *p*-(dimethylamino)benzaldehyde, that of Buttery and Soar a fluorometry with formaldehyde and then ferric chloride, and the others an absorptiometry or fluorometry of tryptophan after its isolation by high-performance liquid chromatography. Furthermore, the interlaboratory means concerning corn, soybean meal, and fish meal samples analyzed in the European ringtest have coefficients of variation amounting to 10.0, 3.9, and 6.2%, respectively, which are very close to those of 8.3, 4.1, and 7.0% found for barley, soybean meal, and fish meal, respectively, by the laboratories of the English ringtest having applied Miller's procedure (Williams *et al.*, 1982). With the same samples analyzed according to the procedure of Buttery and Soar, the percentages were virtually doubled, suggesting some problems with this assay as has been

Table 4. Hydrolysis in the Presence of 4 M NaOH or LiOH: Recovery of Tryptophan and α- and 5-Methyltryptophan and Estimation of Tryptophan after Correction from Recoveries of α- or 5-Methyltryptophan

	recovery (%)				
conditions[a]	Trp	α-MT	5-MT	Trp after correction	ref[b]
A	91.5 ± 2.2	96.2 ± 1.8		95.1 ± 0.7	1
A	91.5 ± 2.2		90.0 ± 1.7	101.7 ± 1.6	1
B	86.0 ± 0.6	93.4 ± 0.4		92.0 ± 1.1	1
B	84.9 ± 0.7		83.3 ± 0.8	102.0 ± 2.1	1
C	85.2 ± 0.8		82.7 ± 1.1	103.0 ± 1.2	2
D				100.6 ± 1.8	2
E	88.8 ± 8.6		86.9 ± 7.2	102.5 ± 3.7	3

[a] Conditions are as follows: (A) hydrolysis of protein-bound [^{3}H]tryptophan in the presence of partially hydrolyzed starch or thiodiglycol, 4 M NaOH or 4 M LiOH, and α- and 5-methyltryptophan; (B) hydrolysis of protein-bound [^{3}H]tryptophan in the presence of a chocolate drink powder, 4 M NaOH, and α- or 5-methyltryptophan; (C) heating of free tryptophan and 5-methyltryptophan in the presence of maltodextrins and 4 M LiOH; (D) hydrolysis of nine feedstuff samples with 4 M LiOH, maltodextrins, and 5-methyltryptophan [tryptophan recovery as corrected for losses of added 5-methyltryptophan and evaluated from data obtained with $Ba(OH)_2$ hydrolysates and taken as reference]; (E) hydrolysis of nine feedstuff samples with 4 M NaOH and 5-methyltryptophan [tryptophan recovery was evaluated from data obtained with $Ba(OH)_2$ hydrolysates and corrected for losses of added 5-methyltryptophan]. [b] References are as follows: (1) Nielsen and Hurrell (1985); (2) Landry *et al.* (1992); (3) Landry and Delhaye (1994).

evoked by Williams *et al.* (1982) and Nielsen and Hurrell (1985). Nevertheless, the assay of liberated tryptophan using ion-exchange chromatography and colorimetry without isolating lysinoalanine or other ninhydrin-positive compounds able to be cochromatographed with tryptophan on an ion exchanger can lead to some inconsistencies, as noted by Delhaye and Landry (1993).

(3) Tryptophan recovery is independent of the type of alkali used as hydrolysis agent, which is in keeping with the fact that only hydroxyl ions are effective for tryptophan liberation but inconsistent with the observations of Lucas and Sotelo (1980).

(4) The recovery of tryptophan is incomplete with the procedure most used for its assay, leading to a variable underestimation averaging 15%. It is noteworthy that five laboratories participating in an American ringtest have found a mean recovery of 85.4% (range 59−102%) on the basis of expected concentration of tryptophan in β-lactoglobulin (Allred and MacDonald, 1988).

(5) The efficiency of tryptophan recovery varies due to correction for losses of added internal standard. The use of 5-methyltryptophan leads to a correct estimation of tryptophan for laboratories 2 and 10 of the French ringtest, in keeping with the literature data, presented in Table 4 and pointing out a slight overestimation under those conditions. The same does not hold for laboratories 3 and 5 of the European ringtest. The underestimation is due to a probable error in the standardization for laboratory 3 and the fact that 5-methyltryptophan is added after hydrolysis in the procedure of Slump *et al.* (1992) used by laboratory 5. It is noteworthy that the two laboratories analyzing tryptophan of five feedstuff samples in the second European ringtest have found a mean recovery of 103.4 ± 4.5% (unpublished results). On the other hand, low recovery is achieved using α-methyltryptophan, confirming the data of Nielsen and Hurrell (1985) (Table 4) and those of the second European ringtest (unpublished results) but in disagreement with the finding of Bech-Andersen (1991).

CONCLUSION

The study presented here is based on about 500 tryptophan determinations for the French and European ringtests and more than 300 determinations for the literature data. It is the first report concerning the quantitative analysis of tryptophan of complex material from alkaline hydrolysis that takes into consideration so many data. Their normalization allows one to elucidate the apparently conflicting observations originating from investigations conducted over 80 years and to achieve a more coherent overview of the effects of conditions used for alkaline hydrolysis prior to tryptophan recovery and to confirm the existence of satisfactory methods for assaying tryptophan of feedstuffs using alkaline hydrolysis.

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