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Autor: Schneider, Hanna / Gerber, Nadine / Friedli-Wunderli, Barbara

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Quantitative Determination of Tryptophan in Wheat Flour, Milk Powder and Meat by High-Performance Liquid Chromatography – Short Note

Hanna Schneider[1], Nadine Gerber[2], Barbara Friedli-Wunderli[3], Caspar Wenk[2] and Renato Amadò[1]
ETH Zurich, CH-8092 Zurich, Switzerland

[1]Institute of Food Science and Nutrition, Food Chemistry
[2]Institute of Animal Science, Nutrition Biology
[3]Present address: Haco AG, 3073 Gümligen, Switzerland

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Introduction

An accurate determination of L-tryptophan is an important issue because this amino acid is essential for man as well as for many animals and must be ingested as part of the diet. The main function of L-tryptophan is as a building unit in protein synthesis. Additionally L-tryptophan is a precursor for serotonin (a neurotransmitter), melatonin (a neurohormone), and niacin and it is essential for brain functions and neuronal regulatory mechanisms by the action of its indole functional group.

L-tryptophan is found as a component of dietary protein. The quantitative determination of this amino acid in food and feed is a challenge for analytical chemists, because of its chemical instability. The indole group is very sensitive to redox reactions, particularly in strong acidic media as generally used for protein hydrolysis (1). Numerous efforts have been made to find a generally applicable method for the analysis of tryptophan (2). Some researchers have proposed direct reactions both with hydrolysed and with intact proteins applying procedures such as second-derivative spectroscopy (3, 4), reactions with *p*-dimethylaminobenzaldehyde (5), *p*-phenylenediamine (6) and ninhydrin in strong acid media (7). 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. Photometric methods can be tedious and suffer from problems such as colour stability and interferences with other food or feed components. According to most suggestions tryptophan is to be measured in hydrolysates by chromatography under time-consuming and special conditions. These include using (i) hydrochloric acid + additives (8–12), (ii) organic acids (13), (iii) enzyme (14), and (iv) bases (15–25). But since the release of tryptophan in the methode i–iii is often reported to be incomplete (17) the alkaline hydrolysis of protein-bound tryptophan has thus become the method of choice. The majority of these procedures involve the following basic steps: (a) alkaline hydrolysis of the sample at 110–125 °C in oxygen-free medium for 16–18 h; (b) dilution of the hydrolysate, with chromatographic buffer (neutralised or not); (c) clarification of the dilute hydrolysate; (d) high-performance liquid chromatographic (HPLC) separation; (e) spectrophotometric or fluorimetric determination (25).

The aims of this investigation were: a) to optimise a method particularly by taking into account for the differences in the ratio tryptophan to internal standard in order to have a simple and accurate method for quantitative determination of tryptophan; b) to evaluate the levels of tryptophan in different meat cuts as well as in wheat flour and milk powder.

Material and Methods

Determination of the protein content

Meat samples were heated to 1050 °C following AOAC 992.15 (26) in a Leco model CN-2000 (Leco Instruments, Michigan, USA) protein/nitrogen analyser calibrated with EDTA (Dumas method). The nitrogen to protein conversion factor considered was 6.25.

Determination of tryptophan

Principle

The food or feed sample is hydrolysed in alkali, neutralised and diluted. Subsequently tryptophan is determined by HPLC with fluorescence detection. α-methyl-tryptophan is used as internal standard.

Chemicals

The following chemicals were used: lithium hydroxide, D (+)-lactose-monohydrate, hydrochloric acid fuming 37 % (Fluka AG, Buchs, Switzerland), ortho-phosphoric acid, DL-tryptophan (Merck, Darmstadt, Germany), methanol Chromosolv® for HPLC (Riedel-de Haen, Buchs, Switzerland), α-methyl-tryptophan (Sigma, Taufkirchen, Germany), water of Nanopur quality (Skan AG, Allschwil, Switzerland).

The α-methyl-tryptophan standard (1 mg/ml) as well as the tryptophan standard (10 mg/1 ml) were dissolved in 0.4 M lithium hydroxide solution and stored at –18 °C until used.

Samples

Several meat samples from different productive livestock, commercial milk powder and wheat flour were analysed. The meat samples were homogenised (Moulinette S, Moulinex, Ecully Cedex, France) at room temperature and stored vacuum packaged (bags polyamide/polyethylene 90 µm, Inauen Maschinen AG, Herisau, Switzerland) at –18 °C until analysed. Milk powder and wheat flour were analysed without prior treatment.

Sample preparation

The amount of sample to be analysed was chosen in a way that it contained approximately 1 mg tryptophan, or 60 mg protein. The appropriate amount was weighed into a 25 ml Pyrex hydrolysis tube (Barloworld Scientific Ltd., Staffordshire, United Kingdom), 1 ml α-methyl-tryptophan standard solution (1 mg/1 ml), 150 mg D (+)-lactose-monohydrate and 19 ml 4 M lithium hydroxide solution were added.

The standard samples contained 100 µl tryptophan solution (10 mg/ml), 1 ml α-methyl-tryptophan solution (1 mg/ml), 150 mg D (+)-lactose-monohydrate and 19 ml 4 M lithium hydroxide solution. The samples were heated in an oven at 105 °C±2 °C for 1 h followed by 16 h at 120 °C±2 °C. During the first hour of hydrolysis the tubes were not tightly closed to completely remove the oxygen and for pressure equalisation in the flasks.

After cooling the samples were shaken and 1 ml hydrolysate was neutralised with 1 ml 4 M hydrochloric acid. The neutralised samples were diluted 1:200 with HPLC eluent (composition see below) and filtered in vials through 0.45 µm pore size membrane (17 mm Titan HPLC-Filter membrane PVDF, Infochroma AG, Zug, Switzerland).

With each sample batch, 4 standard samples containing 1 mg tryptophan and 1 mg α-methyl-tryptophan were analysed as well. The tryptophan concentration was calculated by means of a corresponding response factor. In addition samples with 0.5, 0.75 1.25 and 1.5 mg/ml tryptophan were analysed to calculate the recovery.

Chromatographic conditions

A Hewlett Packard HPLC system (Serie HP1050, Agilent Technologies, Palo Alto, California, USA) equipped with a fluorescence detector (Serie HP1046A, Hewlett-Packard) was used for all analyses. Separation was achieved with a Nucleosil 100-5 C 18 column (250×8×4 mm; Macherey Nagel AG, Oensingen, Switzerland).

A mobile phase consisting of 1.5 g ortho-phosphoric acid, 750 ml water and 250 ml methanol was used. The pH was adjusted to 4.00 with 6 M NaOH solution and the eluent filtered through a membrane (pore size: 0.22 µm, Infochroma).

Separation was performed at ambient temperature at a flow rate of 0.8 ml/min. The injection volume was 20 µl. The fluorescence detector was operated at an excitation wavelength of 228 nm and at an emission wavelength of 350 nm.

Each sample was injected twice; standards were measured at the beginning, in the middle and at the end of a series of analyses.

Calculation

Calculation of the response factor:

$$F = \frac{\text{Area Trp}}{\text{Area IS}}$$

F: Response factor
Trp: Tryptophan
IS: Internal Standard

Calculation of the tryptophan content in sample:

$$m = \frac{1 \cdot \text{Area Trp (sample)}}{F \cdot \text{Area IS (sample)}}$$

$$\%\text{Trp} = \frac{m \cdot 100}{E \cdot 1000}$$

m: Mass tryptophan in the sample
%Trp: Tryptophan content in weight by weight (w/w)
E: Weighed sample in g

Results and Discussion

Optimisation of the method

The method used in this study is based on the method published by VDLUFA (23). In preliminary experiments the method was optimised and an internal standard (α-methyl-tryptophan) was added to the sample before hydrolysis. The reliability of the method was checked by using different amounts of tryptophan at a constant concentration of internal standard.

Figure 1 shows to what extent the ratio tryptophan to internal standard (α-methyl-tryptophan) affects the accuracy and reliability of the determination. Setting a 5 % margin of error as limit of accuracy, none of the values for the samples containing 0.5 and 1.5 mg/ml tryptophan were within this range, whereas half of the values of the samples containing 0.75 and 1.25 mg/ml tryptophan were in the preset

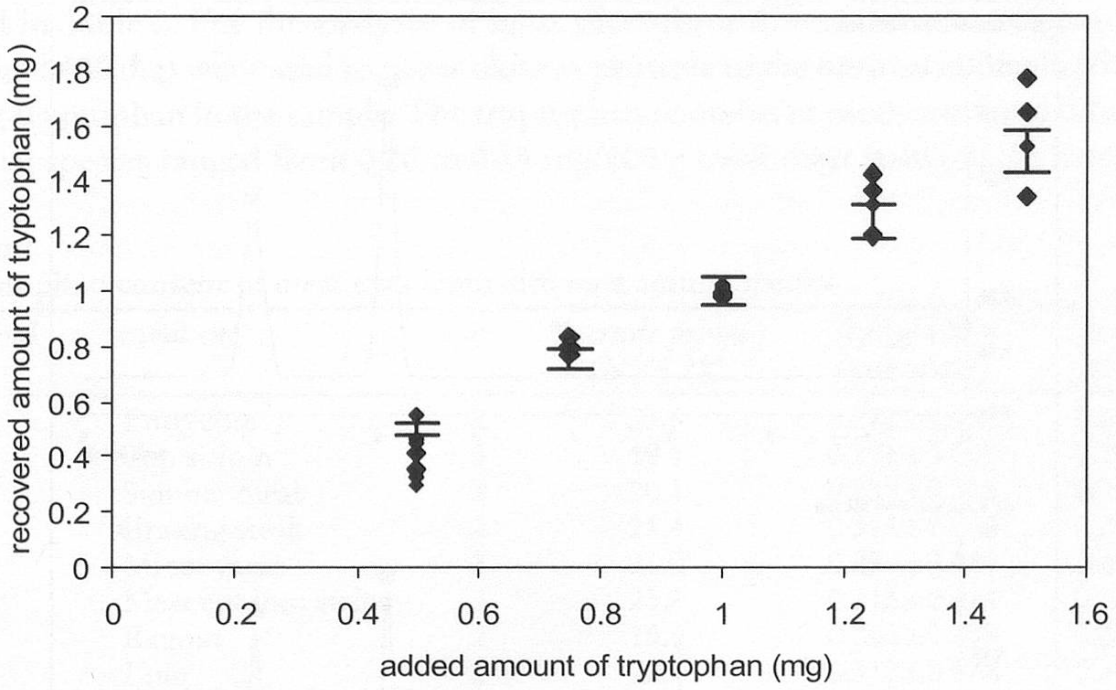

Figure 1 **Recovery rate of tryptophan at different concentration**
(♦ individual recovery rate, I ± 5 % margin of error as limit of accuracy)

range. These results clearly indicate that the applied method provides reliable results only for samples containing approximately 1 mg/ml tryptophan, the same amount as for the internal standard. Based on the above mentioned results three different sample weights were analysed to get as close as possible to the optimal ratio. The findings obtained with standard solutions were confirmed by experiments using different amounts of samples of milk powder and wheat flour for which similar results were found (results not shown).

Tryptophan analysis in food

To establish and test the performance of the method, the amount of tryptophan in milk powder, wheat flour, and meat samples were measured. Figure 2 shows typical chromatograms for a standard solution (A) and for a meat sample (veal rolled breast) (B). Tryptophan and α-methyl-tryptophan were resolved at baseline by applying the procedure to different type of samples. The peak eluting at a retention time of approx. 3 min has not been identified since it did not disturb the quantification of tryptophan in the food samples.

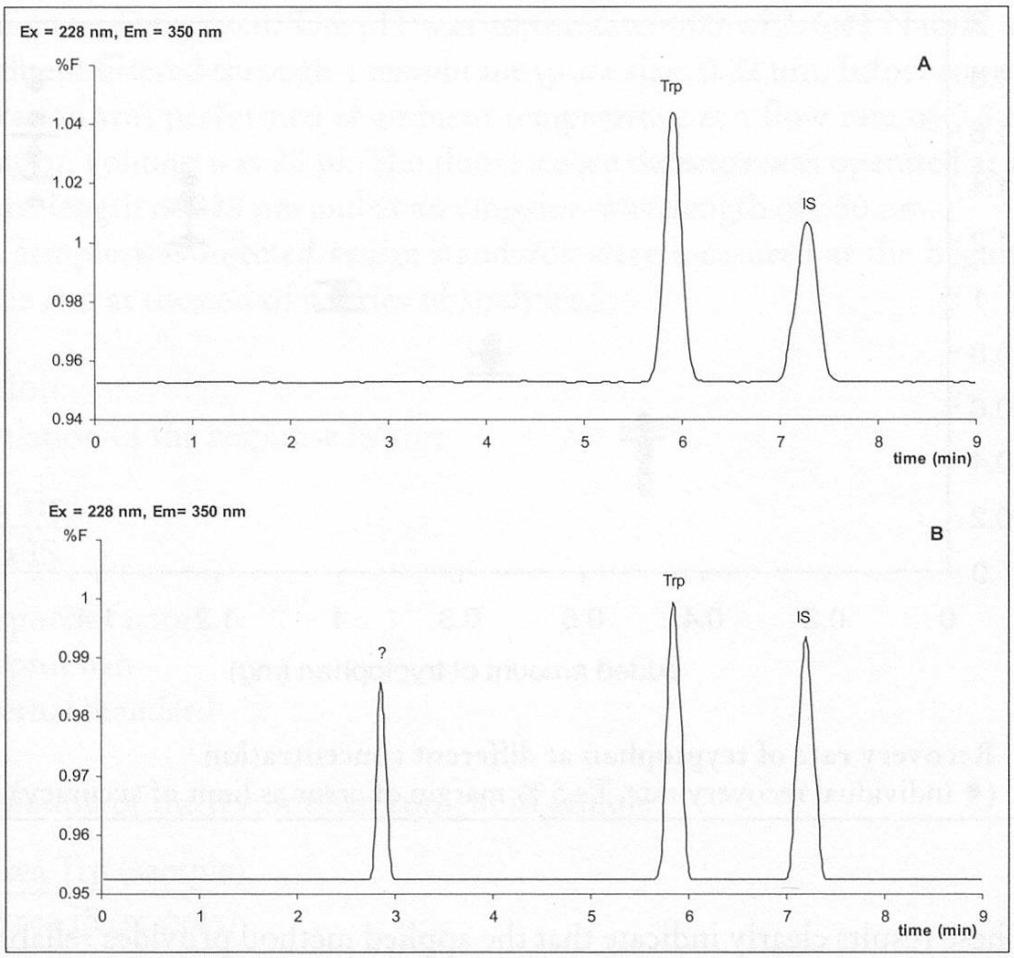

Figure 2 **HPLC chromatograms of tryptophan analyses**
A: Standard solution; B: meat sample (veal rolled breast)
Trp = tryptophan peak, IS = internal standard peak; ? = unknown peak;
Ex = excitation wavelength; Em = emission wavelength

Table 1 shows the data of tryptophan analyses of wheat flour and milk powder. The values obtained were compared with data found in the literature (19, 25, 27–28). Results lay within the range of those previously reported for these food stuffs. The examination of the literature data has revealed a wide variability in the determined tryptophan contents of wheat flour which could be due to the natural variability of the grains as well as to the various analytical methods used.

Table 1
Tryptophan content of different food samples

Food stuff	*Tryptophan (g/100 g product)*	*Literature values, range*
wheat flour (n=13)	0.176±0.009	0.145–0.21 [a]
milk powder (n=5)	0.364±0.015	0.35 [b]

[a] references (19, 25, 27–28); [b] references (28)

The results obtained for the tryptophan analyses with meat samples are complied in Table 2. For the analyses of meat slices three different initial weights (150, 300 and 450 mg) were used to get as close as possible to the optimal concentration of 1 mg tryptophan in the sample. The tryptophan contents of meat cuts from different animal species ranged from 0.20 to 0.35 mg/100 g fresh meat (table 2). In literature

Table 2
Tryptophan content of meat cuts from different animal species

Animal	*meat cut*	*n*	*% crude protein (N×6.25)*	*Trp (g/100 g fresh meat)*	*Literature (28)*
Beef	Entrecôte	2	23.8	0.282±0.035	0.290[a]
	Top sirloin	3	19.1	0.271±0.005	0.260[a]
	Simmer meat	3	20.1	0.259±0.018	0.250[a]
	Brasing steak	2	21.4	0.311±0.042	0.290[a]
	Mince meat	2	21.0	0.254±0.041	0.260[a]
	Meat cut into strips	2	23.7	0.313±0.014	0.280[a]
	Ragout	2	18.9	0.263±0.039	0.280[a]
Veal	Loin	2	22.1	0.310±0.078	0.300[a]
	Chop	3	20.2	0.255±0.014	0.270[a]
	Brasing steak	2	21.0	0.266±0.002	0.280[a]
	Rolled breast	3	18.1	0.228±0.029	
	Mince meat	2	20.7	0.284±0.053	0.270[a]
	Meat cut into strips	2	21.7	0.245±0.026	
	Ragout	2	18.8	0.217±0.035	
Pork	Chop	2	23.5	0.317±0.052	0.300[a]
	Loin	2	22.7	0.348±0.048	0.300[a]
	Brasing steak	2	22.2	0.277±0.038	0.300[a]
	Steak	3	18.2	0.241±0.025	0.250[a]
	Spare ribs	3	18.3	0.264±0.047	0.240[a]
	Mince meat	2	19.7	0.255±0.007	0.260[a]
	Meat cut into strips	2	22.0	0.289±0.015	0.290[a]
	Ragout	2	20.0	0.250±0.007	0.260[a]
Lamb	Gigot	2	20.7	0.255±0.034	
	Chop	2	21.9	0.269±0.040	
	Loin	2	22.0	0.283±0.034	
	Ragout	2	17.6	0.231±0.036	
Horse	Entrecôte	2	22.7	0.299±0.044	
	Filet	2	22.5	0.316±0.054	0.150[a, b]
	Brasing steak	2	21.0	0.302±0.043	
Foal	Entrecôte	2	24.5	0.348±0.058	
	Filet	2	22.3	0.316±0.063	
	Brasing steak	2	21.5	0.292±0.046	
ABison	Entrecôte	2	22.7	0.295±0.043	
Ostrich	Filet	2	20.6	0.289±0.081	
Deer	Entrecôte	2	21.0	0.287±0.049	
Rabbit	Filet	2	22.5	0.314±0.056	
Venison	Ragout	2	22.0	0.288±0.042	
	Escalope	2	23.1	0.312±0.054	
Wild boar	Entrecôte	2	21.0	0.261±0.047	

[a] reference (28); [b] reference (29)

(28) similar results can be found for veal, beef and pork. In contrast, the tryptophan content of horse meat in this study was found to be twice as high compared with other studies (28, 29). However, it has to be considered that in these studies the crude protein content was much lower. Furthermore, the tryptophan contents in study (28) were even calculated and not analysed. Only few or no detailed data about tryptophan content in different meat cuts can be found for bison, foal/horse, rabbit, venison, ostrich and wild boar.

Conclusion

The present results show that the method described provides reliable results only if the amount of tryptophan correspond to the amount of internal standard. In this case, the method has been shown to be suitable for a quantitative routine determination of tryptophan in different meat cuts, wheat flour and milk powder. The HPLC method is easy to perform, and gives highly reproducible results.

Summary

A high performance liquid chromatography method is presented which allows quantification of tryptophan in different food matrices. Tryptophan is liberated from proteins by an alkaline hydrolysis, followed by a neutralisation and appropriate dilution. Separation is performed by HPLC using fluorescence detection. Emphasis is laid on the optimisation of the ratio tryptophan to internal standard (α-methyl tryptophan) and accordingly to the sample weight. These parameters mostly influence the reliability of the results.

The method was established for routine tryptophan analysis in wheat flour, milk powder and particularly meat slices of different origin (veal, pork, beef etc).

Zusammenfassung

Eine HPLC Methode wird beschrieben, die eine einfache quantitative Bestimmung von Tryptophan in verschiedenen Lebensmittel Matrices erlaubt. Die Freisetzung des Tryptophans aus den Proteinen erfolgt durch eine alkalische Hydrolyse, gefolgt von einer Neutralisation und einer entsprechenden Verdünnung der Proben. Die Analyse wird mittels HPLC und Fluoreszenzdetektion durchgeführt. Das Schwergewicht bei der Methodenanpassung lag bei der Optimierung des Verhältnisses Tryptophan zu internem Standard (α-Methyl Tryptophan) und damit der Einwaage der Probe, da diese Parameter den grössten Einfluss auf die Zuverlässigkeit der erzielten Resultate haben.

Die Methode wurde erfolgreich für die routinemässige quantitative Bestimmung von Tryptophan in Weizenmehl, Milchpulver und insbesondere in Fleischproben unterschiedlicher Herkunft (Schwein, Rind Kalb usw.) verwendet.

Résumé

Une méthode de chromatographie en phase liquide à haute performance (HPLC) est présentée, laquelle permet une détermination simple et quantitative de l'acide aminé tryptophane dans des aliments avec des matrices différentes. La libération du tryptophane se fait par une hydrolyse alcaline, succédée par une neutralisation et une dilution appropriée des échantillons. L'analyse est faite par HPLC équipé avec un détecteur de fluorescence. L'adaptation de la méthode a été focalisée sur l'optimisation de la relation tryptophane/standard interne (α-méthyl-tryptophane) et avec ça sur le poids de l'échantillon. Ces deux paramèters ont l'effet le plus prononcé sur les résultats obtenus.

La méthode a été utilisée avec succès pour une détermination de routine du taux de tryptophane dans des échantillons de farine de blé, de poudre de lait et surtout de viande d'origine différente (veau, porc, bœuf, etc.).

Key words

Tryptophan, HPLC, meat, milk powder, wheat flour

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Corresponding address: Hanna Schneider, ETH Zurich, Institute for Food Science and Nutrition, Schmelzbergstrasse 9, CH-8092 Zurich, Switzerland,
Phone: +41 (0)44 632 55 89, e-mail: hanna.schneider@ilw.agrl.ethz.ch