



Separation and determination of the tryptophan enantiomers

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Abstract. Diastereoisomers of L- and D-tryptophan were formed with a chiral reagent 1-thio- β -D-glucose tetraacetate and *o*-phthaldialdehyde and they were separated from the derivatives of the other amino acids that occur in food proteins on an achiral column by high performance liquid chromatography. Mercaptoethanesulfonic acid that is an adequate agent for hydrolyzing proteins made the derivatization with *o*-phthaldialdehyde and 1-thio- β -D-glucose tetraacetate impossible, contrary the reaction completed in the presence of *p*-toluenesulfonic acid, but the oxidative losses during hydrolysis is significant. During boiling, the racemization of tryptophan can be detected above pH = 9 after 12 hours, but the rate of conversion was lower than expected (<1%). The concentration decrease of L-tryptophan after 24 h was 2–5% depending on pH. Besides racemization other reactions e.g. oxidative deterioration may played a role in the loss of L-tryptophan.

Key words and phrases: racemization, tryptophan, mercaptoethanesulfonic acid, *p*-toluenesulfonic acid

1 Introduction

The knowledge of the exact amino acid demand of the animals and the available amino acid content of the fodder is becoming a crucial point in animal nutrition due to its economical and environmental aspects. In the case of the domestic animal species the digestibility and the bioavailability of the D-enantiomer of a given amino acid is usually lower than that of the L-enantiomer. With the knowledge of the ratio of the amino acid enantiomers within the proteins of fodder, the amino acid requirements of the animals could be better satisfied. That could be especially important for essential amino acids like tryptophan (Trp). The determination of the amino acid enantiomers is also a question of importance in the human nutrition when the health effects due to the consumption of D-amino acids are studied.

In order to determine the amount of Trp in food samples the hydrolysis of the proteins is necessary. Under the most often used acidic hydrolysis conditions (6 M hydrochloric acid, 110 °C, 24 h) the amount of this amino acid partially decompose due to oxidative processes and the loss is even higher in real food samples when carbohydrates are present. Hydrolysis in alkaline solutions (e. g. sodium hydroxide) has been reported to preserve almost the whole Trp content of the sample [6]. Later collaborative studies clarified that the use of an internal standard is important in order to avoid the underestimation of the amount of Trp [9]. The AOAC method uses NaOH hydrolysis method [1] if the ratio of the enantiomers is not in the scope of the interest. In the case of the determination of D- and L-Trp, the enhanced degree of the racemization of Trp under alkaline conditions theoretically excludes this sort of solution for hydrolysis. Among acidic hydrolysis methods the highest recoveries were reported when 3 M mercaptoethanesulfonic acid [11] and 3 M *p*-toluenesulfonic acid containing 0.2% tryptamine [10] was used.

Besides the deliberation of the amino acids from the peptide chain the separation of the Trp enantiomers should be accomplished. In the case of high performance liquid chromatography (HPLC) there are three main possibilities: using columns with a chiral stationary phase, using an achiral stationary phase column with a chiral mobile phase, or derivatization with a chiral reagent and separation of the diastereoisomers formed on an achiral stationary phase column. Precolumn derivatization can be accomplished with *o*-phthaldialdehyde (OPA) and N-isobutyryl-L-cysteine (IBLC) [2] or N-isobutyryl-D-cysteine (IBDC) [3], with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) [7], or with OPA and 1-thio- β -D-glucose tetraacetate (TATG) [8].

The primary purpose of the research was to achieve an analytical method

that renders practicable the detection of the racemization of Trp. First the separation of the Trp enantiomers in the form of diastereoisomers was accomplished on an achiral column in the presence of the other amino acids occurring in food proteins. Later on the applicability of acidic hydrolysis methods was investigated to clarify how they can be used prior to derivatization and analysis when protein bound amino acids has to be analyzed. Finally the racemization kinetics of free L-Trp of during heat treatments at different pH was studied.

2 Material and methods

Derivatization and analysis. Diastereoisomers were produced with OPA (*o*-phthalaldehyde) and TATG (1-thio- β -D-glucose tetraacetate) based on the methods of Einarsson et al. [8] and Csapó et al. [4]. 200 μ l sodium-tetraborate buffer (pH = 9.5) was added to 460 μ l hydrolyzed protein solution or free amino acid standard solution containing 0.16 mg/ml amino acid, then 20 μ l derivatization reagent (8 mg OPA and 44 mg TATG were dissolved in 1000 μ l methanol) was added. The solution was mixed and after 6 minute-standing 20 μ l was injected into the HPLC. OPA was obtained from Sigma Chemica Co. St Louis USA, and TATG was purchased from Aldrich-Chemie GmbH, Steinheim, Germany. The separation was performed with a Superspher 60 RP-8e column or with a Purospher RP-18e 125 \times 4 column (MERCK, Darmstadt, Germany); the temperature of the oven was 40 °C. The organic solvents were gradient grade methanol and acetonitril (MERCK, Darmstadt, Germany). The pH of the 39 mM phosphate buffer was set to 7.0 with 6 M hydrochloric acid solution, and ultrapure water for the preparation of the buffer was obtained from a Millipore Direct-Q instrument (Millipore, Molstein, France). The derivatives were detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm). Derivatization and analysis were carried out with a MERCK-Hitachi HPLC containing the following modules: L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, AIA data conversion utility for D-7000 HPLC system manager (MERCK, Darmstadt, Germany).

Two sorts of amino acid standards were used for the HPLC method development. The first solution did not contain Trp. This solution consisted of 40 nmol/ml of the following protein constructed amino acids dissolved in water: D-Asp, L-Asp, D-Thr, L-Thr, D-Ser, L-Ser, D-Glu, L-Glu, D-Ala, L-Ala, D-Val, L-Val, D-Met, L-Met, L-D-, D-L-, L-L-, D-D-Ile, D-Leu, L-Leu, D-Tyr, L-Tyr, D-Phe, L-Phe, D-Lys, L-Lys, D-His, L-His, D-Arg, L-Arg and Gly. Secondary amino acids as Pro are not derivatized by OPA. Cys is deriva-

tized, but the fluorescence quantum yield is very low. The amide group of Asn and Gln hydrolyze during the hydrolysis of proteins and form Asp and Glu, therefore these four amino acids were not included into the standard solution. The second standard solution consisted of 40 nmol/ml L-Trp and 20 nmol/ml D-Trp. The D- and L-amino acids were purchased from Sigma-Aldrich, St Louis, USA).

Hydrolysis. In order to eliminate the reaction of MES-OH and TATG three sorts of trials were applied. The aim was to remove or destroy MES-OH. First 5 ml 0.5 M CuSO₄ solution was added to 5 ml mercaptoethanesulfonic acid solution (0.01 mmol D- and 0.01 mmol L-Trp and 4.5 mmol MES-OH) then the solution was centrifuged at 4000 g for 20 min. The pH of the supernatant was set between 5 and 6 with 4 M NaOH solution.

For the second time the mercaptoethanesulfonic acid solution (3 ml 3 M MES-OH and 0.01 mmol D- and 0.01 mmol L-Trp) was diluted three-fold, then 3 ml oxidising solution (the mixture of one part of 30 (w/v)% H₂O₂ and nine part of 85 (w/v) % formic acid) was added to 1 ml solution. The mixture was heated at 50 °C for 5 minutes. After cooling the remaining performic acid was reacted with 0.52 g sodium-metabisulfite. The same procedure was accomplished with D- and L-alanine (0.01 mmol of each).

For the third time 1 ml aliquots of mercaptoethanesulfonic acid solution (1 ml 3 M MES-OH solution and 0.01 mmol of D- and 0.01 mmol of L-Trp in 5 ml; 0.6 mmol MES-OH in each ml) was placed into a 25-ml-volumetric flask. The pH was set to 2, 6 or 9 with 4 M NaOH (a control sample also was prepared without pH setting) then 20 ml distilled water and 1 ml 0.1138 g /ml (0.612 mmol) iodoacetic acid solution was added and the volume was set with distilled water. Mercaptoethanesulfonic acid was obtained from Fluka Chemie GmbH, Buchs, Switzerland.

In the case of *p*-toluenesulfonic acid hydrolysis 5 ml 3 M *p*-toluenesulfonic acid solution containing 0.2% 3-(2-aminoethyl)indole (tryptamine) was added to the sample containing 15 mg protein. The hydrolysis was carried out in a closed ampoule under nitrogen at 110 °C for 24 h. Lyophilized sheep hemoglobin samples were hydrolyzed with this method. The *p*-toluenesulfonic acid was obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Neutralization was carried out with 4 M NaOH and the solution was diluted 125-fold with water.

Boiling. Free L-Trp-solutions (1 mg L-Trp/ml) with different pH values (pH=3; 5; 7; 9; and 11) were prepared. The solutions were acidified with 6 M hydrochloric acid solution and the alkaline pH was set with 4 M NaOH solution. The ampoules with solutions were sealed after purging with nitrogen for 2 min. The L-Trp solutions were kept at $100\text{ }^{\circ}\text{C}\pm 1\text{ }^{\circ}\text{C}$ for 0; 5; 10; 20; 40; 60 min and 2; 4; 8; 12; 24; 48 hours.

3 Results

The separation of the derivatives of L- and D-Trp. Though the hydrolysis of proteins is prior to the analysis of the amino acids, the studying of the hydrolysis methods cannot be achieved without an analytical method for the separation of the Trp enantiomers. To reach the aim, D- and L- Trp were reacted with OPA and TATG and the resulting diastereoisomers were separated on an achiral stationary phase column following fluorescence detection with high performance liquid chromatography. An amino acid standard solution that contained the amino acids that are present in the food proteins with the exception of Trp and another standard solution of D- and L-Trp was used for method development in order to avoid interferences.

Table 1: Eluent composition applied for the separation of OPA-TATG derivatives of L- and D-Trp from the derivatives of the other amino acids that occur in food proteins

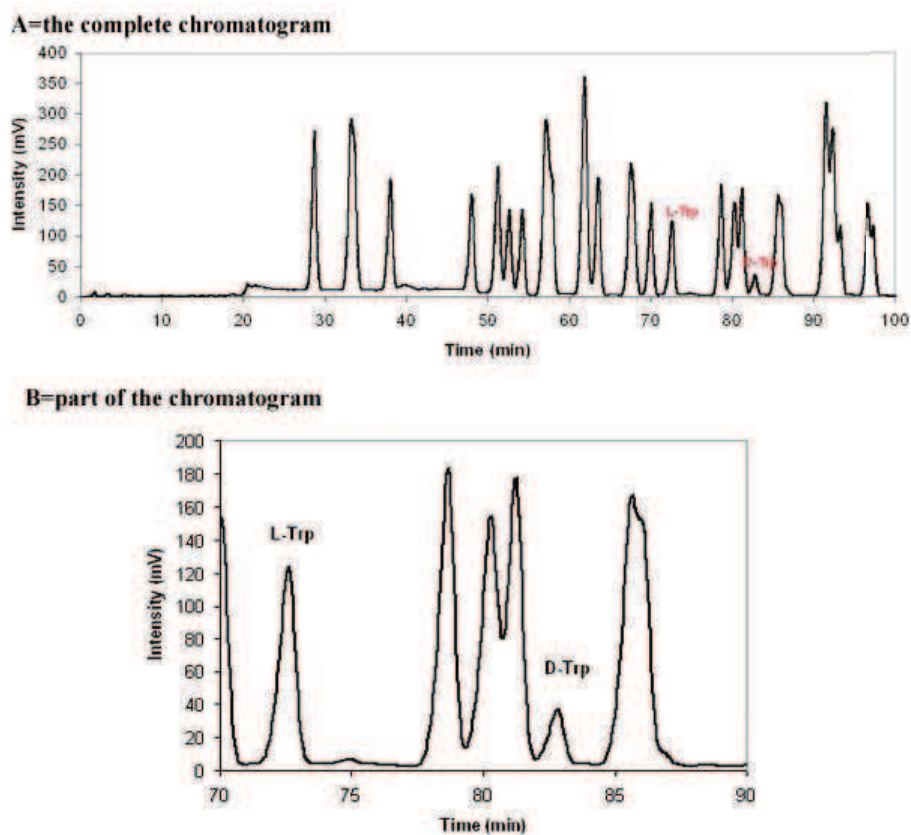
Time (min)	Metanol (v/v%)	Phosphate buffer (39 mM, pH=7.0) (v/v%)	Acetonitril (v/v%)
0	20	80	0
120	20	45	35
130	20	45	35
135	20	80	0
140	20	80	0

(Column: Purospher RP-18e; 125 mm \times 4 mm; flow rate: 1 ml/min)

During the optimization of the separation the sort of the stationary phase; the type (methanol, acetonitril or both) and the ratio of the organic solvent was changed. The best separation (see *Figure 1* for chromatogram containing

both Trp and the other amino acids) was achieved on the RP-18 column and the composition of the mobile phase can be seen in *Table 1*.

Figure 1: Separation of the OPA-TATG derivatives of L- and D-Trp from the derivatives of the other amino acids that are occur in food proteins



(Column: Purospher RP-18e; 125 mm × 4 mm; flow rate: 1 ml/min)

There was a significant difference between the fluorescence factors of the enantiomers. With the same concentration increase the relative fluorescence of L-Trp was 2.4-fold higher than that of D-Trp. The data of calibration graphs

for the enantiomers can be seen in *Table 2*. The detector response was linear between 14 and 336 ng L-Trp / injection, and 14–700 ng D-Trp / injection. The limit of detection was 0.9 ng / injection for L- and 0.7 ng / injection for D-Trp. The limit of quantification for L- and D-Trp was 9.1 and 7.5 ng / injection, respectively. The relative standard deviation (RSD) in the low concentration range (14 ng L-Trp and 28 ng D-Trp / injection) were 1.1 and 4.1%; in the medium concentration range (140 ng L-Trp and 280 ng D-Trp / injection) were 6.8 and 1.9%; and in the high concentration range (276 ng L-Trp and 552 ng D-Trp / injection) were 1.6 and 2.3%, respectively. The number of replicates was 6.

Table 2: The calibration graph data of the OPA-TATG derivatives of the tryptophan enantiomers detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm)

Amino acid	Linear range nmol/ml	Correlation coefficient	Slope (A) (x,y see below)	Intercept (B)	LOQ nmol/ml
L-Trp	3 – 72	0.9998	12.6	–2.6	2.2
D-Trp	3 – 150	0.9996	5.3	–3.0	1.8

X = height of the peak (mV) / concentration (nmol/ml)

Y = concentration (nmol/ml)

Investigation of hydrolysis methods. Before the analyses of real samples different hydrolysis methods were tried out with standard Trp-solutions in order to examine if the following step the derivatization with OPA-TATG can be carried out.

Mercaptoethanesulfonic acid hydrolysis. In the presence of mercaptoethanesulfonic acid (MES-OH) D- and L-Trp did not form derivatives with OPA and TATG. Probably, instead of the bulky molecule of TATG, the MES-OH molecule reacts with OPA and the amino acid, and an achiral derivative of Trp forms. In the case of the other amino acids that occur in the protein MES-OH was applied for both hydrolysis and formation of achiral derivatives with OPA [5]. In the case of Trp OPA/MES-OH derivatives can also be formed and used for the determination of the amount of (L+D) Trp. When the knowledge

of the ratio of the Trp enantiomers is important, the building of MES-OH into the derivative should be eliminated. When hydrolysis is completed and the solution with free Trp is ready to derivatization, MES-OH should be converted into nonactive form with respect to the above-mentioned derivatization. To reach the aim three trials were conducted:

Elimination of MES-OH in the form of metal salt: The copper-mercaptide precipitate was separated from the suspensoid with centrifugation and the clear supernatant was used for derivatization.

Performic acid oxidation of MES-OH: The thiol group of MES-OH was converted into sulfonic acid group. The remaining amount of performic acid was reacted with sodium-metabisulfite.

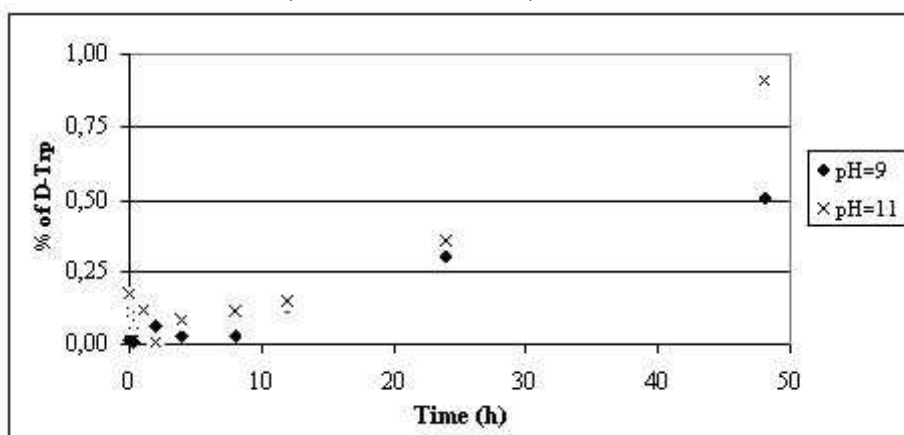
Reaction of MES-OH with iodoacetic acid: In order to block the thiol group, carboxyl-methyl derivative of MES-OH was formed.

In the first and the second cases we cannot detect OPA-TATG derivatives of D- and L-Trp. In the third trial there were some formation of the required derivatives, but the conversion rate of D- and L-Trp were poor. The amount of TATG was increased in order to provide enough TATG for derivatization, because TATG can also react with the remaining iodoacetic acid, but the level of conversion did not increase.

***p*-Toluenesulfonic acid hydrolysis.** First *p*-toluenesulfonic acid was added to standard solutions of L- and D-Trp and it can be ascertained that in the presence of *p*-toluenesulfonic acid the reaction of D- and L-Trp into OPA-TATG derivatives was completed. When samples are heat-treated during hydrolysis the addition of tryptamine is necessary [10] because this molecule is a protecting agent against oxidative conversion of Trp. In the presence of 0.2% tryptamine in the *p*-toluenesulfonic acid solution the derivatization of Trp is blocked due to the amine group of tryptamine. The recovery of *p*-toluenesulfonic acid hydrolysis without tryptamine was reported to be low [10]. The same tendency was observed when sheep hemoglobine was hydrolyzed without tryptamine and the recovery of L+D Trp was $44\pm 2\%$. Despite of the low recovery the ratio of the L- and D-Trp can be established. The next step should be the use of another protecting agent such as 3-(3-indolyl)propionic acid that does not contain amine group but suitable for the protection of the indole ring of Trp from oxidative deterioration.

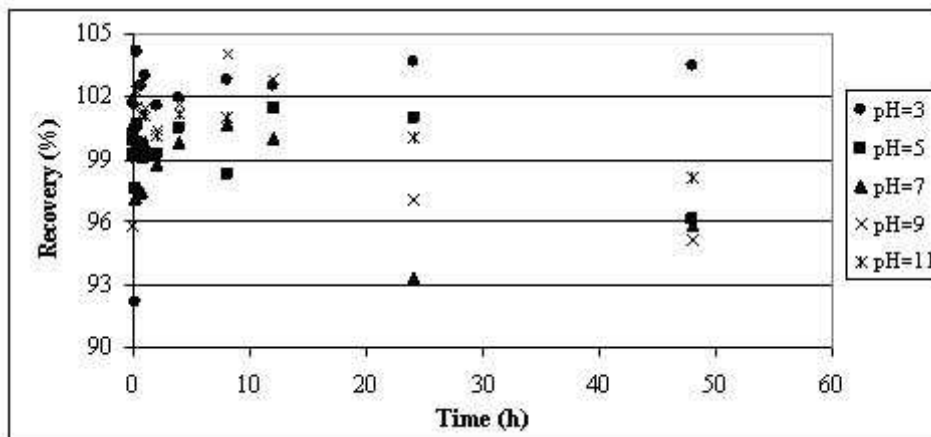
The influence of boiling on the Trp content in the function of pH and time. The loss of L-Trp due to racemization was not significant at lower pH values (pH = 3–7). The amount of the D-Trp increases at pH = 9 and pH = 11 after twelve hours of boiling (*Figure 2*), but the rate of conversion (<1%) is lower than expected.

Figure 2: The increase of D-Trp content during boiling at 100 °C at pH = 9 and pH = 11 (% of Trp content)



After 24 h the amount of L-Trp tended to decline slightly (*Figure 3*). When the boiling exceeds one day, the loss of Trp can be 2–5%. Beside racemization other reactions e.g. oxidative deterioration of Trp indole-ring can be responsible for the loss of L-Trp.

Figure 3: Change in the L-Trp content during heat treatment at 100 °C in aquatic solutions at different pH



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