

NEW POSSIBILITIES FOR THE DETERMINATION OF THE TRYPTOPHAN ENANTIOMERS**NOVE MOGUĆNOSTI ZA ODREĐIVANJE ENANTIOMERA TRIPTOFANA****K. Lóki, Cs. Albert, É. Varga-Visi, P. Sára, Zs. Csapó-Kiss, J. Csapó**

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SUMMARY

Diastereoisomers of L- and D-tryptophan were formed with a chiral reagent 1-thio- β -D-glucose tetraacetate (TATG) and o-phthalaldehyde (OPA) and separated from the derivatives of the other amino acids that occur in food proteins on an achiral column by high performance liquid chromatography. Mercaptoethanesulphonic acid that is an adequate agent for hydrolyzing proteins made the OPA/TATG derivatization impossible, contrary to the reaction completed in the presence of *p*-toluenesulphonic acid. During boiling, the racemization of tryptophan could be detected after 12 hours above pH=9, 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. Beside racemization other reactions e. g. oxidative deterioration may have played a role in the loss of L-Trp.

Key words: tryptophan, hydrolysis, mercaptoethanesulfonic acid, *p*-toluenesulfonic acid, racemization, 1-thio- β -D-glucose tetraacetate

INTRODUCTION

The knowledge of the amount of the amino acid enantiomers in the constituents of the fodder can serve as an additional information on the biological value of the feed. When it contains a significant amount of D-amino acids the proteases cannot cleave effectively the peptide bonds of proteins, decreasing the digestibility of protein, and the absorption of the free D-enantiomer can also be restricted related to its mirror image pair. The absorbed D-enantiomers are not converted completely into the L-form because the efficiency of D-amino acid oxidase system of the mammals is restricted (*Friedman*, 1999). Thus the presence of

the D-amino acids can lead to the loss of nutritious protein source.

The analysis of tryptophan (Trp) is probably the most difficult one among the amino acid quantification methods. In the case of fodder samples the amino acids need to be deliberated from the peptide chain prior to analysis. The hydrochloric acid hydrolysis can be applied in the

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case of all amino acids in the protein with one exception: Trp almost totally decomposes during these harsh conditions. In order to protect Trp from the oxidative deterioration protecting agents with thiol groups were applied (*Freedlender and Haber, 1972; Matsubara and Sasaki, 1969*). Another possibility is using *p*-toluenesulfonic acid with 3-(2-aminoethyl)indol (triptamin) (*Liu and Chang, 1971*) or mercaptoethanesulfonic acid (*Penke et al., 1974*) for hydrolysis. The best recoveries of Trp were obtained in the case of alkaline hydrolysis with barium- or sodium hydroxide (*Miller, 1967*). In the presence of alkaline the racemization of the amino acids has been reported to be accelerated (*Friedman, 1999*) thus prior to the analysis of Trp enantiomers the above mentioned hydrolysis methods are not supposed to be appropriate.

Besides hydrolysis the quantification of the enantiomers should be accomplished. The diastereoisomers of the amino acids can be separated on an achiral stationary phase chromatography column. With the use of *o*-phthalaldehyde (OPA) and 1-thio- β -D-glucose tetraacetate (TATG) the separation of the other amino acids was obtained (*Einarsson et al., 1987*).

The aim of the research was to develop a high performance liquid chromatography method for the determination of the L- and D-Trp content and the selection of the best hydrolysis method in order to cleave the proteins into amino acids prior to analysis. In order to study the applicability of alkaline hydrolysis the pH-dependence of the racemization of Trp was observed at high temperature.

MATERIALS AND METHODS

Hydrolysis

p-Toluenesulfonic acid hydrolysis was carried out using 10 cm³ 0.2% 3-(2-aminoethyl)indole (tryptamine) in 3 M *p*-toluenesulfonic acid solution for a sample containing 0.1 mg Trp. The hydrolysis was completed at 110 °C for 24 h in nitrogen atmosphere. The neutralization was carried out with 4 M NaOH and the solution was diluted 2.5-fold with distilled water.

During mercaptoethanesulfonic acid (MES-OH) hydrolysis 10 cm³ 3 M MES-OH the solution was added to sample containing 0.1 mg Trp. The hydrolysis was carried out at 110 °C for 24 h in nitrogen atmosphere. The pH was set to pH=7 with 4 M NaOH. In this case for the elimination of the reaction of MES-OH and TATG three sorts of trials were applied before derivatization and analysis. Firstly 5 cm³ 0.5 M CuSO₄ the solution was added to 5 cm³ 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. Secondly the mercaptoethanesulfonic acid solution (3 cm³ 3 M MES-OH and 0.01 mmol D- and 0.01 mmol L-Trp) was diluted three-fold, then 3 cm³ oxidizing solution (the mixture of one part of 30 (w/v)% H₂O₂ and nine parts of 85 (w/v) % formic acid) was added to 1 cm³ 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). Thirdly 1 cm³ aliquots of mercaptoethanesulfonic acid solution (1 cm³ 3 M MES-OH solution and 0.01 mmol of D- and 0.01 mmol of L-Trp in 5 cm³; 0.6 mmol MES-OH in each cm³) was placed into a 25-cm³-volumetric flask. The pH was set to 2, 6 or 9 with 4 M NaOH (a control sample was also prepared without pH setting) then 20 cm³ distilled water and 1 cm³ 0.1138 g/cm³ (0.612 mmol) iodoacetic acid solution was added and the volume was set with distilled water.

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. (1997)* and *Csapó et al. (1995)*. The separation was performed using a Superspher 60 RP-8e column or using a Purospher RP-18e 125x4 column; the temperature of the oven was 40 °C. 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).

Boiling

The L-Trp solutions (with the pH values of 3; 5; 7; 9; 11) 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. The initial concentration of L-Trp was 1 mg/cm^3 . The ampoules with solutions were sealed after purging with nitrogen for 2 min.

RESULTS

Studying the acidic hydrolysis methods

Mercaptoethanesulfonic acid (MES-OH) can be applied for both hydrolysis and derivation of amino acids. In the presence of OPA and MES-OH amino acids form achiral derivatives with fluorescence properties (Csapó, 2004). In the case of Trp OPA/MES-OH derivatives can also be formed and can be used for the determination of the amount of (L+D) Trp. The difficulties arise when amino acid enantiomers should be determined and instead of MES-OH a chiral reagent TATG should be incorporated into the molecule. When MES-OH is present 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 present work three experiments were conducted for the elimination of the negative effect of MES-OH on the derivatization. Firstly metal-mercaptide was formed and the copper-mercaptide precipitate was separated from the suspension by centrifugation and the clear supernatant was used for derivatization. Secondly, the thiol group of MES-OH was converted into sulphonic acid group with performic acid oxidation. The remaining amount of performic acid was reacted with sodium-metabisulphite. After these reactions the OPA/TATG derivatization of Trp enantiomers was

not been accomplished: the OPA/TATG derivatives of D- and L-Trp cannot be detected. At the third experiment iodoacetic acid was used in order to block the thiol group of MES-OH with the formation of carboxyl-methyl derivative of MES-OH. After this trial there was some formation of the required derivatives, but the conversion rate of D- and L-Trp was 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.

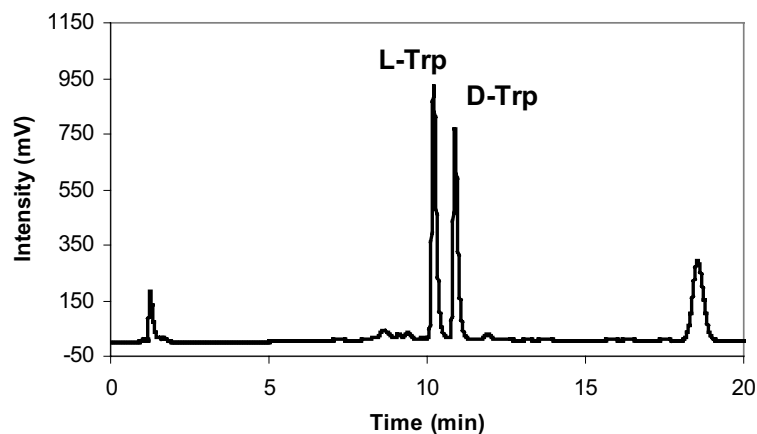
p-Toluenesulfonic acid hydrolysis of proteins gave the second highest recoveries among the acidic hydrolysis methods and there was an absence of thiols therefore *p*-toluenesulphonic acid was thought to be an appropriate reagent for hydrolysis without disturbing the following OPA/TATG derivatization. In the presence of *p*-toluenesulfonic acid the conversion of D- and L-Trp into OPA/TATG derivatives was adequate. It means that despite the lower recovery of *p*-toluenesulfonic acid hydrolysis (Penke *et al.*, 1974, Liu and Chang, 1971) this method should be applied if the separation of the enantiomers is intended to solve on an achiral column as OPA/TATG diastereoisomer derivatives. However, when triptamine was added to the solutions in order to increase the recovery of Trp, precipitation was observed during derivatization, and the recovery drastically decreased. Maybe the amino group of the triptamine also reacted with the derivatization reagents and thus there were not enough reagents for the derivatization of L- and D-Trp. In this manner *p*-toluenesulphonic acid hydrolysis can be used solely in the absence of triptamine.

Precolumn derivatization and separation of derivatives of L- and D-Trp

First diastereoisomers were formed during precolumn derivatization with a chiral reagent TATG and OPA, and the separation was accomplished on an achiral stationary phase column following fluorescence detection with high performance liquid chromatography. The separation of the diastereoisomers can be seen in Figure 1, and the gradient conditions are shown in Table 1.

Figure 1. Separation of the OPA/TATG derivatives of L- and D-Trp (Column: Superspher 60 RP-8e; 125 mm x 4 mm; flow rate: 1 cm³ min⁻¹)

Slika 1. Separacija OPA/TATG derivata L- i D-Trp (Stupac: Superspher 60 RP-8e; 125 mm x 4mm; stopa protoka: 1 cm³ min⁻¹)



In the case of the presence of other protein-constructing amino acids more time and another column was necessary for separation (Figure 2 and Table 2).

Figure 2. Separation of the OPA/TATG derivatives of L- and D-Trp from the other amino acids. (Column: Purospher RP-18e; 125 mm x 4 mm; flow rate: 1 cm³ min⁻¹).

Slika 2. Separacija OPA/TATG derivata od L- i D-Trp iz drugih aminokiselina (Stupac: Purospher 60 RP-18e; 125 mm x 4mm; stopa protoka: 1 cm³ min⁻¹)

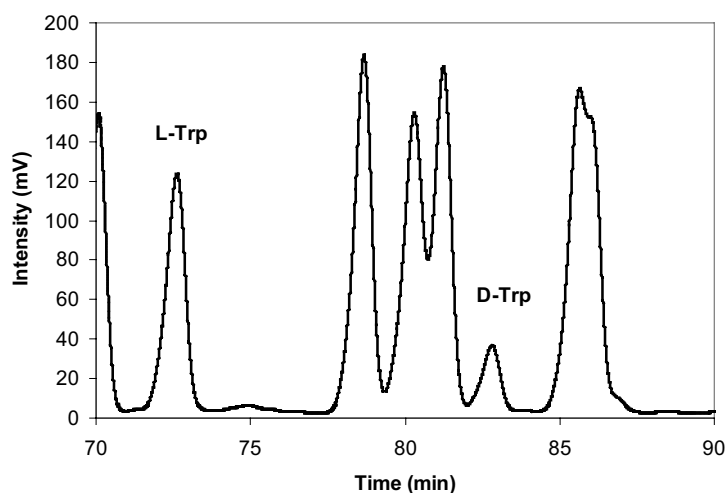


Table 1. Eluent composition applied for the separation of OPA/TATG derivatives of L- and D-Trp (Column: Superspher 60 RP-8e; 125 mm x 4 mm; flow rate: 1 cm³ min⁻¹)

Tablica 1. Sastav eluenta primijenjen za separiranje OPA/TATG derivata L- i D-TRp (Stupac: Superspher 60 RP-8e; 125 mm x 4mm; stopa protoka: 1 cm³ min⁻¹)

Time Vrijeme (min)	Metanol (v/v%)	Phosphate buffer (39 mM, pH=7.0) (v/v%)	Acetonitril (v/v%)
0	28	72	0
10	17	47	36
20	17	47	36
25	28	72	0
30	28	72	0

Table 2. Eluent composition applied for the separation of OPA/TATG derivatives of L- and D-Trp from the other amino acids. (Column: Purospher RP-18e; 125 mm x 4 mm; flow rate: 1 cm³ min⁻¹).

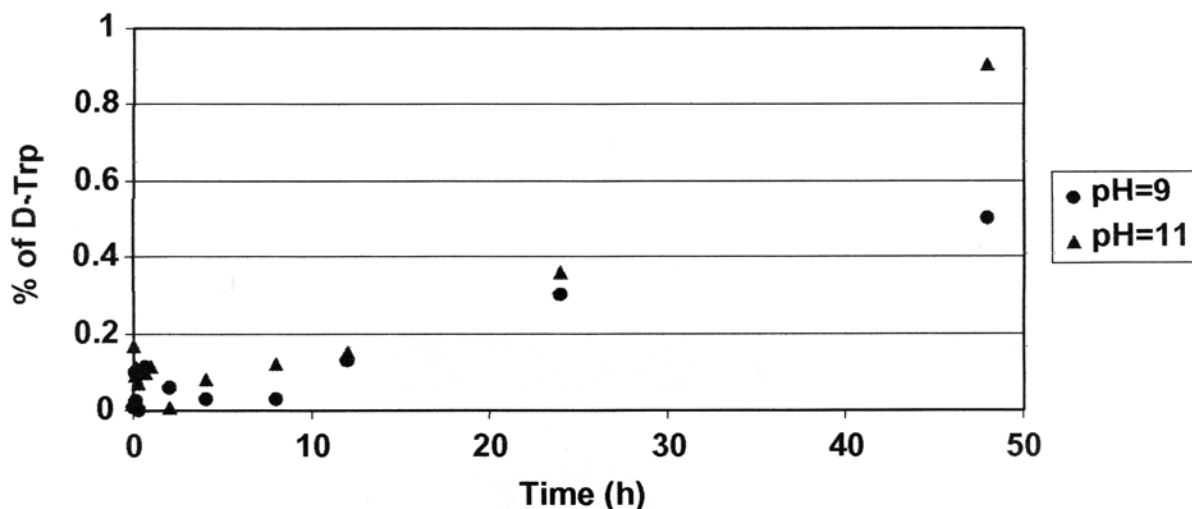
Tablica 2. Sastav eluenta primijenjen za separiranje OPA/TATG derivata L- i D-Trp iz drugih aminokiselina (Stupac: Purospher 60 RP-18e; 125 mm x 4mm; stopa protoka: 1 cm³ min⁻¹)

Time Vrijeme (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

The influence of boiling on the Trp content in the function of pH and time

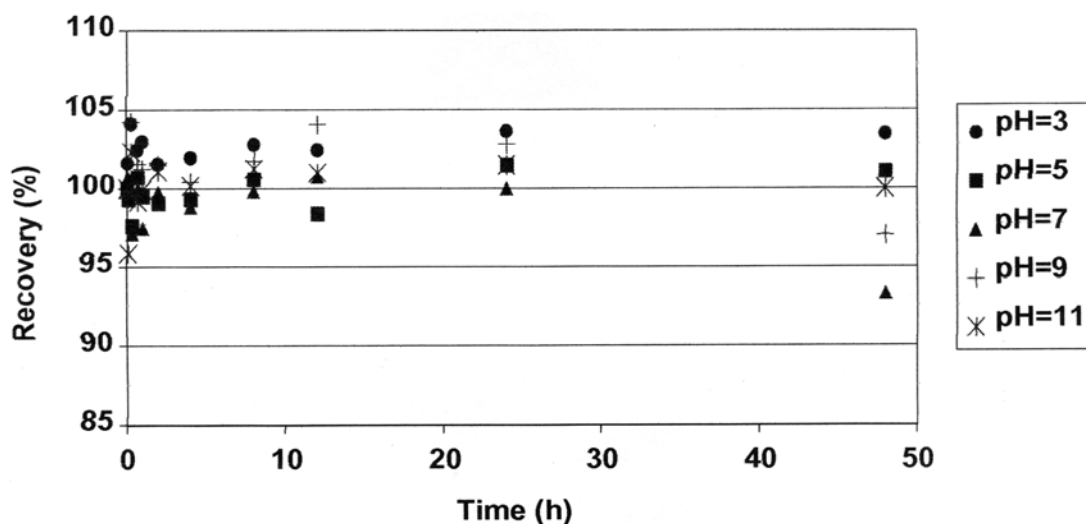
The loss of L-Trp due to racemization was not significant at lower pH values (pH=3-7). The amount of the D-Trp increased at pH=9 and pH=11 after twelve hours of boiling (Figure 3), but the rate of conversion (<1%) was lower than expected.

Fig 3. The increase of D-Trp content during boiling at 100 °C at pH=7 and pH=11 (% of Trp content)
Slika 3. Porast sadržaja D-Trp za vrijeme vrenja na 100 °C uz pH=7 i pH=11 (% sadržaja Trp)



After 24 h the amount of L-Trp tended to decline slightly (Figure 4). When boiling exceeded one day, the loss of Trp could be 2-5%. Besides racemization other reactions e. g. oxidative deterioration of Trp indole ring can be responsible for the loss of L-Trp.

Fig 4. Change in the L-Trp content during boiling at 100 °C at different pH
Slika 4. Promjena u sadržaju L-Trp za vrijeme vrenja na 100 °C uz različiti DH



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SAŽETAK

Dijastereoizomeri L. i D-triptofana formirali su se s kiralnira reagensom L-thio- -D tetracetatom glukoze (TATG) io-phthalaldehydom (OPA) te su odvojeni od derivata drugih aminokiselina, što se nalaze u bjelančevinama u hrani, u kiralnom stupcu (vrlo djelotvorne) tekuće kromatografije visoke performance. Merkaptoetansulfonična kiselina, odgovarajući agens za hidrolizu bjelančevina, onemogućila je derivaciju OPA/TATG suprotno reakciji dovršenoj u prisutnosti p-toluenulfonske kiseline. Za vrijeme vrenja racemizacija triptofana mogla se uočiti nakon 12 sati iznad pH=9, ali stopa konverzije bila je niža od očekivane (<1%). Smanjenje koncentracije L-triptofana nakon 24 sata bilo je 2 do 5%, ovisno o pH. Osim racemizacije druge su reakcije, npr. slabljenje oksidacije, mogle igrati ulogu u gubitku L-triptofana.

Ključne riječi: triptofan, hidroliza, merkaptoetansulfonska kiselina, p-toluenesulfonska kiselina, racemizacija, l-thio- -D-glukozni tetraacetat