KRMIVA

SEPARATION AND DETERMINATION OF SULPHUR CONTAINING AMINO ACID ENANTIOMERS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

SEPARIRANJE I ODREĐIVANJE ENANTIOMERA AMINOKISELINA KOJE SADRŽE SUMPOR POMOĆU TEKUĆE KROMATOGRAFIJE VISOKE PERFORMANCE

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SUMMARY

Performic acid oxidation of cysteine and methionine resulting in the formation of cysteic acid and methionine sulphon has been applied in order to avoid the loss of these two sulphur containing amino acids during the acidic hydrolysis of proteins that is necessary prior to amino acid analysis. The aim of the research was assigned by the increasing demand for the determination of the amount of amino acid enantiomers: the applicability of performic acid oxidation was evaluated from this point of view. Racemization of L-cysteine and L-methionine was found not significant during oxidation with performic acid, therefore this process can be applied before hydrolysis during quantification of cysteine and methionine enantiomers. Additionally, the quantification of cysteic acid and methionine sulphon enantiomers was accomplished in the form of their diastereoisomer derivatives via the development of a reversed phase of high performance liquid chromatography method.

Key words: performic acid oxidation, cysteine and methionine enantiomers, racemization.

INTRODUCTION

The determination of the amount of sulphur containing amino acids in foods and feeds involves some difficulties because under the generally used protein hydrolysis conditions (6 M hydrochloric acid solution, 110 °C, 24 hours) a part of the amino acids undergoes oxidative deterioration (*Martin and Synge*, 1945). In order to prevent these losses the thiol group of these amino acids was suggested to be converted into more stable groups. With performic acid oxidation cysteine and methionine can form cysteic acid and methionine-sulphon (*Schram et al.,* 1954) and the loss of these molecules during hydrolysis is negligible related to that of the initial amino acids. This method has

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been used for decades for the determination of sulphur containing amino acids and cysteic acid can be analyzed rapidly by an ionic exchange liquid chromatography system (*Csapó*, 1982). Nowadays there is an increasing demand for the determination of the amount of the L- and D-enantiomers of the amino acids. The question arises if this sort of analysis is needed, whether the extent of racemization during performic acid oxidation is negligible or not. The purpose of the research was to investigate whether performic acid oxidation could used when the aim was determine the amount of methionine and cysteine enantiomers, and, besides, an RP-HPLC method was developed in order to separate the derivatives of these oxidized amino acids. In a preliminary research the separation of cysteic acid enantiomers had been accomplished (*Varga-Visi et al.*, 2000). In the present work the aim was to extend the separation and the investigation of performic acid oxidation to the other sulphur containing amino acid, methionine in order to determine the amount of methionine and cysteine in one single analysis.

MATHERIALS AND METHODS

Oxidation with Performic Acid

A sample of cysteine and that of methionine (approx. 0.1 mM) was weighed into a vial. Five cm^3 performic acid, produced based on the method of *Hirs* (1956) was added and the mixture which was heated at 50 °C for 15 minutes then it was cooled down immediately and lyophilized at -5 °C. If the sample contains only free amino acids the dried sample is washed with water into a 50 cm^3 volumetric flask. The pH was adjusted to 7 with 4M sodium hydroxide, and the solution was ready for analysis.

Hydrolysis

For protein containing samples the oxidized and lyophilized sample was dissolved in hydrochloric acid (6 M; 5 cm^3) and hydrolyzed at 110°C for 24h. After cooling the solution was neutralized (pH 7) with the sodium hydroxide solution (4 M).

Derivatization and Analysis

Diastereoisomers were produced with OPA (o-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) by the method of *Einarsson et al.* (1987). OPA and TATG were obtained from Sigma (St. Louis, MO, USA). The compounds were separated on a 125 mm x 4 mm i.d. column packed with LiChrospher 100 RP-18. At the beginning of the experiments, the mobile phase consisted of 5 % (v/v) of tetrahydrofuran and 95 % of phosphate buffer (39 mM, pH=7.05), as in the case of the separation of OPA-TATG derivatives of cysteic acid enantiomers (*Varga-Visi, 2000*). The temperature of the oven was 40 °C. The derivatives were detected with a fluorescence detector (λ_{ex} 325nm, λ_{em} 420 nm).

The derivatization and analysis were carried out using a MERCK-Hitachi HPLC comprising L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, and AIA data conversion utility for the D-7000 HPLC system manager.

Reagents were *pro analysis* grade. Solvents (tetrahydrofuran and water) were HPLC gradient grade and purchased from MERCK (Darmstadt, Germany).

RESULTS AND DISCUSSION

Separation of the enantiomers of sulphur containing amino acids

The aim of the method development was to achieve an acceptable resolution within a reasonable range of the retention factor k ($1 < k < 10$). The adequacy of resolution was the most important point in the method development because the amount of the D-enantiomer can be less with two orders of magnitude than the amount of L-enantiomer in foods and feeds. When the separation method of the OPA-TATG derivatives of D- and L-cysteic acid was developed, the type of organic solvent used, the organic solvent/buffer ratio and the stationary phase of the column were optimalized in analytical conditions. In order to separate the OPA-TATG derivatives of D- and L-methionine-sulphon the strength of the mobile phase had to be changed and some part of the

resolution of the first diastereoisomer pair had to be sacrificed to elute the second diastereoisomer pair in time. Increasing the initial tetrahydrofuran volume ratio with only two percent (that means 7 % tetrahydrofuran, 93% phosphate buffer) halved the retention of cysteic acid derivatives while the resolution also dropped significantly, from 2.1 to 1.4. But this value is still acceptable in case of diastereoisomer pairs. Separation of cysteic acid and methionine-sulphon derivatives in one analysis cannot be achieved using isocratic condition in this system thus a gradient program was developed. After an initial period when the cysteic acid derivatives were to be separated (0 - 14 minutes) the ratio of tetrahydrofuran was increased in the eluent. The changes of the resolution and the retention time of the methionine-sulphon derivatives in the function of the tetrahydrofuran-phosphate buffer composition of the mobile phase from the 20^{th} minutes of analysis can be seen in Table 1.

Fine-tuning of the tetrahydrofuran volume ratio from 20 % (v/v) to 16% from the 20 min resulted in a retention increase of L- and D-methionine-sulphon to some extent but the resolution of these two peaks improved considerably (from 0.87 to 1.31).

The final mobile-phase gradient is given in Table 2.

Table 1. The influence of eluent composition on the resolution and retention time of OPA-TATG derivatives of methionine sulphon. See additional information in the text.

Tablica 1. Utjecaj sastava eluenata na vrijeme rastvaranja i retencije OPA-TATG derivata metioninskog sulfona. Vidjeti dodatne podatke u tekstu

Eluent composition % (v/v)		Retention time (min) OPA-TATG derivative of		
Tetrahydrofuran	Phosphate buffer (39 mM, pH=7.05)	L-methionine sulphon	D-methionine sulphon	Resolution
20	80	29.4	29.7	0.87
19	81	29.6	30.1	1.02
18	82	29.9	30.5	1.09
16	84	31.2	32.4	1.31

Table 2. The mobile phase gradient for the separation of sulphur containing amino acids. See additional information in the text

Tablica 2. Gradijent mobilne faze za separaciju aminokiselina koje sadrže sumpor. Dodatni podaci u tekstu

Time - Vrijeme (min)	Gradient composition (v/v%)	
	Phosphate buffer (39 mM. pH=7.05)	Tetrahydrofuran
0	93	7
13	93	7
14.5	85	15
20	84	16
31	84	16
35	60	40
45	60	40
47	93	7
50	93	7

The flow rate was 1 cm^3 min^{-1}. - Stopa protoka: 1 cm^3 min^{-1})

With the use of the above gradient program the OPA-TATG derivatives of acidic amino acids can also be separated besides those of the sulphur containing amino acids (Figure 1).

Figure 1. Separation of OPA-TATG derivatives of cysteic acid, asparatic acid, glutamic acid and methionine-sulphon enantiomers with PR-HPLC. (Resolution: D/L cysteic acid: 1.4; D/L methionine-sulphon: 1.3.)

Slika 1. Separacija OPA-TATG derivata cisteične kiseline, asparaginske kiseline, glutaminske kiseline i metionin-sulfon anantiomera s PR-HPLC

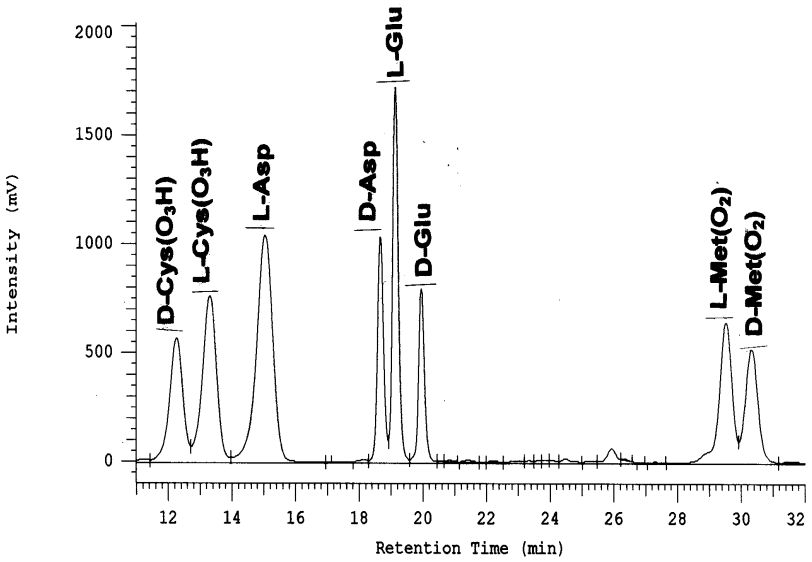
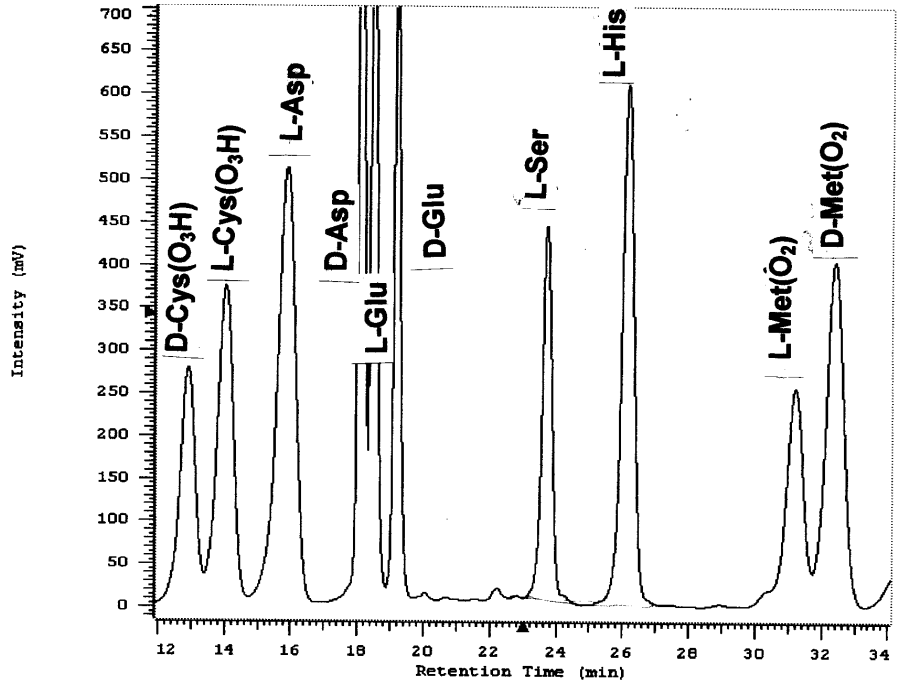


Figure 2. Separation of OPA-TATG derivatives of cysteic acid, asparatic acid, glutamic acid, and methionine-sulphon enantiomers, L-serine and L-histidine with PR-HPLC. (Resolution: D/L cysteic acid: 1.4; D/L methionine-sulphon: 1.3.)

Slika 2. Separacija OPA-TATG derivata cisteične kiseline, asparatične kiseline, glutaminske kiseline i metionin-sulfon enantiomera, L.serina i L- histidina s PR-HPLC



Based on the results of the preliminary research L-serine and L-hystidine derivatives were considered to elute in the same time period as the above amino acid derivatives therefore the possible interference was investigated. Derivatives of L-serine and L-hystidine were separated from the derivatives of the sulphur containing amino acids and separation was also acceptable for the derivatives of the acidic amino acids shown in Figure 2. The detection limit for methionine-sulphon was 0.61 nmol/injection. The detector response was linear between 5.5 and 250 nmol/injection. At 50 nmol methionine-sulphon/injection the *RSD (n=3)* was calculated to be 8.5%.

Investigation of Performic Acid Oxidation

In case of cystein no significant racemization was observed during performic acid oxidation. For the other sulphur containing amino acid L-methionine standard of high optical purity was used to detect whether racemization occurred during oxidation. Solutions of L-metionine were oxidized like samples, and the quantity of D- and L-methionine-sulphon was measured. The D/(D+L)x100 ratio, corrected with the fluorescence factors of the corresponding OPA-TATG derivatives, proved to be less than 10^{-4} . This ratio is not significant when it is compared to the D/(D+L)x100 ratios in food analysis, therefore it can be concluded that the extent of racemization of methionine during oxidation with performic acid is negligible.

To study the rate of conversion, that is the extent of the other losses during performic acid oxidation of the amino acid, L-methionine in standard solutions were oxidized and analyzed. The quantity of the product was determined by use of calibration curves of methionine-sulphon standard solutions. The rate of conversion from methionine to methionine-sulphon seemed to be higher than that of cysteine to cysteic acid (96±3% and 71±3% (n=3) respectively). Certainly the determination of the recovery needs to be accomplished separately in case of each substance under study.

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SAŽETAK

Proces oksidacije performičnom kiselinom cisteina i metionina, što rezultira stvaranjem cisteične kiseline i sulfona metionina primijenjen je kako bi se izbjegao gubitak te dvije aminokiseline koje sadrže sumpor, za vrijeme acidične hidrolize bjelančevina, potrebne prije analize aminokiselina. Cilj istraživanja potakli su sve veći zahtjevi za određivanjem količina enantiomera aminokiselina: primjenjivost oksidacije performične kiseline ocijenjena je s tog gledišta. Racemizacija L-cisteina i L-metionina nije se pokazala značajnom za vrijeme oksidacije s performičnom kiselinom pa se, stoga, taj proces može primijeniti prije hidrolize tijekom kvantifikacije cisteinskih i metioninskih enantiomera. Osim toga, kvantifikacija cisteične kiseline i enantiomera metionskog sulfona izvedena je u obliku derivata njihovih dijastereosomera putem razvoja obrnute faze metode tekuće kromatografije visoke performance.

Ključne riječi: oksidacija performične kiseline, enantiomeri cisteina i metionina, racemizacija