Determination of the Enantiomers of Methionine and Cyst(e)ine in the Form of Methionine-sulphon and Cysteic Acid After Performic Acid Oxidation by Reversed Phase High Performance Liquid Chromatography

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SUMMARY

In the quantitative analysis of cystine, cysteine and methionine content of foods and feeds, the sulphur containing amino acids are often oxidised by performic acid before hydrolysis of the protein to make the determination more punctual. The applicability of the performic acid oxidation to the quantification of the enantiomers of sulphur-containing amino acids has been examined. An reversed phase high performance liquid chromatography (RP-HPLC) method was developed for determination of the amount of cysteic acid and methionine-sulphon enantiomers and the rate of the conversion during oxidation from cystine and cysteine into cysteic acid and from methionine to methionine-sulphon was determined. Racemization of the L-cysteine and L-methionine was negligible during performic acid oxidation, and therefore the process can be applied before hydrolysis of the protein during quantification of enantiomers of sulphur-containing amino acids. The separation between the acidic amino acids (Asp, Glu) and the oxidised from of the sulphur-containing amino acids at the applied gradient system is very good, and the resolution of the peaks were better than 1.4 in every case.

KEY WORDS

racemization, hydrolysis, enantiomers, RP-HPLC

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INTRODUCTION

In case of determination of the total amino acid composition of foods and feeds before the analysis hydrolysis it is necessary to cleave the protein chains into amino acids. Under the classic condition of hydrolysis (6M hydrochloric acid solution, 24 hours, 110°C) amino acids are partially decomposed. The loss of sulfur containing amino acids is significant. Cysteine may be converted into alanine, serine and glicine (Yoritaka et al., 1954) and it can also undergo an oxidative conversion. Methionine also undergo an oxidative decomposition in the presence of the trace of oxygen, may be decomposed into homocysteine, homocystine and glycine, therefore oxidation with performic acid is recommended before hydrolysis (Shram et al., 1954; Hendriks et al., 1998). During this oxidation process cystine and cysteine are converted into cysteic acid, and methionine converted into methionine-sulphon. After oxidation and hydrolysis, cysteic acid can be analyzed rapidly by an ionic exchange liquid chromatography system using the quick method developed by us, but both of cysteic acid and methionine-sulphon can be easily determined by ion exchange column chromatography.

Nowadays not only the knowledge of the amino acid composition but also that of the ratio of D- and L-optical isomers is becoming more and more important in several scientific fields, for example in nutrition research (digestibility studies) and in archaeology (age determination).

The rate of racemization of amino acids depends mostly on temperature, pH and the property of the R-group in the amino acid and the structure of the surrounding amino acids in the peptide chain. The absolute value of the racemization half-time of the amino acids is variable depending on the conditions, but the relative susceptibility to racemization of amino acids is quite stable. If amino acids are ranged based on the increasing half-time this sequence does not change even under different conditions. In this sequence cysteine has one of the shortest relative half-time, it can therefore be used for the indication of racemization if present in sufficient quantities in the sample. Methionine is not so sensitive for racemization than cysteine, but it is among the most sensitive amino acids, moreover the methionine is essential for most of the animals and for human, therefore it is useful to know what percentage of the total methionine is in D-enantiomeric form.

We reached the conclusion that in order to improve the accuracy of the determination of cyst(e)ine and methionine enantiomers, oxidation with performic acid before hydrolysis and analysis by HPLC is to be recommended if oxidation process does not induce racemization.

We have to be aware of the disadvantages of this method: cysteine and cystine cannot be determined separately, the different oxidised form of cysteine and methionine can not be determined, because all of them converted into cysteic acid and methionine-sulphon, and the determination of the other amino acids requires another analysis, because most of the amino acids partly decompose during performic acid oxidation.

Our first aim was to develop a method to determine cysteine and methionine enantiomers in the form of cysteic acid and methionine sulphone enantiomers. First an RP-HPLC method was developed and tested for the separation of cysteic acid enantiomers, after for the separation of methionine-sulphon enantiomers, then we examined if there was any racemization during oxidation, and the rate of conversion from cysteine to cysteic acid and from methionine to methionine-sulphon.

MATERIALS AND METHODS

During the oxidation with performic acid the sample containing about 12 mg cysteine and 15 mg of methionine (one-tenth of the mmolweight) was weighed into a vial. Five ml performic acid (produced based on the method of Hirs, 1956) was added. The mixture was heated at 50°C for 15 minutes then cooled down immediately and lyophilized (-5°C). If the sample contained only free amino acids the dried sample was washed with water into a 50 ml volumetric flask. The pH was adjusted to pH=7 with 4M sodium hydroxide solution, and it was ready for analysis. In case of protein containing samples the oxidized and lyophilized sample was solved in 5 mL 6 M hydrochloric acid solution and hydrolyzed at 110°C for 24h. After that cooling neutralization was carried out with 4 M sodium hydroxide solution and pH was set to pH=7.

During the derivatization, separation and determination of the enantiomers, the initial conditions were the same as we used to determine the other D- and L-amino acids: diastereomers were produced with OPA (o-phthaldialdehyde) and TATG (1-thio-β-D-glucose tetraacetate) based on the method of Einarsson et al. (1987). The separation was performed with a LiChrospher 100 RP-18 125x4 column or with a Superspher 60 RP-8e column; the temperature of the oven was 40 °C. The derivatives were detected with a fluorescence detector (ex.:325nm, em.:420 nm). The initial gradient conditions can be seen in the Table 1.

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

Table 1. Initial gradient conditions for separation of cysteic acid enantiomers

Time (min)	Methanol, %	Phosphate buffer, %♦	Aceto-nitril, %
0	28	72	0
10	28	72	0
70	24	36	40
85	24	36	40
87	28	72	0
90	28	72	0

(Flow rate: 1 mL·min^{-1}, ♦ = 39 mmol·L^{-1}, pH=7,05)

Table 2. The retention time (t_R) and the resolution (R) of methionine-sulphon enantiomers by decreasing concentration of THF

THF, % Phosphate buffer, %	t_R (min)	R
20-80	29,35-29,71	0,87
19-81	29,64-30,12	1,02
18-82	29,93-30,53	1,09
17-83	29,53-30,33	1,20
16-84	31,20-32,39	1,31

for D-7000 HPLC system manager. Reagents were p. a. grade. OPA and TATG were obtained from Sigma (St. Louis, MO, USA). Solvents (acetonitrile, methanol and water) were HPLC gradient grade and purchased from MERCK (Darmstadt, Germany).

RESULTS

First we separated the cysteic acid enantiomers. The derivative of cysteic acid emerged as the first peak before aspartic acid enantiomers using the earlier initial gradient conditions, but enantiomers did not separate from each other. Our aim was to separate them using a two-component-mixture under isocratic conditions. In order to achieve acceptable separation the analytical conditions were varied: the organic solvent/buffer ratio applying less and less strong mixtures, the type of organic solvent used (methanol or acetonitrile or tetrahydrofuran) and the type of the stationary phase of the column (LiChrospher 100 RP-18 or Superspher 60 RP-8e). The concentration and the pH of the buffer, the temperature of the column, and the flow velocity were not changed. At 10% methanol 90% buffer eluent, the resolution accomplished was acceptable, but the retention was too high (k>50). Neither was acceptable separation attained within a reasonable time with acetonitrile-buffer systems. In case of tetrahydrofuran-buffer eluents, we succeeded in achieving adequate resolution between the enantiomers within a reasonable time for both columns. At about the same k values, the resolution (R) was better with the LiChrospher 100 RP-18 125x4 column than with the Superspher 60 RP-8e 125x4 column (2.1; 1.2 respectively). The former column was therefore used for the analysis of the enantiomers under isocratic conditions (eluent: 5% tetrahydrofuran-95% buffer).

If the rate of the THF was increased for 16%, not only the separation of the cysteic acid enantiomers, but the separation of methionine-sulphon enantiomers was adequate. The changes of retention time and resolution of methionine-sulphon enantiomers by decreasing concentration of THF can be seen in the Table 2.

Not only the cysteic acid and methionine-sulphon enantiomers are to be separated but also the other amino acids that occur in foods and feeds. So thus the possible interference from the other amino acids was examined. A standard solution containing D- and L-amino acids was prepared and analyzed under the same conditions as the D- and L-cysteic acid and D- and L-methionine-sulphon standard solutions. D- and L-cysteic acid was separated from the other amino acids. The two cysteic acid enantiomers were the first and the second peak on the chromatogram (Figure 1).

The detection limit was 5 fmol cysteic acid/injection. The detector response was linear between 0.8-1100 pmol/injection. At 0.8 pmol and 580 pmol cysteic acid/injection the RSD (n=3) was calculated at 9.2% and 3.8%, respectively. The two enantiomers of cysteic acid were followed by the four peak of aspartic and glutamic acid enantiomers, and the two well separated peaks of methionine-sulphon enantiomers appeared after this. On the next chromatogram (Figure 2) it can be seen that not only the former compounds, but the peaks of L-serine and L-histidine did not disturb the separation of sulphur-containing amino acid enantiomers.

In order to determine whether any racemization occur during the performic acid oxidation, L-cysteine and L-methionine standards (with high level of optical purity) were oxidized as it was described above, and then the quantity of D-cysteic acid and D-methionine-sulphon was then measured. The (D/D+L)*100 ratio was negligible, the amount of D-cysteic acid and D-methionine-sulphon was not significant (twice as high as the detection limit). This can be explained by the low pH value since the degree of racemization is more significant in alkaline conditions.

To study the rate of conversion during oxidation, L-cysteine and L-methionine standards were oxidized and analyzed. Molar quantity of L-cysteic acid and L-methionine-sulphon after oxidation was determined using calibration curves of cysteic acid and methionine-sulphon standard solutions; this was then divided by the initial molar amount of L-cysteine and L-methionine.

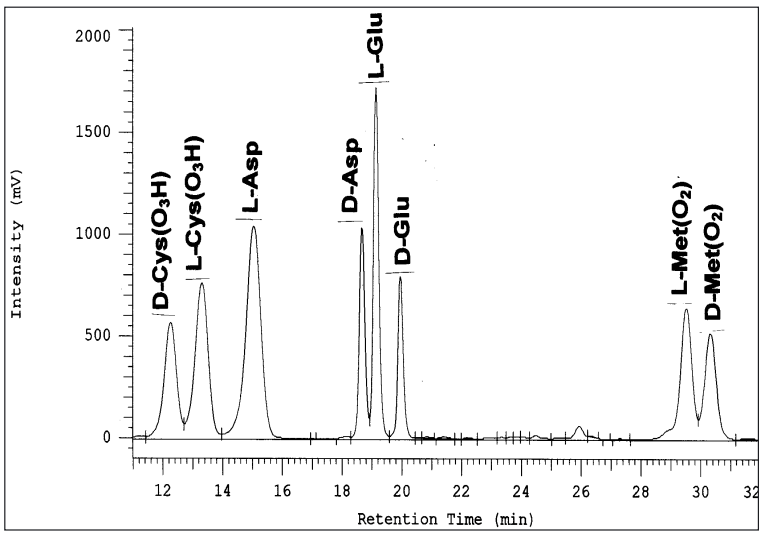

Figure 1.
Separation of the enantiomers of cysteic acid, aspartic acid, glutamic acid and methionine-sulphone by RP-HPLC

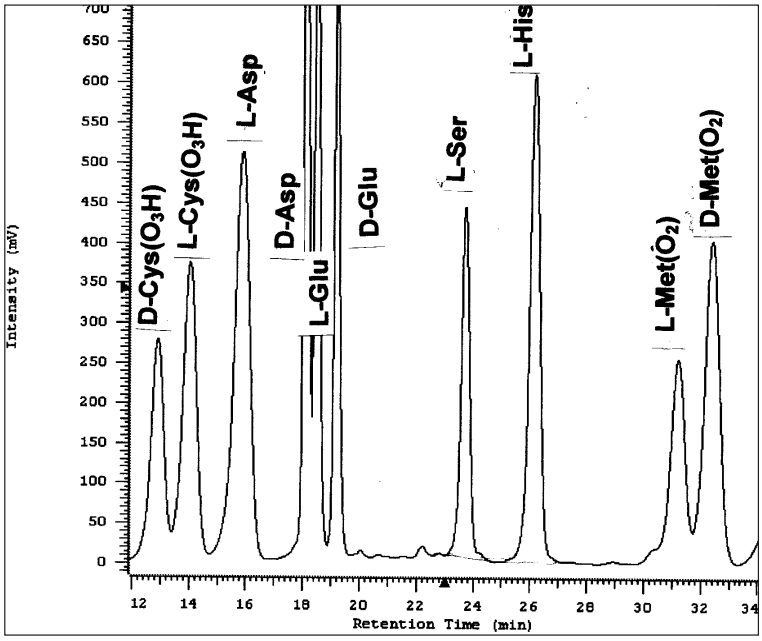

Figure 2.
Separation of the enantiomers of cysteic acid, aspartic acid, glutamic acid, methionine-sulphone as well as L-serine and L-histidine by RP-HPLC

The rate of conversion proved to be 71±3% for cysteine, and 96±3% for methionine, signifying that simultaneous oxidation of standards and samples under the same conditions is necessary. The method was used to determine the amount of L- and D-cysteine in cysteine containing preparations prepared by fermentation and subsequent purification. Samples with low cysteine and high methionine content (meat) and high cysteine and low methionine content (feather) were oxidized, hydrolyzed and then analyzed, both with and without the addition of D-cysteine and D-methionine. Now we are to study the conversion rate of cysteine, cystine and methionine during oxidation with standard addition in different matrixes.

CONCLUSION

In order to determine the quantity of cyst(e)ine and methionine, oxidation of cystine and cysteine into cysteic acid and methionine into methionine-sulphon with performic acid is often applied before hydrolysis. We examined the applicability of this process in case of quantification of cyst(e)ine and methionine enantiomers. An RP-HPLC analytical method was developed for the determination of the amount of cysteic acid and methionine-sulphon enantiomers. The rate of conversion during oxidation from cyst(e)ine into cysteic acid and from methionine into methionine-sulphon was determined. Racemization of L-cysteine and methionine was not significant during oxidation with performic acid, therefore this process can be applied before hydrolysis during quantification of cyst(e)ine an methionine enantiomers.

REFERENCES

Einarsson, S., S. Folestad, and B. Josefsson. (1987). Separation of amino acid enantiomers using precolumn derivatization with o-phthalaldehyde and 2,3,4,6,-tetra-O-acetyl-1-thio-β-glucopyranoside. J. Liquid Crom., 10, 1589.

Hendriks, W.H., Tarttelin, M.F., Moughan, P.J. (1998). The amino acid composition of cat (Felis catus) hair. Anim. Sci. 67, 1. 165-170.

Hirs, C.H.W. (1956). The oxidation of ribonucleare with performic acid. J. Biol. Chem. 219, 611-621.

Shram, E., Moore, S., Bigwood, E.I. (1954). Chromatografic determination of cystine as cysteic acud. Biochem. J. 57, 33-37.

Yoritaka, T., Ono, T. (1954). Determination of methionine in proteins and peptides. Nagasaki Iggakai Zassi, 29, 400.

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