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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SULFUR AMINO ACIDS IN PROTEIN HYDROLYSATES

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SUMMARY

A rapid, quantitative high-performance liquid chromatographic procedure for the determination of methionine and cystine after oxidation to methionine sulfone and cysteic acid is described. The Dns derivatives of the amino acids are separated by reversed-phase chromatography with a phosphate buffer–acetonitrile gradient and detected by UV absorption at 254 nm. The procedure is validated by confirming the methionine and cystine content of ribonuclease A. The average yields of cysteic acid and methionine sulfone from triplicate analyses of ribonuclease A were 98.1% (±3.3) and 106.1% (±2.4) of the theoretical values, respectively.

INTRODUCTION

Sulfur amino acids are nutritionally limiting in soy protein and other legumes. Because of their low levels and the oxidative instability of cystine and methionine, it is important to develop accurate procedures for their determination.

Due to the lability of cysteine and methionine during acid hydrolysis, these amino acids are more accurately determined after oxidation to cysteic acid and methionine sulfone by performic acid treatment as recommended by Moore[1]. However, the standard cation-exchange chromatographic determination of cysteic acid and methionine sulfone is less than optimal because cysteic acid elutes in the void volume[2,3] and is therefore subject to interferences, and methionine sulfone is difficult to resolve chromatographically from aspartic acid and threonine.

Several investigators have reported poor precision for the analysis of sulfur amino acids using cation exchange chromatographic procedures. Porter *et al.*[4] reported a mean absolute deviation of 17.6% for the interlaboratory determination of methionine in cod muscle, compared to an average deviation of 7.4% for the other amino acids. In gelatin, which has a low methionine content, the mean absolute deviation for methionine was 83.3% compared to 7.5% for the other amino acids. Williams *et al.*[5] reported relative standard deviations of up to 38% for the interlaboratory determination of cystine. Sarwar *et al.*[6] in a recent collaborative study

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reported intralaboratory relative standard deviations of up to 19.4% for the determination of methionine and up to 18% for the determination of cystine.

Standard procedures for amino acid analysis by ion exchange in addition to having high variances for the determination of sulfur amino acids, are time consuming and require expensive specialized instrumentation. Bayer *et al.*[7] and Hsu and Currie[8] have recently described the high-performance liquid chromatographic (HPLC) separation of Dns-amino acids. Quantitative analyses were not reported by Bayer *et al.* Hsu and Currie reported approximately 75% of the theoretical yield for the analysis of various peptides.

A rapid, quantitative analytical procedure and validation data are reported here for the determination of methionine and cystine in protein hydrolysates. These results are much closer to theoretical values and are achieved using standard HPLC instrumentation.

EXPERIMENTAL

Performic acid oxidation

The procedure used was essentially that of Moore[1]. Approximately 0.030 g of protein was treated with 10 ml performic acid (88%, w/w, formic acid–30%, w/v, hydrogen peroxide 9:1, v/v) at 0°C for 18 h. To reduce the excess performic acid, 1.5 ml of cool hydrobromic acid (48%, w/w) was added. The solution was then evaporated to dryness under vacuum.

Acid hydrolysis

To the dry oxidized protein sample, 10 ml of 6 *M* hydrochloric acid was added. The sample was transferred to a vacuum tube (Pierce No. 29564), frozen in a dry ice–acetone bath, evacuated below 10^{-5} MPa and the tube sealed. The sample was then hydrolyzed in an oven at 110°C for 22 h. A 5-ml portion of an aqueous solution containing 0.50 mg/ml 3-aminobutyric acid was added as internal standard, mixed, and the sample was transferred to a 25-ml volumetric flask. The pH was adjusted to 7 ± 1.5 with 50% (w/w) aqueous sodium hydroxide and brought to volume with distilled water.

Dns derivatization

Solutions of sodium carbonate buffer in water (at 0.2 *M*; pH 9.7) and 5 mg/ml Dns chloride (Pierce No. 21755) in acetonitrile–water (70:30) were prepared. Aliquots of 100 µl of hydrolyzed sample or standard amino acid mixture were reacted with 1.0 ml of carbonate buffer and 1.0 ml of Dns chloride solution at 55°C in an oven for 10-60 min. If Dns chloride crystals were present after this time, the vial was shaken to dissolve the crystals and reacted in the oven for another 5 min. This step was repeated as long as Dns chloride crystals were still present. The solution was then evaporated under a nitrogen stream to a volume of about 1.5 ml to lower the acetonitrile content. Then 1.0 ml of 16.5% (w/w) phosphoric acid aqueous solution was added and the pH adjusted to 6.2 ± 0.2 with 1 *M* hydrochloric acid.

If the samples were not chromatographed within 24 h, it was necessary to readjust the pH, because of dissociation of acidified carbonate buffer to carbon dioxide and water with a resulting increase in pH due to evaporation of carbon dioxide.

Chromatographic separation

A Perkin-Elmer Series 3 gradient liquid chromatograph with an LC-65T oven–variable wavelength UV detector and a Sigma 10 Data Station were used. The Dns-amino acids were separated by reversed-phase liquid chromatography on a 25 cm × 4.6 mm I.D. column packed with 5-μm particle size Spherisorb hexyl. A number of chromatographic solvent systems were tried. The solvent system finally used was: (A) acetonitrile–0.02 *M* phosphate buffer pH 6.2 (60:40); (B) acetonitrile–0.02 *M* phosphate buffer, pH 6.2 (5:95). The gradient program consisted of three linear segments: from 7% A to 45% A in 30 min; from 45% A to 60% A in 1 min; from 60% A to 70% A in 7 min. The flow-rate was 1.5 ml/min. The column oven temperature was set at 34°C. Detection was at 254 nm and 0.08 a.u.f.s. Injection volume was 60 μl.

Micro-Kjeldahl analysis of ribonuclease A

Total nitrogen was measured using a mercuric oxide–potassium sulfate catalyst[9].

RESULTS AND DISCUSSION

Quantitation

The relative standard deviations of integrated area ratios for methionine sulfone and 3-aminobutyric acid solutions (whether reacted for 10, 20, 30, 40, 50, or 60 min) was less than 1%. The Dns derivatization reaction proceeded quickly and the Dns-amino acids were stable under the reaction conditions. Thus, samples were derivatized by reacting in an oven at 55°C for 30 min as routine procedure.

Internal standardization with 3-aminobutyric acid was used for quantitative calculations. Using this standard, the linearity of the integrated detector response for cysteic acid and methionine sulfone was measured over the concentration range of 0.02 to 0.20 mg/ml for each of the two compounds. The correlation coefficient (linearity of response) for each sulfur amino acid was greater than 0.998.

To obtain quantitative results, shaking of the mixture was done when needed to insure that Dns chloride crystals did not remain after the reaction. If Dns chloride crystals which formed had not been redissolved, Dns-amino acids would have preferentially adsorbed onto the crystals and changed the solution concentrations of the Dns-amino acids. This would have resulted in relative errors of over 30%.

Optimization of separation

The separation was optimized by characterizing the effect of pH and phosphate buffer concentration on the resolution of the 19 amino acids typically found in protein hydrolysates. A buffer concentration of 0.02 *M* with resulting pH of 6.2 was found to be best. Fig. 1 shows the separation of a Dns derivatized mixture of 20 common amino acids plus Dns-ammonia, Dns chloride, and Dns-sulfonic acid. The chromatographic efficiency required for this separation was approximately 10,000 theoretical plates.

The effect of changes in phosphate buffer concentration at pH 6.2 on the retention times of the Dns-amino acids relative to proline is graphically illustrated in Fig. 2. This graph is useful in a practical sense to optimize the resolution of the Dns-amino acids. If two Dns-amino acids are not adequately resolved in a trial separation

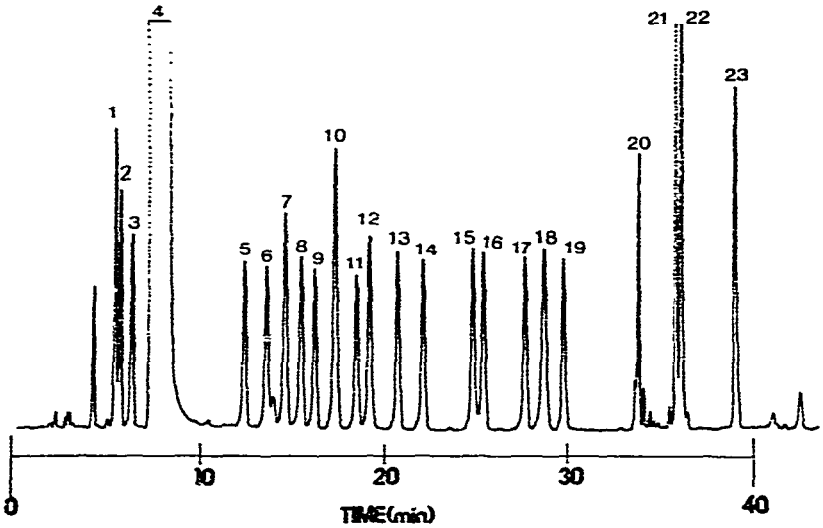

Fig. 1. Separation of Dns-amino acids according to conditions described in text. Peaks: 1 = cysteic acid; 2 = aspartic acid; 3 = glutamic acid; 4 = Dns-sulfonic acid; 5 = serine; 6 = threonine; 7 = glycine; 8 = alanine; 9 = methionine sulfone; 10 = 3-aminobutyric acid; 11 = arginine; 12 = proline; 13 = valine; 14 = methionine; 15 = isoleucine; 16 = leucine; 17 = phenylalanine; 18 = Dns-chloride; 19 = cystine; 20 = Dns amide; 21 = lysine; 22 = histidine; 23 = tyrosine.

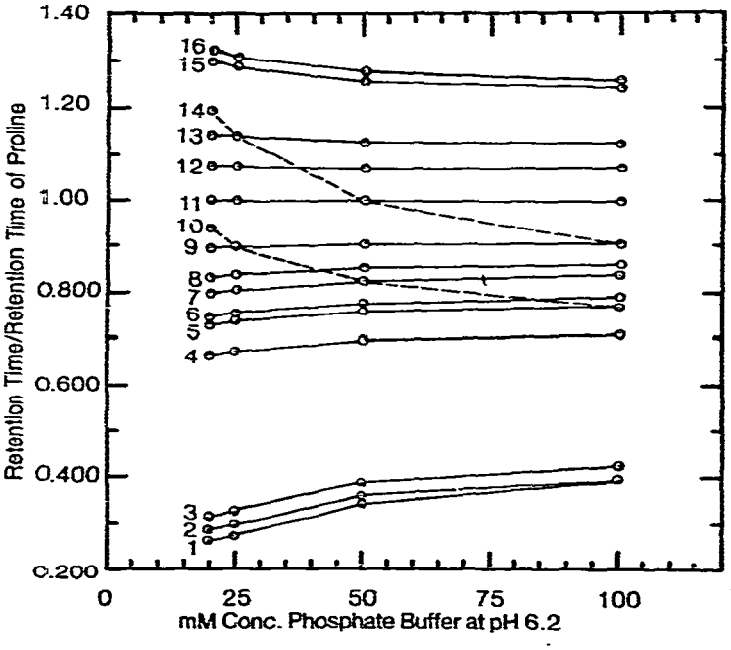

Fig. 2. Effect of buffer concentration on the relative retention times of Dns-amino acids. Plots: 1 = cysteic acid; 2 = aspartic acid; 3 = glutamic acid; 4 = serine; 5 = threonine; 6 = glycine; 7 = alanine; 8 = methionine sulfone; 9 = 3-aminobutyric acid; 10 = unknown; 11 = proline; 12 = valine; 13 = methionine; 14 = arginine; 15 = isoleucine; 16 = leucine.

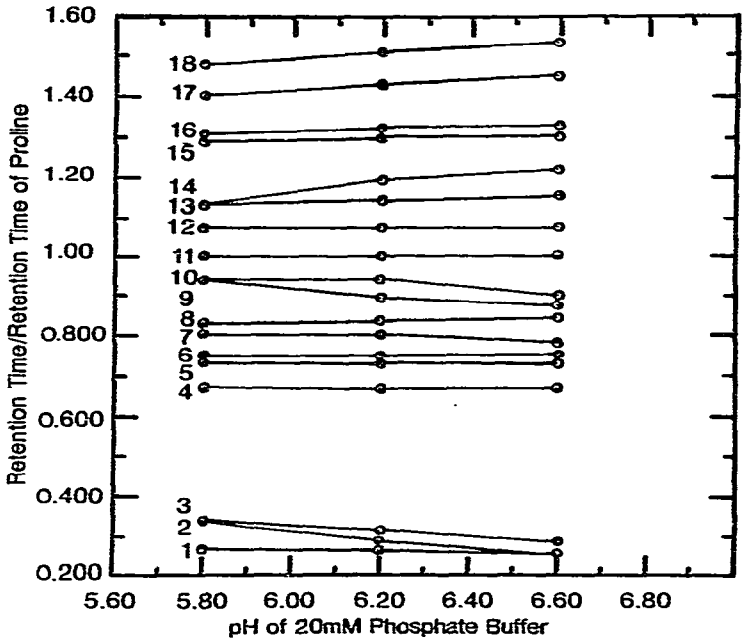

Fig. 3. Effect of pH on relative retention times of amino acids. Plots: 1 = cysteic acid; 2 = aspartic acid; 3 = glutamic acid; 4 = serine; 5 = threonine; 6 = glycine; 7 = alanine; 8 = methionine sulfone; 9 = 3-aminobutyric acid; 10 = unknown; 11 = proline; 12 = valine; 13 = methionine; 14 = arginine; 15 = isoleucine; 16 = leucine; 17 = phenylalanine; 18 = Dns chloride.

run, this graph can be used to determine whether increasing or decreasing the buffer concentration is necessary to improve the chromatographic resolution. Fig. 3 illustrates the effect of changes in pH in a 0.02 *M* phosphate buffer on the relative retention times of the Dns-amino acids. This graph can be used, similarly to the graph in Fig. 2, to optimize separation through pH adjustment.

Detection

Either spectrofluorometric[10] or UV absorption techniques can be used for detection of the eluted Dns-amino acids. In this study, UV detection at 254 nm was used because these detectors are commonly available and the increased sensitivity of spectrofluorometric detection was not needed. The standard amino acid mixture contained approximately $5 \cdot 10^{-7}$ mole/ml of each amino acid, prior to Dns derivatization, and approximately $1 \cdot 10^{-9}$ mole of each amino acid was actually injected onto the column. If sample quantities were limited, detection sensitivity could be improved by about a factor of three by using 220 nm instead of 254 nm as the detection wavelength. Alternatively, detection sensitivity could be improved by at least an order of magnitude by using spectrofluorometric detection.

Validation

To validate the accuracy and precision of the entire analytical procedure, a chromatographically pure, sequenced protein containing a known number of cysteine and methionine residues was analyzed. Three samples of ribonuclease A (Calbiochem

TABLE I

DETERMINATION OF CYSTEIC ACID AND METHIONINE SULFONE CONTENT OF RIBONUCLEASE A

R.S.D. = relative standard deviation.

Sample	*Actual cysteic acid content (g/100 g sample)*	*Adjusted cysteic acid content (g/100 g ribonuclease A)**	*Recovery (as % of theoretical)*	*Actual methionine sulfone content (g/100 g sample)*	*Adjusted methionine sulfone content (g/100 g ribonuclease A)**	*Recovery (as % of theoretical)*
1	8.61	10.06	101.8	4.79	5.60	105.7
2	8.22	9.60	97.2	4.93	5.76	108.7
3	8.08	9.44	95.4	4.72	5.51	104.0
		Mean	98.1		Mean	106.1
		R.S.D. (%)	3.3		R.S.D. (%)	2.4

* This value is calculated based on a Kjeldahl N analysis of 14.98% for the samples and a theoretical N content of 17.50% for ribonuclease A.

No. 55674) were oxidized with performic acid, acid hydrolyzed, and analyzed by HPLC. Table I shows the results of these analyses. Ribonuclease A was analyzed in duplicate for total nitrogen by the method described. The protein content of the samples was calculated based on the nitrogen analyses and the theoretical nitrogen content of ribonuclease A (17.5%, w/w). Since the ribonuclease A was chromatographically pure, it was assumed to be the only source of nitrogen in the samples. The percent theoretical calculations in Table I are based on ribonuclease A containing eight cysteine residues per mole of protein and four methionine residues per mole of protein. The recovery calculated from the cysteine and methionine found is in excellent agreement with the expected content based on nitrogen content and the sulfur amino acid composition of ribonuclease A.

Conclusion

This HPLC procedure to determine total sulfur amino acids in protein hydrolysates is more rapid, both in terms of chromatographic separation time and in sample preparation time, than the standard ion-exchange procedure. Furthermore, this procedure offers increased precision, compared to ion-exchange methods. The separation difficulties encountered with ion-exchange chromatography for cysteic acid and methionine sulfone are also eliminated with this HPLC procedure. Finally, this procedure uses standard HPLC instrumentation instead of expensive specialized instrumentation.

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