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GAS–LIQUID CHROMATOGRAPHY OF AMINO ACIDS

DETERMINATION OF CYSTINE AND CYSTEINE AS N-ACETYL, *n*-PROPYL S-CARBOXYMETHYLCYSTEINATE

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SUMMARY

A procedure is described for the derivatisation of S-carboxymethylcysteine yielding the corresponding N-acetyl, *n*-propyl S-carboxymethylcysteinate which is separated by gas chromatography from similar derivatives of other protein amino acids. Its quantitative determination provides a useful extension to a method for analysis of amino acids by gas chromatography and offers potential as a means of obviating errors in cystine (and/or cysteine) analysis arising from acid hydrolysis of proteins.

INTRODUCTION

A recently described technique[1] for the determination of amino acids by gas chromatographic analysis of their N-acetyl, *n*-propyl ester derivatives appears to offer some important potential advantages over other similar procedures involving different derivatives; in particular, that developed by Kaiser *et al.*[2], which, with modifications[3], is currently used in this laboratory for routine analysis of feedstuffs. The main advantage is that all of the protein amino acids except cystine can be quantitatively determined using a single column, the chromatography being completed in approximately 20 min. The inability to elute the cystine derivative is, however, of little importance because during acid hydrolysis considerable degradation of cystine may occur, and, in consequence, erroneous results for this amino acid are obtained[4].

To obviate such losses it is usual to oxidise cystine and cysteine to cysteic acid, which is stable during subsequent normal acid hydrolysis[5]; however, the latter amino acid may not conveniently be derivatized for use in the above gas chromatographic determinations. An equally satisfactory alternative modification technique[6] involves the reduction of cystine to cysteine followed by conversion of the latter into S-carboxymethylcysteine, which is stable to conventional acid hydrolysis conditions, provided that oxygen is effectively excluded, and which leads to a result being obtained for cysteine and cystine collectively.

A similar procedure but in which the reduction step is omitted yields a value

for cysteine alone, thus enabling an analysis for cystine to be accomplished. It was expected that the S-carboxymethylcysteine would readily be derivatized to yield the corresponding N-acetyl, *n*-propyl ester. The success of such a procedure would obviate the difficulties with cystine both at the hydrolysis step and in subsequent chromatography, provided that sufficiently good resolution from other derivatives is obtained. This report describes the preparation of the S-carboxymethylcysteine and its successful quantitative determination as the N-acetyl, *n*-propyl derivative.

EXPERIMENTAL

Materials and reagents

Amino acids were obtained as a kit from Sigma (St. Louis, Mo., U.S.A.). The internal standard, *trans*-4-(aminomethyl)-cyclohexanecarboxylic (tranexamic) acid, was obtained from A-B Kabi (Stockholm, Sweden) as Cyclokapron.

n-Propanol (BDH, Poole, Great Britain), acetic anhydride, triethylamine and acetone (E. Merck, Darmstadt, G.F.R.) were all re-distilled prior to use. Reagents for esterification and acylation were prepared as described by Adams[1].

Esterification and acylation reactions were conducted in Pyrex screw-top culture tubes (75 × 16 mm) the caps of which were fitted with thin PTFE gasket seals.

Preparation of S-carboxymethylcysteine standard

Sodium iodoacetate solution (0.1 *M*, 500 ml) was adjusted to pH 8 with 3 *N* sodium hydroxide solution, washed twice with hexane (*ca.* 100 ml) so as to remove any free iodine and slowly added, with stirring, to cysteine solution (0.1 *M*, 500 ml) that had also been adjusted to pH 8. During the addition, the pH was monitored and maintained at 8 by dropwise addition of 3 *N* sodium hydroxide. The reaction was carried out at 50° (in a water-bath), the final mixture being maintained at this temperature for a further 90 min. This solution was then concentrated *in vacuo* to *ca.* 150 ml and added to an anion-exchange column, Dowex 1-X8 (OH^-). After washing with water until neutral the required S-carboxymethylcysteine was eluted with 0.5 *N* hydrochloric acid as its hydrochloride salt. Micro-analysis of the free base gave C, 32.9; H, 5.0; N, 7.7%. Required for $C_5H_9O_4SN$: C, 33.5; H, 5.1; N, 7.8%.

A standard solution of amino acids, including S-carboxymethylcysteine hydrochloride, was prepared by dissolving 9.80 mg of the product in an amino acid solution (25 ml) containing 39.04 mg of internal standard per 100 ml of solution.

Derivatization

Standard solution (1 ml), in a reaction tube, was taken to dryness in a current of dry nitrogen at 110°. Following addition of about 50 µl of dimethoxypropane and hydrogen chloride in propanol (8 *M*, 1 ml), the tube was capped and heated at 110° for 20 min. After cooling, the propylating reagent was removed with a current of dry nitrogen at 60° until the presence of only traces of acid could be detected. About 50 mg of anhydrous sodium carbonate were then added, followed by 1 ml of freshly prepared acylating reagent, and the tube was re-capped. This addition of sodium carbonate was found necessary in order to ensure complete acylation of histidine and arginine. The mixture was sonicated for 15 sec so as to remove the white residue that formed on addition of acylating reagent and heated at 60° for 3 min. The mixture

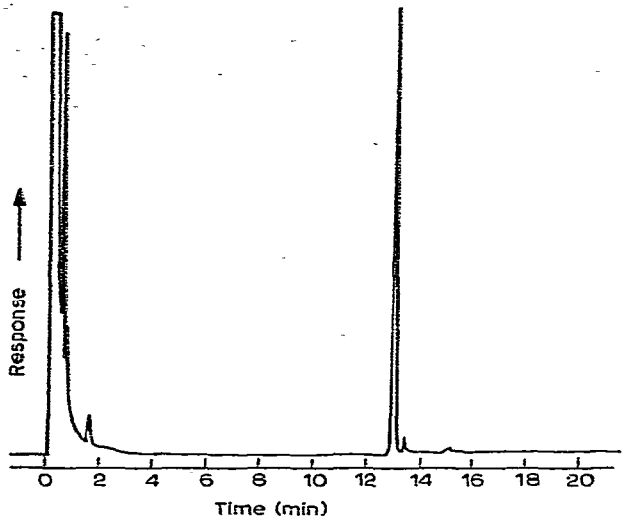

Fig. 1. N-Acetyl, *n*-propyl S-carboxymethylcysteinate chromatographed using 0.31 % (w/w) Carbowax 20M, 0.28 % (w/w) Silar 5CP and 0.06 % (w/w) Lexan on acid-washed Chromosorb W (120–140 mesh). Carrier gas flow-rate *ca.* 25 ml/min; initial temperature 100° for 1 min; programme rate 6°/min to 170°, then at 20°/min to 250°.

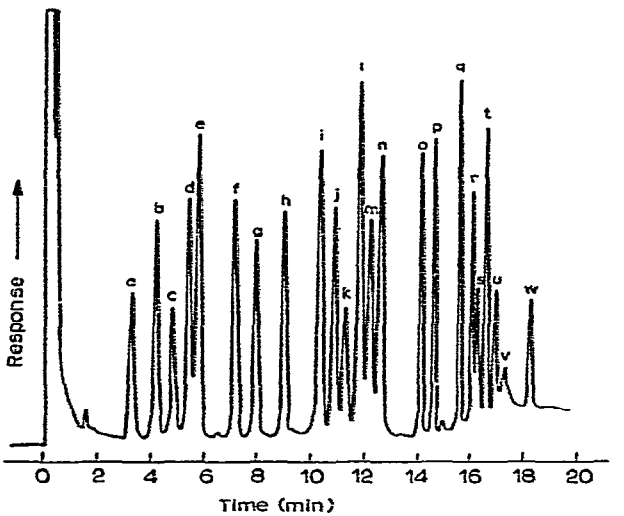

Fig. 2. Mixture of amino acid standards, including S-carboxymethylcysteine, derivatized and analysed using 0.31 % (w/w) Carbowax 20M, 0.28 % (w/w) Silar 5CP and 0.06 % (w/w) Lexan on acid-washed Chromosorb W (120–140 mesh). Carrier gas flow-rate *ca.* 25 ml/min; initial temperature 90° for 1 min; programme rate 6°/min to 170°, then at 20°/min to 250°. a = Alanine; b = valine; c = glycine; d = isoleucine; e = leucine; f = proline; g = threonine; h = serine; i = aspartic acid; j = methionine; k = cysteine; l = phenylalanine; m = hydroxyproline; n = glutamic acid; o = tranexamic acid; p = S-carboxymethylcysteine; q = tyrosine; r = ornithine; s = histidine; t = lysine; u = arginine; v = ?; w = tryptophan.

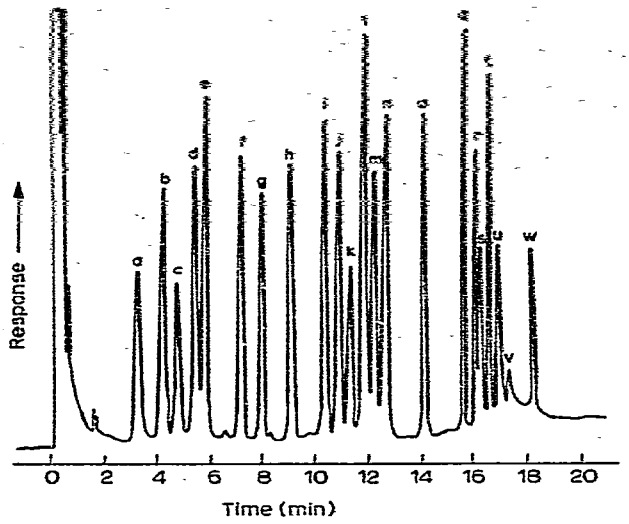

Fig. 3. Mixture of amino acid standards, excluding S-carboxymethylcysteine. Chromatographic conditions and key as in Fig. 2.

was finally taken to dryness in a current of dry nitrogen at 60°, 0.5 ml of dry ethyl acetate added and the solution filtered from sodium carbonate through a fine fibreglass plug. Aliquots of this solution were taken for gas chromatography or stored at 2° until required

Gas chromatography

A Hewlett-Packard 7611 gas chromatograph equipped with flame ionization detectors was used, and was coupled to a Hewlett-Packard 3352B laboratory data system.

The column used was 600 × 2.5 mm I.D. glass, U-shaped and silanised. The column packing was prepared by washing Chromosorb W (120–140 mesh) exhaustively with acid and, after thoroughly drying it, coating the support with 0.31% (w/w) Carbowax 20M, 0.28% (w/w) Silar 5 CP and 0.06% (w/w) Lexan, using the filtration technique. Conditioning was performed as described by Adams[1].

TABLE I

REPEAT ANALYSES OF A SINGLE DERIVATIVE SOLUTION

Run No.	*S-carboxymethylcysteine found (%)*
1	3.94
2	3.95
3	3.93
4	3.94
5	3.98
Mean	3.95
S.D.	0.019
Actual present	3.85

TABLE II

REPEAT ANALYSES OF SEPARATE DERIVATIVE SOLUTIONS

Run No.	*S-carboxymethylcysteine found (%)*
1	4.08
2	3.81
3	3.94
4	4.08
5	3.90
Mean	3.96
S.D.	0.12
Actual present	3.85

Analysis by gas chromatography

An aliquot of S-carboxymethylcysteine stock solution was derivatized as above. A typical chromatogram is shown in Fig. 1. A known amount of the S-carboxymethylcysteine was then added to an aliquot of standard amino acid solution, similarly derivatized, and found to yield the chromatogram shown in Fig. 2. The "unspiked" amino acid mixture yielded the chromatogram shown in Fig. 3.

In order to check the reproducibility and accuracy of the method, a derivative solution was analyzed repeatedly, and separate derivative solutions were analyzed, yielding the results shown in Tables I and II, respectively.

CONCLUSION

S-Carboxymethylcysteine derived from either cysteine or reduced cystine can be successfully derivatized quantitatively, thus permitting a more accurate determination of these amino acids by gas chromatography than has hitherto been possible.

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