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## Note

# Gas–liquid chromatography of amino acids

## A routine determination of cystine/cysteine in fish meal

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The hydrolysis step necessary for the liberation of amino acids from protein material prior to their analysis invariably leads to the partial destruction of *inter alia* cystine and cysteine. Several studies[1–4] have been devoted to establishing techniques to avoid this destruction following the realization that cystine and/or cysteine analysis figures based on a simple acid hydrolysis are unreliable. As a consequence, recognition is frequently[5] given only to those results derived from procedures in which (a) both cystine and cysteine are determined as cysteic acid following a pre-hydrolysis oxidation with performic acid or (b) before the hydrolysis step cystine is first reduced to cysteine, which is subsequently modified to yield either S-carboxymethyl (SCM)[6] or S-carboxyethyl (SCE) cysteine[7,8].

In attempting to integrate a cystine/cysteine analysis into our already established gas chromatographic (GC) procedure[9], the first of these alternative routes seemed unlikely to succeed in view of the observations of Shahrohki and Gehrke[10], who were unable to obtain peaks for the analogous N-trifluoroacetyl *n*-butyl ester derivatives of both cysteic acid and taurine. Doubt arose about the ability of our esterification conditions leading to satisfactory derivatization of the sulphonic acid group found in cysteic acid. However, the carboxylic acid group present in S-carboxyalkylcysteines was expected to esterify as smoothly as any of those found in the other amino acids. Accordingly, our previous investigations on this subject[11,12] were directed towards the establishment of a suitable procedure, based on the formation of either SCM- or SCE-cysteine, and which anticipated the analysis of a complex biological material (*viz.*, fish meal) incorporating both soluble and insoluble proteins. No success was achieved using solvent extraction and freeze-drying techniques to remove interfering contamination that arose from reagents used to effect pre-hydrolysis reduction and addition. Furthermore, clean-up using post-hydrolysis cation-exchange columns was not successful owing to problems in recovering certain amino acids from such columns[13], a phenomenon also reported by James[14] and independently by Boila and Milligan[15].

Following the appearance of a paper on the subject by MacKenzie and Fin-

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layson[16], attention was re-focused on the derivatization of cysteic acid. These workers confirmed an earlier report[17] that derivatization of cysteic acid using isobutyl alcohol (3 *M* in dry hydrogen chloride) and heptafluorobutyric anhydride followed by GC analysis led to a peak whose retention time was unexpectedly short and with a peak height affected by the injection port temperature, reaching a maximum when the latter was 300°C. Mass spectral data on the compound responsible led MacKenzie and Finlayson[16] to the conclusion that whilst the heptafluorobutyryl isobutyl ester (HBB) derivative of cysteic acid was injected, molecular degradation occurred in the injection port to yield the corresponding derivative of dehydroalanine. No statistical evaluation of this process was included in the report, however. A further investigation into this procedure was initiated with a view to incorporating it into our routine amino acid analysis technique.

## EXPERIMENTAL

Cystine/cysteine analysis of fish meals required a pre-hydrolysis oxidation step based on the protocol originally described by Weidner and Eggum[1] and subsequently modified by Mason *et al.*[4].

*Pre-hydrolysis oxidation of fish meal*

Fish meal (*ca.* 30 mg) was accurately weighed into a screw-capped tube and oxidized prior to acid hydrolysis by treatment with freshly prepared and chilled performic acid (2.5 ml). The latter solution was obtained in 10-ml aliquots (or convenient multiples thereof) by mixing 30% hydrogen peroxide (1 ml) with 88% formic acid (9 ml), incubating the mixture at 30°C for 1 h, then chilling (ice–water bath) for *ca.* 15 min before use.

The pre-hydrolysis oxidation mixture was kept at *ca.* 1°C for 16 h. Care was taken to ensure that fish meal particles were thoroughly wetted and immersed in the oxidizing medium during this period.

Following oxidation, the contents of the tube were frozen and the reagents removed *in vacuo* using a freeze-dryer. This procedure, designed to decompose excess of performic acid, was found to be more satisfactory than the previously described[1,4] addition of hydrogen bromide or sodium dithionite. Constant-boiling hydrochloric acid (20 ml) was added to the remaining oxidized meal. Hydrolysis and derivatization were then carried out as described elsewhere[9].

GC analyses were carried out using a silanized glass column (3 m × 2.7 mm I.D.) packed with 3.5% OV-101 on resilanized Supelcoport (100–200 mesh), installed in a Hewlett-Packard 5710A gas chromatograph.

The analytical conditions used were as previously reported[9] except that the injection port temperature was increased to 300°C for cysteic acid analysis. A Hewlett-Packard 3353 Lab Data System was used for acquisition and handling of analytical data. In this method, it is assumed that pre-hydrolysis oxidation of both cystinyl and cysteinyl residues proceeds quantitatively to cysteic acid. Results for cystine (or cysteine) are directly related on a molecular basis to data for cysteic acid and are calculated by adjustment of the latter using the ratio of molecular weights. This operation may be conveniently included in the data handling step.

RESULTS AND DISCUSSION

It is now confirmed that cysteic acid elutes (as dehydroalanine) in the position reported (Fig. 1) and that an injection port temperature of 300°C leads to a satisfactory and reproducible peak. Comparison with standard derivatives chromatographed under the more usual conditions (*i.e.*, injection port at 250°C) revealed that certain other amino acids appear to undergo thermal breakdown, as evidenced by their lower levels. Use of an intermediate injection port temperature (285°C) neither reduced this breakdown nor improved the cysteic acid response. Statistical evaluation of repeated injections under the new conditions (higher injection port temperature) of the same solution of derivatized amino acid standards (Table I) reveals good reproducibility

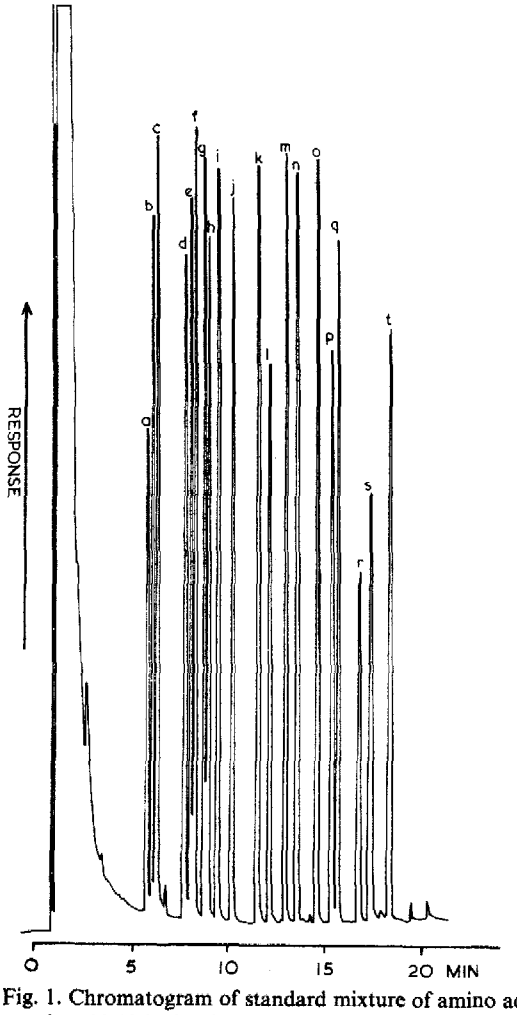



Fig. 1. Chromatogram of standard mixture of amino acid HBB derivatives showing resolution of that of cysteic acid (dehydroalanine) from those of other amino acids. a, Cysteic acid; b, alanine; c, glycine; d, valine; e, threonine; f, serine; g, leucine; h, isoleucine; i, norleucine (internal standard); j, proline; k, hydroxyproline; l, methionine; m, aspartic acid; n, phenylalanine; o, glutamic acid; p, lysine; q, tyrosine; r, arginine; s, histidine; t, SCE-cysteine.

TABLE I

PRECISION OF CHROMATOGRAPHY OF AMINO ACID DERIVATIVES, INCLUDING CYSTEIC ACID

| *Amino acid* | *Mean* (% sample)* | *Standard deviation* | *Relative standard deviation (%)* |
|---|---|---|---|
| Cysteic acid | 5.49 | 0.02 | 0.4 |
| Alanine | 5.66 | 0.01 | 0.2 |
| Glycine | 5.35 | 0.01 | 0.2 |
| Valine | 5.41 | 0.02 | 0.3 |
| Threonine | 6.02 | 0.03 | 0.4 |
| Serine | 5.42 | 0.01 | 0.2 |
| Leucine | 5.54 | 0.01 | 0.2 |
| Isoleucine | 5.65 | 0.03 | 0.5 |
| Proline | 5.25 | 0.01 | 0.3 |
| Hydroxyproline | 5.42 | 0.04 | 0.7 |
| Methionine | 5.30 | 0.03 | 0.5 |
| Aspartic acid | 5.70 | 0.03 | 0.5 |
| Phenylalanine | 6.80 | 0.03 | 0.4 |
| Glutamic acid | 5.00 | 0.02 | 0.4 |
| Lysine | 5.03 | 0.03 | 0.7 |
| Tyrosine | 5.33 | 0.06 | 1.2 |
| Arginine | 4.81 | 0.12 | 2.5 |
| Histidine | 4.87 | 0.18 | 3.6 |

* Mean of six injections of the same standard mixture.

TABLE II

PRECISION OF CHROMATOGRAPHY AND DERIVATIZATION OF AMINO ACID DERIVATIVES, INCLUDING CYSTEIC ACID

| *Amino acid* | *Mean* (% sample)* | *Standard deviation* | *Relative standard deviation (%)* |
|---|---|---|---|
| Cysteic acid | 5.57 | 0.15 | 2.7 |
| Alanine | 5.71 | 0.13 | 2.2 |
| Glycine | 5.18 | 0.07 | 1.3 |
| Valine | 5.50 | 0.10 | 1.8 |
| Threonine | 5.78 | 0.08 | 1.3 |
| Serine | 5.08 | 0.15 | 3.0 |
| Leucine | 5.50 | 0.05 | 0.9 |
| Isoleucine | 5.54 | 0.07 | 1.2 |
| Proline | 5.20 | 0.05 | 0.9 |
| Hydroxyproline | 5.29 | 0.13 | 2.5 |
| Methionine | 5.30 | 0.10 | 1.8 |
| Aspartic acid | 5.75 | 0.11 | 2.0 |
| Phenylalanine | 6.93 | 0.12 | 1.7 |
| Glutamic acid | 5.16 | 0.13 | 2.6 |
| Lysine | 5.05 | 0.17 | 3.4 |
| Tyrosine | 5.27 | 0.31 | 5.9 |
| Arginine | 5.01 | 0.52 | 10.3 |
| Histidine | 4.63 | 0.58 | 12.6 |

* Mean of six derivatizations of the same standard mixture.

and precision achieved in chromatography. Increased deviations exhibited by the later eluting derivatives may be attributed to thermal degradation brought about by their longer residence time in the high-temperature region.

An assessment of the precision shown by both derivatization and chromatography is illustrated by the results in Table II. These represent single injections of six separately derivatized standards and show considerable deterioration in the precision of a number of amino acids, although that of cysteic acid remains acceptable.

The pre-hydrolysis procedure described above was applied to seven samples of a particular fish meal which, after acid hydrolysis, derivatization and analysis, gave chromatograms of the type shown in Fig. 2. The precision with cysteic acid and hence cystine is acceptable (Table III), whereas that of several of the other amino acids is poor. As expected, a response for methionine is absent, as this amino acid is oxidized during the pre-hydrolysis oxidation, the resultant methionine sulphone

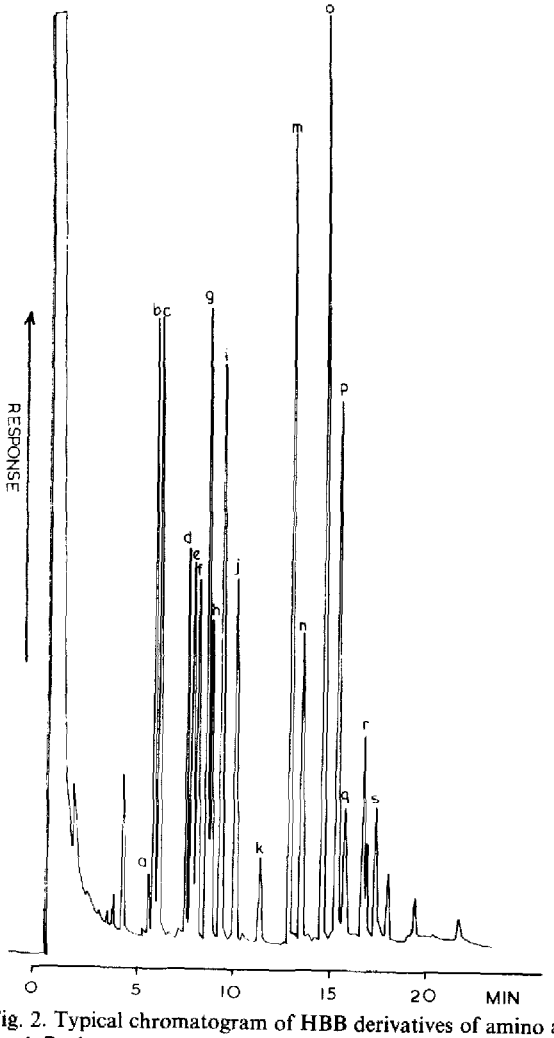
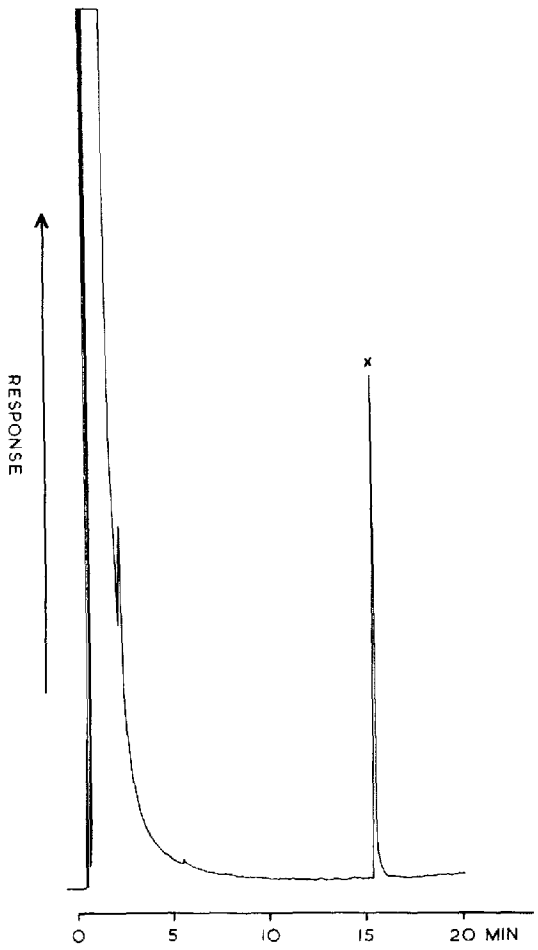



Fig. 2. Typical chromatogram of HBB derivatives of amino acids found in a hydrolysate of anchovy fish meal. Peaks as in Fig. 1.



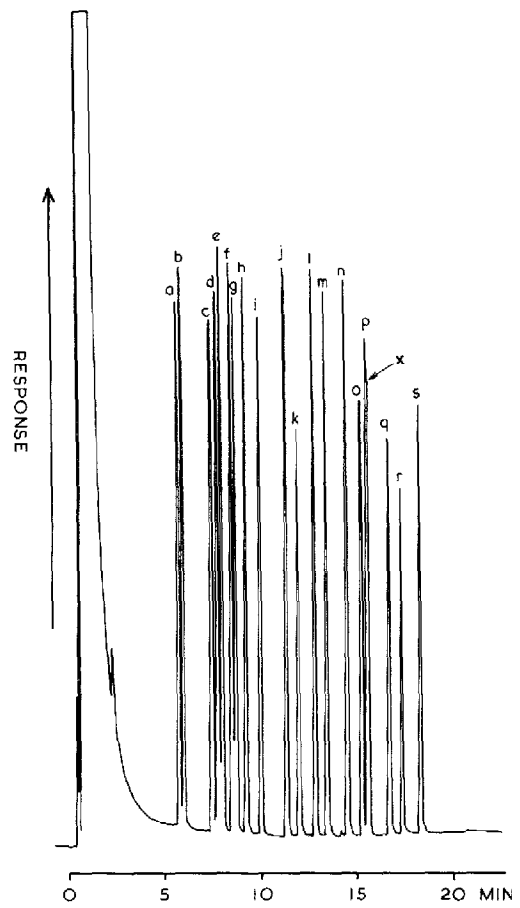




Fig. 3. Chromatogram of HBB derivative of methionine sulphone (x).

Fig. 4. Chromatogram of standard mixture of amino acid HBB derivatives showing the almost identical elution of tyrosine (p) and methionine sulphone (x) HBB derivatives. a, Alanine; b, glycine; c, valine; d, threonine; e, serine; f, leucine; g, isoleucine; h, norleucine (internal standard); i, proline; j, hydroxyproline; k, methionine; l, aspartic acid; m, phenylalanine; n, glutamic acid; o, lysine; p, tyrosine; q, arginine; r, histidine; s, SCE-cysteine; x, methionine sulphone.

derivative (Fig. 2) co-eluting with that of tyrosine (Fig. 3). This latter amino acid, however, together with arginine and histidine, is severely degraded by the oxidation process[4] and quantitation of these amino acids from pre-hydrolysis oxidized materials is unsatisfactory.

## CONCLUSION

The proposed GC technique enables reliable cystine/cysteine analyses of proteinaceous materials (in this case fish meal) to be carried out. Use of this method could possibly be extended to other feedstuffs. On account of the poor precision

TABLE III

PRECISION OF AMINO ACID ANALYSIS OF ANCHOVY MEAL FOLLOWING PRE-HYDROLYSIS TREATMENT WITH PERFORMIC ACID

| *Amino acid* | *Mean* (% sample)* | *Standard deviation* | *Relative standard deviation (%)* |
|---|---|---|---|
| Cysteic acid | 0.95 | 0.02 | 1.8 |
| Cystine** | 0.68 | 0.01 | 1.8 |
| Alanine | 4.20 | 0.09 | 2.0 |
| Glycine | 3.60 | 0.03 | 0.9 |
| Valine | 2.60 | 0.13 | 4.9 |
| Threonine | 2.70 | 0.04 | 1.6 |
| Serine | 2.63 | 0.04 | 1.5 |
| Leucine | 4.50 | 0.05 | 1.1 |
| Isoleucine | 2.06 | 0.06 | 3.0 |
| Proline | 2.41 | 0.04 | 1.5 |
| Hydroxyproline | 0.55 | 0.05 | 9.5 |
| Methionine | – | – | – |
| Aspartic acid | 5.52 | 0.09 | 1.6 |
| Phenylalanine | 2.22 | 0.04 | 1.9 |
| Glutamic acid | 7.53 | 0.13 | 1.7 |
| Lysine | 4.79 | 0.03 | 0.6 |
| Thyrosine | 1.53 | 0.30 | 19.2 |
| Arginine | 3.99 | 0.33 | 8.4 |
| Histidine | 1.33 | 0.07 | 5.6 |

* Mean of seven separate hydrolystes of the same sample.
** Cysteine results calculated of cysteic acid.

achieved for the analysis of certain amino acids following a pre-hydrolysis oxidation, the accomplishment of a simultaneous analysis of all protein amino acids (except tryptophan) from an acid hydrolysate appears to be impracticable, given the present state of the art.

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