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# Rapid derivatization and gas chromatographic determination of amino acids

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## ABSTRACT

Reaction conditions for the derivatization of amino acids with chloroformates were established. In an aqueous reaction medium the reagents are able to esterify not only the side-chain reactive groups but even the carboxylic group. Suitable derivatives for the determination of amino acids by capillary gas chromatography were found to be the N(O,S)-ethoxycarbonyl ethyl esters, which are formed in a few seconds. Six stationary phases were tested with a mixture of derivatized protein amino acids. A moderately polar stationary phase of the OV-1701 type permits the determination of amino acids with good resolution in less than 5 min.

## INTRODUCTION

Determinations of amino acids can nowadays be achieved at levels down to $10^{-18}$ mol using fluorimetric [1–4] or voltametric detection [5–7], combined with high-performance liquid chromatography (HPLC) or capillary electrophoresis [3,4]. However, although one-purpose amino acid analysers are no longer essential, routine automated measurements of amino acids with modern, flexible HPLC instruments are hardly possible with relatively inexpensive systems [7]. Therefore, gas chromatography (GC), with its capillary flexibility, resolving power and speed of analysis and with the instrumental costs being one third of those of HPLC, is worth re-examination. The main problem is the need for derivatization, commonly involving laborious and multi-step procedures taking 1 h or more [8,9], thereby losing the advantage of the speed of GC itself. However, some derivatization procedures require only 15 min [10,11]. Considering both the time of sample preparation and the overall duration of analysis, two procedures can be considered as the most rapid, one based on HPLC (condensation with phenyl isothiocyanate [12]) and the other on GC (condensation with dichlorotetrafluoroacetone [10]). Both permit the determination of a wide range of amino acids within 30 min. An alternative rapid procedure based on treatment of amino acids with fluorenylmethyl chloroformate and HPLC analysis was published recently [13,14].

In this paper a procedure is described that is uniquely rapid. The derivatives can be prepared in a few seconds and the subsequent capillary GC analysis can be performed in a few minutes. The total time of sample preparation and the analysis can

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be as short as 5 min. Other advantages are simple sample handling, the ability to effect derivatization in aqueous solutions and the use of inexpensive reagents.

The derivatization is based on the treatment of amino acids with chloroformates and is an extension of a recent study of chloroformate-induced esterification of carboxylic groups [15].

## EXPERIMENTAL

### *Chemicals*

Methyl and ethyl chloroformate (MCF, ECF), pyridine, chloroform, methanol and ethanol were obtained from Fluka (Buchs, Switzerland). An equimolar solution of seventeen protein amino acids in 0.1 *M* hydrochloric acid (AA-S-18) was purchased from Sigma (St. Louis, MO, U.S.A.) and used together with a complementary solution of the following amino acids in 0.1 *M* hydrochloric acid: asparagine, glutamine, tryptophan, cysteine, ornithine, hydroxyproline and *p*-chlorophenylalanine (internal standard). The concentration of cystine in the AA-S-18 solution was adjusted to equimolarity.

### *Procedures*

Amino acids in residues (not more than 100 μg in total) are treated with 100 μl of water–ethanol–pyridine (60:32:8) and 5 μl of ECF are added and mixed by briefly shaking the tube (3–5 s). Gas evolution (carbon dioxide) usually occurs. Then, 100 μl of chloroform (containing 1% ECF) are added and the derivatives are extracted into the organic phase by striking the tube against a pad for about 5 s. The chloroform layer clears during this process and the aqueous phase turns opaque. An aliquot of the organic phase is injected into the capillary column.

Alternatively, 15 μl of the amino acid solution in 0.1 *M* hydrochloric acid are diluted to 60 μl with water and 40 μl of ethanol–pyridine (4:1) are added. The procedure is then continued as above.

If treatment with MCF is preferred, the solvent used is water (or 20–25 m*M* hydrochloric acid)–methanol–acetonitrile–pyridine (60:16:16:8). Addition of the reagent (5 μl) is followed by addition of 100 μl of chloroform containing 1% of MCF.

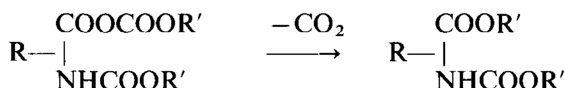
### *Instruments*

A Model 5890 gas chromatograph (Hewlett-Packard) with a flame ionization detector and a Model 3392A integrator was employed throughout with temperature ranges as given in the figure legends. The injector and detector temperatures were 200–250 and 250–300°C, respectively (the lower setting was used with columns coated with the most polar phases). Hydrogen was used as the carrier gas. Six different columns with increasing stationary phase polarity, *i.e.*, from methyl- through phenyl- to cyanopropylsilicones, were tested with the derivatized amino acids; the lengths and inside diameters were as follows: (A) 25 m × 0.32 mm CP-Sil 5 CB [100% dimethylpolysiloxane (DMS), 0.11 μm film thickness] from Chrompack (Middelburg, The Netherlands); (B) 25 m × 0.20 mm HP-5 (5% diphenyl–95% DMS, 0.33 μm) from Hewlett-Packard (Palo Alto, CA, U.S.A.); (C) 15 m × 0.25 mm DB-17 (50% diphenyl–DMS, 0.25 μm) from J & W Scientific (Folsom, CA, U.S.A.); (D) 10 m × 0.25 mm CP-Sil 19 CB (7% phenyl–7% cyanopropyl–DMS, 0.2 μm) from

Chrompack; (E) 15 m × 0.25 mm DB-225 (25% phenyl–25% cyanopropyl–DMS, 0.25 μm); and (F) 30 m × 0.25 mm DB-23 (50% cyanopropyl–DMS, 0.25 μm), both from J & W Scientific. The hydrogen head pressure corresponding to each column is given in the legends to the figures.

## RESULTS AND DISCUSSION

Chloroformates have been used previously, but the treated groups involved only alcoholic and amino groups [11,13,14,16,17], with the exception of the formation of mixed anhydrides as chemical intermediates [18–20]. A process of instantaneous decarboxylation of the intermediate alkoxycarbonyl esters (mixed anhydrides) was applied only with keto acids [21] and even on the macroscale of organic chemistry instantaneous decarboxylation of prepared mixed anhydrides was not observed [22,23]. A surprising finding was that pyridine as a catalyst (and only pyridine among many organic bases tested) is responsible for the instantaneous decarboxylation. The resulting N(O,S)-alkoxycarbonyl alkyl esters are formed by the decarboxylation of the intermediate alkoxycarbonyl esters:




The process of decarboxylation is induced by pyridine provided that the base is present in a molar excess with respect to the reagent, and this is fulfilled under the given conditions. It should be noted that even the reagent starts to decompose when added to the medium. The evolution of carbon dioxide, being accompanied by foaming of the solvent, can also be observed with a blank sample and has nothing to do with decarboxylation of the minute amount of the solute, when present. The decomposition products are thus carbon dioxide, alcohol and hydrogen chloride, which blocks the catalytic activity of pyridine; this is the reason why the pyridine should be present in a molar excess.

The yield of derivatives did not prove to be related to the decarboxylation of the reagent. The phenomenon of reagent decomposition followed by gas evolution does not have to proceed at all, as it does not occur in the instance of an increased ethanol concentration in the medium up to 40% (Fig. 1). Under such condition even higher yields with the hydroxyl-containing aliphatic amino acids and cysteine were observed; the basic amino acids, however, respond in the opposite way. The opposite behaviour takes place when the ethanol concentration is lowered to 20%. From Fig. 1, it is therefore apparent that an ethanol concentration in the middle of the tested range affords optimum results for the various amino acids.

The yield of amino acids from Fig. 1 and also that of H and Q can be partially influenced even by addition of water (100 μl) as a counter phase to the chloroform extraction. The yield of basic amino acids improves (by about 10–15%, the highest with H) and that of the hydroxy amino acids decreases by about 5–10%. However, the response of Q declined by over 30% on addition of water and extraction without an extra water is therefore to be preferred.

Not all the reactive groups in amino acid side-chains are altered by the action of

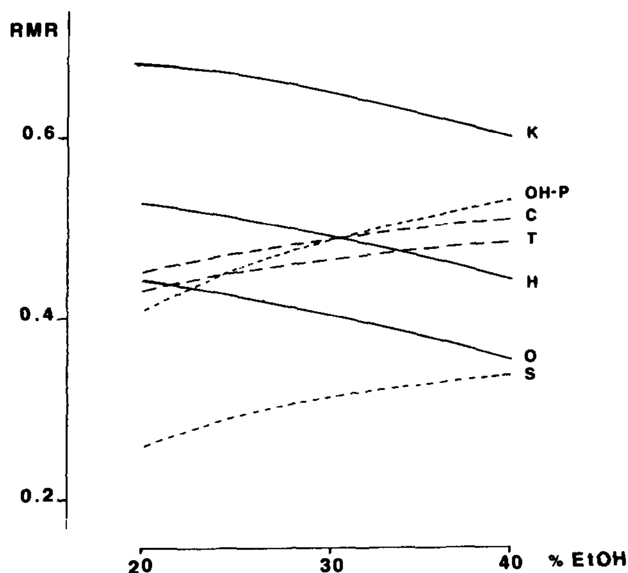



Fig. 1. Influence of changes in water–ethanol (EtOH) volume ratio in the reaction medium on the molar responses of amino acids.

the reagent. The imino group of arginine remains untouched, which is the reason for absorption of this amino acid derivative in the columns tested. The free imino group of indole in the molecule of W and free β-hydroxy groups in T, S and that in OH-P do not prevent the compounds from being eluted from the columns. The sulphydryl group of C, the phenolic group of Y and the imino group of the imidazole ring of H are derivatized. A marked difference in the retention times of the amides N and Q indicates that a dissimilar derivative formation takes place; a GC–mass spectrometric (MS) study is in progress [24]. However, the responses of N, D and C were markedly improved when 1% ECF was added to the chloroform.

Double-derivative formation with E and Q was observed by GC and confirmed

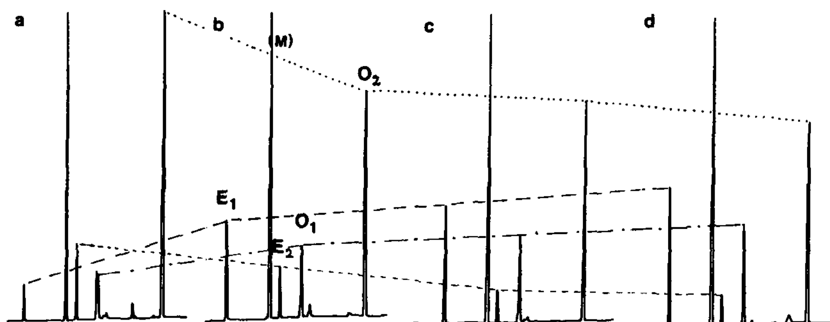



Fig. 2. Influence of acidification or alkalinization of the aqueous reaction medium on the double-derivative formation with E and O. M was added as internal standard. Water as component of the reaction medium (b) was replaced with (a) 100 m$M$ ammonia solution, and (c) 20 m$M$ and (d) 100 m$M$ hydrochloric acid.

TABLE I

REPRODUCIBILITY OF THE PROCEDURE

Mean molar responses (relative to internal standard) (RMR) and relative standard deviations (R.S.D.) were obtained by preparing ten individual samples analysed on the OV-1701 capillary column. Amino acids are sequenced according to elution order on this column.

| Amino acid | Abbreviation | RMR | R.S.D. (%) |
|---|---|---|---|
| Alanine | A | 0.441 | 3.7 |
| Glycine | G | 0.388 | 2.5 |
| Valine | V | 0.671 | 3.3 |
| Leucine | L | 0.718 | 2.5 |
| Isoleucine | I | 0.666 | 2.9 |
| Threonine | T | 0.465 | 3.6 |
| Serine | S | 0.312 | 3.1 |
| Glutamic acid | E | 0.298 | 8.3 |
| Proline | P | 0.713 | 2.7 |
| Asparagine | N | 0.428 | 2.2 |
| Aspartic acid | D | 0.361 | 4.2 |
| Methionine | M | 0.663 | 2.3 |
| Hydroxyproline | OH-P | 0.483 | 4.0 |
| Phenylalanine | F | 1.066 | 2.3 |
| Cysteine | C | 0.493 | 4.6 |
| Glutamine | Q | 0.305 | 5.3 |
| Ornithine | O | 0.407 | 3.4 |
| Lysine | K | 0.658 | 2.7 |
| Histidine | H | 0.486 | 5.8 |
| Tyrosine | Y | 1.061 | 2.6 |
| Tryptophan | W | 0.992 | 3.4 |
| Cystine | C-C | 0.554 | 3.5 |

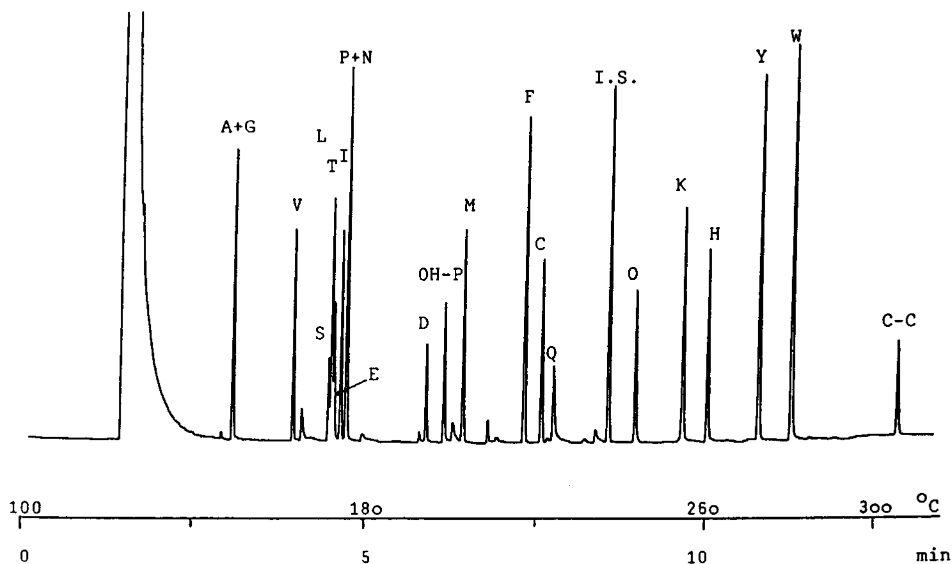



Fig. 3. EOC amino acid ethyl esters separated on a phase of OV-1 type (column A). Hydrogen head pressure, 100 kPa; temperature, increased at 16°C/min.

by MS. The compounds with lower retention are products of an internal cyclization resulting in the formation of pyroglutamic acid (assigned to E) and alkylated 3-aminopiperidinone in the case of O [eluted following F in Figs. 5 and 6 (not assigned)]. The main peak, assigned to O, has the expected structure of the $N^{\alpha}$,$N^{\omega}$-di-EOC ethyl ester. The process of internal cyclization of both can be influenced by acidification or alkalinization of the reaction medium, as shown in Fig. 2. By means acidification with dilute hydrochloric acid the cyclization is promoted, especially with E, so that the ratio of pyroglutamic to glutamic acid diethyl ester changes from 2:1 in water to 3:1 in 20–25 m*M* hydrochloric acid, which is desirable. The decline in O due to the cyclization to the piperidinone is low. In contrast, the use of ammonia is a means of suppressing the cyclization of O; with E, however, both peaks are low and the responses of other amino acids are slightly altered. The use of dilute hydrochloric acid is superior as with E the peak of interest is augmented and the molar responses of other amino acids do not deteriorate. When amino acids were treated with MCF the cyclization was promoted more by a partial replacement of methanol with

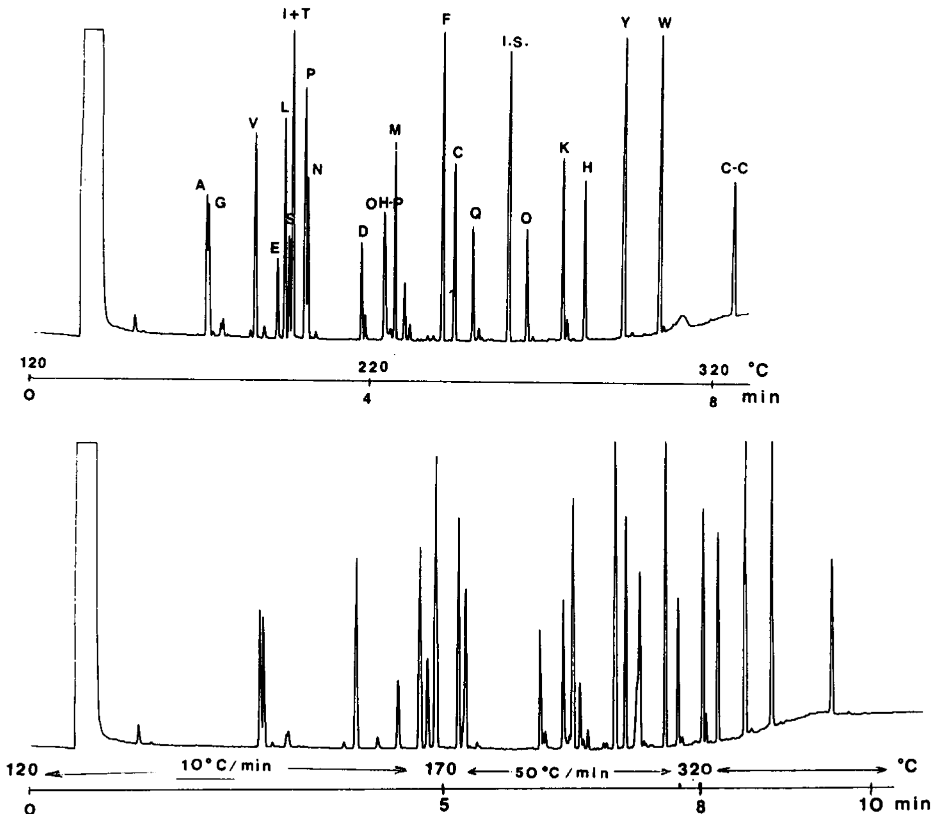



Fig. 4. EOC amino acid ethyl esters separated on a phase of SE-54 type (column B) with linear (25°C/min, top) and multi-linear (10 and 50°C/min, bottom) temperature programming. Hydrogen head pressure, 150 kPa.

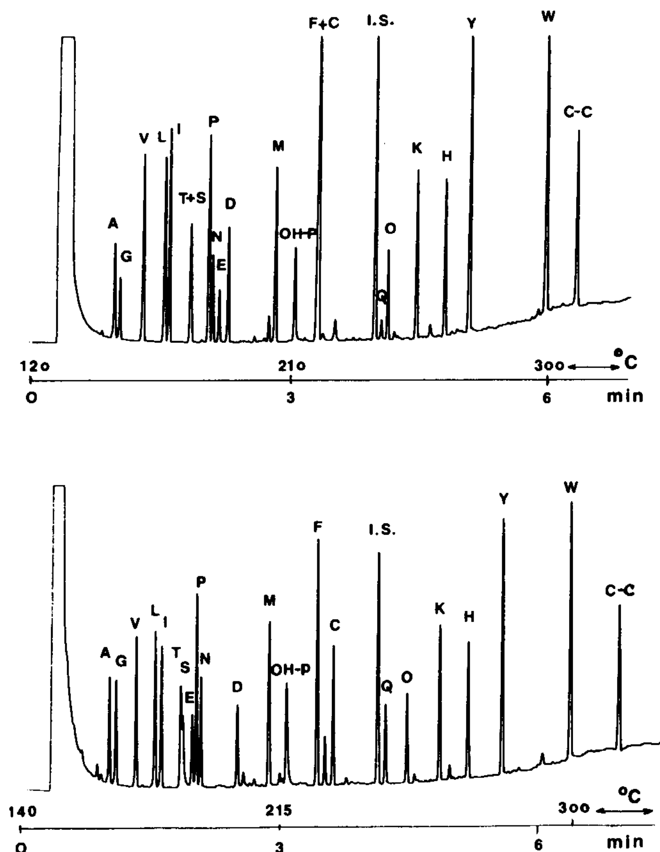



Fig. 5. Amino acids as MOC methyl (top) and EOC ethyl esters (bottom) analysed on a phase of OV-17 type (column C) under conditions optimum for their separation. Temperature, increased at 30 and 25°C/min; hydrogen head pressure, 70 and 60 kPa, respectively.

acetonitrile, as indicated under Experimental. In this way even the yields of T and S were slightly improved.

The reproducibility of derivative formation with ECF under the optimum reaction conditions with dilute (20 m*M*) hydrochloric acid in the medium is given in Table I. The relative standard deviations were lower than 5% on average; higher values were observed with three amino acids, E, Q and H. The values were obtained with the Chrompack CP-Sil 19 CB capillary column.

The subsequent examination of various stationary phases was aimed at obtaining optimum resolution in the fastest possible analysis. First choice the thermally most stable least polar phases OV-1 and SE-54 were tested, followed by the moderately polar phases OV-17 and OV-1701 and finally the most polar, least stable phases with a high abundance of cyanopropyl groups. The results of this study are given in Figs. 3–7.

The chromatograms show clearly that the polarity of the stationary phase markedly influences the resolution of some pairs or groups of amino acids, *e.g.*, the resolution of the pairs A–G, L–I and T–S. With increasing phase polarity the

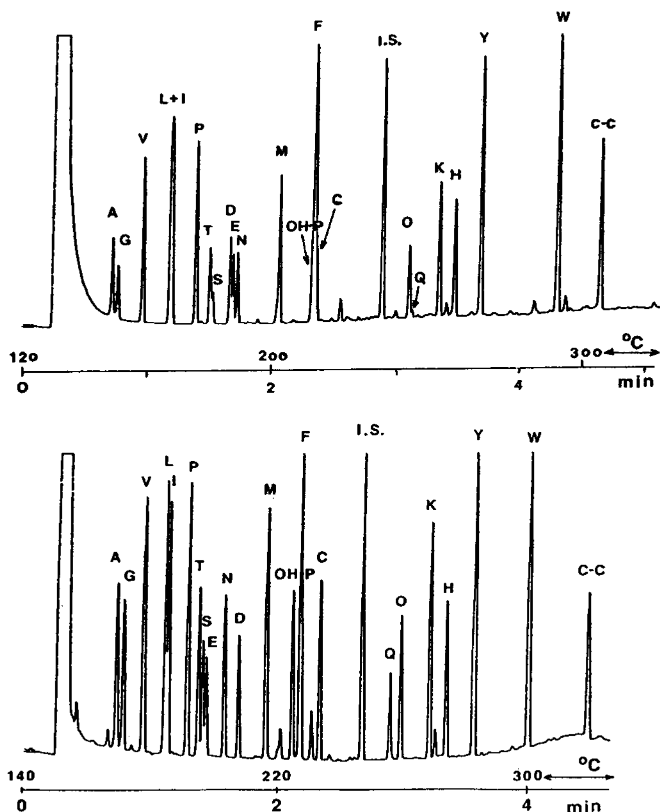



Fig. 6. Amino acids as MOC methyl (top) and EOC ethyl esters (bottom) analysed on a phase of OV-1701 type (column D) under conditions optimum for their separation. Temperature, increased at 40°C/min; hydrogen head pressure, 50 kPa.

resolution A–G continuously improves, from no resolution at all with OV-1, through a good resolution with the moderately polar phases to such a marked shift of G to a higher retention that it moves even behind V (Fig. 7). The opposite occurred with the pair L–I, the best resolution being achieved with the methylsilicone phase (Fig. 3), poorer with the moderately polar phases and failing completely with the most polar phases. This is of course a substantial shortcoming of the use of the polar phases in the analysis of amino acids as their EOC ethyl esters. The retention times of T–S (S–T sequence with the least polar phases) undergo a reversal of elution order with an increase in phase polarity, and a marked shift of both these amino acids with free hydroxy groups to nearly doubled retention times, compared with the L–I pair, can be observed. With the moderately polar OV-17, which otherwise is one of the most powerful, the reversal of the elution order of S–T to T–S only just occurs, so that the resolution of these amino acids fails or is very poor (Fig. 5). Incorporation of a small amount of cyanopropyl groups into the structure of this phase would not be helpful, as even such a low proportion as 7% (Fig. 6) alters the elution order of T–S *vs.* E and P completely.

It is a fortunate combination of phenyl, cyanopropyl and methyl groups in the

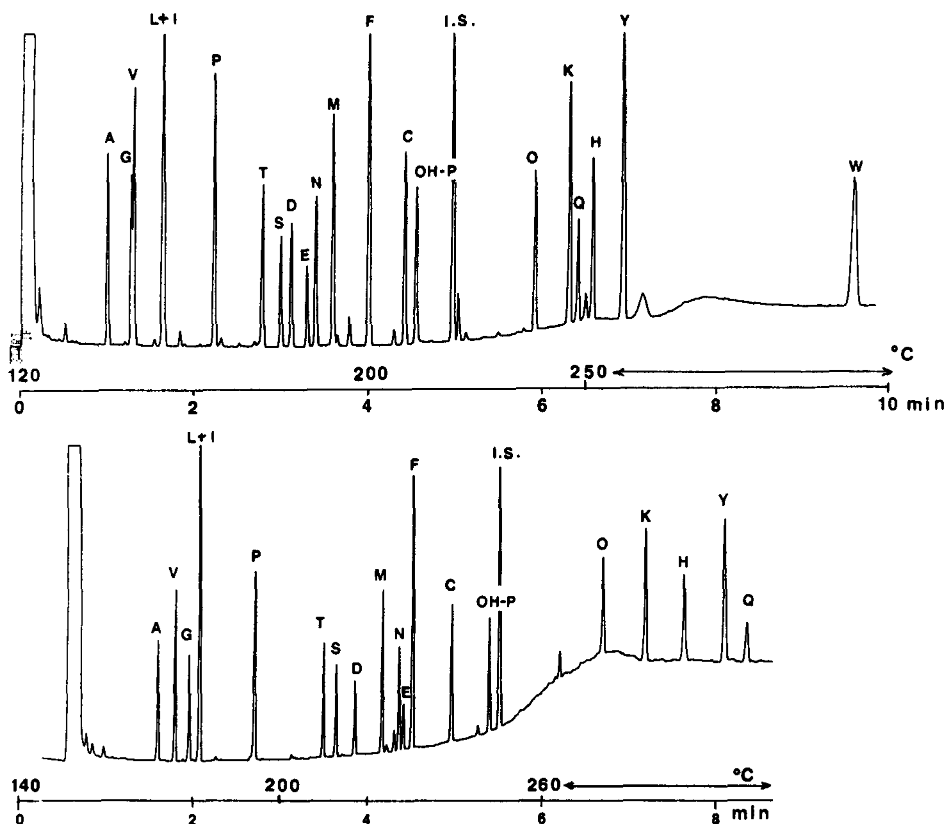



Fig. 7. EOC amino acid ethyl esters analysed on cyanopropyl phases of OV-225 (top) and OV-275 (bottom) type, corresponding to columns E and F. Temperature, increased at 20°C/min (both columns); hydrogen head pressure, 150 (top) and 175 kPa (bottom).

composition of the OV-1701 phase that results in good resolution, even when the separation of the L–I pair starts to deteriorate owing to the presence of cyanopropyl groups in the phase structure, as already mentioned.

The chosen temperature gradient of 40°C/min, permitting a high speed of analysis, is both necessary and optimum, because any change (or starting at a lower temperature) would be at the cost of separating the S–E pair, although the resolution of the L–I pair would be improved.

Other amino acids, *e.g.*, OH-P, N and Q similarly undergo a considerable increase in retention times. OH-P moves from a position between D and M on the least polar phase to between C and I.S., as shown in Fig. 7. In the same direction, N changes from elution with or behind P to elution behind M, and Q changes position from behind C to behind Y (DB-23 column). The influence of phase polarity on the retention behaviour of some amino acids is, therefore, remarkable.

With MOC amino acid methyl esters, which are prepared equally as smoothly as the ethyl esters, no satisfactory resolution on the columns tested could be achieved. The results with OV-1701 (Fig. 6) were clearly inferior to those obtained with the EOC

ethyl esters. The use of OV-17 seems to be promising provided that a column with a higher resolving power is used. However, the response of S is low, because of the lower extraction into the organic layer, and the response of Q is nearly zero, pointing further to the necessity to elucidate the structure of the compound by MS. These limitations are inherent in the procedure and must be taken in account. The inability to elute arginine with the underivatized imino group of its guanidine moiety persists and it is the major obstacle to the general adoption of this approach of amino acid determination via chloroformate treatment.

The stability of the EOC amino acid ethyl esters is good; Q starts to decline first. If storage is intended, it is recommended to separate chloroform from the counter phase and to keep the layer in refrigerator. No changes were observed after storage for 1 week.

Split injection was used for amino acid analyses throughout this study. If a splitless technique is preferred, it would be necessary to take up the chloroform layer, evaporate it to nearly dryness (remaining drop of pyridine) at room temperature and dissolve the residue in a polar solvent with a higher boiling point, perhaps dimethylformamide. This suggestion has not been investigated.

In conclusion, the method presented is unique in the rapidity of sample preparation and speed of GC analysis. Its drawback is the inability to determine arginine, the only protein amino acid which would require another procedure or additional reaction step.

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