GAS CHROMATOGRAPHY OF VOLATILE AMINO ACID DERIVATIVES

IV. MIXED STATIONARY PHASES FOR THE SEPARATION OF N-TRIFLUOROACETYLATED AMINO ACID *n*-AMYL ESTERS

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INTRODUCTION

In previous attempts to find stationary phases for the complete isothermal resolution of mixtures of protein amino acids as their N-trifluoroacetylated *n*-amyl esters we showed that in spite of the wide range of phases tried[1] no single one gave a complete resolution of the most volatile of these amino acid derivatives (those of alanine, valine, glycine, isoleucine, leucine, threonine and serine). The most important factors operating against achieving the desired resolution were the lack of sufficient selectivity on the part of the phases tried and also the use of packed columns giving only 3,000 total theoretical plates with some stationary phases and much less with other phases.

With the second group of amino acid derivatives (those of cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid and glutamic acid) a phase was found which gave the necessary resolution[2], but on further study tyrosine, which had not been included in this investigation, was found to be insufficiently separated from aspartic acid[3].

These difficulties were largely overcome by the use of mixtures of packings prepared from different stationary phases and by using columns with a higher total number of theoretical plates. The choice of stationary phases was deliberately restricted to those with high thermal stability and low bleed-rates.

MATERIALS AND METHODS

Apparatus

A D6 gas density balance gas chromatograph (Griffin & George Ltd., Alperton, Middlesex) was used. Nitrogen (99.9 % "White spot" from British Oxygen Co. Ltd., and "high-purity oxygen-free" from Air Products Ltd.) was used as carrier gas. Some work was also done with the D1 single column modification of this instrument, which permitted the use of higher operating pressures, and had a flash heater on the inlet. The final results were checked on a MicroTek MT 220 dual-column gas chromatograph with flame-ionisation detectors to prove their general application.

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Columns

In order to increase the total number of theoretical plates, glass columns were made from Pyrex tubing of 0.4 or 0.25 cm internal diameter, with a "paper-clip" configuration and in this way columns of any required length could be used. The sector plates that normally hold the columns in the oven of the D6 chromatograph were fitted with column-top fittings which had been modified by brazing on at the lower end, $^1/_8$ in. "Swagelok" male couplings drilled out to accept the ends of the glass column. The ends of the column inserted into the couplings were made gas tight with two $^1/_4$-in. "Viton" O-rings and locked in position with nuts. A similar arrangement was used to fit the same glass columns into the D1 modification of the instrument.

Column packings

Since the preparation of Silocel C22 as used previously was time-consuming, "10-mesh cuts" of Anakrom acid and base washed and vacuum siliconized (ABS) were used. These are commercially available and were found to give high efficiency columns. Anakrom has a lower packing density and surface area per g than Silocel C22, but for equivalent weights of stationary phase in the column the separations achieved were found to be comparable.

The stationary phases were dissolved in a suitable solvent (usually ethyl methyl ketone) in a 1 l round-bottomed flask with four dimples in the side to promote mixing. The correct weight of Anakrom ABS (90–100 or 100–110 mesh) (Analabs, through Gas Chromatography Ltd., Maidenhead, Berks.) was added, and the solvent was evaporated off under reduced pressure using first a water pump and then an oil pump on a rotary evaporator. The flask was turned slowly by hand to prevent the production of "fines". The packings were prepared from single stationary phases only, and mixed phases were made up subsequently by blending together the calculated proportions of the dried packings by weight. These blended packings were filled into the column through a funnel attached at one end, while suction was applied from an oil-pump at the other end. The packing was compacted by vibration and gentle tapping. The column and the glass yarn used to plug the ends of the column were cleaned with a chromic acid–sulphuric acid mixture, washed free of acid with distilled water, dried, deactivated with 5 % dimethyldichlorosilane in toluene for 2 h, rinsed twice with toluene, washed with methanol and dried before using.

Preparation of derivatives

Modifications were introduced in methods previously described[1]. Owing to difficulty in esterifying lysine hydrochloride with amyl alcohol[3] the amino acids (0.5–2.0 mg of each) were first dissolved in trifluoroacetic acid (0.2 ml) in a B 14 test tube. Amyl alcohol (2 ml) was then added and dry HCl gas was bubbled continuously through the reaction mixture at 108° ± 2° for 25 min. The alcohol was then removed with the rotary evaporator using first a water pump then an oil pump. As the amyl ester of arginine gives no volatile trifluoroacetylated derivative on standing in trifluoroacetic anhydride at room temperature it was necessary to use higher temperatures[4]. The amino acid esters were taken up in a minimum of methyl alcohol and an aliquot transferred by means of a microlitre syringe to a small Pyrex tube. The alcohol was removed with the rotary evaporator or alternatively by standing the tube in an oven at 70°. Trifluoroacetic anhydride (0.1 ml) was added and the tube sealed and

placed in the oven at 140° for 5 min. The tube was then cracked open, the TFA anhydride removed on the rotary evaporator and the residue taken up in a known volume of dry ethyl methyl ketone for injection onto the column.

RESULTS AND DISCUSSION

In isothermal gas chromatography of the most volatile amino acid derivatives it was found necessary to restrict the amino acids to alanine, valine, glycine, threonine, isoleucine, leucine and serine. The next most volatile derivatives, those of cysteine and proline, have much longer retention times than those of leucine and serine.

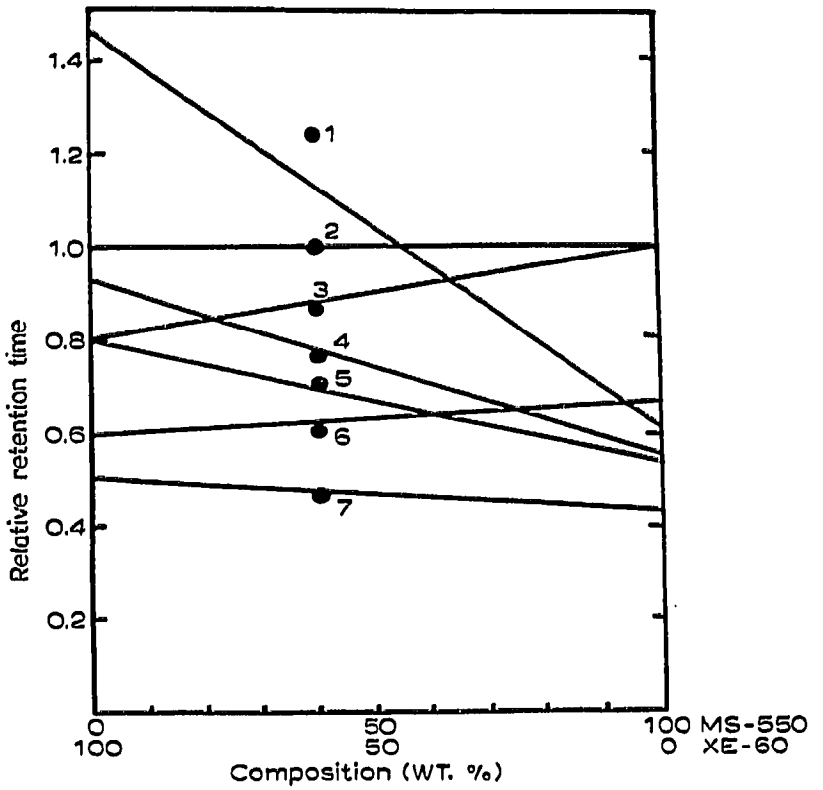

Fig. 1. The relationship between the relative retention time (leucine = 1.0) of the trifluoroacetylated *n*-amyl esters of seven amino acids and the composition (wt. %) of the mixed stationary phase XE-60 and MS-550 at 140°. 1 = Serine; 2 = leucine; 3 = isoleucine; 4 = glycine; 5 = threonine; 6 = valine; 7 = alanine.

The selection of mixed stationary phases was made by plotting the retention times of the derivatives relative to the leucine derivative on one stationary phase against the relative retention times of the same derivatives on a second phase using data obtained at the same temperature. This procedure eliminates combinations that will not improve resolution and also indicates the best proportions to be used in combinations that are likely to be effective. Three such combinations were found to give complete resolution of the derivatives of this first group of seven amino acid derivatives:

61 % XE-60 and 39 % Duo-seal pump oil;
27 % XE-60 and 73 % XF-1105;
60 % XE-60 and 40 % MS-550.

The individual packings were prepared to contain 5 % of the stationary phase.

Fig. 1 shows the result of applying such a graphical method for this third mixed phase. Serine is the only amino acid to give a relative retention time deviating considerably from the expected value. Packings containing Duo-seal pump oil and XF-1105 were not developed further, since these stationary phases were found to bleed considerably at temperatures above 150°. Quite appreciable bleed-rates which are often not very noticeable with the gas density balance detector are unsatisfactory with the flame-ionisation detector due to the base-line drift which becomes apparent at higher sensitivities.

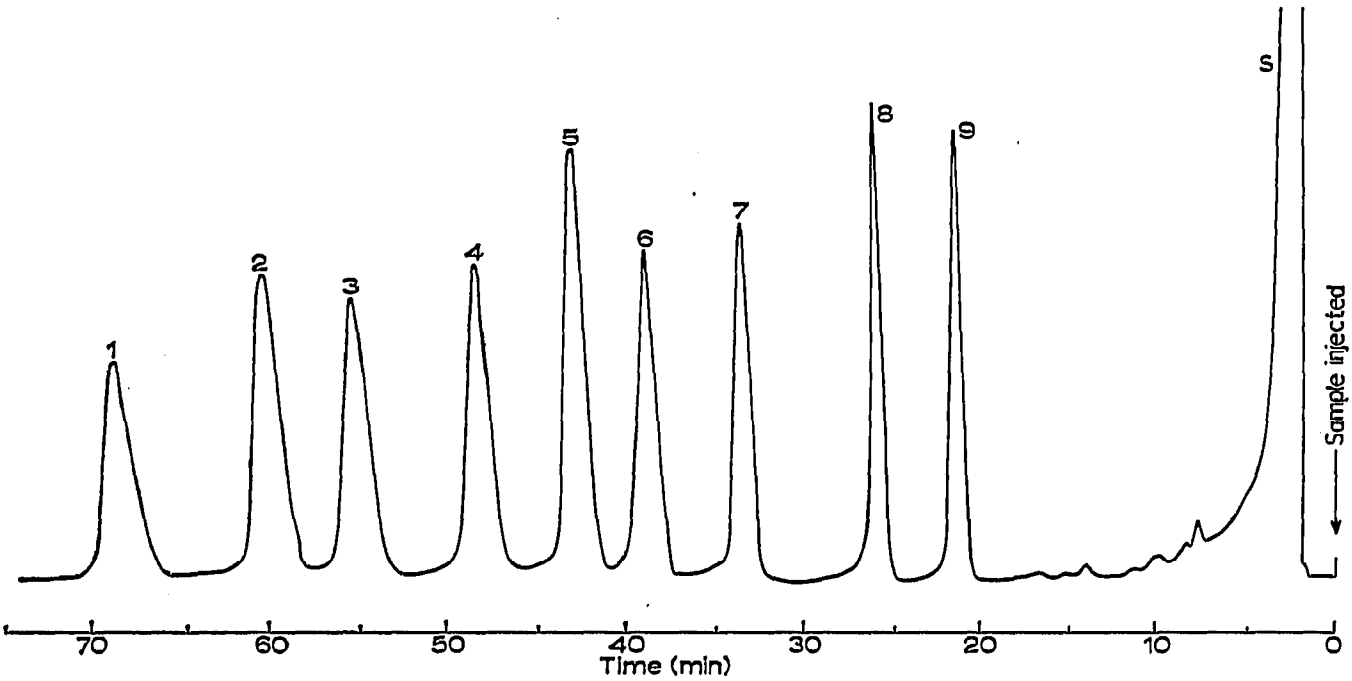

Fig. 2. Separation of a mixture of 9 trifluoroacetylated amino acid *n*-amyl esters on Anakrom ABS 90–100 mesh with the mixed phase XE-60 and MS-550. The packings were prepared separately to contain 5% w/w of the stationary phase and the column was filled with a blend of 60% XE-60 and 40% MS-550. The glass column was 5 m long with internal diameter 0.25 cm. Column temperature: 135°; gas flow: 24 ml N_2/min; inlet pressure: 25 p.s.i. D6 chromatograph with gas density balance detector. Sample size: 2.0 μl. Attenuation: × 1. Total number of theoretical plates for the leucine derivative (45 μg): 7300. 1 = Serine; 2 = β-amino-*n*-butyric acid; 3 = leucine; 4 = isoleucine; 5 = glycine; 6 = threonine; 7 = valine; 8 = alanine; 9 = α-amino-isobutyric acid. S = Solvent.

We found very little difference in performance between the columns filled with the blended packings and those produced by filling consecutive sections of the columns with different packings (segmented packings: see HAAHTI, VANDENHEUVEL AND HORNING[5]). However, it was found that the blended packings were more convenient, because it was easier to achieve the desired proportions and to alter the proportions if they required adjustment. It was also found that columns prepared from blended packings gave slightly higher efficiencies. When "mixed-film" packings were prepared by dissolving both stationary phases in the same solvent in the desired proportions prior to coating onto the support they were not always found to give the relative retention times that were expected. They gave slightly lower efficiencies and also the proportions of the stationary phases could not be altered.

Fig. 2 shows the separation of the first group of amino acids on a column packed with a mixture of the stationary phases XE-60 and MS-550. Two non-protein amino

acids, α-amino-isobutyric acid and β-amino-*n*-butyric acid are included, as these may be conveniently used as internal standards. It is interesting to note the difference in retention times for this pair of isomers. For certain quantitative work it may be preferable to add a known volume of a solution containing an external standard to the final reaction mixture. Naphthalene or ethyl benzoate are suitable external standards and emerge before the α-amino-isobutyric acid peak. Biphenyl and bibenzyl[1] do not have suitable retention times for this mixed column.

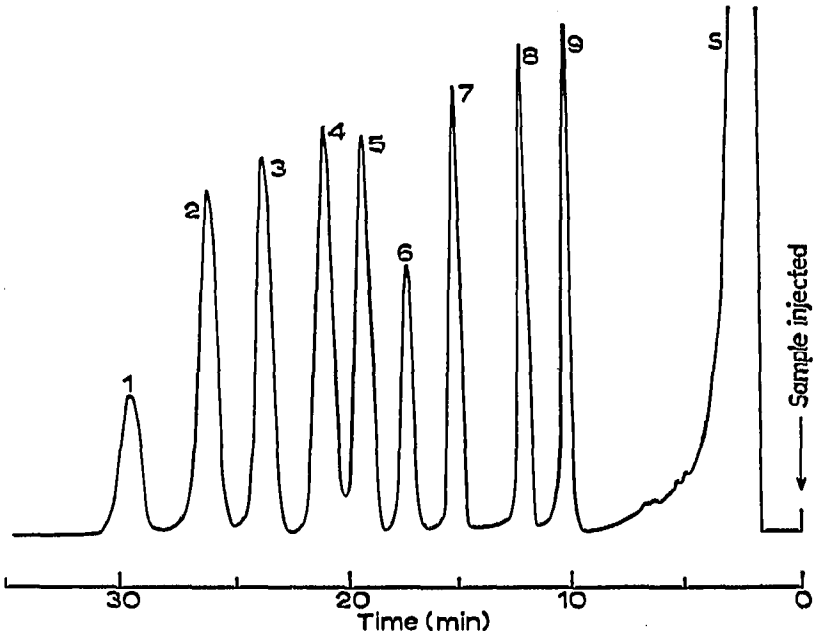

Fig. 3. Separation of same mixture as in Fig. 2 diluted eight-fold with methyl ethyl ketone. Column packing as for Fig. 2. Glass column 320 cm long with internal diameter 0.25 cm. Column temperature: 135°; gas flow: 24 ml/min. MicroTek MT 220 chromatograph with flame ionization detector. Sample size: 0.2 μl. Attenuation: 0.1 × 32. Total number of theoretical plates for the leucine derivative (~ 0.5 μg): 5100.

The relative retention times of these derivatives (relative to leucine taken as 1.0) vary with temperature. If the relative retention times are plotted against temperature, straight lines are obtained, whose slopes vary considerably. In practice, these temperature effects may be exploited to optimize the separation of these seven protein amino acids. With the mixed phases studied the separation of glycine, threonine and valine always presented the greatest difficulties and it was found that by raising or lowering the temperature by only a few degrees it was possible to adjust the separation to give the best resolution. Changing the gas flow-rate was not found to improve separations. The highest efficiencies were obtained with nitrogen gas flow rates of 38 ml/min and 25 ml/min for 0.4 and 0.25 cm internal diameter columns respectively.

The gas density balance detector was used to obtain the separation in Fig. 2. In Fig. 3 the same sample of mixed amino acids was separated with the MicroTek MT 220 gas chromatograph. In this separation the absolute amount of sample applied to the column was reduced to take advantage of the high sensitivity of the flame ionization detector and the total time taken has been reduced from 71 min to 31 min. The relative peak heights in Figs. 2 and 3 are not the same. This is due to the fundamental differences between the two types of detector used.

For the second group of derivatives (those of cysteine, proline, hydroxyproline, methionine, phenylalanine, aspartic acid, glutamic acid, tyrosine, lysine, arginine, tryptophan and cystine) the major problem was to get adequate resolution of cysteine, proline and hydroxyproline without extending the time of analysis too much for the less volatile derivatives such as lysine, arginine and tryptophan. To separate the amino acid derivatives in this group a mixed phase was finally developed after carrying out the procedure of plotting relative retention times as shown in Fig. 1. For convenience aspartic acid was taken as 1.0. Only one mixed phase was found to give adequate resolution: 53 % QF-1 and 47 % MS-710. Both these stationary phases have high maximum operating temperatures and low rates of bleeding, so that they are suitable for use with highly sensitive detectors. Fig. 4 shows the separation of 11 amino acids on this mixed phase at 170°. The two non-protein amino acids, ethionine and ornithine are included and may be used as internal standards. All the amino acids shown in Figs. 2 and 3 have very short retention times under these conditions and

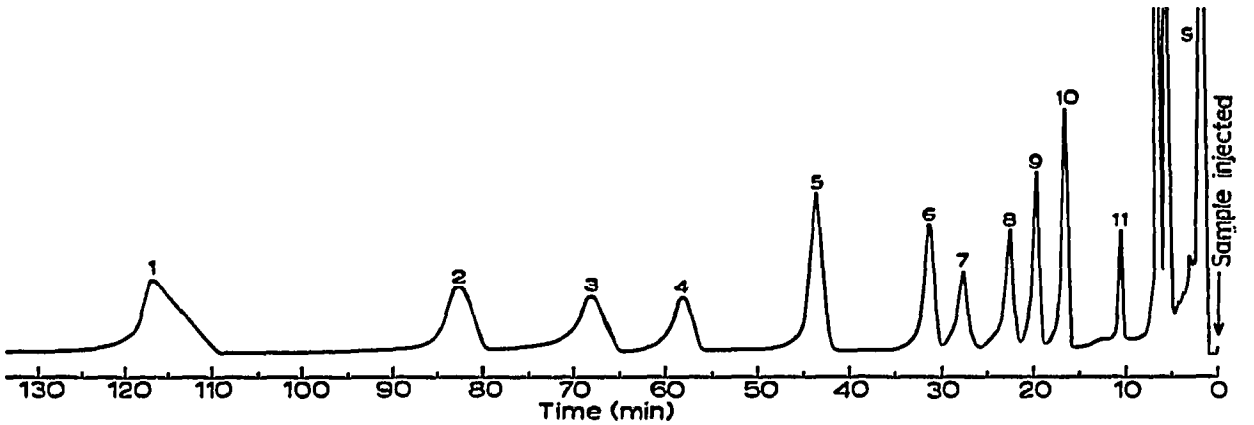

Fig. 4. Separation of a mixture of 11 trifluoroacetylated amino acid *n*-amyl esters on Anakrom ABS 100–110 mesh with the mixed phase QF-1 and MS-710. The packings were prepared separately to contain 5 % w/w of the stationary phase and the column was filled with a blend of 53 % QF-1 and 47 % MS-710. The glass column was 320 cm long with internal diameter 0.4 cm. Column temperature: 170°; gas flow: 38 ml N_2/min; inlet pressure: 16 p.s.i. D6 chromatograph with gas density balance detector. Sample size: 2.0 μl. Attenuation: × 1. Total number of theoretical plates for the aspartic acid derivative (40 μg): 3500. 1 = Lysine; 2 = glutamic acid; 3 = ornithine; 4 = tyrosine; 5 = aspartic acid; 6 = phenylalanine; 7 = ethionine; 8 = methionine; 9 = hydroxyproline; 10 = proline; 11 = cysteine. S = Solvent.

emerge as an unresolved set of peaks after the solvent but before cysteine. On this column the peaks of arginine and tryptophan are not completely resolved. They have a relative retention time of 4.5 (aspartic acid taken as 1.0). No identifiable volatile derivative has been obtained from histidine as the trifluoroacetylated *n*-amyl ester derivative. However, other workers have shown peaks for the trifluoroacetylated histidine methyl[6–8] and butyl[9] esters. The retention time of the cystine derivative is estimated to be about 6 h under these conditions and it may be advantageous to reduce cystine to cysteine[6] prior to making the derivative. Attempts to make a volatile derivative with cysteic acid using the methods described here have failed. The isothermal separation of the protein amino acids as their trifluoroacetylated methyl ester derivatives was carried out by MAKISUMI AND SAROFF[7] and these authors found it necessary to separate the amino acids in three groups, each at a different temperature.

Although no survey of stationary phases comparable to that carried out for the

trifluoroacetylated amino acid *n*-amyl esters has been made for the corresponding methyl esters, our results on a limited number of different columns indicate that at any one temperature the retention times of many of the methyl derivatives are about one third of those of the corresponding amyl derivatives. Similar results were also

TABLE I

RETENTION TIMES OF TRIFLUOROACETYLATED ESTERS OF ASPARTIC AND GLUTAMIC ACIDS

5% w/w QF-1 and 5% w/w MS-710 both on Silocel C22, 90–100 mesh acid washed and deactivated[1], blended in the proportion 60:40 and packed into two stainless steel tubes[1] with total length 182 cm. Gas flow: 38 ml N_2/min; column temperature: 185°.

N-TFA ester	R_T (*min*)
Aspartic acid	
dimethyl	3.3
methyl-amyl	8.5
diamyl	22.5
Glutamic acid	
dimethyl	5.5
methyl-amyl	15.2
diamyl	41.0

found by MAKISUMI, NICHOLLS AND SAROFF[10]. They quote a ratio of 1:3.4 for the relative retention times of the N-TFA leucine methyl and amyl esters. However, where two ester groups on the same molecule are involved the retention times of the methyl derivatives are about $^1/_7$ of the corresponding amyl derivatives. Table I shows

TABLE II

RETENTION TIMES OF TRIFLUOROACETYLATED CYSTINE ESTERS

5% w/w SE-30 on Anakrom ABS 90–100 mesh packed into a glass column 152 cm long with 0.4 cm internal diameter. Gas flow: 38 ml N_2/min; column temperature: 210°.

N,N'-Di-TFA cystine ester	R_T (*min*)
Dimethyl	6.8
Diethyl	10.0
Dipropyl	14.5
Dibutyl	28.0
Diamyl	48.0

the increase in retention time for the dimethyl, the monomethyl-monoamyl and the diamyl esters of both aspartic and glutamic acids. Table II shows the increase in retention time for the homologous series of esters of cystine. In practice it is found that the methyl ester derivatives may be conveniently chromatographed with the column maintained at a temperature 30° lower than that required for the amyl esters. Using various stationary phases for isothermal separations the trifluoroacetylated methyl ester derivatives show a different order of emergence from the column to that of the trifluoroacetylated amyl esters, (*cf.* MAKISUMI AND SAROFF[7] and IKEKAWA[11] with refs. 1, 2 and 3). Where we have carried out isothermal and temperature-pro-

grammed separations on the same column, no difference was found in the order of emergence of our amyl ester derivatives.

It may be added that the trifluoroacetylated amino acid esters of pentan-2-ol could be resolved into two peaks on packed columns. These derivatives are more volatile than the corresponding pentan-1-ol esters. Fig. 5 shows the double peak

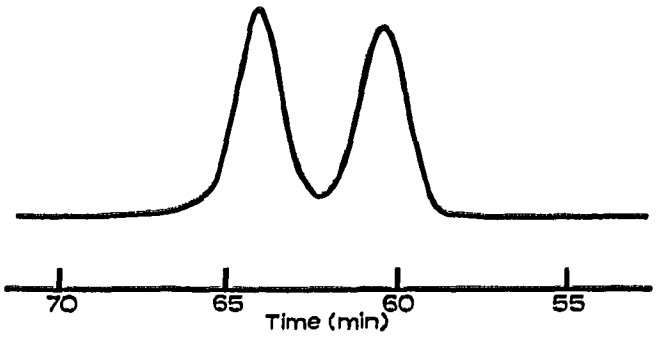

Fig. 5. Resolution into two peaks of the trifluoroacetylated D,L-valine D,L-pentan-2-ol ester. Same column in MicroTek MT 220 as used for Fig. 3. Column temperature: 100°; gas flow: 27 ml N_2/min. Total number of theoretical plates: 8500.

obtained with valine. No allocation of stereoisomers to the peaks has been made. Using capillary columns, GIL-AV, CHARLES AND FISCHER[12] and POLLOCK AND OYAMA[13] showed a similar resolution with the trifluoroacetylated octan-2-ol and butan-2-ol amino acid esters, respectively.

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SUMMARY

A gas chromatographic separation of the trifluoroacetylated *n*-amyl esters of α-amino-isobutyric acid, alanine, valine, threonine, glycine, isoleucine, leucine, β-amino-*n*-butyric acid and serine in that order has been achieved isothermally using mixed stationary phases. With a different mixed phase at a higher temperature the following amino acids were separated: cysteine, proline, hydroxyproline, methionine, ethionine, phenylalanine, aspartic acid, tyrosine, ornithine, glutamic acid and lysine. The difficulties with other protein amino acids are discussed.

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