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KOVÁTS' INDICES OF TRIMETHYLSILYLATED AMINO ACIDS ON FUSED-SILICA CAPILLARY COLUMNS

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SUMMARY

Trimethylsilyl derivatives of protein amino acids were separated by high-resolution gas chromatography on three fused-silica capillary columns, coated with SE-54, SP-2100 and Carbowax 20M, respectively Kováts' retention indices were also calculated by using *n*-alkanes as standards and tabulated Excellent reproducibility of the index values were obtained The usefulness of the method in actual amino acid analysis of peptides and proteins was demonstrated by analyzing the HCl-hydrolyzate of lysozyme

INTRODUCTION

During the past two decades, a great deal of effort has been made to analyze amino acids by gas chromatography (GC) Amino acids, however, must be derivatized prior to their GC analysis. About 100 different derivatization methods have been suggested and successfully used for this purpose[1–3] Zomzely *et al*.[4] introduced the N-trifluoroacetyl (N-TFA) *n*-butyl esters as a possible derivative Later, Kaiser *et al*.[5] extensively investigated these derivatives for the GC analysis of amino acids Many other N-acyl alkyl ester derivatives have also been utilized for the same purpose[6–13] The use of thiohydantoin derivatives in GC has been reported, especially for the sequence analysis of peptides[14,15]. Trimethylsilylation is another derivatization method, which was introduced by Rühlmann and Giesecke[16]. Several authors have used this method for the GC analysis of amino acids utilizing various silylation reagents[17–23]

Most of the papers listed above have reported separations using conventional packed columns GC with capillary columns, however, offers great advantages over GC with packed columns[24–27] This technique has also been applied to GC analysis of amino acids[7 9 11–13 15 28–31] Several review articles have appeared dealing with GC of amino acids[1 3 15 32–34].

In this paper, we describe the GC Kovats' retention indices[35] of trimethylsilylated amino acids on three fused-silica capillary columns[36] coated with SE-54, SP-2100 and Carbowax 20M, respectively

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EXPERIMENTAL*

Apparatus

A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a flame ionization detector was used. The injection port and detector were maintained at 250°C. Injections were made manually. Helium was used as the carrier gas for all separations at an inlet pressure of 75 kPa. The split ratio was 1:100. The following three fused-silica capillary columns (wall-coated open tubular) (Hewlett-Packard) were used (each 12 m × 0.2 mm I.D.):

(1) SE-54 (5% phenyl, 1% vinyl, methyl silicone gum: siloxane deactivated) The measured efficiency was *ca.* 5400 theoretical plates per meter based on the pentadecane peak at 120°C (capacity factor, $k' = 6.16$; linear velocity $\bar{\mu} = 39.9$ cm/sec). "Trennzahl" (TZ) between tetradecane and pentadecane was 23.3 The ratio of 2,6-dimethylphenol (DMP) to 2,6-dimethylaniline (DMA) was 1.00

(2) SP-2100 (methyl silicone fluid, Carbowax 20M deactivated) The measured efficiency was *ca.* 4400 plates per meter based on the pentadecane peak at 130°C ($k' = 6.58$; $\bar{\mu} = 33.3$ cm/sec). TZ was 20.0 The ratio of DMP to DMA was 1.00

(3) Carbowax 20M (polyethylene glycol, Carbowax 20M deactivated) The measured efficiency was *ca.* 5000 theoretical plates per meter, based on the pentadecane peak at 100°C ($k' = 2.55$; $\bar{\mu} = 33.4$ cm/sec). TZ was 18.7.

Materials

Amino acids were purchased from Vega Biochemicals (Tucson, AZ, U.S.A.) Lysozyme was obtained from Sigma (St. Louis, MO, U.S.A.). Bis(trimethylsilyl)-trifluoroacetamide (BSTFA), acetonitrile and constant-boiling HCl were purchased from Pierce (Rockford, IL, U.S.A.) *n*-Alkanes were from Supelco (Bellefonte, PA, U.S.A.).

Trimethylsilylation

A sample of *ca.* 1 mg of each amino acid was placed in a PTFE-capped hypovial (Pierce) and trimethylsilylated with 0.4 ml of a mixture of BSTFA and acetonitrile (1:1) by heating for 45 min at 140°C.

Hydrolysis of lysozyme with HCl

A sample of *ca.* 10 mg of the native protein was hydrolyzed with 0.6 ml of constant-boiling HCl in evacuated and sealed tubes at 110°C for 24 h. After hydrolysis samples were dried *in vacuo* and then trimethylsilylated as above

RESULTS AND DISCUSSION**

The Kováts' retention index system[35] has generally been accepted as the best way to characterize the retention behavior of chromatographically separated com-

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* Certain commercial equipment, instruments or materials are identified in this paper in order adequately to specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified are necessarily the best available for the purpose.

** Abbreviations for amino acids follow IUPAC–IUB recommendations [see *Biochem. J.*, 126 (1972) 773]. C10, etc., represents an *n*-alkane with ten carbon atoms. pC corresponds to picocoulomb (see Fig. 17).

pounds[37–39]. In most cases, *n*-alkanes are used in GC as standards for this purpose

In the present work, we separated trimethylsilylated protein amino acids by GC on three fused-silica capillary columns and also determined the Kovats' indices of these compounds using *n*-alkanes with an even number of carbon atoms as standards *n*-Alkanes were added to original samples prior to injection The gas holdup time was determined by using methane, which was injected together with the sample Two silicone stationary phases, SE-54 and SP-2100, and one polar stationary phase, Carbowax 20M (see ref 40 for McReynolds constants of these phases), were used for separation of amino acids and determination of their retention index values Ideally, symmetrical peak shapes have to be obtained for precise determination of Kovats' indices The Carbowax 20M column, however, did not provide symmetrical peaks for all the amino acids Some of them particularly those with long retention times, yielded tailing peaks Despite this fact, retention indices of all the amino acids were also determined on this polar column in order to provide sufficient confidence in the identification by matching retention indices The use of columns of different polarity greatly increases the usefulness of the retention index system for identification purposes Overloading of the columns was avoided, because it causes leading peak shapes, and the position of the peak maximum shifts resulting in incorrect index values

According to definition, Kovats' indices are determined at constant temperature In this paper, we have determined the retention indices of amino acids mainly at constant temperature. It is however, impossible to determine the index values of all the amino acids in a single run at a certain constant temperature, because of the large differences in their retention times Thus, index values were determined at different constant temperatures corresponding to a reasonable retention time for individual amino acids and an acceptable resolution Retention indices obtained by using a temperature program were also given

Major error sources in retention index determinations are incorrect temperature measurements and inaccurate measurement of the retention times In this paper, the oven temperature was controlled by a microprocessor Retention times were also measured by the microprocessor which reported these values to the nearest 0 001 min[27] Injections were made manually Mean retention index values and standard deviations were calculated from at least ten measurements As the results below show, excellent reproducibility was obtained, particularly on SE-54 and SP-2100 columns

Separations and Kovats indices

On SE-54 column Four different temperatures, 85°C, 120°C, 150°C and 190°C, were used for measurement of the retention index values of all the amino acids on this column. Chromatograms obtained at these temperatures are given in Figs 1–4, respectively Fig. 5 shows the separation of all the amino acids in a single run by using a temperature program

Figs 1–4 also include *n*-alkanes used for calculation of index values Mean index values and standard deviations obtained from at least ten measurements are given in Table I. All the amino acids gave symmetrical peaks on this column, providing accurate determination of the index values Standard deviations varying from 0 03 to 0 12 depending on individual compounds were achieved. Temperature programming yielded slightly higher standard deviations for some amino acids

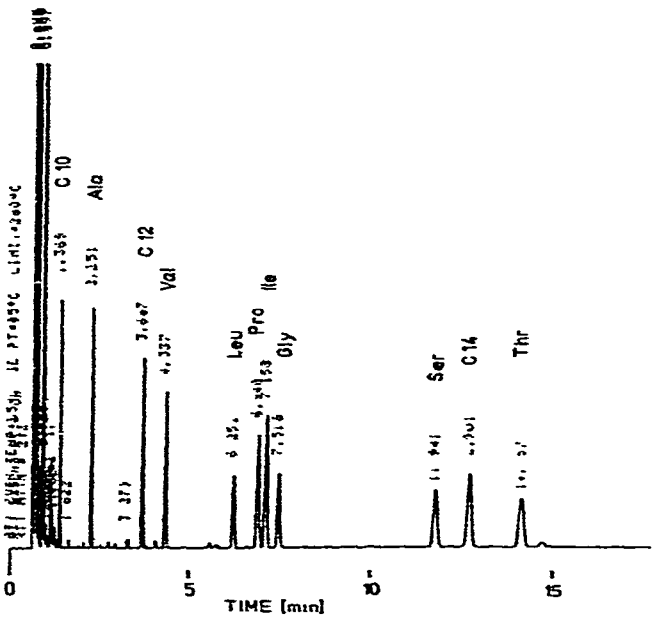


Fig. 1. Separation of some trimethylsilylated amino acids. Column, fused-silica SE-54, 12 m × 0.2 mm I.D.; temperature 85°C. For other column details see Experimental.

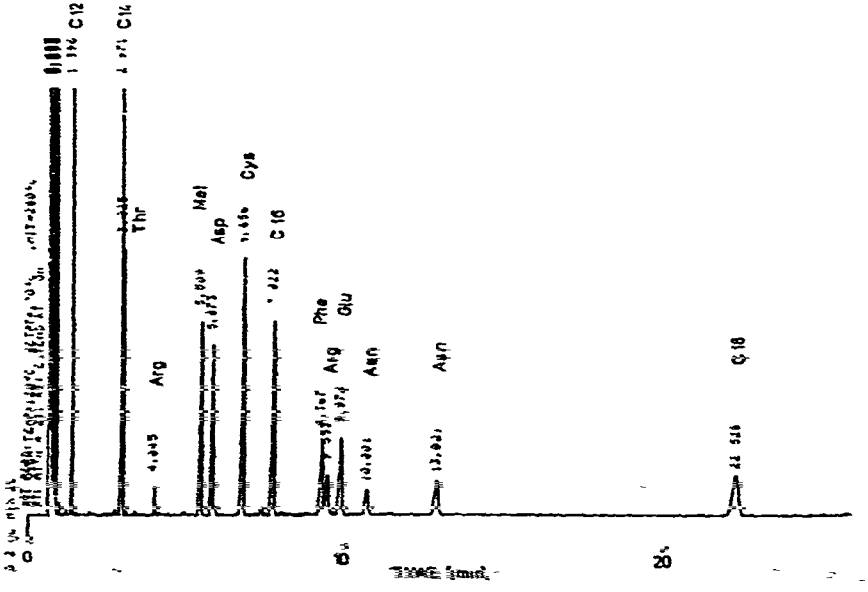


Fig. 2. Separation of some trimethylsilylated amino acids. Column details as in Fig. 1 except temperature 128°C.

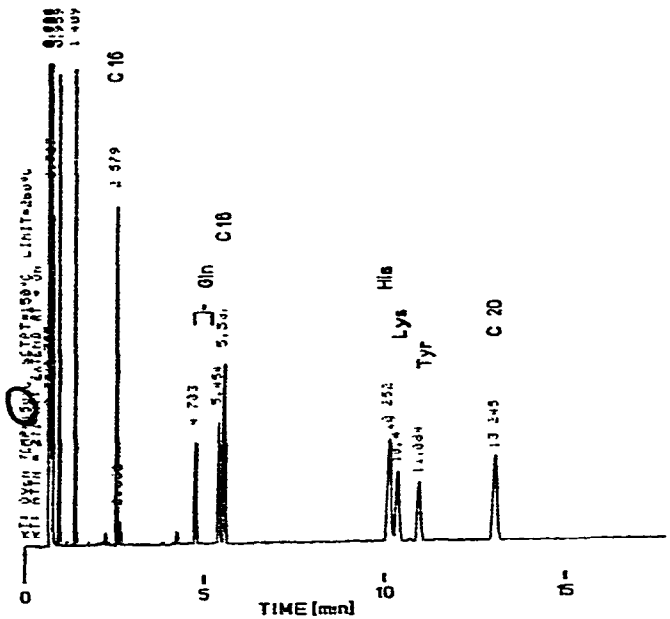


Fig 3 Separation of some trimethylsilylated amino acids Column details as in Fig 1 except temperature 150°C

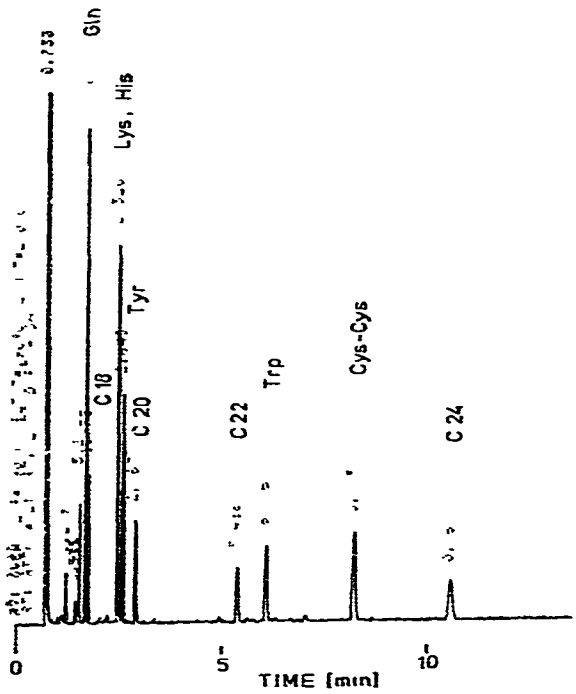


Fig. 4 Separation of some trimethylsilylated amino acids. Column details as in Fig 1 except temperature 190°C.

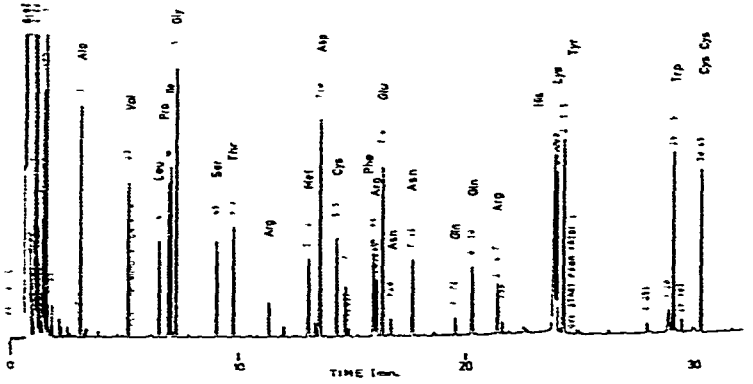


Fig. 5 Separation of trimethylsilylated amino acids Column details as in Fig 1 except temperature programmed at 4°C/min from 70°C to 170°C, then at 8°C/min from 170°C to 250°C

TABLE I

KOVÁTS' INDICES (*I*) (MEAN ± STANDARD DEVIATION) OF AMINO ACIDS ON SE-54 COLUMN

Amino acid	$I_{85°C}$	$I_{130°C}$	$I_{150°C}$	$I_{190°C}$	*Temp program (Fig 5)*
Ala	1110 27 ± 0 07				1119.33 ± 0 07
Val	1227 77 ± 0 05				1234.50 ± 0 05
Leu	1287 56 ± 0 06				1299 65 ± 0 07
Pro	1304 19 ± 0 05				1316 04 ± 0 10
Ile	1309 25 ± 0 03				1320 60 ± 0 07
Gly	1316 87 ± 0 05				1328.30 ± 0 06
Ser	1388.23 ± 0 04				1388 61 ± 0 05
Thr		1404 11 ± 0 05			1416 30 ± 0 07
Arg		1467.67 ± 0 09			1479 89 ± 0 11
Met		1531 10 ± 0 04			1541 58 ± 0 06
Asp		1543 89 ± 0 04			1557 09 ± 0 06
Cys		1574.31 ± 0 07			1580 37 ± 0 06
Phe		1635.10 ± 0 09			1640 06 ± 0 13
Arg		1638 81 ± 0 10			1646 83 ± 0 06
Glu		1647 08 ± 0 07			1655 51 ± 0 09
Asn		1662.50 ± 0.11			1671.72 ± 0 10
Asn		1697.85 ± 0 06			1707.08 ± 0 06
Gln			1762.35 ± 0 11		1774 99 ± 0 11
Gln			1794 37 ± 0 11		1800
Arg			—		1848 24 ± 0.17
His			1942.25 ± 0.10	1941 81 ± 0 11	1947 08 ± 0 08
Lys			1947 43 ± 0.12	1941.81 ± 0.11	1952.97 ± 0.09
Tyr			1960 08 ± 0 06	1959.22 ± 0 05	1964 36 ± 0 07
Trp				2237.31 ± 0 10	2235 62 ± 0 20
Cys–Cys				2327.19 ± 0 09	2332.95 ± 0 17

Asn and Gln gave two peaks due to trimethylsilylation to different extents[23] (Figs. 2, 3 and 5) Three peaks of Arg (Fig 5) correspond to its decomposition products The third peak represents ornithine The other two decomposition products were not identified.

On SP-2100 column. SP-2100 is a methyl silicone phase whose polarity is slightly less than that of SE-54 (ref. 40)

Retention indices obtained on this column are given in Table II Figs 6–9 show the actual separations at different temperatures Separation of all the amino acids using a temperature program is demonstrated in Fig 10 These chromatograms also show the positions of *n*-alkanes used for index calculations

The elution order of the amino acids is the same as that on the SE-54 column, with one exception. Tyr elutes earlier than Lys (compare Figs 3 and 8, and Figs 5 and 10) On this column, Lys is completely separated from His with a difference of *ca.* 37 index units at 150°C (Fig 8 and Table II), whereas SE-54 column does not give a complete separation of these amino acids (Fig. 3 and Table I). On the other hand, a better separation of Tyr from Lys was obtained on SE-54 (*ca* 13 index units *versus ca* 4 index units at 150°C, Tables I and II, respectively, compare also Figs 3 and 8).

Two compounds, Trp and Cys–Cys, gave somewhat broad peaks on SP-2100 column (Figs. 9 and 10) All the other amino acids are represented by symmetrical peaks. A repeatability of 0 04–0.15 index units was achieved for most amino acids on this column (Table II) Higher standard deviations were obtained only for His, Trp (both 0 28) and Cys–Cys (1 00) This is probably due to the fact that these compounds

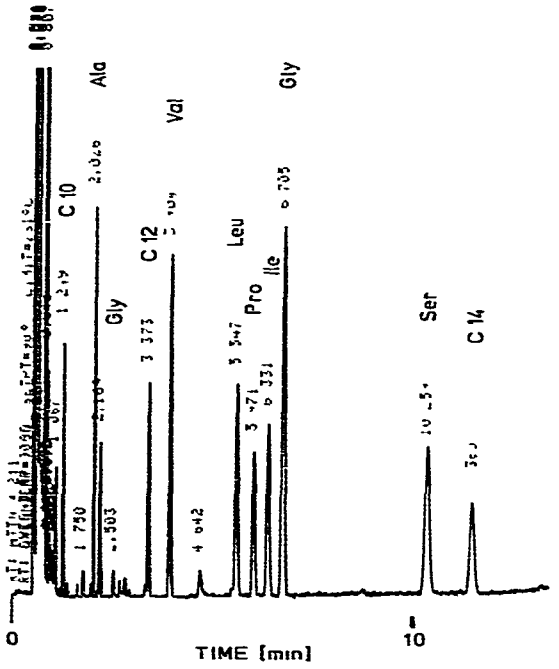


Fig. 6 Separation of some trimethylsilylated amino acids Column, fused-silica SP-2100 12 m × 0 2 mm I D ; temperature 90°C For other column details see Experimental

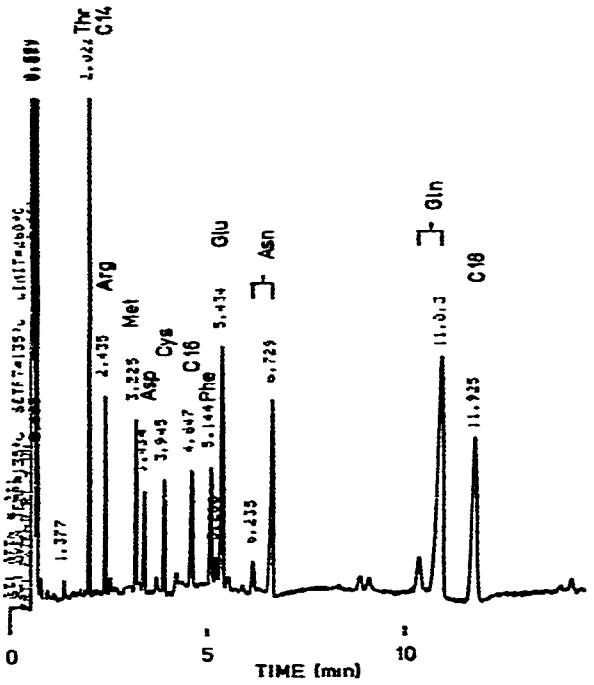


Fig. 7. Separation of some trimethylsilylated amino acids. Column details as in Fig. 6 except temperature 135°C.

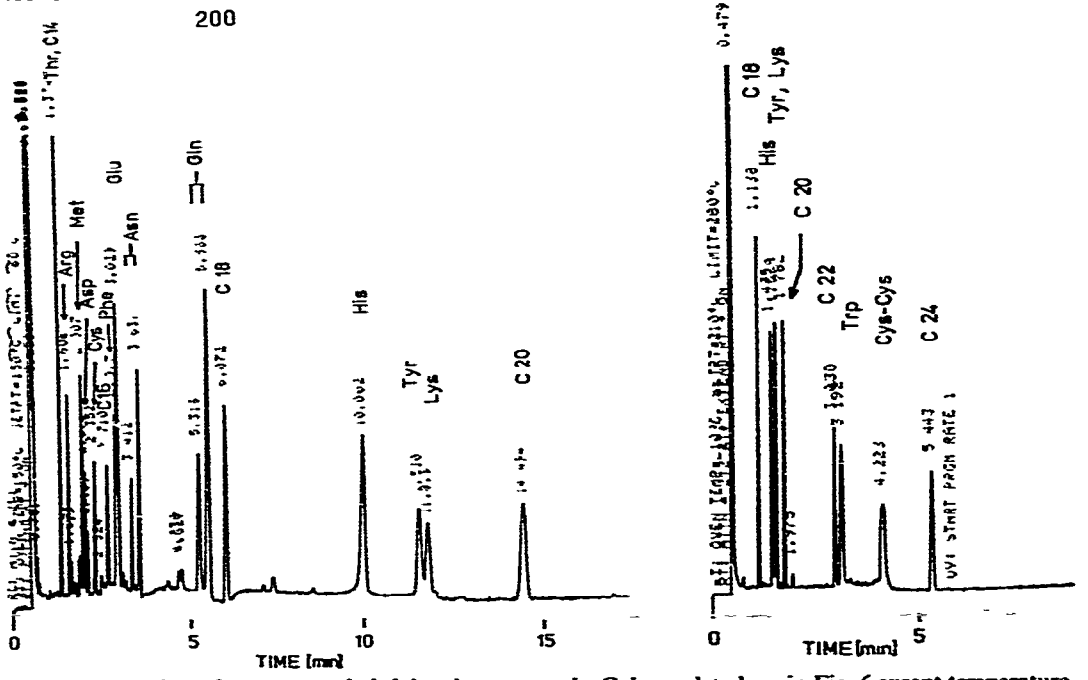


Fig. 8. Separation of some trimethylsilylated amino acids. Column details as in Fig. 6 except temperature 185°C.

Fig. 9. Separation of some trimethylsilylated amino acids. Column details as in Fig. 6 except temperature 210°C.

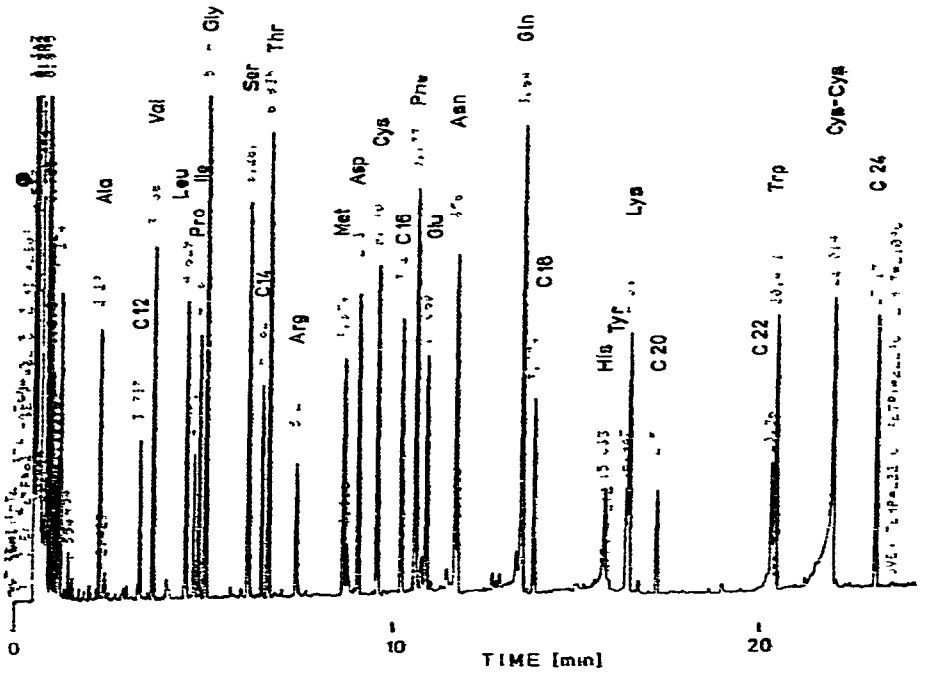


Fig. 10 Separation of trimethylsilylated amino acids. Column details as in Fig. 6 except temperature programmed at 6°C/min from 80°C to 250°C

TABLE II

KOVÁTS' INDICES (*I*) (MEAN ± STANDARD DEVIATION) OF AMINO ACIDS ON SP-2100 COLUMN

Amino acid	$I_{90°C}$	$I_{135°C}$	$I_{150°C}$	$I_{210°C}$	*Temp. program (Fig. 10)*
Ala	1106.11 ± 0.09				1113.37 ± 0.20
Val	1225.60 ± 0.07				1229.37 ± 0.13
Leu	1284.85 ± 0.05				1294.37 ± 0.08
Pro	1296.99 ± 0.05				1308.31 ± 0.14
Ile	1306.52 ± 0.06				1316.34 ± 0.05
Gly	1315.88 ± 0.04				1326.30 ± 0.20
Ser	1383.95 ± 0.11				1383.44 ± 0.04
Thr		1400	1400		1411.67 ± 0.14
Arg		1448.37 ± 0.15	1451.46 ± 0.07		1458.22 ± 0.03
Met		1516.78 ± 0.08	1518.48 ± 0.06		1527.51 ± 0.13
Asp		1531.51 ± 0.07	1528.16 ± 0.08		1547.12 ± 0.07
Cys		1563.23 ± 0.10	1562.44 ± 0.07		1571.22 ± 0.05
Phe		1622.47 ± 0.07	1624.82 ± 0.05		1624.42 ± 0.08
Glu		1634.25 ± 0.15	1629.40 ± 0.07		1642.34 ± 0.10
Asn		1664.12 ± 0.06	1660.13 ± 0.04		1674.29 ± 0.17
Asn		1680.55 ± 0.05	1676.14 ± 0.13		1689.41 ± 0.11
Gln		1773.65 ± 0.07	1768.75 ± 0.09		1776.96 ± 0.10
Gln		1784.82 ± 0.15	1780.34 ± 0.14		1784.27 ± 0.06
His			1916.60 ± 0.28	1921.05 ± 0.13	1920.38 ± 0.10
Tyr			1949.42 ± 0.09	1950.38 ± 0.08	1954.20 ± 0.08
Lys			1953.89 ± 0.07	1950.38 ± 0.08	1958.35 ± 0.14
Trp				2218.50 ± 0.28	2208.39 ± 0.27
Cys–Cys				2314.22 ± 1.00	2320.37 ± 0.41

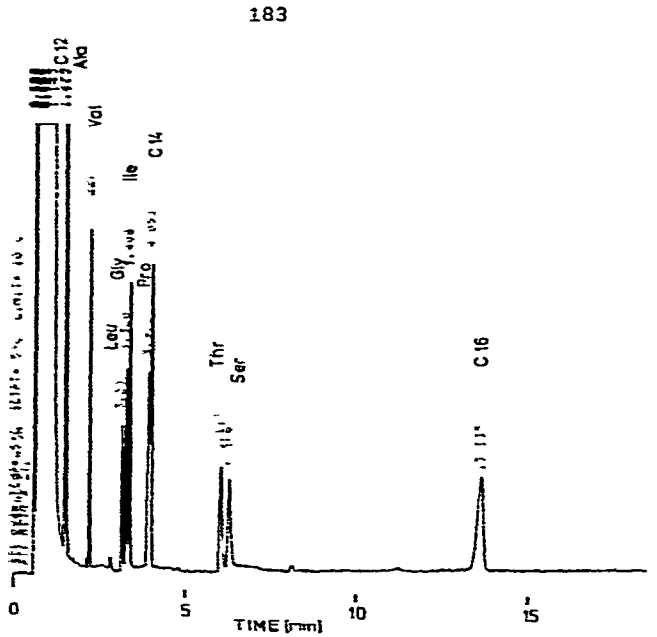
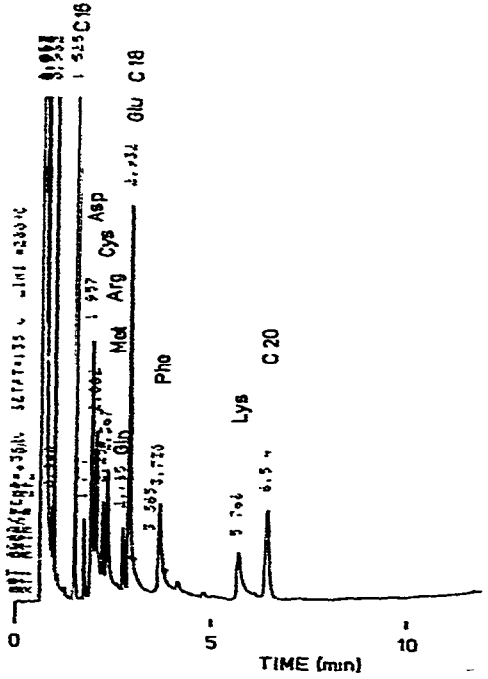


Fig. 11. Separation of some trimethylsilylated amino acids. Column, fused-silica Carbowax 20M 12 m × 0.2 mm I.D., temperature 75°C. For other column details see Experimental.



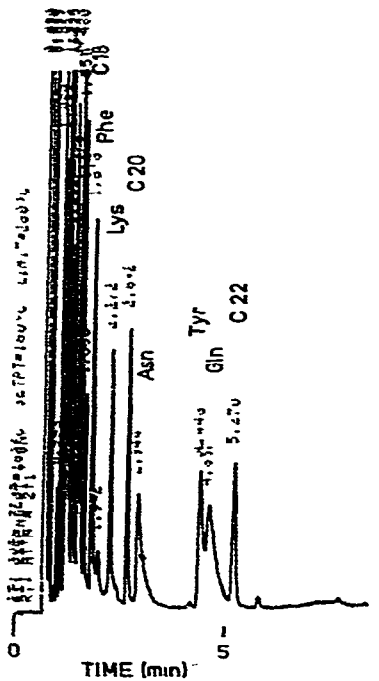


Fig. 12. Separation of some trimethylsilylated amino acids. Column details as in Fig. 11 except temperature 135°C.

Fig. 13. Separation of some trimethylsilylated amino acids. Column details as in Fig. 11 except temperature 160°C.

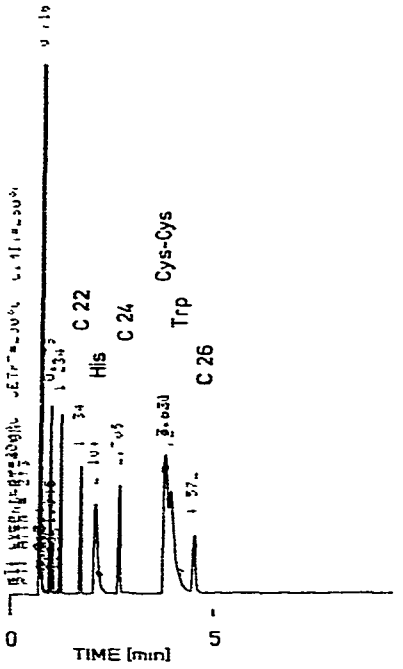


Fig 14 Separation of some trimethylsilylated amino acids Column details as in Fig 11 except temperature 200°C

TABLE III

KOVÁTS' INDICES (I) (MEAN ± STANDARD DEVIATION) OF AMINO ACIDS ON CARBOWAX 20M COLUMN

Amino acid	$I_{75\,C}$	$I_{135\,C}$	$I_{160\,C}$	$I_{200\,C}$
Ala	1206 10 ± 0 10			
Val	1289 72 ± 0 07			
Leu	1358 58 ± 0 12			
Gly	1364 90 ± 0 03			
Ile	1369 55 ± 0 05			
Pro	1396 61 ± 0 07			
Thr	1469 26 ± 0 09			
Ser	1476 09 ± 0 06			
Asn		1653 32 ± 0 15		
Asp		1683 85 ± 0 14		
Cys		1703 19 ± 0 06		
Arg		1726 72 ± 0 20		
Met		1741 00 ± 0 16		
Gln		1786 61 ± 0 28		
Glu		1800		
Phe		1863 65 ± 0 11	1863 83 ± 0 07	
Lys		1971 52 ± 0 16	1944 21 ± 0.10	
Asn			2028 71 ± 0 13	
Tyr			2151 59 ± 0 15	
Gln			2165 40 ± 0 20	
His				2293 85 ± 0 91
Cys–Cys				2537 21 ± 0 78
Trp				2548 52 ± 0 26

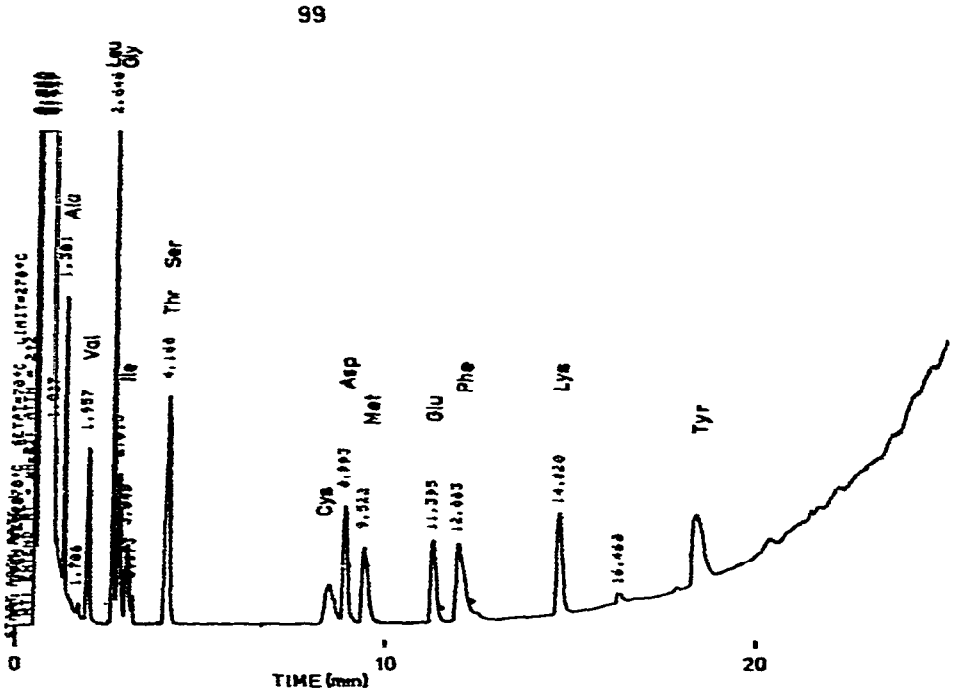


Fig. 15 Separation of some trimethylsilylated amino acids. Column details as in Fig. 11 except temperature programmed at 5°C/min from 70°C to 200°C.

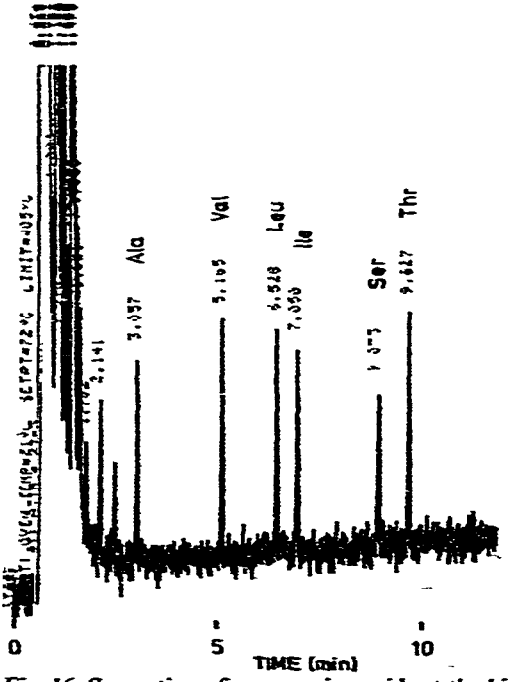


Fig. 16. Separation of some amino acids at the highest sensitivity setting of the detector. Column details as in Fig. 5. Amount injected for each amino acid was *ca.* 0 6 pmol.

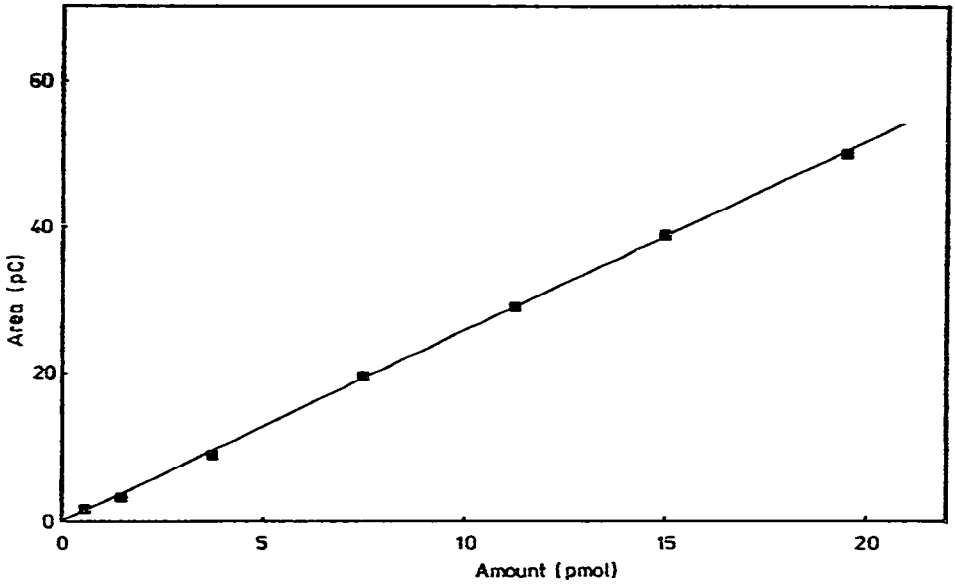


Fig 17 Linearity of the detector response for Ser Column details as in Fig 5

yielded broad and tailing peaks on this column. Standard deviations were slightly higher for some amino acids when temperature programming was used.

On Carbowax 20M. As expected, this highly polar column has a totally different selectivity from the other two columns toward trimethylsilyl (TMS) derivatives of amino acids as Figs 11–14 and Table III clearly demonstrate The majority of the amino acids gave "tailing" peaks. As mentioned above, symmetrical peaks are required for accurate determination of index values. Nevertheless, index values of the amino acids separated on this column were also calculated and tabulated (Table III)

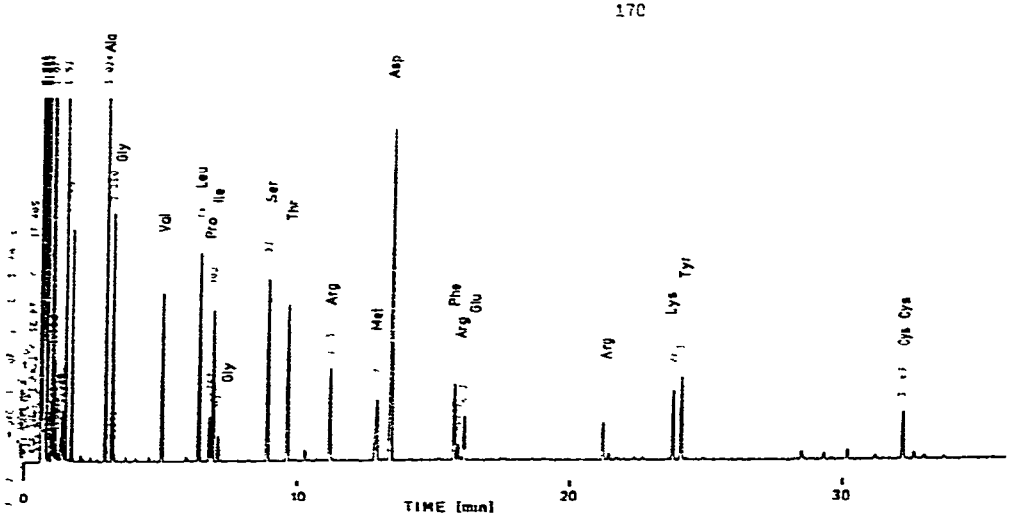


Fig. 18 Separation of the HCl-hydrolyzate of lysozyme Column details as in Fig. 1 except temperature programmed at 4°C/min from 70°C to 250°C

An additional column with a different polarity and selectivity can, of course, be very useful for identification purposes Standard deviations were higher on this column than those on SE-54 and SP-2100 columns. Fig. 15 shows the separation of some amino acids using a temperature program

Sensitivity

The method described here provides a high sensitivity. Fig. 16 shows the separation of six amino acids at the highest sensitivity setting of the detector. Each peak represents *ca.* 0.6 pmol of an amino acid. A signal-to-noise ratio of 2 (minimum detection limit) corresponds to *ca.* 0.1 pmol. Detector response for all amino acids was found to be linear from 0 1 to 20 pmol injected as shown in Fig 17 for Ser

CONCLUSIONS

As the results above show, the SE-54 column is best suitable for separation of TMS derivatives of the amino acids among the columns tested here All the amino acids including the most troublesome ones, such as Arg, His, Lys, Trp and Cys–Cys, gave symmetrical peaks. Also, the best reproducibility of the index values was achieved on this column. The superior performance of the SE-54 column in comparison to the SP-2100 column may be due to the type of column deactivation (see Experimental) or simply to the slightly higher polarity of the SE-54 stationary phase[40].

In addition, we recommend the use of short (12 m), narrow-bore (0.2 mm I D) fused-silica capillary columns for GC analysis of trimethylsilylated amino acids. According to our experience, TMS derivatives of amino acids, particularly those with long retention times, tend to decompose on longer (25 m) columns. This is particularly potentiated for troublesome amino acids such as Arg, His, Lys, Trp and Cys–Cys. These compounds decompose either partly or completely on 25-m SE-54 and SP-2100 columns. No peaks were obtained on a 25-m Carbowax 20M column

The usefulness of the methodology described here is clearly demonstrated in Fig 18 where the separation of the HCl-hydrolyzate of lysozyme is shown. Because of its excellent resolving power and very high sensitivity, this method could be well applied to amino acid analysis of peptides and proteins

REFERENCES

1 K Blau, in H. A. Szymanski (Editor), *Biomedical Applications of Gas Chromatography*, Vol 2, Plenum, New York, 1968 p 1.
2 P Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139
3 K Blau, in J M Rattenbury (Editor), *Amino Acid Analysis*, Wiley, New York, 1981, p 48
4 C. Zomzely, G. Marco and E. Emery *Anal Chem.*, 34 (1962) 414
5 F. E. Kaiser, C. W Gehrke, R. W. Zumwalt and K. C. Kuo, *J Chromatogr.*, 94 (1974) 113
6 C W Moss, M. A. Lambert and F. J Diaz, *J Chromatogr* 60 (1971) 134
7 J C. Cavadore, G Nota, G Prota and A. Previero, *Anal Biochem.*, 60 (1974) 608
8 M. Makita, S Yamamoto and M Kōno, *J. Chromatogr.*, 120 (1976) 129
9 C F. Poole and M Verzele, *J Chromatogr.*, 150 (1978) 439
10 S. L. MacKenzie and D Tenaschuk, *J. Chromatogr.*, 171 (1979) 195
11 J Desgres, D Boisson and P. Padieu, *J. Chromatogr.*, 162 (1979) 133
12 R. F Adams, F. L. Vandemark and G J. Schmidt, *J Chromatogr Sci.*, 15 (1977) 63
13 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 167 (1978) 187.
14 J J Pisano, T. J Bronzert and H. B Brewer, Jr., *Anal Biochem.*, 45 (1972) 43.

15 Z. Deyl, *J Chromatogr* 127 (1976) 91
16 K Rühlmann and W Giesecke, *Angew Chem* 73 (1961) 113
17 E D Smith and H Sheppard Jr *Nature (London)* 208 (1965) 878
18 D L Stalling, C W Gehrke and R W Zumwalt *Biochem Biophys Res Commun*, 31 (1968) 616
19 C W Gehrke, H Nakamoto and R. W Zumwalt, *J Chromatogr* 45 (1969) 24
20 K Bergstrom, J Gurtler and R Blomstrand *Anal Biochem*, 34 (1970) 74
21 C W Gehrke and K Leimer *J Chromatogr* 53 (1970) 201
22 R W Zumwalt K Kuo and C W Gehrke, *J Chromatogr* 57 (1971) 193
23 C W Gehrke and K Leimer *J Chromatogr* 57 (1971) 219
24 G Schomburg H Husmann and F Weeke *J Chromatogr* 99 (1974) 63
25 G Schomburg and H Husmann *Chromatographia* 8 (1975) 517
26 M Novotny, *Anal Chem*, 50 (1978) 16A
27 R R Freeman *High Resolution Gas Chromatography* Hewlett-Packard Palo Alto CA 1979
28 J Johnson J Eyem and S Sjoquist *Anal Biochem* 51 (1973) 204
29 R J Pearce *J Chromatogr* 136 (1977) 113
30 H Frank G J Nicholson and E Bayer *J Chromatogr Sci* 15 (1977) 174
31 I Abe T Kohno and S Musha *Chromatographia* 11 (1978) 393
32 J J Pisano *Methods Enzymol* 25 (1972) 27
33 W Jennings *Gas Chromatography with Glass Capillary Columns* Academic Press New York 1980 p 231
34 H Jaeger, H Frank H U Klor and H Ditschuneit in W Jennings (Editor) *Glass Capillary Gas Chromatography —The Applications* Marcel Dekker New York 1980
35 E Kovats *Adv in Chromatogr* 1 (1965) 229
36 R D Dandeneau and E H Zerenner *J High Resolut Chromatogr Chromatogr Commun* 2 (1979) 351
37 G Schomburg and G Dielmann, *J Chromatogr Sci* 11 (1973) 151
38 L S Ettre *Chromatographia* 6 (1973) 489
39 L S Ettre *Chromatographia* 7 (1974) 39
40 W O McReynolds, *J Chromatogr Sci* 8 (1970) 685