Gas-Liquid Chromatography of Trifluoroacetylated Amino Acid Methyl Esters

By A. DARBRE and A. ISLAM

Department of Biochemistry, King's College, University of London

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The gas-liquid chromatography of amino acids has for some years promised to become a useful method for the analytical chemist. Extensive reviews deal with the various methods adopted by workers to convert the amino acids into suitable volatile derivatives and the techniques involved in obtaining a separation of these derivatives by means of gas-liquid chromatography (Potteau, 1965; Weinstein, 1966; McBride & Klingman, 1967; Blau, 1968). Principally because of the ease of preparation and universal application to all amino acids, the method most widely adopted has been to convert the amino acids into the trifluoroacetylated ester derivatives. The use of trifluoroacetic anhydride for application to amino acids was first introduced by Weygand & Csendes (1952), and Bayer (1958) first showed that TFA*-amino acid methyl esters were suitable for gas-liquid chromatography.

The TFA-amino acid methyl esters are the most volatile derivatives of this class, and because of the danger of losses when carrying out quantitative work Darbre & Blau (1965) worked with the *n*-pentyl esters and Lamkin & Gehrke (1965) with the *n*-butyl esters. Such losses may be prevented by using the method described below. An important advantage in the use of the methyl esters is that chromatography may be effected at a lower temperature than when the higher esters are used, and this is particularly important for the least volatile derivatives. Lower temperatures reduce column bleed and with it 'noise', base-line drift and the risk of breakdown of the derivatives on the column. Also, the methyl esters of the amino acids are easily prepared in a single-step chemical operation. There are difficulties in preparing the esters of higher alcohols. Thus in preparing the TFA-amino acid *n*-butyl esters Gehrke, Lamkin, Stalling & Shahrokhi (1965) found it best to prepare the methyl esters before converting these into the butyl esters.

The usefulness of isothermal conditions for the separation of volatile amino acid derivatives is limited, as shown by Makisumi & Saroff (1965) for the TFA-amino acid methyl esters and by Darbre & Blau (1967) for the TFA-amino acid *n*-pentyl esters. It is not usually practical to separate at any single temperature more than eight or nine amino acid

derivatives. This is because of the limitations imposed by increasing peak width with increasing retention time as shown on the recorder chart and also the wide range of volatilities involved between the various derivatives. The use of temperature programming has enabled several groups of workers to separate 15 or more TFA-amino acid methyl esters in a single run (Ikekawa, 1963; Cruickshank & Sheehan, 1964; Hagen & Black, 1965), but these workers used polar stationary phases of polyester type. Such phases have been implicated in the breakdown of trifluoroacetylated esters of cysteine and hydroxy amino acids during gas-liquid chromatography (Darbre & Blau, 1966). For this reason in searching for a suitable stationary phase we have restricted ourselves not only to silicone-type phases but also those that have good thermal stability. No single phase was found to give all the separations required and a mixed phase was developed.

Materials and methods. Stage 1. The amino acids (total 2 mg.) are placed in a B 14 test tube with 2 ml. of dry methanol and placed in an oil bath at $70 \pm 2^\circ$ with slow continuous bubbling of dry HCl through a capillary tube for 30 min. The alcohol is removed with the rotary evaporator first with a water pump, to prevent losses by 'bumping', then with an oil pump to evaporate the mixture to dryness.

Stage 2a. Trifluoroacetic anhydride in excess (0.2 ml.) is then added and the stoppered tube is kept at room temperature for at least 30 min. The excess of trifluoroacetic anhydride is removed with the rotary evaporator by immersing the B 14 tube in an ice bath and vacuum applied with the oil pump for no longer than 4 min. If a longer time is used there is danger of some loss of the more volatile derivatives. The residue is taken up in methylene dichloride (100 μ l.) and a sample injected on to the column with a 1 μ l. syringe. The method described is satisfactory for most amino acids. Histidine, however, gives a very small yield. Arginine does not give a volatile derivative (see Stalling & Gehrke, 1966; Darbre & Blau, 1967). In order to acylate arginine it is necessary to proceed as described in stage 2b.

Stage 2b. The residue from stage 1 is taken up in methanol and transferred to a small Pyrex tube with a bulb at the end. The methanol is removed, either

* Abbreviation: TFA, trifluoroacetyl.

by standing the tube in an oven at 70–80° or by placing it in a B14 tube and using the rotary evaporator. Trifluoroacetic anhydride (0.2ml.) is added and the tube is sealed and placed in the oven at 140° for 10min. After cooling, the tube is cracked open and the excess of trifluoroacetic anhydride removed by placing it in a B14 tube on the rotary evaporator. The same precautions against losses of the more volatile amino acids are taken as described in stage 2a. The residual derivatives are dissolved in methylene dichloride. This method is successful with all protein amino acids, except histidine, which still gives a low yield (about 5%).

The stationary phase consists of a mixture of XE 60, QF-1 and MS 200 (46%, 27% and 27% respectively by weight). XE 60 was obtained from Applied Science Laboratories Inc. (State College, Pa., U.S.A.), DC-QF-1-0065/10 000 was from Midland Silicones Ltd. (Reading, Berk., U.S.A.) and silicone fluid MS 200 was from Hopkin and Williams Ltd. (Chadwell Heath, Essex). It is best to make up the mixed phase as a 1% stock solution in butan-2-one. Glass columns (3.25m. × 2.5mm. internal diam.) are packed with deactivated Diatoport S (80–100 mesh) coated with 2.5% (w/w) of mixed stationary phase. The gas chromatograph used is a MicroTek MT 220 fitted with dual flame ionization detectors. The columns are normally conditioned at 230° overnight. However, the column is usable

after about 2hr. at this temperature. A good column should give 500 plates/ft. with a 1μg. injection of TFA-leucine methyl ester at 100° giving a retention time of about 15min. We have achieved 800 plates/ft.

Results and discussion. Fig. 1 shows the separation of 22 amino acids. A number of small peaks due to TFA artifacts are usually seen after the solvent peak. All tryptophan samples so far tested have given a small additional peak, which has not been identified. This appears before lysine (peak 20 in Fig. 1). However, Ikekawa (1963), Cruickshank & Sheehan (1964) and Makisumi & Saroff (1965) show one peak only for the tryptophan methyl ester. Ornithine is not obtained as a breakdown product from arginine (see Stalling & Gehrke, 1966); if it were present it would appear after tyrosine. The small peak after phenylalanine corresponds to the mono-TFA-homoserine methyl ester. This is only seen when some di-TFA-homoserine has been hydrolysed by traces of moisture. Histidine only shows on a chromatogram when a large excess is taken. Two peaks are given; the first one comes off the column just before *E* and is about 10% of the size of the second peak, which appears after *G* in Fig. 1. They may represent the di-TFA- and the mono-TFA-histidine methyl ester respectively.

The programming of the chromatographic separation is capable of considerable variation. The

Fig. 1. Separation of TFA-amino acid methyl esters by gas-liquid chromatography. Instrument details are as follows: MicroTek MT220 fitted with dual flame ionization detectors; glass tubing (3.25m. × 2.5mm. internal diam.) packed with 2.5% (w/w) mixed stationary phase XE 60, QF-1 and MS 200 in the proportions 46%, 27% and 27% respectively (by weight) on deactivated Diatoport S (80–100 mesh); inlet heater block temperature 230°; detector block temperature 250°; nitrogen gas flow 14 ml./min.; sample size 0.4 μl.; attenuation $10^2 \times 8$, except for the peaks 19, 20 and 23 as indicated. Programming details are as follows: *A*, initial temperature 100°, programmed at 1°/min.; *B*, programmed at 3°/min.; *C*, held at 140°; *D*, programmed at 5°/min. to 210°; *E*, held at 210°; *F*, programmed at 5°/min. to 230°; *G*, held at 230°. Peaks: 1, solvent; 2, α-aminoisobutyric acid; 3, alanine; 4, valine; 5, glycine; 6, isoleucine; 7, threonine; 8, leucine; 9, norleucine; 10, serine; 11, proline; 12, aspartic acid; 13, cysteine; 14, homoserine; 15, hydroxyproline; 16, methionine; 17, glutamic acid; 18, phenylalanine; 19, tyrosine; 20, lysine; 21, tryptophan; 22, arginine; 23, cystine. Each peak represents 10 mμmoles of original amino acid taken, except 5 mμmoles for proline, hydroxyproline, phenylalanine, tyrosine and tryptophan.

total chromatographing time can be reduced considerably by using faster programming rates, but some loss of resolution occurs, particularly between isoleucine and threonine and between methionine and glutamic acid.

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