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GAS–LIQUID CHROMATOGRAPHY OF TRIFLUOROACETYLATED AMINO ACID METHYL ESTERS

DETERMINATION OF THEIR MOLAR RESPONSES WITH THE FLAME IONIZATION DETECTOR

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SUMMARY

The molar responses of trifluoroacetylated amino acid methyl esters were determined with the flame ionization detector. Linearity of response with carbon number was shown for amino acids possessing the same reactive groups. Non-linearity of response could be used to show either incomplete derivatization or breakdown of the derivative during gas chromatography. The non-effective carbon numbers of the compounds were calculated and shown to be due to specific groups in the molecules.

INTRODUCTION

The response of hydrocarbons in the flame ionization detector (FID) is generally accepted as being almost directly proportional to the number of carbon atoms in the molecule and independent of molecular structure. PERKINS *et al.*[1] noted excellent linearity of response between the relative responses per mole and carbon number for a variety of hydrocarbons and compounds containing alcoholic, ether, ester and amine groups with a maximum number of twelve carbon atoms in any molecule. Lower responses were obtained for compounds containing certain functional groups when compared with hydrocarbons possessing the same number of carbons, but they concluded that the FID could be used for quantitative analysis, if appropriate calibration factors were employed. Thus if the calibration factor for a class of compound were determined, this could then be subsequently applied to all members of that class.

In earlier papers[2,3] the qualitative separation of the protein amino acids as their trifluoroacetylated methyl ester derivatives was described using gas–liquid chromatography with the FID. Although this detector gives a linear response over a wide range of concentrations (about 10^7) (ref. 4), it does not give a quantitative

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result in absolute terms. Thus when studies were made on the quantitative derivatization of the amino acids, a method was required for correlating the peak area of the derivative observed on the chromatogram with the absolute amount injected and which eventually reached the FID after passing through the column.

The gas density balance (GDB) detector[5] was used to solve this problem. Despite its low level of sensitivity (about 1 μg) it had the great advantage of responding quantitatively to volatile compounds which were present in the effluent carrier gas emerging from the column. Gas chromatography (GC) of samples under identical conditions in two different instruments, one fitted with a GDB detector and the other with an FID, was carried out. In this way the quantitative amount of amino acid derivative determined by means of the GDB detector was used to determine the molar response in coulombs per mole obtained with the FID.

EXPERIMENTAL

Apparatus

A gas chromatograph Pye series 104, Model 24, fitted with dual FID (W.G. Pye Ltd., Cambridge, Great Britain) was used with a Honeywell 10 mV 1 sec Electronik strip chart recorder (Honeywell Controls Ltd., Greenford, Middx., Great Britain). Integration of the peak areas was carried out with the Kent Chromalog 2 electronic integrator (Kent Instruments Ltd., Luton, Beds., Great Britain).

The D6 gas chromatograph with the Martin GDB detector[5] (Griffin & George Ltd., Alperton, Middx., Great Britain) was modified in order to have sample injection and chromatography conditions similar to those obtaining in the Pye chromatograph. The D6 inlet system was replaced by a Pye injection head with inlet heater. Direct on-column injection was then possible with the same micro-syringe in both instruments. A pin-valve flow controller was introduced for the carrier gas, which after passing through the U-shaped column was led by stainless-steel capillary tubing (1 mm I.D.) to the detector in the bottom of the oven. A Honeywell–Brown Elektronic 1 mV 1 sec recorder was used in conjunction with the Instron digital mechanopotentiometric integrator (Instron Ltd., High Wycombe, Bucks., Great Britain).

Nitrogen (99.9% "white spot" from British Oxygen Co. Ltd., and "high purity oxygen-free" from Air Products Ltd.) was used as carrier gas.

Materials

Chemicals and stationary phases were obtained as follows: N-trifluoroacetyl (TFA) norleucine methyl ester was prepared by the method of MAKISUMI AND SAROFF[6] and was also later obtained from BDH Chemicals Ltd. (Poole, Dorset, Great Britain). Hydrocarbons and amino acids were obtained from BDH Chemicals Ltd. except biphenyl (Hopkin & Williams, Chadwell Heath, Essex, Great Britain), isoleucine (*allo*-free) (Calbiochem Ltd., London W1H 1AS, Great Britain), 2,3-diaminopropionic acid mono-HCl (Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, Great Britain), 2,4-diaminobutyric acid di-HCl and 2,6-diaminopimelic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks, Great Britain), HP Chromosorb W, 80–100 mesh (Perkin-Elmer Ltd., Beaconsfield, Bucks., Great Britain), silicone gum XE 60 (Applied Science Laboratories Inc., State College, Pa., U.S.A.), silicone oil MS-200, 100 cS (Hopkin & Williams Ltd.), silicone (fluoro)-FS 1265 (QF-1) (F & M Scientific Corporation, Avondale, Pa 19311, U.S.A.).

Preparation of columns

The stationary phase consisted of three mixed silicones[2] and the coating of the support material in a flat-bottomed dish was previously described[3]. The glass columns for both instruments were similar, approximately 3.25 m × 2.5 mm I.D.; they were coiled in the Pye chromatograph and U-shape in the Griffin and George chromatograph. Details were given for pre-treating both the column and glass thread and packing the column[7].

Preparation of derivatives

The weighed amount of amino acid (2 mg) was placed in a test tube with a B 14 ground glass joint. About 2 ml of 4 *M* dry methanolic HCl were added and the stoppered tube placed in an oven at 70 ± 2° for 90 min. Methionine was esterified for 30 min only to give an optimum yield. The use of 4 *M* methanolic HCl evolved from quantitative studies (to be published). This was conveniently prepared by adding slowly the required amount of acetyl chloride to dry methanol cooled in an acetone–solid carbon dioxide bath. After incubation the contents of the tube were taken to dryness with the rotary evaporator first with a water pump to prevent losses by "bumping", then with an oil pump. Trifluoroacetylation was subsequently carried out by adding 0.1 ml of TFA anhydride and allowing the stoppered tube to stand at room temperature for 20 to 30 min. Excess reagent was removed with the rotary evaporator at room temperature. When the more volatile derivatives were present in the solution, evaporation was carried out as previously described, with the tube immersed in an ice-water bath at 0° to prevent losses due to excessive volatility[2,3].

RESULTS

In order to determine the quantitative response of the GDB detector under the same isothermal conditions to be used for amino acids, standard mixtures of biphenyl, bibenzyl, and phenylbenzoate in butan-2-one were chromatographed isothermally at various temperatures ranging from 120 to 150°. The peaks were well resolved and peak area values obtained with the Instron integrator. Using the formula of PHILLIPS AND TIMMS[8] the following relationship could be obtained

$$q_u = q_s \cdot \frac{A_u}{A_s} \cdot \frac{M_s - m}{M_s} \cdot \frac{M_u}{M_u - m}$$

where

q_u = weight of the vapour of the compound having molecular weight M_u

q_s = weight of the vapour of the internal standard having molecular weight M_s

A_u and A_s = the respective integral responses or peak areas

m = molecular weight of the carrier gas.

Injections were made with a 10-μl microsyringe and the total volume injected was obtained by determining the volume remaining in the needle[9]. Between 40 and 200 μg of each compound were injected. In one experiment, using biphenyl as the standard, recoveries for six replicate determinations gave a mean of 97.5 % bibenzyl (coefficient of variation 1.96 %) and 97.2 % phenylbenzoate (coefficient of variation 1.09 %). These and similar results indicated that it was possible to estimate compounds within experimental error of ± 3 %.

Norleucine was selected for use as a non-protein amino acid standard because it was well separated from protein amino acids on the mixed silicone stationary phase. A standard solution of pure N-TFA norleucine methyl ester was prepared by weighing and dissolving in dry butan-2-one containing biphenyl. For comparison, a known amount of norleucine was converted to the N-TFA methyl ester derivative by the two-stage process described in the EXPERIMENTAL section, and made up to a known volume with butan-2-one containing biphenyl. Aliquots of these two solutions were chromatographed under identical conditions. The results are given in Table I, where the percentage recoveries range from 98.5 to 100.6%, with coefficients of variation from 0.59 to 1.51%.

TABLE I

YIELD OF TFA NORLEUCINE METHYL ESTER MEASURED AFTER GAS CHROMATOGRAPHY USING THE GAS DENSITY BALANCE DETECTOR

The TFA norleucine methyl ester was chromatographed with biphenyl internal standard on a glass column (3.25 m × 2.5 mm I.D.) packed with HP Chromosorb W, 80–100 mesh, coated with 2.5% (w/w) mixed stationary phase XE-60, QF-1 and MS200 in the proportions 46%, 27% and 27%, respectively (by weight). Column temperature 130°. Nitrogen carrier gas flow 30 ml/min through column, and 30 ml/min to reference side of detector. Attenuation: × 2 or × 5. Sample sizes: 5–10 μl.
Six determinations were made with each sample.

Starting product	*Mean % yield*	*% Coefficient of variation*
Norleucine	(a) 100.5	1.51
	(b) 99.4	1.42
TFA norleucine methyl ester	(a) 98.5	1.30
	(b) 100.6	0.59

Using as nearly as possible the identical conditions of GC it was possible to directly compare the response obtained with the FID. Because of the difference in sensitivity between the two detectors, 10-μl aliquots were injected for the GDB and 0.5-μl for the FID. From the absolute yield obtained with the GDB it was possible to determine the molar response given by the FID, *i.e.* the quantity of electricity produced by one mole of compound[10]. For N-TFA norleucine methyl ester the molar response was 1.26 C/mole (Table II). Derivatives of other amino acids were prepared under conditions which were established to give maximum yields. The amino acids were studied either singly or in mixtures with the FID using norleucine and biphenyl as internal standards. Where similar retention times made this impossible, biphenyl was replaced by bibenzyl or *n*-hexadecane. It was essential for constancy of response with the FID to control carefully all the conditions of chromatography particularly the air, hydrogen and carrier gas flows. Variations in atmospheric pressure[11] were not recorded but controls were carried out daily with a hydrocarbon mixture and N-TFA norleucine methyl ester.

In Table II, column 1 all the compounds studied are listed in groups according to their structure and their corresponding molar response factors in coulombs per mole are given in column 2. GDB determinations were made only with alanine,

TABLE II

MOLAR RESPONSES OF HYDROCARBONS AND TFA AMINO ACID METHYL ESTERS WITH THE FLAME IONIZATION DETECTOR

Molar response factors and % coefficients of variation were obtained from eight replicates for methionine, proline, valine, isoleucine, leucine and cysteine and ten for all other compounds. Norleucine was the primary internal standard. Pye gas chromatograph with dual FID. Stationary phase and column as in Table I. Column temperatures between 80 and 180°. Nitrogen carrier gas flow 30 ml/min. Attenuation between 5.0×10^{-10} and 2.0×10^{-9}A for f.s.d. Sample sizes between 0.5 and 2.0 μl.

Compound	*Molar response factor (C/mole)*	*Coefficient of variation (%)*	*Number of carbon atoms*	*Effective carbon number*	*Corrected response (C/g atom carbon)*
(A) Hydrocarbons					
1 Biphenyl	2.64	0.95	12	12	0.220
2 Bibenzyl	3.14	1.27	14	14	0.224
3 *n*-Hexadecane	3.60	1.65	16	16	0.225
(B) Monoamino monocarboxylic acids (homologous series)					
4 Glycine	0.33	1.21	5	1.3	0.254
5 Alanine	0.57	1.25	6	2.3	0.248
6 α-Amino *n*-butyric acid	0.83	1.29	7	3.3	0.252
7 Norvaline	1.03	0.82	8	4.3	0.240
8 Norleucine	1.20	—	9	5.3	0.238
9 α-Amino *n*-octanoic acid	1.78	0.98	11	7.3	0.244
(C) Monoamino dicarboxylic acids (homologous series)					
10 Aspartic acid	0.78	0.78	8	3.4	0.229
11 Glutamic acid	1.00	1.44	9	4.4	0.227
12 α-Amino adipic acid	1.18	0.98	10	5.4	0.219
13 α-Amino pimelic acid	1.46	0.83	11	6.4	0.228
14 α-Amino suberic acid	1.66	1.10	12	7.4	0.224
(D) Diamino monocarboxylic acids (homologous series)					
15 2,3-Diamino propionic acid	0.65	0.99	8	2.7	0.241
16 2,4-Diamino butyric acid	0.84	0.61	9	3.7	0.227
17 Ornithine	1.14	0.78	10	4.7	0.243
18 Lysine	1.33	1.32	11	5.7	0.233
(E) Other amino acids					
19 Methionine	1.03	1.92	8	4.3	0.240
20 Proline	1.08	0.76	8	4.3	0.251
21 Valine	1.05	1.06	8	4.3	0.244
22 Isoleucine	1.29	1.66	9	5.3	0.243
23 Leucine	1.27	0.79	9	5.3	0.240
24 Phenylalanine	2.00	1.19	12	8.3	0.241
25 Cysteine	0.72	1.97	8	2.7	0.267
26 Serine	0.64	0.81	8	2.7	0.237
27 Threonine	0.86	1.20	9	3.7	0.232
28 Hydroxyproline	1.11	1.65	10	4.7	0.236
29 Tyrosine	1.78	1.40	14	8.7	0.205

valine, glycine, isoleucine and leucine and in each case the yield of derivative was within the range of error 100 ± 3 %. The FID response was more accurately reproducible and freshly-prepared amino acid derivatives were chromatographed and their molar responses determined using norleucine as the primary standard. The percentage coefficients of variation in Table II, column 3 were based on eight or ten replicate

determinations as shown. For each replicate a weighed sample of amino acid was taken through the two-stage process of derivatization and finally taken up in butan-2-one with internal standards for GC.

The hydrocarbons selected were those which gave retention times similar to those of the amino acid derivatives. They were easily available in pure form. Their molar response factors are plotted against the number of carbon atoms in the molecule (Fig. 1, line A). The regression equation[12] for the hydrocarbon line A passing

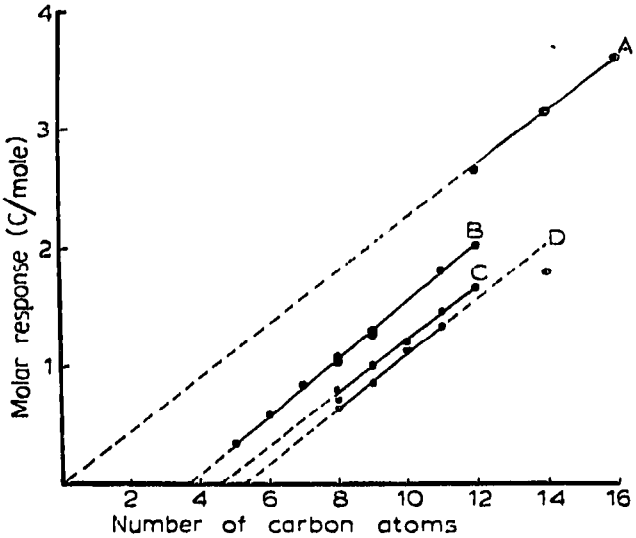

Fig. 1. Relationship between molar response and carbon number with the FID. Regression equations for hydrocarbons, line A, $y_1 = bx = 0.224x$; monoamino monocarboxylic acids, line B, $y_1 = 0.967 + 0.242\ (x - 7.667)$; monoamino dicarboxylic acids, line C, $y_1 = 1.216 + 0.220\ (x - 10)$; diamino monocarboxylic acids, line D, $y_1 = 0.990 + 0.234\ (x - 9.5)$. The numbers correspond with the compounds listed in Table II.

through the origin is represented by $y_1 = bx$, where y_1 is the estimated value in C/mole, $b = \Sigma xy/\Sigma x^2$, x is the carbon number and y is the molar response. The regression coefficient b has the value 0.224 C for each gram atom of carbon in the molecule.

The regression line B (Fig. 1) for the homologous series of monoamino monocarboxylic acids (Group B in Table II) is given by the regression equation $y_1 = a + bx$ where y_1, b and x are as before and a is the value of the ordinate where the line crosses the y axis. By extrapolation the regression line cuts the abscissa at the value for 3.68 carbon atoms and this value may be used as an estimate of the non-effective carbon number for this group of compounds. The monoamino dicarboxylic acids (Group C in Table II) give the regression line (Fig. 1, C) cutting the abscissa at 4.56 carbon atoms. The diamino monocarboxylic acids, listed in Table II, Group D, have the highest non-effective carbon number 5.27 as shown (Fig. 1, D). The other amino acids in Table II, Group E when plotted coincide closely with the linear plots in Fig. 1. Thus, compounds 19 to 24 give points which fall along line B for the monoamino monocarboxylic acids. Amongst the compounds 25 to 29 which possess two TFA groups and one ester group only cysteine and tyrosine deviate considerably from line D in Fig. 1. The corrected response values in the last column of Table II are calculated from the molar response factors and the corresponding effective carbon numbers. All the values lie within the range 0.254 (glycine) to 0.219 C/g atom carbon (α-amino adipic acid) except those for cysteine and tyrosine.

DISCUSSION

From the results in Table I it is seen that the conversion of norleucine to its N-TFA methyl ester derivative was quantitative ± 3 % as determined by means of the GDB detector. The derivative appeared to be completely stable under the conditions of GC used. Using the same samples under similar conditions with the FID the molar response could be measured. The molar response factors for the FID in Table II gave coefficients of variation which varied between 0.61 % for 2,4-diaminobutyric acid and 1.97 % for cysteine. These coefficients of variation indicated the overall reproducibility of amino acid derivatization, response of the FID and subsequent integration of the electrical charge collected. They also showed that if there was any breakdown of the compounds during GC due to the temperature conditions or to interaction with the column packing or the stationary phases then this occurred to a reproducible extent.

In calculating the regression line (Fig. 1, A) for the hydrocarbons it was assumed that this would lead to the origin. The relative molar response values of hydrocarbons are considered to be linearly dependent on the number of carbon atoms in the molecule[13–16]. However, variations from linearity have been shown[17–19]. PERKINS *et al.*[1] showed that the FID response was linear within experimental error with the regression line going back to the origin for alkanes, alkenes and aromatic compounds possessing up to 12 carbons in the molecule. The three hydrocarbons in Table II gave a mean response of 0.224 C/g atom carbon. This may be compared with values of 0.230 to 0.240 C/g atom carbon calculated from the figures given by STERNBERG *et al.*[20] for nine hydrocarbons possessing between one and eight carbons in the molecule. In his analysis of the molar responses given by the FID, ETTRE[17] pointed out that aromatic hydrocarbons and cycloparaffins give lower than the theoretically expected values. This may have occurred here for biphenyl, bibenzyl and *n*-hexadecane. We were unable to use hydrocarbons with lower carbon numbers for comparison as these were too volatile for chromatography under the same conditions as the amino acid derivatives.

The results for the homologous series of amino acids in Table II, Group B (Fig. 1, B) show linearity of response with the carbon number of the molecule. Because hydrocarbon compounds giving similar molar response values to these amino acid derivatives were not chromatographed, the extrapolated value of 3.7 carbon atoms (Fig. 1) was used as a measure of the reduction of response given by the FID, caused by the presence of both one methyl ester and one TFA group on the molecule. BOČEK AND JANÁK[21] reviewed studies made on the response of the FID to fundamental groups. Oxygenated aliphatic hydrocarbons[22] have been particularly well examined, but no report could be found on molecules having the TFA and ester groups reported here. Carbon in the C=O group had a non-effective carbon number equal to 1.0 (refs. 1, 20). Esters were shown by various workers to have a response relative to hydrocarbon effectively diminished by values of 0.45 (refs. 22, 23), 1.0 (ref. 1) and by 1.2 to 1.5 (ref. 20) carbon atom per molecule. Thus with the mono-TFA amino acid methyl esters a reduction of carbon number (–COO Me = 1.0) would leave a non-effective carbon number of 2.7 for the group $-NH\cdot CO\cdot CF_3$. PERKINS *et al.*[1] proposed a non-effective carbon value of 0.5 for aliphatic amines, whilst ACKMAN[24] suggested a value of 1.0 for some aliphatic amines, anilines and nitrogen-

containing heterocyclics. Also multiple halogen substitution on a single carbon atom was reported to lead to a diminished response with the FID[20]. The amino acids (Table II, Nos. 19–24) which carried the same reactive groups fell on or close to line B, Fig. 1. The response given by methionine was not reduced by the presence of the thio–ether bond, unlike oxygen ethers which showed a decrease in effective carbon number equal to 1.0 (refs. 1, 20, 22, 25) because their stability made ion formation less probable. This suggested that the thio–ether bond was not as stable in the flame as the oxygen–ether bond. Amino acid isomers showed a higher response with an increase in branching of the carbon chain in the order isoleucine>leucine>norleucine and valine>norvaline. This was also shown with hydrocarbon compounds[17].

The compounds in Table II, Group C (Fig. 1,C) each possess two ester groups and one TFA group and showed a total of 4.6 non-effective carbon atoms. If allowance is made for two ester groups each with one non-effective carbon the grouping $-NH\cdot CO\cdot CF_3$ would have a non-effective carbon number of 2.6 (*cf.* 2.7 for Group B).

In Table II, Group D (Fig. 1, D) the derivatives possess one ester and two TFA groups, with a non-effective carbon number 5.3. Making a reduction for the ester group this leaves a non-effective carbon number of 4.3 for two $-NH\cdot CO\cdot CF_3$ groups. In this connection, PERKINS *et al.*[1] showed that the presence of two amino groups did not alter the effective carbon number of compounds more than did a single amino group. Thus the presence of two $-NH\cdot CO\cdot CF_3$ groups did not lead to an additive effect. It is premature at this stage of our studies to calculate the effective carbon number for the group ($-CF_3$) by making a hypothetical reduction of, say, 0.7 for the presence of the imino group and 1.0 for the carbonyl group. Further FID studies with compounds possessing various combinations of these chemical groupings would be of interest. The hydroxy amino acids (Table II, Nos. 26–28) showed similar molar response values, thus indicating that –N-TFA and –O-TFA groups had similar effects with FID. It should be noted that cysteine (Table II, No. 25) with one –N-TFA and one –S-TFA group gave a higher response (0.267 C/g atom C) than those other compounds possessing two TFA groups, and in Fig. 1 falls above line D. This cannot be explained. Studies on molar responses for sulphur compounds have been mostly restricted to CS_2 and these showed little or no response with FID[1,20,25–29]. DOUGLAS AND SCHAEFER[30] showed that previous reports on the lack of response with FID were due to the oxidation of CS_2 to SCO, SO_2 and CO. With suitable hydrogen gas-flow rate conditions CS_2 gave a response less than 1% that of CH_4. BLADES[31] obtained conditions for response of 0.16 relative to CH_4. With the gas-flow conditions of the FID used here it was unlikely that the sulphur present in cysteine or methionine gave any measurable effect on the FID, otherwise an increase in the non-effective carbon number would have resulted in higher corrected molar response values than those quoted in Table II.

Tyrosine (Table II, No. 29; Fig. 1, D) gave a low molar response factor when compared with other compounds having two TFA groups (Table II, Nos. 15–18, 25–28). Further investigation showed that the tyrosine derivative was breaking down on the column used in this work. This was confirmed by chromatography on other silicone columns, when a higher molar response factor could be obtained. It has proved useful to use the maximal molar responses which could be obtained relative to N-TFA norleucine methyl ester as a method for selecting the conditions for obtaining the maximum yields of our amino acid derivatives.

The appropriate non-effective carbon numbers obtained here may be applied to the molar response values which were calculated from single determinations for five TFA amino acid methyl, *n*-butyl and *n*-pentyl esters[32]. These values were obtained under different chromatography conditions by relating the radioactivity of the derivative to the response observed on the chromatogram. The corrected responses (C/g atom carbon) compare well with those given in Table II in this paper.

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