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Note

Capillary gas chromatography of some mono-, di- and tricarboxylic acid isobutyl ester derivatives*

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Many derivatisation procedures have been described for gas–liquid chromatographic (GLC) analysis of carboxylic acids[1,2]. For instance, methyl esters[3,4], *n*-butyl esters[5,6] and silyl derivatives[7,8] have been extensively used to achieve separation of carboxylic acids.

In the process of assaying amino acids as the N(O,S)-heptafluorobutyryl (HFB) isobutyl esters by GLC[9,10], we have identified compounds of other chemical classes which have been derivatised by either or both of the reagents depending on the functional groups in the compounds. Hydroxyl, sulphydryl, and amino groups are acylated and carboxylic and other acid groups are esterified although some acids such as the sulphonic acids are not generally sufficiently stable to survive the usual chromatographic conditions[11]. In particular, we have identified a number of mono-, di- and tricarboxylic acids when assaying biological samples without prior clean-up by ion-exchange chromatography.

Since only a few of the carboxylic acids that we have identified coelute with amino acids and these compounds are not necessarily present in significant amounts in all samples, it can be advantageous, especially when only a small amount of sample is available, to assay a sample directly, either for amino acids or carboxylic acids, without risking loss during ion-exchange chromatography. Although fatty acids also represent a potential source of interference, we have not experienced any problem in this regard when analysing plant-derived fluids such as phloem and xylem sap.

In this report, we present information on the relative retention times of the HFB isobutyl ester derivatives of a number of aliphatic and aromatic carboxylic acids, and some hydroxycarboxylic acids. Our procedure has also been applied to the separation and identification of some Krebs-cycle intermediates in a biological sample.

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EXPERIMENTAL

Reagents

Heptafluorobutyric anhydride 99% (HFBA) was obtained from Fluka (Buchs, Switzerland) and the isobutanol–HCl was prepared by mixing 1 ml of isobutanol with 0.27 ml of acetyl chloride (99%; Aldrich, Milwaukee, WI, U.S.A.).

Esterification and acylation

Esterification and acylation were performed as previously described[9,10]. A 25-μl volume of a carboxylic acid solution (2.5 μmole/ml) was mixed with an equimolar quantity of either octadecane or stearylamine as an internal standard and submitted to the derivatisation procedure.

GLC and mass spectrometry

GLC was performed using a Varian Vista 6000 gas chromatograph equipped with a capillary injection system and dual flame ionization detectors and operated in the split mode (1:50). The column used was a 15 m $\times$ 0.25 mm I.D. SPB-1 fused-silica capillary column (Supelco, Oakville, Canada). The oven temperature was maintained at 70°C for 2 min and then increased to 270°C at a rate of 4°C/min. The masses of the derivatives were confirmed using a Finnigan Model 3300 mass spectrometer operated in the chemical ionization mode (CI) using methane as the reagent gas.

RESULTS AND DISCUSSION

The retention times relative to octadecane and stearylamine and also the derivative masses are given in Table I for various classes of carboxylic acids: aliphatic carboxylic acids: aromatic carboxylic acids: aliphatic carboxylic acids with aromatic groups or with other acidic and/or basic functions and eight Krebs-cycle acids.

With this derivatisation procedure, more than one peak was obtained for each of 3-hydroxypropionic acid, hydroxyphenylacetic acid and fumaric acid. It is not possible now to conclude whether these observations are related to the purity of the sample or to an artefact of the derivatisation procedure. It is, however, reasonable to suggest that maleic acid might be produced by epimerisation during the derivatisation of fumaric acid, especially since the two peaks observed had virtually identical mass spectra.

Some carboxylic acids either coelute with or are not sufficiently resolved from certain amino acids. These are benzoic acid with threonine, mesoxalic acid with leucine, malic acid with hydroxyproline (not shown in Table I), 2-ketovaleric acid with methionine, 2-ketoglutaric acid with aspartic acid and glutaconic acid with phenylalanine. These compound pairs cannot be accurately assayed without first fractionating the sample by, for example, ion-exchange chromatography on Dowex 50.

The predominant ions of the chemical ionisation spectra of a number of simple, keto- and methyl-substituted dicarboxylic acids are described in Table II. The spectra are unremarkable and simply reflect the well known pattern of fragmentation of the carboxylic acid ester. The specific series of ions obtained for the isobutyl ester of a dicarboxylic amino acid has been described previously[12]. The simple dicarboxylic isobutyl esters behave similarly, except that an ion representing M $-$ 83 is consist-

TABLE I

MASSES AND RELATIVE RETENTION TIMES OF HEPTAFLUOROBUTYRYL ISOBUTYL ESTERS OF CARBOXYLIC ACIDS AND AMINO ACIDS

Compound	*Mass**	*RRT***	
		C_{18} = *1*	*Stear* = *1*
Lactic acid	342	0.107	0.073
3-Hydroxypropionic acid***	342	0.232	0.158
2-Ketoisocaproic acid	186	0.250	0.170
Ala		0.269	0.183
Gly		0.285	0.194
Oxalic acid	202	0.337	0.229
Val		0.383	0.260
Benzoic acid	178	0.401	0.273
Thr		0.406	0.276
Ser		0.422	0.287
Malonic acid	216	0.442	0.301
Nicotinic acid	179	0.448	0.305
Leu		0.458	0.311
Mesoxalic acid	230	0.459	0.312
Methylmalonic acid	230	0.467	0.318
Ile		0.474	0.322
Pro		0.566	0.384
Succinic acid	230	0.589	0.401
Fumaric acid***	228	0.610	0.414
Methylsuccinic acid	244	0.614	0.418
2-Hydroxyphenylacetic acid	404	0.655	0.445
p-Hydroxybenzoic acid	390	0.674	0.459
Malic acid	442	0.688	0.468
Glutaric acid	244	0.709	0.482
2-Ketovaleric acid	258	0.726	0.493
Met		0.730	0.496
Cinnamic acid	204	0.760	0.517
Tartaric acid	654	0.790	0.537
Asp		0.802	0.545
2-Ketoglutaric acid	258	0.802	0.546
Anthranilic acid	389	0.805	0.547
p-Hydroxymandelic acid	616	0.826	0.561
Adipic acid	258	0.836	0.568
Glutaconic acid	242	0.847	0.576
Phe		0.850	0.578
3-Methyladipic acid	272	0.876	0.596
3,4-Dihydroxyphenylacetic acid	616	0.889	0.605
p-Hydroxyphenyllactic acid***	630	0.922	0.627
Glu		0.941	0.640
Lys		1.011	0.687
Tyr		1.035	0.704
Suberic acid	286	1.069	0.727
2-Phenylpyruvic acid	220	1.070	0.728
Oxaloacetic acid	244	1.107	0.753
Arg		1.135	0.772
2-Aminopimelic acid	483	1.161	0.790
Citric acid	556	1.169	0.795
His		1.183	0.804
Ferulic acid	446	1.210	0.823
Isocutric acid	556	1.214	0.826
Aconitic acid	342	1.220	0.830
Cys		1.525	1.037

* Molecular ion of the derivatives.

** Retention time relative to octadecane (C_{18}) and stearylamine (stear).

*** Additional minor peaks obtained.

TABLE II

CHEMICAL IONIZATION MASS SPECTRA OF DICARBOXYLIC ACID ISOBUTYL ESTERS

Ion	*Acid (% of base ion)*									
	Oxalic	*Malonic*	*Methyl-malonic*	*Keto-malonic*	*Succinic*	*Methyl-succinic*	*Glutaric*	*2-Keto-glutaric*	*Adipic*	*3-Methyl-adipic*
M + 1	16.5	24.3	30.7	29.4	76.3	53.9	46.7	15.5	100.0	32.0
M − C_4H_7	61.3	42.9	50.1	81.0	57.0	25.4	24.4	39.2	19.9	4.6
M − OC_4H_7	26.5	–	–	30.3	–	–	–	9.5	–	–
M − OC_4H_9	–	–	–	–	36.8	41.0	99.3	8.5	97.7	100.0
M − 83	100.0	34.0	25.8	100.0	14.4	3.8	48.6	26.9	–	–
M − $COOC_4H_9$	–	–	–	–	24.5	21.9	–	9.7	22.7	13.5
M − C_4H_7 − C_4H_8	14.2	100.0	100.0	21.5	48.0	9.2	–	100.0	–	–
M − C_4H_7 − OC_4H_9	–	–	–	–	–	–	100.0	–	–	
M − C_4H_8 − OC_4H_9	4.4	–	11.5	–	100.0	100.0	–	47.0	77.3	50.7

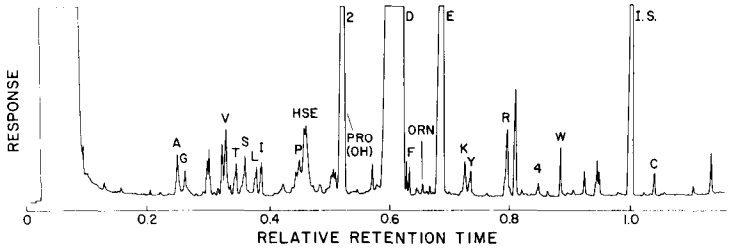

Fig. 1. Chromatogram illustrating separation of constituents of crude pea xylem sap as heptafluorobutyryl isobutyl ester derivatives. Peaks: A = alanine; G = glycine; V = valine; T = threonine; S = serine; L = leucine; I = isoleucine; P = proline; HSE = homoserine; 2 = malic acid; PRO (OH) = hydroxyproline; D = aspartic acid; F = phenylalanine; ORN = ornithine; E = glutamic acid; K = lysine; Y = tyrosine; R = arginine; 4 = aconitic acid; W = tryptophan; I.S. = internal standard, stearylamine; C = cysteine.

ently obtained for the lower-molecular-mass homologues but not for adipic acid and 3-methyladipic acid. Presumably, the two carboxylic acid groups must be sufficiently close to permit the formation, by cyclisation, of a stable ion or neutral fragment. This loss can best be rationalised as representing the loss of two isobutyl moieties from the molecular ion to form a neutral fragment which subsequently forms an adduct ion, *i.e.* $[M - C_4H_8 - C_4H_8] + C_2H_5$. Alternatively, the same mass can be represented as the loss of two butyl moieties from the adduct ion M + 29, *i.e.* $[M + C_2H_5] - C_4H_8 - C_4H_8$. Whatever the more probable mechanism, this ion aids in the identification of simple dicarboxylic acid isobutyl esters and to distinguish them from dicarboxylic amino acids.

The tricarboxylic acid, in addition to the series of ions observed for the dicarboxylic acids, are characterised by the loss of a fragment of mass 185, corresponding to $(C_4H_8 + C_4H_8 + OC_4H_9)$.

Fig. 1 illustrates an amino acid analysis of pea xylem sap without prior clean up by ion-exchange chromatography. A major non-amino acid component was identified as malic acid. When the acidic–neutral fraction was analysed (Fig. 2), the predominant component was confirmed to be malic acid and other components were present only in relatively minor amounts.

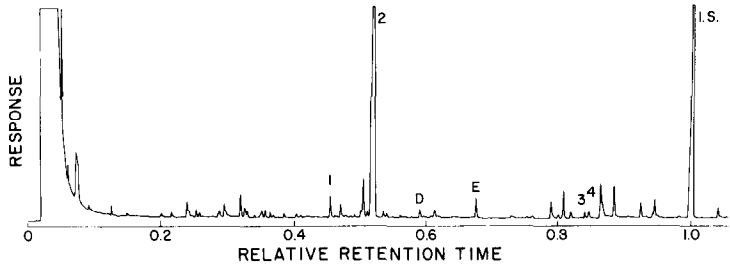

Fig. 2. Chromatogram illustrating separation of constituents of the acidic–neutral fraction of pea xylem sap as the heptafluorobutyryl isobutyl ester derivatives. Peaks: 1 = succinic acid; 2 = malic acid; D = aspartic acid; E = glutamic acid; 3 = citric acid; 4 = aconitic acid; I.S. = internal standard, stearylamine.

In summary, most of the common carboxylic acids do not interfere with assaying amino acids as the N(O,S)-HFB isobutyl ester derivatives. Furthermore, reference to the information presented in Table I, and knowledge of the mass spectral characteristics have enabled us to rapidly identify carboxylic acids when assaying samples such as pea xylem sap in a crude form.

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