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Note

Gas–liquid chromatography of N-trifluoroacetyl *n*-butyl esters of amino acids

A comparison of high-performance and acid-washed Chromosorb W as the support material

BRIAN THOM and JOHN W. PARSONS

Department of Soil Science, Aberdeen University, Meston Walk, Aberdeen (Great Britain)

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The ideal solid support for gas–liquid chromatography (GLC) should have a large surface area with a strong affinity for the liquid phase but remaining inert to the components of the sample. Chemical activity of the support material is due either to the presence of trace metals which can be removed by acid or alkali washing or to the presence of Si–OH groups. Deactivation of the latter is usually achieved by silanization and is of particular importance in the separation of polar compounds.

Islam and Darbre[1] compared a number of solid supports coated with the same mixed silicone liquid phase for the separation of N-trifluoroacetyl (N-TFA) methyl derivatives of amino acids. The only non-silanized material included, acid-washed Celite 560, gave low efficiency compared to the silanized support. Gehrke *et al.*[2] used ethylene glycol adipate (EGA) on Chromosorb G for the separation of N-TFA *n*-butyl esters but eventually replaced the support with acid-washed Chromosorb W which produced a better separation[3]. Neither Chromosorb G nor Chromosorb W were silanized.

The *n*-butyl esters are polar compounds but are less polar than the methyl esters so we decided to investigate the difference, if any, in the separation of the *n*-butyl esters on silanized as opposed to non-silanized Chromosorb W.

MATERIALS AND METHODS

Apparatus

A Pye gas chromatograph, Series 104 Model 64, was used with a dual column oven, separately controlled injection point heaters and detector oven, dual hydrogen flame ionization detectors and a linear temperature programmer. The amplifier was connected to a Telsec recorder.

White spot nitrogen was used as carrier gas and all gases were passed through molecular sieve 5A mixed with drierite.

Reagents

All amino acids were chromatographically pure grade. A standard solution (1 mg/ml) was prepared in 0.1 *M* HCl and aliquots were taken for derivatization as required.

Butanol was of 'chromatographic grade' and methylene chloride and acetonitrile were labelled 'special for IR spectrometry'. Before use, butanol and methylene chloride were refluxed over anhydrous calcium chloride for several hours and then redistilled from an all-glass system directly into their bottles, which were then double stoppered and sealed with Parafilm to exclude moisture. 3 *M* HCl in butanol was prepared by bubbling anhydrous HCl gas (99.99% purity, BDH, Poole, Great Britain) into redistilled butanol.

Stabilized grade EGA and 80–100 mesh AW Chromosorb W were obtained from Phase Separations, Queensferry, Great Britain. The silanized, acid-washed support, 80–100 mesh HP Chromosorb W, was obtained from BDH.

Preparation of chromatograph columns

Prior to use, both 1.5 m × 4 mm I.D. all-glass columns, the glass-wool plugs and fluted flask used for coating the stationary phase were cleaned and silanized by the method of Darbre and Blau[4].

The Chromosorb W packings, after drying at 140° for 12 h, were coated with the desired amount of EGA using acetonitrile as solvent, according to the method of Roach and Gehrke[3]. The columns were then conditioned in the chromatograph at 220° for various periods of time.

Preparation of derivatives

The direct esterification–acylation procedure (macro method) of Roach and Gehrke[5] was used. The acylation reaction was carried out in acylation tubes fitted with PTFE screw caps and PTFE-coated rubber septa, supplied by Serva, Micro-Bio Labs., London, Great Britain. To prevent blow-out of the septum it was supported on the outside by an aluminium disc with a small hole in the centre to allow sampling and held in position by the screw cap.

A Tecam Driblock, DB-3H, bored to accept the tubes was used to heat them at 150° for 5 min. Esterification was carried out on the same Driblock at 100°.

RESULTS

Fig. 1 shows the separation achieved when the mixture of seventeen amino acids was injected directly on to a column of 0.65% (w/w) EGA on HP Chromosorb W. Only sixteen peaks were obtained, indicating that two of the amino acid derivatives had failed to separate. The order of the amino acids on the chromatograph was determined by spiking individual derivatives during runs of all seventeen amino acids. Injection of singly derivatized standards alone, further ascertained the order by noting retention times under specified operating conditions.

Table I shows the order and retention times of the amino acids as they emerged from the column.

Similar experiments were carried out using 0.65% (w/w) EGA on AW Chromosorb W, and the separation achieved is shown in Fig. 2, while the order of emergence and retention times are shown in Table I.

Several other chromatograms were run on HP Chromosorb W with *n*-butyl stearate and cysteine present in the mixture. Figs. 3 and 4 show the positions of these compounds relative to the other amino acids.

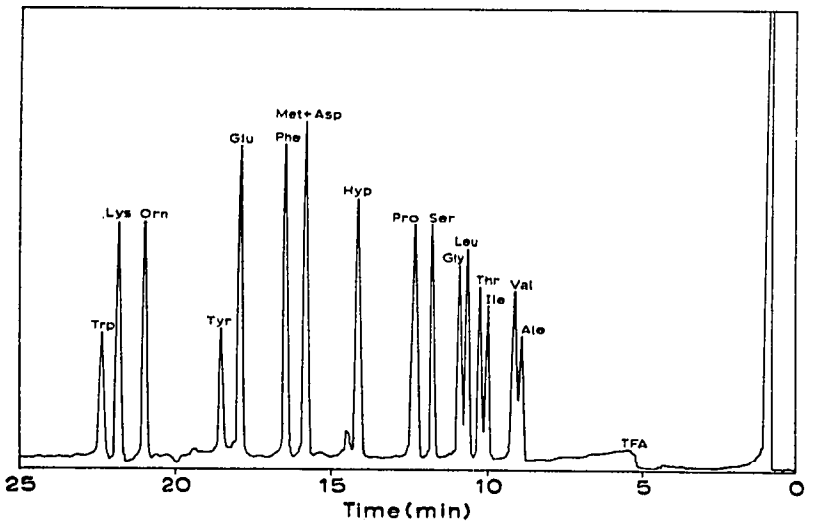

Fig. 1. GLC of standard amino acid solution, N-TFA *n*-butyl esters. Column: 1.5 m × 4 mm I.D., glass, 0.65 % (w/w) EGA on dried 80–100 mesh HP Chromosorb W. Sample: 3.5 μg total amino acids injected (1.4 μl). Conditions: nitrogen flow-rate, 20 ml/min; temperature, programmed at a rate of 8 °/min, initial temperature 60 °, final temperature 220 °; injection port temperature, 200 °; detector oven temperature, 250 °. Attenuation, $5 \cdot 10^3$. Chart speed, 1 cm/min.

TABLE I

RETENTION DATA FOR 0.65 % (w/w) EGA ON AW CHROMOSORB W AND HP CHROMOSORB W

The figures shown are the actual retention times (*ART*) in min and the relative retention times (*RRT*), taking the glutamic acid derivative as 1.00. Column conditions: see legends to Figs. 1 and 2.

Amino acid	*HP Chromosorb W*		*Amino acid*	*AW Chromosorb W*	
	ART	*RRT*		*ART*	*RRT*
Alanine	8.9	0.50	Alanine	8.0	0.41
Valine	9.1	0.51	Valine	9.2	0.47
Isoleucine	10.0	0.56	Glycine	9.8	0.50
Threonine	10.2	0.57	Isoleucine	10.5	0.53
Leucine	10.6	0.59	Leucine	11.3	0.57
Glycine	10.9	0.61	Proline	12.0	0.61
Serine	11.8	0.66	Threonine	12.6	0.64
Proline	12.3	0.69	Serine	13.8	0.70
Hydroxyproline	14.1	0.79	Methionine	15.7	0.80
Methionine + aspartic acid	15.9	0.89	Hydroxyproline	16.3	0.83
Phenylalanine	16.4	0.92	Phenylalanine	16.7	0.85
Glutamic acid	17.9	1.00	Aspartic acid	17.4	0.88
Tyrosine	18.5	1.03	Glutamic acid	19.7	1.00
Ornithine	21.0	1.17	Tyrosine	21.2	1.08
Lysine	21.3	1.19	Ornithine	22.7	1.15
Tryptophan	22.4	1.25	Lysine	23.8	1.21
			Tryptophan	24.5	1.24

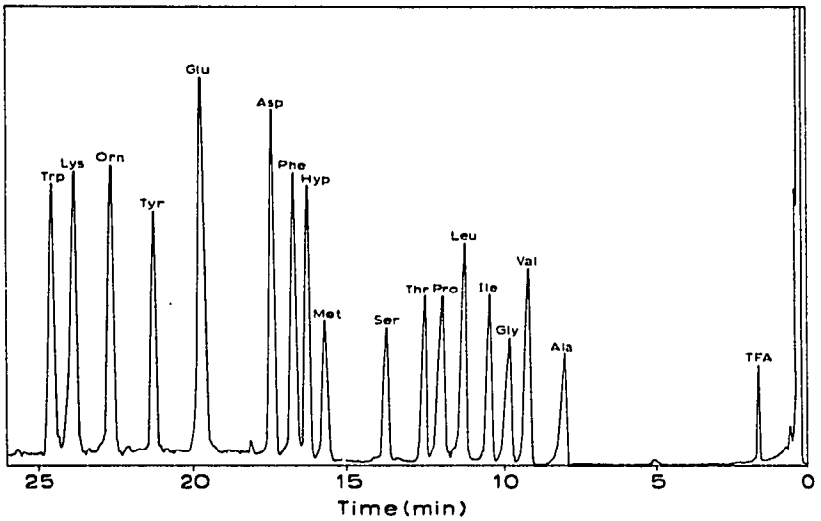

Fig. 2. GLC of standard amino acid solution, N-TFA *n*-butyl esters. Column: 1.5 m × 4 mm I.D., glass, 0.65 % (w/w) EGA on dried 80–100 mesh AW Chromosorb W. Sample: see legend to Fig. 1. Conditions: nitrogen flow-rate 50 ml/min; temperature, programmed at a rate of 6 °/min, initial temperature 60 °. Attenuation $1 \cdot 10^4$.

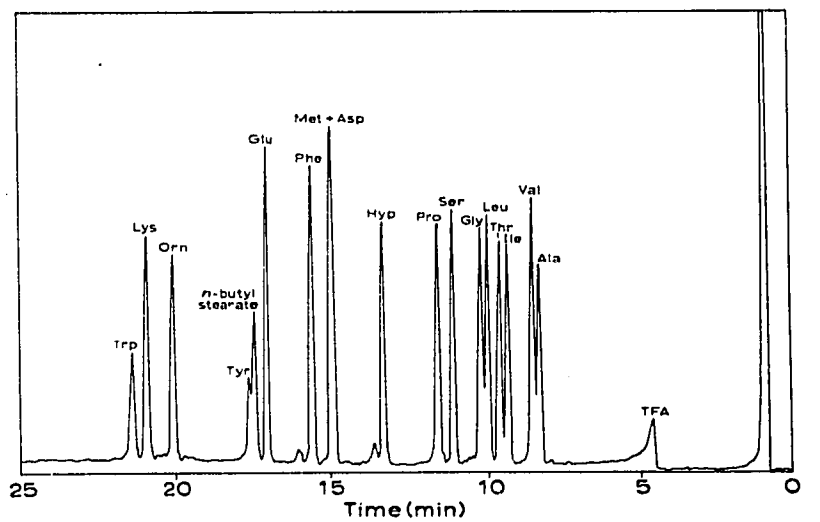

Fig. 3. GLC of standard amino acid solution + *n*-butyl stearate, N-TFA *n*-butyl esters. Attenuation $1 \cdot 10^4$. For further conditions see legend to Fig. 1.

DISCUSSION

The chromatograms shown in Figs. 1 and 2 clearly show the effect of the different pre-treatments of the packings used. The separation efficiency and order of emergence of the derivatized amino acids can only be put down to differences in the surfaces of the two supports used. Contrary to what may have been expected

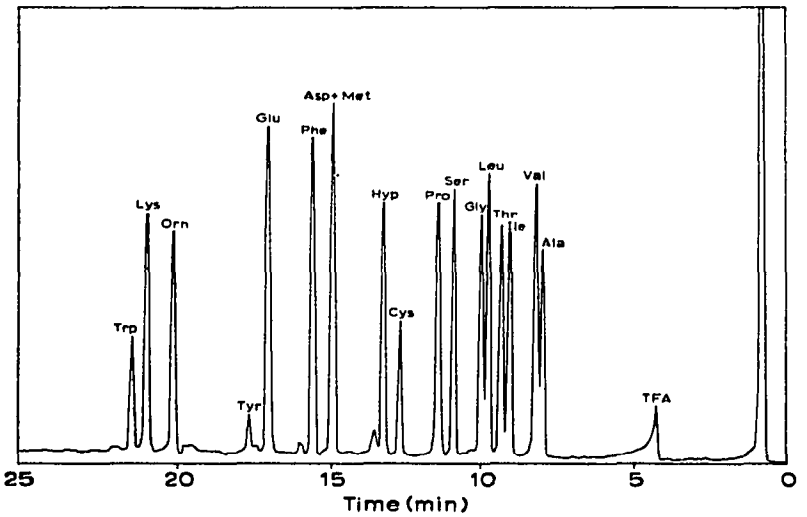

Fig. 4. GLC of standard amino acid solution + cysteine, N-TFA *n*-butyl esters. Attenuation, $1 \cdot 10^4$. For further conditions see legend to Fig. 1.

the least deactivated support, namely the AW Chromosorb W, gave the most reproducible resolution of the seventeen amino acids. HP Chromosorb W gave poorer resolution of the first six amino acids and no separation of methionine and aspartic acid.

On the HP support, however, cysteine was well separated from all other amino acids and emerged as a discrete peak between proline and hydroxyproline. Roach and Gehrke[3] show cysteine to appear between serine and methionine on a corresponding column with AW Chromosorb W. On some of the chromatograms shown with this non-silanized support, cysteine ran very close to methionine and occasionally existed as a doublet peak.

On HP chromosorb W, *n*-butyl stearate, often used as an internal standard along with ornithine and tranexamic acid[6], came off the column before tyrosine. Gehrke *et al.*[6] show *n*-butyl stearate to emerge as a single peak after tyrosine on the non-silanized AW Chromosorb W.

Many batches of HP material coated independently with EGA gave poorer resolution of the first six peaks, *i.e.* the most volatile derivatives. Alanine and valine were prone to run together and emerge as one peak or as a poorly resolved doublet. A similar tendency was apparent with the pairs isoleucine and threonine, and leucine and glycine. When this occurred, variation of operating conditions such as the introduction of an initial isothermal period, alteration of the temperature programme or a change in the carrier gas flow-rate often improved their resolution.

Similar tendencies were noted with AW Chromosorb W, but continued conditioning of the column at elevated temperatures generally improved resolution until complete separation of all seventeen amino acids was achieved. Columns used after only several hours conditioning produced doublet peaks for proline and threonine, and hydroxyproline and methionine, while glycine ran close to isoleucine and phenylalanine to aspartic acid. 24–48 h was usually sufficient to

give complete separation of glycine and isoleucine, and phenylalanine and aspartic acid, but longer periods were often necessary for complete resolution of the first two pairs mentioned.

The improved resolution after conditioning at 220° was attributed to loss of EGA by bleed off (upper temperature limit 190°), which was initially excessive at high temperatures, suggesting a lower load of stationary phase may enhance resolution after reduced conditioning periods. Support for this suggestion may be found in the work of Stefanovic and Walker[7] who reported that increased loadings of EGA on AW Chromosorb W caused the peaks of glycine and isoleucine, proline and threonine, and phenylalanine and aspartic acid to merge. They further reported the hydroxyproline peak to emerge between methionine and phenylalanine at 0.5% (w/w) EGA, at 1% (w/w) EGA no separation of hydroxyproline and methionine and at 2% (w/w) EGA the emergence of hydroxyproline ahead of methionine. Increasing the EGA load has the exact opposite effect of prolonged conditioning and affects the same pairs of amino acids.

The effect of different loadings of EGA (0.325–4.0%) on HP Chromosorb W was also investigated. No difference in the overall pattern or individual order of amino acids occurred but 0.325% (w/w) load caused skewing of peaks while column efficiency became seriously impaired with the heavier loadings (>2.0%). Thin films of the polyester EGA should therefore be used at all times.

Zumwalt *et al.*[8] have suggested that preheating the AW Chromosorb W packing at 140° for 24 h may be unnecessary. To test this, a number of chromatograms was run on AW Chromosorb W again with 0.65% (w/w) EGA as stationary phase, but initial resolution was found to be poorer than that of the preheated support. However, after a longer than normal conditioning time, separation was markedly improved.

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