

## GAS CHROMATOGRAPHY OF THE METHYL ESTERS OF THE AMINO ACIDS AS THE FREE BASE AND BY DISSOCIATION OF THEIR ACID SALTS

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The dissociation of ammonium salts into free acid and base is well known<sup>1</sup>. Early attempts to apply the principle of dissociation of the ammonium salts of esters of amino acids to gas phase analysis resulted either in failure or very poor results<sup>2,3</sup>. Recent experiments in our laboratory have demonstrated that acid salts of fifteen of the twenty common amino acid esters are amenable to gas phase analysis by virtue of their ease of dissociation under the influence of temperature.

Comparative studies were carried out on the amino acid esters in the form of the free base and the salts of hydrochloric and acetic acids. The starting derivative for all of the studies was the methyl ester hydrochloride of the amino acid, usually in the crystalline form<sup>4</sup>.

### *Free base*

In this study amino acid esters in the form of the free base were prepared as a reference with which to compare the gas chromatographic behaviour of the acid salts. Gas chromatography of amino acid esters as the free base has been reported<sup>3,5</sup>. Disadvantages of these methods resulting from the use of sodium hydroxide<sup>6</sup> and ammonia<sup>3</sup> were eliminated by the following method of preparation. The free bases were prepared by shaking a methanolic solution of the chloride salt (0.25 M) with about 20% of its volume of anhydrous Dowex-1 in the hydroxide form. Careful preparation of the resin, to exclude most of the free hydroxyl ions, was carried out by washing the resin in the hydroxide form with deionized water until the pH of the effluent was lower than 8, then dehydrating the resin by continuous washing with anhydrous (0.04% water) methanol.

Anhydrous solutions of the free bases of amino acid esters prepared under these conditions were found to be stable for periods varying from hours to days. For example, the methyl esters of glycine and leucine showed less than 5% loss of yield after standing for 18 to 24 hours at room temperature. After 72 hours, the yield from the methyl ester of proline was decreased by 45%.

The free bases, as well as the salts, were chromatographed either in a laboratory-

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constructed apparatus using nitrogen as a carrier gas and a hydrogen flame ionization detector, or in a commercial apparatus (American Instrument Co., Silver Spring, Md.) with helium as carrier gas and a thermistor detector. The most suitable column packing was found to be 2% neopentyl glycol succinate on Fluoropak 80 (The Fluorocarbon Co., Anaheim, Calif.). When Chromosorb W was used as inert support, considerable tailing of the peaks occurred, whereas Fluoropak gave sharp peaks as illustrated in Fig. 1. In addition to stability in solution, the methyl esters of the amino acids exhibited equivalent stability during the course of a gas phase analysis. Estimates of yields in excess of 90% for glycine, leucine and proline were made using the

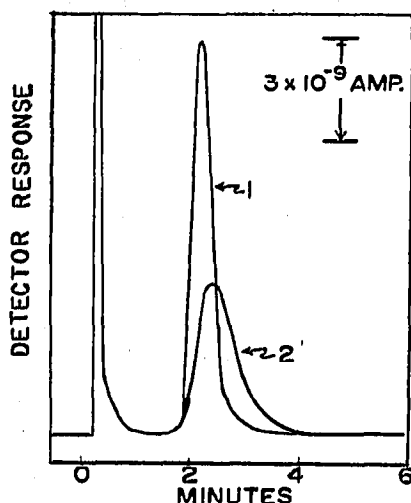


Fig. 1. Gas chromatograms of aspartic acid dimethyl ester. *Curve 1*, free base; *Curve 2*, chloride salt. The curve for the acetate salt was almost the same as that for the free base. Conditions for chromatography were: column and preheat temperature 175°; 6 ft.,  $\frac{3}{16}$  in. I.D. glass column packed with 2% neopentyl glycol succinate on Fluoropak 80; carrier gas: nitrogen at 60 c.c./min; detector: hydrogen flame ionization.

hydrogen flame detector, the effective carbon data of STERNBERG *et al.*<sup>6</sup> and methyl decanoate<sup>7</sup> as a primary standard. Diketopiperazine formation in the column was ruled out by a study comparing the behavior of the methyl ester of glycine and glycine anhydride.

The data presented in Table I show that of the twenty common amino acids cysteine, histidine, tyrosine and tryptophan could not be chromatographed satisfactorily in the form of the free base of the methyl ester. Mixtures of the amino acid derivatives chromatographed under the conditions of Table I presented no difficulties except that the pairs glycine-alanine, leucine-isoleucine and phenylalanine-hydroxyproline were unresolved. (Serine and cysteine were also unresolved when the acetate salts were employed. See below.)

#### Chloride salts

The gas chromatographic behavior of the amino acid methyl ester hydrochlorides as compared with their free bases are summarised in Table I, while a typical peak is shown in Fig. 1. It can be seen that considerable differences exist between the behavior of the various amino acids. Whereas no amino acid ester gave a satisfactory peak at a temperature below about 170°, above this temperature, the esters of alanine, glycine,

TABLE I

GAS CHROMATOGRAPHIC BEHAVIOR OF AMINO ACID METHYL ESTERS AS THE FREE BASE, CHLORIDE AND ACETATE SALTS

Column: 6 ft.,  $\frac{3}{16}$  in. I.D., packed with 2% neopentyl glycol succinate on Fluoropak 80. Gas flow 60 c.c./min. Sample size 0.25  $\mu$ mole. The recorded temperature was that of the preheater and column.

	Free base <sup>a</sup>		Acetate salt <sup>a</sup>		Hydrochloride salt		Peak area
	Temp. (°C)	Retention time (min)	Temp. (°C)	Retention time (min)	Temp. (°C)	Retention time (min)	
Leucine <sup>b</sup>	120	4.3	120	4.3	120	4.7	10% of free base
	175				175	0.1	80% of free base
Proline	115	5.2	115	5.2	115	5.5	10% of free base
	175	0.6	175	0.6	175	0.7	40% of free base
Serine <sup>c</sup>	150	3.6	150	3.6	150	—	no peak
	175	2.4	175	2.4	175	2.9	small broad peak
Aspartic acid <sup>d</sup>	150	4.8	150	4.8	150	6.7	broad peak
	175	2.0	175	2.0	175	2.3	30% of free base
							40% of free base
Phenylalanine <sup>e</sup>	155	9.0	155	9.0	155	11.0	40% of free base
	180	6.9	180	6.9	180	7.1	80% of free base
Hydroxyproline	175	5.7	175	5.7	175	6.5	broad peak
	195	2.9	195	2.9			
Cysteine	175	negative result	175	1.9	175	2.0	equal to acetate
Cystine	180	6.7	180	6.7	180	6.7	80% of free base
Lysine <sup>f</sup>	180	3.5	180	negative result	180	negative result	

<sup>a</sup> Except for cysteine, lysine and arginine, all the above amino acid esters gave similar (to within 10%) peak areas for both the free base and the acetate salt.

<sup>b</sup> Alanine, glycine, valine and isoleucine gave results similar to those of leucine.

<sup>c</sup> Threonine gave results similar to those of serine.

<sup>d</sup> Glutamic acid gave results similar to those of aspartic acid.

<sup>e</sup> Methionine gave results similar to those of phenylalanine.

<sup>f</sup> Arginine gave results similar to those of lysine.

valine, leucine, isoleucine, methionine, phenylalanine, cysteine and cystine gave peaks of good yields although the retention times were slightly longer than the corresponding free bases. Even at these elevated temperatures, proline, hydroxyproline, serine, threonine, aspartic and glutamic acids gave poor yields while lysine, arginine, tyrosine, histidine and tryptophan gave negative results.

The nature of the inert support for the stationary phase has an important influence on the behavior of the amino acid derivatives. Under conditions which produced peaks of good yield for leucine and phenylalanine on columns packed with Fluoropak 80, negative results were obtained with Chromosorb W as the inert support.

#### Acetate salts

Acetate salts were prepared from the corresponding chlorides by shaking a solution of the ester hydrochloride in methanol (0.25M) with approximately 20% of its volume of anhydrous Dowex-1 in the acetate form. The results summarized in Table I indicate that when the free base of an amino acid ester and its corresponding acetate salt both gave peaks, the areas and retention times were similar. This demonstrates that even at temperatures as low as 120°, the phenomenon occurring in the column was dis-

sociation of the salt and chromatography of the free base. With the esters of lysine and arginine no such dissociation was apparent. It is of interest to note that whereas cysteine methyl ester gave negative results in the form of the free base, peaks of good yield were obtained with the corresponding acetate salt.

The consistent behavior of the acetate salts of the amino acid esters as compared with the corresponding chlorides suggests that the acetate salts dissociate more readily at the temperatures employed and are therefore more amenable to gas phase analysis. Combined use of the free base and acetate salt of the methyl esters of the amino acids makes facile the gas phase analysis of all of the common amino acids except histidine, tyrosine and tryptophan.

#### SUMMARY

When anionic exchange resins were employed to generate the free basic form of the methyl esters of the amino acids, these derivatives were stable for periods of time long enough for gas chromatography. Dissociation of the acetate salts of the amino acid esters into the free base and acetic acid occurred in gas chromatographic columns under the influence of temperatures varying from 120° to 200°. A combination of the use of the amino acid esters as the free base and by dissociation of their acid salts made possible the gas chromatography of the derivatives of all of the common amino acids except histidine, tyrosine and tryptophan.

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