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Note

Rapid formation of amino acid isobutyl esters for gas chromatography*

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The analysis of amino acids by gas–liquid chromatography (GLC) requires the preparation of appropriate derivatives, frequently involving two separate reactions such as esterification followed by acylation. Esterification has been achieved by procedures such as methylation followed by interesterification[1,2], and by direct esterification[3]. Direct esterification involving a double esterification reaction has also been used[4]. In all these procedures, the advantages of minimising the number of manipulations and/or reducing the derivatisation time are self-evident.

We have previously reported[5] the preparation and GLC separation of the N-heptafluorobutyryl (HFB) amino acid isobutyl esters. The esterification procedure used, essentially that of Roach and Gehrke[3], involved heating the reaction mixture for 1 h at 110°. Satisfactory esterification of all the protein amino acids was obtained. We now report further studies on the effect of temperature on the formation of the amino acid isobutyl esters and the reduction of the time required for esterification.

EXPERIMENTAL

Reagents

Standard amino acid mixtures were obtained from Hamilton (Whittier, Calif., U.S.A.) and heptafluorobutyric anhydride (99% purity) was obtained from Fluka (Buchs, Switzerland). All other reagents were prepared as previously described[5].

Esterification

Direct esterification was performed as previously described[5] except that temperatures of 110, 120 and 130° were used. At each temperature separate samples (in triplicate) were heated for 10, 15, 20 and 30 min. Acylation of the amino acid esters was then performed by heating for 10 min at 150° as previously described[5].

Chromatography

All analyses were performed using a Hewlett-Packard Model 7611 gas chromatograph equipped with dual flame ionisation detectors. The column packing (3% SE-30 on Gas-Chrom Q, 100–120 mesh) was obtained from Applied Science Labs.

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(State College, Pa., U.S.A.). Pyrex columns (approximately 3.5 m × 2.5 mm I.D., thin-walled) were filled with the stationary phase by gentle tapping under suction and conditioned overnight with a carrier gas (nitrogen) flow-rate of 30 ml/min. The chromatographic conditions were: temperature program, 90–240° at 4°/min; injector temperature, 250°; detector temperature, 280°; air flow-rate, 300 ml/min; hydrogen flow-rate, 30 ml/min; nitrogen flow-rate, 30 ml/min. Quantitation was performed using an Infotronics Model CRS208 automatic digital integrator.

RESULTS AND DISCUSSION

The molar responses, relative to the internal standard pipecolic acid, resulting from esterification of the amino acids for various times and temperatures are shown in Table I. The data are expressed as a percentage of the maximum response obtained to make comparison easier. Heating for 10 min at 110° was clearly insufficient to produce quantitative esterification of most amino acids and the response of isoleucine was particularly low. Heating for a longer time, as expected, gave improved responses. However, the derivatisation of alanine, glycine and valine was not complete, even after 30 min. Those amino acids (*e.g.*, proline and tyrosine) which gave a good response after only 10 min heating were relatively unaffected by heating for a longer time.

Increasing the reaction temperature to 120° markedly improved the responses obtained after 10 min heating, particularly for valine, serine, leucine, isoleucine and

TABLE I

RELATIVE MOLAR RESPONSE OF AMINO ACID DERIVATIVES AS A FUNCTION OF ESTERIFICATION TIME AND TEMPERATURE

Amino acid	*Temperature*											
	110°				*120°*				*130°*			
	Time (min)											
	10	*15*	*20*	*30*	*10*	*15*	*20*	*30*	*10*	*15*	*20*	*30*
Ala	94.5	93.7	94.2	96.1	96.4	100.0	99.2	98.1	96.6	98.1	98.5	99.0
Gly	96.8	95.6	94.0	96.4	96.2	99.6	99.6	98.6	98.8	99.6	100.0	99.8
Val	90.8	94.7	96.9	97.1	96.7	99.5	100.0	100.0	98.6	98.6	98.2	98.7
Thr	96.8	98.5	98.8	99.4	99.8	100.0	99.9	100.0	99.5	99.2	100.0	99.5
Ser	96.3	97.8	99.5	100.0	99.6	99.0	99.5	99.5	99.6	98.5	99.7	99.2
Leu	97.6	97.8	98.2	98.4	98.5	99.0	100.0	100.0	99.0	99.6	99.2	99.8
Ile	80.1	91.5	97.1	98.2	90.8	97.7	99.1	99.0	97.3	98.0	100.0	99.7
Pro	99.4	99.2	99.1	99.2	99.7	99.8	100.0	99.6	99.8	99.2	99.4	99.1
Met	98.1	98.5	97.9	99.1	99.8	99.1	99.4	100.0	98.1	100.0	99.4	89.2
Asp	100.0	98.6	98.1	98.1	96.0	98.9	98.8	98.1	98.4	100.0	97.5	99.5
Phe	98.2	99.7	99.4	98.8	98.4	99.4	100.0	99.0	98.6	99.7	96.5	97.2
Glu	96.4	97.6	98.7	99.1	97.8	96.9	98.9	98.0	98.2	100.0	97.1	99.7
Lys	94.4	98.3	99.5	99.7	97.6	98.7	99.8	99.7	100.0	98.7	97.6	98.5
Tyr	99.1	99.5	100.0	99.3	97.2	99.8	99.0	98.6	98.2	98.6	98.2	98.2
Arg	98.5	99.2	98.7	98.1	98.0	98.7	100.0	98.5	96.3	97.5	93.9	95.1
His	93.2	98.1	100.0	99.5	98.6	100.0	100.0	99.2	99.5	98.6	98.6	99.1
Cys	99.2	99.2	98.3	98.9	99.8	98.4	99.5	100.0	98.5	98.0	97.5	97.0

histidine, although derivatisation of alanine, glycine, valine and isoleucine was not complete. A longer reaction time again improved the derivatisation of most amino acids although a slightly lower value was obtained for arginine after 30 min.

A further increase in reaction temperature to 130° gave more quantitative derivatisation of some amino acids after 10 min (*e.g.*, glycine, valine and isoleucine), but the response of arginine was lower. Increasing the reaction time improved the derivatisation of most amino acids but the responses for arginine were substantially lower after 20 min and 30 min while the methionine response was lower after 30 min. Also, the arginine and cystine responses were generally lower at 130° than at 120° presumably because of degradation at the higher temperature.

The choice of optimum esterification conditions is governed by the need for more rigorous conditions to derivatise isoleucine completely. A similar observation was made by Roach and Gehrke in reference to the *n*-butyl ester of isoleucine[3]. A further restriction is the apparent degradation of arginine and methionine at 130°. We therefore chose 20 min at 120° as the reaction conditions for esterification in preference to our earlier procedure of 1 h at 110°. When a large number of samples are prepared, the shorter esterification time is less significant because, presumably, samples would be processed in batches. However, when only a few samples are to be processed, the shorter esterification time facilitates rapid analysis.

In order to minimise the apparatus required, acylation was also performed at 120° and 130°. However, incomplete acylation of arginine resulted and thus the acylation temperature was maintained at 150°.

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