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Note

Pyrolysis–gas chromatography of separated zones on thin-layer chromatograms

III. Application to the determination of some amino acids

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Amino acids occur naturally along with peptides and proteins in vegetaion, fruit, milk and meat and are also found in preparations derived from these products. They may be produced from hydrolysis of peptides and proteins, as for example in studies of amino acid sequences in such polymeric substances. Determination of one or more amino acids in a commercial product can sometimes provide useful information about quality as well as basic composition. Chromatographic methods, particularly based on ion-exchange as in commerical amino acid analysers, are widely used for their separation and determination. Thin-layer chromatography (TLC) can provide a versatile, inexpensive alternative for the separation but the technique is somewhat restricted in utility by problems associated with determination of the separated constituents.

In this paper, a new approach to the determination of amino acids separated by TLC is offered. It makes use of pyrolysis–gas chromatography (Py–GC) as set out in previous papers[1,2]. Five amino acids, selected for structural and compositional variation are separated by TLC and conditions established for the reliable determination of each by Py–GC. They are L-cystine, L-glutamic acid, L-methionine, L-proline and D,L-leucine. A gas chromatograph coupled to a mass spectrometer was used as an aid to identification of some of the pyrolysis products on which the determinations are based.

EXPERIMENTAL

Reagents

Amino acids were taken from an Amino Acid Reference Collection obtained from B.D.H., Poole, Great Britain. Standard solutions of each amino acid in water or 0.1 *M* NaOH were prepared at concentrations of 0.100 and 1.00 g l^{-1}. Solutions containing all 5 amino acids were also prepared so that each was at a concentration in the range 0.100 to 1.00 g l^{-1}.

Analytical Reagent grade solvents were used as supplied by Fisons Scientific Apparatus, Loughborough, Great Britain.

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Apparatus

Plates for TLC, the apparatus for Py–GC and for Py–GC–mass spectrometry (MS) are described in Part II[2]. The carrier gas for Py–GC was nitrogen at a flow-rate of 50 ml min^{-1} and for Py–GC–MS it was helium at the same flow-rate.

Thin-layer chromatography

The solvent system for separation of the amino acids was *n*-butanol–acetic acid–water (4:1:1) as described by Opienska-Blauth *et al.*[3]. The chromatogram was developed in the ascending mode for 10 cm in a closed tank saturated with solvent vapour at 25°C. Iodine vapour in a closed system at room temperature was used as revealing agent.

Pyrolysis–gas chromatography

Experiments similar to those described for the vitamins in Part II[2] were conducted with the amino acids either in solution or adsorbed on thin-layer substrate.

Mass spectrometry of pyrolysis products

Each amino acid was treated in the way described in Part II[2] and under the same experimental conditions except for the pyrolysis temperature which was chosen to be optimum for the particular test substance. In addition glutamic acid, proline and leucine were each mixed with water-saturated silica gel and the experiments repeated.

RESULTS AND DISCUSSION

The experiments in which each amino acid was introduced into the pyrolysis chamber in solution was used to establish the furnace temperature for optimum production of the decomposition product on which the determination was to be based. A selection of the pyrograms taken from the series conducted with methionine is reproduced in Fig. 1. It is seen that the pyrograms obtained at the lower end of the

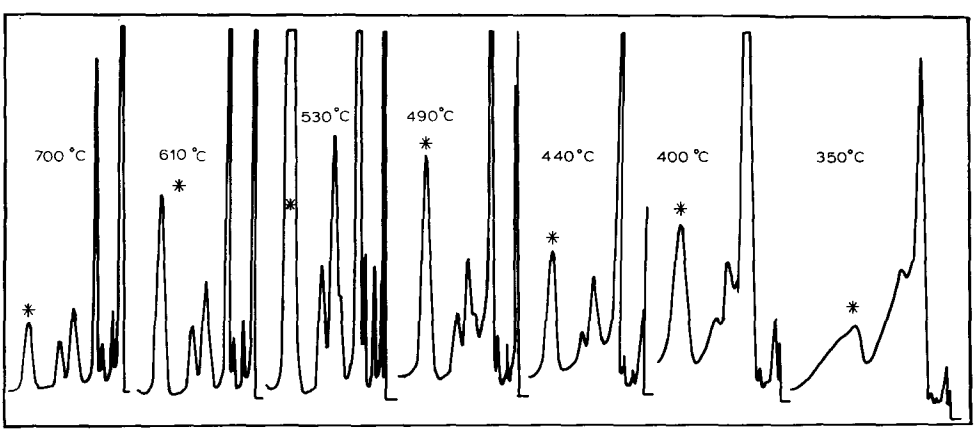

Fig. 1. Effect of changing temperature of pyrolysis on the pyrograms produced from L-methionine introduced into the furnace in aqueous solution. The pyrolysis product chosen for quantitative measurement is marked by an asterisk. The GC oven temperature was 170°C.

temperature range are not well developed. The peak, on which the measurement is based, grows in height and area as the temperature is increased. However, above temperatures of around 530°C there is a decline in yield of this particular product with increasing furnace temperature. The decline arises from the production of increasing yields of lower molecular weight products as the temperature rises. The behaviour of methionine is typical of that observed for the other amino acids studied and the vitamins reported earlier[2].

The results of the experiments with solutions of each amino acid are summarised in Table I. The table sets out the optimum pyrolysis temperatures for each amino acid and the oven temperature found to be most satisfactory for optimum resolution and sensitivity. A linear relation exists between the amount of each amino acid and the height of the peak produced by the GC detector for the chosen product of pyrolysis over the sample ranges recorded in column 3 of the table. Statistical tests indicated the reproducibility was good, relative errors never exceeding 3% for 3 to 5 replicate measurements. The temperature settings presented in Table I were confirmed to be optimum for pyrolysis of each amino acid on the thin-layer substrate.

TABLE I

SUMMARY OF DATA DERIVED FROM THE Py–GC OF SOLUTIONS OF THE AMINO ACIDS

The GC retention times are given for the pyrolysis products used for quantitative determination.

Amino acid	*Solvent*	*Sample range (μg)*	*Pyrolysis temp. (°C)*	*GC oven temp. (°C)*	*GC retention time (min)*
L-Cystine	Water	0.4–2.5	530	170	2.1
L-Glutamic acid	0.1 *M* NaOH	0.6–4.0	530	180	2.1
L-Methionine	Water	0.2–2.0	530	170	4.5
L-Proline	0.1 *M* NaOH	0.2–2.0	450	180	2.0
D,L-Leucine	Water	0.2–2.0	410	180	7.5

However, yields of the selected products were different. The ratios of yields from amino acid on the solid substrate relative to that from aqueous solution for the chosen pyrolysis products were 1.15, 3.20, 0.71, 0.74 and 0.61 for cystine, glutamic acid, methionine, proline and leucine in that order. Thus it is seen that yields are in some instances enhanced and in other suppressed by pyrolysis on thin-layer substrate. This is as might be expected from the general comments made in the previous paper[2]. Typical pyrograms for each amino acid pyrolysed on thin-layer substrate are set out in Fig. 2.

For each of the amino acids, experiments were conducted to identify the pyrolysis product on which the determination is based. The results of this work are summarised in Table II, column 5. The Py–GC–MS experiments and GC retention time data point to the products being thiirane (ethylene sulphide) and acetaldehyde for cystine and glutamic acid, respectively. With methionine, the product is ethenyl methyl sulphide as deduced from the Py–GC–MS measurements only. (The substance was not available for confirmation by retention time studies.) In the Py–GC–MS studies with proline and leucine pyrolysis products with GC retention times greater than about 1 min did not appear to reach the mass spectrometer. (Changing the

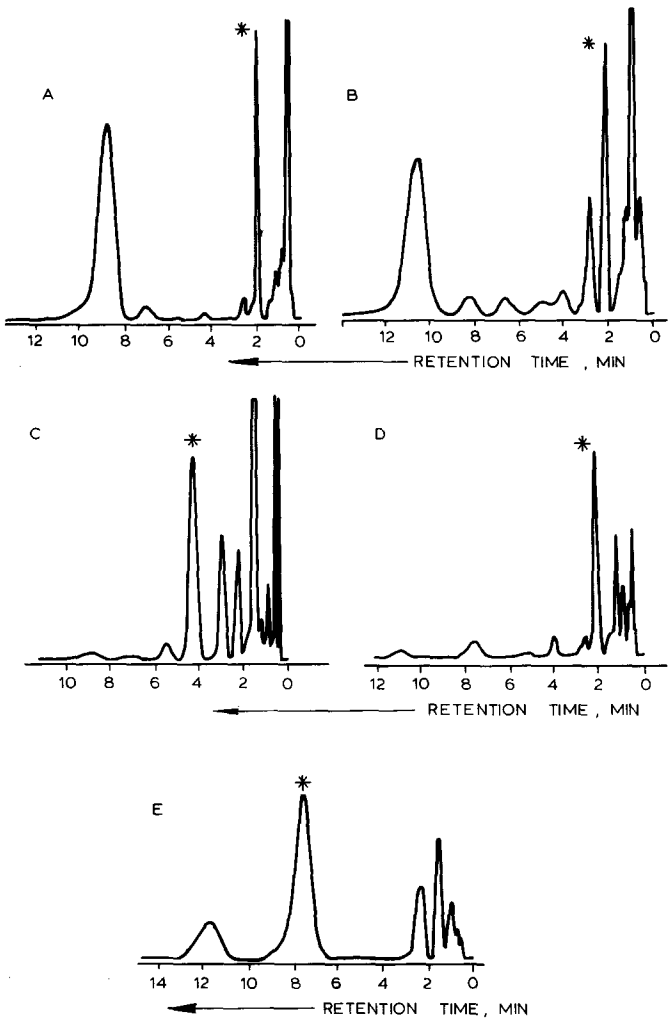

Fig. 2. Pyrograms obtained under optimum conditions on thin-layer substrate. Peaks chosen for quantitative measurement are marked by asterisks. A, proline; B, glutamic acid; C, methionine; D, cystine; E, leucine.

physical conditions of the sample to be pyrolysed as described in the experimental section did not lead to any useful mass pyrogram.) This result would suggest that the high concentration of fragments in these experiments, relative to that in the Py–GC studies leads to polymerisation or other secondary reactions which efficiently remove volatile components before the detector is reached. Retention time data suggest that the product measured following pyrolysis of proline may be one or both isomers of *n*-butene. In the case of leucine, the product determined remains unknown.

When amino acid mixtures were subjected to TLC, separated zones were located using iodine vapour as revealing agent. Mean R_F values for solvent travel of 10 cm on the chromatogram were 0.07, 0.32, 0.43, 0.24 and 0.53 for cystine, glutamic acid, methionine, proline and leucine, respectively. Neither chromatographic devel-

TABLE II

DATA RELEVANT TO THE DETERMINATION OF EACH AMINO ACID PYROLYSED ON THIN-LAYER SUBSTRATE

Amino acid	*Sample range (μg)*	*Limit of detection (μg)*	*Maximum sampling rate ($hour^{-1}$)*	*Product determined*
L-Cystine	0.2–3.0	0.1	5.0	Thiirane
L-Glutamic acid	0.4–2.0	0.4	4.6	Acetaldehyde
L-Methionine	0.2–2.7	0.1	6.0	Ethenylmethyl sulphide
L-Proline	0.4–3.0	0.3	5.0	*n*-Butene?
D,L-Leucine	0.2–3.0	0.1	4.3	?

opment nor use of the revealing agent had an effect on the yield of pyrolysis product chosen for the determination of each amino acid. The lower limit of determination of a particular amino acid is set by the limit of detection (Table II) of the zone on the thin-layer chromatogram as found in the work with the vitamins[2]. The rate at which determinations can be made depends on the retention times of the least volatile pyrolysis product; sampling rates, estimated on this basis are set out in Table II. Sample ranges over which each of the five amino acids are known to give a response directly proportional to the amount of sample are also set out in Table II. For each amino acid in these ranges, reproducibility was good; relative errors of 3% or less were attained in the middle of each range with careful working and attention to experimental conditions as discussed in Part II[2].

For the determination of these amino acids separated by TLC from each other or from other substances, the technique presented here possesses some useful advantages. Because of the good reproducibility it can provide reliable results without the need for several replicate determinations as may be the case in applying the elution technique or densitometry when accurate results are required[4]. Errors arising from losses by elution and subsequent separation from thin-layer substrate are avoided as is the problem of finding a solvent compatible with the method of determination. Under conditions strictly specified the pyrogram of an amino acid can be regarded as a fingerprint. This can be compared with pyrograms from separated components and thus provide information about the character and quality of the substance in the thin-layer zone. The amino acids examined in this work have been separated from each other and from many substances by thin-layer methods[3,5]. Where such separations are possible on silica gel and probably other as yet untested stationary phases, it follows that the method of determination described here can be used. However, where clean-up of sample and derivatisation are readily accomplished the technique offered here is, of course, not competitive as it stands with GC, GC–MS or liquid chromatographic analysis, particularly where many amino acids are to be determined in a single sample, *e.g.* protein hydrolysate. It can be expected to be of more use where TLC would offer a convenient separation of one or a few named amino acids from a complex mixture of substances without resort to elaborate sample pre-treatment or if an independent method was required for comparative non-routine purposes.

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