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PYROLYSIS–GAS CHROMATOGRAPHY OF SEPARATED ZONES ON THIN-LAYER CHROMATOGRAMS

II. APPLICATION TO THE DETERMINATION OF SOME WATER-SOLUBLE VITAMINS

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SUMMARY

Using the furnace and gas chromatograph described previously, it is shown that amounts down to *ca.* 0.2 μg of each of the vitamins B_5, B_6 and C, separated from each other by thin-layer chromatography, can be determined with a relative error of 2–3%. Advantages and limitations of the technique as applied to quantitative thin-layer chromatography are discussed.

INTRODUCTION

The pyrolysis–gas chromatographic (Py–GC) technique described in Part I[1] has been applied to the determination of calcium D-pantothenate (vitamin B_5), pyridoxine hydrochloride (vitamin B_6) and L-ascorbic acid (vitamin C). Taking solutions of each vitamin separately, suitable conditions for pyrolysis and separation of volatile pyrolysis products by GC have been determined. Then it is shown that each vitamin can be determined by the Py–GC technique while adsorbed on a thin-layer stationary phase which may or may not have undergone chromatographic development. Mixtures of vitamins are also determined following their separation by thin-layer chromatography (TLC). Advantages and limitations of the Py–GC technique revealed by this work are discussed. GC coupled with mass-spectrometry (MS) was used as an aid to identification of the pyrolysis products on which determination of each vitamin was based.

EXPERIMENTAL

Chemicals and solutions

The vitamins were obtained from Sigma (St. Louis, MO, U.S.A.) and reagent-grade solvents from Fisons (Loughborough, Great Britain). Standard solutions of each vitamin were prepared in double distilled water; they contained either 0.100

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$\mu g/\mu l$ or 1.00 $\mu g/\mu l$ of the appropriate vitamin. Mixtures, in which the concentration of each vitamin was from 0.1 $\mu g/\mu l$ to 1.0 $\mu g/\mu l$, were also prepared in water. The solutions were kept cool and in the dark when not in use and renewed every few days.

Thin-layer plates

The plates were prepared in our laboratory from silica gel G (Merck, Darmstadt, G.F.R.) on a backing of aluminium (0.1 mm thick) sheet (British Aluminium, London, Great Britain). Before coating, the aluminium sheets were heated at 600°C for 12 h to remove any surface organic matter. A coating 0.25 mm thick was used. The plates were dried and activated at 100°C for 30 min before use.

Apparatus

The furnace for pyrolysis and the gas chromatograph including the column are described in Part I. A Pye-Unicam series 104 chromatograph connected through a flow-splitter membrane separator to an AEI MS 20 mass spectrometer (GEC-AEI (Electronics), Manchester, Great Britain) was used in the GC–MS studies. The furnace for pyrolysis was connected to the top of the GC column which was the same as that used in Py–GC.

Thin-layer chromatography

The solvent system for separation of mixtures of the vitamins was glacial acetic acid–acetone–methanol–benzene (5:5:20:70, v/v) ternary mixture as used by Gaenshirt and Malzacher[2]. The development was by ascending mode in a closed tank saturated with solvent vapour at 25°C. The developed plates were air-dried and a revealing agent was applied; iodine vapour was used to locate vitamins B_6 and C and after their zones were removed, ammoniacal silver nitrate (0.1–0.5 *M*) was sprayed on the plate to reveal vitamin B_5.

Pyrolysis–gas chromatography

Known amounts of each vitamin in solution or adsorbed on thin-layer substrate were introduced into the pyrolyser hot-zone, and the volatile products of pyrolysis were swept onto the chromatography column by the nitrogen carrier gas, flow-rate 50 ml/min. The separate procedures for liquid or solid sample introduction and the furnace modifications to accommodate either type are described in Part I. The solid samples for pyrolysis were prepared in one of two ways.

(1) Standard solution of vitamin was spotted on to a small square (*ca.* 6 × 6 mm) of thin-layer plate (silica gel G with aluminium backing) which was then air-dried before insertion into the furnace.

(2) Standard solutions of mixtures of the vitamins were separated by TLC as described above, the zones located by the appropriate revealing agent and each zone punched out from the dried plate as a disc, 6 mm in diameter, using a lever-operated punch.

Pyrolysis–gas chromatography with mass spectrometric detection

Each vitamin (*ca.* 5 mg) was introduced into the furnace in solid form in a shallow aluminium cup (6 mm diameter, 1 mm deep). The pyrolysis temperature depended on the compound and was that considered to be optimum in the Py–GC

studies. The carrier gas was helium, flow-rate 50 ml/min, and the gas chromatograph oven temperature was 180°C. The mass separator was maintained at 200°C, and the mass range 20–200 was scanned. The electron impact energy was 68.2 eV and the current 50 μA.

RESULTS AND DISCUSSION

Pyrograms obtained by direct injection of aqueous solutions differ from those adsorbed and dried on adsorbent for TLC (Fig. 1). However, with the present experimental arrangement and test substances the major differences in the number of resolved peaks and their relative areas is largely confined to products with short retention times, *i.e.* less than *ca.* 2 to 3 min. The differences among the products are partly due to differences in the amount of water present and its accessibility to the decomposing compound. Water will produce a peak of low retention time on a Porapak Q column but it may also, through interaction with reactive intermediate pyrolysis products, give rise to others. Pyrolysis products from vitamin C with retention times greater than 3 min appear to be more affected by the change in form of sample introduction, perhaps partly due to a more significant role for water in its pyrolysis. However, the yields of all substances undergoing pyrolytic decomposition can be expected to be a function of heat-up time[3]. This in turn is determined by the thermal conductivity of the material introduced into the furnace. Fortunately any differences in this property of the system due to changes in the amount of adsorbed substance to be determined on thin-layer substrate are negligible at the microgram level. (The same is true for the substance introduced in liquid form if the volume of solvent is constant.)

From the GC–MS studies, the products of pyrolysis, on which quantitative measurement by Py–GC was based, were identified as isobutanal, acetaldehyde and acetic acid for vitamins B_5, B_6 and C, respectively. Confirmation was obtained by checking their retention times in the Py–GC apparatus. However, from the pyrograms for vitamin C it is evident that the acetic acid peak contains a second, only partially resolved, still unknown, component. Fortunately its presence is not deleterious to the determination of the vitamin using the peak height.

The experiments conducted with standard solutions provide a convenient means of finding the optimum conditions for pyrolysis of a specified compound so as to maximise the yield of a well-resolved pyrolysis product in the gas chromatograph. This is done by studying the change in yield as the furnace temperature is altered by means of the Variac controller. Carrier gas flow-rate, column packing and oven temperature are all amenable to convenient study by this method. Optimum operating conditions thus found for the determination of each vitamin are set out in Table I. Furthermore the range of weights of substance to be determined can readily be delineated and the relation between peak height or area and amount established. In this way the data collected in Tables II–IV were obtained for the three vitamins. A good rectilinear relation exists between peak height and amount of each vitamin, and the standard deviations indicate good reproducibility for replicate determinations.

Results of experiments in which the vitamins have been pyrolysed on thin-layer substrate are collected in Tables V–VII. It is seen that they are not significantly influenced by the history of the vitamin on the thin-layer substrate. The determi-

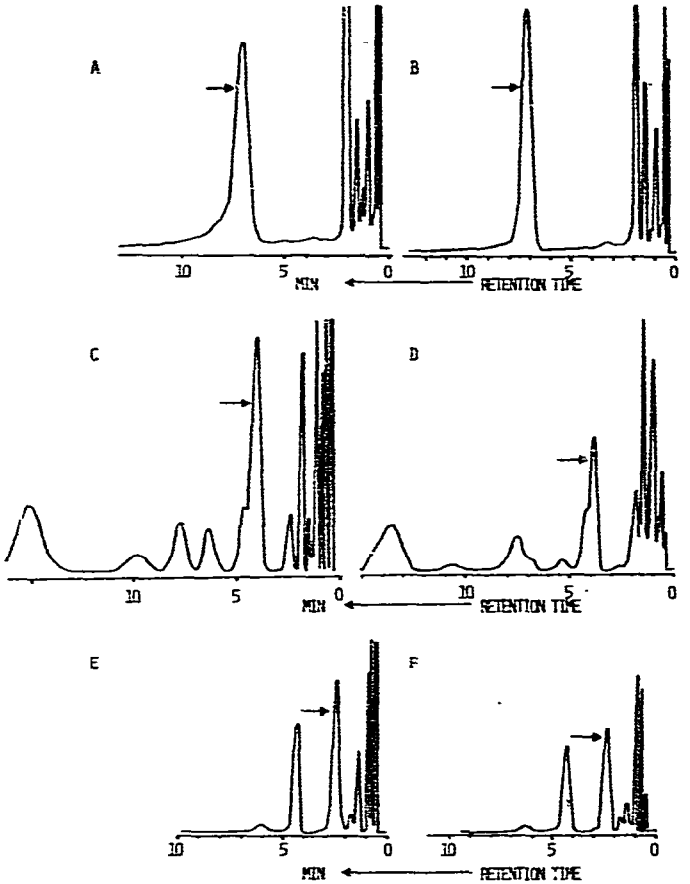

Fig. 1. Pyrograms for samples of calcium D-pantothenate (A and B), L-ascorbic acid (C and D) and pyridoxine hydrochloride (E and F). A, C and E were obtained for samples introduced in aqueous solution; B, D and F for samples adsorbed on thin-layer substrate. The peaks marked by arrows represent those used for measurement purposes.

nations carried out on developed chromatograms relate to mixtures of the vitamins separated by TLC before pyrolysis. (The mean R_F values for vitamins B_5, B_6 and C were 0.55, 0.11 and 0.35, respectively, for solvent travel of 10 cm.) In these experi-

TABLE I

CONDITIONS AND DATA RELATING TO THE DETERMINATION OF THE VITAMINS BY Py–GC

Vitamin	*Optimum pyrolysis temp. (°C)*	*GC oven temp. (°C)*	*Product* determined*	*GC retention time (min)*
B_5	580	150	Isobutanal	7.2
B_6	580	180	Acetaldehyde	2.1
C	530	150	Acetic acid	3.9

* Pyrolysis product on which the determination of the vitamin is based.

TABLE II

PYROLYSIS OF CALCIUM D-PANTOTHENATE USING THE DIRECT INTRODUCTION PROCEDURE FOR SOLUTIONS

For operating conditions see Table I.

Sample weight (μg)	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	16	0.6	3
0.4	29	0.5	8
0.6	46	1.2	7
0.8	59	0.8	6
1.0	74	0.9	8
1.4	102	2.1	6
1.8	135	0.6	3
2.0	148	1.4	8
2.5	183	1.0	3
3.0	223	0.6	3

ments the vitamins were located on the chromatogram using a revealing agent, and in each instance it can be concluded that the latter did not affect the yield of the pyrolysis product that was made the basis of the determination. The standard deviations obtained from replicate determinations indicate that good reproducibility can be expected over the sample ranges investigated. The day-to-day reproducibility depends on careful control of all operating temperatures and gas flow-rates including, of course, those to the flame ionisation detector (FID), and it can be checked by injection of a standard mixture such as methanol–isopropanol into the furnace[3]. The main sources of experimental error then relate to sample handling and volumetric transfer of solutions to the thin-layer plates.

Introduction of a sample on a solid support into the furnace takes *ca.* 30 sec and *ca.* 2.5 min is needed to restore the baseline of the detector output signal; hence

TABLE III

PYROLYSIS OF PYRIDOXINE HYDROCHLORIDE BY INJECTING STANDARD AQUEOUS SOLUTION INTO THE FURNACE

For operating conditions see Table I.

Sample weight (μg)	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	25	0.0	3
0.4	51	0.3	3
0.6	72	0.3	3
0.8	100	0.6	3
1.0	128	2.6	8
1.5	172	1.7	8
2.0	236	2.6	8
2.5	296	3.4	5
3.0	352	4.1	8

TABLE IV

PYROLYSIS OF VITAMIN C BY INJECTION OF AQUEOUS SOLUTIONS INTO THE FURNACE

For operating conditions see Table I.

Sample weight (μg)	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	16	0.6	6
0.4	33	1.4	8
0.6	47	2.2	8
0.8	64	1.4	10
1.0	82	1.2	10
1.2	96	1.2	3
1.4	113	2.3	3
1.6	129	1.5	3
1.8	144	1.5	3
2.0	160	0.8	5
2.5	201	3.0	3
3.0	241	2.5	3

these operations are not particularly time-consuming. The next sample preferably should not be introduced into the pyrolyser until the least volatile product has passed through the detector, otherwise valuable qualitative information from the profile of pyrolysis products (pyrogram) may be lost. Thus the retention time of the least volatile pyrolysis product will determine the sampling rate, which would be *ca.* 5, 6 and 3 per hour for vitamins B_5, B_6 and C in that order. Sometimes more than one peak in the pyrogram can be made the basis of the determination, *e.g.* for vitamins B_6 and C, and the ratios can be used as a check on the purity of the zone from the thin-layer chromatogram.

The sensitivity of the method is dependent on the limit of detection of the

TABLE V

PYROLYSIS–GAS CHROMATOGRAPHY OF CALCIUM D-PANTOTHENATE ON THIN-LAYER SUBSTRATE WITH AND WITHOUT CHROMATOGRAPHIC DEVELOPMENT

For operating conditions see Table I.

Sample weight (μg)	*Without development*			*With development*		
	Mean peak height (mm)	*Standard deviation (mm)*	*Number of measurements*	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	25	1.0	3	26	1.5	3
0.5	69	1.2	4	69	1.2	3
0.8	110	2.5	5	113	2.6	3
1.0	138	1.5	3	139	1.5	3
1.3	181	4.6	4	180	5.5	3
1.7	236	5.6	3	238	4.9	3
2.0	275	5.5	3	275	4.0	3

TABLE VI

PYROLYSIS–GAS CHROMATOGRAPHY OF PYRIDOXINE HYDROCHLORIDE ON THIN-LAYER SUBSTRATE WITH AND WITHOUT CHROMATOGRAPHIC DEVELOPMENT

For operating conditions see Table I.

Sample weight (μg)	*Without development*			*With development*		
	Mean peak height (mm)	*Standard deviation (mm)*	*Number of measurements*	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.2	27	0.6	3	28	0.6	3
0.4	46	1.0	3	45	1.5	3
0.6	66	1.0	4	66	0.8	4
0.8	90	1.5	3	90	1.7	3
1.0	115	1.3	7	114	2.0	3
1.5	177	2.5	4	177	3.1	3
2.0	230	2.2	7	230	3.1	3
2.5	289	3.5	5	288	3.5	3
3.0	341	3.4	4	340	4.5	3

separated constituent of a mixture on the thin-layer chromatogram; this, in turn, depends on the chemical revealing agents or physical methods available. The preferred method will generally be a physical one such as fluorescence in UV light. A chemical revealing agent must be such that it will not undergo pyrolysis to give products which would obscure those from the substance to be determined.

The technique described here may be compared with long-standing techniques for quantitative TLC based on elution or *in situ* densitometric scanning procedures. Problems associated with reproducible, preferably quantitative, elution of the zone from the chromatogram in a state of purity are avoided and unlike densitometry, the surface uniformity and grain density of the thin-layer are unimportant[4]. Further-

TABLE VII

PYROLYSIS–GAS CHROMATOGRAPHY OF VITAMIN C ON THE THIN-LAYER SUBSTRATE WITH AND WITHOUT CHROMATOGRAPHIC DEVELOPMENT

For operating conditions see Table I.

Sample weight (μg)	*Without development*			*With development*		
	Mean peak height (mm)	*Standard deviation (mm)*	*Number of measurements*	*Mean peak height (mm)*	*Standard deviation (mm)*	*Number of measurements*
0.1	15	0.5	4	13	1.5	3
0.3	40	1.0	4	40	0.6	3
0.5	63	2.1	6	64	1.0	3
0.8	106	0.8	4	106	1.1	3
1.0	127	2.2	4	128	1.5	3
1.4	179	3.1	4	179	3.6	3
2.0	260	3.4	6	260	5.0	3

more, a single measurement can give a result of good accuracy whereas replicate determinations are needed to give mean values of comparable reliability in applying methods based on elution or densitometry. This can be ascribed, at least in part, to the mode of measurement which is based on the total constituent and not just a fraction of it as in reflective densitometry and in some elution methods. Two general points can be made in comparison with the Iatroscan instrument[5,6] in which TLC is carried out on adsorbent-coated thin silica rods and the developed rods are passed through a hydrogen flame to produce gaseous decomposition products which are determined by the FID principle. The rods have a very low loading capacity so that sample loading can present problems not experienced in operating the technique described here. Furthermore, unlike the present technique, the detector is non-discriminating with respect to the pyrolysed substance relying entirely on TLC for discrimination.

Apart from restrictions on location of zones on developed chromatograms, the main additional limitations to general application of the technique described here relate to materials for TLC and to the need to generate at least one volatile pyrolysis product. Backing plates, stationary phases and binding agents for TLC made from organic substances obviously cannot be used. Materials for backing plates are further restricted to those readily cut with minimum disturbance to the supported thin layer. However, thin-layer material could be removed mechanically, but less conveniently, from a glass backing to a spoon device for insertion into the furnace. Lack of suitable volatile decomposition products produced by pyrolysis might be overcome by supplementary use of suitable chemical reagents, *e.g.* oxidising agents, as an aid to break-up of stable molecules but, as yet, this possibility remains unexplored.

The proposed technique uses a furnace of simple construction to interface TLC and GC so that a quantitative analysis can be performed on mixtures containing involatile constituents for which either GC or Py–GC alone would be insufficient. Its application is illustrated by the separation and determination of vitamins B_5, B_6 and C and it follows that it should be applicable also to the determination of one or all of these substances in any matrix provided each can be separated[7] from other constituents of the sample by TLC.

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