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A TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHIC TECHNIQUE FOR THE RESOLUTION OF MONOCARBONYL DINITROPHENYLHYDRAZONES

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SUMMARY

Carbonyl dinitrophenylhydrazones have been separated by class (alkanone, alkanal, alk-2-enal and alka-2,4-dienal) using adsorption thin-layer chromatography and on the basis of chainlength using partition techniques. A two-dimensional technique is now described in which the two separations can be effected on one plate. The problem that initial class separation demands a small sample to prevent overloading, yet secondary chainlength separation following Carbowax 400 impregnation demands a large sample to allow facile detection, has been solved by employing a novel chromatographic concentration step between separations.

A complete separation can be achieved during one working day.

INTRODUCTION

Since carbonyl compounds, formed as a result of autoxidation, contribute significantly to the flavour and aroma of fats and fat-containing foods, their generation, isolation and analysis in such systems has been extensively studied. Amongst the methods used in these studies, thin-layer chromatography (TLC) of the 2,4-dinitrophenylhydrazones (DNPs) has been extensively exploited as it offers the possibility of quick and easy fractionation of extremely small quantities of the isolated derivatives.

Although ONOE[1] and later DHONT AND DE ROOY[2] had used TLC to separate the DNPs of a limited range of carbonyl compounds, it was not until 1963 that SCHWARTZ AND PARKS[3], BADINGS AND WASSINK[4] and URBACH[5] employed TLC for the examination of the wide variety of carbonyls formed in autoxidizing lipids.

SCHWARTZ AND PARKS[3] proposed the use of magnesium oxide to resolve DNPs into classes by adsorption chromatography. BADINGS AND WASSINK[4] proposed class separation by a zinc carbonate adsorption technique or complex formation by silver

nitrate impregnated kieselguhr; for chainlength separation they proposed partition between Carbowax 400 and petroleum ether, amongst other systems. For resolution of complex mixtures into individual components, they used a three stage separation, recovering groups of components by solvent elution of selected bands for re-running in the subsequent systems. URBACH[5] achieved class separation by adsorption chromatography on alumina or on silver nitrate impregnated plates, and chainlength separation by partition on 2-phenoxyethenol impregnated plates. She employed two-dimensional systems for resolution of complex mixtures.

In addition to these papers, many others have appeared advocating different adsorbents or partition systems[6], application to other carbonyl types[7–13] and improvements in technique[14], but fundamentally, it remains true that classes are separated by adsorption and chainlengths by partition chromatography.

In this laboratory, the most satisfactory techniques investigated have been magnesia adsorption chromatography for class separation, and Carbowax 400 partition chromatography for chainlength separation.

A two-dimensional technique is described in which separation is effected first by class and then, after impregnation with Carbowax, on a chainlength basis. The problem that a small sample must be applied in the first dimension (to prevent overloading) and that a large sample is required for the second dimension (to allow facile detection) has been solved by employing a chromatographic concentration step between separations.

EXPERIMENTAL

Materials

Magnesium oxide (B.D.H.)
Microcell T 38 (Johns Manville)
Carbowax 400.
Solvents: petroleum ether 40–70°, SVR and chloroform, distilled to remove non-volatile residue.
TLC Sample Streaker (Applied Science Laboratories)
TLC spreader and associated apparatus (Desaga)

Preparation of TLC plates

The magnesium oxide used for the adsorbent layer is dried overnight at 110°. Magnesium oxide (20 g) and Microcell T 38 (20 g) are slurried with water (120 ml) and rolled in a ball mill for ½ h to ensure intimate mixing and grinding of coarse particles. The slurry so prepared is sufficient to coat five plates 200 × 200 mm. The plates are air-dried for 1 h, activated for 1 h at 60° and then stored until required in a desiccator containing freshly dried silica gel.

Development of chromatogram

Fig. 1 represents the various stages of development of the chromatogram. The sample is applied as a streak running longitudinally in the direction of spreading of the adsorbent layer, *i.e.*, the first solvent is run at right angles to the spreading direction. The sample streak is applied at least 5 cm from the bottom of the plate, commencing 4 cm from the left hand edge and terminating 3 cm from the right hand edge. The

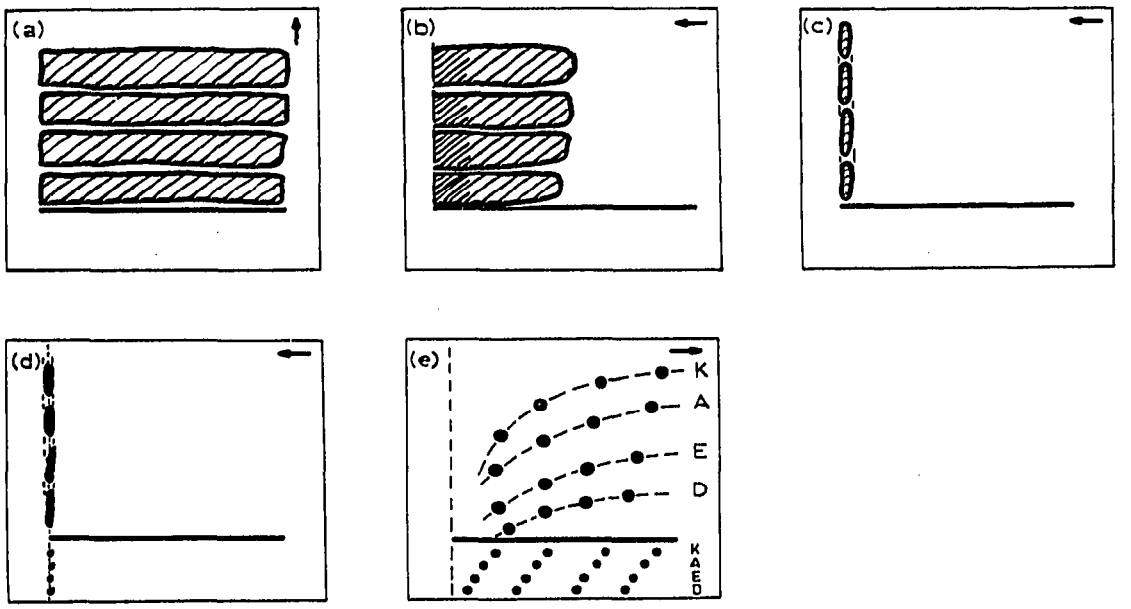

Fig. 1. Diagrammatic representation of the two-dimensional separation of dinitrophenylhydrazones (direction of solvent travel indicated by arrow). (a) Stage 1, class separation; (b) Stage 2, first concentration; (c) Stage 3, second concentration; (d) Stages 4 and 5, impregnation and marker application; (e) Stage 6, chain length separation. K = ketones; A = anals; E = enals; D = dienals.

plate is immediately developed in a tank lined with filter paper saturated in the developing solvent (petroleum ether–chloroform (85:35). The solvent is allowed to rise for approximately 12 cm after which the plate is removed and the solvent front marked by small nicks at the edges.

For the second development, the right hand edge becomes the bottom of the plate and the separated bands are chromatographed in a solvent consisting of chloroform–SVR (80:20). Development is continued until the solvent front just reaches the top limit of the original streak. After brief drying, the plate is developed a second time in the same direction in the same solvent. As all DNPs have nearly unit R_F value in this solvent, they are now all concentrated into fairly compact bands near the top of the plate. A third development is given in the same direction in a solvent consisting of 20% Carbowax 400 in chloroform, development being continued until the solvent front moves just past the line of DNPs. This treatment serves to impregnate the plate with stationary phase and further compacts the DNP bands.

After brief air drying, mixtures of known DNPs are spotted on to the 5 cm section below the original sample streak, the plate is turned through 180° and the chainlength resolution effected by development in petroleum ether. Fig. 2 shows a typical separation achieved.

DISCUSSION

Whilst the technique described appears complex, there is really little actual work involved; the whole fractionation can be completed within one working day and, provided certain precautions are observed, the separations achieved are remarkably clear.

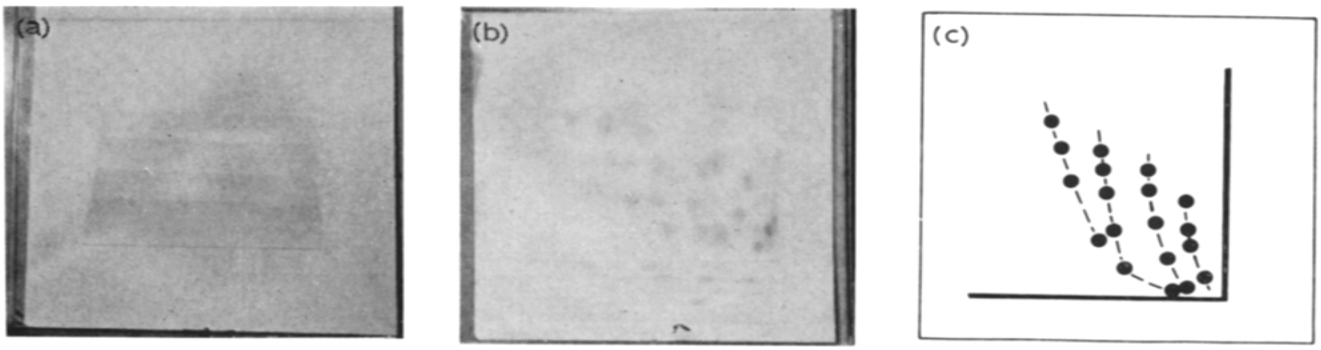

Fig. 2. TLC separation of dinitrophenylhydrazones. (a) Stage 1, class separation; (b) Stage 6, final chromatogram of 19 DNPs; (c) diagram of (b). From left to right: ketones, $C_{3,5,7,11}$; anals, $C_{1,3,5,7,9,11}$; enals, $C_{3,5,7,9,11}$; dienals, $C_{6,8,9,11}$.

The main requirement for success is that the DNP classes must be well separated in the first dimension, and the bands produced must be as near to linear as possible. The most important factors involved in the class separation are careful preparation of the absorbent, even coating of the plate and sample application in a narrow streak of uniform concentration along the length of the streak. This latter factor is important since the R_F value of the various classes is influenced by concentration, a high concentration leading to a higher R_F value. Quite spectacular improvement in clarity of class resolution can be achieved by applying a thin (1 mm) even line of sample and, to achieve this ideal, the use of a mechanical streaker has been found to be virtually mandatory.

The maintenance of linear bands during the first development appears to be dependent upon two factors, *viz.* the use of a filter paper lining to the solvent tank to ensure rapid equilibration of the plate with the solvent atmosphere prior to development, and application of the sample streak at least 5 cm above the bottom of the plate. Such sample applications ensure an appreciably lower rate of flow of solvent by the time it reaches the sample, and thus allow a more even development. When the sample was applied closer to the plate edge, the separated bands always were curved, and usually of higher R_F value near the edges of the plate. Moreover, the 5 cm sample-free band is useful in that it is later used for the application of known DNPs to assist identification of the unknown spots.

Usually adequate class separation can be achieved with one solvent development. Occasionally, the adsorbent activity tends to be too high with resultant lower R_F values and lesser separation. A second development can serve to improve resolution.

For reasons unknown, overnight drying of the magnesium oxide was found to reduce the tendency of the spots to tail during the chainlength separation. With this exception, there appears to be no other critical factor which must be observed to effect clean fractionation once the class separation has been achieved.

In the interest of speed, it has been found convenient to use a large volume of Carbowax solution during the impregnation step and so start the solvent travel from well up the plate. This is possible because two developments with chloroform–alcohol move the DNPs nearly to the desired starting line.

Separation into classes is not absolute. Whilst, in general, the R_F values decrease in the order ketones, alkanals, alk-2-enals and alka-2,4-dienals, the shortest chain-

length members have R_F values approximately equal to that of one class lower, *i.e.* acetone DNP runs with the alkanals etc. Likewise, the chainlength separation is to some extent influenced by the class. However, after two-dimensional separation there are no compounds inseparable within the chainlength limitations of the Carbowax plate. Homologues fall along a smooth curve.

With the impregnation technique described it is possible to separate compounds differing by one carbon atom up to 9 for alkanones, 10 for alkanals, 12 for alk-2-enals and 12 for alka-2,4-dienals. No doubt the limit for chainlength separation could be increased somewhat by applying a more concentrated Carbowax solution. At the other end of the scale, the resolution of the short chain members can be increased if necessary by running the plate a second time in petroleum ether. Qualitative identification remains facile because the markers on the side of the plate receive the same treatment.

Sensitivity of detection can be increased by spraying with a solution of 10% potassium hydroxide in 80% aqueous ethanol. This offers the further advantage that the classes develop different colours, *viz.* alkanones—grey-brown to brown, alkanals—brown to reddish brown, alk-2-enals—pink-brown and alka-2,4-dienals—mauve.

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REFERENCES

1 K. ONOE, *J. Chem. Soc. Japan, Pure Chem. Sect.*, 73 (1952) 337; *C. A.*, 47 (1953) 3757d.
2 J. H. DHONT AND C. DE ROOY, *Analyst*, 86 (1961) 74.
3 D. P. SCHWARTZ AND O. W. PARKS, *Microchem. J.*, 7 (1963) 403.
4 H. T. BADINGS AND J. G. WASSINK, *Neth. Milk Dairy J.*, 17 (1963) 132.
5 G. URBACH, *J. Chromatog.*, 12 (1963) 196.
6 L. M. LIBBEY AND E. A. DAY, *J. Chromatog.*, 14 (1964) 273.
7 E. F. L. J. ANET, *J. Chromatog.*, 9 (1962) 291.
8 W. Y. COBB, *J. Chromatog.*, 14 (1964) 512.
9 D. P. SCHWARTZ, M. KEENEY AND O. W. PARKS, *Microchem. J.*, 8 (1964) 176.
10 W. Y. COBB, L. M. LIBBEY AND E. A. DAY, *J. Chromatog.*, 17 (1965) 606.
11 G. A. BYRNE, *J. Chromatog.*, 20 (1965) 528.
12 P. W MEIJBOOM, *J. Chromatog.*, 24 (1966) 427.
13 H.-J. STAN AND J. SCHORMÜLLER, *J. Chromatog.*, 43 (1969) 103.
14 D. P. SCHWARTZ, J. SHAMEY, C. R. BREWINGTON AND O. W. PARKS, *Microchem. J.*, 13 (1968) 407.