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CLASS SEPARATION OF FLAVOUR VOLATILES BY LIQUID CHROMATOGRAPHY ON SILICA GEL AT 1°

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SUMMARY

A simple technique is described for the fractionation of complex flavour mixtures by liquid chromatography on silica gel using low boiling solvents at 1°. Its application to the examination by combined gas chromatography–mass spectrometry of the volatiles from peas and bananas is outlined.

INTRODUCTION

The use of the gas chromatograph directly coupled to a fast-scanning mass spectrometer has, in recent years, become the principal technique for the identification of volatile food flavour constituents. However, it is now apparent that the complex nature of volatile food flavours is such that, even with the customary use of high resolution capillary columns, a considerable proportion of the flavour constituents is not fully resolved by the gas chromatograph, and mixed mass spectra result. Only in those cases where the spectra are especially characteristic can mixed spectra be interpreted, and at best the resulting evidence is by itself not completely acceptable for the unequivocal identification of the components. Unless components of a mixture are first fully resolved by the gas chromatograph the full potential of the combined use of the gas chromatograph and the mass spectrometer is not being realised.

Greater separation of components prior to their mass spectral scanning is obviously required and in this laboratory has been effected in two ways: (a) by pre-fractionation of the mixture into classes by liquid chromatography and (b) by the development of a workable system to transfer "single peaks" from one high resolution column to another of different polarity[1]. The first approach has been employed in current investigations of the volatiles from frozen peas and bananas.

Alcohols were found to predominate in the volatiles from peas[2], but their identification by combined gas chromatography–mass spectrometry (GC–MS) was made difficult by the presence of minor unresolved non-alcoholic components. The preliminary separation of the alcohols as a group required a simple technique capable of handling the small amount of volatiles available from peas. Thin layer chromatography (TLC) at low temperature, as suggested by STAHL[3], proved unsatisfactory due to the loss of the more volatile constituents (boiling below approximately 100°).

To prevent this loss of volatiles the adsorbent was enclosed in a narrow bore plastic tube, which could be readily sectioned to recover the fractions. The literature shows that flexible tubing (cellophane[4–6] and teflon[7]) has been used previously to contain the adsorbent and has also been sectioned to recover the separated zones[5,6]. Techniques have been described which use dry columns of adsorbent developed by the solvent front moving horizontally[6], or vertically upwards[8] or downwards[9].

To minimize the loss of the more volatile components, the use of low boiling solvents was considered vital. Monofluorotrichloromethane (b.p. 24.7°) and methyl ethyl ether (b.p. 7.9°) were chosen for column development and the elution of fractions respectively, but the two were also mixed for development. The temperature of operation was reduced to 1°. Since the risk of contamination was very real in view of the small amounts of material involved, teflon tubing was chosen, and a grade of neutral silica gel was selected with a negligible content of extraneous matter[10].

EXPERIMENTAL

Glassware

All glassware was cleaned by chromic acid mixtures, rinsed with distilled water and oven dried.

Solvents

Since methyl ethyl ether was not readily available commercially it was prepared as follows: Ethyl chloride (300 ml) and sodium methoxide in methanol (sodium 115 g in methanol 1500 ml) were charged at 0° into a stirred stainless steel bomb which was slowly heated. When the reaction began at 55° the heating was discontinued and the temperature rose to a maximum of 140°. The reaction mixture, cooled to —20°, was transferred to a 3 l flask, from which the crude ether was distilled through a reflux condenser at 20° to reduce the carry over of methanol, and thence to a further condenser cooled by solid carbon dioxide. The ether was redistilled through a column 20 in. by 0.6 in. packed with 1/8 in. glass helices. The distillate containing about 1 % methanol was stood over calcium chloride overnight and the remaining traces of methanol were removed by percolation through a column of Linde Molecular Sieve 4A.

Monofluorotrichloromethane ("Isceon 11" Monsanto Chemicals Aust. Ltd.) was fractionally distilled through the above column. Carbon tetrachloride, the main impurity in the commercial product, was thereby reduced to less than 10 parts per million.

Adsorbents

Silica gel (HR Merck) was partially deactivated by the addition of 25 %, w/w of water. Silver nitrate impregnated silica gel (Adsorbosil CABN, Applied Science Labs.) was stored over phosphorus pentoxide until required.

Apparatus and method

The teflon tube, 9 in. in length, 1/4 in. O.D. 3/16 in. I.D. was thoroughly washed in hot soapy water followed by distilled water and then oven dried. For support and protection from contamination the teflon tube was slid into a glass tube of similar length. The column was packed as follows: One end of the teflon tube was plugged with 1/8 in. of glass wool, a few crystals (*ca.* 1 mg) of azobenzene added above

the plug, and with gentle suction and vibration the adsorbent was added until 1 in. remained unfilled. Subsequent operations were conducted in a cold room at 1°. A charge (30–100 μl) of the flavour concentrate was added to the top of the packing, quickly followed by the remainder of the packing which was retained by a second glass wool plug.

The loaded column was inverted and stood in a beaker to which the developing solvent was added to a depth of 1/4 in. Development began when the ascending solvent front reached the 1 in. level and was complete, usually in about 4 h, when the orange colour of the azobenzene appeared in the upper glass wool plug. The column (Fig. 1A) was removed from its glass tube and cut into selected sections of 1/2 or 1 in.

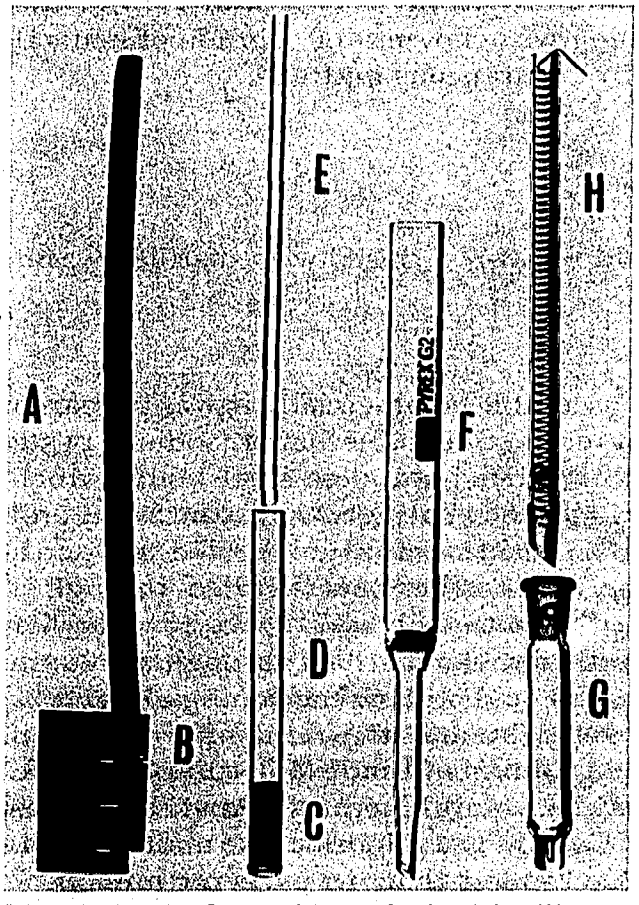

Fig. 1. (A) Teflon tube packed with silica gel; (B) template for sectioning column; (C) column section; (D) glass holder for column section; (E) extruding rod for packing; (F) filter tube for eluting fraction; (G) collection tube; (H) fractionating column.

with a scalpel and the sheet metal template (B). Cut sections (C) were transferred with forceps to glass tubular holders (D) and the packing, still wet with solvent, extruded by means of flat-ended rods (E) into sintered glass filter tubes (F). Each fraction was eluted from the adsorbent with four additions each of 0.5 ml of methyl ethyl ether, the combined washings being collected in tubes (G). The ether was distilled off through simple wetted-wall fractionating columns (H) each of which consisted of a 25 SWG Nichrome wire helix closely fitting a 5 mm I.D. glass tube. For simplicity no reflux head was employed since adequate reflux was provided by partial condensation of the ether on the column wall. The distillation rate was adjusted by the depth

of immersion of the tubes in a water bath a 25°. When distillation had ceased in about 1 h the column hold-up (about 0.1 ml) was allowed to drain back into the tube, which was then disconnected from the column. Most of the remaining solvent was driven off by careful warming with the fingers. When the amount of recovered material was small each tube was stoppered and centrifuged to gather the material in the capillary cavity in the thickened base. Fractions were stored in the same tubes at —20° until examined by GC–MS.

The technique employing silver nitrate impregnated silica gel, applied as described below, was identical to the above except that development of the column was made with a 3:5 mixture of "Isceon 11" and methyl ethyl ether.

APPLICATIONS

As a preliminary trial to examine the order of separation of five classes of compounds, 50 μl of a mixture of hexan-1-ol, pentan-1-al, heptan-2-one, ethyl hexanoate and dodecane was chromatographed on silica gel as described. Fractions recovered from 1 in. sections of the column were examined by gas chromatography and the distribution of the five compounds among the fractions was indicated to be as follows:

0–1 inch: discarded
1–2 inch: hexanol only
2–3 inch: pentanal, heptanone; hexanol
3–4 inch: heptanone, pentanal
4–5 inch: heptanone, pentanal (small)
5–6 inch: ethyl hexanoate, heptanone (small)
6–7 inch: ethyl hexanoate only
7–8 inch: nil
8–9 inch: dodecane.

The result showed a complete separation of three of the components. Hexanol overlapped slightly with pentanal and heptanone which separated only slightly, the ketone running ahead and becoming the major component in the 4–5 in. fraction. The overlap between ketone and ester appeared to be slight. The hydrocarbon obviously ran with the solvent front.

The above result was sufficiently encouraging to immediately apply the technique to actual flavour mixtures. Experience with such mixtures has shown that the resolution between classes is improved if the column is not heavily loaded. A charge of 30 μl is considered the optimum for 3/16 bore columns as used above.

Pea volatiles

As indicated above the technique was developed primarily to aid the identification by GC–MS of the alcoholic constituents of the volatiles from frozen peas. The total pea volatile alcohols were separated as a fraction on silica gel essentially as described. Their spectra then showed removal of the interfering background, which was found to be due largely to a range (C_{10}–C_{15}) of branched and straight chain paraffins (also separated as a fraction).

Although this pre-separation of the alcohols served to strengthen greatly the mass spectra evidence for most of the saturated alcohols and for *cis*-hex-3-en-1-ol, it

also revealed the presence of other unsaturated alcohols, many only in trace amounts and some with peaks incompletely resolved. In view of their possible role in contributing to the overall pea flavour, stronger evidence for their identification was essential. Their further separation from the much more abundant saturated alcohols was therefore attempted using silver nitrate impregnated silica gel as the adsorbent. The total alcohol fraction above was chromatographed on silver nitrate–silica gel as described. This effectively spread the alcohols between eight fractions, the examination of which by GC–MS resulted in the positive identification of nine unsaturated alcohols and the tentative identification of three.

The composition of the eight fractions showed that complete separation between saturated and unsaturated alcohols as classes was not fully realised, as the carbon number, the presence of methyl branches, whether the hydroxyl is primary or secondary, and whether double bonds are *cis* or *trans* influence the rate of travel on the column. However a clean separation was effected between saturated and unsaturated primary straight-chain alcohols of the same carbon number. For instance, hexan-1-ol, a major component, ran ahead of *trans*-hex-3-en-1-ol, which was followed and partly overlapped by *cis*-hex-3-en-1-ol, another major component. The above *trans* isomer was indicated in the gas chromatogram of the total alcohol fraction as a slight shoulder on the tail of the large hexan-1-ol peak. Yet in two fractions from the silver nitrate column it was fully resolved to the base line and in one of these in sufficient concentration to give a strong clear mass spectrum and an informative infra red spectrum. Similar separations were observed for saturated and unsaturated alcohols of other chain lengths.

Banana volatiles

In connection with studies in this laboratory on banana ripening the need arose for a clear knowledge of the identity and relative amounts of the volatile saturated alcohols present in the ripe fruit. Although investigation of the volatiles from banana has been extensive, the complex nature of the mixture, especially the predominance of esters, made it impossible to extract this information from the published data (reviewed by WICK *et al.*[11]). Pre-fractionation on silica gel has made the investigation of this point a relatively simple matter. A concentrate of the volatiles from ripe bananas (30 μl) was chromatographed on silica gel as described and the column divided into seven fractions. The first fraction (1–2 in.) contained exclusively the saturated alcohols, which, being relatively few in number and well resolved, presented no difficulties in identification by their mass spectra. The esters formed a broad band (2 1/2–5 in.) well resolved from the alcohols. Within this band separation of some esters according to type had taken place and in several instances the separation of two esters of identical GC retention times had been achieved. Details of this work will be published elsewhere.

DISCUSSION

In a recently published discussion on the division of flavour volatiles into groups[12], two objections to the use of thin layer or column chromatography for this purpose were: the possibility of chemical changes to sensitive compounds induced by the adsorbent, and the difficulty of removing the solvent without loss of the more

volatile components. In adopting the present technique the authors recognize the possibility of isomerization due to a double bond shift and the hydrolysis of sensitive esters. For these reasons silica gel was chosen in preference to alumina. Its extensive deactivation and use at lower than normal temperatures would both weaken any such activity. In the application of this technique to flavour mixtures no instance of chemical change due to the adsorbent has been observed. Lower esters are apparently unaffected, since both methyl and ethyl acetates were found in fractions of banana volatiles separated in this way.

With the use of low boiling solvents the loss of the lower boiling volatiles becomes a much less serious problem. Methanol is clearly recoverable in high yield. Even acetaldehyde was observed in fractions of pea volatiles.

Besides the prime advantage of making possible a greater overall resolution of flavour constituents, this separation by liquid chromatography often effects a considerable concentration of minor components in certain fractions. This is especially so when one class of compound or even a single compound predominates. In addition the classification of fractions according to their location on the column is valuable supporting evidence for identification, and may well provide the vital clue for the interpretation of mass spectra. This has been the experience in this laboratory especially in the interpretation of the mass spectra of alcohols and esters when the intensity of the molecular ion is often low.

The successful use of silver nitrate impregnated silica gel for the specific separation of unsaturated alcohols has suggested the possible use of other adsorbents with specific activity. For instance sulphur compounds are of considerable interest to the flavour chemist, but their occurrence usually as very minor components poses great difficulties in their separation and identification. Their pre-separation as a class or into several classes would appear to be possible on specific adsorbents such as weak Raney metal catalysts[13] or supports impregnated with mercury salts[14]. Possible extension of the described technique in this direction is being examined.

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