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CHEMICAL REACTIONS WITH NANOGRAM QUANTITIES OF COMPOUNDS COLLECTED FROM GAS CHROMATOGRAPHIC EFFLUENT

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SUMMARY

Structural information about compounds, which are only in nanogram quantities in a complex mixture, can be obtained with the aid of gas chromatography (GC). Individual compounds can be efficiently trapped from the GC effluent into glass capillaries and there treated with a variety of reagents before re-chromatography. Information for identification can be quickly obtained, to confirm or augment information from gas chromatography–mass spectrometry, such as presence of a functional group, position and geometry of double bonds, or conversion to a more useful derivative. The reagent solution (1 μl) is injected into the capillary, where it dissolves the trapped material, and after reaction, the whole solution is re-injected into a capillary column.

The apparatus required is very simple, consisting of nothing more expensive than a GC outlet splitter, a microlitre syringe with a fine needle and an on-column injector.

Examples are given of epoxidation of alkenes, borohydride reduction of carbonyl compounds, bromination and formation of methyl esters.

For solid substances, melting points can be determined with great accuracy. Many reactions are rapid and the transfers are near quantitative.

INTRODUCTION

Because of its resolution and sensitivity, gas chromatography (GC) is useful not only for analysis but also for detection and identification. The usual arrangement for identification is the GC–mass spectrometer, but GC–mass spectrometry (MS) does not always give sufficient information for a full identification. Mass spectrometry frequently cannot locate the position of double bonds, nor can it distinguish between *Z*- and *E*-isomers. The mass spectrum may be featureless or unable to distinguish between several isomeric structures. Reactions before chromatography, or on-column can, in very simple ways, give information about the class of compounds encountered and their functional groups, or convert them to more stable or more volatile derivatives, as well as provide some idea of molecular weight.

These advantages have been exploited in many areas of chemistry, none more so than in the study of insect substances. Various authors have described methods

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for hydrogenation[1–4], ozonolysis[2–6], epoxidation[7], reduction[8,9], hydrolysis[9] and esterification[9] on microgram samples, or have used extraction loops[10,11] to remove classes of compounds from the mixture.

We have described the methods we have used for injecting small pieces of biological tissue for GC analysis without the intervention of solvents[12], and for identifying nanogram quantities of highly volatile C_1–C_5 compounds[13,14]. The examples we have used are practical ones, drawn from our experience in the study of insect pheromones. In continuing our investigations in this field we have sought to reduce the scale of earlier methods below the microgram level and to add to the range of reactions available.

It is possible to carry out a surprising number of reactions on nanogram quantities of chromatographable compounds where the reaction is reproducible, quantitative and gives simple products. These methods, of value in pheromone studies, could have equal application in other fields where sample size is limited. We have recently described a method whereby compounds can be trapped from a mixture emerging from a GC column and made to undergo a number of reactions, such as ozolysis, reduction, epoxide cleavage, and hydrogenation, and the reaction products re-chromatographed, all without the intervention of solvent and on quantities below the nanogram level[15].

There are many other reactions which could swell the armoury of nano-scale chemistry which can only be conveniently performed in a solvent. If the usual quantity of solvent is used, it must either be evaporated off with loss of some of the solute, or only a small portion re-injected, with consequent loss of sensitivity. We therefore sought ways in which nanogram quantities of compound could be isolated from a complex mixture, caused to undergo reaction in a solvent, and the reaction products re-chromatographed for separation and identification. We find that this can be done in some cases by carrying out the reaction in 1 μl of solution inside a glass capillary and illustrate its application with some reactions we have explored.

EXPERIMENTAL

Apparatus and chromatographic conditions

The analytical work was performed with a Carlo Erba Fractovap 4160 gas chromatograph with an on-column cold injection system and a flame ionisation detector. A 25 m × 0.32 mm I.D. fused-silica capillary column with bonded OV-1 stationary phase (0.4 μm, film thickness) was used. The oven temperature was programmed from 40 to 300°C at 10°C/min. Helium was used as the carrier gas at 2 ml/min.

The preparative work was performed with a Pye 104 gas chromatograph with a flame ionization detector using either a packed column of 2.75 m × 4 mm, 10% PEG 20M on Chromosorb W (100–120 mesh) or a 1 m × 4 mm, 15% FFAP on Chromosorb W (100–120 mesh). Nitrogen was used as the carrier gas at a flow-rate of 50 ml/min.

The melting points were determined using a Reichert hot stage microscope.

Trapping technique

The GC effluent was passed through a splitter of the design of Baker *et al.*[16] to give a 95:5 (outlet:flame ionisation detector) split ratio. The heater on the collecting

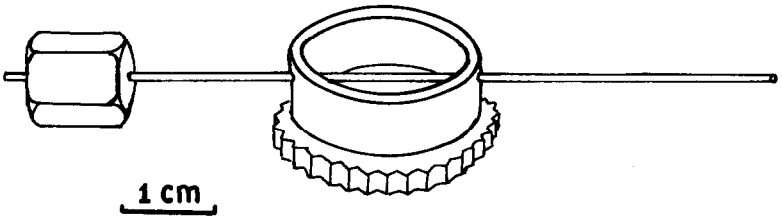

Fig. 1. Arrangement of glass capillary (45 mm × 0.45 mm I.D.) through a plastic cap filled with liquid nitrogen for trapping from the GC effluent.

arm of the splitter was maintained at 200°C and the outlet closed when not in use, with a silicone rubber septum, held in place with a 6-mm hexagonal coupling nut. Approximately 5 sec before a desired peak appeared, the blanking septum was replaced with one holding a 45 mm × 0.5 mm O.D. (0.45 mm I.D.) glass capillary which has been threaded through two holes in a 15-mm diameter polyethylene cap from a specimen tube (Fig. 1). The plastic cap was filled with liquid nitrogen. After elution of the peak to be collected, the capillary tube was removed from the splitter and the plastic cap and the trapped material in it used in the appropriate experiments.

For rapid change-over of collecting capillaries, the screw fitting on the end of the splitter was replaced by a male Luer fitting and the glass capillaries attached through a female Luer joint containing a soft silicone rubber septum with a hole to take the capillary. The glass capillaries were prepared by baking in an oven at 230°C for 30 min before use and handled with clean hands.

Insertion and removal of solution

The reagent solution was added to the trapped material in the capillary with a 5 μl syringe (SGE, London, U.K.) fitted with a 75 mm × 0.23 mm O.D. steel

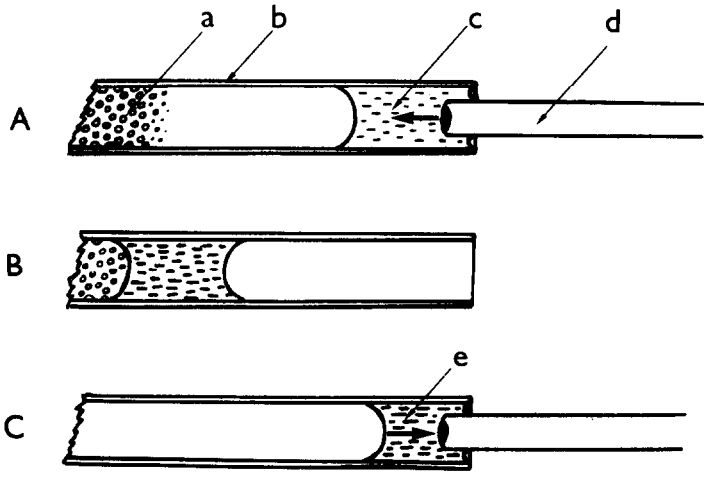

Fig. 2. Insertion and removal of reagent solution. (A) The reagent solution (c) (1 μl) added by inserting the tip of the syringe needle (d) just inside the glass capillary (b). (B) The reagent solution is run down to the trapped material (a) by tipping the capillary. (C) The reaction mixture (e) is tipped back to the end of the capillary and withdrawn with the syringe.

needle. The solution was added by inserting the tip of the needle just inside the capillary, injecting 1 μl of solution, removing the needle and tipping the capillary to cause the column of liquid to run down to the region of the trapped sample (Fig. 2). When all the sample had dissolved, the column of liquid was tipped back to the end of the capillary, the tip of the needle was inserted again, and the column drawn back up into the syringe. If the column of liquid is left in the tube for any length of time, it should be away from the end of the tube where evaporation can be rapid.

Epoxidation

A quantity of an alkene (50–500 ng) was trapped into a glass capillary as described above. A solution of *m*-chloroperbenzoic acid in hexane (1 μl, 0.05 M) was added with the syringe and mixed with the trapped alkene by tipping the capillary from side to side, then removed and immediately injected onto the cold (40°C) capillary column through the on-column injector, and investigated by temperature programming to 300°C at 10°C min.

Alkenes used were solutions (350 ng/μl) of *Z*- and *E*-6-pentadecene, *Z*- and *E*-7-pentadecene, *Z*- and *E*-8-heptadecene, *Z*- and *E*-9-nonadecene and *Z*-9-tricosene.

Sodium borohydride reduction

A solution of sodium borohydride in tetrahydrofuran (1 μl, 0.03 *M*) was injected into the glass capillary containing trapped carbonyl compound as in the example above. The capillary was kept at room temperature for 10 min. The syringe was rinsed several times with distilled water, the solution in the capillary was drawn up into the syringe and the solution injected onto a capillary column at 40°C and temperature programmed.

Esterification

Diazomethane was prepared from N-methyl-N′-nitro-N-nitrosoguanidine (1 mg) by the method of Fales *et al.*[17] and extracted into tetrahydrofuran (100 μl).

Samples of long chain carboxylic acids (500 ng) were trapped into glass capillaries from a packed 15% FFAP column. Diazomethane in tetrahydrofuran (1 μl) was injected into the capillary and mixed with the acid to dissolve it and almost immediately withdrawn by the syringe and injected onto the cold capillary column.

RESULTS AND DISCUSSION

Trapping technique

The individual GC peaks were trapped separately in glass capillaries. The trapped material was removed from the glass capillary, for rechromatography, by injecting 1 μl of solvent using a syringe with a fine needle (0.23 mm O.D.) and withdrawing the liquid back into the syringe. The trapped material can be rechromatographed with 85 ± 5% efficiency. The trapping efficiency depended on the boiling point of the sample. The size of the glass capillary was important. A narrower capillary altered both the flow rate and the split ratio of the effluent splitter and more effluent was vented to the flame ionisation detector. Trapping was less efficient from larger capillaries. The optimum size of the capillary was 45 mm × 0.5 mm (0.45 mm

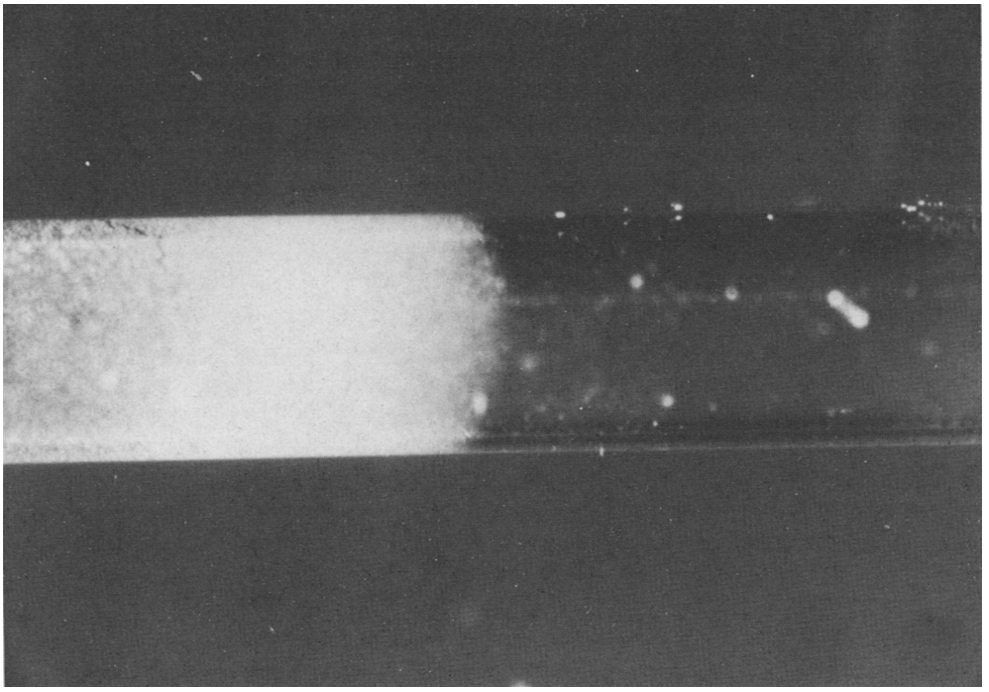

Fig. 3. A few micrograms of myristic acid trapped into a capillary tube from GC effluent. Gas flow from right. The myristic acid condensed in the cooled part to the left.

I.D.). Lower flow-rates of the carrier gas gave better trapping efficiencies. Flow-rates above 50 ml/min decreased the trapping efficiency and no trapping was observed above 80 ml/min.

Nanogram to microgram quantities of pure material were made available by this method for microreactions or rechromatography on a different stationary phase. The method was particularly useful to isolate the volatile constituents, after the direct injection of a piece of biological tissue by a solid injection technique[12]. The rechromatography of a particular GC peak on a different stationary phase is useful to check the homogeneity of the peak.

Similar methods of trapping substances from the GC effluent into glass capillaries have been described by Brownlee and Silverstein[18], Stanley and Kennet[4], and Cronin and Gilbert[3].

Melting points of trapped solids

The trapped substances from the GC effluent were restricted usually to a small region (normally about 1 cm) inside the glass capillary tube. This was demonstrated by the trapping of solid substances. Fig. 3 shows a few micrograms of myristic acid trapped from the effluent of the FFAP column. Melting points of solids amenable to gas chromatography can be determined with great accuracy using only a few micrograms or less of material. The glass capillary with the trapped substance was placed directly on the hot stage of the melting point apparatus. The melting points observed were very sharp because of the high purity of the samples collected from

TABLE I

REACTIONS CARRIED OUT IN CAPILLARIES

The products were examined on a OV-1 fused-silica capillary column. The temperature was programmed from 40 to 300°C at 10°C/min.

Substance (100 ng)	*Reagent (1 µl)*	*Reaction time (min)*	*Product*	*Yield (%)*
6-Pentadecene	*m*-Chloroperbenzoic acid in hexane	3	6,7-Epoxypentadecane	100
7-Pentadecene			7,8-Epoxypentadecane	100
8-Heptadecene			8,9-Epoxyheptadecane	100
9-Nonadecene			9,10- Epoxynonadecane	100
9-Tricosene			9,10-Epoxytricosane	100
3-Octanone	Sodium borohydride in tetrahydrofuran	10	3-Octanol	90
6-Pentadecene + pentadecane	Bromine in hexane	1	(Alkenes removed) (Alkanes remain)	100
8-Heptadecene + heptadecane				
9-Nonadecene + nonadecane				
Myristic acid	Diazomethane in tetrahydrofuran	1	Methyl myristate	*
Palmitic acid			Methyl palmitate	*
Stearic acid			Methyl stearate	*
Benzoic acid			Methyl benzoate	*

* Expected to be 100%.

the GC effluent. This must be the lowest quantity of material used for a melting point determination. It therefore provides a new, non-destructive way for comparing micro-amounts of natural products with authentic materials. A few of the reported insect pheromones, for example, are solids[19,20].

Epoxidation

One of the methods available to determine the position and geometry of alkene bonds is epoxidation. The epoxides can be readily prepared by the reaction of the alkene with *m*-chloroperbenzoic acid[21]. Electron impact (EI)–MS of simple epoxides can locate the position of the oxirane ring[22]. For more complicated epoxides, chemical ionisation (CI)–MS has been successfully employed[23].

Epoxidation of alkenes by peracids is a stereospecific *cis* addition. While the separation of *E*- and *Z*-isomers of alkenes by GC has been limited to special conditions and compounds[24,25], the conversion of the *E*- and *Z*-alkenes into the corresponding epoxides allows base line separations, even on packed columns[26]. The *trans*-epoxide elutes faster than the *cis*-epoxide when chromatographed on a polar or a non-polar stationary phase.

The epoxidation reaction has been used generally to examine milligram quantities of alkenes. In the present study the technique was refined to epoxidize nanogram quantities of alkenes, restricting the total reaction volume to 1 µl. The alkenes were trapped in glass capillary tubes and 1 µl of *m*-chloroperbenzoic acid in hexane added.

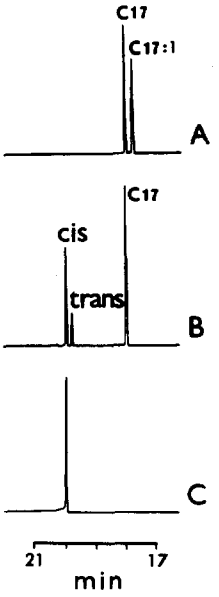

Fig. 4. Separation of *E*- and *Z*-8-heptadecenes by epoxidation. Chromatograms on the OV-1 capillary column with the oven temperature programmed from 40 to 300°C at 10°C/min. (A) Approximately 100 ng each of heptadecane and *Z*- and *E*-8-heptadecenes in 0.5 μl of hexane; (B) both peaks of (A) were trapped, epoxidized and reinjected; (C) *Z*-8-heptadecene from the Dufour gland of *M rubra* trapped, epoxidized and reinjected.

Hexane was found to be more suitable than dichloromethane as solvent for on-column injection (40°C). This method used to determine the *E*- and *Z*-isomer ratio of a number of synthetic alkenes (Table I). The results obtained with a mixture of heptadecane and 8-heptadecene are shown in Fig. 4. When the mixture was trapped and epoxidized the 8-heptadecene peak completely disappeared to yield the two corresponding epoxides, while the heptadecane peak remained untreated. Similarly, when 8-heptadecene from the Dufour gland of the ant *Myrmica rubra* was epoxidized, only a single peak, co-chromatographing with the epoxide of synthetic *Z*-8-heptadecene, was obtained (Fig. 4C). This confirmed that 8-heptadecene from *M. rubra* was 100% *Z*-isomer. In many cases of insect pheromones the characteristic *E*- to *Z*-ratio gives the maximum biological activity.

Sodium borohydride reduction

The identification of aldehydes and ketones has been aided by their reduction to alcohols with sodium borohydride and subsequent GC analysis of the products[8]. A saturated solution of $NaBH_4$ in ethanol or water has been used to reduce 1 μg samples in the syringe barrel and products larger than hexanol have been identified[9].

Water and ethanol are not suitable for on-column injection. Tetrahydrofuran (THF) or dimethoxyethane were better suited although the solubility of $NaBH_4$ in THF is poor (100 mg/100 g). The reduction of 3-octanone to yield 3-octanol was over 90% complete in 10 min (Table I).

Bromination

A method to identify the unsaturated peaks in a GC profile is valuable to the micro-chemist. Bromination results in the complete elimination of all unsaturated peaks from a GC trace, leaving the saturated peaks intact. Morgan and Wadhams[12] used bromine in carbon disulphide to brominate the components of the Dufour glands of the ant *M. rubra*. Many workers have found the method to be useful[27]. O'Keefe *et al.*[28] have utilized bromination in solution to subtract unsaturated fatty acid methyl esters in lipid identifications.

The bromination was extended in the present study to nanograms of material trapped in glass capillaries. If sufficient bromine is added to the trapped alkene, a yellow colour of bromine can be observed in the solution under a dissecting microscope. Fig. 5 shows an example of application of the technique to a mixture of heptadecane, 8-heptadecene, nonadecane and 9-nonadecene. The unsaturated peaks were readily identified by their disappearance on bromination. The brominated products, other than those from very low molecular weight alkenes, elute so slowly, no peaks are ever observed.

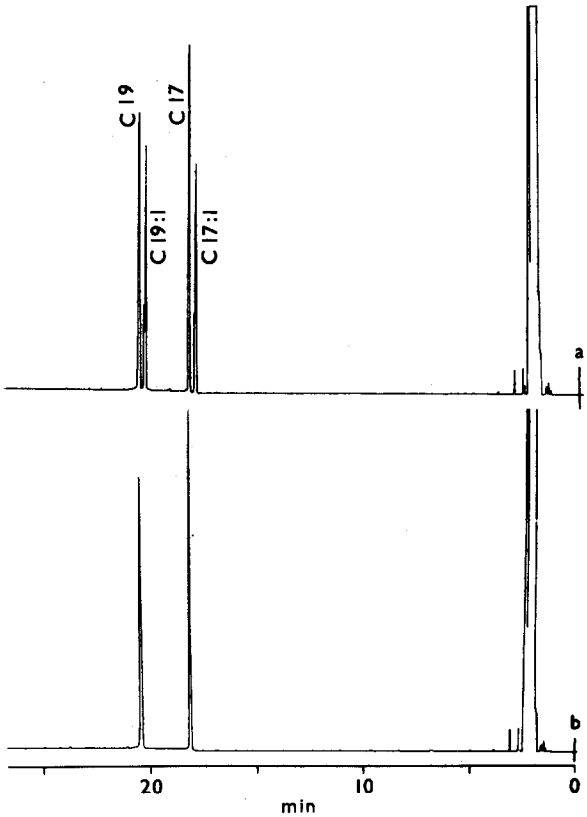

Fig. 5. Bromination of alkenes. Chromatograms on the OV-1 capillary column with the oven temperature programmed from 40 to 300°C at 10°C/min. (a) A mixture of heptadecane, *Z*- and *E*-8-heptadecene, nonadecane and *Z*- and *E*-9-nonadecene (100 ng each) was trapped and reinjected. (b) The same mixture reinjected after treatment with bromine.

Esterification with diazomethane

Lower members of aliphatic monocarboxylic acids are sufficiently volatile to be separated directly by GC on a stationary phase. However, adsorption onto the chromatographic support due to the highly polar character of carboxyl function often leads to tailing of the peaks. Esterification is frequent used to convert relatively non-volatile and polar carboxylic acids into less polar derivatives with better GC characteristics. The esters also give better mass spectra than their parent acids. Dabre[29] and Ma and Ladas[30] have reviewed the wide variety of techniques available for the esterification of fatty acids. The preferred method using methanol–boron trifluoride is not suitable for the present technique because boron trifluoride is destructive towards GC columns.

The fatty acids were trapped in glass capillaries from an FFAP column, and treated with diazomethane in THF. The reactions were instantaneous and quantitative (Table I). Huwyler *et al.*[31] used a comparable method to identify the fatty acids found in the trail pheromone of the ant *Lasius fuliginosus* on a microgram scale. The present technique reduces samples to much smaller quantities.

We have now used a number of reagents in reaction gas chromatography, some of which, such as sodium borohydride leave involatile residues on the column, but the quantities are so small, no deterioration of column performance has been noted over several months of use.

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