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Note

Microchemical methods for the identification of volatile pheromones

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Many investigators are actively pursuing studies of volatile substances from biological tissues, in particular the pheromones present in insects and higher animals, including vertebrates. The substances are usually collected by solvent extraction or distillation. Both these methods have serious drawbacks: the solvent can introduce impurities or mask short retention components in gas chromatography (GC), and distillation may lose the more volatile components, requires relatively large amounts of material for manipulation, or a process of adsorption and desorption on a material such as Tenax GC.

In our studies of volatile insect secretions we find the use of organic solvents must be avoided and have developed techniques for structure determination without them. We have already described a method for the direct introduction of a gland or piece of biological tissue into the gas chromatograph by a solid sampling technique[1]. We describe here techniques whereby even samples which have volatility similar to common solvents may be directly removed from a gland or reservoir and gas chromatographed, with the aid of reaction GC to determine functional groups and arrive at a complete structural identification on nanogram samples or less. It avoids the need to trap material by adsorption and desorption.

Essentially, glandular liquid is drawn by capillary action into a fine glass tube which is introduced into the GC column by the solid sampling technique already described[1]. The addition of aqueous reagents or the removal of classes of compounds by reaction loops can lead to full chemical identification. The techniques are described by reference to investigations of the poison gland and Dufour gland secretions of the common red ant, *Myrmica rubra* L.

EXPERIMENTAL

GC was performed on a 5 ft. × 1/4 in. O.D. glass column packed with Porapak Q, 120–150 mesh (Waters Assoc.), in a Pye Unicam Model 64 gas chromatograph with flame ionization detectors (FID). The oven temperature was 167° isothermal, with the injection heater at 220°. The carrier gas was nitrogen or helium at a flow-rate of 50 ml/min.

Individual glands from ant workers were dissected out, washed with water and

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blotted dry, then pierced with a 50–70-μm diameter hard glass capillary. The diameter appears to be critical. The contents of the gland were drawn into the tube by capillarity. The filled portion of the tube was dropped into a 1.5-cm length of soda glass melting-point tubing sealed at one end. The contents of 1–10 glands were collected in the larger tube which was then sealed and introduced into the gas chromatograph by the solid sampling technique described in an earlier paper[1].

Reactions were performed by placing an aqueous solution of reagent in a similar glass capillary in the large tube with the gland contents, sealing and centrifuging to expel the materials from the capillary and mix them, or alternatively adding finely powdered reagent, *e.g.* sodium borohydride or sodium hydroxide to the larger tube before sealing.

For removal of alcohols, the column effluent was passed through a post-column loop[2] of 6 × 1/4 in. O.D. glass of which the first 3 in. were packed with 20% boric acid on Porapak Q followed by 3 in. of Porapak Q and then led to the detector. For removal of aldehydes[3], a similar loop was filled for 3 in. with 5% *o*-dianisidine on Supersorb (80–100 mesh) followed by 3 in. of Porapak Q.

RESULTS AND DISCUSSION

By the avoidance of solvents with the solid sampling technique[1], it was realized that the glandular secretions of *M. rubra* contained a portion of very volatile

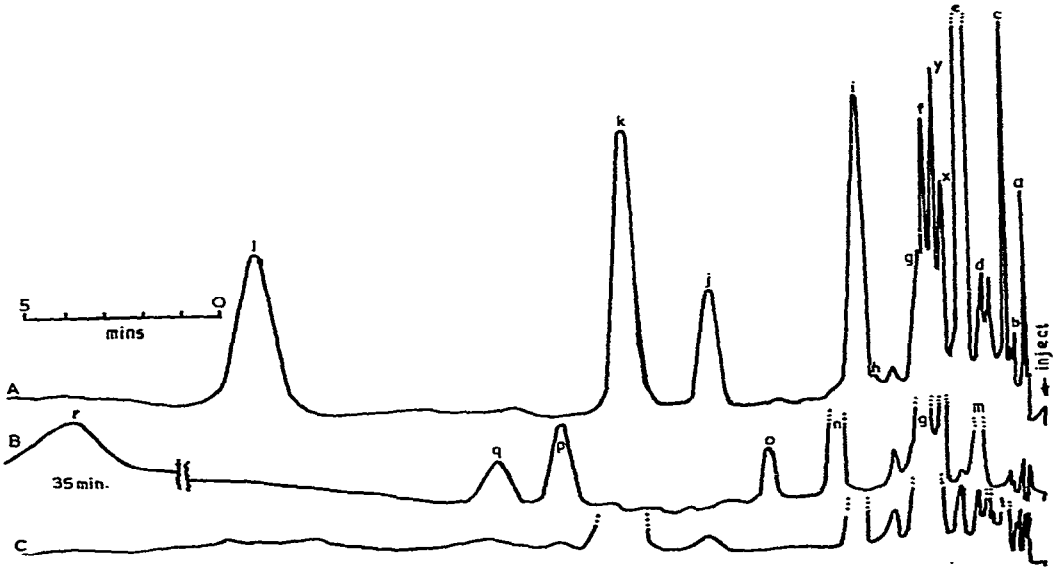

Fig. 1. A, Contents of 3 poison gland reservoirs of *M. rubra* chromatographed on Porapak Q at 167° and at a helium flow-rate of 50 ml/min, attenuated ×50. B, Contents of 6 poison reservoirs chromatographed as in A, after treatment with solid $NaBH_4$. C, Contents of 8 poison reservoirs after passage through an *o*-dianisidine extraction loop. Peak identification: a = pressure disturbance on crushing sample; b = methane followed by ethane; c = water peak; d = methanol; e = ethanal; f = butane; g = ethanol; h = propanal; i = acetone; j = 2-methylpropanal; k = butanone; l = 2- or 3-methylbutanal; m = reagent peak; n = 2-propanol; o = 1-propanol; p = 2-methylpropanol; q = 2-butanol; r = 2- or 3-methylbutanol; x and y are unidentified components.

substances. At the low levels (ng per individual) involved, contamination with tissue chemicals and pyrolysis products of the cells surrounding the gland became significant when the whole tissue sample was heated in the injector. The technique of withdrawing the material from the reservoir of the gland with a capillary was developed and found to be easily reproducible and very sensitive. There is no selective loss of more volatile compounds. Because compounds elute from Porapak in approximate order of molecular size, retention times immediately gave some indication of the nature of the compounds separated. Reaction with sodium borohydride in the sealed ampoule before chromatography removed all peaks due to aldehydes and ketones and replaced those peaks with others for the corresponding alcohols. Use of the *o*-dianisidine extraction loop removed aldehydes, but not ketones, through imine formation. Reaction with solid sodium hydroxide removed aldehydes and α,β-unsaturated ketones, presumably through condensation reactions and partially removed alcohols. Alcohols were selectively removed by the boric acid loop. It was necessary to have uncoated Porapak after the boric acid or dianisidine absorbants to prevent these materials from bleeding into the detector at the high temperature and high sensitivity

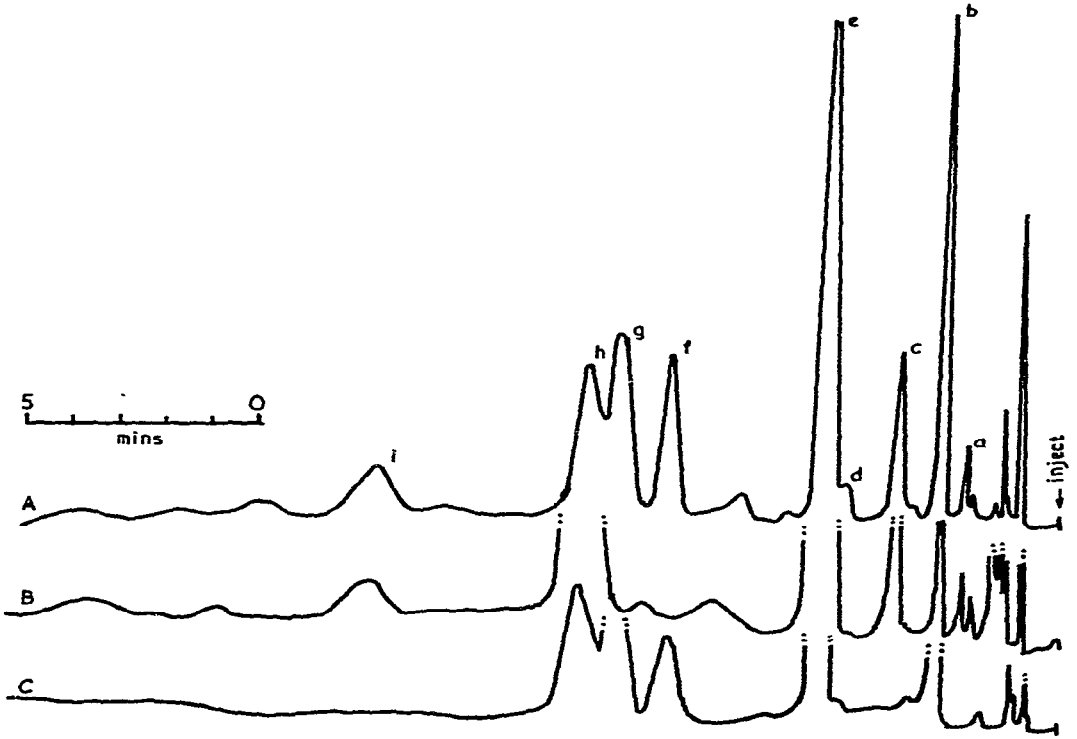

Fig. 2. A, Contents of 9 Dufour glands of *M. rubra*; chromatographic conditions as in Fig. 1, except attenuation (×200). B, Contents of 3 Dufour glands chromatographed under the same conditions as in Fig. 1. after treatment with powdered NaOH. C, Contents of 10 Dufour glands after passage through a boric acid extraction loop. Identified components: a = methanol; b = ethanal; c = ethanol; d = propanal; e = acetone; f = 2-methylpropanal; g = butenone; h = butanone; i = 1-butanol. Pressure disturbance on crushing and water peak are observed at short retention time, as in Fig. 1.

used. No deterioration in the effectiveness of the subtraction loops was observed after some 30 runs with the small samples used.

By use of these techniques eleven principal constituents of the poison gland and Dufour gland secretions of *M. rubra* in the C_1–C_5 range were identified, all of which are present in the 1–50-ng range per insect. These results are illustrated in Figs. 1 and 2. In all cases identifications were checked on authentic standards, singly and in mixtures, for both retention times and response to the reagents. The biological significance of the compounds is discussed elsewhere[4,5].

In the poison gland secretion, very small amounts of methane, ethane and propane were also recognized from their retention times, but no reaction, or lack of reaction, evidence was used to confirm the identifications. Two significantly large peaks in the C_4 region for the poison gland secretion (Fig. 1) were not identified. Neither peak corresponded in retention to any of the most probable candidates (isobutane, isobutene, 1- or 2-butenes, etc.).

The technique is applicable to substances in either aqueous or non-aqueous medium, since the poison gland secretion is essentially an aqueous protein solution, and the Dufour gland secretion is a non-aqueous mixture principally composed of higher hydrocarbons[6]. The method has been described chiefly for the identification of oxygenated compounds, but should be equally applicable to other classes with the use of suitable reagents[7]. The quantities used did not permit taking coupled GC–mass spectra, but no modification is required where sufficient material is available for mass spectra.

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