The Electroantennographic Detector — a Selective and Sensitive Tool in the Gas Chromatographic Analysis of Insect Pheromones

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A gas chromatographic detector is described which uses an insect antenna as a sensing element and permits the continuous monitoring of pheromones and other compounds with olfactory activity. In high-resolution gas chromatography allowing separations of positional and geometrical isomers, the electroantennographic detector gives precise information on the retention times of active compounds and permits the analysis of insect pheromones with exceedingly small amounts of material.

Insects perceive many odors with receptors located at their antennae, and measurements of the electrical signals associated with olfaction have been made for many years. The electroantennogram (EAG) [1], a recording of the potential changes which can be measured between base and tip of the antenna as a result of chemical stimulation, has provided important information on the sensitivity and specificity of olfactory receptors [2, 3]. Although more insight into the mechanisms of odor perception can be gained by recording from single receptor cells, EAG information has been of invaluable service in the study of the chemistry of insect sex pheromones, 1. to predict active structures from responses to related molecules, and 2. to monitor biological activity during isolation and purification of attractants from the natural sources [4–6]. A direct monitoring system has been described in which the gas chromatography (GC) effluent is continuously collected in a flask which is vented over an antennal preparation at regular intervals [4]. This setup, which combines sophisticated instrumentation of both gas chromatography and electrophysiology, has been used successfully in the identification of the sex pheromones of several noctuid moths [7, 8]. Many researchers have preferred to collect GC fractions by hand and to determine their EAG activity in a separate step, although this procedure consumes more time and leads to some loss of GC resolution.

In high-resolution capillary GC with typical peak half-widths of a few seconds, neither the repeated sampling nor the manual trapping method are practical or do justice to the separation capabilities. We have found, however, that the rise times obtained in capillary GC are short enough to give good EAG responses without the need for pulsation of the stimulus. The EAG setup can thus be directly coupled to the gas chromatograph as a true GC detector which gives precise information on the retention times of compounds with olfactory activity. Continuous monitoring has the additional advantage that signals from mechanoreceptive and temperature-sensitive cells are largely eliminated. We have now built an extremely simple system which gives reliable EAG information on the GC effluent directly at the chemist's laboratory bench.

The sensing element of the electroantennographic detector (EAD) is an excised insect antenna of which the last few distal segments have been removed [9]. It is placed between two chloridized silver wires which are attached to the amplifier inputs (Fig. 1 *). The end of each wire is spoon-shaped to accept a small drop of 0.75% NaCl solution which establishes electrical contact and holds the antenna in place by surface tension. This setup is small enough that it can be inserted into the end of a glass tube of 7 mm diameter which is supplied with a humidified stream (300 ml/min) of synthetic air. This prevents contamination from the laboratory atmosphere during detector operation. The glass tube which runs directly across the top of the GC oven is provided with a water jacket for cooling. By maintaining the temperature of the water jacket and of the wash bottle providing air humidification

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* Fig. 1 see Table on page 724 a.

a few degrees above ambient (ca. 30°), the two saline drops holding the antenna are kept from drying out.

To prevent condensation on cooling, the column effluent (1 ml/min) is diluted with nitrogen (30 ml/min) in the GC oven through the inlet normally used for hydrogen in flame ionization detector (FID) operation. This mixture leaves the oven through a modified FID jet and enters the glass tube through a hole 15 cm up-stream from the antenna. The signal generated by the antenna is measured with a high impedance (10^{12} Ohm) voltage follower circuit; baseline drift is eliminated, when necessary, with a high-pass filter of a corner frequency of 0.01 Hz or less. A regular GC strip-chart recorder with a pen response of 25 cm/0.3 sec is fully satisfactory for recording EAD-detected GC peaks.

Application of the EAD in the analysis of insect sex pheromones is illustrated with the European grapevine moth, *Lobesia botrana* (Schiff.). This tortricid species is an important grape pest in the warmer climates of Europe, notably the Mediterranean area. Earlier EAG data pointed to (E,Z)-7,9-dodecadienyl acetate (EZ-dda) as a possible sex pheromone structure, and this compound was found highly attractive to *L. botrana* males after synthesis [10]. Mass fragmentographic and mass spectrometric evidence has since been obtained that this compound is indeed produced by *L. botrana* females [11]. A mixture of the four isomeric 7,9-dodecadienyl acetates can be separated on a capillary column, as shown by the GC trace obtained with the FID (Fig. 2 a). With electroantennographic detection using the antenna of a *L. botrana* male, a chromatogram of a mixture containing equal amounts of all four geometrical isomers shows a major peak at the retention time of EZ-dda (Fig. 2 b). Only a small response is obtained from the (Z,E)-isomer and none from the (Z,Z) and (E,E) isomers. The inactivity of the compounds emerging after EZ-dda may not be fully representative due to antenna adaptation. After stimulation with 10 pg of EZ-dda, a rest period of 3 min is usually required to obtain a peak of the same size with a second stimulus. We have not yet investigated what adaptation effects take place when the antenna is successively stimulated with two different chemicals, but we could confirm the low activity of the two late eluting isomers by diverting the flow of effluent carrier gas up to

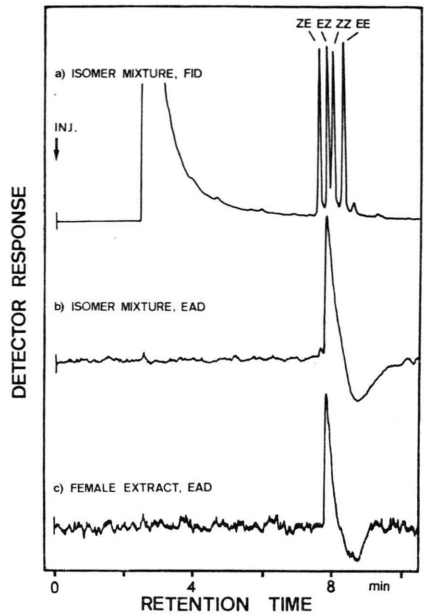

Fig. 2. Chromatograms obtained with the electroantennographic detector (EAD) using a *Lobesia botrana* male antenna, in comparison with the flame ionization detector (FID). UCON 50 HB 5100 glass capillary column, 50 m × 0.3 mm at 180°, helium head pressure 1.5 atm., inlet splitter at 100 ml/min. Actual split ratio of (E,Z)-7,9-dodecadienyl acetate (EZ-dda) is 1:12.5. a. Mixture of EZ-dda and its geometrical isomers, amount injected 250 ng each in 1 µl hexane, FID. b. Same mixture, 250 pg each in 1 µl hexane, EAD. c. *L. botrana* female extract, 0.1 female equivalent in 1 µl diethyl ether, EAD.

the time of their appearance in the chromatogram. This was accomplished simply by placing a vacuum tube over the exit of the GC effluent which removed all unwanted material. Of special interest is also the fact that virtually no signal was obtained from the solvents hexane or ether.

To obtain an indication on the retention time of active materials produced by the female, a diethyl ether extract was made from abdominal tips of virgin *L. botrana* females and injected without further purification. It gave a peak only at the retention time of EZ-dda (Fig. 2 c). It appears, therefore, that this compound is not only the most active of the four possible geometrical isomers, but also the most active, perhaps the only pheromonal compound present in *L. botrana* female extracts.

The dose-response curve of the *L. botrana* EAD to EZ-dda is given in Fig. 3. Signal intensities vary considerably from one antenna to another; stimulation with 10 pg gave responses between 1.0 and 11.4 mV with an average of 3.5 mV. On the other hand, ratios of peak heights with different amounts

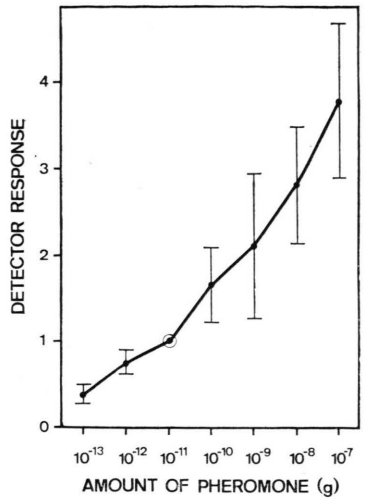

Fig. 3. Response of the EAD with a *Lobesia botrana* male antenna to (E,Z)-7,9-dodecadienyl acetate (EZ-dda). Values are actual amounts of EZ-dda leaving the GC column as a peak under the conditions given in Fig. 2. Responses (± std. deviation) are normalized for 1.0 at 10^{-11} g.

of stimulus are fairly reproducible, and so each response was normalized by dividing its value by that of a preceding 10 pg standard peak which was followed by a 3 min disadaptation period. The sensitivity of the *L. botrana* EAD is very high; with a good antennal preparation, a signal-to-noise ratio of 3:1 or better could still be obtained with 0.1 pg EZ-dda. The EAD is thus clearly more sensitive than the FID or even the mass fragmentographic detector, and ranks among the most sensitive GC detectors known. Between 0.1 pg and 100 ng, the signal intensity is approximately linear with the logarithm of the amount of stimulant as reported for the silkmoth [2]. In our case no flattening of the curve at high levels is obtained, as the entire sample is vaporized in the GC injector. Stimulations with amounts larger than 1 ng seem to cause irreversible adaptation. As long as the stimuli are kept low, antennal preparations can often be used for up to two hours without deterioration and permit the recording of a large number of chromatograms.

Although the EAD is primarily intended for the biological monitoring of gas chromatograms, the direct coupling of the EAG measurement to GC, similarly to the coupling of a mass spectrometer to GC, can be used for rigorous purification of samples prior to the determination of biological activity, and to eliminate the contribution of impurities to the EAG. We have measured the EAG activity of the three non-pheromonal isomers of EZ-dda and found that, similarly to the three Bombykol isomers in the silkmoth [2], all give response-curves of a shape similar to EZ-dda, but about 100 to 1000 times more material is needed to obtain the same signal. Since isomeric impurities in synthetic samples are often in the percent range, measurements on compounds with low activity which can contain highly active impurities are not reliable without on-line purification.

The high sensitivity of the EAD permits the determination of retention times of biologically active compounds with exceedingly small amounts of material. We have obtained EAD-chromatograms of female extracts of several tortricid species, using never more than 0.1 female equivalent per injection of which over 90% were still wasted in the inlet splitter. The possibility of obtaining chemical information on the pheromone composition from individual insects opens new avenues in pheromone research, *e. g.* for the investigation of genetics of pheromone biosynthesis or the study of field-collected insects which cannot be reared in the laboratory. As an example, we were able to obtain evidence for the sex pheromone structure of the eye-spotted budmoth, *Spilonota ocellana*, as (Z)-8-tetradecenyl acetate, from the extract of 7 females and using the antennae of only two males. This chemical was previously found attractive to *S. ocellana* males in the field [12], but its occurrence in nature has so far not been reported.

The direct linkup with high resolution GC adds a new dimension to electrophysiological measurements. It should in principle be adaptable to other sensory recording techniques, such as single cell recordings in insects or electroolfactograms obtained from vertebrates. The electroantennographic detector described leads to a sensitivity and precision previously not achieved in pheromone analysis.

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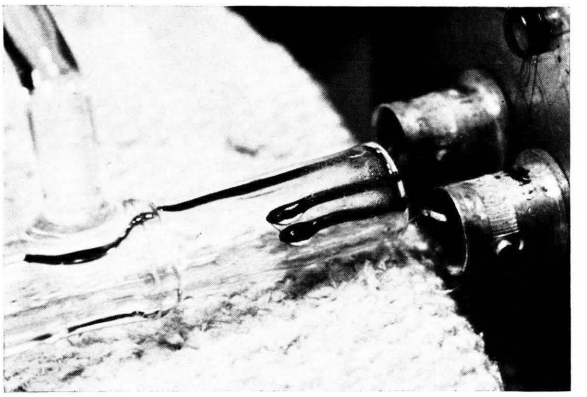

Fig. 1. The electroantennographic detector consisting of an insect antenna placed between two drops of saline solution at the tips of chloridized silver electrodes. Diameter of silver wires is 1.2 mm, antenna shown is of a *Lobesia botrana* male.

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