

# Coupled Gas Chromatography – Single Cell Recording: a New Technique for Use in the Analysis of Insect Pheromones

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A method is described for the continuous biological monitoring of the effluent from a high resolution gas chromatographic column using a single cell recording technique. The system can be used to provide precise information on the retention times of compounds with olfactory activity that are not detected with a coupled gas chromatograph – electroantennogram system.

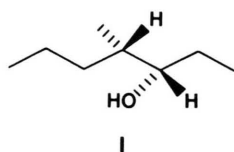
## Introduction

The perception of olfactory stimuli by insects is mediated largely through receptors located on their antennae. The electroantennogram (EAG) technique [1], which measures changes in potential between the base and tip of the antenna as a result of chemical stimulation, has been widely used to monitor biological activity during the isolation of insect pheromones [2]. Several systems have been described which directly combine the EAG technique with the resolution of gas chromatography [3–5]. The coupled high resolution GC-EAG technique, in which the column effluent is monitored simultaneously by EAG and gas chromatographic detectors provides a highly sensitive and precise tool for pheromone studies.

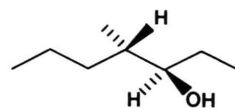
Although the EAG can provide some information on the specificity of insect olfactory receptors, a more detailed understanding of the perception of olfactory stimuli can be obtained by recording from single olfactory cells. Multicomponent pheromone systems are being increasingly implicated in insect chemical communication and specialised receptor cells keyed to different components of the pheromone mixture have been found in several families of Lepidoptera [6, 7]. Similarly, single cell studies of bark beetles of the genus *Ips* have shown that the individual components of the aggregation pheromone are perceived by separate groups of specialised olfactory cells [8].

The large European elm bark beetle, *Scolytus scolytus* (F.), aggregates in response to a male produced pheromone [9] which consists of a mixture

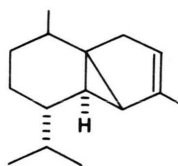
of beetle and host metabolites. A number of behaviourally active compounds have been identified from air entrainment extracts of the volatiles associated with beetles boring into elm, including the male beetle metabolites (–)-threo (**I**) and (–)-erythro-4-methyl-3-heptanol (**II**) [5]. **I** and **II** are key components of the aggregation pheromone and their activity in the field is synergised by the host metabolite  $\alpha$ -cubebene (**III**) [10, 11].



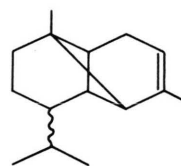
**I**



**II**



**III**



**IV**

Electrophysiological studies have shown that each of the (–) stereoisomers of 4-methyl-3-heptanol (**I** and **II**) interact with separate groups of specialised olfactory cells [12]. Similarly, other compounds implicated in the chemically-mediated behaviour of *S. scolytus* are also perceived by separate groups of cells [13, 14]. In addition, single cell recording (SCR) techniques have shown the presence of a small proportion of cells which do not respond to any of the compounds so far identified. Although these cells can be stimulated by the total air entrainment extracts, the coupled GC-EAG technique has

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In this paper a coupled high resolution GC-SCR system is described which has been developed in order to locate the additional active components. The use of the technique in the identification of these compounds from the air entrainment extracts will be presented elsewhere.

The coupled GC-SCR system is shown in Fig. 1. The Carlo Erba 2151 AC gas chromatograph was equipped with a flame ionisation detector (FID) (12) and fitted with a 21 m  $\times$  0.3 mm i.d. SE-30 fused silica column (8). The carrier gas was hydrogen. The effluent from the capillary column was split approximately equally between the FID and the line

to the antennal preparation by means of a low volume all glass splitter constructed from glass lined stainless steel tubing (GLT) and deactivated fused silica tubing. Thus the end of the column was inserted into a GLT "T" piece (9) (Scientific Glass Engineering Ltd.) and two equal lengths of fused silica tubing were inserted into the other two arms. One of these (10) led to the FID, while the other (11), which was heated to 120–130 °C, was taken through the oven wall and directed the remainder of the column effluent into a purified airstream (300 ml/min) which passed continuously over the preparation. This latter fused silica transfer line was carried in a length of thin walled 1/16th inch stainless steel tubing, one end of which was securely clamped to the body of the GC. This arrangement afforded some rigidity and protection to the transfer line while retaining a degree of flexibility. In order to

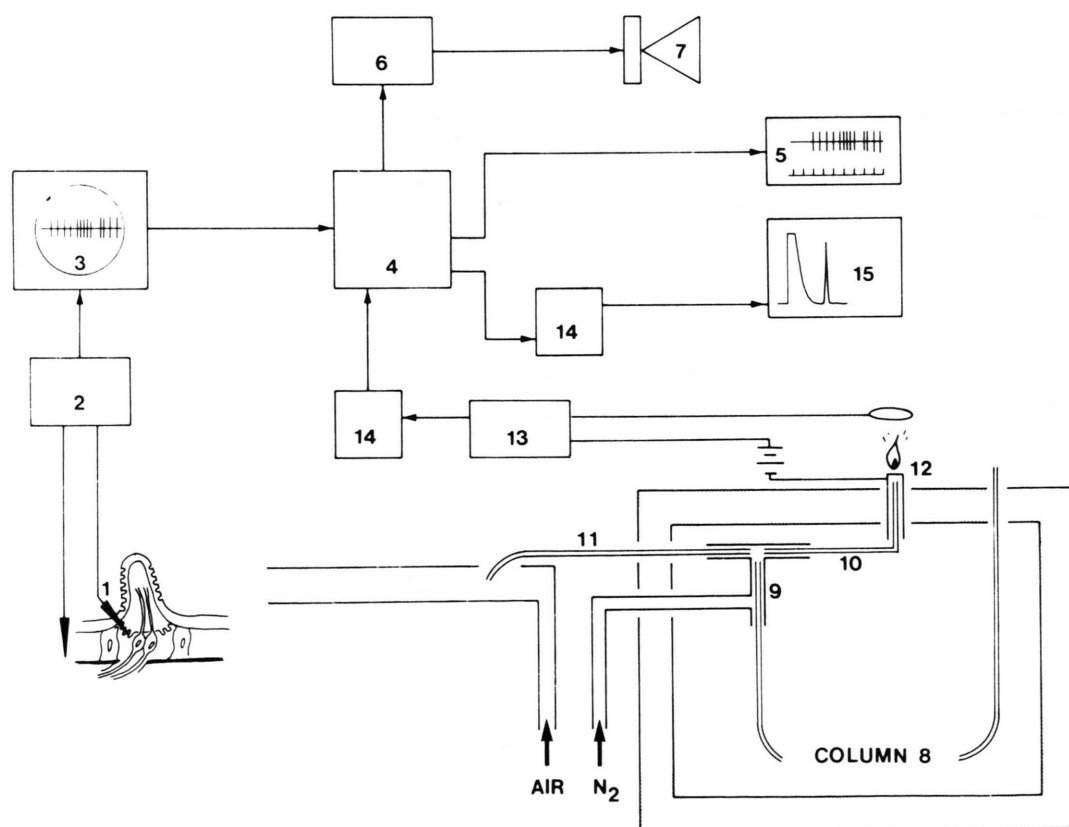


Fig. 1. Diagram of the coupled GC-SCR system. 1. Recording electrode; 2. AC coupled amplifier; 3. oscilloscope; 4. tape recorder; 5. mingograph 34 T ink jet recorder; 6. audio amplifier; 7. loudspeaker; 8. capillary column; 9. glass lined tubing (G.L.T.) 'T' piece; 10. deactivated fused silica transfer line to F.I.D.; 11. deactivated fused silica transfer line to insect preparation; 12. flame ionisation detector (F.I.D.); 13. F.I.D. amplifier; 14. phase lock loop; 15. chart recorder.

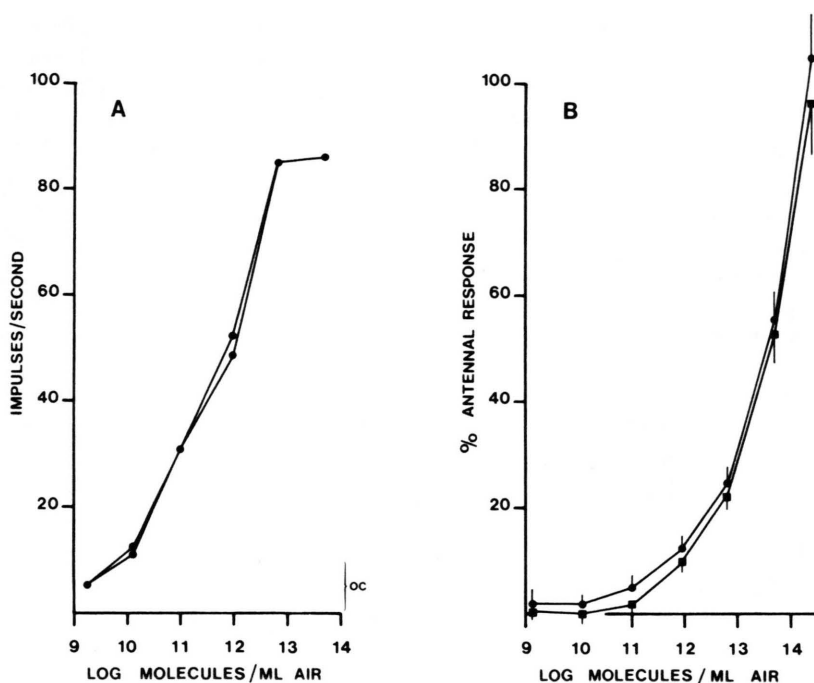


Fig. 2. (A) Dose response curve of a typical olfactory cell (single cell recording) specialised to  $\alpha$ -cubebene. This was recorded from a female *S. scolytus* preparation. Samples were presented twice to the preparation except for the two highest stimulus concentrations of  $\alpha$ -cubebene which were presented once. The responses are shown as the number of impulses elicited during the first 1 s after stimulation. Other compounds (OC) tested against the preparation included 4-methyl-3-heptanol isomers, multistriatin mixed isomers (34.7%  $\alpha$ -multistriatin),  $\beta$ -pinene and limonene. (B) EAG responses of male (■) and female (●) *S. scolytus* to  $\alpha$ -cubebene. Samples were presented twice to each preparation and each point is the mean response (10 preparations)  $\pm$  S.E. normalised with respect to the ( $\pm$ )-threo-4-methyl-3-heptanol standard.

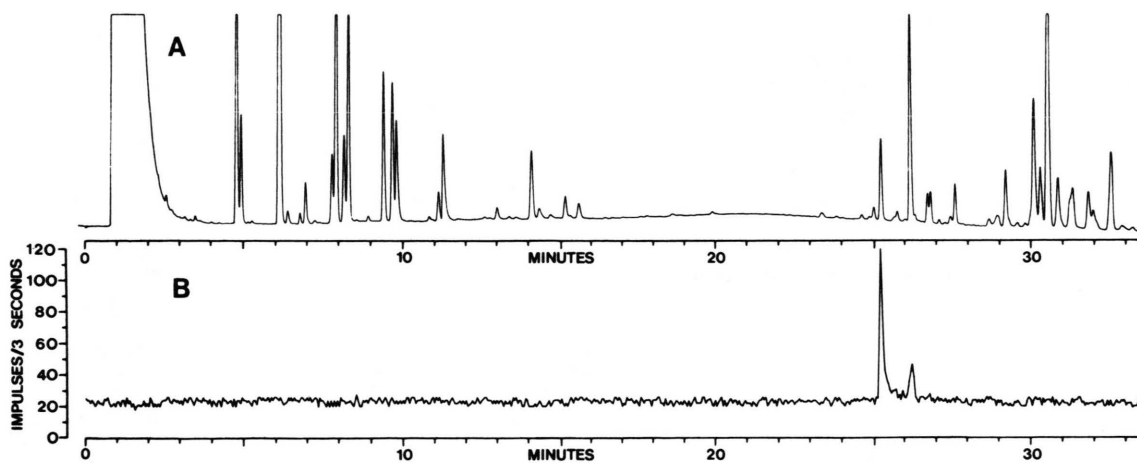


Fig. 3. Coupled GC-SCR. (A) Gas chromatogram of an air entrainment extract of the volatiles associated with female *S. scolytus* boring into English elm. GC conditions: 21 m  $\times$  0.3 mm i.d. SE-30 fused silica column programmed from 40–75  $^{\circ}$ C at 2  $^{\circ}$ /min and then from 75–115  $^{\circ}$ C at 4  $^{\circ}$ /min with hydrogen as the carrier gas, inlet pressure 0.5 kg/cm $^2$ . (B) Response of an olfactory cell specialised to  $\alpha$ -cubebene, to stimulation with the air entrainment extract. The cell was recorded from an *S. scolytus* female preparation. The impulses elicited from the cell were summed over 3 s intervals. The increase in activity of the cell at 25.2 min corresponded to the elution from the capillary column of  $\alpha$ -cubebene. The weak response shown by the cell at 26.2 min corresponded to the elution of  $\alpha$ -ylangene from the column.

maximise the FID response and to reduce residence time in the transfer line,  $N_2$  (60 ml/min) was added as a make up gas.

Recordings from olfactory cells associated with individual sensilla were made using tungsten micro-electrodes [15]. The indifferent electrode was positioned in the mouthparts of the insect and the recording electrode (1) was brought into contact with the surface of the antennal club until impulses were recorded. The signals were amplified with an AC coupled amplifier (2), displayed on an oscilloscope (3) and stored on magnetic tape (4). Permanent copies of the responses were made on a Mingograph 34T ink jet recorder (5). Once a cell was located its olfactory specificity was determined by stimulation with a series of test compounds which were injected into the airstream which passed continuously over the preparation [13].

The signals from the FID amplifier (13) were fed through a phase lock loop (14) to a TEAC A3440 tape recorder (4). This system was used to upgrade a relatively inexpensive AC tape recorder into a DC recorder of limited band width (0–1500 Hz) [16]. The output from the recorder was monitored on a chart recorder (15).

The impulses given by the individual olfactory cells were summed over 3 s intervals and then plotted against the FID response.

The EAG system has been previously described [13]. After subtraction of the response obtained from a pentane control, the responses elicited by the test samples were normalised with respect to the response obtained from stimulation with  $2.5 \times 10^{11}$  molecules/ml of air of ( $\pm$ )-threo-4-methyl-3-heptanol.

The stimulus concentrations for both EAG and single cell studies, were determined using a GC technique. The concentration was estimated as the number of molecules/ml of air which stimulated the antenna [13].

## Results and Discussion

A major use of the coupled high resolution GC-EAG technique is to provide rapid and precise information on the retention times of physiologically active compounds in complex natural product extracts. We have found some limitations in the application of this technique to the study of bark beetle aggregation pheromones. In *S. scolytus*, most,

if not all, the individual components of the pheromone complex are perceived by groups of specialised olfactory cells and the proportions of the different cell types present on the antenna varies considerably. Thus 20–25% of the cells recorded

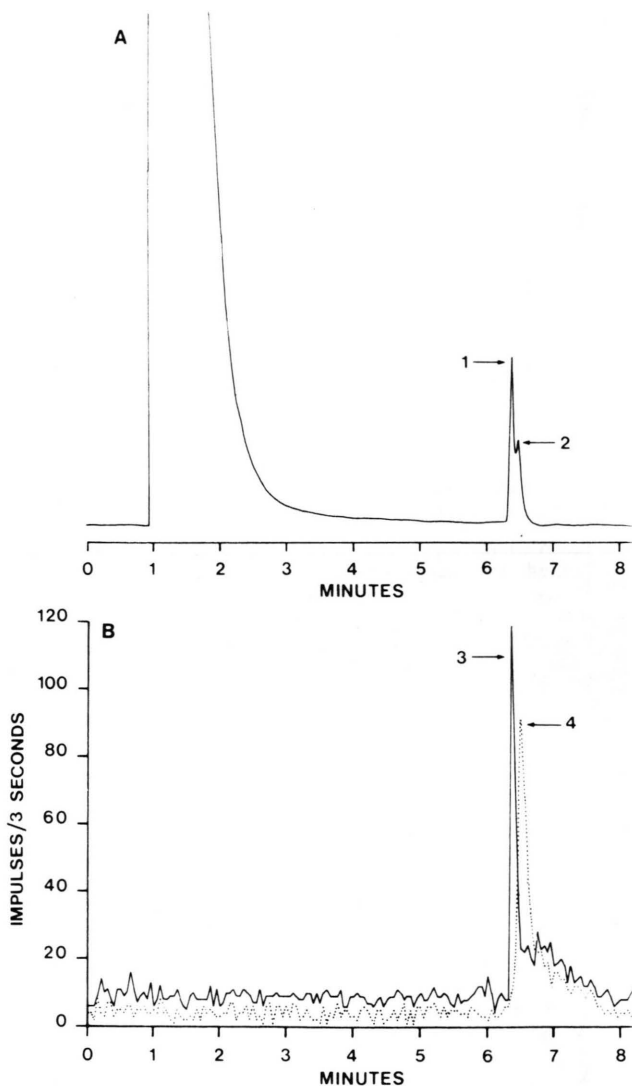


Fig. 4. Response of (A) flame ionisation detector and (B) two olfactory cells from a female *S. scolytus* preparation to (1) ( $\pm$ )-threo- and (2) ( $\pm$ )-erythro-4-methyl-3-heptanol. The cell (3) was specialised to the ( $-$ )-threo stereoisomer while the cell (4) was specialised to ( $-$ )-erythro-4-methyl-3-heptanol. The impulses recorded from each cell were summed over 3 s intervals. Total sample injected onto the GC was 6.7 ng ( $\pm$ )-threo- and 3.3 ng ( $\pm$ )-erythro-4-methyl-3-heptanol. The inlet split ratio was 5:1 and the column effluent was split approximately equally between the FID and the insect preparation. GC conditions: 21 m  $\times$  0.3 mm i.d. SE 30 fused silica column at 50 °C with hydrogen as the carrier gas, inlet pressure 0.5 kg/cm<sup>2</sup>.

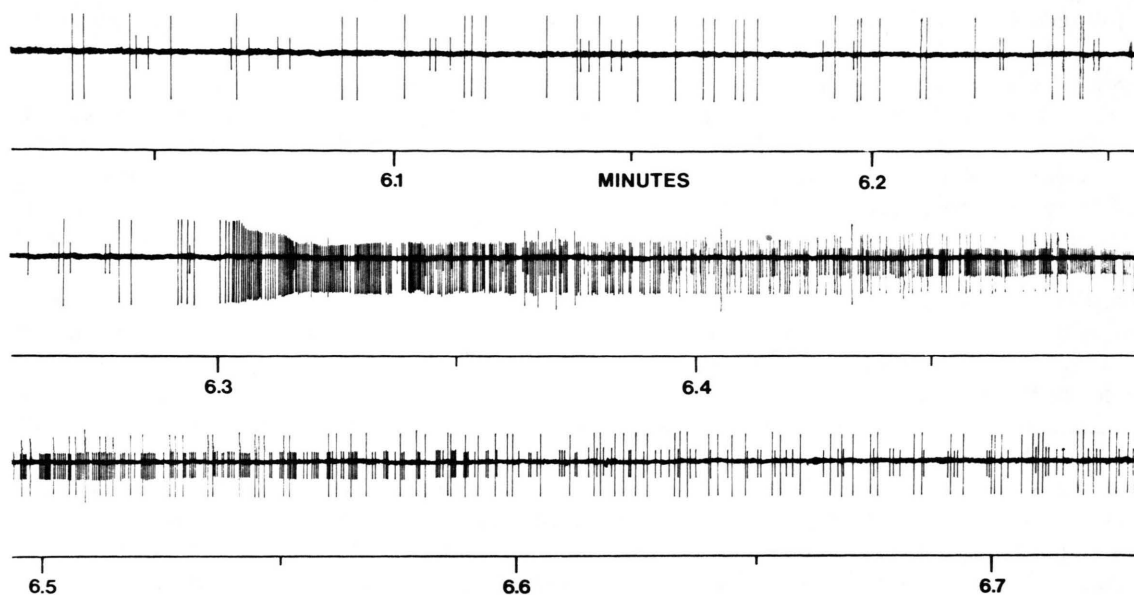


Fig. 5. Responses of the two olfactory cells over the period of elution from the GC column of the 4-methyl-3-heptanol isomers (see Fig. 4). The cell with the larger amplitude was specialised to (–)-threo-4-methyl-3-heptanol while the cell with the smaller amplitude was specialised to the (–)-erythro stereoisomer.

from the whole antenna are specialised to each of the (–) stereoisomers (**I** and **II**) of 4-methyl-3-heptanol but only 2–3% are specialised to the host synergist  $\alpha$ -cubebene (**III**). The EAG and SCR threshold concentrations for (–)-threo (**I**) and (–)-erythro-4-methyl-3-heptanol (**II**) are similar [13]. In contrast, the EAG threshold concentration for  $\alpha$ -cubebene (**III**) is 2 orders of magnitude greater than that obtained using the SCR technique (Fig. 2). The differences in EAG sensitivity between  $\alpha$ -cubebene and the 4-methyl-3-heptanol stereoisomers presumably reflects the differences in the proportions of the cell types present on the antenna, since the amplitude of the EAG response is thought to be directly related to the number of receptors responding [17]. Thus although significant responses to the 4-methyl-3-heptanol stereoisomers are observed with the GC-EAG technique [5], no responses were obtained with  $\alpha$ -cubebene since the stimulus concentrations required to elicit a detectable EAG response from  $\alpha$ -cubebene are not attainable without severely overloading the capillary column with a consequent reduction in efficiency.

These limitations do not apply to the GC-SCR system. Threshold concentrations of individual olfactory cells in *S. scolytus* are usually of the order

of  $10^9$ – $10^{10}$  molecules/ml of air and such stimulus concentrations are readily obtainable under high resolution GC conditions. Fig. 3 shows the response elicited from a cell, specialised to  $\alpha$ -cubebene, to stimulation with an air entrainment extract of the volatiles associated with female *S. scolytus* boring into English elm. The increase in the activity of the cell at 25.2 min corresponded with the elution from the capillary column of  $\alpha$ -cubebene, the key compound for the cell. Thus the GC-SCR system provides a sensitive biological detector that is independent of the proportions of the different cell types present on the antenna. The overall sensitivity of the system has not been fully examined. However, stimulation of a pair of cells specialised to (–)-threo (**I**) and (–)-erythro-4-methyl-3-heptanol (**II**) with 200 pg of the mixed isomers resulted in a signal to noise ratio of 6:1. This amount of material is close to the detection limits of the GC system.

The selectivity of the coupled GC-SCR system is shown by the responses of a pair of cells to stimulation with a mixture of (±)-threo- and (±)-erythro-4-methyl-3-heptanol (Fig. 4). The actual responses of the cells over the period of stimulation by the 4-methyl-3-heptanol isomers are shown in Fig. 5. The cell giving spikes of larger amplitude was specialised



to (–)-threo-4-methyl-3-heptanol. It showed an increase in the level of activity at 6.3 min which corresponded with the elution from the GC column of (±)-threo-4-methyl-3-heptanol. The cell with the smaller amplitude spikes was specialised to (–)-erythro-4-methyl-3-heptanol and showed maximum impulse frequency between 6.45 and 6.5 min which corresponded with the elution from the capillary column of the (±)-erythro isomer. Thus, despite the fact that there is incomplete separation on the GC column of (±)-threo and (±)-erythro-4-methyl-3-heptanol the peaks corresponding to the key compounds for each cell can be readily distinguished.

The stabilities of the SCR preparations are high and provided that stimulus concentrations are kept low, recordings may be continued for several hours. In general, each cell responds only to its key compound in the air entrainment extracts. However the  $\alpha$ -cubebene cell preparation shown in Fig. 3 also responded to the component which eluted at 26.2 min. This compound (**IV**), which is either  $\alpha$ -ylangene or its stereoisomer  $\alpha$ -copaene [18] is structurally related to  $\alpha$ -cubebene. Although the adaptation effects that occur when a cell is successively stimulated under the coupled GC-SCR conditions have not yet been fully investigated, the weak response of the  $\alpha$ -cubebene cell to **IV** was confirmed by diverting the transfer line (11) from the airflow passing over the antenna until after the elution of  $\alpha$ -cubebene from the capillary column. The response elicited from the cell by stimulation with **IV** under these conditions did not differ greatly

from that elicited after the cell had been previously stimulated with  $\alpha$ -cubebene.

The examples of the use of the GC-SCR technique presented above have all related to our present study of bark beetle aggregation pheromones. However, the technique should also be applicable to other Coleoptera and other insect Orders. Although the method is primarily intended for the biological monitoring of gas chromatograms, the specificity of the olfactory cells is apparently such that the system may be used, in conjunction with the GC retention times, as a specific detector in its own right. Thus this technique could prove of value in the analysis of interspecific communication between sympatric species. In addition, since small isomeric impurities in synthetic samples are frequently difficult to remove without GC purification, the direct coupling of the SCR technique to the GC allows the study of the responses of individual olfactory cells to isomers of very high purity.

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