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A New Method to Improve Olfactory Responses to GC Effluents

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Abstract

Biological detectors such as the human nose or an insect antenna represent extremely attractive detectors for gas chromatography (GC) since they are specifically tuned to perceive biologically relevant compounds. However, these detectors are nonlinear, noisy and often perform poorly under standard GC conditions since they are adapted to detect transient rather than long-lasting stimuli. These drawbacks can be overcome if the chemical stimulus, delivered by the gas chromatograph, is re-shaped (modulated) before reaching the biological detector. We describe a pulsing system that improves the performance of an insect olfactory system when stimulated with the effluent from a GC capillary column. Chemicals eluted from the GC column are trapped and rapidly desorbed within a thermal modulator in order to transform the continuous effluent into a succession of short-pulsed stimuli. The output from this modulator was directed to an insect antenna, from which electrophysiological responses were recorded. The system was evaluated with adult male *Spodoptera littoralis* (Lepidoptera: Noctuidae) stimulated with the conspecific female sex pheromone. Results obtained with this new approach indicate that both sensitivity and reliability of the biological detector are improved compared with the classical technique. Possible developments of this new technique are discussed.

Introduction

Classical gas chromatographic systems utilize a physical detector, e.g. a flame ionization detector (FID), to monitor the elution of molecules. Although these provide an accurate estimate of the elution time and quantities of the individual components in a mixture, they are of limited value in the location and identification of biologically active components in complex natural product extracts such as natural fragrances, perfumes or plant odours.

In contrast, the human nose or the olfactory receptors located on insect antennae are tuned to detect biologically relevant stimuli. In the case of insects, these receptors are associated with the perception of volatile semiochemicals involved in intra- and inter-specific communication, and the location and selection of suitable food sources or oviposition sites. Whilst the receptors associated with the perception of these semiochemicals are specific and more sensitive than physical detectors, they also differ in a number of respects in that they: (i) encode chemical concentrations on a logarithmic scale rather than on a linear scale; (ii) show a variable hysteresis (adaptation or fatigue); and (iii) respond to concentration changes rather than to the absolute concentration (responses are phasi-tonic). In addition, biological detectors are generally short-lived, especially when electrical signals are recorded from dissected preparations. This imposes limitations on the GC analysis time and the number of replicates, and can cast doubts on accuracy of the recorded responses. Such biological preparations must be checked regularly to evaluate their responsiveness, e.g. by recording responses to a standard stimulus. Nonetheless, systems combining high-resolution gas chromatography with an electroantennogram detector (GC-EAG) have proved invaluable in the identification of insect pheromones (Arn *et al.*, 1975). Indeed, this technique is now used routinely in a number of laboratories and commercial systems are available. However, the GC-EAG has a number of limitations, particularly in the location of physiologically active components from plants, since plant-derived semiochemicals are thought to be perceived by smaller populations of olfactory receptors than pheromones (Wadhams, 1990; Anderson *et al.*, 1996; Marion-Poll and Thiéry, 1996). For purely physiological reasons, stimulation of these receptors is less likely to evoke large EAG responses. In addition, it has recently been suggested that the 'shape' of the odour stimulation as delivered by the GC is not optimal

for stimulation of the olfactory system (Marion-Poll and Thiéry, 1996).

The latter problem can be overcome if the chemical stimulus, delivered by the gas chromatograph, is re-shaped by periodic sampling before it reaches the biological detector. Indeed, this approach was utilized by Moorhouse *et al.* (1969), working with packed GC columns. Components eluting from the GC column were trapped in a glass vial (15 s collection period) before being delivered to the antenna as a single pulse. With the advent of high-resolution capillary columns, this pulsed approach was largely superceded (except Cork *et al.*, 1990) by systems in which the GC column effluent was continuously monitored by both FID and EAG detectors (Arn *et al.*, 1975). Although this allowed full advantage to be taken of the GC resolution capabilities, it also resulted in reduced GC-EAG detection levels for some semiochemicals.

A number of systems, using fast valves (Wells, 1985) or thermal modulation (Phillips *et al.*, 1985), have been described that would allow pulsed delivery of the GC effluent to the EAG detector with minimal loss of GC resolution. The latter authors showed that a GC column could be used as a modulator by subjecting it to cycles of different temperatures. Thermal modulators have also been used to perform multiple injections and have proved efficient for continuous analysis of environmental pollutants in a variety of experimental situations (Phillips *et al.*, 1986; Mitra and Phillips, 1986, 1989).

In this paper we investigated the use of a thermal modulator, placed at the output of a GC column, to improve the detection capabilities of an insect antenna. We tested this approach on a model insect, *Spodoptera littoralis* (Lepidoptera: Noctuidae), stimulated with a high-boiling-point pheromone component. Results obtained with the classical GC-EAG technique were compared with recordings obtained with the experimental thermal modulator system. EAG responses were improved by the modulation of the GC effluent, both by reducing the detection threshold and by increasing the reliability of the results.

Materials and methods

The GC-EAG system was based on a Varian 3300 gas chromatograph equipped with an sample programmable injector (SPI 1093, Varian, France), an apolar BPX5, fused silica column (25 × 0.32 m i.d., 0.5 μm, SGE, USA), a variable split (OSS-2, SGE) and an FID. Injector and oven temperatures were programmed separately. The injector temperature was maintained at 48°C for 0.1 min and then increased at 180°C/min to 220°C. The oven temperature was held at 40°C for 1 min and then increased in two steps to 120°C at a rate of 20°C/min and then at 15°C/min to 220°C.

The split ratio was set at 60:40 (EAG:FID). Both transfer line sections following the effluent splitter were prepared from deactivated fused silica tubing (SGE 0.3 mm dia.). At

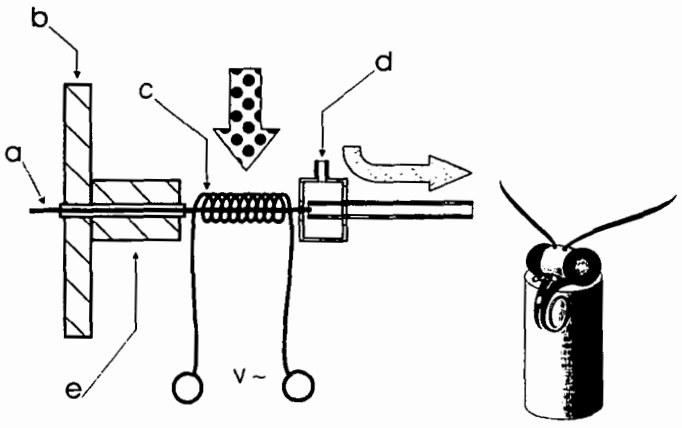

Figure 1 Diagram of the modulator. **(a)** GC capillary column driving the output effluent; **(b)** oven; **(c)** heating coil; **(d)** humidified air inlet and mixing chamber; **(e)** heating element for the output GC column, adjusted to 220°C. Large arrows: black dots = compressed air flowing continuously over the coil; left to right = humidified air blowing on the insect's antenna.

the outlet of the oven, a temperature-controlled transfer line (Syntech, NL) was adjusted to the final oven temperature (220°C). The column effluent was introduced into a humidified and filtered airstream (55 l/h; at room temperature) directed over the insect's antenna.

A prototype thermal modulator was positioned at the end of the transfer line, before the humidified and purified airstream (Figure 1). The modulator consisted of the last 2 cm of the GC transfer line, wrapped with a heating coil (isolated wire used for transformers, 0.2 mm dia.). A pulse generator (Syntech, The Netherlands) was used to deliver voltage pulses at regular intervals (see below) through the wire. The voltage intensity was adjusted to control the heating capacity of the wire. Compressed air was continuously passed over the exposed column and coil, in order to cool the modulator between heating pulses and to improve the adsorption efficiency.

Male *S. littoralis* originated from a culture maintained in the laboratory on an artificial diet. For electrophysiological recordings, each insect was immobilized between two pieces of rigid foam. EAGs were recorded using glass capillary electrodes filled with 0.1 M KCl. A reference electrode was inserted into the cephalic capsule whilst the recording electrode was positioned over the cut end of one antenna. Electrical signals were amplified with a conventional pre-amplifier (WPI 750, USA) in DC mode and not further filtered. Under these conditions, EAG responses could be recorded for >6 h from the same insect.

For the classical GC-EAG system the coil was removed from the end of the fused silica transfer line and the GC column effluent was directed into the purified and humidified airflow passing continuously over the insect preparation.

(*Z,E*)-9,11-Tetradecenyl acetate (Z9E11–14:Ac), the

major pheromone emitted by *S. littoralis* females, was used to compare both GC-EAG systems. Samples (0.1 pg to 100 ng) were injected in 1 μl of hexane. With the split ratio used, this was equivalent to 0.060 pg to 60 ng of pheromone reaching the antenna. At least three stimulations were performed for each concentration and each insect preparation was tested with the entire series of concentrations if possible.

The FID and EAG signals were evenly sampled at 10 Hz with a Varian GC data acquisition system (Star, Varian; precision 24 bits) and stored into binary data files. Results were analysed from the raw data, exported as text files to a spreadsheet program (Excel, Microsoft) and to a custom designed electrophysiological data analysis program with interactive measure capabilities, Awave32 (Marion-Poll, 1995). Results were analysed using analysis of variance procedures (SAS 6.11, SAS Institute, NC), in order to evaluate the effects of thermal modulation on the EAG responses.

Results

Classical GC-EAG recordings

The amplitudes of the EAG response elicited from male *S. littoralis* antennae to stimulation with serial dilutions of the major pheromone component Z9E11–14:Ac are shown in Figure 4. The EAG detection limit was 1 pg. At this concentration the FID showed no detectable response. However, estimated from the 100 ng sample, the retention time of Z9E11–14:Ac was 14 min with a peak width at the base of 10–20 s. The FID peaks were bell-shaped and symmetrical.

With this method, a number of EAG recordings were difficult to analyse because of random baseline fluctuations. These variations originated not from the recording equipment but from the biological preparation. The use of a high-pass filter, with a corner frequency properly adjusted (10–100 Hz), removed most of these variations but altered the shape and amplitude of the responses. In such noisy recordings, responses could be clearly characterized only if their frequency content was different from the random baseline variations, i.e. if the responses generated exhibited a fast onset.

Modulated GC-EAG recordings

Electrophysiological responses were considered as modulated if the response during the elution of the stimulus was pulsed, i.e. consisted of a succession of depolarizations that followed the same cycling period as that imposed on the modulator. A fully modulated response (Figure 2) consisted of a succession of depolarizations with a complete return to the baseline level between successive responses. An undermodulated response consisted of a series of depolarizations superimposed on a depolarized baseline that followed the time course of the GC peak elution.

Time parameters and modulation

Modulated responses were obtained by adjusting four parameters on the thermal modulator: heat intensity, cooling temperature, heat pulse duration and total period duration. With the current system, optimum results were obtained under the following conditions: 2 V potential across the coil (so that it was glowing when the current was

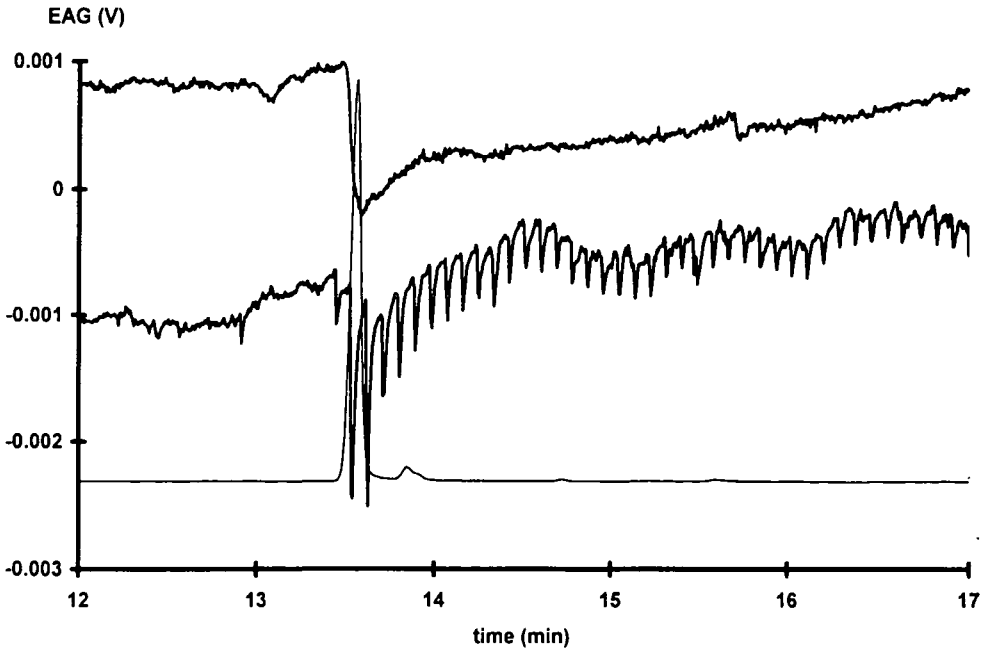

Figure 2 Response of *Spodoptera littoralis* to 100 ng of Z9E11–14:Ac. Top curve: unmodulated response. Middle curve: modulated response; frequency, 5 s; duration, 0.5 s; intensity, 1 V. Lower curve: FID signal.

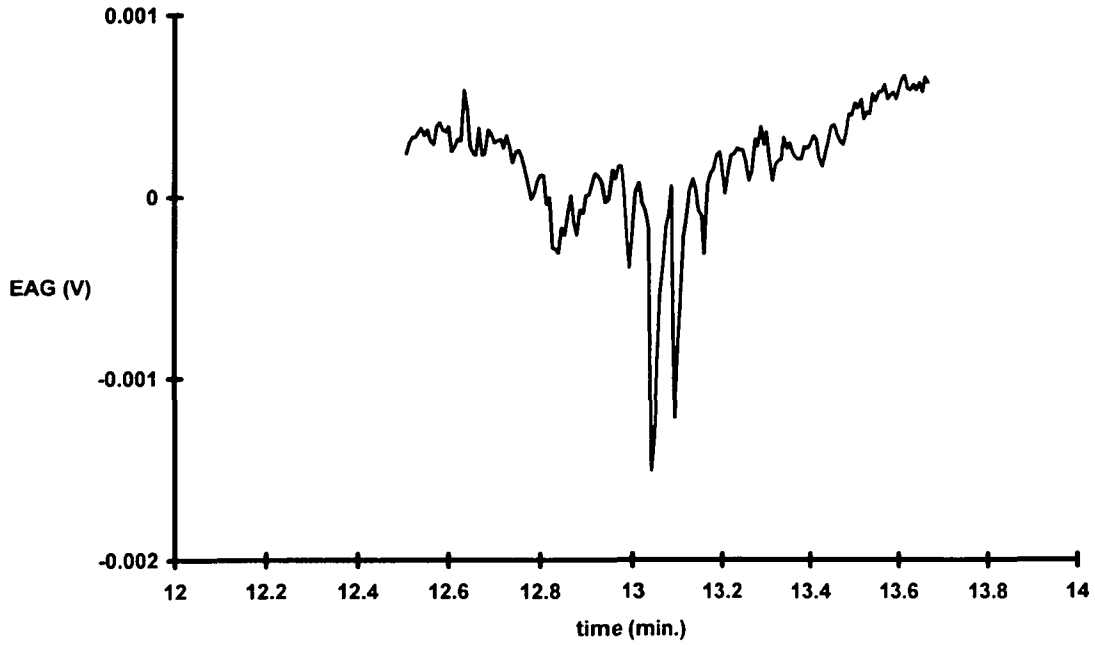

Figure 3 Response of *Spodoptera littoralis* to 1 pg of Z9E11–14:Ac. Frequency, 3 s; duration, 0.8 s; intensity, 2 V.

on), a constant flow of humidified compressed air directed over the coil at room temperature, heating pulses of 0.8 s and a total period of 3 s. Under such conditions, EAG responses were perfectly synchronized with the FID response (Figure 3). Other parameters gave mixed results, ranging from no detectable EAG peaks during the heat pulses, equivalent to the classical situation (no modulation), to EAG peaks superimposed on a classical EAG (under-modulation).

Duration and shape of the modulated responses

Initial studies compared responses, with and without modulation, to high stimulus concentrations (100 ng) of Z9E11–14:Ac (Figure 2). The classical GC-EAG system showed a strong response at the onset of the GC peak, followed by a long-lasting depolarization. Modulated EAG responses consisted of a series of sharp EAGs whose overall envelope (maximal amplitude of each individual response) followed the time course of the unmodulated response. Under these experimental conditions, additional, small amplitude EAGs were consistently observed after the elution of the GC peak (Figure 2). At lower doses, EAGs were observed during the elution of the peak even when no measurable signal was delivered by the FID (Figure 3).

Responses to Z9E11–14:Ac with thermal modulation

Modulated response amplitudes were estimated by measuring the maximum EAG in a series (Figure 4). Experiments were done with the same modulation parameters as in Figure 3 (period, 3 s; heating pulse, 0.8 s). These results were compared with the previous EAG responses to Z9E11–14:Ac obtained in the absence of modulation. The variance analysis indicates that thermal modulation and concentration are two highly significant factors (modulation effect: $F = 18.93$, df = 1, $P > 0.0001$; concentration: $F = 74.01$, df = 1, $P > 0.0001$) with no interaction between them ($F = 0.72$, df = 1, $P > 0.4$). The corresponding regression curves ($AX + B$) are parallel (modulation: $A = 0.10$; no modulation: $A = 0.12$; $P > 0.4$) but with different intercept factors (modulation: $B = 1.65$; no modulation: $B = 1.08$; $P > 0.0001$).

Although the dose–response curves are parallel, the modulated responses are shifted to the left. Thus, for the same stimulus, the amplitudes of the responses were ~0.5 mV higher and the detection threshold was at least an order of magnitude lower. EAG responses to 0.1 pg of Z9E11–14:Ac were still detectable with thermal modulation of the effluent, whilst the detection limit with classical GC-EAG system was 1 pg. In addition, modulated responses are easier to detect and to characterize, particularly when the baseline of the recordings is noisy.

Discussion

In this paper we present a new GC output thermal modulator that delivers sampled volatiles as short pulses of odour to the EAG detector. Results obtained with this new technique are compared with those obtained with a classical GC-EAG output stimulus design.

This approach has a number of advantages over the classical GC-EAG system. Since receptors are only exposed to short-duration stimulations interspersed with periods without stimulation, they are less prone to adaptation

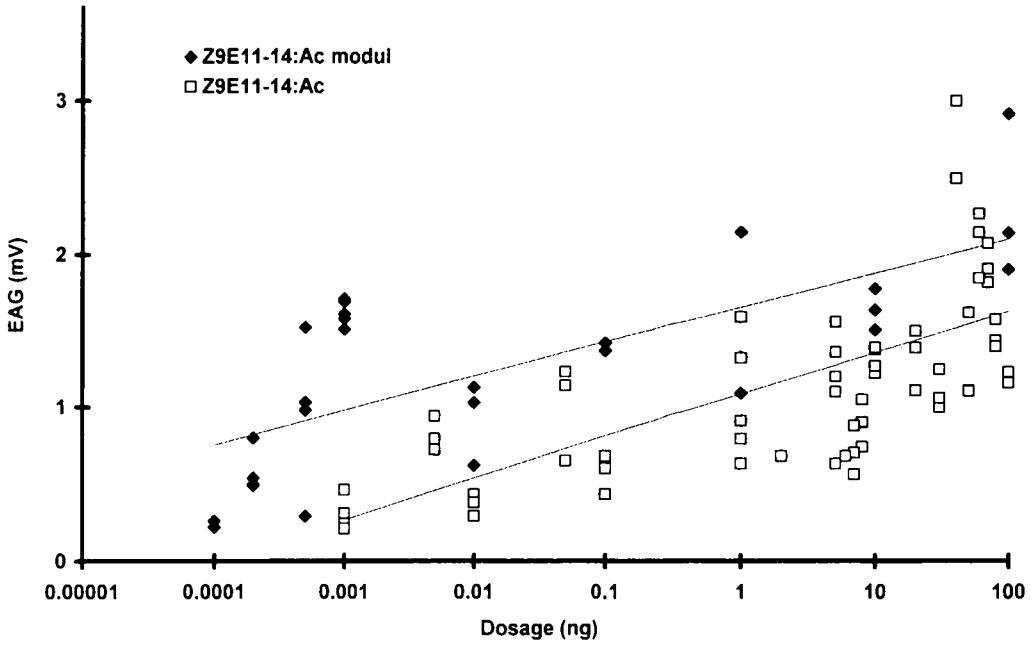

Figure 4 Dose–response curves of *Spodoptera littoralis*, stimulation with Z9E11–14:Ac. ◆ EAG responses to modulated stimulus; □ EAG responses to unmodulated stimulus (period, 3 s; heat pulse, 0.8 s; potential, 2 V).

than under the classical GC-EAG system. In addition, by trapping the eluted components during the cold cycle and releasing them during the heating pulse, the modulator concentrates the column effluent, thereby effectively increasing the stimulus concentration. These factors combine to increase the sensitivity of the electrophysiological detector by at least an order of magnitude and improve reliability at low stimulus concentrations.

At the lowest stimulus concentrations only a single EAG was elicited with the modulated system. However, with higher concentrations a number of EAG responses were observed which continued even after the FID peak had returned to the baseline. These effects could be associated with limitations in the design of the current modulator, resulting either in incomplete desorption of volatiles during the heating phase or in compounds 'sticking' to the deactivated transfer line. It is interesting to note that, in some studies using the coupled GC-single cell recording technique, increased levels of spontaneous activity were also observed after the elution of the active component, suggesting that there is indeed some adsorption and re-release of compounds from the deactivated fused silica transfer lines (Campbell *et al.*, 1990). Alternatively, the post-event EAG responses could be associated with the splitter system introducing a dissymetry in the elution of compounds directed to the insect's antenna, or to tailing of the GC peak. Since the FID performs poorly at low concentrations, such tailing would not be observed on the FID response. Further work is required to determine the cause of the post-stimulation EAG responses observed with high stimulus concentrations.

In order to modulate efficiently the output of a GC column, a modulator should optimize both the trapping capacity and the desorption efficiency. These factors are linked to a series of related experimental variables, such as cycle duration, temporal ratio of cooling/heating phases and their respective temperatures, and trapping efficiency of the modulator's column (with or without phase). These parameters could not all be varied independently with the current prototype system. For example, heat intensity and duration were limited by the coil, which burned out when too much heat was applied. This problem occurred not only when the net intensity of the current applied to the coil was too high, but also when the duration of the heating pulse/cooling phases were not correctly adjusted. Thus, heat could build up in the present system by thermal inertia. The temperature of the modulator column was not monitored directly and could not be predicted by the temperature of the coil since, judging by the distribution of the glow emitted when the heating current was close to the maximum, the heat seemed to be distributed unevenly across the length of the coil.

Although considerable work is still required to optimize the system particularly with regard to pulse duration, these preliminary studies have shown the feasibility of the modulated delivery system. Pheromone molecules were efficiently trapped during the cooling phase and released with no apparent thermal degradation during the heating phase. The modulated system retains the advantages of the original pulsed technique (Moorhouse *et al.*, 1969) whilst the short pulse durations still utilize the full GC resolution capabilities. This latter aspect is of particular importance

since the accurate location of electrophysiological activity in complex natural product extracts is essential to the identification of semiochemicals by GC–mass spectrometry (Pickett, 1990).

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References

Anderson, P., Larsson, M., Löfqvist, J. and **Hansson, B.S.** (1996) *Plant odour receptor neurones on the antennae of the two moths* Spodoptera littoralis *and* Agrotis segetum. Entomol. Exp. Appl., 80, 32–34.

Arn, H., Städler, E. and **Rausfcher, S.** (1975) *The electroantennographic detector: a selective and sensitive tool in the gas chromatographic analysis of insect pheromones*. Z. Naturforsch., 30C, 722–725.

Campbell, C.A.M., Dawson, G.W., Griffiths, D.C., Pettersson, J., Pickett, J.A., Wadhams, L.J. and **Woodcock, C.M.** (1990) *Sex attractant pheromone of damson-hop aphid* Phorodon humuli *(Homoptera, Aphididae)*. J. Chem. Ecol., 16, 3455–3465.

Cork, A., Beevor, P.S., Gough, J.E. and **Hall, D.R.** (1990) *Gas chromatography linked to electroantennography: a versatile technique for identifying insect semiochemicals*. In McCaffery, A.R. and Wilson, I.D. (eds), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York, pp. 271–279.

Marion-Poll, F. (1995) *Object-oriented approach to fast display of electrophysiological data under Windows*. J. Neurosci. Methods, 63, 197–204.

Marion-Poll, F. and **Thiéry, D.** (1996) *Dynamics of EAG responses to host plant volatiles delivered by a gas chromatograph*. Entomol. Exp. Appl., 80, 120–123.

Mitra, S. and **Phillips, J.B.** (1986) *High capacity desorption modulators for gas chromatography*. J. Chromatogr. Sci., 26, 620–623.

Mitra, S. and **Phillips, J.B.** (1989) *Automated on-line analysis using thermal desorption modulators*. Anal. Instrum., 18, 127–145.

Moorhouse, J.E., Yeadon, R., Beevor, P.S. and **Nesbitt, B.F.** (1969) *Method for use in studies of insect chemical communication*. Nature, 223, 1174–1175.

Phillips, J.B., Luu, D. and **Pawliszyn, J.B.** (1985) *Multiplex gas chromatography by thermal modulation of a fused silica capillary column*. Anal. Chem., 57, 2779–2787.

Phillips, J.B., Luu, D. and **Lee, R.P.** (1986) *Thermal desorption modulation as a replacement for sample-injection in very small diameter gas chromatography capillary columns*. J. Chromatogr. Sci., 24, 396–399.

Pickett, J.A. (1990). *Gas chromatography–mass spectrometry in insect pheromone identification: three extreme case histories*. In McCaffery, A.R. and Wilson, I.D. (eds), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York, pp. 299–309.

Wadhams, L.J. (1990). *The use of coupled gas chromatography: electrophysiological techniques in the identification of insect pheromones*. In McCaffery, A.R. and Wilson, I.D. (eds), Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York, pp. 289–298.

Wells, G. (1985) *Design and performance of mass-flow modulated detection for gas chromatography*. J. Chromatogr., 319, 263–272.

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