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GC × GC/TOF MS technique—A new tool in identification of insect pheromones: Analysis of the persimmon bark borer sex pheromone gland

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Abstract

Conventional gas chromatography with electroantennographic detection (GC-EAD) and two-dimensional (GC × GC) gas chromatography using a time-of-flight mass spectrometric detector (TOFMS), were combined to analyse the female sex pheromone gland extract of the persimmon bark borer, *Euzophera batangensis*. GC-EAD analysis produced two EAD responses in GC areas where no compounds were detected by FID detection. GC × GC/TOFMS analysis of this area indicated the presence of several chemicals, including (*Z*9,*E*12)-tetradeca-9,12-dien-1-ol and (*Z*9)-tetradec-9-en-1-ol, pheromone components of closely related *Euzophera* species. Spectral characteristics, retention behaviour and the ability to elicit GC-EAD responses imply that both identified unsaturated alcohols are candidates for *E. batangensis* sex pheromone components. GC × GC/TOFMS facilitated the analysis of complex matrices on a subnanogram level and was shown to have great potential as a powerful tool in the analysis of insect pheromones.

Keywords: GC × GC/TOFMS; GC-EAD; (*Z*9,*E*12)-tetradeca-9,12-dien-1-ol; (*Z*9)-tetradec-9-en-1-ol; *Euzophera batangensis*; Pheromone identification

1. Introduction

Since identification of the first insect (the silkworm moth) sex attractant [1] more than 1000 Lepidopteran sex pheromones have been characterised to date [2]. Pheromones are usually produced in tiny amounts that vary from picograms to nanograms. As a consequence, pheromones are rarely isolated in quantities sufficient to carry out the full range of spectroscopic analyses (^{1}H and ^{13}C NMR, infrared, and mass spectrometric) routinely used in structural organic analysis. Identifications usually rely on mass spectrometry (MS) coupled with gas chromatography. An integral part of pheromone analysis is GC coupled with electroantennographic detection, GC-EAD [3], where a male antenna (connected by means of two electrodes to an amplifier) is used as a biological detector responding exclusively to physiologically active (i.e. pheromonal) chemicals in the GC effluent stream. The GC-EAD analysis thus helps to focus the identification effort only on those components that have biological activity. Quite often, however, GC-EAD indicates activity in chromatogram areas where FID or MS detects no chemical(s) [4]. In such cases, the unknown compound has to be identified by other methods, e.g. by thorough electrophysiological investigation of male antenna response specificity that narrows the choice to a few structures which must be synthesized and tested [5]. Another problem in the process of pheromone identification arises with co-elution of several compounds. The repeated GC-EAD analysis on another column with different polarity, which solves the problem, requires more time and additional material for analysis.

GC × GC/TOFMS is a recently developed analytical technique that offers a solution to the co-elution problem and provides high sensitivity and selectivity. In principle, the method consists of two GC systems (GC × GC) equipped with columns of different polarity connected by an interface with an integrated cryogenic trap. The cryogenic trap repeatedly condenses compounds eluting from the primary column and releases them periodically as short pulses to the secondary column. Parameters like duration and frequency of both condensation and injection pulses are variable and allow precise tuning of the instrument according to the requirements of the analysis. Since the

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GC × GC produces very narrow peaks (down to 50 ms, depending on the frequency of cryogenic modulation) a TOFMS detector with a high acquisition rate (up to 500 spectra per second) is required. The pulsed nature of the TOFMS source of ionisation further enhances the system accuracy by avoiding spectral skewing common in a continuous ionisation mode. GC × GC with TOFMS detection thus operates with a high precision independent of concentration range [6–16].

The persimmon bark borer [17], *Euzophera batangensis* Caradja, is a small moth from the Pyralidae family distributed widely in East Asia (China, Korea, Japan) [18,19]. The originally harmless polyphagous insect became a serious pest of the jujube tree (*Ziziphus jujuba*) in North China [20], especially in areas where girdling, a common horticultural practice, is routinely used to enhance fruit harvest. *E. batangensis* females lay eggs in fresh girdles and hatched larvae feed on the tree cambium. The vigour of infested trees is decreased and they eventually die. Since *E. batangensis* produces several generations per year, the economic loses could be high. Due to *E. batangensis* endophytic behaviour, there is no efficient method to control the pest. Identification of the sex pheromone could lead to significant improvement in pest management strategies.

In this paper, we report the advantages of application of the GC × GC/TOFMS method to the analysis of hexane extracts of the *E. batangensis* sex pheromone gland.

2. Experimental

2.1. Insects

Larvae of the persimmon bark borer *E. batangensis* were collected in jujube orchards near Shijiazhuang City (Hebei province, North China). In the laboratory, larvae were maintained under a 14:10 light: dark regime, 25 °C and 50% humidity on an artificial diet for *Ostrinia nubilalis* [21] until pupation. Pupae were sexed and kept separately. Emerged females were maintained in the rearing room, males were maintained at a low temperature (5 °C) until use.

2.2. Pheromone gland extraction

Pheromone glands of 1–2 day old females were excised during the calling period (the end of the scotophase). Calling females were cooled to −20 °C for at least 5 min prior to gland excision. Pheromone glands were extracted in hexane for 2 h (ca. 10 μL of hexane per abdominal tip). Extracts were stored at −20 °C until analysis.

2.3. GC-EAD

Isolated antennae of 2–7 days old males were used in GC-EAD experiments. Samples were injected splitless into a 5890A Hewlett-Packard gas chromatograph equipped with a DB-5 (J & W Scientific, Folsom, CA, USA; 30 m × 250 μm i.d. × 0.25 μm film) column. The column effluent stream was split by a Graphpack 3D/2 four-arm splitter conducting the eluting compounds to FID and EAD detectors. The GC was operated at an initial temperature of 50 °C for 2 min, then ramped at a rate of 10 °C/min to 270 °C (with 10 min hold). The temperature of GC inlet and detector were set to 200 and 260 °C, respectively. A series of *n*-alkanes (C_{14}–C_{20}; Sigma-Aldrich) was co-injected with authentic samples to determine Kováts indices (I_K) of EAD-active compounds.

2.4. GC × GC/TOFMS

The GC × GC/TOFMS analyses were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA) equipped with a non-moving quad-jet cryomodulator. A slightly polar DB-5 column (J&W Scientific, Folsom, CA, USA; 30 m × 250 μm i.d. × 0.25 μm film) was used for GC in the first dimension. The second dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX, USA; 2 m × 100 μm i.d. × 0.1 μm film). Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program for the primary GC oven was as follows: 50 °C for 2 min, then 50–300 °C at 10 °C/min, and finally 10 min hold at 300 °C. The program in the secondary oven was 5 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration and the cool time between stages were set at 3.0, 0.4 and 1.1 s, respectively. The transfer line to TOFMS detector source was operated at 260 °C. The source temperature was 250 °C with a filament bias voltage of −70 eV. The data acquisition rate was 100 Hz (scans/s) for the mass range of 29–400 amu. The detector voltage was 1750 V. One microlitre samples were manually injected splitless. The inlet temperature was 200 °C. The purge time was 60 s at a flow of 60 mL/min. Data were processed and consecutively visualised on 2D and 3D chromatograms using LECO ChromaTOF^{TM} software. As in GC-EAD experiments, series of *n*-alkanes (C_{14}–C_{20}; Sigma-Aldrich) was co-injected with authentic samples to determine Kováts indices (I_K) of analytes.

2.5. Chemicals

Synthetic standards were prepared in our laboratory. All standards were purified by flash chromatography and dissolved in trace analysis grade hexane (Fluka).

2.6. Quantitative analysis

To determine the detection threshold of the system, synthetic (*Z*9,*E*12)-tetradeca-9,12-dien-1-ol (9*Z*,12*E*-14:OH) was diluted in hexane in concentrations ranging from 10 pg to 2 ng per μL. One microlitre of a respective dilution was injected into the GC × GC/TOFMS under the same conditions as stated above. The respective peaks were integrated manually and a calibration curve was constructed by plotting the peak areas against the concentration.

3. Results

GC-EAD analyses of *E. batangensis* gland extract (one female equivalent; one FE) consistently showed one major

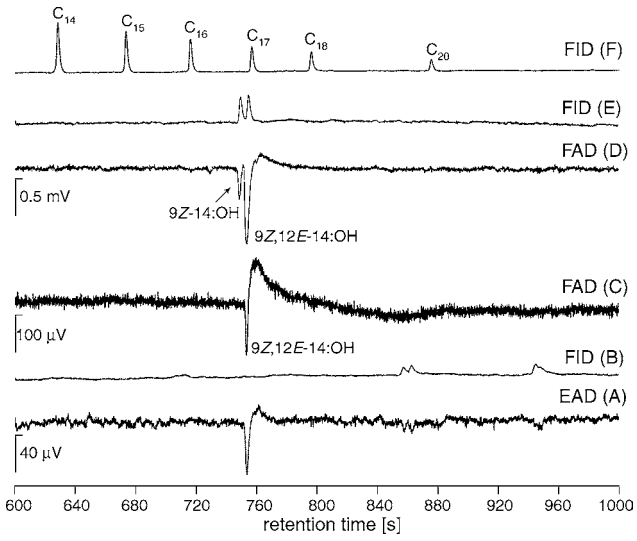

Fig. 1. Sections of GC-EAD/GC-FID traces on DB-5 phase. (A/B) Hexane extract of *E. batangensis* female pheromone gland (∼1 FE). (C) Synthetic 9*Z*,12*E*-14:OH (100 pg). (D/E) Co-injection (10 ng) of synthetic 9*Z*,12*E*-14:OH and 9*Z*-14:OH. (F) *n*-Alkane (C_{14} to C_{20}) standards.

EAD response (Fig. 1A). The respective compound eliciting the EAD response was not FID-detectable (Fig. 1B). Since Pyralidae usually utilize linear aliphatic C_{12}–C_{14} acetates, alcohols or aldehydes with one or more double bonds [2] we determined the Kováts index of the EAD activity ($I_{K,\,EAD}$) and compared it with tabular [22] I_K values of saturated and monounsaturated alcohols, acetates and aldehydes. The EAD Kováts index determined with the DB-5 column ($I_{K,\,EAD} = 1681$) did not closely correspond with any tabular [22] I_K value suggesting that the prominent EAD activity is not associated with a C_{12}–C_{18} saturated or monounsaturated alcohol, acetate or aldehyde. This observation indicated the possibility that additional double bonds might be present within the active molecule. Further GC-EAD experiments with available diunsaturated synthetic standards showed a perfect match of the retention time of the EAD response of the unknown compound from the pheromone gland extract with the retention time of the EAD response of synthetic 9*Z*,12*E*-14:OH (Fig. 1C). This compound has been reported as a pheromone component of other species of the genus *Euzophera* and many other Lepidopteran species [2]. However, one-dimensional GC/MS analysis of the extract did not provide any relevant mass spectrum to confirm such identification, since the MS signal was deeply hidden in the background noise. On the other hand, the GC × GC/TOFMS analysis of the area corresponding with EAD activity disclosed several compounds (Fig. 2A) including 9*Z*,12*E*-14:OH. Fig. 2A also illustrates nicely the high "wall" of the column bleed that would hide the analyte peaks in standard 1D GC analysis, but is well separated in the second dimension of the GC × GC experiment. The mass spectrum and two-dimensional retention times (Fig. 2C; Table 1) of the synthetic 9*Z*,12*E*-14:OH were identical with the compound found in the gland extract. The detection threshold of GC × GC/TOFMS for synthetic 9*Z*,12*E*-14:OH was determined to be approximately 10 pg. Such sensitivity almost approaches the sensitivity of GC-EAD where 10 pg elicited significant EAD activity. Quantitative analysis (with construction of a calibra-

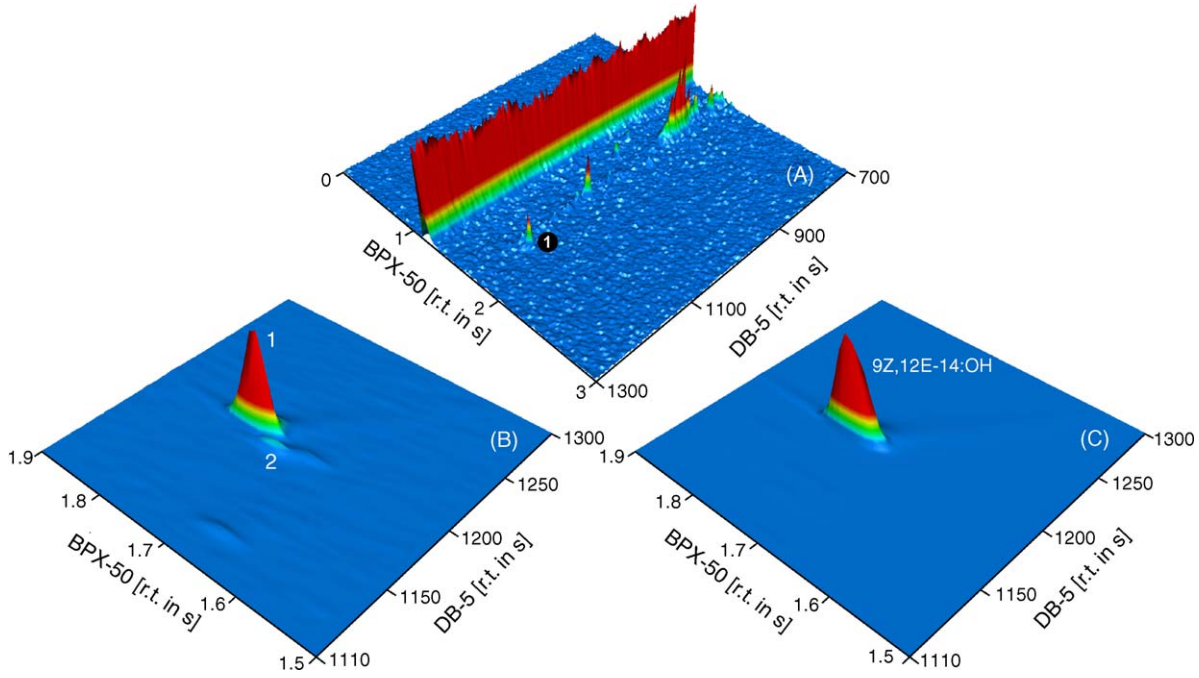

Fig. 2. GC × GC/TOFMS chromatograms (3D-surface plots) at mass 31. (A) Analysis of *E. batangensis* female pheromone gland (∼1 FE). The small peak labelled (1) matches (NIST, EAD and retention behaviour) of 9*Z*,12*E*-14:OH. The well-separated "wall" of chemical noise (column bleed, traces of solvent, etc.) demonstrates the power of comprehensive two-dimensional GC. (B) Zoomed-in EAD-active region of pheromone gland extract. The peak labelled (1) depicts the main pheromone component; the very small peak labelled (2) corresponds to 9*Z*-14:OH. (C) Zoomed-in EAD-active region of analysis of synthetic 9*Z*,12*E*-14:OH (100 pg).

Table 1
Retention parameters of standards and analytes

Compound	I_K (1st dimension)	Ret. time[a,c] (s; 1st dimension)	Ret. time[b,c] (s; 2nd dimension)
Standards			
*Z*9-14:OH	1676	1188	1.700
*Z*9,*E*12-14:OH	1681	1194	1.750
Analytes			
Peak 2	1676	1190	1.711
Peak 1	1681	1194	1.761

[a] DB-5 (30 m × 250 μm i.d. × 0.25 μm film).
[b] BPX-50 (2 m × 100 μm i.d. × 0.1 μm film).
[c] Temperature program: see Section 2.

Table 2
9*Z*,12*E*-14:OH detection threshold and pheromone gland quantitative analysis

Concentration (*C*; pg)	Peak area (*A*)
Synthetic 9*Z*,12*E*-14:OH[a]	
2000	198667 ± 12662
1000	96782 ± 12423
500	40336 ± 4506
100	9737 ± 1136
10	937 ± 158
Pheromone gland extract [1 FE]	
115[b]	8690

[a] Analyses triplicated for each concentration.
[b] Calculated from regression equation:
$A = (99.908 \pm 2.887) \times C - (2842 \pm 2961)$; $r^2 = 0.9893$.

tion curve) determined the amount of 9*Z*,12*E*-14:OH in the one pheromonal gland to be approximately 100 pg (Table 2).

The intimate inspection of 3D-surface plots of GC × GC/TOFMS chromatograms in the EAD activity area showed in addition to 9*Z*,12*E*-14:OH (peak 1; Fig. 2B), also traces of another pheromone-like compound eluting slightly earlier than 9*Z*,12*E*-14:OH in both dimensions (peak 2; Fig. 2B, Table 1). The mass spectrum of this compound together with two-dimensional retention parameters was identical with (*Z*9)-tetradec-9-en-1-ol (9*Z*-14:OH), suggesting the possibility that *E. batangensis* might have a two-component pheromone. The presumable physiological function of 9*Z*-14:OH was confirmed by separate GC-EAD experiments with synthetic 9*Z*-14:OH (co-injected with 9*Z*,12*E*-14:OH; Fig. 1D and E) and with more concentrated gland extract (5 FE; Fig. 3). Measurably but substantially weaker EAD responses were elicited with 10 ng of 9*Z*-14:OH in comparison with 9*Z*, 12*E*-14:OH.

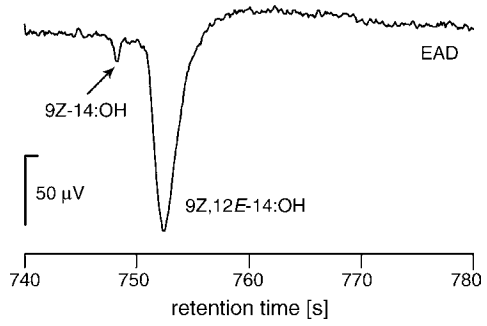

Fig. 3. Sections of GC-EAD/GC-FID traces: hexane extract of *E. batangensis* female pheromone gland after concentration (~5 FE). The antennal response of both presumable sex pheromone components is clearly visible.

4. Discussion

For about a decade, two-dimensional chromatography has been used for analysis of volatile organic compounds in many applications and has shown to be superior to 1D GC. The main advantages of 2D GC are the ability to separate co-eluting compounds and significant improvement of detection sensitivity [23]. Both features are very valuable in the identification of volatile compounds active in chemical communication, since active odour mixtures might be rather complex and they are usually produced in quite low quantities. This is also true in the field of insect chemical communication, where it has been shown that a single molecule of Lepidopteran female sex pheromone can trigger a response in the male olfactory receptor cell and very few molecules are required to affect male behavioural responses [24]. The small amounts of semiochemicals produced by pheromone glands make the analysis of insect semiochemicals challenging. Using standard GC/MS, we quite often faced a situation where a biologically active compound was present, however the MS spectrum remained hidden in the chromatographic background noise [4]. This was also true for the initial GC/MS analysis *of E. batangensis* sex pheromone. The application of the new technique, GC × GC/TOFMS represents a significant improvement of analytical discrimination power, since we obtained MS spectra not only for the major EAD active peak, but also for the minor one. In general, the main source of increased sensitivity in GC × GC/TOFMS is the modulator. The magnitude of the sensitivity gain depends on an appropriate choice of parameters such as the modulation period, hot-pulse duration, cool time and peak width in the second column [25,26] that narrows peaks arriving at the detector. Another source of increased sensitivity, especially important in our analysis, was the second dimension separation of the background from the biologically active compounds. The background components (solvent traces, septum and column bleed, etc.) have significantly different retention behaviour and elute from the second dimension column much earlier [23]. The high "wall" of column bleed was well separated in the second dimension allowing MS analysis without unwanted interference. The discrimination power of GC × GC/TOFMS was also enhanced by a new data processing routine. The GC × GC/TOFMS experiment involves MS analysis of numerous "cut-outs" of the primary column chromatogram. Any analysis thus can be seen as a series of 2D-chromatograms eluting one after another [23]. Depending on modulation parameters and peak widths, each compound eluting from the primary column shows up as several consecutive narrow peaks. These complex patterns are usually converted into a contour plot with the primary retention plotted along the *X*-axis, and the secondary retention plotted along the *Y*-axis (2D display), and eventually detector response

on the *Z*-axis (3D display). On such chromatograms, compounds characterised by the same mass spectrum appear as one spot. As the quantity of compounds can be colour coded, the colour of individual spots continuously changes from peak base to its top. Such coloured contour plots make searching for possible active compounds much easier, especially when a display based on characteristic ion current is selected instead of a total ion current (TIC). The recognition of spot patterns allows the experimenter to efficiently compare different chromatograms and search quickly for any differences. The GC × GC/TOFMS also makes identification based on retention parameters more reliable than with 1D GC. It provides identification capabilities similar to performing two separate 1D GC separations, as required by some analytical methods. The identification by two dimensional retention coordinates and mass spectral match between an analyte and a spectrum, offered either by a library or obtained by an injection of available standard, is very robust and the possibility of misidentification is negligible.

Our GC-EAD and GC × GC/TOFMS of pheromone gland extract of *E. batangensis* revealed two EAD active compounds. Their spectra and retention behaviour suggest that they both bear alcohol functionality and have linear C_{14}-chains. Our data indicate that the minor compound is monounsaturated, while the major one has two double bonds in positions 9 and 12. A close match of I_K, 2D retention behaviour and EAD activity of unknown compounds with values for *Z*9,*E*12-14:OH and *Z*9-14:OH suggest that these compounds are likely candidates for *E. batangensis* sex pheromone. *Z*9,*E*12-14:OH has been found as a part of the sex pheromone communication system in Lepidopteran species of the family Noctuidae (*Spodoptera exiqua*, *Rynchaglea scitula*) and in many species of the Pyralidae family, including major stored product pests, like *Cadra cautella, Ephestia kuehniella, Plodia interpunctella* and many others [2]. Within the genus *Euzophera*, the sex pheromones are known in three species, *E. pinguis, E. punicaella, E. semifuneralis*, and 9*Z*,12*E*-14:OH is present as a pheromone component in all of them [27-29]. These facts together with our results from GC × GC/TOFMS strongly enhance the probability that 9*Z*,12*E*-14:OH is also a major pheromone component in *E. batangensis*.

9*Z*-14:OH is a constituent of a great number of Lepidopteran sex pheromones [2]. It also forms a part of the sex pheromone of the closely related species *E. semifuneralis* [29] and has been found to be an attractant for congeneric species *E. bigella* [30]. Therefore the possibility that 9*Z*-14:OH also plays a role in the *E. batangensis* pheromonal communication system is quite high. The attractiveness of identified compounds was comparable with that of calling females in preliminary field observations. Though detailed behavioural experiments are needed to confirm 9*Z*,12*E*-14:OH and 9*Z*-14:OH as pheromone components in *E. batangensis*, our experiments clearly show the high efficiency of GC × GC/TOFMS in pheromone identification. The fact, that we were able to identify not only the major pheromone-like component, but also the minor one from a limited amount (∼10) of sex pheromone glands proved the potential of this analytical technique in the field of insect chemical communication research.

5. Conclusions

This study showed the suitability of the GC × GC/TOFMS system for insect pheromone analyses. Based on the results of GC-EAD and GC × GC/TOFMS we suggest that (9*Z*,12*E*)-tetradeca-9,12-dien-1-ol and (9*Z*)-tetradec-9-en-1-ol are candidates for female sex pheromone components in *E. batangensis*. Preliminary field observations do not contradict the suggested hypothesis.

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