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ANALYSIS OF INSECT PHEROMONES BY QUADRUPOLE MASS FRAGMENTOGRAPHY AND HIGH-RESOLUTION GAS CHROMATOGRAPHY

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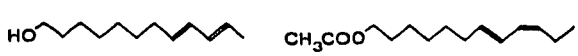
SUMMARY

A sensitive technique is described for the analysis of insect pheromones. Insect extracts are separated into fractions by column chromatography and are analyzed directly by quadrupole mass fragmentography on high-resolution glass capillary columns and by mass spectrometry. Applications are illustrated with two important pests. In the codling moth, *trans*-8,*trans*-10-dodecadien-1-ol was found to be present at a level of 3.5 ng per female, and in the European grapevine moth *trans*-7,*cis*-9-dodecadienyl acetate at a level of 1.6 ng. Both compounds appear to be present in high isomeric purity in the female insect and are absent in the corresponding males.

INTRODUCTION

Pheromones are compounds released by organisms to transmit messages to individuals of the same species. The sex pheromones dealt with in this study are produced by female insects in a gland located at the tip of the abdomen and serve to attract the males for mating. Synthetic pheromones are receiving considerable attention as tools for monitoring insect pests and as potential pest control agents. Owing to the minute amounts of materials present, the isolation and identification of sex pheromones is usually a difficult and tedious process. It involves rearing on a large scale, extraction from thousands of insects and an extensive clean-up in connection with a suitable bioassay technique.

Indications of the structures of several sex pheromones have been obtained by the electroantennogram technique on gas chromatographic fractions of female extracts and on series of synthetic model compounds[1]. With this technique, which requires only a few insects, the sex pheromone structures of two important pests, the codling moth *(Laspeyresia pomonella)* and the European grapevine moth *(Lobesia botrana)* were proposed as *trans*-8,*trans*-10-dodecadien-1-ol [1](I) and *trans*-7,*cis*-9-dodecadienyl acetate [2](II), respectively.



(I) (II)

These compounds were later found to be identical with the pheromones produced by the female insects[3,4]. Using these two insects as examples, we present in this paper a rapid and sensitive method for detecting and quantifying insect pheromones and analogues in biological material.

EXPERIMENTAL

Preparation and partial purification of insect extracts

The extraction and purification scheme is shown in Fig. 1. Abdominal tips (250 or 500) of 3- to 4-day-old laboratory-reared female moths were excised, placed in a vial containing 5 ml of diethyl ether and extracted by grinding with a glass rod and sonifying using a low-energy ultrasonic bath. Male extracts were similarly prepared for reference purposes. The extracts were filtered through small plugs of glass-wool in disposable Pasteur pipettes and the residues washed with three portions of diethyl ether. The total volume of ether did not exceed 10–12 ml. The clear filtrates were carefully concentrated to 0.3 ml with a gentle stream of nitrogen. The concentrates were transferred into 75 × 10 mm silica gel (70–230 mesh, Merck, Darmstadt, G.F.R.) columns topped with 5 mm of anhydrous sodium sulphate. The columns were eluted successively with 15-ml portions of 0, 3, 8 and 20% solutions of diethyl ether in pentane, diethyl ether and finally with a 20% solution of methanol in diethyl ether. The pentane and diethyl ether were Merck analytical grade, percolated through basic alumina (Woelm, Eschwege, G.F.R.).

All fractions were concentrated at room temperature with a stream of nitrogen and brought to a concentration of 1 moth-equiv./μl with *n*-hexane. The fractions were stored in PTFE-capped vials, wrapped with aluminium foil and kept in a freezer until required for analysis.

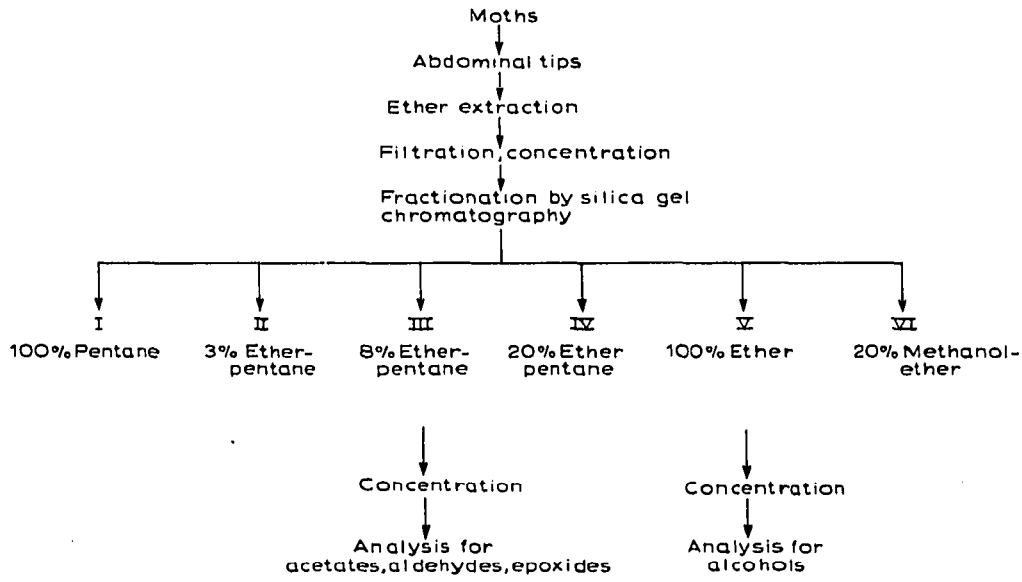


Fig. 1. Insect extraction and purification scheme.

Prior to mass spectrometric analysis, fractions requiring further purification were subjected to sweep co-distillation (Fig. 2). A suitable aliquot containing 50–150 moth-equiv. was diluted with diethyl ether to 0.5 ml and slowly injected into the sweep co-distillation column. The vaporizer section of this column was heated to 180° in the injection port of a gas chromatograph and supplied with a stream of nitrogen. The volatiles were swept into a glass trap maintained at −15° and condensed together with the solvent. In order to rinse the condensate from the trap into the sample retainer, three additional injections of 0.5 ml of diethyl ether were made at 1-min intervals. Prior to analysis, the solution was concentrated to 5–10 μl while care was being taken not to allow samples evaporate completely to dryness.

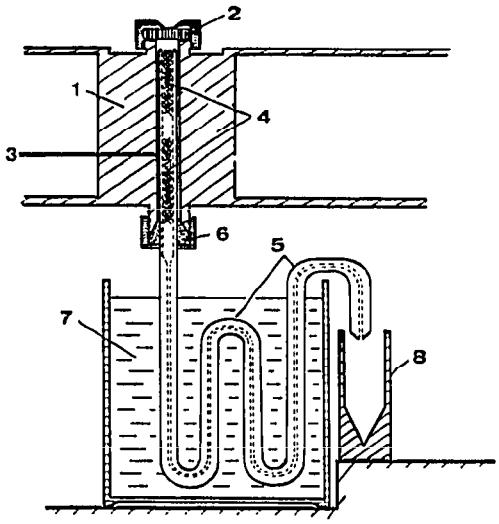


Fig. 2. Sweep co-distillation apparatus. 1 = Vaporizer block, 180°; 2 = septum; 3 = nitrogen, 15 ml/min; 4 = vaporizer (glass, 6 mm O.D., 4 mm I.D.) with silanized glass-wool; 5 = trap (60-cm glass tube, 6 mm O.D., 1 mm I.D.); 6 = Swagelok, Teflon ferrule; 7 = ice–salt bath, −15°; 8 = sample retainer.

Instrumentation and analysis

A high-resolution glass capillary column (Ucon 50 HB 5100, 50 m × 0.3 mm I.D., Grob type, commercially available from H. Jäggi, CH-9043 Trogen, Switzerland) was mounted in a Carlo Erba 2101 gas chromatograph equipped with an inlet splitter. A flexible platinum capillary (50 cm × 0.15 mm I.D., Engelhard Industrien, Zürich, Switzerland), heated to 170° with a Nichrome wire coil, served as interface to the Finnigan 1015 D quadrupole mass spectrometer (MS). At one end it was fused to the glass capillary column, and at the other into a short glass tube and introduced through the direct probe inlet into the ion source of the MS.

The column was operated at 170° and the inlet of the gas chromatograph at 200°. For best results, the glass vaporizer insert with silanized glass-wool had to be replaced after each sample injection. A pressure of 1.2 atm of helium was required in order to produce an optimal carrier gas velocity of 25 cm/sec (platinum capillary interface connected to the MS). Using a flame ionization detector (FID) (interface omitted), the same velocity was obtained with a pressure of 1.4 atm of helium.

Sample introduction was effected by splitless injection. The operation sequence is shown in Fig. 3. Prior to injection of a sample, the ion source was switched off, the carrier gas inlet pressure increased to 2.5 atm, the column cooled to room temperature and the vaporizer insert replaced. The sample was slowly injected with the inlet splitter closed. Two minutes after injection, the splitter was re-opened (70 ml/min) and the carrier gas pressure reduced to 1.2 atm. After 4 min, oven heating was started. When the oven control unit indicated 170°, the ion source was turned on and recording and retention time measurements were initiated.

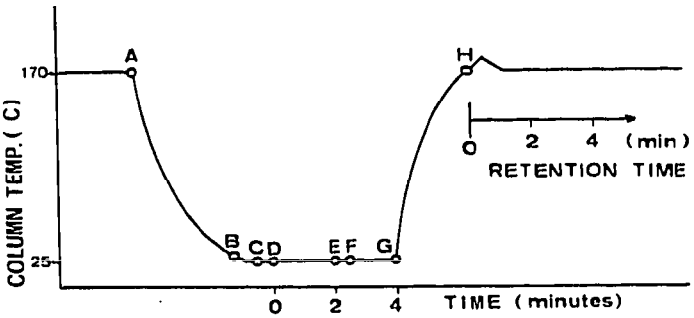


Fig. 3. Operating sequence for splitless injection. A = End of previous analysis, ion source off, cooling started and column inlet pressure increased (2.5 atm); B = glass vaporizer insert replaced; C = inlet splitter closed; D = sample injected; E = inlet splitter opened (70 ml/min); F = column inlet pressure reduced (1.2 atm); G = column heating started; H = oven control indicating 170°, ion source on, recording and retention time measurements initiated.

For mass fragmentography (mass specific detection), the MS was operated with unit resolution under the conditions listed in Table I. The precision mass meter was set at the selected ion, representative of the compound of interest. Ion intensities were recorded (1–5 mV) after suitable electronic filtering. Regular mass spectrometric scans (*m/e* 35 to 250 in 1 sec) were recorded at 5-sec intervals before, during and after elution of a compound of interest. Differential mass spectra for the component were compared with spectra of synthetic standards.

TABLE I

OPERATING CONDITIONS FOR THE QUADRUPOLE MASS SPECTROMETER (FINNIGAN 1015 D)

Parameter	*Value*	*Parameter*	*Value*	
Electron energy (eV)	23	Electron multiplier (V)	3100	
Filament current (μA)	600	Pre-amplifier (A/V)	10^{-7}	
Ion energy (V)	8	Filter (a.m.u./sec)	10	(scanning, 300)
Extractor (V)	10	Recorder (mV)	1–10	(scanning, 100)
Lens (V)	90			

RESULTS AND DISCUSSION

Purification of insect extracts

Owing to the instability and volatility of pheromonal compounds, a rapid clean-up is essential. In order to reduce losses, low-boiling solvents were used and

elution volumes and column dimensions were kept to a minimum. Insect extracts were partially purified by column chromatography. A group separation was carried out by adjusting the polarity of the elution solvents in such a manner that acetates and alcohols were completely separated into two chromatographic fractions. Acetates, such as dodecyl, dodecenyl and dodecadienyl acetates, appeared in the 8% diethyl ether–pentane fraction and alcohols, such as dodecadienols, in the 100% diethyl ether fraction (see Fig. 1). The former fraction also contained aldehydes and epoxides. By adding known amounts of synthetic compounds to the extraction solvents, recoveries of several pheromones and analogues were established to range from 65 to 95% at levels of 200–1000 ng (Table II). No isomerization and no contamination of neighbouring fractions were observed.

TABLE II

RECOVERIES OF PHEROMONAL COMPOUNDS BY PURIFICATION SCHEME

Compound	*Level (ng)*	*Recovery (%)*
n-Dodecyl acetate	1000	95*
cis-8-Dodecenyl acetate	1000	95*
cis-9-Dodecenyl acetate	500	83*
trans-9-Dodecenyl acetate	500	77*
trans-7,*cis*-9-Dodecadienyl acetate	1000	95*
trans-7,*cis*-9-Dodecadienyl acetate	200	65*
trans-7,*cis*-9-Dodecadien-1-ol	1000	65**
trans-8,*trans*-10-Dodecadien-1-ol	1000	72**
n-Dodecan-1-al	1000	95*
11-Undecen-1-al	1000	95*

* Recovered in 8% ether fraction.
** Recovered in 100% ether fraction.

Complete mass spectrometric analysis may require the injection of up to 100 moth-equiv. The presence of non-volatile insect co-extractants can prevent the required concentration of fractions prior to mass spectrometric analysis. Few techniques are available to separate volatiles from the bulk of co-extractants with acceptable yields at low concentrations. A technique used for that purpose in pesticide analysis is sweep co-distillation or forced volatilization[5], and its application for the further clean-up of insect extracts was investigated. *trans*-7,*cis*-9-Dodecadienyl acetate and the corresponding alcohol were recovered by this technique with a yield of 65% at the 200-ng level and similar yields were obtained from extracts of the grapevine moth.

Gas chromatographic separation

The large number of possible positional and geometrical isomers of the class of compounds investigated requires gas chromatographic columns of the highest separation power. High-resolution glass capillary columns that only recently became

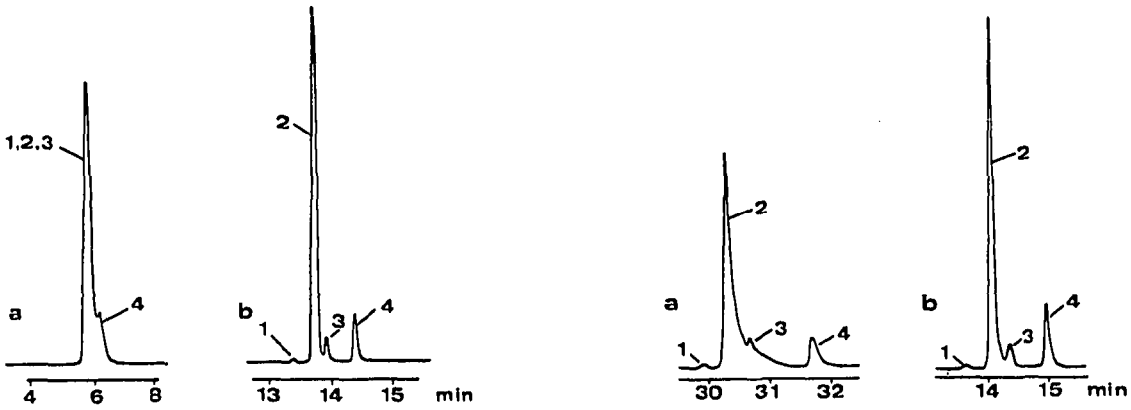


Fig. 4. FID chromatograms showing separation of 7,9-dodecadienyl acetate isomers: (a) by a packed column (Carbowax 20M, 3%, 2 m × 2 mm I.D., 4000 theoretical plates); (b) by a glass capillary column (Ucon 50 HB 5100, 50 m × 0.3 mm I.D., 140,000 theoretical plates). 1 = *cis*-7,*trans*-9 isomer; 2 = *trans*-7,*cis*-9 isomer; 3 = *cis*-7,*cis*-9 isomer; 4 = *trans*-7,*trans*-9 isomer.

Fig. 5. Mass fragmentograms (*m/e* 164) of 7,9-dodecadienyl acetate isomers. Separation on a glass capillary column (Ucon 50 HB 5100, 50 m): (a) with commercial interface (glass-lined stainless-steel capillary, column temperature 150°); (b) with platinum capillary interface (column temperature 170°).

available commercially possess the required separation power and therefore are ideally suited for the analysis of insect extracts. Fig. 4b shows a chromatogram obtained with a 50-m capillary column from a mixture of the four isomers of 7,9-dodecadienyl acetate. A polar stationary phase (Ucon 50 HB 5100) was found to be superior in separating the four isomers than a non-polar stationary phase (silicone SF 96). A packed column did not resolve all isomers (Fig. 4a).

Owing to the minute amounts of pheromone (1–10 ng) present in an insect, injection with stream splitting would be wasteful. By splitless injection similar to a technique described by Grob and Grob[6], samples of up to 10 μl could be introduced without impairing the vacuum system of the mass spectrometer or the gas chromatographic separation.

The interface to the mass spectrometer becomes a critical factor when using capillary columns. The use of the standard interface supplied with the instrument, consisting of a stainless-steel capillary and a micro-valve resulted in loss of separation (Fig. 5a). By direct interfacing from the column end to the ion source with a platinum capillary[7], full separation could be restored (Fig. 5b), which was the same as that when using a FID (Fig. 4b).

Mass spectra of long-chain acetates and alcohols

Electron impact mass spectra of the proposed pheromones for the codling moth and the European grapevine moth are presented in Fig. 6. Recording at low electron energy (23 eV) was found to enhance the intensity of ions in the higher mass range. The spectra are typical of long-chain acetates and alcohols. Both compounds show molecular ions (*m/e* 182 and 224, respectively) of some intensity, as expected

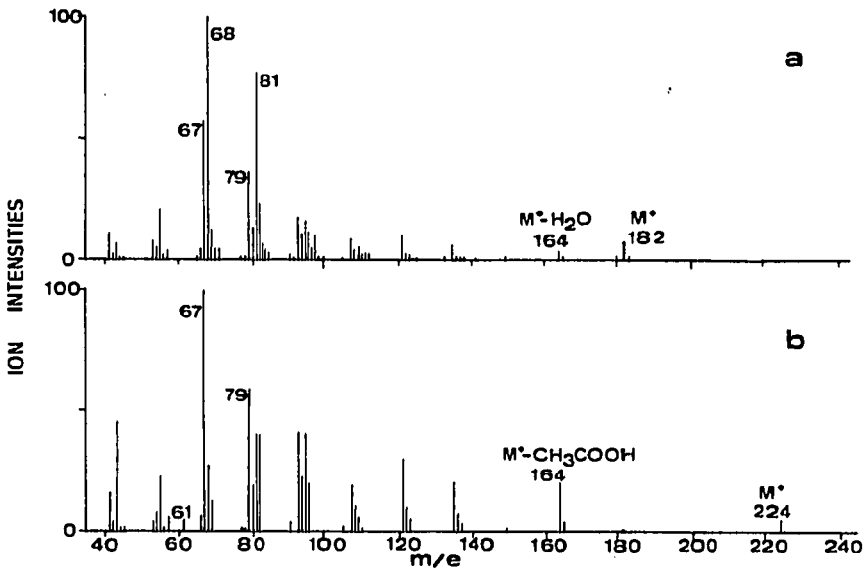


Fig. 6. Mass spectra of synthetic pheromonal compounds: (a) *trans*-8,*trans*-10-dodecadien-1-ol (pheromone of the codling moth); (b) *trans*-7,*cis*-9-dodecadienyl acetate (pheromone of the grapevine moth).

for compounds with two double bonds. The more saturated analogues, however, show a very weak or no molecular ion. Saturated and unsaturated long-chain acetates have prominent ions at $M^+ - 60$ resulting from the loss of CH_3COOH from the molecular ion. In alcohols, the same ions are prominent, in this case resulting from the loss of H_2O from the molecular ion. A weak but typical ion for acetates is at *m/e* 61 ($CH_3COOH_2^+$); otherwise the spectra of acetates and corresponding alcohols are very similar in the lower mass range. Geometrical isomers of these compounds generally yield very similar spectra, but positional isomers show significant differences in the relative intensity of ions in the lower mass range. The mass spectra of long-chain acetates and alcohols were discussed in more detail by Budzikiewicz *et al.*[8].

Mass fragmentography

The small amounts and low concentrations of pheromones in an insect extract require highly specific and sensitive detection methods when a direct analysis is desired. The usual gas chromatographic detectors are not suitable for the direct and specific detection of long-chain acetates and alcohols in biological extracts because these compounds contain no strongly electron capturing groups and no hetero-atoms other than the bulk of the co-extractants.

Mass fragmentography (mass specific detection) has been used for the analysis of drugs, their metabolites, pesticides and their by-products[9,10]. By monitoring selected ions representative of a compound of interest, this technique allows far lower detection limits than the amounts required to obtain complete mass spectra. In this study, the ions used for detecting proposed pheromones were selected from the spectra in Fig. 6. Interference from co-eluting components can be minimized by monitoring ions in the higher mass range. Therefore, the molecular ion *m/e* 182 was

selected for *trans*-8,*trans*-10-dodecadien-1-ol and the $M^+ - CH_3COOH$ ion (*m/e* 164) for *trans*-7,*cis*-9-dodecadienyl acetate, although ions of higher intensity for both compounds are available in the lower mass range. The detection limits obtained were better than 200 and 100 pg, respectively.

The possible presence of the mono-unsaturated pheromone analogues was investigated by monitoring the ion at *m/e* 166. This value corresponds to the $M^+ - CH_3COOH$ ion for dodecenyl acetates and to the $M^+ - H_2O$ ion of dodecenols. The molecular ions (*m/e* 226 and 184, respectively) of both compounds were too weak for mass fragmentography.

Codling moth sex pheromone

The extracts of male and virgin female moths (250 each) were subjected to column chromatography and the 100% ether fractions were analyzed for the presence of *trans*-8,*trans*-10-dodecadien-1-ol. Fig. 7 shows chromatograms of both extracts obtained with flame ionization detection (5 moth-equiv. injected). Both sexes show a series of common peaks but the female extract contains a peak with the retention time of *trans*-8,*trans*-10-dodecadien-1-ol, which is absent in the males. The mass fragmentograms of these extracts (*m/e* 182, 2 moth-equiv.) are presented in Fig. 8. They show a peak at the retention time of *trans*-8,*trans*-10-dodecadien-1-ol (16.45 min) in the female extract only, relatively free from other co-extractants. By co-injection, this peak co-chromatographed with the synthetic standard. Although no other isomers were present, the chromatogram seems to indicate a high isomeric purity of the female component. By co-injection with a synthetic sample using an impurity peak as internal

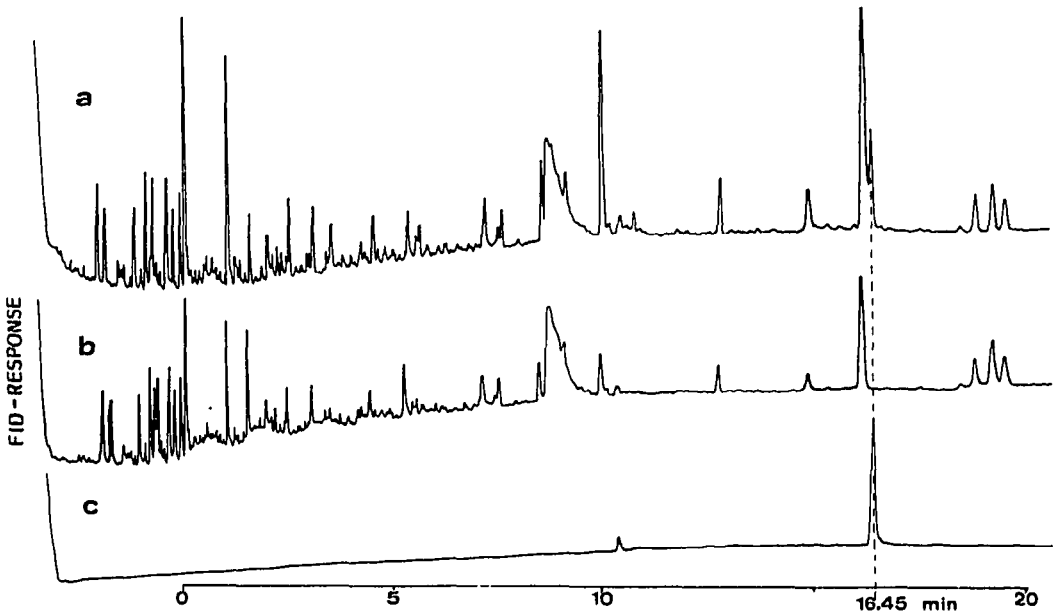


Fig. 7. FID chromatogram of codling moth extracts: (a) female moth, 100% ether fraction (5 moth-equiv.); (b) male moth, 100% ether fraction (5 moth-equiv.); (c) synthetic *trans*-8,*trans*-10-dodecadien-1-ol.

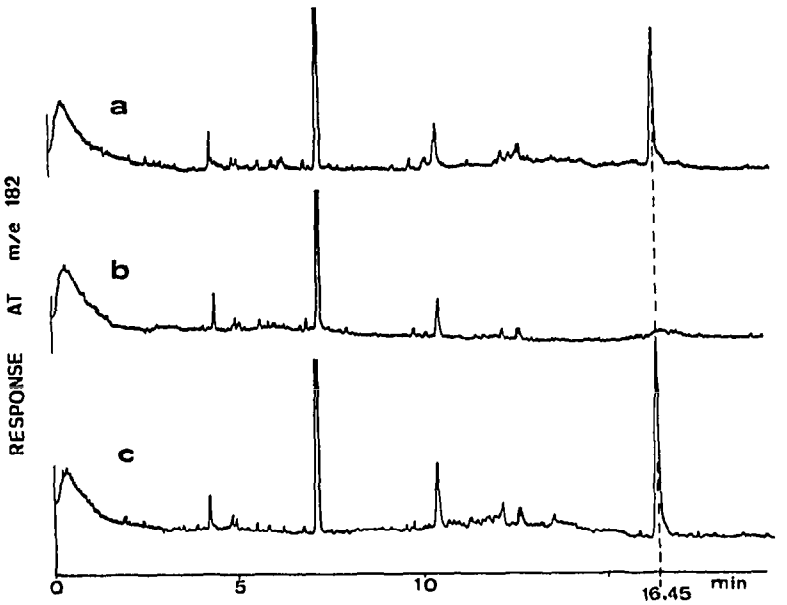


Fig. 8. Mass fragmentograms (*m/e* 182) of codling moth extracts: (a) female moth, 100% ether fraction (2 moth-equiv.); (b) male moth, 100% ether fraction (2 moth-equiv.); (c) synthetic *trans*-8, *trans*-10-dodecadien-1-ol (recovered in 100% ether fraction).

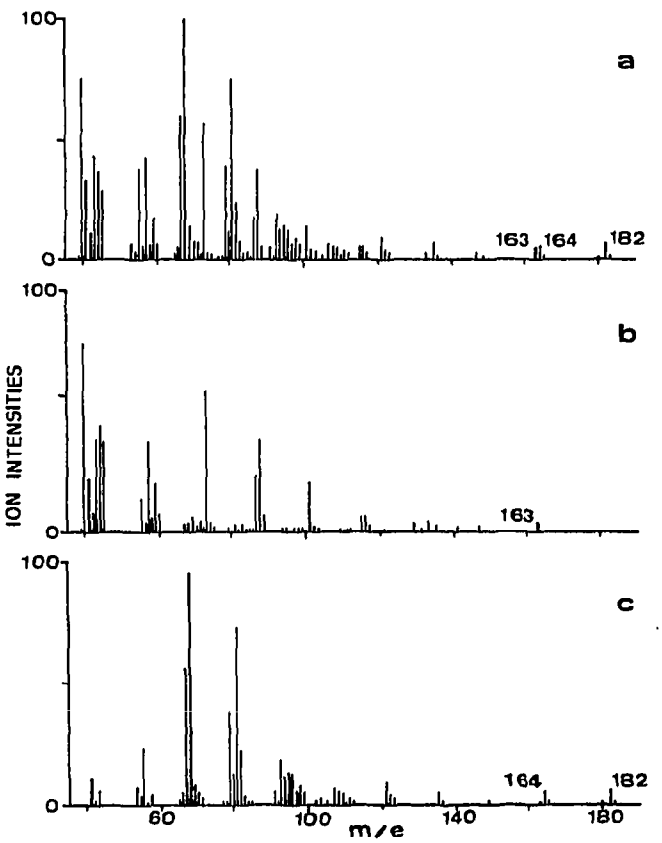


Fig. 9. Mass spectrum of codling moth female component: (a) spectrum obtained during elution at 16.45 min; (b) background spectrum after elution at 16.60 min; (c) differential mass spectrum (a – b).

standard, the amount of *trans*-8,*trans*-10-dodecadien-1-ol per female was determined to be 3.5 ng, taking into account a 70% clean-up recovery. This is in good agreement with the 5 ng per female obtained by Beroza *et al.*[3] using computerized GC–MS.

The presence of *trans*-8,*trans*-10-dodecadien-1-ol in the female extract was further confirmed by mass spectrometry. After concentration of the sample, 50 moth-equiv. were injected and complete mass spectra were recorded at short (3–5 sec) intervals around the retention time of the female component. Fig. 9 shows mass spectra obtained during and after elution of the female component. A differential mass spectrum (Fig. 9c, spectrum a — spectrum b) is essentially identical with that of a synthetic standard (Fig. 6a). Signals at *m/e* 163 in the spectra of the female component are due to dimethyl phthalate ($M^+ = 194$; $M^+ - OCH_3 = 163$) eluting immediately before *trans*-8,*trans*-10-dodecadien-1-ol. Dimethyl phthalate was detected in both male and female moth extracts but not in blank samples. It is frequently used as a plastifier; however, efforts to trace it to the containers used for insect rearing were unsuccessful.

Mass spectra obtained at high filament currents ($> 800\ \mu A$) and low electron energies (< 23 eV) showed special features. Signals at *m/e* 185 and 187 were concluded to be due to vaporization of rhenium from the filament. Ions at *m/e* 180 ($M^+ - 2$) in the spectra of *trans*-8,*trans*-10-dodecadien-1-ol and the female component indicate dehydrogenation of this compound. Nevertheless, Fig. 10 shows excellent agreement between the partial differential spectra of the female component and that of the synthetic standard obtained under these conditions.

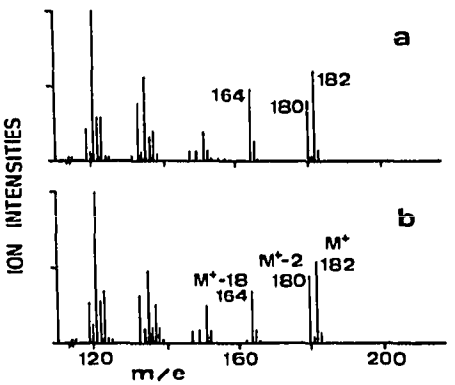


Fig. 10. Differential mass spectra (*m/e* 115–200) at high filament currents: (a) codling moth female component at 16.45 min; (b) synthetic *trans*-8,*trans*-10-dodecadien-1-ol.

Pheromone analogues present in the female have recently become important as possible biosynthetic precursors or as attractant synergists[11]. The presence of mono-unsaturated analogues was investigated by analyzing the 100% ether fraction of male and female moth extracts for *trans*-8- and *trans*-10-dodecen-1-ol. Mass fragmentograms (*m/e* 166, $M^+ - H_2O$) of the female extract and synthetic standards are shown in Fig. 11. They indicate a level of less than 0.1 ng of the two compounds per female moth, less than 1/25th of the amount of *trans*-8,*trans*-10-dodecadien-1-ol present. The same level was determined in male moths.

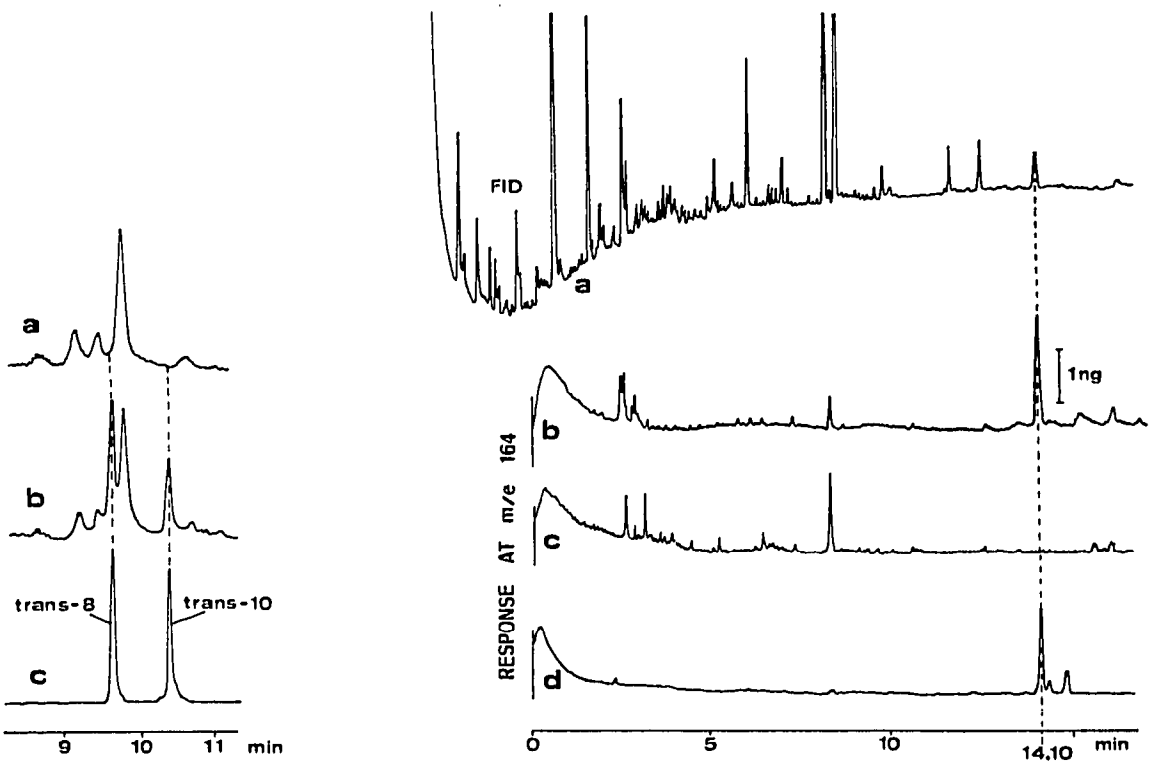


Fig. 11. Partial mass fragmentograms (*m/e* 166) of codling moth extracts: (a) female moth, 100% ether fraction (10 moth-equiv.); (b) same, co-injected with synthetic standards; (c) synthetic *trans*-8- and *trans*-10-dodecen-1-ol (5 ng each).

Fig. 12. Grapevine moth extract. (a) FID chromatogram of female moth, 8% ether fraction (5 moth-equivalents). Mass fragmentograms (*m/e* 164); (b) female moth, 8% ether fraction (1 moth-equiv.); (c) male moth, 8% ether fraction (1 moth-equiv.); (d) synthetic *trans*-7,*cis*-9-dodecadienyl acetate.

Sex pheromone of the European grapevine moth

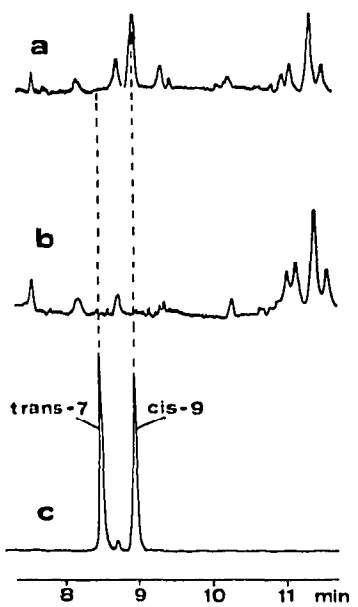
Male and virgin female moths (500 each) were extracted and the 8% ether fractions were analyzed for the presence of *trans*-7,*cis*-9-dodecadienyl acetate. Fig. 12a shows the FID chromatogram of a 5-female moth aliquot with a minor peak at the retention time (14.1 min) of *trans*-7,*cis*-9-dodecadienyl acetate. This peak was not found in male extracts. The mass fragmentogram (*m/e* 164, $M^+ - CH_3COOH$, Fig. 12b) of the female extract shows a main peak that co-chromatographed with a synthetic standard (Fig. 12d). The amount of *trans*-7,*cis*-9-dodecadienyl acetate determined by co-injection with a synthetic sample using an impurity peak as internal standard was 1.6 ng per female abdominal tip (male < 0.02 ng). Closer inspection of the female chromatogram (Fig. 12b) and comparison with that of a standard (Fig. 12d) revealed a high isomeric purity of at least 97% for the female component. These findings have been reported previously[4].

On gas chromatography, *trans*-7,*cis*-9-dodecadienyl acetate proved to be extremely sensitive to the presence of other co-extractants. Repeated injection led to

peak broadening and loss of resolution, unless the glass vaporizer insert of the chromatograph was changed each time. Obviously, the oily deposits that formed in the vaporizer tube prevented proper vaporization of the subsequent injections. For these reasons, injection of larger aliquots (up to 150 moth-equiv.) for complete mass spectrometric analysis was possible only after sweep co-distillation of the extract. This technique proved to be extremely helpful for removal of interfering non-volatile co-extractants. The differential mass spectrum obtained for the female component (retention time 14.1 min) was identical with that of a synthetic standard of *trans*-7,*cis*-9-dodecadienyl acetate[4].

The presence of mono-unsaturated analogues was investigated by analyzing the 8% ether fractions. The mass fragmentograms (*m/e* 166, $M^+ - CH_3COOH$) in Fig. 13 indicate levels of at most 0.2 ng of *cis*-9-dodecenyl acetate per female and of 0.02 ng per male moth. The *trans*-7-dodecenyl acetate levels for the male and female moth were below 0.02 ng.

The presence of *trans*-7,*cis*-9-dodecadien-1-ol (alcohol analogue) was investigated by analyzing aliquots of the 100% ether fraction. In Fig. 14 mass fragmentograms (*m/e* 182, M^+) for male and female moths extracts and of a synthetic sample are presented. The separation of the geometrical isomers in the synthetic sample is similar to that of the acetate isomers (Fig. 4b). Fig. 14 indicates a level of less than 0.1 ng of alcohol analogue per female and male abdominal tip.



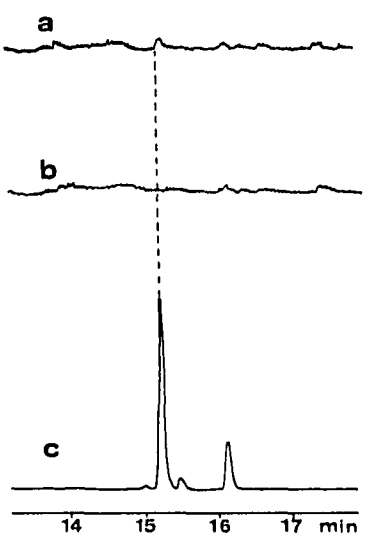


Fig. 13. Partial mass fragmentograms (*m/e* 166) of grapevine moth extracts: (a) female moth, 8% ether fraction (5 moth-equiv.); (b) male moth, 8% ether fraction (5 moth-equiv.); (c) synthetic *trans*-7- and *cis*-9-dodecenyl acetates.

Fig. 14. Partial mass fragmentograms (*m/e* 182) of grapevine moth extracts: (a) female moth, 100% ether fraction (10 moth-equiv.); (b) male moth, 100% ether fraction (10 moth-equiv.); (c) synthetic *trans*-7,*cis*-9-dodecadien-1-ol.

CONCLUSIONS

Quadrupole mass fragmentography and mass spectrometry with high-resolution gas chromatography proved to be extremely specific and sensitive for confirming the presence and structures of pheromones in insects. Although all positional isomers could not be eliminated because of a lack of synthetic standards, the mass spectrometric and gas chromatographic data obtained in this study provide corroborative evidence of the presence of *trans*-8,*trans*-10-dodecadien-1-ol in codling moth females and *trans*-7,*cis*-9-dodecadienyl acetate in grapevine moth females and the identity of these compounds with the natural sex pheromones of these insects. They further indicate that analogues of these compounds can only be present in minor amounts in possible pheromone blends of these species.

The techniques described offer a rapid analysis of insect extracts and, using inert glass capillary columns, a minimum risk of breakdown and isomerization. The technique is highly sensitive and information on the presence and amounts of the pheromonal compounds can be obtained from injections of as little as 1 moth-equiv. The clean-up procedure prior to analysis still involves 200–500 moths, but it can probably be scaled down further. This will become important in the investigation of pheromone blends of local races when only a limited number of field-collected insects are available.

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