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Partial elucidation of *Trichogramma* putative sex pheromone at trace levels by solid-phase microextraction and gas chromatography–mass spectrometry studies

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Abstract

Virgin females of the minute parasitoid wasp *Trichogramma turkestanica* produce about 2 pg/h of two putative sex pheromonal compounds. These compounds could be successfully sampled during 20–50 h with SPME from 1.8 mL vials, containing 50–110 wasps and analysed by GC–MS. Accurate mass measurements at the 1 ng scale were possible with an internal standard of sulphur. One compound with MW 236 was identified as a $C_{17}H_{32}$ hydrocarbon while the other compound was the corresponding allylic alcohol with composition $C_{17}H_{32}O$. The alcohol could be silylated on-fibre and its mass spectrum suggested the presence of a conjugated 2,4-diene moiety. A miniaturised solvent extraction system for SPME needles, using 5 μL of acetone in a microtube was developed. After reaction of the extracted volatiles with the dienophile 4-methyl-1,2,4-triazoline-3,5-dione (MTAD), GC–MS of the MTAD adducts confirmed the presence of a diene. Interpretation of the combined mass spectral data, in combination with retention indexes of both compounds on non-polar and polar columns, suggested 2,6,8,12-tetramethyltrideca-2,4-diene and 2,6,8,12-tetramethyltrideca-2,4-dien-1-ol as most probable structures. These compounds have not been described previously. Biogenetically, they are most likely polyketides made up of a C_4 starter unit that has been elongated with C_2 and C_3 units. Further biological and synthetic studies are necessary to prove their role as sex pheromone, confirm the proposed structures and determine the correct stereochemistry of the double bonds and the methyl groups.

Keywords: *Trichogramma turkestanica*; Parasitoid wasps; SPME; Miniaturised solvent extraction; Derivatisation; Silylation; MTAD; Sex pheromones; GC–MS; Mass spectral fragmentation

1. Introduction

Trichogramma (Hymenoptera, Trichogrammatidae) wasps are tiny ubiquitous insects that parasitise eggs of over 400 other insect species [1]. *Trichogramma* species are commercially applied as a form of biological crop protection on several million ha per year [1–3]. They are effective as they kill pest insects before these can damage the crop. The adults of these minute wasps are ca. 0.5 mm in length and 8 μg in weight with a homogeneous morphology. These characteristics make the classification of the species in this genus difficult. Morphological characterisation, allozymic analysis and DNA profiling have been used to identify *Trichogramma* species [4–8]. An alternative potentially very specific way of identifying wasps at the species level is through their sex pheromones [9]. There is behavioural evidence for the existence of both substrate-borne [10] and volatile [11] sex pheromones in *Trichogramma* species. This was confirmed by biological experiments with *Trichogramma turkestanica* Meyer 1940, and this species was, therefore, selected for further study.

Preliminary biological and analytical experiments carried out by us showed that populations of virgin females always produced two volatiles that were neither present in populations of only males nor in populations of both males and

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females. Males exposed to volatiles produced by virgin females but physically separated from those same females by a porous glass barrier showed the same casting behaviour as males exposed to hexane extracts of virgin females (data not shown). The casting is a behavioural courtship response preceding copulation. The minute amounts of the two volatiles released by the wasps could only be sampled by the sensitive and clean solid-phase microextraction (SPME) technique. Although, the obtained mass spectra were of good quality, they did not allow the elucidation of the structures. Therefore, further derivatisations followed by GC–MS studies were necessary. A problem that was encountered during this study was the inability to carry out certain derivatisations on the SPME needle. In this paper, we report on a microchemical method for solvent extraction of SPME needles followed by derivatisation and the application thereof in mass spectral studies of the putative sex pheromone constituents of *T. turkestanica* parasitoid wasps.

2. Experimental

2.1. Parasitoid wasps

Individuals of *T. turkestanica* (line MB39) were used. This line was collected from Lepidoptera eggs in tomato fields in Mora, Alentejo Province (Southern Portugal) and reared in the Laboratory of Entomology of Wageningen University, since 1992. Voucher specimens are kept by B. Pintureau in INSA–INRA (Villeurbanne, France). In Wageningen, cultures were maintained on UV-killed eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) at 22 ± 2 °C, relative humidity 50 ± 20%, with a 16 h light/8 h dark regime. In order to obtain virgin individuals, parasitised eggs (≈650) of *E. kuehniella* were individually placed in glass test tubes (75 mm length, 10 mm diameter), containing a tiny drop of honey (Mellona) and covered with a cotton plug. This was done 5 days after the eggs had been exposed to *Trichogramma*, when the parasitised eggs become black. The females used for parasitising were 0–4 days old. Eggs were incubated at 23 ± 1 °C, RH 50 ± 20%, with a 16 h light/8 h dark regime. After adult emergence (ca. 12 days after parasitism had occurred, ≈55% of the total number of eggs), wasps of 1–2 days old were separated into males and females with a binocular (25×). The average of female/male ratio was 1.75. By means of an aspirator they were collected one by one into a pipette tip. For each SPME sampling 60–115 specimens were collected. Individuals in pipette tips were anaesthetised with CO_2 for ca. 1 min and introduced in glass test tubes. Next, the wasps were transferred into glass screw vials (1.8 mL, Phase Separations), containing a tiny drop of honey. About 5–10% of the wasps are lost during these procedures. The vials were closed by a cap fitted with a PTFE-lined rubber septum. All materials used to collect wasps into vials had been washed with acetone and afterwards heated at 100 °C for 45 min. Plastic parts were then removed and the remaining materials were heated at 150 °C for another 45 min.

2.2. SPME sampling

SPME samplings followed by GC or GC–MS were repeated 20–25 times over a number of years. Volatiles from wasps were collected by SPME needles with 100 μm polydimethylsiloxane (PDMS) coated fibres (Supelco). The needle was inserted through the septum into the vial, containing 50–110 wasps and the fibre was exposed for 20–50 h. The vials stayed in vertical position inside a water bath at 25 ± 2 °C with a light regime corresponding to the season of sampling. Cleaning of the fibres was done just before sampling by inserting the needle into a GC injector at 250 °C during 5–15 min.

2.3. Solvents and chemicals

Acetone and dichloromethane were of analytical grade and used as such. 4-Methyl-1,2,4-triazoline-3,5-dione (MTAD 95%) was obtained from Aldrich. Pristane was obtained from Sigma. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) was obtained from Fluka. The ginger oil was hydrodistilled in our laboratory from fresh Indonesian ginger.

2.4. Gas chromatographic equipment

Most of the preliminary SPME studies, the silylation and the MTAD reaction were analysed on a HP6890 GC–MS system equipped with a J&W DB-5 column (30 m × 0.25 mm i.d. and 0.25 μm film thickness). The injection temperature was 250 °C. The injection was splitless during 1 min, after 1 min a split ratio 1:50 was maintained, carrier gas He, linear velocity 36 cm/s; a constant flow was maintained during the entire run. The initial column temperature was 50 °C. After 2 min, the column was ramped at 10 °C/min to 250 °C and remained at this temperature for 10 min. The mass spectrometer was operated in the 70 eV electron ionisation (EI) mode with scanning from *m*/*z* 30 at 4 spectra/s. Actual measurements started 2.5 min after injection to protect the filament of the mass spectrometer.

For the model experiments with ginger oil the mass spectrometer was equipped with a J&W DB-1 column, 20 m × 0.18 mm i.d. and 0.4 μm film thickness. Injections were carried out in the split mode (split ratio 1:50).

For accurate mass measurements SPME injections were accomplished on a Varian 3400 GC system connected to a Finnigan MAT 95 mass spectrometer. The GC was equipped with an SGE BP-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness). During the splitless injection, the column temperature was kept at 30 °C, then ballistically heated to 60 °C and finally programmed to 260 °C at a rate of 4 °C/min. The mass spectrometer was operated in the 70 eV EI mode with scanning from *m*/*z* 24–300 at 0.7 s/decade.

To obtain accurate mass measurements of the small amounts, the mass spectrometer was tuned to full sensitivity at a resolution of $M/\Delta M = 1000$. During the GC run sulphur (S_8) was introduced into the ion source and used as the internal mass reference. Due to the large mass defect of ^{32}S (mass 31.97207 u), the ions for S_n ($n = 2$–8) do not interfere with those of normal organic compounds.

For the determination of the retention indexes a HP6890 gas chromatograph equipped with one split/splitless injection system, a 1:1 inlet splitter, two columns and two flame ionisation detectors was used. The two columns were a J&W DB-1, 60 m × 0.25 mm i.d., 0.25 μm film thickness and a Restek Stabilwax, 60 m × 0.25 mm i.d., 0.25 μm film thickness, respectively. Splitless injection, carrier gas H_2, inlet pressure 20 psi (1 psi = 6894.76 Pa), linear velocity 35 cm/s; temperature programme, 50 °C (0-min hold) to 238 °C (8-min hold) at 4°/min; injection temperature 220 °C; detection temperature 260 °C. All indexes are linear retention indexes. They were calculated by comparing the retention times of A and B with those of a series of C_7–C_{23} alkanes.

2.5. Silylation

After sampling the wasps during 48 h, the SPME needle was withdrawn from the vial and inserted in a 1.8 mL vial, containing 5 μL BSTFA with 1% TMCS. The needle was not brought in direct contact with the liquid reagent. After 10 min, the SPME needle was withdrawn from the vial and inserted into the injector of the GC–MS system.

2.6. Solvent extraction of SPME needles

The bottom 12-mm of a melting point tube with i.d. ≈1.0 mm was cut off with a glass cutting tool and inserted in a block of polystyrene. The polystyrene block was placed on a laboratory jack. The microtube was filled with ≈6 μL of acetone by means of a GC syringe. The SPME needle with the sampled volatiles was put in a clamp in such a way that the needle was positioned directly above the opening of the microtube and the PDMS fibre was extruded. By means of the laboratory jack and by lowering the adjustable needle guide of the SPME the fibre was submersed in the acetone until the tip of the fibre reached the bottom of the micro tube. The extraction was carried during 15 min. During this period, the needle was occasionally moved 1-mm up and down. After this period, the needle was withdrawn from the acetone solution. The remaining ≈4 μL of acetone with extracted volatiles can be used for a subsequent derivatisation step.

2.7. Derivatisation of wasp volatiles with MTAD

After sampling the wasps during 48 h, the SPME needle was extracted as described in section 2.6 above. To the remaining 4 μL of acetone with extracted volatiles, 2 μL of a 0.1% solution of MTAD in CH_2Cl_2 was added. The solution was mixed with a thin glass rod. After 5 min, the volume of the solution had decreased to 4 μL due to evaporation. This volume was injected splitless into the GC–MS system and mass spectra were recorded.

3. Results and discussion

3.1. Initial experiments

From the very first experiment onwards, it was clear that the virgin females with their adult weight of 8 μg produced only minute quantities of the putative sex pheromonal compounds. Thus, if the compounds would be novel, the elucidation of their structures would prove challenging. Sampling was done by SPME with a 100 μm PDMS fibre in a 1.8 mL autosampler vial. The SPME samplings of virgin *T. turkestanica* females were reproducible over 20–25 samplings that were carried out over a number of years. The total ion current (TIC) profile after GC or GC–MS measurements always showed the presence of two significant peaks A and B (Fig. 1). SPME samplings of empty vials with honey were clean and showed very few background peaks. SPME samplings of *T. turkestanica* males or mixed populations of both males and females showed many peaks also present in the headspace of virgin females but never any trace of peaks A or B. Through external calibration by injecting similar compounds, the amounts of A and B were estimated at ≈1 and ≈3 ng, respectively. Assuming that most of the compounds are absorbed by the PDMS fibre, this means that the production of each compound is around 1–3 pg/h per virgin female. Hexane washings of vials or females after sampling never showed larger quantities of A and B than present on the SPME fibres. High quality mass spectra could only be recorded after sampling 50–100 virgin females during 1–2 days. The mass spectra of peaks A and B can be found in Fig. 2A and B,

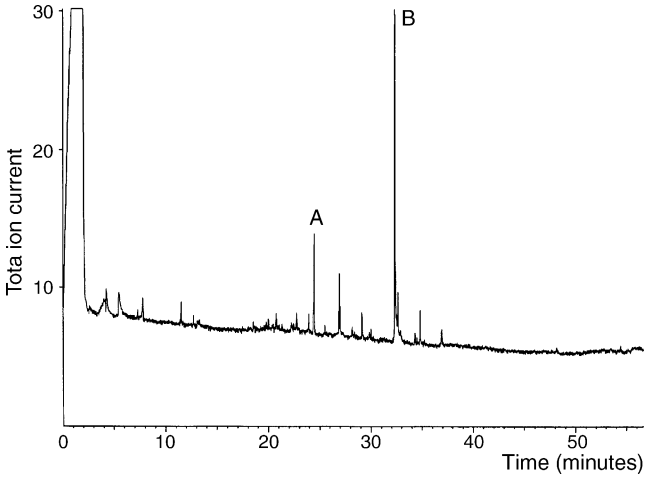


Fig. 1. Total ion current chromatogram of an SPME extract of ≈60 virgin *T. turkestanica* females after 22 h of sampling. The peaks marked A and B are the putative sex pheromonal compounds.

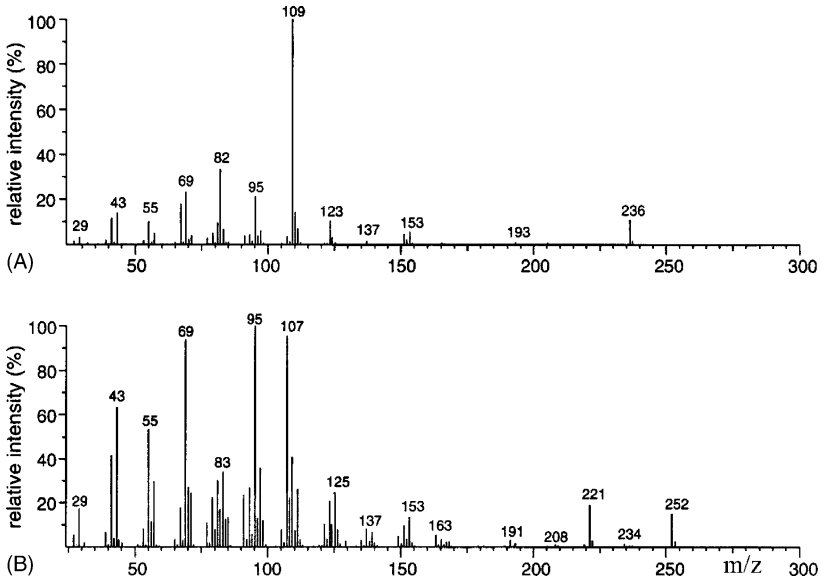


Fig. 2. Mass spectra (EI) of compounds A and B.

respectively. The molecular ion of A was observed at *m*/*z* 236 with the prominent base peak at *m*/*z* 109. The mass spectrum of B showed the molecular ion at *m*/*z* 252 and more prominent fragmentation with major ions at *m*/*z* 69, 95 and 107. A comparison of the mass spectra suggested that A and B were closely related, with B being an alcohol or epoxide derivative of A. The spectra did not occur in either the commercial US National Institute of Standards and Technology (NIST) and Wiley mass spectral libraries or in our own volatile natural products library of 3000 compounds.

3.2. Accurate mass measurements

By using both, a primary standard (PFK) and a secondary standard (S_8) it was possible to perform accurate mass measurements with a resolving power of 1000 with GC–MS of peaks consisting of 1–3 ng A or B. The results have been summarised in Tables 1 and 2.

The molecular composition of both A and B shows that both compounds possess a total of two double bonds and/or rings. The elemental composition of the base peak of A C_8H_{13} indicates the loss of a neutral fragment of C_9H_{19}, i.e. a fully saturated fragment. This suggests that the two double bonds are clustered in one part of the molecule. The fragment ions at *m*/*z* 151, 137, 123 and 95 correspond to the fragment ion at *m*/*z* 109 with fewer or more carbons from the saturated part of the molecule. Furthermore, the even mass ion of *m*/*z* 82 suggests a 3,5-diene- or a methyl-substituted 2,4-diene moiety, since, reference spectra of 2,4-dodecadiene and 4,6-hexadecadiene [12], show prominent ions of *m*/*z* 68 and 96, respectively (Fig. 3). The formation of the base peak of composition $C_8H_{13}^+$, via the loss of a C_9H_{19} fragment by allylic cleavage, necessitates the presence of a methyl group at C-7 (3,5-diene) or C-6 (2,4-diene; Fig. 4). Substitution at other positions (C-2 to C-6 for a 3,5-diene or C-2 to C-5 for a 2,4-diene) would shift the even mass fragment ion from *m*/*z* 82 to

Table 1
Accurate mass measurements results for peak A

Mass	Rel. Int. (%)	Delta* (mmu)	Composition
69.0705	25.5	−0.1	C_5H_9
82.0759	35.8	2.4	C_6H_{10}
95.0838	23.1	2.3	C_7H_{11}
109.1025	100.0	−0.8	C_8H_{13}
123.1158	11.5	1.6	C_9H_{15}
151.1490	5.7	−0.3	$C_{11}H_{19}$
153.1647	7.5	−0.4	$C_{11}H_{21}$
236.2492	14.1	1.2	$C_{17}H_{32}$ $[M]^+$

* Delta = calculated mass − observed mass.

Table 2
Accurate mass measurements results for peak B

Mass	Rel. Int. (%)	Delta* (mmu)	Composition
69.0686	100.0	1.8	C_5H_9
95.0818	90.0	4.3	C_7H_{11}
107.0863	85.0	−0.2	C_8H_{11}
125.1002	28.0	−3.6	$C_8H_{13}O$
153.1607	20.0	3.6	$C_{11}H_{21}$
191.1765	4.7	3.5	$C_{14}H_{23}$
221.2264	22.0	0.5	$C_{16}H_{29}$
252.2458	20.0	−0.5	$C_{17}H_{32}O$ $[M]^+$

* Delta = calculated mass − observed mass.

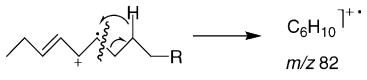


Fig. 3. Origin of the even mass fragment ion of *m*/*z* 82 for a 3,5-alkadiene. A 2,4-alkadiene with a methyl group at C-2 to C-4 would also give a fragment ion of *m*/*z* 82.

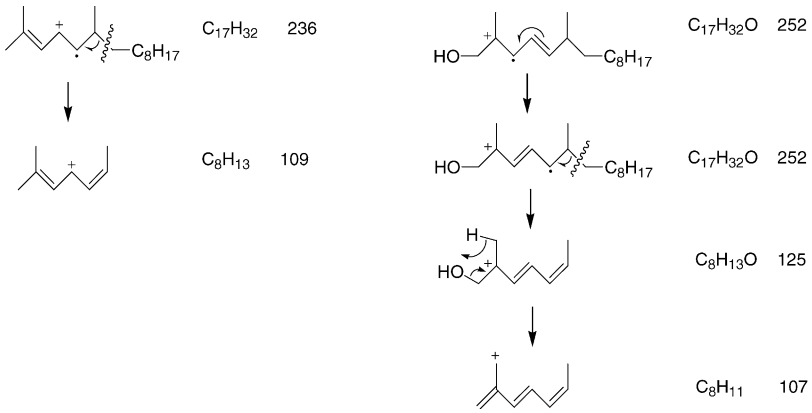
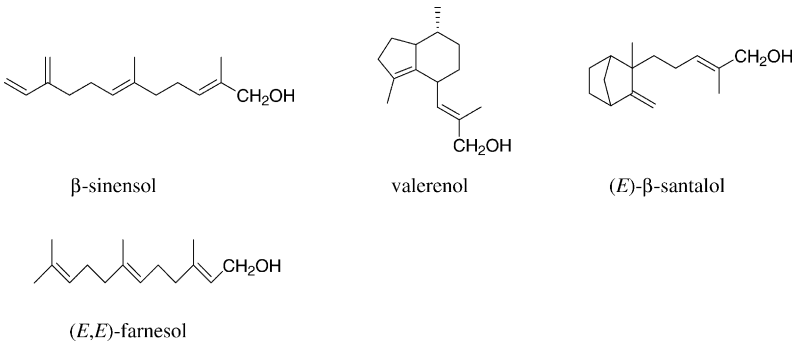


Fig. 4. Formation of the base peak of *m*/*z* 109 in A and fragment ions of *m*/*z* 125 and *m*/*z* 107 in B.

96. Finally, the small but distinct ion at *m*/*z* 193 could point to a methyl group at the $\omega - 1$ position.

In the spectrum of B, the presence of a large $[M-31]^+$ ion in combination with the absence of an appreciable $[M-18]^+$ is distinctive. The loss of CH_2OH is usually diagnostic of a primary alcohol but the lack of loss of water suggests the presence of a double bond or double bonds on the carbons closest to the CH_2OH group (Fig. 4). A non-substituted 2-en-1-ol or 2,4-dien-1-ol moiety can be ruled out, as for example the mass spectra of 2-decen-1-ol and 2,4-decadien-1-ol [12] show an intense $[M-18]^+$ ion and no $[M-31]^+$ ion. A methyl substitution at C-2, however, often gives rise to an $[M-31]^+$ ion at a higher intensity than the $[M-18]^+$ ion (e.g. valerenol, β-sinensol and (*E*)-β-santalol), while a methyl substitution at C-3 results in an $[M-18]^+$ ion more intense than the $[M-31]^+$ ion (e.g. (*E*,*E*)-farnesol). Assuming the same structure for A and B, this would imply a 2,6-dimethyl-2,4-dien-1-ol moiety. The ion in the spectrum of B at *m*/*z* 107 is formed by the loss of water from the allylic cleavage ion of *m*/*z* 125 (Fig. 4). The base peak at *m*/*z* 95 can be explained by the loss of formaldehyde from the ion of *m*/*z* 125.



3.3. Retention indexes

To obtain more information on the polarity and general structure of A and B the retention indexes (*I*) were determined on a non-polar dimethylpolysiloxane column (DB-1) and a polar polyethylene glycol column (wax phase). On the DB-1 column, the *I*-values were 1511 and 1766 for A and B, respectively. On the polar column, the *I*-values were 1579 and 2317 for A and B, respectively. Several useful observations can be made from these data. First the *I* of A on the DB-1 column is 189 units lower than that of heptadecane (*n*-C_{17}), the corresponding *n*-alkane with the same number of carbon atoms. In our extensive collection of *I*-values of sesquiterpene hydrocarbons, the differences with the corresponding *n*-alkane (*n*-C_{15}) vary from +55 to −152. The lowest values are given by tricyclic sesquiterpenes with one double bond like α-cubebene. However, cyclic structures are incompatible with the mass spectral data. Khorasheh et al. have reported *I*-values of a large number of non-cyclic C_9–C_{26} monoalkyl and polymethyl alkanes and alkenes on dimethylpolysiloxane [13]. From their extensive set of data, it can be concluded that (1) a double bond has a limited but mostly slightly *I*-lowering effect, (2) the position of the methyl side group(s) has a limited variable effect on the *I*, and (3) the number of side groups has a pronounced cumulative *I*-lowering effect. This last effect has been summarised in Table 3. Thus, the *I* of compound A on the non-polar phase suggests that it is a tetramethyl substituted C_{13} hydrocarbon. The observed 189 *I* units difference with the corresponding *n*-alkane is, for instance, similar to that of pristane (2,6,10,14-tetramethylpentadecane) whose *I* is 1710, i.e. 190 units lower than nonadecane (*n*-C_{19}). Second,

Table 3
Retention index lowering effect of 1–4 methyl side chains in alkanes

Number of methyl side chains	Substituent effect ($\Delta I \pm$ S.D. relative to corresponding *n*-alkane with same carbon number)	*N* (number of compounds)
0	0.0	–
1 (total number of carbons 14–20)	42.9 ± 10.0	45
2 (total number of carbons 12–20)	76.0 ± 9.5	18
3 (total number of carbons 14–20)	132.9 ± 17.4	15
4 (total number of carbons 20–22)	189.3 ± 17.3	9

the difference of only 68 *I* units for A on the two columns is unusually small. For most non-aromatic sesquiterpene hydrocarbons the difference varies between 115 and 260 units. This again suggests a highly branched linear structure. Third, also the difference in *I* between A and heptadecane on the polar column, −121 units, is an extreme value when one compares it to similar ΔI values of sesquiterpenes. For non-aromatic sesquiterpene hydrocarbons the values vary from −39 for tricyclic structures with one double bond to +277 for monocyclic compounds with three double bonds. For pristane, a value of −231 was determined ($I = 1669$). The value of −121 again rules out a cyclic structure and it is 110 units higher than that of pristane due the presence of two double bonds.

The values for B relative to heptadecane are less extreme. Many sesquiterpene alcohols show similar values relative to pentadecane and the difference in *I* of B ($\Delta I = 551$) on the two columns is not in contradiction with the presence of an alcohol or aldehyde function. It is too large for an epoxide function. Finally the ΔI between A and B on the non-polar column is 255. When epoxides are compared with the corresponding hydrocarbons typically ΔI values are 90–150. The difference between primary alcohols and hydrocarbons, however, is around 250–280, a value similar to what was found for A and B. Thus, on the basis of their mass spectra and retention index on two different columns, it was concluded that A is a non-cyclic hydrocarbon with two double bonds and four methyl side chains and B the corresponding primary alcohol with the alcohol function next to the double bond.

3.4. Silylation

To confirm the presence of an alcohol function, a derivatisation with a silylating agent was carried out. It is known that silylations can be carried out on the SPME fibre by keeping the fibre in a vial, containing volatile silylating agent [14,15]. After sampling the wasps in the usual manner, the fibre with the sampled A and B was briefly inserted in a vial, containing BSTFA vapour. Afterwards, the needle was directly inserted into the injector GC–MS system where any silylated products are thermally desorbed. The resulting chromatogram showed a peak for unchanged A but the peak of B was no longer visible. Instead of B, a peak with the molecular ion at *m*/*z* 324 and the base peak at *m*/*z* 169 (Fig. 5) was observed. The molecular ion at *m*/*z* 324 confirms the presence of an alcohol function in B. From the isotope pattern of the base peak, it was clear that this fragment ion contained silicon. This indicates an ion, such as $C_6H_8O{-}Si(CH_3)_3]^+$, which supports the conclusion that the two double bonds are present in the first six carbons of the chain, if one counts the carbon with the hydroxyl group as carbon 1. A stable trimethylsilyl oxonium ion is postulated for the prominent base peak at *m*/*z* 169. Due to the stabilising effect of the trimethylsilyl group, such oxonium ions can be isolated and are even stable at room temperature [16]. A plausible explanation of the formation of the base peak in the mass spectrum of silylated B is given in Fig. 6. The mass spectrum confirms the conclusions drawn earlier from the mass spectra of underivatised A and B. The ion at *m*/*z* 97 in the mass spectrum of underivatised B, corresponding with the ion at *m*/*z* 169 in silylated B, is a minor fragment due to the lack of a stabilising effect [H versus trimethylsilyl (TMS)].

3.5. Solvent extraction of SPME needles

To confirm the presence of two conjugated double bonds at the low nanogram scale, a derivatisation with MTAD was attempted. This reagent is an extremely strong dienophile and reacts fast and selectively at room temperature with conjugated double bonds according to a Diels–Alder mechanism [17,18]. The reaction is also clean and leads only to one stable product. For conjugated dienes up to 20 carbons, the product is volatile enough to be investigated by GC–MS and the fragmentation pattern is highly diagnostic. It has been successfully applied in the study of pheromones [19]. Unfortunately, it was not possible to carry out this reaction on fibre. First MTAB is non-volatile, so, it has to be brought in contact with the fibre via a solution. To avoid extraction of A and B from the PDMS fibre into the MTAB containing solution, the solvent should be polar. However, MTAB is not very stable in aqueous solutions and is probably too polar to be absorbed into the PDMS. In any case, in none of the attempts any peaks possibly belonging to MTAB derivatives of A and B could be observed.

Thus, it was decided to develop a method for the solvent extraction of SPME needles. A study of the literature revealed that SPME on-fibre derivatisations prior to GC are frequently carried out [20–22] but never after off-line desorption. Solvent desorption of SPME needles prior to HPLC is also common [23,24] but this is never followed by derivatisation. The method should (1) allow a near 100% extraction from the SPME fibre, (2) have a final volume of not more than 4 μL which corresponds with the maximum volume that can be introduced splitless into a standard GC–MS system, (3) not introduce any high-boiling impurities and (4) use an extraction solvent that does not destroy the SPME fibre and is compatible with MTAD. The method was developed with ginger oil as model substrate. Ginger oil contains a number of C_{15}

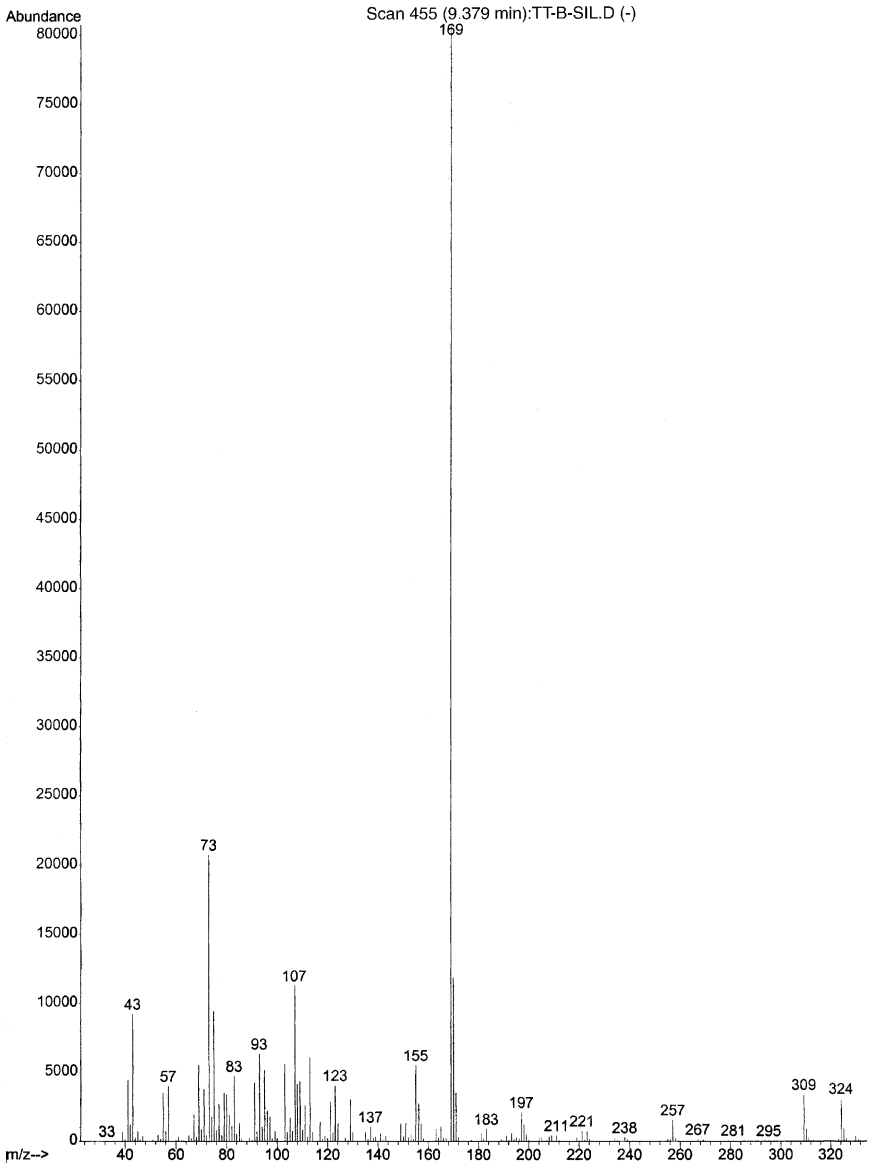


Fig. 5. Mass spectrum (EI) of silylated B.

hydrocarbons with similar polarity to compound A and B and some of these compounds were expected to react with MTAB [25]. From a melting point tube, a microtube was made with internal dimensions 10 mm × 1 mm (internal volume 8 μL). This tube nicely fitted the 100 μm PDMS fibre of the SPME needle (Fig. 7). Initially, 6 μL of 1-propanol was used as extraction solvent but this solvent did not show a 100% extraction within 15 min and was also not volatile enough for a spontaneous reduction of the volume during the extraction. Non-polar solvents like tertiary-butyl methyl ether and dichloromethane could not be used because they dissolved the PDMS coating. Eventually, acetone was found to give a near quantitative extraction of the fibre in 15 min without dissolving the fibre. Some movement of the needle during the extraction was necessary for a good yield. This could be easily accomplished by turning the adjustable needle guide up

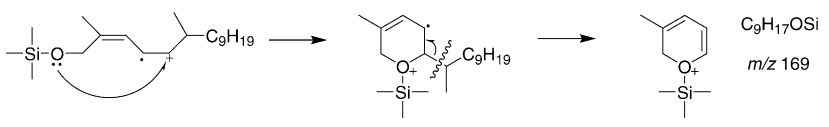


Fig. 6. Plausible fragmentation pattern for silylated B leading to the postulated stable oxonium ion base peak at *m/z* 169.

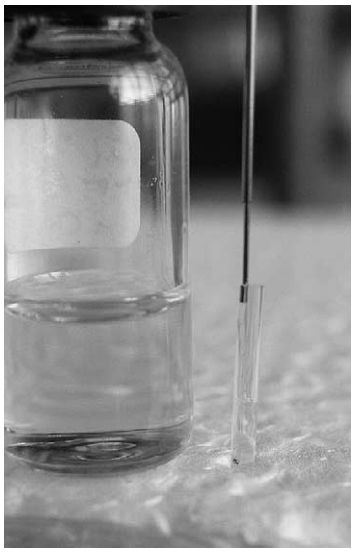


Fig. 7. SPME needle placed in 8 μL extraction vial with 3 μL acetone being present.

and down. During the 15 min extraction approximately 2 μL of acetone evaporated which was convenient as it allowed the direct addition of 2 μL of MTAB solution to the volatiles in acetone.

A 1-min reaction time in combination with a small grain of solid MTAB gave a full conversion of myrcene, α-zingiberene and α-farnesene without affecting any other peaks of ginger oil. At higher retention times, extra peaks corresponding to the MTAD reaction products appeared. The linear structures myrcene and α-farnesene showed as expected their molecular ions at *m*/*z* 249 and 317, respectively, and both gave diagnostic fragment ions at *m*/*z* 180 and 123. The cyclic α-zingiberene also showed its molecular ion at *m*/*z* 317 but due to the fact that the adduct is tricyclic the diagnostic base peak now appeared at *m*/*z* 179. If a longer reaction time in combination with a larger excess of MTAD was used, many more peaks disappeared and results were less clear. Thus, a short reaction time in combination with a 0.1% solution of MTAD in dichloromethane was chosen for testing the presence and location of a diene moiety in compounds A and B.

3.6. Derivatisation of wasp volatiles with MTAD

Having developed a method for the solvent extraction of SPME needles, it was applied on A and B obtained from parasitoid wasps. After the extraction of the needle with acetone, A and B could be detected by GC–MS in the acetone extract, with B still being the largest peak visible (results not shown). To the next acetone extract of wasp volatiles, some MTAB in dichloromethane was added and after 5 min the remaining solution was injected splitless in the GC–MS system. This time no peaks for A and B could be observed but instead two small peaks shifted to 9.97 and 10.18 min higher retention times were visible. Two weak averaged spectra (Fig. 8) could be obtained and they were correlated to MTAD derivatives of peak A and B, proving that both A and B contain a conjugated pair of double bonds. In the mass spectrum of the earlier eluting peak, essentially only two peaks were visible at *m*/*z* 194 (base peak) and *m*/*z* 137. The molecular ion at *m*/*z* 349 was not detected. The fragment ion at *m*/*z* 194 would support the loss of a saturated $C_{11}H_{23}$ side chain from the MTAD adduct of compound A. Further loss of methyl isocyanate, typical for MTAD fragmentations, generates ions at *m*/*z* 137.

The spectrum of the MTAD adduct of B was more informative. The molecular ion (*m*/*z* 365) was not detectable, but the presence of the CH_2OH group next to the double bond could be confirmed by the ion at *m*/*z* 334 $[M-31]^+$ in the spectrum. The base peak at *m*/*z* 210 is the result of the loss of the $C_{11}H_{23}$ side chain from the molecular ion of the MTAD adduct of B. Further loss of methyl isocyanate gives the ion of *m*/*z* 153. The fragment ion at *m*/*z* 180 can be explained by the loss of formaldehyde from the base peak. Finally also this fragment ion can lose methyl isocyanate giving rise to an ion at *m*/*z* 123. The proposed fragmentation pattern of the MTAD adduct of B is summarised in Fig. 9. Thus, the MTAD spectra confirm the presence of conjugated diene system and the earlier structural hypotheses. They also confirm that on either carbon 2, 3, 4 or 5 a methyl group must be present but do not allow any conclusion about the exact position, nor do they confirm that there is methyl side chain at C-6. With regard to these positions the spectra of the underivatised products are more informative.

3.7. Biosynthetic considerations

On the basis of all the above data the two skeletons that most likely represent A and B are 2,6,8,12-tetramethyltridecane or 2,6,10,12-tetramethyltridecane. The presence of a minor fragment ion at *m*/*z* 151 in combination with the absence of a fragment ion at *m*/*z* 179 slightly favours the 2,6,8,12-tetramethyltridecane skeleton (Fig. 10). Similar branched compounds have, for instance, been identified in the plumage of birds [26]. Among others, Jacob et al. reported a 2,6,10-trimethyl-C_{13} and a 2,6,8,12-tetramethyl-C_{16} compound from *Pelecanus crispus* and *P. onocrotalus*, respectively. The stereochemistry of the double bonds as well as that of the two chiral centres remains undetermined (Fig. 10).

Although, at first glance the proposed structure appears of terpenoid origin, we nonetheless, propose that it is of polyketide origin. A possible biogenetic pathway could start from isobutyric acid (C_4 unit forming carbons 11–13 and the last methyl side chain in the proposed structure) to which are subsequently attached a malonate (C_2), a methylmalonate (C_3), a methylmalonate (C_3), a malonate (C_2) and finally, a methylmalonate (C_3). Various reductions and eliminations of water can lead to the two proposed structures. This mechanism and many examples of branched pheromones of non-terpenoid origin, have been described by Francke and Schulz [27].

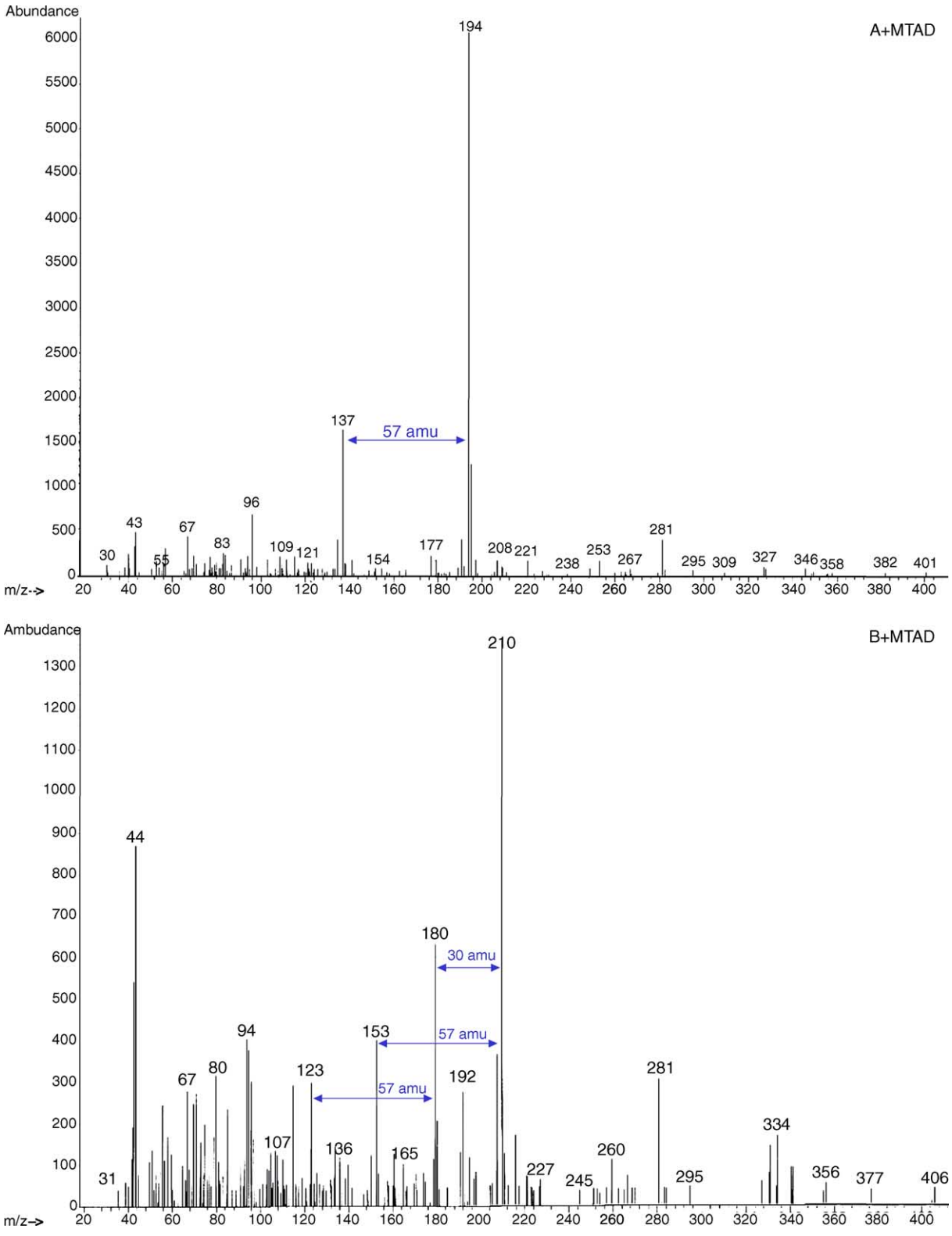


Fig. 8. Mass spectra (EI) of MTAD derivatives of peak A and B. The ion at *m*/*z* 281 is an impurity.

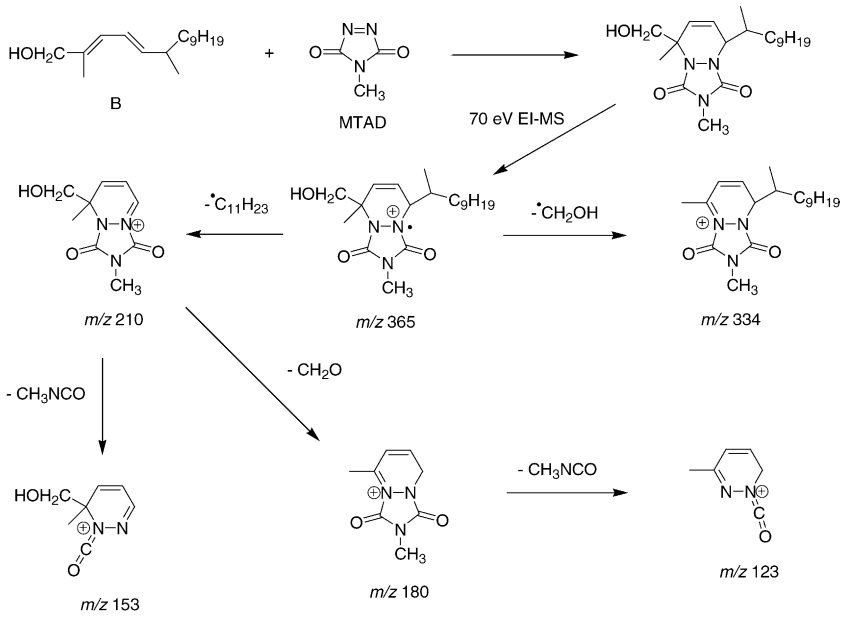


Fig. 9. Probable mass spectral fragmentation of adduct of B with MTAD.

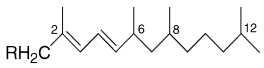


Fig. 10. Structural proposal for A (R = H) and B (R = OH). The stereochemistry of the two double bonds and the two chiral centres at C-6 and C-8 remains undetermined.

4. Conclusion

It was shown that virgin *T. turkestanica* females produce two compounds in the low picogram per hour range that are neither produced by conspecific males nor by mixed populations of males and females. These compounds are thought to be (part of) the sex pheromone of this minute wasp species. The compounds could be well sampled by SPME. It was demonstrated that by use of an internal standard of sulphur accurate mass measurements of 1 ng of compound can be obtained with SPME GC–MS. The elemental composition of the two compounds was determined as $C_{17}H_{32}$ and $C_{17}H_{32}O$. From these compositions and the fragmentation patterns, a partial structure proposal could be made. SPME on-fibre silylation followed by GC–MS proved the presence of an alcohol group and showed that the two double bonds were present within a six carbon fragment. A detailed study of the retention indexes of both compounds on a polar and non-polar column made it possible to deduce the presence of linear C_{13} skeleton substituted with four methyl groups. A method was developed for the desorption of SPME needles and subsequent off-line derivatisation at a 1 ng level. With this new method—through reaction with the dienophile MTAD—the presence of a conjugated diene in both compounds was proven. On the basis of all evidence, partial tentative structures were proposed for the two compounds. If the proposed structures are correct, these compounds have not been isolated or described previously. Further proof for the basic skeleton should be obtained through catalytic hydrogenation followed by GC–MS. Two attempts to do this on-line [28] with Pd in the liner and H_2 as carrier gas have so far not been successful. It will now be attempted to do this off-line with the newly developed SPME desorption method. For final proof of the structures, including the stereochemistry of the double bonds and the chiral centres, chemical synthesis will be necessary. Once the two compounds are available in larger amounts through synthesis, a bioassay should substantiate whether they are truly sex pheromonal compounds.

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