Solid Phase Micro Extraction Technique Used for Collecting Semiochemicals. Identification of Volatiles Released by Individual Signalling *Phyllonorycter sylvella* Moths

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The SPME (solid phase micro extraction) technique was used in the collection of volatiles released by calling females of the 4–6 mm long tentiform leafminer moth *Phyllonorycter sylvella* (Lepidoptera, Gracillariidae). The volatiles released by the calling *P. sylvella* females were identified by GC-MS as a mixture of *Z*10-tetradecenyl acetate (92%), *E*10-tetradecenyl acetate (2%) and *Z*8-tetradecenyl acetate (6%). The amount of volatiles released by one calling female during three hours and collected on a polydimethylsiloxane fibre, was as large as the amount extracted from the glands of 20 females. The SPME technique gives the opportunity of continuously following the release of behaviour mediated signals from weak scented living organisms.

Introduction

There has long been a need for simple and sensitive odour collection techniques for studying insect semiochemicals. The entrainment technique, in which the volatiles released from the biological objects are concentrated, via an air stream, on a porous polymer as Porapak Q, Tenax GC or active charcoal (Borg-Karlson, 1990), has freqently been used also for the collection and enrichment of moth volatiles. The polymers are desorbed by heat or by rinsing the polymer with an organic solvent. The latter procedure gives a sample that can be used repeatedly both for analyses and for biological tests. However, the need for long periods of entrainment, the possible discrimination of compounds of low volatility, and the large solvent peak limit the range of detectable volatiles and the possibility to study short time changes in living organisms.

Recently, the solid phase micro extraction technique (SPME) has been developed and made commercially available (Zang and Pawliszyn, 1993; Zang and Pawliszyn, 1995; Górecki and Pawliszyn, 1995). A number of applications have already been published, dealing with environmental research, volatiles in food and beverage, fungi, and water contaminants (Mindrup, 1995; Pelusio *et al.*, 1994 and references therein).

The SPME technique gives us the opportunity to collect volatiles from an individual insect, even a very small one. The scent collection from small moths such as species from the genus *Phyllonorycter* has earlier been extremely time- and individual-consuming, as the volatiles have been collected by dissections of the glands of numerous females, followed by extraction in super-clean organic solvents (Mozuraitis, unpublished results). In this paper we present our first results using the SPME technique on signalling *Phyllonorycter* moths.

The Biological Object

Most of the leafminer moths of the genus *Phyllonorycter* are monophagous or oligophagous insects, feeding on one or a few host-plant species (Kuznetzov, 1975). The *Phyllonorycter* moths are convenient model objects for investigations of interspecific interactions, as the form, colour, and location of a mine on a host leaf make it possible to identify the species before adult emergence and

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collect pupae of single species for experimental needs. Unlike in many other moth species, the caterpillars of the phyllonoryctids feed inside the leaf and are not able to change the nourishment place selected by the adult female. This biological feature makes it possible in advance to determine the approximate population density and the various species occurring at the place where field tests will be performed. Little is known about the chemical communication systems, of phyllonoryctids, thus only for a few species of this genus the pheromones are identified (Mozuraitis, unpublished results; Arn *et al.*, 1992). Due to the size of the moth great efforts are needed to identify pheromone components using conventional extraction techniques.

Materials and Methods

Phyllonorycter sylvella (Hbn.) (Lepidoptera, Gracillariidae) was selected as a model species for collection of the volatiles released by the females during the peak of their signalling activity, using the solid phase micro extraction technique. *P. sylvella* moths feed on different species in the genus *Acer* (Ivinskis *et al.*, 1985). Leaves with *P. sylvella* mines were collected from the maple *Acer platanoides* L. near Vilnius (Eastern Lithuania) in October 1994, just before the shedding of leaves. The leaves collected were placed in wooden boxes on a 6–8 cm layer of moistened peat and were kept outdoors during the winter. In the spring, the pupae in the mines were placed in vials of a volume of 32 cm^3 and activated in laboratory conditions at a temperature of 14±2 °C during scotophase and 20±2 °C during photophase. The light:dark regime was 14:10 hours. Following emergence, the adults were collected daily immediately after the beginning of the photophase and were then sexed and placed in individual holding vials provided with a solution of 5% (w/v) sucrose in water.

Sorption of volatiles from Phyllonorycter moths

Pheromone components from single *P. sylvella* females were collected as follows: The moth (4–6 mm long) was placed in a glass tube (10x80 mm). The tip of the syringe with the cleaned (see below) SPME fibre (100 µm polydimethylsiloxane) was placed a few mm from the protruded abdominal glands of the calling female. The sorption went on for 2–3 hours at a temperature of 12–14 °C. When the sorption from the females was finished, dodecane (100 ng) and an undecenyl acetate (5 ng) dissolved in hexane were added by syringe onto the inner wall of the glass tube as internal standards and collected on the SPME fibre for six minutes. The volatiles from five calling females were collected and analysed one by one.

Solvent extraction of abdominal glands of Phyllonorycter moths

The abdominal glands of 100 calling females were excised, cut and extracted twice in 10 µl of pentane for 15 min at room temperature. The extract was concentrated and a few drops of hexane (pa Merck) were added. The solution was stored at –20 °C until GC-MS-analyses were performed. Voucher specimens are kept at the Department of Chemistry, Organic Chemistry, KTH.

Desorption and cleaning procedure of the SPME fibre

The reference compound, 10 ng *Z*10-tetradecenyl acetate was dissolved in hexane and added by syringe onto the inner wall of a glass vial of the same size as for the sorption of moth volatiles. Sorption was made during 15 min. *Z*10-Tetradecenyl acetate was totally desorbed from the fibre at injector temperatures of 200, 225, and 250 °C and splitless times of 30 and 60 seconds. Before the collection periods, the purity of the SPME fibre was checked through two subsequent injections in a split/splitless GC-injector for 30 seconds. Routine conditioning of the fibre was done at 225 °C for 10 min in a GC injector, splittless mode with helium as the carrier gas.

Identification of pheromone components

The constituents released were identified by using the Finnigan SSQ 7000 GC-MS system, including a Varian 3400 GC. One DB-5 and one DB-wax silica capillary column (30 m, id 0.25 mm, film thickness 0.25 µm) were used with a temperature programme of 80 °C (1 min), followed by 10 °C/min to 140 °C, and thereafter by 1 °C/min up to 200 °C. Splitless injection for 30 seconds at an injector temperature at 220 °C (helium 5 psi). After injection, the fibre was heated at 225 °C for 10

minutes. Peaks from non-calling and calling females were compared and only those coming from signalling moths were measured. Mass spectral data and retention times of selected peaks on both columns were compared with corresponding data for synthetic standards. *Z*8-Tetradecenyl acetate, *E*10-tetradecenyl acetate, and *Z*10-tetradecenyl acetate were purchased from Flora Co., (Tartu, Estonia) and the SPME-equipment from Supelco-Aldrich.

Results and Discussions

Three compounds from the signalling *P. sylvella* females were identified for the first time (Table I). The sorption of volatiles released by one calling female during three hours gave a mixture of *Z*8-tetradecenyl acetate (6%), *E*10-tetradecenyl acetate (2%), and *Z*10-tetradecenyl acetate (92%). In all, five females were analysed. The individual variation in the scent production among females was low. Around 100 individuals of the *Phyllonorycter* moths were nessecary to make an extract sample concentrated enough for the identification prodecure by GC-MS. Injection of a sample, representing 20 females, showed a similar composition (8%, 2% and 90%, respectively), and the same amounts in the GC-MS analyses (Table I) as did the SPME technique.

*Z*10-Tetradecenyl acetate has also been identified in *P. ulmifoliella* (Hbn.) and confirmed as a sex pheromone by field tests (Mozuraitis *et al.)* and known as sex attractant for males of *P. orientalis* (Kumata) (Ando *et al.,* 1977), *P. klemanella* (F.) (Booij and Voerman, 1984) and *P. ringoniella* (Matsumura) (Sugie *et al.,* 1986). With the last two species the compound is more effective in binary mixtures with either of *E*11-tetradecenyl acetate and *E*4, *Z*10-tetradecadienyl acetate. Other species in the genus *Phyllonorycter* release related mono- and di-unsaturated acetates with twelve and fourteen hydrocarbons (Mozuraitis *et al.* unpublished results). However, different host plants and different signalling rhythms of the moth species with the same pheromone may prevent competition and confusion among species. *Z*8- and *E*10-tetradecenyl acetate was neither found as an attractant nor identified earlier in the genus Phyllonorycter.

The SPME technique is shown to be a most valuable tool for collection of volatiles produced by weak scented moths. This makes it possible to investigate variations and differences in signalling compound composition among different geographical or host-plant races, among populations or on other conspecific levels. The collection of volatiles from undisturbed insects is essential, as the scent production may vary with the stages in their life cycles. The SPME technique also allows us to collect the pheromones during the whole signalling period of a female, which lasts for several days. The technique is extremely easy and rapid to use. Thus, it will enhance the possibility for continuous studies of both quick and slow changes in the chemical communication systems among undisturbed insects.

Table I. Pheromone components isolated from calling *Phyllonorycter sylvella* moths by solvent extraction and the SPME technique. n=number of moths, SD=standard deviation.
* *Z*10-Tetradecenyl acetate was estimated to ca. 50 ng in the samples by GC-MS analysis using an authentic sample.

Compound	SPME Retention time (min + SD) DB-WAX	SPME Relative amounts% Mean, n=5	SPME SD Relative amounts	EXTRACTION OF GLANDS Relative amounts%
*Z*8-Tetradecenyl acetate	30.78±0.01	6	1.1	8
*E*10-Tetradecenyl acetate	30.98±0.01	2	0.3	2
*Z*10-Tetradecenyl acetate	31.74±0.01	92*	1.2	90

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