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SPME collection and GC-MS analysis of volatiles emitted during the attack of male *Polygraphus poligraphus* (Coleoptera, Curculionidae) on Norway spruce

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Abstract: Tree mortality caused by bark beetles has increased in recent decades in both Europe and North America. In a large recent outbreak in central Sweden the bark beetle *Polygraphus poligraphus* was often found together with the spruce bark beetle *Ips typographus* in killed trees. To increase the understanding of the aggregation behavior of *P. poligraphus* we used solid phase microextraction (SPME) to collect volatile organic compounds (VOCs) released from single *P. poligraphus* males, with and without added females, colonizing Norway spruce stem sections and analyzed the sampled compounds by combined gas chromatography and mass spectrometry (GC-MS). High amounts of terpinen-4-ol, a substance found in the hindguts of *P. poligraphus* males in earlier studies, were released by colonizing males. The emission of both enantiomers of terpinen-4-ol was monitored by GC-MS over time as the males aged in the absence and presence of females. Single males emitted (*R*)-(–)-terpinen-4-ol for up to 60 days in high enantiomeric purity but the enantiomeric excess (ee) varied between males, and also for the same individual, over time from 96.3% to 99.3% ee. In the presence of females, males also emitted terpinen-4-ol for up to 50 days but now in lower amounts and with lower enantiomeric purity varying from 67.7% ee to 99.3% ee. Small quantities of other volatile compounds were emitted from the colonizing beetles including *cis*- and *trans*-4-thujanol, both of which were previously shown to be present in the hindguts of males. In earlier studies frontalin was found to attract *P. poligraphus*, but in our study it was not identified among emitted compounds from colonizing beetles.

Keywords: analysis; enantiomers; forest pest; pheromone; terpinen-4-ol; volatile organic compounds.

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1 Introduction

Europe has lost more than 150 million m^3 of timber due to bark beetle attacks over the last 50 years. Moreover, the amount of damage caused by these insects in both Europe and America has increased in recent decades [1–3]. This trend is expected to continue due to the effects of climate change on bark beetle voltinism and forest vitality [4, 5]. In addition, bark beetle damage has been occurring at progressively more northerly latitudes [3]. Central Sweden recently experienced a large bark beetle outbreak and at least 3 million m^3 of timber were lost between 2008 and 2011 in the province of Västernorrland (Wulff, personal communication). Unexpectedly, not only the spruce bark beetle *Ips typographus* (L.), the major tree-killing bark beetle in Europe, but also the bark beetle *Polygraphus poligraphus* (L.) were present in many of the killed trees (Schroeder and Wulff, personal communication). This raises questions about the roles these species play in killing trees.

Semiochemicals such as pheromones are essential in the attack strategies of many tree-killing bark beetles [6, 7]. As with most polygamous bark beetle species, males of *P. poligraphus* are the host-selecting sex and therefore also emitters of the aggregation pheromone [8]. Many bark beetle species use oxygenated terpenoids as aggregation pheromones. GC analyses of volatiles from *P. poligraphus* hindguts after feeding from phloem tissue of Norway spruce, *Picea abies*, revealed three male-specific compounds [9]. One of these was identified as terpinen-4-ol [the (*R*)-enantiomer of which is shown as **1** in Figure 1] but the other two could not be identified. In the same

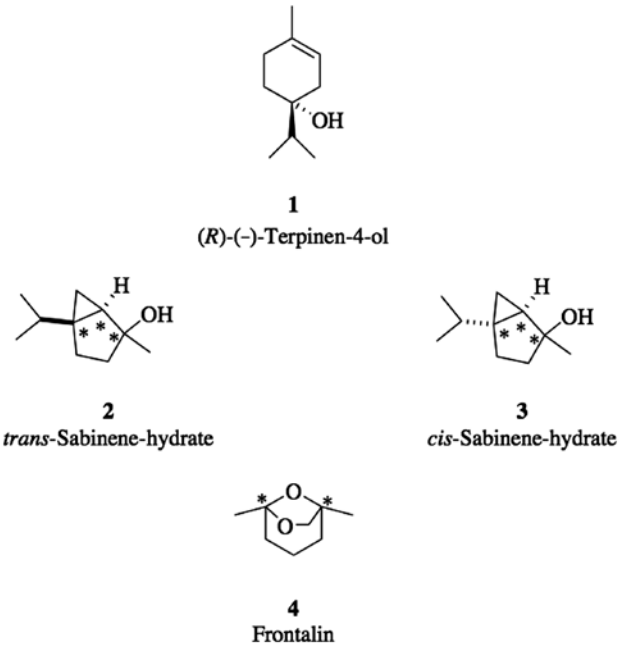

Figure 1: (*R*)-(–)-Terpinen-4-ol (**1**) and *trans*- and *cis*-sabinene hydrate (**2, 3**), which are synonyms of *trans*- and *cis*-4-thujanol, respectively, were previously identified among the VOCs from *P. poligraphus* males [10, 11]. Frontalin (**4**) exhibited activity in field trapping of *P. poligraphus* [10, 11].

year, Brummer identified the latter two compounds as the terpene alcohols *trans*- and *cis*-4-thujanol (**2** and **3** in Figure 1) [10]. Subsequently (*R*)-(–)-terpinen-4-ol (**1** in Figure 1) in high enantiomeric purity (99.35% ee) was found in male *P. poligraphus* beetles [11]. In the latter work, the volatile terpene alcohol was collected by head-space extraction from the hindguts of male beetles feeding on billets freshly cut from Norway spruce and analyzed by GC-FID using a chiral column.

Early field trials using racemic terpinen-4-ol failed to attract *P. poligraphus* but male-infested bark was highly attractive to both males and females [9]. (*R*)-(–)-Terpinen-4-ol of lower optical purity (75% ee) was tested in the field but yielded somewhat lower trap catches than authentic male-infested bark [12]. The bicyclic ketal frontalin (**4** in Figure 1) also exhibited biological activity when used as bait and yielded catches comparable to those achieved with (*R*)-(–)-terpinen-4-ol of moderate enantiomeric purity (75% ee). There appeared to be a synergistic effect between the two compounds because the trap catches increased when frontalin was used together with (*R*)-(–)-terpinen-4-ol [12]. To our knowledge, frontalin has never been identified among VOCs from *P. poligraphus*. However, frontalin has been identified as an aggregation pheromone in other bark beetle species such as *Dendroctonus brevicomis* and *D. frontalis*, and also as a multifunctional pheromone in *D. ponderosae* [13–15].

SPME has proven to be a powerful and general technique for isolating volatiles because it is fast, simple to perform, usually requires no solvent, and can achieve detection limits in the parts per trillion ranges for certain compounds. The method presents some advantages over traditional analytical methods [16] and has been used successfully in numerous environmental, food, flavor, pheromone, pharmaceutical, clinical and forensic applications [17–19]. However, its sensitivity to external factors such as vial shape, sampling time, and the condition of the fiber complicates its use in quantitative analysis [20].

In this work we used SPME to collect VOCs emitted by single *P. poligraphus* males over time as they colonized spruce stem sections and then identified the collected compounds by GC-MS. The effects of time since initiation of colonization, and access to added females, on the emissions were also investigated, with particular emphasis on the influence of these variables on the enantiomeric ratio of the emitted terpinen-4-ol.

2 Materials and methods

2.1 Experiments

All experiments were performed in a laboratory environment at Mid Sweden University during June and July 2012. Five different stem sections (length 35–45 cm, diameter 7–10 cm) cut from the same living spruce tree were used in the experiments. The stem sections were stored at 5 °C until used. Newly emerged *P. poligraphus* adults were introduced to the stem sections in 1.5 ml Eppendorf microtubes (Sarstedt, Nümbercht, Germany), which were nailed on to the stem sections to collect volatiles released from the holes bored by the colonizing beetles. In all of the experiments, the SPME fiber was placed in the cut end of the micro tube and the gap between the SPME fiber and the opening was sealed with aluminum foil. All experiments were performed at room temperature (20–22 °C). The stem sections were exposed to natural daylight normally between the hours of 09:00 and 15:00 and otherwise kept without direct natural daylight. VOC sampling over a period of 1 h and GC-MS analysis were normally performed once per day in each experiment using an SPME fiber conditioned for 10 min at 250 °C. Sampling was conducted for 10–13 days however, in two cases it was continued until terpinen-4-ol could no longer be detected. In most of the experiments, the enantiomeric composition of the emitted terpinen-4-ol was monitored by GC-MS over time. A total of 16 microtubes containing beetles (either single males or males and females) were placed on four of the five stem sections. In experiments including females, the females were introduced to the male, one by one, normally starting on day three or four after the male's entry into the microtube. Five replicates were performed using single males (on three different stem sections), two replicates with one male and one female (on the same stem section), one replicate

with one male and two females, one replicate with one male and three females, three replicates with one male and four females (on two different stem sections), and four replicates with one male and six females (on three different stem sections). To identify compounds related to defense substances released by the wounded spruce tissue, background emissions samples were collected from a fifth stem section onto which two empty microtubes were nailed and entrance holes were predrilled using a 2 mm drill to penetrate the outer and inner bark of the stem to mimic a beetle attack. Both of these background replications were sampled on days 1, 7, 15, and 16 after drilling.

In a separate experiment, the VOCs emitted by two live *P. poligraphus* males and two females that were walking on wet paper in a plastic box were sampled by SPME and analyzed. Samples were collected from males and females in isolation and from both groups together.

For all experiments, the SPME fiber carrying the collected VOCs was injected into a GC-MS for chemical analysis as presented below.

2.2 Insects

Between February and March of 2012, standing Norway spruce trees that had been colonized by *P. poligraphus* during the previous summer were cut in the province of Medelpad in central Sweden. Infested stem sections (45 cm long) were placed in emergence cages in a climate chamber (20 °C, day length 20 h) and emerging *P. poligraphus* individuals were collected on a daily basis. The beetles were sexed based on the presence of the two median tubercles on the frons of males and the long hairs of females [9]. They were then stored for a few days up to three weeks on moist paper at 5 °C until used in the experiments.

2.3 SPME

VOCs were collected using 65-µm polydimethylsiloxane/divinylbenzene (PDMS/DVD, pink 57 326-U) or 75-µm Carboxen™ PDMS SPME (CAR/PDMS, black, 57343-U) fibers (Supelco, Bellefonte, PA, USA). While both fibers exhibited good, reproducible and similar adsorptive capacities for the compounds of interest, the 65-µm PDMS/DVD fiber was selected for use in subsequent experiments. Sampling times of 1 and 2 h were tested; 1 h was found to be sufficient to obtain good and reproducible results in the subsequent assays and was therefore selected as the standard sampling time.

2.4 Chemicals

(*R*)-(−)-Terpinen-4-ol (50% ee) and α-terpineol were purchased from TCI (Portland, OR, USA), estragole, 3-methyl-2-buten, and sabinene hydrate from Sigma-Aldrich (St. Louis, MO, USA). Frontalin was purchased from Contech (Delta, BC, Canada) and Synergy Semiochemicals (Burnaby, BC, Canada).

2.5 Chemical analysis

The SPME samples were manually injected into the GC-MS and the fiber was held in the injector for 10 min to fully desorb VOCs.

To determine the range of compounds emitted under various conditions, samples were analyzed using an Agilent 7890A GC (Agilent Technologies, Santa Clara, CA, USA) with a FactorFour VF-5ms column (30 m×0.25 mm×0.25 µm; 5% phenylmethylpolysiloxane phase (Varian, Palo Alto, CA, USA) coupled to an MS-240 ion trap (Agilent) operating in electron impact mode (EI, 70 eV). These samples were injected using a multimode injector operating in splitless mode for 2.51 min at 250 °C with the transfer line temperature set at 280 °C. The SPME fiber was conditioned when left for 10 min in the injector before used for the next injection. Compounds were analyzed using helium as mobile phase (flow rate = 1.0 ml/min) and a temperature program in which the column was initially held at 40 °C for 1 min, then warmed at 5 °C/min to 250 °C, then at 20 °C/min to 300 °C, and finally held at 300 °C for 5 min.

To determine the enantiomeric composition of the emitted terpinen-4-ol, samples were analyzed on a Hewlett-Packard 6890N GC using a HP 5973 mass spectrometer (MS) operating in electron impact (EI, 70 eV) ionization mode for detection. The mobile phase (flow rate = 1.0 ml/min) was helium, the split/splitless injector was operated in splitless mode for 2.51 min at 250 °C, the transfer line was maintained at 230 °C and the aux temperature was set to 200 °C. The SPME fiber was conditioned when left for 10 min in the injector before used for the next injection. The GC was equipped with a BETA DEX™ 120 column (30 m×0.25 mm×0.25 µm; Supelco Park, Bellefonte, PA, USA), and separations were performed using a temperature program in which the column was initially held at 50 °C, then warmed to 100 °C at 10 °C/min and finally heated to 120 °C at 0.5 °C/min.

2.6 Data analysis

The raw MS data were analyzed using the Workstation 7.0.0 program (Agilent) and compounds were identified by comparing fragmentation patterns observed in the mass spectra to literature data and the Wiley and NIST MS mass spectral libraries together with reverse and forward match values. The identities of identified compounds were also secured by comparing their retention times and mass spectra to those of authentic samples.

3 Results

The *P. poligraphus* males began boring into the spruce stem sections around 3 h after their introduction, and an increasing GC peak area for terpinen-4-ol over the following days was recorded. In most cases, the GC peak area of terpinen-4-ol detected during the first three to eight days were much greater than those for other VOCs (see Figure 2A). The GC-MS total ion count (TIC) chromatogram of host plant VOCs sampled from a stem section with mechanically drilled holes, and no added *P. poligraphus* males, indicates that spruce produces only very small quantities of terpinen-4-ol (Figure 2B). Some of the other host plant's VOCs were identified as α-terpineol, *trans*-sabinene hydrate, *cis*-sabinene hydrate, chavicol and cubenol (Figure 2B).

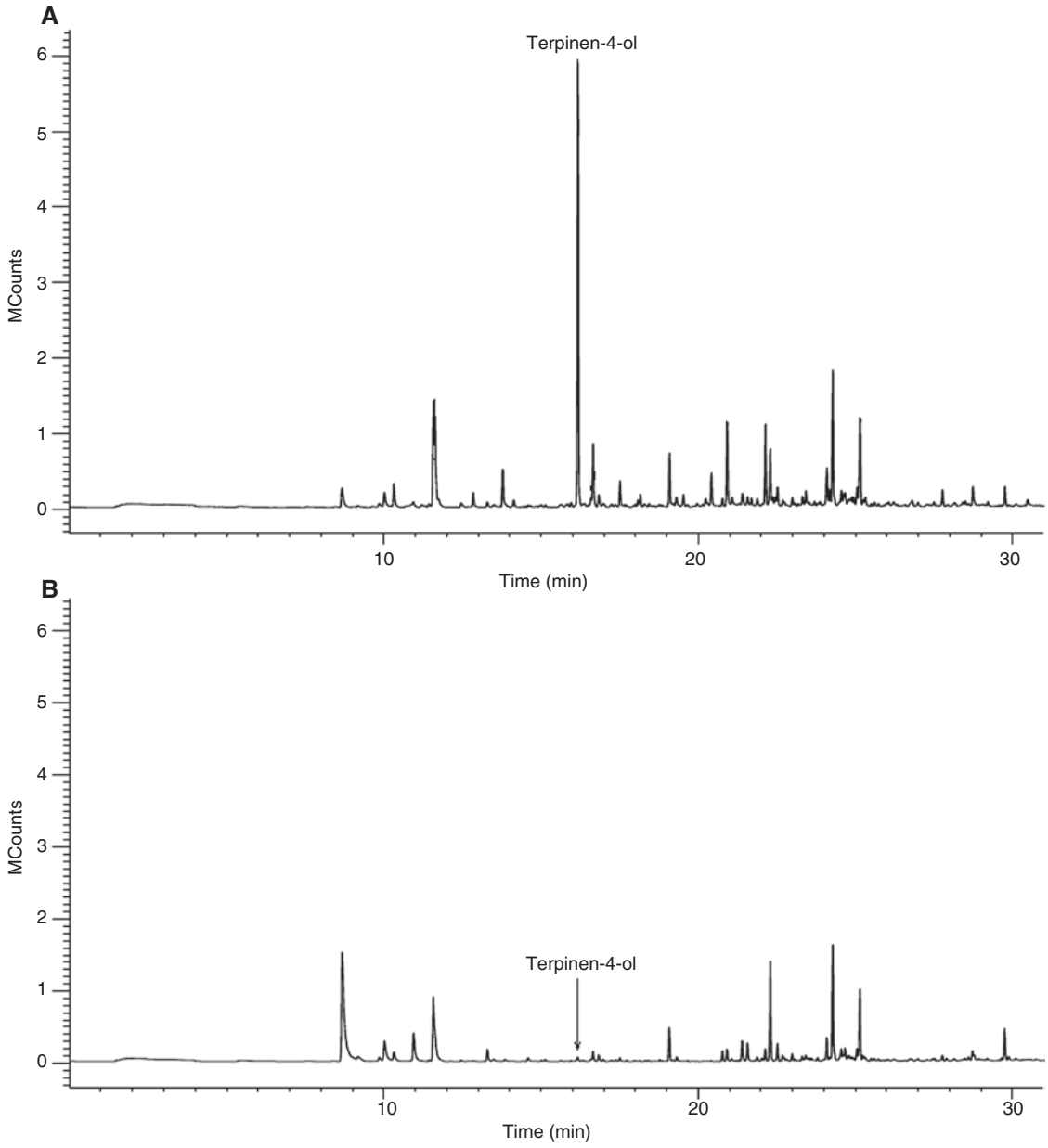

Figure 2: (A) A representative GC-MS chromatogram (FactorFour VF-5ms column) showing that terpinen-4-ol was the major compound isolated by SPME sampling on day 3 after the introduction of a single *P. poligraphus* male onto a spruce stem section. (B) A chromatogram (FactorFour VF-5ms column) showing VOCs emitted by a stem section taken from the same tree as above that had a hole drilled into it but was not exposed to *P. poligraphus* (sampled on the day the hole was drilled).

Table 1 shows minimum, maximum and mean GC-MS peak areas for selected VOCs collected from single *P. poligraphus* males, single males with one added female, and with six added females in spruce stem sections. The GC peak areas of several compounds varied over time, but the recorded GC-peak area of terpinen-4-ol always was much greater than those of the other VOCs. However, the addition of six females caused the emissions of some host plant VOCs to increase, including cubenol and chavicol (see Table 1).

Comparisons to an authentic sample revealed that no frontalin was detected among the compounds emitted by single *P. poligraphus* males, single males with added females or in the background emissions from the spruce stem sections.

The data presented in Figure 3 show that the presence of females reduces males' emissions of terpinen-4-ol. But even when six females were added, the males' production of terpinen-4-ol was greater than that of the mechanically damaged stem sections in the absence of beetles. The

Table 1: GC-MS peak areas (FactorFour VF-5ms column) for selected VOCs sampled by SPME of spruce stem sections exposed to single *P. poligraphus* males (M), a single male in the presence of one female (M+1♀), or a single male with six females (M+6♀).

Replicates	Compounds	Days sampled	Minimum value of GC-MS peak area	Maximum value of GC-MS peak area	Mean value of GC-MS peak area
M replicate one	Terpinen-4-ol	8	4.56E+05	1.56E+07	3.20E+06
	α-Terpineol	8	0.00E+00	2.52E+05	4.16E+04
	trans-Sabinene hydrate	8	0.00E+00	5.00E+05	7.18E+04
	cis-Sabinene hydrate	8	0.00E+00	1.16E+06	1.72E+05
	Cubenol	8	0.00E+00	5.07E+05	1.22E+05
	Chavicol	8	0.00E+00	3.66E+05	7.23E+04
M replicate two	Terpinen-4-ol	7	2.70E+05	1.22E+07	3.15E+06
	α-Terpineol	7	0.00E+00	7.34E+04	2.82E+04
	trans-Sabinene hydrate	7	0.00E+00	1.45E+05	2.08E+04
	cis-Sabinene hydrate	7	0.00E+00	5.25E+05	1.12E+05
	Cubenol	7	8.97E+04	7.78E+05	2.78E+05
	Chavicol	7	0.00E+00	2.46E+05	5.41E+04
M+1♀ replicate one	Terpinen-4-ol	6	2.96E+05	1.07E+07	3.14E+06
	α-Terpineol	6	4.02E+04	1.57E+06	3.70E+05
	trans-Sabinene hydrate	6	0.00E+00	2.95E+05	8.71E+04
	cis-Sabinene hydrate	6	0.00E+00	2.74E+05	5.43E+04
	Cubenol	6	0.00E+00	7.74E+05	2.65E+05
	Chavicol	6	4.43E+04	4.53E+05	1.71E+05
M+1♀ replicate two	Terpinen-4-ol	7	3.72E+05	8.51E+06	2.80E+06
	α-Terpineol	7	0.00E+00	1.14E+05	2.39E+04
	trans-Sabinene hydrate	7	0.00E+00	0.00E+00	0.00E+00
	cis-Sabinene hydrate	7	0.00E+00	5.07E+05	1.24E+05
	Cubenol	7	0.00E+00	3.76E+05	1.49E+05
	Chavicol	7	0.00E+00	2.08E+05	6.04E+04
M+6♀ replicate one	Terpinen-4-ol	7	0.00E+00	3.16E+06	1.39E+06
	α-Terpineol	7	0.00E+00	1.27E+06	2.90E+05
	trans-Sabinene hydrate	7	0.00E+00	1.74E+05	5.46E+04
	cis-Sabinene hydrate	7	0.00E+00	1.38E+05	5.30E+04
	Cubenol	7	1.13E+05	4.52E+06	1.01E+06
	Chavicol	7	2.81E+04	6.10E+06	1.07E+06
M+6♀ replicate two	Terpinen-4-ol	7	0.00E+00	8.40E+06	2.23E+06
	α-Terpineol	7	0.00E+00	3.61E+05	1.15E+05
	trans-Sabinene hydrate	7	0.00E+00	0.00E+00	0.00E+00
	cis-Sabinene hydrate	7	0.00E+00	5.34E+05	1.05E+05
	Cubenol	7	0.00E+00	1.82E+06	5.10E+05
	Chavicol	7	0.00E+00	1.39E+06	3.74E+05

All replicates were from the same stem sections.

male emissions of terpinen-4-ol increased over the first few days of the sampling period but then decreased. The terpinen-4-ol emissions of single males were much higher during the initial period than those of males accompanied by females, and continued for several weeks.

An optimized chiral GC protocol using a BETA DEX™ 120 column was developed to achieve good baseline separation for the two enantiomers of terpinen-4-ol. The separation achieved using this protocol with a commercial sample of terpinen-4-ol is shown in Figure 4A. The application of these GC conditions to an SPME sample obtained from a *P. poligraphus* male that had access to females revealed that the male produced an unexpected large amount of (*S*)-(+)-terpinen-4-ol relative to the (*R*)-(–)-enantiomer (Figure 4B).

Some of the *P. poligraphus* males accompanied by females emitted substantially higher proportions of (*S*)-(+)-terpinen-4-ol than males in the absence of females (Table 2). Moreover, the enantiomeric excess of emitted terpinen-4-ol varied over time for all males, regardless of the presence of females. Females were added, one by one, as soon as the males began emitting terpinen-4-ol; this usually occurred four days after the males were first placed on the stem. At this point, the emissions of terpinen-4-ol were normally high enough to permit determination of both enantiomers with high accuracy.

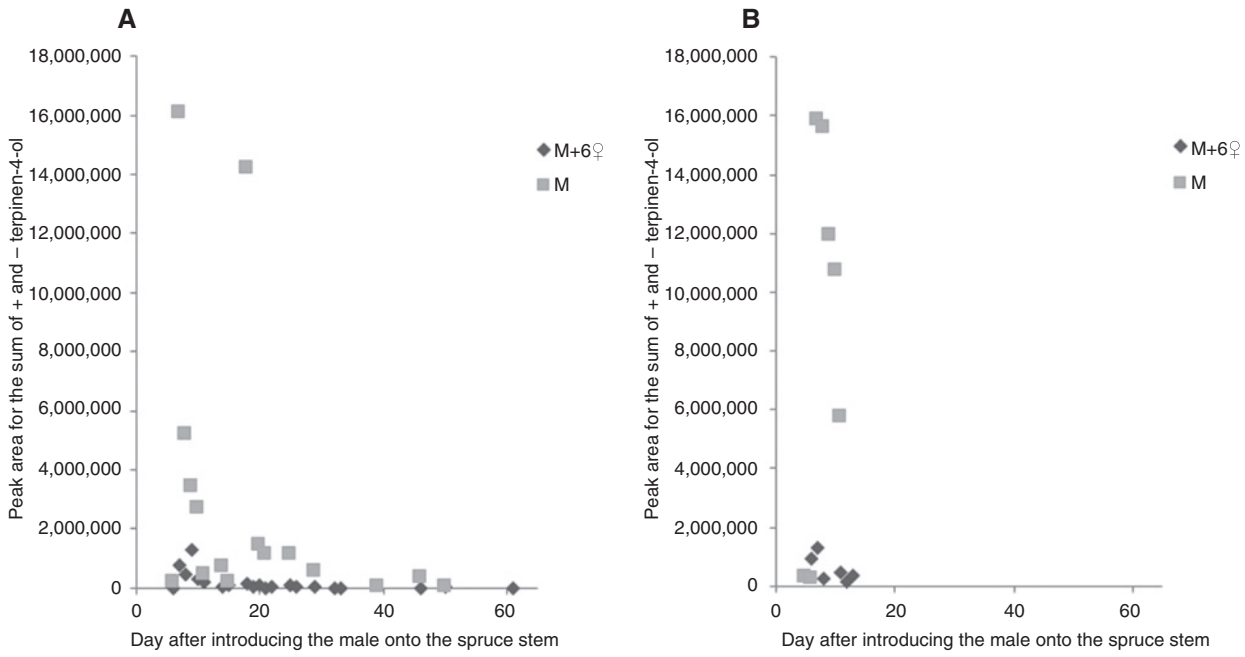

Figure 3: Summed GC-MS peak areas (BETA DEX™ 120 column) for (+)-terpinen-4-ol and (-)-terpinen-4-ol emitted by a single *P. poligraphus* male (M) and a male in the presence of six females (M+6♀). The data were gathered in two separate replicates (3A and B) conducted on two spruce stem sections from the same tree.

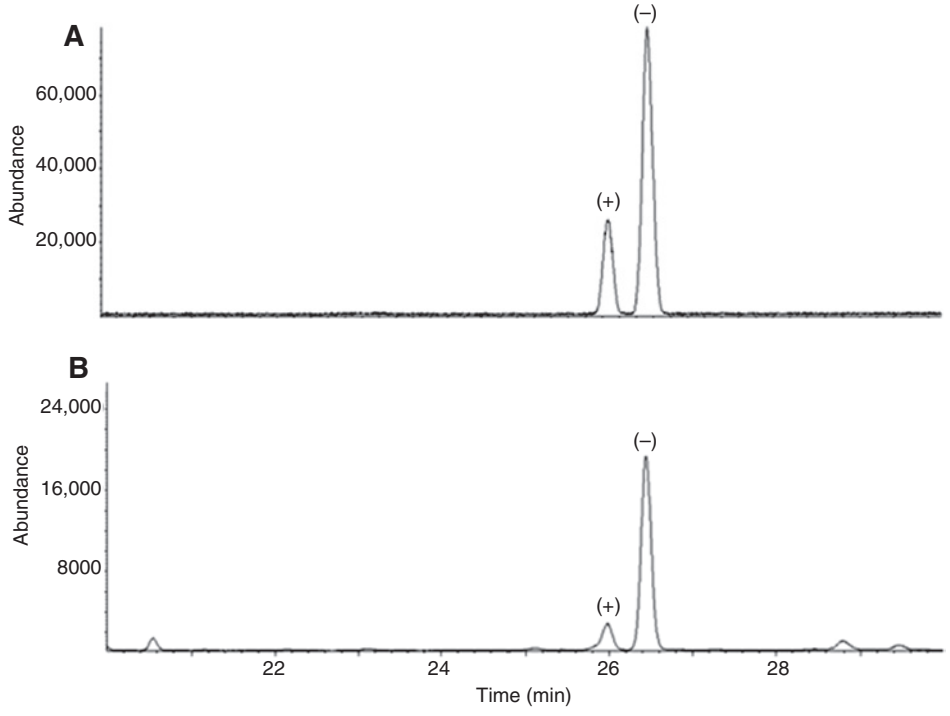

Figure 4: Chiral GC-MS chromatogram (BETA DEX™ 120 column) of (A) a synthetic mixture of terpinen-4-ol enantiomers (ee = 50%), and (B) of terpinen-4-ol with 78% ee collected during a sampling involving a male of *P. poligraphus* with access to females.

The background emissions of terpinen-4-ol from by hand drilled spruce stem sections without *P. poligraphus* were not high enough (see Figure 2B) to permit analysis of the enantiomeric purity of the alcohol.

No detectable VOC emissions were produced by *P. poligraphus* males or females, whether separately or together, when placed on wet paper in a plastic box without access to the host plant.

Table 2: Percentage of (*S*)-(+)-terpinen-4-ol in the terpinen-4-ol emitted by three *P. poligraphus* males without females, a male in the presence of two females (M+2♀), three females (M+3♀), four females (M+4♀), and six females (M+6♀) over time (BETA DEX™ 120 column).

P. poligraphus replicates	Days sampled	(*S*)-(+)-terpinen-4-ol (%)$_{min}$	(*S*)-(+)-terpinen-4-ol (%)$_{max}$	(*S*)-(+)-terpinen-4-ol (%)$_{mean}$
Male 1	7	0.34	1.14	0.80
Male 2	7	0.34	1.64	0.85
Male 3	7	1.00	1.84	1.25
Male 4+2♀	8	0.00	0.93	0.61
Male 5+3♀	9	0.00	16.00	6.58
Male 6+4♀	8	0.00	11.36	3.27
Male 7+6♀	6	0.00	0.47	0.08

Males 1 and 2 were placed on the same stem section while male 3 was placed on a separate section. Both sections originated from the same tree. Male 4 and male 6 were placed on the same stem section while male 5 and 7 were placed on a different section. Both stem sections originated from the same spruce tree.

4 Discussion

Our results show that much higher amounts of terpinen-4-ol are released from *P. poligraphus* males colonizing stem sections of spruce than from the background signal from the stem itself. This is in agreement with earlier studies demonstrating high amounts of terpinen-4-ol in hindguts of *P. poligraphus* males (see Figures 2, 3 and Table 1). However, it is important to note that previous SPME analyses have shown that terpinen-4-ol also occurs naturally in spruce [17, 21].

Our results also show that even when up to six females were added, males continued to emit terpinen-4-ol at levels that substantially exceeded the background signal (see Table 1). The GC-MS peak areas corresponding to the amount of terpinen-4-ol emitted by male *P. poligraphus* appear to be inversely related to the presence of females to at least some extent (see Table 1 and Figure 3). In particular, there were differences between single males and males accompanied by six females with respect to both the amount of terpinen-4-ol emitted and the duration of emission (see Figure 3). This may also be due to individual differences between males, but it may also suggest that males who have not had the opportunity to mate emit more of the pheromone to increase and/or prolong their attractiveness to females.

It has been reported that when *cis*- and *trans*-4-thujanol were added to mixtures of frontalin and terpinen-4-ol, only (-)-*cis*-4-thujanol increased the catch of *P. poligraphus* [12]. Brummer found that both *cis*- and *trans*-4-thujanol were male-specific volatile compounds in extracts of *P. poligraphus* [10]. However, our results did not reveal any strong emission of these compounds from males (see Table 1). Further experiments and field trials will therefore be required to resolve this discrepancy. Wajs et al recently identified both of these compounds in sap- and heartwood samples of Norway spruce by SPME sampling and GC-MS analysis [21]. We also identified α-terpineol, cubenol and chavicol among the compounds adsorbed on our SPME fibers (see Table 1), all of which have previously been detected in small quantities in spruce [21]. Terpinen-4-ol was released at higher quantities (i.e. larger GC peak areas) than any other VOCs, with the exception of cubenol and chavicol in the experiments with males accompanied by six females (see Table 1). The substantial emissions of the latter two compounds under these conditions may be due to the high number of mated females establishing maternal galleries in the bark and phloem for egg laying and thus enhance the release of host tree substances. Another possibility is that these compounds are emitted by the beetles and may function as repellents.

Frontalin exhibited biological activity on *P. poligraphus* when used as bait, and a synergistic effect was observed when it was used together with (*R*)-(−)-terpinen-4-ol [12]. Surprisingly, we could not identify frontalin among the VOCs emitted from colonizing single males nor from males with females and the role of this compound in *P. poligraphus* aggregation [12] is thus unclear.

The enantiomeric composition of the terpinen-4-ol emitted by *P. poligraphus* males varied over time (see Table 2). Most males emitted (*R*)-(−)-terpinen-4-ol with high enantiomeric excess (>96% ee). However, the ee varied between males and over time for the same male, from 96.3% to 99.3% (see Table 2). Interestingly, some males accompanied by females emitted (*R*)-(−)-terpinen-4-ol of similar ee to males without females while others emitted (*R*)-(−)-terpinen-4-ol with much lower ee values. (See Figure 4B and Table 2). While the enantiomeric excess of the emitted terpinen-4-ol seems to depend on

the presence of females, it may also be influenced by the status of the microorganism(s) involved in its production as well as the beetles' physiological circumstances and genetic factors. Further investigations will thus be required to clarify this issue.

In field trials, racemic terpinen-4-ol failed to attract *P. poligraphus* [9] and the trap catches achieved using (*R*)-(−)-terpinen-4-ol of low optical purity (75% ee) were lower than those for male-infested bark [12]. These results are in accordance with our results that the beetles emitted (*R*)-(−)-terpinen-4-ol with high enantiomeric excess (>96% ee) and thus suggest that the enantiomeric composition of the aggregation pheromone is important for its attractiveness. The variation in the enantiomeric purity of the aggregation pheromone observed in this work suggest that further biological assays should be performed using enantiomerically pure (*S*)-(+)- and (*R*)-(−)-terpinen-4-ol in order to determine their effectiveness individually and when mixed in different ratios.

Several bark beetle species use compounds ingested from their host plants as precursors for pheromone production [17]. For example, Chararas *et al*. provided evidence that the attractants of the bark beetle *Phloeosinus armatus*, terpinen-4-ol and α-terpineol, are produced from sabinene by a bacterium isolated from the beetles' hindguts [22]. The site of semiochemical production in *P. poligraphus* is unknown, but it is possible that it too relies on a microorganism in the male beetles' hindguts to produce pheromones from host plant compounds or they are de novo produced as most bark beetle pheromone components, except for *cis*- and *trans*-verbenol [23]. It would therefore be interesting to concentrate the terpinen-4-ol produced by the host plant and compare its enantiomeric purity to that of the emitted material. It would also be useful to feed *P. poligraphus* with specific potential pheromone precursors to determine the origins of its emitted compounds [24]. Efforts in this direction are currently underway in our laboratories.

To summarize, we have found that SPME is a useful and simple technique for sampling volatile compounds emitted by males of *P. poligraphus* that are attacking spruce stem sections. The emissions of terpinen-4-ol and its enantiomeric composition were followed over time for individual males with and without females. Single males emitted (*R*)-(−)-terpinen-4-ol of high enantiomeric purity for up to 60 days. The enantiomeric purity of the emitted alcohol varied between males and also for the same individual over time, from 96.3% to 99.3%. In the presence of females, males emitted the aggregation pheromone for up to 50 days, but in lower amounts and with lower and even more pronounced variation of the enantiomeric purity (67.7% ee to 99.3% ee).

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