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Abstract

The extraction of small lipophilic molecules (SLMs) in the soil-root interface that play a role in belowground ecological interactions between plants and insect herbivores was investigated. Polydimethylsiloxane (PDMS) microtubing has been shown to absorb root SLMs selectively in low-disturbance setups, where analytes were extracted from the polymer with methanol. This technique was adapted to isolate SLMs that diffuse in the vapour phase in soil and sand and under various experimental parameters, extracting with a plug of diethyl ether pushed through the length of the silicon tubing. Moisture level had a substrate-dependent effect on the recovery rate of analytes that were applied as synthetic blends of known belowground SLM semiochemicals in the media. Higher amounts of two selected SLMs, (E)-caryophyllene and (-)-thujopsene, were extracted from sand, and increased polymer and solvent volume, as well as sampling duration, resulted in more of these two SLMs recovered by extraction. It was also shown that PDMS tubes lose no extraction capacity after repeated use. The signature compound (E)-caryophyllene was successfully isolated from the rhizosphere of maize plants infested with Diabrotica v. virgifera larvae by extracting the silicon tubing with diethyl ether. Because the tubes are preconditioned to reduce the presence of contaminants, such extracts can be directly analysed by GC and GC-MS and used in electrophysiological and behavioural assays. After further modifications, non-invasive, in situ PDMS probes can be developed that extract SLMs from plant rhizosphere for the study of belowground chemical ecology processes.

Keywords	soil chemistry; belowground interaction; chemical ecology; semiochemical; diffusion; absorption
Taxonomy	Natural Products, Gas Chromatography, Sample Preparation, Analytes, Detection Technique, Mass Spectrometry

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Dear Editor-in-Chief,

Please find enclosed our manuscript, “Novel use of PDMS tubing for in-soil capture of plant natural products” by József Vuts et al., which we would like to submit for publication as a regular research paper in Journal of Chromatography B. We have defined a novel use of PDMS tubing in rhizosphere chemical ecology research, producing extracts directly analyzable by GC-based methods.

We believe our findings would appeal to the readership of Journal of Chromatography B.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

All authors have approved the manuscript and agree with its submission to Journal of Chromatography B.

Please address all correspondence to: Dr. József Vuts (jozsef.vuts@rothamsted.ac.uk).

We look forward to hearing from you at your earliest convenience.

Sincerely yours,

József Vuts, PhD

- Semiochemicals govern belowground interactions, but their extraction is difficult
- We adapted a method to isolate small lipophilic molecules (SLMs) from soil matrices
- We show ether extraction of silicon tubing (PDMS) produces samples enriched in SLMs
- Performance of the extraction method is described for various sampling parameters
- PDMS is a non-invasive, in situ method to provide samples for chemical ecology assays

Abstract
The extraction of small lipophilic molecules (SLMs) in the soil-root interface that play a role in belowground ecological interactions between plants and insect herbivores was investigated. Polydimethylsiloxane (PDMS) microtubing has been shown to absorb root SLMs selectively in low-disturbance setups, where analytes were extracted from the polymer with methanol. This technique was adapted to isolate SLMs that diffuse in the vapour phase in soil and sand and under various experimental parameters, extracting with a plug of diethyl ether pushed through the length of the silicon tubing. Moisture level had a substrate-dependent effect on the recovery rate of analytes that were applied as synthetic blends of known belowground SLM semiochemicals in the media. Higher amounts of two selected SLMs, (*E*)-caryophyllene and (-)-thujopsene, were extracted from sand, and increased polymer and solvent volume, as well as sampling duration, resulted in more of these two SLMs recovered by extraction. It was also shown that PDMS tubes lose no extraction capacity after repeated use. The signature compound (*E*)-caryophyllene was successfully isolated from the rhizosphere of maize plants infested with *Diabrotica v. virgifera* larvae by extracting the silicon tubing with diethyl ether. Because the tubes are preconditioned to reduce the presence of contaminants, such extracts can be directly analysed by GC and GC-MS and used in electrophysiological and behavioural assays. After further modifications, non-invasive, *in situ* PDMS probes can be developed that extract SLMs from plant rhizosphere for the study of belowground chemical ecology processes.

Keywords: soil chemistry; belowground interaction; chemical ecology; semiochemical; diffusion; absorption

Novel use of PDMS tubing for in-soil capture of plant natural products

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1 Introduction

Small lipophilic molecule (SLM) semiochemicals (naturally occurring behaviour- and development-modifying chemical signals, e.g. pheromones and other allelochemicals) govern a wide range of ecological interactions within and across trophic levels, e.g. between plants and their associated insect, pathogen and weed pests. Whilst the role and identity of volatile SLMs in aboveground multitrophic interactions has been well characterised by numerous chemical ecology studies, by comparison, there are significant gaps in knowledge of similar SLMs involved in belowground interactions [1], i.e. in the rhizosphere (the zone of chemical, biological and physical influence generated by root growth and activity). The abundance of live biomass and the diversity of organisms in the rhizosphere is a source of a breadth of natural products with ecological functions. Bacteria, fungi, plants, arthropods and other soil-dwelling invertebrates have mostly been studied in isolation from the rhizosphere, resulting in the description of a plethora of SLMs without the characterisation of their semiochemical roles. This is in part due to the methodological difficulties that accompany the isolation, bioassay-guided fractionation and identification of SLMs from soils. As soil is a three-state system, consisting of a solid phase of minerals and organic matter (the soil matrix), as well as a porous phase that holds gases (the soil atmosphere) and water (the soil solution), the diffusion of SLMs is affected by soil pH [2] as well as their ability to cross state boundaries while interacting with substrate particles and water [3].

Over the past two decades, a number of invasive and non-invasive techniques have been developed for the *in vitro* and *in vivo* isolation of rhizosphere SLMs from artificial and natural soil ecosystems. Freezing cleaned maize (*Zea mays* L., Poaceae) roots in liquid nitrogen after herbivory by the western corn rootworm (*Diabrotica v. virgifera* LeConte) (Coleoptera: Chrysomelidae) larvae, followed by collection of volatile compounds from powdered tissue using solid-phase microextraction (SPME), led to the identification of (*E*)-caryophyllene as a cue used by entomopathogenic nematodes to locate their herbivore hosts [4]. Headspace sampling methods, commonly used to collect aboveground plant volatiles [5], were also developed to sample compounds from live roots *in situ*. Vacuum pumps were used to remove air continuously from the root zone of *Citrus paradisi* Macf. × *Poncirus trifoliata* L. Raf. (Rutaceae) plants infested with the weevil *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae), with Super Q adsorbent polymer being placed in the airflow to trap volatile SLMs emitted by the roots [6]. This way, pregeijerene was identified in the adsorbent extracts from both greenhouse- and field-grown trees, which attracted natural enemies (entomopathogenic nematodes) of the herbivore larvae.

Polydimethylsiloxane (PDMS)-based applications exploit the property of PDMS to absorb lipophilic compounds selectively [7]. PDMS is used as the stationary phase in capillary columns for gas chromatography (GC), and as enrichment material in SPME or stir-bar sorptive extraction (SBSE). PDMS/divinylbenzene-coated SPME microfibres have been used to collect volatile SLMs *in situ* from near the roots of pot-grown broccoli plants (*Brassica oleracea* L. var. *italica*) (Brassicaceae) infested with cabbage fly larvae (*Delia radicum* L.) (Diptera: Anthomyiidae) [8]. Analysis of the volatiles after thermal desorption from SPME fibres revealed the presence of different sulphur-containing compounds that characterised the headspace of infested broccoli roots. Other techniques relied on the absorption of SLMs into the PDMS coating of SBSE rods [9], [10] and permeation of non-polar analytes through the wall of PDMS microtubing [11], [12]. Kallenbach et al. [13] used short pieces of PDMS to extract volatiles from aboveground plant organs, which were subsequently analysed via thermal desorption, and the same procedure was applied to the rhizosphere [14]. Mohney et al. [15] inserted long pieces of PDMS tubing into the root zone of *Tagetes* spp. (Asteraceae), and by pushing a plug of methanol through the tubing and collecting the solvent in an HPLC vial, they could extract root-exuded thiophenes (MW 216 and 248) with allelopathic properties, which are generally distributed by soil mycorrhizal networks [16]. Using *in situ* PDMS microtubing placed in the root zone

of *T. patula* and extracting the tubing with methanol by slowly pushing it through with a syringe pump, the spatial and temporal heterogeneity in thiophene production was later demonstrated [17]. The method by Mohney et al. [15] utilised the three-step transfer phenomenon of SLM vapour across the PDMS polymer, i.e. 1) dissolution of the molecules in the polymer, 2) diffusion of the molecules across the polymer under a concentration gradient, and 3) release of the vapour from the polymer at the opposite side of the membrane [18].

The approach described in [17] allows the non-invasive, repeated sampling of the root zone for lipophilic compounds. However, to study the chemical ecology of belowground ecological interactions using GC, coupled GC-mass spectrometry (GC-MS), coupled GC-electroantennography (GC-EAG) or bioassays, methanol is not a desirable solvent, because SLMs need to be extracted with a less-polar solvent from methanol extracts prior to analysis, and it is potentially toxic to test organisms. Here, we explore, for the first time, the use of PDMS tubing as a novel approach that enables isolation and biological studies to be rationalised through collection of re-usable biological samples directly analysable by GC-based methods, and without the need of a pump system. Diethyl ether is an established solvent for use in chemical ecology research to extract a range of polymers [5], hence is used in this study. We also determine some key sampling parameters in growth media spiked with synthetic standards, and in plant rhizosphere.

2 Materials and methods

2.1 Experiments with synthetic compounds

Synthetic compounds were selected based on their published bioactivity in the rhizosphere (see references in Table 1). All compounds were obtained from Sigma-Aldrich (Gillingham, UK).

Polydimethylsiloxane (PDMS) tubing (1 mm i.d. x 0.4 mm wall thickness) was obtained from VWR International Ltd (Lutterworth, UK). Prior to each experiment, PDMS tubing was cut into 28 cm pieces and cleaned either by soaking in acetonitrile:methanol 4:1 for 3 h [19] or in methanol for 24 h [15], and the cut lengths then placed in a glass vessel and baked in an oven at 180°C under nitrogen for 1.5 h.

Sand (0.25-0.71 mm grain size) or soil (pH 5.5-6.0; 75% medium-grade peat, 12% sterilised loam, 3% medium-grade vermiculite; 10% 5 mm lime-free grit; <2 mm particle size after sieving), heat-sterilised for 48 h in an oven at 80°C, was mixed with distilled water (pH 9) to reach the required level of moisture content (v/v). The middle 6 cm section of a PDMS tube was positioned on a ca. 1 cm thick bed of medium in a 250 mL glass beaker such that the rest of the tube ran alongside the opposite walls and a 1 cm piece of both the ends reached over the beaker rim. While holding the tube in place, the beaker was filled up completely with medium, which was then compacted with a metal spoon. Synthetic compounds were delivered into the medium in hexane (100 µL) by first making a 5 mm diam. hole into the medium with a glass rod, then inserting a glass micropipette (100 µL, BLAUBRAND® intraMARK, Wertheim, Germany) held inside the rod, down to the bottom of the hole and injecting the solution. After withdrawal of the delivery device, the hole was filled back with the medium and the experiment run at 20°C. It was expected that after solvent evaporation, the solutes vaporised in the test media.

At the end of each experiment, PDMS tubes were extracted with redistilled diethyl ether by inserting the narrow end of a Pasteur pipette (150 mm, Fisher Scientific UK Ltd, Loughborough, UK) into one end of the tube, placing the other end into a glass vial (1.1 mL, Thermo Fisher Scientific, Hemel Hempstead, UK) and administering diethyl ether into the Pasteur pipette. The remaining solvent in the tube was extruded using a ca. 1 mL bolus of air from a pipetting bulb.

The following experiments were repeated four-six times:
1) *Effect of sand moisture level (0, 5, 10, 15 and 20% v/v) on the recovery of (E)-caryophyllene and (-)-thujopsene*. Applied amount of each compound: 1 mg. Sampling duration: 2 h. Number of PDMS tubes/replicate: 1. Extraction: 1 x 1 mL diethyl ether/tube.
2) *Effect of soil moisture level*. As for experiment 1 but using soil instead of sand.
3) *Effect of sand moisture level (0, 5, 10, 15 and 20% v/v) on the recovery of allyl isothiocyanate, (RS)-limonene, methyl benzoate, (E)-2-nonenal, (S)-bornyl acetate, methyl eugenol, phenethyl isothiocyanate, β-ionone and (-)-caryophyllene oxide*. Applied amount of each compound: 1 mg. Sampling duration: 2 h. Number of PDMS tubes/replicate: 1. Extraction: 1 x 1 mL diethyl ether/tube. Analytes dissolve in the relatively large volume of PDMS (absorption); thus, there is little chance of competitive interactions among multiple metabolites [7].
4) *Effect of soil moisture level*. As for experiment 3 but using soil instead of sand.
5) *Effect of applied dose on the recovery of synthetic compounds in sand*. Moisture level: 15% (v/v). Applied amounts: 100 ng, 10 µg or 1 mg, with the highest dose still ca. two orders of magnitude lower than that used in similar studies [2]. Number of PDMS tubes/replicate: 1. Extraction: 1 x 1 mL diethyl ether/tube.
6) *Effect of applied dose on the recovery of synthetic compounds in soil*. As for experiment 5, but using soil instead of sand, and a moisture level of 5% (v/v).
7) *Effect of volume of solvent on the recovery of (E)-caryophyllene and (-)-thujopsene in sand*. Moisture level: 15% (v/v). Applied amount of each compound: 1 mg. Sampling duration: 2 h. Number of PDMS tubes/replicate: 1. Extraction: 1, 2, 3, 5 or 10 x 1 mL diethyl ether/tube.
8) *Effect of sorbent volume (total tube length) on the recovery of (E)-caryophyllene and (-)-thujopsene in sand*. Moisture level: 15% (v/v). Applied amount of each compound: 1 mg. Sampling duration: 2 h. Number of PDMS tubes: 1, 2 or 3. Extraction: 1 x 1 mL diethyl ether/tube.
9) *Effect of sampling duration on the recovery of (E)-caryophyllene and (-)-thujopsene in sand*. Moisture level: 15% (v/v). Applied amount of each compound: 1 mg. Sampling duration: 0.5, 2 or 4 h. Number of PDMS tubes/replicate: 1. Extraction: 1 mL diethyl ether/tube. Determination of sampling duration was based on [13].
10) *Effect of PDMS tube re-use on the recovery of (E)-caryophyllene and (-)-thujopsene in sand*. Moisture level: 15% (v/v). Applied amount of each compound: 1 mg. Sampling duration: 2 h. Number of PDMS tubes/replicate: 1. Extraction: 1 mL diethyl ether/tube. Each tube was used three times, with the cleaning procedure ([15]) undertaken between each use. Cleaned tubes, placed in empty glass beakers the way described above, were also extracted by pushing 1 mL diethyl ether through each of them to check for compound residues and thus for the evaluation of the efficiency of each cleaning process.

2.2 Experiments in plant rhizosphere

To test the efficacy of the PDMS tubing to extract SLMs from plant rhizosphere, a 130 cm piece of PDMS tube was coiled around a 19 cm long glass tube (5 mm diam.) bent into a U shape (8-3-8 cm sections). The increased sorption surface was used in anticipation of small amounts of SLMs, notably (*E*)-caryophyllene, being released from the roots of maize attacked by *Diabrotica v. virgifera* LeConte (Coleoptera: Chrysomelidae) larvae [4]. Larvae were collected in a field near Bonyhád, Hungary, by pulling out maize plants and inspecting the root ball for 2nd and 3rd instars. After transferring to the Rothamsted laboratory, 15-20 larvae were added to a 250 mL glass beaker filled with soil. Each beaker contained two corn seedlings. Larvae were kept under artificial light (15L:9D photoperiod) at 23°C, 60±10% RH, and watered daily. The probe was inserted into a 250 mL glass beaker with 15% moisture sand, into which a 16-day-old maize plant (cv Delprim), previously grown in soil, was transplanted. The treatment consisted of placing five 2nd instar larvae on top of the sand in each beaker, which eventually tunnelled into the medium. Non-infested plants served as control. After five days, the tubing was

extracted with 2 mL diethyl ether as above. Five replicates were done. To estimate volatile release/dry weight, maize roots were washed with distilled water, dried at 172 °C for 24 h and then weighed. n=4, mean (±SE) weight=0.21±0.02 g.

2.3 Sample analysis

Eluted diethyl ether samples were analysed using an Agilent 6890N GC equipped with a cool on-column injector, a flame ionization detector (FID), and either a 50 m × 0.32 mm i.d. non-polar HP-1 column (0.52 µm film thickness) or a 10 m × 0.53 mm i.d. HP-1 column (2.65 µm film thickness) (J & W Scientific, Folsom, CA, USA). The oven temperature was maintained at 30°C for 1 min, then programmed to increase at 5°C/min to 150°C, then held for 0.1 min, then programmed to increase at 10°C/min to 250°C and then held for 20 min. The carrier gas was hydrogen. The identity of peaks in PDMS extracts was confirmed by comparison of their gas chromatographic (GC) and mass spectrometric (GC-MS) properties with those of authentic standards, and by GC peak enhancement using authentic samples. GC-MS conditions: a Micromass Autospec Ultima magnetic sector mass spectrometer (Waters, Milford, MA) attached to an Agilent 6890N GC (fitted with a 50 m x 0.32 mm i.d. x 0.52 µm film thickness HP-1 column, J & W Scientific), and equipped with a cool-on-column injector. Ionization was by electron impact (70 eV, 220°C). The GC oven temperature was maintained at 30°C for 5 min and then programmed to increase at 5°C/min to 250°C, with a 70-min run time. A log-to-base 10 transformation was applied to the amount (µg) of compounds to account for some heterogeneity of variance over the treatments, with an adjustment of 0.001 to account for zero observations. ANOVA, providing an F-test for the overall difference between treatments, was followed by application of Fisher's least significant difference (LSD) test ($p<0.05$) for the statistical separation of means, or a two-sample t-test ($p<0.05$) for comparisons between mean amounts of two compounds. The Genstat (2015, 18th edition, VSN International 140 Ltd, Hemel Hempstead, UK) statistical package was used for the analysis. Estimation of quantities of compounds was achieved using the single-point external standard method with a series of C_7-C_{22} alkanes.

To determine the total amount of (*E*)-caryophyllene and (-)-thujopsene absorbed into the volume of PDMS, sand with 15% moisture content was spiked with 10 µg of each compound and left at 20°C for 2 h. The 28 cm long tube was then removed from the medium and sand particles were rinsed off with distilled water. The tube was cut up to four equal pieces and a piece was put into an empty Tenax tube (Sigma-Aldrich, Gillingham, UK) for immediate analysis, while the remaining pieces were sealed individually into glass ampoules under nitrogen and kept at -20°C until analysis. Tenax tubes were inserted into the OPTIC PTV unit of a GC (30->250°C ballistically at a rate of 16°C/s) equipped with a 50 m × 0.32 mm i.d. HP-1 column (0.52 µm film thickness) and FID, and with GC oven conditions as described above. Five replicates were done.

To determine expected recovery (extraction yield), the formula $\eta=1/((\beta/K_{ow})+1)$ was used, where η is the extraction yield (recovery), β is the phase ratio of the static extraction system and is defined as V_{medium}/V_{PDMS}, and K_{ow} is the octanol–water partition coefficient. β was calculated using the following parameters: V_{medium} = 250 mL, V_{PDMS} = [r (0.09 cm)^2 × π × length within medium (26 cm)]-[r of internal hole (0.05 cm)^2 × π × length within medium (26 cm)] = 0.46 mL. The log K_{ow} values were in part extracted from [20]: (*E*)-caryophyllene = 4.73, (-)-thujopsene = 6.12, methyl benzoate = 2.2, β-ionone = 4, methyl eugenol = 3.45, allyl isothiocyanate = 2.15, (*RS*)-limonene = 4.23, phenethyl isothiocyanate = 3.47, (-)-caryophyllene oxide = 3.94, (*S*)-bornyl acetate = 4.3, (*E*)-2-nonenal = 3.18.

3 Results

3.1 Experiments with synthetic compounds

The moisture content of sand had a significant effect on the recovery of (*E*)-caryophyllene and (-)-thujopsene, with higher moisture levels resulting in higher recovery rates (Fig. 1A). There was no difference in the extracted amounts between the two sesquiterpenes at any of the moisture levels (two-sample t-test, df=8; 0%: t=-1.54, p=0.163; 5%: t=-1.55, p=0.159; 10%: t=-1.20, p=0.265; 15%: t=-1.68, p=0.132; 20%: t=-0.45, p=0.662). The other synthetic compounds in sand all showed a moisture-dependent recovery pattern similar to that observed for the two sesquiterpenes (Fig. 3A). The amounts of synthetic compounds in PDMS extracts was also moisture-dependent (Table 2).

In soil, more (*E*)-caryophyllene and (-)-thujopsene (Fig. 1B), as well as methyl benzoate, (*E*)-2-nonenal and (*S*)-bornyl acetate (Fig. 3B), were collected at the two lowest (0 and 5%) moisture levels, although the increase was not significant. However, there was a significant increase at 0 and 5% for methyl eugenol, phenethyl isothiocyanate and β-ionone (Fig. 3B). (*RS*)-Limonene showed the highest, although not significant, recovery level at 5%, and (-)-caryophyllene oxide at 0 and 20%, whereas the recovery pattern for allyl isothiocyanate resembled that measured in sand (Fig. 3B). The relative abundance of recaptured compounds in PDMS extracts was moisture-dependent (Table 2).

The amount of (*E*)-caryophyllene and (-)-thujopsene collected from the PDMS tubes in sand increased with increasing solvent volume, sorbent volume and sampling time (Fig. 1C, D and E, respectively).

The dose of synthetic compound used per experiment had a profound effect on compound recovery (Table 3). At the highest dose (1 mg), all compounds were detected in PDMS extracts from both sand and soil. In sand, all constituents of PDMS extracts produced a detectable FID signal even at the lowest applied dose (100 ng), except (-)-caryophyllene oxide; in soil, however, only (-)-thujopsene could be detected at this dose. Also, at the 10 µg applied dose in soil, only (-)-thujopsene, (*E*)-caryophyllene and (*RS*)-limonene could be detected by GC in the PDMS extracts.

There was no significant loss of recovery of (*E*)-caryophyllene and (-)-thujopsene after three repeated uses of the same PDMS tube (ANOVA, d.f.=2, p=0.51) (Fig. 1F). Neither compound could be detected in extracts prepared from cleaned tubes.

The predicted recovery (η) values were as follows: (*E*)-caryophyllene = 0.0086, (-)-thujopsene = 0.0111, methyl benzoate = 0.0040, β-ionone = 0.0073, (*E*)-2-nonenal = 0.0058, methyl eugenol = 0.0063, allyl isothiocyanate = 0.0039, (*RS*)-limonene = 0.0077, phenethyl isothiocyanate = 0.0063, (-)-caryophyllene oxide = 0.0072, (*S*)-bornyl acetate = 0.0079.

Increasing the moisture level to 5% in sand caused the η for (*E*)-caryophyllene and (-)-thujopsene to exceed their expected values (Fig. 2A). This was also true for all the other compounds, except β-ionone and (-)-caryophyllene oxide, which only exceeded their expected recovery values at 10% or above (Fig. 4). In soil, only (*RS*)-limonene at 5 and 10% and methyl benzoate at 5% moisture level exceeded the expected recovery values (data not shown).

Of the 10 µg (*E*)-caryophyllene and (-)-thujopsene injected into the sand, in total 1.568±0.166 and 1.8±0.214 µg, respectively, could be detected in the PDMS polymer by GC thermal desorption, equalling to 15.7 and 18% recovery (extraction yield).

The cleaning procedure affected the level of contamination present in silicon tube extracts, the methanol soaking [15] resulting in smaller contaminant peaks in extracts prepared following the cleaning procedure (Fig. 5).

3.2 Experiments in plant rhizosphere

An average amount of 1.38±0.24 ng (*E*)-caryophyllene was recovered from the rhizosphere of maize infested with *D. virgifera virgifera* larvae, whereas this compound was not observed in control samples. Accounting for the expected recovery of (*E*)-caryophyllene from 15% moisture sand and correcting with tube length, the estimated average release of the compound from damaged maize roots over the experiment was 78.2 ng/g dry weight.

4 Discussion

The development of *in situ*, non-invasive sampling methods to study chemical processes in the rhizosphere is key to understand ecological interactions among soil-dwelling organisms, and between them and non-living matter. Such complex interactions shape how healthy ecosystems function and how crop plants perform [21]. Here, we report on the assessment of a range of parameters for a technique with the potential to be developed into low-cost non-invasive probes that sample the rhizosphere to uncover the chemical drivers of crop-pest ecology and allelopathy [22].

As PDMS has a hydrophobic surface, lipophilic organic solvents diffuse into the polymer causing swelling. The swelling ratio for diethyl ether was reported to be 1.38 after 24 h soaking, during which time it has reached an equilibrium [23], whereas we measured this ratio to be only 1.07 after a short (~10 s) elution with a 1 mL plug of diethyl ether. According to Lee at al. [23], solvents that cause significant swelling (swelling ratio >1.28) extract organic compounds from the PDMS with high efficacy, and the 1.07 value compares only with solvents of lower extraction ability. The amount of volatiles extractable from the polymer might be increased if the solvent plug is retained inside the tubing for longer, i.e. by slowing down its flow-through rate with a glass narrowing attached to one end, as test compounds had likely reached equilibrium concentrations by the end of an average 2 h experiment [13]. However, our pilot experiments suggest that 0.5 mL diethyl ether in a 50 cm long PDMS tube completely evaporates within 5.96±0.07 min. Extraction yield of absorbed compounds could be increased either by using larger volumes of solvent or extending the extraction time; a syringe pump system used in [17] aimed to account for the latter. A first-order one-compartment model [18] outlines the analyte mass accumulation within the PDMS as a function of time. The first of the three distinguished phases is the linear region, where the analyte mass collected is directly proportional to its concentration in the sample and the time for which the polymer is exposed to the sample. The relationship between extraction yield and sampling duration in this study shows a strong resemblance to the linear region of the model, indicating that equilibrium has not been reached and recovery can be further increased by increasing the sampling time.

A similar relationship can be modelled between extraction yield and volume of solvent used to extract the analytes, which appears to arrive at the equilibrium phase only at 10 × solvent volume, i.e. from a 2.17 to a 21.7 $V_{solvent}/V_{sorbent}$ ratio. Sorbent volume, on the other hand, can be further increased, as it shows a linear relationship with recovery within the parameter values tested. High sorbent:medium ratio was used in [15] to extract thiophenes from soil. The same authors could reach ca. 10% recovery after 24 h incubation from sand spiked with a synthetic thiophene.

Recovery can also be influenced by the diffusion rate of test compounds both in the polymer and the media. The analyte concentration on the surface of the sorbent exposed to the sample is higher than on the other surface, and the difference depends on the partition coefficient and diffusion rate [18], [24], influenced by sorbent chemical composition and wall thickness (silicon microtubing is available in different wall thicknesses). (*RS*)-Limonene was the most abundant analyte in the PDMS extracts [ca. 4 × the amount of (*E*)-caryophyllene and (-)-thujopsene] in this study, which might be due to its lower molecular weight and thus higher volatility than that of the two sesquiterpenes (MW 136 vs 204, resp.). All the other test compounds contained at least one heteroatom, which makes them more polar, thus less able to dissolve into the PDMS matrix, hence their lower recovery rate compared to

(*RS*)-limonene (Fig. 6). Certainly, this might have reduced the extraction yield of allyl isothiocyanate, methyl benzoate and (*E*)-2-nonenal, all with the same or similar MW as (*RS*)-limonene, whereas larger molecules may simply diffuse more slowly, hence their relatively lower recovery. Methyl eugenol, despite its similar mass compared to, e.g., (*S*)-bornyl acetate, produced lower extraction yield at certain moisture levels, which could be due to decomposition during diffusion from the source to the polymer [2], [25], or solubilisation into the soil solution [26], which might stand for some of the other oxigenated chemicals [27]. Degradation of linalool in sand medium is influenced by pH conditions [2], acidic, but not basic, environments facilitating it. It can thus be speculated that the use of pH 9 water in this study had little effect on the chemical properties of analytes, but the impact of pH on the fate of soil SLMs needs to be investigated in the future.

The amount of (*E*)-caryophyllene measured from infested maize root tissue of the same cultivar used by Rasmann et al. [4] was around 6 ng/h, whereas what was actually released into the rhizosphere remains unknown. We attempted to measure levels of this compound directly from the growth substrate to get a more realistic view of the plant`s response to root damage by a specialist herbivore. To maximise absorption, based on previous experiments with synthetic (*E*)-caryophyllene, sand at 15% moisture level was used, and relatively low, but significant amounts were extracted from the medium with the tubing. Using dynamic headspace sampling, maize (cv. Delprim) root (*E*)-caryophyllene emissions were ca. 2.3 ng/h [28]. Other plants have also been shown to release (*E*)-caryophyllene from their roots in varying amounts: *Centaurea stoebe* L. (Asteraceae), for example, emits ca. 3 µg/g dry weight/h [29].

Although adsorption of SLMs onto colloids within the media could slow down or stop both vertical and horizontal diffusion [30], higher water levels can decrease their adsorption onto substrate particles [3]. This may explain the higher general compound recovery in higher moisture sand, but not in soil. In the latter, increasing moisture content decreased extraction yields, which might be accounted for by the observation that adsorption of SLMs in the presence of water is a phenomenon dependent on the mineralogical composition of the substrate (e.g. soil colloids) [3], or the presence of natural surfactants that assist in transporting relatively water-insoluble SLMs into the soil via micellization [31]. Certain SLMs were also found to diffuse further in sand at higher moisture levels, which contributed to their higher recovery on the sampling device deploying Tenax polymer [2]. However, the opposite trend was found with (*E*)-caryophyllene, arguably because low moisture levels enhance the horizontal diffusion of this lipophilic molecule in the gaseous phase, the extent of which is reduced with increasing moisture content [30]. Our contrasting results might be explained by the negative effect of substrate water content on molecular adsorption onto particles, e.g. silicates in sand [3], [32]. The dramatic increase in absorption into the PDMS polymer of all test compounds from 0 to 5% moisture level in sand can be attributed to the decrease in vertical diffusion of molecules due to a thin layer of water slowing down evaporation into the aboveground atmosphere [30]. Although water that covers pores slows down volatile diffusion markedly in substrates as compared to air [33], the usual sampling duration in our experiments (2 h) could allow enough time for the synthetic compounds to diffuse through >3.5 cm wet sand before reaching the PDMS polymer. The highest (*E*)-caryophyllene concentration in Som et al. experiments was measured 13.5 cm from the source (5 mg released) after 18 h, the recovery from the probe with Tenax polymer steadily increasing up to this point [2]. Our experiments also point at the positive effect of sampling duration on extraction yield.

The recovery of test compounds in soil was significantly lower than in sand, which could be because of 1) the more heterogenous composition of soil that may have retained the diffusing molecules by sorbing them into particles of organic matter more intensively as the moisture level rose [26], [32], or 2) the less constant porosity of the soil medium, creating air spaces of different size, density and distribution, and thereby affecting the diffusion of molecules. In the rhizosphere, however, the diffusion and fate of SLMs is not only affected by their interactions with moisture, soil colloids and

minerals, but also with microorganisms such as fungi and bacteria [34], [35]. *Fusarium* spp. (Ascomycota: Nectriaceae) were shown to metabolize and transform soil volatiles and speculated that these compounds might also be adsorbed onto fungal hyphae by yet unknown mechanisms, thereby acting as a possible sink for bioactive volatiles [34]. Weidenhamer et al. [17] also emphasize the nature of heterogeneity in allelochemical concentrations in the soil substrate, with less extent of degradation of them by microorganisms when released at high doses. This sets a challenge for the development of non-invasive, *in situ* soil probes that need to accommodate the influence of the dynamically changing abiotic and biotic environment on their sampling capability, by creating a patchy distribution of SLMs.

Our results indicate that diethyl ether extraction of PDMS tubes can produce biological samples suitable for multiple analysis by GC and GC-MS, and consequently also for direct use in behavioural and electrophysiological assays. As the solvent evaporates quickly, it is not likely to exert major (if any) negative effects on test organisms, and owing to the clean-up procedure prior to sampling, contaminants are less likely to interfere with chromatographic and biological signals. Sampling efficiency can be improved by increasing sorbent and solvent volume, and sampling time. The choice of experimental media (composition, texture, structure) in lab assays has a significant influence on the extraction yield. In summary, the described extraction method may lead to the development of *in situ*, non-invasive probes capable of repeated field sampling, which can be left in place after installation and without the need for a pump system (see [17]). Optimisation processes will need to consider characteristics of the media, such as porosity, moisture level, pH or temperature, as their combined effect under field conditions is expected to influence the enrichment of analytes from rhizodeposits into the PDMS polymer. It will also be possible to obtain information about the spatial distribution of soil SLMs by using a network of PDMS probes. However, it must be noted that such probes will only provide snapshots of the temporal dynamics of rhizosphere natural products, the resolution of which depending on sampling duration and hence analyte accumulation in the polymer [36].

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Additional information

The raw dataset is available at https://doi.org/10.6084/m9.figshare.12248921.v1.

Author statement

József Vuts: Conceptualization, Investigation, Methodology, Data curation, Project administration, Supervision, Roles/Writing - original draft. **Diego M. Magalhães**: Investigation, Methodology, Data curation. **Ariane L. Soares**: Investigation, Methodology. **Akash A. W. Ratnayaka**: Investigation, Methodology. **John C. Caulfield**: Data curation, Formal analysis, Validation. **Michael A. Birkett**: Conceptualization, Methodology, Supervision, Funding acquisition, Resources, Writing - review & editing.

compound	purity (%)	KI*	MW	vapour pressure (kPa, 25°C)	reference
allyl isothiocyanate	95	855	99	0.670	37
(*RS*)-limonene	97	1027	136	0.207	37
methyl benzoate	99	1073	136	0.051	37
(*E*)-2-nonenal	97	1139	140	0.034	37
(*S*)-bornyl acetate	95	1276	196	0.030	37
methyl eugenol	98	1374	178	0.004	37
phenethyl isothiocyanate	99	1428	163	0.670	37
(*E*)-caryophyllene	98.5	1430	204	0.002	4
(-)-thujopsene	97	1444	204	0.003	38
β-ionone	96	1471	192	0.007	37
(-)-caryophyllene oxide	95	1584	220	0.0009	37

*on a HP-1 non-polar column

Table 1. Synthetic compounds used in the PDMS experiments. KI=Kováts index, MW=molecular weight

Compound	Substrate moisture level (v/v %)									
	0		5		10		15		20	
	Sand	soil	sand	soil	Sand	soil	sand	soil	sand	soil
	p value									
	0.218	0.528	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	0.021
allyl isothiocyanate	a	a	cd	b	c	c	abc	bc	a	ab
(*RS*)-limonene	a	a	d	b	d	c	d	c	b	c
methyl benzoate	a	a	cd	b	cd	b	cd	ab	a	ab
(*E*)-2-nonenal	a	a	bc	b	bc	ab	bc	ab	a	b
(*S*)-bornyl acetate	a	a	abc	b	bc	ab	bc	ab	a	b
methyl eugenol	a	a	a	b	a	a	a	a	a	a
phenethyl isothiocyanate	a	a	ab	b	ab	a	abc	a	a	ab
β-ionone	a	a	a	b	a	a	ab	a	a	ab
(-)-caryophyllene oxide	a	a	a	a	ab	a	abc	a	a	ab

Table 2. Comparison between the amounts of synthetic compounds collected by PDMS tubes at each applied moisture level in sand and in soil. Compounds with the same letters in each column are not significantly different (ANOVA, Fisher`s protected least significance test, $p<0.05$)

compound	Dose					
	100 ng		10 µg		1 mg	
	Sand	soil	sand	soil	sand	soil
(*E*)-caryophyllene	0.041±0.013	0.0±0.0	1.377±0.387	0.024±0.014	24.084±1.088	1.830±1.099
(-)-thujopsene	0.041±0.014	0.002±0.002	0.975±0.272	0.025±0.014	28.206±1.472	1.946±1.162
allyl isothiocyanate	0.021±0.008	0.0±0.0	0.329±0.079	0.0±0.0	26.39±2.943	3.235±0.426
(*RS*)-limonene	0.117±0.029	0.0±0.0	0.961±0.186	0.22±0.101	72.395±7.363	12.358±1.33
methyl benzoate	0.078±0.021	0.0±0.0	0.41±0.108	0.0±0.0	32.852±5.182	1.404±0.752
(*E*)-2-nonenal	0.033±0.014	0.0±0.0	0.25±0.733	0.0±0.0	25.266±3.334	0.705±0.392
(*S*)-bornyl acetate	0.04±0.012	0.0±0.0	0.239±0.598	0.0±0.0	23.289±2.753	1.596±0.709
methyl eugenol	0.008±0.005	0.0±0.0	0.027±0.027	0.0±0.0	9.449±1.847	0.036±0.016
phenethyl isothiocyanate	0.045±0.019	0.0±0.0	0.256±0.082	0.0±0.0	15.798±2.442	0.045±0.019
β-ionone	0.007±0.004	0.0±0.0	0.025±0.025	0.0±0.0	11.535±2.442	0.062±0.021
(-)-caryophyllene oxide	0.0±0.0	0.0±0.0	0.016±0.016	0.0±0.0	12.785±2.876	0.118±0.017

Table 3. The effect of applied dose on the recovery of synthetic compounds (mean µg ±SE). The length of individual PDMS tubes was 28 cm, and each experiment was conducted in 250 mL glass beakers in five replicates. Sand at 15% and soil at 5% moisture level (v/v) was used, the sampling lasted for 2 h at 20°C, and each tube was rinsed with 1 mL diethyl ether.

Figure legends

Fig. 1. The effect of different factors on the recovery of (*E*)-caryophyllene (black bars) and (-)-thujopsene (grey bars) in sand and soil. The length of individual PDMS tubes was 28 cm, and each experiment was conducted in 250 mL glass beakers in five replicates at 20°C. A) Sand moisture level. B) Soil moisture level. C) Solvent volume. D) Sorbent volume (total tube length). E) Sampling duration. F) Repeated use. Columns with the same letter within one diagram are not significantly different at α=0.05, ANOVA. ns=not significant

Fig. 2. Recovery (extraction yield, η) of (*E*)-caryophyllene and (-)-thujopsene in sand (A) and soil (B) as a function of substrate moisture level. The grey straight lines are the expected η for (*E*)-caryophyllene and (-)-thujopsene.

Fig. 3. The effect of moisture content of medium (v/v%) on the recovery of synthetic compounds. See Fig. 1. for experimental conditions. A: sand experiment, B: soil experiment. Columns with the same letters are not significantly different for each compound (ANOVA, LSD, $p<0.05$). ANOVA p values A: allyl isothiocyanate 0.006, (*RS*)-limonene 0.004, methyl benzoate <0.001, (*E*)-2-nonenal <0.001, (*S*)-bornyl acetate <0.001, methyl eugenol <0.001, phenethyl isothiocyanate <0.001, β-ionone <0.001, (-)-caryophyllene oxide <0.001; ANOVA p values B: allyl isothiocyanate 0.399, (*RS*)-limonene 0.167, methyl benzoate 0.180, (*E*)-2-nonenal 0.156, (*S*)-bornyl acetate 0.131, methyl eugenol <0.001, phenethyl isothiocyanate 0.005, β-ionone <0.001, (-)-caryophyllene oxide 0.183

Fig. 4. Recovery (extraction yield, η) of nine synthetic compounds in sand. The grey solid line is the expected η for each compound.

Fig. 5. Representative GC chromatograms of PDMS diethyl ether extracts immediately after cleaning by soaking in either 4:1 acetonitrile:methanol for 3 h (upper trace) or methanol for 24 h (lower trace). n=3

Fig. 6. Relationship between molecular weight, K_{ow} (octanol–water partition coefficient), PSA (polar surface area) and recovery. The compounds along the horizontal axis are in ascending order of molecular weight from left to right (MW 99 to 220).

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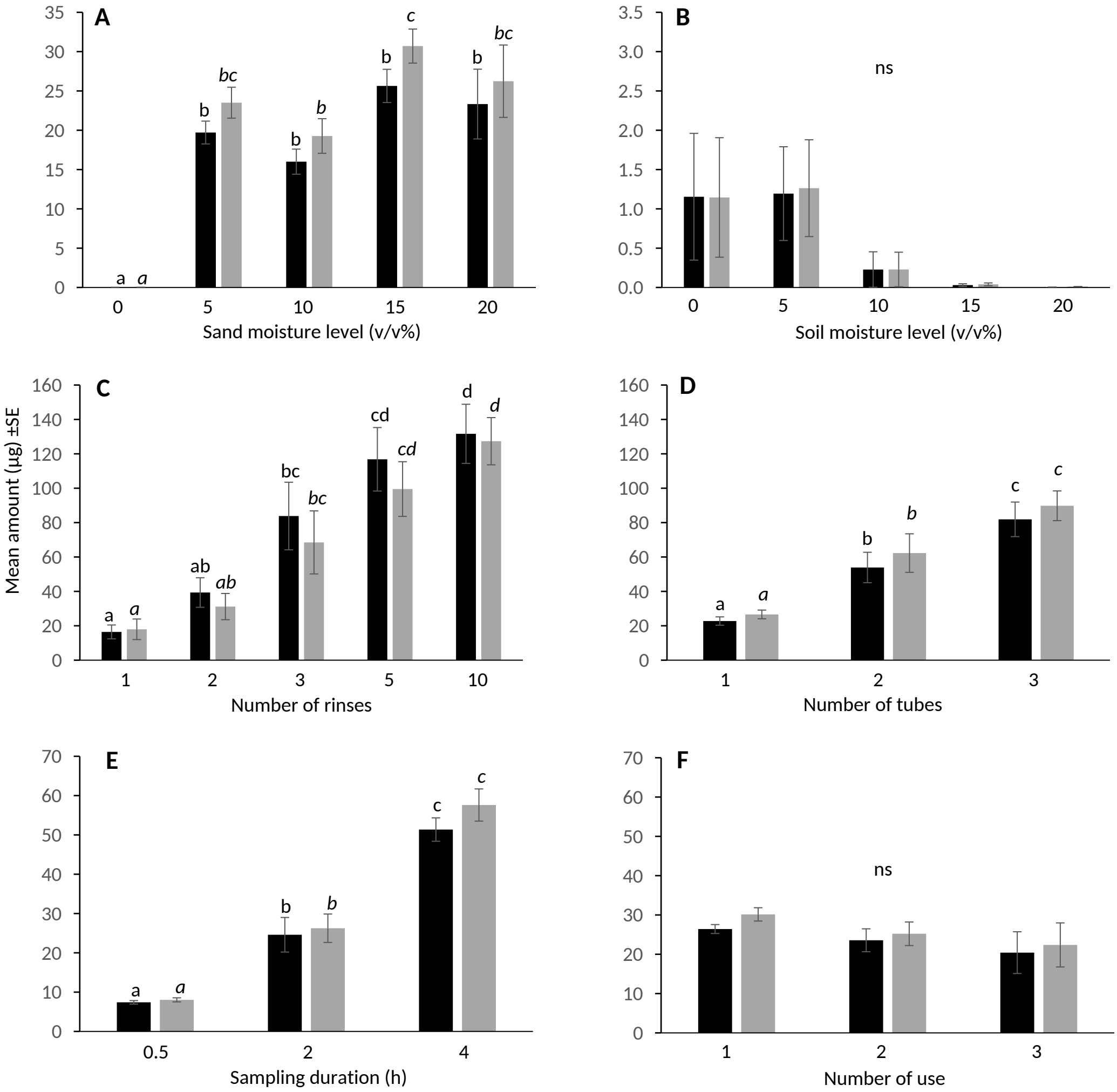
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Sand moisture level (v/v%)
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Fig. 1

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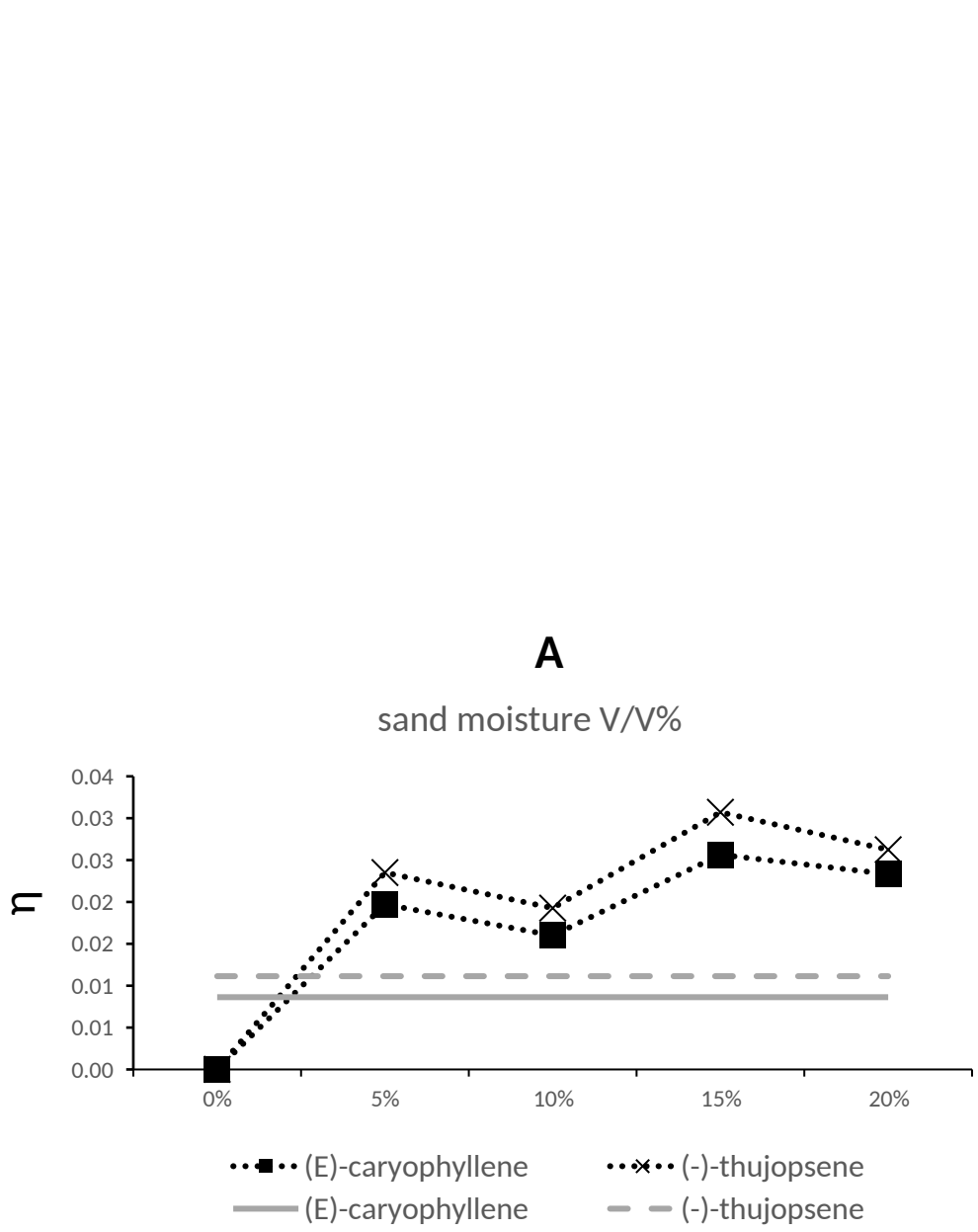
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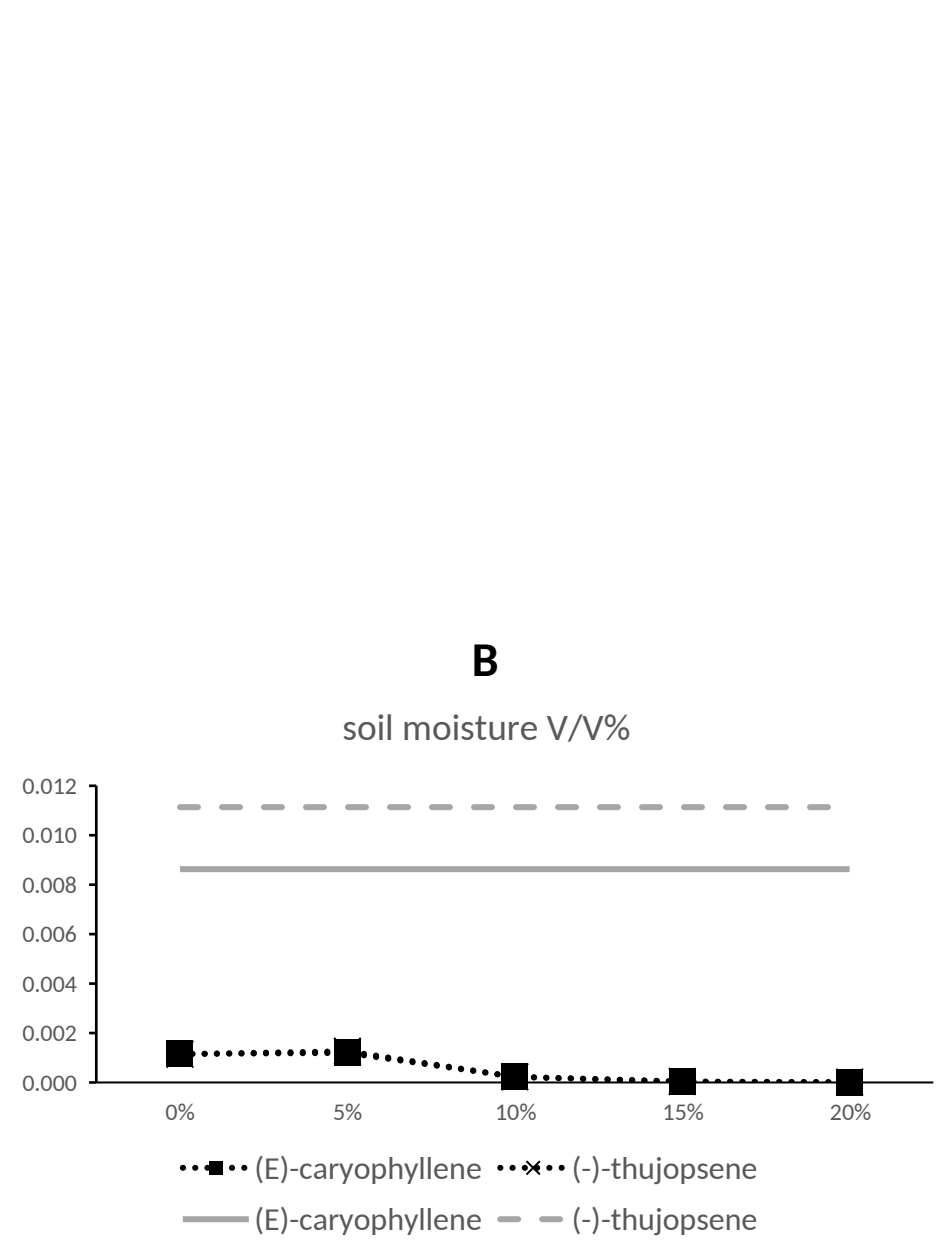
0.03
0.03
0.02
0.02
0.01
0.01
0.00
0%
5%
10%
15%
20%
(E)-caryophyllene
(-)-thujopsene
(E)-caryophyllene
(-)-thujopsene

B


soil moisture V/V%
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0%
5%
10%
15%
20%
(E)-caryophyllene
(-)-thujopsene
(E)-caryophyllene
(-)-thujopsene

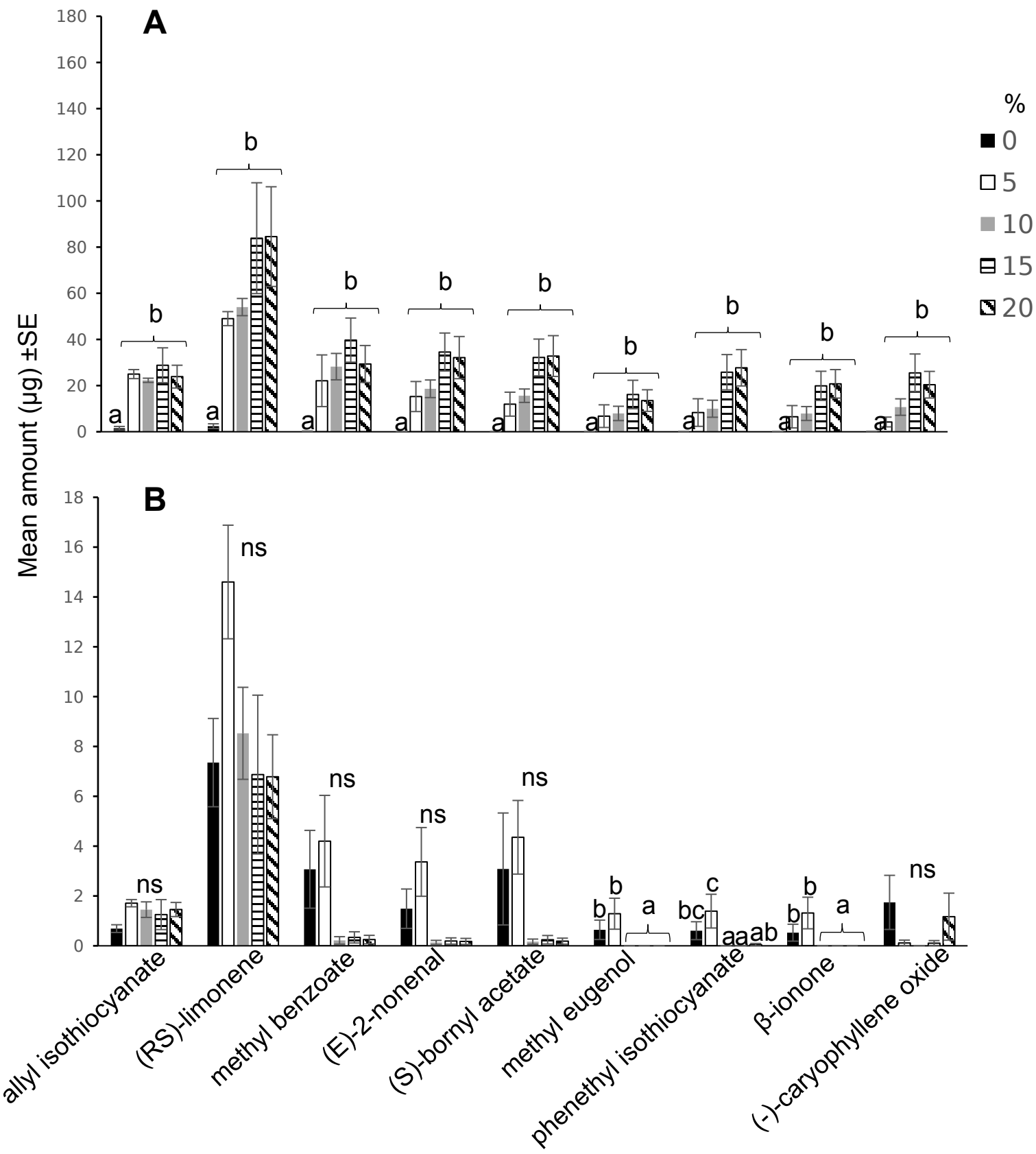
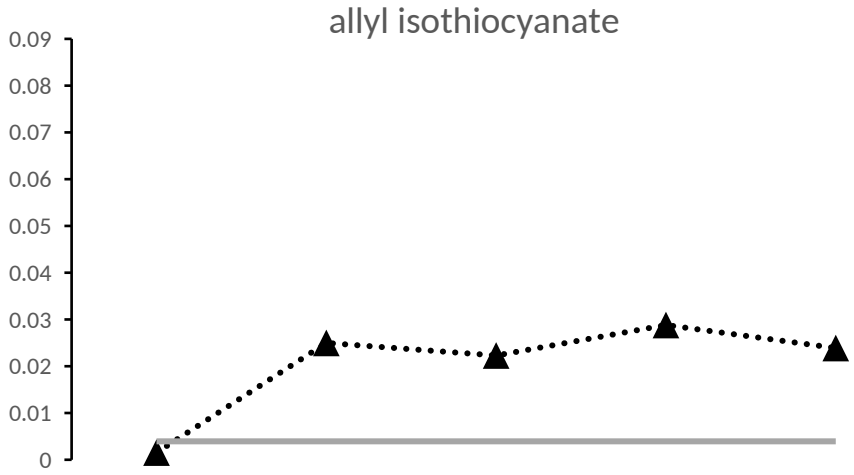
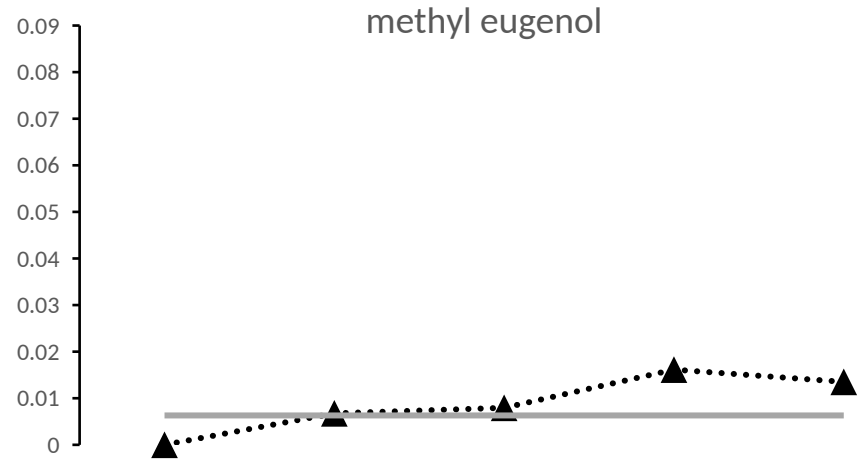

A
B
Mean amount (µg) ±SE
%
0
5
10
15
20
a
b
ns
allyl isothiocyanate
(RS)-limonene
methyl benzoate
(E)-2-nonenal
(S)-bornyl acetate
methyl eugenol
phenethyl isothiocyanate
β-ionone
(-)-caryophyllene oxide

Fig. 2

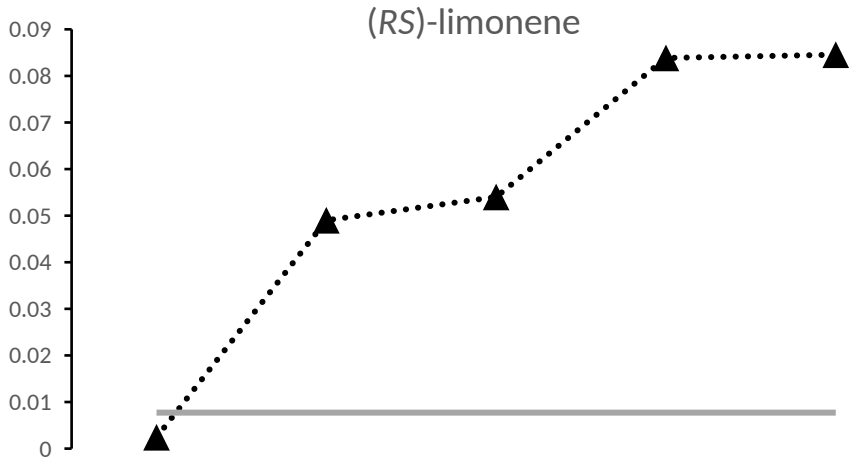
allyl isothiocyanate



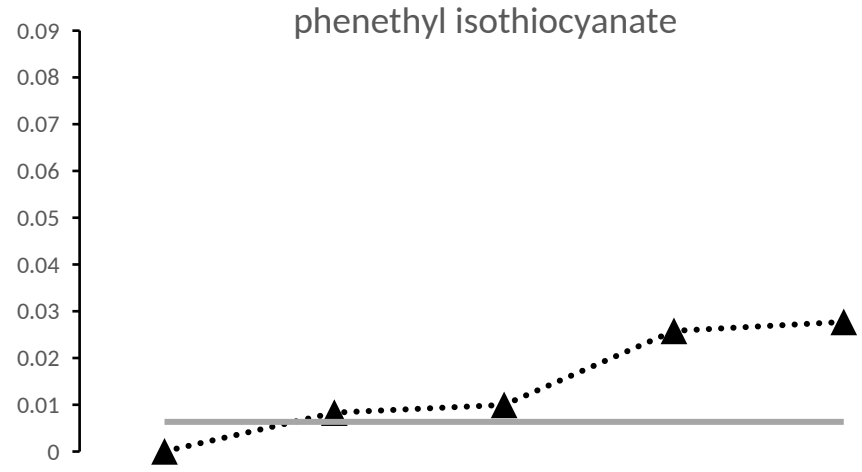
methyl eugenol



(*RS*)-limonene

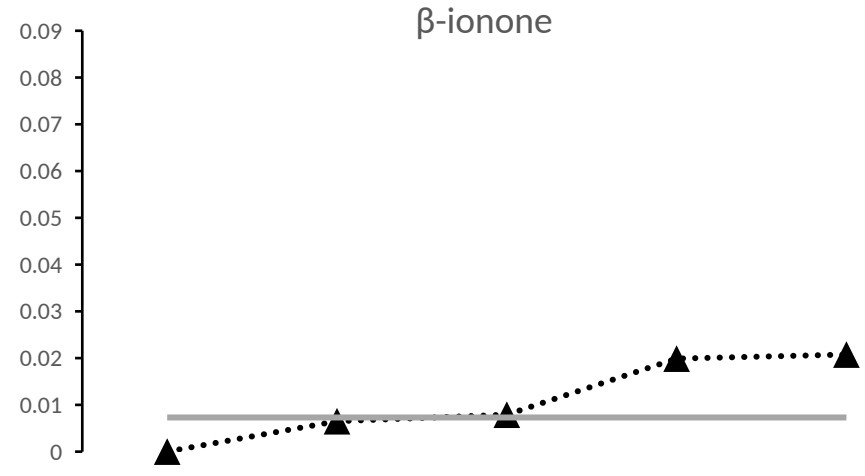


phenethyl isothiocyanate

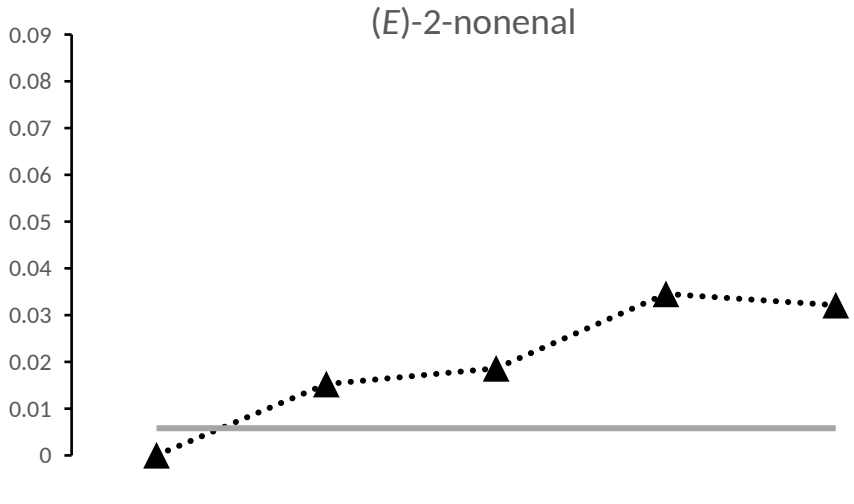


methyl benzoate

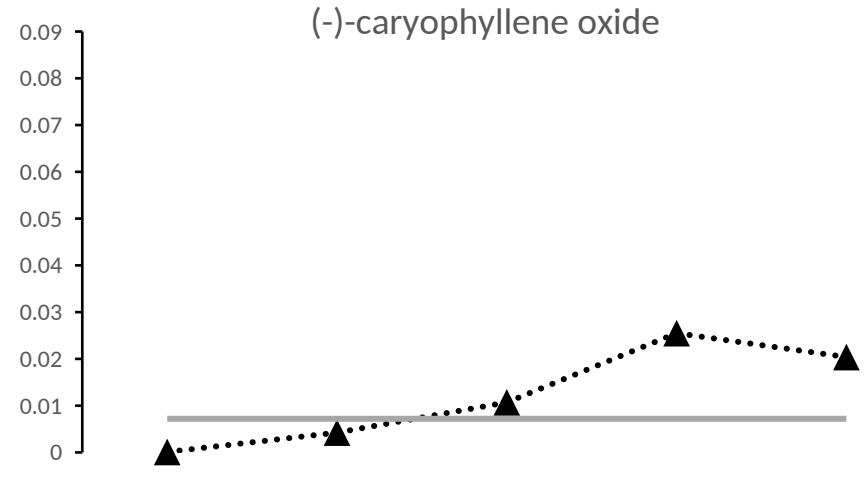
β-ionone



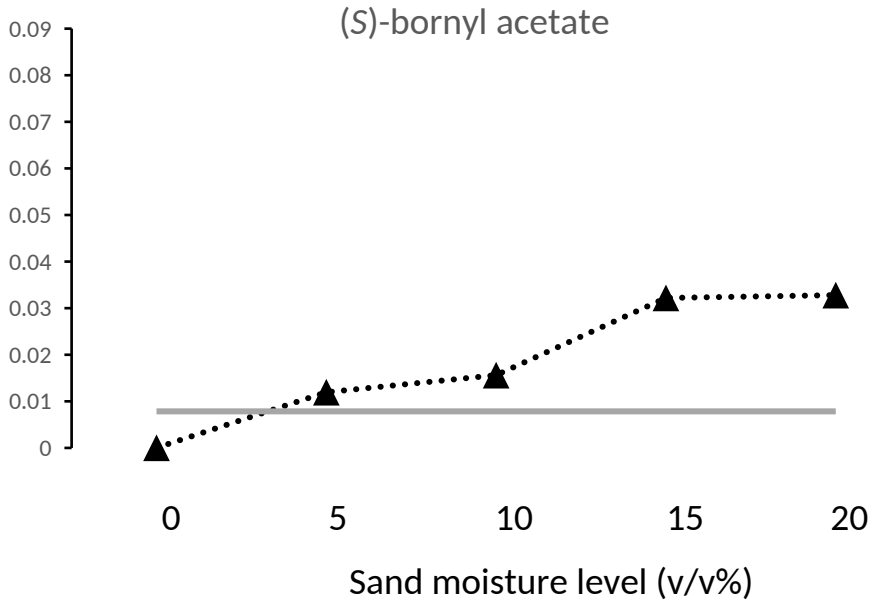
(*E*)-2-nonenal



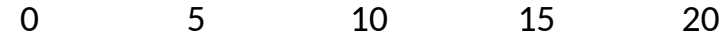
(-)-caryophyllene oxide

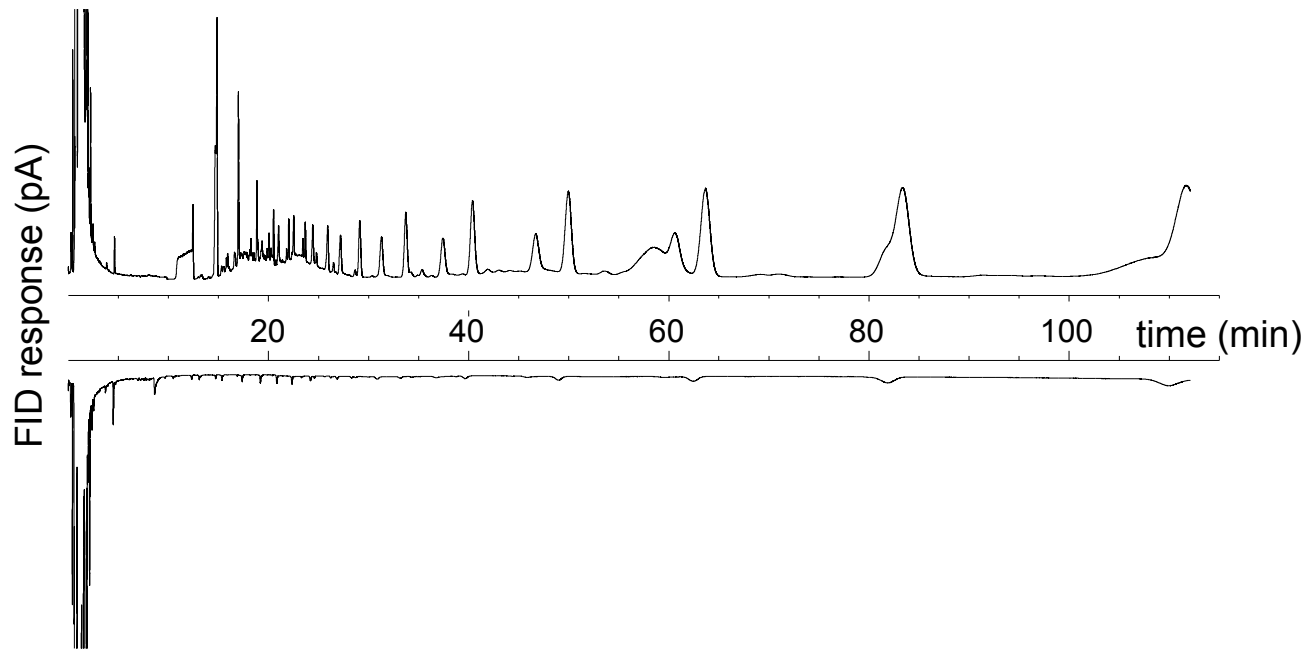


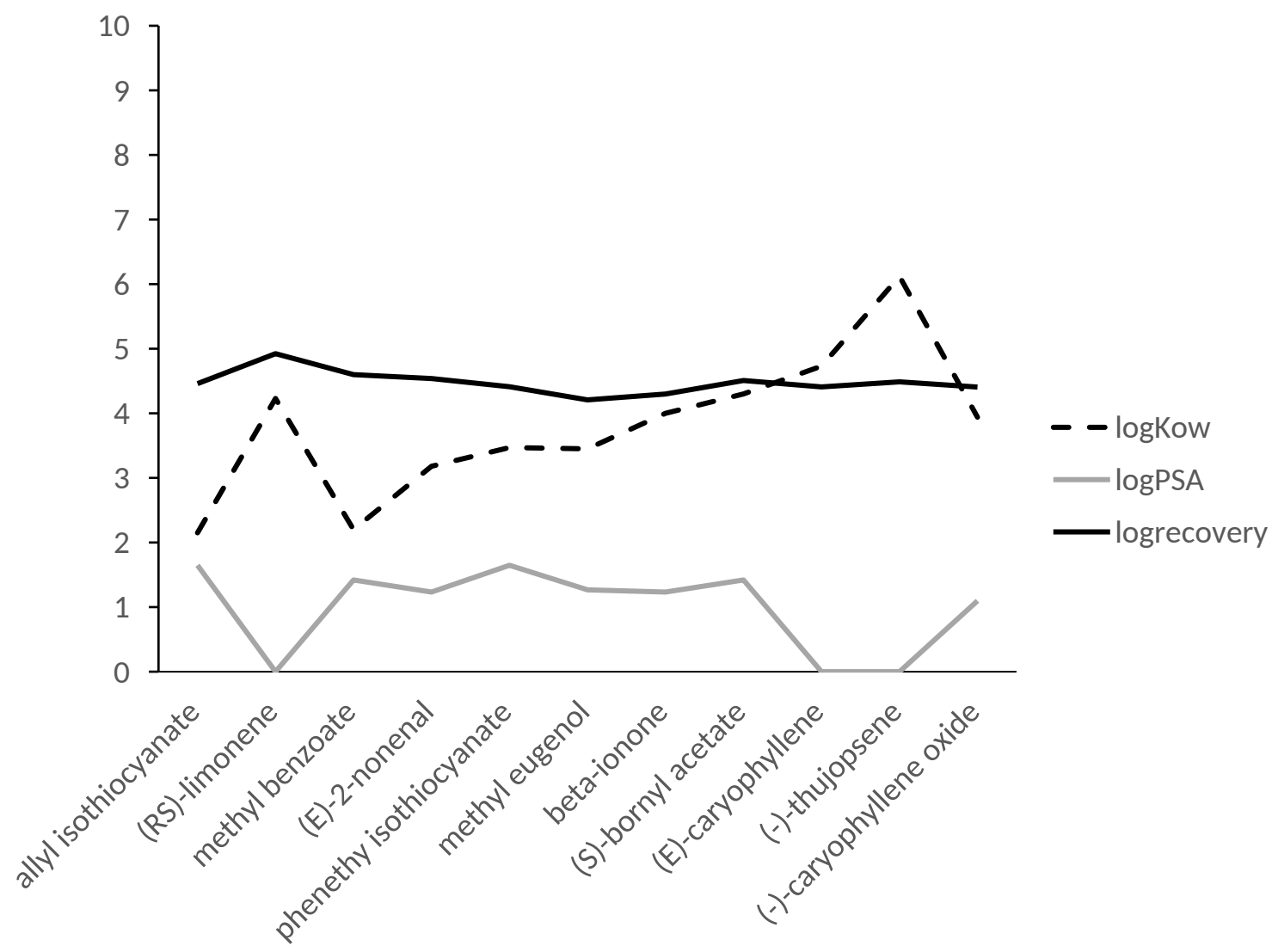
(*S*)-bornyl acetate



η




FID response (pA)
20
40
60
80
100
time (min)


10
9
8
7
6
5
4
3
2
1
0
logKow
logPSA
logrecovery
allyl isothiocyanate
(RS)-limonene
methyl benzoate
(E)-2-nonenal
phenethy isothiocyanate
methyl eugenol
beta-ionone
(S)-bornyl acetate
(E)-caryophyllene
(-)-thujopsene
(-)-caryophyllene oxide

Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: