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## Article

# Identification of Bioactive Plant Volatiles for the Carob Moth by Means of GC-EAD and GC-Orbitrap MS

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**Abstract:** The aim of this study was to validate a workflow that allows structural identification of plant volatiles that induce a behavioral response in insects. Due to the complexity of plant volatile emissions and the low levels at which these bioactive components tend to occur, gas chromatography-electroantennography (GC-EAD) was applied as the prime differentiator technique, i.e., to indicate particular peaks of interest in the chromatogram. In a next step, the analysis was repeated under identical conditions using GC-Orbitrap mass spectrometry (MS). Combining electron impact (EI) ionization and chemical ionization (CI) with the superior spectral resolution and mass accuracy of the technique enabled straightforward identification of these unknowns, with high confidence in a minute amount of time. Moreover, because of the intrinsic sensitivity of the technique, components that occur at trace amounts but may induce disproportional large behavioral responses are evenly well-identified. We were able to positively identify  $\beta$ -caryophyllene as a bioactive compound in female carob moths. Behavioral attraction was negatively correlated with the amount of  $\beta$ -caryophyllene in host fruits. In an oviposition experiment on filter paper,  $\beta$ -caryophyllene was stimulated in the range of 40–100 ng, while concentrations above 200 ng inhibited oviposition.

**Keywords:** unknown unknowns; GC-Orbitrap MS; carob moth; biomarkers;  $\beta$ -caryophyllene; GC-EAD; attraction; oviposition



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## 1. Introduction

Insect pest are responsible for up to 20% of crop losses worldwide [1], and losses are projected to increase by 10% to 25% per degree of global mean surface warming [2]. However, as the effects of the ecological and climate change crises become more apparent by the day [3,4], use of pesticides is an increasingly untenable solution [5], and we are still looking for sustainable alternatives of crop protection. Semiochemical-based behavioral manipulation methods have therefore been increasingly implemented in pest management [6,7]. In moth pests, for instance, long-range female sex pheromones are well-established for monitoring, mass-trapping, and mating disruption [6]. However, female sex pheromones only affect males, while females play the most prominent role in population dynamics [8]. Behavioral manipulation methods will thus be more efficient if they target not only males but also females [9]. For the development of such new strategies, knowledge of biological active compounds is essential. However, natural stimuli such as host plant odors are often complex mixtures containing hundreds of compounds. Finding and identifying those that

are biologically active is difficult and time-consuming. Here, we investigate a combination of electrophysiological, analytical, and behavioral techniques with the aim to improve the efficiency of this process.

Both female and male moths perceive host plant volatiles via specialized olfactory receptor neurons, and use this information to discriminate between suitable and unsuitable food sources, rendezvous sites for mating, or larval food plants [10–13]. These sensory systems, often both highly selective and extremely sensitive, can be used to quickly screen complex odor mixtures. Signals from receptor neurons are recorded in real time by sending the effluent from a classic gas chromatography (GC) analysis over the antenna of a living insect, such that bioactive signals are unambiguously detected. The setup is called GC-EAD (GC-electroantennographic detection) [14].

This quick screening can effectively be combined with advanced mass spectrometry (Orbitrap MS). Since its commercialization in 2015, GC-Orbitrap MS has revolutionized the identification of unknown unknowns in various application domains. The particular detection principle of the instrument, in which mass fragments are detected at high resolution in an orbital ion trap, allows to achieve unmatched spectral resolution and mass accuracies. In untargeted mode, the technique has been applied in food analysis [15], environmental analysis [16], metabolomics [17], exposomics [18], etc. In the present study, we applied GC-EAD combined with GC-Orbitrap MS for the first time for in situ identification of volatile components that evoke electrophysiological and behavioral responses in carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae). This species is a destructive polyphagous pest that attacks the fruits of a variety of commercial produce, such as pomegranate, dates, and pistachio, both pre- and post-harvest. It is recognized as the most important pest in the date industry in the United States [19,20], a key pest of almond in Australia [21], and frequently a problem in stored fruits and nuts in Europe [22]. In the Middle East, *E. ceratoniae* is the most damaging pest of pomegranate, *Punica granatum* L. (Lythraceae), in almost all pomegranate production areas, and can cause 30–80% yield loss [23–27]. Pomegranate, its main host in the Middle East, continuously flowers during the growing season, and different phenological stages, from fresh flowers to mature fruits, are present when carob moths are active. In the Middle East, carob moth activity starts mid-season (June–August), when most pomegranate fruits are of mature size [25,27,28] and peels of many fruits are naturally cracking [24,29,30]. It is, however, unknown which odor compounds from the different phenological stages influence orientation and oviposition. The carob moth–pomegranate combination is a highly relevant experimental system because the egg-laying and larval feeding activity occur within the fruits, rendering commercial insecticides ineffective. In addition, an efficient sex pheromone attractant is still lacking in carob moth pest management (Supplementary Materials S1).

We developed and tested a workflow starting with pre-screening for sensory activity by GC-EAD followed by GC-Orbitrap MS. In total, we were able to detect 11 peaks that produced positive GC-EAD responses. As a proof-of-principle, the proposed workflow was employed to identify and investigate the role of a component that elicited a pronounced EAG response, whilst giving rise to only a minor signal in the Orbitrap MS chromatogram.

## 2. Materials and Methods

**Standards.**  $\beta$ -caryophyllene (CAS 87-44-5,  $\geq 98.0\%$ ) and C8–C20 alkane mix (~40 ppm in hexane, diluted 10 $\times$ ) were purchased from Sigma-Aldrich (Dorset, UK). Standards of caryophyllene were prepared in hexane at different concentrations for peak confirmation and quantification. Pentadecane (CAS 629-62-9,  $\geq 98.0\%$ ) was used as an injection control standard at a concentration of 10 ppm in hexane.

**Insects.** For all experiments, female carob moths were used, reared from larvae that had been collected in commercial pomegranate orchards located in Chandab, Iran (35°25'13" N, 51°56'04" E, 1141 m elevation), during 2013 and 2014.

**Plants.** Pomegranate, *Punica granatum* L. (Lythraceae), fruits and flowers in various stages (Supplemental Materials S2) were obtained from the cultivar Galu-Barik and picked

from the same orchard in Chandab (Iran) as the insects. Pistachio (*Pistachia vera* L. (Anacardiaceae)) fruits were collected from cultivar Akbari, grown in a commercial pistachio orchard in Qazvin, Iran (35°54'07'' N, 50°03'06'' E and 1201 m elevation).

**Collection of Plant Volatiles.** Headspace volatiles for behavioral, electrophysiological, and chemical analysis were collected from 2 kg of pomegranate fruits, 300–400 g of pomegranate flowers, or 1 kg of pistachio fruit placed in 4 L glass jars. Charcoal-filtered air was pulled through the jar with a vacuum pump (22 h at 0.5 L/min), and the headspace was collected on an adsorbent trap (60 mm L × 6 mm I.D.), containing 50 mg of Tenax-GR 60/80 (Restek, Bellefonte, PA, USA). Control samples were collected using an empty jar. Tenax traps were conditioned at 200 °C for 30 min under a stream of N<sub>2</sub> before use. After sampling, traps were extracted with 2 mL of n-hexane (Merck, Darmstadt, Germany). Samples were sealed and stored at –20 °C prior to chemical analysis.

**Bioassays.** Sensory and analytical data from insects can only be interpreted in light of associated behavioral responses. We, therefore, conducted both wind tunnel experiments (to measure attraction behavior) and oviposition experiments (to measure acceptance behavior). Details of the assays can be found in Supplementary Materials S3.

**Coupled Gas Chromatography-Electroantennogram Detection (GC-EAD).** To determine which volatile compound(s) in the plant extracts is detected by the carob moth, GC-EAD experiments [31] were conducted. A 2- to 3-day-old mated female carob moth was mounted in a plastic pipette tip and its antenna was immobilized with a small strip of parafilm, pressing the antennal base against the head. The amplitude of the EAD was measured using an IDAC-4 amplifier equipped with a high-impedance (>10<sup>9</sup> Ohm) head stage and captured with GC-EAD/2014 software (Syntech, Kirchzarten, Germany). See Supplementary Materials S4 for details.

**Gas Chromatography-Orbitrap Mass Spectrometry (GC-Orbitrap MS).** Confirmatory analyses on cracked pomegranate extracts were carried out using a Thermo Scientific Trace 1300 GC, again equipped with an instant-connect Cold-On-Column injector, but now hyphenated to a Thermo Scientific Q-Exactive Orbitrap MS (Interscience, Breda, The Netherlands). Separation was achieved using a 30 mL × 0.25 mm I.D., 0.25 µm df Rxi-5Sil MS capillary column (Restek), preceded by a 2 mL × 0.53 mm I.D. retention gap. Injection volume was 1 µL. Helium was used as a carrier gas at 1.2 mL/min in constant flow mode. The GC oven was programmed from 55 (1 min) to 85 °C (1 min) at 55 °C/min, and subsequently to 165 °C at 2 °C/min to achieve separation of all target analytes. Finally, the oven was heated to 300 °C at 20 °C/min to regenerate the column. Secondary cooling was switched off after 21 s. The Orbitrap MS was applied in both EI (70 eV) and PCI (methane, 1.5 mL/min) full-scan mode between 35 and 550 *m/z*. Mass resolution was set at 6 × 10<sup>4</sup> (FWHM at 200 *m/z*), AGC target at 1 × 10<sup>6</sup>, and emission current at 50 µA. The filament delay was 6.00 min, the source temperature was 250 (EI) and 200 °C (PCI), quadrupole temperature was 280 °C, and MS transfer line temperature was 250 °C. Lock mass was 207.0235 *m/z*. The instrument was tuned separately for EI and PCI analysis according to routine settings, as proposed by the manufacturer. Data were acquired with Chromeleon CDS (Thermo Scientific, Waltham, MA, USA) and subsequently processed using Thermo Scientific Compound Discoverer 3.2 and Mass Frontier 8.1 software. NIST 2020 (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used as the EI spectral library (unit mass).

**Component Identification.** In order to provide a reliable identification of these bioactive components, a standardized workflow for peak identification using GC-Orbitrap MS was applied. Initially, all data were acquired in EI mode (70 eV), allowing comprehensive fragmentation of all analytes for subsequent library export. In order to determine Kovats retention indices (RI), first, the C<sub>8</sub>–C<sub>20</sub> alkane mix was injected and retention times of the alkanes were recorded [32]. Next, the extracts were measured under identical experimental conditions and RIs were calculated for each of the target compounds found with GC-EAD. Once collected, data were processed using the software provided with the GC-Orbitrap instrument. First, Compound Discoverer 3.2 was used in EI mode for high-resolution

spectral deconvolution and NIST library export, including RI scoring for each individual peak in the chromatogram. For each library hit, a high-resolution filtering (HRF) score was calculated by Compound Discoverer. This score indicates to which extent the elemental composition of each of the fragment ions in the NIST candidate spectra can be retrieved from the high-resolution spectrum associated with the peak under investigation [33]. The subsequent match factor includes both the HRF and library search index for each candidate.  $\Delta$ RI values were provided separately and interpreted manually. Molecular ion confirmation, which is key in the identification of unknowns, was achieved by means of a repeat analysis in PCI mode. Due to its soft nature, PCI is able to preserve the molecular ion, which is essential in order to determine (or confirm) the elemental composition of the native analyte. Typical adducts of the applied CI gas were automatically confirmed in the high-resolution spectra, and a results table was compiled that included the proposed elemental composition of all molecular ions. EI and PCI identifications were compared to provide hits that occur in both lists. As a final step in the workflow, Mass Frontier 8.1 was used to match the theoretical fragmentation of the proposed chemical structures (using common fragmentation and rearrangement rules), as derived from the elemental composition of the molecular ion, with the experimental fragmentation pattern obtained during EI analysis and expressed as Fragment Ion Search (FISh) scores [34].

Statistical Analysis. All analyses were performed in R, v3.3.1 (R Core Team 2016).

### 3. Results

#### 3.1. Identification of Bioactive Compounds

GC-EAD analysis of cracked pomegranate volatiles revealed at least 11 peaks that generated an EAG response (Supplementary Materials S5). Table 1 summarizes candidate molecules and associated identification criteria. Note that the table also includes some green leaf volatiles that did not generate an electrophysiological response but were considered valuable in the context of this investigation (see the Discussion Section). Last but not least, definitive confirmation of component identity, using both retention and spectral verification, was achieved for  $\beta$ -caryophyllene, i.e., the component with the most consistent response in the GC-EAD analyses, by analyzing an authentic standard solution containing the native component.

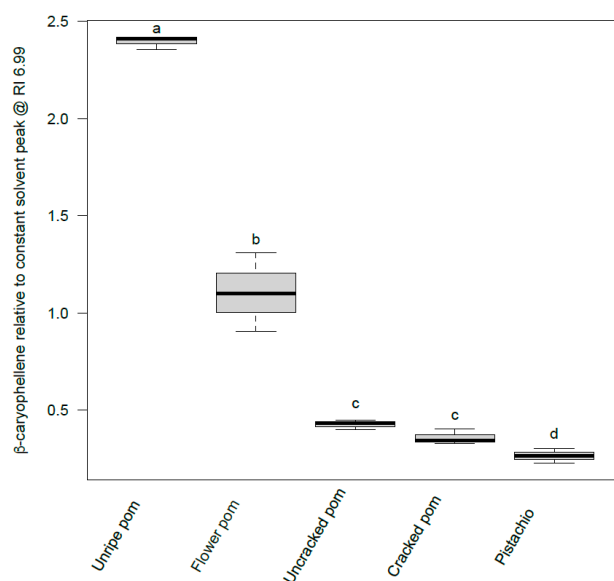
**Table 1.** Component identification from cracked pomegranate headspace extract by means of GC-Orbitrap MS workflow.

Peak	RT (min)	EAD	Candidate	TIC (Area/10 <sup>6</sup> )	MF <sup>1</sup>	HRF <sup>2</sup>	RI <sup>3</sup>	$\Delta$ RI <sup>4</sup>	PCI <sup>5</sup>	A <sub>ppm</sub> <sup>6</sup>	FISh <sup>7</sup>
1	6.26	N	Hexanal	9.79	C6H12O	98.8	n/a *	n/a	+	0.45	+
2	7.06	N	<i>trans</i> -3-Hexen-1-ol	5.69	C6H12O	99.3	834	18	-	n/a	+
3	7.53	N	<i>cis</i> -3-Hexen-1-ol	496	C6H12O	99.8	861	4	-	n/a	+
4	9.48	Y	$\alpha$ -Pinene	969	C10H16	98.3	945	3	+	-0.67	+
5	<b>34.08</b>	<b>Y</b>	<b><math>\beta</math>-Caryophyllene</b>	<b>244</b>	<b>C15H24</b>	<b>99.9</b>	<b>1424</b>	<b>5</b>	<b>+</b>	<b>0.78</b>	<b>+</b>
6	34.75	Y	<i>cis</i> - $\alpha$ -Bergamotene	3110	C15H24	99.7	1435	20	+	0.65	+
7	35.16	Y	$\beta$ -Cedrene	455	C15H24	99.7	1442	21	+	0.57	+
8	35.78	Y	<i>cis</i> - $\beta$ -Farnesene	468	C15H24	99.8	1452	12	+	0.48	+
9	35.92	Y	Isogermacrene D	468	C15H24	99.7	1455	7	+	0.09	+
10	36.23	Y	$\alpha$ -Humulene	574	C15H24	99.8	1460	6	+	0.49	+
11	37.83	Y	9-epi-(E)-Caryophyllene	478	C15H24	99.8	1486	8	+	0.21	+
12	38.67	Y	Pentadecane **	1380	C15H32	n/a	1500	0	-	n/a	+
13	39.18	Y	Valencene	438	C15H24	99.5	1509	9	+	-0.03	+
14	39.95	Y	Ethyl-4-ethoxy benzoate	916	C11H14O3	98.0	1523	1	+	-0.28	+

n/a = not available. \* RI could not be calculated using the C8–C20 alkane mix because the compound elutes before C8. \*\* Component used as internal standard. <sup>1</sup> Molecular formula of the candidate compound. <sup>2</sup> High-resolution filtering score as determined by Compound Discoverer. <sup>3</sup> Experimental Retention Index (RI) as determined by Compound Discoverer. <sup>4</sup> Difference between experimental RI and tabulated RI for a similar column in NIST 2020. <sup>5</sup> Result of Positive Chemical Ionization analysis. '+' means that the molecular formula as depicted in the MF column was confirmed. <sup>6</sup> Mass accuracy in ppm as calculated by Compound Discoverer for the molecular ion in the PCI analysis. <sup>7</sup> Fragment Ion Search score as determined by Mass Frontier 8.1.

### 3.2. Biological Analysis

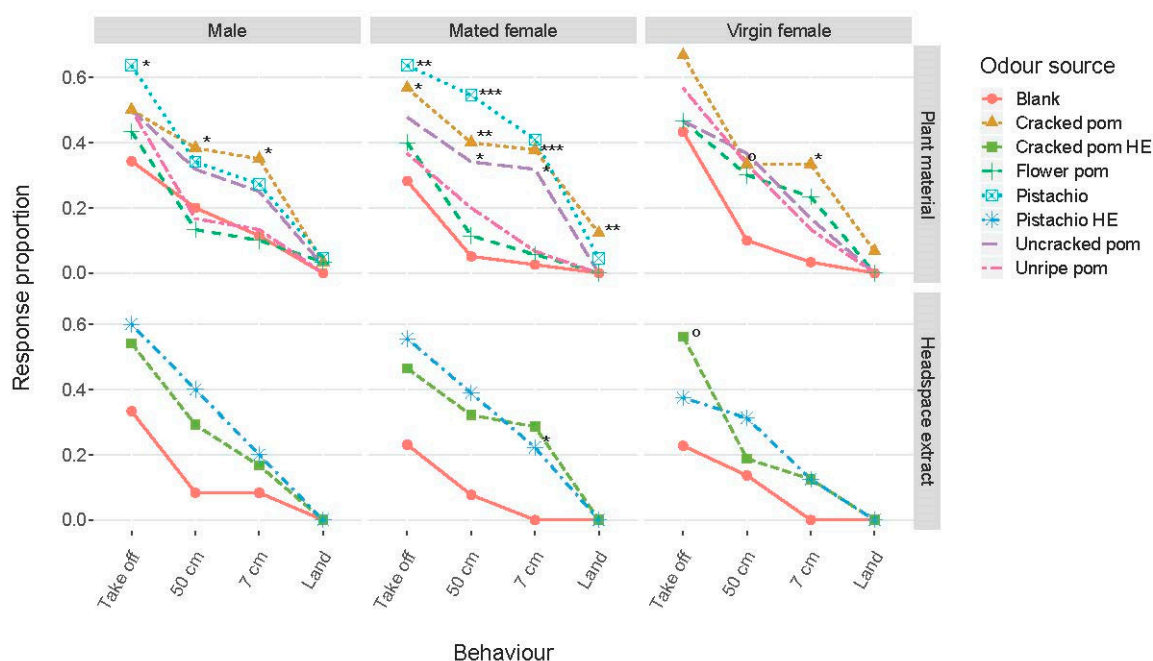
After positive confirmation of  $\beta$ -caryophyllene as the most consistently electrophysiological active compound in the headspace of cracked pomegranate, its presence and concentration were determined in all headspace extracts. We found that  $\beta$ -caryophyllene was present in all pomegranate samples as well as in that of pistachio (Supplementary Materials S6). Unripe pomegranate and pomegranate flowers contained relatively high amounts, whereas the extracts of pistachio and ripe pomegranate, whether cracked or uncracked, contained lower levels of  $\beta$ -caryophyllene (Figure 1). The amount of  $\beta$ -caryophyllene relative to the solvent peak at a retention time of 6.99 min was significantly different among different plant materials tested ( $F = 264.11$ , d.f. = 4, 10;  $p < 0.0001$ ; Supplementary Materials S7).



**Figure 1.** Amounts of  $\beta$ -caryophyllene in the headspace extracts of pomegranate and pistachio fruits and flowers relative to the constant peak in the solvent (retention time: 6.99). Plant materials with different letters are significantly different. Three samples were analyzed per plant sample.

In the wind tunnel experiment, behavioral attraction to headspace extracts was negatively correlated with the amount of  $\beta$ -caryophyllene in host fruits and flowers (Figure 2). Only mated females (i.e., ready to oviposit) actually landed on cracked pomegranates. Virgin females did not start flying towards any of the odor sources, and males only flew upwind to cracked pomegranate but never landed (Figure 2, Supplementary Materials S3).

In an oviposition experiment on filter paper,  $\beta$ -caryophyllene was stimulated in the range of 40–100 ng, while concentrations above 200 ng inhibited oviposition. The control *cis*-3 hexen-1-ol did not show this inhibition (Supplementary Materials S3).



**Figure 2.** Behavioral responses of mated female and virgin female and male carob moths to a series of plant materials (pomegranate and pistachio) and their headspace extracts (HE) in wind tunnel bioassays. Differences between the odor sources and controls at each behavioral step are depicted using the following codes: \*\*\*  $p \leq 0.001$ , \*\*  $p \leq 0.01$ , \*  $p \leq 0.05$ , °  $p \leq 0.1$ .

#### 4. Discussion

The identification of unknown components is a tedious and time-consuming process. The use of GC-Orbitrap MS and supporting software Compound Discoverer and Mass Frontier accelerates this process significantly. Key in this respect is the accuracy of the mass measurements, which is typically  $<1$  ppm, and drastically reduces the number of candidate element compositions that is able to match the mass-to-charge ratio of the molecular ion. As each elemental composition may give rise to a multitude of chemical structures, it is fair to conclude that the list of seemingly valid elemental compositions should be as small as possible prior to starting the identification exercise. In that respect, pre-screening with GC-EAD is an important step. Additionally, Kovats RI is a valuable tool, even in combination with Orbitrap MS data. Indeed, when isomeric components, such as terpenoids, are to be confirmed, (high resolution) spectral matching alone is not enough for positive identification (see Table 1). In fact, these components are better designated as ‘unknown knowns’, requiring retention verification for definitive confirmation. In case of  $\beta$ -caryophyllene, the calculated Kovats RI was 1424, which is close to the 1420 average value for non-polar columns from the NIST v20 database (as summarized by NCBI (2020)), and well within the  $\pm 10$  RI that can be expected for terpenoids on a 5% phenyl column [35].

In the current proof-of-principle, GC-EAD and GC-Orbitrap MS were used sequentially. However, it is easily possible to combine EAD and MS in parallel [36,37] and omit the need to compare chromatograms [38]. In combination with Orbitrap MS, the method becomes even more powerful.

The odor of pomegranate contains a large amount of secondary plant compounds, mainly green leaf volatiles (GLV) and terpenoids [39–41]. We identified 11 of these compounds that were evoking a response in the antenna of female carob moth. GLVs were also present but not causing consistent GC-EAD responses. This might be related to the low levels present (see Table 1). The low GLV levels (except for *cis*-3-hexen-1-ol) were not unexpected, since these compounds are mainly induced by stresses, such as senescence [42] and in particular herbivorous attack [43,44]. However, we cannot fully exclude the possibility that the low levels of green odors we found were the result of losing some of these more

volatile compounds during the relatively long sampling phase (22 h) or during the evaporation under a gentle nitrogen stream needed to sufficiently increase the concentrations in the eluate from the trap for analytical and electrophysiological measurements.

All identified compounds have known ecological roles. Pentadecane is present in flower odors of many plant families [45], and is also found in pomegranate [41]. It can act as a repellent [46]. Pentadecane levels are changing during the development of grapevines [47], and differ between pomegranate plant parts [41]. However, since the component was used as an injection standard, no conclusions can be drawn regarding its presence in our headspace samples. Ethyl-4-ethoxy benzoate has antibacterial properties, is involved in root competition, and is highly elevated under drought stress [48,49]. These ecological links make the compounds probably good indicators of plant health. All other active compounds were terpenoids, and in particular sesquiterpenes. Over 55,000 terpenoids are known [50]. They are produced by a family of synthetases [51,52] and fulfil a wide range of ecological functions, such as direct and indirect defenses against pathogens and herbivores, pollinator attraction, and inter-plant communication and root competition [53]. What all the identified compounds from pomegranate have in common is that they are informative about plant status [54–56].  $\beta$ -Caryophyllene is a good example. It is a sesquiterpene common in essential oils and flower odors of a wide variety of higher plants [44] and was also identified in pomegranate [57,58] and pistachio [59].  $\beta$ -Caryophyllene was present in different phenological stages of pomegranate as well as in ripe pistachio. Concentrations of  $\beta$ -caryophyllene in the headspace of the unattractive hosts, unripe pomegranate, and pomegranate flowers, were respectively 5 and 2 times higher than in attractive fruits (Figure 1), which may contribute to the lack of attraction of, in particular, mated female carob moths to these plant sources in the wind tunnel (Figure 2) and in the field [26].

Our oviposition experiments also showed that lower levels of  $\beta$ -caryophyllene induced more egg laying than higher levels. This pattern is observed more often [41] and falling levels of  $\beta$ -caryophyllene during development has been documented as well [57,60,61]. The compound, therefore, may serve well as in indicator of fruit ripeness and host suitability. This is confirmed by our bioassay results. Orientation was associated with low levels of the compound and oviposition was inhibited at higher levels.

The identified  $\beta$ -caryophyllene has already been successfully used to trap *L. botrana* in field cages when used in a blend containing synthetic plant compounds [62]. It may be used alone or in combination with the long-range sex pheromone in monitoring or attract-and-kill methods in integrated pest management. For carob moth pest management, exploration of the possibility to reduce infestation by perfuming mature pomegranates with (relatively) high concentrations of compounds are recommended. Alternatively, it may be possible to breed pomegranate cultivars with altered levels of secondary plant compounds [51,63]. This was recently demonstrated for  $\beta$ -caryophyllene by Salvagnin et al. [43]. It should be noted, however, that altering the levels of secondary plant compounds might have unexpected side effects on other pests or pathogens [53,64]. Still, the ability to quickly and with high confidence identify difficult to characterize compounds will be invaluable for the development of sustainable pest management strategies.

## 5. Conclusions

The aim of this study was to explore the possibilities for efficient structural identification of unknown plant volatiles that induce behavioral responses in insects. We demonstrated the effectiveness of combining GC-EAD recordings and high-resolution Orbitrap-GC-MS with a fixed and efficient workflow that allows fast identification of unknown unknowns. The possibility to quickly identify bioactive compounds is of crucial importance for the development of new methods for sustainable pest management now that it is becoming increasingly clear that the use of traditional pesticides is an untenable solution.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/app11188603/s1>, S1: Carob moth biological background, S2: Pomegranate sample information, S3: Carob moth wind tunnel and oviposition results, S4: GC-EAD setup details, S5: GC-EAD active



peak identification and associated raw data, S6: quantification of  $\beta$ -caryophyllene in headspace extracts from flowers and fruits at different stages of development, S7: raw data and scripts.

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