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Profiling and characterization of volatile secretions from the European stink bug *Graphosoma lineatum* (Heteroptera: Pentatomidae) by two-dimensional gas chromatography/time-of-flight mass spectrometry

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ABSTRACT

An efficient method combining the headspace solid-phase microextraction (HS-SPME) sampling procedure and comprehensive two-dimensional gas-chromatography/time-of-flight mass spectrometry (GC × GC/TOF-MS) was established to study the volatile secretion components of stink bugs (Heteroptera: Pentatomidae). The combined power of this approach is illustrated by the identification of fifty-seven compounds in the secretion of a European stink-bug representative, *Graphosoma lineatum*. (*E*)-4-oxohex-2-enal and (*E*)-dec-2-enal were found to be the major components in the adult bug secretions followed by lower amounts of *n*-alkenal (C_5–C_{12}), *n*-alkenyl acetate (C_5–C_{11}), *n*-alkane (C_{11}–C_{17}) homologs, dienals and other compounds. More than thirty known compounds have been identified that had not been described before in *G. lineatum* adults. Of these compounds, (*E*)-4-oxohex-2-enal is of particular interest, since its isolation and identification, while calling some previous reports into question, clearly demonstrates a potential ability of our approach to yield artifact-free secretion profiles.

1. Introduction

A wide variety of insect species have developed chemical defense mechanisms that significantly contribute to their widespread success in ecosystems [1,2]. Typical representatives of such species are the pentatomid bugs (Heteroptera: Pentatomidae), commonly known as "stink bugs", because in response to disturbance or aggression they produce large quantities of foul-smelling odorous volatiles. The pentatomids are one of the four largest families of Heteroptera comprising approximately 4500 species worldwide [3]. Their volatile secretions released from exocrine glands, such as the metathoracic glands (MTG) in adults or dorsal abdominal glands in nymphs [4,5], act mainly as defensive means against predators/parasitoids and/or as aggregation/alarm pheromones [1,6]. Since chemical defense mechanisms have a profound impact on the entire biology of an insect species (e.g. adaptations in morphology, physiology, niche use, behavior, etc.) [1], considerable research effort has been directed at isolating and characterizing the volatile secretion components in a variety of stink bugs [7–10]. However, the data are difficult to compare, mainly owing to the use of diverse experimental designs and analytical methods, so that numerous questions concerning the role that most of the components may play in chemical communication systems within the Pentatomidae family still remain to be answered. It is often unclear which chemicals are defensive against predators, which elicit a dispersal behavior among conspecifics and whether those chemical identities change as the insects pass through different life stages. Apart from this, minor constituents have yet to receive the same level of attention as the major ones to provide more detailed chemical information about the systems.

One of the stink bugs widely distributed in Europe is the striated shield bug *Graphosoma lineatum* (Linneaus), a 1–1.2 cm long bug occurring on umbelliferous plants. The adults of *G. lineatum* are mostly recognized by their conspicuously red (epidermis) and black (melanized cuticle) striated coloration. Previous attempts to identify the compounds secreted by *Graphosoma* have involved a variety of analytical approaches including a solvent extraction of either whole insects or MTGs [7,8], trapping the volatile compounds in MeOH or using a solid-phase microextraction (SPME) followed by conventional (one-dimensional) gas chromatographic (GC) separation combined with mass spectrometry (MS) [9,10]. While most of these studies [7,8,10] indicate that, besides hydrocarbons, the composition of *G. lineatum* secretions primarily includes saturated aldehydes and (*E*)-alk-2-enals with either (*E*)-dec-2-enal [7,8] or (*E*)-hex-2-enal [10] being the most abundant components, some investigators have found a predominance of (*Z*)-alkenals over the corresponding (*E*)-isomers and a relatively large amount of furanones [9,10].

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The fundamental differences between the reported analytical results indicate that there are still important gaps in our knowledge concerning the chemistry of *G. lineatum* secretion constituents. A part of the irreproducibility in the previous analyses can certainly be attributed to factors such as variable rearing conditions, age, gender, geographic origin, and food availability as well as the different processing and analytical methods used. It should also be emphasized that an analysis of complex stink-bug secretion mixtures by one-dimensional GC may fail or be unsatisfactory considering the known limitation of one-dimensional GC and/or GC/MS techniques, which are inherently unable to separate and identify the multitude of compounds that are present in low concentrations and can co-elute.

Two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GC × GC/TOF-MS) is considered one of the most powerful and versatile separation tools among the chromatographic methods, reducing the problem of co-eluting peaks and providing high sensitivity and selectivity [11,12]. Over the past few years, an increasing number of laboratories have explored the use of GC × GC/TOF-MS for the analysis of petrochemicals, agrochemicals, and food as well as other environmentally and biologically relevant compounds [13–16]. The advantages and limitations of using this technique as well as some theoretical and practical aspects have been summarized in recent reviews [17,18]. However, the literature evaluating the performance of this technique in insect chemistry is rather limited [19,20] and, to the best of our knowledge, the approach has not yet been applied in the analysis of heteropteran insect secretions.

Whereas the utility of combining GC × GC with TOF-MS in the study of complex mixtures has been well-established, it is worth mentioning that mass spectrometry alone is often insufficient to distinguish between structural isomers which exhibit identical mass spectra [21]. In such cases, preparative-scale GC (prep-GC) [22,23] is a valuable technique to obtain the pure compound of interest in sufficient quantity in order to provide its further spectroscopic (NMR spectroscopy, FTIR spectroscopy, etc.) characterization and/or complete structure elucidation. Through the use of prep-GC, a variety of pheromone components [24,25], insect-induced plant volatiles [26] and/or other isomeric products [27] have been isolated and unambiguously identified.

In the present study, we have reinvestigated the volatile secretions produced by the model stink bug, *G. lineatum*, to demonstrate, for the first time, the advantages and unprecedented resolving power of using the GC × GC/TOF-MS technique for the separation and identification of stress-induced volatile components of stink bugs. The objectives of this study were to: (i) evaluate the feasibility of using a HS SPME-based procedure for the collection of volatiles produced by living stink bugs, (ii) show the capabilities of GC × GC/TOF-MS technique to profile male- and female-produced secretions, and (iii) establish whether 5-ethylfuran-2(5*H*)-one really does occur in *G. lineatum* secretions. Our analytical approach is expected to allow a more complete characterization of the primary secretion components, and the results might contribute to the formation of a reliable compound base for further behavioral studies and help in elucidating the mechanism underlying the stink-bug defense against predators.

2. Experimental

2.1. General

The NMR spectra were recorded on a *Bruker Avance II-500* spectrometer (500.0 MHz for ^{1}H and 125.7 MHz for ^{13}C) in $CDCl_3$. The NMR spectra were referenced to TMS.

2.2. Sample preparation

Live, wild stink bugs, *G. lineatum*, were collected near Prague, Czech Republic. The adults were divided by sex, maintained until needed in plastic containers at 25 ± 2 °C under a 16:8 photoperiod, reared on wild chervil seeds and fed with tap water. The volatile secretions were collected separately from males (N = 8) and females (N = 8). Each individual was squeezed with the help of tweezers until a typical strong "stink bug smell" could be detected and quickly placed in a 4-ml glass vial sealed with a Teflon cover with a rubber septum [28]. The sheath of the SPME fiber was inserted to the vial 5 min after putting the stressed animal into the vial. The control animals, without squeezing them, were carefully inserted with the tweezers into the vial.

The volatile compounds were extracted from the headspace (HS) using a manual SPME sampler with a 2-cm StableFlex fiber assembly (Supelco, Bellefonte, PA, USA) coated with a triple phase 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) at 25 °C. The selection of the DVB/CAR/PDMS fiber was based on its known wide-sampling capacity and sensitivity for the entire range of compounds of different volatilities and polarities [29]. This fiber was also chosen as the best for the collection of volatile mixtures of a similar type as those expected for *G. lineatum* [7–10], such as e.g. some plant volatiles [30,31], coleopteran insect secretions [32], and honey [16]. An extraction temperature of 25 °C was used, because this temperature is close to insect defense natural conditions. After a 15-min extraction (this point will be returned to in Section 3.1), the SPME device was immediately inserted into the injection port of the GC × GC/TOF-MS system and the fiber thermally desorbed at 260 °C for 5 min. The fiber was conditioned according to the manufacturer's recommendation prior to use.

2.3. Chemicals

A mixture of *n*-alkanes (C_8–C_{20}) dissolved in *n*-hexane for retention index determinations was supplied by Supelco (Bellefonte, PA, USA); the *n*-pentane for GC-analysis >99% was purchased from Fluka. Most of the other reference compounds were acquired from Sigma–Aldrich Co.: hexanal, nonanal, (*E*)-pent-2-enal, (*E*)-hex-2-enal, (*E*)-hept-2-enal, (*E*)-oct-2-enal, (*E*)-non-2-enal, (*E*)-dec-2-enal, (*E*,*E*)-deca-2,4-dienal, (*E*,*E*)-hexa-2,4-dienal, (*E*,*E*)-octa-2,4-dienal, (*Z*)-dec-2-enal, limonene, nonan-2-one, tridecan-2-one, tridec-1-ene, acetophenone, (*E*)-hex-2-en-1-ol, 2-ethylhexan-1-ol, nonylbenzene, dihydromyrcenol, cyclohexan-1,4-dione, 2-ethylfuran, 2-acetylfuran, benzene and 1-phenylnonane. The acetates, i.e. heptyl acetate, (*E*)-pent-2-enyl acetate, (*E*)-hept-2-enyl acetate, (*E*)-oct-2-enyl acetate, (*E*)-dec-2-enyl acetate, (*E*)-undec-2-enyl acetate, and (*Z*)-dec-3-enyl acetate, were either obtained from the Research Institute for Plant Protection (IPO-DLO, Wageningen, Netherlands) or prepared from the corresponding alcohols previously in our laboratory [33]. Authentic samples of (*E*)-4-oxohex-2-enal and 5-ethylfuran-2(5*H*)-one were synthesized according to the described procedures [34,35]. The ^{1}H and ^{13}C NMR data of the synthesized compounds (see Supplementary information) were consistent with those reported.

2.4. GC × GC/TOF-MS analysis

The GC × GC/TOF-MS analyses were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA), coupled to Agilent 6890N gas chromatograph with split–splitless injector, 7683 Series autosampler and time of flight mass spectrometer LECO Pegasus III. A weakly polar DB-5 column (5% phenyl–95% methylpolysiloxane, J&W Scientific, Folsom, CA, USA; 30 m × 250 μm i.d. × 0.25 μm film) was used for GC in the first dimension. The second-dimension analysis was performed on a

polar BPX-50 column (50% phenyl–50% methylpolysiloxane) SGE Inc., Austin, TX, USA; 2 m × 100 μm i.d. × 0.1 μm film). Helium was used as a carrier gas at a constant flow of 1 mL/min. The temperature program in the first column commenced at 50 °C (held for 2 min), was raised to 300 °C at 10 °C/min and held at 300 °C for 10 min. The program in the secondary oven was 5 °C higher than in the primary one and was operated in an iso-ramping mode. The modulation period was set at 4.0 s. The transfer line to the TOF-MS detector source was operated at 260 °C. The source temperature was 250 °C with a filament bias voltage of −70 eV. The data-acquisition rate was 100 Hz (scans/s) for the mass range of 29–400 amu. The detector voltage was 1470 V. The total ion chromatograms (TIC) and/or analytical ion chromatograms (AIC) were processed and consecutively visualized on 2D plots using the LECO ChromaTOF^{TM} (v. 2.32) automated data processing software.

2.5. Preparative GC chromatography

The preparative GC chromatography was performed with an AT 6890N gas chromatograph (Agilent Technologies, Wilmington, DE, USA), configured with a liquid-nitrogen-cooled EPCPTV inlet (Gerstel, Mühlheim, Germany) and FID detector. Sample volumes of 5 μL were injected twice with an AT 7683B autosampler. The injector starting temperature was −20 °C, which was held for 30 s. During that time, 20 mL/min of helium flow was applied. The inlet pressure was adjusted to 0.5 psi. After 30 s, the split valve was closed with the liner being flash-heated at 12 °C/s to 350 °C and held for 2 min. For GC separation, an HP-1 fused silica capillary column (30 m × 0.53 mm ID, 0.88 μm) coated with 100% dimethylpolysiloxane stationary phase (Hewlett Packard, Palo Alto, CA, USA) was used along with helium as a carrier gas in the constant flow mode at 4 mL/min. The column temperature was held at 40 °C for 1 min, programmed at 5 °C/min to 60 °C, then at 30 °C/min to 280 °C and held for 1 min.

The effluent continued to the Preparative Fraction Collector PFC (Gerstel), where the analyte was captured by cooling the effluent in the trapping capillary at −80 °C. The transferline temperature was held at 270 °C.

2.6. Identification of the volatile components

After the GC × GC/TOF-MS data acquisition by ChromaTOF software, the samples were subjected to a data processing method where the individual peaks were automatically detected on the basis of a 100:1 signal to noise ratio. The initial tentative identifications of the secretion components were made by comparing the obtained deconvoluted spectra with those found in the NIST/Wiley mass spectra database libraries [36,37]. Based on previous findings [13,38], similarity and reverse factors above 750 and 800, respectively, were considered to be a good match with the library spectrum. A series of *n*-alkanes (C_8–C_{22}) was analyzed under the same experimental conditions as those used for the samples (not taking into consideration the effect of second-dimension retention) to establish the first-dimension retention indices (LRI_{exp}) of the analytes [13]. The confirmation of the tentatively identified compounds was performed by comparing the calculated LRI_{exp} with those available in reference libraries (LRI_{lit}) [36,37], as well as by comparing the experimental retention times and mass spectra of the compounds with those of contemporaneously analyzed reference standards.

2.7. Data processing

The average peak areas of each component were calculated from deconvoluted TIC peak areas based on eight replicates, for which the analysis of male and female samples was performed. The internal standard (*n*-decanal) diluted in pentane was added to each sample by being injected directly into the vial, and the instrumental response to *n*-decanal was determined by using known amounts of this compound. The averaged peak area data were normalized [29] versus the internal standard area, and the areas of the major fifty-seven peaks representing >0.1% of a relative peak area (and present in all individuals) were selected and re-standardized to 100%. Although this approach does not allow a semi-quantification of all of the mixture components whose responses remain unknown, it provides a reasonable comparison of their representation in male and female samples.

Because the relative peak areas represent compositional data, they were transformed according to Aitchison's formula [19,39,40]: $Z_{ij} = \log[A_{ij}/g(A_j)]$, where A_{ij} is the area of peak i for bug j, $g(A_j)$ is the geometric mean of all of the peak areas for bug j and Z_{ij} is the transformed area of peak i for individual j. The values of the $\log_{10}$-transformed relative peak areas for each compound were summarized by standard descriptive statistics using the Shapiro–Wilk test ($N = 8$, $\alpha \geq 0.05$) to evaluate the fit of the data to a normal distribution and expressed as the mean ± SD. The means were then back-transformed to obtain the mean relative percentages. Because of these transformations, the upper and lower SE values were not necessarily symmetrical around the mean. The nonparametric two-sample Kolmogorov–Smirnov method ($\alpha \geq 0.05$) was used to test the null hypothesis that the $\log_{10}$-transformed male and female bug data were from the same distribution. The statistical analyses were performed using the Statgraphics Centurion^{R} software version XV (Manugistics, Inc., Rockville, MD, USA).

3. Results and discussion

3.1. HS SPME conditions

With the fiber type (DVB/CAR/PDMS) and extraction temperature (25 °C) having been pre-selected (see Section 2.2), the optimization of the extraction time was accomplished by testing the effect of a time variation from 2 min to 60 min on the recovery of six of the target compounds, namely (*E*)-hex-2-enal, (*E*)-oct-2-enal, (*E*)-dec-2-enal, (*E*)-4-oxohex-2-enal, (*E*,*E*)-hexa-2,4-dienal, *n*-tridecane, and an internal standard, *n*-decanal. Each point in the extraction time dependency (Fig. 1) was constructed from three repetitions. It was observed (Fig. 1) that for four compounds (*n*-tridecane, *n*-decanal, (*E*)-dec-2-enal and (*E*,*E*)-hexa-2,4-dienal) the equilibrium was still not reached after 60 min, while for (*E*)-hex-2-enal, (*E*)-oct-2-enal, and, particularly, (*E*)-4-oxohex-2-enal the amount adsorbed on the fiber increased for only up to 15–20 min of extraction, after which time it started to diminish, probably owing to desorption/competition effects [41–43]. Since the extraction time of 15 min showed the maximum response for the target compound, (*E*)-4-oxohex-2-enal while representing a compromise between the sensitivity and analysis time for most of the other volatiles, this time was selected for all experiments.

3.2. Qualitative analysis of volatiles

Volatiles were collected from the headspace over individual living male and female *G. lineatum* bugs. While the GC × GC/TOF-MS analysis of the control headspace *G. lineatum* extracts obtained from unmolested bugs did not show any detectable compounds, more than 100 compounds were detected in blends released by disturbed bugs. On the basis of such criteria as the mass spectral match factor (S) of measured deconvoluted mass spectra to the NIST 05 library data $S > 750$, signal-to-noise ratio ≥ 100, and linear retention index differences $\Delta I = LRI_{exp} - LRI_{lit} \leq \pm 20$ index units

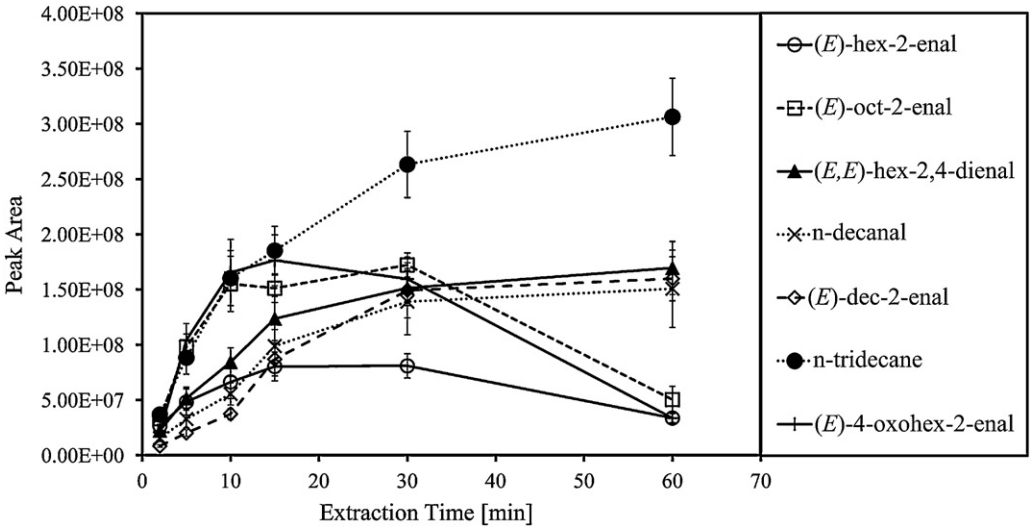

Fig. 1. The effect of extraction time on headspace amounts of selected *G. lineatum* volatiles using a DVB/CAR/PDMS fiber at 25 °C; each data point represents an average of three individual runs.

(LRI_{exp}: linear retention indices calculated for the first dimension of the GC × GC/TOF-MS analysis, LRI_{lit}: linear retention indices reported in the literature for the DB-5 GC column or equivalents), the number of compounds confidently identified in both sexes of *G. lineatum* was reduced to 57. The identity of 42 of the 57 compounds was additionally confirmed using pure analytical standards.

The compounds identified in *G. lineatum* males and females along with the first-(1D RT) and second-(2D RT) dimension retention times, LRI_{exp}, similarity (S), unique mass (U) values, methods of identification and relative percent areas are presented in Table 1. The retention times given for each of the compounds in Table 1 refer to the most intense peak in the series of peak modulations belonging to the same compound. Further, the illustrative Analytical Ion Chromatogram (AIC) for a *G. lineatum* male-emitted volatile secretion sample is shown in Fig. 2A. This figure demonstrates that the GC × GC/TOF-MS system allowed an efficient chromatographic separation of the peaks in both chromatographic dimensions. It can be observed that the majority (≈90%) of the identified compounds showed similarity matches $S > 800$. Plotting the LRI_{exp} against those of the databases measured with a mono-dimensional configuration resulted in a straight line (Fig. S1) with a high coefficient of determination ($r^2 = 99.87\%$), a slope very close to 1 (0.9975) and a standard error of estimation (SEE) of 6.84. Since not all of the standards were available, this linear correlation was used as an additional tool to support the identification task performed. For certain compounds that were part of a homologous series, their positions in the series (Fig. S2) are also of significance, providing an additional possibility to confirm compound identity.

Although most of the identifications proposed by the MS library were well supported by the retention index calculation, some deviations were observed. We find it surprising that compound No. 19 (Table 1) with its recorded mass spectrum characterized by the molecular ion (M^+) at $m/z = 112$ (Fig. 2B) and $LRI_{exp} = 978$ on DB-5 column was tentatively identified by the NIST MS library as 5-ethylfuran-2(5*H*)-one (i.e. in accordance with two previously reported assignments [9,10]) but did not match the retention index of the synthesized 5-ethylfuran-2(5*H*)-one ($m/z = 112$, $LRI = 1054$ on DB-5 column). Therefore, the peak corresponding to compound No. 19 was isolated from the mixture by preparative GC using secretions produced by twenty insect individuals. The isolated compound (≈2 mg) was identified as (*E*)-4-oxohex-2-enal from its NMR spectra. The verification that the compound was indeed (*E*)-4-oxohex-2-enal and not 5-ethylfuran-2(5*H*)-one was obtained from a comparison of the MS and retention time data of isolated compound No. 19 with those of synthesized (*E*)-4-oxohex-2-enal and 5-ethylfuran-2(5*H*)-one samples. The findings that (*E*)-4-oxohex-2-enal is one of the major constituents while 5-ethylfuran-2(5*H*)-one is entirely absent in the *G. lineatum* secretions are in strong disagreement with the reported results [9,10]. As demonstrated in Fig. 2B and C, the mass spectra of (*E*)-4-oxohex-2-enal and 5-ethylfuran-2(5*H*)-one are virtually identical. Therefore, the commercial MS database searching and matching alone is insufficient to distinguish between these structural isomers.

The inventory of the compounds identified in the *G. lineatum* secretions as presented in Table 1 shows a preference of the *Graphosoma* species to synthesize oxygen-containing compounds together with aliphatic hydrocarbons, which conforms to the general pattern for other Pentatomoidea [4,6]. Of the 57 compounds identified in this study, the compounds previously detected in *G. lineatum* secretions include some *n*-alkanes, alk-2-enals, alk-2-enols, alk-2-enyl acetates and limonene [8–10]. These compounds, along with some dienal isomers [44,45] and other compounds, such as e.g. (*E*)-hex-2-enyl butyrate, tridec-1-ene [44], 3-methyltridecane, cyclohexane-1,4-dione [46] and even (*E*)-4-oxohex-2-enal [6,47–49], were also occasionally found in other heteropteran bug families [50–52]. However, only approximately one third of the volatiles listed in Table 1 have been previously reported as constituents of *G. lineatum* secretions, while 39 (Table 1, compounds in bold) of the 57 identified volatiles are reported here for the first time.

The recognition and identification of α,β-unsaturated oxo aldehydes (*E*)-4-oxohex-2-enal and (*E*)-4-oxopent-2-enal in the volatile secretion of both *G. lineatum* sexes appear to be particularly important and indicate that these compounds might be more significant as defensive components of adult *G. lineatum* bugs than has been suggested from the earlier data [7–10]. As a component of defensive secretion of *Thasus neocalifornicus* (Coreidae), (*E*)-4-oxohex-2-enal was shown to be highly toxic to predators such as mantids and tarantulas [55] and is generally known for its mutagenic and cytotoxic properties by reacting with deoxyguanosine [53,54]. On the other hand, (*E*)-4-oxopent-2-enal, has not yet been reported in the volatile secretions within the Pentatomidae family or other heteropteran bugs. The identification of (*E*)-4-oxopent-2-enal using GCxGC/TOF-MS demonstrates the resolving power of this technique. Although (*E*)-4-oxopent-2-enal (#18, Fig. 2A) co-eluted with much more abundant (*E*)-hex-2-enal (#4, Fig. 2A) in

Table 1
The identification of oxygen-containing compounds and hydrocarbons in the volatile secretions of *G. lineatum* adults.

ID	1D RT[a] (min)	2D RT[a] (s)	Compound[b]	LRI_{exp}[c]	S[d]	U[e]	Method[f]	% peak area[g]	
								Male	Female
Aldehydes									
1	8.23	2.08	*n*-Hexanal*	804	880	56	A,B,C	0.1 (0.07)	0.1 (0.06)
2	14.92	2.32	***n*-Nonanal***	1108	974	57	A,B,C	0.2 (0.04)	0.2 (0.14)
3	7.45	2.29	**(*E*)-Pent-2-enal***	765	853	84	A,B,C	tr[h]	tr[h]
4	9.27	2.31	(*E*)-Hex-2-enal*	851	948	43	A,B,C	5.0 (1.12)	4.2 (2.44)
5	11.67	2.38	(*E*)-Hept-2-enal*	959	853	83	A,B,C	0.2 (0.10)	0.1 (0.07)
6	14.20	2.40	(*E*)-Oct-2-enal*	1074	836	70	A,B,C	3.2 (1.00)	4.1 (1.08)
7	16.13	2.47	(*E*)-Non-2-enal*	1163	924	70	A,B,C	0.9 (0.41)	0.7 (0.44)
8	18.43	2.49	(*E*)-Dec-2-enal*	1275	863	70	A,B,C	20.7 (7.72)	22.3 (2.70)
9	20.18	2.53	**(*E*)-Undec-2-enal**	1363	847	70	B,C	5.8 (1.86)	5.3 (3.29)
10	22.15	2.54	**(*E*)-Dodec-2-enal***	1473	884	70	A,B,C	0.1 (0.05)	tr[h]
11	8.20	2.13	**(*Z*)-Hex-3-enal**	802	941	41	B,C	0.2 (0.06)	0.3 (0.20)
12	14.73	2.38	**(*E*)-Non-4-enal**	1099	867	67	B,C	0.3 (0.06)	0.3 (0.07)
13	18.00	2.21	(*Z*)-Dec-2-enal*	1254	835	70	A,B,C	1.6 (0.38)	2.1 (0.87)
14	16.90	2.46	**(*Z*)-Dec-4-enal**	1197	850	55	B,C	4.9 (2.99)	4.2 (2.05)
15	10.65	2.51	**(*E,E*)-Hexa-2,4-dienal***	913	904	81	A,B,C	0.7 (0.62)	0.3 (0.23)
16	15.18	2.57	**(*E,E*)-Octa-2,4-dienal** *	1119	819	81	A,B,C	tr[h]	tr[h]
17	19.07	2.67	**(*E,E*)-Deca-2,4-dienal***	1325	836	81	A,B,C	1.3 (0.99)	1.6 (0.99)
18	9.42	2.89	**(*E*)-4-Oxopent-2-enal**	863	805	98	C	1.2 (0.53)	1.3 (0.48)
19	12.15	3.05	**(*E*)-4-Oxohex-2-enal***	978[i]	790	112	A,B,C	22.9 (3.57)	23.8 (0.48)
Ketones									
20	14.60	2.39	**Nonan-2-one***	1093	884	58	A,B,C	0.5 (0.26)	0.6 (0.46)
21	14.27	2.98	**Acetophenone***	1078	783	105	A,B,C	tr[h]	tr[h]
22	22.57	2.37	**Tridecan-2-one***	1499	799	58	A,B,C	0.1 (0.03)	0.1 (0.02)
23	13.27	3.21	**Cyclohex-2-ene-1,4-dione**	1033	836	110	C	0.9 (0.09)	0.7 (0.11)
24	13.25	3.10	**Cyclohexan-1,4-dione***	1032	866	97	A,B,C	0.5 (0.06)	0.3 (0.11)
25	10.67	2.82	**2-Acetylfuran***	914	801	95	A,B,C	0.1 (0.03)	tr[h]
26	11.10	2.42	**3-Methylpent-3-en-2-one**	923	796	55	B,C	tr[h]	tr[h]
27	11.13	2.33	**(*E*)-Hept-3-en-2-one**	936	870	55	B,C	0.1 (0.09)	0.1 (0.07)
28	13.20	2.47	**4-Methylhex-4-en-3-one**	1023	801	55	C	0.3 (0.05)	0.4 (0.08)
Esters									
29	10.67	2.29	**(*E*)-Pent-2-enyl acetate***	914	826	43	A,B,C	0.1 (0.02)	0.1 (0.01)
30	15.00	2.25	**Heptyl acetate***	1110	864	43	A,B,C	0.2 (0.11)	0.1 (0.02)
31	15.82	2.41	**(*E*)-Hept-2-enyl acetate***	1149	779	67	A,B,C	0.3 (0.04)	0.2 (0.10)
32	17.13	2.45	(*E*)-Oct-2-enyl acetate*	1209	873	54	A,B,C	4.1 (0.37)	2.3 (0.27)
33	20.93	2.48	(*E*)-Dec-2-enyl acetate*	1406	916	54	A,B,C	4.2 (2.39)	6.1 (2.76)
34	16.77	2.35	**(*Z*)-Hex-2-enyl butanoate**	1193	839	57	B,C	0.9 (0.25)	0.7 (0.13)
35	20.58	2.37	**(*Z*)-Dec-3-enyl acetate***	1388	866	43	A,B,C	0.3 (0.06)	0.2 (0.07)
36	22.73	2.52	**(*E*)-Undec-2-enyl acetate***	1506	920	82	A,B,C	tr[h]	tr[h]
Alcohols									
37	9.68	2.16	(*E*)-Hex-2-en-1-ol*	869	834	41	A,B,C	0.2 (0.14)	0.1 (0.05)
38	13.22	2.22	**2-Ethylhexan-1-ol***	1032	882	57	A,B,C	0.3 (0.09)	0.3 (0.23)
39	15.50	2.51	(*Z*)-Oct-2-en-1-ol	1134	762	57	C	tr[h]	tr[h]
40	16.47	2.29	***n*-Nonanol***	1177	862	70	A,B,C	tr[h]	tr[h]
41	14.20	2.20	**Dihydromyrcenol***	1074	847	59	A,B,C	0.1 (0.03)	0.1 (0.01)
Hydrocarbons									
42	14.80	2.00	*n*-Undecane*	1102	952	71	A,B,C	1.2 (0.01)	1.2 (0.10)
43	16.97	2.01	*n*-Dodecane*	1202	912	170	A,B,C	2.4 (1.30)	2.3 (0.88)
44	19.00	2.03	*n*-Tridecane*	1305	869	71	A,B,C	7.1 (6.36)	6.5 (0.31)
45	20.87	2.03	*n*-Tetradecane*	1401	935	57	A,B,C	0.3 (0.18)	0.2 (0.12)
46	22.62	2.04	*n*-Pentadecane*	1502	927	57	A,B,C	0.3 (0.10)	0.2 (0.01)
47	24.33	2.05	***n*-Hexadecane***	1602	902	57	A,B,C	tr[h]	tr[h]
48	25.93	2.05	*n*-Heptadecane*	1698	869	57	A,B,C	tr[h]	tr[h]
49	10.45	1.84	**(*E*)-Non-2-ene**	902	891	56	B,C	tr[h]	0.1 (0.07)
50	18.88	2.14	**Tridec-1-ene***	1299	904	55	A,B,C	2.0 (1.70)	2.0 (0.85)
51	20.72	2.05	**(*E*)-Tetradec-4-ene**	1394	910	57	B,C	0.1 (0.04)	0.1 (0.05)
52	20.33	1.99	**3-Methyltridecane**	1374	885	57	B,C	0.2 (0.07)	0.1 (0.03)
53	13.40	2.13	Limonene*	1038	804	68	A,B,C	0.1 (0.02)	0.1 (0.05)
54	6.07	1.84	**Benzene***	701	834	78	A,B,C	0.2 (0.02)	0.2 (0.02)
55	24.00	2.46	**Nonylbenzene***	1583	841	92	A,B,C	tr[h]	tr[h]
Others									
56	6.58	1.82	**2-Ethylfuran***	727	889	81	A,B,C	2.3 (0.35)	1.9 (1.11)
57	6.95	1.94	**2-Vinylfuran**	744	867	65	B,C	2.6 (0.53)	3.0 (0.47)

[a] The 1D-RT and 2D-RT retention times in the first and second dimension, respectively.
[b] The compounds identified for the first time in *G. lineatum* secretions are in bold while those identified using standard compounds are marked by an asterisk.
[c] The retention indices on the first-dimension DB-5 column determined using C_8–C_{20} *n*-alkanes as references.
[d] Leco's similarity factor of the unknown compared with the spectrum of the MS database.
[e] Unique mass ions (identified by the automated data processing).
[f] The method used for the identification: A, the mass spectrum and retention index were consistent with those of an authentic standard; B, the mass spectrum and retention index were consistent with those of the NIST database; C, the mass spectrum was consistent with that of the NIST database (tentative identification).
[g] The relative amount of each component was determined as the percentage of the total and reported as the mean ($N = 8$) with standard deviation (in parentheses).
[h] tr: traces, <0.08%.
[i] Ref. [44] reports a LRI value of 976 measured on a CP Sil 8 column.

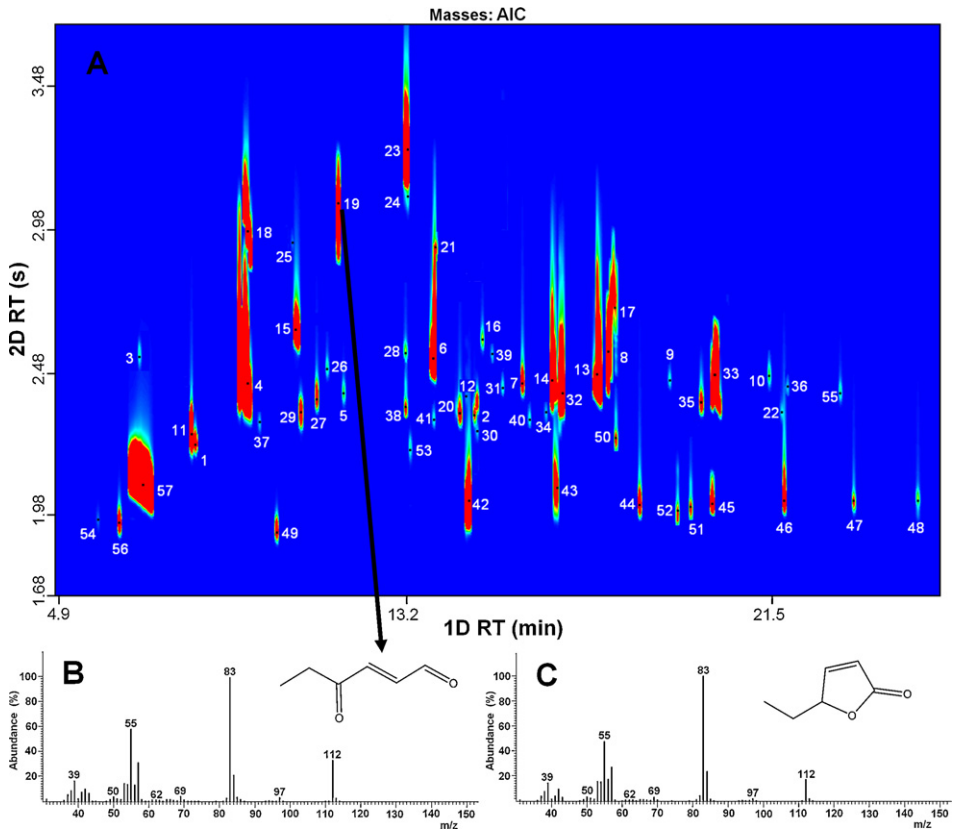

Fig. 2. An analytical ion chromatogram contour plot showing the 2D distribution of the volatile oxygenates and hydrocarbons in the secretions of an adult *G. lineatum* male (A), the mass spectra of the isolated (*E*)-4-oxohex-2-enal (B) and synthesized 5-ethylfuran-2(5*H*)-one (C); the numbered peaks relate to the ID numbers of the compounds listed in Table 1.

the first dimension (non-polar column) and was only partly separated in the second dimension (polar column), clear spectra of both compounds were obtained by applying ChromaTOF software deconvolution procedure (Fig. S3).

3.3. Semi-quantitative comparison of male vs. female secretions

Our main primary aim was to verify whether there are sex-specific differences in the composition of the volatile secretions by applying a qualitative screening method. We followed a common practice [43,56] of using the deconvoluted Total Ion Chromatogram (TIC) signal for semi-quantification along with the use of an internal standard, an approach frequently applied in cases where reference materials are not easily available and/or their cost is considerably high. A compound-specific unique mass was not exploited for integration and semi-quantification due to the inability of the ChromaTOF software to constantly assign the same unique mass to an identical compound for the same set of analyzed samples in repeatedly performed experiments. Previous studies [16,57] have already described this phenomenon. Since the TIC-based semi-quantification can produce distorted results in case of chromatographic co-elutions (which cannot be completely avoided even with GC × GC), we used deconvoluted TIC peak areas for the semi-quantification of the compounds (an example is shown in Fig. S4). Thus, peak areas of the compounds identified from the headspace of live bugs ($N = 8$ for each sex) were integrated from the deconvoluted TIC profiles and normalized by dividing each peak area by that of the internal standard (*n*-decanal) in corresponding runs. In order to reduce the within-sample variance, data were further pre-processed expressing the relative peak areas to *n*-decanal as a percentage of the total area; this kind of "internal normalization" should correct for the variance associated with the sampling steps.

To determine whether the relative amounts of the 57 selected peaks had undergone any statistically significant fluctuation when changing the bug sex type, we compared the distribution of the $\log_{10}$-transformed relative raw percentages of all 57 peaks for eight individuals of each sex type using the Kolmogorov–Smirnov test ($\alpha \geq 0.05$). Supporting Information Table S1 summarizes the logarithms of relative peak areas obtained for each compound identified in the secretions of eight male and eight female bugs. The results (DN = 0.0855, two-sided large sample K–S statistic = 1.2914 and approximate *P* value = 0.07119) showed no statistically significant difference between the two distributions at a confidence level of 95%. However, potential qualitative (or quantitative) differences between trace components in the secretions would not have been detected in the current study, since chemicals were only included in the analyses if their relative concentration in the mixture was ≥0.1%. Table 1 shows the semi-quantitative data (percentage of total volatile composition) calculated for each of the volatiles and samples under study.

In the absence of establishing a headspace equilibrium or access to reliable relationships between the chromatographic peak response and absolute abundance of all of the components in the headspace or access to isotope-labeled MS standards, the relative percent peak areas listed in Table 1 can only be regarded as a fingerprint to make a semi-quantitative comparison of individual compounds in the male and female samples.

The results of this study showed that the predominant secretion components identified in both sexes of adult *G. lineatum* were (Table 1) in the order of the greatest proportion: (*E*)-dec-2-enal ≈ (*E*)-4-oxohex-2-enal (~20–23% relative peak area of 57 identified compounds as sampled by HS-SPME) > *n*-tridecane ≈

(*E*)-undec-2-enal (~6–7%), followed by (*E*)-dec-2-enyl acetate and (*Z*)-dec-4-enal (4–6%). Remarkably, the rough percentages of (*E*)-dec-2-enal, (*E*)-4-oxohex-2-enal and *n*-tridecane estimated here are similar to those found for the same compounds (described also as major components) in defensive secretions of the neotropical stink bug *Chinavia ubica* [45].

Although the results reported here are indicative rather than quantitative, they provide a further insight into the possible function of *G. lineatum* secretions. Considering (i) the lack of distinct differences between the sexes of *G. lineatum* in the chemical character and relative percentages of the volatile secretion components, (ii) the secretion is released in response to disturbance and (iii) at least some of the secretion components are known irritants (e.g. (*E*)-alk-2-enals, *n*-tridecane) or toxic compounds (α,β-unsaturated oxo aldehydes) [54,55], our results support the hypothesis [5,21] that the secretion primarily acts to deter predators. The quantification of major components will be a major issue for future work.

4. Conclusions

The HS-SPME analysis coupled with GC × GC/TOF-MS has been evaluated as an effective method for profiling the headspace composition of the total volatile excretions of the model stink bug *G. lineatum*, which minimizes the creation of secondary products. To our knowledge, 39 of the 57 identified volatiles have never been described in the secretion of *G. lineatum* before.

The recognition and identification of a relatively large amount of (*E*)-4-oxohex-2-enal in adult bugs indicate a previously unrecognized significance of this compound in adult *G. lineatum* secretions. Also, the identification of a variety of new minor constituents by means of our approach is a convincing illustration of its potential. Since the approach used was shown to avoid the chemical modifications and artifact generations that can occur in conventional methods, it seems to be useful for the routine characterization of a wide variety of stink-bug secretions. More work should be done especially in the standardization of the procedures in order to reduce variability and compare the results from different laboratories in a more efficient way. Further work focused on the analysis of the volatile secretion profiles of other heteropteran bugs, including the more economically important species, is currently under way.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.11.043.

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