Analysis of defensive secretion of a milkweed bug *Lygaeus equestris* by 1D GC-MS and GC×GC-MS: sex differences and host-plant effect

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Abstract

The composition of defensive secretion produced by metathoracic scent glands was analysed in males and females of a milkweed bug *Lygaeus equestris* (Hemiptera: Heteroptera: Lygaeidae) using gas chromatography with mass spectrometric detection (GC-MS). The bugs were raised either on cardenolide-containing *Adonis vernalis* or on control sunflower in order to determine whether the possibility to sequester cardenolides from their host plants as an alternative antipredatory defence would affect the composition of defensive scent-gland secretion. Separation of the secretion using one-dimensional GC-MS combined with a low-polar SLB-5ms column was not sufficiently effective, and especially substances with high relative contents were co-eluted. Use of a method of comprehensive two-dimensional GC hyphenated with MS with an orthogonal polarity columns enabled separation and identification of a substantially greater number of substances, including those formerly co-eluted, i.e. (*E*)-2-octen-1-ol and (*E*)-2-octenal, decanal and 3-octen-1-ol acetate, (*E,E*)-2,4-hexadienyl acetate, 2,6-dimethyl-7-octen-2-ol and 1-octanol, which play important roles in antipredatory defence and intraspecific communication. Profiles of the composition of defensive secretions of males and females raised on sunflower were closely similar, with predominant presence of (*E*)-2-octenal, (*E*)-2-octen-1-ol, decanal and 3-octen-1-ol acetate. The secretion of bugs raised on *Adonis* was more sexually dimorphic, and some chemicals, e.g. (*E,E*)-2,4-hexadienyl acetate and 2-phenylethyl acetate were dominant in males, but absent in females. Compared to bugs from sunflower, bugs raised on *Adonis* exhibited lesser willingness to release scent-gland secretion. This corresponded with the lower overall intensity of the peaks obtained for chemicals detected in the scent-gland secretion of the bugs raised on *Adonis* and with the absence of some chemicals that have supposedly antipredatory function, e.g. (*E*)-2-hexenal, (*E*)-4-oxo-hex-2-enal, 2,4-octadienal. Taken together, our results suggest that there might be a trade-off between the two lines of

chemical defence, with the bugs raised on toxic host plants investing less to the defensive components of their scent-gland secretion.

Introduction

Chemical defense belongs to the most widespread antipredatory strategies [1,2] and particularly insects employ a diverse arsenal of chemicals to defend themselves against predators [3-5]. Among insects, the true bugs (Hemiptera: Heteroptera) are characterized by ancestral and almost universal presence of chemical defenses, as well as by their diversity across various true bug taxa and frequent association with aposematic coloration and mimicry [6,7]. Chemical defense of adult true bugs consists in a secretion produced by metathoracic scent glands and released upon an attack of a predator [8]. The scent-gland secretion of true bugs is a complex mixture of compounds, some of which could be toxic or irritating to predators, others may act as chemical warning signals perceived either by smell (highly volatile compounds) or by taste or chemesthesis [6,9,10]. Short-chain aldehydes, oxo-aldehydes, ketones, alcohols, organic acids, esters etc. occur most frequently in defensive secretion of various true bug taxa [6,11,12]. The composition of the scent-gland secretion is nevertheless taxon-specific and sexually dimorphic [13], and also differs between adults and larvae, which possess a different scent-gland system than the adults (dorsoabdominal scent-glands) [14]. Moreover, the relative content of individual compounds may depend on the season, physiological state and diet [15].

Several procedures have been proposed for sampling volatile secretions of true bugs, most of which require killing the insects (for dissection and extraction of sufficient quantities of secretion) [14,16-19]. These methods are especially suitable for the study of large species (e.g. shield bugs, Pentatomidae) of which only a few individuals are needed [12,20,21]. Several studies have been focused on non-lethal sampling methods, but these procedures usually do not involve any irritation of the insects that would simulate a predator attack and ensure the discharging of the defensive secretion [19,22]. A method when an individual is squeezed with tweezers has been used [23], but the highly volatile analytes can be lost before the individual is transferred to a container with SPME fiber. In our previous study [11], a non-lethal sampling method based on irritation of

true bugs by pressing them with the plunger of a syringe has been developed and successfully applied to the analysis of defensive secretions of *Pyrrhocoris apterus, Pyrrhocoris tibialis* and *Scantius aegyptius* (Heteroptera: Pyrrhocoridae) by gas chromatography with mass spectrometric detection (GC-MS), combined with a mid-polar column (Rtx-200). This sampling procedure is simple, involves negligible loss of volatile compounds and effectively simulates an attack a vertebrate predator (e.g., bird or lizard).

Defensive secretion of true bugs is a highly complex mixture of diverse compounds demanding high separation efficiency. GC-MS is the most appropriate technique for the identification of these compounds. Nevertheless, one-dimensional GC-MS (1D GC-MS) can encounter difficulties with the separation and identification of all the micro- and macro-components during one analysis [24]. Therefore, comprehensive two-dimensional GC hyphenated with MS (GC×GC-MS) could be more effective. This technique allows sample separation simultaneously on two columns of orthogonal polarities so that almost co-eluting peaks could be separated [25]. Commonly, a non-polar column is used for the first dimension and a short, polar one (usually a polyethylene glycol phase) for the second dimension [26]. The most efficient systems are the GC×GC systems equipped with a cryogenic modulation technique, which utilize repeated freezing and evaporating steps of sample fractions eluting from the first column as a sampling procedure for the second separation column [27].

In addition to endogenously produced scent-gland defensive secretion, some species of true bugs sequester toxic cardenolides (cardiac glycosides) from their host plants to protect themselves against predators [28-30]. Cardenolides are specific inhibitors of a membrane-bond enzyme Na/K-ATPase, which is essential for many physiological functions in animals, and they are therefore highly toxic for a wide range of animal taxa [31]. Several insect taxa have nevertheless evolved a physiological resistance thanks to the strongly reduced binding affinity of their Na/K-ATPase to cardenolides [32,33], and they are able to employ cardenolides as an

antipredatory chemical defense. Sequestration of host-plant cardenolides is characteristic for milkweed bugs (Heteroptera: Lygaeidae: Lygaeinae) [30,34,35]; for example, *Oncopeltus fasciatus* sequesters cardenolides from the seeds of *Asclepias* spp. (Apocynaceae; [36]). Sequestered cardenolides are stored in a special system of elaborate subcuticular storage compartments [34,35], from which they are released upon an attack of a predator.

Remarkably, sequestration of cardenolides is a widespread and ancestral trait in the Lygaeinae and occurs also in the Palaearctic species *Lygaeus equestris* [33]. Although *L. equestris* has a specific association with *Vincetoxicum hirundinaria* (Apocynaceae), its host-plant spectrum includes various other plants [37]. Among them, a cardenolide-producing *Adonis vernalis* (Ranunculaceae) is an important host plant for *L. equestris*, and the bugs can sequester cardenolides from this evolutionary novel host-plant species. Milkweed bugs, including *L. equestris*, are characterized by their red-and-black aposematic color patterns, and they are unpalatable for both vertebrate [9,38] and arthropod [39,40] predators. As both the synthesis of defensive chemicals and their sequestration from host plants are generally regarded as being costly for herbivorous insects [1,41]; the recent meta-analysis [5] supports this view only for synthesized chemicals though, it is likely that in those species that use both lines of chemical defense, there would be a trade-off between investments into each of them. In polyphagous species, the individuals feeding on a toxin-containing host plants may thus invest less into the synthesis of autogenous defensive chemicals.

In this study, we have analyzed the secretions of metathoracic scent glands in males and females of a milkweed bug *L. equestris* by 1D GC-MS and GC×GC-MS in combination with a commercial cryogenic modulator. A non-lethal method of mechanical irritation of the true bugs and a solid phase microextraction (SPME) [11] was used for secretion sampling. Specifically, we have compared the composition of secretion between the bugs raised either on a toxic, cardenolide-containing host plant *A. vernalis* or on a control sunflower to test (1) whether there

are any intraspecific differences in composition of the secretion connected with feeding on different host plants, and (2) whether the potential differences would indicate a trade-off between the chemical defense based on autogenous scent-gland secretion and the defense based on sequestered host-plant cardenolides.

Materials and Methods

Instrumentations, separation conditions and evaluation

The 1D GC-MS analyses were performed using a gas chromatograph with a quadrupole mass spectrometer GCMS-QP2010 Ultra instrument (Shimadzu, Kyoto, Japan). The instrument was equipped with a mid-polar Rtx-200 column - 20 m × 0.15 mm i.d., 0.15 μm film thickness (trifluoropropylmethyl polysiloxane stationary phase, Restek, USA) or with low-polar SLB-5ms column - 30 m × 0.25 mm i.d., 0.25 μm film thickness (5% dipheny/95% polydimethylsiloxane stationary phase, Supelco, USA). Helium (99.999%, Linde, Czech Republic) was used as a carrier gas at a constant linear flow rate of 35 cm s^{-1}. Splitless-mode injection (1 min) with SPME liner at 250 °C was employed for analysis of the true bug's secretion. For measurement of the standard solutions, 1 μl of the sample was injected by an auto-sampler (AOC-20i, Shimadzu) in the split mode (split ratio, 1:50).

The oven temperature gradient was set as follows: 35 °C was held for 3 min, ramped at 5 °C min^{-1} to 130 °C, then ramped at 20 °C min^{-1} to 300 °C and maintained for 5 min (the total run time was 35.50 min).

The mass spectrometer was operated in the full scan mode (*m*/*z* 35 - 500). The ion source and interface temperatures were 200 and 250 °C, respectively.

A Shimadzu gas chromatograph with quadrupole mass spectrometer GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) equipped by a cryogenic modulator Zoex ZX1 (Zoex Corporation, Houston, USA) with a two-stage loop liquid nitrogen modulator was used for the GC×GC-MS

analyses. The low-polar column SLB-5ms - 30 m × 0.25 mm i.d., 0.25 μm film thickness (5% diphenyl/95% polydimethylsiloxane stationary phase, Supelco, USA) was used for the first dimension and polar Supelcowax 10 - 1 m × 0.1 mm i.d., 0.1 μm film thickness (poly(ethylene glycol) stationary phase, Supelco, USA) for the second dimension. Another 2 m of the second dimension column was used as a loop.

The cryogenic modulation conditions were: 400 kPa inlet column pressure, modulation period 4 s, duration of hot pulse 375 ms and temperature 350 °C of hot jet. All the other parameters were the same as for the 1D GC-MS measurement.

1D GC and GC×GC data were collected and evaluated by GCMS solution software version 2.7 (Shimadzu, Kyoto, Japan) and transformation and visualization of GC×GC data were carried out by GC Image software (version 2.0, Zoex Corporation, Houston, USA).

Secretion components were identified by comparing the obtained spectra with those in the NIST 2008 Mass Spectra Library, and the identification was confirmed by comparing the retention times and indices and mass spectra with those obtained from measurements of reference compounds. Identification by library reference was used for substances for which a standard was not available, but only if the match factor in the NIST 2008 database was higher than 85%. OriginPro 2015 (Origin Lab corporation, Northampton, MA, USA) software were used for data processing.

Chemicals

A mixture of *n*-alkanes (C6 – C10) dissolved in pentane used for retention index determination was purchased from Fluka (Buchs, Switzerland) and α-pinene (≥ 95 %) from Merck. Other reference compounds were supplied by Sigma-Aldrich (Munich, Germany): (*E*)-2-hexen-1-ol (≥ 95 %), (*E*)-2-octenal (≥ 94 %), (*E*)-2-nonenal (≥ 93 %), hexyl acetate (99%), 1-decyne (98%), (*Z*)-3-hexenyl acetate (≥ 98 %), (*E*)-2-decenal (analytical standard), 3-methyl-1-butanol acetate

(isoamyl acetate) (≥ 97 %), benzaldehyde (≥ 99 %), nonanal (97%), (*E*)-2-hexenal (98%), (*E*)-2-octen-1-ol (97%), decanal (≥ 98 %), 2,4-octadienal (96%), (*E,E*)-2,4-decadienal (analytical standard), (*R*)-(+)-limonene (97%), (*E,E*)-2,4-hexadienal (≥ 95 %) and diluents also from Sigma-Aldrich: dichloromethane (anhydrous, ≥ 99.8 %), pentane (anhydrous, ≥ 99 %).

Milkweed bug cultures and sample preparation

Adults of *L. equestris* were originally obtained from an *A. vernalis* habitat north of Lebus, Germany and maintained in the laboratory on sunflower (*Helianthus annuus*; sunflower further on) seeds for multiple generations at 26-28 °C, with a 16:8 h day/night cycle. The bugs were supplied with water from 2 ml Eppendorf tubes plugged with cotton. For the experiment, the eggs of *L. equestris* were transferred to a diet of either pure sunflower seeds or a mixture of suflower and seeds of *A. vernalis*. After reaching adult stage, the bugs were subjected to the chemical analysis.

A non-lethal mechanical irritation method [11] was used for sampling the defensive secretion. The bugs were stressed by pressing them with the syringe plunger, which regularly resulted in release of the scent-gland secretion. One bug individual was placed in a syringe with a barrel volume of 12.5 mL (Eppendorf, Hamburg, Germany), and the syringe was tempered (at 40 °C for 1 minute) in an incubating shaker (bioSan, Riga, Lithuania). Then the bug was carefully compressed with the plunger of a syringe. The bug could not move and the syringe tip was then closed with a rubber stopper. The compression lasted until drops of liquid appeared on ventro-lateral parts of metathorax of the bug. Then the rubber stopper was removed from the syringe tip, 5 mL of air was drawn in and a SPME fiber coated with triple phase 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) located in the manual SPME holder (Supelco, Bellefonte, PA, USA) was immediately inserted into the tip of the barrel. The syringe with the bug and the SPME fiber was placed in the incubated shaker again.

The analyte sorption on the SPME fiber lasted 90 minutes at 40 °C, to increase the vapor pressure.

The SPME fiber was thoroughly cleaned and conditioned for 15 min before and after each analysis in an external syringe oven at 250 °C under vacuum. Before each analysis of a defensive secretion, control analyses of the SPME fiber itself without the sorption part and with the sorption part in the clean vacant syringe were performed. The analyses of secretions obtained from the individual bugs within each of the four groups (males and females raised either on *Adonis* or sunflower) were carried out in triplicate. In parallel, a measurement with a non-irritated bug by the same procedure as for the defense secretion sampling (except for pressing the plunger) was performed as a blank.

Samples of the reference compounds were prepared by dilution of the individual analytes in dichloromethane, except for a mixture of alkanes which were diluted in pentane. The concentration was 0.1 mg mL^{-1} but the final dilution of each standard was individually modified to obtain good reproducibility of the retention times.

Results and discussion

One-dimensional GC-MS analysis of the *L. equestris´*s defensive secretion

For the pilot sampling of the defensive secretion of *L. equestris* males raised on sunflower seeds and its subsequent GC analysis, we employed the methodology described by Krajíček et al. [11]. As the sampling method by SPME fibre yielded peaks with very high response intensity, only one individual was used for collection of the secretion, instead of three, as described by Krajíček et al. [11]. The sample was separated on a medium polar Rtx-200 column, which is considered to be universal. As can be seen in Fig. 1, individual components of the defensive secretion were not adequately separated on this column, compared to the defensive secretions of the true bug species analysed in a previous study [11] and the spectrum had substantially fewer peaks. This

fact was most obvious in the area around 13 – 15 min, where there was only one peak in the chromatogram. According to the data obtained in separation of the standards on the Rtx-200 column under the same conditions [11], it can be assumed that substances, such as nonanal, (*E*)-2-octenal, 2,4-octadienal, or decanal, are co-eluted in this area. Consequently, in the next experiment, a substantially less polar SLB-5ms column was employed, on which much better separation was obtained for the same experimental conditions as on column Rtx-200 (see Fig. 1), and thus all the subsequent 1D GC-MS analyses were performed on this column.

The chromatograms obtained for males and females from both host plants are depicted in Fig. 2, and the composition of the defensive secretion (based on three measurements within each group) is summarised in Tab. 1.

The numbers next to the individual peaks correspond to all the identified, important/relevant components in the defensive secretions. In order to eliminate misleading results, we list in both the table and the figure individual components of some peaks, although they were identified only later by GC×GC-MS and correspond to a single peak (circled in the Fig. 2) in the 1D GC-MS system.

Two-dimensional GC×GC-MS analysis of the defensive secretion of *L. equestris*

Because of co-elution of some components of the defensive secretion in the 1D GC-MS system, analysis using GC×GC-MS in an orthogonal system of a low-polarity SLB-5ms column in the first dimension and a polar Supelcowax 10 column in the second dimension under the conditions described above was performed with the same sampling method as for 1D GC-MS. Secretions from males and females of *L. equestris* raised on toxic *A. vernalis* or control sunflower were measured, and the resultant contour plots are depicted in Fig. 3. The main components of the defensive secretion of this species were determined in the same way as for 1D GC-MS (see Tab. 2).

The use of the SLB-5ms column both in 1D GC-MS and in the first dimension of GC×GC-MS enabled subsequent comparison of the obtained spectra. The use of GC×GC-MS facilitated the identification of a larger number of analytes than the use of 1D GC-MS and, in particular, it was possible to identify the individual components of the peaks, which were co-eluted in the 1D system, exhibited the highest intensities and probably represented the most important components of the defensive secretion. This corresponded to the peak with retention time in the 1D-GC-MS system of 16.9 min, which could be separated using the GC×GC-MS system and identified as (*E*)-2-octenal and (*E*)-2-octen-1-ol at times 15.90 and 16.20 min, respectively. Unfortunately, the peak eluting at time 18.7 min (in 1D GC-MS) could not be completely separated into its individual components even using GC×GC-MS, but they could at least be identified. In this case, the substances decanal and 3-octen-1-ol acetate were identified (both at time 20.30 min in Fig. 4). The above four analytes were substances with the greatest relative contents in the defensive secretion and they are likely to play an important role in either antipredatory defence or intraspecific communication of Heteroptera. Especially aldehydes, e.g. C_6 and C_8 (*E*)-2-alkenals and (*E*)-4-oxo-2-alkenals, belong among substances that are important for defence against predators [42].

For the peak eluting in the 1D GC-MS system at time 15.5 min, the separation in the GC×GC-MS system was not sufficiently effective; however, it was possible to identify the individual components 1-octanol, 2,6-dimethyl-7-octen-2-ol and (*E,E*)-2,4-hexadienyl acetate (see the differences between sexes). On the other hand, at retention times of 12.72 and 14.78 min (for GC×GC-MS), it was possible to completely separate the pair benzaldehyde and (*E*)-4-oxo-hex-2-enal and the pair (*R*)-(+)-limonene and 2-ethyl-1-hexanol.

In the contour plot obtained using GC×GC-MS, it is possible to clearly compare the intensities in responses of individual components of the defensive secretion. The contour plots depicted in Fig. 3 were obtained under the same conditions adjusted by the evaluating GC Image software.

It is thus possible to observe marked differences in the intensities of the responses of the individual peaks between males and females and especially between individuals raised on different host plants.

Because of the usage of the same sampling method, it is possible to directly compare our results with those reported by Krajíček et al. [11] for three true bug species from the family Pyrrhocoridae. Analytes that belonged amongst the most intense and thus probably the most important components of the defensive secretion of *L. equestris* were also identified in at least one of the three pyrrhocorid species analysed in this study. These analytes were (*E*)-2-octenal, decanal and (*E*)-2-octen-1-ol. Other 13 substances that occurred in the secretion of sunflower-raised *L. equestris* matched the compounds found in at least one of the three studied pyrrhocorid species including (*E,E*)-2,4-hexadienal, 3-methyl-1-butanol acetate, 2-ethyl-cyclohexanone, 2-hexen-1-ol acetate, and undec-2-enyl acetate [11] .

The composition of metathoric scent-gland secretion was analysed in several other species of milkweed bugs (Lygaeinae), though using different methods of secretion sampling and analysis, and therefore the results are not entirely comparable. Nevertheless, short-chained aldehydes (namely (*E*)-2-hexenal and (*E*)-2-octenal), alcohols and acetates were dominant also in secretions of metathoric scent-glands in *Lygaeus kalmii*, *Oncopeltus fasciatus*, *O. cingulifer*, *O. unifasciatellus*, *Tropidothorax cruciger*, and *Spilostethus rivularis* [42-44].

Differences in the composition of defensive secretion between males and females

There were minimal differences between males and females of *L. equestris* raised on sunflower seeds. With regard to type of chemicals, the compositions were practically identical in both sexes. Differences were found especially in ratios of the intensities of the largest peaks. In males, the predominant components of the secretion were decanal and 3-octen-1-ol acetate and, compared to the females, the peak of cyclooctanol was more intense, while in females, the

predominant peaks were those corresponding to (*E*)-2-octen-1-ol and (*E*)-2-octenal. Most of the simple alcohols belong particularly in the group of insect attractants and, in various ratios, especially the above two alcohols indicate that these substances also have a pheromonal function [45]. In addition, on an average of all the measurements, about a one third larger amount of branched cyclohexanones was identified in females (these were allyl-cyclohexanones and, based on the spectral library, most probably 2-pentyl-cyclohexanone and 2-butyl-cyclohexanone). These substances are usually considered to be pheromones and mostly also smell pleasantly to humans [46].

The differences between females and males raised on seeds of *A. vernalis* were found not only in the intensity of the response of individual components, but also in the presence or absence of some of the chemicals. In females, the intensity of obtained peaks was substantially lower than in males. This phenomenon is probably caused by the fact that the amount of cardenolides sequestered from host plants by females is often greater than in males [47]. The greatest difference in the composition of the defensive secretion is represented by the peak at a time of 15.5 min (for 1D GC-MS) found only in males (almost the largest in GC×GC-MS). Identification of the components of this peak was completely impossible when using 1D GC-MS and adequate separation was not achieved even with GC×GC-MS (see Fig. 5). Most probably, three substances are co-eluted here and, on the basis of point spectra at the three most intense points in the blob, three analytes were identified as 1-octanol, 2,6-dimethyl-7-octen-2-ol and (*E,E*)-2,4-hexadienyl acetate. The last one is present in the largest amounts in this peak.

Another important difference between males and females raised on *A. vernalis* was the absence of 2-phenylethyl acetate in females' chromatograms. This compound was the fourth largest peak in the 1D GC-MS chromatograms from males, and in the GC×GC-MS contour plots it was amongst the four most intense peaks. Both these acetates, found only in males, were also found only in secretion of males of other milkweed bugs (e.g. *Neacoryphus bicrucis*) as

pheromones attracting both sexes [43,44,48]. The presence of these acetates in males raised on *A. vernalis* also supports the hypothesis that toxins sequestered from host plants suppress the defensive role of the metathoracic scent glands in milkweed bugs, and therefore the glands can serve their other function in producing pheromones [6,49].

Effect of host-plant toxicity on composition of defensive secretion

When pressed with the plunger during the secretion sampling, the bugs raised on *A. vernalis* showed a substantially low willingness to release the scent-gland secretion, and they frequently released a sequestered fluid (probably containing cardenolides; [34]) instead. This fluid was also sampled using SPME fibres in the same way as the scent-gland secretion. When the chromatogram of the sequestered fluid was compared with a blank, (see Fig. 6) only very few volatile components could be detected. According to GC×GC-MS chromatograms of sequestered fluid it was possible to identify only a small amount of octanal, nonanal, decanal and also α-pinene (α-pinene was also sometimes found in the blank and it is thus probably derived from food). Releasing the sequestered fluid as a response to pressure corresponds with the presence of an elaborate system of subcuticular storage compartments [34], which allows for the quick discharge of the fluid upon a predator attack.

More importantly, scent-gland secretion of the bugs raised on *A. vernalis* contained smaller number of chemicals compared to the secretion of sunflower-raised bugs. Moreover, for most of the chemicals that were present in secretions of bugs from both host plants, the overall intensity of the peaks obtained was lower in the bugs raised on *A. vernalis* than in the bugs from sunflower (Tab. 2, Fig. 3). Out of 13 chemicals that were dominant in the secretion of sunflower-raised bugs of both sexes (Tab. 2), only 2 (cyclooctanol and α-pinene) were equally dominant in the secretion of bugs raised on *A. vernalis*. Six others were present in much lesser amount (e.g. (*E*)-2-octenal, decanal and 3-octen-1-ol acetate) and 6 were entirely absent (e.g.

(*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, 2,4-octadienal and (*E*)-2-octen-1-ol). Contrastingly, out of the chemicals dominant in the secretion of the bugs raised on *A. vernalis*, only 4 (e.g. 2-phenylethyl acetate and (*E,E*)-2,4-hexadienyl acetate) were absent in secretion of sunflower-raised bugs, all of them being sex specific and present only in males (see above). In general, the secretion of individuals raised on *A. vernalis* contained mostly esters, variously branched alcohols, and some aldehydes and, amongst substances eluting later, also higher carboxylic acids, whereas the secretion of bugs raised on sunflower was dominated by aldehydes and oxo-aldehydes and also some alcohols and esters.

The absence (or low amount) of some of the aldehydes (3-hexenal, (*E*)-2-hexenal, (*E*)-2-octenal, heptanal, octanal, 2,4-octadienal, (*E*)-2-nonenal, (*E,E*)-2,4-decadienal) and oxo-aldehydes ((*E*)-4-oxo-2-hexenal) in the secretion of bugs raised on *A. vernalis* is striking, as they are considered to be an important constituents of true-bug antipredatory defence, being aversive [50] or toxic for arthropod predators [6,16,51] and aversive for vertebrate predators, specifically for lizards [52], geckos [53], house shrews and passerine birdsThese results suggest that there might be a trade-off between the two lines of chemical defence, with the bugs raised on cardenolide-producing host plant investing less to the defensive components of their scent-gland secretion. On the other hand, it seems that the metathoracic scent glands, which are somewhat reduced in Lygaeinae and are frequently regarded as having mostly pheromonal function [6], might represent a more flexible system that interacts with the alternative line of defence based on sequestration of host-plant chemicals.

Conclusions

Composition of the defensive secretion of males and females of a milkweed bug *Lygaeus equestris* was analysed using 1D GC-MS and GC×GC-MS techniques. The bugs were raised

either on a toxic, cardenolide-producing host plant or on a control sunflower. The secretion was sampled by slightly compressing the bugs with the plunger of a syringe and subsequent SPME technique. Separation in a 1D GC-MS system with a mid-polar column Rtx-200 column and consequently with the low-polar SLB-5ms column was not sufficiently effective for some substances, which were co-eluted. The use of GC×GC-MS with an orthogonal polarity columns enabled separation or at least identification of the formerly co-eluting substances, such as (*E*)-2-octen-1-ol, (*E*)-2-octenal, decanal, 3-octen-1-ol acetate, (*E,E*)-2,4-hexadienyl acetate, 2,6-dimethyl-7-octen-2-ol and 1-octanol, which play an important role in antipredatory defence or intraspecific communication. Dominant components in the secretion of bugs raised on sunflower were (*E*)-2-octenal, (*E*)-2-octen-1-ol, decanal and 3-octen-1-ol acetate. Chromatograms obtained for the defensive secretions of sunflower-raised males and females displayed practically the same pattern of the identified substances and differences were observed only in their relative contents. Substantially greater intersexual differences were observed in the bugs raised on *A. vernalis*. Several components were identified only in the secretion of males, particularly (*E,E*)-2,4-hexadienyl acetate and 2-phenylethyl acetate with potential pheromonal function.

The bugs raised on *Adonis* exhibited lesser willingness to release scent-gland secretion than the bugs from sunflower, and also the intensity of peaks obtained for the compounds detected in the secretion of *Adonis*-raised bugs was lower. Moreover, some of the compounds that were dominant in the secretion of sunflower-raised bugs were entirely absent in the secretion of bugs raised on *Adonis*. These included chemicals with antipredatory defensive or signalling function, e.g. (*E*)-2-hexenal, (*E*)-4-oxo-hex-2-enal, 2,4-octadienal. The results indicate a trade-off between the two lines of chemical defence, with the bugs raised on toxic host plants investing less to the defensive components of their scent-gland secretion.

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Figure 1. Comparison of chromatograms obtained by SPME-GC-MS analysis of the defensive secretion of *L. equestris* males raised on sunflower seeds performed on different columns. Sampling of the secretion was made by compression with the plunger of a syringe and SPME sorption on a DVB/CAR/PDMS fiber.

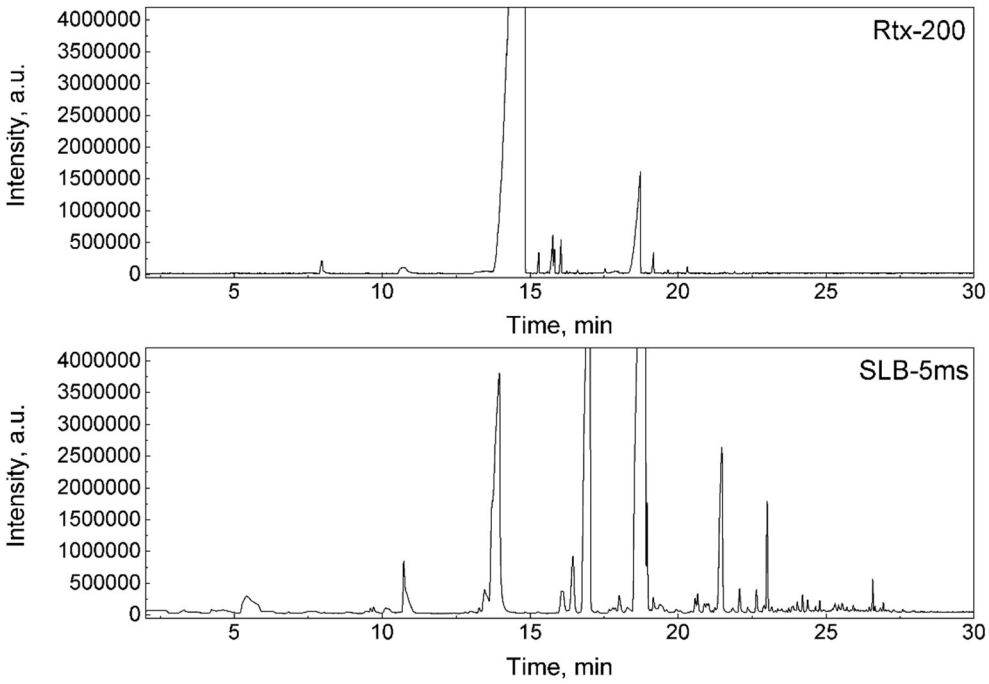
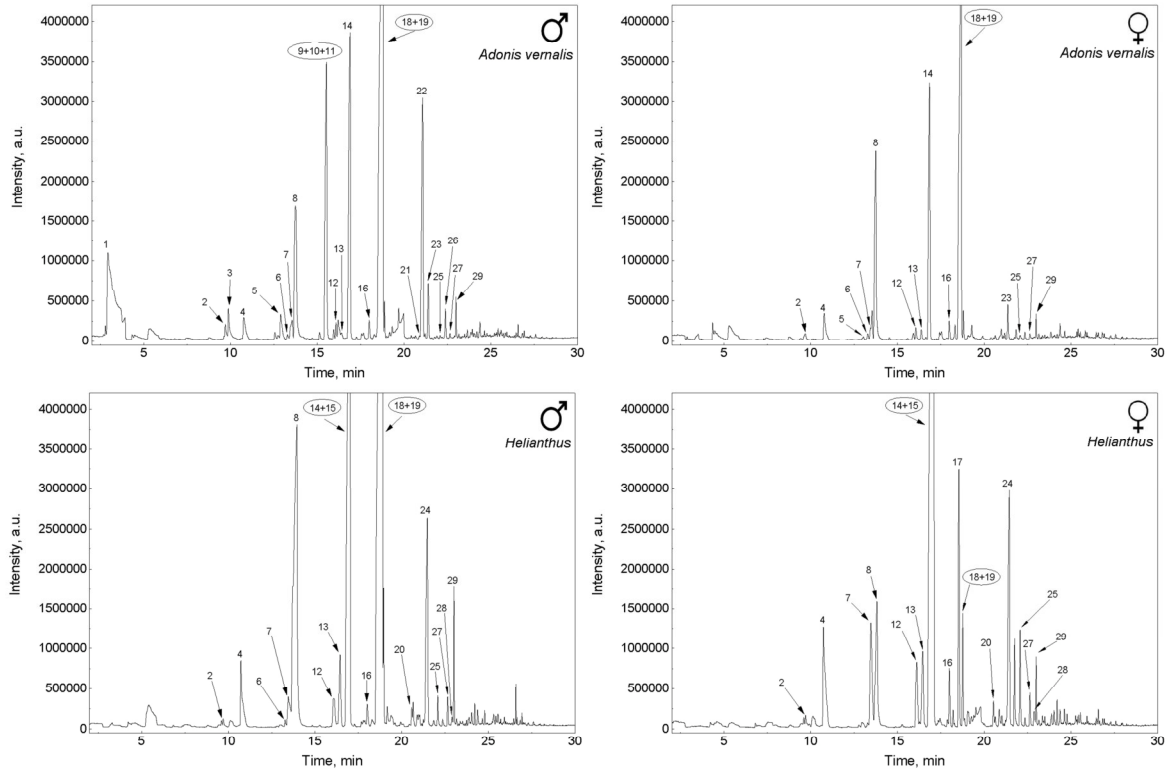


Figure 2. SPME-GC-MS analysis of the defensive secretion of *L. equestris*. Comparison of males and females raised on different host plants: toxic *A. vernalis* and control sunflower. Sampling of the secretion was made by compression with the plunger of a syringe and SPME sorption on a DVB/CAR/PDMS fiber. Numbering of the peaks corresponds to Tab. 1.



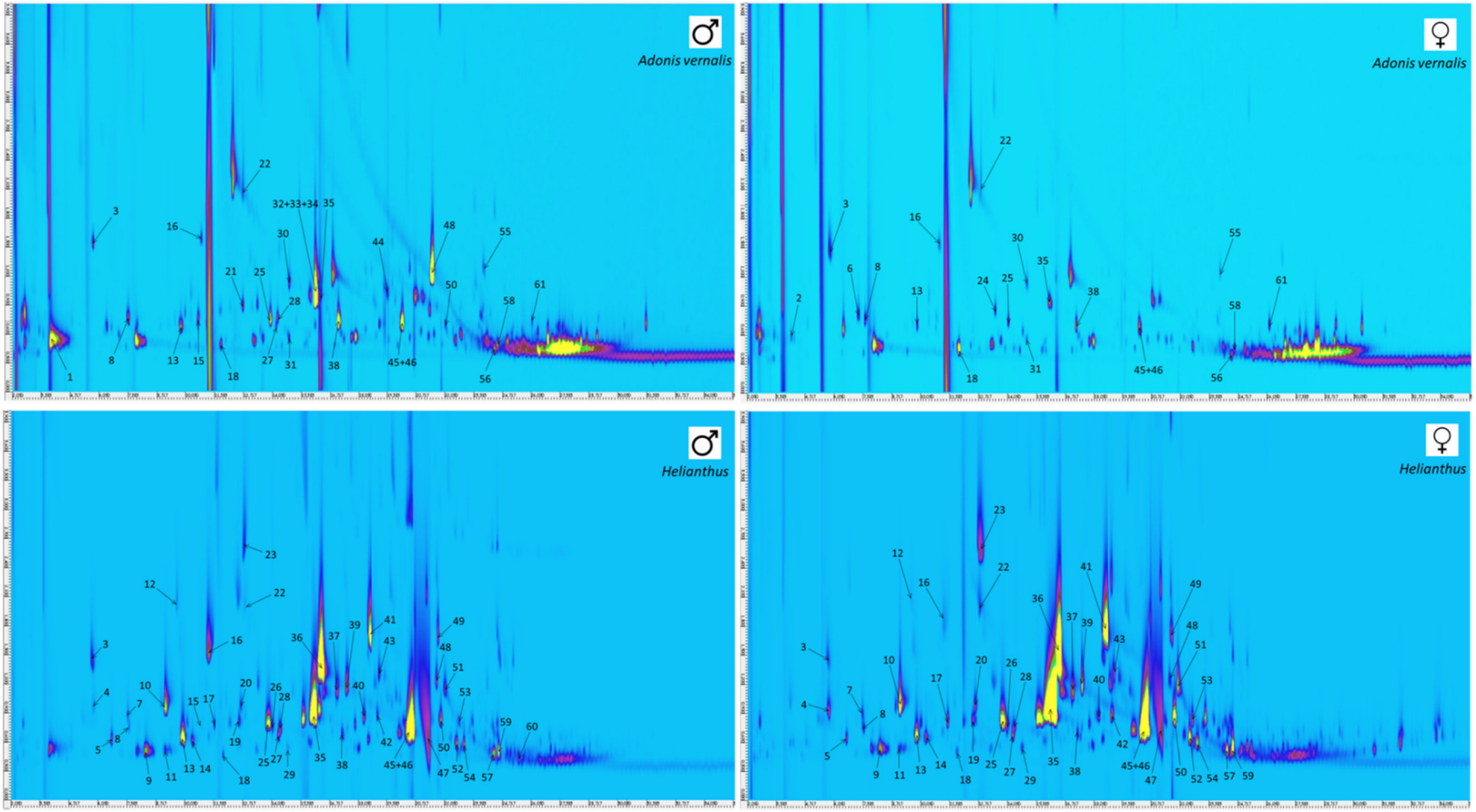


Figure 3. SPME-GC×GC-MS analysis of the defensive secretion of *L. equestris*. Comparison of males and females raised either on toxic *Adonis vernalis* or control sunflower (*Helianthus annuus*) Sampling of the secretion was made by compression with the plunger of a syringe and SPME sorption on a DVB/CAR/PDMS fiber. Numbering of the peaks corresponds to Tab. 2.

Figure 4. Cut-out of the contour plot obtained for the defensive secretion of the males of *L. equestris* raised on sunflower seeds. Identification of co-eluted compounds.

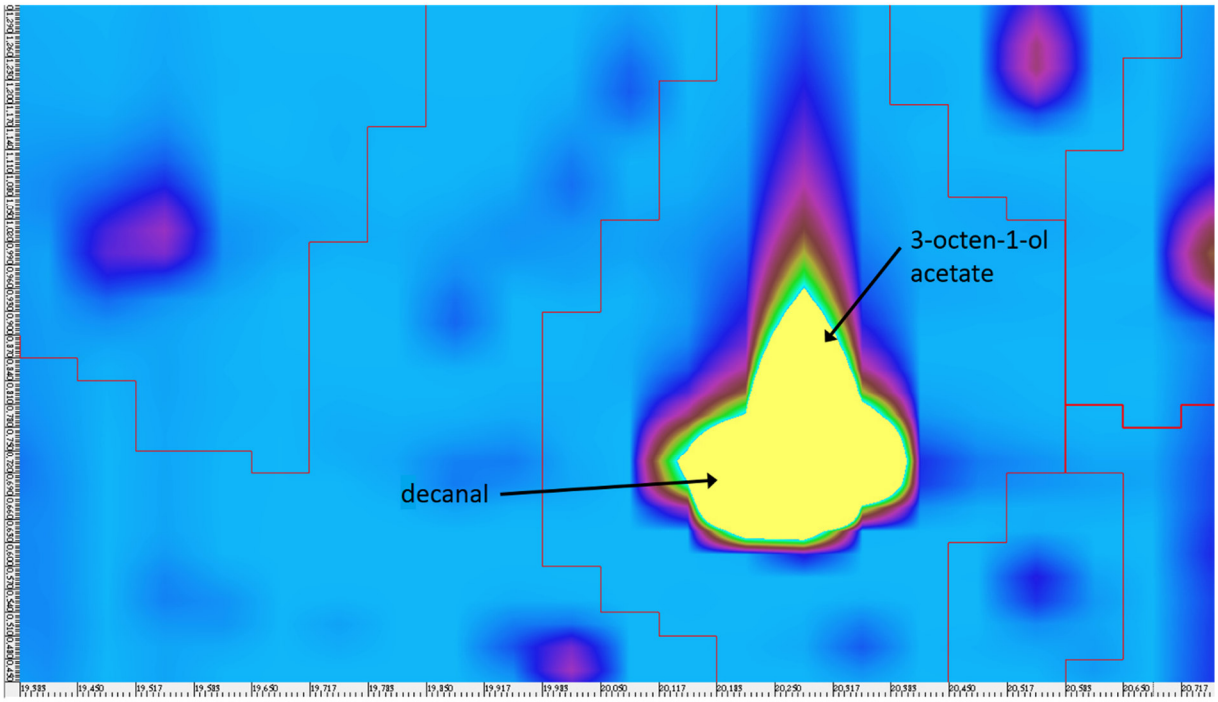


Figure 5. Cut-out of the contour plot obtained for the defensive secretion of *L. equestris* males raised on *A. vernalis* seeds. Identification of co-eluted compounds.

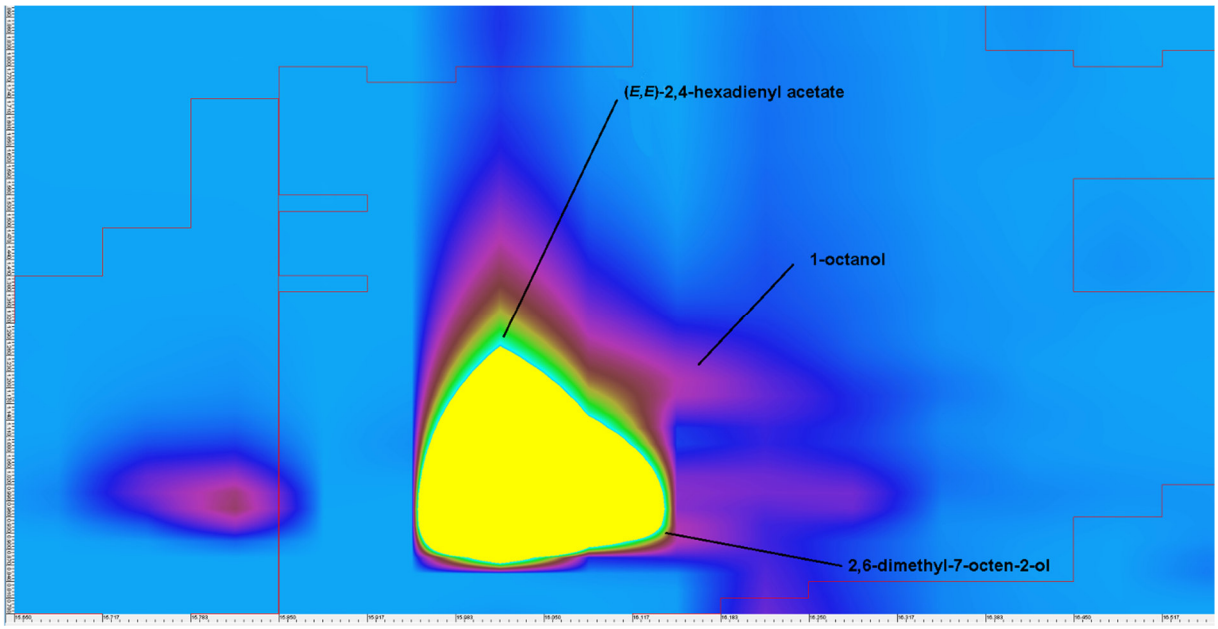


Figure 6. Comparison of the blank, sequestered fluid and scent-glands secretion. SPME-GC-MS analysis of the scent-gland secretion of the male *L. equestris* raised on sunflower (*Helianthus annuus*) seeds and the blank and the sequestered fluid released by male *L. equestris* raised on *A. vernalis*. Sampling of the secretion was made by compression with the plunger of a syringe, for blank was this step omitted. SPME sorption was made on a DVB/CAR/PDMS fiber.

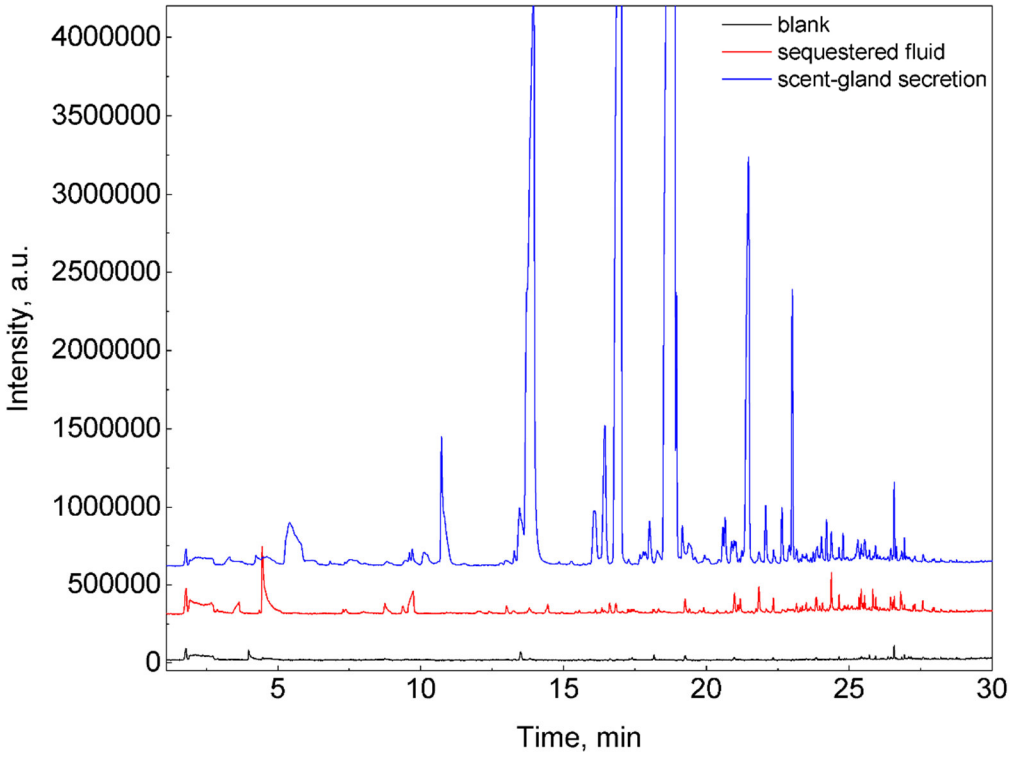


Table 1. Number of the peak in Fig. 2, retention times obtained by 1D GC-MS and identification method of the compounds identified in the defensive secretions of males and females of *L. equestris* raised either on toxic *Adonis vernalis* or control sunflower (*Helianthus annuus*).

Number of peak	**t_{ret} (min)**	**Compound**	**Male$_{Helianthus}$**	**Female$_{Helianthus}$**	**Male$_{A.vernalis}$**	**Female$_{A.vernalis}$**	**Identification**
1	3.06	1-Methyl-1,3-cyclopentadiene	-	-	++	-	B
2	9.69	3-Methyl-1-butanol acetate	+	+	+	+	A
3	9.87	2,4-Hexadien-1-ol	-	-	+	-	B
4	10.72	(*E,E*)-2,4-Hexadienal	+	++	+	+	A
5	12.88	2-Ethyl furan	-	-	+	+	B
6	13.27	(*Z*)-3-Hexenyl acetate	+	-	+	+	A
7	13.46	3-Methyl-4-methylene-hexane	+	++	+	+	B
8	13.88	Cyclooctanol	++	++	++	++	B
9		(*E,E*)-2,4-Hexadienyl acetate	-	-	+++	-	B
10	15.53	2,6-Dimethyl-7-octen-2-ol	-	-	++	-	B
11		1-Octanol	-	-	++	-	B
12	16.09	1-Ethyl-1-methyl-cyclopentane	+	+	+	+	B
13	16.46	5-Ethyl-2(5H)-furanone	+	+	+	+	B
14	16.90	(*E*)-2-Octenal	+++	+++	+++	++	A
15		(*E*)-2-Octen-1-ol	+++	+++	-	-	A

16	17.99	2,4-Octadienal	+	+	+	+	A
17	18.54	4-Ethylcyclohexanol	-	++	-	-	B
18	18.70	3-Octen-1-ol acetate	+++	++	+++	+++	B
19		Decanal	+++	++	+++	+++	A
20	20.55	2-Propyl-cyclohexanone	+	+	-	-	B
21	20.89	1-Ethyl-cyclopentanol	-	-	+	-	B
22	21.07	2-Phenylethyl acetate	-	-	++	-	B
23	21.37	3-Methyl-4-undecene	-	-	+	+	B
24	21.46	2-Ethyl-cyclohexanone	++	++	-	-	B
25	22.07	(*E*)-2-Decenal	+	+	+	+	A
26	22.39	Butanoic acid, undec-2-enyl ester	-	-	+	-	B
27	22.64	4,5-Dimethyl-2-cyclohexen-1-one	+	+	+	+	B
28	22.87	(*E,E*)-2,4-Decadienal	+	+	-	-	A
29	22.99	(*E*)-2-Decenyl acetate	++	+	+	+	B

$t_{ret_}$ is the retention time of the relevant substance, + the plus symbol indicates the presence of a compound, and the higher number of pluses indicates a larger peak area, - the minus symbol indicates the absence of the compound.

The methods used for the identification: A - retention time and mass spectrum of the relevant substance was compared with the reference compound; B - the mass spectrum of the relevant substance was compared with NIST 2008 Mass Spectra Library.

Table 2. Number of the peak in Fig. 3, retention rimes in the first and second dimension obtained by GC×GC-MS and identification method of the compounds identified in defensive secretions of males and females of *L. equestris* raised either on toxic *Adonis vernalis* or control sunflower (*Helianthus annuus*)

Number of peak	t_{ret_1} **(min)**	t_{ret_2} **(min)**	**Compound**	**Male**$_{Helianthus}$	**Female**$_{Helianthus}$	**Male**$_{A.vernalis}$	**Female**$_{A.vernalis}$	**Identification**
1	3.78	0.51	1-Methyl-1,3-cyclopentadiene	-	-	++	-	B
2	4.05	0.54	3-Methyl-butanal	-	-	-	+	B
3	5.78	1.47	3-Methyl-1-butanol	+	+	+	+	B
4	5.85	0.93	2-Methyl-2-butenal	+	+	-	-	B
5	6.58	0.63	Isobutyl acetate	+	+	-	-	B
6	7.12	0.81	2-Hexanone	-	-	-	+	B
7	7.32	0.84	3-Hexenal	+	+	-	-	B
8	7.45	0.72	Hexanal	+	+	+	+	B
9	8.25	0.51	1,3-Octadiene	+	+	-	-	B
10	9.05	0.96	(*E*)-2-Hexenal	++	++	-	-	A
11	9.05	0.51	2,4-Octadiene	+	+	-	-	B
12	9.58	2.04	(*E*)-2-Hexen-1-ol	+	+	-	-	A
13	9.92	0.63	3-Methyl-1-butanol acetate	++	++	+	+	A
14	10.30	0.59	2-*n*-Butyl-furan	+	+	-	-	B

15	10.61	0.75	Heptanal	+	-	+	-	B
16	11.05	1.53	(*E,E*)-2,4-Hexadienal	++	+	+	+	A
17	11.25	0.81	2-Methylbut-2-en-1-yl acetate	+	+	-	-	B
18	11.78	0.45	α-Pinene	+	+	+	++	A
19	12.52	0.81	3-Ethyl-phenol	+	+	-	-	B
20	12.58	0.96	(*E*)-2-Heptenal	+	+	-	-	B
21	12.72	0.87	2-Ethyl furan	-	-	+	-	B
22	12.72	2.01	Benzaldehyd	+	+	+	+	A
23	12.72	2.61	(*E*)-4-Oxo-2-hexenal	+	++	-	-	B
24	13.45	0.84	6-Methyl-5-hepten-2-one	-	-	-	+	B
25	13.85	0.75	Cyclooctanol	++	++	++	+	B
26	14.04	0.75	Octanal	+	+	-	-	B
27	14.32	0.66	Hexyl acetate	+	+	+	-	A
28	14.38	0.72	2-Hexen-1-ol acetate	+	+	+	-	B
29	14.72	0.63	5-Decyne	+	+	-	-	B
30	14.78	1.11	2-Ethyl-1-hexanol	-	-	+	+	B
31	14.78	0.51	(*R*)-(+)-Limonene	-	-	+	+	A
32	15.98	1.20	(*E,E*)-2,4-Hexadienyl acetate	-	-	+++	-	B
33	16.11	1.14	2,6-Dimethyl-7-octen-2-ol	-	-	++	-	B
34	16.12	0.90	1-Octanol	-	-	++	-	B

35	15.90	0.90	(*E*)-2-Octenal	+++	+++	++	++	A
36	16.20	1.35	(*E*)-2-Octen-1-ol	+++	+++	-	-	A
37	16.98	1.11	3-Propyl-cyclohexene	+	+	-	-	B
38	17.25	0.66	Nonanal	+	+	++	++	A
39	17.45	1.17	2,4-Octadienal	++	++	-	-	A
40	18.25	0.84	3-Nonen-2-one	+	+	-	-	B
41	18.58	1.68	2-Butyl-cyclohexanone	++	+++	-	-	B
42	18.91	0.84	(*E*)-2-Nonenal	+	+	-	-	A
43	18.98	1.00	2-Pentyl-cyclohexanone	+	+	-	-	B
44	19.38	1.29	(+)-Menthol	-	-	+	-	B
45	20.30	0.65	Decanal	+++	+++	++	++	A
46	20.30	0.91	3-Octen-1-ol acetate	+++	+++	+	++	B
47	21.12	0.63	2-Octenoic acid	++	++	-	-	B
48	21.65	1.23	2-Phenylethyl acetate	+	+	+++	-	B
49	21.72	1.68	5-Butyldihydro-2(3H)-furanone	+	+	-	-	B
50	21.78	0.78	(*E*)-2-Decenal	+	++	+	-	A
51	22.05	1.11	2-Decen-1-ol	+	+	-	-	B
52	22.45	0.63	(*E*)-Non-3-enyl acetate	+	+	-	-	B
53	22.65	0.84	(*E,E*)-2,4-Decadienal	+	+	-	-	A
54	22.92	0.57	Undec-2-enyl acetate	+	+	-	-	B

55	23.70	1.26	*n*-Decanoic acid	-	-	+	+	B
56	24.32	0.39	Tetradecane	-	-	+	+	B
57	24.32	0.45	Butanoic acid, 1-ethenylhexyl ester	+	+	-	-	B
58	24.45	0.45	Dodecanal	-	-	+	+	B
59	24.52	0.48	(*E*)-2-Decenyl acetate	+	+	-	-	B
60	25.51	0.42	1-Dodecen-3-ol	+	-	-	-	B
61	26.05	0.72	*n*-Dodecanoic acid	-	-	+	+	B

t_{ret_1} is the retention time of the relevant substance in the firts dimension, t_{ret_2} is the retention time of the relevant substance in the second dimension, + the plus symbol indicates the presence of a compound and the higher number of pluses indicates a larger peak area, - the minus symbol indicates the absence of the compound.

The methods used for the identification: A - retention time and mass spectrum of the relevant substance was compared with the reference compound; B - the mass spectrum of the relevant substance was compared with NIST 2008 Mass Spectra Library.