

**COMPOUNDS IN ABDOMINAL AND METATHORACIC SCENT
GLANDS OF NYMPHS AND ADULTS OF *GRAPHOSOMA
LINEATUM* (LINNAEUS, 1758) (HET., SCUTELLERIDAE)
UNDER LABORATORY CONDITIONS**

**Reza Farshbaf Pour Abad*, Shahrzad Azhari*,
Djavanshir Djozan** and Mir Jalil Hejazi***

* Department of Plant Protection, Faculty of Agriculture, University of Tabriz, 51664, Tabriz- IRAN. E-mail: rfpourabad@yahoo.com

** Department of Analytical Chemistry, Faculty of Chemistry, University of Tabriz, Tabriz- IRAN.

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ABSTRACT: The contents of metathoracic glands of two sexes and different ages of adult *Graphosoma lineatum* (L.) were analyzed at laboratory conditions. Insect rearing were carried out on the seeds of three species of umbelliferous plants (Parsley, *Petroselinum crispum*; Carrot, *Daucus carota*; and coriander, *Coriandrum sativum*). The scent gland compounds of 3- 15- and 25-days old adults were extracted by solid-phase microextraction (SPME) and analyzed by gas chromatography coupled with mass spectrometry (GC-MS). In this study, SPME and direct injection were applied to evaluate commercial and common fiber. The results showed that SPME method and common pencil fiber were effective in detection of scent gland extracts. Different components such as tridecane, 3-methyl pentanol, *cis*-1,2- dimethyl cyclopentane, hexane, heptane, (*E*)-2-hexenal, ethyl cyclohexane, limonene, (*Z*)-4-hexen-1-ol acetate, undecane, dodecane, (*E*)-2-decenal, 1-tridecene, tetradecane, (*E*)-5-decenyl acetate, pentadecane and hexadecane were detected in scent glands which tridecane and 3-methyl pentanol were the major components. Comparison of chromatographic peak area obtained in the same experimental conditions revealed that the abundance of some compounds varied considerably by the sex and age of insects.

KEY WORDS: Chemical analysis, *Graphosoma lineatum*, Insect, Scent glands.

Stink bugs are characterized by the production of strong-smelling defensive substances when they are disturbed (Remold, 1962; Carayon, 1971; Staddon et al., 1987; Aldrich, 1988; Aldrich et al., 1997). The stripped bug, *Graphosoma lineatum* (L.) is one of the most important pests of the umbelliferous plants such as parsley, coriander and carrot. This species recorded from East Azarbaijan, Tehran, Khorassan and Golestan provinces in Iran (Yazdanian et al., 2006). Eggs of this species are used as a substitution host for rearing of egg parasitoids which are used in biological control of the sunn pest, *Eurygaster integriceps*. Scent glands in insects are exocrine glands with epidermal structure and covered by cuticle. The scent glands in adults and nymphs are located in metathoracic segment of the body, and abdomen respectively (Staddon, 1979). The main physiological role of scent glands in Heteroptera is to synthesize and store the

scent compounds for subsequent release of volatiles (Carayon, 1971). One of the major functions of these scent materials is defensive, but they perform other functions such as alarm, mating, aggregation and so on (Staddon, 1979). The defensive chemicals, which are produced and stored in large metathoracic glands, are composed of blends of short-chain saturated, mono- and diunsaturated esters, aldehydes, and 4-oxo-alkenals, with the blends being qualitatively similar among species (Aldrich, 1988). Complementary studies are needed to be done in order to determine exact functions of them, making it possible to use them in pest management programs. This research was performed to evaluate the scent substances of abdominal and metathoracic glands of 4 and 5 instars and adults of the bug *G. lineatum* (L.) under laboratory conditions. The effects of different host plants on composition of scent chemicals were also determined.

MATERIALS AND METHODS

Insect Rearing: A laboratory colony of *G. lineatum* was established using one male and one female. They were let to reproduce by five generations to get a net line of species. Insects reared on umbelliferous seeds. Seeds of three host plants (coriander, parsley and carrot) were cleaned and screened and attached by a natural glue to the inside walls of two kinds of plastic containers (45 × 22 × 30 cm and 30 × 22 × 8 cm; length, width, and height, respectively) with screen-covered net on the top for ventilation. The insects were left in the containers to feed freely of seeds. Insects reared in an environmentally controlled room at 25 ± 2 °C and 60 ± 5% relative humidity, with a 16:8 L:D. Photoperiod provided using 32 Watts fluorescent lights above umbelliferous seeds in the insectarium of Plant Protection Department, Faculty of Agriculture, University of Tabriz. Nymphs in 4 and 5 instars and 3-, 15-, and 25-days old adults were selected and separated for the extractions. These bugs were analyzed by GC system transferred. Abdominal glands of nymphs and metathoracic gland contents of adults were analyzed from 5 individuals per each stage. For this, a new and effective analytical method developed through our previous study (Djozan et al., 2005) was used.

Chemicals and Reagents. All chemicals were in pro-analysis grade and were provided by E. Merck (Germany).

Apparatus: Monitoring of the analytes was performed with a gas chromatograph model GC-15A, Shimadzu (Japan), equipped with a FID, a hydrogen generator model, OPGU-1500s, and a split/splitless injector with a 1 mm internal diameter glass liner. The column used for the separations was 30 m × 0.25 mm capillary column coated with a 0.25 µm film of SPB-50 (SUPELCO, UK). A HB pencil lead of 0.35 mm diameter and 60 mm length (rotring 0.5) was mounted in the home-made SPME device and the exposed fiber was trimmed to 2 cm. Thermal conditioning of pencil lead fibers was conducted in a Carbolite furnace (Bemaford, Sheffield, England). A SPME manual sampling holder (SUPELCO,

UK) was used for the delivering of volatile contents of scent gland to the sample vials.

Determination of optimal conditions of GC: Standard chemical solutions including hexanal, octenal, dodecane, tridecane, pentadecane and nonane were selected as a control. About 0.04 μl of head space of any solution injected into GC by Hamilton syringe and chromatogram of these samples were recorded. Then, a mixture of these solutions was injected into system and different temperature conditions were examined and optimal condition was recorded. So, the best temperature conditions for a fraction were selected on the bases of chromatograms. GC conditions were as the following: **Column type** [(CBP5 (SE-52,54, L= 25m, ID= 0.33 mm, film thickness= 0.5 μm , Shimadzu Hicap)], **Injector** [(splitless, splitter opened after 1 min (desorption time), temperature (desorption temperature)= 350 $^{\circ}\text{C}$)], **Detector** [(flam ionization detector (FID), temperature= 350 $^{\circ}\text{C}$)], **Carrier Gas** [(He (99.99%), velocity= 30 cm/min)], **Makeup gas** [(He (99.999 %), flow rate= 30 ml/min)], **Column temperature program** [40 $^{\circ}\text{C}$ (hold 1 min) 10 $^{\circ}\text{C}/\text{min}$ 250 $^{\circ}\text{C}$ (hold 4 min)].

Determination of optimal injection method: Substances of abdominal glands of nymphs and metathoracic glands of adults were extracted by insulin syringe and added to 10 μl of normal pentane. Then, 0.2 μl of this solution was injected into GC system by using a Hamilton syringe. By comparing chromatograms recorded by SPME method, the best method of injection was selected.

Determination of optimal fiber of SPME: In this experiment, Supelco fiber was compared with pencil lead fiber. Each fiber was separately exposed to headspace of the extracted substance for 15 minutes and then was injected into GC. Chromatograms which were compared on the bases of the number and heights of pikes and then suitable fiber were selected.

Analytical procedures: Scent gland volatile organic compounds were extracted from the prepared samples using head-space solid-phase microextraction (HS-SPME) method. Home-made pencil-lead fiber was plunged into 2 ml vial containing analytes dissolved into 200 μl of n-pentane, and exposed to the headspace of them for 15 min until stable position was reached. SPME fiber was retracted and introduced into GC injector valve using split mode with 1/10 rate. The analytes were injected into GC injection port using split mode with 1/10 rate. Monitoring of analytes was carried out with capillary GC-FID and GC-MS.

Analysis of data: Due to insufficient replications and abnormality of data for analyzing, nonparametric methods including χ^2 and Whitney-Wilcoxon were used. Treatments were compared on the bases of sub-curve surface amounts in each pike.

RESULTS

Analysis of adults and nymphs scent substances: Comparing the chromatograms obtained from standard solutions, emission time of main compounds from adult and 4th and 5th instar scent glands were determined by GC-MS (Figure 1). In the remained stages, retention time of control compounds was used to determine the emission time of main compounds and to identify the scent substances.

Table 1 shows the determined compounds in the scent glands of adults and nymphs reared on three hosts. Most of the compounds were alkanes and the rest were compounds such as aldehydes, alcohols, esters and alkenes. Comparing the amounts of compounds, the (*Z*)-4-hexen-1-ol acetate amount was so less. The main compounds detected in nymphs were 3- methyl pentanol, hexane and tridecane, meanwhile the low amounts of other detected compounds in adults were found in nymphs too. In developed stages, both Ethyl cyclohexane and tridecane were observed in high quantity. High amounts of 3-methyl pentanol and hexane were detected in nymphs whereas, they were low in adults.

Evaluation of performance of SPME, direct injection, commercial and common fiber: In this evaluation two methods including SPME and direct injection were compared. Results showed that SPME method was better than direct injection because of containing higher and more pikes with the best qualities. Since in SPME method there was no need for solvent, the pikes of solvent was not present and leads to increase accuracy of identification. In addition, the probability of denaturation of scent substances due to reaction with solvent was reduced (Figure 2).

Furthermore, the performance of commercial and common fiber was evaluated. Common fiber which was used for determining the number and clearance pikes of compounds had a good quality. Using common pencil fiber, 16 compounds were detected while using commercial fiber (Supelco) the number of detected compounds was only 8 (Figure 3). Because of the effectiveness of SPME method and common pencil fiber for identification of compounds, they were recommended for using in main analysis.

Effects of host plants in abundance and types of compounds: Host plant type had no effect on type of compounds extracted from scent glands of the sunn pest adults and nymphs. Almost all compounds detected from scent glands of adults and nymphs reared on different host seeds were the same having differences in their quantity.

Effects of gender and age of adult insects, and nymphal stages on type and frequency of compounds: Comparing the chromatograms obtained by injection of samples of scent glands from 3-days old male and female adults reared on different hosts showed the similarity in compounds and difference in amounts between sexes.

Coincided chromatograms from 3-, 15- and 25-days old male and female adults and 4th and 5th instar nymphs reared on different hosts in the same scale showed that the quantity of compounds were different whereas their types were similar in all cases. Host, gender and age of insects had no effects on compounds type, but their quality were affected.

As a conclusion, compound quantity decreased due to the insect's aging (short pikes). In nymphs, two pikes of 3-methyl pentanol and hexane were higher than those of adults. In the remained cases, pikes obtained from adults were higher than those of nymphs. Figure 4 shows the chromatograms from scent glands of female adults and those of nymphs injected into GC-MS.

DISCUSSION

Detected compounds from scent glands of adults and nymphs of *Graphosoma lineatum* were revealed by other researchers in different amounts from other bugs with different behavioral functions. Zarbin et al. (2000) showed that study in *Piezodorus guildinii* some compounds such as (*E*)-2-hexenel and (*E*)-4-oxo-2-hexenal had the same role as the alarm compounds. In *Dichelops melacanthus*, tridecane was the major component followed by lesser and approximately equal to the amounts of (*E*)-4-oxo-2-hexenal and (*E*)-2-octenal. Other compounds including (*E*)-2-hexenal, decane, (*E*)-2-hexenyl acetate, undecane, (*E*)-4-oxo-2-octenal, dodecane, (*E*)-2-octenyl acetate, 1-tridecene, tetradecane and pentadecane were also identified (Marques et al., 2007).

The attractive aggregation pheromone from males of the bean bug, *Riptortus clavatus*, has been identified as a blend of (*E*)-2-hexenyl (*E*)-2-hexenoate, (*E*)-2-hexenyl (*Z*)-3-hexenoate and tetradecyl isobutyrate. Octadecyl isobutyrate was also detected in airborne volatiles. In field experiments, the attractiveness of tetradecyl isobutyrate to *R. clavatus* was increased by adding the octadecyl isobutyrate. These results suggest that octadecyl isobutyrate is one of the components of the attractive aggregation pheromone of *R. clavatus* and may acts as a synergistic composition in this bug (Yasuda et al., 2007).

In *Chlorochroa uhleri*, *Ch. sayi*, and *Ch. ligata* (Hemiptera: Pentatomidae), tridecane was the most abundant component, with lesser and approximately equal amounts of (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, and an ester component, (*E*)-2-octenal (Ho et al., 2001).

Nagnan et al. (1994) detected 11 compounds from scent glands of *Lincus spurcus* and *L. malveolu*; the (*E*)-2-hexenal, (*E*)-2-octenal and undecane were the main compounds which confirm the results of the current research.

The mixture of (*E*)-2-hexenel and tridecan was a more effective repellent than individually using of these components (Gunawardena and Herath, 1991). The outcomes of these researchers showed that some compounds have a kairomonal role for *Trissolcus basalus*. They detected compounds such as *n*-dodecane and *n*-tridecane which confirm our results on *G. lineatum*.

In this study, the amounts of ethyl cyclohexane and tridecan; and in nymphs, 3-methyl pantanol and hexane were more in male and female insects. Detected

compounds in adults and nymphs were the same, but differed in quantity. For example, in nymphs 3-methyl pentanol and hexane were in maximum amount but low in adults. These results were similar to those from Aldrich (1988) on scent glands of adults and nymphs of different pentatomid bugs (*Chlorochroa uhleri*, *Ch. Sayi* and *Ch. ligata*). (*E*)-2-hexenal, dodecane, tridecane, pentadecane and (*E*)-2-decenal were observed in both study. Aldrich (1995) suggested that these compounds have a defensive role in insects. In the soybean stink bug *Piezodorus guildinii*, the chemical composition of the metathoracic scent gland secretions were identified as a mixture of (*E*)-2-hexenal, (*E*)-4-oxo-2-hexenal, (*E*)-2-octenal, undecane, *n*-dodecane, *n*-tridec-1-ene and *n*-tridecane. In this study, (*E*)-2-hexenal and (*E*)-4-oxo-2-hexenal were detected as major compounds with alarm pheromone function (Zarbin et al., 2000).

In the present study, detected compounds of males and females were similar and were the same which has been reported for other several pentatomid species (e.g. Aldrich 1988 and Ho et al., 2001). Thus, these compounds appear to be highly conserved, being shared both within and between genera, and as well as between bug families (Aldrich, 1995). The results showed that quantity of male and female compounds were equal, and in spite of a minor difference, the extracts from nymphs were similar to adults (Ho et al., 2001). In other studies such as Nagnan et al. (1994), (*E*)-2-hexenoic acid in females of *L. spurcus* and *L. malveolus* were five times more than that in males. The relative amounts of the volatile compounds hexyl butyrate, (*E*)-4-oxo-2-hexenal, and (*E*)-2-hexenyl butyrate were 100, 44, and 4 percent, in females, and 83, 37, and 3 percent in male's metathoracic scent glands of *Lygus hesperus* respectively (Byers, 2006).

Results of this study as well as Aldrich's (1988) showed that compounds detected in males and females were similar, but can not play a role in sexual activity of insects, unless there should be a distinct difference between sexes. Aldrich et al. (1978) detected (*E*)-2-hexenal in *Nezara viridula* scent glands and revealed that this compound has cumulative pheromone role. They suggested that the amount of this compound is so important due to smaller amount have cumulative but larger amounts have alarm role. As Aldrich (1988), we also showed that the type of compounds in 4th and 5th instar nymphs differed only in amounts. Coincidence of decreasing compounds with developing nymphs indicates the defensive rule of scent materials, because as the insect grows, other defensive mechanisms appear in bugs. Aldehydes and esters are strongly bad smell and injurious compounds that could play as a repellent. Similarity in nymph's and adult's scent gland compositions suggests that these compounds may not involve as a sex pheromone because nymphs don't need any sex pheromone. *N*-alkane mixed with (*E*)-2-hexenal had more repellency on insects (Kral et al., 1999). The egg parasitoid wasp, *Trissolcus basalis*, uses defensive compounds of its host such as a kairomone (Matiachi et al., 1993). The function of the hydrocarbons is less clear, but they may serve as solvents and as controlled-release materials for more volatile aldehydes such as (*E*)-2-decenal and (*E*)-2-hexenal (Remold, 1962). Ishiwatari (1976) reported that the differences in scent components of different species may be considered as cause of species-specificity

of the aggregate formation. He reported that *trans*-2-hexenal was considered as an aggregation pheromone and also he showed repellent function of this compound in the same insect. This bifunctional effect of *trans*-2-hexenal is related to the amount of substances used.

In the true bugs, different pheromones such as sex-, attractant-, aggregation-, and alarm- pheromones have been reported by various researchers. Identification of these infochemicals can be an important means for monitoring the time of pest population emergence, pest density, identification of new pests, and developing an efficient control program (Demirel, 2007). It is clear that more studies are needed for complete identification of all components of scent gland substances and additional researches should be carried out in order to understanding the function of them separately as well as in composition with other chemicals presence in scent substances.

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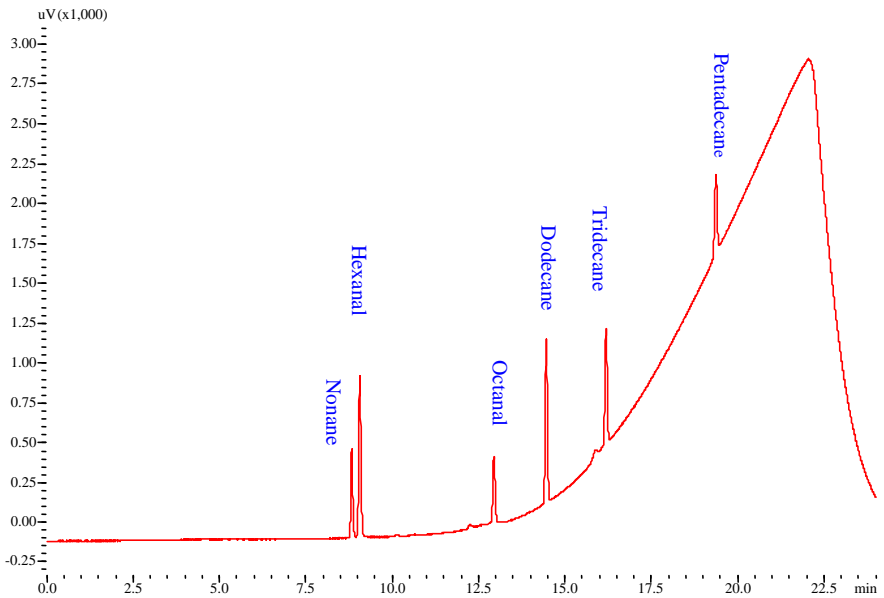


Figure 1. Chromatogram of mixed control compounds injected to GC-MS.

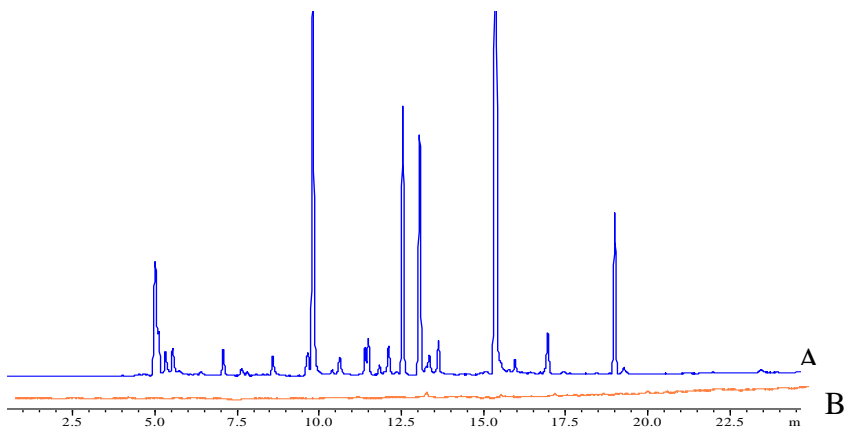


Figure 2. Chromatograms of comparing SPME (A) and direct injection (B) methods for detection of scent substances from scent glands of *Graphosoma lineatum*.

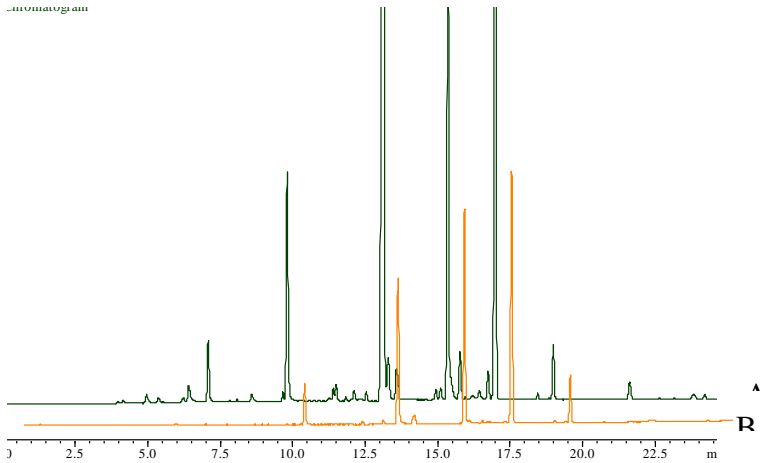


Figure 3. Comparing chromatograms from injection of scent gland substances of *Graphosoma lineatum* to GC-MS using common pencil fiber (A) and commercial fiber (B).

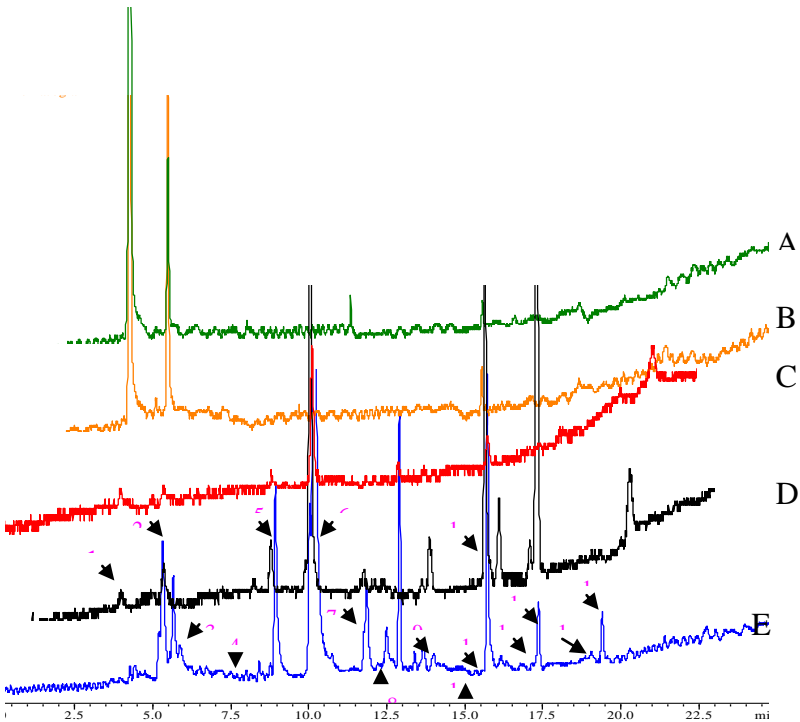


Figure 4. Comparing the chromatograms of female's and nymphal scent gland compounds of *Graphosoma lineatum* injected into the GC-MS. A. 5th instar nymphs; B. 4th instar nymphs; C. 25-days old adults; D. 15-days old adults; E. 3-days old adults; (1) 3-methyl pentanol; (2) *cis*-1,2-dimethyl cyclopentane; (3) hexane; (4) heptane; (5) (*E*)-2-hexenal; (6) Ethyl cyclohexane; (7) limonene; (8) Undecane; (9) Dodecane; (10) (*E*)-2-decenal; (11) 1-tridecene; (12) tridecane; (13) tetradecane; (14) (*E*)-5-decenyl acetate; (15) Pentadecane; (16) hexadecane.

Table 1. Compounds identified from scent glands of adults and nymphs reared on three hosts.

	Compound name	Saturation/unsaturation	Chemical group
1	3-methyl pentanol	saturated	Alcohol
2	<i>cis</i> -1,2-dimethyl cyclopentane	saturated	Aromatic alkanes
3	Hexane	saturated	Alkanes
4	Heptane	saturated	Alkanes
5	(<i>E</i>)-2-hexenal	unsaturated	Aldehyde
6	Ethyl cyclohexane	saturated	Aromatic alkanes
7	Limonene	unsaturated	Aromatic alkanes
8	(<i>Z</i>)-4-hexen-1-ol acetate	unsaturated	Alcohol
9	Undecane	saturated	Alkanes
10	Dodecane	saturated	Alkanes
11	(<i>E</i>)-2-decenal	unsaturated	Aldehyde
12	1-tridecene	unsaturated	Alkenes
13	Tridecane	saturated	Alkanes
14	Tetradecane	saturated	Alkanes
15	(<i>E</i>)-5-decenyl acetate	saturated	ester
16	Pentadecane	saturated	Alkanes
17	Hexadecane	saturated	Alkanes