J. Crop Prot. 2014, 3 (3): 357-367

Research Article

Identification and analysis of the male labial gland secretions of three species of *Bombus* (*Thoracobombus*) (Hymenoptera: Apidae)

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Abstract: The males of the three species of bumblebees, *Bombus* (*Thoracobombus*) *ruderarius* (Müller), *B.* (*T.*) *persicus* Radoszkowski and *B.* (*T.*) *mesomelas* Gerstaecker collected from Vikan village, Qazvin province and their male labial gland secretions were analyzed by gas chromatography/ mass spectrometry (GC/MS) and components of their extracts were identified. The major compounds were a complex mixture of alkenols, alkenals, fatty acids, hydrocarbons, wax type esters and steroids. The main component of male labial gland secretions of *B. ruderarius*, *B. persicus* and *B. mesomelas* were determined to be 9-hexadecenol (42.1%), Z-13-octadecen-1-yl acetate (81.8%) and Z-12-pentacosene (34.4%), respectively. Results showed that if detection of species in male bumblebees would be difficult by using morphologic characters, then identification could be confirmed by detecting main component of male labial gland of these bees.

Keywords: Labial glands, bumblebees, chemotaxonomy, *Bombus*, *Thoracobombus*, Iran

Introduction

The studies on chemical communication of bumblebees has led to many interesting results in the last decades. Marking pheromones of bumblebee males are produced by the cephalic part of the male's labial gland (Kullenberg *et al*., 1973). During the premating behaviour, males scent-mark their territories to attract conspecific females for mating (Patrolling behavior) (Bergman, 1997). The patrolling behaviour is the most common type of premating behaviour among the bumblebee and cuckoo bumblebee species. Since Frank (1941) described this flight path activity of male bumblebees in detail, there has been a growing interest in this peculiar behaviour of male bumblebees: a long-lasting, energy consuming daily flight activity (Bertsch, 1984) along fixed routes marked by scent produced in the cephalic part of the labial glands (Kullenberg *et al*., 1973).

Calam (1969) was the first to demonstrate that the secretions of the labial cephalic glands of male bumblebees are highly specific. Since then the cephalic gland secretions of many species have been studied (Bergstrom and Svensson, 1973a,b; Svensson and Bergstrom, 1979; Cederberg *et al*., 1984; Descoins *et al*., 1984; Bergstrom *et al*., 1985; Bertsch, 1997a, b; Pamilo *et al*., 1997). These contributions have been reviewed recently by Bergman (1997), Terzo *et al*. (2003) and Bertsch *et al*. (2004a, b). Each bumblebee species produces a specific blend of compounds for scent marking their territories (Valterova and Urbanova, 1997). In some

Handling Editor: Dr. Ali Asghar Talebi

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Received: 24 August 2013, Accepted: 15 February 2014
Published online: 15 February 2014

closely related species the morphological similarities and colour variations within the species make it difficult to determine the species unambiguously (Bertsch, 1997a, b; Pamilo *et al*., 1997). This fact led researchers to use characteristics other than morphological or morphometric ones for the separation of the taxa. An analysis of the males' labial gland secretion seems to be a good tool for distinguishing between closely related species and it was used in the past for taxonomic purposes (Svensson and Bergstrom, 1977).

While variability in color is sometimes great (Rasmont *et al*., 2005), the pheromonal composition is more or less stable within a species regardless of the locality of occurrence. Thus, a thorough redescription of the compositions of male sexual pheromones for their use in taxonomical and phylogenetic studies is needed. This necessity became greater with the development of sensitive analytical tools that allow identification of minor and trace components of complex mixtures in the gland extracts. The older literature often reported only one or a few major pheromonal components, and these data are not satisfactory for chemotaxonomic purposes (Bergstrom, 1981).

The objective of this study was detecting male species of collected Iranian bumblebee specimens by extracting the main contents of labial gland secretions.

Materials and Methods

Biological material

Males of individual species were collected during the years 2010–2012 in the following localities: ***B. ruderarius,*** Ghazvin, Verk, 1.viii.2011, 1♂; Ghazvin, Vikan, 1.viii.2011, 2 ♂♂; Ghazvin, Alamoot, 1.viii.2011, 2 ♂♂; Chaloos, Kamarbon, 30.vii.2011, 3 ♂♂; Chaloos, Duna, 27.vii.2011, 3 ♂♂; Chaloos, Klardasht, Roodbarak, 17.viii.2011, 1 ♂; Ardabil, Sarein, Alvares, 31.vii.2011, 3 ♂♂; Ardabil, Moeil, 31.viii.2011, 2 ♂♂; 6.viii.2009, 1 ♂; Ardabil, Meshkinshahr, Shirvan Derrehci, 6.viii 2011, 13 ♂♂. ***B. persicus***, Ghazvin, Verk, 1.viii.2011, 2 ♂♂; 8.viii.2011, 6 ♂♂; Tehran, Lalan, 19.viii.2011, 2 ♂♂; Tehran, Zaaygan, 24.vii.2011, 1 ♂; Chaloos, Kamarbon, 4.viii.2011, 8 ♂♂, Chaloos, Klavengah, 18.viii.2011, 5 ♂♂; Ardabil, Meshkinshahr, Shabil and Ghotursuee, 28.viii.2011, 2 ♂♂., ***B. mesomelas***, Alborz, Jadehchalus, Duna, 30.vii.2011, 2 ♂♂; East Azerbaijan, Tabriz, Kandovan village, 1.viii.2011, 2 ♂♂; Ardabil, Meshkinshahr, Salavat village, 3.viii.2011, 2 ♂♂; Ardabil, Sarein, Alvares, 1.viii.2011, 3 ♂♂; Ardabil, Meshkinshahr, Shirvan Derrehci, 6.viii.2009, 12 ♂♂; Ardabil, Meshkinshahr, Shabil and Ghotursuee, 28.viii 2011, 9 ♂♂.

For the chemical analyses, the collected living insects were transported to the laboratory and then kept in a freezer until the labial glands were dissected. The cephalic part of the labial glands was dissected from the head of males in frozen condition and placed in vials (glands from 5 males per vial) containing 20 µl hexane per gland (total 100 µl per vial). After 15 min of shaking and 2 h standing in the refrigerator, the hexane extract was filtered and stored in a freezer (-18 °C) before analysis.

Chromatography

The samples were analyzed using a gas chromatograph–mass spectrometer (GC–MS) Fisons MD 800 with electron-impact ionisation (70 eV). For the separation of components, a DB-5 column (5% fenylmethyl silicone), 30 m × 0:25 mm film thickness 0.25 lm was used. A splitless injector mode (220 °C) and helium carrier gas (flow 0.94 ml/min) were used. The temperature program of analyses was 70 °C; 2 min; 10 °C/min to 320 °C; 15 min (Urbanova *et al*., 2004). Compounds were identified by comparing their mass spectra with those of the NIST Library (National Institute of Standards and Technology, USA) and by retention times and chemical ionization measured on an ion-trap instrument with MeCN used as a reagent gas

for the determination of the C = C bond positions (Oldham and Svatos, 1999).

Results

Species diagnosis

***Bombus ruderarius*:** Body length 15 to 17 mm; wingspan from 34 to 38 mm; under stereomicroscope (40 ×) hair of the clypeus and around antenna black, hair of the vertex black and yellow; hair of anterior thoracic collar dark yellow and Interalar band black, the scutellum yellow and black with dominance of black; hair of T1 in the middle brown with low density, marginal sides with a mass of yellow hair, hairs of T2 and T3 black, T4 and T5 yellow, marginal sides of T6 with yellow hair and in median part with black hair, T7 black. The general form of the genitalia as Fig. 1-a, inner corner located near mid-point of its length without any inwardly-directed hooks, the volsella is nearly triangular in the distal section, gonostylus with the distinct interio-basal process that is associated with many long branched hairs, head of penis valve nearly straight from dorsal aspect.

Bombus mesomelas: Body length 15-17 mm; wingspan 30-33 mm; the clypeus marginally with some white and black hair, area around antenna with a mix of white and black hair more black, hair of the vertex white, around of that black hair; hair of anterior thoracic collar and the scutellum white and dense, hair of interalar band black; hair of T1 and T2 white with cream hair medially, in T3 hair of primary rows cream and terminal row white, T4-T6 is like T3 but hair of primary rows orange and terminal row yellow, hair of T7 black to dark brown. The general form of the genitalia as Fig. 1-b, the volsella with the anterio-apical corner indeed bearing a single combined inwardly-directed backside process, gonostylus is nearly triangular with two processes, head of penis valve curved outwards.

Bombus persicus: Body length 15-17 mm; wingspan 28-32 mm; hair of around clypeus black and white, hair of around antenna black and white with dominant black, the vertex hair a mix of brown and pale yellow ; hair of anterior thoracic collar white, interalar band black with hair on front margins white and terminal margin black, hair of the scutellum black, hair of T6 and T7 dark brown. The general form of the genitalia as in Fig. 1-c, inner margin of the volsella with dense hair, inner corner located near mid-point of its length without any inwardly-directed hook, gonostylus like a thin strip, with a long distinct interio- basal process, but not associated with long hair, head of penis valve somewhat curved outwards, with a posterior–apical corner.

Chemical compounds

The labial glands of species including *B. ruderarius*, *B. persicus* and *B. mesomelas* males contained a mixture of alkenols, alkenals, fatty acids, hydrocarbons, wax type esters and steroid. Typical chromatograms of the male cephalic labial gland secretions of *B. ruderarius*, *B. persicus* and *B. mesomelas* are shown respectively in Figs. 2, 3 and 4. The compounds are summarized in Table 1.

In labial glands of male *B. ruderarius*, the major compound was 9-hexadecen-1-ol (z) (Fig. 2, peak 1 at 16.468 min, 42.1% of total peak area) and considerable amounts of 9-tricosene(z) (Fig. 2, peak 2 at 20.307 min, 35.9%), tricosene (Fig. 2, peak 3 at 21.955 min, 11.8%) and less amounts of the other components including 1.14-tetradecanediol (at 15.183 min, 0.32%), hexadecenal (at 16.425 min, 0.22%), Z-9-Hexadecen-1-ol acetate (at 17.716 min, 2.69%), ethyl, 9. hexadecenoate (at 17.925 min, 1.49%), 1-heptadecanol (at 18.12 min, 0.72%), 2-hexyle-1- octanol (at 18.32 min, 0.09%) icosane (at 18.595 min, 0.2%), docosane (at 18.780 min, 0.26%), 7-docosene (at 19.123 min, 0.11%), 11-tricosene (at 19.389 min, 0.31%), tricosane (at 20.511 min, 2.4%), z-12- pentacosane (at 22.141 min, 1.27%) and pentacosadiene (at 22.367 min, 0.12%) that show in Tabel

1. The mass spectera of main component (9-hexadecen-1-ol (z)) is shown in Fig. 5. The main components in the male labial gland secretions of many bumble bee species are 1-alcohols, therefore it is likely that they play a major role in communication. The alcohols in the labial gland secretions are not very stable; if specimens are stored for long-term at -25 °C the alcohols are converted into carboxylates. 9-tricosen, the second component according to amount is a common component that is present in labial gland secretions of many bumblebees and was found in the other two species tested.

In *B. persicus*, the major compound was Z- 13- octadecen- 1-yl acetate (Fig. 3, peak 2 and 3, corresponding to 19.517 and 19.568 min, overall 81.8% of total peak area) and considerable amounts of 11-hexadecen-1-ol (Fig. 3. Peak 1. at 18.429 min. 12.02% of total peak area) and the other components, with minor quantity, included tetradecanol (at 17.81 min, 1.33%), 1-eicosanol (at 20.211 min, 0.15%), 5-eicosene (at 20.422 min, 3.28%), eicosene (at 20.502 min, 1.86%) and docosane (at 21.144 min, 0.24%), 9-tricosene(z) (at 21.342 min, 0.32%). The mass spectera of main component Z- 13-octadecen- 1-yl show in Fig, 6. The corresponding acetates are often found only in minor amounts or as traces in pheromonal component. Indeed, sometimes they are completely absent and occur only because of an aging process of the prepared glands. But in this species, acetate is as the main component with most deal in labial gland secretions and plays an important copulative role in *B. persicus.*

In *B. mesomelas*, the major compounds were Z-12- pentacosane (Fig. 4, peaks 3 and 4, corresponding to 21.963 and 22.023, overall 34.4% of total peak area) and considerable amounts of 9-hexacosene (Fig. 4. Peak 5 and 6, corresponding with 23.493 and 23.552 min, overall 20.42%), 9- tricosene (Fig. 4. Peak 1, at 20.366 min, 17.543%), tricosene (Fig. 4, Peak 2, at 20.519 min, 12.41%) and heptacosene (Fig. 4. Peak 8, at 24.121 min, 11.5%), the other components with minor quantity included Pentadecanal (at 18.601 min, 0.68%), 1-heptadecanol (at 18.790 min, 0.8%), eicosene (at 19.42 min, 0.14%), Henicosane (at 19.51 min, 0.11%), docosane (at 19.62 min, 0.12%), 9-eicosen-1-ol (at 21.11 min, 0.8), 1-eicosanol (at 21.148 min, 0.65%), hexacosane (at 23.654 min, 0.09%), Nonacosane (at 24.92 min, 0.12%) and the final composition was not known (at 25.464 min, 4.261%). The mass spectera of main components of Z-12-pentacosane is shown in Fig. 7.

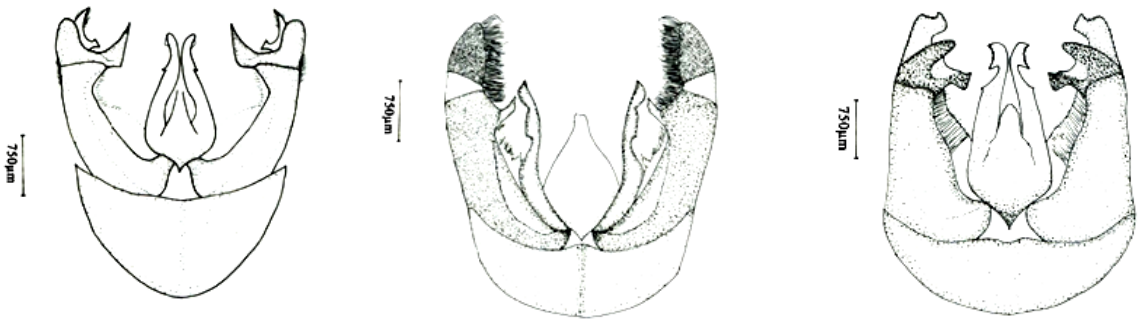

Figure 1 Genitalia pattern of 3 male species of subgenus *Thoracobombus*, a: *B. ruderarius*, b: *B. persicus*, c: *B. mesomelas* (original).

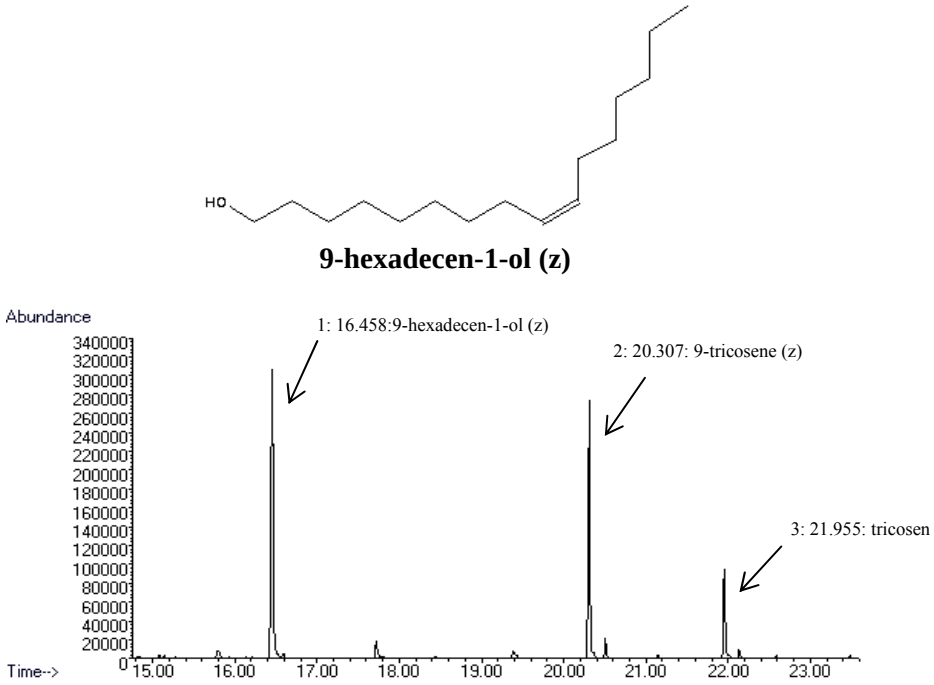

Figure 2 Gas chromatogram of the labial gland secretion of *Bombus* (*Thoracobombus*) *ruderarius* males 15 up to 23 min retention time (Coding is just for great peak).

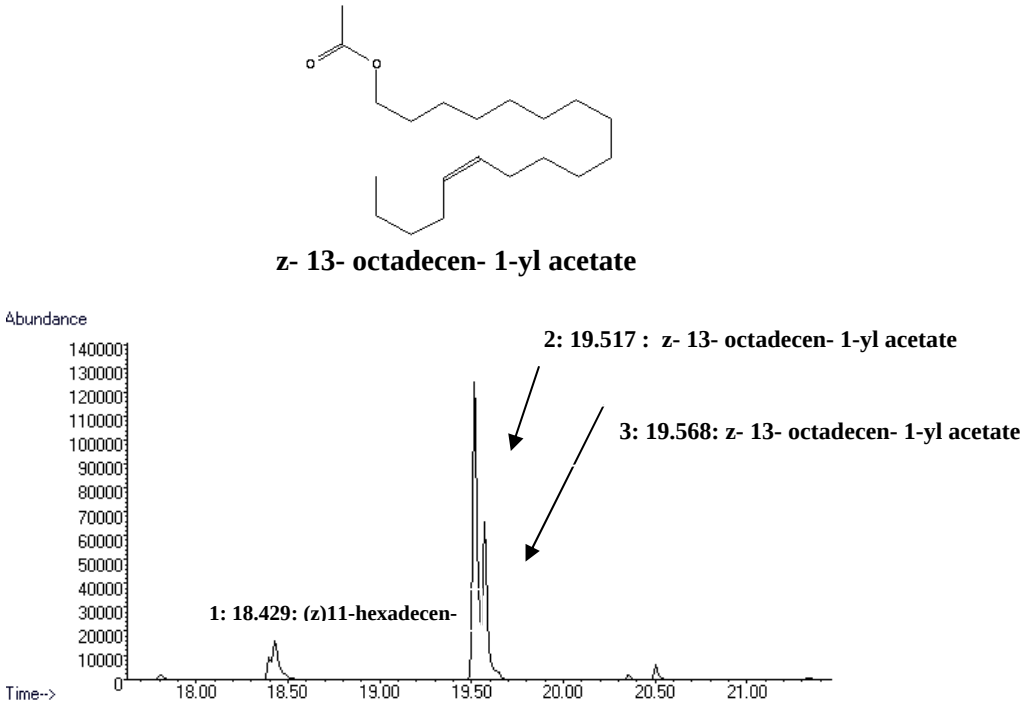

Figure 3 Gas chromatogram of the labial gland secretion of *Bombus* (*Thoracobombus*) *persicus* males 18 up to 21 min retention time (Coding is just for great peak).

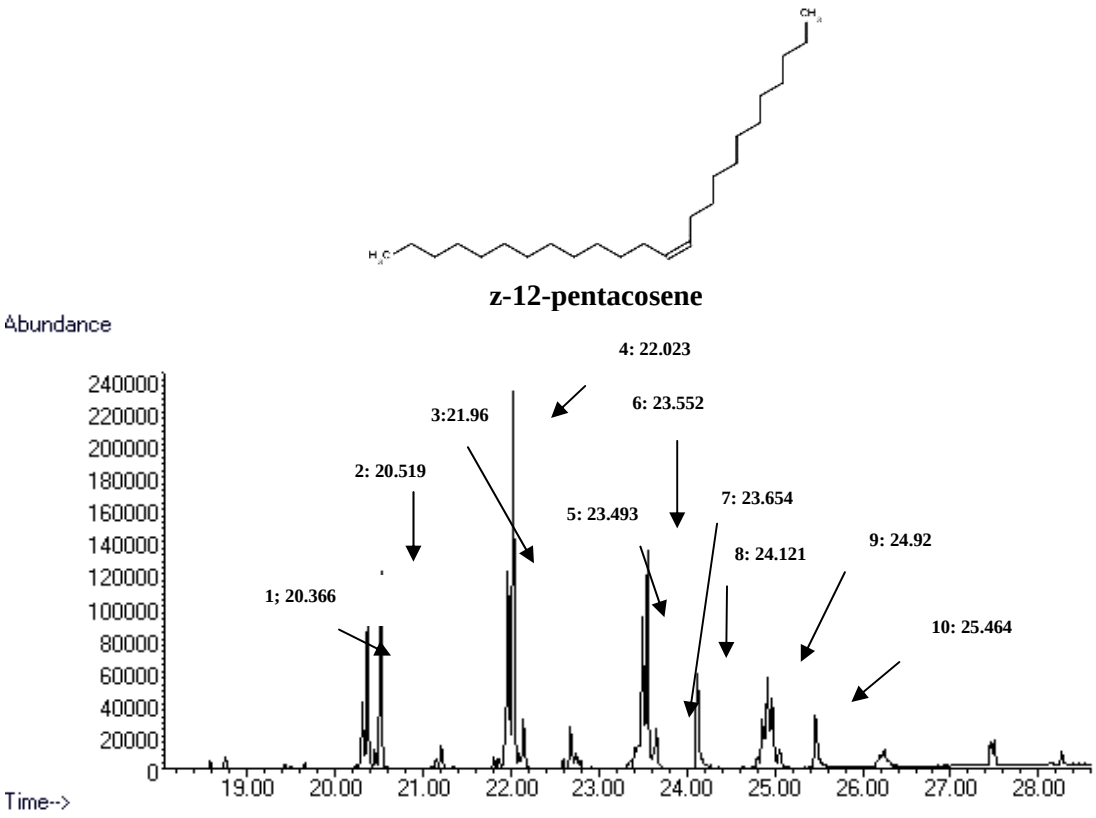

Figure 4 Gas chromatogram of the labial gland secretion of *Bombus* (*Thoracobombus*) *mesomelas* males 15 up to 23 min retention time (Coding is just for great peak).

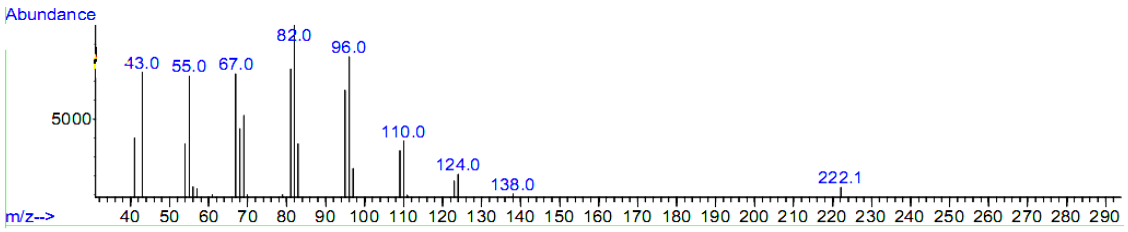

Figure 5 Mass spectra of 9-hexadecen-1-ol (z).

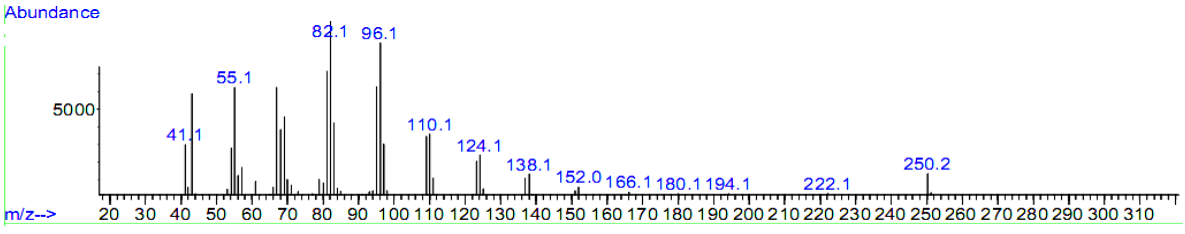

Figure 6 Mass spectra of Z- 13- octadecen- 1-yl acetate.

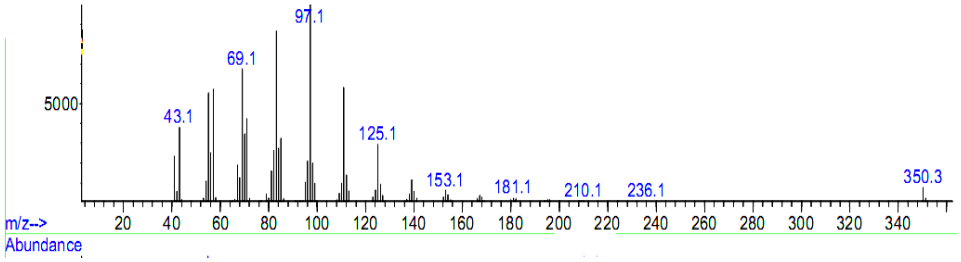

Figure 7 Mass spectra of Z-12-pentacosene.

Table 1 Composition of the male cephalic labial gland secretions of *Bombus* (*Thoracobombus*) *ruderarius*, *B.* (*T.*) *persicus* and *B.* (*T.*) *mesomelas* from Iran. Relative proportions of compounds in order of their retention times (RT).

N	Components	*Bombus ruderarius*	*Bombus persicus*	*Bombus mesomelas*
	ALCOHOL			
1	2-HEXYL-1. OCTANOL	0.09		
2	9-HEXADECEN-1-OL (Z)	42.10*		
3	11-HEXADECEN-1-OL (Z)		12.02	
4	1-HEPTADECANOL	0.72		0.80
5	1.14-TETRADECANEDIOL	0.32		
6	TETRADECANOL		1.33	
7	HEPTACOSANOL			
8	9-EICOSEN-1-OL,CIS			0.80
9	1-EICOSANOL		0.15	0.65
	ESTER			
10	Z-9-HEXADECEN-1-OL ACETATE	2.69		
11	Z-13-OCTADECEN- 1-YL ACETATE		81.80*	
	ALKANS AND ALKENS			
12	DOCOSANE	0.26	0.24	0.12
13	ETHYL, 9.HEXADECENOATE	1.49		
14	HENICOSANE			0.11
15	TRICOSANE	2.40		
16	7-DOCOSENE	0.11		
17	Z-12- PENTACOSANE	1.27		34.40*
18	9-TRICOSENE(Z)	35.90	0.32	17.54
19	11-TRICOSENE	0.31		
20	5-EICOSENE	11.80	3.28	
21	TRICOSENE			12.41
22	EICOSENE		1.86	0.14
23	9-HEXACOSENE			20.42
24	ICOSANE	0.20		
25	HEXACOSANE			0.09
26	HEPTACOSENE			11.50
27	PENTACOSADIENE	0.12		
28	NONACOSANE			0.12
	ALDEHYDE			
29	PENTADECANAL			0.68
30	HEXADECENAL	0.22		

***main component.**

Discussion

In some labial gland secretions of male bumble bees saturated uneven numbered straight chain hydrocarbons (pentacosane, tricosane, heptacosane, etc) are characteristic, the C23-C29 alkanes are normally accompanied by mono-unsaturated alkenes and the same is true about Species tested in this article. These hydrocarbons are generally not considered to belong to the biologically active compounds of the species, but are often treated as contamination from the cuticular hydrocarbons (Oldham *et al*., 1994). It is well established that complex mixtures of species-specific (Howard, 1993) cuticular hydrocarbons are the primary chemical cue involved in species and kin recognition systems (Howard and Blomquist, 1982; Bonavita-Cougourdan *et al*., 1987).

In all three species of subgenus *Thoracobombus* tested, none of the compounds Isoprenoids that have in many labial gland secretions of Subgenera *Bombus*, were found. A number of compounds are obtained in two or three species. For example 9-tricosene was present in all three species. The work of Bergman (1997) and Kindl *et al*. (1999) argues for the use of major compounds of the male cephalic gland secretion in bumblebees as sexual pheromones. Accordingly the combination of our tests were compared with the work of Terzo *et al*. (2007), Terzo *et al*. (2005), Valterova and Urbanova (1997) and the identity of the three species *B. ruderarius*, *B. mesomelas* and *B. persicus* from Iran was confirmed.

Acknowledgments

This research was carried out by financial support of Yasouj University. Our special thanks to Klara Valterova for her helpful comments. Also we thank Mrs. Mozhgan Karimpirhayati for her assistance in sampling trips.

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تشخیص و تجزیه ترشحات غدد لبی جنس نر سه گونه از زنبورهای مخملی *Bombus* (*Thoracobombus*) (Hymenoptera: Apidae)

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دریافت: ۲ شهریور ۱۳۹۲؛ پذیرش: ۲۶ بهمن ۱۳۹۲

چکیده: زنبورهای نر مخملی سه گونه *B.* (*T.*) *persicus* ،*B.* (*Thoracobombus*) *ruderarius* (Müller) Radoszkowski و*B.*(*T.*) *mesomelas* Gerstaecker از روستای ویکان، استان قزوین جمع‌آوری و ترکیبات ترشحات غدد لب پایین آن‌ها به‌وسیله دستگاه کروماتوگرافی گازی همراه با طیف‌سنجی جرمی تجزیه و شناسایی شد. بیشتر ترکیبات آن شامل آلکانول‌ها، آلکن‌ال‌ها، اسید چرب، هیدروکربن‌ها، استرهای مومی و استریدها بود. ترکیب اصلی ترشحات غدد لب پایین در سه گونه *B. ruderarius* ،*B. persicus* و *B. mesomelas* به‌ترتیب ۹-hexadecenol (۴۲/۱٪)، Z-13-octadecen-1-yl acetate (۸۱/۸٪) و Z-12- pentacosene (۳۴/۴٪) تعیین شد. نتایج نشان داد، درصورتی‌که شناسایی گونه در جنس نر زنبورهای مخملی با ویژگی‌های مورفولوژیک دشوار باشد، می‌توان از شناسایی ترکیب اصلی محتویات غدد لبی نمونه‌های نر این زنبورها برای تأیید شناسایی گونه این زنبورها استفاده کرد.

واژگان کلیدی: غدد لبی، زنبورهای مخملی، طبقه‌بندی شیمیایی، *Thoracobombus* ، *Bombus*، ایران